UNIVERSITY OF TASMANIA

# Physiological Responses of Bluethroat Wrasse, *Notolabrus tetricus*, Horseshoe Leatherjacket, *Meuschenia hippocrepis* and Greenback Flounder, *Rhombosolea tapirina*, to Low Temperature Transport

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**Declaration of Originality**

I declare, to the best of my knowledge, that the material contained within this thesis is original except where due acknowledgment is given. It has not been accepted for the award of any other degree or diploma.

James D. Findlay
29 June, 2005

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# ABSTRACT

The study reviewed the techniques used in the global trade of live fish. Based on this review and field and laboratory experiments, methods were identified to improve the survival of bluethroat wrasse, *Notolabrus tetricus*, horseshoe leatherjacket, *Meuschenia hippocrepis* and greenback flounder, *Rhombosolea tapirina*, during capture, holding and transport. Investigation of the physiological responses of fish exposed to lowered temperatures was a central theme including description of cold thermal tolerance and the effects of lowered temperature on oxygen consumption.

The Critical Thermal Minima and Incipient Lethal Temperature were determined for bluethroat wrasse and greenback flounder. Greenback flounder exhibited remarkable thermal tolerance with a $TL_{50}$ (24hr) of 2.3°C (acclimation temperature 15°C).

A flow-through respirometer was used to measure oxygen consumption. Bluethroat wrasse exhibited 20% greater oxygen consumption than greenback flounder under normoxic conditions. In both species, a reduction in temperature of 10°C more than halved the oxygen consumption as predicted by typical biological $Q_{10}$ values.

Greenback flounder maintained or increased oxygen consumption during graded hypoxia (i.e. a non-conformer) and exhibited a critical oxygen tension of less than 25% oxygen in air-saturated seawater. Oxygen consumption of bluethroat wrasse decreased during graded hypoxia (i.e. a conformer) but no significant increase in blood lactate concentration was observed.

Lowered temperatures induced and maintained coma in horseshoe leatherjackets and bluethroat wrasse, however mean coma-inducing temperatures were generally <0.5°C higher than the $TL_{50}$. The induction of coma in bluethroat wrasse did not significantly reduce oxygen consumption beyond the level predicted by temperature alone and did not result in a significant increase in blood lactate. The risk of exceeding thermal tolerance when applying coma-inducing temperatures needs to be carefully assessed before use.

Feeding history had a marked impact on thermal tolerance. Greenback flounder deprived of food for 72hr exhibited greater than 90% survival when exposed to temperatures of 3°C whereas fish deprived of food for only 24hr exhibited less than 10% survival at the same low temperature. The magnitude of specific dynamic action (SDA) in greenback

flounder was 1.88 times higher than the routine rate of oxygen consumption and total duration was 52hr at 15°C.

Oxygen extraction by bluethroat wrasse was significantly reduced during lowered temperature and hypoxia. The ventilation rate and ventilation stroke volume of bluethroat wrasse generally decreased with lowered temperature and afferent oxygen tension.

The study demonstrated that food deprivation and temperature reduction are powerful tools to improve the survival of fish during transport.

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# INTRODUCTION



## OVERVIEW

As fish stocks continue to decline on a global scale there has been, and will be, an inevitable swing toward quality rather than quantity within the seafood industry in pursuit of the highest possible economic returns from the reduced level of harvest. Increases in the living standards within many countries that consume large quantities of seafood per capita have also contributed to the worldwide increase in demand for higher quality products.

Live products are considered to be the ultimate product within a seafood industry that is actively seeking higher quality and value-added products. Both the Australian domestic and worldwide demand for live fish is growing. However, development of techniques to economically supply and develop the live fish market has not kept pace with increasing demand. It will only be through dedicated research that scientific knowledge will be gained to assist industry to attempt to meet this void.

The major economic constraint within the live fish trade, especially within the Australian industry, is the fish to water ratios currently employed during the transport of live fish. Scientific investigation of the physiological responses of fish to various treatments could greatly assist the development of improved transport methods by reducing the amount of water required for a given weight of fish. In particular, the use of lowered water temperatures to reduce oxygen demand of poikilothermic fish is a focus for current and future research.

## VALUE ADDED PRODUCTION

The importance of live seafood to Australia's seafood industry has increased markedly over the last decade with an export value in excess of A$366 million in 2000-01

(ABARE 2002) with 11% of this value being derived from live fish and the remainder from live crustaceans. The high price markets of Hong Kong and Japan are the largest receivers of Australia's live seafood (McGilvray & Chan 2003). Other export markets (e.g. Korea and Taiwan) and the domestic market are also significant and have continued to expand rapidly in recent years.

Consumers are willing to pay a premium price for fish that are alive at the point of sale (Singh & Daud 2001). For example, the average sale price of live plaice, *Pleuronectes platessa*, (Midling *et al.* 1996) and bluethroat wrasse, *Notolabrus tetricus*, (Steffens 1995) is over three times the average price of the fresh chilled product. The sale price of live horseshoe leatherjacket, *Meuschenia hippocrepis*, is generally around six times that of the fresh chilled product (Findlay 1995) and Norwegian markets pay up to 100 times the price of fresh chilled herring, *Clupea harengus*, if the herring are sold alive or immediately after death (Midling *et al.* 1996).

Fish that are alive immediately prior to preparation and cooking are considered to be the superior raw material. Fish cooked immediately after death has a fresh taste (although the pre-death level of exhaustion and stress affects the precise qualities of such fish). At room temperature, if fish are not cooked within several minutes after death, lactic acid, adenosine diphosphate (ADP) and calcium ions ($Ca^{2+}$) begin to accumulate and rigor mortis sets in (Pitcher & Hart 1982, Gregory 1998).

Rigor is a linking of the actin and myosin molecules to form actomyosin and the rupture of some myocommata results (Pitcher & Hart 1982, Seki & Watanabe 1984, Montero & Borderias 1989, Ando *et al.* 1993, Gregory 1998, Erikson 1999, Torrissen *et al.* 1999). Fish cooked during rigor are rigid and tough. The continuing accumulation of $Ca^{2+}$ activates lysosomal enzymes that resolve rigor approximately one hour after death. Fish cooked at this stage taste fresh and tender and it is at this stage that most fishers aim to market their 'wet' product using ice to prolong the biochemical process. After this point, decomposition continues with further hydrolysis of protein (by both endogenous and bacterial enzymes), release of ammonia and increasing pH (Gregory 1998). Soon after, methylamines (usually trimethylamine) are released. It is these amine products that give fish its classically 'fishy' smell. Enzymatic degradation of methylamines leads to toughening of the flesh prior to the fish becoming unsuitable for consumption (Pitcher & Hart 1982).

In discussing the quality of wet fish, Pitcher & Hart (1982) stated that, "The key to good quality products is first-class raw materials. To obtain these it is necessary to have good knowledge of the handling and storage properties of fish backed up by good practice on board ship and in the factory." Their statement is certainly no less

prescriptive for live seafood products, however our knowledge of efficient and effective practices is limited.

## HISTORY

The practice of keeping and storing fish alive after capture from the wild originated as a means of maintaining freshness in the absence of refrigeration or other methods of preservation. Live fish have been held and traded in China for over 3000 years (Huss 1995) and presumably much of what we now know as aquaculture had its genesis in the holding of live, wild-caught fish.

The live fish industry can hardly be said to be a modern concept in value-adding. For at least the past 200 years, fishers have been catching, transporting, holding, and selling live fish in significant, commercial quantities. Traditionally, fish species supplied alive to European markets have included both pelagic species such as saithe, *Pollachius virens*, herring, sprat, *Sprattus sprattus,* common mackerel, *Scomber scombrus* and demersal species such as cod, *Gadus morhua* and plaice (Midling *et al.* 1996).

As early as 1850, Norwegian fishers stored live cod onboard their sailing vessels in wells flushed through perforations in the hull while under sail. These live cod were transferred to wooden boxes along the Norwegian coastline where they were held for extended periods. When weather permitted, the Norwegians would transport live cod to England where they would receive prices up to 100 times greater than that for salted cod (Midling *et al.* 1996).

In recent times the live fish industry has been experiencing a renaissance. There are now a large number of species sold live worldwide (Huss 1995). Currently, the Australian live seafood industry utilises at least 12 species of crustaceans, 10 species of finfish and several species of molluscs. The vast majority of the Australian industry is focussed on export, for the most part, to Asian markets. Within the Asian markets the Australian industry has a clean, green image (i.e. few, if any, chemicals are used to assist transport) (Franklin 1995).

The Australian domestic market is mainly centred in Brisbane, Sydney and Melbourne where Asian restaurants are concentrated. Many of these restaurants provide retail sales of fish selected from live holding tanks in addition to offering these fish for immediate preparation and consumption in the restaurant.

Considerable potential exists for expansion and development of the Australian live seafood industry. Development of this value-adding industry is consistent with

Australia's fisheries policy objectives of economically efficient, and ecologically sustainable, industry development.

## THE PROBLEM

Today, as in the past, the ability to economically capture, hold and transport live seafood is obviously fundamental to the profitable supply of live products to the consumer. However, the costs incurred during this process are significant and thus, any mechanism that can reduce these costs while maintaining supply can provide significant competitive advantages within the marketplace. Concomitantly, any reduction in the mortality of animals during capture, holding and transport will reduce the impact of fishing upon stocks through minimising any additional harvest to allow for this mortality.

It should also be noted that while efficient and cost-effective methods are obviously valuable for the industry as a whole, they are particularly crucial to the development of the low to medium value product sector because of the lower profit margins realised from this sector.

In addition to methodological difficulties, the transport of live fish raises significant and growing ethical concerns. To avoid potential criticisms, it is important that the industry employs humane techniques and implements a code of practice that deals with proper care and handling of live fish (Terchunian *et al.* 1999).

The ability to hold live products for extended periods has the potential to eliminate supply peaks and troughs that are a common and substantial problem affecting market supply and price, for many seafood products. Indeed, such holding would represent another latter day marriage of wild harvest and aquaculture.

The path from harvest to consumption of live seafood is comprised of several critical steps (Figure 1). When one considers that much of Australia's live fish industry is focussed on export markets many thousands of kilometres away, the challenges to the successful and economic supply of these markets are all the greater. It should be noted that the economic impacts of mortality during this process are proportionally greater as one moves closer toward the point of consumer purchase.

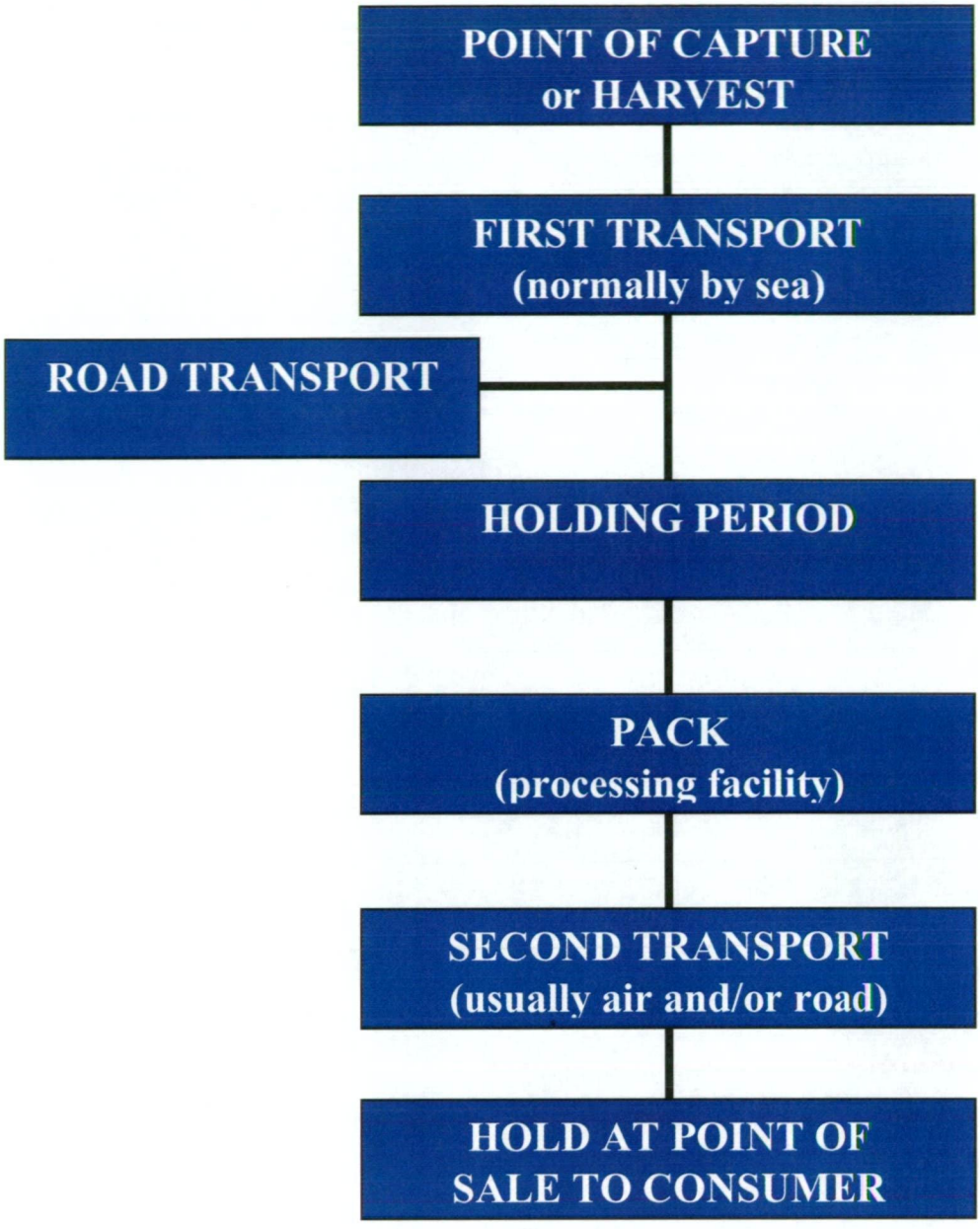



**Figure 1 Generalised pathway for supply of live fish to consumers**

The protocols developed by industry to maintain fish alive during capture, holding and transport through to the point of sale to the consumer are largely the result of haphazard, *in situ* trials. This has led to highly variable and often poor outcomes. These results reflect the genesis of the methods currently in use. It is generally perceived by Australian industry and researchers that impacts on supply (including various sources of mortality) and concurrent logistical problems, in particular transportation, are the principle constraining factors in the realisation of the full commercial potential of live seafood (Figure 2). Considerable potential exists for elucidation of cost-effective methods that greatly reduce these constraints.

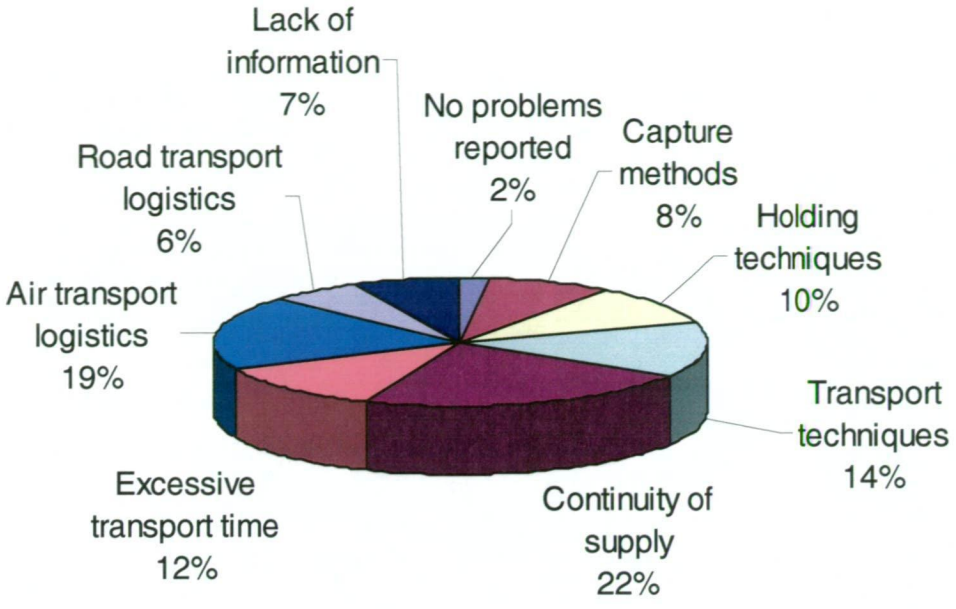



**Figure 2 Problems reported in the Australian live seafood industry adapted from Anon. (1995a,b)**

As seen in Figure 2, capture and holding methods each account for around 10% of the problems reported by industry with transport techniques and logistics accounting for over 70% of the problems with survival and product quality.

## SPECIFIC AIMS

As a reflection of the relative magnitude of the problems faced by industry and the potential for scientific understanding to address some of these problems, this study reviewed current capture, holding and transport methods used in live fish supply and explored the utility of lowered temperatures during transport as a means to reduce respiratory oxygen demand and hence improve survival rates and/or reduce transport costs/constraints.

The specific aims of this study were to:

1) review current and historical methods of capture, holding and transport for supply of live fish;
2) develop appropriate techniques in the context of live fish transport and subsequently describe the thermal tolerance of several species of temperate, marine fish;
3) describe the physiological and behavioural responses of several fish species to experimentally reduced water temperatures and oxygen tension including description of the effects of body size, sex and feeding history on ventilation, oxygen extraction, oxygen consumption, blood lactate and survival; and
4) assess the merits of lowered water temperature during transport as part of the live fish supply chain.

# CHAPTER 2

# REVIEW OF CAPTURE, HOLDING AND TRANSPORT METHODS



## INTRODUCTION

The production of live fish can be broken down into three main steps - capture, holding and transport. Capture, handling, holding and transport have all been shown to have the potential to markedly increase the levels of physiological stress indicators and/or result in mortality of fish (Flos *et al.* 1988, Foo & Lam 1993, Schreck *et al.* 1989, Waring *et al.* 1992, Jeon *et al.* 2000a,b). Thus, methods and equipment used for the capture, holding and transport of live fish must seek to minimise stress and physical damage to promote survival.

Dedicated research into the methods of live fish supply has often delivered remarkable advances to industry. For example, in 1939, the Norwegian Live Fish Sales Organisation was founded and was soon actively participating in formal research and development through scientific projects (Sundes 1957b). Development of new methods through both dedicated research and trial and error resulted in considerable improvements in efficiencies. Soon after World War II, researchers defined successful techniques allowing fish to water ratios of 1:1 for up to 18 hours.

This allowed live fish to be transported long distances across inland Europe at a greatly reduced cost (Midling *et al.* 1996).

As a modern example of the success of dedicated research into live fish protocols Rimmer (1995) observed zero mortality of barramundi, *Lates calcarifer*, during 14 hours of transport in 1:2 fish to water ratio. At that time ratios of about 1:4 were standard for this species. Obviously zero mortality could not be expected in commercial settings but such improvements demonstrate the considerable commercial gains that can be realised through dedicated scientific research. These changes in the fish:water ratio reported by Rimmer (1995) represent a 40% reduction in the overall weight/volume of any shipment (for the same yield of product at destination) and this means a 40% reduction in the transport cost. As further evidence of the advances that have been made in live fish transport, new systems designed for high-density transport of plaice have raised capacities from 1:19 (fish:water) to an amazing 3:1 (Midling *et al.* 1996).

In light of these and other advances a review of the current practices and techniques used in live fish supply is timely. Each of the steps (capture, holding and transport), will be discussed in detail later in this chapter, but it should be noted that 'handling' of fish is a necessary part of the production process.

#### *Handling*

Handling methods can greatly affect product quality and survival of fish (Solomon & Hawkins 1981, de Guingand 1994, 1995, Midling *et al.* 1996). For example, rough gloves or coarse netting on 'dip' nets used during handling can cause physical damage leading to mortality and/or a reduction in value. The live fish industry can borrow heavily from accepted aquaculture practice with respect to handling procedures. Broadly speaking, efficient and careful handling is possibly more crucial in the live fish industry than in many aquaculture processes due to the time-critical nature of the process and the virtual absence of time for recovery from stress and physical damage. At every stage of production, potential exists for improved handling methods to reduce stress and physical damage while concomitantly increasing survival. Fishers and processors used to dealing with 'wet' or frozen product need to be educated in effective live fish handling practices. In particular, it is very important that fishers and processors minimise the amount of air exposure to reduce drying and compromised gas exchange (Rimmer *et al.* 1994). Even relatively short periods of aerial exposure have been observed to result in considerable increases in the levels of various stress indicators (Waring *et al.* 1996, Barton *et al.* 1998, Davis *et al.* 2001).

A literal 'hands-on' approach to handling is to be avoided wherever possible. This is because the force generally needed to subdue fish that have not been anaesthetised greatly increases the risk of physical damage. Moreover, it is vital that fishers and processors do not insert fingers under the operculum and into the gills or gill rakers of fish nor place fingers into the nares or eye sockets. Similarly, the lifting of fish by only the lower jaw has been reported by aquaculturists and fishers to cause considerable damage to fish including internal bleeding and spinal damage in some species.

## CAPTURE METHODS

A full review of the advantages/disadvantages, modifications and potential improvements of fishing gears for use in the live fish industry is beyond the scope of this thesis. However, a summary of past and current fishing methods used in the supply of live fish and some conclusions on their relative merits are provided below.

Capture methods used in the supply of live fish greatly affect survival and product quality (Rimmer *et al.* 1994, Midling *et al.* 1996). Many authors have concluded that capture is the major contributor to stress indicator levels during the live fish supply chain (Miles *et al.* 1974, Kreiberg & Powell 1991, Pottinger 1998) and the effects of various stressors differ and that stressors act in cumulative manner in a wide number of species (Barton *et al.* 1986, Faulkner & Moberg 1997, Mugnier *et al.* 1998, Vijayan & Leatherland 1988).

However, Davis *et al.* (2001) also observed that, in sablefish, *Anoplopoma fimbria*, the major contributor to the level of stress indicators was capture but that an additional stressor (e.g. increased post-capture water temperature during holding) resulted in significantly increased mortality without a significant effect on the levels of a range of stress indicators. Sharpe *et al.* (1998) observed that a wide range of different handling and laboratory procedures all resulted in remarkably similar levels across a range of stress indicators in juvenile chinook salmon in a hatchery when each stressor was applied in isolation. Therefore, caution must be exercised when assuming that capture is the major stressor and when implying survival probabilities from physiological indicators of stress. It is likely that stress indicators do not adequately describe actual physiological stress when they are approaching maximum observed values (i.e. the indicator level becomes asymptotic despite subsequent additional stressors that may lead ultimately to mortality). Moreover, a review of the literature clearly shows that it is common for various stress indicators to respond differently to a given series of graded or different stressors and therefore the predictive capacity of any one indicator is questionable.

Farrell *et al.* (2000) compared the levels of physiological stress indicators and metabolic exhaustion of coho salmon, *Oncorhynchus kisutch*, captured using purse seine, gill net and trolling methods. Very small differences were observed in the levels of stress indicators and all fish exhibited severe metabolic exhaustion. Unfortunately the authors did not compare physical damage or mortality rates across these methods. However, from these data it might be concluded that the method of capture would not greatly affect the suitability of any of these methods for supply of live fish however such a conclusion is contradicted by the observations of dedicated live fish suppliers and researchers. Such findings also call into question the applicability of common physiological indicators of stress with respect to live fish supply if not used in conjunction with mortality estimates and/or other indicators (e.g. physical damage, post-capture behaviour).

Similarly, the feeding history of rainbow trout, *Oncorhynchus mykiss*, has been shown to have a marked impact on the levels of some stress indicators (in particular blood glucose and glycogen) when fish were exposed to a standardised stressor (Vijayan & Moon 1992). The differential effect of feeding history on blood glucose and glycogen levels was also observed in juvenile striped bass in response to handling stress (Reubush & Heath 1997a) but in not adult striped bass (Reubush & Heath 1997b). Thus, caution should be exercised when undertaking comparisons of some stress indicators during live supply procedures to ensure that feeding history, age or other factors do not confound the results.

Current capture methods used to harvest fish for live sale are generally the same techniques and gear used to harvest fish for a wet fish or frozen market. This situation is understandable when one considers that the bulk of the current live fish industry had its genesis as a value-adding mechanism within established fisheries. Modifications and changes to fishing methods offer considerable potential for improvement in the quality of fish harvested for the live fish trade.

For example, in 1988 the Barents Sea cod fishery collapsed. Following this collapse, quotas were introduced representing a reduction of up to 85% of previous annual catch for some fishermen. In response, fishermen made considerable efforts to improve the sale price of their catch. The value-adding method chosen by most fishers was live capture and on-growing of cod. The gear used during this process was the Danish seine. Vessels using Danish seine had always delivered cod of premium quality for the 'wet' and salted cod market but its use in the capture of fish for the live fish market caused several problems (Midling *et al.* 1996).

Unlike the live cod production of the past using 8-10m vessels equipped with traps or line gear to take a few hundred kilograms of catch, these Danish seiners were 25-

30m in length and typical catches were 10-15t per trip. Unlike the trap or line fishers the seiners experienced significant mortalities at every stage - capture, transportation and in holding pens. Mean overall mortality was estimated at more than 50% (dead and dying cod were removed, quality graded and processed for human consumption). While these considerable mortalities did not represent a complete loss, the price the fishers could get for landed live product was at least double that of dead cod and this difference in value was sufficient for fishers to look closely at capture techniques to improve survival. The changes made by this, and other fleets, to improve survival of live fish and take advantage of the higher prices pad for live fish are discussed below.

Before proceeding with a presentation of the more common methods of capture for live fish supply and specific changes that have been implemented to improve efficiency, it should be noted that the high value-adding nature of the live fish trade poses special challenges for fisheries managers. Under the output controls on fishing (e.g. total allowable catch limits and individually transferable quotas) increasingly used to manage fisheries over recent years, there is an obvious incentive for non-reporting of any discarded fish. Given the particular economic advantage of 'high grading' in the case of live fish harvesting, the incentive for non-reporting is very high. For example, if fishers using a particular type of gear can catch fish quickly but only a proportion of these fish are suitable for live transport then there is an obvious incentive to dump the rest of the catch, some or all of which may be dead or moribund. Therefore, it is important that effective quota decrementation and compliance procedures are in place to ensure that the total take of the fishers is managed not just landings at the wharf. In this way, live fish production can concomitantly provide both enhanced economic and conservation advantages and fishers are encouraged to fish by methods that ensure a higher survival rate in the catch.

### *Gear types*

#### *Trawl (e.g. beam, otter, and pair)*

Trawling is a very common fishing method throughout the industrial world. Trawling describes the pulling of a net through the water by a boat(s). The exact details of the net and method vary widely across different species and environments but the basic principle is that the net 'strains' or 'sieves' the water mass ultimately catching fish contained therein. The catch collects toward the closed end ('codend') of the net and the net is then retrieved from the water along with the catch. Trawling is able to catch large quantities of fish relatively quickly. The amount of fish caught in a single 'shot' can be in the order of tens or even hundreds of tonnes.

While obviously dependent upon the species and environment, the efficiency of capturing fish by trawl is generally high but the method has the potential to impact on several key features of successful live fish production and the utility of trawling for supply of live fish is questionable.

To be successful, the trawl speed of the net must be faster than the sustained swimming speed of the target species but not necessarily the burst speed. Many fish species will only become trapped in the codend once they are exhausted from swimming to escape the confines of the trawl. Thus, many fish caught by trawl nets are exhausted prior to retrieval and are therefore less likely to survive during the live fish supply process. This conclusion is supported by the observations of Olla *et al.* (1997) who observed that the speed and duration of trawling altered the levels of stress indicators and stress induced mortality rates of juvenile walleye pollock, *Theragra chalcogramma* and adult sablefish.

In addition, Turunen *et al.* (1996) observed that trawling caused rapid exhaustion of vendace, *Coregonus albula*. These authors observed that fish with high levels of physiological stress indicators after capture by trawling had a higher mortality rate post-capture than individuals whose indicators were at lower levels. However, vendace captured using purse seine that had similarly high stress indicator levels did not have high mortality rates. The authors concluded that the higher mortality rates observed for trawled vendace must be due to a combination of physiological and physical factors in particular suppression of ventilation.

As fish accumulate in the codend, weight and water pressure can combine to cause physical damage to individuals. In addition, this pressure can prevent opercular movements and hence ventilation. These problems can occur both during trawling and the lifting or retrieval of the catch. In addition to physical damage through compression against other individuals, the codend of most fish trawl nets is usually made from braided or twisted polyamid and/or polyethylene knotted to form the net lattice. While the actual materials are fairly lenient, it is the knotted construction, which can cause considerable damage to fish during both the actual trawls and during lifting.

These problems affect the appearance, flesh quality and survival of fish caught using trawl gear. The impact of these problems can be mitigated through some modifications such as reducing the length of time the net is towed during each shot.

Various fishers have recognised the difficulties when trawling for live fish supply and attempted to modify their methods to improve quality and survival. For example, Norwegian cod trawlers approximately halved their codend-lifting bag

from around 600kg to around 300kg when fishing to supply live cod to market. They also lined the lifting bag with canvas to further reduce the pressure on the fish. These modifications reduced both the immediate mortality rate and the number of 'floaters' (i.e. fish that have lost equilibrium and float belly up at the surface due to accumulated gas in the gut cavity) (Isaken & Midling 1995). Codend fish pumps may assist in the retrieval of trawled catches to supply fish to market alive by reducing physical pressure, air exposure and handling damage.

Oddson *et al.* (1994) observed that trawl duration had a considerable impact on the survival rate of Pacific halibut, *Hippoglossus stenolepis*. An increase of trawl duration from 30 to 120 min. reduced the survival rate from 80% to 55%. The increase in trawl duration had a similar impact on some physiological stress indicators but not all of those examined.

Muenker *et al.* (2000) compared the life status of a wide range of species at time of landing following 70 trawling operations in the North and Baltic Seas. Trawl duration varied from 30min. to 6hr. Trawl depth varied from 15 to 250m. Total weight of catch per haul varied from 100 to 3500kg. Different species exhibited markedly different mortality rates however demersal species were, in general, far more robust to the impacts of trawl capture than pelagic species. Overall the mortality rate in the catch increased with increasing depth of trawl, trawl duration and size of catch.

Due to the problems discussed above trawling is not well suited to the supply of live fish. At best, the supply of live product captured using the trawl method could be considered as an adjunct to fishing operations to supply wet or frozen product.

*Line (e.g. hand, pole, longline, dropline, trolling)*

The capture of fish using a hook or toggle attached to a line is an ancient fishing technique that has proved to be extremely versatile in its application across many species and environments. In general, fish are attracted to bite on a hook through various means at which time the hook penetrates the fish and the fish is retrieved using the attached line. The exact nature of the gear varies widely from a single hook on a metre or two of line to pelagic longlines in the order of one hundred kilometres in length with as many as 4000 hooks attached.

Line fishing is generally labour intensive and does not return high quantities of fish per unit time or labour relative to some other methods such as trawling and seining. It does however produce very high quality fish because of the minimal physical damage relative to other capture methods. The level of exhaustion during capture is determined by the duration and intensity of struggling once hooked. In general, the

duration is relatively short (unlike sportfishing, commercial fishermen generally retrieve their catch as quickly as possible once hooked). For example, the time from hooking to landing in a tuna poling operation can be a matter of seconds. In some cases, line fishing is more passive and fish can be hooked for an extended period before retrieval (e.g. longlining).

Paust and Svennson (1986) observed that the proportion of rockfish, *Sebastes alutus*, alive at the time of retrieval of a longline significantly decreased when soak time was increased from two hours to four hours. A significant increase in the mortality of black marlin, *Makaira indica*, with increasing soak time of pelagic longlines has also been observed (Campbell *et al.* 1996a,b). Therefore, it would appear that the fishers using line fishing to capture fish for supply alive to market should seek to avoid long periods prior to retrieval of the catch.

Fish hooks can cause significant physical damage especially when they lodge in soft tissues (e.g. gut) or gills of fish. For example, hook damage to gills will generally render a fish unsuitable for live transport due to bleeding and reduced gas exchange efficiency. Line fishing methods and hook design can greatly affect the frequency of 'deep' or 'gut' hooking. There is a range of possible gear and technique modifications that can reduce hook damage. This includes the use of circle, barbless and conical pointed hooks.

Due to the labour intensive nature of line fishing, its application as an economical fishing method is necessarily reduced. However line fishing is particularly suited to capture of fish for live supply when the species, habitat and profit margins allow it to be applied. For example, in northern Australian waters, coral trout, *Plectropomus* spp., and other reef species are caught on heavy monofilament hand-held lines. Fishers work over reefs from small boats associated with a mothership. The small scale of each individual's operation and high value product (up to A$300/kg) allow great control over the exact method of harvest, holding and transport while maintaining an acceptable economic efficiency.

*Trap (including funnel/fyke nets, pots, stownets, veranda nets)*

Trapping of fish is probably one of the earliest methods of fishing. Fish are either attracted to or inadvertently drawn into the apparatus that ultimately prevents, or makes difficult, their escape. Trap apparatus vary greatly in size and exact operation. For example, some of the Mediterranean funnel traps used to capture northern bluefin tuna, *Thunnus thynnus*, are over one hundred kilometers long and use no 'bait' to attract the fish at all. At the other extreme small baskets and hand basins are baited with bread or dough and used to catch mullet, *Mugil cephalus*, in South East

Asia. Like line fishing, trap fishing is very versatile in its application to differing species and environments.

Fish can be attracted to enter a trap through use of bait or even other fish of the same species (e.g. females to attract males). Other types of traps use migration/movement of fish to direct fish into the trap using physical barriers. Traps offer considerable advantages for the capture of fish for live supply. Traps generally cause little physical damage provided due attention is given to the construction material. Fish in traps generally do not struggle to escape until the trap is retrieved or harvesting is initiated and are therefore in prime condition for live holding and transport. A study by Brown *et al.* (1998) showed that stress indicators in lake whitefish, *Coregonus clupeaformis*, and white sucker, *Catostomus commersoni*, captured in traps were considerably lower than their counterparts taken by gill net supports this observation. Indeed, these workers stated, "Except for plasma cortisol concentrations, white sucker appeared unaffected by trapnetting".

One problem with the use of food to attract fish into traps is that fish may be able to consume significant quantities of food once they enter the trap. This results in increased post-prandial oxygen demand and reduced water quality due to excretion during holding and transport after capture. This issue is discussed in more detail in Chapter 5.

In Australia, traps have been used to capture many different species including bream, *Acanthropagrus australis* and *A. butcheri*, luderick, *Girella tricuspidata*, eel, *Anguilla anguilla*, ocean leatherjackets, *Nelusetta ayraudi*, yellowtail kingfish, *Seriola lalandi*, snapper, *Pagrus auratus*, and dolphinfish, *Coryphaena hippurus*, on a commercial scale. The techniques used in these fisheries are immediately applicable to live fish supply with only minor modification. Surprisingly, trapping is not widely practised for the supply of live fish at present.

*Encircling/Surrounding Nets (including purse seine, beach seine, cast net, lift nets)*

Surrounding nets have been, and continue to be, used widely throughout the world to catch fish. Their design and operation varies with environment and species but broadly speaking their successful application requires fish to be aggregated in a discrete area around which the net is set thereby confining the fish within the encircled net. The net is then drawn or lifted to reduce the volume of water contained until the fish are densely confined and ultimately harvested.

This confinement and harvesting process introduces panic and crowding problems along the same lines as that experienced by trawled fish, albeit to a lesser extent. Another problem that is more critical is the potential for reduced levels of dissolved

oxygen within the tightly packed fish in the confines of the net. Following large purse seine or beach seine sets, fish may be held within the net for many hours while harvesting occurs. Some types of encircling net (e.g. cast net and lift nets) involve very short periods of confinement prior to landing.

Encircling nets were the capture methods used in some of the earliest large-scale, industrial live fish production. In Norway, as early as the 19th century, herring, sprat and mackerel were captured and held alive using beach seines and around the start of the twentieth century purse seining was introduced and continues to be the method of choice among Norwegian fishers for the capture of species that are supplied live to the market (Midling *et al.* 1996).

*Entangling Net (including gill net, drift net, trammel, ring net, entangling seine)*

Entangling nets are used world wide to target many species. While the exact design and method vary considerably, the principle behind such gear is that fish become entangled after contact. While such nets can be highly efficient, their application as a capture method for use in live fish supply requires considerable modification over traditional methods.

de Guingand (1995) noted that considerable mortalities occurred prior to and after retrieval of fish caught in gill nets. That author noted that gill net fishers often had 'soak' times (i.e. the length of time between setting and retrieval of the net) of around 12hr. At time of retrieval it was observed that many fish were very lethargic and that the opercula were often prohibited, or severely restricted, from movement whilst entangled in the net. As a result, it was suggested that ventilation (de Guingand (1995) used the term *respiration* but that term is applied to a different process in this thesis) would be restricted. The author also noted considerable physical damage to fish caught in gill nets including to the gills.

Farrell *et al.* (2000) observed that the levels of physiological stress indicators and metabolic exhaustion of coho salmon significantly increased when soak time of gill nets increased. From these observations it is apparent that some modifications, such as a reduction in soak time and careful selection of netting material especially its diameter, can improve the survival of fish captured by entangling nets.

It must be noted that entangling nets, almost by definition, demand considerable handling of fish to remove them from the net. The cutting of fish out of the net rather than untangling offers considerable advantages in reducing handling stress (Rimmer *et al.* 1994) but obviously incurs damage to the net.

Despite their shortcomings, entangling nets are commonly used in the capture of fish for live supply. Around Tasmania, Australia, gill nets are used extensively to capture banded morwong and bluethroat wrasse. In Norway, live plaice are traditionally supplied from gill netting operations (Midling *et al.* 1996).

*Chemicals*

The use of chemicals as part of fishing operations has a very long history especially within Australia. Aboriginal tribes have used various naturally occurring plant extracts for many thousands of years to debilitate and capture fish. These chemicals act by either removing oxygen from the water or by direct effect on the fish's normal metabolic processes.

In modern times, chemicals such as cyanide and rotenone have been used to capture fish, in particular, fish supplied to the aquarium trade. Rotenone (the active ingredient of the pesticide commonly known as Derris dust) acts to inhibit electron transfer within the NADH-Q reductase complex thereby inhibiting ATP production (Stryer 1988) and thus, respiration. Cyanide reacts with the ferric form of heme $a_3$ to block electron flow in cytochrome oxidase (Stryer 1988) and, like rotenone, blocks ATP production. Anaesthetics are also used to capture fish. Their action is discussed in detail later in this chapter.

Such fishing methods are generally extremely destructive because of their non-discriminatory nature on all organisms that rely on gills for gas exchange. The other difficulties with these methods include obvious food safety issues and the fact that while many fish appear to be alive at time of entry to the market, they are moribund as a result of exposure to these chemicals and soon die especially when exposed to further stress during transport.

*Electrofishing*

Electrofishing utilises direct, alternating or pulsed currents to 'stun' fish by causing tetanic spasms in muscles. Electrofishing is not widely practiced on a commercial basis generally because of its relatively limited efficiency and human safety issues.

Smith *et.al.* (1994) observed that electrofishing had no significant effect on the long-term survival of chinook salmon, *Oncorhynchus tshawytscha*, during migration upriver relative to controls that had been captured at the same site using barrier nets as a form of fish trap. Mitton & McDonald (1994) observed that typical electrofishing techniques using pulsed direct current (pDC) resulted in increases in blood and muscle lactate concentrations but had no immediate effect on mortality rate of rainbow trout. The authors observed some skeletal damage when fish of less than 600g bodyweight were shocked with pDC at the extreme high end of the range used (600V, 40s).

It would appear that electrofishing is a technique that may offer significant advantages for the capture of fish to supply the live fish market. Electrofishing generally results in no long lasting physical damage and no external damage to reduce product presentation. The stunned fish are easily handled for a short period immediately post-capture without the need for chemical anaesthesia. Fish recover relatively quickly (especially when using pulsed and direct current) and thus stress and exhaustion are minimised. However, only direct current produces a state of electronarcosis or galvanonarcosis (i.e. muscle relaxation) (Ross & Ross 1984).

While electrofishing is not widely used in the marine environment, there are several references that describe the use of electrofishing in the marine environment to stun and capture fish and crustaceans (e.g. Phillips & Scolaro 1980, Halsband & Halsband 1984, Reynolds 1996). Effective electrofishing in the marine environment requires changes to the apparatus and currents applied and these added costs limit its use in the marine environment however future technological advances may see greater scope for its use in the collection of fish to be sold alive.

In summary, with a few exceptions, large or industrial scale capture methods such as trawling and seining are not particularly suited to the supply of live fish to market and the application of entangling nets requires significant modifications to be successful. Fishing by line and trap are the capture techniques most suited to use in operations exclusively supplying a live fish market (Rimmer *et al.* 1994). However, it must be noted that not all species or habitats are suited to capture by such methods.

One factor, which must be considered irrespective of the particular fishing method, is the effects of rapid pressure changes on the survival of captured fish. The impacts of these pressure changes can be severe and are commonly referred to as 'barotrauma'.

***Barotrauma***

Rimmer *et al.* (1994) and de Guingand (1995) defined barotrauma simply as "overinflation of the swimbladder", and its cause as, "rapid ascent through the water column in the process of capture". Barotrauma imposes considerable stress on various organs. In extreme cases, the swim bladder and ultimately the peritoneum will burst. In less severe cases, symptoms include abnormal protrusion of the eyes, oesophageal and swim bladder protrusion from the mouth and/or anus (Plate 1). It must be noted that barotrauma affects all fish including those species without a swimbladder and those that have a swim bladder connected to the oesophagus by a *ductus pneumaticus*. Albeit, such species do not exhibit the extreme overt symptoms of fish with a closed swim bladder however exopthalmia remains an obvious indicator of trauma in all species. Thus, while the effects of barotrauma vary

between species this condition is relatively common where fish are harvested from water greater than a few metres in depth.

In species with a physoclistous swim bladder, the production and absorption of gas in the swim bladder are both relatively slow processes (Tytler & Blaxter 1973, Sand & Hawkins 1974). Considering that water pressure decreases by approximately one atmosphere for every ten metre decrease in depth, during capture, fish can be moved through this extreme pressure gradient much faster than the gas exchange mechanisms of most species can resolve the expansion of the gas in the swim bladder. The decrease in pressure causes the volume of the swim bladder to increase markedly. The relationship between volume (V) and pressure (P) under changing conditions is described by the equation:

$$V_1P_1=V_2P_2.$$

In addition to increasing internal pressure due to expanding gases, fish being hauled through, and lifted from, the water (e.g. in trawl or seine nets) would have to contend with the added strain from dynamic water pressure and weight of other fish in the catch.

**Plate 1. Bluethroat wrasse showing signs of barotrauma. Gas-filled anal protrusion is clearly visible.**

As water pressure decreases, the expanding swim bladder displaces other internal organs and may eventually burst releasing the gas into the gut cavity. For example, the swim bladders of cod have been found to burst when the surrounding pressure is reduced by 60% during capture (Midling *et al.* 1996). If the peritoneum bursts during capture the gas is released and the gut cavity and swim bladder will flood with water. The result is a fish that is negatively buoyant.

By categorically ranking the severity of barotrauma in harvested fishes, de Guingand (1995) and Rimmer & Franklin (1997) observed the significant effect it had on mortality. The significance of barotrauma and the resultant considerable mortality may well prohibit the supply of deep-water caught fish to the live fish market. de Guingand (1995) correctly questioned the use of deep-water harvest of fish in the live fish market because of barotrauma problems.

Fishers have developed a number of techniques to improve the survival of fish exposed to barotrauma including the puncturing of the gut cavity and swim bladder to aspirate accumulated gas (Rimmer *et al.* 1994, de Guingand 1995, Midling *et al.* 1996, Rimmer & Franklin 1997). de Guingand (1995) reported that in most trials, the survival of banded morwong, *Cheilodactylus spectabilis*, improved by only 10-30% when the swim bladder was aspirated. Swim bladder deflation using a hypodermic syringe to pierce the swim bladder had no significant effect on mortality of red snapper, *Lutjanus campechanus*, during either short-term (24-48hr) or longer term (30-40 days) holding after capture by hook and line from water depths greater than 20m (Render & Wilson 1996). Regardless of whether or not the swim bladder was deflated, the mortality of red snapper increased with increasing water depth. Similarly, Rimmer & Franklin (1997) observed only slight increases in survival of coral trout and no improvement in bluethroat wrasse when the swim bladders of barotrauma-affected fish where aspirated using hypodermic needle.

The absence of any clear benefit from aspiration of the swim bladder may be due to the fact that many fish exhibit ruptured coeliac arteries and significant blood loss following barotrauma. Furthermore, this puncturing process necessarily involves considerable handling and the actual puncture wound provides a point of entry for opportunistic pathogens. Poorly practised, this technique could ultimately kill as many fish as it saves. The efficacy of this labour intensive practice is therefore questionable. Decompression, where possible, may offer a more effective solution however severely affected fish rarely survive irrespective of treatment (Rimmer *et al.* 1994).

***Aquaculture***

Aquaculture has a number of distinct advantages with respect to the successful supply of live fish (Bremmer *et al.* 1996). For a variety of reasons the harvest of fish in an aquaculture setting is generally more flexible and less problematic in its method. As there is often a need in aquaculture to carefully capture fish alive outside harvest periods (e.g. for grading), techniques for high quality live harvests are generally well established. Of concern to the successful production of aquaculture fish for the live fish market is obvious fin erosion and the greater incidence of abnormal phenotypic expression. As the live fish trade often sees consumers selecting and purchasing live fish, it may be necessary to conduct thorough inspection and grading of cultured products in order to remove fish with aesthetically displeasing physical conditions including abnormalities.

Included in the benefits of aquaculture are advantages such as greater control of acclimation temperature and feeding history. Aquaculture also provides the

possibility for selective breeding to produce fish that are more tolerant of the stressors experienced during live transport (Pottinger & Pickering 1997).

In addition, repeated exposure to a particular stress event (e.g. handling) over extended periods reduces the magnitude of the stress indicator response relative to that of fish that have not been previously exposed (Barry *et al.* 1996). Thus, the routine husbandry procedures of aquaculture can condition fish to the types of stressors likely to be experienced during capture, packaging, transport and later holding at the point of sale.

## HOLDING METHODS

For the purposes of this thesis, holding can be defined as the maintenance of fish post-capture that does not include the movement of fish from one place to another. For example, net pens into which captured fish are placed are an example of 'holding'. As stated in Chapter 1, post-capture 'holding' of fish onboard vessels can be considered a type of transport given the greater similarities with what is conventionally referred to as transport (e.g. higher densities due to similar constraints on space and weight) than holding as defined above.

However, one operation that blurs this distinction is the Japanese practice of breeding, growing or holding fish in converted bulk carriers and tankers. These vessels are often moored in sheltered bays but can move their live cargo significant distances to improve water parameters (e.g. temperature) and travel to markets. Another aberration is the development of towable net pens that serve the dual purpose of transport and holding of live Pacific herring (Kreiberg & Solmie 1987).

Holding allows recovery from the acute stressors experienced during capture and/or transport. Generally, the process of capture results in extended periods of elevated levels of stress indicators well beyond the capture episode (Wells 1987). Peaks in physiological stress indicators have most often been observed to occur well after the capture event (i.e. stress indicators often peak several hours after capture) (Davidson *et al.* 1997, DeAlteris & Valley 1999). The time taken for stress indicators to recover to basal or 'pre-capture' levels has been observed to generally take between 1-6 days across a wide range of indicators, species and capture/holding methods (Oddson *et al.* 1994, Turunen *et al.* 1996, Cubero & Molinero 1997, Pottinger 1998). Obviously, packing and transporting fish at high densities immediately after capture is likely to result in much higher mortality rates than if the fish were allowed to adequately recover from the capture event before transport.

Holding as a part of the supply chain can serve a number of other purposes. These include:

- collection of sufficient suitable specimens for economic shipment;
- production levelling;
- continuity of supply;
- weight increase/growout;
- grading;
- quality assurance;
- depuration and gastric evacuation;
- collection of eggs; and
- preparation of fish for transport.

The difficulties faced by operators during holding periods are generally less acute than those during other stages of production. In many respects holding is very similar to the main stages of aquacultural production and shares many of the same challenges. These include, *inter alia*, the need to mitigate the effects of aggression, cannibalism, predation, physical damage, stress and disease. It should be noted that unlike many aquacultured fish, wild caught fish held alive:

- are not 'domesticated';
- often exhibit high rates of excretion and oxygen consumption immediately following capture and/or transport; and
- may not resume feeding readily or at all during holding.

As for methods and equipment used in the capture, handling and transport stages, holding methods and equipment should be designed to minimise stress and physical damage. As the holding duration may be considerably longer than that experienced during transport, it is more important that due attention be given to more chronic stressors such as osmotic and toxic stressors.

Holding equipment and methods can be broken into two main forms, near-shore and onshore.

#### *Near-shore*

Near-shore operations generally involve net pens, yards, cages or coffs held in sheltered bays, harbours or rivers in which fish are placed following capture. Cages can be very simple. For example, woven palm baskets are used to hold fish in rivers in China (Huss 1995). The exact detail of the design of the structures and materials used has been shown to have a significant effect on the level of physical damage and survival of fish (Rimmer *et al.* 1994, de Guingand 1995, Farrell *et al.* 2000).

Fish may be held in the apparatus used for capture and/or transport. For example, in the 1800s, herring, sprat and mackerel were traditionally caught in beach seines and the seine was then staked to form a holding pen within which the catch was held for several weeks before being harvested. Soon after the end of the World War II,

Norwegian fishermen began towing net pens to the fishing grounds and transferring fish caught there to these pens before towing them back to shore (Midling *et al.* 1996). This method is now relatively common throughout the world for many species including saithe, herring, sprat, mackerel, northern bluefin tuna, yellowfin tuna, *Thunnus albacares* and southern bluefin tuna, *Thunnus maccoyii*.

Just as live fish holding methods have borrowed heavily from techniques used in aquaculture, researchers endeavouring to address problems in live fish holding have developed new aquaculture techniques and equipment. One example involves the development of new cod holding cages in response to considerable mortalities in cod immediately after initial transfer to net cages following capture and transport. The newly arrived cod would aggregate at the bottom of the net to the point where their weight would often cause the net to be pulled down several metres. Divers described these fish as "blunt" (i.e. they did not respond overtly to external stimuli) (Midling *et al.* 1996).

Midling *et al.* (1996) observed that mean dissolved oxygen levels within this aggregation were 6.3 ml $O_2.l^{-1}$, far in excess of the lethal levels of 0.8 ml $O_2.l^{-1}$ (Sundes 1957). It was therefore proposed that the cause of the mortalities was suffocation due to the weight of other cod preventing ventilatory movements. In order to deal with this problem, new net pens were built which had a rigid perforated floor through which a pump pushed water to both increase flow and suspend the cod. This modification greatly reduced post-transport mortality and after two days the cod were then transferred to a traditional pen without further problems of this nature (Midling & Isaken 1995). These technologies have since been transferred to other species and are now being used as the basis for construction of cages for ongrowing purpose for species like halibut, *Hippoglossus hippoglossus* and wolf-fish, *Anarhichas minor* and *A. lupus* (Midling *et al.* 1996).

#### *Onshore*

Onshore systems generally consist of some form of tank. Water is provided on either a flow-through or recirculating basis. Generally tanks offer much greater control over water quality parameters such as temperature, oxygen levels, suspended matter and toxic waste products than near-shore systems (Huss 1995). In the case of recirculating systems, several authors warn against shock loading of biological filtration systems (e.g. Rimmer *et al.* 1994, Steffens 1995). Shock loading of bio-filters is a common problem in live transport holding because fish are often received in large shipments following an absence of fish from the holding system.

The other main difficulty with onshore systems is thermal stress due to temperature differences between the holding system and the ambient water from which the fish

were caught or in which they were transported. Some holding systems incorporate temperature control units that allow operators to adjust the water temperature to minimise thermal shock. Such systems also allow *in situ* chilling of fish prior to packaging and transport.

Feeding may or may not occur during holding. Feeding is often not considered in cases where fish are held for relatively short periods (e.g. from a few days and up to two weeks), however, fish can lose considerable amounts of weight during the holding period. In some cases operators use extended holding periods to fatten fish before sale.

In summary, holding is generally the least problematic stage of live fish production. Techniques for successful holding of fish for live supply can borrow heavily from accepted aquacultural methods.

## TRANSPORT METHODS

Transport is the most critical constraint on successful development of the live fish industry. The fundamental principle of transport is that the smaller and lighter the package the cheaper it is to transport. Every litre of water weighs approximately one kilogram and occupies 1000 $cm^3$. Therefore, the obvious method to reduce transport costs is a reduction in the amount of water in the shipment relative to the amount of live fish.

Different modes of transport offer various advantages and disadvantages for live fish transport and each has potential for improvement. The main modes are sea, road and air transport. Each of these modes is discussed below.

### *Sea Transport*

For most species, the use of boats to transport live fish is an almost ubiquitous part of the supply chain. Early transport of large quantities of live fish was undertaken exclusively by sea. As a result, sea transport of live fish has a long history but technological advances have been few.

From around 1850, Norwegian cod fisherman transported cod captured during the last two weeks of each trip in wells perforated with one inch holes ('wet wells') and fitted with perforated baffles. Fishermen reported that the fish would die if the vessel stopped sailing (Midling *et al.* 1996). Today, most onboard holding systems remain very simple with most vessels capturing live fish still fitted with 'wet wells' or onboard 'holding' wells. Even with the addition of venturi scoops (for holding

wells) and pumps (to supply water while the vessel is at rest) the vast majority of vessels still rely solely on flow-through systems.

Midling *et al.* 1996 reported on the effectiveness of minor changes to onboard holding systems. It had been observed that cod which were not 'floating' (as a symptom of barotrauma), tended to aggregate at the bottom of the holding tank in densities estimated to approach 1:1. On the basis of this observation, onboard tanks were modified to provide water through a perforated false bottom. This modification decreased the density of fish in the bottom of the tank to 1:3 (Midling *et al.* 1996). Midling *et al.* (1996) claimed that mortality in the redesigned tanks was reduced to zero. While it is difficult to conceive of zero mortality being realised during transport in a commercial setting on an ongoing basis, it is obvious that relatively minor modifications of sea transport systems can offer considerable and cost-effective advantages.

Towable net pens are used to transport live pacific herring and live tuna. As a mode of transport and holding it offers relatively high survival and allows very large catches to be transported by vessels much smaller than those required if the catch were to be held alive onboard. Mortality of about 3% has been reported during such transport of pacific herring (Kreiberg & Solmie 1987). Mortality of southern bluefin tuna transported using towable net pens is generally not greater than 2% (*pers comm.* Mr Brian Jeffriess, Australian Tuna Boat Owners Association).

Whether the live fish are transported aboard the vessels or towed behind, a fundamental requirement of successful sea transport is the constant supply of sufficient clean, oxygenated water. While this statement is equally applicable to all forms of transport it is particularly critical during sea transport immediately after capture when stress indicators are peaking, respiratory rates are extremely high and excess mucus production is common (mucus degrades water quality and may inhibit gas exchange).

As stress indicators peak, fish are in a high state of readiness and are generally more 'skittish' or nervous than at other times and are subsequently more prone to incurring physical damage through panic. This, in addition to the movement of the vessel causing water to move within the transport container, means that sea transport systems need to be carefully designed to minimise further stress, ensure high quality water supply and minimise physical damage.

***Road transport***

Road transport is an almost ubiquitous part of the production process for live fish. Road transport ranges from bicycles used to transport several live fish contained in small buckets in mainland China (Huss 1995) to specialised trucks transporting up to 50t of live salmon as a single shipment in the United States of America (Terchunian *et al.* 1999).

Road transport is a major vector for live fish transport in the United Kingdom (Anon. 2003) and the United States of America (Rimmer & Franklin 1997). There have been major developments in the equipment and techniques to improve efficiency and survival used during road transport (Rimmer & Franklin 1997, Forsberg *et al.* 1999). These have included:

- matching truck size and design to specific needs;
- use of insulated tanks;
- transporting fish in dark or low light conditions;
- designing loading and unloading system to minimise fish handling;
- oxygen supply during loading and transport (often using liquid rather than gaseous oxygen);
- ram ventilators for gas exchange;
- water agitation;
- water temperature reduction;
- pre-transport tempering of fish to simulated transport conditions; and
- establishment of species and size specific loading densities.

As a general rule, road freight is generally much cheaper per kilogram than airfreight and is far more flexible especially with respect to timing and location. Its obvious limitation is international travel from continent to continent.

Relative to air transport, road transport generally allows more robust transport systems to be used which incorporate temperature control and aeration and/or oxygen. Moreover, road transport can sometimes take advantage of suitable water en-route to undertake water exchanges. The major advantage of road transport in Australia is that trucks can pick up from remote locations and deliver direct to markets and retailers.

In summary, while it is relatively slow, road transport is both cheaper and far more flexible (especially with respect to packaging and itinerary) than air transport and is likely to play a very important and continuing role in live fish supply.

***Air Transport***

Air transport has the advantage that it is the fastest form of transport available to the live fish industry. Speed is an obvious advantage in live fish transport because of the time-limited nature of the survival of fish once packed. Thus, operators must trade-off the increased cost of airfreight with the increased speed allowing proportionally greater densities of fish within the shipment.

The difficulties with airfreight of live fish are numerous. For example, in 1995 an Australian airline, suspended all live fish shipments because of leaking containers. Many carriers in Europe have also ceased the transport of live seafood because of similar problems. Leakage of transport packaging is an obvious problem for aircraft. Leaking water can cause considerable damage to the aircraft in both the short- and long-term. The likelihood of rough handling of goods needs to be taken into consideration by live fish suppliers when determining the best type of packaging for air transport. In general, the stronger the packaging material the greater its weight and/or construction cost. The current industry-preferred method of plastic bags in polystyrene boxes needs to be improved to eliminate leakage if live fish as freight are to be more widely accepted by air carriers.

Many baggage handlers do not follow requests to store live fish shipments in a cool place out of direct sunlight. This often results in water temperatures increasing and mortalities occurring. One live fish supplier in New Zealand reported fluctuations in survival from 10-90% that were ultimately traced to variable temperatures during transit due to improper handling. Many live fish operators have taken to including disposable temperature recorders in order to monitor handling practices in transit. Such recorders have provided invaluable proof of incorrect handling and allowed operators to successfully seek remuneration for what are often considerable losses.

Many of the airfreight carriers that do transport live fish will not permit the carriage of pure oxygen either overlaid in bags or in compressed cylinders. While there are obvious safety concerns for airlines, this prohibition causes considerable problems for the cost-effective transport of live fish.

Other considerations on the use of air transport include the fact that air carriers generally charge higher premiums on freight for which arrival is time critical and International Air Transport Association regulations prohibit travel times of more than 48 hours for live fish regardless of packaging (Terchunian *et al.* 1999).

While there are significant obstacles to successful air transport of live fish, smaller air carriers are increasing their role in domestic transport of live fish in Australia.

Light aircraft are successful for similar reasons to road transport. They are more willing to handle non-standard sized packages and devote more care to the specific cargo. Many smaller carriers often allow the use of oxygen in shipments.

At present, air transport is the only viable option for Australian exporters of live fish to the major Asian markets. However, given the difficulties involved with air transport, considerable incentive exists for cost-effective improvements to the current techniques and equipment used.

## PACKAGING EQUIPMENT AND TECHNIQUES

### *Equipment*

Equipment for transporting live fish ranges from basic artisanal systems such as buckets or sealed barrels/drums through to very sophisticated systems installed on transport trucks that filter and recycle water, add oxygen and regulate temperature (Schoemaker 1991). Even minor changes to the equipment used has been shown to markedly increase survival. For example, transport of delta smelt, *Hypomesus transpacificus*, at the same densities using cylindrical polyethylene bags rather than rectangular coolers increased survival rates by 2-4 times across various durations between 4 and 48hr (Swanson *et al.* 1996).

#### *Basic systems*

In some instances live fish are transported successfully using very basic equipment. For example, a bucket on the back of a bicycle is a common live fish transport method in China. In the African Congo live fish are regularly transported in aluminium containers covered with palm leaves and water hyacinth to prevent fish from jumping out and to reduce evaporation (Huss 1995). The water in these containers is changed periodically and an almost constant visual check is kept on the fish during transport.

#### *Bagging*

The most common type of packaging for air transport involves the use of double polyethylene bags held inside polystyrene boxes. Such packaging offers a relatively inexpensive and disposable system but is labour intensive and only moderately successful in keeping fish alive at the fish to water ratios normally used by operators marketing live fish for human consumption (unlike the aquarium trade that tends to use more water per kilogram of fish) (Franklin 1995).

One interesting development has been 'breathing bags' made of specialised membranes that allow for the exchange of air (including oxygen) and carbon dioxide while retaining water. Air space is not needed in these bags since gases move freely

from the surrounding air into the bag while carbon dioxide diffuses out. Of particular interest is the fact that diffusion through these bags increases with increasing temperature (i.e. these bags will at least partially compensate for increased respiration due to increasing temperature). Breathing bags can be doubled and heat-sealed and still function efficiently. They can also be reused. The major drawback is the initial cost of the bag compared to standard polyethylene bags (Terchunian *et al.* 1999).

*Sophisticated/Specialised systems*

Some significant developments have occurred over the last decade within the packaging of live fish for transport. These developments have included sophisticated humidified, sealed transport units capable of filtering water, supplying oxygen, removing carbon dioxide and conditioning the internal environment.

High humidity environments produced by water sprays or trickle arrangements have also been developed to transport live fish, however the utility of these techniques tends to be species dependent. Other systems use rigid plastic frames to angle fish so that only the head immersed in water constantly. These systems can provide near saturated oxygen levels and effective carbon dioxide stripping greatly reducing the volume of water required. However, as mentioned previously few airlines will accept oxygen supply/generation systems and there is the obvious cost to recover such systems after arrival at their destination. For these reasons most exporters of live fish for human consumption still persist with disposable and less directly expensive transport equipment.

*Light and Motion Stimuli During Transport*

Rimmer (1995) suggests that decreasing the light exposure of the fish during transport offers considerable advantages. However, it should be noted that while opaque packaging will reduce the level of external visual stimulation not all fish are less active in low light environments and thus the benefits of opaque packaging need to be assessed for each individual species. Light levels can have dramatic effects on the behaviour and activity of some fish species during holding and transport (Findlay 1994, Jo & Kim 1998).

Another factor that has been reported to affect survival of fish during transport is water motion. Greater stress responses have been observed in some fish species exposed to water motion similar to that which may be experienced during transport (Winkler 1987, Takashima *et al.* 1983, Schreck *et al.* 1989, Steffens 1995, Evans & Fewtrell 1996). The thermal tolerance of mosquito fish, *Gambusia affinis*, was narrower for fish exposed to water slosh during transport compared to fish that had been transported in containers that prevented water slosh (Winkler 1987). It should be noted however that different responses to water motion may be observed in

species from different environments and that water motion of some form is almost impossible to avoid during transport. Furthermore, water movement can be beneficial as a means of increasing gas exchange efficiency.

### *Techniques*

There is considerable variation in the techniques used prior to and during transport. Techniques include deprivation of food, anaesthesia, other chemical/drug treatment, addition of oxygen and chilling. Each of these techniques offers particular advantages and disadvantages.

#### *Deprivation of Food*

Deprivation of food of fish is a well-known technique within aquaculture processes to improve survival during acute stress. However its use as part of live fish transport protocols has not yet been widely accepted and adopted. This is particularly true in operations that are not dedicated live fish producers. In such operations the holding and maintenance of live fish to permit gastric evacuation (usually requiring several days of food deprivation) is a considerable added complication to what is already a labour- intensive process.

The theoretical advantages of food deprivation prior to transport are considerable and include avoidance of increased post-prandial oxygen demand and high rates of excretion (including possible regurgitation). However, there is an obvious cost in establishing systems to hold fish during purging that must be considered relative to these benefits.

To get around the delays involved in holding fish to allow gastric evacuation some live reef fish exporters in the Pacific islands have taken to giving recently caught marine fish a freshwater bath for 2-3 minutes to induce vomiting before packaging the fish for live transport (Chan 2000). These operators have reported improved water quality in shipments when this technique has been applied but no empirical data were available on actual survival rates.

#### *Anaesthesia*

Anaesthetics are chemical or physical agents that, with increasing exposure or concentration, first calm (sedate) an animal, then cause it to successively lose mobility, equilibrium, consciousness and finally reflex action (Wedemeyer *et al.* 1990). The generalised stages of anaesthesia are given in Table 1.

| *Stage* | *Description* | *Behaviour, ventilation rate and reflex action* |
|---|---|---|
| *0* | Normal | Reactive to external stimuli; opercular rate and muscle tone normal |
| *1* | Light sedation | Slight loss of reactivity to external stimuli visual and tactile stimuli; opercular rate slightly decreased; equilibrium normal |
| *2* | Deep sedation | Total loss of reactivity to external stimuli except strong pressure; slight decrease in opercular rate; equilibrium normal |
| *3* | Partial loss of equilibrium | Partial loss of muscle tone; swimming erratic; increased opercular rate; loss of spinal reflexes |
| *4* | Total loss of equilibrium | Total loss of muscle tone and equilibrium; slow but regular opercular rate; loss of spinal reflexes |
| *5* | Loss of reflex reactivity | Total loss of reactivity; opercular movements slow and irregular; heart rate very slow; loss of all reflexes |
| *6* | Medullary collapse (asphyxia) | Opercular movements cease; cardiac arrest usually follows quickly |

**Table 1. Stages of anaesthesia as described by Summerfelt & Smith (1990)**

Anaesthetics are frequently used within aquaculture and research settings to facilitate handling, reduce damage and/or reduce stress (Fergusson 1988). Encouragement of quiescence through the use of anaesthetics offers considerable advantages to live fish transport through reduction of activity and thereby respiration. For example, Itazawa & Takeda (1982) reported that the oxygen consumption of carp, *Cyprinus carpio*, was halved when an anaesthetic ($CO_2$) was applied.

A reduction of respiration increases the survival of fish during transport and/or increases the carrying capacity of a given volume of water during transport thereby concomittantly reducing the cost per kilogram (McFarland 1960, Jirasek *et al.* 1978, Ferreira *et al.* 1982, Sado 1985). Use of anaesthetics during transport has also been shown to reduce the levels of stress indicators in fish (Barton & Peter 1982, Sado 1985).

The physiological process of anaesthesia in fish is not well understood (Blasiola 1977, Smith 1982, Ross & Ross 1984) but it is accepted that chemical anaesthetics

depress the central nervous system (CNS) acting first on the cerebral cortex, then the medullary respiratory centre and spinal cord (Summerfelt & Smith 1990). Depression of the CNS produces valuable effects within the transport process including narcosis and muscular relaxation (Carrie & Simpson 1988). Different anaesthetics vary in their exact mode of action to induce these affects on the CNS.

While the Australian live seafood industry has a 'clean and green' image (Franklin 1995), several fish anaesthetics including MS222 (tricaine methanesulphonate) and benzocaine (ethyl p-aminobenzoate) are believed to be in use. Neither MS222 nor benzocaine are currently registered for use in food fish in Australia and such use is illegal. In addition to the risk to the consumer there are also risks to the live fish supplier from direct contact with water containing the anaesthetic. For example, MacKinlay *et al.* (1994) reported that the use of MS222 as an anaesthetic during the large-scale Pacific salmon tagging programs in the United States was stopped in the 1980s due to health concerns for those conducting the tagging (the use of MS222 in this tagging program has since recommenced under revised guidelines on contact).

Some anaesthetics are approved for use within aquaculture but the withholding period required before consumption is well in excess of the typical holding period at the final destination for fish transported for retail sale alive. For example, the United States Food and Drug Administration (USFDA) imposes a 21-day minimum withholding period on fish treated with MS222.

The reported benefits of anaesthesia vary markedly between studies and anaesthetics. For example, metomidate has been observed to reduce the level of the plasma corticosteroids in chinook salmon after handling relative to untreated fish (Kreiberg & Powell 1991). Swanson *et al.* (1996) observed that the addition of MS222 to transport containers containing delta smelt increased survival by around 50%. Benzocaine in concentrations ranging from 5-20ppm did not significantly reduce mortality of matrinxa, *Brycon cephalus*, during transport (Urbinati *et al.* 2001). In addition, small differences in the dose of benzocaine provoked pronounced differences in levels of physiological indicators and physical injuries were highest in the tanks that contained low doses of benzocaine..

Rainbow trout immersed in water containing 2-phenoxyethanol at a concentration 150ppm for a little as one hour caused a significant increase in the serum cortisol concentration. Moreover, both anaesthetised and unanaesthetised trout exhibited similar elevations in the concentration of serum cortisol when the water was agitated (Takashima *et al.* 1983). These observations led the authors to conclude that 2-phenoxyethanol would not reduce stress during transport.

MS222 (also known as tricaine, tricaine methanesulfonate, sandoz, finquel, metacaine, 3 aminobenzoic acid ethyl ester and ethyl m-aminobenzoate) is an anaesthetic that has been widely used in aquatic animals (Ohr 1976, Summerfelt & Smith 1990) including during the transport of live fish (Webb 1958, Kelly 1999). MS222 has been reported to interfere with the uptake of oxygen across the gills of fish (Davis *et al.* 1982, Bourne 1984, Ross & Ross 1984, Fergusson 1988, Thomas & Robertson 1991). It appears that MS222 initiates a cascade of physiological responses that may be maladaptive relative to the available oxygen concentration of the water (e.g. increase ventilation rate and volume) and its use in live fish transport is therefore questionable.

The proprietary product known as AQUI-S$^{TM}$ is a food-grade anaesthetic containing 50% isoeugenol. AQUI-S is approved for use in food fish in Australia and the United States under a 'Generally Regarded As Safe' (GRAS) status (Jerret 1992). AQUI-S$^{TM}$ is already in use as part of 'rested harvest' procedures within the Australian salmonid industry and has been trialed during harvest of caged southern bluefin tuna. Templeton (1996) reports that AQUI-S$^{TM}$ provides improved handling of salmonids and that fish harvested using this product exhibit improved flesh quality and whole fish appearance. Andersen *et al.* (1997) concluded that AQUI-S is as effective as MS222 as an anaesthetic for rainbow trout. Wagner *et al.* (2002) observed that levels of physiological stress indicators in the same species were significantly lower for fish treated with AQUI-S than either MS222 or carbon dioxide and costs of use were also lower than both of these other anaesthetics. From these results it is apparent that AQUI-S may offer considerable advantages for use as an anaesthetic during live fish transport.

Carbon dioxide ($CO_2$) is considered an anaesthetic of low regulatory priority within the context of food safety (Anon. 1997, 1999) and is commonly used as a fish anaesthetic in research and aquaculture settings. $CO_2$ is either applied directly as a gas bubbled through water (Takeda & Itzawa 1983) or through addition of sodium bicarbonate and acid to water (Booke *et al.*1978, Mishra *et al.* 1983, Iwama *et al.* 1991, Gelwicks *et al.* 1998). The anaesthetic effects of $CO_2$ are limited to a very short duration (i.e. a few minutes) post-application (Booke *et al.*1978, Yoshikawa *et al.* 1988, Anderson et al. 1997). Hyperactivity is also commonly reported when using $CO_2$ as an anaesthetic (Iwama *et al.* 1991)

Generally, the addition of $CO_2$ to water causes hypercapnia and decreases blood pH of aquatic vertebrates. A lowering of the pH of blood reduces the oxygen affinity of haemoglobin in most animals. This effect is known as the 'Bohr effect'. In some fish, cephalopods and crustaceans an increase in $CO_2$ or a decrease in blood pH causes not only a reduction in the oxygen affinity of haemoglobin but also a

reduction in oxygen capacity known as the 'Root Effect' (Eckert *et al.* 1988). Itazawa & Takeda (1982) observed that if oxygen was bubbled through the water at the same time as $CO_2$ then the supply of oxygen to tissues could be maintained despite hypercapnia.

$CO_2$ does offer some potential advantages in addition to anesthesia. $CO_2$ lowers pH in water thereby reducing the concentration of toxic un-ionised ammonia. Densities of aerobic bacteria are also greatly reduced when $CO_2$ is added (Steffensen 1989).

Given the limited duration of effect post-application, $CO_2$ would need to be applied constantly during transport to result in anaesthesia. The harmful effects of $CO_2$ within the live transport setting (outlined above) suggest that it should not be used to encourage quiescence during transport.

Even though anaesthetics may produce anaesthetic stress (Barton & Peter 1982, Bourne 1984, Ross & Ross 1984, Summerfelt & Smith 1990), it is widely accepted that anaesthetics provide a net benefit to stress reduction during transport and handling procedures (Ross & Ross 1984). Numerous authors suggest that stages 1 & 2 of anaesthesia (as detailed in Table 1) are best suited to routine handling and transportation (e.g. Durve 1975, Ferreira *et al.* 1984, Ross & Ross 1984, Sado 1985). At stage 3 and beyond fish typically lose equilibrium and will actively struggle to maintain balance. This increase in activity results in an increased respiratory rate with a subsequent increase in oxygen consumption, carbon dioxide and ammonia production. Thus, in a transport context, sedation beyond the point at which fish begin to lose equilibrium may lead to higher mortality (Ferreira *et al.* 1984).

Chemical anaesthetics largely act by depression of the CNS and therein affect the function of the respiratory centre in the medulla. If ventilation does not meet the aerobic oxygen demand, an oxygen debt will rapidly accumulate. Hyperventilation (indicative of oxygen debt) is commonly observed in fish recovering from anaesthesia (Summerfelt & Smith 1990). If this debt is not recovered the fish will die. Thus, careful consideration needs to be given to the potential to overdose and the potential for a lethal accumulated oxygen debt if anaesthetics are to be used within live transport.

*Use of Chemicals/Drugs*

In addition to anaesthetics there are a number of potential uses for other chemicals/drugs within live fish supply. Steffensen (1989) observed that consumption of oxygen by bacteria within experimental apparatus can be considerable and must be considered when calculating respiratory demands of fish.

Obviously the use of antibiotics and/or disinfectants in a commercial setting would reduce the oxygen demand by micro-organisms within the transport container.

Chemicals that 'condition' the water within the transport system provide obvious benefits. In particular, chemicals that buffer the environment to pH change and/or bind ammonia are obviously beneficial to reduce the toxicity of accumulated waste products. Zeolite is commonly used in live transport of aquarium fish to reduce the concentration of ammonia (Teo *et al.* 1989). Soda lime has also been used to reduce dissolved carbon dioxide (Rimmer & Franklin 1997) during transport.

Some retailers selling 'minnows' as live bait for recreational fishers in the United States add aspirin (salicylic acid or acetylsalicylate) tablets to the water containing the purchased fish. To my knowledge there is no scientific literature on the benefits of aspirin within live transport processes however the following information provides some insight on why it may improve survival rates. It is known that not all oxygen consumed is used in the catabolic reaction of proteins, lipids and/or carbohydrates as part of respiration. It is also known that aspirin inhibits the synthesis of prostaglandins by acetylating the terminal amino sub-unit of cyclooxygenase (Stryer 1988). The immediate precursors of prostaglandins are synthesised in membranes from $C_{20}$ fatty acids and are released from membrane phospholipids by phospholipases. The result is the formation of a cyclopentane ring with four oxygen atoms being introduced by the cyclooxygenase component of prostaglandin synthase. All of the oxygen atoms introduced into prostaglandin come from molecular oxygen (i.e. that oxygen also available for respiration) (Stryer 1988). As aspirin blocks the first step of prostaglandin production the molecular oxygen is not consumed and is therefore available for respiration.

It must be remembered that prostaglandins have a wide range of effects, with one of their key functions being the regulation of vasodilation or constriction in response to epinephrine (adrenaline) secreted as part of the sympathetic 'flight or fight' response to a stressor (Eckert *et al.* 1988). While inhibition of part of the typical 'fight or flight' response may at first appear beneficial within live transport, the overall benefits are not clear. Even so, the use of aspirin within live transport is worthy of further investigation.

*Addition of Oxygen*

Lack of oxygen is the most acute potential stressor within most live transport processes and provision of additional oxygen (rather than additional water) provides obvious advantages to the likely survival of fish. Staurnes *et al.* (1994) observed that, when oxygen saturation was maintained at normoxic levels, the effects of simulated transport at ratios of 1:2 and 1:1 (fish:water) on stress indicators in cod

were only moderate at worst and that changes in some indicators were negligible. This observation reinforces the concept that oxygen availability is the most critical consideration in successful live fish transport.

Assuming complete mixing, water in an open system will establish a level of dissolved oxygen in equilibrium with the air (or other gas) at its boundary. The time taken to reach equilibrium is dependent upon the surface area of the water and amount of mixing. The solubility of oxygen in water increases with decreasing temperature. Therefore, there are a range of mechanisms available in an open system to increase the amount of oxygen available to fish. These include:

- cooling of water;
- increasing the concentration of oxygen in adjacent gas (may include use of oxygen bubbled through water via compressed gas or oxygen producing chemicals);
- mixing (water and/or air); and
- aeration (to increase surface area and mixing).

Within a closed system the constraints are somewhat different. The oxygen within the system is finite and maximum at the time of closure of the system. Therefore, techniques are limited to those that increase the amount of oxygen in the package at time of closure. Such techniques include:

- use of cooled water;
- use of water super-saturated with oxygen (generally achieved by bubbling oxygen through the water);
- replacing air in the sealed container with oxygen; and
- addition of oxygen producing chemicals.

While too little oxygen is clearly a problem for successful transport of live fish, too much oxygen dissolved in water can also be a major problem. Hyperoxia occurs when oxygen becomes super-saturated in water, most commonly as a result of rapid pressure or temperatures changes resulting in supersaturation of total dissolved gases. Diffusion of oxygen into the blood stream of fish living in hyperoxic conditions can result in a phenomenon commonly referred to a gas bubble disease (GBD). GBD is a non-infectious physically-induced process that can be caused by supersaturation of nitrogen or other components of the total dissolved gases in addition to oxygen.

GBD is characterized by the formation of gas bubbles in the blood (emboli), tissues (emphysema) and the body cavities of fish, such as behind the eyes (causing exophthalmia) or between layers of skin tissue. Small bubbles can form within the vascular system, blocking the flow of blood (haemostasis) and causing tissue death. If blockages occur in the gills, GBD can result in death by asphyxiation and GBD has been blamed for mass fish kills (Bouck 1980, Domitrovic *et al.* 2000).

While tolerance to hyperoxia varies between species, Deufel & Mohr (1983) stated that, for most species, there is a risk of GBD once oxygen saturation exceeds 105%. Domitrovic *et al.* (2000) observed no macroscopic symptoms and rare microscopic symptoms of GBD among dozens of species examined until oxygen saturation levels exceeded 107%. Ryan *et al.* (2000) stated that GBD was rare in non-salmonids until oxygen saturation exceeded 120%. Some species have been reported to show no signs of GBD even when oxygen saturation exceeded 300% (Anon. 1995c). History of exposure to gas supersaturation, temperature and salinity have all been shown to alter the sensitivity of fish of the same species (Bouck 1980).

*Brackish Water*

The use of brackish water (i.e. water with a salinity between 3 and 20ppt) during holding and transport has been shown to significantly decrease levels of stress indicators and/or increase the survival rate during transport of a number of freshwater and saltwater species including muskellunge, *Esox masquinongy* (Miles *et al.* 1974), spot, *Leiostomus xanthurus* (Hales *et al.* 1990), red drum, *Sciaenops ocellatus* (Weirich & Tomasso 1991), freshwater-acclimated striped bass, *Morone saxatilis* (Harrell 1992, Cech *et al.* 1996, Brick & Cech 2002), freshwater-acclimated hybrid striped bass *M. saxatilis* X *M. chrysops* (Reubush & Heath 1997), dolphinfish (Morgan *et al.* 1996), coho salmon (Jeon *et al.* 2000b) and pejerry, *Odontesthes bonariensis* (Tsuzuki *et al.* 2001).

Other, less direct findings also support the use of brackish water during transport. For example, the thermal tolerance range of juvenile blue tilapia, *Oreochromis aureus*, peaked when fish were treated in isosmotic salinity water (in this case 11.6 ppt) (Zale & Gregory 1989). Kutty *et al.* (1980) also observed that isosmotic salinity significantly increased thermal tolerance. Furthermore, while the use of brackish water (5ppt NaCl) did not attenuate the levels of various stress indicators in walleye, *Stizostedion vitreum*, it did reduce the time taken for recovery to pre-stress levels (Barton & Zitzow 1992, 1994). Similarly, Young & Cech (1993) observed a similar pattern of faster recovery but absence of attenuation in stress indicator levels of striped bass following net confinement when the fish were immersed in brackish water (10ppt NaCl).

The apparent beneficial effects of brackish water are not clearly understood (Barton & Zitzow 1994). For example, oxygen consumption in freshwater acclimated Adriatic sturgeon (*Acipenser naccarii*) increased by 30% when the fish were placed in brackish (mildly hypertonic) water (McKenzie et al. 2001a,b). Similarly, Stauffer (1986) and Chung (1997) reported that salinity did not alter thermal tolerance of Mozambique tilapia, *Sarotherodon mossambicus* (≈ *Oreochromis mossambicus*) and

tropical salt marsh fish, *Cyprinodon dearboni* respectively. Such findings are somewhat contradictory with the numerous reports of higher survival in many species during brackish water transport and care needs to be taken in application of this method in different species.

Ion exchange has been shown to be markedly affected by capture and handling stress in a number of species (Wedemeyer 1972, Vinogradov & Klerman 1987, Reubush & Heath 1997). It may be that the use of water that contains dissolved salts and other ions in concentrations approaching that of blood during holding (soon after capture/transport) and/or transport would reduce this exchange and its' harmful physiological effects.

*Chilling*

Low temperature produces effects resembling anaesthesia. Indeed this process is sometimes referred to as 'cold anaesthesia' (Yoshikawa *et al.* 1988). The FDA lists the use of ice to chill live fish and 'reduce metabolism' as a treatment with a low regulatory priority (Anon. 1999). Chilling as a technique for improving live fish transport is discussed in detail in Chapter 4 but for completeness in this review of current transport methods a short discussion is warranted here.

Numerous authors (e.g. Mittal & Whitear 1978, Winkler 1987, Eckert *et al.* 1988, Summerfelt & Smith 1990, Terchunian *et al.* 1999) have reported that as temperature decreases there are a range of concomitant benefits that improve survival of aquatic poikilotherms during transport. These include:

- increased solubility of oxygen (and $CO_2$) in water;
- increased oxygen affinity of haemoglobin;
- reduced tissue oxygen demand/metabolism;
- reduced response to external stimuli and/or reduced levels of stress indicators;
- reduced activity; and
- reduced mucus production compared with chemical anaesthetics.

While the benefits can be easily described in broad terms, like anaesthetics, there is wide species and individual variation in the optimal temperature regime for cold anaesthesia (Mittal & Whitear 1978, Yokoyama *et al.* 1989, Huss 1995). Within a live transport setting it can be difficult to maintain a prescribed temperature (Yoshikawa *et al.* 1988) and, in the extreme, chilling can cause 'cold shock' which is typified by changes in body fluids, blood electrolytes and haematology (Summerfelt & Smith 1990) and may lead to death.

The use of ice adjacent to the transport container has been a common technique to reduce temperature during live fish transport. However melting ice is no less expensive to transport than the same volume of additional water (Rodman 1963).

*Combinations*

Various treatments and techniques can be combined to address a range of physiological and physical stressors. For example, Swanson *et al.* (1996) compared the survival of delta smelt using three different water treatments (i.e. 8ppt NaCl, 8ppt NaCl + MS222, 8ppt NaCl + NovAqua (a commercial polymer water conditioner)). The addition of the anaesthetic, MS222, increased survival by around 50% relative to NaCl alone. The addition of NovAqua approximately doubled the survival of delta smelt relative to NaCl alone (27.9% to 54.8%) after 72hr in the transport container. The authors attributed this increase to the reduction of osmotic stress due to the polymers contained in NovAqua.

However, while combined treatments may confer greater benefits there is also the potential for deleterious synergistic reactions between treatments that are beneficial in isolation. For example, Teo *et al.* (1989) observed that when clinoptilolite (a zeolite ammonia remover), tris buffer and 2-phenoxyethanol (an anaesthetic) were used in combination the mortality of guppy, *Poecilia reticulata*, was significantly greater than when any one of these treatments was used in isolation. Similarly, Harrell (1992) observed that stress indicator levels in striped bass following transport were higher in fish treated with both brackish water and MS-222 than when salt-alone was used. It is apparent that various water quality and/or physiological parameters do not operate in isolation and care needs to be taken when combining treatments.

## SUMMARY

Live fish transport (and the equipment and techniques used therein), has a relatively long history. Developments and improvements have occurred in a somewhat step-wise fashion with the greatest advances being the result of dedicated cooperative research involving both industry and researchers.

There remains scope for significant advances in live fish supply practices to assist the development of an ecologically sustainable and economically efficient industry that can also satisfy ethical concerns about animal welfare. In particular, there appears to be merit in:

- the promotion of line and trap fishing as the preferred methods of capture for live fish supply;
- encouraging greater information exchange between aquaculture and live fish operators with respect to holding and handling methods;

- developing a better understanding of the necessary period of food deprivation prior to transport of live fish; and
- investigating the application of lowered water temperatures during transport.

It is these final two aspects that are the focal points of the current study.

# CHAPTER 3

# GENERAL METHODS AND MATERIALS



## INTRODUCTION

To avoid repetition in following chapters, provided below are descriptions of the:

- species studied;
- capture, holding and transport methods utilised;
- main experimental systems; and
- data analysis undertaken.

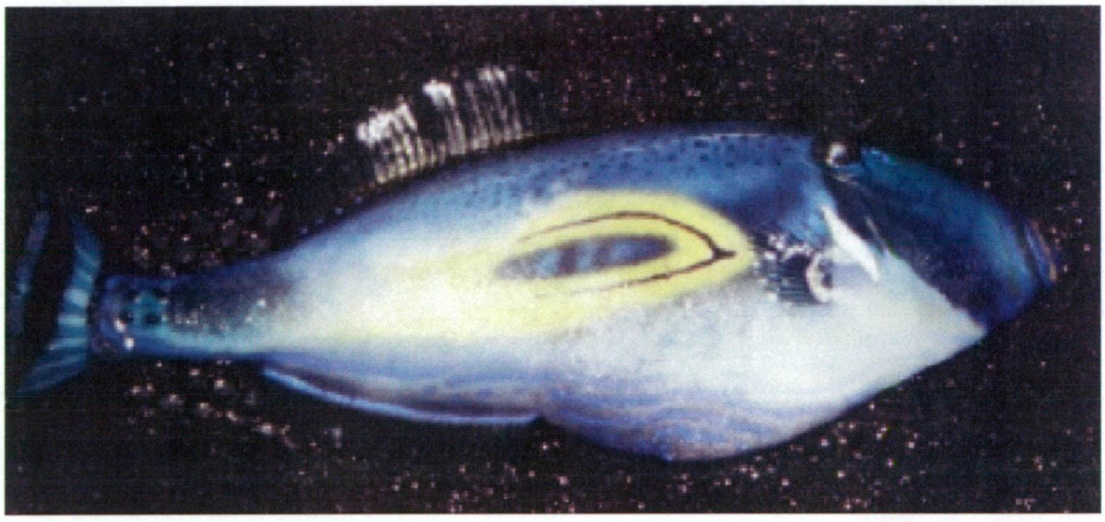
## SPECIES STUDIED

Three temperate, demersal, marine species were chosen. These species were chosen because most work (especially within Australia) has centred on freshwater and tropical marine species and hence there is little information regarding the live transport of temperate marine species. While experimental specimens of two of the species (i.e. horseshoe leatherjacket and bluethroat wrasse) were sourced from wild populations and specimens of the third (i.e. greenback flounder) were sourced from aquaculture production, all three species are found in close association in the wild. A comparison of their physiological tolerances will assist development of protocols for other species which share a habitat (i.e. do different species from the same habitat exhibit similar or markedly different thermal tolerances?)

***Horseshoe Leatherjacket***

The horseshoe leatherjacket, *Meuschenia hippocrepis* Quoy & Gaimard, is an attractive monocanthid fish that inhabits the coastal temperate waters of Tasmania, Victoria, South Australia and southern Western Australia (Grant 1987). This species is easily distinguished by the black 'horseshoe' found mid-body on both sexes (Plate 2). Growing to approximately 2.5kg (Grant 1987) and 51cm total length (May & Maxwell 1986), this species is the second largest member of its genus and a popular table fish throughout most of its range. Members of this genus are very inquisitive, often accompanying divers and make attractive aquarium subjects.

**Plate 2 Horseshoe leatherjacket, *Meuschenia hippocrepis***



No reference could be found which described external, sexually dimorphic characteristics. Dissection of many specimens of horseshoe leatherjackets resulted in the observation that the lateral caudal spines of horseshoe leatherjackets with male gonads, were considerably larger and clearly protruded from the skin surface while those of individuals exhibiting female gonads, had much smaller spines. This feature was used to identify the sexes.

Horseshoe leatherjackets used in the experiments were 24-42cm in length and 300-1100g in weight.

***Bluethroat Wrasse***

The bluethroat wrasse, *Notolabrus tetricus* Richardson (=*Pseudolabrus tetricus*), is an abundant labrid fish around the southern Australian coastline and is particularly abundant in Tasmanian coastal waters. Juveniles and females are greenish or reddish brown, darker on the back and white on ventral surface (Plate 3). Adult males are greenish-blue to reddish orange with a broad white vertical bar on the body and are readily distinguished by their dark blue chin and throat (Kuiter 1993) (Plate 4). The

species has a maximum reported total length of 50cm (May & Maxwell 1986) (although Gommon *et al.* (1994) states a maximum total length of "around 60cm), with all large individuals being male. No reference to a maximum weight could be found.

**Plate 3 Female Bluethroat Wrasse, *Notolabrus tetricus***

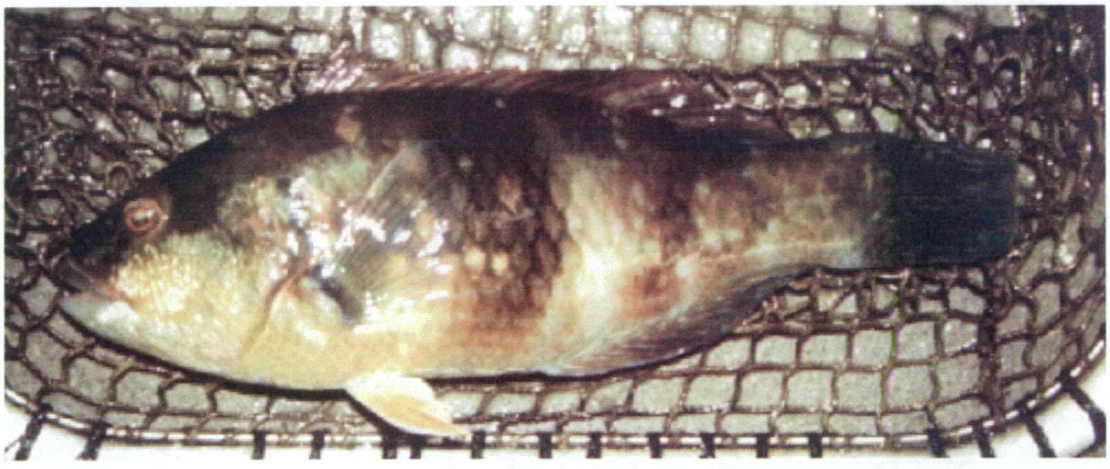
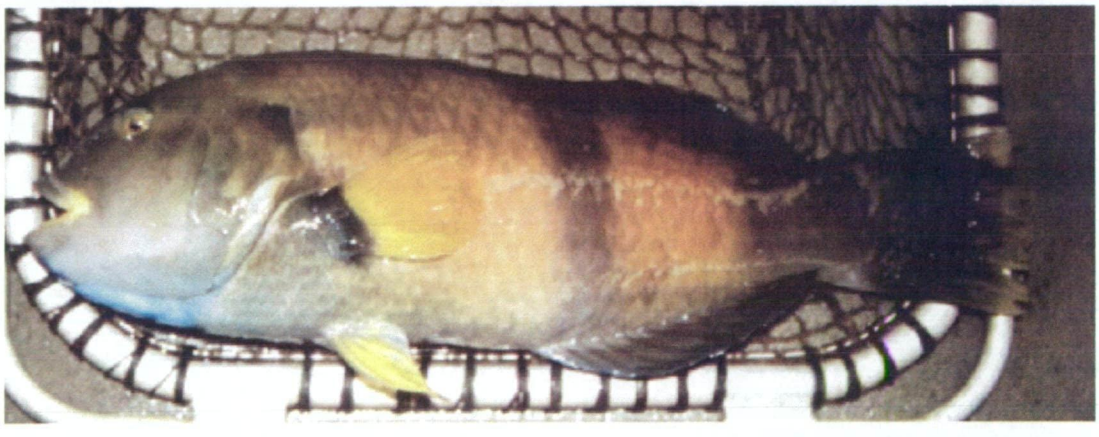


**Plate 4 Male Bluethroat Wrasse, *Notolabrus tetricus***



A notable observation was that during the course of extended holding several larger, presumably female, individuals began to change colouration to that of a male. This occurred in the absence of males in the holding tank. While most but not all of the 500 species of the 60 genera of the family labridae that have been studied have been shown to be protogynous hermaphrodites (Lejeune 1987, Nakazono & Kusen 1991, Nelson 1994), no reference can be found of this species being a protogynous hermaphrodite and given that the reported juvenile colouration is the same as the

female colouration it is not possible to assume that these animals were female. However, observations in captivity during the course of experiments were highly suggestive that this species is indeed a protogynous hermaphrodite and that sex change is precipitated by the absence of a male rather than size or age alone.

Bluethroat wrasse used in the experiments were 16-48 cm in length and 200-2300g in weight.

***Greenback Flounder***

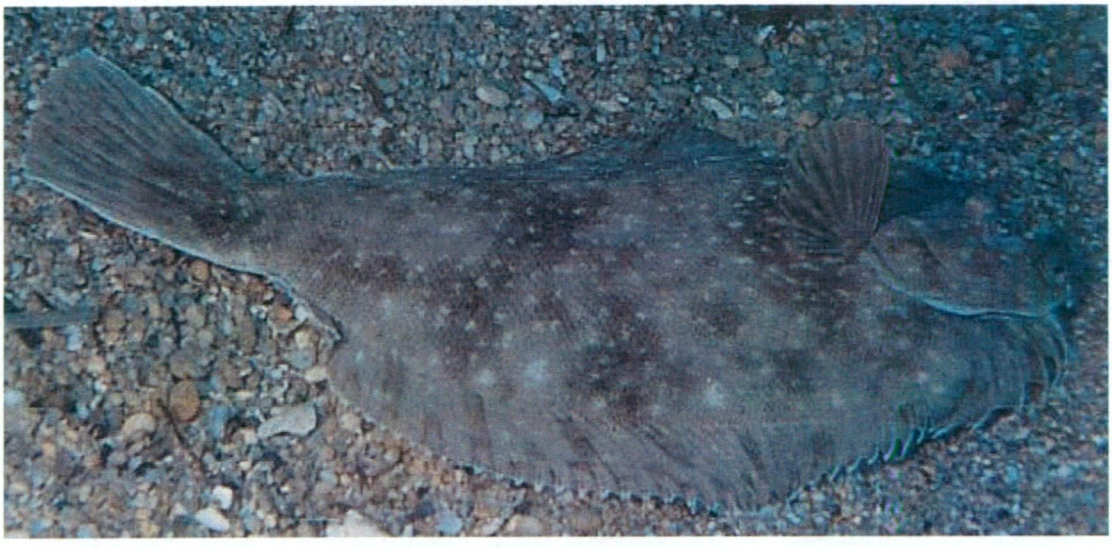
The greenback flounder, *Rhombosolea tapirina* Günther, is an important commercial species in the southern states of Australia. Belonging to the flatfish family Pleuronectidae, this species is typically found lying on sand or silt bottoms. Greenback flounder reach a maximum weight in the wild of around 600g (Grant 1987) and maximum total length of 45cm (May & Maxwell 1986). Greenback flounder are also an important recreational species as they enter shallow waters at night where they are speared with the assistance of lights used to first find them lying on the bottom.

Body colouration is dependent in part upon the colour of the substrate on which the animal is living but the upper surface is usually greyish-green to brown and all fins are edged with white.

No reports could be found detailing external sexually dimorphic characteristics. However, a technique was demonstrated to me by a fellow doctoral student at the National Key Centre for Aquaculture using a bright light to 'candle' the gonad area. If present, the elongated male gonads were distinguishable from the more rounded and dense female gonad. Because this method relied on the gonad being reasonably well-developed only 70% of fish greater than 100g were able to sexed with any degree of confidence. When mature, small amounts of milt can be expressed from the male while swelling around the mid-dorsal region is evident in females. The life cycle of greenback flounder has been closed in culture (Crawford 1984) and live supply to Asia shows strong potential as a market for greenback flounder.

Greenback flounder used in the experiments were 14-26cm in length and 45-200g in weight.

**Plate 5 Greenback flounder, *Rhombosolea tapirina***



Source: Marine Life Society of South Australia Website, http://www.mlssa.asn.au.

## CAPTURE, HOLDING AND TRANSPORT METHODS

### *Capture*

Specimens of horseshoe leatherjacket and bluethroat wrasse were captured by line fishing from anchored boats at Hebee Reef and surrounding waters in the vicinity of the mouth of the Tamar River, Tasmania, Australia (Figure 3).

During line fishing, water depth and surface temperature was monitored through the use of a portable echosounder (Humminbird™ 400ID) or fixed unit (Humminbird™ Wide Eye). In an effort to minimise barotraumas, fishing was not conducted in waters greater than 10m deep and most activity occurred in waters less than 5m deep.

Line fishing equipment consisted of rod, reel and copolymer monofilament or gelspun polyethylene line with a single conical-pointed, barbless 1/0 longshank hook tied approximately 30cm above a 20-40g lead weight. The bait consisted of small

of squid. After fish were hooked they were retrieved in a steady manner to minimise potential hook damage.

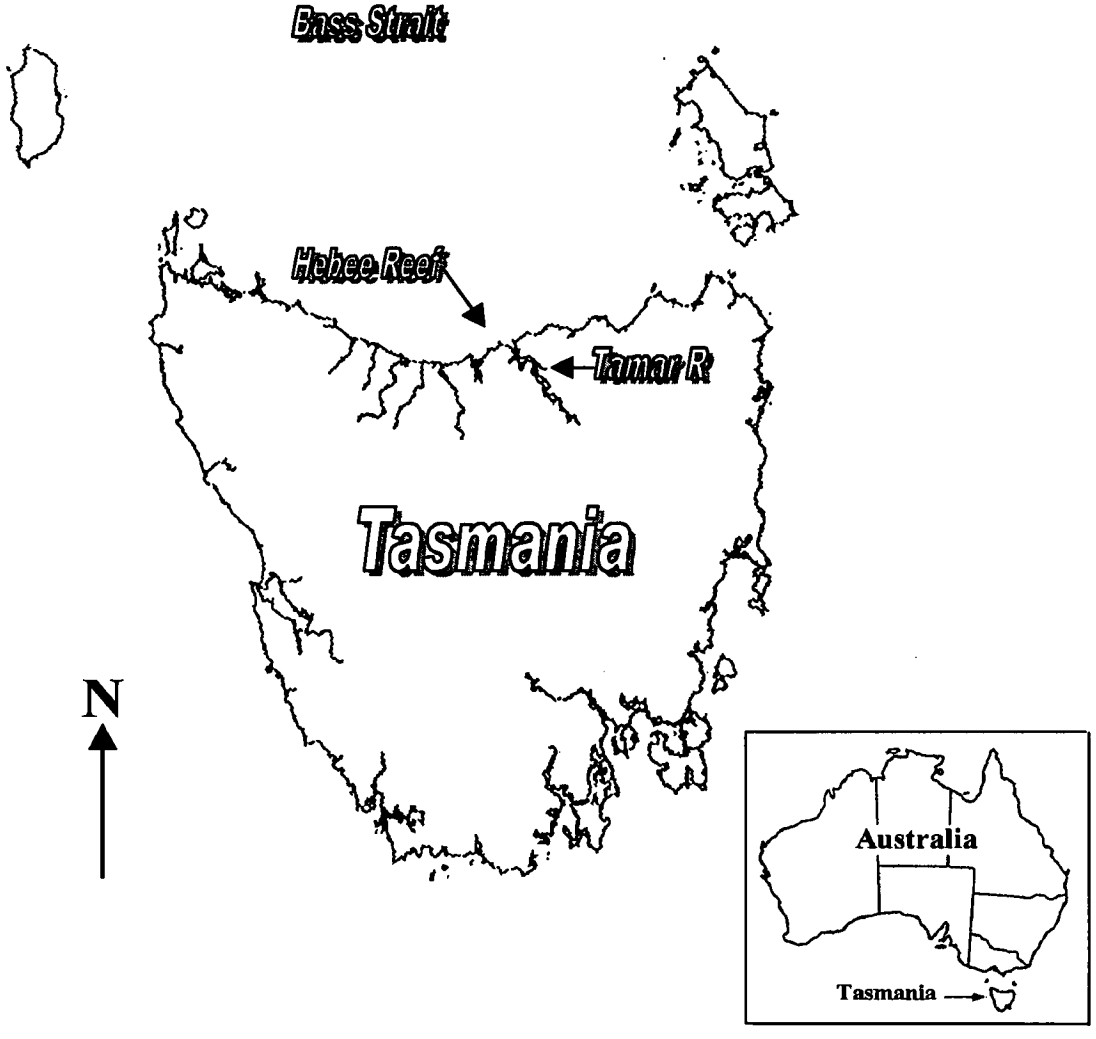



**Figure 3 Site of capture for horseshoe leatherjackets and bluethroat wrasse**

At each fishing location catch rates of bluethroat wrasse while line fishing generally declined relatively quickly and the vessel was moved regularly to maintain catch rates. The actual distance of the move generally only needed to be around 15-20m to realise renewed high catch rates. One or two large male wrasse were frequently caught among the first few fish captured from each location followed by a succession of

female/juvenile bluethroat wrasse of various sizes. It was rare to catch more than two males from any one location. The composition of the catch and decline in catch rates is consistent with localised depletion on a fine-scale of small territorial patriachal 'harems'. Divers have also reported harems for this species.

Relatively few fish showed obvious significant physical damage during hooking and landing (estimated to be <5%). All fish that were foul hooked (i.e. hooked elsewhere in the body other than the mouth) or fish that were obviously bleeding at time of capture were released.

In addition to line fishing, two fish traps were also deployed. Mortality of trap caught fish while being held aboard the vessel was very low and much less than that observed for fish taken by line fishing.

It is important to note that data recording during fishing was not attempted due to the rough conditions experienced, small vessel (<5m), small crew (1 or 2 persons only) and the need to ensure short overall fishing times to minimise for mortality within the onboard holding tank and maximise capture rates.

Experimental specimens of greenback flounder were obtained from cultured specimens bred and held at the National Key Centre for Aquaculture.

***Sea Transport***

Initially, fish were removed from the hook with no or minimal direct handling. Retained fish were held in a 200-l plastic container onboard the vessel. Oxygen was supplied via a pressurised bottle and diffuser. Oxygen saturation was monitored at all times during sea transport via a WTW OXI323 meter and probe. If oxygen saturation exceeded 102% at any time for more than a few moments, supply of oxygen was ceased until such time as levels had fallen back between 90-100%. Frequent water exchange was provided via a small submersible pump and/or by buckets. Fish to water ratios (by volume) in excess of 1:1 were maintained for up to three hours. Water motion during onboard holding was considerable both as a result of water changes and motion of the vessel.

The number of bluethroat wrasse with overt symptoms of barotrauma appeared to increase considerably with both size of fish and water depth. Large male bluethroat wrasse from deeper water (8-10m) appeared to exhibit this condition more often than not. It was common for large male 'floaters' to die within one hour of capture while being held in the onboard tank. In contrast, no external signs of barotrauma were observed in any horseshoe leatherjackets.

Regurgitation of bait and other food items (e.g. brittle stars) was common during the first 30min. after capture while in the transport tanks onboard the vessel. Regular water changes were required to maintain reasonable water quality. Any onboard systems used in a commercial context for these species should provide a ready means of clearing regurgitated matter from the system.

***Road Transport***

It was necessary to transport captured wrasse and leatherjackets to the National Key Centre for Aquaculture by road. A 1000-l cylindrical transport tank was filled with seawater and the fish transported by light truck. Transit time from loading to unloading of fish was never greater than 90min. Fish were supplied with oxygen via a submerged diffuser connected to a pressurised oxygen cylinder. Oxygen saturation was monitored at all times during road transport via a WTW OXI323 meter and probe. If oxygen saturation exceeded 102% at any time for more than a few moments, supply of oxygen was ceased until such time as levels had fallen back between 90-100%. Stocking density was relatively low and dissolved oxygen never fell below 90% of air-saturation. No mortalities were recorded during road transport.

***Holding***

Fish were carried onboard the boat until they were transferred to a holding cage in the river or transported directly by road to the National Key Centre for Aquaculture (Launceston, Tasmania, Australia) as described above.

On some occasions logistic constraints prevented the transport of the captured fish immediately back to the National Key Centre for Aquaculture. On these occasions the fish were held inside the river mouth at Low Head. This site was less than 500m from the entrance to the river. In the absence of floods, the salinity at this site would be very similar to that at Hebee Reef.

The river holding cage was cylindrical in shape some 2m deep and 1.2 m in diameter. The mesh was knotless salmon cage netting. The cage was weighted and positioned approximately mid-water in water 8-10m deep. Fish placed in the holding cage were then left there between one and three days before collection and road transport. Mortality data during this stage of holding were not collected due to known theft of fish from the cage by other persons, possible escape due to predator damage (most likely by seals) to the cage, and predation (including possible cannibalism).

Once transported to the National Key Centre for Aquaculture the fish were held in a 4000-l square recirculating seawater tank. This holding tank was fitted with a temperature control system, biofilter, solids settlement sump and aeration. The water temperature of the tank was initially set at the surface temperature of the water at the

location of capture/holding. The holding temperature was then gradually changed to 15°C. Other studies have generally used either ambient laboratory conditions (e.g. Lomholt & Johansen 1979) or the mean environmental temperature (e.g. Fernandes *et al.* 1995) as the acclimation temperature. An acclimation temperature of 15°C in this study is considered to be a reasonable estimate of mean environmental temperature.

Water quality was monitored and regular exchanges of 25% of the tank volume were undertaken to ensure that a high water quality was maintained throughout holding. Three days after transport, fish were offered food (squid, clams and mussels) followed by a mixture of these foods with pelletised trout and salmon food on subsequent days. Bluethroat wrasse readily accepted these pellets but horseshoe leatherjackets were never observed to consume any pellets. The minimum holding period prior to use in experiments was three weeks. Stocking rates of fish to water never exceeded 1:200 (weight for weight).

Horseshoe leatherjackets rapidly resumed feeding once transported to holding facilities. Most specimens accepted food offered by hand within three days of arrival in the holding tanks. Feeding resumption is socially facilitated with fish from previous trips stimulating feeding in new fish. This in turn reduced the time to initial captive feeding. The relative ease with which these fish become relatively "domesticated" is possibly an indicator of low stress levels in captivity.

Several minor problems with handling and holding of live horseshoe leatherjackets became apparent. The first was the risk to handlers from biting and caudal and dorsal spines. Much of the head area of the horseshoe leatherjacket is comprised of large jaw musculature and the single fused tooth in each of the upper and lower jaws possess considerable shearing power capable of causing considerable damage to handlers. Leatherjackets are renowned in the aquarium trade as substrate biters. Horseshoe leatherjackets displayed only low levels of substrate biting over the year they were held at the National Key Centre for Aquaculture. However, it was still necessary to shield submerged power cords and temperature probes from damage.

Both horseshoe leatherjackets and bluethroat wrasse exhibited persistent, intra-specific, territorial aggression particularly after introduction of new specimens to the holding tank. In an effort to reduce this aggression, several one-metre lengths of 300mm diameter PVC pipe were placed within the holding tank. These new structures considerably reduced observed aggression and subsequent physical damage to fish (Plate 6).

While more readily damaged during territorial battles, bluethroat wrasse also exhibited rapid healing of wounds. However, bite injuries on horsehsoe leatherjackets were

30% (9 of 31) of the horseshoe leatherjackets being held were euthanased following severe parasitic infections. A parasitic survey of these fish suggested that the opportunistic pathogens (i.e. *Euronema* spp., *Vibrio* spp. and *Flexibacter* spp.) were mainly responsible. In an effort to reduce mortality due to infection, damaged horseshoe leatherjackets were treated with a freshwater bath containing 100ppm trimethoprim for one hour. However, this treatment was only moderately successful and many fish did not recover despite treatment. The overall long-term mortality of horseshoe leatherjackets during holding was around 5%.

Some bluethroat wrasse were held for more than 12 months. Overall mortality of bluethroat wrasse during holding was less than 2%. Most of these mortalities were the result of fish jumping out of the holding tank.

**Plate 6 Lengths of 300mm diameter PVC pipe used to reduce territorial aggression**

Flounder were held in 1000l, recirculating, semi-oval, 'Reln' tanks within a temperature controlled 'reefer' shipping container. Water quality was monitored and maintained through water exchange when necessary. Flounder were fed on a

commercial, pellet diet. Mortality of flounder during holding was rare and the few deaths that did occur were due to fish jumping out of the holding tank.

## EXPERIMENTAL SYSTEMS

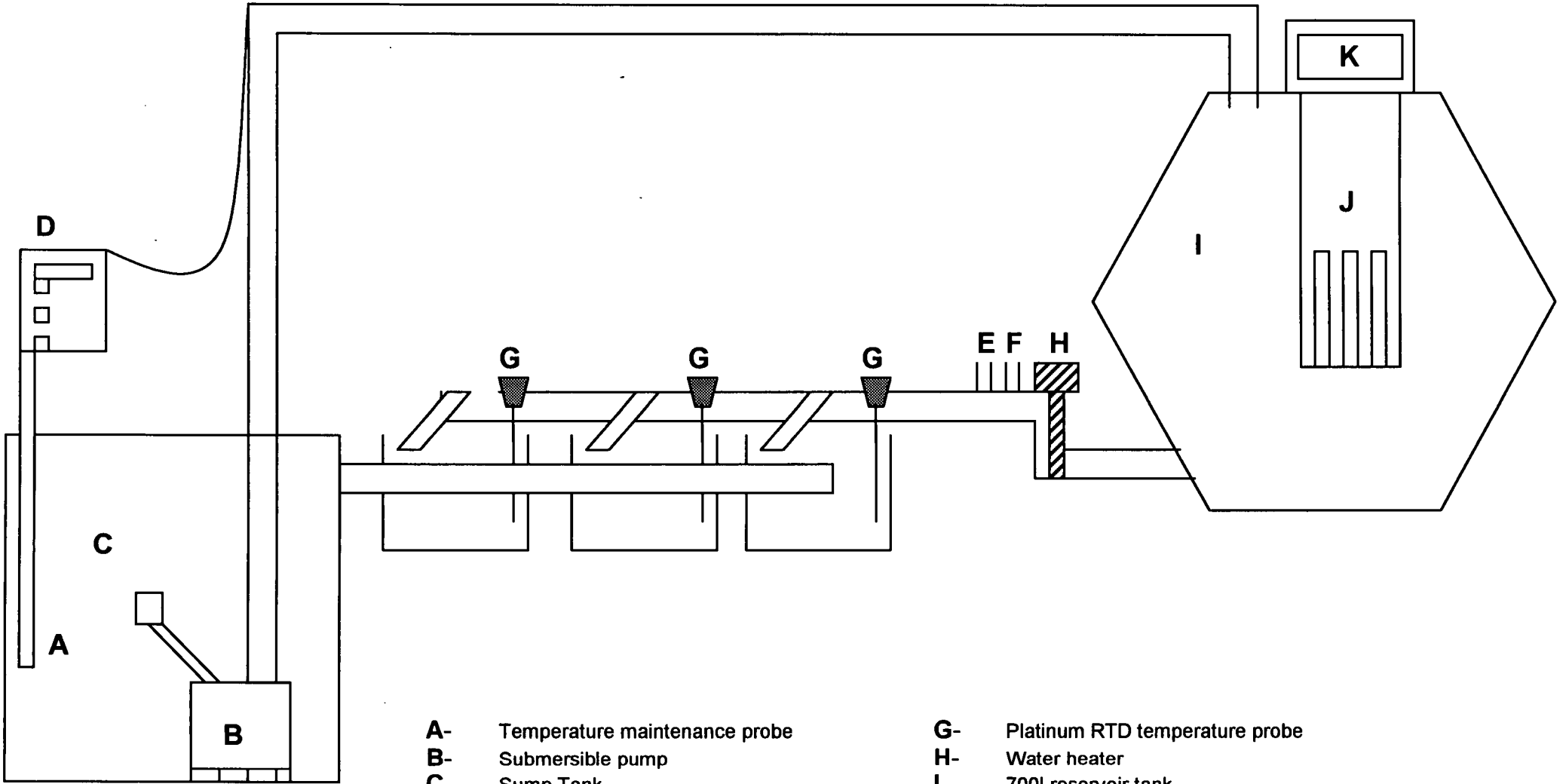
The room housing the experimental apparatus was an internal room (i.e. no exterior windows) and all internal windows were lined with opaque, black plastic to minimise uncontrolled variables such as ambient or artificial lighting or other visual stimuli.

### *Open, flow-through tanks*

The open, flow-through experimental system was used during thermal tolerance trials. It consisted of three 45l translucent plastic tanks arranged in parallel (i.e. with isolated inlet and outlet pipes) supplied with seawater from a common 700l header tank. The system could also be operated in series if required. A refrigeration unit connected to a titanium coil within the header tank was used to chill the water while a submersible aquarium type heater within the outlet pipe of the header tank provided the main heat source. The desired water temperature (in increments of 0.5°C) could be set using a Phasefale™ temperature control unit wired to a temperature probe and the heating and cooling elements of the system.

Gate valves controlled flow rates into each experimental tank. A graduated cylinder and stopwatch were used to measure flows in each experimental tank. The flow rates for all three tanks were experimentally equalised. A dissolved oxygen meter was used to check that the flow rate was sufficient to maintain oxygen saturation at or very near 100% saturation. Translucent plastic lids covered all experimental tanks. Platinum electrodes in each tank recorded the water temperature and these data were logged using a 'Datataker DT50' data logger (with PCMCIA card for additional memory). All attached probes reported data as continuous millivolt outputs and the logging regime was determined through original DeTerminal software programs.

A submersible pump in the sump tank was used to return the water to the header and mix and aerate the water within the sump. An aerator was also placed within the header tank to ensure oxygen saturation and minimise carbon dioxide levels. A schematic diagram and photograph of the experimental system are shown at Figure 4 and Plate 7 respectively.




**A-** Temperature maintenance probe
**B-** Submersible pump
**C-** Sump Tank
**D-** Phasefale temperature control
**E-** pH probe-WTW pH 323
**F-** Oxygen probe-WTW OXI 323
**G-** Platinum RTD temperature probe
**H-** Water heater
**I-** 700l reservoir tank
**J-** Platinum cooling coil
**K-** Heat exchanger

**Figure 4 Schematic diagram of open flow through used to study thermal tolerance**

**Plate 7 Photograph of the experimental system used in thermal tolerance experiments.**

***Flow-through respirometer***

While there are many different apparatus described in the literature to measure fish oxygen consumption these can generally be classified into four basic types as follows:

1. Closed or static systems which enable researchers to monitor the reduction of oxygen concentration within a single fixed volume (e.g. Humboldt & Provencal 1809, Heath & Pritchard 1965, Job 1969a, Sutcliffe *et al.* 1975, Caulton 1977, Lomholt & Johansen 1979, Jobling 1982, Verheyen *et al.* 1985, Forster 1990, Carlson & Parson 2001, Brick & Cech 2002). Some designs incorporate a blank control chamber (e.g. Marais *et al.* 1976, Subrahmanyam 1980, Cech *et al.* 1985). Closed systems include apparatus that generate a flow against which the fish swim (e.g. Steffensen *et al.* 1984, Martinez-Palacios & Ross 1986).

2. Flow-through systems which allow the measurement of the difference in oxygen concentration between afferent and efferent water using the Fick principle (Fick 1870) (e.g. Ege & Krogh 1914, Spoor 1946, Beamish & Mookherjii 1964, Brett 1965, Heath & Pritchard 1965, Jones 1971, Webb 1971a,b, Evans 1972, Caulton 1978, Cech *et al.* 1979, Lomholt & Johansen 1979, Ultsch *et al.* 1980, Jobling 1982, Hughes *et al.* 1983, Umezawa *et al.* 1983, LeMoigne *et al.* 1986, Atzuma & Itzawa 1993, Rantin *et al.* 1993, Fernandes *et al.* 1995, Oikawa & Itazawa 1995, Macisaac *et al.* 1997, Jo & Kim 1998, Kalinen *et al.* 1999, Kidder & Bell 1999).

3. van Dam-type respirometers which are essentially modified flow-through systems and measure oxygen consumption as the product of the difference in oxygen concentration between inspired and expired water and the volume of water. However, the inspired and expired water are separated by a physical barrier rather than mixed as in flow-through systems (e.g. van Dam 1938, Davis & Cameron 1971, Cech & Wohlschlag 1973, Burggren & Randall 1978, Campagna & Cech 1981, McKim & Goeden 1981, Daxboeck *et al.* 1982).

4. Manometric or volumetric system (e.g. Scholander *et al.* 1943, Oikawa & Itazawa 1995).

Somewhat surprisingly there are few direct comparisons of different apparatus described in the literature. It is therefore difficult to empirically compare the performance of these various apparatus. Most comparisons that have been reported are limited to either theoretical comparisons (Steffensen 1989, Cech 1990) or only two system variations (Gerkhe 1993).

Oikawa & Itazawa (1995) compared three different systems (i.e. flow-through, intermittent and manometry) used in estimation of oxygen consumption in carp. The main conclusion from these authors was that each system resulted in different levels of activity in carp and hence different measured metabolic rates. Hence it is clear that inter-study comparisons need to take the apparatus into account when trying to draw conclusion about species or treatments.

Unfortunately, Oikawa & Itazawa (1995) did not include a closed respirometer in their comparison but various other authors (Forstner 1983, Steffensen 1989, Cech 1990) recommend that researchers avoid closed respirometry, especially during long-term studies, as it results in confounding accumulation of $CO_2$ and other waste products as well as large changes in ambient oxygen concentration (closed systems essentially simulate many live transport container systems). The main problem with closed respirometry is that fish are exposed to graded hypoxia that is likely to have

different but equally problematic implications for conforming and non-conforming species. The oxygen consumption of conformers will be under-estimated by closed respirometry. Conversely the oxygen consumption of many non-conformers increases during slight to moderate hypoxia and closed respirometry is therefore likely to over-estimate consumption in these species.

A further problem in the use of closed respirometry involves the potential for gas supersaturation when the temperature of the water is increased. Indeed, Cech (1990) refers to the benefits of using transparent containers to "make it easier to detect bubbles, which must be removed because the air they contain is rich in oxygen...". In addition to the potential errors in respirometry due to bubble formation, gas supersaturation results in a risk of gas bubble disease as discussed in Chapter 2. These criticisms of closed respirometry resulted in the rejection of a closed system for this present study.

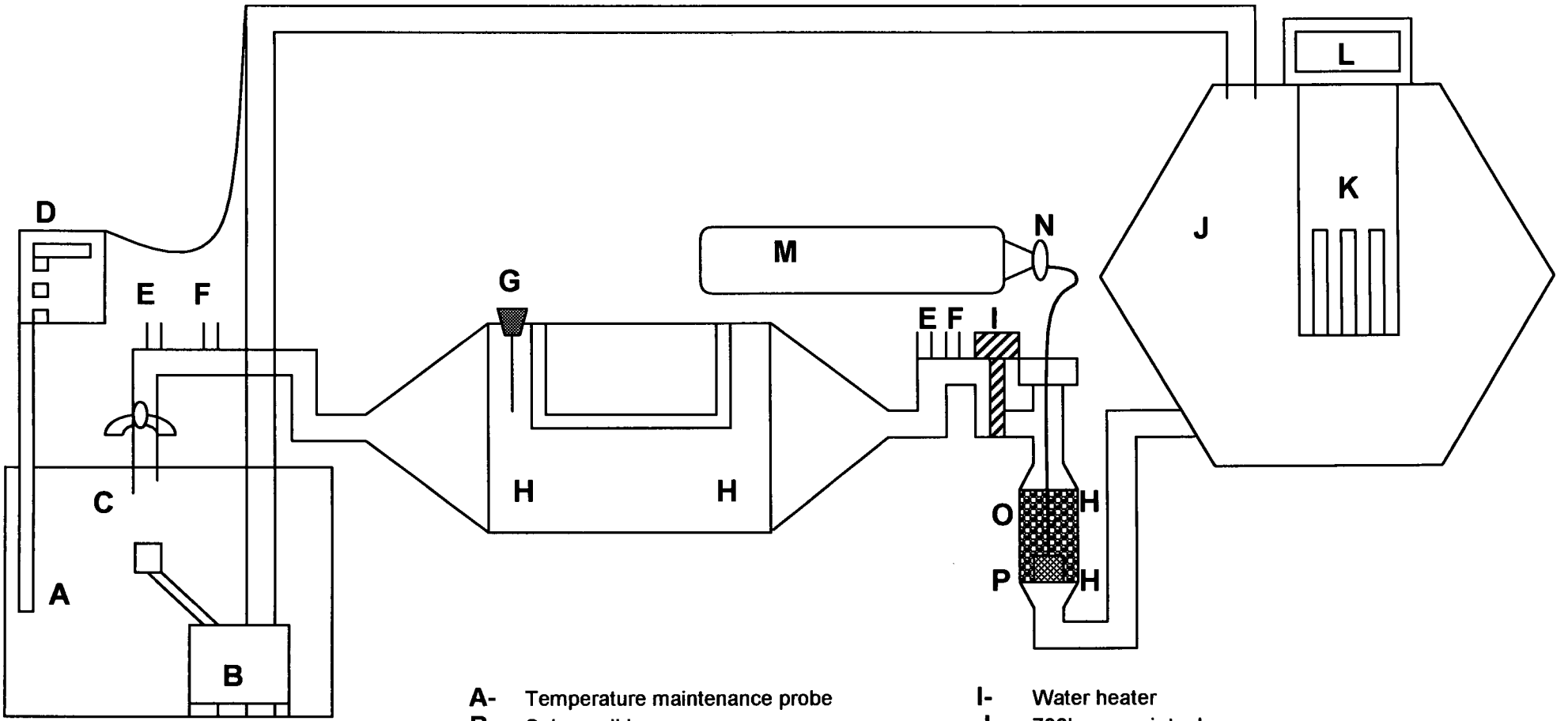
Steffensen (1989) states that, due to difficulties in correcting for the washout effect, that intermittent flow is preferable to flow-through systems. However, the author fails to provide sufficient support for rejection of flow-through systems in favour of intermittent flow. By applying the author's own calculations, intermittent flow does nothing more than provide repeated (albeit smaller) errors that, ultimately, must be summed and will then equal those present in the flow-through system. Intermittent flow greatly increases the complexity of correction based on the Fick Principle and has the potential to increase activity through large variation in water flow. Never-the-less, intermittent flow respirometers avoid many of the problems associated with closed systems and in recent years such systems have seen growing use (e.g. Franklin *et al.* 1995, Oikawa & Itazawa 1995, Herskin 1999)

Furthermore, Steffensen (1989) assumed that the sole reason for the lag in recorded oxygen consumption behind a change in activity is solely due to the reservoir or wash-out effect. However, this lag also incorporates hysteresis between sensory and ventilatory systems and respiration. Hysteresis in ventilation by humans in response to respiration changes is considerable (Eckert *et al.* 1988) and its effect upon the lag in measured oxygen consumption in fish cannot be discounted at present. If hysteresis is significant it will cause acute problems for systems using intermittent flow. In conclusion, flow-through systems should not be rejected in favour of intermittent flow and a flow-through system was chosen for this study.

The basic design of the flow-through respirometer used in this study closely follows the design detailed by Cech *et al.* (1979). This design is easily modified for use as a fully closed flow-through system (or "Brett" type), intermittent system or static, closed system. Within the experimental system, the respirometer replaced the three

open flow-through tanks in the system described above. A schematic diagram and photograph of the respirometer is shown at Figure 5 and Plate 8.

In addition to experimental manipulation of temperature and water flow (as for the three tank system), the partial pressures of dissolved gases (e.g. oxygen and carbon dioxide) within the respirometer could be experimentally manipulated by incorporating a gassing column between the header tank and chamber containing the fish. The concentration of gases and pH of water was monitored through the use of calibrated and corrected dissolved oxygen (WTW OXI323) and pH (WTW pH323) meters in-line on the afferent and efferent pipes of the respirometer. The dissolved oxygen and pH meters produced millivolt outputs that were connected to the data logger.




**A-** Temperature maintenance probe
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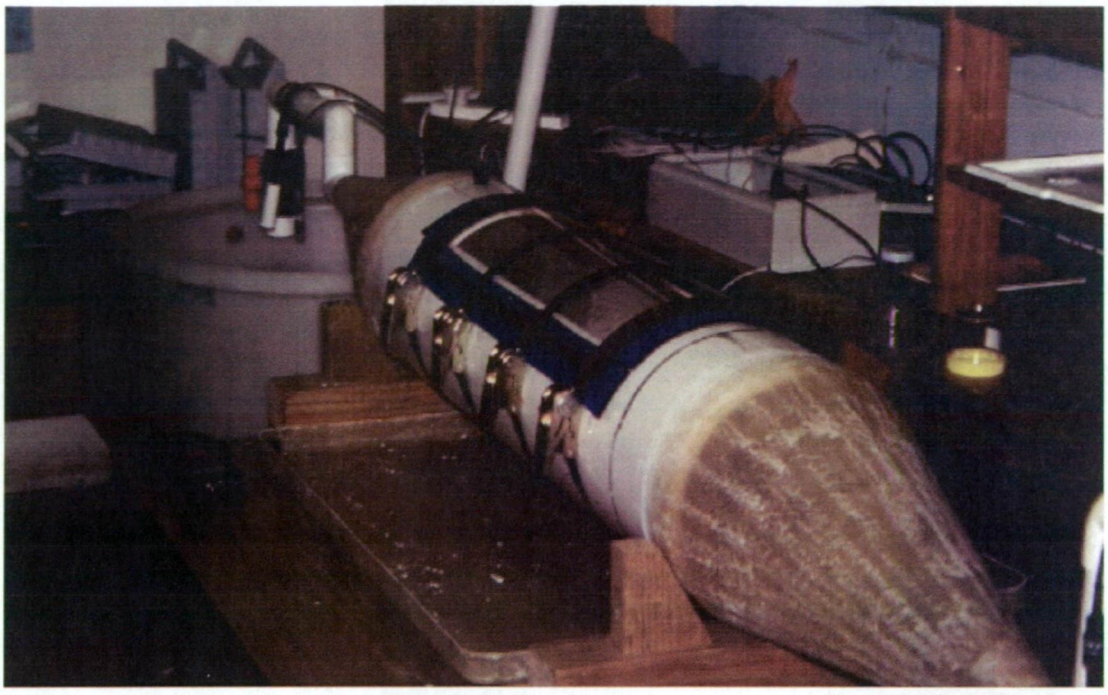
**H-** Baffle plates
**I-** Water heater
**J-** 700l reservoir tank
**K-** Platinum cooling coil
**L-** Heat exchanger
**M-** Gas cylinder
**N-** Regulator
**O-** Glass marbles
**P-** Diffuser stone

**Figure 5 Schematic diagram of respirometer**

**Plate 8 Respirometer used to measure oxygen consumption.**



Within all respirometers stratification and eddies of non-mixed water can result in measurement errors (Steffensen 1989, Cech 1990) and while this is generally less problematic in flow-through systems, perspex baffles bounded the chamber containing the fish to assist mixing of water. In addition, smooth-walled cones with a gradual taper were used in transition areas between sections of different diameter. The design of the system also allowed all air to be vented from the portion of the system between the dissolved oxygen probes. This feature avoided any potential interchange across gas-water boundaries, affecting the measurements.

A clear viewing window made from curved perspex allowed direct behavioural observations. During most experiments, this viewing window was covered with opaque material to reduce visual stimulation.

The temperature and oxygen concentration within the fish chamber could be monitored directly through probes attached to rubber bungs in the viewing window. In addition, catheters and tubes for direct sampling from the chamber and/or from the fish were also inserted using the same system.

Initially, it was intended that a van Dam type respirometer would also be trialed for comparison to the results from the flow-through design however, despite several applications, the Animal Ethics Committee of the University of Tasmania did not grant approval for use of this apparatus.

Oxygen concentration readings were taken via calibrated and difference-corrected oxygen probes (WTW OXI-323) before and after the chamber containing the fish(es). These readings were then paired after correcting for the time required for water to move between the two probes (i.e. washout time). The oxygen consumed could then be calculated by subtracting the efferent reading from the afferent reading and multiplying by the rate of water flow past the fish.

As noted by Niimi (1978), it is vital to adjust for the lag in the measured response of the respirometer. The appropriate pairings of afferent and efferent readings were determined by correcting for the washout time (calculated by dividing the volume (l) between the two probes by the rate of flow (l/min)). The rate of flow was controlled by a gate valve on the inlet pipe and was calculated using the average of three measurements of the time taken to fill a one litre volumetric flask from the outlet pipe. The time was measured using a stopwatch.

The oxygen consumption as measured in the respirometer was calculated as follows:

$$\mathbf{O_2\ Consumption\ (mg\ O_2.kg^{-1}.hr^{-1}) = ((A_t - E_{t+w}) \times R \times 60) \div Wt}$$

where:

| | | |
|---|---|---|
| $A_t$ | = | mean concentration of oxygen in afferent water (mg $O_2.l^{-1}$) for a given minute at time t |
| $E_{t+w}$ | = | mean concentration of oxygen in efferent water (mg $O_2.l^{-1}$) for a minute at time t+w |
| w | = | washout time (min.) calculated by dividing the volume (112.708l) between the probes by the rate of flow ($l.min^{-1}$) |
| R | = | rate of water flow through the respirometer ($l.min^{-1}$) |
| Wt | = | total wet weight of fish in respirometer (kg) |

The level of oxygen consumption within the system was generally undetectable in the absence of fish and was usually within the small error associated with the probes. The oxygen consumption of the blank system (i.e. without fish) was measured before and after each trial and all experimental consumption figures were adjusted by the average of these values if there was detectable consumption. Once the system consumption exceeded 1-2mg $O_2.hr^{-1}$ in the absence of any fish, the system was drained, disinfected with chlorine in freshwater and allowed to air dry before further trials were undertaken.

Noting the concerns raised by Steffensen (1989) with regard to the difficulty in correcting for the washout effect during flow-through respirometry, preliminary trials

were undertaken to test the validity of the washout correction applied in this study (at *w* above). These trials are described below.

***Testing the Correction for Washout Effect***

Greenback flounder were chosen for the investigation of the washout effect correction (described above) because they spend most of their time sitting on the bottom rather than swimming in the water column. As a result of this behaviour, the effect of different water flow rates on the exercise rate (and hence respiration and oxygen consumption) was expected to be considerably lower for greenback flounder relative to the other two species.

Ten flounder acclimated to 15°C were starved for 24hrs and placed in the respirometer and left for 24hrs at an experimentally constant temperature of 15°C (Day 1). During Day 2 the temperature of the water flowing through the respirometer was cyclically cooled and warmed between 13°C and 15°C for seven hours on each of three consecutive days. On each day, a different water flow rate was applied (i.e. 2, 4 & 6 $l.min^{-1}$). During these trials the oxygen consumption was measured and calculated using the formula described above with three different washout correction factors (i.e 56, 28 and 19 minutes respectively). The oxygen consumption values derived from the formula for each different rate of flow are shown in figure 6. It should be noted that the WTW OXI323 dissolved oxygen meters used in this study have an integrated pressure sensor to correct for pressure change corresponding with different flow rates.

The time difference between the peak in oxygen consumption and peak in water temperature was compared for each rate of flow. There was no significant difference in the lag in oxygen consumption between different rates of flow (P=0.8311, d.f.=2). This result supports the washout correction formula used in this study.

However, the results of this experiment (Figure 6) clearly show that a lag does exist between changes in water temperature and oxygen consumption. Moreover, the shape of the oxygen consumption plot is not simply a phase shift of the temperature plot smoothed by hysteresis as may be expected for a poikilothermic organism. These observations will be discussed in Chapter 6.

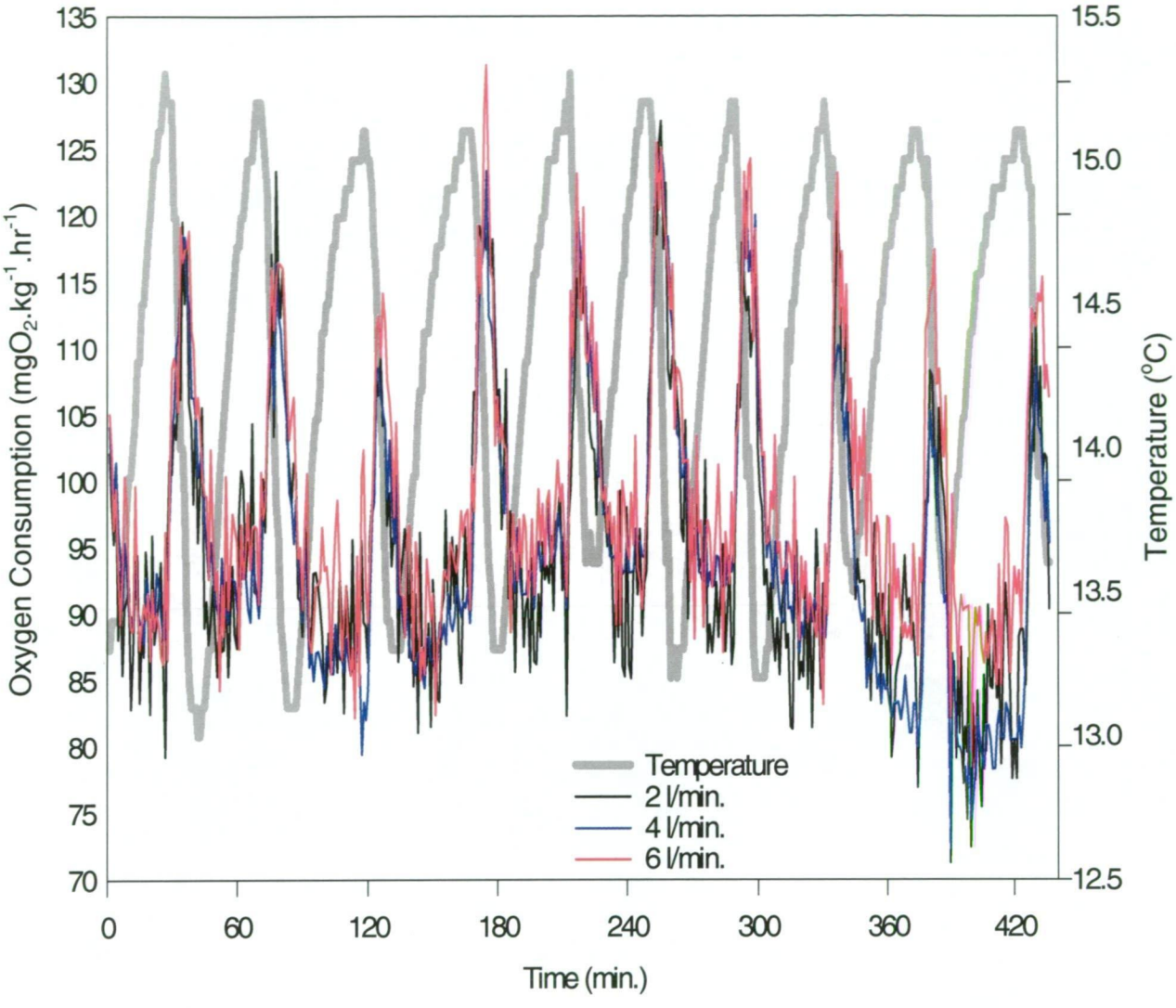



**Figure 6 Fine scale oxygen consumption of greenback flounder at three different rates of water flow.**

## DATA ANALYSIS

The logged data were downloaded to a laptop PC using DeTerminal and DeCopy software and then merged into an Excel spreadsheet. All data were analysed using either SAS 6.11, Data Analysis Toolpak of Excel '98 or SigmaPlot 6.1. A significance level of 0.05 was used for all tests of significance.

# CHAPTER 4

# THERMAL TOLERANCE



## INTRODUCTION

The habitats in which fish occur range in temperature from –1.86°C in high latitude, marine waters to greater than 40°C in waters such as desert springs, streams and very shallow tropical waters (Hazel 1993). However, in the wild it is uncommon for fish to be exposed to rapid fluctuations in temperature and any significant changes in temperature generally involve time periods of weeks or even months.

Fish exhibit a range of behavioural and physiological responses to temperature change. The principle method of thermoregulation in poikilothermic fishes is to actively seek out water of a temperature at or near some preferred level (Eckert *et. al* 1988, Siemen & Stauffer 1989, Heath *et al.* 1993). During live fish supply processes and, in particular transport, changes in temperature can be marked and sudden. As a result of their confinement, fish in transit are denied the opportunity to seek favoured temperatures to mitigate against such changes. Therefore, live fish suppliers must, at a minimum, ensure temperatures remain within the limits for survival. Indeed, failure of many live fish shipments is attributable to fish being left in an exposed position on airport tarmacs in very high temperatures and full sun during air transport (Anon. 1995 a,b).

While it is readily accepted that the success of live shipment is fundamentally dependent upon ensuring that the temperature of the shipment does not breach upper or lower lethal limits, it is the application of lowered temperature (within lethal limits) that is investigated in this thesis.

As noted in earlier chapters, dissolved oxygen is probably the most critical limiting factor in the cost-effective supply of live fish. There are only two ways to reduce the constraints of oxygen delivery within live fish transport. These are to increase the quantity of oxygen available and/or reduce the demand for oxygen.

Like all gases, the solubility of oxygen in water is increased as temperature decreases. Therefore, in an open system, the oxygen available in a given volume of water increases with decreasing temperature (Fry 1957, 1967, 1971, Hutchinson 1957) Figure 7. However, it is in the area of reducing oxygen demand during transport where temperature reduction offers the greatest potential benefits.

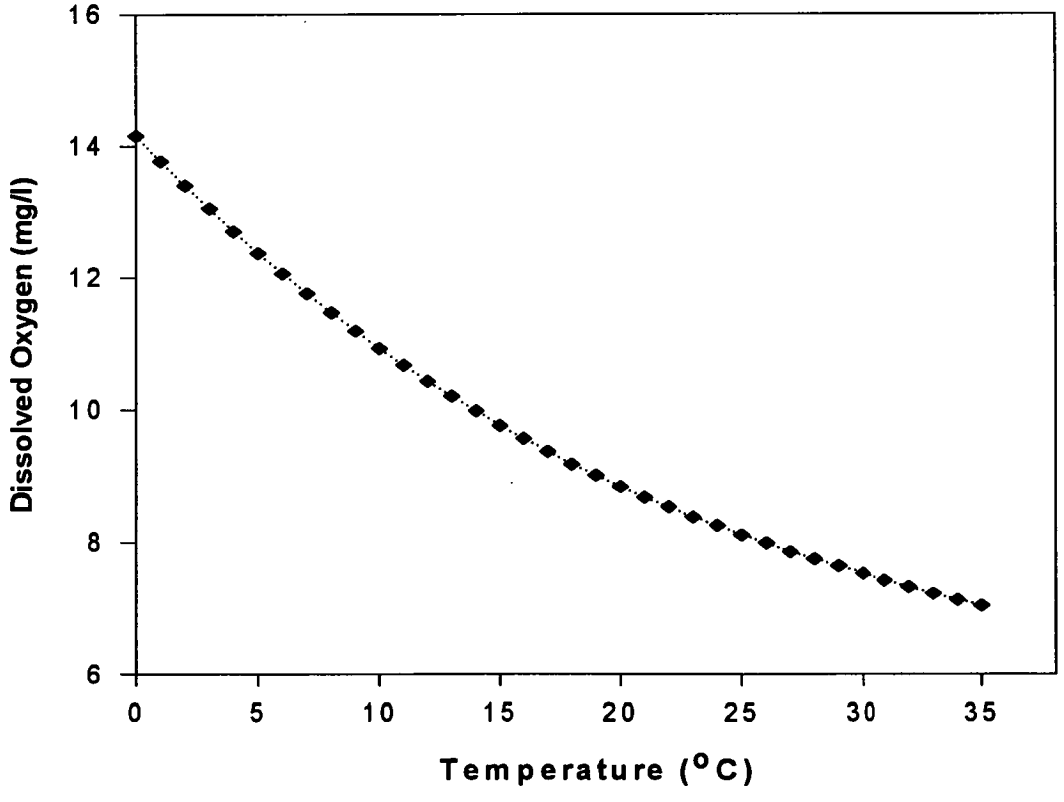



**Figure 7 Change in saturation levels of dissolved oxygen in freshwater with temperature. The graph indicates that the amount of dissolved oxygen contained in a given volume of freshwater at 5°C is 26.7% greater than the same volume at 15°C. Adapted from Hutchinson (1957).**

Enzymes catalyse most chemical processes in living organisms, including fish. Enzymatic reaction rates are highly temperature-dependent (Eckert *et al.* 1988). The temperature dependence of a reaction is described by the *Arrhenius equation:*

$$\mathbf{k = Ae^{-Eq/RT}}$$

where $k$ is the velocity constant of the reaction, $A$ is the constant related to collision frequency of molecules, $E_q$ is the activation energy and $e$ is the base of natural log, 2.72, R is Rydberg's constant and T is the temperature (°C).

When considering the influence of temperature on the rate of a reaction a temperature difference of 10°C has become a standard, arbitrary span over which to determine the temperature sensitivity, or temperature quotient, of a biological function. It is important to note that $Q_{10}$ has no theoretical basis but instead is an entirely empirical value. The $Q_{10}$ is calculated using the altered *van't Hoff equation:*

$$\mathbf{Q_{10} = (\eta_2/\eta_1)^{10/(t2-t1)}} \qquad \textbf{or} \qquad \mathbf{Q_{10} = \eta_{(t+10)} / \eta_t}$$

for temperature intervals of precisely 10°C

where $\eta_1$ and $\eta_2$ are the metabolic rates at temperatures $t_1$ and $t_2$ respectively.

For a given enzymatic reaction, the $Q_{10}$ differs over different temperature ranges. However, in general, chemical reactions have $Q_{10}$ values of around two to three, while purely physical processes, such as diffusion, have lower temperature sensitivities (i.e. closer to one) (Eckert *et al.* 1988). Thus, decreases in kinetic energy (and therein temperature as its measure) reduce the rate of all chemical reactions and exponentially decrease metabolic reactions that consume oxygen.

The vast majority of oxygen consumed metabolically by most animals is used for respiration (Eckert *et. al* 1988). The term respiration is used inconsistently in the literature but is defined for the purposes of this thesis as the catabolic reaction in which proteins, lipids and/or carbohydrates are oxidised to generate cellular energy in the form of adenosine triphosphate (ATP). By definition, a temperature reduction that results in a reduction in the rate of respiration will reduce the oxygen demand.

The effect of small increases in activity resulting in large increases in respiration and, subsequently, oxygen consumption and metabolic waste production is well known (Krogh 1914, Spencer 1939, Spoor 1946, Higginbotham 1947, Graham 1949, Prosser *et al.* 1957, Basu 1959, Brett 1965). Even when external stimuli are essentially eliminated some fish still exhibit a considerable amount of spontaneous activity (Beamish & Mookherji 1964).

Fish, like all poikilothermic animals, exhibit reduced activity in response to decreases in temperature. Reductions in temperature can cause extreme lethargy and induction of pseudo-hibernation or coma in fish. Such states of decreased activity greatly reduce the oxygen demands normally generated by activity and movement.

Krogh (1914) defined standard metabolism as the "nearest attainable approximation to the basal metabolism which would be obtained when all organs were absolutely at rest." Obviously, in live transport the closer the metabolic rate of the fish to standard metabolism the lower respiration and oxygen demand are likely to be. The difficulty in attaining and measuring standard metabolism in fish is eliminating spontaneous activity.

Investigators who encouraged quiescence have recorded lowered rates of oxygen consumption such that these rates were considered to be close approximations of true standard metabolism (Graham 1949, Job 1955, Mann 1956, Pritchard *et al.*1958, Hickman 1959, Moss & Scott 1961).

The anaesthetic-like effects of reduced water temperature on fish are well known (Doudoroff 1942, Randall & Hoar 1971, Mittal & Whitear 1978, Yoshikawa *et al.* 1989). The use of lowered temperature to induce a state of pseudo-hibernation or coma prior to packaging reduces handling time and associated stress and potential physical damage (Terchunian *et. al* 1999). However, induction and maintenance of coma requires careful temperature control and the most appropriate temperature regime varies with different species. As a result of this variability, Huss (1995) considered that the use of chilling to induce coma should be considered an experimental technique for most species.

The affinity of haemoglobin for oxygen is labile and it increases with decreasing temperature (Eckert *et. al* 1988). Thus, gas exchange from water to blood is more efficient at lower temperatures reducing the required ventilation rate and its resultant oxygen demand.

In addition to reducing the respiratory demands of fish in the transport container, lowered temperatures reduce growth and replication of microscopic organisms such as bacteria, algae and other protozoans. Bacterial oxygen demand in transport systems can contribute significantly to the total biological oxygen demand (BOD) of a shipment and subsequently increase the mortality of fish (Steffensen 1989, Huss 1995, Terchunian *et al.* 1999).

Beyond reducing bacterial BOD, the use of temperature to reduce bacterial proliferation has other advantages. Some species of bacteria can clog fish gills during transport (Terchunian *et. al* 1999) thereby reducing gas exchange efficiency and consequently increasing respiratory demand as the fish increases ventilation to compensate for this reduction.

Opportunistic pathogens such as *Vibrio* spp. can more readily infect fish which are physically damaged and immunologically compromised as is often the case with fish during live fish supply processes. Such infections can greatly reduce product quality and may result in added mortality.

In addition to assisting in improved dissolved oxygen balance, reduced rates of reaction as a result of chilling reduce the amount of toxic waste products from these reactions such as ammonia, nitrite and carbon dioxide which are toxic to fish (Huss 1995, Terchunian *et al.* 1999).

Lowered water temperature has been observed to reduce stress responses and indicator levels in fish. For example, when exposed to the same stress inducing treatment (handling and confinement), levels of several stress indicators were two to four times higher in roach, *Rutilus rutilus*, acclimated and held in water at 16°C relative to those of fish acclimated and held in water at 5°C (Pottinger 1999).

In summary, lowered body temperature (within lethal limits):

- decreases metabolic oxygen demand in teleosts (Brett 1962, Beamish & Mookherjii 1964, Job 1969, Spitzer *et al.* 1969, Beamish 1970, Edwards *et al.* 1970, Edwards 1971, Fry 1971, Cech & Wohlschlag 1973, Caulton 1978, Cech *et al.* 1979, Ott *et al.* 1980, Priede & Holliday 1980, Jobling 1982, Kasim 1982, Hughes *et al.* 1983, Martinez-Palacios & Ross 1986, Eckert *et al.* 1988, Fernandes & Rantin 1989, Fernandes *et al.* 1995, Huss 1995, Terchunian *et al.* 1999);
- increases oxygen availability in an open system (Fry 1957, 1967, 1971, Hutchinson 1957);
- improves handling (de Guingand 1995);
- reduces stress (Steffens 1996, Pottinger 1999);
- increases efficiency of oxygen supply to tissues (Eckert *et al.* 1988);

- decreases production of harmful metabolic waste products (Huss 1995, Terchunian *et al.* 1999); and
- decreases proliferation of bacteria and other microscopic organisms (Steffensen 1989, Huss 1995, Terchunian *et al.* 1999).

While the potential benefits of reduced temperature within live fish transport are considerable, as stated earlier, low temperatures can be fatal. There are wide differences in the temperature tolerances and optimal acclimation rate of various species (Huss 1995) and these vary with the acclimated temperature (Steffens 1996, Terchunian *et al.* 1999).

The term *acclimation* is used in this thesis to describe the specific physiological changes in response to a laboratory condition (such as water temperature in holding tanks). *Acclimatisation* is the gradual, compensatory biochemical adaptation that occurs over the course of several weeks to altered environmental conditions in a natural setting (Eckert *et. al* 1988). The issue of temperature acclimatisation or adaptation in fishes has received considerable attention in the literature. For reviews of this work the reader is referred to Brett (1970), Hochacka & Somero (1984) and Hazel (1993).

The effect of temperature acclimation has been described at molecular, tissue and whole animal levels. Within lethal limits, the effect of temperature reduction at different acclimation temperatures remains relative. That is to say that a given reduction in temperature (say 5°C) will have a similar effect on responses of a given species of teleost at different acclimation temperatures (Brett 1952, Stauffer *et al.* 1984a,b, Rantin & Petersen 1985, Heath *et al.* 1993, Steffens 1995).

It is reasonable to expect that modification of enzymes is involved with acclimation and the modification of enzymes can take up to four weeks (Love 1980). Thus, in live fish supply processes it is generally impractical to acclimate fish to a lower temperature prior to shipment and as can be seen above largely unnecessary. It is important to understand that it is the difference in the temperatures that is often more significant than the absolute levels when determining the most appropriate transport temperature.

Extremes of temperature, beyond the tolerance of the organism are ultimately lethal. Bennett & Judd (1992) recognised that temperature and time acted in combination with respect to determining survival. These authors listed three zones to describe thermal tolerance:

1. *Lethal Zone* – conditions intolerable for any period of time, death due to cold a certainty.
2. *Resistance Zone* – fish may survive for limited periods; death is a function of temperature and time.
3. *Tolerance Zone* – death is no longer a function of temperature: survival time infinite.

Critical thermal determination and incipient lethal temperature determination are the two main methods of describing thermal tolerance listed in the scientific literature. Critical thermal determination involves change of the water temperature until some identifiable endpoint is reached. The dynamic Critical Thermal Temperature is the mean of the temperatures at which the endpoint was observed. The endpoint may be loss of equilibrium (e.g. Cox 1974) or death (e.g. Bennett & Judd 1992, Steffens 1996). The lower Critical Thermal Temperature is most often referred to as the Critical Thermal Minima, $CT_{Min}$.

The use of death as an endpoint would appear to pose significant problems in the description of $CT_{Min}$. The determination of the point of death due to cold exposure in fish is very difficult and is best determined by a failure to recover. A continuous decrease in temperature without attempted recovery leaves considerable margin for error in determination of the point of death.

As death is the outcome of primary interest in the context of live transport it was decided to retain death as the reference point in the dynamic temperature approach. However, in this study the continuous temperature decrease was replaced with a series of minimum temperature points that, once reached, would trigger the gradual warming of the water back to the acclimation temperature.

This change in method makes calculation of $CT_{Min}$ statistically invalid because the $CT_{Min}$ descriptive statistic is calculated as the mean temperature at which the reference condition is reached (e.g. death or loss of equilibrium). Under a recovery protocol not all fish in the trial may reach this reference point and as such will not be included in calculation of the $CT_{Min}$. However, if there is a reasonable spread of minimum temperatures between 0 and 100% mortality the value of $CT_{Min}$ calculated using this protocol will retain some merit. Indeed this statistic may be of more value than that calculated using continuous temperature change (and death as the endpoint) in the context of developing live transport methods. An alternative descriptive statistic to describe thermal tolerance under a dynamic temperature regime will be presented later in this section.

The rate of temperature change varies considerably among workers making comparisons of the impact of rate of change difficult. In addition, Reynolds *et al.* (1976) noted that results may be affected by the time lag between water and body temperature. Elliot (1981) suggested that the prime requirement is that there should be no significant time lag between internal fish temperature and water temperature however this is not feasible for many focal species and hence the potential for a lag in body temperature following changes in water temperature must be taken into account when designing and comparing experiments.

The rate of thermal exchange of body temperature with change in ambient temperature is governed by Newton's law of excess temperature which states that the instantaneous rate of thermal exchange is directly proportional to the slope between ambient and body temperature. The expression is $Y=Y_o e^{-kt}$ where $Y_o$ is the initial excess body temperature, Y is the body temperature (°C) at time t, and k is the coefficient of thermal exchange.

In obligate ectotherms there will be a time delay in body temperature behind changes in water temperature. The magnitude of this delay has been observed to differ according to a number of factors including body mass, surface area (Stevens & Fry 1974, Mueller 1976, Spigarelli *et al.* 1977, Elliot 1981), thermal inertia of tissue heat capacity, physiological factors (Crawshaw 1976) and chemical anaesthesia (Mueller 1976). The half-life for thermal exchange for a 3.6kg lake trout, *Salvelinus namaycush*, was 13 min while that of 30g alewife, *Alosa pseudoharengus*, was one min (Spigarelli *et al.* 1977). When determining the rate of temperature change to be applied during experiments consideration should be given to thermal inertia (i.e. high rates of change are likely to give poor estimates in larger fishes).

The Incipient Lethal Temperature, ILT, is defined as the temperature beyond which an organism can no longer live for an indefinite time (i.e. the temperature of the finite division line between the zone of tolerance and the zone of resistance) (Fry 1971). Beyond the ILT, death is a function of both temperature and time. For a given temperature beyond the ILT, the "effective time" is the period of time required to bring about the lethal effect. Naturally, there is an upper incipient lethal temperature ($ILT_{Max}$) and a lower incipient lethal temperature ($ILT_{Min}$).

ILT determination has classically involved abruptly shifting the fish from the acclimation temperature to the test temperature. Rather than endeavour to measure ILT against what may ultimately be an indefinite period by definition, most workers have described a value for the temperature that is lethal to 50% of the fish (Hoff & Westman 1966, Elliot 1981, Bennett & Judd 1992, Steffens 1996) for a given period of exposure (e.g. 24hrs). This value is referred to as both the $LD_{50}$ (Fry 1971) and

the $TL_{50}$ (Bennett & Judd 1992). Just as the dynamic method confounds temperature with time, the incipient lethal or static method adds handling stress and potential thermal shock to the physiological effect of the temperature variable (Reynolds *et al.* 1976).

The $TL_{50}$ value is determined by first recording the survival for a range of temperatures and interpolating a line of best fit for data points between 0 & 100% survival (including the first point for 0% survival and the first 100% survival point). The temperature equivalent to 50% survival (i.e the $TL_{50}$) can be calculated derived by solving the equation for the regression line for temperature ($x$) when the survival ($y$) is set at 50%.

The $ILT_{Max}$ generally increases linearly with increasing acclimation temperature, until a plateau is reached. The $ILT_{Min}$ values, however, may either be linear (Woo & Fung 1980, Rantin & Petersen 1985, Fernandes & Rantin 1986) or non-linear (Brett 1952).

By definition the ILT is a function of time and temperature. As noted the determination of $ILT_{Min}$ uses an experimentally constant lowered temperature over a fixed period. However, once below the ILT there is no theoretical difference between constant and variable temperature in that both can be represented as a function of temperature and time (e.g. with degree.minutes as the units). Thus, once below the ILT the 'dose' of either a constant or variable temperature that is lethal to 50% of the fish can be described and compared. For experiments in which a dynamic temperature change was applied both a $TL_{50}$ and $CT_{Min}$ value were calculated.

Both $ILT_{Min}$ and $CT_{Min}$ are species-specific (Doudoroff 1942, 1945, Brett 1952, Reynolds *et al.* 1976, Chung 1980, Elliot 1981, King *et al.* 1985, Yoshikawa *et al.* 1989, Chung & Mendez 1993, Elliot *et al.* 1994, Richardson *et al.* 1994, Elliot 1995, Bennett *et al.* 1997, Smith & Fausch 1997, Walsh *et al.* 1997, Currie *et al.* 1998) and affected by a range of factors including genetic strains (Bulger & Schultz 1979, *et al.* 1985, Procarione & King 1993, Meffe *et al.* 1995, Myrick & Cech 2000), and thermal history (Brett 1952, Woo & Fung 1980, Elliot 1981, Jain & Garg 1984, King *et al.* 1985, Rantin & Petersen 1985,Yoshikawa *et al.* 1989, Elliot 1991, Al-Habbib & Yacoob 1993, Procarione & King 1993, Meffe *et al.* 1995, Korhonen & Lagerspetz 1996, Bennett *et al.* 1997, Lyytikaeinen *et al.* 1997, Smith & Fausch 1997, Tsuchida *et al.* 1997, Walsh *et al.* 1997, Currie *et al.* 1998, Bravo *et al.* 1999, Chippari-Gomes *et al.* 1999, Aho & Voranen 2001, Chung 2001).

Only four studies have estimated both $ILT_{Min}$ and $CT_{Min}$ for a given species (i.e. Pitkow 1960, Elliot 1981, Bennett & Judd 1992, Steffens 1996) and, of these, only Bennett and Judd (1992) and Steffens (1996) made a statistical comparison between the two methods of describing thermal tolerance. Those studies found that a significant difference did exist between the $TL_{50}$ and the $CT_{Min}$ for pinfish, *Lagodon rhomboides* and bluethroat wrasse respectively. In both studies the $CT_{Min}$ was significantly lower than the $TL_{50}$.

There are three possible explanations for the difference in $CT_{Min}$ and $TL_{50}$ observed by Bennett and Judd (1992) and Steffens (1996). The first is that during static exposure the fish succumb initially to 'cold shock' or primary chill coma. This syndrome was first described by Doudoroff (1942) and is the final stage just prior to death in response to sudden exposure to cold water beyond the incipient lethal level. Pitkow (1960) describes this stage as being characterised by convulsions, loss of equilibrium, complete paralysis and a state of apparent death wherein the fish would revive only if warmed within a few minutes.

Doudoroff (1942) postulated that death may in fact be due to a breakdown in the central nervous system (CNS) and therefore death should be increasingly delayed in larger fish. Respiration is regulated within the medulla oblongata of the CNS via the superior cervical ganglion of the autonomic nervous system (Eckert *et al.* 1988). Pitkow (1960) demonstrated that respiration did not recommence in fish that had been exposed to three plus times the $TL_{50}$ cold exposure and prior exposure to decreased oxygen content only increased the number of dead. Subsequently he concluded that death due to primary chill coma is highly suggestive of anoxic damage to a cold-depressed respiratory centre thereby reinforcing Doudoroff's (1942) assumption that the cause of primary chill coma was due to CNS malfunction/breakdown. Hence, it is the shock of the sudden temperature change rather than the temperature alone that results in the $TL_{50}$ being significantly higher than the $CT_{Min}$.

The second explanation is another interaction between temperature and time. When determining $CT_{Min}$, the temperature is lowered at a constant rate, in the cases in question the rate was one degree Celsius per hour ($1°C.\ hr^{-1}$). The possibility that the fish were capable of partial acclimation to lower temperatures, as the temperature decreased, cannot be discounted.

Finally, both of these studies defined the endpoint as death. The difficulty in defining this endpoint using continuous temperature reduction may have resulted in an over-estimation of the dynamic temperature reduction required to deliver the end point. That is to say that the fish is moribund at a higher temperature but not

observed to have reached the end-point until a later time at which the temperature is lower. Chung (1997) identified similar problems in using death as an end-point by using different rates of heating when estimating $CT_{Max}$ of tropical salt marsh fish. In that study, different rates of heating had significantly different $CT_{Max}$ values demonstrating the problem with defining the temperature that resulted in death.

Temperatures not so acute as to inflict primary chill coma may still cause death after prolonged exposure to that temperature as fish enter secondary chill coma. Brett (1952) experimenting as to the correctness of earlier assumptions of Doudoroff (1942, 1945) concluded that secondary chill coma was due to osmoregulatory failure. In Brett's (1952) experiment, sockeye salmon, *Oncorhynchus nerka*, were acclimated to 20°C in both fresh and sea-water diluted to 9.9 ppt. There was no significant difference observed in rapid and delayed cold-deaths at 0.2°C in either media. At 0.7°C an indication of greater resistance among the last surviving members in the saline solution was present. At 3.2°C an increase in the resistance was observed but not to the point of eliminating death from low temperature in part of the sample. Brett (1952) concluded that, at the lowest levels of temperature, a medium of saltwater (slightly hypertonic) does not alter the course of death from that observed in freshwater. Such a medium did however, reduce the lethal effects of low temperature for delayed cold-death when resistance times exceed 17 hours. Therefore, at least in part secondary chill coma is a symptom of loss of osmoregulatory control.

Obviously, in order to realise the maximum potential benefits of lowered temperature within live fish transport one must have a strong understanding of the thermal tolerance of the species across different acclimation temperatures and temperature reduction regimes. Moreover, if coma is used within live transport then a close understanding of the best induction and safe maintenance regime must also be acquired. With the exception of one study (Steffens 1996) and local environmental data, little is known about the thermal tolerance and most appropriate transport regimes for the three species in this study.

Steffens (1996) studied the ILT and $CT_{Min}$ of bluethroat wrasse acclimated at three different temperatures, 10, 15 & 20°C. A summary of the results from that study is shown below in Table 2.

| *Acclimation* | *10°C* | *15°C* | *20°C* |
|---|---|---|---|
| $CT_{Min}$ | 3.64°C | 4.33°C | 6.78°C |
| $TL_{50}$ *(24hrs)* | 5.0°C | 6.0°C | 8.3°C |

**Table 2 Critical thermal minima and the lower incipient lethal temperature of bluethroat wrasse described by Steffens (1996) for three different acclimation temperatures.**

It is noted that dissolved oxygen levels are likely to decline during transport and that reduced partial pressures of oxygen in water were was observed by Pitkow (1960) to reduce the thermal tolerance of fish. However, as stated earlier, the reason for studying thermal tolerance is to enable manipulation of transport settings to reduce oxygen consumption and thereby reduce the impacts of oxygen depletion during transport. In this context it is appropriate to initially study thermal tolerance in isolation from any compounding effects of oxygen depletion.

There is little data on the influence of the sex of fish on the determination of lower lethal temperature and most authors do not comment on this element of their experiments. Pitkow (1960) did observe that male guppies, *Lebistes reticulatus*, were less cold tolerant than females however, it should be noted that the females used in that study were at least twice the weight of males. Johnson (1976) also observed that male mosquito fish had a narrower range of thermal tolerance than females. In addition, several authors have noted sex differences in preferred temperature that is suggestive of a similar difference in thermal tolerance. For example, male guppy, *Poecilia reticulata,* and *P. latipinna*, preferred lower temperatures to females and juveniles (Johansen & Cross 1980, Stauffer *et al.* 1985). Male blackchin tilapia, *Sarotherodon melanotheron*, displayed a preference for lower temperatures than females (Stauffer 1986).

While it is accepted that weight influences thermal inertia in fish there is little information on the influence of weight in determining lower lethal temperature when not potentially confounded by sex differences. Brett (1952) found that smaller Pacific salmon (*Oncorhynchus* spp.) were more susceptible than larger specimens to lowered temperature but not to higher temperatures. Steffens (1996) found no significant effect of sex or weight on the lower lethal temperature in bluethroat wrasse.

The aims of this chapter are to describe the thermal tolerance (i.e. ILT and $CT_{Min}$) of horseshoe leatherjackets, bluethroat wrasse and greenback flounder and establish a reference data set for the development of effective transport protocols using reduced

temperature. The study compared the two main methods for describing thermal tolerance and examined differences in thermal tolerance between three species that occur in close association in the wild.

## METHODS AND MATERIALS

The open, flow-through, three-tank apparatus described in Chapter 3 was used during the measurement of thermal tolerance and coma induction.

All fish were acclimated at 15°C for at least two weeks prior to experimental use and deprived of food for 24hr before placement within the experimental tanks. Fish used in any trial were not re-used for at least one month after being returning to the holding tanks and the majority of fish were not used more than once. To minimise the potential effects of handling-induced stress, all fish were weighed, measured and sexed after the experimental trial was complete.

Due to differences in the sex ratio of bluethroat wrasse and horseshoe leatherjackets captured it was not possible to develop balanced experimental designs with respect to sex and size ratio. Due to the large variations in the sizes of animals used both within and between species it was impossible to standardise the number and mass of fish in each aquaria. "Floor space" limited the number of greenback flounder that could be added to each aquaria further restricting the ability to statndardise the mas of fish in each aquaria between trials. Within each trial the mass of fish in each aquaria was balanced as far as possible and, as noted in Chapter 3, dissolved oxygen levels were monitored to ensure high saturation levels (≈100% of air saturated levels) at all times in all trials.

### *Control Trials*

Control experiments were undertaken with each species using a constant water temperature of 15°C for at least 72hr duration prior to returning fish to the acclimation tank (at 15°C) where they were monitored for a further 24hr. No mortalities were recorded during any of the control experiments.

### *Thermal Tolerance*

#### *Static Exposure*

Experiments to determine the $TL_{50}$ of each species over several exposure periods were undertaken. Fish were removed from the acclimation tank and placed in the experimental system that had been pre-chilled to a pre-determined experimentally constant temperature.

It is acknowledged that this method required handling of the fish immediately before and after the exposure period of trial and that animals were given no time to acclimatise to the system. Due to logistical constraints, construction of a preferred, parallel two-temperature system was precluded. Such a system would have eliminated the need for handling immediately before exposure to experimental temperatures.

Fish were left in the experimental system for 2, 12 or 24hr before removal and return to holding tanks at 15°C. All mortality was recorded following the initial placement in the experimental system until some 24hr after return to the acclimation tank. This 24hr period was included post-exposure to allow fish that were moribund as a result of the exposure and subsequently died during the following 24hr to be included in the mortality results. A period of 24hr was chosen as a representation of a reasonable minimum time that a fish might have to survive post-transport in a commercial setting. The $TL_{50}$ value was calculated as described earlier in this chapter.

*Dynamic Exposure*

Experiments to determine the $TL_{50}$ and $CT_{Min}$ of each species were conducted with the same apparatus and general procedure as described above except that fish were placed in the system in seawater at the acclimation temperature of 15°C. The system was immediately set to chill until the target minimum was reached at which time the water in the system was warmed. Once the acclimation temperature had been regained the fish were removed and returned to the acclimation tank. All mortalities were recorded from the time the fish were placed in the experimental system until 24hr after transfer to the acclimation tank. A $CT_{Min}$ value was calculated as the mean of the lowest temperatures to which all deceased animals were exposed. A $TL_{50}$ value was calculated as for static exposure trials.

Again it is acknowledged that this method involved handling and no time to 'adjust' to the system but this procedure was followed in an effort to best ensure reasonable comparison with the static exposure protocol.

*Down and Hold Exposure*

To test the effect on survival of gradual cooling and warming rates when applied in conjunction with an extended period of lowered, constant temperature, the cooling and warming periods of a $CT_{Min}$ trial were separated by an extended period (24hr) of experimentally constant minimum temperature.

For each trial, fish were added to the system and water was chilled from 15°C down to a pre-determined set point. Once the set point was reached the lowered water temperature was kept experimentally constant for 24hr. After this hold period the

water was warmed to 15°C and fish were transferred to acclimation tank and monitored for a further 24hr.

As in all previous temperature exposure experiments all mortalities were recorded from the time the fish were placed in the experimental system until 24hr after return to the acclimation tank. As each of these trials required over 5 days from start to finish this protocol was trialed for flounder only, due to time limits.

The range of temperature exposure regime trials across the three species is summarised in Table 3.

| *Exposure Regime* | *Species* | *Time (hr) at Min. Constant Temp.* | *Approx. Min. Temp. Range (°C)* | *No. of Trials Across Min. Temp.Range* | *No. of Fish in Each of the 3 Aquaria* | *Approx. Range in Mass (g) in Each Aquaria* | *No. of Fish in Each Trial* | *Total No. of Fish Used in All Trials* |
|---|---|---|---|---|---|---|---|---|
| Static | HSLJ | 2 | 5.0 - 7.5 | 3 | 1 or 2 | 700-1100 | 3-5 | 14 |
| Static | BTW | 2 | 4.0 - 8.5 | 9 | 1 or 2 | 700-2300 | 3-4 | 39 |
| Static | BTW | 12 | 5.5 - 10.5 | 9 | 1 or 2 | 700-2300 | 3-4 | 40 |
| Static | BTW | 24 | 6.5 - 11.0 | 9 | 1 or 2 | 700-2300 | 3-4 | 37 |
| Static | GBF | 2 | 0.0 - 7.5 | 12 | 3 or 4 | 400-700 | 10 | 120 |
| Static | GBF | 12 | 0.5 - 7.5 | 12 | 3 or 4 | 400-700 | 10 | 120 |
| Static | GBF | 24 | 0.5 - 7.5 | 12 | 3 or 4 | 400-700 | 10 | 120 |
| Dynamic | BTW | N/A | 4.5 - 8.0 | 6 | 1 or 2 | 700-2300 | 3-5 | 24 |
| Dynamic | GBF | N/A | 1.0 - 7.0 | 6 | 1 or 2 | 400-700 | 10 | 60 |
| 'Down & Hold' | GBF | 24 | 1.0 - 7.5 | 12 | 3 or 4 | 400-700 | 10 | 120 |

**Table 3 Summary of methods for thermal tolerance trials. Species abbreviations are: HSLJ= horseshoe leatherjacket, BTW= bluethroat wrasse & GBF= greenback flounder.**

*Note on Analysis*

In each trial, the paired values for temperature and time for each of the three tanks were compared to ensure there was no significant difference in the temperature regime between tanks in any given trial. Provided this test was satisfied, the temperature data for all three tanks in each trial was averaged and the percentage mortality in each tank represented a single replicate of that average temperature exposure for a total of three replicates in each trial for use in tests of significance. For graphical representation, the percentage mortality for the sum of fish in all three aquaria were used due to the low and unbalanced numbers of fish in each aquaria (1-4). Hence these graphical values of % mortality represented aggregate values rather than means with standard deviations but rather a single point value for reference.

***Coma Induction***

*Static Exposure*

In order to avoid the potential impacts of direct observations on the results of thermal tolerance trials separate trials were conducted to describe coma induction times. Initially, fish were treated as per the static exposure tolerance trials and the elapsed time for coma induction following immersion in chilled water was recorded, from observations at one minute intervals, for various constant lowered temperatures. Observations at intervals of one minute were used rather than constant observations to standardise tank disturbance despite the potential for a resultant loss of sensitivity in the data.

For bluethroat wrasse and horseshoe leatherjacket a fish was considered to be in a coma when it displayed a complete loss of equilibrium and failed to respond to external stimulation. As greenback flounder are relatively sedentary animals that rest on the bottom of the tank for extended periods as part of normal behaviour, loss of equilibrium could not be used to define coma. In the case of greenback flounder a fish was considered to be in a coma when it failed to respond to external stimulation. External stimulation was applied in the form of approach and gentle touching with the handle of a dip net. The problem in comparing data between species using these different observational definitions of coma is apparent but unavoidable.

*Dynamic Exposure*

As for coma induction observations during the static (i.e. constant) temperature trials (described immediately above), coma induction and thermal tolerance trials using dynamic temperature exposure were separated to avoid the potential for necessary observation during coma induction trials affecting the results of thermal tolerance trials. Fish were treated as per dynamic thermal tolerance trials. The temperature at which coma was first observed was recorded based on observations at one-minute intervals. Again these intervals were used to standardise tank disturbance. Due to

the more gradual change in temperature during dynamic trials, the effect of one-minute intervals on the sensitivity of the data is not considered to have any significant impact on the results.

## RESULTS

### *Mortality*

*Static Exposure*

Horseshoe Leatherjacket

The survival of 14 horseshoe leatherjackets (in total), plotted by sex and mass (g), following exposure for two hours to three constant temperatures between 5 & 7.5°C is shown at Figure 8. Unfortunately insufficient specimens of horseshoe leatherjacket could be collected to enable any further testing. Thus, any conclusions from the data must be viewed with caution due to the small sample size (n=14). Noting that each fish is a pseudo-replicate within the design, in undertaking the analysis each individual fish was treated as a replicate (nb this was not the case for other species). Within the temperature range tested, mean exposure temperature did not significantly affect survival of individual horseshoe leatherjackets (P =0.7802, df=2, no significant difference in any pairing within Tukey's and LSD tests).

Acknowledging the need for constraint in the strength of conclusions due the small sample size, the survivorship of males was significantly lower than that of females (P=0.0070, df=1, Tukey's and LSD tests determined a significant difference). However, it must also be noted that males are generally larger than females. Thus, the difference in survival may not be a sex difference but rather a size difference or some interaction of these effects. Insufficient sample size precluded meaningful analysis of size or sex/size interaction effects on survival in horseshoe leatherjackets.

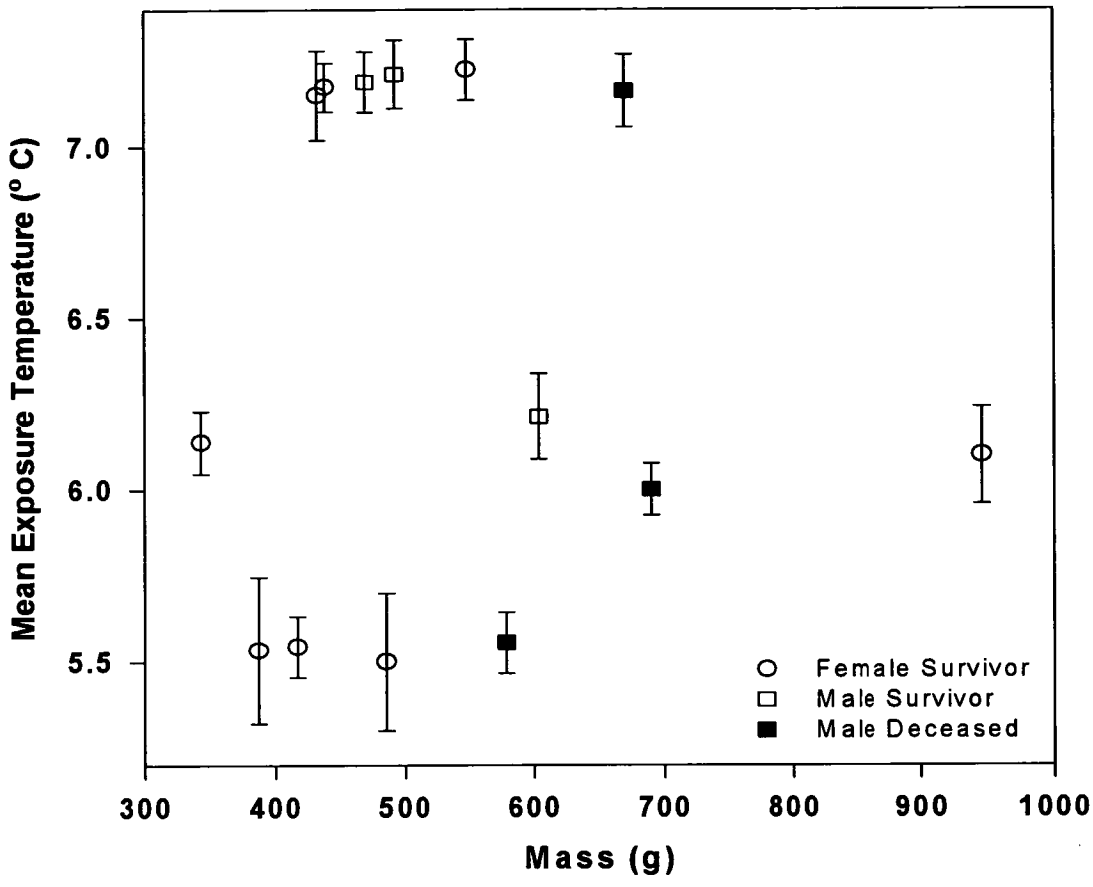



**Figure 8 Survival of horseshoe leatherjackets following 2hr exposure to various lowered static temperatures (n=14). Temperatures shown +/- 1 s.d.**

Bluethroat Wrasse

A total of 39 bluethroat wrasse were exposed for two hours to nine different constant temperatures between 4°C and 8.5°C across nine trials. The temperature data for all three tanks in each trial was averaged and the percentage mortality in each tank represented a single replicate of that temperature for a total of three replicates in each trial. Survival decreased with decreasing temperature within the experimental range (Figure 9). Exposure temperature had a significant effect on the survival of fish during trials (P=0.0001, d.f. =8). Both Tukey's and LSD tests grouped the upper five trials in order of descending temperature (temperatures from 7.9°C to 6.0°C) together but distinct from the four cooler trials (temperatures from 5.7°C to 4.4°C).

A total of 40 bluethroat wrasse were exposed for 12 hours to temperatures between 5.5°C and 10.5°C in nine different trials. Survival decreased with decreasing

temperature within the experimental range. Exposure temperature had a significant effect on the survival of fish (P=0.0001, d.f. =8). Both Tukey's and LSD tests grouped the data in consecutive groupings of descending mean exposure temperature.

A total of 37 bluethroat wrasse were exposed for 24 hours to temperatures between 6.5°C and 11.0°C in nine different trials. The temperature and survival data were treated as above. Survival decreased with decreasing temperature within the experimental range. Exposure temperature had a significant effect on the survival of fish during trials (P=0.0001, d.f .=8). Both Tukey's and LSD tests grouped the data in consecutive groupings of descending mean exposure temperature.

As seen in Figure 9, at similar temperatures the results of the two hour exposure trials were clearly different to the 12hr and 24hr exposure trials. In the two hour exposure trials, the 100% survival temperature is lower than the zero survival temperature in each of the 12hr and 24hr exposure plots.

There was no significant difference in survival between sexes (P=0.7808, d.f.=115) or at different sizes (P=0.1701,d.f.=115).

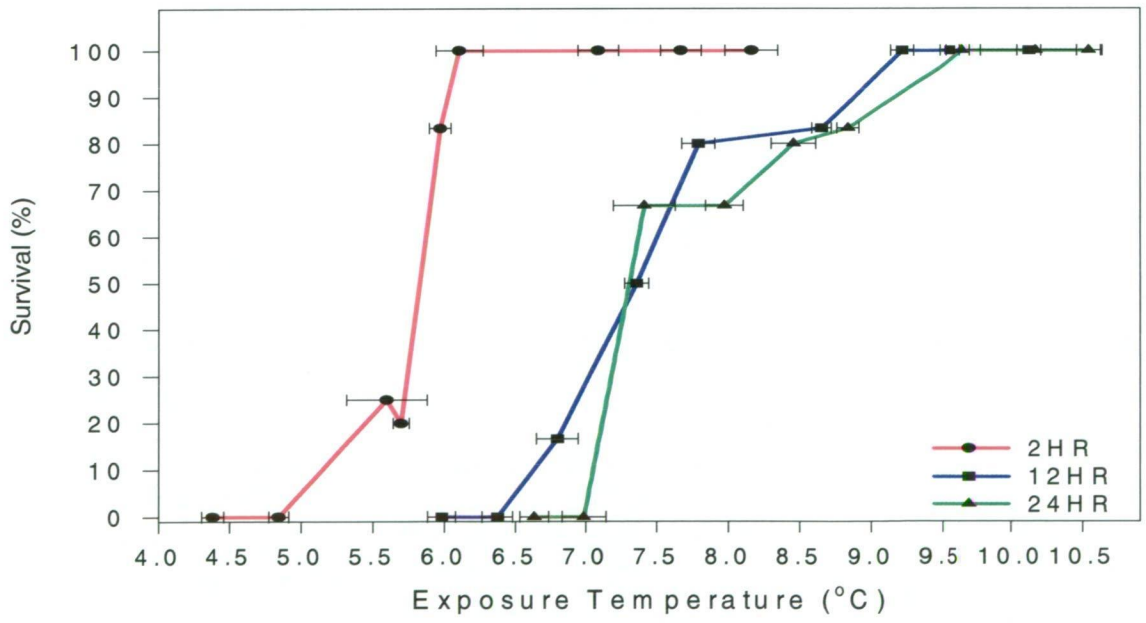



**Figure 9 Survival of bluethroat wrasse following exposure to a range of static lowered temperatures for durations of 2, 12 and 24hr. Temperatures are shown +/- 1 s.d.**

Greenback Flounder

Some initial trials using flounder were disregarded as comatose fish that became buried under other tank members most often died. To eliminate this source of variation during trials using temperatures that induced coma, fish were moved apart if they became buried.

A second interesting observation during early rejected trials was that all greenback flounder that exhibited a bulbous gut died when exposed to temperatures as little as 5°C lower than the acclimation temperature. This swelling was the mature gonad of the female flounder. As a result of this observation all fish that exhibited this condition were rejected as experimental animals for the purposes of the trials reported below.

A total of 120 greenback flounder were exposed for two hours to temperatures between 0.0°C and 7.5°C in 12 different trials. The temperature and survival data were treated as above. Survival decreased with decreasing temperature within the experimental range (Figure 10). Exposure temperature had a significant effect on the survival of fish during trials (P=0.0001, d.f. =11). Both Tukey's and LSD tests grouped the data in various groupings of descending mean exposure temperature. Tukey's and LSD groupings suggest a survival break point of approximately 3°C.

A total of 120 greenback flounder were exposed for 12 hours to temperatures between 0.5°C and 7.5°C in 12 different trials. The temperature and survival data were treated as above. Survival decreased with decreasing temperature within the experimental range (Figure 10). Exposure temperature had a significant effect on the survival of fish during trials (P=0.0001, d.f. =11). Both Tukey's and LSD tests grouped the data in various groupings of descending mean exposure temperature.

A total of 120 greenback flounder were exposed for 24 hours to temperatures between 0.5°C and 7.5°C in 12 different trials. The temperature and survival data were treated as above. Survival decreased with decreasing temperature within the experimental range (Figure 10). Exposure temperature had a significant effect on the survival of fish during trials (P=0.0001, d.f. =11). Both Tukey's and LSD tests grouped the data in various groupings of descending mean exposure temperature.

Only around two-thirds of the flounder could be sexed with reasonable confidence. Consequently, no attempt was made to analyse the survival data related to sex in greenback flounder. The relationship between size and survival was analysed and no significant relationship was observed (P=0.3017, d.f.=359).

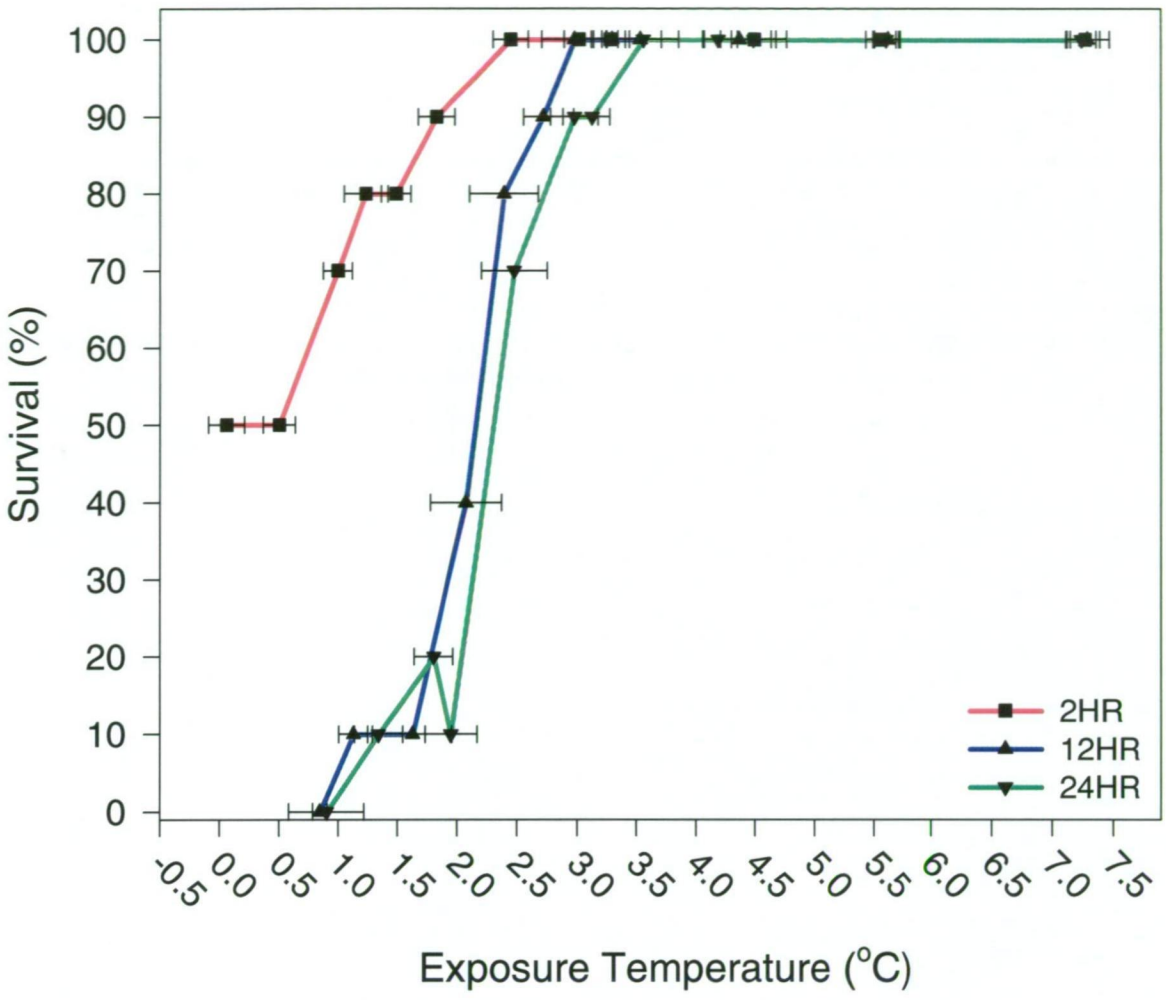



**Figure 10 Survival of greenback flounder following exposure to a range of static lowered temperatures for durations of 2, 12 and 24hr**

*Dynamic Exposure*

Bluethroat Wrasse

A total of 24 bluethroat wrasse were exposed to gradually cooled temperatures commencing at the acclimation temperature and declining to various mean minimum temperatures between 4.5°C and 8.0°C before warming again to the acclimation temperature. The minimum temperature data for all three tanks was averaged and the percentage mortality in each tank represented a single replicate of that temperature for a total of three replicates in each trial. Survival decreased with decreasing mean minimum temperature within the experimental range (Figure 11). The mean minimum temperature had a significant effect on the survival of bluethroat wrasse exposed to this temperature regime (P= 0.0035, d.f.=5).

It should be noted that mean minimum temperature and overall rate of cooling were not independent due to the reduced cooling efficiency of the experimental system at lower mean minimum temperatures. In these trials the rate of cooling (dT/dt) varied between 1.5°C.hr$^{-1}$ and 2.5°C.hr$^{-1}$. The slower rates of both cooling and warming coincide with lower mean minimum temperatures and lower survival (Figure 11).

Greenback Flounder

A total of 60 greenback flounder were exposed to gradually colder temperatures commencing at the acclimation temperature and declining to various mean minimum temperatures between 1.0°C and 7.0°C before warming again to the acclimation temperature. The minimum temperature and mortality data were treated as above.

Some mortality was recorded at the lowest minimum temperatures that the system was capable of but survival was not less than 50% in any trials (Figure 11). The mean minimum temperature had no significant effect on the survival of greenback flounder exposed to this temperature regime (P=0.1564, d.f.=5). However, LSD tests did separate the lowest two exposure temperatures as significantly different to the warmer temperatures. Again it should be noted that mean minimum temperature and overall rate of cooling and warming were not independent.

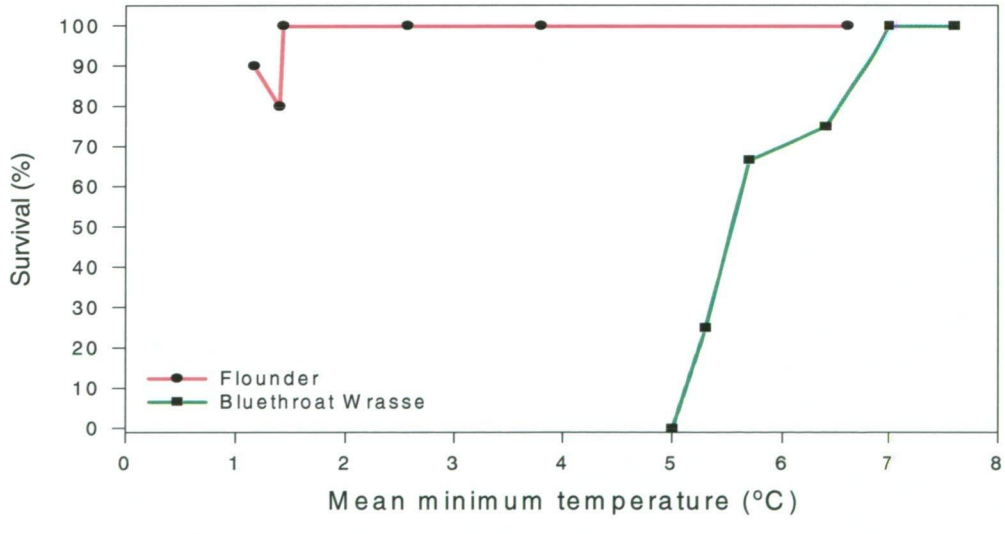



**Figure 11 Survival of bluethroat wrasse and greenback flounder following dynamic reduced temperature exposure. The thermal tolerance of these two species was markedly different.**

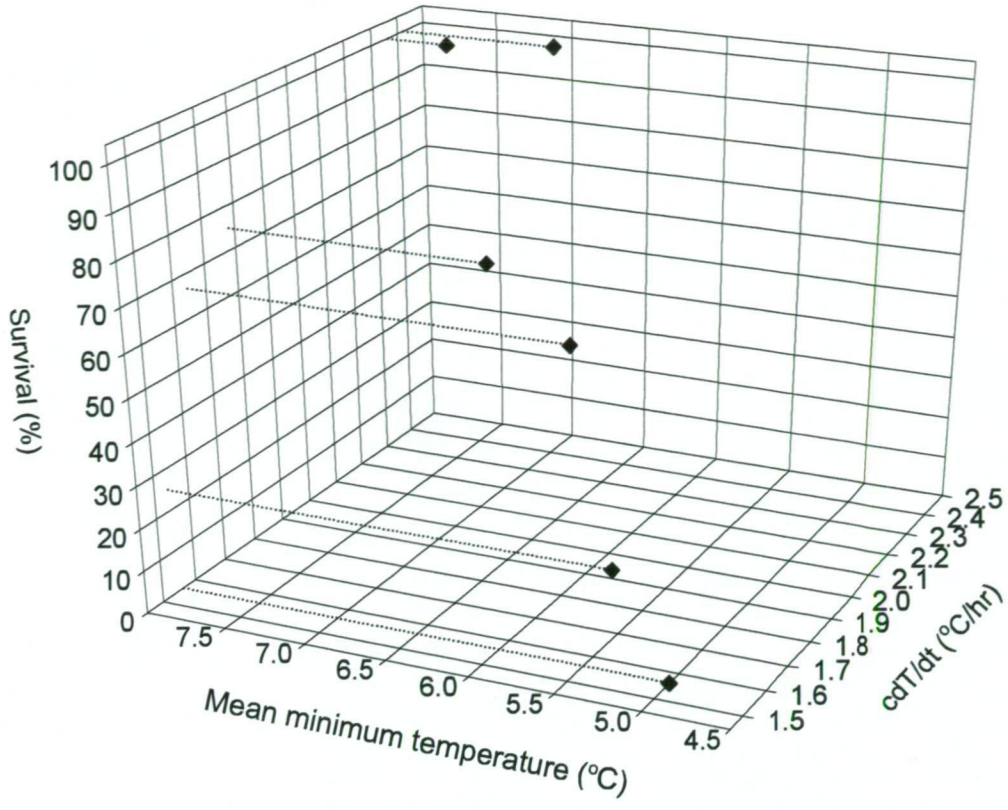



**Figure 12 Survival of bluethroat wrasse during dynamic temperature exposure across various rates of cooling (cdT/dt). The system interaction between mean minimum temperature and rate of cooling is clear. Hence the effects of rate of change and the minimum temperature cannot be isolated within the experimental regime.**

*Down and Hold Exposure*

Greenback Flounder

A total of 120 greenback flounder were exposed to the temperature regime of gradual cooling followed by maintenance of an experimentally constant minimum temperature for 24 hr followed by gradual warming back to the acclimation temperature. A significant difference was observed in flounder survival at different minimum temperatures (P=0.0001, d.f.=9) (Figure 13). In Figure 13 the results of the static and dynamic experiments for greenback flounder are also shown for comparison. The survival of flounder exposed to the down and hold protocol was significantly greater than that of the static 24hr exposure trials (P=0.0071, d.f.=1). The mortality of flounder during down and hold trials was markedly higher than that of the 2hr static exposure trials at similar minimum temperatures.

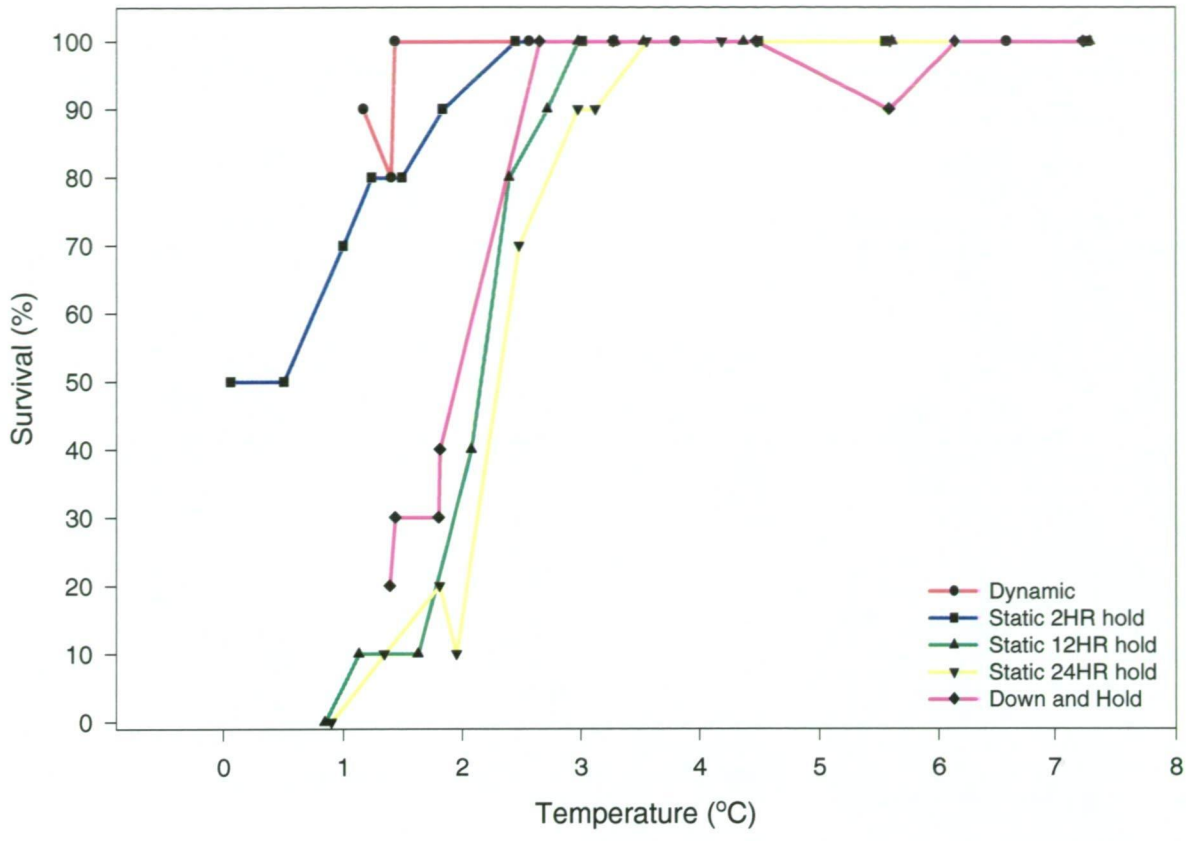



**Figure 13 Survival of greenback flounder following 'down & hold' temperature exposure. For comparison the survival of greenback flounder during static and dynamic trials is also shown.**

A summary of the thermal tolerance of bluethroat wrasse and greenback flounder across the three methods of exposure is given in Table 4.

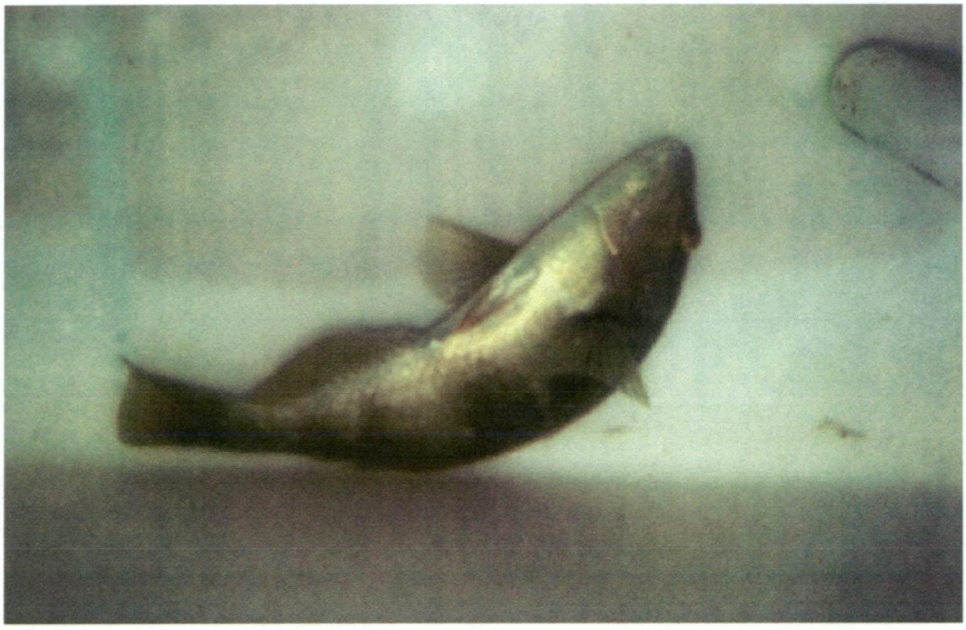
| | *Bluethroat Wrasse* | *Greenback Flounder* |
|---|---|---|
| $TL_{50}$ *(2hr)* | 5.8°C | 0.3°C |
| $TL_{50}$ *(12hr)* | 7.4°C | 2.2°C |
| $TL_{50}$ *(24hr)* | 7.3°C | 2.3°C |
| $TL_{50}$ *(Dyn)* | 5.5°C | ? > 50% at min.°C |
| $TL_{50}$ *(D&H)* | n.a. | 1.9°C |
| $CT_{Min}$ *(Dyn)* | 5.33°C (s.d.=0.46) | 1.32°C (s.d.=0.14) |
| $CT_{Min}$ *(D&H)* | n.a. | 1.73°C (s.d.=0.83) |

**Table 4 Summary of results for descriptors of thermal tolerance for bluethroat wrasse and greenback flounder. The marked difference in the thermal tolerance of the two species is clear. A $TL_{50}$ could not be determined for greenback flounder during dynamic trials as more than 50% of fish survived in all trials.**

***Coma Induction***

Both horseshoe leatherjackets and bluethroat wrasse consistently exhibited lateral curvature prior to and following loss of equilibrium and coma during both static and dynamic exposure protocols (Plate 9). However, fish exhibited considerably more thrashing and overt panic during dynamic trials than static trials before curvature and subsequent coma was observed.

**Plate 9** **Female bluethroat wrasse in coma. The fish in this photograph shows the loss of equilibrium and curvature of the body and that was typical of both bluethroat wrasse and horseshoe leatherjackets during coma.**



*Static Exposure*

Horseshoe Leatherjacket

There was a significant difference (P=0.0046, d.f.=3) in the elapsed time prior to coma induction at different exposure temperatures (Figure 14).

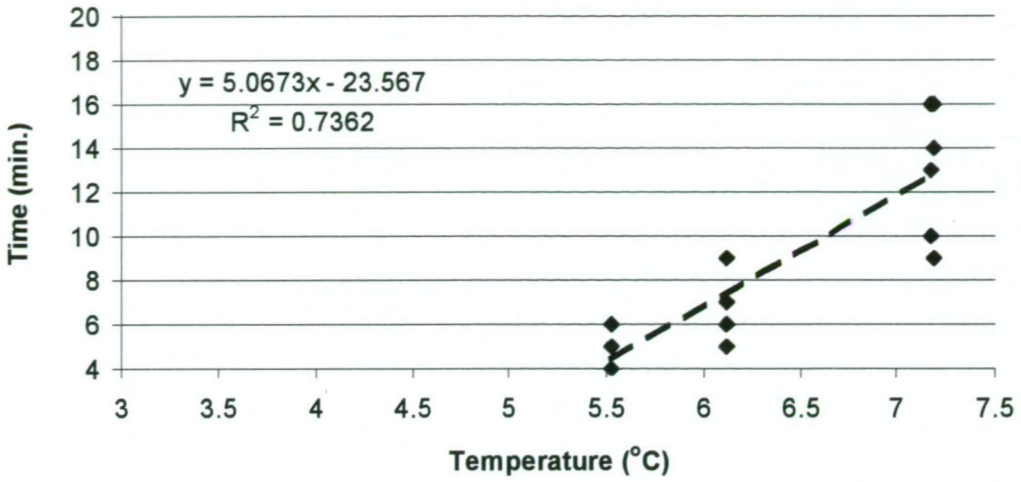



**Figure 14 Time elapsed before induction of coma in horseshoe leatherjackets following immersion in chilled water**

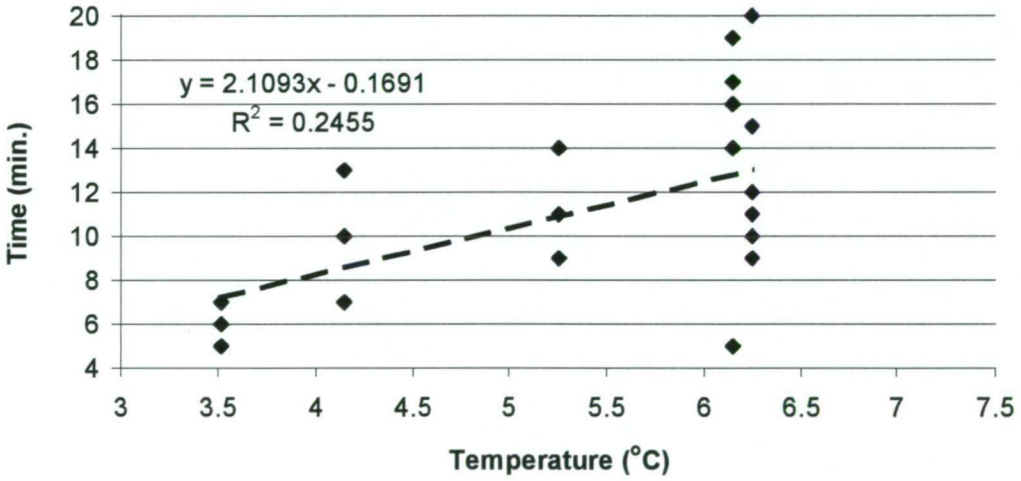



**Figure 15 Time elapsed before induction of coma in bluethroat wrasse following immersion in chilled water**

Bluethroat Wrasse

There was no significant difference (P=0.0949, d.f.=3) in the elapsed time prior to coma induction at different exposure temperatures (Figure 15).

Greenback Flounder

Coma induction in greenback flounder during static trials was very difficult to determine and no attempt was made to analyse the data. Fish appeared to drop in and out of coma over a period with 'non-coma' periods extending in duration after the initial period. The observations suggested that flounder were rapidly acclimating to the temperature or otherwise able to ameliorate its effects.

*Dynamic Exposure*

Horseshoe Leatherjacket and Bluethroat Wrasse

The mean temperatures at which coma was induced in horseshoe leatherjackets and bluethroat wrasse are shown in Table 5. There was no significant difference in the dynamic coma induction temperature in bluethroat wrasse and horseshoe leatherjackets (P=0.0914, d.f.=1)

| *Species* | *Mean Coma Temp.* | *Std Dev.* | *Range* | *Rate (dT/dt)* |
|---|---|---|---|---|
| ***Horseshoe Leatherjacket (n=9)*** | 7.2°C | 0.1643 | 6.9-7.3°C | 2.17°C.hr$^{-1}$ |
| ***Bluethroat Wrasse (n=15)*** | 7.6°C | 0.3841 | 6.9-8.4°C | 2.20°C.hr$^{-1}$ |

**Table 5 Summary of coma induction results for horseshoe leatherjackets and bluethroat wrasse for dynamic low temperature exposure. There was no significant difference in the mean coma induction temperatures between species (P=0.0914).**

Greenback Flounder

Coma induction in greenback flounder during dynamic trials was very difficult to determine and no attempt was made to analyse data. As temperature decreased, fish appeared to fall in and out of coma with periods of coma increasing in frequency and duration as temperature continued to decrease.

## DISCUSSION

### *Thermal Tolerance*

The current study has generated information on the thermal tolerance of three species of temperate fish, two of which had not been previously studied (i.e. horseshoe leatherjacket and greenback flounder). The results for the third species, bluethroat wrasse, differ somewhat from those of the only other study of this species by Steffens (1996). The results of this study and Steffens (1996) are shown in Table 6.

| | *This Study* | *Steffens (1996)* |
|---|---|---|
| $CT_{Min}$ | 5.33°C (s.d.=0.46) | 4.33°C |
| $TL_{50}$ *(24hr)* | 7.3°C | 6.0°C |

**Table 6 Comparison of the thermal tolerance values of the present study and Steffens (1996). Both the $CT_{Min}$ and $TL_{50}$ (24hr) values are markedly different and likely to be explained by differences in the methods in particular identification of point of death.**

The exposure protocols used by Steffens (1996) to determine the $CT_{Min}$ are sufficiently different from those used in this study to explain these differences. In particular, Steffens (1996) declared death without endeavouring to recover the fish and as such may have under-estimated mortality at any given temperature during both ILT and $CT_{Min}$ trials. That is to say that moribund fish at a given temperature were not declared dead until temperature had been further decreased. Due to the difficulty in determining the point of death during continuously decreasing temperature, the use of death as an endpoint without use of a recovery protocol is obviously questionable in the studies of Bennett & Judd (1992) and Steffens (1996). Such protocols have obvious ramifications if used to describe thermal tolerance for the purposes of live transport in commercial settings and should not be used for this purpose.

The reason for the difference in the results of ILT experiments on bluethroat wrasse in this study compared with Steffens (1996) is more difficult to explain. Certainly the use in this study of the additional 24hr period after exposure for the purposes of determining overall mortality may explain some of this result. Fish that were moribund at the end of the exposure period but not dead would not be included in the $TL_{50}$ as calculated by Steffens (1996).

As death below the ILT is a function of both temperature and time it is likely that the effect of moribund fish on the result would be significant. This is because those temperatures at the upper end of the lethal range are likely to have the longest lethal time and result in more fish being moribund rather than declared as dead at the end of the exposure period.

The other factor that may explain at least some of the difference in the $TL_{50}$ results for bluethroat wrasse in this study compared to those of Steffens (1996) is the fact that Steffens (1996) used only three different temperatures to describe the line between 0 and 100% survival (inclusive). As shown in this study (which had six temperature values between 0 & 100% survival inclusive), the slope of the line used to calculate the $TL_{50}$ is very steep between 0 and 100% survival. The use of only three points could have resulted in a lack of sensitivity in the data for the purposes of interpolation and hence may explain the difference in the results of this study and Steffens (1996).

For ease of comparison, the survival results for all three species during static exposure experiments are shown in Figure 16. While the limited number of trials undertaken with horseshoe leatherjackets limit the strength of conclusions, the results suggest that this species does not differ markedly in its thermal tolerance than the bluethroat wrasse a close neighbour in its habitat. However, both these species exhibit widely different thermal tolerance to that shown by the greenback flounder that also occurs in close proximity in the wild.

The difference in the tolerance of these species may reflect differing broad-scale range of greenback flounder relative to the ranges of the other two species (i.e. the overlapping range of these species is small and represents 'warm' water habitat for flounder but a 'cold' water habitat for the other species). There is no evidence in the literature to support this suggestion and these species seem to have a broad area of overlap across a range of latitudes both above and below that of the study area.

An alternate explanation is that the greater tolerance of greenback flounder to lower temperatures is a function of a fine-scale 'range' or rather behavioural difference. Flounder are the target of considerable recreational fishing effort using spears and spotlights in shallow water (<1m) sandflats at night. Spearing does not occur during the day in these areas when visibility is likely to be better. This suggests that flounder venture into these shallow sandflat areas predominantly under the cover of darkness.

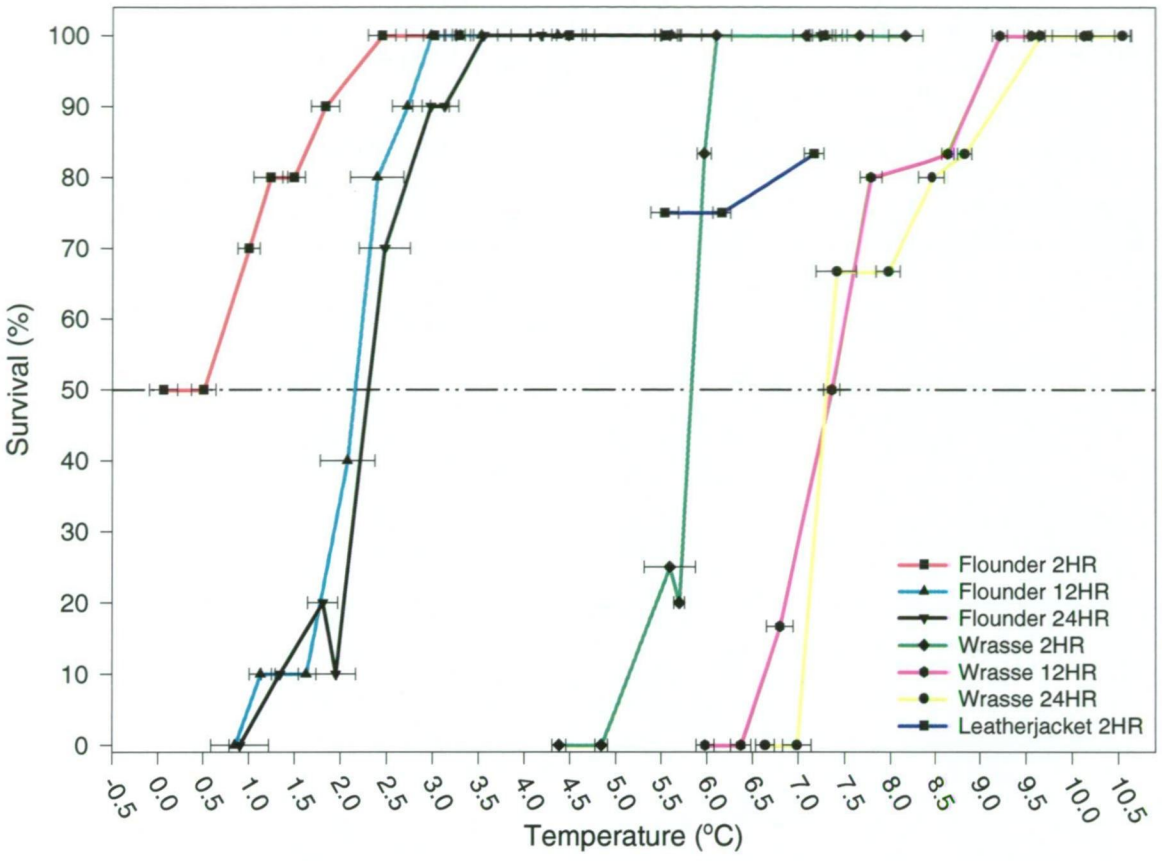



**Figure 16 Summary of survival results for horseshoe leatherjackets, bluethroat wrasse and greenback flounder following static low temperature exposure. Temperature is shown +/- 1 s.d. Times given in legend are exposure duration. The differences in the thermal tolerance of greenback flounder and bluethroat wrasse is clear across all exposure durations.**

It is well known that, unlike deeper waters, shallow waters exhibit relatively rapid temperature fluctuations as a function of air temperature (assisted by wave action). In waters surrounding Tasmania it is not unusual for night time air temperatures to be below 0°C during winter months. This creates the situation where a fish that wished to enter very shallow waters (i.e. <30cm depth) at night could be exposed to relatively rapid temperature fluctuations when swimming from deeper water into the colder, shallow waters. While water temperatures in shallow waters would be unlikely to approach zero, fish traveling from deeper into shallower water would never-the-less need to be able to tolerate a relatively wide temperature range and

rapidly adjust to changing temperatures. It would appear that greenback flounder are indeed capable of tolerating rapid temperature changes and low water temperatures in very shallow water at night.

This is not to say that this temperature change would be instantaneous and fish would be able to regulate how quickly they changed temperature by moving into shallower waters gradually (e.g. over a period of a few hours) and could still avoid areas of extreme low temperature by avoiding very shallow water. A need for at least some short period of 'adaptation' is possibly supported by the increased survival of flounder when exposed to the more gradual changes of the down and hold protocol (i.e. changes in the order of 2°C per hour) when compared to the sudden changes of the static protocol.

As stated it is not possible for fish to acclimatise to such temperature gradients within the matter of a few hours and it is accepted that acclimatisation generally takes time in the order of weeks or even months (Eckert *et al.* 1988). There appear to be two mechanisms that may explain the ability of greenback flounder to tolerate rapid and large decreases in temperature.

First, while the metabolic rate in most ectotherms varies with body temperature, decreasing 50-67% for every 10°C decrease in ambient temperature, the metabolic rates of some poikilotherms exhibit remarkable temperature independence. For example, some intertidal invertebrates that experience large swings in ambient temperature have metabolic rates with a $Q_{10}$ of about 1.0 so the rate of metabolism shows no change with temperature changes as large as 20°C. These animals appear to possess enzyme systems with extremely broad temperature optima. Such systems may result from staggering of the temperature optima of multiple enzymes in a reaction so that a drop in the rate of one step in the reaction chain is compensated for by an increase in the rate of other steps (Eckert *et al.* 1988).

A second alternate mechanism is that single enzymes may be capable of forming multiple, effective enzymes with slightly different structures. Such enzymes are known as isoenzymes or isozymes (Stryer 1988). Isoenzymes enable processes to continue in the face of changing conditions that inhibit one or more of the suite of tetramers but some tetramers remain active to catalyse the biochemical reaction. One or both of these mechanisms may explain the ability of flounder to tolerate large, rapid decreases in ambient temperature.

In what is now a well-known accident, it was discovered that winter flounder survived sub-zero water temperatures when a tank containing these species was accidentally frozen. This accident subsequently led to the identification of an 'anti-

freeze' gene. I was unable to find any references to winter flounder routinely venturing into shallow water in high latitudes to support a hypothesis that such a gene is beneficial to these fish for this reason and may be similarly present in greenback flounder.

It should be noted that the greenback flounder used in the current study were the product of captive spawning and grow-out. These greenback flounder had never lived in the wild and had been held in near constant temperature for their entire life (around 18°C). It is difficult to suggest that the different acclimatisation conditions of these greenback flounder compared to the other species studied explain the much broader cold tolerance of greenback flounder used in these trials. Rather, it is more readily assumed that this cold tolerance is also inherent in the wild population and may indeed be more 'developed' in wild populations through acclimatisation to conditions in the wild.

***Sensitivity***

Below the threshold temperature at which the first mortalities were observed, the rate of mortality increased very quickly with relatively small decreases in temperature. This was especially evident in those trials with relatively long exposure periods (i.e. 24hrs) where the temperature difference between 100% and 0% survival was around 2°C. This finding is consistent with the results of other workers (Fry 1971, Bennett & Judd 1992, de Guingand 1995, Steffens 1996) and has a range of ecological implications. It may provide useful information to those studying the ecological valences of various aquatic and marine ecosystems including assessment of translocation risks or the effects of thermal pollution and oceanic upwellings.

This observation also has important implications for the use of chilling within a commercial live fish transport process. As mentioned in earlier chapters the results of commercial *ad hoc* trials with chilling have been mixed and often unpredictable. The sensitivity of fish survival to relatively small changes in temperature at the extreme ends probably explains some of these results especially when the logistical constraints of packaging with respect to temperature control and/or insulation are considered.

The results of this study lead me to agree with the conclusions of de Guingand (1995) that while there are likely to be commercial benefits derived from chilling of live fish, extreme care must be taken not to exceed thermal minima. In a commercial setting this may mean that the target temperature is a couple of degrees above the theoretical optimum temperature to provide a reasonable buffer against mortality-inducing temperature fluctuation. If transporting fish through regions of low ambient temperature then a chilled product may need to be carefully handled and packaged to

avoid over-chilling just as the product needs to be protected from exposure to high ambient temperatures. The marked difference in survivorship of fish exposed for two hours compared to those exposed for 12 or 24hr does provide some comfort in a commercial setting that short periods of cold exposure below the target will not necessarily result in mortality.

***Duration of Exposure***

As expected the results of this study clearly showed an interaction of time and temperature once temperatures were lowered beyond the tolerance zone for each species. While this result is not surprising it has obvious implications for live fish transport. Operators will need to carefully consider the length of time the product is likely to be in transit before attempting to identify the most appropriate chilling temperature.

The increased survival of greenback flounder when gradually chilled and warmed relative to the rapid changes experienced during the static trials of the same hold duration and temperature is not immediately surprising. Without additional information it could be assumed that the gradual changes ameliorate the effects of thermal shock presumed to be present during static trials. However, the results of the two hour static trials show that survival of these fish is markedly higher than that of the down and hold trials at similar hold temperatures. Thus, mitigation of thermal shock does not sufficiently explain the increased survival of the fish exposed to gradual change and the results suggest that greenback flounder are undertaking at least partial acclimation over this very short period (i.e. <10hr) of gradual change applied in the down and hold protocol. This result has not been observed in other studies and short periods of gradual change have not been shown to change the thermal tolerance of other species beyond those directly attributable to thermal shock. The mechanism that allows greenback flounder to exhibit this response was not investigated but could be linked to the enzymatic mechanisms discussed earlier.

A review of literature revealed a wide variation in the period of exposure used to describe ILT. As is clear from the differing results of this study when different periods of exposure were applied this non-standard approach makes inter-study comparison almost impossible. It is recommended that a standard approach to exposure period (e.g. 12hr), should be adopted by all researchers of thermal tolerance.

***Observations of note***

The total mortality of all female greenback flounder with mature gonads when exposed to temperatures as little as 5°C lower than the acclimation temperature suggests any live shipment of greenback flounder should be graded to remove such

animals. While this process may be time-consuming and the proportion of the total shipment that exhibits this condition may be quite low, the death of animals during transport greatly increases the risk of other animals dying due to reduced water quality (Terchunian *et al.* 1999).

Within the constraints of the limited sample size, male horseshoe leatherjackets exhibited significantly higher mortality rates than females when exposed to the same temperature regime. The average size of the males captured and used during this study was greater than the females and the males that died were at the upper end of the size range. The potential for the difference in survivorship to be attributable to size differences is noted but such a causative relationship is inconsistent with literature on other species. Most authors have reported either no difference related to size or that larger individuals of a given species have a wider temperature tolerance than smaller individuals.

The result does indicate that further work should be undertaken to test this observation prior to finalising commercial chilling methods for horseshoe leatherjackets. If a sex difference is affirmed then operators should either establish chilling protocols based on males as the lowest common denominator in mixed sex shipments, or sort shipments by sex and use different protocols for each sex.

Some initial trials were disregarded for the purposes of data analysis due to the death of most greenback flounder that became buried under other tank members while comatose. The reason why these fish often died was not investigated but two likely hypotheses are as follows. First, fish that became buried may have been prevented from ventilatory movements of the operculum by the weight of the fish above. This phenomenon has been observed in cod during capture and transport (Midling *et al.* 1996).

A second possibility is that significant gas exchange in greenback flounder can occur across the skin. Such exchange has been observed in other fish (LeMoigne *et al.* 1986, Cech 1990). Unfortunately, the University of Tasmania's Ethics Committee would not approve the use of a modified van Dam respirometer as described by Cech (1990) to investigate to the significance of gas exchange across the skin of greenback flounder especially during coma.

Regardless of the mechanism this observation has important implications for design of transport containers and packing. If temperatures are likely to induce coma then transport containers must be designed to minimise the likelihood of individuals becoming buried. This presents an interesting challenge for the design of containers for greenback flounder and other flatfishes that may show a similar inhibition.

***Coma Induction***

The time taken to induce coma in both bluethroat wrasse and horseshoe leatherjackets was significantly different at temperatures that differed by less than one degree. Fish that were exposed to gradual temperature decrease during dynamic trials and to higher coma inducing temperatures during static trials (and subsequently took longer period to fall into coma) displayed longer periods of agitation and hyperactivity. Fish exposed to anaesthetic concentrations of carbon dioxide in water often exhibit hyper-activity (MacKinlay *et al.* 1994). Such hyperactivity was also observed during the current study when developing anaesthetic regimes for surgery to implant canuli and ventilation electrodes (presented in Chapter 6).

If seeking to use coma-inducing temperatures in experimental or commercial contexts it recommended that very low temperatures for a short period under a static application be used to induce coma so as to minimise this agitated or hyper-active stress response. Higher, coma maintaining temperatures can then be applied.

***Conclusions***

This study has described and compared the thermal tolerance of the three species and provided a strong basis on which to proceed with the development of commercial methodologies for use of chilling during transport of these species. The study also provides a comparison of the various methods used to describe thermal tolerance and their application in both scientific and commercial settings.

The temperatures that induce coma are very close to (within tenths of a degree) or below those that correspond to less than 100% survival for the 12hr and 24hr periods. Therefore, the use of temperatures that induce coma cannot be recommended for prolonged periods for the species studied due to the high risk of mortality. Thus, it is concluded from these results that coma induction and maintenance is unlikely to be beneficial in most commercial settings for the species studied despite the likely disproportional decrease in oxygen consumption that may correspond with coma.

While outside the scope of this thesis, the extreme low temperature tolerance and responses to low temperature exposure of greenback flounder provide should prompt further work to describe the biochemical and behavioural basis for this capability. Such work may have considerable scientific and commercial value especially through transgenic or biomedical applications.

As noted in Chapter 2, some workers have described the application of lowered-water temperature as 'cold anaesthesia'. It is worthwhile comparing the observations of the current lowered-water temperature study to a list of the desirable

characteristics of an anaesthetic proposed by Ross & Ross (1984) and Bell (1987). This list and comparison is provided in Table 7.

| *Desirable Characteristic* | *Coma Inducing Temperature* | *Low, Non-Coma Inducing Temperature* |
|---|---|---|
| ***Effective at low doses*** | Dose in the context of chilling is a function of temperature and time and therefore this criterion is difficult to apply directly.<br>Indirectly in the context of health or cost concerns regarding chemical then chilling compares very favourably against this criteria. | Dose in the context of chilling is a function of temperature and time and therefore this criterion is difficult to apply.<br>Indirectly in the context of health or cost concerns regarding chemical then chilling compares very favourably against this criterion. |
| ***Wide safety margin between the effective and lethal dose*** | No – the results of this study showed that coma inducing temperatures are very close to those that are ultimately lethal. | Yes – the use of lowered temperatures marginally above those that induce coma appears to offer a wide safety margin given appropriate insulation. |
| ***Fast acting*** | Dependent on the temperature | Function of the thermal inertia but relatively fast |
| ***Does not cause hyperactivty during induction*** | Coma-inducing temperatures do cause hyperactivity in two of the species studied especially if using gradual reduction or exposure temperatures very near the maximum coma-inducing temperature | No activity generally declines with temperature |
| ***Soluble in water***<br>***Inexpensive*** | This is not directly comparable<br>The cost of chilling and maintenance of low temperatures in water is dependent on a range of factors but is probably not greater than the cost of most chemical anaesthetics. | This is not directly comparable<br>The cost of chilling and maintenance of low temperatures in water is dependent on a range of factors but is probably not greater than the cost of most chemical anaesthetics. |
| ***Quick recovery*** | Yes – probably related almost solely on the thermal inertia | Yes – probably related almost solely on the thermal inertia |
| ***No lasting residue***<br>***Safe for human operators.*** | No lasting residue<br>Chilled water is comparatively safe (especially in smaller volumes) and is easily contained without harmful fumes. | No lasting residue<br>Chilled water is comparatively safe (especially in smaller volumes) and is easily contained without harmful fumes |

**Table 7 Comparison of low temperature exposure and the desirable characteristics of an anaesthetic as described by Ross & Ross (1984).**

# CHAPTER 5

# EFFECTS OF FEEDING ON RESPIRATORY PHYSIOLOGY



## INTRODUCTION

A post-prandial increase in oxygen consumption has been observed for many fish species (Muir & Niimi 1972, Hamada & Ida 1973, Beamish 1974, Brett 1962, 1976, Miura *et al.* 1976, Tandler & Beamish 1979, Jobling 1982, Lucas & Priede 1992). This increase is thought to be the result of increased metabolic consumption of oxygen during the nutritive process and is mostly attributed to a post-absorptive effect (Jobling 1981). In the literature this phenomenon has been termed the specific dynamic effect (Kleiber 1961), heat increment and calorigenic effect of feeding (Garrow 1974), however specific dynamic action (SDA) is the most commonly used term.

There are three parameters of interest with regard to the study of SDA: peak, duration and magnitude (function of the period and level of increase above the 'resting rate'). Various factors have been shown to affect these parameters including:

- ration size (both absolute and relative to body size) (Averett 1969, Muir & Niimi 1972, Hamada & Ida 1973, Beamish 1974, Schalles & Wissing 1976, Caulton 1978, Tandler & Beamish 1979, Vahl & Davenport 1979, Jobling & Davies 1980);
- temperature (Saunders 1963, Jobling 1980, Jobling & Davies 1980); and

- composition of the meal (Jobling & Davies 1980).

While a range of factors can affect the peak, duration and magnitude of post-prandial oxygen increase, in general terms there is a rapid increase in oxygen consumption to a peak a few hours after feeding. The peak rate of post-prandial oxygen consumption occurs in the first quarter of the total duration (Saunders 1963, Muir & Niimi 1972, Beamish 1974, Vahl & Davenport 1979, Jobling 1980, Jobling & Davies 1980). Beyond this point there is a gradual tapering off with increasing time post-feeding before oxygen consumption returns to the pre-feeding rate (Jobling 1981, 1982).

The post-prandial peak rate is generally around double 'low routine' or 'standard' resting rates for most species studied (Saunders 1963, Muir & Niimi 1972, Beamish 1974, Irvin 1974, Hettler 1976, Schalles & Wissing 1976, Vahl & Davenport 1979, Jobling & Davies 1980, Jobling 1981) and is certainly less than those rates observed in actively swimming fish of the same species. While post-prandial oxygen consumption typically reaches an asymptote considerably below the maximum metabolic rates, feeding has been shown to reduce the scope for activity (Muir & Niimi 1972, Vahl & Davenport 1979).

Due to its effect on metabolic rate and oxygen demand, feeding is clearly an important consideration when determining the optimal methods of live fish capture, holding and transport. For example, if no regard is given to the need to deprive fish of food before transport, the rate of oxygen consumption during transport could be markedly higher than that of the same mass of fish deprived of food.

While some recent literature (de Guingand 1995, Huss 1995, Terchunian *et al.* 1999) suggests that fish should be deprived of food prior to live transport to increase survival, the recommendation appears to be based on anecdotal evidence rather than empirical analysis. Few authors investigating live fish transport physiology have investigated the effects of feeding on various key parameters. Indeed, most make no mention of the recent feeding history of experimental animals.

It is clear that in the context of minimising oxygen consumption, fish should be deprived of food prior to transport. The general effects of feeding on oxygen consumption are well known. However, there are two important outstanding questions in the context of live fish transport and the chilling of transport water: what effect, if any, does feeding have on thermal tolerance and what is the appropriate deprivation period? The work described in this chapter aimed to answer these two questions for greenback flounder and investigate a technique for estimating the minimum deprivation period before transport that is applicable in a commercial setting.

***What effect, if any, does feeding have on thermal tolerance?***

As discussed in Chapter 2, there are two ways to reduce the risk of mortality due to hypoxic conditions during transport. Simply, these are to increase the supply of dissolved oxygen or decrease the demand for dissolved oxygen. As established earlier, both of these elements can be pursued through reduction of temperature within the limits of thermal tolerance.

It might be reasonable to assume that, in direct accordance with the temperature change effects observed for biological $Q_{10}$, lowering the temperature will reduce any given metabolic rate, including that component attributable to post-prandial processes. Indeed, most authors (e.g. Muir & Niimi 1972, Beamish 1974, Vahl & Davenport 1979, Jobling 1980, Jobling & Davies 1980, Joblng 1981) have observed that the peak rate and magnitude of post-prandial oxygen consumption is lower when temperature is reduced. If this assumption is true then the only question facing live fish supply is how long should fish be deprived of food prior to transport to avoid the period of post-prandial increase in oxygen consumption?

However, two sets of observations led me to question whether thermal tolerance was affected by recent feeding history. First, as stated earlier, feeding has been shown to reduce the scope for activity (Muir & Niimi 1972, Tyler 1977,Vahl & Davenport 1979). Second, Chiba (1965) and Warren & Duodoroff (1971) observed that fish reduced their intake of food when the concentration of dissolved oxygen was lowered. These observations suggest that, in addition to simply increasing overall metabolic rate, post-prandial metabolic processes will 'compete' with other metabolic components under conditions when oxygen demand outstrips supply.

Hence, recent feeding could result in a net oxygen debt during low temperature exposure that could otherwise be tolerated and/or increase the rate of oxygen debt accumulation beyond the net oxygen demand and supply breakpoint.

***What is the appropriate food deprivation period?***

Regardless of the impact of feeding history on thermal tolerance there remains strong anecdotal evidence supporting the practice of starving fish prior to transport to avoid the effects of post-prandial elevation in oxygen consumption.

Obviously, one option to describe the appropriate deprivation period prior to transport is to undertake respirometry under a range of feeding and temperature conditions for each species of interest. In line with such an approach the SDA for

greenback flounder held at 15°C and fed the typical ration of a commercial pellet diet (1% body weight) was measured using the respirometer as described later in this chapter. However, while this approach will give direct information on the appropriate deprivation period prior to transport (for a given set of circumstances), this solution is labour intensive, very costly and unlikely to be practical in commercial settings or even in some laboratories. Hence, there is considerable benefit in developing techniques that do not involve sophisticated and expensive equipment to estimate the appropriate starvation period prior to transport.

A hypothesis put forward by Beamish (1974) provides the basis of an alternative solution to respirometry to estimate the duration of post-prandial increase in oxygen consumption. Beamish (1974) hypothesised that the duration of post-prandial oxygen consumption increase was linked to the time of passage of food through the gut, with the elevated rate persisting fractionally longer than the evacuation of the last of the food. This hypothesis is supported by the observations of Jobling & Davies (1979, 1980) that, in plaice, the duration of post-prandial elevation of oxygen consumption was approximately 25% longer than the time taken for complete gastric evacuation.

Jobling and Davies (1979, 1980) observed that, at least in plaice, the duration of increased post-prandial oxygen consumption could be estimated from the gastric evacuation time using a relatively simple linear equation. Thus, in a commercial setting, the duration of gastric evacuation could provide a reasonable basis on which to determine the approximate deprivation period that should be applied before transport. Moreover, determination of the period of gastric evacuation will allow avoidance of the main period of excretion and thereby allow better water quality to be maintained during shipment.

Numerous factors modify gastric evacuation rates in fishes. These include:

- temperature (Molnar *et al.* 1967, Brett & Higgs 1970, Elliot 1972, Jobling *et al.* 1977, Jobling & Davies 1979,1980, Flowerdew & Grove 1979, Jobling 1980, Gwyther & Grove 1981, Grove *et al.* 1985, Grove 1986, Bagge *et al.* 1995, De Silva & Andersen 1995, Specziar 2002);
- meal size (Tyler 1980, Beamish 1972, Elliot 1972, Jones 1974, Flowerdew & Grove 1979, Grove & Crawford 1980, Gwyther & Grove 1981, Grove *et al.* 1985, Saether & Jobling 1997);
- fish size (Swenson & Smith 1973, Jones 1974, Jobling *et al.* 1977, Persson 1979, 1981, Grove *et al.* 1985);
- meal composition (Windell 1967, Kionka & Windell 1972, Windell *et al.* 1972, Jones 1974, Grove *et al. 1978*, Jobling 1980, Flowerdew & Grove 1979, Hofer *et al.* 1982, Weisberg & Lotrich 1982); and

- feeding frequency (Rozin & Mayer 1964, Tyler 1970, Laurence 1971, Nobel 1973, Jones 1974, Possompes *et al.* 1975, Hofer *et al.* 1982, Grove *et al.* 1985).

Not surprisingly, these factors overlap with those affecting post-prandial oxygen consumption. Of interest in the current study is the effect of temperature on gastric evacuation rate. Most studies have found that gastric evacuation rate decreases exponentially with decreasing temperature (Grove 1986, Jobling *et al.* 1977, Jobling & Davies 1979,1980, Jobling 1980,1981, 1982, DeSilva & Andersen 1995, Flowerdew & Grove 1979, Gwyther & Grove 1981). Therefore the application of lowered temperature during transport to fish that have not been deprived of food before packing would result in an extended gastric evacuation time and period of heightened post-prandial oxygen demand.

Early work on the gastric evacuation rates of fish was based on direct observation of individual consumption and invasive techniques to measure food remaining in the gut after a given time. While direct observations of gastric evacuation maybe practical in a commercial setting when circumstances are relatively limited, direct observations are very time-consuming and always fatal to the fish (Talbot 1985) and hence such an approach will be constrained in it's ability to generate data in empirical research settings when a range of circumstances are to be investigated. Hence it is important that less time consuming and less invasive techniques be explored for use in research into gastric evacuation.

***X-radiography***

During the 1980s, a non-invasive technique utilising X-radiography was developed to study the passage of gut contents. In brief, X-radiography detects a marker incorporated into the feed at a known concentration as it passes through the alimentary system. Since its development, X-radiography has been widely applied in qualitative and quantitative studies of feeding and gastric evacuation in fishes. The reader is referred to Fange & Grove (1979), Talbot (1985) and Jobling (1993) for detailed reviews of this work.

Talbot & Higgins (1983) first described a modification of previous methods that used particulate rather than 'dissolved' markers (such as the radioisotope $^{131}I$ as described by Storebakken *et al.* (1981) and chromic oxide). This modification greatly increased the power of X-radiographic studies to deliver meaningful quantitative results on the amount of material in the gut. The particulate method was successful in studies of the gastric evacuation rates of Atlantic salmon (Talbot & Higgins 1983, Talbot 1985) and dab, *Limanda limanda* (Gwyther & Grove 1981). However, when this method was applied to Arctic charr, *Salvelinus alpinus* (Jorgensen & Jobling

1988) and cod (dos Santos & Jobling 1991), preferential retention of the particulate marker led to overestimation of the duration of gastric evacuation.

The study reported in this chapter aimed to determine the gross effects of feeding on thermal tolerance, measure the SDA of greenback flounder and investigate the use of gastric evacuation duration in estimation of SDA duration. The utility of X-radiographic techniques for estimating gastric evacuation of greenback flounders was also investigated.

## METHODS AND MATERIALS

### *Effect of Feeding on Thermal Tolerance*

For several months before commencing the trials to investigate the effects of feeding on thermal tolerance, experimental specimens of greenback flounder were held in tanks with a constant water temperature of 15°C. The fish in these tanks were fed a daily ration approximately equal to 1% of the total mass of fish in the tank. Fish were fed once per day.

Two different sets of trials were undertaken to investigate the effects of feeding on thermal tolerance of greenback flounder. The first four trials were run using the 'down & hold' regime (as described in Chapter 4) with 10 flounder per trial that had been deprived of food for 72hr. The survival of fish deprived of food for 72hr was then compared to the survival data for fish deprived of food for 24hr in the 'down and hold' trials presented in Chapter 4. In addition, a $TL_{50}$ and $CT_{Min}$ were calculated for the 72hr deprivation trials.

In the second set of experiments, two fish deprived of food for 12hr and two fish deprived of food for 72hr were placed in each of the three open flow-through tanks described in Chapter 3. Fish from each feeding regime were distinguished using dorsal markings. Using the 'down & hold' regime with a hold temperature of 3°C, the survival of fish from each group was compared. This experiment was repeated and mortality data for 12 fish from each deprivation period was collected.

### *Specific Dynamic Action*

Two groups of ten flounder held in 15°C sea water were deprived of food for 72hr and then fed a single typical daily ration (1% body weight) of the commercial pellet diet over a fifteen minute period and oxygen consumption of each group was then measured every 20s for 96hr post-feeding using the flow through respirometer described in Chapter 3.

***Gastric Evacuation and X-radiography***

Direct observations of gastric evacuation were made as part of validation experiments for X-radiography. For greenback flounder 100-200g in weight, no. 8.5 'Ballotini' glass spheres (0.4mm diameter) were chosen as the X-ray dense marker. As a guide to preparing marked feeds, McCarthy *et al.* (1993) recommended that no more than 100-150 Ballotini spheres should be present in the gut of the experimental animals at any time so as to minimise overlap during counting. Based on this recommendation and the typical daily ration of the greenback flounder to be used in the trial, 3g beads were added to each 100g of the existing diet. In this ratio, the beads and pellets from the greenback flounder's existing commercial diet (supplied by Pivot Pty Ltd, Tasmania) were blended together and re-pelletised. The reconstituted pellets were of the same size and moisture content as the commercial pellets.

A calibration curve was then prepared for the marked diet by counting the number of spheres displayed on X-ray films of a range of samples of the diet of known dry weight. A formula for the relationship between density of spheres and dry weight of food was derived from the regression line of the calibration curve.

For X-radiography to be successful in gastric evacuation experiments, the marker must not be preferentially excreted or retained relative to the food. Thus, the next step was to conduct a validation trial to determine if this prerequisite was satisfied.

For the validation trial, 24 fish kept at 15°C were deprived of food for 72hr and then two fish in each of the twelve 50l circular tanks were fed a known weight of the marked diet. Consumption of all food presented was confirmed by visual observation. Fish were offered food repeatedly over a four-hour period.

Every four hours after the cessation of feeding, the fish in one tank were removed, anaesthetised and X-rayed whole. These fish were then given an overdose of anaesthetic (benzocaine) before an incision was made along the ventral surface and the gut exposed. The gut was tied, using waxed thread, at the oesophagus and anus and at six approximately equidistant points along its length before removing it from the body cavity. The gut was arranged to enable clear identification of the various sections and was then X-rayed. The gut contents were then gently scraped out and the contents were lyopholised and weighed.

The actual dry weight of the gut contents was then compared to the dry weight predicted by X-ray counts of the spheres in both whole fish and the dissected gut.

The number of spheres in each portion of the gut was also counted to monitor their progress through the gut with time.

## RESULTS

### *Effect of Feeding on Thermal Tolerance*

During low temperature exposure treatments, the survival of greenback flounder deprived of food for 72hr was significantly higher than that of fish deprived of food for 24hr (P=0.0002, d.f.=1, different Tukeys and LSD groupings) (Figure 17).

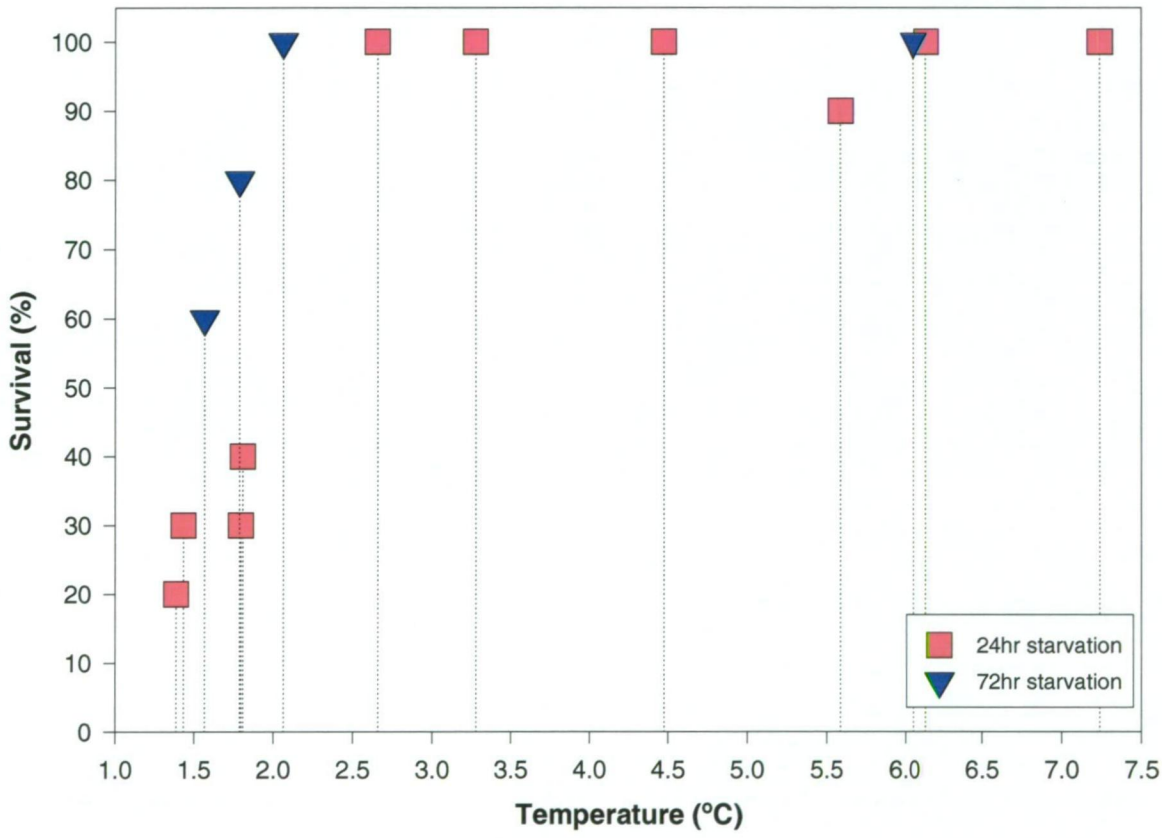



**Figure 17 Survival of greenback flounder deprived of food for either 24 or 72hr after exposure to down and hold lowered temperature regime.**

$TL_{50}$ and $CT_{Min}$ values were calculated from the mortality data for the fish deprived of food for 72hr. These values are given in Table 8 together with the values for fish deprived of food for 24hr (as provided in Chapter 4) for comparison. There was no

significant difference in the $CT_{Min}$ values for 24 and 72 hr deprivation periods (P=0.7837, d.f.=1).

| | *24hr Deprivation* | *72hr Deprivation* |
|---|---|---|
| $TL_{50}$ | 1.9°C | 1.5°C |
| $CT_{Min}$ *(std dev., n)* | 1.73°C (0.83, 29) | 1.64°C (0.11, 6) |

**Table 8 $TL_{50}$ and $CT_{Min}$ values for greenback flounder deprived of food for 24 & 72hr**

In the second set of trials the mortality of fish deprived of food for 72hr was significantly lower than that of fish deprived of food for only 12hr ($\chi_{calc}$=10.08, $\chi_{tab}$=3.84, d.f.=1). Indeed all twelve of the fish deprived of food for 72hr survived but only one of the 12 fish deprived of food for only 12hr survived.

***Specific Dynamic Action***

Post-prandial oxygen consumption of greenback flounder at 15°C is shown in Figure 18. There was no significant difference in the oxygen consumption of the two groups of ten flounder over time (P=0.9818, d.f.=1, nested ANOVA). Both groups of flounder showed a rapid increase in oxygen consumption after feeding. Peak oxygen consumption occurred around six hours after feeding. This peak was 1.88 times the routine oxygen rate for fish starved for 72hr. Between six and 18 hours post-feeding, oxygen consumption declined gradually. Between 18hr and 26hr post-feeding oxygen consumption declined more quickly but the rate of decline then decreased continuously for the next 24hr. By 52hr post-feeding, oxygen consumption had fallen back to levels not significantly different (P=0.2210, d.f.=1) from routine levels for greenback flounder deprived of food for 72hr at 15°C.

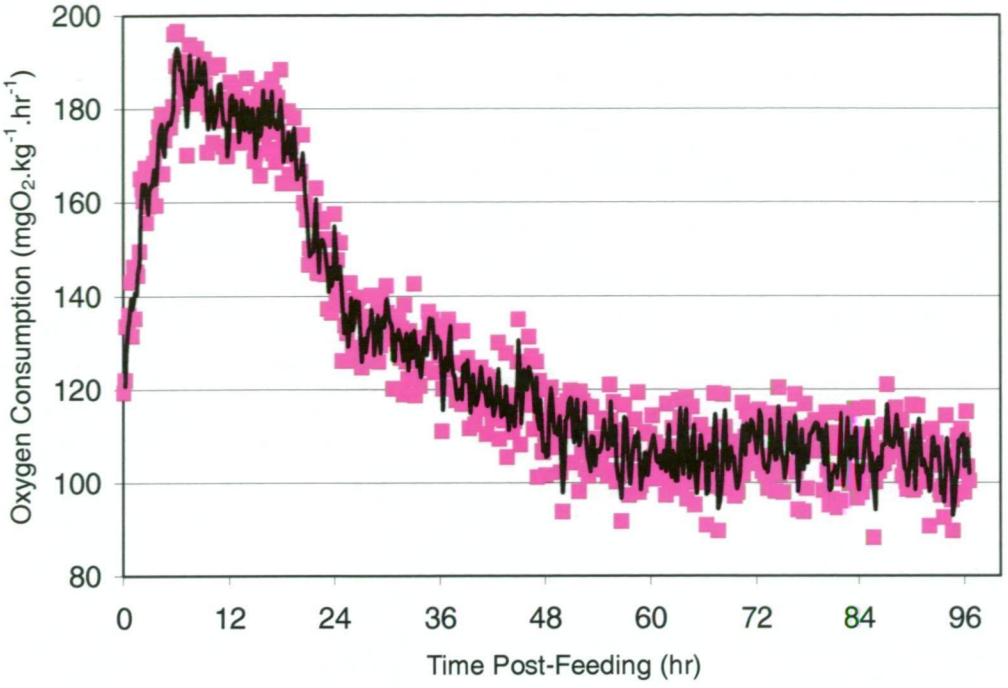



**Figure 18 Post-prandial oxygen consumption of greenback flounder at 15°C. Points are combined group averages (two groups of ten flounder) over ten minute time periods. Trendline (black line) is the moving average.**

***Gastric Evacuation and X-radiography***

*Dissection Results*

The remaining dry weight of gut contents as a percentage of total dry weight of food consumed is shown in Figure 19. While noting the poor fit of the data to the regression line during the first 20hr post-feeding, extrapolating from the equation of the line suggests that complete gastric evacuation would be achieved in 39.5hr at 15°C.

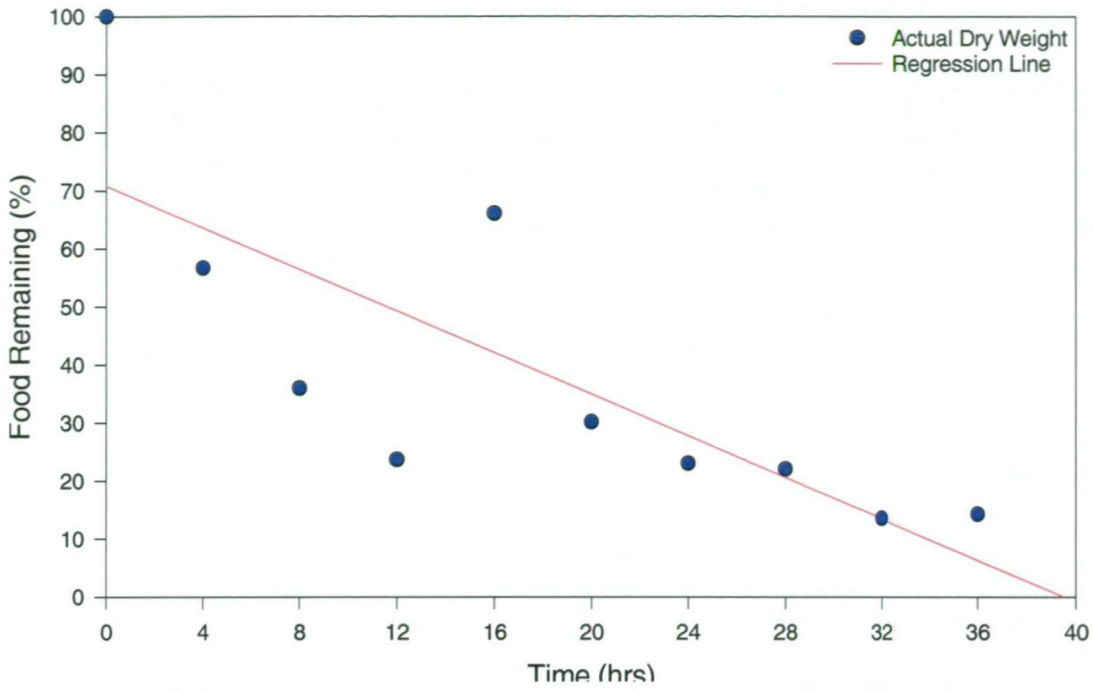



**Figure 19 Gastric evacuation of greenback flounder as determined from dissection results. These results suggest that gastric evacuation would be complete after 39.5hr.**

*Ballotini Sphere Calibration*

The calibration curve for the diet used is shown at Figure 20 including a linear regression and 95% confidence intervals. From this figure the following equation for predicting dry weight of gut contents was derived:

$$\text{Predicted Dry Weight (g)} = \frac{\text{No. of spheres counted on X-ray}}{115.45 \text{ (spheres/g)}}$$

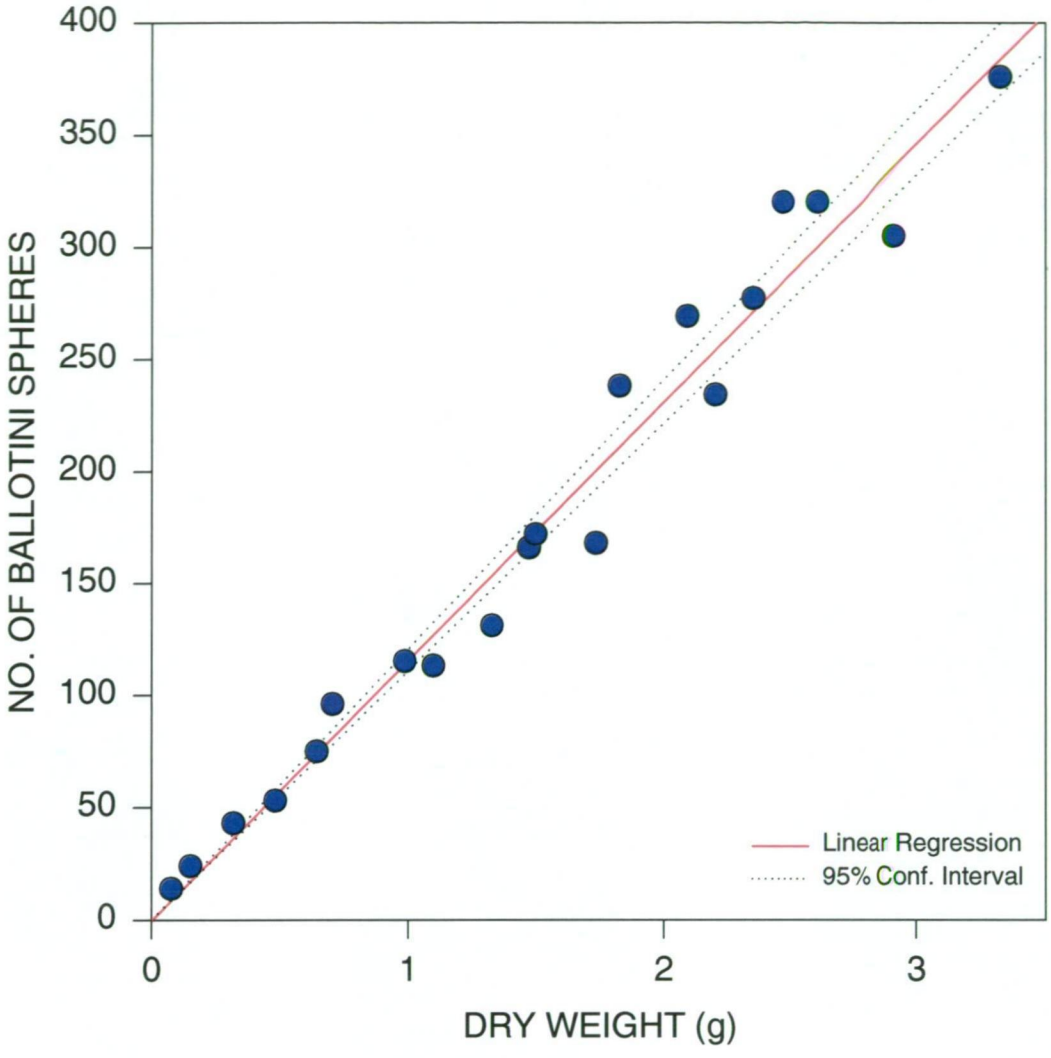



**Figure 20 Calibration curve for Ballotini spheres incorporated in diet. Size 8.5 Ballotini spheres (0.4mm diam.) were added to feed at a rate of 3% by weight.**

*Comparison of Actual and Predicted Gastric Evacuation*

The dry weights predicted from both X-rays of whole fish and dissected guts were compared to the actual dry weight. Analysis of variance (including Tukeys and LSD comparisons) determined no significant difference between actual and either of the predicted weights (P=0.5658, d.f.=2) (Figure 21). Looking at the figure this statistical outcome was somewhat surprising and comparisons were re-run using analysis of covariance and Student paired *t*-tests to confirm this result. All tests confirmed that there was no significant difference in the predicted and actual dry weights.

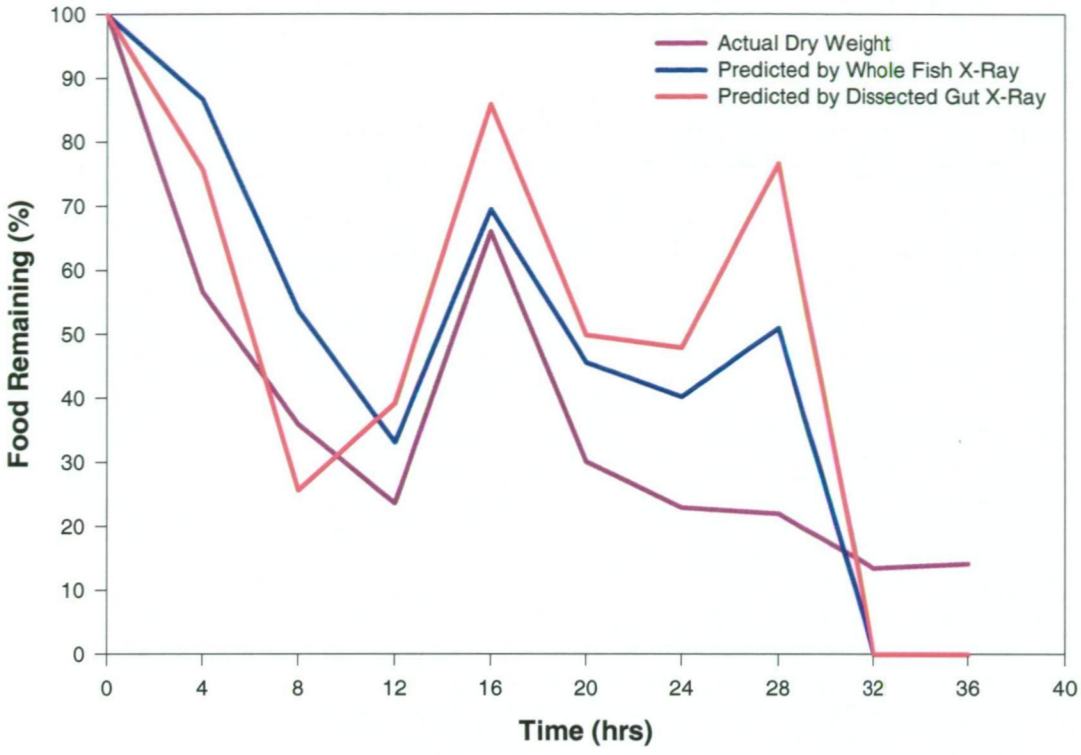



**Figure 21 Comparison of gastric evacuation results predicted by X-ray (whole fish and dissected gut) and actual dry weight of gut contents.**

*Comparison of X-ray Counts from Whole Fish and Dissected Gut*

A comparison of counts of Ballotini spheres from X-rays from whole fish and dissected gut (Figure 22) revealed some problems with the methods. First, the majority of pairings exhibited higher counts for dissected gut than counts for whole fish.

Second, some counts were higher for whole fish and this loss of spheres was attributed to regurgitation during the anaesthesia overdose prior to dissection. One fish from each pair processed at times 4, 8 and 28hr post-feeding regurgitated some food during anaesthesia.

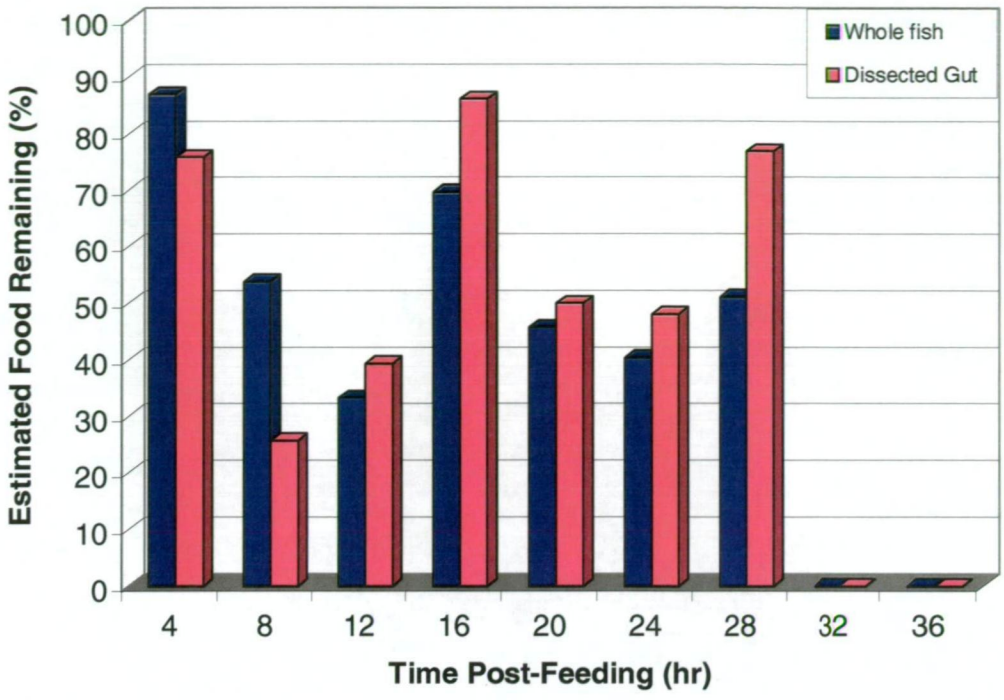



**Figure 22 Comparison of gastric evacuation estimates from X-rays of whole fish and dissected gut.**

*Progression Beads Through the Gut*

In the counts of beads within each section of the gut over time there was a general pattern of retention of beads in the foregut (i.e. anterior half) followed by relatively rapid progression and excretion at around 24hr post-feeding (Figure 23).

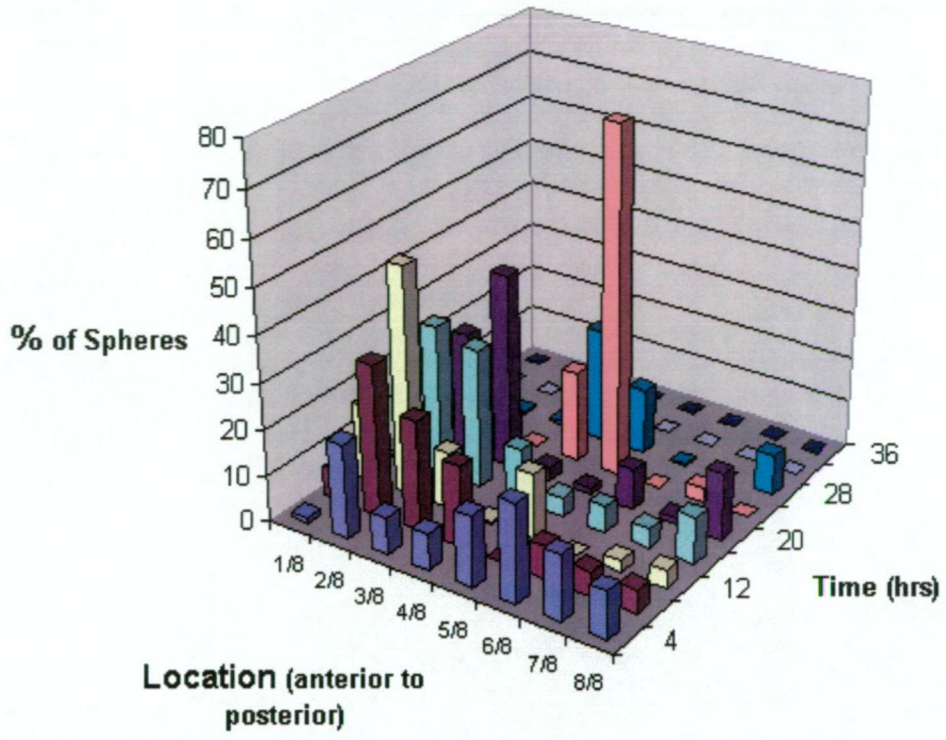



**Figure 23 Progression of no. 8.5 Ballotini glass spheres through the gut of greenback flounder up to 36hr post-feeding. The gut was divided into eight sections (1/8 = anterior to 8/8 = posterior).**

## DISCUSSION

### *The Effect of Feeding on Thermal Tolerance*

The results of this study show that time since feeding significantly alters cold thermal tolerance of greenback flounder. This is the first time that investigation of this effect has been reported for any species of fish. This is a valuable finding both within the development of live fish transport techniques and the broader scientific study of thermal tolerance of fish.

The effect of recent feeding history on thermal tolerance reported here constrains inter-study comparison of thermal tolerance experiments and requires that authors carefully consider this effect when designing experiments to describe thermal tolerance. In the context of live transport it emphasises the need for deprivation periods that allow for complete gastric evacuation and provide for the additional period required for oxygen consumption to fall back to routine rates.

The cause of the effect of feeding on thermal tolerance is unknown. Two possible explanations are as follows. The first is that the post-prandial increase in metabolic

oxygen demand results in net oxygen debt occurring at an earlier stage during low temperature exposure regimes and/or the debt accumulates at a higher rate in fish that have been recently fed than in deprived of food fish. Second, it is possible that, as the systems to deliver oxygen to tissues become constrained by lowering temperature, the body reduces the flow of blood and oxygen to the alimentary system. This could result in the accumulation of toxins that are harmful to fish and result in the heightened mortality rates for a given low temperature exposure.

The value of $CT_{Min}$ for fish deprived of food for 72hr was not significantly different from the value for fish deprived of food for only 24hr despite the fact that the survival rates of these experiments were significantly different. Thus, the use of $CT_{Min}$ as a descriptive statistic for thermal tolerance when death is used as the endpoint is again questioned (as in Chapter 4).

***Specific Dynamic Action***

The specific dynamic action of greenback founder closely followed the general pattern reported by many authors for other species of fish (e.g. Saunders 1963, Muir & Niimi 1972, Beamish 1974, Vahl & Davenport 1979, Jobling 1980, 1981, 1982, Jobling & Davies 1980). SDA peaked in the first few hours at nearly double (1.88 times) the routine metabolic rate for fish deprived of food for 72 hr. Eighty percent of the magnitude of SDA was recorded during the first half of the duration (i.e the first 26 hours). These results clearly demonstrate the benefits of depriving fish of food before transport to markedly reduce oxygen consumption. Indeed depriving fish of food for even 24hr before transport would reduce oxygen consumption by around 45% relative to fish that were fed immediately prior to transport. In economic terms this is a considerable benefit to a commercial live fish operator.

With access to a respirometer it is relatively straightforward to measure SDA but such an approach is not practical in a commercial setting and alternate mechanisms need to be investigated for estimating the appropriate period of food deprivation.

***Gastric Evacuation in Greenback Flounder***

Direct observations show that gastric evacuation was virtually complete 39hr after feeding. It is clear that direct observation can be used to measure gastric evacuation and from this the duration of SDA could be estimated using a ratio of gastric evacuation to total duration of post-prandial elevation of oxygen consumption such as that calculated from Jobling & Davies (1980) (i.e. 0.79:1). Using this ratio, the estimated duration of SDA for the trials reported in this chapter would have been 50hr. This corresponds very closely with the actual SDA duration (51 hr 51 min.) measured using the respirometer. While this proxy estimation process for SDA is less than robust, it would appear that in a commercial setting, use of deprivation

period around 25% longer than the gastric evacuation time is likely to provide a meaningful reference point for the appropriate period of food deprivation before transport.

The use of a straight multiplier (i.e. $y = ax$) of gastric evacuation time to estimate SDA duration rather than include a non-zero constant in the linear equation (i.e. $y = ax + b$) is appropriate as it will make the estimate of SDA duration more robust to changing circumstances such as temperature or ration size. It is possible that alternate equations (e.g. $y=x^a$) for estimation of SDA duration from gastric evacuation time may provide a better correlation than the simpler linear equation but due to the failure of X-radiography to provide a workable estimate of gastric evacuation this data was unavailable in the present study.

***Use of X-radiography to Study Gastric Evacuation in Greenback Flounder***

For the purposes of expanding the research into gastric evacuation and SDA (including further testing of the proxy for SDA duration from gastric evacuation time described above), it was hoped that X-radiographic techniques for measuring gastric evacuation would avoid the need to sacrifice the large numbers of fish required if direct observations were used. While the results did not find a significant difference in the actual gastric evacuation rate compared to that predicted by X-radiography, the technique was considered to provide a relatively poor estimate of gastric evacuation rate for the purposes of inclusion with respirometry experiments to investigate metabolic rates in association with gastric evacuation.

There were problems associated with the comparison of counts of spheres for the same fish before and after dissection. First, where counts were higher for whole fish, loss was attributable to regurgitation during anaesthesia and prior to dissection. Second, as a result of gut coiling and subsequent overlapping of spheres, the majority of pairings exhibited higher counts on X-ray films of dissected guts. This second problem can be avoided by reducing the density of beads in the prepared feed. However, the former problem is more difficult to resolve and may require careful adjustment of anaesthetic concentrations and handling procedures.

The progression of glass beads through the gastric tract suggests that greenback flounder retain the beads in the foregut before a subsequent and relatively rapid evacuation of beads from the intestinal tract. The staged progress of beads through the gut is suggestive of retained functional ability of the stomach despite the absence of sphincters. This finding contrasts with that of Grove *et al.* (1985) who observed a considerably reduced role for the stomach in the dab (another member of the family Pleuronectidae).

### *Conclusion*

It is clear that fish need to be deprived of food prior to chilling and transport if significant increases in oxygen consumption during specific dynamic action are to be avoided. While SDA is relatively simple to describe using a respirometer, such techniques are impractical in most commercial and many research settings. Initial results suggest that gastric evacuation time appears to offer considerable potential as a basis of a proxy for SDA duration.

The results presented in this chapter strongly suggest that researchers should pay close attention to the effect of feeding on studies of thermal tolerance. All publications should clearly describe, as a minimum, the food deprivation period and temperature. The extensive review of literature on thermal tolerance presented in Chapter 4 shows that few researchers provide any information on feeding history.

Use of X-radiography to study gastric evacuation in greenback flounder appears problematic and other methods should be assessed. If X-radiography is to be used in greenback flounder it should be constrained to studies involving estimates of intake rather than evacuation.

### *Next Steps*

While the results presented in this chapter regarding the use of gastric evacuation time as a multiplier base for an estimate of SDA duration are promising, the application of such a proxy in a commercial live transport requires further investigation. In particular the information presented in this thesis and in the work of Jobling & Davies (1980) is based on two species of pleuronectid fishes and other families or species of fish may show quite different multipliers of gastric evacuation time are necessary to provide a reasonable estimate of SDA duration.

# CHAPTER 6

# RESPIROMETRY



## INTRODUCTION

In 1956, Winberg wrote, "..there is no need for additional accumulation of data on metabolic rate of fish under conditions of weak activity or nearly resting because not one of the basic problems of practical interest can be solved in that manner." Obviously this statement was not made with the challenges of live transport of fish in mind. The encouragement of quiescence is a focal point in the improvement of live fish transport techniques. An understanding of the oxygen consumption of fish under various conditions can greatly accelerate the development of new and improved transport methods and remove the frustrations of a trial and error approach.

### *Respiratory Oxygen Consumption*

Aerobic catabolic processes (e.g. aerobic glycolysis) consume molecular oxygen in the production of energy (ATP) from proteins, lipids and sugars during respiration. Carbon dioxide and water are produced as products of these reactions (Eckert *et al.* 1988). Energy from ATP is the major source of chemical energy used by animal cells to drive biochemical reactions. Thus, under aerobic conditions, the oxygen consumed is proportional to the metabolic rate and the oxygen consumed by an animal can be used to estimate its metabolic rate.

When animal tissue receives less oxygen than is required to produce the required amount of energy the energy will be produced by anaerobic glycolysis. Under anaerobic respiration, the rate of production of pyruvate by glycolysis exceeds the oxidation of pyruvate by the citric acid cycle. Under anaerobic respiration the rate of formation of NADH by glycolysis is greater than the rate of oxidation by the respiratory chain. Continued glycolysis to release ATP depends on the availability of $NAD^+$ for the oxidation of glyceradelhyde 3-phosphate. The accumulation of both NADH and pyruvate is reversed by lactate dehydrogenase, which oxidises NADH to $NAD^+$ as it reduces pyruvate to lactate.

Lactate is a dead end in metabolism. It must be converted back into pyruvate before it can be metabolized. Conversion of lactate to pyruvate and then to glucose occurs in the liver and is referred to as gluconeogenesis. This cycle of glycolysis and gluconeognesis is known as the Cori cycle and this cycle is facilitated by the transport of glucose and lactate in the blood (Eckert *et al.* 1988, Stryer 1988). The production of lactate allows ATP production but creates an oxygen debt. Ultimately, if a shortfall in oxygen supply to tissues persists, the fish will continue to accumulate lactic acid, lose equilibrium and die (Heath & Pritchard 1965).

In most species of fish, the vast majority of molecular oxygen is supplied by diffusion of dissolved oxygen across the gills. This diffusion is passive and its rate is proportional to the gradient of oxygen tension between the water and blood. Increasing the flow of water over the gills during opercular or ram ventilation increases the gradient and is the main response to increasing oxygen demand (Eckert *et al.* 1988). However, increased ventilation incurs a metabolic cost.

Hypoxic conditions are not typically encountered in aquatic environments (Butler & Metcalfe 1983). However, fish sometimes encounter low oxygen levels in the environment. For example, fish migrating upstream in high densities for breeding or those living in marginal river pools may encounter waters low in oxygen (Maier 1977, Esteves 1988, Lloyd 1992). For useful reviews of oxygen in water and its impact on fish the reader is referred to Fry (1971), Holeton (1980) and Heath (1990).

Fish oxygen consumption differs considerably between species and ranges from 10-1400 mg $O_2.kg^{-1}.hr^{-1}$ (Terchunian *et al.* 1999) although most fish species studied have recorded values between 50 and 400 mg $O_2.kg^{-1}.hr^{-1}$. Table 9 is a summary of routine oxygen consumption and extraction results for a range of species across various studies.

It should be noted that different species exhibit marked differences in their oxygen demand per unit weight and their ability to cope with lowered levels of dissolved

oxygen in water. Some species such as carp, lungfish and eels are capable of surviving in water with very low oxygen tension or anoxic water for considerable periods. Carp in particular are noted for their extreme tolerance of hypoxia (Lomholt & Johansen 1979, Butler & Metcalfe 1983). N'Goma (1993) attributed the tolerance of carp to biological adaptations to cope with the wide yearly variations in the levels of dissolved oxygen in their habitat.

Within a given species there are a range of factors that affect oxygen demand. As discussed in earlier chapters, changes in temperature have generally resulted in changes in the oxygen consumption of poikilothermic animals by increasing or decreasing metabolic rate and activity in response to higher or lower temperatures respectively within the bounds of lethal range.

The thermal history of fish of the same species will affect their oxygen consumption at a given temperature. Cold-acclimated fish use more oxygen than warm acclimated fish when exposed to the same temperature (Gibbson & Fry 1954, Job 1955, 1959, 1969b, Ananthakrishnan & Kutty 1974, Davis 1968, Kutty 1972, Jobling 1982, Kasim 1982). Moreover, the lethal oxygen concentration has been observed to be significantly higher in fish acclimated to lower temperatures (Ananthakrishnan & Kutty 1974, Kutty & Peer Mohamed 1975, Kasim 1982). This is an important consideration in live transport methods as both the oxygen demand and critical oxygen tension will be higher in fish that are acclimatised or acclimated to a lower temperature (e.g. during winter).

| *Common Name* | *Species* | *Weight (kg)* | *Temp (°C)* | *$O_2$ Tension of Afferent Water* | *$O_2$ Cons. ($mgO_2/kg/hr$)* | *$O_2$ Extraction (%)* | *Source* |
|---|---|---|---|---|---|---|---|
| Bass, European Sea | *Dicentrachus labrax* | Juvenile | | Normoxia | 126.2 | | Herskin 1999 |
| Bass, Largemouth | *Micropterus salmoides* | 0.23-0.47 | 20 | Normoxia | 68 | | Cech *et al.* 1979 |
| | | | 20 | Severe Hypoxia | 63 | | |
| | | | 25 | Normoxia | 103 | | |
| | | | 25 | Moderate Hypoxia | 107 | | |
| | | | 25 | Severe Hypoxia | 89 | | |
| | | | 30 | Normoxia | 151 | | |
| | | | 30 | Severe Hypoxia | 153 | | |
| | | | 30 | Severe Hypoxia | 94 | | |
| Bream, Red Sea | *Pagrus major* | | 18.5-22.9 | Normoxia | 114.6-233 | | Mitsunaga *et al.* 1999 |
| Bullhead | *Ictalurus nebulosus* | 0.186 | 20 | Normoxia | 85 | 68 | Saunders 1962 |
| | | | | Severe Hypoxia | 85 | 13 | |
| Carp | *Cyprinus carpio* | 0.8-1.2 | | Normoxia | 69 | 80 | Lomholt & Johansen 1979 |
| | | | | Severe Hypoxia | 88 | 72 | |
| Carp | *Cyprinus carpio* | 0.174 | 20 | Normoxia | 100 | 72 | Saunders 1962 |
| | | | | Sever Hypoxia | 100 | 46 | |
| Carp | *Cyprinus carpio* | 2 - 6 | 10 | Normoxia | 40 | 72-87 | Garey 1967 |
| | | | | Moderate Hypoxia | 40 | 52-80 | |
| Catfish | *Ictalurus punctatus* | 0.004-0.009 | 24 | Normoxia | 205 | 47 | Gerald & Cech 1970 |
| Catfish, Far Eastern | *Silurus asotus* | | 15-19.9 | Normoxia | 35.2-96.4 | | Jo & Kim 1998 |
| | | | 20-24.9 | Normoxia | 78.6-127.9 | | |
| | | | 25-29.9 | Normoxia | 120.1-231.7 | | |
| | | | 30-34.9 | Normoxia | 197.7-352.3 | | |
| Ciclid, Graham's | *Oreochromis alcalicus grahami* | | 37 | Normoxia | 593 | | Frankln *et al.* 1995 |

| *Common Name* | *Species* | *Weight (kg)* | *Temp (°C)* | *$O_2$ Tension of Afferent Water* | *$O_2$ Cons. ($mgO_2$/kg/hr)* | *$O_2$ Extraction (%)* | *Source* |
|---|---|---|---|---|---|---|---|
| Cod, Atlantic | *Gadus morhua* | | 5 | Normoxia | 35.5 | | Schurmann & Steffensen 1997 |
| | | | 10 | Normoxia | 57.0 | | |
| | | | 15 | Normoxia | 78.2 | | |
| Cod, Siberian | *Arctogadus borisovi* | | | Normoxia | 40.9 | | Drud jordan *et al.* 2001 |
| Dogfish | *Scyliorhynus stellaris* | 0.24-3.9 | | Normoxia | 57 | 47 | Piiper & Schumann 1967 |
| Dogfish, Pacific | *Squalus suckleyi* | 2-4.3 | 8 | Normoxia | 27 | 58 | Hanson & Johansen 1970 |
| | | | | Moderate Hypoxia | 44 | 37 | |
| Dogfish | *Scyliorhynus stellaris* | 2-5 | 18 | Normoxia | 94 | 64 | Piiper *et al.* 1977 |
| Dragonet | *Callionymus lyra* | 0.1 | 11.5 | Normoxia | 72 | 30.7 | Hughes & Umezawa 1968 |
| Goldfish | *Carassius auratus* | 0.1 | 25 | Normoxia | 107 | 48.3 | Dejours *et al.* 1968 |
| Hagfish | *Eptatretus cirrhatus* | | 11 | Normoxia | | 9.9 | Forster 1990 |
| Mexican mojarra | *Cichlasoma uropthalmus* | 0.091-0.116 | 20-36 | Normoxia | 31-4500 | | Martinez-Palacios & Ross 1986 |
| Oscar | *Astronotus ocellatus* | | 28 | Normoxia | | 108 | Muusze *et al.* 1998 |
| Porgy | *Pagrus major* | 0.574 | 21 | Normoxia | 92 | 72.5 | Azuma & Itzawa (1993) |
| Rainbow Trout | *Oncorhynchus mykiss* | 0.5-1 | 15 | Normoxia | 119 | 55 | Holeton & Randall 1967 |
| | | | | Severe Hypoxia | 119 | 20 | |
| Shark, Bonnethead | *Sphyrna tiburo* | | | Normoxia | 234.6 | | Carlson & Parson 2001 |
| Shark, Blacknose | *Carcharinus acronotus* | | | Normoxia | 437.2 | | Carlson & Parson 2001 |
| Shark, Florida smoothhound | *Mustelus norrisi* | | | Normoxia | 161.4 | | Carlson & Parson 2001 |
| Skate, Little | *Raja erinacea* | | | Normoxia | 48.3 | | Hove & Moss 1997 |

| *Common Name* | *Species* | *Weight (kg)* | *Temp (°C)* | *$O_2$ Tension of Afferent Water* | *$O_2$ Cons. ($mgO_2/kg/hr$)* | *$O_2$ Extraction (%)* | *Source* |
|---|---|---|---|---|---|---|---|
| Starry Flounder | *Platichthys stellatus* | 1-8 | 10 | Normoxia | 39 | 55 | Watters & Smith 1973 |
| | | | | Moderate Hypoxia | 30 | 45 | |
| Sturgeon | *Acipenser transmontanus* | 0.82-1.06 | 15 | Normoxia | 72 | 49 | Burggren & Randall 1978 |
| | | | | Moderate Hypoxia | 53 | 21 | |
| Sucker | *Catostomus commersoni* | 0.25 | 20 | Normoxia | 85 | 56 | Saunders 1962 |
| | | | | Severe Hypoxia | 85 | 7 | |
| Trahira | *Hoplias malabricus* | 0.312 | 25 | Normoxia | 74 | | Rantin *et al.* 1993 |
| | | | | Severe Hypoxia | 83 | | |
| Tariputanga | *Hoplias lacerdae* | 0.375 | 25 | Normoxia | 74 | | Rantin *et al.* 1993 |
| | | | | Severe Hypoxia | 89 | | |

**Table 9** **Summary of routine oxygen consumption and extraction results for various species. In this table, treatments that exposed fish to water with a partial pressure of oxygen less than 50mm of Hg were described as severe hypoxia, those with partial pressures of 50-100mm of Hg as moderate hypoxia and the remainder as normoxia. Please note that the formula of Green & Carritt (1967) for conversion of ml $O_2$ to mg $O_2$ has been applied to some data to allow ready comparison.**

In addition to temperature, there are a number of other factors that have been shown to affect the rate of oxygen consumption by fish. These include:

- salinity (Job 1959, 1969a,b, Brett 1962, Kryuchkov & Kasimov 1990);
- mass (Job 1955, Winberg 1956, 1961, Fry 1957, Sundes 1957a, Pritchard *et al.* 1958, Basu 1959, Hickman 1959, Beamish & Mookherjii 1964, Glass 1969, Edwards *et al.* 1970, Edwards 1971, Caulton 1978, Jobling 1982, Martinez-Palacios & Ross 1986, Kikuchi *et al.* 1990, Grantner & Taborsky 1998);
- activity (Krogh 1914, Spencer 1939, Spoor 1946, Higginbotham 1947, Graham 1949, Prosser *et al.* 1957, Basu 1959, Brett 1962, 1965, Jones 1971, Webb 1971a,b, Jobling 1982, Lucas & Priede 1992);
- sex (Idler & Clemens 1959, Brett 1965);
- reproductive state (Terchunian *et al.* 1999);
- diurnal rhythms (Steffensen 1989, Terchunian *et al.* 1999);
- nutritional state (as discussed in Chapter 5); and
- oxygen tension (Brett 1962, Jones 1971, Cech *et al.* 1979, Lomholt & Johansen 1979, Jobling 1982, Rantin *et al.* 1993, Fernandes *et al.* 1995)

Of these parameters, temperature, activity and nutritional state seem to have the greatest scope to impact on oxygen demand of a given mass of a given species.

Some fish species exhibit rates of oxygen consumption that are directly dependent upon ambient oxygen tension (conformers) while others exhibit oxygen consumption that is independent of ambient oxygen tension (non-conformers) until a critical oxygen tension is reached. These differences are important considerations in the transport of live fish that are likely to be exposed to hypoxia. In a closed transport system (e.g. polyethylene bags) the total available oxygen is maximal when the bag is sealed containing the fish and then declines as a result of consumption through respiration resulting in graded hypoxia. Non-conforming species will continue or even increase oxygen consumption in response to graded hypoxia until some critical tension is reached whereas conformers will exhibit a decline in oxygen consumption with graded hypoxia that is compensatory within the context of live transport (i.e. oxygen consumption during transport will decline as oxygen is depleted from the container).

Many species increase the active gill surface area (known as lamellar recruitment) in response to hypoxia (Butler & Metcalfe 1983). In addition, increases in the consumption of oxygen in response to increasing temperature or maintenance of consumption by non-conformers in response to graded hypoxia, generally also involves an increase in the rate of flow of water over the gills. Increasing the rate of flow across the gills in non-ram ventilating fishes requires an increase in the rate and/or volume of ventilatory movements of the opercula that are impacted upon by

factors that increase oxygen demand. For example, in curimbata, *Prochilodus scrofa*, gill ventilation increased as a result of increases in both the rate and breath volume in response to increasing temperature or hypoxic conditions (Fernandes *et al.* 1995). Similarly, oxygen uptake has been maintained by rainbow trout during extreme hypoxia (<5% saturation) by increasing gill ventilation to up to 13 times the rate in normoxia (Jones *et al.* 1970). Oxygen extraction per unit volume of water flow generally declines as ventilation increases (Saunders 1962, Holeton & Randall 1967, Lomholt & Johansen 1979). Thus, the efficiency of ventilation declines with increasing rate.

The relationship between the effects of temperature and hypoxia on oxygen uptake and ventilatory responses has been studied in fish acclimatised at different temperatures and exposed to a range of oxygen levels (Spitzer *et al.* 1969, Ott *et al.* 1980, Hughes *et al.* 1983, Fernandes & Rantin 1989). Many species show differing ventilatory responses to similar conditions. The ventilatory response of fish to changes in temperature or oxygen tension can be used to predict the physiological responses of fish to conditions likely to be encountered during live transport.

It is well known that the optimisation of ventilation to maintain oxygen transfer and satisfy oxygen requirements of fish is controlled via peripheral oxy-chemoreceptors, central catecholamine responses and mechano- and proprioceptor reflexes acting on the respiratory centre of the medulla and modulated in the mid- and fore-brain (Burleson *et al.* 1992, Taylor 1992, Randall 1993, Milson 1993). However, it is not fully understood how these controls affect the breathing frequency and breath volume.

Most studies have been directed toward ventilatory control in fish exposed to hypoxia (Fritsche & Nilsson 1993, Randall 1993) and little information is available on the effect of temperature changes (Roberts & Rowell 1988). Many authors have observed that gill ventilation increases during hypoxia are largely due to increases in breath volume rather than frequency (Gerald & Cech 1970, Steffensen *et al.* 1984, Fernandes & Rantin 1989, Rantin *et al.* 1992). This response is considered to represent an energy saving over increased frequency to deliver the same rate of flow over the gills (Dejours 1988, Rantin *et al.* 1992).

*Estimation of Oxygen Consumption*

The subject of respirometry and its applications in fish has been widely discussed and reviewed. The techniques used by other researchers to estimate respiratory oxygen demand in fish have been presented in chapter 3. For a detailed summary of the history and methods used in fish respirometry the reader is referred to Cech (1990).

Steffensen (1989) considered that standard metabolism is unlikely to be accurately measured in respirometry trials using any extended period due to the probability of at least some spontaneous activity. Therefore that author recommended the measurement of routine rather than standard metabolism of fish should be the goal of respirometry.

Routine oxygen consumption may be defined as the oxygen consumed for fish in which the only movements are spontaneous (Krogh 1914). This has been the most commonly measured state of activity. The difficulty in measuring standard metabolism (i.e. absolute rest) in fish is the requirement to eliminate spontaneous activity. Some investigators who encouraged quiescence have taken the standard rate to be the lowest amount of oxygen consumed over a given period (e.g. Graham 1949, Job 1955, Mann 1956, Pritchard *et al.* 1958, Hickman 1959, Moss & Scott 1961) however, for the purposes of this study these reference levels are largely semantics and for the purposes of comparison levels recorded in this study were compared to routine levels in other studies. The relative change in oxygen consumption in response to changing temperature and graded hypoxia is of greatest relevance in this current study.

Unlike the researchers of thermal tolerance, researchers of oxygen consumption in fish have recognised, almost without exception, the potential effects of nutritional state. As a result, estimation of metabolism generally involves use of fish deprived of food for several days before any measurements of oxygen consumption are taken.

*Oxygen Extraction*

The efficiency of oxygen extraction from water can be a valuable physiological indicator. Oxygen extraction has been measured by researchers using a comparison of the oxygen concentration of inspired and expired water using mask type apparatus similar to a van Dam respirometer (Lomholt & Johansen 1979) or via buccal and opercular cannulation (Burrgren 1978, Burrgren & Randall 1978, Fernandes *et al.* 1995).

*Ventilation*

Description of ventilation rates can complement data on oxygen consumption and extraction in the comprehensive study of respiratory physiology. Three main methods of estimating ventilation are reported in the literature. These are:

- acoustic transducer (Fernandes *et al.* 1995);
- electrodes and resistance (Burggren & Randall 1978); and
- mask experiments with flow meter on expired water (Lomholt & Johansen 1979).

No inter-method comparison could be found nor extensive discussion of the relative merits of each method.

### *Lactate*

In addition to measuring oxygen debt through respirometry, the level of lactate in the blood can be used as a measure of the level of anaerobic respiration. For many species the concentration of blood lactate has correlated well with other measures of anaerobic respiration. However, it should be noted that several studies have failed to observe a clear correlation between oxygen debt and lactate accumulation. Heath & Pritchard (1962, 1965) observed lactate accumulation but no oxygen debt in bluegill sunfish following hypoxia. Indeed, Heath & Pritchard (1965) observed a decrease in oxygen consumption after hypoxic stress in bluegill sunfish. In contrast, Blazka (1958) observed compensatory oxygen consumption consistent with oxygen debt in brown trout, *Salmo trutta*, post-hypoxia but did not observe a rise in lactate levels.

### *The Present Study*

The aims of this study were to describe the effect of temperature change and hypoxia on the respiratory physiology of bluethroat wrasse and greenback flounder. Changes were measured in oxygen consumption, oxygen extraction, ventilation and lactate accumulation in response to temperature changes and hypoxic exposure. Changes in these parameters in response to these treatments are then discussed in the context of live transport and the broader physiology of these fishes.

## METHODS AND MATERIALS

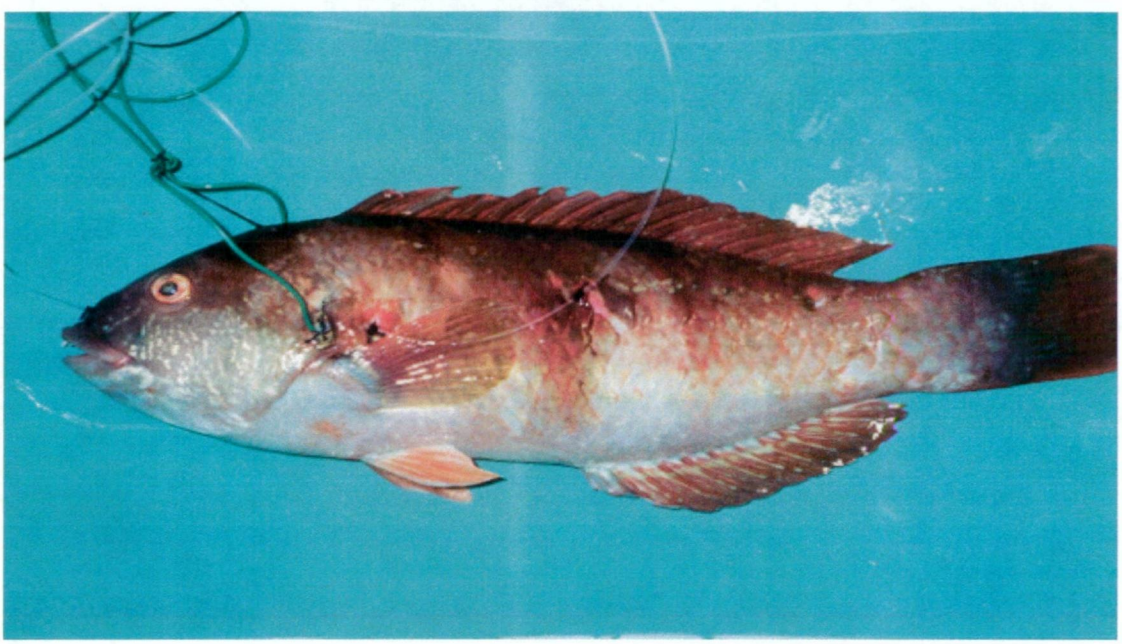
### *Ventilation*

Bluethroat wrasse were deprived of food for at least 24hr but not more than 48hr before being anaesthetised by immersion in 15°C seawater containing benzocaine at a concentration of 400ppm for five minutes. Fish were then placed on a wet sponge and seawater containing an anaesthetic (125ppm benzocaine) was flushed across the gills via a small pump connected to a flexible plastic hose inserted into the mouth. Once it had passed over the gills the water was then drained into a 20l bucket (containing the pump) before re-circulation. Oxygen gas was bubbled through the water in the bucket via a diffuser connected to an oxygen cylinder. The water temperature was maintained at 15°C using a temperature-controlled bath.

Electrodes were prepared by soldering 2m of insulated electrical wire to a small piece of copper sheet (approx. 5mm x 8mm). While the fish was anaesthetised one copper electrode was firmly tied to the posterior edge of each operculuni using surgical thread. To facilitate anchoring of the electrode a small hole was made in the

hard edge of each operculuni using a bevelled hypodermic needle. A small hole was also drilled through the electrode. The thread passed through both of these holes and around the edge of the operculuni several times before being tied off. Various locations along the posterior edge were initially trialed. Placement of the electrode as shown in Plate 10 was found to be the most effective.

**Plate 10.** **Bluethroat wrasse showing location of electrodes to measure ventilation. The cannulae to sample inspired and expired (lateral) water are also visible however the cannula through the operculuni to sample expired water is on the other side of the fish and is obscured in this photo.**



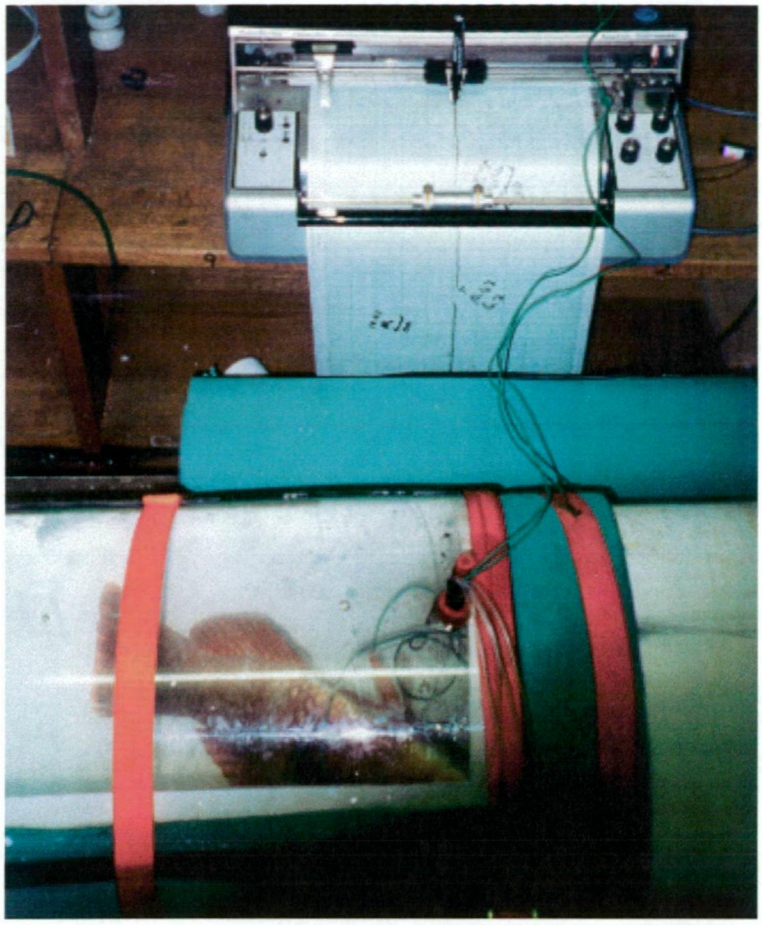
The wires trailing from the electrodes were bridled to the dorsal ridge immediately behind the head using surgical thread. This bridle arrangement created a 'towing point' that prevented the wires from pulling on the opercula as the fish swam around. The wires were in turn connected to a rheostat and subsequently to a flat bed chart recorder (Houston Instrument OmniScribe).

The fish was then placed into the respirometer and allowed to recover from the anaesthetic for 24hr. A 0.1V direct current was passed through the wires and the impedance was recorded as a trace on the chart recorder. The distance between the terminating electrodes altered the resistance and hence the ventilatory movements of the opercula were recorded as a series of peaks and troughs on the flat bed recorder. Validation of the trace with regard to opercular movement was made visually. The shape, height (i.e. distance between opercula) and frequency (i.e. ventilation rate) of

the peaks and troughs were manually measured and counted. It was assumed that breath volume was proportional to the magnitude of opercular movement. Records of the ventilatory movements were made under various conditions. Some observations were made during the anaesthetic recovery period immediately post-surgery but all other measurements were made following the 24hr recovery period. Records of the ventilatory movements during normoxic (air saturated), hypoxic (25% of $O_2$ in air-saturated seawater) and reduced temperature conditions were made. Temperature reduction involved gradual cooling at approximately two degrees per hour. Hypoxic conditions were imparted by bubbling nitrogen gas through the water prior to it flowing into the chamber containing the fish.

In order to obtain sufficient detail in the trace and to discriminate between peaks, the flat bed chart recorder was set at a scroll speed of $10cm.min^{-1}$. Due the amount of paper that would be generated at this speed it was impractical to allow the flat bed recorder trace to run continuously during gradual temperature decrease (i.e. total duration of around seven hours). Instead, data collection was intermittent with a 10min. recording-period every hour (corresponding to approximately a 2°C change in temperature) (Plate 11).

**Plate 11 Comatose female bluethroat wrasse with electrodes and cannulae attached to measure ventilation rates, magnitude and oxygen extraction.**



***Oxygen Consumption***

Respirometry to measure oxygen consumption of fish involved a flow-through respirometer and the technique described in Chapter 3. The respirometer measured the difference between the oxygen concentration of water entering the chamber containing the fish and the water leaving the chamber.

The time allowed for the fish to adjust and settle after placement in the respirometer varies considerably between studies from a few hours (Lomholt & Johansen 1979) to as long as 40hr (Jobling 1982). Most studies allow fish to acclimate to the respiratory chamber for at least 10hr after handling to avoid high lactic acid subsequent to handling (Black 1957, Beamish & Mookherjii 1964) and allow the fish to acclimate to its new surroundings (Burggren & Randall 1978). In this study fish were acclimated to the respirometer for 24hr before any measurements were commenced. The only exception was during measurements of oxygen consumption and ventilation immediately post-surgery to investigate the effects of extended anaesthesia.

Oxygen consumption measurements were made for both bluethroat wrasse and greenback flounder under normoxic and graded hypoxic conditions across a range of temperatures using the flow-through respirometer. In addition to respirometry, direct sampling of blood to allow direct testing for oxygen concentration was trialed. This method involved the insertion of small catheters or cannulae into major blood vessels. The surgery involved use of the same basic techniques and equipment described for placement of electrodes to record ventilation. Multiple sites of insertion were trialed, including the major caudal vessels and those leading from the heart to the brain. Despite firmly securing the flexible tubing to the skin of the fish to take the pressure off the points of insertion into the blood vessels both catheters and cannulae tore out of the blood vessels readily once the fish recovered fully from the anaesthetic. This prevented or severely limited blood sampling in most fish and the approach was abandoned.

***Oxygen Extraction***

Using the deprivation of food, anaesthetic and surgery techniques described earlier in this section, flexible silicon cannulae were attached to the fish to sample the water before and after it had passed over the gill. The first cannula was inserted into the buccal cavity between the mouth and gills to sample inspired water as described by Fernandes *et al.* (1995) for curimbata. Two cannulae were attached to sample expired water. The first 'expired' cannula was stitched onto the body of the fish so that its opening faced the anterior end of the fish immediately behind the posterior edge of the operculuni. The second 'expired' cannula was positioned on the posterior edge of the operculuni by drilling a small hole through the hard edge of the operculuni with a beveled hypodermic needle. Once all three cannulae were in place the fish was placed in the respirometer tube and allowed to recover for 24hr before treatment and sampling.

The cannulae were connected to 1ml syringes via a tight fitting hypodermic needle inserted into the tubing. Only silicon tubing was used as rubber tubing is more permeable to gases and should not be used for sampling for gas analysis (Fry 1957). All air was removed from the tubing and the cannulae were positioned to siphon water from the respirometer to avoid air entering the tubing when not in use for sampling.

Only bluethroat wrasse were used for these trials. This species is not a ram ventilator and water is moved across the gills predominantly by opercular movement. To sample inspired water the corresponding syringe was drawn to fill while the opercula were moving outwards away from the gills. To sample expired water the corresponding syringes were drawn simultaneously as the opercula were moving inwards toward the gills.

Once filled, the three syringes were immediately capped using small rubber stoppers and the partial pressure of oxygen (mm of Hg) in the water was then measured using a blood gas analyser (Radiometer BMS3Mk2 Blood Micro System) in a temperature controlled water bath. The blood gas analyser was calibrated before and after each group of three measurements using air-saturated seawater. Any difference in the calibration values before and after each sample group was used to adjust the values of the sample group as follows:

Sample 1 = no change from original reading
Sample 2 = original reading + (Calibration 1 – Calibration 2)/2
Sample 3 = original reading + (Calibration 1 – Calibration 2)

As the three samples had to be read one at a time with rinsing between samples there was a small time difference between reading each sample (i.e. 1-2 mins). A trial was conducted to determine whether or not this short time delay affected the measurement of oxygen concentration. Three simultaneous samples were drawn under hypoxic conditions in the absence of any fish. The oxygen concentration of the three syringes was then tested and the order of testing noted along with the result. This process was repeated ten times. It was found that the order of testing did affect the reading of partial pressure of dissolved oxygen and a correction factor of 0, +0.55 and +1.1 mm of Hg was applied to the three samples in their order of testing. The order of testing was chosen on an *ad hoc* (i.e. not strictly random) basis and noted to allow for correction.

Once the partial pressure of oxygen in each sample had been measured and corrected for calibration drift and order of measurement, the oxygen concentration of the water in each sample was calculated using the formula below:

$$C_{O2\,(WS)} = \frac{P_{O2\,(WS)} \times C_{O2\,(ASSW)}}{P_{O2\,(ASSW)}}$$

where:

$C_{O2\,(WS)}$ = Concentration of oxygen in the sample (mg $O_2.l^{-1}$)
$P_{O2\,(WS)}$ = Partial pressure of oxygen in the sample (mm of Hg)
$C_{O2\,(ASSW)}$ = Concentration of oxygen in air-saturated seawater at the sample temperature (mg $O_2.l^{-1}$)
$P_{O2\,(ASSW)}$ = Partial pressure of oxygen in air-saturated seawater (mm of Hg)

The concentration of oxygen in air-saturated seawater at the sample temperature was determined from tables by Hutchinson (1957) giving oxygen concentration in pure

water across a range of temperatures and correcting these values for the salinity of seawater using data provided by Sverdrup *et al.* (1942). The effect of altitude was assumed to be negligible at the laboratory's elevation based on tables from Rawson (1944). The partial pressure of oxygen in air-saturated seawater was determined from the blood gas analyser following calibration of the measurement using a series of standard solutions of known oxygen concentration. The blood gas analyser was zeroed using a solution of $Na_2SO_3$.

***Lactate***

In order to examine the effects of different temperatures and partial pressures of dissolved oxygen on blood lactate concentration, bluethroat wrasse were first exposed to three different regimes. These were:

1. 'Normal'
During this treatment, fish were placed in the respirometer and exposed for two hours to air-saturated seawater at 15°C.

2. 'Coma'
During this treatment, fish were placed in the respirometer and coma was induced using experimentally constant low water temperatures (using a 'static' protocol as described in Chapter 4) and maintained for two hours. Afferent seawater was air-saturated.

3. 'Hypoxia'
During this treatment, the fish were placed in the respirometer and exposed for two hours to seawater with partial pressure of oxygen at 25% of that of air-saturated seawater.

At the completion of the treatment regime fish were immediately anaesthetised by placing the fish in a 20l bucket of seawater containing 500ppm benzocaine. After two minutes fish were removed from the anaesthetic and placed in cradle prior to drawing blood from the caudal vessels using a 23g needle attached to a 1ml blood gas syringe containing dry lithium heparin. The blood was immediately transferred to eppendorf tubes which were sealed and placed on ice. The blood was centrifuged at 60*g* for 3mins (Eppendorf Centrifuge 5403) and the plasma transferred to clean eppendorf tubes and stored at –20°C.

Plasma samples were tested for lactate concentration using the following materials and technique. Most of the reagents used were sourced from the Lactate Kit (Cat. 826-A), Sigma Aldrich Pty Ltd. To reduce the cost of testing, a modified technique to that prescribed in the kit, was applied. The modified technique described below

allows the number of samples analysed by each kit to be multiplied by seven thereby reducing the cost of testing.

Standard solution – PCA was prepared by adding 20.8ml of 70% PCA to 1L of double distilled water (DDW) and then neutralised with 60µl.ml$^{-1}$ 5M $K_2CO_3$ for storage. 50µl of cold lactate standard (400mg.l$^{-1}$, Sigma Cat. 826-10) was then added to 100µl of 0.6M perchloric acid (PCA) to make the standard solution.

β-nicotinamide adenine dinucleotide (NAD) solution – to 1 vial of β-NAD (Sigma Cat. 260-110), 2ml glycine buffer, 4ml DDW, 100µl lactate dehydrogenase (Sigma Cat. 826-6) was added and the vial placed on ice. Glycine buffer was prepared by adding 0.79g glycylglycine and 0.15g L-glulamic acid to 8ml DDW and titrated to pH 10 with approximately 0.77ml of 10M sodium hydroxide.

Sample solutions - 50µl of plasma was added to 100µl cold 0.6M PCA and shaken to mix in the eppendorf. The solution was placed on ice for 5mins and then centrifuged at 8000g for 3mins before being placed on ice again.

For each sample, 57µl of the corresponding sample solution and 800µl of NAD solution was added to a cuvette. A reference standard was prepared by adding 57µl and 800µl of NAD solution to a cuvette. The blank was prepared by adding 57µl of PCA and 800µl of NAD solution to a cuvette. All prepared cuvettes were placed at room temperature for 45mins.

The absorbance of each sample was read using a Unicam 8625 UV/VIS Spectrameter ATI UNICAM. The absorbance was zeroed using the blank and the reference standard was read every eight samples to allow for correction of any drift as a result of continued reaction during the short time delays between reading samples. No more than 20 samples were tested in any run to avoid extended delays in reading.

The concentration of lactate in each sample cuvette was calculated by multiplying the absorbance value by 65.1mg.100ml$^{-1}$.

Absorbance was read at 340nm since the difference in the absorption spectra between the oxidised form of NAD ($NAD^+$) and the reduced form (NADH) is greatest at that wave-length (Lehninger 1975). The addition of NAD ($NAD^+$) converts lactic acid to pyruvate and releases NADH through oxidative phosphorylation. It is the change in concentration of $NAD^+$ and NADH that is measured by the testing procedure.

## RESULTS

### *Ventilation*

*Hypoxia*

In the five fish tested, hypoxia significantly reduced the mean ventilation rate (P=0.0355, d.f.=4) compared to the ventilation rate of the same fish during normoxic conditions (Figure 24). Hypoxia also significantly decreased the mean ventilatory magnitude (distance between the opercula) during each ventilatory cycle (P=0.0004, d.f.=4).

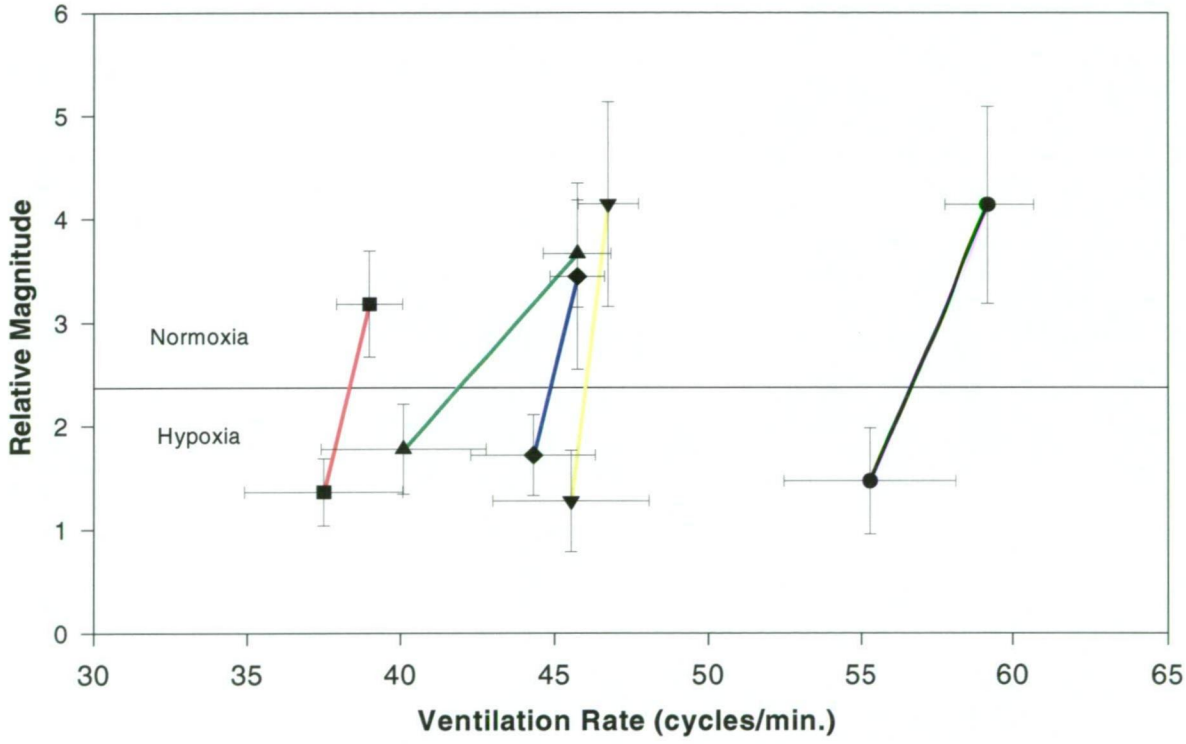



**Figure 24 Ventilation rate and magnitude of bluethroat wrasse during normoxia and hypoxia. Different symbols denote paired readings for each individual fish (n=5). All values are shown +/- 1 s.d.**

Hypoxic conditions significantly increased the variability in the mean rate of ventilation (P=0.0001, d.f.=4) but significantly decreased the variation in the ventilatory magnitude (P=0.0085, d.f.=4).

*Chilling*

Initially, there was a gradual reduction in the ventilation rate as temperature decreased. The lowest ventilation rates were observed when temperatures reached 8.5-9.5°C (Figure 25). Below 7.5°C, the ventilation rate was observed to increase dramatically in most cases. Indeed the ventilation rate at these low temperatures sometimes exceeded that observed at 15°C.

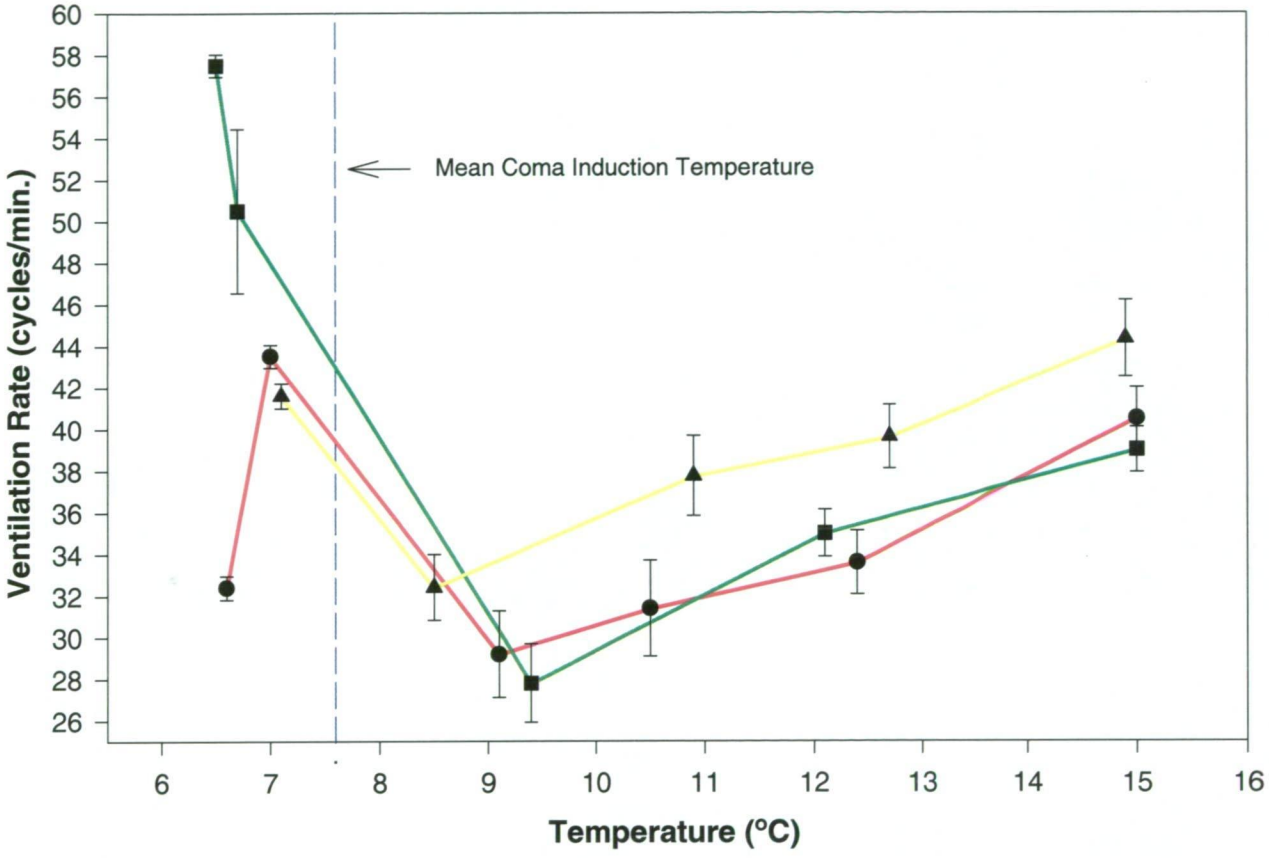



**Figure 25 Effect of declining temperature on ventilation rate of bluethroat wrasse. Different symbols denote individual fish (n=3). Values for ventilation rate are shown +/- 1 s.d.**

The relative magnitude of opercular movements was relatively constant or increasing with declining temperature from 15°C to approximately 10.5°C (Figure 26). Below 10.5°C, the relative magnitude decreased with temperature and was lowest at the lowest temperatures. It should be noted that few, if any, ventilatory movements were visible to the naked eye at low, coma-inducing temperatures.

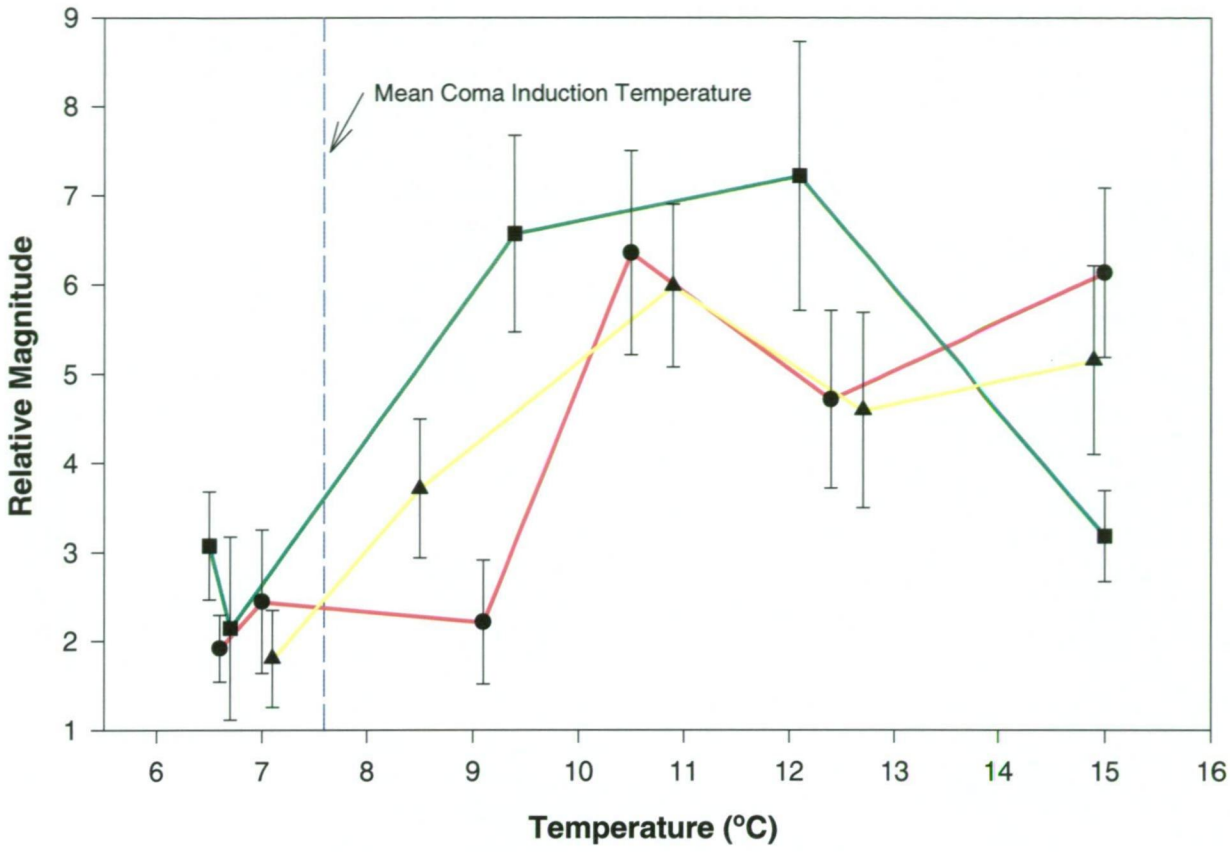



**Figure 26 Effect of declining temperature on ventilation magnitude of bluethroat wrasse. Different symbols represent individual fish. Relative magnitude values are shown +/- 1 s.d.**

As shown in Figure 25 & Figure 26 the ventilatory rate and magnitude differ in the pattern of change with temperature. In order to examine the relationship between ventilatory rate and magnitude as temperature changes, the paired values for rate and magnitude were plotted in Figure 27 and pooled into four temperature bands of 2.5°C intervals. The area of the plot was then arbitrarily divided into four quadrants representing the four combinations of high/low ventilation rate and magnitude. Included in this figure were the paired values from the five fish used in the hypoxia trials described above during normoxic conditions at 15°C prior to exposure to hypoxic conditions.

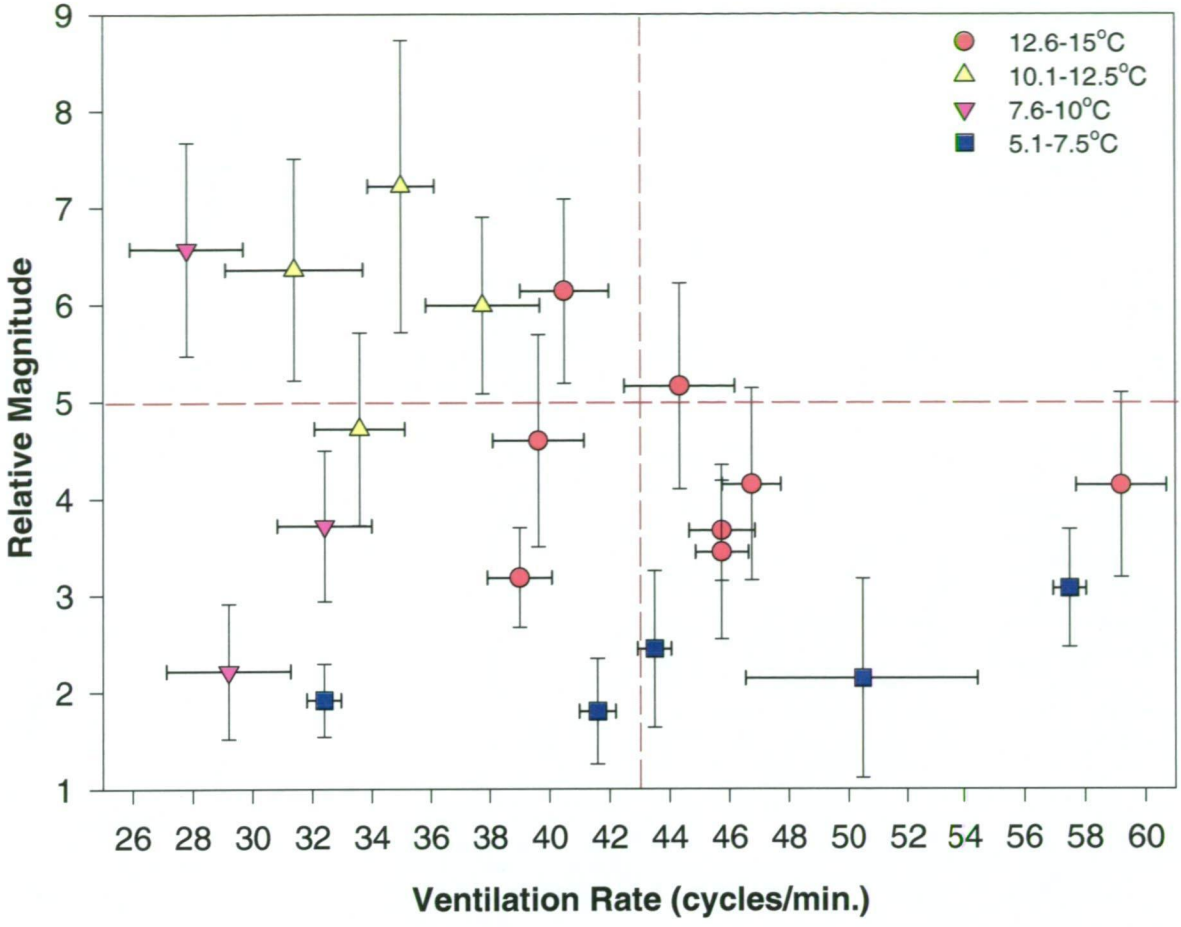



**Figure 27 Changes in ventilation rate and magnitude with declining temperature. The area of the graph has been arbitrarily divided into four quadrants representing the four combinations of high/low ventilation rate and magnitude. All values are shown +/- 1 s.d.**

Figure 27 illustrates a generalised pattern in the distribution of pairings at different temperatures. None of the pairings strongly exhibited both high ventilatory rate and magnitude. At the lowest temperatures ventilation rate was often high but magnitude was at its lowest. Temperatures ranging from 10.1-12.5°C generally decreased the ventilatory rate relative to the higher temperature band but showed an increase in the magnitude. Temperatures in the 7.6-10°C range generally exhibited the lowest ventilatory rate and were in the low region for magnitude. It should be noted that these temperatures correspond with low temperatures approaching but not exceeding the mean coma-inducing temperature for bluethroat wrasse.

As noted in Chapter 4, gradual temperature reduction often induces hyperactivity in bluethroat wrasse. During these periods of hyperactivity the ventilatory trace was

unreadable. However, immediately after these episodes there was typically a period of around thirty seconds during which the ventilation trace would show large magnitude ventilation peaks approximately three times higher in magnitude than those peaks typically recorded before and shortly after these episodes.

The ventilatory magnitude was typically at its lowest at temperatures approaching and below those that induced coma. However, single, large-magnitude spikes (e.g. up to six times greater in magnitude than the typical height at higher temperatures) were observed on an irregular basis. On average one such peak was observed every 2-3 minutes. These 'coughs', 'yawns' or opercular flares replaced a normal sized peak in the ventilatory rhythm and were presumed to have a ventilatory function if not necessarily direct causation. These opercular flares have been observed in many species of fish (Carlson 1982).

*Anaesthesia*

Ventilatory traces were attempted for all fish post-anaesthesia but in all but two cases these traces were either unreadable and/or abandoned due to high levels of hyperactivity following anaesthesia as discussed in Chapter 4. For two fish, ventilatory data was collected over the first half-hour and compared to that recorded 24hr post-surgery.

While it is recognized that the sample size is very small some general patterns were apparent in the data collected from these fish. The ventilatory rate in the first 20min. post-anaesthesia was nearly double that observed after 24hr. However, at 25min. the ventilatory rate was below the rate at both 30min. and 24hr post-exposure in both fish. By 30min. post-exposure both fish had a ventilatory rate near that recorded 24hr post-surgery (Figure 28).

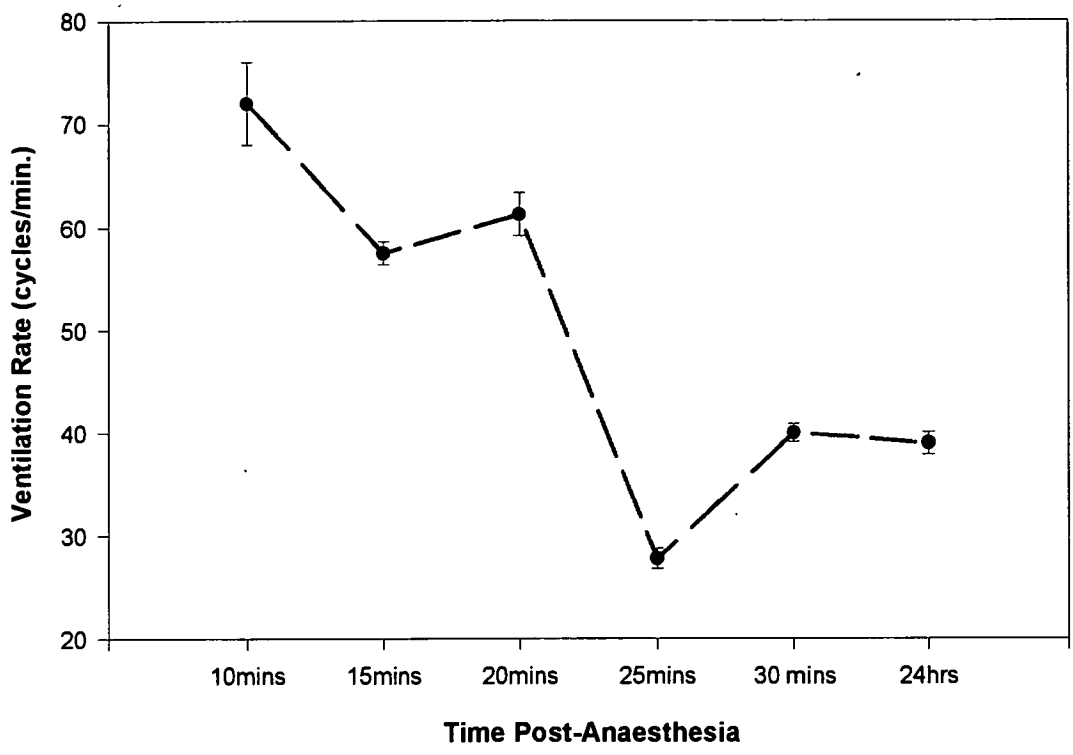



**Figure 28 Ventilation rate of bluethroat wrasse following extended anaesthesia. Ventilation rate is shown +/- 1 s.d.**

The mean magnitude of ventilation in the first half-hour post-anaesthesia was not markedly different from that recorded after 24hr however the variance was considerably higher at most sample times during the first half-hour (Figure 29).

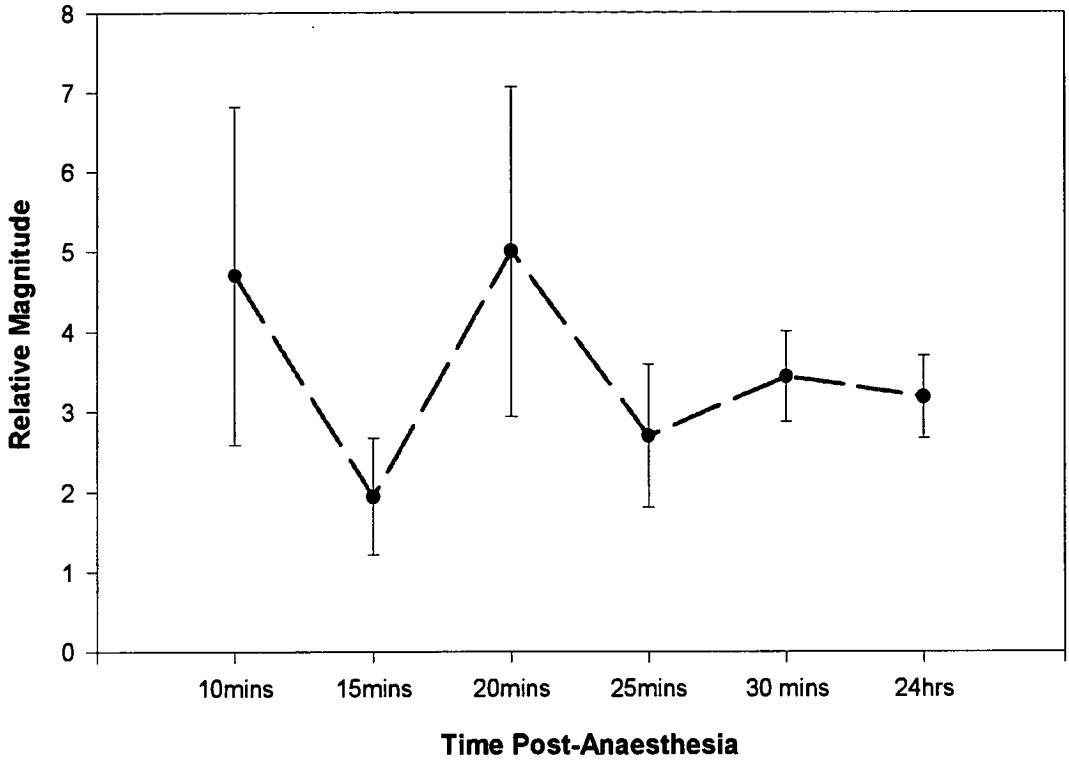



**Figure 29 Magnitude of ventilation of bluethroat wrasse following extended anaesthesia. Relative magnitude is shown +/- 1 s.d.**

For the purposes of comparison, the rate and magnitude of ventilation were plotted as paired values (Figure 30). The same arbitrary lines of division as those placed on Figure 27 were also plotted. Post-anaesthesia ventilation did not exhibit sustained high rate concomitantly with high magnitude but did exhibit wide variation in the magnitude of ventilation.

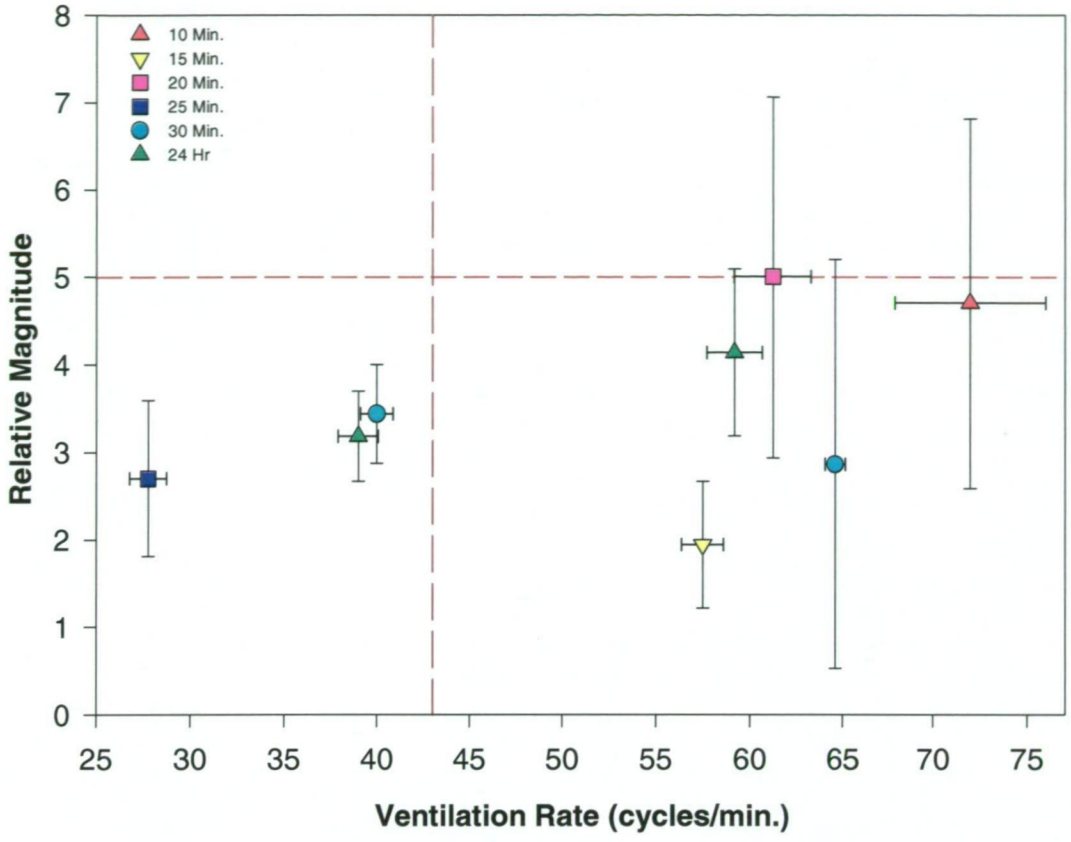



**Figure 30 Combined data on ventilation rate and magnitude following anaesthesia of bluethroat wrasse. For reference the quadrants from Figure 26 have been transposed on this figure. All values are shown +/- 1 s.d.**

All anaesthetised bluethroat wrasse (n=33) exhibited the curvature of the body during initial anaesthetic exposure. As for cold-induced coma, approximately 90% of anaesthetised blue throat wrasse showed curvature that was concave on the left-hand side of the body.

Initial work to develop the most appropriate concentrations and exposure periods for benzocaine to anaesthetise fish also revealed that lower initial concentrations of benzocaine (e.g. 200ppm) induced hyperactivity similar to that observed in fish exposed to gradual temperature reduction when the temperature approached coma-inducing levels.

### *Oxygen Consumption*

Oxygen consumption for 12 individual bluethroat wrasse under normoxic conditions during temperature reduction is shown in Figure 31.

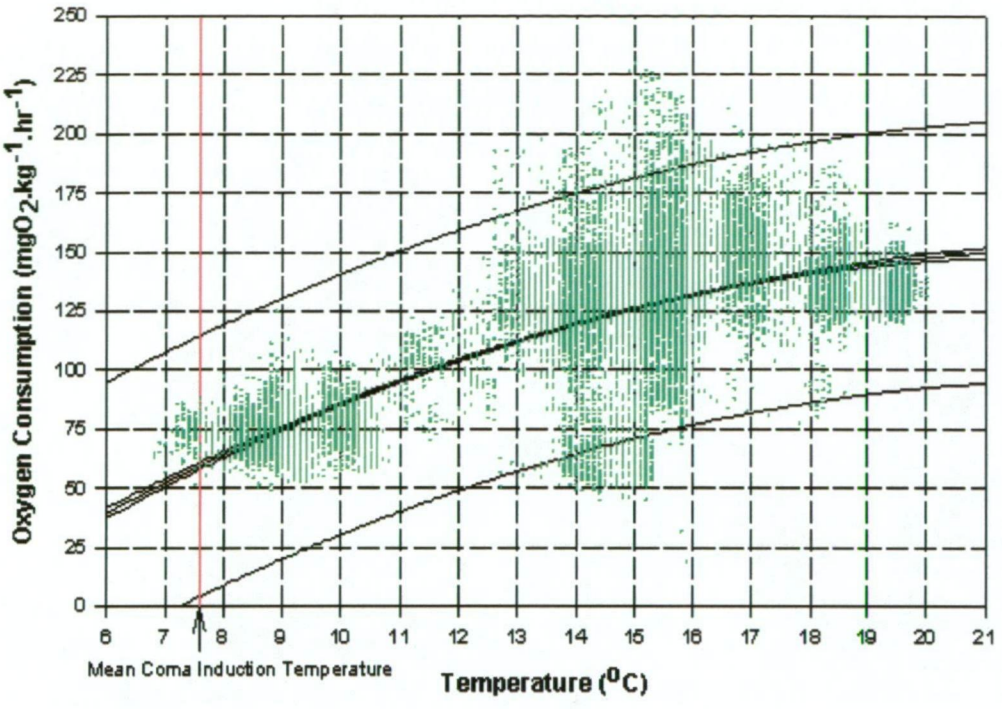



**Figure 31 Oxygen consumption values of 12 individual bluethroat wrasse across a range of temperatures. The equation of the second order polynomial regression line shown is $y = -0.37933x^2 + 17.597x - 52.4791$. The correlation coefficient of this line is $r^2 = 0.3809$. The 95% confidence interval and prediction interval are also shown. Noting that oxygen consumption cannot be less than zero, the equation of the regression line using a constrained y-intercept of zero is $y = -0.0948x^2 + 9.6991x$. This alternate regression line has a correlation coefficient of $r^2 = 0.3741$.**

To assist interpretation, the mean oxygen consumption across five arbitrary reference points in the temperature range is presented below (Table 10). The $Q_{10}$ (7.5-17.5°C) for bluethroat wrasse was 2.05.

| *Median Temperature (°C)* | *Data Range Used* | *N* | *Mean Oxygen Consumption ($mgO_2.kg^{-1}.hr^{-1}$)* | *Standard Deviation* | *% of Consumption at 15°C* |
|---|---|---|---|---|---|
| ***7.5*** | 7.3-7.7 | | 73.14 | 6.82 | 65 |
| ***10*** | 9.8-10.2 | | 79.19 | 10.03 | 71 |
| ***12.5*** | 12.3-12.7 | | 117.46 | 25.08 | 105 |
| ***15*** | 14.8-15.2 | | 131.85 | 37.83 | 100 |
| ***17.5*** | 17.3-17.7 | | 148.37 | 19.56 | 133 |

**Table 10 Mean oxygen consumption of bluethroat wrasse at five arbitrary reference temperatures. Mean oxygen consumption was calculated from the oxygen consumption values for all temperatures +/-0.2°C from the median temperature.**

The oxygen consumption for five groups of ten greenback flounder across a range of temperatures from 4-20°C under normoxic conditions is shown in Figure 32.

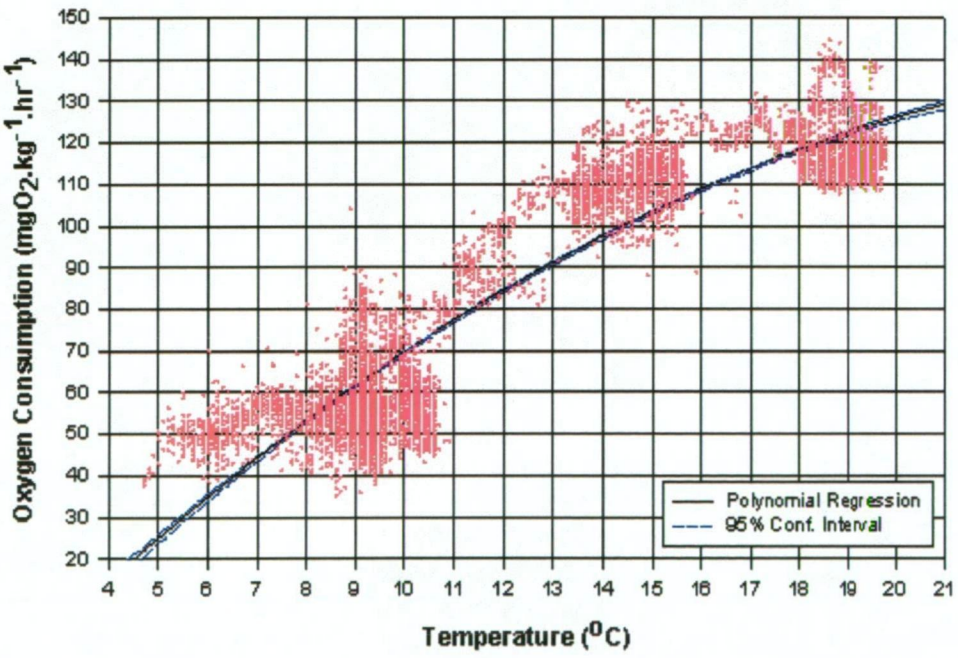



**Figure 32 Oxygen consumption of greenback flounder across a range of temperatures. The equation of the second order polynomial regression line shown is $y = -0.2189x^2 + 12.2203x - 30.5$. The correlation coefficient of this line is $r^2 = 0.8508$. Noting that oxygen consumption cannot be less than zero, the equation of the regression line using a constrained y-intercept of zero is $y = -0.0396x^2 + 7.2718x$. This alternate regression line has a correlation coefficient of $r^2 = 0.8429$.**

To assist interpretation, the mean oxygen consumption across six arbitrary reference points in the temperature range is presented below (Table 11). The $Q_{10}$ (7.5-17.5°C) for greenback flounder was 2.56.

| *Median Temperature (°C)* | *Data Range Used (°C)* | *N* | *Mean Oxygen Consumption ($mgO_2.kg^{-1}.hr^{-1}$)* | *Standard Deviation* | *% of Consumption at 15°C* |
|---|---|---|---|---|---|
| ***5*** | 4.8-5.2 | | 47.57 | 4.98 | 43 |
| ***7.5*** | 7.3-7.7 | | 54.97 | 4.30 | 49 |
| ***10*** | 9.8-10.2 | | 61.15 | 8.70 | 55 |
| ***12.5*** | 12.3-12.7 | | 101.76 | 9.62 | 91 |
| ***15*** | 14.8-15.2 | | 111.94 | 6.70 | 100 |
| ***17.5*** | 17.3-17.7 | | 122.79 | 3.34 | 110 |

**Table 11 Mean oxygen consumption of greenback flounder across six arbitrary reference temperatures. Mean oxygen consumption was calculated from the oxygen consumption values for all temperatures +/-0.2°C from the median temperature.**

The data in Table 10 and Table 11 indicate that the oxygen consumption of bluethroat wrasse is higher than that of the greenback flounder across the temperature range applied under normoxic conditions. The variation in the oxygen consumption of bluethroat wrasse (at a given temperature) is also markedly higher.

The oxygen consumption by bluethroat wrasse declined during graded hypoxic exposure at 15°C (Figure 33). This pattern is consistent with that of an oxygen conformer. Conversely, oxygen consumption by greenback flounder increased slightly but not significantly relative to normoxic consumption. This pattern is consistent with a non-conformer with a critical oxygen tension not less than 25% saturation at 15°C.

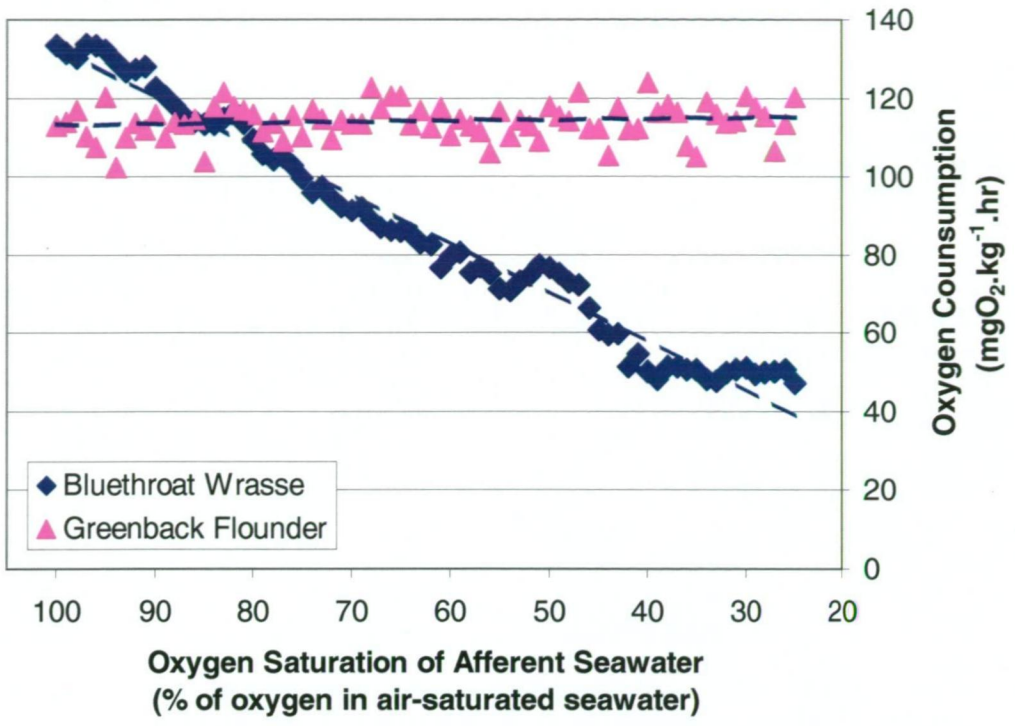



**Figure 33 Mean oxygen consumption of bluethroat wrasse (n=6) and greenback flounder (n=20) during graded hypoxic exposure at 15°C.**

***Oxygen Extraction***

The mean percentage of inspired oxygen consumed under normoxic and hypoxic conditions (25% of oxygen saturation in air) was measured at 8.5 and 15°C using two groups of three bluethroat wrasse at each temperature (Figure 34). Data was analysed using a one-way ANOVA (using arcsine transformed % data) rather than a two way ANOVA due the significant difference (P=0.0001, d.f.=1) in the absolute level of dissolved oxygen at the two different temperatures. The percentage of inspired oxygen consumed was significantly different (P=0.0001, d.f.=3) under the four different treatments (i.e. 15°C normoxia, 15°C hypoxia, 8.5°C normoxia, 8.5°C hypoxia) when measured at both the lateral and opercular expired water sites. Tukey's and LSD distinguished between the means of all treatment pairings at both sites except the two levels of oxygen saturation at 8.5°C. The percentage of oxygen removed from inspired water at 8.5°C under normoxic and hypoxic conditions was very low (<3%).

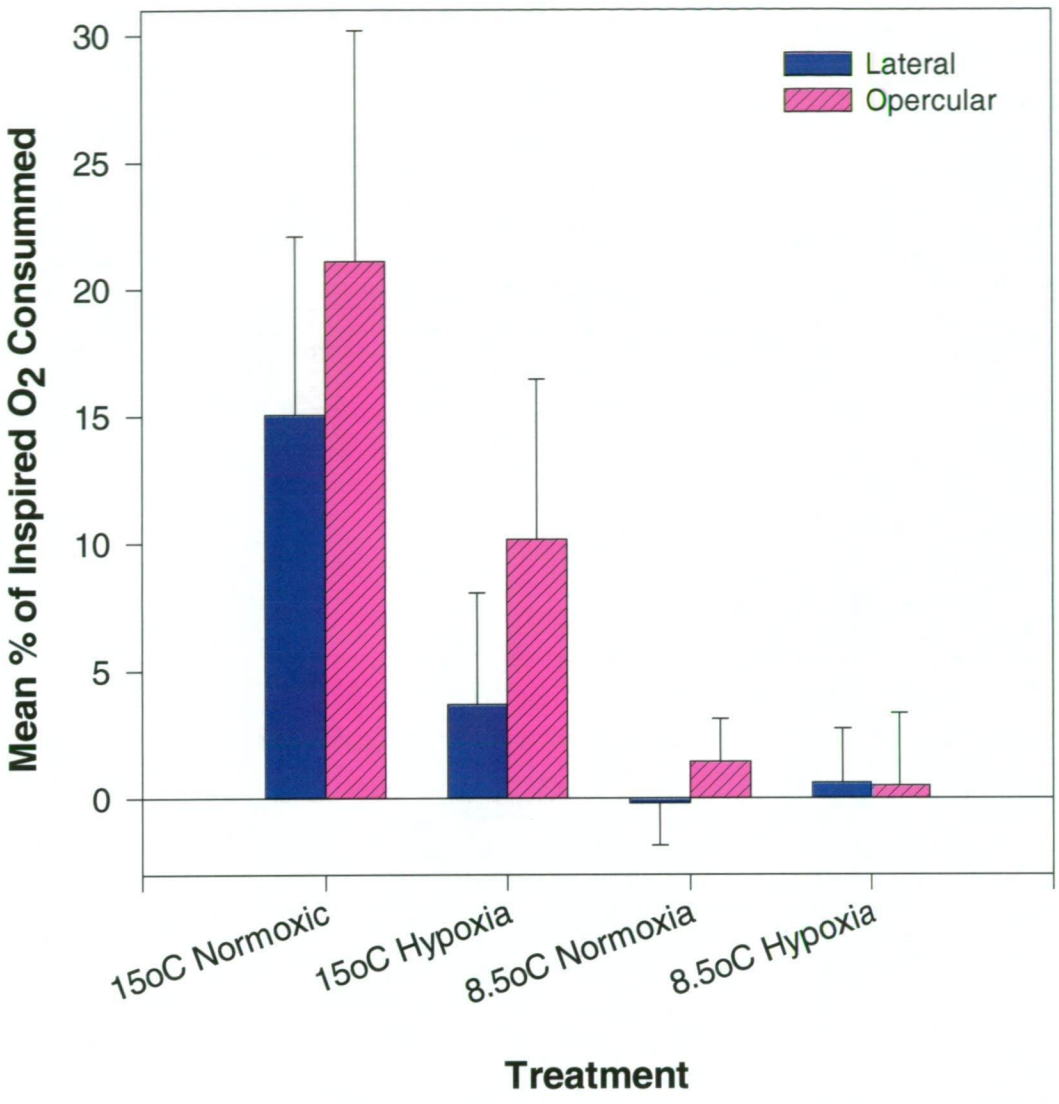



**Figure 34 Effect of lowered temperature and hypoxia on oxygen extraction of bluethroat wrasse. Error bars are plus one standard deviation.**

At 15°C, opercular cannula measurements were significantly higher that those measured at the lateral cannula (P=0.03298, d.f.=5) under both normoxic and hypoxic conditions. However, at 8.5°C no significant difference was observed (P=0.45795, d.f.=5).

***Fine Scale Changes in Oxygen Consumption with Temperature***

In Chapter 2 I reported on an experiment to validate the calculation to correct for the washout effect of the flow-through respirometer. The fine-scale changes in oxygen consumption of greenback flounder in response to small cyclical changes in water temperature are shown in Figure 35. There was a lag in the peak oxygen consumption behind that of the peak in temperature of around 3-4 minutes. The troughs however are separated by around 10-12 minutes. In Figure 35, oxygen consumption clearly does not follow the same cyclical pattern as changes in temperature. While small changes in temperature clearly produce changes in oxygen consumption, these lag behind temperature change and exhibit a different plot shape.

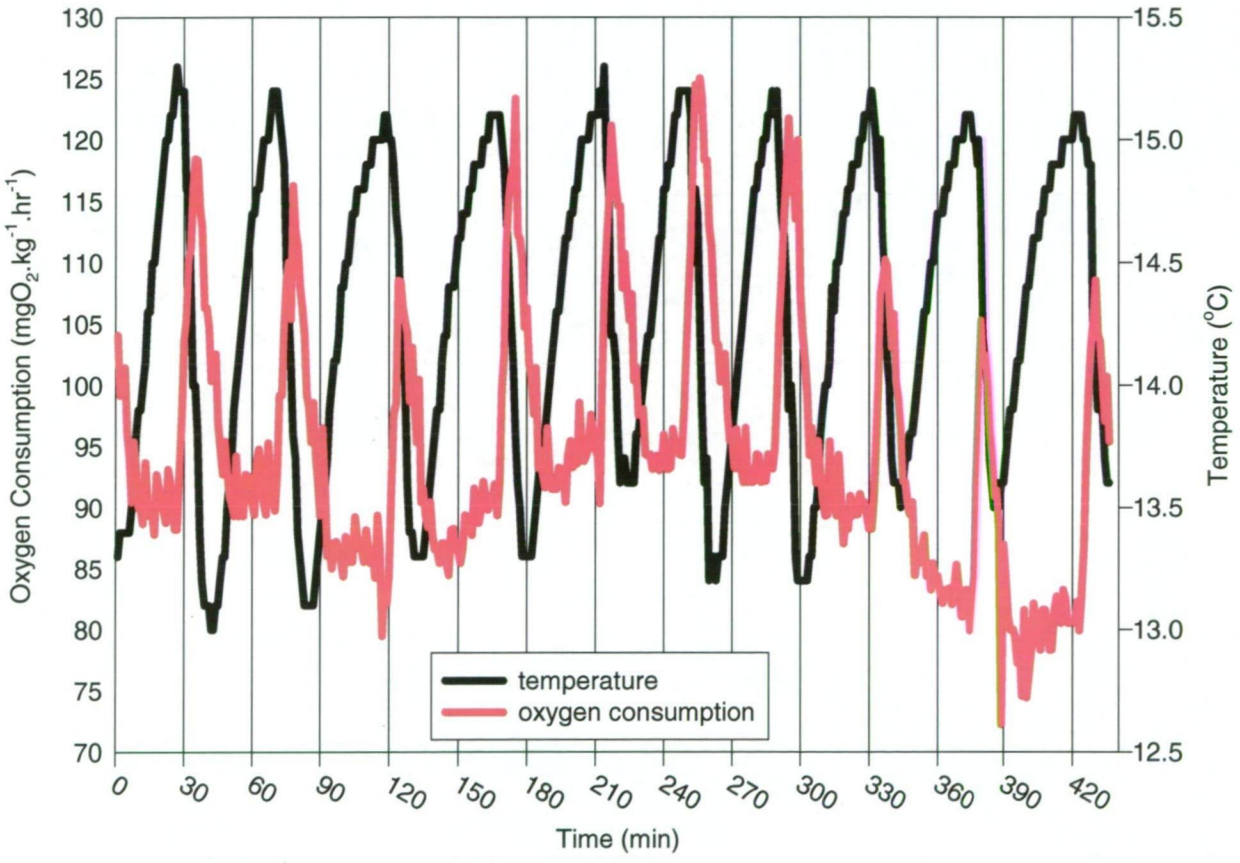



**Figure 35 Fine-scale oxygen consumption of greenback flounder in response to changes in temperature.**

### *Lactate*

Blood lactate concentration was measured for 23 bluethroat wrasse across the three treatments. While there was a general trend of higher blood lactate concentration in animals in cold-induced coma or exposed to hypoxic conditions, there was no significant difference in the blood lactate levels between any of the treatments (P=0.3322, d.f.=2, no significantly different Tukeys or LSD groupings). The results of this experiment are shown in Table 12.

| *TREATMENT* | *NORMOXIA 15°C* | *NORMOXIA COMA* | *HYPOXIA 15°C* |
|---|---|---|---|
| ***Mean Conc. of Lactate*** ***(mg.100ml$^{-1}$)*** | 6.56 | 7.64 | 10.23 |
| ***Std Dev.*** | 3.56 | 3.50 | 6.17 |

**Table 12 Mean blood lactate concentration in bluethroat wrasse exposed to normoxia, hypoxia and coma-inducing temperature**

## DISCUSSION

### *Temperature effects*

With $Q_{10}$ values of 2.05 and 2.56 respectively, the oxygen consumption of both bluethroat wrasse and greenback flounder decreased with decreasing temperature as would be expected for poikilothermic animals. This result of declining oxygen consumption with declining temperature is consistent with all reviewed studies for fish except the results of Caulton (1977) and Job (1969a). These two authors used closed respirometry for the closely related redbreast tilapia, *Tilapia rendalli* and Mozambique tilapia respectively and observed little change in oxygen consumption over a temperature range of greater than 13°C. The results of Job (1969a) and Caulton (1977) were later rejected by Caulton (1978) on the basis that the results were the result of problems with oxygen depletion during closed respirometry.

Despite a likely differential in oxygen consumption due to the difference in the mean body mass of the two species, greenback flounder exhibited lower oxygen consumption and it is suggested that this difference is mainly due to the differences in activity of the two species. Greenback flounder were more sedentary and rested on the bottom for extended periods whereas, bluethroat wrasse swam around or actively maintained position in the respirometer. It has been generally observed that the metabolic rates of most flatfish species studied have been relatively low (Wood *et al.* 1979).

Bluethroat wrasse exhibited relatively high variation in the oxygen consumption values for a given temperature. Indeed this variation of oxygen consumption was considerably higher than that observed for greenback flounder. Other authors have observed similarly high variation in oxygen consumption at a given temperature for other species such as carp (Lomholt & Johansen 1979). Lomholt & Johansen (1979) suggested that high levels of spontaneous activity are responsible for much of this variation and the direct observations of bluethroat wrasse in this study support this conclusion. The relatively large size range of bluethroat wrasse (200-2300g) may have also contributed to this variation. Never-the-less, such variation may at least partially explain why commercial operators spoken to during the course of the research have reported that survival in shipments of live bluethroat wrasse is highly unpredictable.

As might be expected, ventilation rate and magnitude in bluethroat wrasse decreased with temperature except where very high ventilation rates were observed beyond the mean coma induction temperature. This high ventilation corresponded with the lowest ventilatory magnitude and therefore ventilatory flow was expected to be minimal.

Bluethroat wrasse exposed to temperatures in the 7.6-10°C range generally exhibited the lowest opercular movement rate coupled with low magnitude opercular movements. It should be noted that these temperatures correspond with lowered water temperatures approaching but not exceeding the mean coma-inducing temperature and it would appear to represent the temperature range at which ventilatory supply is most efficient relative to demand.

Oxygen extraction by bluethroat wrasse during cold-induced coma was not markedly lower than that predicted by the temperature alone and thus coma induction as a means to reduce oxygen consumption by reducing activity did not appear to offer significant advantages despite the spontaneous activity exhibited by this species. Activity declined considerably with temperature and quiescence was often achieved by temperatures several degrees higher than the coma inducing temperature.

Oxygen consumption measured in water sampled via the cannula through the operculuni was generally higher than that along the lateral surface of the body directly posterior of the gill and therefore, it is suggested that cannulation of the opercula is the preferred method. It is possible that the method or location of cannulation under- estimated the extraction rate by partially drawing water that had not passed over the gills of the fish. It is expected that mask experiments would

resolve this uncertainty. However, such experiments were not permitted under the guidelines of the University of Tasmania Animal Ethics Committee.

Davis & Waters (1969) and Piiper *et al.* (1977) commented on the difficulty in obtaining a representative sample of exhalent water using a single cannula. Burggren (1978) used x-ray analysis to show that almost all exhalent water is expelled by sturgeon in a discrete stream along the lower one-third of each opercular slit. On the basis of this information Burggren & Randall (1978) tried various sites along this section of the operculuni but observed only relatively small variation (1-2mm of Hg) between sites.

Oxygen extraction rates for bluethroat wrasse during normoxic conditions at the acclimation temperature of 15°C were in the low range of observed values for other species. By way of comparison, carp extract over 80% of inspired oxygen under normoxic conditions and maintain greater than 70% extraction even when exposed to hypoxic water with an oxygen saturation of less than 20% (Lomholt & Johansen 1979). Extraction rates of sturgeon under normoxic conditions at 15°C were 50% (Burggren & Randall 1978) still more than double that observed for bluethroat wrasse under the same conditions.

Exposure to temperatures approaching the mean coma inducing temperature reduced oxygen extraction to very low levels (<5%) under both normoxic and hypoxic conditions. It is likely that temperature effects on ventilation explain this result. The use of mask experiments is likely to provide a more effective regime for studying the extraction by fish during or near coma with concomitantly low ventilation.

***Effects of Hypoxia***

Hypoxia decreased both the ventilatory rate and magnitude of bluethroat wrasse and presumably total gill ventilation exhibited a similar decrease. This result is contrary to the observed responses for species such as carp (Peyraud & Serfaty 1964, Lomholt & Johansen 1979), curimbata (Fernandes *et al.* 1995), trahira, *Hoplias lacerdae*, tariputanga, *H. malabricus* (Rantin *et al.* 1993), rainbow trout (Jones *et al.* 1970), bonnethead shark, *Sphyrrna tiburo*, blacknose shark, *Carcharinus acronotus* (Carlson & Parson 2001) and tench, *Tinca tinca* (Butler & Metcalfe 1983). All of these species showed increases in ventilatory rate and magnitude and activity.

However, the response of bluethroat wrasse to hypoxia is consistent with the responses dogfish, *Scyliorhynus canicula* (Taylor & Butler 1982), Florida smoothhound shark, *Mustelus norrisi* (Carlson & Parson 2001), epaulette shark, *Hemiscyllium ocellatum* (Routley *et al.* 2002), sturgeon, *Acipenser transmontanus*

(Burggren & Randall 1978), cod (Schurmann & Steffensen 1992, 1994) and oscar, *Astronotus ocellatus* (Muuze *et al.* 1998).

Butler & Taylor (1975) observed that dogfish were unable to maintain oxygen consumption rates under hypoxic conditions and supposed that this was a result of the reduced scope of ventilatory movement in elasmobranchs relative to teleosts that prevented increased ventilation volume to maintain oxygen supply (the findings of Carlson and Parson 2001 are not consistent with this hypothesis however). Burggren & Randall (1978) observed that while sturgeon exhibited a reduced ventilation volume there was no subsequent payback of oxygen debt after return to normoxia that would be consistent with the production of lactate during anaerobic respiration. This led the authors to suggest that the sturgeon was responding to hypoxia by reduced energy expenditure rather than switching to anaerobic respiration. As bluethroat wrasse exhibited similar ventilatory responses to hypoxia as the sturgeon and lactate production during hypoxia was not significantly higher than normoxic levels it is also suggested here that bluethroat wrasse may reduce activity during exposure to hypoxia.

The habits of bluethroat wrasse may provide some reason why this species exhibits such a response under hypoxic conditions. Bluethroat wrasse often shelter in small caves, crevices and 'holes' in the reef and some species of labrid fish have been observed by divers to 'sleep' in such places in relatively high densities. It is possible that dissolved oxygen declines in these micro-environments and that such fishes have developed physiological responses of decreased activity in response to this 'self-imposed' hypoxia. If so, greater numbers of fish would be able to inhabit the reef before competition for such niches became constraining due to hypoxia. Such physiological adaptation would obviously assist the live transport of bluethroat wrasse as it is compensatory to depletion of oxygen during transport.

Oxygen extraction by bluethroat wrasse under hypoxic conditions was approximately half that during normoxic conditions at 15°C. In all studies reviewed the extraction of oxygen decreased for all species under hypoxic conditions (e.g. Jones *et al.* 1970, Lomholt & Johansen 1979, Butler & Metcalfe 1983), including sturgeon (Burggren & Randall 1978) and dogfish (Taylor & Butler 1982). Indeed, the oxygen extraction of sturgeon was also approximately halved during similar hypoxic exposure to that applied to bluethroat wrasse in this study.

Greenback flounder did not exhibit reduced oxygen consumption in response to graded hypoxia and can therefore be described as a non-conformer. This result is consistent with the observations for plaice, a related flatfish (Edwards *et al.* 1970, Wood *et al.* 1975, Jobling 1982). The near constant oxygen consumption of

greenback flounder during initial hypoxia followed by a slight increase is typical of many non-conformers (Beamish 1964, Lomholt & Johansen 1979).

***Anaesthesia***

Immediately after extended anaesthesia and surgery, the ventilation rate (but not ventilation magnitude) was almost double that observed 24hr post-surgery. This ventilation rate declined quickly over the proceeding 30min to be similar to that 24hr post-surgery. Hyperventilation is commonly observed in fish recovering from anaesthesia and is considered an indicator of oxygen debt (Summerfelt & Smith 1990). It should be noted that sturgeon exhibited an elevated ventilation rate immediately after surgery and Burggren & Randall (1978) attributed this increase to acclimation to new surroundings rather than oxygen debt.

***Fine Scale Changes in Oxygen Consumption with Temperature***

While it could be expected that oxygen consumption in poikilothermic animals may be readily predicted by temperature there were both phase and shape differences in the fine-scale changes in oxygen consumption of greenback flounder with temperature. As detailed in Chapter 2, the correction applied to the washout effect is considered to be valid and thus the washout effect is not proposed as a significant explanation for this observation.

There are a number of explanations for the observed inertia in oxygen consumption relative to temperature change. One obvious source of such inertia is differential hysteresis of the oxygen probe used and the separate platinum temperature probe used to record temperature. The manufacturers of the oxygen probe claim that polarisation time of the electrode is zero however this claim was not tested and thus cannot be rejected. However, due to the asymmetry of the oxygen consumption recorded it is difficult to believe that hysteresis within the equipment alone is sufficient to explain the results alone. Equipment hysteresis alone should simply result in a right shift of phase but have no effect on the general shape of the oxygen consumption plot relative to temperature.

Hysteresis of the physiological responses of greenback flounder to changes in tissue temperature (and hence respiration rate) may also explain the inertia in oxygen consumption in response to changes in water temperature. The lag in oxygen extraction rate behind respiratory changes in humans is considerable (Eckert *et al.* 1988) and is considered a likely explanation, at least in part, for the lag observed in greenback flounder.

In obligate ectotherms there may be short-term differences in body and water temperature. The rate of change has been shown to be influenced by morphology,

mass (Stevens & Fry 1974, Spigarelli *et al.* 1977, Elliot 1981), thermal inertia of tissues and physiological factors that differentially influence the rates of warming and cooling (Crawshaw 1976). The half-life for thermal exchange in a 3.6kg lake trout was 13 minutes while that of a 30g alewife was only one minute (Spigarelli *et al.* 1977). The body size (approx. 100g) and morphology of greenback flounder (i.e. flatfish with a relatively high body surface area:mass) used in this study suggest that thermal half-life would be considerably closer to that of the alewife than of the lake trout. Regardless, it can reasonably be presumed that the thermal half-life of 100g greenback flounder is significant and indeed may approximate the time lag between the peaks of temperature and oxygen consumption.

The anticipated oxygen debt arising from the initial inertia following an increase in temperature and physiological factors that differentially influence the rates of warming and cooling such as those reported by Crawshaw (1976), are probably sufficient to explain the asymmetry of the increase and decrease in the rate of oxygen consumption.

***Lactate***

No significant difference was found between the blood lactate concentrations of any of the treatments (n.b. both cold temperature coma-induction and hypoxic exposure did result in increases in blood lactate concentration). Increasing the number of replicates may find a significant difference however this difference is expected to be relatively small.

Assuming the results are indeed representative and there was no significant difference in the blood lactate resulting from the various treatments then there are several possible hypotheses to explain these results. One possible explanation is that the treatments were not of sufficient severity or duration to induce detectable net anaerobic respiration. Indeed, oscar show similar ventilatory and activity responses to hypoxic as bluethroat wrasse and this species does not show any significant increase in blood lactate until dissolved oxygen is less than 6% air-saturation (Muusze *et al.* 1998).

It is possible that the testing process was unable to successfully detect a difference that did exist however the testing process used in this study has been successfully used in the same laboratory. Indeed this process has detected differences in the lactate concentration of fish exposed only to confinement for one hour without coma induction or hypoxia (Evans & Fewtrell 1996). The issue of confinement raises the possibility that the lactate levels were high or maximal in all treatments and the additional stressors or coma and hypoxia did not affect the levels significantly. While it should be remembered that such indices are relative, the blood lactate levels

for bluethroat wrasse were remarkably low compared to many species (see summary by Kirk (1976)) and this explanation is not favoured.

It is possible that the total time of hypoxic exposure and immediate sampling was insufficient to allow lactate to move from the tissues into the blood. Heath & Pritchard (1965) observed that blood lactate levels in cutthroat trout peaked some two hours after return of fish to normoxia following hypoxic exposure. Similarly, Milligan (1994) observed that blood lactate levels did not peak until 2-4 hours after strenuous exercise in rainbow trout. Furthermore Black (1957) suggests that exposure to lowered temperatures will reduce the diffusion rate of lactic acid from the tissues to the blood stream. However, subsequent work by Black *et al.* (1962) observed a very rapid diffusion of lactate from the muscles into the blood of exercised rainbow trout with peaks occurring a few minutes after exercise and hence this explanation is not favoured.

Goldfish, *Carassius auratus*, are very tolerant of anoxic and hypoxic conditions and exhibit modified anaerobic respiration (Van Waversveld 1989). This novel anaerobic metabolic pathway produces ethanol and carbon dioxide (Van den Thillart & Kesbeke 1978, Shoubridge & Hochachka 1980, Mourik *et al.* 1982, Van den Thillart *et al.* 1983, Van den Thillart & Van Waarde 1985). A reaction involving alcohol dehydrogenase, pyruvate dehydrogenase and lactate dehydrogenase converts lactate into ethanol and carbon dioxide (Mourik *et al.* 1982, Van den Thillart 1982). Very few authors have investigated or commented on the possibility that the absence of a correlation between oxygen debt and lactate could be explained by the presence of the same or similar anaerobic metabolic pathway as that observed in goldfish. The potential for such a physiological process to assist explanation of the results of this study cannot be discounted.

While there are many possible explanations listed, the most likely explanation is that lactate was retained within white muscle rather than passing into the blood stream. Retention of the vast majority of lactate in the white muscle has been observed in rainbow trout (Laberee & Milligan 1999a,b) and winter flounder (*Pseudopleuronectes americanus*) (Girard & Milligan 1992) and such retention would most readily explain the results of this experiment.

***Summary***

The routine metabolic rate of greenback flounder was significantly lower than that of bluethroat wrasse. Therefore, it can reasonably be expected that greenback flounder would require less oxygen during transport and hence this species could be packed at higher densities than bluethroat wrasse.

Decreasing temperature reduced oxygen consumption of both bluethroat wrasse and greenback flounder. Indeed, a reduction of 10 degrees at least halved the consumption of oxygen by both species. The induction of coma did not significantly reduce oxygen consumption relative to that predicted by the lowered temperature alone.

Exposure to hypoxia showed that greenback flounder were non-conformers while bluethroat wrasse were conformers. This result is important because while bluethroat wrasse showed a higher metabolic rate under normoxic conditions than greenback flounder their rate declined in a compensatory manner in response to declining dissolved oxygen. Thus, in a typical transport system (i.e. declining oxygen over time) both species may exhibit very similar total demands on oxygen supply. Lactate production in bluethroat wrasse was also in the low range compared to many species and this is a favourable observation for fish to be supplied alive to consumers.

# CHAPTER 7

# GENERAL DISCUSSION

The results of this study demonstrate that temperature reduction is a powerful tool in manipulating the physiology and hence respiratory 'budget' of fishes and one that appears to offer particular merit in improving techniques for transporting live fish. A reduction in temperature by 10°C more than halved the oxygen consumption of bluethroat wrasse and greenback flounder. Thus, a given fish to water ratio can be at least doubled if the transport temperature can be reduced by 10°C.

However, this study showed that temperature reduction must be applied carefully due to the small difference between temperatures that minimise oxygen consumption with high survival and those that result in high or even total mortality. Indeed less than 0.5°C can be the difference between total survival and total mortality in a group of fish. This finding suggests that while temperature reduction should be applied to reduce oxygen consumption during transport, this reduction should be based on an understanding of the thermal tolerance of the fish and knowledge of the lower lethal limits as a function of temperature and time. The results of this study for two species of fish that are close neighbours in their environment, reinforces the findings of other studies that thermal tolerance is strongly species-specific and assumptions about thermal tolerance based on shared habitat are dangerous.

The effect of feeding on reducing thermal tolerance is an important finding in the development of live fish transport techniques and study of thermal tolerance in fishes. It raises significant doubt about the comparison of existing data on various species and requires a standardised approach to feeding in the study of thermal tolerance of fishes. This result has immediate application in the development of protocols to improve live fish transport by reinforcing the desirability of starving fish prior to transport.

Cold-induced coma could be readily induced in the laboratory and, in the case of bluethroat wrasse, carefully maintained for extended periods. However, the proximity of coma-inducing temperatures to lethal limits raises doubts as to the practicality of coma induction and maintenance in a commercial setting. For example, the mean coma inducing temperature for bluethroat wrasse was less than 0.3°C higher than the $TL_{50}$ (24hr). Moreover, the induction of coma did not appear to deliver significant gains through reduced oxygen consumption of bluethroat wrasse beyond that predicted by the temperature alone. These findings suggest that coma induction may have limited application in these species for the purposes of

transport. One potential use is the cold induction of coma for packing of fish to reduce handling stress and elevated initial oxygen consumption. Subsequent transport temperatures would need to be higher to minimise mortality due to cold exposure.

Greenback flounder demonstrated a tolerance to water temperatures greater than ten degrees below the acclimation temperature and a high value of respiratory $Q_{10}$ (i.e. 2.65). Thus, low temperature transport can reduce the oxygen demand of a given mass of fish to one third (or less) that of the same mass of fish transported at say 15°C. Conversely, this corresponds to a tripling of the ratio of fish to water.

The responses of greenback flounder and bluethroat wrasse to hypoxia provided an interesting comparison. Greenback flounder, like most fish species, exhibited oxygen consumption patterns consistent with non-conformity (i.e. oxygen consumption did not decline with hypoxia). However, oxygen consumption of bluethroat wrasse conformed with declining dissolved oxygen during hypoxia. Therefore, while the oxygen consumption of bluethroat wrasse was approximately 20% higher than that of greenback flounder under normoxic conditions, the oxygen consumption of bluethroat wrasse declines during hypoxia and is likely to approach or even fall below that of greenback flounder under relatively moderate hypoxia (at the same temperature). Of course it is important to remember that oxygen consumption is not a reliable indicator of metabolic rate during anaerobic respiration but the absence of significant lactate production in bluethroat wrasse during hypoxia supports a conclusion that bluethroat wrasse reduce metabolic oxygen demand in response to hypoxia.

This is a valuable finding in the case of bluethroat wrasse. More generally it should be noted that oxygen conformers are likely to allow higher packing density than non-conformers with similar or even moderately higher metabolic rates.

## FUTURE DIRECTIONS

Obviously, the next step for this research would be to trial various thermal regimes during simulated transport conditions based on the thermal tolerance and oxygen consumption results of this study for these two species. As a starting point, I would recommend use of:

- a period of deprivation from food not less than 25% longer than the time for gastric evacuation;
- gradual cooling prior to packaging at a rate not greater than 2°C per hour; and

- transport temperatures of 3.0°C and 9.0°C for greenback flounder and bluethroat wrasse respectively (assuming an acclimation temperature of 15°C) provided temperature fluctuations were minimal in both size and duration.

The methods used in this study to describe thermal tolerance and oxygen consumption are readily applicable for commercial or scientific investigation of live transport protocols for other species. It would appear however, that oxygen consumption *per se* does not warrant significant attention as the predictions provided by biochemical $Q_{10}$ values more than warrant the application of lowered temperatures during transport. Thus, the main focus of studies on other species should be to determine the thermal tolerance (as a function of both temperature and time) to identify the optimal temperature regime for high-density transport.

Some initial trials exposing greenback flounder to very low temperatures resulted in significant mortality of fish that became buried under other tank members. This observation suggests that gas exchange across the skin of flounder may be a significant source of oxygen in these fish especially during depression of ventilation during exposure to lowered temperature. Use of a van Dam respirometer would allow investigation of the relative contribution of gas exchange across the skin and gills. In addition this observation presents a challenge to the design of systems to transport greenback flounder to avoid fish becoming buried if lowered temperatures are to be applied. The use of dividers within containers to isolate individual fish would address this problem.

The recovery of greenback flounder from coma despite persistence of temperatures that induced coma was remarkable. This is the first time such observations have been made for this species. However, similar results have been observed for other flatfish found in the northern hemisphere. The now well-known case of the attempted transgenic conference of low temperature resistance from winter flounder to cultured Atlantic salmon and the resulting marked increases in growth of these transgenic salmon may warrant investigation of the genetic basis for cold-resistance in greenback flounder.

The disparity of methods used in the study of thermal tolerance in fishes is a considerable constraint in inter-study and inter-species comparison (e.g. use of death as an endpoint in continuous temperature reduction trials). A review of the methods and the problems associated with each is overdue.

This study did not look at the usefulness or otherwise of the application of chemicals during transport. However, the effect of the drug Aqui-S™ on both thermal

tolerance and oxygen consumption should be studied to determine if such a product may provide advantages to live fish transport.

As discussed briefly in Chapter 2, the use of aspirin during transport of minnows for bait has been observed in the United States of America. Aspirin inhibits the synthesis of prostaglandins (Stryer 1988) and in so doing reduces the metabolic consumption of oxygen that is part of this process. As a result, the use of aspirin warrants attention in future studies of oxygen consumption in fish. It had been intended to investigate the effects of aspirin on thermal tolerance and oxygen consumption of fish however an absence of adequate testing facilities to monitor effective dosage (i.e. dose of aspirin in blood) precluded such experiments.

## CONCLUSION

In his opening address to the National Seafood Centre's Live Fish Transport Workshop held in Hobart, Australia in 1995, Mr Peter Dundas-Smith, Executive Director of the Fisheries Research and Development Corporation, stated that the ultimate goal of live fish transport could be the successful delivery of live southern bluefin tuna to the Japanese markets after capture in Australia.

While this study does not deliver this goal now it has established a foundation for an approach to develop of methods that might ultimately deliver such a pinnacle in live fish transport. In the meantime, the results are immediately applicable to development of improved live fish transport techniques for the three species studied and other less demanding species.

# REFERENCES


ABARE, (2002), *Australian Fisheries Statistics 2001*, Canberra.

Aho, E. & Vornanen, M.; (2001), Cold acclimation increases basal heart rate but decreases its thermal tolerance in rainbow trout (*Oncorhynchus mykiss*), *Journal of Comparative Physiology*, **171**, 173-179.

Al-Habbib, O.A.M. & Yacoob, M.P. (1993), Effects of acclimation and experience to changing heat and cold shock temperature on lethal temperature and thermal tolerance of *Gambusi affinis* (Baird and Girard) (Poeciliidae), *Cybium*, **17**, 265-272.

Ananthakrisnan, K.R. & Kutty, M.N., (1974), Mortality and breathing rate at high ambient temperatures in the cichlid fish, *Tilapia mossambica* (Peters), *Indian Journal of Experimental Biology*, **12**, 55-59.

Andersen, W.G., McKinley, R.S. & Colavecchia, M., (1997), The use of clove oil as an anesthetic for rainbow trout and its effects on swimming performance, *North American Journal of Fisheries Management*, **17(2)**, 301-307.

Ando, M., Toyohara, H., Shimizu, Y. & Sakaguchi, M., (1993), Post-mortem tenderization of fish muscle due to weakening of pericellular connective tissue, *Bulletin of the Japanese Society of Scientific Fisheries*, **59(6)**, 1073-1076.

Anon., (1995a), *Preliminary Report - Status of the Live Seafood Industry in Australia,* National Seafood Centre, Brisbane.

Anon., (1995b), *Live Seafood Handling: Strategies for Development,* National Seafood Centre, Brisbane.

Anon., (1995c), *Introduction to Fish Health Management*, United States Fish and Wildlife Service, USDOI Printing Office, Miami.

Anon., (1997), *New Animal Drugs of Low Regulatory Priority (LRP) Used n Aquaculture*, Guide 1240.4200, United States Department of Health and Human Services, Food and Drug Administration, Center for Veterinary Medicine.

Anon., (1999), *Guide to Drug, Vaccine and Pesticide Use in Aquacultural Extension Service*, Publication B-5085, United States Department of Health and Human Services, Food and Drug Administration, Center for Veterinary Medicine.

Anon., (2003), *Report on the Welfare of Farmed Fish*, Farm Animal Welfare Council, London.

Averett, R.C., (1969), *Influence of temperature on energy and material utilization by juvenile coho salmon*, Ph.D.Thesis, Oregan State University.

Azuma, T. & Itzawa, Y., (1993), Respiration of a marine teleost, the porgy, under normoxic resting condition, *Nippon Suisan Gakkaishi*, **59,** 621-626.

Bagge, O.; Nielsen, E. & Steffensen, J.F.; (1995), Consumption of food and evacuation in dab (*Limanda limanda*) related to saturation and temperature. Preliminary results, *ICES Council Meeting papers,* ICES, Copenhagen.

Barry, T.P., Parrish, J.J. & Malison, J.A., (1996), *Effects of early handling on the development of corticosteroid stress response in rainbow trout (Oncorhynchus mykiss)*, Wisconsin University Sea Grant Institute.

Barton, B.A. & Zitzow, R.E., (1992), Physiological stress responses of walleye (*Stizostedion vitreum*), to handling and their recovery in saline versus fresh water, In, *Aquaculture '92: Growing Toward the 21st Century*, Orlando, Florida (USA), 21-25 May 1992, pp 35-36.

Barton, B.A. & Zitzow, R.E., (1994), Evaluating recovery of walleye to handling stress in saline versus fresh water, In, *High Performance Fish: Proceedings of an International Fish Physiology Symposium at the University of British Columbia in Vancouver Canada, July 16-21, 1994*, Fish Physiology Association, Vancouver, BC (Canada), pp 401-407.

Barton, B.A. and Peter, R.E., (1982), Plasma cortisol stress response in fingerling rainbow trout, *Salmo gairdneri* Richardson, to various transport conditions, anesthesia and cold shock. *Progressive Fish Culturist*, **20**, 39-51.

Barton, B.A., Peter, R.E. & Paulencu, C.R., (1980), Plasma cortisol levels of fingerling rainbow trout (*Salmo gairdneri*) at rest, and subject to handling confinement, transport and stocking, *Canadian Journal of Fisheries and Aquatic Sciences*, **37**, 805-811.

Barton, B.A., Rahn, A.B. Feist, G., Bollig, H. & Schreck C.B., (1998), Physiological stress responses of the freshwater chondrostean paddlefish (*Polydon spathula*) to acute physical disturbances, *Comparative Biochemistry and Physiology, A.*, **120**, 355-363.

Barton, B.A., Schreck, C.B. & Sigismondi, L.A., (1986), Multiple acute disturbances evoke cumulative physiological stress responses in juvenile Chinook salmon, *Transactions of the American Fisheries Society*, **115**, 245-251.

Basu, S.P., (1959), Active respiration of fish in relation to ambient concentrations of oxygen and carbon dioxide, *Journal of the Fisheries Research Board of Canada*, **16**, 175-212.

Beamish, F.W.H., (1964), Respiration of fishes with special emphasis on standard oxygen consumption. III. Influence of oxygen, *Canadian Journal of Zoology*, **42**, 355-366.

Beamish, F.W.H., (1970), Oxygen consumption of largemouth bass, *Micropterus salmoides*, in relation to swimming speed and temperature, *Canadian Journal of Zoology*, **48**, 1221-1228.

Beamish, F.W.H., (1972), Ration size and digestion in large mouth bass, *Micropterus salmoides* Lacepede, *Canadian Journal of Zoology*, **50**, 153-164.

Beamish, F.W.H., (1974), Apparent specific dynamic action of largemouth bass, *Micropterus salmoides*, *Journal of the Fisheries Research Board of Canada*, **31**, 1763-1769.

Beamish, F.W.H. & Mookherjii, P.S., (1964), Respiration of fishes with special emphasis on standard oxygen consumption, *Canadian Journal of Zoology*, **42**, 161-175.

Bell, G.R., (1987), An outline of anesthetics and anesthesia for salmonids, a guide for fish culturists in British Columbia, *Canadian Technical Reports of Fisheries and Aquatic Sciences*, No. 1534.

Bennett, W.A. & Judd, F.W., (1992), Comparison of methods for determining low temperature tolerance: experiments with pinfish, *Lagodon rhoboides*, *Copeia*, **4**, 1059-1065.

Bennett, W.A., Currie, R.J., Wagner, P.F. & Beitinger, T.L., (1997), Cold tolerance and potential overwintering of the red-bellied piranha, *Pygocentrus nattereri*, in the United States, *Transactions of the American Fisheries Society*, **126**, 841-849.

Berka, R., (1986), *The transport of live fish, A review*, EIFAC Technical Paper 48, FAO, Rome.

Black, E.C., (1957), Alterations in the blood level of lactic acid in certain salmonid fishes following muscular activity. I Kamloops trout, *Salmo gairdneri*, *Journal of the Fisheries Research Board of Canada*, **14**, 117-134.

Black, E.C., Connor, A.R., Lam, K.C. & Chiu, W.G., (1962), Changes in glycogen, pyruvate and lactate in rainbow trout (*Salmo gairdneri*) during and following muscular activity, *Journal of the Fisheries Research Board of Canada*, **19**, 409-436.

Blasiola, G.C., (1975), Quinaldine sulphate, a new anesthetic formulation for tropical marine fishes, *Journal of Fish Biology*, **10**, 113-119.

Blazka, P., (1958), The anaerobic metabolism of fish, *Physiological Zoology*, **31**, 117-128.

Booke, H.E., Hollender, B. & Lutterbie, G., (1978), Sodium bicarbonate, an inexpensive fish anesthetic for field use, *Progressive Fish Culturist*, **40**, 11-13.

Bouck, G.R., (1980), Etiology of gas bubble disease, *Transactions of the American Fisheries Society*, **109(6)**, 703-707.

Bourne, P.K., (1984), The use of MS-222 (Tricaine methanesulphonate) as an anaesthetic for routine blood sampling in three species of marine teleosts, *Aquaculture*, **36**, 313-321.

Bravo, D.E & Chung, K.S., (1999), Ecophysiological behaviour of *Petenia kraussel* exposed to different temperatures and salinities, In, Nelson, J. & MacKinley, D., (eds), *Special Adaptations in Tropical Fish*, Towson University, Baltimore.

Bremmer, H.A., Goodrick G.B. & Paterson, B.D., (1996), Conditions of adaptation for live sales, In, *Proceedings of the Conference of IIR Commission C2, Bordeaux Colloquim- Refrigeration and Aquaculture*, Paris, France, pp. 89-100.

Brett, J.R. & Higgs, D.A., (1970), Effect of temperature on the rate of gastric digestion in fingerling sockeye salmon, *Oncorhynchus nerka, Journal of the Fisheries Research Board of Canada*, **27**, 1767-1779.

Brett, J.R., (1952), Temperature tolerance in young Pacific Salmon, Genus *Oncorhynchus, Journal of the Fisheries Research Board of Canada*, **9**, 265-323.

Brett, J.R., (1962), Some considerations in the study of respiratory metabolism in fish, particularly salmon, *Journal of the Fisheries Research Board of Canada*, **19**, 1025-1038.

Brett, J.R., (1965), The relation of size to rate of oxygen consumption and sustained swimming speed of sockeye salmon (*Oncorhynchus nerka*) *Journal of the Fisheries Research Board of Canada*, **22**, 1491-1470.

Brett, J.R., (1970), Temperature, In, Kinne, O. (ed.), *Marine Ecology*, John Wiley & Sons, New York.

Brett, J.R., (1976), Feeding metabolic rates of young sockeye salmon, *Oncorhynchus nerka*, in relation to ration level and temperature, *Fisheries and Marine Service Research and Technical Report*, 675.

Brick, M. E. & Cech, J.J. Jr., (2002), Metabolic responses of juvenile striped bass to exercise and handling stress with various recovery environments, *Transactions of the American Fisheries Society*, **131**, 855-864.

Brown, S.B., Mills, K.H. & Hara, T.J., (1998), Capture- induced changes in plasma cortisol thyroid hormones, glucose and electrolytes in lake whitefish (*Coregonus clupeaformis*) and white sucker (*Catostomus commersoni*) from soft water lakes, Biology and Management of Coregonid Fishes, Proceedings of the Sixth International Symposium, *Advances in Limnology*, **50**, 273-282.

Bulger, A.J. & Schultz, R.J., (1979), Heterosis and interclonal variation in thermal tolerance in unisexual fishes, *Evolution*, **33**, 848-859.

Burggren, W.W. & Randall, D.J., (1978), Oxygen uptake and transport during hypoxic exposure in the sturgeon, *Acipenser transmontanus*, *Respiratory Physiology*, **34**, 171-183.

Burggren, W.W., (1978), Gill ventilation in the sturgeon, *Acipenser transmontanus*: Unusual adaptations for bottom dwelling, *Respiratory Physiology*, **34**, 261-278.

Burleson, L.M., Smatresk, N.J. & Milson, W.K., (1992), Afferent inputs associated with cardioventilatory control in fish, In, Hoar, W.S., Randall, D.J. & Farrell, A.P., (eds), *Fish Physiology, Vol XII B*, Academic Press, New York.

Butler, P.J. & Metcalfe, J.F., (1983), Control of Respiration and Circulation, In, Rankin, J.C., Pitcher, T.J. & Duggan, R.T. (eds), *Control Processes in Fish Physiology*, Croom Helm Ltd, London.

Campagna, C.G. & Cech, J.J.Jr, (1981), Gill ventilation and respiratory efficiency of Sacramento blackfish, *Orthodon microlepidotus* Ayres, in hypoxic environments, *Journal of Fish Biology*, **19**, 581-591.

Campbell, R., Whitelaw, W. & McPherson, G., (1997a), *Domestic Longline Fishing Methods and the Catch of Tunas and Non-Target Species off North-Eastern Queensland, (1st Survey: October-December, 1995)*, Report to the Eastern Tuna and Billfish Management Advisory Council, Australian Fisheries Management Authority, Canberra.

Campbell, R., Whitelaw, W. & McPherson, G., (1997b), *Domestic Longline Fishing Methods and the Catch of Tunas and Non-Target Species off North-Eastern Queensland, (2nd Survey: May-August 1996)*, Report to the Eastern Tuna and Billfish Management Advisory Council, Australian Fisheries Management Authority, Canberra.

Carlson, J.K. & Parsons, G.R., (2001), The effects of hypoxia on three sympatric shark species: physiological and behavioural responses, *Environmental Biology of Fishes*, **61**, 427-433.

Carlson, R.W., (1982), Some characteristics of ventilation and coughing in bluegill *Lepomis macrochirus* Rafinesque, *Environmental Pollution*, **29**, 35-56.

Caulton, M.S., (1977), The effect of temperature on routine metabolism in *Tilapia rendalli* Boulenger, *Journal of Fish Biology*, **11**, 549-553.

Caulton, M.S., (1978), The effect of temperature and mass on routine metabolism in *Sarotherodon (Tilapia), mossambicus* (Peters), *Journal of Fish Biology*, **13**, 195-201.

Cech, J.J.Jr., (1990), Respirometry, In, Schreck, C.B. & Moyle, P.B., (eds), *Methods in Fish Biology*, American Fisheries Society, Maryland.

Cech, J.J. Jr., Bartholow, S.D., Young, P.S. & Hopkins, T.E., (1996), Striped bass exercise and handling stress in freshwater: Physiological responses to recovery environment, *Transactions of the American Fisheries Society*, **125**, 308-320.

Cech, J.J.Jr. & Wohlschlag, D.E., (1973), Respiratory responses of the striped mullet, *Mugil cephalus* L., to hypoxic conditions, *Journal of Fish Biology*, **5**, 421-428.

Cech, J.J.Jr., Campagna, C.G. & Mitchell, S.J., (1979), Respiratory responses of the largemouth bass (*Micropterus salmoides*) to environmental changes in temperature and dissolved oxygen, *Transactions of the American Fisheries Society*, **108**, 166-171.

Cech, J.J.Jr., Massingill, M.J., Vondracek, B. & Linden, A.L., (1985), Respiratory metabolism of mosquito fish, *Gambusia affinis*: effects of temperature, dissolved oxygen and sex difference, *Environmental Biology of Fishes*, **13**, 297-307.

Chan, P., (2000), The industry perspective: Wholesale and retail marketing aspects of the Hong Kong Live Reef Food Fish Trade, *Live Reef Fish Bulletin*, **7**, Secretariat of the Pacific Community, Noumea.

Cheetham, J.L.; Garten, C.T. Jr; King, C.L. & Smith, M.H., (1976), Temperature tolerance and preference of immature channel catfish (*Ictalurus punctatus*), *Copeia*, **1976**, 609-612.

Chiba, K., (1965), A study on the influence of oxygen consumption on the growth of juvenile carps, *Bulletin of the Freshwater Fisheries Research Laboratory,* **15**, 35-47.

Chippari-Gomes, A.R.; Gomes, L.C. & Baldisserotto, B.; (1999), Lethal temperatures for silver catfish, *Rhamdia quelen*, fingerlings, *Journal of Applied Aquaculture*, **9**, 11-22.

Chittenden, M.E., (1972), Responses of young American shad, *Alosa sapidissima* to low temperatures, *Transactions of the American Fisheries Society*, **101**, 680-685.

Chung, K.S., (1980), Thermal tolerance of some tropical marine fish: Preliminary study, *Boletim do Instituto Oceanografico,* **29**, 107-108.

Chung, K.S.; (1997), Thermal tolerance of the tropical fish *Cyprinodon dearborni* (Atheriniformes: Cyprinodontidae) under several temperature and salinity regimes, *Revista do Biologia Tropical*, **45**, 1541-1545.

Chung, K.S.; (2001), Critical thermal maxima and acclimation rate of the tropical guppy *Poecilla reticulata*, *Hydrobiologia*, **462**, 253-257.

Chung, K.S. & Mendez, S.; (1993) Comparative thermal tolerance of some tropical fishes of Venezuela, *Ciencia (Maracaibo),* **1**, 1-7.

Claireaux, G.; Webber, D.M.; Kerr, S.R. & Boutilier, RG, (1995), Physiology and behaviour of free-swimming Atlantic cod (*Gadus morhua*) facing fluctuating temperature conditions, *Journal of Experimental Biology*, **198**, 49-60.

Congleton, J.L.; (1980), Observations on the responses of some Southern California tidepool fishes to nocturnal hypoxic stress, *Comparative Biochemistry and Physiology A,* **66(4),** 719-722.

Cox, D.R., (1974), Effects of Three Heating Rates on the Critical Thermal Maximum of Bluegill, In, Gibbons, J.W. & Sharitz, R.R. (eds), *Thermal Ecology*, National Technical Information Service, Illinois.

Crawford, C.M., (1984), Preliminary results of experiments on the rearing of Tasmanian flounders, *Rhombosolea tapirina* and *Ammotretis rostratus*, *Aquaculture*, **42(1)**, 75-81.

Crawshaw, L.I., (1976), Effect of temperature change on mean body temperature and gill ventilation in the carp, *American Journal of Physiology,* **231**, 837.

Cubero, L. & Molinero, A., (1997), Handling, confinement and anesthetic exposure induces changes in the blood tissue immune characteristics of gilthead sea bream, *Diseases of Aquatic Organisms*, **31**, 89-94.

Currie, R.J.; Bennett, W.A. & Beitinger, T.L.; (1998), Critical thermal minima and maxima of three freshwater gamefish species acclimated to constant temperatures, *Environmental Biology of Fish*, **51**, 187-200.

Davidson, G.W., Thorarensen, H.T., Lokman, M. & Davie, P.S., (1997), Stress of capture and captivity in kahawai, *Arripis trutta* (Bloch and Schneider) (Perciformes: Arripidae), *Comparative Biochemistry and Physiology A*, **118**, 1405-1410.

Davis, J.C & Cameron, J.N., (1971), Water flow and gas exchange at the gills of rainbow trout, *Salmo gairdineri*, *Journal of Experimental Biology*, **54**, 1-18.

Davis, J.C. & Waters, K., (1970), Evaluation of opercula catheterization as a method for sampling water expired by fish, *Journal of the Fisheries Research Board of Canada*, **27**, 1625-1635.

Davis, J.C., (1968), The influence of temperature and activity on certain cardiovascular and respiratory parameters in adult sockeye salmon, M.Sc. Thesis, University of British Columbia.

Davis, K.B., Parker, C.P. & Suttle, M.A., (1982), Plasma corticocosteroids and chlorides in striped bass exposed to tricaine methanesulfonate, quinaldine, etomidate and salt, *Progressive Fish Culturist*, **44,** 205-207.

Davis, M.W., Olla, B.L. & Schreck, C.B., (2001), Stress induced by hooking, net towing, elevated sea water temperature and air in sablefish: lack of concordance between mortality and physiological measures of stress, *Journal of Fish Biology*, **58**, 1-15.

DeAlteris, J.T. & Valley, K.J.L., (1999), Physiological response of scup, *Stenotomus chrysops*, to a simulated trawl and escape event, *Marine Technology Society Journal*, **33**, 25-34.

de Guingand, P., (1994), Keeping fish in top nick, *Fishing Today*, 7, 16-17.

de Guingand, P., (1995), Commercial applications of current research in live fish transport, In, Anon., *Live Seafood Handling: Strategies for Development*, National Seafood Centre, Brisbane.

Dejours, P., (1988), *Respiration in Water and Air. Adaptation, Regulation and Evolution*, Elsevier Scientific Publishing, Amsterdam.

Dejours, P., Armand, J. & Verriest, G., (1968), Carbon dioxide dissociation curves of water and gas exchange of water breathers, *Respiratory Physiology*, **5**, 23-33.

de Kinkelin, P. & Hedrick, R.P., (1991), International veterinary guidelines for the transport of live fish or fish eggs, *Annual Review of Fish Diseases*, 27-40.

De Silva, S.S. & Anderson, T.A., (1995), *Fish Nutrition in Aquaculture*, Chapman and Hall Pty Ltd, Melbourne.

Deufel, J. & Mohr, A., (1983), Limits for oxygen use in aquaculture, *Optimierung von Betriebsparametern der Aquakultr*, **39**, 1-11.

Dewar, H. & Graham, J.B., (1994), Studies of tropical tuna swimming performance in a large water tunnel. 1. Energetics, *Journal of Experimental Biology*, **192**, 13-31.

Domitrovic, H.A., Bechara, J.A., FloresQuintana, C., Roux, J.P. & Gavilan, G., (2000), A survey study of gas supersaturation and fish gas bubble disease in the Parana River below Yacyreta Dam, Argentina, *Revista de ictiologia Corrientes*, **8(1-2)**, 29-40.

dos Santos, J. & Jobling, M., (1991), Gastric emptying in cod, *Gadus morhua* L.: emptying and retention of indigestible solids, *Journal of Fish Biology*, **38**, 187-197

Doudoroff, P., (1942), The resistance and acclimatization of marine fishes to temperature changes. 1. Experiments with *Girella nigricans* (Ayres), *Biological Bulletin*, **83**, 219-225.

Doudoroff, P., (1945), Experiments with *Fundulus* and *Atherinops*, *Biological Bulletin*, **88**, 194-206.

Drud Jordan, A.; Jungersen, M. & Steffensen, J.F. (2001), Oxygen consumption of East Siberian cod; no support for the metabolic cold adaptation theory, *Journal of Fish Biology*, **59**, 818-823.

Durve, V.S., (1975), Anesthetics in the transport of mullet seed, *Aquaculture*, **5**, 53-63.

Eckert, R., Randall, D. & Augustine, G., (1988), *Animal Physiology: Mechanisms and Adaptations (3rd ed.)*, W.H. Freeman and Company, New York.

Edwards, R.R.C., (1971), An assessment of the energy cost of gill ventilation in the plaice (*Pleuronectes platessa* L.), *Comparative Biochemistry and Physiology*, **40**, 391-398.

Edwards, R.R.C., Blaxter, J.H.S., Gopalan, U.K. & Mathew, C.V., (1970), A comparison of the standard oxygen consumption of temperate and tropical bottom living marine fish, *Comparative Biochemistry and Physiology*, **34**, 491-495.

Ege, R. & Krogh, A., (1914), On the relation between the temperature and respiratory exchange in fishes, *International Reviews in Hydrobiology and Hydrography*, 7, 48-55.

Elliot, A.; (1995), A comparison of thermal polygons for British freshwater teleosts, *Freshwater Forum*, **5**, 178-184.

Elliot, J.M., (1972), Rates of gastric evacuation in brown trout, *Salmo trutta* L. *Freshwater Biology*, **2**, 1-18.

Elliot, J.M., (1981), Some Aspects of Thermal Stress on Freshwater Teleosts, In, Pickering, A.D., (ed.), *Stress in Fish*, Academic Press, London,.

Elliot, J.M., (1991), Tolerance and resistance to thermal stress in juvenile Atlanic salmon, *Salmo salar*, *Freshwater Biology*, **25**, 61-70.

Elliot, J.M., Elliot J.A. & Allonby, J.D., (1994), The critical thermal limits for the stone loach, *Noemacheilus barbatulus*, from three populations in North-west England, *Freshwater Biology*, **32**, 593-601.

Erikson, U., (1999), Rigor measurements, In, Kestin, S.C. & Warriss, P.D., (eds), *Proceedings of the International Conference on Farmed Fish Quality, 7-9 April 1999*, Blackwell Science Ltd, Oxford, 448pp.

Esteves, F. de A., (1988), *Fundamentos de Limnologia*, Interciencia/FINEP, Rio de Janeiro, Brazil.

Evans, D.O.; (1972), Correction for lag in continuous-flow respirometry, *Journal of the Fisheries Research Board of Canada*, **29**, 1214-1216.

Evans, L. & Fewtrell, J., (1996), Water Motion: Stress effect during live transport of silver bream, In, Bremner, A., Davis, C. & Austin, B., (eds.), *Making the most of the catch, Symposium Proceedings*, AUSEAS, Brisbane.

Fange, R. & Grove, D.J., (1979), Digestion, In, Hoar, W.S., Randall, D.J. & Brett, J.R., (eds.), *Fish Physiology, Vol.VIII*, Academic Press, New York.

Farrell, A.P., Gallaugher, P., Clarke, C., DeLury, N., Kreiberg, H., Parkhouse, W. & Routledge, R., (2000), Physiological status of coho salmon (*Oncorhychus kisutch*),

captured in commercial non-retention fisheries, *Canadian Journal of Fisheries and Aquatic Sciences*, **57**, 1668-1678.

Faulkner, I.N., and G.P. Moberg., (1997), Effects of short term management stress on the ability of GnRHa to induce gonadotropin secretion in male white sturgeon, *Acipenser transmontanus, Aquaculture,* **159**, 159-168.

Feldmeth, C.R., Stone, E.A. & Brown, J.H., (1974), An increased scope for thermal tolerance upon acclimating pupfish (*Cyprinodon*) to cycling temperatures, *Journal of Comparative Physiology*, **89**, 39-44.

Fergusson, H.W., (1988), *Fish Diseases - Proceedings of the Post-Graduate Committee in Veterinary Science 106*, University of Sydney Press, Sydney.

Fernandes, M.N. & Rantin, F.T., (1986), Lethal temperatures of *Oreochromis nicoticus* (Pisces, Cichlidae), *Revue of Brasilian Biology,* **46,** 589-595.

Fernandes, M.N. & Rantin, F.T., (1989), Respiratory responses of *Oreochromis niloticus* (Pisces, Cichlidae), to environmental hypoxia under different thermal condition, *Journal of Fish Biology*, **35**, 509-519.

Fernandes, M.N., Barrionuevo, W.R. & Rantin, F.T., (1995), Effects of thermal stress on respiratory responses to hypoxia of a south American Prochilodontid fish, *Prochilodus scrofa, Journal of Fish Biology*, **46**, 123-133.

Ferreira, J.T., Smit, G.L. & Schoonbee, H.J., (1982), The use of benzocaine hydrochloride as an aid in the transport of fish, *Aquaculture*, **4**, 169-174.

Fick, A., (1870), Uber die Messung des Blutquantums in der Hertzventrikeln, *Sitzer. Physik. Med. Ges. Wurzburg*, **16,** Cited In, Steffensen, J.F., (1989), Some errors in respirometry of aquatic breathers: how to avoid and correct for them, *Fish Physiology and Biochemistry*, **6,** 49-59.

Findlay, J.D., (1994), *Schooling Dynamics of Pelagic Baitfish*, Honours Thesis, University of Queensland, Zoology Library.

Findlay, J.D., (1995), Live transport of the horseshoe leatherjacket, In, Anon., *Live Seafood Handling: Strategies for Development*, National Seafood Centre, Brisbane, 1995.

Flos, R. Reig, L., Torres P. & Tort, L., (1988), Primary and secondary stress responses to grading and hauling rainbow trout, *Salmo gairdneri, Aquaculture*, **71**, 99-106.

Flowerdew, M.W. & Grove, D.J., (1979), Some observations of the effects of body weight, temperature, meal size and quality on the gastric emptying time in turbot, *Scopthalmus maximus* (L.), using radiography, *Journal of Fish Biology*, **14**, 229-238.

Foo, J.T.W. & Lam, T.J., (1993), Serum cortisol response to handling stress and the effect of cortisol implantations on testosterone level in tilapia, *Oreochromis mosambicas*, *Aquaculture*, **115**, 145-158.

Forsberg, J.A., Barton, B.A. & Summerfelt, R.C., (1999), Effects of ram-air ventilation during transportation on water quality and physiology of walleye fingerlings, In, Barry, T., Barton, B. & MacKinlay, D., *Stress in Fish, Symposium Proceedings*, Towson University, Baltimore.

Forster, M.E., (1990), Confirmation of the low metabolic rate of hagfish, *Comparative Biochemistry and Physiology A*, **96**, 113-116.

Forstner, H., (1983), An automated multiple-chamber intermittent flow respirometer, In, Gnaiger, E. & Forstner, H., (eds), *Polarographic Oxygen Sensors, Aquatic and Physiological Applications*, Springer-Verlag, Berlin.

Franklin, B., (1995), An overview of current packaging and transport techniques, In, Anon., *Live Seafood Handling: Strategies for Development*, National Seafood Centre, Brisbane.

Franklin, C.E.; Johnston, I.A.; Crockford, T. & Kamunde, C., (1995), Scaling of oxygen consumption of Lake Magadi tilapia, a fish living at 37 degree C, *Journal of Fish Biology*, **46**, 829-834.

Fritsche, R. & Nilsson, S., (1993), Cardiovascular and ventilatory control during hypoxia, In, Rankin, J.C. & Jensen, F.B., (eds), *Fish Ecophysiology*, Chapman & Hall, London.

Fry, F.E., (1957), The aquatic respiration of fish, In, Brown, M. E., (ed.), *The Physiology of Fishes, Volume 1*, Academic Press, New York.

Fry, F.E.J., (1967), Responses of Vertebrate Poikilotherms to Temperature, In, Rose, A.H., (ed.), Thermobiology, Academic Press, London.

Fry, F.E.J., (1971), The Effect of Environmental Factors on the Physiology of Fish, In, Hoar, W.S. & Randall, D.J., (eds), *Fish Physiology Vol IV*, Academic Press, New York.

Garey, W.F., (1967), *Gas exchange, cardiac output and blood pressure in freeswimming carp (Cyprinnus carpio)*, Ph.D. Thesis, State University of New York.

Garrow, J.S., (1974), *Energy Balance and Obesity in Man,* North-Holland Ltd, London.

Gehrke, P.C., (1993), *Physiological ecology of spangled perch, Lecopotherapon unicolor (Gunther 1859), (Percoidei, Teraporidae), with special emphasis on cardiovascular responses to temperature and dissolved oxygen,* PhD Thesis, Dept of Zoology, University of Queensland.

Gelwicks, K.R., Zattf, D.J. & Bobbitt, J.P., (1998), Efficacy of carbonic acid as an anesthetic for rainbow trout, *North American Journal of Fisheries Management*, **18**, 432-438.

Gerald, J.W. & Cech, J.J.Jr., (1970), Respiratory responses of juvenile catfish (*Ictalurus punctatus*), to hypoxic conditions, *Physiological Zoology*, **43**, 47-54.

Gibbson, E.S. & Fry, F.E.J., (1954), The performance of lake trout, *Salvelinus namaycush* at various levels of temperature and oxygen pressures, *Canadian Journal of Zoology*, **32**, 252-360.

Girard, S.S. & Milligan, C.L., (1992), The metabolic fate of blood-borne lactate in winter flounder (*Pseudopleuronectes americanus*) during recovery from strenuous exercise, *Physiological Zoology*, **65(6)**, 1114-1134.

Glass, N.R., (1969), Discussion of calculation of power function with special reference to respiratory metabolism in fish, *Journal of the Fisheries Research Board of Canada*, **26,** 2643-2650.

Gommon, M.F., Glover, J.C.M. & Kuiter, R.H., (1994), *The Fishes of Australia's South Coast*, State Print, Adelaide.

Graham, J.M., (1949), Some effects of temperature and oxygen pressure on the metabolism and activity of the speckled trout, *Salvelinus fontinalis*, *Canadian Journal of Research Section D Zoological Sciences*, **27**, 270-288.

Grant, E.M., (1987), *Fishes of Australia,* E.M. Grant Publishers Pty Ltd, Brisbane.

Grantner, A. & Taborsky, M., (1998), The metabolic rates associated with resting, and with performance of agonistic, submissive and digging behaviours in the cichlid fish, *Neolamprologus pulcher* (Pisces: Cichlidae), *Journal of Comparative Physiology B*, **168(6)**, 427-433.

Green, E.J. & Carritt, D.E., (1967), New tables for oxygen saturation of seawater, *Journal of Marine Research*, **25**, 140-147.

Gregory, N.G., (1998), *Animal Welfare and Meat Science*, CABI Publising, Oxon, 304pp.

Griffiths, J.S. & Alderdice, D.F., (1972), Effects of acclimation and acute temperature experience on the swimming speed of juvenile coho salmon, *Journal of the Fisheries Research Board of Canada*, **29**, 251-264.

Grove, D.J. & Crawford, C., (1980), Correlation between digestion rate and feeding frequency in the stomachless teleost, *Blennius pholis* L., *Journal of Fish Biology*, **16**, 235-247.

Grove, D.J., (1986), *Gastrointestinal physiology: Rates of food processing in fish*, In, Nilsson, S. & Holmgren, S., (eds), *Fish Physiology: Recent Advances*, Croom Helm Pty Ltd, Sydney.

Grove, D.J., Loizides, L. & Nott, J., (1978), Satiation amount, frequency of feeding and gastric emptying rate in *Salmo gairdneri, Journal of Fish Biology,* **12**, 507-516.

Grove, D.J., Moctezuma, M.A., Flett, H.R.J., Foott, J.S., Watson, T. & Flowerdew, M.W., (1985), Gastric emptying and the return of appetite in juvenile turbot, *Scopthalmus maximus* L., fed on artificial diets, *Journal of Fish Biology*, **26**, 339-354.

Gwyther, D. & Grove, D.J., (1981), Gastric emptying in *Limanda limanda* (L.), and the return of appetite, *Journal of Fish Biology*, **18**, 245-261.

Hales, L.S. Jr., Lay, C.C. & Helfman, G.S., (1990), Use of low-salinity water and gel-coating to minimize handling mortality of spot, *Leiostomus xanthurus* (Perciformes: Sciaeanidae), *Aquaculture*, **90**, 17-27.

Halsband, E. & Halsband, I. (1984), *Electrofishing*, Canadian Translations in Fisheries and Aquatic Sciences no. 5048, 235 pp

Hamada, A. & Ida, T., (1973), Studies on specific dynamic action of fishes: I Various conditions affecting values of measurement, *Bulletin of the Japanese Society of Scientific Fisheries*, **39**, 1231-1235.

Hanson, D. & Johansen, K., (1970), Relationship of gill ventilation and perfusion in Pacific dogfish, *Squalus suckleyi, Journal of the Fisheries Research Board of Canada*, **27**, 551-564.

Harrell, R.M., (1992), Stress mitigation by use of salt and anesthetic for wild striped bass captured for brood stock, *Progressive Fish-Culturist*, **54(4)**, 228-233.

Hazel, J.R., (1993), *Thermal Biology,* In, Evans, J.H., (ed.), *The Physiology of Fishes,* CRC Press, Boca Raton.

Heath, A.G. & Pritchard, A.W., (1962), Changes in the metabolic rate and blood lactic acid of bluegill sunfish, *Lepomis macrochirus*, Raf., following severe muscular activity, *Physiological Zoology*, **35**, 323-329.

Heath, A.G. & Pritchard, A.W., (1965), Effects of severe hypoxia on carbohydrate energy stores and metabolism in two species of fresh-water fish, *Physiological Zoology*, **38,** 325-334.

Heath, A.G., (1990), *Water Pollution and Fish Physiology*, CRC Press, Boca Raton.

Heath, A.G., Turner, B.J. & Davis, W.P., (1993), Temperature preferences and tolerances of three fish species inhabiting hyperthermal ponds on mangrove islands, *Hydrobiologia,* **259**, 47-55.

Herskin, J., (1999), Effects of social and visual contact on the oxygen consumption of juvenile sea bass measured by computerized intermittent respirometry, *Journal of Fish Biology*, **55**, 1075-1085.

Hettler, W.F., (1976), Influence and temperature and salinity on routine metabolic rate and growth of young Atlantic menhaden, *Journal of Fish Biology,* **8**, 55-65.

Hickman, C.P.Jr., (1959), The osmoregulatory role of the thyroid gland in the starry flounder, *Platichthys stellatus, Canadian Journal of Zoology*, **37**, 997-1060.

Higgenbotham, A.C., (1947), Notes on the oxygen consumption and activity of catfish, *Ecology*, **28**, 462-464.

Hochachka, P.W. & Somero, G.N., (1971), Biochemical Adaptation to the Environment, In, Hoar, W.S. & Randall, D.J., (eds), *Fish Physiology*, Academic Press, New York.

Hochachka, P.W. & Somero, G.N., (1984), *Biochemical Adaptation*, Princeton University Press, New Jersey.

Hofer, R., Forstner, H. & Rettenwander, R., (1982), Duration of gut passage and its dependence on temperature and food consumption in roach, *Rutilus rutilus (L.),* laboratory and field experiments, *Journal of Fish Biology*, **20**, 289-301.

Hoff, J.G & Westman, J.R., (1966), The temperature tolerances of three species of marine fishes, *Journal of Marine Research,* **24,** 131-140.

Holeton, G.F. & Randall, D.J., (1967), The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of rainbow trout, *Journal of Experimental Biology*, **46**, 317-327.

Holeton, G.F., (1980), Oxygen as an environmental factor in fishes, In, Ali, M.A., (ed.), *Environmental Physiology of Fishes*, Plenum Press, New York.

Hove, J.R.; Moss, S.A., (1997), Effect of MS-222 on response to light and rate of metabolism of the little skate *Raja erinacei*, *Marine Biology*, **128**, 579-583.

Hughes, G.M. & Umezawa, S., (1968), On respiration in the dragonet *Callionymus lyra* L., *Journal of Experimental Biology*, **49**, 565-582.

Hughes, G.M., Albers, C., Muster, D. & Gotz, K.H., (1983), Respiration of the carp, *Cyprinus carpio* L., at 10 and 20°C and the effects of hypoxia, *Journal of Fish Biology*, **22**, 613-628.

Humboldt & Provecal, (1809), Recherches sur la respiration des poisons, *Mem. Phys. Chim. Soc d'Arcueil*, **2**, 559, Cited In, Steffensen, J.F., (1989), Some errors in respirometry of aquatic breathers: how to avoid and correct for them, *Fish Physiology and Biochemistry*, **6,** 49-59.

Huss, H.H., (1995), *Quality and quality changes in fresh fish*, FAO Fisheries Technical Paper 348, FAO, Rome.

Hutchinson, G.E., (1957), *A treatise on Limnology*, Wiley, New York.

Idler, D.R. & Clemens, W.A., (1959), The energy expenditures of Fraser River sockeye salmon during the spawning migration to Chilko and Stuart Lakes, *International Pacific Salmon Fisheries Commission Progress Report*, 6.

Irvin, D.N., (1974), Temperature tolerance of early developmental stages of Dover sole *Solea solea* (L.), In, J.H.S., Blaxter, (ed), The Early Life History of Fish, Springer-Verlag, Heidelberg, pp. 449-463.

Isaken, B. & Midling, K.O., (1995), Fishing strategy, gear modifications and new holding tanks in order to keep seine fish alive, *Presentations at the Fourth Asian Fisheries Forum*, Beijing.

Itazawa, Y. & Takeda, T., (1982), Respiration of carp under anesthesia induced by mixed bubbling of carbon dioxide and oxygen, *Bulletin of the Japanese Society of Scientific Fisheries*, **48**, 489-493.

Iwama, G.K., Yesaki, T.Y. & Ahlborn, D., (1991), The refinement of the administration of carbon dioxide gas as a fish anesthetic: The effects of varying the water hardness and ionic content in carbon dioxide anesthesia, *ICES Council Meeting Papers*, ICES, Copenhagen.

Jain, S.K. & Garg, S.K., (1984), Thermal tolerance limits of the Indian murrel *Channa punctatus*, *Indian Journal of Ecology*, **11**, 309-312.

Jeon, J-K, Kim, P-K, Park, Y-J, Myoung, J-G, Kim, J-M., (2000a), Changes of serum cortisol concentration and stress responses in coho salmon (*Oncorhynchus kisutch*), to netting, *Journal of the Korean Fisheries Society*, **33**, 115-118.

Jeon, J-K, Kim, P-K, Park, Y-J, Myoung, J-G, Kim, J-M., (2000b), Stress responses of coho salmon, *Oncorhynchus kisutch*, to transport in fresh water or salt water, *Journal of the Korean Fisheries Society*, **33**, 119-123.

Jerret, A.R., (1992), Methods for sedating, anaesthetising and euthanasing aquatic organisms, *NZ Patent Application 244531*.

Jirasek, J., Adamek, Z. & Giang, P.M., (1978), The effect of administration of the anaesthetics MS 222 (Sandoz) and R 7464 (Propoxate) on oxygen consumption in the tench (*Tinca tinca* L.), *Zivocisna Vyroba*, **23(11)**, 835-840.

Jo, J.Y. & Kim, Y.H., (1998), Oxygen consumptiopn of Far Eastern catfish, *Siluris asotus*, on the different water temperatures and photoperiods, *Journal of the Korean Fisheries Society*, **32**, 56-61.

Job, S.V., (1955), The oxygen consumption of *Salvelinus fontinalis*, University of Toronto Biological Series, No. **61**.

Job, S.V., (1959), The metabolism of *Plotosus anguilaris* (Bloch), in various concentration of salt and oxygen in the medium, *Proceedings of the Indian Academy of Science, Vol. L, Section B*, **5**, 267-288.

Job, S.V., (1969a), The respiratory metabolism of *Tilapia mossambica* (Teleostei), I., The effect of size, temperature, salinity and partial pressure of oxygen, *Journal of Marine Biology*, **2**, 121-126.

Job, S.V., (1969b), The respiratory metabolism of *Tilapia mossambica* (Teleostei), II. The effect of size, temperature, salinity and partial pressure of oxygen, *Journal of Marine Biology*, **3**, 222-226.

Jobling, M. & Davies, P.S., (1979), Gastric evacuation in plaice, *Pleuronectes platessa* L.: effects of temperature and meal size, *Journal of Fish Biology*, **14**, 539-546.

Jobling, M. & Davies, P.S., (1980), Effects of feeding on the metabolic rate and the specific dynamic action in plaice, *Pleuronectes platessa* L., *Journal of Fish Biology*, **16**, 629-638.

Jobling, M., (1980), Effects of starvation on proximate chemical composition and energy utilisation of plaice, *Pleuronectes platessa* L., *Journal of Fish Biology*, **17**, 325-334.

Jobling, M., (1981), The influence of feeding on the metabolic rate of fishes: A short review, *Journal of Fish Biology*, **18**, 385-400.

Jobling, M., (1982), A study of some factors affecting rates of oxygen consumption of plaice, *Pleuronectes platessa*, *Journal of Fish Biology*, **20**, 501-516.

Jobling, M., (1993), Bioenergetics: Feed intake and energy partitioning, In, Rankin, J.C. & Jensen, F.B., (eds), *Fish Ecophysiology*, Chapman & Hall, London.

Jobling, M., Gwyther, D. & Grove, D.J., (1977), Some effects of temperature, meal size and body weight on gastric evacuation time in the dab *Limanda limanda* (L.), *Journal of Fish Biology*, **10**, 291-298.

Johansen, P.H. & Cross, J.A., (1980), Effects on sexual maturation and sex steroid hormone treatment on the temperature preference of the guppy, *Poecilia reticulata* (Peters), *Canadian Journal of Zoology*, **58**, 586-588.

Johnson, C.R., (1976), Diel variation in the thermal tolerance of *Gambusia affinis affinis* (Pisces: Poeciliidae), *Comparative Biochemistry and Physiology*, **55**, 337-340.

Jones, D.R., (1971), The effect of hypoxia and anaemia on the swimming performance of rainbow trout (*Salmo gairdneri*), *Journal of Experimental Biology*, **55**, 541-551.

Jones, R., (1974), The rate of elimination of food from the stomachs of haddock, *Melanogrammus aeglefinus*, cod, *Gadus morhua*, and whiting, *Merlangius merlangus*, *Journal of the Conseil International pour l'Exploration de la Mer*, **15**, 225-243.

Jorgensen, E.H. & Jobling, M., (1988), Use of radiographic methods in feeding studies: a cautionary note, *Journal of Fish Biology*, **32**, 487-488.

Kalinin, A.L.; Glass, M.L. & Rantin, F.T.; (1999), A comparison of directly measured and estimated gill ventilation in the Nile tilapia, *Oreochromis niloticus*, *Comparative Biochemistry and Physiology A*, **122**, 207-211.

Kapoor, B.G., Smit, H & Verighina, I.A., (1975), The alimentary canal and digestion in teleosts, *Advances in Marine Biology*, **13**, 109-239.

Kasim, H.M., (1982), Effect of Temperature on Respiration of *Labeo fimbriatus* (Bloch), *Indian Journal of Fisheries*, **29**, 85-93.

Kelly, K., (1999), The use of chemicals in the live fish export industry, *Queensland Fisherman*, **3**, 22.

Kidder, G.W. & Ball, A., (1999) Oxygen consumption of the killifish, *Fundulus heteroclitus*, *Bulletin of the Mount Desert Island Biological Laboratory*, **38**, 20-21.

Kikuchi, K., Takeda, S., Honda, H. & Kiyono, M., (1990), Oxygen consumption and nitrogenous excretion of starved Japanese flounder, *Paralichthys olivaceus*, *Nippon Suisan Gakkaishi*, **56**, 1891.

King, T.L., Zimmerman, E.G. & Beitinger, T.L., (1985), Concordant variation in thermal tolerance and allozymes of the red shiner, *Notropis lutrensis*, inhabiting tailwater sections of the Brazos River, Texas, *Environmental Biology of Fishes*, **13**, 49-57.

Kionka, B.C, & Windell, J.T., (1972), Differential movement of digestible and indigestible food fractions in rainbow trout, *Salmo gairdneri*, *Transactions of the American Fisheries Society*, **101**, 112-115.

Kirk, W.L., (1976), Effects of sodium chloride on blood lactate and pH of channel catfish during recovery from hypoxia, *Progressive Fish Culturist*, **38**, 48-50.

Korhonen, I.A. & Lagerspetz, K.Y.H., (1996), Heat shock response and thermal acclimation in *Asellus aquaticus*, *Journal of Thermal Biology*, **21**, 49-56.

Kreiberg, H & Solmie, A., (1987), A production-scale towable net pen for efficient high-volume transport of Pacific herring: Design and comparative performance, *Aquacultural Engineering*, **6**, 289-299.

Kreigberg, H. & Powell, J., (1991), Metomidate sedation reduces handling stress in Chinook salmon, *World Aquaculture*, **22**, 58-59.

Krogh, A., (1914), The quantitative relation between temperature and standard metabolism in animals, *Intern. Z. Physik. Chem. Biol.*, **1**, 491-508, Cited In,

Steffensen, J.F., (1989), Some errors in respirometry of aquatic breathers: how to avoid and correct for them, *Fish Physiology and Biochemistry*, **6,** 49-59.

Kryuchkov, V.I. & Kasimov, R.Y., (1990), Respiratory processes in juvenile beluga, *Huso huso*, and Russian sturgeon, *Acipenser gueldenstaedti*, in waters of differing salinity, *Journal of Ichthyology*, **30,** 22-30.

Kuiter, R.H., (1993), *Coastal Fishes of South-Eastern Australia*, Crawford House Press, Bathurst.

Kutty, M.N., (1972), Respiratory quotient and ammonia excretion in *Tilapia mossambica*, *Marine Biology*, **16,** 126-133.

Kutty, M.N. & Peer Mohamed, M., (1975), Metabolic adaptation of mullet, *Rhinomugil corsula* (Hamilton), with special reference to energy utilization, *Aquaculture*, **5**, 253-270.

Kutty, M.N., Sukumaran, N. & Kasim, H.M., (1980), Influence of temperature and salinity on survival of the freshwater mullet, *Rhinomugil corsula* (Hamilton), *Aquaculture*, **20**, 261-274.Laberee, K. & Milligan, C.L., (1999a), Lactate transport across sarcolemmal vesicles isolated from rainbow trout white muscle, *Journal of Experimental Biology*, **16**, 2167-2175.

Laberee, K. & Milligan, C.L., (1999b), *Transport of lactate in rainbow trout white muscle using giant sarcolemmal vesicles*, In, MacKinlay, D., Howard, K. & Cech, J. Jr, *Fish Performance Studies*, Department of Fisheries and Oceans, Vancouver, Canada.

Laurence, G.C., (1971), Digestion of larval largemouth bass, *New York Fish and Game Journal*, **18**, 52-56.

Lecomte-Finiger, R., (1981), Experimental study on the thermal resistance of elvers from the Gironde estuary and the Mediterranean Sea, *Cahiers Laboratory Hydrobiology*, **12**, 17-22.

Lehninger, A.L., (1975), *Biochemistry*, Worth Pty Ltd, New York.

Lejeune, P., (1987), The effect of local stock density on social behaviour and sex change in the Mediterranean labrid *Coris julis*, *Environmental Biology of Fishes*, **18(2)**, 135-141.

Le Moigne, J.; Soulier, P.; Peyraud-Waitzenegger, M. & Peyraud, C., (1986) Cutaneous and gill $0_2$ uptake in the European eel (*Anguilla anguilla*) in relation to ambient $P_{0_2}$, 10-400 Torr, *Respiration Physiology*, **66**, 341-354.

Leonard, J.B., Norieka, J.F., Kynard, B. & McCormick, S.D., (1999), Metabolic rates in an anadromous clupied, the American shad (*Alosa sapidissima*), *Journal of Comparative Physiology*, **169**, 287-295.

Lloyd, R., (1992), *Pollution and Freshwater Fish*, Oxford Press, Oxford.

Lomholdt, J.P. & Johansen, K., (1979), Hypoxia Acclimation in Carp – How its Affects $O_2$ Uptake, Ventilation, and $O_2$ Extraction from Water, *Physiological Zoology*, **52**, 38-49.

Love, R.M., (1980), *The Chemical Biology of Fish, Volume 2*, Academic Press, London.

Lucas, M.C. & Priede, I.G., (1992), Utilisation of metabolic scope in relation to feeding and activity by individual and grouped zebrafish, *Brachydanio rerio* (Hamilton-Buchanan), *Journal of Fish Biology*, **41**, 175-190.

Lutterschmidt, W.I. & Hutchinson, V.H.; (1997), The critical thermal maximum; History and critique, *Canadian Journal of Zoology*, **75**, 1561-1574.

Lyytikaeinen, T.; Koskela, J. & Rissanen, I., (1997), Thermal resistance and upper lethal temperatures of underyearling Lake Inari Artic charr, *Journal of Fish Biology*, **51**, 515-525.

Macisaac, P.F.; Goff, G.P. & Speare, D.J., (1997), Comparison of routine oxygen consumption rates of three species of pleuronectids at three temperatures, *Journal of Applied Ichthyology*, **13**, 171-176.

MacKinlay, D.D., Johnson, M.V.D. & Celli, D.C., (1994), Evaluation of stress of carbon dioxide anesthesia, In, *High Performance Fish: Proceedings of An International Fish Physiology Symposium*, University of British Columbia, Vancouver, Canada, pp. 421-424.

Maier, M.H., (1977), *Estudo de variacao sazonal das condicoes fiscias e quimicas ao longo de um trecho do Rio Moji-Gaucu-Cachoeira das Emas, Estado de Sao Paulo*, M.Sc. Thesis, University of Sao Paulo.

Mann, K.H., (1956), A study of the oxygen consumption of five species of leech, *Journal of Experimental Biology*, **33**, 615-626.

Martinez-Palacios, C.A. & Ross, L.G., (1986), The effects of temperature, body weight and hypoxia on the oxygen consumption of the Mexican mojarra, *Cichlasoma urophthalmus* (Guenther), *Aquaculture and Fisheries Management*, **17**, 243-248.

May, J.L. & Maxwell, J.G.H., (1986), *Trawl fish from temperate waters of Australia*, CSIRO Division of Fisheries Research, Tasmania, 492pp.

McCarthy, I.D., Houlihan, D.F., Carter, C.G. & Moutou, K., (1993), Variation in individual food consumption rates of fish and its implications for the study of fish nutrition and physiology, *Proceedings of the Nutrition Society*, **52**, 427-436.

McCauley, R.W.& Binkowski, F.P., (1982), Thermal tolerance of the alewife, *Transactions of the American Fisheries Society*, **111**, 389-391.

McCormick, J.H., Hokanson, K.E.F. & Jones, B.R., (1972), Effects of temperature on growth and survival of young brook trout, *Salvelinus fontinalis*, Journal of the Fisheries Research Board of Canada, **29**, 1107-1112.

McFarland, W.N., (1960), The use of anaesthetics for the handling and transport of fishes, *California Fish and Game*, 46, 407-431.

McGilvray, F. & Chan, T., (2003), Market and industry demand issues in the live reef fish trade, *SPC Live Reef Fish Information Bulletin #11*, April 2003.

McKenzie, D.J., Cataldi, E., Romano, P., Owen, S.F., Taylor, E.W. & Bronzi, P., (2001), Effects of acclimation to the brackish water on the growth, respiratory metabolism, and swimming performance of young-of-year Adriatic sturgeon, *Canadian Journal of Fisheries and Aquatic Sciences*, **58**, 1104-1112.

McKenzie, D.J., Cataldi, E., Romano, P., Taylor, E.W., Cataudella, S. & Bronzi, P., (2001), Effects of acclimation to the brackish water on tolerance of salinity challenge by young-of-year Adriatic sturgeon (*Acipenser naccarii*), *Canadian Journal of Fisheries and Aquatic Sciences*, **58**, 1113-1121.

Meffe, G.K.; Weeks, S.C.; Mulvey, M. & Kandl, K.L., (1995), Genetic differences in thermal tolerance of eastern mosquitofish (*Gambusia holbrooki*; poecillidae) from ambient and thermal ponds, *Canadian Journal of Fisheries and Aquatic Sciences*, **52**, 2704-2711.

Midling, K., Beltestad, A. & Isaken, B., (1996), *Live Fish Technology*, In, Bremner, A., Davis, C. & Austin, B., *Making the most of the catch, Symposium Proceedings*, AUSEAS, Brisbane.

Midling, K.O. & Isaken, B., (1995), New net pen construction to increase survival rates during acclimatisation of seine net captured cod (*Gadus morhua*), for aquaculture, *Proceeding of the Fourth Asian Fisheries Forum*, Beijing.

Miles, H.M., Loehner, S.M., Michaud, D.T. & Salivar, S.L., (1974), Physiological responses of hatchery reared muskellunge (*Esox masquinongy*), to handling, *Transactions of the American Fisheries Society*, **103**, 336-342.

Milligan, L., (1994), *Recovery process: Metabolic pathway*, In, *High Performance Fish: Proceedings of an International Fish Physiology Symposium at the University of British Columbia*, Fish Physiology Association, Vancouver.

Milson, W.K., (1993), Afferent inputs in regulating ventilation in vertebrates, In, Bicudo, J.E.P.W., (ed), *The Vertebrate Gas Transport Cascade, Adaptations to Environment and Mode of Life*, CRC Press, Boca Raton.

Mishra, B.K., Kumar, D. & Mishra, R., (1983), Observations on the use of carbonic acid anesthesia in fish fry transport, *Aquaculture*, **32**, 405-408.

Mitsunaga, Y., Sakamoto, W., Arai, N. & Kasai, A., (1999), Estimation of the metabolic rate of wild sea bream, *Pagrus major*, in different water temperatures, *Nippon Suisan Gakkaishi*, **65**, 48-54.

Mittal, A.K. & Whitear, M., (1978), Letter to the editor, A note on cold anesthesia of poikilotherms, *Journal of Fish Biology*, **13**, 519-520.

Mitton, C.J.A. & McDonald, D.G., (1994), Consequences of pulsed DC electrofishing and air exposure to rainbow trout, *Canadian Journal of Fisheries and Aquatic Sciences*, **51**, 1791-1798.

Molnar, G., Tamassy, E. & Tolg, I., (1967), The gastric digestion of living predatory fish, In, Gerking, S.D., (ed.), *The Biological Basis of Freshwater Fish Production*, Blackwell Scientific, Oxford.

Montero. P. & Borderias, J., (1989), Distribution and hardness of muscle connective tissue in hake (*Merluccius merluccius* L.) and trout (*Salmo irideus* Gibb), Zeitschrift fur Lebensmittel-Untersuchung Und-Forschung A-Food Research and Technology, **189(6)**, 530-533.

Morgan, J.D., Balfry, S.K., Vijayan, M.M. & Iwama, G.K., (1996), Physiological responses to hyposaline exposure and handling and confinement stress in juvenile dolphin (mahi mahi: *Coryphaena hippurus*), *Canadian Journal of Fisheries and Aquatic Sciences*, **53**, 1736-1740.

Moss, D.D. & Scott, D.C., (1961), Dissolved-oxygen requirements of three species of fish, *Transactions of the American Fisheries Society*, **90**, 377-393.

Mourik, J., Raeven, P., Steur, K. & Addink, A.D.F., (1982), Anaerobic metabolism of red skeletal muscle of goldfish, *Carassius auratus* (L.): Mitochondrial produced acetaldehyde as anaerobic electron acceptor, *FEBS Letts*, **137**, 111-114.

Mueller, R., (1976), Investigation on the body temperature of freshwater fishes, *Archives of Fisheries*, **27(2)**, 1-28.

Muenkner, W., Kuhlmann, H. & Oehlenschlaeger, J., (2000), Investigations on the sensitiveness of sea water fish on board, Part 2: Demersal and pelagic fish species of the North and Baltic Sea, *Information of the Fischwirtsch. Fischereiforsch*, **47**, 97-101.

Mugnier, C., Fostier, A., Guezou, S., Gaignon, J-L. & Quemener, L., (1998), Effect of some repetitive factors on turbot stress response, *Aquaculture International*, **6**, 33-45.

Muir, B.S. & Niimi, A.J., (1972), Oxygen consumption of the euryhaline fish aholehole (*Kuhlia sandvicensis*), with reference to salinity, swimming and food consumption, *Journal of the Fisheries Research Board of Canada*, **29**, 67-77.

Muira, T., Suzuki, N., Nagoshi, M. & Yamamura, K., (1976), The rate of production and food consumption of the Biwamusa, *Oncorhynchus rhodurus*, population in Lake Biwa. *Researches Population Ecology Kyoto University*, **17**, 135-154.

Muusze, B., Marcon, J., van den Thillart, G. & Almeida-Val, V., (1998), Hypoxia tolerance of Amazon fish – respiromentry and energy metabolism of the cichlid, *Astronotus ocellatus*, *Comparative Biochemistry and Physiology*, **120**, 151-156.

Myrick, C.; (1999) Temperature, genetic, and ration effects on juvenille rainbow trout (*Oncorhynchus mykiss*), Bioenergetics, *Science and Engineering*, **60**, 433.

Myrick, C.A. & Cech, J.J. Jr; (2000), Temperature influences on California rainbow trout physiological performance, *Fish Physiology and Biochemistry*, **22**, 245-254.

Nakazono, A. & Kusen, J.D., (1991), Protogynous hermaphroditism in the wrasse *Choerodon azurio*, *Nippon Suisan Gakkaishi*, **57(3)**, 417-420.

Nelson, J.S., (1994), *Fishes of the World, 3rd Edition*, John Wiley & Sons Inc., New York. 600 p.

N'Goma, G., (1993), *Ecoulement du poisson vivant et du poisson frais-congele de la Cuvette Congolaise*, FAO Fisheries Circular 867, FAO, Rome.

Niimi, A.J., (1978), Lag Adjustment Between Estimated and Actual Physiological Responses Conducted in Flow-Through Systems, *Journal of the Fisheries Research Board of Canada*, **35**, 1265-1269.

Nobel, R.L., (1973), Evacuation rates of young yellow perch, *Perca flavescens* (Mitchell), *Transactions of the American Fisheries Society*, **102**, 759-763.

Oddson, G., Pikitch, E.K., Dickhoff, W. & Erickson, D.L., (1994), Effects of towing, sorting and caging on physiological stress indicators and survival in trawl caught and discarded Pacific halibut (*Hippoglossus stenolepis* Schmidt 1904), In, *High Performance Fish: Proceedings of an International Fish Physiology Symposium at the University of British Columbia in Vancouver Canada, July 16-21, 1994*, Fish Physiology Association, Vancouver, BC (Canada), pp. 437-442.

Ohr, E.A., (1976), Tricaine methanesulfonate – I. pH and its effects on anesthetic potency, *Comparative Biochemistry and Physiology*, **54C**, 13-17.

Oikawa, S. & Itazawa, Y., (1995), Comparison of oxygen consumption of larval and juvenile carp determined by three different methods, *Fisheries Science*, **61**, 487-490.

Olla, B.L., Davis, M.W. & Schreck, C.B., (1997), Effects of simulated trawling on sablefish and walleye pollock: the role of light intensity, net velocity and towing duration, *Journal of Fish Biology*, **50**, 1181-1194.

Ott, M.E., Heisler, N. & Ultsch, G.R., (1980), A re-evaluation of the relationship between temperature and the critical oxygen tension in freshwater fishes, *Comparative Biochemistry and Physiology*, **67**, 337-340.

Pankhurst, N.W. & Sharples, D.F., (1992), Effects of capture and confinement on plasma cortisol concentrations in snapper, *Pagrus auratus*, *Australian Journal of Marine and Freshwater Research*, **43**, 345-356.

Pankhurst, N.W., Wells, R.M.G. & Carragher, J.F., (1992), Effects of stress on plasma cortisol levels and blood viscosity in blue mao mao, *Scorpis violaceus* (Hutton), a marine teleost, *Comparative Biochemistry and Physiology*, **101**, 335-339.

Paust, B. & Svensson, J., (1986), Quality Handling of Hook-Caught Rockfish, Alaska Sea Grant College Program, *University of Alaska Fairbanks*, Fairbanks.

Perry, S.F. & Thomas, S., (1991), The effects of endogenous or exogenous catecholamines on blood respiratory status during acute hypoxia in rainbow trout (*Oncorhynchus mykiss*), *Journal of Comparative Physiology*, **161**, 489-497.

Persson, L., (1979), The effect of temperature and different food organisms on the rate of gastric evacuation in perch (*Perca fluviatilis), Freshwater Biology,* **9**, 99-104.

Persson, L., (1981), The effect of temperature and meal size on the rate of gastric evacuation in Perch, *Perca fluviatilis,* fed on fish larvae, *Freshwater Biology,* **11**, 131-138.

Peyraud, C. & Serfaty, A., (1964), Le Rythme respiratoire de la carpe (Cyprinus carpio L.), et ses relations avec le taux de l'oxygene dissous dans le biotope, *Hydrobiologica*, **23**, 165-178.

Phillips, B.F. & Scolaro, A.B., (1980), An electrofishing apparatus for sampling sublittoral benthic marine habitats, *Journal of Experimental Marine Biology and Ecology*, **47(1)**, 69-75.

Pickering, A.D., Pottinger, T.G., Sumpter, J.P., Carragher, J.F. & LeBail, P.Y., (1991), Effects of Acute and Chronic Stress on the Levels of Circulating Growth Hormone in rainbow Trout, *Oncorhynchus mykiss, General and Comparative Endocrinology*, **49**, 232-239.

Piiper, J. & Schuman, D., (1967), Efficiency of $O_2$ exchange in the gills of dogfish, *Scyliorhinus stellaris, Respiratory Physiology*, **2**, 135-148.

Piiper, J., Meyer, M., Worth, H. & Willmer, H., (1977), Respiration and circulation during swimming activity in the dogfish *Scyliorhynus stellaris, Respiratory Physiology*, **30**, 221-239.

Pitcher, T.J. & Hart, P.J.B., (1982), *Fisheries Ecology,* Croom Helm Pty Ltd, Sydney.

Possompes, B.P., Bergot, P. & Luguet, P., (1975), Mise au Point d'une methode d'etude du transit gastro-intestinal chez la truite arc-en-ciel, *Salmo gairdneri* R.: Influence du nombres de repas, des quantites ingerees et de la temperature d'acclimation. *Annuals in Hydrobiology,* **6**, 131-143.

Pottinger, T.G. & Pickering, A.D., (1997), Genetic basis to the stress response: selective breeding for stress tolerant fish, In, *Fish Stress and Health in Aquaculture-Society of Experimental Biology Seminar Series No. 62*, Cambridge University Press, Cambridge, pp. 171-193.

Pottinger, T.G., (1998), Changes in blood cortisol, glucose and lactate in carp retained in anglers' keepnets, *Journal of Fish Biology*, **53**, 728-742.

Pottinger, T.G., Yeomans, W.E. & Carrick T.R., (1999), Plasma cortisol and 17 beta-oestradial levels in roach exposed to acute and chronic stress, *Journal of Fish Biology*, **54**, 525-532.

Priede, I.G. & Holliday, F.G.T., (1980), The use of a new tilting respirometer to investigate aspects of metabolism and swimming activity of the plaice (*Pleuronectes platessa* L.), *Journal of Experimental Biology*, **85**, 295-309.

Pritchard, A.W., Florey, E. & Martin, A.W., (1958), Relationship between metabolic rate and body size in an elasmobranch (*Squalus suckleyi*), and in a teleost (*Ophiodon elongatus*), *Journal of Marine Research Sears Found Marine Research*, **17**, 403-411.

Procarione, L.S. & King, T.L., (1993), Upper and lower temperature tolerance limits for juvenile red drums from Texas and South Carolina, *Journal of Aquatic Animal Health*, **5**, 208-212.

Prosser, C.L., Barr, J.M., Lauer, C.Y. & Pinc, R.D., (1957), Acclimation of goldfish to low concentrations of oxygen, *Physiological Zoology*, **30**, 137-141.

Randall, D.J. & Hoar, W.S., (1971), Special Techniques, In, Hoar, W.S. & Randall, D.J., (eds), *Fish Physiology (Volume VI)*, Academic Press, New York.

Randall, D.J. & Shelton G., (1963), The effects of changes in environmental gas concentrations on the breathing and heart rate of a teleost fish, *Comparative Biochemistry and Physiology*, **9**, 229-239.

Randall, D.J., (1993), The regulation of breathing in aquatic vertebrates, In, Bicudo, J.E.P.W., (ed), *The Vertebrate Gas Transport Cascade. Adaptations to Environment and Mode of Life*, CRC Press, Boca Raton.

Rantin, F.T. & Petersen, J.A., (1985), Thermal tolerance of South American cichlid, *Geophagus brasilliensis, Revue D'Hydrobiologie Tropicale*, **18,** 221-226.

Rantin, F.T., Glass, M.L., Kalinin, A.L., Verzola, R.M.M. & Fernandes, M.N., (1993), Cardio-respiratory responses in two ecologically distinct erythrinids (*Hoplias malabricus* and *Hoplias lacerdae*), exposed to graded environmental hypoxia, *Environmental Biology of Fishes*, **36**, 93-97.

Rantin, F.T., Kalinin, A.L., Glass, M.L. & Fernandes, M.N., (1992), Respiratory responses to hypoxia in the relation to mode of life of two erythrinid species (*Hoplias malabricus* and *Hoplias lacerdae*), *Journal of Fish Biology*, **41**, 805-812.

Render, J.H. & Wilson C.A., (1996), Effects of gas bladder deflation on mortality of hook-and-line caught and released red snappers: implications for management, In,

*Biology, fisheries and culture of tropical groupers and snappers, ICLARM conference proceedings 48*, Manila (Philippines), pp 244-253.

Reubush, K.H & Heath, A.G., (1997a), Effects of recovery water salinity on secondary stress responses of hybrid striped bass fingerlings, *Progressive Fish-Culturist*, **59**, 188-197.

Reubush, K.H & Heath, A.G., (1997b), Secondary stress responses to acute handling in striped bass (*Morone saxatilis*), and hybrid striped bass (*Morone chrysops* x *Morone saxatilis*), *American Journal of Veterinary Research*, **5**, 1451-1456.

Reynolds, J.B., (1996), Electrofishing, In, Murphy, B.R. & Willis, D.W., (eds), *Fisheries Technique, 2nd Edition*, American Fisheries Society, Bethesda, p. 221-253.

Reynolds, W.W., Thomson, D.A. & Casterlin, M.A., (1976), Temperature and salinity tolerances of larval Californian grunion, *Leurethes tenius* (Ayres): A comparison with gulf grunion, *L. sardina* (Jenkins & Evermann), *Journal of Experimental Biology and Ecology*, **24**, 73-82.

Richardson, J.; Boubee, J.A.T. & West, D.W.; (1994), Thermal tolerance and preference of some native New Zealand freshwater fish, *New Zealand Journal of Marine and Freshwater Research*, **28**, 399-407.

Rimmer, M., (1994), Live Fish Transport, In, Anon. (ed), *Abstracts from Australian Barramundi Farming Workshop*, Department of Primary Industries, Brisbane.

Rimmer, M., (1995), Development of Live Fish Transport Techniques, In, Anon. (ed), *Live Seafood Handling: Strategies for Development*, National Seafood Centre, Brisbane.

Rimmer, M.A. & Franklin, B., (1997), Development of Live Fish Transport Techniques, Fisheries Research and Development Corporation Project Reports 93/184-195, Canberra.

Rimmer, M., Paterson, B. & de Guingand, P., (1994), A guide to live fish capture and handling, *Australian Fisheries*, **53,** 19-21.

Roberts, J.L. & Rowell, D.M., (1988), Periodic respiration of gill-breathing fishes, *Canadian Journal of Zoology*, **66**, 182-190.

Robertson, L., Thomas, P., Arnold, C.R. & Trant, J.M., (1987), Plasma Cortisol and Secondary Stress Responses of Red Drum to Handling, Transport, Rearing, Density and a Disease Outbreak, *The Progressive Fish Culturist*, **49,** 1-12.

Rodman, D.T., (1963), Anaesthetising and air transporting young white sturgeons, *Progressive Fish Culturist*, **25,** 71-78.

Ross, L.G. and Ross, B., (1984), *Anaesthetic and Sedative Techniques for Fish*, Institute of Aquaculture, University of Stirling, Scotland.

Routley, M.H., Nilsson, G.E. & Renshaw G.M.C., (2002), Exposure to hypoxia primes the respiratory and metabolic responses of the epaulette shark to progressive hypoxia, *Comparative Biochemistry and Physiology*, **131**, 313-321.

Rozin, P. & Mayer, P., (1964), Some factors influencing short-term food intake of the goldfish, *American Journal of Physiology*, **206**, 1430-1436.

Ryan, B.A., Dawley, E.M. & Nelson, R.A., (2000), Modeling the effects of saturated dissolved gas on resident biota in the main-stem Snake and Columbia rivers, *North American Journal of Fisheries Management*, **20(1)**, 192-204.

Sado, E.K., (1985), Influence of the anaesthetic quinaldine on some tilapias, *Aquaculture*, **46**, 55-62.

Saether, B.J. & Jobling M., (1997), Gastrointestinal evacuation of inert particles by turbot, *Psetta maxima*: evaluation of the X-radiographic method for the use in feed intake studies, Aquatic Living Resources, **10**, 359-364.

Sand, O. & Hawkins, A.D., (1974), Measurement of swimbladder volume and pressure in the cod, *Norway Journal of Zoology*, **22**, 31-34.

Satchell, G.H., (1961), The response of the dogfish to anoxia, *Journal of Experimental Biology*, **38**, 531-543.

Saunders, R.L., (1962), The irrigation of the gills in fishes. II. Efficiency of oxygen uptake in relation to respiratory flow, activity and concentrations of oxygen and carbon dioxide, *Canadian Journal of Zoology*, **40**, 817-862.

Saunders, R.L., (1963), Respiration of the Atlantic cod, *Journal of the Fisheries Research Board of Canada*, **20**, 373-386.

Schalles, J.F. & Wissing, T.E., (1976), Effects of dry pellet diets on the metabolic rates of bluegill (*Lepomis macrochirus*), *Journal of the Fisheries Research Board of Canada*, **33**, 2443-2449.

Schoemaker, R., (1991), *Transportation of live and processed seafood*, INFOFISH Technical Handbook 3, Kuala Lumpur.

Scholander, P.F., Haugaard, N. & Irving, l., (1943), A volumetric respirometer for aquatic animals, *Review of Scientific Instruments*, **14**, 48-51.

Schreck, C.B., Solazzi, M.F., Johnson, S.L. & Nickelson, (1989), Transportation stress affects performance of coho salmon, *Oncorhynchus kisutch*, *Aquaculture*, **82**, 15-20.

Schurmann, H. & Steffensen, J.F.; (1992), Lethal oxygen levels at different temperatures and the preferred temperature during hypoxia of the Atlantic cod, *Gadus morhua* L, *Journal of Fish Biology*, **41**, 927-934.

Schurmann, H. & Steffensen, J.F.; (1994), Spontaneous swimming activity of atlantic cod *Gadus morhua* exposed to graded hypoxia at three temperatures, *Journal of Experimental Biology*, **197**, 129-142.

Schurmann, H. & Steffensen, J.F.; (1997), Effects of temperature, hypoxia and activity on the metabolism of juvenile Atlantic cod, *Journal of Fish Biology*, **50**, 1166-1180.

Seki, N. & Watanabe, T., (1984), Connectin content and its post-mortem changes in fish muscle, *Journal of Biochemistry*, **95(4)**, 1161-1167.

Serfaty, A. & Raynaud, P., (1958), Le reflex aerocardiaque chez la carpe commune (*Cyprinsu carpio* L.), at le phenomene d'echappement, *Hydrobiologia*, **12**, 38-42.

Sharpe, C.S., Thompson, D.A. Blankenship, H.L. & Schreck C.B., (1998), Effects of routine handling and tagging procedures on physiological stress responses in juvenile Chinook salmon, *Progressive Fish-Culturist*, **60**, 81-87.

Shoubridge, E.A. & Hochaka, P.W., (1980), Ethanol: Novel end-product of vertebrate metabolism, *Science*, **209**, 308-309.

Singh, T. & Daud, W.J.W., (2001), Live handling and marketing of tilapia, In, Suasinhe, S. & Singh, T., (eds), *Tilapia:production , marketing and technological developments, Proceedings of the Tilapia 2001 International Technical and trade Conference on Tilapia*, INFOFISH, Kaula Lumpur.

Smith, L.S., (1982), *Introduction to Fish Physiology*, T.F.H. Publications.

Smith, R.K. & Fausch, K.D.; (1997), Thermal tolerance and vegetation preference of Arkansas darter and Johnny darter from Colorado plains streams, *Transactions of the American Fisheries Society*, **126**, 676-686.

Smith, S.D., Maule, A.G. & Poe, T.P. (1994), The effects of incidental electrofishing of adult spring Chinook salmon (*Oncorhynchus tshawytscha*), In, *High Performance Fish: Proceedings of An International Fish Physiology Symposium*, University of British Columbia, Vancouver, Canada, pp. 443-444.

Solomon, D.J. & Hawkins, A.D., Fish capture and transport, In, Hawkins, A.D., (ed.), *Aquarium Systems*, Fisheries Laboratory, Lowestoft, 1981.

Specziar A., (2002), An *in situ* estimate of food consumption of five cyprinid species in Lake Balaton, *Journal of Fish Biology*, **60**, 1237-1251.

Specziar A., (2002), *In situ* estimates of gut evacuation and its dependence on temperature in five cyprinids, *Journal of Fish Biology*, **60**, 1222-1236.

Spencer, W.P., (1939), Diurnal activity rhythms in freshwater fishes, *Ohio Journal of Science*, **39**, 119-132.

Spigarelli, S.A., Thommes, M.M.& Beitinger, T.L., (1977), The influence of body weight on heating and cooling of selected Lake Michigan fishes, *Comparative Biochemistry and Physiology*, **56**, 51.

Spitzer, K.W., Marvin, D.E. & Heath, A.G., (1969), The effect of temperature on the respiratory and cardiac responses of bluegill sunfish to hypoxia, *Comparative Biochemistry and Physiology*, **30**, 83-90.

Spoor, W.A., (1946), A quantitative study of the relationship between the activity and oxygen consumption of the goldfish; and its application to the measurement of respiratory metabolism in fishes, *Biological Bulletin*, **91**, 312-325.

Stauffer, R.R. Jr, (1986), Effects of salinity on preferred and lethal temperatures of the Mozambique tilapia, *Oreochromis mossambicus* (Peters), *Water Resources Bulletin*, **22**, 205-208.

Stauffer, R.R. Jr, (1986), Ontogenetic changes in the preferred temperatures of Blackchin Tilapia, *Sarotherodon melanotheron, Archives of Hydrobiologia,* **105,** 397-402.

Stauffer, J., J.R., Hocutt, C.H. & Goodfellow, W.F., (1985), Effects of sex and maturity on preferred temperatures: A proximate factor for increased survival of young *Poecilia latipinna, Archives of Hydrobiologia,* **103,** 129-132.

Stauffer, J., J.R., Lispi, D.R. & Hocutt, C.H., (1984a), The preferred temperatures of three *Semotilus* species, *Archives of Hydrobiologia,* **101,** 595-600.

Stauffer, J., J.R., Lispi, D.R. & Hocutt, C.H., (1984b), Interrelationships among preferred, avoided and lethal temperatures of three fish species, *Archives of Hydrobiologia*, **100,** 159-169.

Staurnes, M., Sigholt, T., Pedersen, H.P. & Rustad, T., (1994), Physiological effects of simulated high-density transport of Atlantic cod (*Gadus morhua*), *Aquaculture*, **119**, 381-391.

Steffens, A.M., (1995), *Stress: The Invisible (and Forgotten?), Factor in Live Fish Transport*, In, Anon., *Live Seafood Handling: Strategies for Development*, National Seafood Centre, Brisbane.

Steffens, A.M., (1996), *The Determination of the Lower Incipient Lethal and Critical Thermal Minimal Temperature of Blue-throat Wrasse (Notolabrus tetricus)*, Master of Applied Science (Fisheries), Thesis, Australian Maritime College, Launceston.

Steffensen, J.F., (1989), Some errors in respirometry of aquatic breathers: how to avoid and correct for them, *Fish Physiology and Biochemistry*, **6,** 49-59.

Steffensen, J.F., Johansen, K. & Bushnell, P.G., (1984), An automated swimming respirometer, *Comparative Biochemistry and Physiology A*, **79**, 437-440.

Steffensen, J.F.; Johansen, K.; Sinderg, C.D.; Soerensen, J.H. & Moeller, JL; (1984), Ventilation and oxygen consumption in the hagfish, *Journal of Experimental Marine Biology and Ecology*, **84**, 173-178.

Steffensen, J.F.; Lomholt, J.P. & Johansen, K.; (1982), Gill ventilation and $O_2$ extraction during graded hypoxia in two ecologically distinct species of flatfish, the flounder (*Platichthys flesus*) and the plaice (*Pleuronectes platessa*), *Environmental Biology of Fishes*, 7, 157-163.

Stevens, E.D.& Fry, F.E.J., (1974), Heat transfer and body temperatures in non-thermoregulatory teleosts, *Canadian Journal of Zoology*, **52**, 1137-1143.

Storebakken, T., Austreng, E. & Steenberg, K., (1981), A method for determination of feed intake in salmonids using radioactive isotopes, *Aquaculture*, **24**, 133-142.

Strange, R.J., (1980), Seawater and confinement alters survival and cortisol concentration in juvenile chinook salmon, *Copeia*, **1980,** 351-353.

Stryer, L., (1988), *Biochemistry*, W.H. Freeman and Company, New York,.

Summerfelt, C. & Smith, P., (1990), Anesthesia, surgery and related techniques, In, Schreck, C., Moyle, P., (eds), *Methods for Fish Biology*, American Fisheries Society.

Sundes, G., (1957a), On the transport of live cod and coalfish, *Journal of the Conseil International pour l'Exploration de la Mer,* **22**, 191-196.

Sundes, G., (1957b), Notes on the energy metabolism of the cod (*Gadus callaris*), and the coalfish (*Gadus virens*), in relation to body size, *Report on Norwegian Fishery and Marine Investigations (Fiskeridirektoratets skrifter)*, **11**, 1-10.

Swanson, C., Mager, R.C., Doroshov, S.I. & Cech, J.J. Jr., (1996), Use of salts, anesthetics, and polymers to minimize handling and transport mortality in delta smelt, *Transactions of the American Fisheries Society,* **125**, 326-329.

Swenson, W.R. & Smith, L.L., (1973), Gastric digestion, food consumption, feeding periodicity and food conversion efficiency in walleye (*Stizostedion vitreum vitreum), Journal of the Fisheries Research Board of Canada,* **30**, 1327-1336.

Takashima, Y., Wan, Z., Kasai, H. & Asakawa, O., (1983), Sustained anesthesia with 2-phenoxyethanol in yearling rainbow trout, *Journal of Tokyo University Fisheries, Suisandai Kempo,* **69**, 93-96.

Takeda, T. & Itzawa, Y., (1983), Examination of possibility of applying anesthesia by carbon dioxide in the transportation of live fish, Bulletin of the Japanese Society of Scientific Fisheries, **49**, 725-732.

Talbot, C. & Higgins, P.J., (1983), A radiographic method for feeding studies on fish using metallic iron powder as a marker, *Journal of Fish Biology,* **23**, 211-220.

Talbot, C., (1985), Laboratory methods in fish feeding and nutritional studies, In, Tytler, P. & Calow, P., (eds), *Fish Energetics*: *New Perspectives*, Croom Helm, London.

Tandler, A. & Beamish, F.W.H., (1979), Mechanical and biochemical components of apparent specific dynamic action of largemouth bass, *Micropterus salmoides* Lacepede, *Journal of Fish Biology,* **14**, 343-350.

Taylor, E.W., (1992), Nervous control of the heart and cardiorespiratory interactions, In, Hoar, W.S., Randall, D.J. & Farrell, A.P., (eds), *Fish Physiology, Vol XII B*, Academic Press, New York.

Taylor, E.W., Short, S. & Butler, P.J., (1977), The role of the cardiac vagus in the response of the dogfish, *Scyliorhynus canicula,* to hypoxia, *Journal of Experimental Biology*, **70**, 57-75.

Templeton, J., (1996), Seafood Research: A New Zealand Perspective, In, Bremner, A., Davis, C. & Austin, B., (eds), *Making the most of the catch Symposium Proceedings*, AUSEAS, Brisbane.

Teo, L.H., Chen, T.W. & Lee, B.H., (1989), Packaging of the guppy, *Poecilia reticulata*, for air transport in a closed system, *Aquaculture*, **78**, 321-332.

Terchunian, A. V., Kunz, N. A. & O'Dierno, L. J. (1999), (eds), *APEC Air Shipment of Live and Fresh Fish and Seafood Catidelines - A Manual on Preparing, Packaging and Packing Live and Fresh Fish and Seafood Air Shipments along with Customs and Inspection Guidelines for Six APEC Member Economies,* First Edition, APEC Fisheries Working Group.

Thomas, P. & Robertson, L., (1991), Plasma cortisol and glucose stress responses of red drum (*Sciaenops ocellatus*), to handling and shallow water stresses and anesthesia with MS-222, quinaldine sulfate and metomidate, *Aquaculture*, **96**, 69-86.

Torrissen, O.J., Sigurgisladottir, D. & Slind, E., (1999), Texture and technological properties of fish, In, Kestin, S.C. & Warriss, P.D., (eds), *Proceedings of the International Conference on Farmed Fish Quality, 7-9 April 1999*, Blackwell Science Ltd, Oxford, 448pp.

Tsuchida, S., Tabata, S. & Nagai, A., (1997), Temperature preference and tolerance of young herring (*Clupea pallasii* Valenciennes) in Mangokuura, Miyagi Prefecture, *Journal of the School of Marine Science and Technology, Tokai University,* **43**, 117-129.

Tsuzuki, M.Y, Ogawa, K., Strussmann, C.A., Maita, M. & Takashima, F., (2001), Physiological responses during stress and subsequent recovery at different salinities in adult pejerry *Odontesthes bonariensis*, *Aquaculture*, **200(3-4)**, 349-362.

Turunen, T., Suuronen, P., Hyvaerinen, H. & Rouvinen, J., (1996), Physiological status of vendace (*Coregonus albula* L.), escaping from a trawl codend, *Nordic Journal of Freshwater Research*, **72**, 39-44.

Tyler, A.V., (1977), Evidence for the assumption of independence between gastric evacuation rate and swimming activity using Atlantic cod, *Gadus morhua, Journal of the Fisheries Research Board of Canada,* **34**, 2411-2413.

Tyler, R.V., (1970), Rates of gastric emptying in young cod, *Journal of the Fisheries Research Board of Canada,* **27**, 1177-1189.

Tytler, P. & Blaxter, J.H.S., (1973), Adaptation by cod and saithe to pressure changes, *Netherlands Journal of Sea Research*, **7**, 31-45.

Ultsch, G.R., Margaret, E.O. & Heisler, N., (1980), Standard metabolic rate, critical oxygen tension and aerobic scope for spontaneous activity of trout (*Salmo gairdneri*), and carp (*Cyprinus carpio*), in acidified water, *Comparative Biochemistry and Physiology*, **67**, 329-335.

Umezawa, S.; Adachi, S. & Taneda, K.; (1983), Group effect on oxygen consumption of the ayu (*Plecoglossus altivelis*) in relation to growth stage, *Japanese Journal of Ichthyology*, **30**, 261.

Urbinata, E.C., Cesar, P. & Carneiro, F., (2001), Metabolic and hormonal responses of matrinxa, *Brycon cephalus*, (Teleost: Characidae), to transport stress under influence of benzocaine, *Journal of Aquaculture in the Tropics*, **16**, 75-85.

Vahl, O. & Davenport, J., (1979), Apparent specific dynamic action of food in the fish, *Blennius pholis, Marine Ecology- Progress Series* **1**, 109-113.

van Dam, L., (1938), *On the utilization of oxygen and regulation of breathing in some aquatic animals*, Ph.D. Thesis, University of Groningen.

Van den Thillart, G. & Kesbeke, F., (1978), Anaerobic production of carbon dioxide and ammonia by goldfish, *Carassius auratus*, (L.), *Comparative Biochemistry and Physiology*, **59**, 393-400.

Van den Thillart, G. & Van Waarde, A., (1985), Teleosts in hypoxia: aspects of anaerobic metabolism, *Molecular Physiology*, **8**, 393-409.

Van den Thillart, G., (1982), Adaptation of fish energy metabolism to hypoxia and anoxia, *Molecular Physiology*, **2**, 49-61.

Van den Thillart, G., Van Berge-Henegouwen, M. & Kesbeke, F., (1983), Anaerobic metabolism of goldfish, *Carassius auratus* (L.): Ethanol and $CO_2$ excretion rates and anoxia tolerance at 20, 10 and 5°C, *Comparative Biochemistry and Physiology*, **76**, 295-300.

Van Waversveld, J., Addink, A.D.F. & Van den Thillart, G., (1989), Simultaneous direct and indirect calorimetry on normoxic and anoxic goldfish, *Journal of Experimental Biology*, **142**, 325-335.

Vasal, S. & Sundararaj, B.I., (1978), Thermal tolerance and preference of the Indian catfish *Heteropneustes fossils*, *Environmental Biology of Fish*, **3**, 309-315.

Vijayan, M.M. & Leatherland J.F., (1988), Effect of stocking density on the growth and stress-response in brook charr, *Salvelinus fontinalis*, In, *Proceedings of the*

*Aquaculture International Congress and Exposition*, Vancouver Trade and Convention Centre, Vancouver, British Columbia, Canada, September 6-9, p. 79.

Vijayan, M.M. & Moon, T.W. (1992), Acute handling stress alters hepatic glycogen metabolism in food-deprived rainbow trout (*Oncorhynchus mykiss*), *Canadian Journal of Fisheries and Aquatic Sciences*, **49**, 2260-2266.

Vinogradov, G.A. & Klerman, A.K., (1987), Ion exchange in freshwater fishes under stress, *Journal of Ichthyology*, **27**, 307-312.

Wagner, E., Arndt, R. & Hilton, B., (2002), Physiological stress responses, egg survival and sperm motility for rainbow trout broodstock anesthetized with clove oil, tricaine methanesulfonate or carbon dioxide, *Aquaculture*, **211(1-4)**, 353-366.

Wallace, R.K. Jr, (1977), Thermal acclimation, upper temperature tolerance, and preferred temperature of juvenile yellowtail snappers, *Ocyurus chrysurus* (Bloch) (Pisces:Lutjanidae), *Bulletin of Marine Science*, **27**, 292-298.

Walsh, S.J., Haney, D.C. & Timmerman, C.M.; (1997) Variation in thermal tolerance and routine metabolism among spring-and stream dwelling freshwater sculpins (Teleostei: Cottidae) of the southeastern United States, *Ecology of Freshwater Fish*, **6**, 84-94.

Waring, C.P., Stagg, R.M. & Poxton, M.G., (1992), The effects of handling on flounder (*Platichthys flesus* L.), and Atlantic Salmon (*Salmo salar* L.), *Journal of Fish Biology*, **41**, 131-144.

Waring, C.P., Stagg, R.M. & Poxton, M.G., (1996), Physiological responses to handling in the turbot, *Journal of Fish Biology*, **48**, 161-173.

Warren, C.E. & Doudoroff, P., (1971), *Biology and Water Pollution Control* W.B. Saunders, Philadelphia.

Watters, K.W.Jr. & Smith, L.S., (1973), Respiratory dynamics of the starry flounder *Platichthys stellatus* in response to low oxygen and high temperature, *Marine Biology*, **19**, 133-148.

Weatherley, A.H., (1973), Effects of constant illumination and hyperoxia on thermal tolerances of goldfish, *Comarative Biochemistry and Physiology*, **45**, 891-894.

Webb, P.W., (1971a), The swimming energetics of trout. I. Thrust and power output at cruising speeds, *Journal of Experimental Biology*, **55**, 489-520.

Webb, P.W., (1971b), The swimming energetics of trout. II. Oxygen consumption and swimming efficiency, *Journal of Experimental Biology*, **55**, 521-540.

Webb, R., (1958), Distribution of bluegill treated with tricaine methanesulfonate (MS-222), *Progressive Fish Culturist*, **20(1)**, 42-44.

Wedemeyer, G.A., (1996), Transportation and handling- Principles of salmonid culture, *Developments in Aquaculture and Fisheries Science*, **29**, 727-758.

Wedemeyer, G.A., Barton, B.A. & Macleay, D.J., (1990), Stress and acclimation, In, Schreck, C. & Moyle, P., (eds), *Methods for Fish Biology*, American Fisheries Society, Maryland.

Weirich,C.R. & Tomasso, J.R., (1991), Confinement- and transport induced stress on red drum juveniles: Effect of salinity, *Progressive Fish-Culturist*, **53(3)**, 146-149.

Weisberg, S.B. & Lotrich, V.R., (1982), Ingestion, egestion, excretion, growth and conversion efficiency for the mummichog, *Fundulus heteroclitus (L.), Journal of Experiments in Marine Biology and Ecology*, **62**, 237-249.

Wells, R.M.G. (1987), Stress response imposed by fish capture and handling: A physiological perspective, *Food Technology (Aust.)*, **39**, 479-481.

Winberg, G.G., (1956), Rate of metabolism and food requirements of fishes, *Transactions in the Journal of the Fisheries Research Board of Canada*, 194.

Winberg, G.G., (1961), New information on metabolic rate in fishes, *Transactions in the Journal of the Fisheries Research Board of Canada*, 362.

Windell, J.T., (1967), Rates of digestion in fishes, In, Gerking, S.D., (ed.), *The Biological Basis of Fish Production*, Blackwell Scientific, Oxford.

Windell, J.T., Hubbard, J.D. & Horak, D.C., (1972), Rate of gastric digestion in rainbow trout, *Salmo gairdneri*, fed three pelleted diets, *Progressive Fish Culturist*, **34**, 156-159.

Winkler, P., (1985), Persistent differences in thermal tolerance among acclimation groups of a warm spring population of *Gambusia affinis* determined under field and laboratory conditions, *Copeia*, **2**, 456-461.

Winkler, P., (1987), A method to minimize stress during fish transport, *Progressive Fish Culturist*, **49**, 154-155.

Woo, N.Y.S. & Fung, A.C.Y., (1980), Studies of the biology of the Red Sea bream *Chrysophrys major*. 1. Temperature tolerance, *Marine Ecology - Progress Series,* **3**, 121-124.

Yokoyama, Y., Yoshikawa, H., Ueno, S. & Mitsuda, H., (1989), Application of $CO_2$ anesthesia combined with low temperature for long term anesthesia in carp, *Nippon Suisan Gaikkaishi*, **55,** 1203-1209.

Yoshikawa, H., Ueno, S. & Mitsuda, H., (1989), Short and long term cold anesthesia in carp, *Nippon Suisan Gaikkaishi*, **55,** 491-498.

Young, P.S. & Cech, J.J. Jr., (1993), Physiological stress responses to serial sampling and confinement in young-of-the-year striped bass, *Morone saxatilis* (Walbaum), *Comparative Biochemistry and Physiology A*, **105**, 239-244.

Zale, A.V. & Gregory, R.W., (1989), Effect of salinity on cold tolerance of juvenile blue tilapias, *Transactions of the American Fisheries Society*, **118**, 718-720.