



| **The Significance of Stressors on Black Bream in the Gippsland Lakes**

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

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In Memory of Mum and Dad

Summary

The Gippsland Lakes and Lake Tyers are two adjacent estuarine systems approximately 15 km apart and located at about 38°S in south-eastern Australia. The Gippsland Lakes is an estuary of 366 km² permanently open to the sea and supported by a much-modified catchment of 20,600 km². Lake Tyers is a small riverine estuary intermittently open to the sea of approximately 25 km² and fed by a mostly undeveloped catchment of 470 km². Black bream (*Acanthopagrus butcheri*) occupy both estuaries and normally prefer to remain in their native estuary. Black bream from Lake Tyers have a greater growth rate and a “superior condition” than those in the Gippsland Lakes.

Analyses are presented of a study of black bream sourced from the Gippsland Lakes and Lake Tyers. The primary aims of the project were to investigate the potential of black bream as a valuable ecological indicator for chronic stress by: (1) developing an acute stress behavioural response test as an indicator of underlying chronic stress , (2) evaluating the potential of this behavioural test, various condition indices and changes in interrenal cells in the kidney to compare chronic stress in black bream from the two estuaries, and (3) investigating differences in elemental composition of otoliths and in genetic polymorphism between the estuaries as causes of differences in chronic stress..

In Chapter 2, a stimulus-startle behavioural test was developed to identify acute stress response to 30 s air exposures such as experienced by fish during catch-and-release practices for undersized fish. Undersized fish from Lake Tyers were collected from a seine net during commercial fishing and acclimated in 2000 L tanks for 3 weeks. The response time to seek cover under a hide in custom-made channel apparatus was measured following a stimulus-response at 0, 6 and 12 hours after air exposure compared with that of fish not so exposed as

controls. The cumulative impacts of one, two and three identical air exposures 3 h apart were compared. Fish were then retained in holding tanks for 3 months for observation and mortality compared. The response time in the stimulus-response test ranged from <1 min to >30 min. The response time and mortality were proportional to the number of air exposures. Fish that died first displayed a dark colour, then became higher in the water column and finally died floating on the surface. The dark colouration was only apparent when fish were in the water. This indicated that the stimulus-response test predicted mortality and could be a useful short-time test for acute stress.

In Chapter 3, the stimulus-response test was used to compare the impact of chronic stress on the reaction times of black bream from the Gippsland Lakes and Lake Tyers. Undersized fish were collected as before and acclimated for up to a year in tanks. A further chronic stress was imposed on some fish: one month of cage confinement at either 14 or 32 kg m⁻³, unlike controls. Caged and control fish were subjected to the stimulus-response test after one air-exposure and their health assessment index (HAI), condition factor (K) and organosomatic indices for spleen (SSI), liver (HSI) and gonads (GSI) calculated. Gippsland Lakes fish had a significantly lesser proportion of fish with a response time of <1 min the stimulus-response test, a greater proportion of fish with enlarged spleens and a greater fork length, weights of whole and gutted fish, spleen, liver and gonads weights than Lake Tyers fish, but no significant difference in HAI, K, SSI, HSI or GSI or sex ratio. There was only one marginally significant criterion between caged and control fish: SSI. The differences in response time and SSI indicated that Gippsland Lakes fish might suffer a greater level of chronic stress than Lake Tyers fish, but inadequate replication made conclusions tentative.

In Chapter 4, interrenal hypertrophy and hyperplasia in the head kidney (as a previously used measure of chronic stress in fish) were compared between wild-caught black bream from the

Gippsland Lakes and Lake Tyers. The head kidney was fixed, processed and embedded in paraffin wax before being sectioned and examined microscopically. Hyperplasia but not cell or nuclear hypertrophy was found in fish from the Gippsland Lakes relative to those from Lake Tyers. Gippsland Lakes fish had a lesser condition factor than in Lake Tyers fish and green bile colour, suggesting lack of recent feeding, unlike the yellow bile colour of Lake Tyers fish. Taken together, this suggests greater chronic stress in Gippsland Lakes fish. This could be because of differences in environmental factors or genetically determined differences in black bream in responses to perceived stress.

In Chapter 5, the elemental composition of otoliths was compared between black bream from the Gippsland Lakes and Lake Tyers using EDXS (electron-dispersive X-ray spectroscopy) in an ESEM (environmental scanning electron microscope). Multivariate analysis of atom% for all elements and their ratios, however, clustered otoliths from both estuaries together, with a few outliers. Of the ten main elements found, there were no significant differences in atom% C, N, O, Na, P, S, K and Ca or their ratios, e.g. S:Ca. For Cl and Sr and the Cl:Ca and Sr:Ca, however, atom% was greater in otoliths from Gippsland Lakes fish than in those from Lake Tyers, as might be expected from Gippsland Lakes being permanently open to the ocean and having a more developed catchment, as Sr is a recognised marker for pollution. Thus differences in environmental factors could have contributed to differences in chronic stress.

In Chapter 5, the genetic composition of black bream was compared between the Gippsland Lakes and Lake Tyers using both microsatellite and mitochondrial primers used previously for *Acanthopagrus* species. DNA was extracted from muscle or fin clippings and amplified by PCR (polymerase chain reaction). ISSR (inter-simple sequence repeats) with six pairs of primers for microsatellites resulted in Gippsland Lakes and Lake Tyers fish being grouped separately by multivariate analysis, suggesting genetic differences. PCR-RFLP (PCR-

restriction fragment length polymorphism) of the mitochondrial D-loop with the endonuclease *DdeI* grouped 9/47 samples from Gippsland Lakes fish and 4/47 samples from Lake Tyers, suggesting that only they were maternally *A. butcheri*, while the others were maternally yellowfin bream (*A. australis*) or hybrids. Thus differences in genetics could have contributed to differences in chronic stress.

This study has had the following major outcomes:

1. the development of a short-term stimulus-response response test that predicted longer-term mortality
2. evidence for catch-and-release fishing rendering undersized black bream more likely to die and more susceptible to predation
3. evidence for greater chronic stress in Gippsland Lakes black bream than in Lake Tyers black bream that could be due to differences in both environment and genetics.

Thus it has contributed to the knowledge and understanding of black bream in the Gippsland Lakes and Lake Tyers estuaries and has provided evidence for the suitability of black bream as a valuable ecological indicator in estuaries.

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Chapter 1. General Introduction

1-1 An Integrative Approach

The environmental health of the Gippsland Lakes and their environs has been investigated by many studies (fisheries - Brown, 1976; Longmore, 1990; Coutin *et al.*, 1997; Fabris *et al.*, 1999; Coutin, 2000, Jenkins *et al.*, 2010, Roberts *et al.*, 2011; Williams *et al.*, 2012, 2013; water quality - Chessman and Marwood, 1988; EPA, 1974, 1983, 1986, 1990, 1993; Robinson, 1995; catchment effects – Aldrick *et al.*, 1992; Erskine *et al.*, 1990; Grayson *et al.*, 1994; possible climate change effects - Gillanders *et al.*, 2011, Booth *et al.*, 2011; climate change and black bream- Jenkins *et al.*, 2010, Gillanders *et al.*, 2011). However, a holistic overview of all such environmental impacts on the Gippsland Lakes is not available. A method with the potential to integrate the findings of many studies is the study of stress in key bioindicator species. By defining and identifying significant multiple stress factors impacting on a valued component from within the Gippsland Lakes ecosystem, summative effects could be quantified (Foran and Ferenc, 1999).

For the Gippsland Lakes, the fish black bream (*Acanthopagrus butcheri* Munro, Family Sparidae) offers many attributes of a good bioindicator as a valued ecological component (Suter *et al.*, 1999). With a natural life-span in excess of 29 years (Morison *et al.*, 1998), there is a life-long preference to inhabit its natal estuary (Chaplin *et al.*, 1998, Potter and Hyndes, 1999; Sarre *et al.*, 2000; Walther *et al.*, 2010; Williams *et al.*, 2012, 2013). in the Gippsland Lakes, black bream are exposed to commercial and recreational fishing; a changing estuarine environment supported and influenced by a much modified and similarly changing catchment and, unlike neighbouring estuaries, an artificial permanent opening to the sea. Furthermore, it has been predicted that climate change and catchment modification will

result in greater salinities and lesser stratification of water in the Gippsland Lakes and therefore will have a negative impact on black bream (Jenkins *et al.*, 2010; Williams 2013).

Indigenous to a naturally stressful estuarine environment, black bream should acclimate to such changes (Webb *et al.*, 2008); however, this fish is stress-sensitive to acute stressors such as handling and confinement associated with angling, resulting in a rapid inhibitory effect on gonadal steroidogenesis (Haddy and Pankhurst, 1999). Greater commercial prices paid historically for black bream caught in Lake Tyers, an adjoining estuary, when compared to their Gippsland Lakes counterparts because of their “superior condition” (Hall, 1984,) give an early indication of a possible difference in black bream growth between these two estuaries. Coutin (2000) similarly indicates that black bream caught in Lake Tyers were generally larger than the same-aged fish originating from the Gippsland Lakes. Gippsland Lakes black bream have displayed variable growth and episodic recruitment (Coutin *et al.*, 1997; Morison *et al.*, 1998; Williams *et al.* 2013). In fish, a reduced growth rate is a recognised indicator of an animal suffering chronic stress (Adams, 1990). In western Australian estuaries, there is evidence to indicate that growth-rates in juvenile black bream are significantly influenced by their surrounding environment rather than by any genetic differences (Partridge *et al.*, 2004). For Gippsland Lakes black bream, the relative influences of genetic versus environmental factors are unknown.

In order to quantify potential responses to stressors, a holistic or integrated approach is required that measures whole-animal performance, such as changes in resistance to disease, condition, behaviour, and ultimately survival (Wedemeyer and McLeay, 1981; Wedemeyer *et al.*, 1990; Barton *et al.*, 2002). In this study, genetic, histological and hard-part analyses were conducted along with performance tests to compare black bream sourced from within the two estuaries: Gippsland Lakes and Lake Tyers. These are adjacent estuarine systems sharing a

common fishery history (Coutin *et al.*, 1997; Coutin, 2000) but differing in catchments and degree of human impact, which may have resulted in different accumulated stress loads in the organisms of the respective ecosystems. This review therefore covers the literature on the two estuarine systems and their fisheries; black bream, the most common target of amateur and commercial fishermen, and measurement of stress, particularly in fish.

1-1-1 Two Adjacent Estuarine Systems: The Gippsland Lakes & Lake Tyers

History and Morphology

Both the Gippsland Lakes and Lake Tyers are located on the southeast coast of Australia, at an approximate latitude of 38°S (Figure 1-1a, b). The Gippsland Lakes are coastal lagoons formed by depositional barriers that sealed them off from the sea some 6,000-20,000 years ago (Bird, 1961, 1978, CSIRO, 2001). Lake Tyers was formed by the submergence of a coastal stream system after sea level rises some 8,000 years ago (Hall, 1984). Today, the entrances to the sea for both systems are less than 15 km apart.

Human involvement

For some 40,000 years, Aboriginal people have inhabited the Gippsland region (Synan, 1989). Land surrounding the Gippsland Lakes and Lake Tyers was territory to the Kurnai – Gunai tribes and these water bodies provided these peoples with a source of fish (Adams, 1981; Hall, 1984). Areas of the catchments to these estuarine systems, during this period, were also modified by fire (EGCMA, 2005). The late 1830s through to 1844 saw the first European contact with the region, leading to occupation of most of the fertile plains surrounding the Gippsland Lakes for grazing (Wakefield 1969; Adams, 1981; Synan, 1989; EGCMA, 2005). Today's distribution of settlement, use of natural resources, and pattern of removal of native forest in the catchments is the result of the occupation of this region by

European settlers (EGCMA, 2005). Much of the diverse catchment has been modified since the 1840s (Fabris *et al.*, 1999; EGCMA, 2005), and varies from brown coal open-cut mining for Victoria's electricity supply in the Latrobe Valley, to various forms of agriculture (e.g. market gardening, dairy and dry-land farming) (Fabris *et al.*, 1999). There is a commercial fishery based at the small town of Lakes Entrance (pop. 5,600), which is a popular tourist resort with 20,000 visitors per year, many of them keen anglers.

1-1-2 Gippland Lakes - the Artificial Entrance

In 1889, a permanent channel 115 m wide was opened at Lakes Entrance to connect the Gippsland Lakes to the sea (Bird, 1961). The transformation of the Gippsland Lakes from a seasonally closed estuary to one permanently open to the sea significantly altered the habitats and environmental factors operating in all regions of the lakes (Bird, 1978).

The Gippsland Lakes comprise Lakes Wellington, Victoria and King (Figure 1-1b) and have a combined area of approximately 366 km² (Bird, 1978; Fabris *et al.*, 1999). Fresh water enters this system via five major rivers: the Latrobe, Avon, Mitchell, Nicholson and Tambo Rivers, which are fed by a 20,600 km² catchment (Bird, 1978; Fabris *et al.*, 1999). In 1889, with the opening of the artificial channel at Lakes Entrance, a significant marine influence developed at the eastern end of the lakes system (Longmore, 1988).

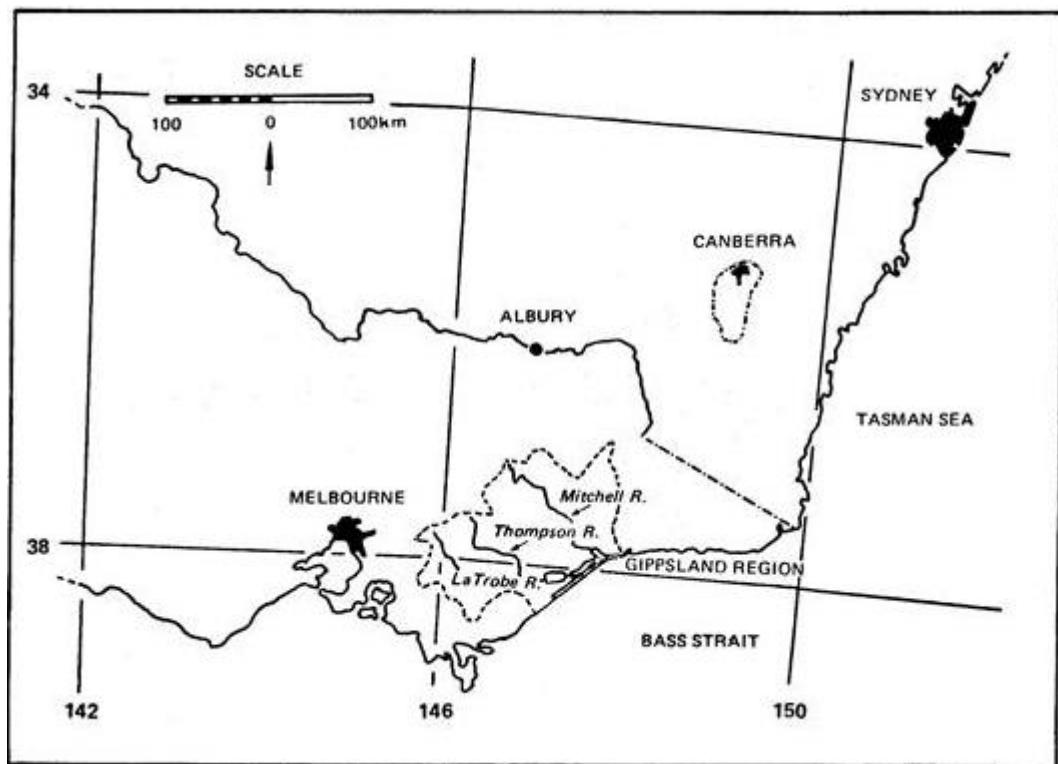


Figure 1-1a: Location of the Gippsland Region (Bek and Bruton, 1979).

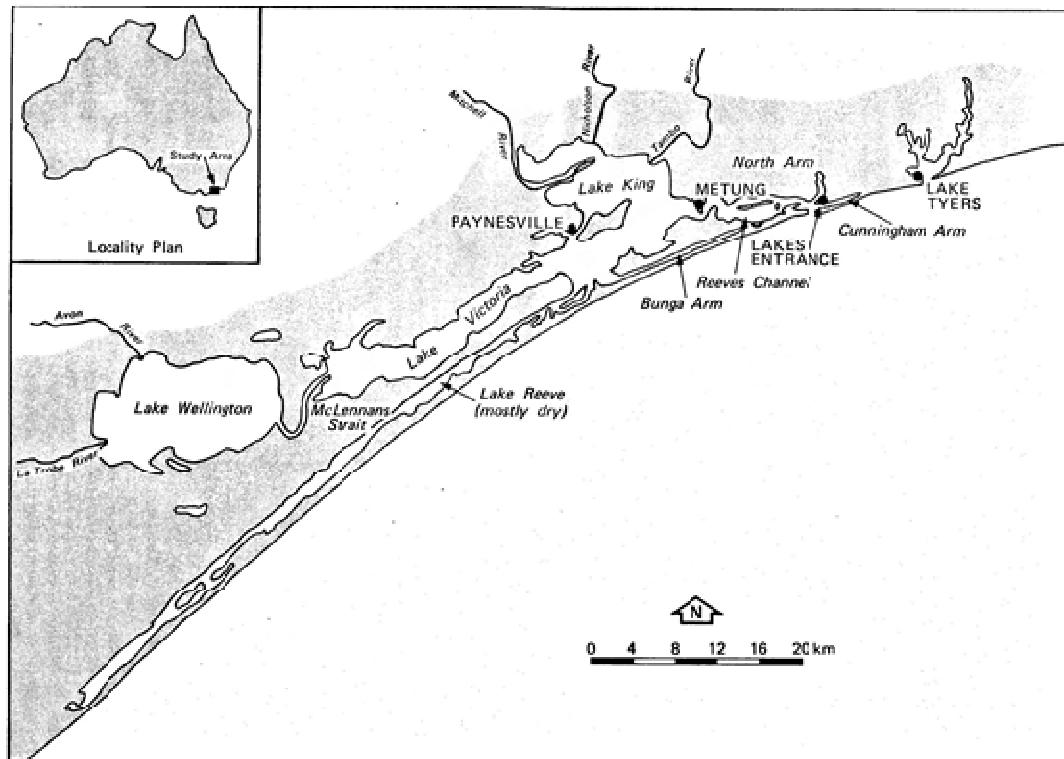


Figure 1-1b: Map of Gippsland Lakes System. (Hall and MacDonald, 1985)

Residence times of water in the Gippsland Lakes vary from a few days during storm flows to virtually no turnover during drought (Harris *et al.*, 1998). Salt-wedge intrusion into the westernmost end of the Gippsland Lakes (Lake Wellington) depends on the rate of evaporation relative to freshwater flows from catchments (Lester, 1983; Black, 1989; Black and Hatton, 1989; Longmore, 1989; Newell, 1991; Lake Wellington CSMPCWG, 1993). Significant seasonal vertical stratification in the water column influences temperature, salinity, and dissolved oxygen concentrations in both Lakes King and Victoria (Bek and Bruton, 1979; Longmore, 1988; Longmore *et al.*, 1990).

1-1-3 Lake Tyers

Lake Tyers, by contrast, is a small riverine estuary, consisting of a main lake and two major arms: the Toorloo and Nowa Nowa Arms, which receive fresh water from the Stoney and Boggy Creeks respectively (Figure 1-1b) (Hall, 1984; Hall and MacDonald, 1985b; MacDonald, 1997a). This estuary has an area of approximately 25 km² that is periodically closed to the sea by sand bars and therefore often experiences wide fluctuations in salinity, dissolved oxygen concentrations and temperature, along with turbidity, and growth of aquatic vegetation (Hall, 1984; NREC, 1991; MacDonald, 1997a).

Lake Tyers is supported by a separate 470 km² catchment (Hall, 1984; Hall and MacDonald, 1985b), comprising mostly state-managed eucalypt forest and native woodland. When compared with the neighbouring Gippsland Lakes catchment, the Lake Tyers catchment has only experienced minimal clearing (Hall, 1984; EGCMA, 2005). Some clearing near the entrance of the estuary has allowed for agricultural, residential and tourist development (Hall, 1984; MacDonald, 1997a), while within the catchment and around the main lake, some forest has also been cleared for pasture development to support cattle and sheep grazing (Hall, 1984).

Within the Lake Tyers catchment is the “Aboriginal Trust Estate” land, which ranges along the western shore of the Nowa Nowa Arm and along the northern shore of the main lake (MacDonald, 1997a). Over half of this land is still forest, with the rest mainly cleared for pasture (Hall, 1984). With about 80% of East Gippsland classified as public land and the Lake Tyers catchment being in this region, the catchment is little changed from its form of 200 years ago (EGCMA, 2005).

1-1-4 The Fishery: Gippsland Lakes and Lake Tyers

The Gippsland Lakes commercial fin fishery

In the Gippsland Lakes, organised commercial netting began in the 1870s (MacDonald, 1997b) and dramatically expanded in 1878 when fishermen such as William Carstairs, a major figure in the development of the commercial fishery, relocated along with others to the Gippsland Lakes from Queenscliff, Point Nepean, Geelong, Port Albert and Western Port. They relocated to take advantage of the opening of the railway from Oakleigh to Sale, which created a more reliable mode of transporting fish fit for human consumption to the Melbourne market (Synan, 1989). Prior to this, Chinese commercial fishermen, operating from Bancroft Bay (Metung), overcame the transportation problem by drying and smoking fish they caught (Synan, 1989).

The main target species were black bream (*Acanthopagrus butcheri*), yellow-eye mullet (*Aldrichetta forsteri*) and, at times, Australian salmon (*Arripis trutta*) and garfish (*Hyporhamphus regularis*) (MacDonald, 1997b). Since 1914, uninterrupted commercial catch data has been collected from the Gippsland Lakes. In 1976, to conserve fish stocks, the Gippsland Lakes fin fishery became a limited entry fishery (MacDonald, 1997b). The two

main commercial methods have always been mesh (gill) netting and haul seining (MacDonald, 1997b).

Lake Tyers commercial fin fishery

Lake Tyers has been fished almost exclusively by fishermen based in the Gippsland Lakes, the fishery being developed at the same time as an adjunct to the larger Gippsland Lakes fishery (Hall, 1984). Due to the fluctuating nature of the catches, commercial fishing in Lake Tyers has always been limited, and many professionals opted for the larger numbers and more reliable catches of the Gippsland Lakes (Hall, 1984).

During the early 1970s, a closed season to commercial fishing was imposed by the State Government, and access to the estuary only allowed for a 5-month period from just after Easter to early September (autumn- spring) (MacDonald, 1997a). Prior to 1977, a commercial licence for the Gippsland Lakes allowed access to Lake Tyers, but after this time a special endorsement was required (Hall, 1984). The main fishing methods used were mesh (gill) netting and two varieties of haul seining: the estuary seine (for bream and other bottom-dwelling fish) and the garfish seine for surface-schooling garfish (MacDonald, 1997a). In Lake Tyers, black bream has always been the main target species, although luderick (*Girella tricuspidata*) were at times caught in large numbers. Other species caught were river garfish (*Hyporhamphus regularis*), yellow-eye mullet, sea mullet (*Mugil cephalus*), tailor (*Pomatomus saltator*) and silver trevally (*Pseudocaranx dentex*) (MacDonald, 1997a).

Of historical interest, and relevant to this study, was the general recognition in the industry that black bream sourced from Lake Tyers commanded a greater market price (up to 33%) than black bream caught from the Gippsland Lakes because of their superior condition

(healthy appearance) (Hall, 1984). In 2003, however, to fulfil an election promise, the Victorian State Government permanently closed Lake Tyers to commercial fishing.

The Gippsland Lakes recreational fin fishery

Recreational fishing in the Gippsland Lakes has occurred for over a century by rod and line or handline (MacDonald, 1997b). Fishery management has been through licence, bag and size limits, and gear restrictions (MacDonald, 1997b). Black bream are the most sought-after species by recreational anglers in the Gippsland Lakes (MacDonald, 1997b).

Lake Tyers recreational fin fishery

For over 100 years, recreational fishing has occurred in Lake Tyers, and this has been almost exclusively by rod and line from either a boat or the shore (MacDonald, 1997a). The most sought-after species are black bream and river garfish, with yellow-eye mullet, luderick, Australian salmon, dusky flathead, tailor, trevally, snapper and King George whiting also caught (MacDonald, 1997a). Management of the fishery has been through licence, size and bag limits, and gear restrictions (MacDonald, 1997a).

1-2 Black Bream and its Biology

1-2-1 Australian *Acanthopagrus* Species

Black bream (*Acanthopagrus butcheri* (Munro), Sparidae), previously known as *Mylio butcheri*, (Munro) after Munro's 1949 classification of "Australian silver bream," are endemic to Australian waters (Figure 1-2, Table 1-1) (Weng, 1971, Norriss *et al.*, 2002). Black bream share Australian waters with six closely related species, including five of the same genus and one belonging to the genus *Rhabdosargus* (Norriss *et al.*, 2002). Table 1-2 lists the species closely related to *A. butcheri* along with their common names, distributions and differentiating characters.



Figure 1-2: Black bream (*Acanthopagrus butcheri*, Family Sparidae).

Table 1-1: Black bream Taxonomic Details

Valid name: *Acanthopagrus butcheri* (Munro, 1949)

Synonymy: *Mylo australis* (Gunther, 1859 [as cited by Munro (1940)])
Mylo butcheri (Munro, 1949)

Common names: Black bream, Perth bream, grunter, southern black bream, Gippsland bream, southern yellowfin bream, blue-nosed bream.

Classification:

Taxon	Scientific name	Common name
Class:	Teleostomi	
Subclass:	Actinopterygii (now a Class - http://www.fishesofaustralia.net.au/home/species/674#classification)	Ray-finned fishes
Order:	Perciformes	Perches and allies
Suborder:	Percoidei	
Family:	Sparidae	Breams
Genus:	<i>Acanthopagrus</i>	
Species:	<i>butcheri</i>	Black bream and others

Description:

Acanthopagrus butcheri is distinguished from *A. australis* (yellowfin bream) by the dark brown colour of the body and lack of canary yellow colouration on anal and ventral fins (they are usually brown-grey). Fin ray counts are homogenous throughout its range and *A. butcheri* cannot be distinguished from other *Acanthopagrus* spp. on this basis.

Modal formula:- D. XI + 12 ; A. 111 + 9 ; V. 1 + 5 ; P. 15.

There are some slight differences in head proportions and lengths of anal fin spines and rays between *A. butcheri* and *A. australis*, as detailed below.

Scales: Lateral line scales 50-62 with mean and mode of 55 (187 specimens), diagnostic as mode greater than in *A. australis* (52) and other *Acanthopagrus* spp. (50).

Five scale rows above lateral line; 13-17 scale rows below lateral line with mean of 15-16, diagnostic as all other *Acanthopagrus* spp. have 13 or fewer rows.

Scales differ from those of *A. australis* in having a wider ctenoid margin containing an additional series of spines.

The double bifurcation of the lateral line tubules distinguishes *A. butcheri* from species other than *A. australis*.

Gill-rakers modal formula 7 + 9 = 16. *A. australis* is identical.

Source: Norriss *et al.* (2002).

Table 1-2: Species closely related to *Acanthopagrus butcheri* in Australian waters. (Source: Norriss *et al.*, 2002)

Scientific name	Common names	Distribution
<i>Acanthopagrus australis</i> (Gunther)	Yellow fin bream, eastern black bream, black bream, silver bream, blue-nose bream	Distributed from Queensland (Townsville) to Victoria (Mallacoota) ¹ .
<i>Acanthopagrus latus</i> (Houttuyn)	Western yellowfin bream	Found in western Australia (Shark Bay northwards).
<i>Acanthopagrus berda</i> (Forskal)	Pikey bream, bream, black bream	Distributed from Western Australia (Exmouth Gulf) around northern Australia to central Queensland. Commonly found in tropical mangrove estuaries.
<i>Acanthopagrus palmaris</i> (Whitley)	North west black bream	Found in Western Australia (Shark Bay northward).
<i>Rhabdosargus sarba</i> (Forskal)	Tarwhine, silver bream	Commonly found in coastal waters of eastern and western Australia. On the east coast, from Queensland to Victoria (Gippsland Lakes). In western Australia, from Albany to Coral Bay.

¹*Acanthopagrus australis* also found in Gippsland Lakes (Roberts *et al.*, 2011).

1-2-2 Geographical Distribution

Black bream is found in estuaries and river mouths, from Myall Lakes in New South Wales, around the southern part of the Australian Continent to the Murchison River in Western Australia (Rowland, 1984; Norriss *et al.*, 2002). The lack of suitable habitat across the Great Australian Bight makes black bream rare in this region of Australia (Kailola *et al.*, 1993). It is also found in Tasmanian rivers and islands off the South Australian coast (Norriss *et al.*, 2002).

1-2-3 Coastal Migration and Genetic Studies

In Victorian coastal waters, coastal migration is not common, as tagging studies have shown only 6/149 fish relocations to other estuaries greater than 50 km apart since 1994 (Burridge *et al.*, 2004). Also, in Western Australia, captive-bred black bream that were tagged and released into the Swan Estuary had no emigration from the estuary after 31 months (Norriss *et al.*, 2002).

Recently, fish otoliths, which are paired hard structures found within the inner ear of teleosts and assist in balance and hearing, are being utilized to describe fish movement and their environmental history. For decades otoliths have been used to supply data for the determination of annual age and growth in order to calculate the demographic rates of populations in single-species management strategies (see Growth and Recruitment below) (Sponaugle, 2010). Identification of fish movement and history has come about through the development of techniques to analyse the microstructure of otoliths, which are composed of biogenic calcium carbonate, typically in the form of aragonite, deposited on a protein matrix (Campana, 1999; Popper & Lu, 2000; Campana & Thorrold, 2001; Elsdon *et al.*, 2008; Webb *et al.*, 2012). Otoliths daily accrete new crystalline and protein material from the surrounding

endolymph onto their exterior surface (Campana 1999). For black bream, this daily accretion is ca. 5 µm (Elsdon and Gillanders, 2006). Within these accreted layers are the major constituents of aragonite: carbon, oxygen and calcium; also present are minor and trace elements that reflect the environment to which fish were exposed and provide a chemical chronology of a fish's life, which consequently can then be used to determine its history and movements (Campana and Neilson 1985; Campana 1999; Elsdon *et al.*, 2008; Webb *et al.*, 2012). In a temporal study of wild black bream sourced from two South Australian estuaries, Elsdon and Gillanders (2006) found that summer versus winter fish movements could be identified by comparing otolith strontium to calcium concentration ratios (Sr : Ca) with those values found in the surrounding water at specific sampling sites. A more detailed technical and historical chronology of otolith microstructure development can be found in Campana (1999) and Elsdon *et al.* (2008); with recent examples of this development in Walther & Limburg (2012) and Webb *et al.* (2012).

In Western Australia, the older technique of allozyme studies indicated that black bream in differing estuaries are reproductively isolated (Chaplin *et al.*, 1998) and confined to individual estuaries (Potter and Hyndes, 1999). Similarly, black bream populations in Western Australia are isolated from those of south-eastern Australia (Burridge *et al.*, 2004). By contrast, an allozyme study undertaken by Farrington *et al.* (2000) found genetic homogeneity in black bream from differing estuaries in south-eastern Australia. Burridge *et al.* (2004), incorporating both a wider geographic sampling range and data from mitochondrial DNA as well as from allozymes, concluded that low levels of gene flow exist in black bream of south-eastern Australia, but are essentially restricted to adjacent estuaries and consistent with a one-dimensional stepping-stone model. Using more modern DNA analysis by microsatellites, Roberts *et al.* (2011) showed that hybridization between marine yellowfin bream (*Acanthopagrus australis*) and estuarine black bream (*Acanthopagrus*

butcheri) has occurred in south-eastern Australia. In the Gippsland Lakes, the presence of introgressed fish indicated that hybrid swarms had persisted for a considerable time, with recent samples producing genetic diversity of 8.2 to 9.2 alleles per locus and expected heterozygosity (H_e) ranging from 0.66 to 0.70, though producing little allele differentiation ($F_{ST} = 0.003$) when compared across sampling times of 69, 67, 14 and 10 years (Roberts *et al.*, 2011).

1-2-4 Black Bream Habitat

Black bream is essentially a demersal estuarine species (MacDonald, 1997b; Sarre and Potter, 1999) that prefers to remain in its natal estuary throughout its life cycle (Sarre and Potter, 1999). But lifecycle strategies within each estuary can vary, depending on the estuary's individual hydrological conditions and on physiological aspects of the fish such as the status of the mouth (Lenanton and Hodgkin, 1985; Norriss *et al.*, 2002) and eye development (Shand *et al.*, 2000 a,b). Larger juveniles and adults tolerate a wide salinity range from seawater to almost freshwater (Starling 1988; Potter *et al.*, 1996). In Victoria, the Gippsland Lakes system (Figure 1-3) offers the largest area of suitable habitat and environmental conditions for black bream (MacDonald, 1997b). Within this system, distribution of this fish is closely related to seasonal fluctuations in amount of freshwater inflow and the physical characteristics of the estuarine system (Coutin *et al.*, 1997). For example, when there is little movement of fresh water through the catchments, resulting in low river flows and high salinity levels, black bream are found more extensively throughout the lakes – at least 10 km upstream in the Mitchell, Tambo and Nicholson Rivers, which adjoin Lake King in the eastern regions of this system (Coutin *et al.*, 1997) and a similar distance upstream in the Latrobe River adjoining Lake Wellington in the western catchment during years of drought (pers. obs.) (Figure 1-2). Alternatively, during periods of flooding resulting from high rainfall

in the catchments, black bream are found around Reeves Channel and Cunningham Arm (Coutin *et al.*, 1997) at the extreme eastern end of the system, and are located near the permanent entrance to the sea (Figure 1-2).

In these favourable regions, black bream are found at locations that provide shelter and places where they can hide, namely rocky river beds, snags or artificial structures such as jetties (Hobday and Moran, 1983; MacDonald, 1997b; Norriss *et al.*, 2002). Juvenile fish are often found in seagrass beds (Rigby, 1982, 1984; Gunthorpe, 1997), using the grass as shelter, and also as a location where there is a diverse and abundant supply of small prey (Rigby, 1982). Gunthorpe (1997) summarizes the habitat requirements for black bream at each of its life stages as being: spawning – pelagic; gametes – pelagic; larvae – pelagic; juvenile – seagrass, reeds; adult – sediment, fringing vegetation and wetlands.

1-2-5 Feeding

Black bream are opportunistic feeders (Day, 1981; Rigby, 1984; Coutin *et al.*, 1997; Gunthrope, 1997; Sarre *et al.*, 2000; Shand *et al.*, 2000a; Norriss *et al.*, 2002; Partridge *et al.*, 2004), with diets depending on the biota of individual estuarine systems (Sarre, *et al.*, 2000, Sarre and Potter, 2000). Larval and post-larval black bream (up to approximately 40 mm standard length (SL) are recognised pelagic feeders of zooplankton (Rigby, 1982; Ramm, 1986; Rigby, 1984; Willis, 1991; Newton, 1996; Gunthorpe, 1997; Sarre and Potter 1999). They typically consume a wide range of (generally) animal species, although Sarre *et al.* (2000) indicated that in the Western Australian Nornalup/Walpole Estuary, black bream consumed significant volumes of the seagrass *Ruppia megacarpa*. Such diverse feeding is physiologically supported by the occurrence of both incisor and molar teeth (Sarre *et al.*, 2000).

Rigby (1982, 1984) found that for black bream in the Gippsland Lakes, feeding behaviour and diets differed between small and large fish. Small black bream (<150 mm TL) fed predominantly on polychaetes, with secondary components of their diets being bivalves and amphipods. In large bream (>150 mm TL), bivalves were a common food source, along with more mobile prey such as fish, crabs and shrimps. Kailola *et al.* (1993) stated that bivalve and gastropod molluscs, crabs, polychaete worms and small fish are significant components of their diet.

Ontogenetic changes in diet were observed in black bream from Western Australian estuaries (Sarre *et al.*, 2000; Norriss *et al.*, 2002). With increase in body size, black bream in estuaries on the lower west coast (e.g. Swan River and Moore River estuaries) consumed decreasing proportions of small soft-bodied and/or thin-shelled prey such as amphipods, small bivalve molluscs and polychaetes and consumed increasing proportions of larger prey such as the bivalve *Xenostrobus securis*, decapods and teleosts (Sarre *et al.*, 2000). Black bream in estuaries on the south coast (Nornalup/Walpole and Wellstead estuaries) displayed an abrupt change in diet when fish attained ca 200 mm (Sarre *et al.*, 2000). For example, in the Wellstead Estuary, fish <200 mm TL fed predominantly on amphipods and to a lesser extent the macroalga *Cladophora* sp. Fish >200 mm TL ingested mainly other macrophytes and in particular the macroalga *Chaetomorpha* sp. Such changes in diet spread food resources amongst different-sized black bream in each of these estuaries (Sarre *et al.*, 2000).

A shift from pelagic to benthic feeding has also been observed in hatchery-reared small black bream (50-80 mm S.L.), which correlated with a change in eye physiology (Shand *et al.*, 2000 a, b). The timing of these changes is variable and is possibly an adaptive feature that allows continued opportunistic feeding from different regions of the water column in often environmentally unstable estuaries (Shand *et al.*, 2000a, b). Observations by Shand *et al.*

(2000 a, b) suggest the fish length at which such feeding strategies are activated varies from 15 – 80 mm S.L.

1-2-6 Growth and Recruitment

Otolith (ear stone) analysis has identified black bream originating from the Gippsland Lakes as being a long-lived, slow-growing fish, with a maximum recorded age of 29 years (Morison *et al.*, 1998; Norriss *et al.*, 2002). Morison *et al.* (1998) considered sectioned otoliths more accurate than other methods used to estimate the age of black bream in the Gippsland Lakes, after validating annual increments from two-year classes over a four-year period. For example, Morison *et al.* (1998) found that scale annuli from other studies of the same population under-estimated age, and von Bertalanffy growth functions consistently over-estimated the size-at-age of younger fish and under-estimated the age-at-size for older black bream. Sarre and Potter (2000) subsequently validated the accuracy of the otolith method when determining the age of black bream in Western Australian studies (where black bream had a maximum age of 21 years).

In Western Australia, black bream growth rate varied between estuaries with different environmental conditions (Figure 1-3a). Fish also ingested different types of food in each of these estuaries (Sarre and Potter, 2000; Sarre *et al.*, 2000). In a separate Western Australian study, Partridge *et al.* (2004) found that growth of juvenile black bream was significantly influenced by quantity and quality of food available, density of black bream and salinity of their environment, rather than by any genetic differences.

In Victoria, Coutin *et al.* (1997) and Coutin (2000) found that black bream originating from Lake Tyers had a growth rate different from those originating from the Gippsland Lakes (see Figure 1-4b) because fish from Lake Tyers were generally larger than Gippsland Lakes black

bream of the same age (as estimated from otoliths). Each of these estuarine systems, however, displayed considerable variability in growth of individual fish of the same age (Coutin *et al.*, 1997). Morison *et al.* (1998) found that the growth rate of Gippsland Lakes bream was similar to that of fish obtained from the Moore River Estuary, but less than that for those in the Swan Estuary in Western Australia. MacDonald (1997b) also showed that the growth rate of black bream was less in Gippsland Lakes fish than at other locations.

Norriß *et al.* (2002) summarized key features associated with black bream recruitment, these being:

1. Temporal variation in recruitment
2. Fluctuation in recruitment of each year class within each estuary/lake /river system.
3. Very little recruitment between systems.

In Victorian estuaries, Jenkins *et al.* (2010) found that recruitment was highly variable and episodic, being significantly influenced by freshwater flow and salinity structure, but uniquely controlled in each estuary by characteristics of the catchment, channel topography and entrance opening and closing.

In the Gippsland Lakes, Hobday and Moran (1983) found significant variation in year class abundance, with dominant classes resulting from spawning during relatively dry springs and above average water temperatures, whereas weak classes coincided with high spring river flows and below average water temperatures. Morison *et al.* (1998) agreed that annual recruitment was highly variable, episodic, and found that both the commercial and recreational fisheries in the Gippsland Lakes in 1993-1996 were dependent on black bream

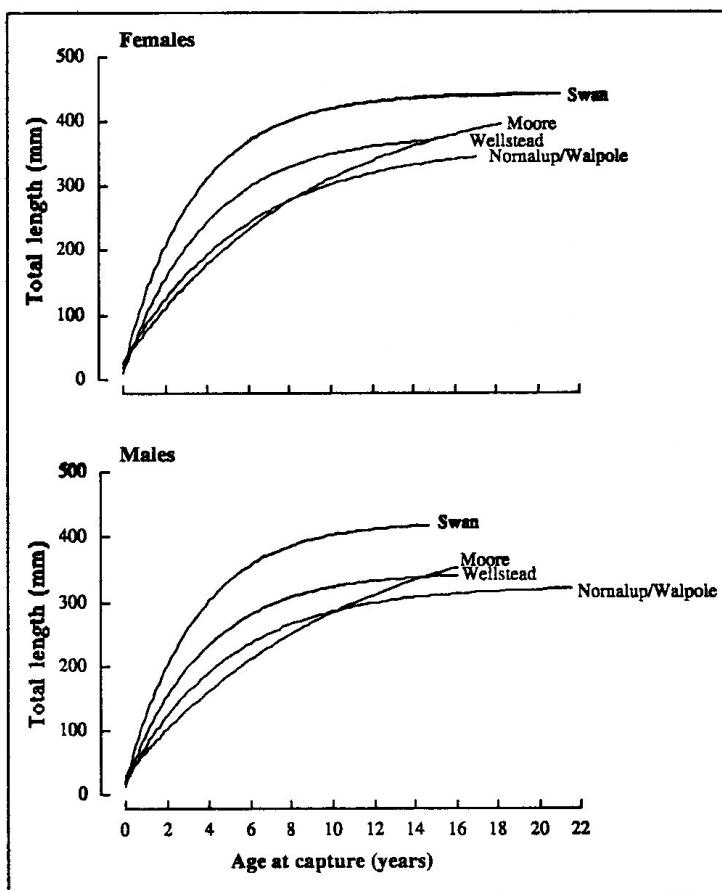


Figure 1-3(a): Growth rates of black bream from various estuaries in Western Australian estuaries (Source: Jenkins *et al.*, 1999).

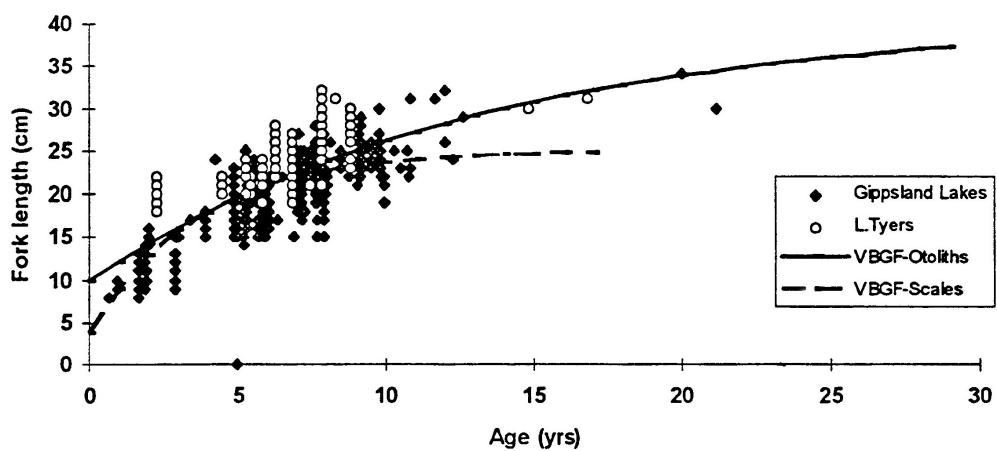


Figure 1-3(b): Age/length and growth characteristics of black bream from Lake Tyers and the Gippsland Lakes (Coutin *et al.*, 1997).

originating from only two spawning seasons, in 1987 and 1989. Within each year class, Morison *et al.* (1998) also observed inconsistent growth, which was reflected in the 3–13 years age-range for fish meeting the legal-minimum-length of 23.5 cm fork length (F.L.) requirement for both fisheries at the time of their study. In an attempt to predict year class strength, Walker *et al.* (1998) developed an environment-recruitment model based on water temperature and river flow along with age structure data obtained from previous studies in the Gippsland Lakes. Jenkins *et al.* (2010) similarly found that recruitment was episodic and the population dominated by a few year classes, with greatest recruitment occurring when the main tributary rivers had intermediate flows (greater than approximately 3000 Ml d⁻¹). When comparing estuaries, Jenkins *et al.* (2010) suggested that variability in recruitment was a consequence of fresh water inflows and therefore salinity structure determined by the characteristics of individual catchments, channel topography and entrance opening and closing. When comparing two spawning seasons, Williams *et al.* (2012) also identified the importance of freshwater flows for spawning activity and concentrations of larvae; more importantly, these researchers found that only locations with a halocline functioned successfully as larval nursery habitats, despite black bream spawning in many locations.

1-2-7 Reproduction

Sex inversion

In the Family Sparidae, modes of reproduction are diverse (Norriiss *et al.*, 2002), with features such as protogynous, protandrous, simultaneous and rudimentary hermaphroditism being reported (Buxton and Garrot 1990; Norriiss *et al.*, 2002). For black bream, conflicting reports also exist on sex change behaviour (Norriiss *et al.*, 2002). Sex inversion has been observed in black bream populations inhabiting estuaries of south-eastern Australia (Rowland and Snape, 1994; Haddy and Pankhurst, 1998; Norriiss *et al.*, 2002), but not those inhabiting estuaries of

south-western Australia (Sarre, 1999). Norris *et al.* (2002) indicated that ovotestes had been identified in black bream from the Gippsland Lakes.

From observations made in Hoyers Lake in New South Wales, Rowland and Snape (1994) have proposed that protogynous hermaphroditism is labile in black bream, this condition being induced by selective pressures, either individually or in a cumulative fashion, from environmental conditions and intense fishing pressure (Rowland and Snape, 1994; Norris *et al.*, 2002). By contrast, Sarre (1999) found no evidence of such sex change in the Western Australian black bream populations sourced from several estuaries, although many of those Western Australian estuaries were also exposed to variable environmental conditions and heavy fishing pressure (Sarre and Potter, 2000).

Size at first sexual maturity

In the Gippsland Lakes, Scott *et al.* (1974) reported that male black bream spawned for the first time at around 3 years old, 12 months before females. Coutin *et al.* (1997) calculated that female black bream attained a total length of 13 cm at first maturity, with all females spawning at a total length of 25 cm. In Western Australia, size and age at first maturity varied markedly in different estuaries (Sarre and Potter, 2000; Partridge *et al.*, 2004). For example, in the Swan River Estuary, sexual maturity was attained at the end of the second year (26.5 cm total length (T.L.), but not until the end of the fourth year in the Moore River Estuary at 14.5 cm T.L.) (Partridge *et al.*, 2004). This difference was related to relative growth rate, as black bream at the end of their second year attained a total length of ca. 26.5 cm T.L. in the Swan River Estuary, while those in the Moore River Estuary were only ca. 14.5 cm T.L. (Partridge *et al.*, 2004). At 30 cm T.L., all male and female black bream in the Swan River Estuary had fully matured (Norriess *et al.*, 2002).

Fecundity

Black bream is a multiple spawner and has a high fecundity, with females having the potential to release 3×10^5 and 3×10^6 eggs in a season (Butcher, 1945; Weng, 1971; Cadwaller and Backhouse, 1983; Kialola *et al.*, 1993; Potter *et al.*, 1996; Jenkins *et al.*, 1999; Sarre and Potter, 1999; Smith *et al.*, 2009). Coutin *et al.* (1997) indicated that fecundity increased with fish length, and under normal summer conditions in the Gippsland Lakes, the small pelagic eggs with a diameter of 0.762 mm hatched in 1-2 days following spawning (Butcher, 1945). In aquaculture, Jenkins *et al.* (1999) indicated that a seasonal fecundity of 3.8×10^3 eggs g⁻¹ (from an average fish weight of 1.8 kg) was achieved. Each egg has a diameter of 0.7-0.8 mm and contains a single spherical oil globule of 0.20 - 0.25 mm (Figure 1-4). In aquaculture conditions at 26°C, hatching is approximately 20 hours after spawning (Jenkins *et al.*, 1999).

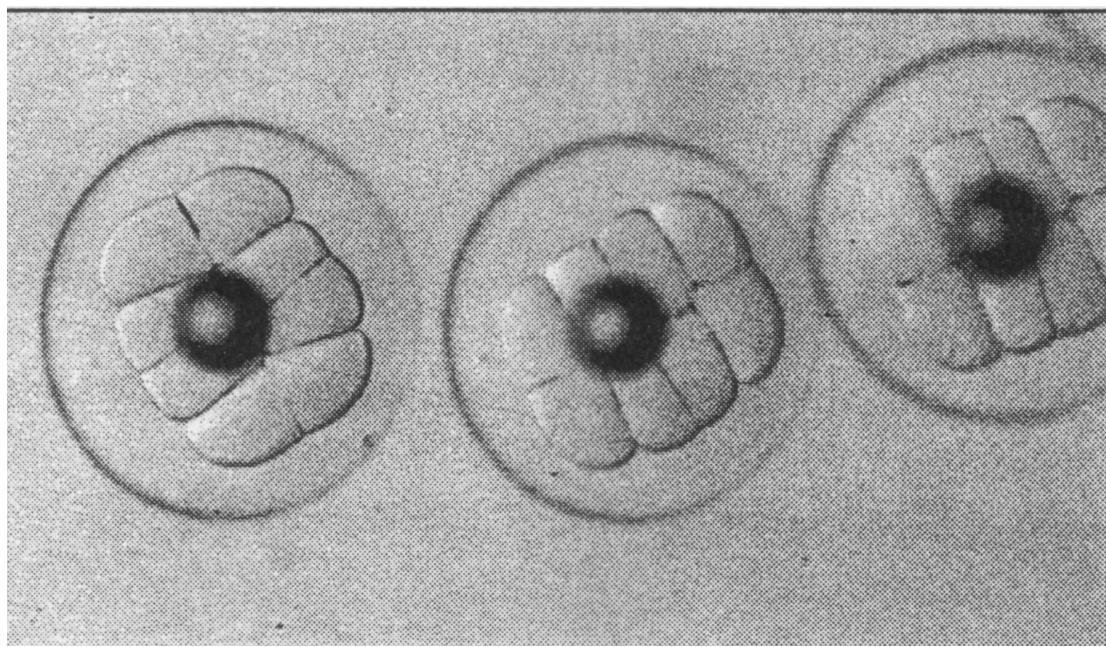


Figure 1-4: Black bream eggs ranging in size from 700 to 800 µm containing an oil globule with a size range of 200-250 µm. (Source: Jenkins *et al.*, 1999).

Spawning

In sparids, reproductive development is fundamentally the same between euryhaline and stenohaline species. Each has an annual reproductive cycle displaying asynchronous gonad development along with a daily spawning pattern occurring over a 2-5 month period (Haddy and Pankhurst, 1998). The pattern and duration of reproductive development in black bream is consistent with those of other *Acanthopagrus* species (Abu-Hakima, 1984; Pollock, 1985; Chang and Yueh, 1990; Haddy and Pankhurst, 1998).

On either side of The Great Australian Bight, black bream are reproductively active over a significant range of environmental conditions (Haddy, and Pankhurst, 1998; Sarre and Potter, 2000; Kanandjembo *et al.*, 2001; Young and Potter, 2002). In the Gippsland Lakes, the gonadosomatic index (GSI: gonad weight as percentage of body weight) for black bream was greatest in October when analysed from 1993-1996 (Figure 1-5) (Coutin *et al.*, 1997). Other studies have also reported greatest proportions of fish with mature gonads in October and November (Butcher, 1945; Gorman, 1962; Hobday and Moran, 1983), though Cadwallader and Backhouse (1983) and Kailola *et al.* (1993) reported the spawning season to be from August to January (midwinter – early summer). For black bream in the Gippsland Lakes, water characteristics such as salinity and temperature, along with the availability of food, are critical for successful spawning (Longmore *et al.*, 1990; MacDonald, 1997b).

The timing of spawning, and its location in the Gippsland Lakes, varies on a seasonal basis and is correlated with fluvial discharge (Norriess *et al.*, 2002; Jenkins *et al.*, 2010; Williams *et al.*, 2012). For example, Butcher (1945) indicated that there was no preference for breeding site, and that spawning locations varied with fluctuating salinity - the optimum salinity range being 11- 18 ppt (parts per thousand). Longmore *et al.* (1990) reported black bream spawning

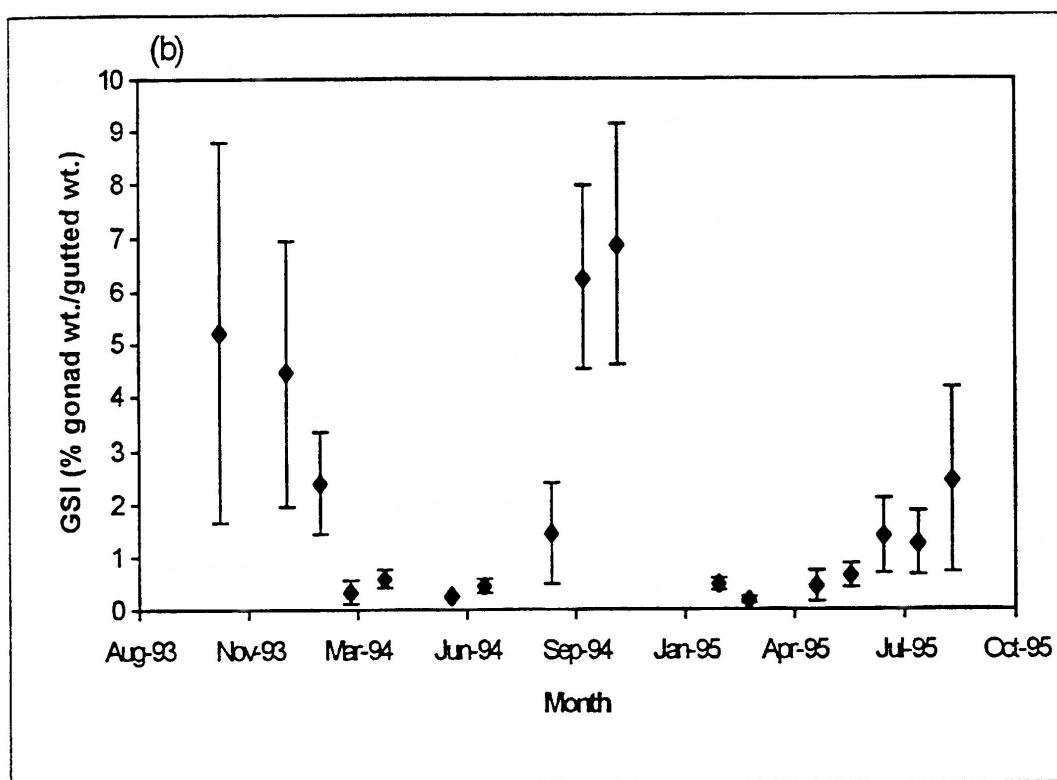
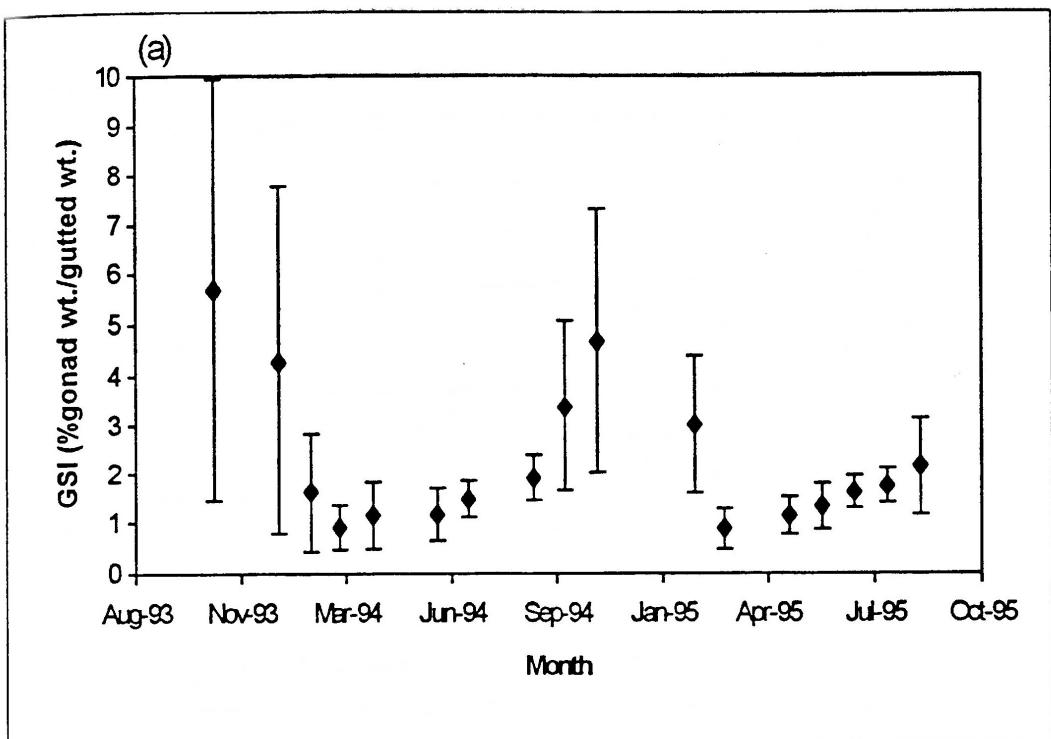


Figure 1-5: Monthly variations in gonadosomatic indices (GSI) in (a) female and (b) male black bream from the Gippsland Lakes (Coutin *et al.*, 1997)

at locations with an optimal salinity of 19-21 ppt and a water temperature of 21°C, which links the importance of water temperature in the Gippsland Lakes to the onset of spawning. Temperature is also recognised as being influential in the onset of spawning in other sparids (e.g. Kojima, 1981; Scott and Pankhurst, 1992).

For spawning locations east of the Gippsland Lakes, such as Lake Tyers (Figure1-2), spawning is believed to occur earlier, and for those systems west, later than in the Gippsland Lakes (Norriß *et al.*, 2002).

1-2-8 Mortality

A stressed fish population experiences a greater mortality rate than an unstressed fish cohort (Webb *et al.*, 2005). In a polluted environment, where contaminants induce metabolic alterations, and those alterations exceed a "scope for activity" then metabolic alterations above and below narrow limits of their scope result in mortality (Priede, 1985; Webb *et al.*, 2005). Furthermore, fish that have to cope with chronic, low-level contaminants in many environments also have to deal with elevated levels of nutrients causing algal blooms (Webb *et al.*, 2005) and harassment associated with bird predation (Reside and Coutin, 2001; Norriß *et al.*, 2002). Barton *et al.* (1986) indicated that multiple stressors evoked a cumulative stress response. In the Swan-Canning Estuary in Western Australia, black bream have to deal with elevated levels of nutrients causing algal blooms (Webb *et al.*, 2005), which are frequent in summer and autumn months and have caused massive fish mortalities (Webb *et al.*, 2005).

Bird predation is also important as a natural stressor. Black bream in the barred river systems of the Culham Estuary suffer high mortality from predatory birds during their upstream spawning run (Norriß *et al.*, 2002). Similarly, in the Gippsland Lakes, depending on seasonal fluctuations in bird numbers, predation of black bream by Great Cormorants (*Phalacrocorax*

carbo carbooides) in certain years has probably equalled or exceeded the combined commercial and recreational catches (Coutin, 2000; Reside and Coutin, 2001). With a 29-year natural life span in the Gippsland Lakes, adult black bream should have a low rate of natural mortality (Morison *et al.*, 1998). However, in those years when high predatory bird numbers are large in the Gippsland Lakes, high levels of mortality would be expected (Reside and Coutin, 2001).

1-3 Stress

1-3-1 Historical Setting

The concept of stress was understood by Hippocrates (ca 460 – 377 BC) (Reite, 1985; Johnson *et al.*, 1992; Barton, 1997). It was not until the seminal work by Cannon (1929) on the fight/flight response, and the subsequent description of homeostasis by Cannon (1935), along with observations made in medical research by Hans Selye, that the first encompassing theory of stress, the General Adaptation Syndrome (GAS) (Selye, 1936, 1950, 1973; Johnson *et al.*, 1992; Barton, 1997; Pickering, 1998) was formulated. Selye became aware of the non-specificity and similarity of physiological and endocrinological responses in experimental animals irrespective of the stimulus, and it was these observations that were the core of the GAS (Barton, 1997; Pickering, 1998). Brett (1958) extended Selye's definition into a fisheries context and Mazeaud *et al.* (1977) extended the GAS concept from responses of the individual organism to those of the population.

Attempts have continued to develop an integrated theory of stress. For example, Moberg (1985) offered an alternative to the GAS in which a stimulus is perceived by the central nervous system, which then responds by a stress response, resulting in a pre-pathological

condition followed by a pathological condition if the organism cannot cope with the change in biological function.

Despite its shortcomings, the GAS has been beneficial in stimulating researchers to investigate biological stress in a systematic way (Pickering, 1998). Also, the GAS paradigm is still utilised as a useful working conceptual framework (Barton, 1997). For example, Schreck (2000), when discussing the cumulative and long-term effects of stress in fish, used the GAS as a starting point. Roberts and Rodger (2001), when introducing and describing the “pathophysiology and systematic pathology of teleosts”, identified the significance of stress factors by describing them in the context of the GAS. Similarly, Barton *et al.* (2002), when describing the responses associated with the intensity of a stressor (see definition below), also indicated that they are consistent with the GAS.

1-3-4 General Adaptation Syndrome (GAS)

Over several decades Hans Selye observed non-specificity and similarity of responses regardless of disturbance and proposed the first major encompassing theory of stress - the three stage General Adaptation Syndrome (GAS) (Selye, 1936, 1950, 1973; Barton, 1997; Roberts and Rodger, 2001) namely (Figure 1-7):

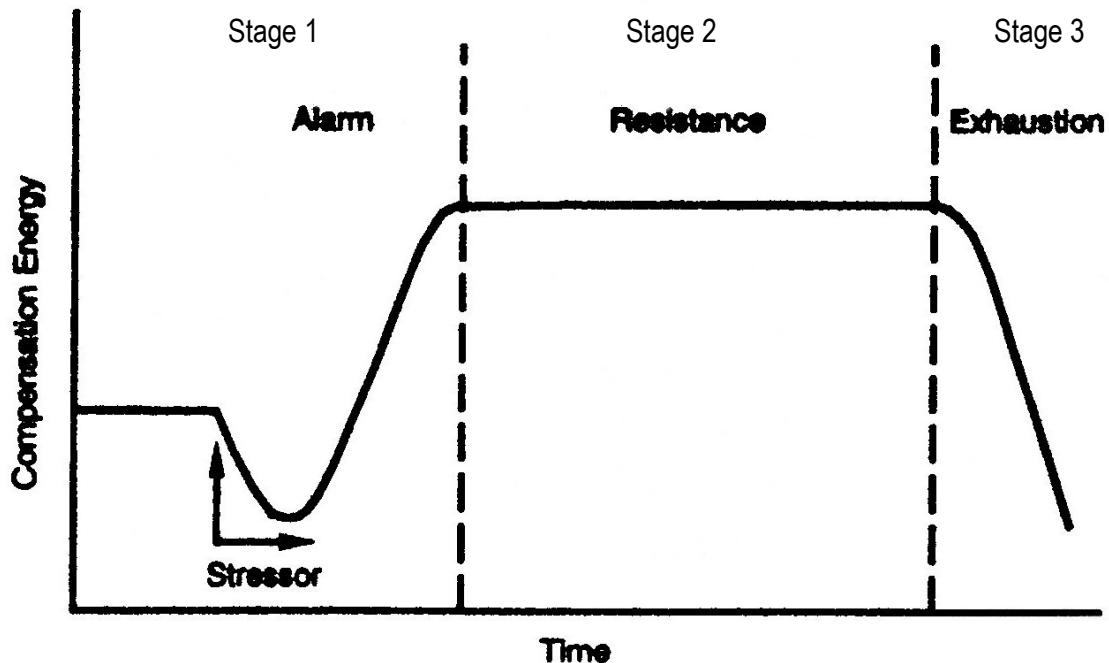


Figure 1-7: Conceptual representation of the three stages of the General Adaptation Syndrome illustrating how energy expenditure for compensating stressor effects changes with time. (Source: Beyers and Rice 2002).

Stage one: The first stage is an alarm reaction in response to a stressor.

Stage two: The stage of resistance is where the organism adjusts or compensates or adapts to regain homeostasis.

Stage three: The stage of exhaustion, where compensation or adaptation has ceased to be adequate and homeostasis is not achieved, possibly leading to a pathological condition or mortality.

Attempts have been made to generalise the GAS to the ecosystem level (e.g., Rapport *et al.*, 1985), and population level (e.g., Shuter, 1990).

1-3-2 Stress in fish

Historical Setting

Early work on stress in fish can be linked to studies such as Huntsman (1938) and a series of investigations by Black in the 1950s (Black, 1956, 1957 a,b,c,; Black and Barret, 1957; Black 1958), each primarily associated with delayed mortalities linked with exhaustive exercise and hypoxic conditions (Barton, 1997; Pickering, 1998). Early behavioural stress studies were associated with the importance of visual perception (Schreck, 1981), for example the stress associated with visual isolation and territorial behaviour (Kalleberg, 1958; Yamagishi, 1962).

The next 20 years saw an increasing number of physiological studies in fish, mainly focussing on the economically important Pacific salmon *Oncorhynchus* sp. of North America (Pickering, 1998). Behavioural studies at this time were centred on three areas: (1) anaesthesia, (2) elimination of visual awareness, and (3) conditioning or training (Schreck, 1981). A symposium in 1980 saw the first collection of information on the biology associated with stress in fish (Pickering, 1981).

From 1980, stress physiology became a key subject area due to rapid developments in endocrinological techniques (Pickering, 1998) for investigating fish metabolism, reproduction and the immune system (Randall and Perry, 1992; Pickering, 1993; Iwama *et al.* 1997; Reid *et al.* 1998; Mommsen *et al.*, 1999; Barton *et al.*, 2002) and a broadening range of fish species (Pickering, 1998). Many published reviews indicate that a major focus of research was to support aquaculture development (e.g. Barton and Iwama, 1991; Iwama *et al.*, 1997; Pickering, 1998 and Barton *et al.* 2002). According to Barton *et al.* (2002), few reviews deal with stress responses in wild fish and hormonal manipulations (but examples are Mazeaud *et al.*, 1977; Pickering, 1981; Gamperl *et al.*, 1994; Wendelaar Bonga, 1997; Fabbri *et al.*,

1998; Mommsen *et al.*, 1999; Schreck, 2000; Gallo and Civinini, 2003). Even less information is available on fish physiological responses to environmental disturbance associated with natural or man-made stressors (examples are Cairns *et al.*, 1984; Adams, 1990, 2002; Niimi, 1990; Brown, 1993; Hontela, 1997; Whyte *et al.*, 2000; van der Oost *et al.*, 2003; Schmitt *et al.*, 2005; Hinck *et al.*, 2006).

By the late 1990s, some still considered behavioural studies on fish as under-utilised in practical situations (e.g. Schreck *et al.*, 1997). In certain disciplines such as toxicology, however, behaviour has continued to be explored for practical outcomes (e.g. Little, 2002). In recent years, literature on animal welfare issues regarding fish has become more prominent (e.g. Chandroo *et al.*, 2004) and there has been ongoing investigation of behaviour as a link between fish physiology and ecology (Marcucella and Abramson, 1978; Olla *et al.* 1980; Beitinger, 1990; Schreck, 1990; Sherer, 1992; Lima, 1998; Brown and Laland, 2003; Kelly and Magurran, 2003; Scott and Sloman, 2004; Wiedenmayer, 2004; Portz *et al.*, 2006). Genetics (e.g. via the consequence of selective breeding in aquaculture) is now being used to support this (e.g. Overli *et al.* 2005; Schjolden and Winberg, 2007; Kittilsen *et al.* 2009; Vallejo *et al.* 2009; Volckaert *et al.* 2012; Drangsholt *et al.* 2014).

1-3-3 Defining and Measuring Stress Responses in fish

Stress is difficult to define both physiologically and behaviourally (Barton, 1997). It has been suggested that the term “stress” should be abandoned because a unified concept over-emphasises similarities and ignores differences (Ladewig, 2000). Nevertheless, terminology to be used throughout this study requires clear and consistent definitions. Therefore, the following definitions will be used throughout:

Stress: *Stress can be considered as a state of threatened homeostasis, with homeostasis being re-established by a complex suite of adaptive responses (Chrousos 1998).*

Stressor- *A stressor is something that stimulates a stress response* (Shuter, 1990). It may be ‘real’ (e.g. of physical or chemical origin), or ‘perceived’ (e.g. presence of a predator) (Figure 1-6). When attempting to identify common patterns of response to different stressors, some investigators (e.g. Shuter, 1990) have further subdivided stressors into Types 1 and 2. Following the Shuter (1990) approach, Type 1 stressors are detectable by individual fish and are spatially localized (e.g. natural predation). They produce strong tropic reactions (avoidance or attraction) in individuals leading to rapid changes in the spatial distribution of the population (Shuter, 1990). These distributional changes can themselves induce further population effects prior to those impacts that arise from direct exposure of individual fish to the stressor. Type 2 stressors are either spatially localized or pervasive over space, but not detectable by individual fish (e.g. exploitation and some contaminants). Here, changes are also made in the spatial distribution of the population, but occur in concert with the changes produced by the exposure.

Stress response: *Stress responses are the physiological or behavioural manifestations that can be measured indicating a stressed state.* The stress response can also be considered as an integrated reaction to adverse conditions, which includes behavioural changes as well as biochemistry and physiology (Pickering, 1998).

1-3-5 Different Interpretations of Acute and Chronic Stress

Many definitions have been proposed for acute and chronic stress. Rand and Petrocelli (1985) proposed that for aquatic toxicology tests, acute stress be defined as that occurring over 4 days or less and chronic stress be defined as that occurring on a continuous basis. According

to Barton *et al.* (2002), this problem is further complicated when attempting to differentiate between acute and chronic stress at the sub-lethal level of response. Acute exposures to

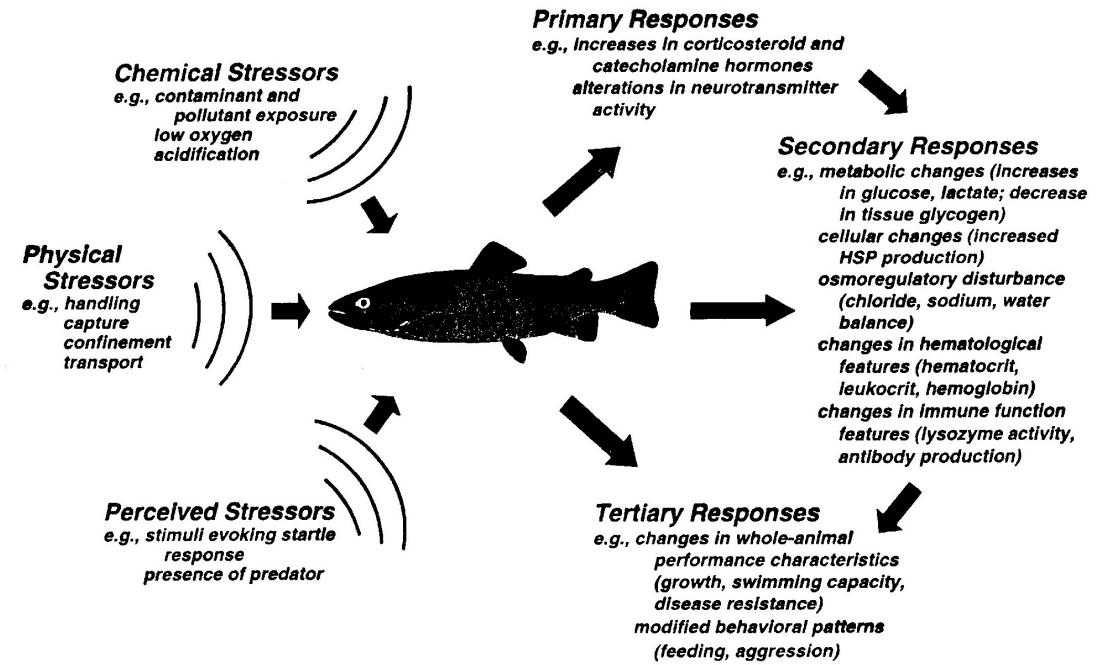


Figure 1-6: Physical, chemical, and other perceived stressors act on fish to elicit physiological and related effects, which are grouped as primary, secondary, and tertiary responses. In many cases, the primary and secondary responses may directly affect secondary and tertiary responses respectively, as indicated by the arrows (Source: Barton *et al.*, 2002).

stressors and responses to them range from seconds to minutes up to hours; chronic disturbances and responses to them range from hours to weeks and months and possibly throughout a lifetime (Barton *et al.*, 2002). Adams (1990) has not made this distinction; acute stress is lethal, and usually occurs quickly in response to short-term perturbations such as chemical spills or significant changes in environmental factors such as water temperature or dissolved oxygen (Figure 1-8). This lethal outcome may be the result of either single or several rapid exposures to a stressor, with the time of response ranging from immediate to

hours to days. Chronic stress is sublethal, and is the consequence of either continuous or periodic exposure to low level stressors with periods ranging from weeks to years. Dhabhar and McEwan (1997) have defined acute and chronic stress in the context of responses in the General Adaptation Syndrome (GAS); acute stress results from brief or short-lived disturbances that manifest in physiologically adaptive responses to regain homeostasis; chronic stress, however, challenges compensatory mechanisms to the extent that maladaptive or pathological conditions result - which can be either sublethal or ultimately lethal, depending on whether or not there is an adequate adaptive capacity to allow recovery from stress. In this study, the stressors that are introduced offer a good opportunity to evoke an acute stress response (e.g. to air exposure in a dip-net) or a chronic response (e.g. to different levels of confinement for a prolonged period) (e.g. Barton *et al.*, 1986).

1-3-6 Direct and Indirect Chronic Stress

Sublethal chronic stress responses can be subdivided into either direct or indirect components (Adams, 1990). Direct chronic stress response is manifested at greater levels of biological organisation such as changes in growth and reproduction, and usually originates when a stressor impacts on metabolism, which then affects functional cellular components such as enzymes and membranes, or alternatively damages essential activities such as respiration, circulation, osmoregulation, immune response and hormone regulation (Figure 1-8). Indirect chronic stress similarly affects growth and reproduction, but does so essentially via the food chain. If available energy is reduced by stress, maintenance and repair processes may be compromised with the consequence of there being insufficient energy available for growth and reproduction.

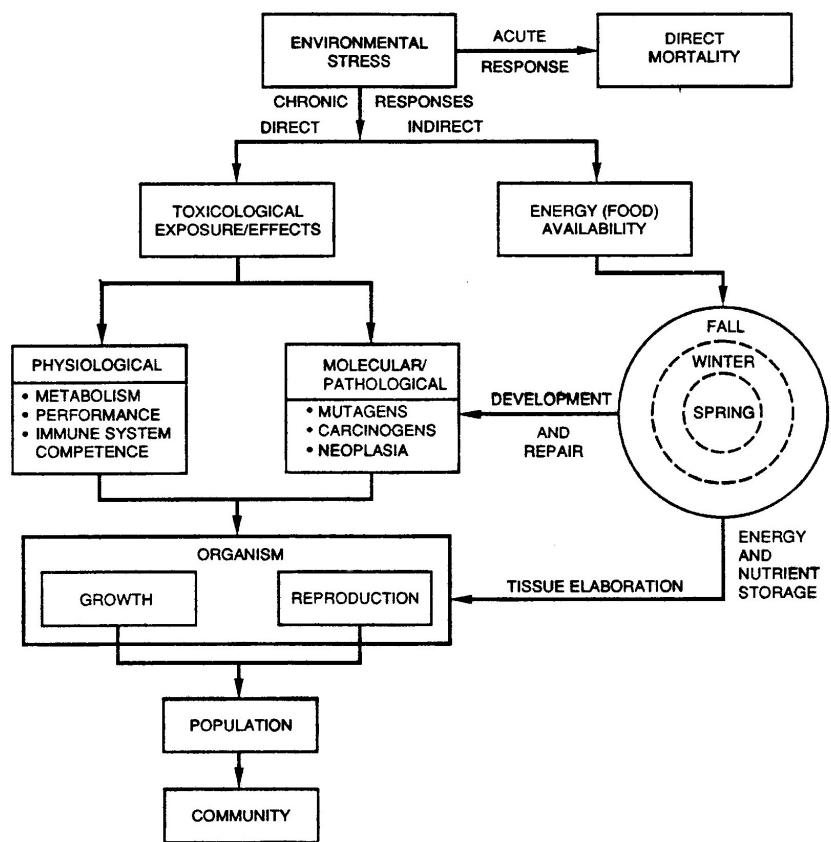


Figure 1-8: Responses of fish to direct and indirect chronic environmental stress (Adams, 1990).

1-3-7 Primary, Secondary and Tertiary Responses

The concept of identifying the effects of stress on fish at the primary (endocrine) and secondary (metabolism and osmoregulation) levels was developed by Mazeaud *et al.* (1977). Further development by Wedemeyer and McLeay (1981) added tertiary or whole-animal responses allowing the inclusion of performance indicators. In reality, such grouping is simplistic; stress, depending on its magnitude and duration, can affect fish at all levels of biological organisation (Adams, 1990). Because of both the inter-related and often inter-regulated levels of biological organisation, a more holistic view of stress responses in fish is now considered appropriate (Barton *et al.*, 2002).

In summary, Barton *et al.* (2002) classified primary, secondary and tertiary responses as follows (Figure 1-6).

Primary responses:

Involvement of the initial endocrine responses, which include the release of catecholamines from chromaffin cells.

Stimulation of the hypothalamic-pituitary-interrenal (HPI) axis (homologue of hypothalamo-pituitary-adrenal axis, culminating in the release of corticosteroid hormones into circulation.

Secondary responses:

Changes in plasma and tissue ion and metabolite levels and haematological characteristics associated with physiological adjustments, i.e. in metabolism, respiration, acid-base status, hydromineral balance and immune function.

Tertiary responses:

Changes in whole-animal performance, such as changes in growth, condition, and overall resistance to disease, metabolic scope for activity, behaviour and survival.

1-3-8 Factors influencing physiological responses

The physiological stress response in fish is initiated by the recognition by the central nervous system (CNS) of a real or perceived threat (a stressor) (Barton *et al.*, 2002), which suggests that the induction of the physiological stress response has a psychogenic component (Schreck 1981; 1990) for Type 1 stressors (see above).

Physiological responses of fish to stressful encounters are affected by their genetics, developmental and nutritional state, and the surrounding environment, with genetics in particular causing potentially wide variation in the endocrine response to the same stressor (Barton *et al.*, 2002). Furthermore, it is unknown whether a low corticosteroid response indicates that the fish is “less stressed” or “as stressed” or has a different capacity to respond to stress compared with others (Barton *et al.*, 2002).

Variation in response to stress occurs among fish species (Barton and Iwama, 1991; Vijayan and Moon, 1994; Ruane *et al.*, 1999; Barton *et al.*, 2002); among strains within the same species (Woodward and Strange 1987; Iwama *et al.*, 1992); and even in the same population (Pottinger *et al.*, 1992). The stage of fish development also affects the ability to respond to stress (Barton *et al.*, 2002); for example, the onset of maturity may attenuate the primary stress response (Pottinger *et al.*, 1995; Barton *et al.*, 2002).

Finally, how a fish responds to stress is influenced by the length of time between discrete stressors, by the cumulative effect of multiple stressors, and by the severity of continuous

stressors (Barton, 2000). Both Schreck (2000) and Barton *et al.* (2002) indicate that if a stressor is unrelenting, fish either eventually compensate for it or they die. The long-term consequence of exposure to a stressor is an allostatic load on the fish, which is the price of accommodating stress (Sterling and Eyer, 1988; McEwen and Stellar, 1993; Schreck, 2000). Barton *et al.* (2002) view successful compensation to a stressor in terms of acclimation or habituation. In studies on stress in fish, it is therefore important to know whether fish are naïve or have possibly been exposed to other stressors in their environment prior to any study (Barton *et al.*, 2002). Since much of this is unknown in wild fish, interpreting their physiological responses can be problematic, especially coupled with variation among fish species and within species by factors such as maturity, gender and condition.

1-3-9 Factors influencing behavioural responses

A behavioural response to an external stimulus depends on the stimulus first being sensed and perceived, either directly or indirectly (Schreck *et al.*, 1997) via the neuroendocrine system, involving catecholamines and glucocorticosteroid hormones (Mazeaud *et al.*, 1977; Barton and Iwama, 1991; Schreck *et al.*, 1997), resulting in a cascade of physiological responses (primary and secondary responses) ultimately affecting tertiary (whole-animal) reactions such as behaviour (Schreck *et al.*, 1997).

The subsequent change in behaviour is the first line of defence to increase the chances of survival or decrease metabolic costs incurred by maintaining physiological homeostasis (Olla *et al.*, 1980; Schreck *et al.*, 1997). Therefore, such changes are involved with success in habitat selection, competition, predator avoidance, food acquisition, orientation and migration, reproduction and learning (Little *et al.*, 1985; Little, 2002; Schreck *et al.*, 1997). The behaviour of fish is therefore a structured sequence of quantifiable actions (Keenleyside,

1979; Little, 2002) that are the culmination of genetic, biochemical and physiological processes (Little *et al.*, 1993; Little, 2002).

1-3-10 Evaluating the Stress Response in Fish

Physiological responses of fish to environmental stressors are usually measured by plasma cortisol, glucose, lactate, chloride (and other ions), osmolality, or various haematological features (Barton *et al.*, 2002). Such measurements vary depending on the genotypes, early life history, nutritional status and environment of the fish (Barton *et al.*, 2002) and again have problems of use in wild fish populations. Conventional methods of evaluating effects of environmental stress (chronic stress) on fish, such as laboratory bioassays that utilise only a single stress response, or one level of biological organisation (e.g. cortisol), generally lack ecological realism because many environmental factors can influence stress responses at all levels of biological organization (Adams, 1990; Adams and Ryon, 1994; Adams *et al.*, 1999; Adams *et al.*, 2000; Adams and Greeley, 2000). To overcome this, a biological indicator approach has been developed, whereby a suite of selected stress responses is measured at several levels of biological organization ranging from subcellular and biochemical to ecosystem level responses; these are also segregated along gradients of toxicological and ecological relevance, and response time (Adams, 1990; Adams and Ryon, 1994; Adams *et al.*, 1999; Adams *et al.*, 2000; Adams and Greeley, 2000). Such a biological indicator approach, therefore, can be used to assess sublethal stress effects in fish, to give early warning of stress, and to obtain insights into causal relationships between stressors and effects manifested at higher levels of biological organization (Adams, 1990; Adams and Ryon, 1994; Adams *et al.*, 1999; Adams *et al.*, 2000; Adams and Greeley, 2000).

Stress Measurement

Tests for glucose, lactate, chloride, and osmolality can be performed with simple assay kits, and easy-to-use meters (Wedemeyer *et al.*, 1990; Iwama *et al.*, 1995). Cortisol measurement usually involves radioimmunoassay or enzyme-linked immunosorbent assay (ELISA) techniques (Barton *et al.*, 2002). Clinical kits for these assays are available but should be calibrated and verified for the taxonomic group being studied (Barton *et al.*, 2002) and relatively few species have specified ranges or responses. Using such methods requires considerable time to work up and commercial kits meant for use on mammals may not be suitable for fish.

Heat shock proteins (HSP) have been used as stress indicators in mammals and are usually detected by gel electrophoresis followed by Western blotting or ELISA, or both (Forsyth *et al.*, 1997; Vijayan *et al.*, 1997; Iwama *et al.*, 1998). Determination of indolamines and catecholamines (neurotransmitters) is undertaken by high performance liquid chromatography (HPLC) (Barton *et al.*, 2002). As with methods for cortisol, significant time and effort is required to set these methods up and verify them for particular fish species.

Stress Measurement and Complicating Factors

The biological significance of the stress response can be difficult to interpret if modified by genetic, developmental and environmental factors when their influence on the magnitude and duration of the stress response is unknown (Barton *et al.*, 2002). Apparent inconsistency in results may exist between indicators for primary and secondary responses (e.g. cortisol and glucose) for the same fish species when responding to an identical stressor (Barton *et al.*, 2002). This emphasises the importance of not relying on a single physiological indicator in stress response studies, and the need for multiple indicators and appropriate controls (Barton

et al., 2002). The interpretation of results may also be complicated by the time lag between perception of the stressor and manifestation of a measurable physiological response (Barton *et al.*, 2002).

Responses to acute stressors may be evaluated by physiological measurements, but this may not always be the case when attempting to monitor fish experiencing sublethal chronic stress (Barton *et al.* 2002). In this situation, physiological processes commonly adapt to compensate for the chronic stress, and blood chemistry parameters, such as plasma cortisol, may appear normal, but not reflect the state of chronic stress (Schreck, 1981, 2000; Adams, 1990; Barton *et al.*, 2002). An alternative approach to evaluate chronic stress is therefore required.

1-3-11 Chronic Stress and Histological Changes

In terms of biological organisation, histological changes in teleosts appear as medium-term responses with regard to sub-lethal stressors (Bernet *et al.*, 1999; Adams and Greeley, 2000; Silva and Martinez, 2007); such changes in various tissues and organs such as the kidney, gills and intestine therefore represent alternative methods to detect effects of stressors, especially chronic ones (Bernet *et al.*, 1999; Silva and Martinez, 2007).

Integrated into the endocrine system of fish is the anterior kidney (also known as the head kidney) where interrenal cells (Nandi 1962) synthesize corticosteroids, chiefly cortisol (Barton *et al.*, 2002; Silva and Martinez, 2007). A rise in plasma cortisol levels, in response to some stressor stimulus, implies an increase in interrenal cell activity (Silva and Martinez, 2007). Continued synthetic and secretory activity of the interrenal tissue during chronic stress results in hypertrophy (increase in size) and hyperplasia (increase in abundance) of interrenal cells (Barton *et al.*, 2002). Chronic stress can then be identified *post hoc* by counting and measuring interrenal cells following standard histological preparation (Barton *et*

al., 2002). Continual interrenal activity also down-regulates the hypothalamic-pituitary-interrenal (HPI) axis as a result of negative feedback by cortisol; this results in a diminished subsequent cortisol response in chronically stressed fish after they encounter a second acute stressor when compared with controls (Hontela 1997; Barton *et al.*, 2002). Monitoring hypertrophy and hyperplasia of the interrenal cells assists in assessing stressed states in fish subjected to social stressors, including high rearing density (Noakes and Leatherland 1977; Fagerlund *et al.* 1981), and heavy metals (Norris *et al.* 1997) and other environmental pollutants (Ram and Singh 1988; Servizi *et al.* 1993). Therefore, chronically stressed fish, when subjected to a second acute stressor, may have a much reduced corticosteroid response when compared with controls (Hontela 1997).

1-3-12 *Tertiary Responses and Performance Tests*

Tertiary or whole-animal responses to stress, such as behaviour, growth, survival and changes in health and condition, indicate the extent to which stress may affect fish performance (Barton *et al.*, 2002). Therefore, measurements of performance tests associated with tertiary responses (Schreck, 1990) can be a powerful tool for assessing stress, because they incorporate several lower levels of biological organization and are therefore integrative (holistic) in nature (Schreck, 1990; Wedemeyer *et al.*, 1990). Tertiary stress responses can indicate that unfavourable environmental conditions have exceeded tolerance limits of fish (Wedemeyer *et al.*, 1990). A performance test identifying a change at a tertiary response level also indicates changes at lower levels of biological organisation (Adams, 1990; Barton *et al.*, 2002). Therefore, the ecological significance can be identified by changes in a tertiary response and more sensitive stressor identification (high toxicological relevance) by lower levels of biological organisation in a later study if limitations exist in resources and infrastructure (Adams, 1990; Adams *et al.*, 2000). The determination of the tertiary response

is very relevant to the general problem of habitat-based predictive methodologies for environmental impact assessments (Wedemeyer *et al.*, 1990).

Performance tests are bioassays that test the ability of a fish to undertake a certain physiological or behavioural activity. A stressed animal can be identified and measured by its impaired performance when exposed to a second stressor or when attempting to undertake another task (Schreck, 1990). The performance test can also assess synergistic effects created by different forms of stressors (Schreck, 1990). The response in a performance test can also be influenced by other variables, such as genetics, stage of development, prior history, present environment, and severity and duration of the stressor (Schreck, 1981; Barton, 1988; Schreck, 1990). Therefore according to Wedemeyer *et al.* (1990), performance testing is based on the presumption that (i) a stress load that exceeds acclimation limits is debilitating and reduces performance capacity; (ii) reduced performance capacity can be measured as reduced tolerance of subsequent stress; and (iii) the probability of survival of the population will be reduced to the extent that reduced performance capacity impairs growth, survival or reproduction of individual fish.

Shelter Seeking

Behavioural responses to assess the effects of stress on individual fish and populations have not been used as widely as other aspects of the stress response (Wedemeyer *et al.*, 1990; Schreck *et al.*, 1997). Some behavioural responses have a qualitative component that makes them difficult to measure (e.g. susceptibility to predation), but others can be used to quantify whole-animal performance, such as the avoidance by Chinook salmon of a harsh stimulus such as a bright light in a trough apparatus containing a hide (Sigismondi and Weber 1988).

Stress can disrupt shelter-seeking behaviour in fish (Sigismondi and Weber, 1988; Schreck *et al.*, 1997). Shelter can be sought in response to a negative phototaxis, a positive thigmotaxis, as a fright response to chemosensory detection of a predator or noxious scent, as a response to a visual fright stimulus, or thermal stress (Schreck *et al.*, 1997). For example, the time to seek shelter after exposure to an overhead light was greater in Chinook salmon that first received handling stress than in controls not so handled (Sigismondi and Weber 1988). Also exposure to a contaminant, the antisapstain fungicide 2-(thiocyanomethylthio) benzothiazole, caused shelter-seeking behaviour in salmonids to be an inappropriate response, in that disoriented fish sought cover in an area of abundant predators (Kruzynski *et al.*, 1994).

Condition factor

The coefficient of condition (K or often Cf), or Fulton's condition factor (Bagenal and Tesch, 1978; Busaker *et al.*, 1990; Barton *et al.*, 2002) is a convenient way of comparing the relative well-being of fish populations (Everhart *et al.*, 1975). It is also a measure of robustness. The condition factor (K) is a function of fish fresh weight divided by the cube of the length, and approaches unity with a scaling constant. K is usually expressed in metric units (e.g. $K = g \text{ mm}^{-3} \times 10^5$; Anderson and Neumann 1996; Barton *et al.*, 2002). Theoretically, values for K assume a constant length exponent of 3 but usually this exponent ranges between 2.5 and 3.5 (Carlander 1969) depending on the species, body shape and its life stage (Barton *et al.*, 2002).

When comparing fish populations, variables that influence the condition factor are sex, season of year, stage of maturity and size of the fish (Everhart *et al.*, 1975; Barton *et al.*, 2002) and the presence of disease or exposure to stressors (Barton *et al.*, 2002). With these variables considered, changes in condition factor reflect the nutritional or energy status of the fish (Lambert and Dutil 1997; Grant *et al.*, 1998; Grant and Brown 1999; Barton *et al.*, 2002) where: (1) a change in feeding behaviour and food consumption may be identified by

declines in reserves, such as liver glycogen or visceral fat deposits, (2) starvation leads to proteolysis, and (3) changes in body water content reflect changes in energy reserves and organ biochemistry (Adams *et al.*, 1985; Cunjak and Power 1986; Lambert and Dutil 1997; Barton *et al.*, 2002).

Organosomatic Indices

Ratios of organ weight to body weight have been used in stress studies on fish (Goede and Barton 1990). They are easy to measure and are based on the assumption that a lesser than normal value indicates energy diversion away from organ growth in order to combat a stressor (Barton *et al.*, 2002).

The most frequently used organosomatic index is the ratio of liver weight to body weight, called the liver-somatic index (LSI) or hepatosomatic index (HSI) (Goede and Barton, 1990; Barton *et al.*, 2002). A change in liver-somatic index to liver glycogen value is valid for chronic stress loading; however, it may not always be valid for an acute stress event such as handling (Goede and Barton, 1990).

Liver-somatic index values fall with an increase in water acidity (Lee *et al.*, 1983), altered flow rates (Barnes *et al.*, 1984), multiple handling (Barton *et al.*, 1987) and fish starvation (Barton *et al.*, 1988). By contrast, liver-somatic index values increase after exposure to contaminants, in particular petroleum hydrocarbons (Fletcher *et al.*, 1982; Fabacher and Baumann, 1985; Baumann *et al.*, 1991). The resulting increased liver weight is due to an expansion in liver cell size (hypertrophy) and number (hyperplasia), which is an adaptive response that enhances the capacity of the liver to detoxify foreign compounds via the induction of the mixed-function-oxidase system (Heath, 1987; Goede and Barton, 1990; Barton *et al.*, 2002).

Other organ weight ratios utilised in fish stress studies include the viscerosomatic index (VSI), the gonadosomatic index (GSI) and splenosomatic index (SSI) (Goede and Barton, 1990; Barton *et al.*, 2002). The organosomatic indices such as LSI, and in particular GSI, vary with season, energy availability, sex and state of sexual maturation (Goede and Barton, 1990; Barton *et al.* 2002). The presence of parasites in organs may also confound the interpretation of an organosomatic index (Barton *et al.* 2002). The use of an organosomatic index assumes that there is no disproportionate relationship in the fish size:liver ratio (Goede and Barton, 1990).

Necropsy-based Indicators

If fish experience long-lasting or severe stress, compensatory mechanisms are exceeded and pathological conditions develop (Moberg, 1985). Goede and Barton (1990) described a necropsy-based condition assessment method which can be used alone or in combination with physiological and haematological measurements (Iwama *et al.* 1995; Barton *et al.* 2002). Later Adams *et al.* (1993) modified this method by assigning numerical values to the necropsy categories so as to provide a quantitative health assessment index (HAI) that could then be compared statistically. The HAI has been used to evaluate wild fish populations of warm- and cold-water fishes exposed to environmental disturbances (e.g. Adams *et al.*, 1993; Barton 1994; Schlenk *et al.*, 1996; Bergstedt and Bergerson 1997; Coughlan *et al.*, 1996; Raymond and Shaw 1997; Robinson *et al.*, 1998); Steyermark *et al.*, 1999; Sutton *et al.*, 2000; McKinney *et al.*, 2001) and, in some cases, extended or modified to incorporate extra site-specific observations (e.g. Steyermark *et al.*, 1999; McKinney *et al.*, 2001).

The necropsy-based condition assessment method, and the subsequent HAI, are based on a number of assumptions: (1) tissue and organ functions changes restore or maintain homeostasis in fish under stress, (2) the fish is normal if the appearance of all organs and

tissues is normal, (3) the fish is responding to changes brought about by an environmental stressor if the appearance of an organ or tissue system departs from the normal control condition, and (4) there is a gross change in structure of organs and tissues if a change in function persists in response to continuous stress (Barton *et al.*, 2002).

1-3-12 Stress Responses & Black Bream

Physiological Responses: Physiological tests to date have utilised the plasma cortisol response exhibited by black bream to ascertain its potential and to enhance the value of the test for improved productivity in aquaculture (e.g. Haddy and Pankhurst, 1998; Haddy and Pankhurst, 1999; Haddy and Pankhurst, 2000a,b; Hobby *et al.*, 2000). Authors, therefore, have focused on the acute stress response associated with those stressors common to aquaculture. In these studies, plasma steroid concentrations were measured by radioimmunoassay (RIA), using the reagents and protocols described in Pankhurst and Carragher (1992).

Typical of many non-salmonids, black bream have plasma cortisol levels that increase from approximately 5 ng ml^{-1} within 5 minutes of capture to approximately 40 ng ml^{-1} within 1 hour of confinement, and which remain above 20 ng ml^{-1} after 24 hours (Haddy and Pankhurst, 1999). Hobby *et al.* (2000) indicated that rainbow trout showed a large elevation of cortisol in response to stress ($35\text{-}900 \text{ ng ml}^{-1}$). For more information on pre- and post-stress corticosteroid levels in a number of fish families and species, see Barton and Iwama (1991).

Capture: Black bream have displayed a range of cortisol values at capture, from the undetectable ($<0.3 \text{ ng ml}^{-1}$) to 27.5 ng ml^{-1} (Haddy and Pankhurst, 1999). Basal levels of cortisol at capture were less for male than female fish ($1.9\pm0.2 \text{ ng ml}^{-1}$ and $2.8\pm0.4 \text{ ng ml}^{-1}$

respectively) (Haddy and Pankhurst, 1999). At capture, cortisol levels in black bream did not change with time of day, gonad stage or season (Haddy and Pankhurst, 1999). However, in a later study, captive black bream displayed a seasonal variation in plasma cortisol concentration over a range of salinities (5, 20 and 35 ppt), with low values being recorded in August and high values in September and November for fish caught in Tasmania (Haddy and Pankhurst, 2000). This seasonal variation was reduced when female bream were held in a constant salinity of 35 ppt, resulting in no significant variation in plasma cortisol concentration over this period. For male fish, no significant variation was recorded at 5 ppt.

Handling

Wild black bream maintained in captivity without handling displayed basal cortisol levels similar to those found in animals sampled in the wild (Hobby *et al.*, 2000). Cortisol levels increased rapidly after handling and remained high (Haddy and Pankhurst, 2000; Hobby *et al.*, 2000). Daily handling caused a sustained elevation in cortisol levels in all fish (Haddy and Pankhurst, 2000).

Confinement

Confinement of black bream resulted in elevated plasma cortisol levels that peaked for male fish at 15 minutes and females at 1 hour (Haddy and Pankhurst, 1999). Cortisol levels then decreased after 24 hours to less than peak values (Haddy and Pankhurst, 1999). As an example of this cortisol profile, fish demonstrated values of 5 ng ml⁻¹ within 5 minutes of capture, approximately 40 ng ml⁻¹ within 1 hour of confinement, and then values remained above 20 ng ml⁻¹ after 24 hours (Haddy and Pankhurst, 1999). Haddy and Pankhurst (1999) considered three possibilities for the decline in plasma cortisol values from their peak after 24 hours. These are: (1) inter-renal exhaustion (Sumpter, 1997), (2) down-regulation of the

hypothalamic-pituitary-interrenal axis (Bradford *et al.*, 1992) or (3) a limited degree of recovery from stress, suggesting that tank confinement was less stressful than the initial capture and handling. Plasma cortisol concentrations in excess of 200 ng ml⁻¹ have been sampled from black bream after they had been captured, handled and then transported (Hobby *et al.*, 2000). Black bream held in captivity, and therefore confined but not exposed to disturbance such as handling, displayed plasma cortisol levels similar to basal levels in black bream caught in the wild (Hobby *et al.*, 2000).

Heat Shock Proteins (hsp70) and Field Sampling

Webb and Gagnon (2008) investigated the usefulness of hsp70 expression to identify anthropogenic stress in black bream under field conditions. They utilised gill, liver and muscle from fish sourced from a highly variable estuarine environment in order to find which organ provided the best inter-site discrimination. Inter-site differences were detected in hsp70 levels in gill and white muscle, while liver showed no inter-site difference. Although there was large inter-fish variability in hsp70 levels in each sample, white muscle provided the best discriminatory power to elucidate inter-site variation, with only 11 fish per site being required to identify significant inter-site differences in white muscle; for gill and liver samples 14 and 21 fish per site, respectively, were required. Webb and Gagnon (2008) indicated that, because of high inter-tissue and inter-individual variability, field measurements of hsp70 should be complemented by evidence of changes in other biomarkers of fish health.

Objectives of this research: A Summary

The Setting

This research was funded by a “Gippsland Lakes Ecosystem Research Scholarship” offered by RMIT University. To comply with the scholarship, research was conducted on black bream, a valued economic component of the Gippsland Lakes region. The intention of this research was to see if black bream could also be a valued ecological monitoring tool by: (i) Identifying its suitability, and, if possible (ii) laying a foundation for the development of black bream as a bioindicator to monitor the environmental health of the Gippsland Lakes.

Chapter 2: Acute Stress - Recovery and Cumulative Responses

The use of black bream as a bioindicator of environmental health has the potential to be confounded by handling associated and expected with catch-and-release of undersized fish in commercial and recreational fishing in the Gippsland Lakes. Post-release recovery and cumulative response to common and expected stressors in commercial and recreational fishing also offers direct benefit to fisheries management. This chapter aimed to measure the response of Gippsland Lakes black bream to an acute stress (air exposure) and the cumulative effect of repeated exposures. The purpose was two-fold: (1) to identify the effect of the stress events and (2) to compare a short behavioural test (shelter-seeking) with a long-term conventional test (mortality) for use in identifying the impact of differing stressors on black bream.

Chapter 3: Chronic Stress - Behaviour

In an inter-estuarine tank-based study, black bream were obtained from the Gippsland Lakes and an adjacent estuarine system, Lake Tyers. The aim was to identify if a difference in

chronic stress loading (high density) carried by fish from different multi-stressor environments changed the response to an acute stress (air exposure). The stress loading was to be measured by behaviour (tertiary response).

Chapter 4: Histological Indicators of Stress

This was an inter-estuarine field study where black bream were again sampled from the Gippsland Lakes and Lake Tyers. The aim was to compare chronic stress loading in fish sourced from these differing environments by hypertrophy and hyperplasia of interrenal tissue (located in the anterior kidney) as a consequence of any extended period of cortisol synthesis. Fulton's condition factor was also to be determined from length and weight measurements from all fish sampled.

Chapter 5: Otolith Elemental Analysis

The comparative response to chronic stress of black bream from the two estuaries could be influenced by movement of fish between the Gippsland Lakes and Lake Tyers estuaries. Otolith elemental composition can reflect the estuary where the fish normally live. Otolith samples were sourced from black bream from the Gippsland Lakes and Lake Tyers for elemental analysis. The purpose was two-fold: (1) to identify any inter-estuarine movement that could interfere with identifying chronic stress loading via otolith elemental signatures, and (2) to determine the value of EDXS (electron-dispersive X-ray spectroscopy) in an ESEM (environmental scanning electron microscope) as a non-destructive otolith elemental analysis technique.

Chapter 6: Genetics

The responses of black bream from different estuaries could be affected by differences in genetics. Samples of fin clippings and muscle were sourced from black bream from Gippsland Lakes and Lake Tyers for genetic analysis using differential primers in the polymerase chain reaction (PCR). The purpose was to compare the genetics of the two populations to assess if differences could modulate the chronic stress response.

Chapter 7: Overview and Conclusion

An integrative overview assesses how well black bream may function as a bioindicator to monitor the health of the Gippsland Lakes.

Chapter 2. Acute Stress

2-1 Introduction

2-1-1 Black bream and the Gippsland Lakes Fishery

Gippsland Lakes black bream (*Acanthopagrus butcheri*, Family Sparidae) supports a major commercial and recreational fishery, with 90% of Victoria's annual commercial catch (Morison *et al.*, 1998) and over 50% of the recreational effort and catch (Henry and Lyle, 2003; Grixti *et al.*, 2008) originating from this location. It is the fish of choice targeted by recreational anglers using the Gippsland Lakes region and other Victorian estuaries (Hall and MacDonald, 1985(a), 1985(b); Conron and Coutin, 1995; Coutin *et al.*, 1997; MacDonald, 1997b; Grixti *et al.*, 2008). The importance of the fishery is demonstrated by the continuous collection of commercial catch data for black bream in the Gippsland Lakes since 1914 (MacDonald, 1997b) and the collection of data on the recreational catches from the 1970's onwards. Numbers of black bream collected commercially or by recreational fisheries have fluctuated over the years; for example, over a 26 year period (1978 to 2004) there was a 420 tonne range difference in annual commercial catches. This has funded a periodic debate on whether or not the trends in catch data reflect the influences of habitat change or fishing pressure in the Gippsland Lakes (MacDonald, 1997b).

In any case, fishery managers have repeatedly sought to set catch trends in black bream by the adjustment of legal minimum lengths (Table 2-1), as well as through use of other strategies such as daily bag limits, limiting entry to the fishery, gear restrictions, area closures and seasonal and weekend closures (MacDonald, 1997b; Grixti *et al.*, 2008). Size limits have forced both the recreational and commercial sector to release or discard undersize black bream. In any case, fishery managers have repeatedly sought to set catch trends in black

bream by the adjustment of legal minimum lengths (Table 2-1), as well as through use of other strategies such as daily bag limits, limiting entry to the fishery, gear restrictions, area closures and seasonal and weekend closures (MacDonald, 1997b\; Grixiti *et al.*, 2008). Size limits have forced both the recreational and commercial sector to release or discard undersize black bream.

Table 2-1: History of black bream legal minimum lengths (LML) based on total length (TL) for the Gippsland Lakes (Sources: Coutin et al., 1997; MacDonald, 1997; Anon, 2004; Cooper, 2006).

Dates	Comments
Early 1900's	LML was 21.5 cm TL.
1946-1950	LML was raised in stages to 26.5 cm TL.
1966	LML was lowered to 24.0 cm TL.
1975	LML was raised to 25.5 cm TL (Gippsland Lakes only).
1996	LML was raised to 26.0 cm TL
2003-2004	LML was raised to 28.0 cm TL.
2005-2006	LML remains at 28.0 cm TL (Gippsland Lakes only). ^A

^A 26.0 cm TL. for other Victorian locations.

Australia-wide, it has been estimated that between 30% and 50% of total recreational angler catch is released or discarded (West and Gordon, 1994; Henry and Lyle, 2003). In the commercial sector, minimum legal length compliance accounts for most discarding practices. For those species with no length requirements, discarding was generally market-driven and size-based (Gray *et al.*, 2001; Gray and Kennelly, 2003). Henry and Lyle (2003) estimated that 8.2 million bream (*Acanthopagrus* spp.) were discarded in 2000/2001 alone. At this time, the majority of black bream recreationally caught in Victoria (840,000) were undersize and were released (Grixti *et al.*, 2008). The fate of black bream released or discarded back into

the Gippsland Lakes is unknown. Therefore, the effect of releases on fish stocks was also unknown at the time of this study in 2007. Grixti *et al.*, (2008) subsequently utilised the 72 h (3day) PRS monitoring time first used in this study with black bream caught in the Glenelg River, SE Victoria and showed that mortality varied from 11-89% but did not conduct other stress tests.

2-1-2 Fish Release Assumptions

Existing fishery management regulations around size limits for recreational fishers rely on the assumption that the majority of fish survive in the fishery when released (Wydoski, 1977; Diggles and Ernst, 1997; Broadhurst *et al.*, 1999; Broadhurst and Barker, 2000; Ayvazian *et al.*, 2002; Broadhurst *et al.*, 2005). A similar assumption exists in the commercial sector when selection technology is incorporated into fishing gear, i.e. fish escaping are not seriously damaged, are minimally stressed and make a complete recovery after escape (Chopin and Arimoto, 1995). The objective in providing for discards from commercial gear is the same, namely the maintenance of fishery stocks and populations of non-target fish species (Van der Haegen, 2004). Escape from selection gear often occurs only after fish are subjected to a wide number of capture stressors and possibly damage from other fish, debris or the fishing gear (Chopin and Arimoto, 1995). This reduces the probability that the survival assumptions are met.

2-1-3 Direct and Indirect Mortality

Muoneke and Childress (1994) classified direct mortality as mortalities occurring within 24 h of fish being released, and delayed mortalities as the deaths of fish that exhibit little evidence of injury or appear healthy and vigorous when released.

Direct mortality following release is obvious if predators (e.g. birds) are seen catching a released fish, or if a fish is rendered immobile due to severe physical injury.. However, mortality is uncertain when a number of crowded fish in a bunt or cod-end of a net are stung to varying degrees by similarly-trapped jellyfish (e.g. *Catostylus mosaicus*) (Gray and Kennelly, 2003) or other species, or crushing injuries occur. Chopin and Arimoto (1995) state that fish escaping or released from fishing gears suffer immediate as well as delayed mortalities owing to physical injury, predation and disease. Although some mortalities can be attributed to one event, most deaths in recreational fisheries result from cumulative sublethal disruptions evoked by combinations of factors involved when catching and releasing fish (Kwak and Henry, 1995; Broadhurst *et al.*, 2005). Chopin and Arimoto (1995) tabulated the mortality of fish escaping or released from commercial, recreational and research fishing gear (Table 2-2). Their data indicate mortality rates ranging from 0 to 100%, depending on species and fishing method.

Indirect mortality in recreational fishing mortalities depends on angling and environmental factors (Muoneke and Childress 1994; Bartholomew and Bohnsack, 2005; Arlinghaus *et al.*, 2007; Grixti *et al.*, 2008), in particular, on hooking location (where the hook point penetrates the tissue), size of fish, passive or active angling techniques, angler experience and bait type (Grixti *et al.*, 2008). In a study of black bream, hooking location and resulting injuries determined survival over a 72 hour period (Grixti *et al.*, 2008). Shallow-hooked fish had a 95% chance of survival, whereas deep-hooked fish had a 74% chance of survival and survival decreased as fish length increased; for deep-hooked fish, there was a 20% greater chance of survival if hooks were not removed; survival was less (58%) if bleeding occurred (80% with no bleeding (Grixti *et al.*, 2008).

2-1-4 Effect of Handling and Air Exposure on Mortality

Capture and handling are significant stressors to fish (Billard *et al.*, 1981; Barton and Iwama, 1991; Pickering, 1992; Wendelaar Bonga, 1997; Barnett and Pankhurst, 1998). In both commercial and recreational fisheries, it is a common practice for undersized and by-catch fish to encounter a period of air exposure prior to release, during events such as hook removal, identification, sorting, length and weight measurements, and photo opportunities (Ferguson and Tufts, 1992; Meka and McCormack, 2005). In teleost fish, the majority of gas and ion transfers take place across the delicate secondary lamellae located on gill filaments (primary lamellae). The lamellae are largely supported by the water flowing between them and, with few exceptions, the lamellae of most species collapse and adhere to the gill filaments if exposed to air, thus creating a near complete barrier to gas exchange, producing anoxia (Boutilier, 1990; Ferguson and Tufts, 1992). Air exposure has also been identified as creating abnormal slowness of heart action in fish (Cooke *et al.*, 2001). After capture and handling, air exposure is considered a significant additional stressor, potentially reducing the survival of released fish (Ferguson and Tufts, 1992; White *et. al.* 2008).

Table 2-2: Mortalities of fish escaping from fishing gears (Source: Chopin and Arimoto, 1995)

Fishing gear	Species	Mortality (%)	Comments	Reference
Surrounding gear	<i>Scomber</i> sp.	50-90	Simulated purse seine experiment.	Lockwood <i>et al.</i> (1983)
Seine nets	Cod, haddock	0, <10	Fish retrieved at surface	Soldal and Isaksen (1993)
Seine nets	Striped bass	1-17	Beach seine. Mortalities of released fish reduced through improved handling techniques.	Dunning <i>et al.</i> (1989)
Seine nets	Freshwater drums	84.7	Beach seine. Estimated mortality after release due to stress and injury.	Fritz and Johnson (1987)
Trawls	Striped bass	1-16	Otter trawl. Mortalities of released fish reduced through improved handling techniques.	
Trawls	Gadoids		Otter trawl and Danish seine; 39-100% surface tagged fish, 12-65% surface non-tagged fish, 13-50% bottom tagged fish, 4-32% bottom non-tagged fish.	Hislop and Hemmings (1971)
Trawl	Various	Varied	Discarded fish study in shrimp trawls. Mortality rates depended on time on deck but no fish survived 20 min on deck.	Wassenberg and Hill (1989)
Trawls	Haddock, whiting	9-27, 10-35	Codend mortality. Figures quoted from tables. Large variation between species and years.	Sangster and Lehmann (1993)
Trawls	<i>Melangogrammus</i> sp.		Otter trawl. Dead and injured fish found in the wake of the trawl (163-169 dead fish h ⁻¹ tow)	Zaferman and Screbrov (1989)
Trawls	Gadoids	14-100	Otter trawls. Large variation in mortality between cages, species and years	Main and Sangster (1990)
Trawls	Haddock, whiting	9-27, 10-35	Otter trawl.	Anonymous (1993)
Trawls	Cod, haddock	0, 1-32	Otter trawl codend.	Soldal <i>et al.</i> (1991)
Trawls	King and Tanner crab	21-22	Otter trawl. Non-target catch	Stevens (1990)
Trawls	Lobster	21	Non-~ catch. Mortality varied depending on moult condition.	Smith and Howell (1987)
Trawls	Atlantic halibut	65	65% mortality after 48 h compared with 23% mortality for longline-caught fish.	Neilson <i>et al.</i> (1989)
Trawls	<i>Clupea harengus</i>	85-90, 75-85	Diamond mesh mortality, sorting grid mortality	Suuronen <i>et al.</i> (1993)
Trawls	Scup, flounder, cod	0-50,0-150	Otter trawl	DeAlteris and Reifsteck (1993)
Dredges	<i>Pecten</i> sp.	78-88	Boat-operated scallop dredge. Mortality from gear, predation and disease	McLoughlin <i>et al.</i> (1991)
Dredges	<i>Placopecten</i> sp	10-17	Boat-operated scallop dredge	Caddy (1973)
Gillnets and entangling nets	Pacific salmon	80-100	Cumulative mortality in captive fish	Thompson <i>et al.</i> (1971)
Gillnets and entangling nets	Pacific salmon	80	Cumulative mortality due to scale damage and stress	Thompson and Hunter (1973)
Gillnets and entangling nets	<i>Clupea</i> sp	1.9	Mortality was v high but attributed to diseases	Hay <i>et al.</i> (1986)
Hooks and lines	<i>Oncorhynchus</i> sp.	12-69	Catch and release mortality estimates	Vincent-Lang <i>et al.</i> (1993)
Hooks and lines	<i>Oncorhynchus</i> sp.	34-52, 40-86	Coho salmon, Chinook salmon	Parker <i>et al.</i> (1959)
Hooks and lines	<i>Salmo</i> sp.	0	No mortalities after 3 days but measurable stress	Wydowski <i>et al.</i> (1976)
Hooks and lines	Rainbow trout	39.3-5	Hook swallowed bait, artificial lure	Barwick (1985)
Hooks and lines	Cutthroat trout	0.3, 3	One time hooked mortality, multiple hooking	Schill <i>et al.</i> (1986)
Hooks and lines	Trout	0-8.6	Angling mortality	Dotson (1982)
Hooks and lines	Smallmouth bass	0, 11	Artificial lures, live bait	Clapp and Clark (1989)
Hooks and lines	<i>Esox</i> sp.	3	Angling mortality	Schwalme and Mackay (1985)
Hooks and lines	Chinook salmon	9-32	Trolling, small fish had higher mortalities	Wertheimer (1988)
Hooks and lines	Pacific salmon	41	Trolling, 34% immediate mortality and 7% delayed mortality	Milne and Ball (1956)

2-1-5 Handling and Stress Response

Commercial and recreational fishing require the handling and often the confinement of fish. Black bream are sensitive to stressors such as handling and confinement, which produce rapid inhibitory effects on gonadal steroidogenesis (Haddy and Pankhurst, 1999).

Other indirect effects of stress, even if not severe enough to cause death directly, may still lead to mortality if fish behaviour is affected or compromised, in particular that behaviour associated with predator avoidance/evasion (Sylvester, 1972; Coutant *et al.*, 1979; Olla *et al.*, 1992a, 1992b, 1995, 1997, 1998; Schreck *et al.*, 1997). The response reflects both the severity and the duration of the stressor (Strange *et al.*, 1978; Barton *et al.*, 1980; Foo and Lam, 1993; Barton, 1998), and may be species-specific (Wedermeyer *et al.*, 1990). Multiple stressors can also evoke a cumulative stress response (Barton *et al.*, 1986) that ultimately may impair the ability to avoid predators (Sigismondi and Weber, 1988; Mesa, 1994). In salmonids, handling increased the vulnerability to predation (Olla and Davis, 1989; Olla *et al.*, 1992a; Olla *et al.*, 1995; Schreck *et al.*, 1997), with recovery depending on stock, species and level of stress that was imposed (Schreck *et al.*, 1997).

Corticosteroid responses to stress have been investigated for many fish species (Barton, 1997), including black bream (Haddy and Pankhurst, 1999; 2000a; 2000b; Hobby *et al.*, 2000). When collected from blood as plasma cortisol (Table 2-3), corticosteroid is recognised by many as a stress indicator of choice (Barton, 1997). However, studies in recovery of predator evasion behaviour in salmonids indicate that significant variability exists in the usefulness of cortisol levels as an indicator of recovery from stress (Olla *et al.*, 1992a, 1995). In some cases Olla *et al.*, (1992a, 1995) found that there was close agreement, whilst in others there was not, with results depending on stock, species and the level of stress imposed.

Elevated levels of plasma cortisol do, however, suppress the immune response in fish (Balm, 1997) and so significantly diminish the ability of fish to deal with an infection challenge (Pickering and Duston, 1983; Woo *et al.*, 1987; Pickering and Pottinger, 1989; Johnson and Albright, 1992; Grutter and Pankhurst, 2000).

2-1-6 Potential of behavioural tests for measurement of stress

In fish, physiological mechanisms are responsible for initiating and maintaining behavioural sequences (Schreck *et al.* 1997). Therefore, once validated against physiological indicators, behavioural tests can be used rather than chemical markers as a non-lethal means of assessing whole-fish effects of stress. Juvenile Chinook salmon (*Oncorhynchus tshawytscha*) showed lethargic behaviour after multiple 30 s air exposures in a dip-net (Sigismundi and Weber 1988) and similarly Mesa (1994) showed that they exhibited lethargic behaviour, disorientation and occasional injury. Rainbow trout exposed to 60 s air exposure varied in the time taken when exercised to exhaustion (Ferguson and Tufts 1992). Rock bass (*Ambloplites rupestris*), after 30 s simulated angling and 30 s or 180 s air exposure, experienced bradycardia (Cooke *et al.*, 2001). Exhaustively-angled bonefish (*Albula* spp.), when exposed to air for 33 s, had problems maintaining equilibrium and remained motionless after release, predisposing them to attack by predators (Cooke and Philipp 2004). These relatively simple and inexpensive measures have not so far been used with black bream.

2-1-7 Aims

The purpose of this study was to provide further and broader insight into the impact of air exposure on black bream (*Acanthopagrus butcheri*) in order to assist with an understanding of the fate of fish released after capture by commercial and recreational fisheries.

Table 2-3: Examples of mean (\pm SE) plasma cortisol concentrations in selected juvenile freshwater fishes before and 1 h after being subjected to an identical 30-s aerial emersion (handling stressor). All species were acclimated to their respective environmental preferences and are listed in increasing order of the magnitude of the 1 h post-stress cortisol response (Barton *et al.*, 2002).

Species	Cortisol (ng/ml)	
	Pre-stress	Post-stress
Pallid sturgeon <i>Scaphirhynchus albus</i>	2.3 \pm 0.3	3.0 \pm 0.3
Hybrid sturgeon <i>S. albus X platorynchus</i>	2.2 \pm 0.4	3.2 \pm 0.3
Paddlefish <i>Polyodon spathula</i>	2.2 \pm 0.6	11 \pm 1.8
Arctic grayling <i>Thymallus arcticus</i>	1.1 \pm 0.3	26 \pm 4.4
Rainbow trout <i>Oncorhynchus mykiss</i>	1.7 \pm 0.5	43 \pm 3.5
Common carp <i>Cyprinus carpio</i>	7.4 \pm 2.9	79 \pm 14
Brook trout <i>Salvelinus fontinalis</i>	4.0 \pm 0.6	85 \pm 11
Yellow perch <i>Perca flavescens</i>	3.4 \pm 1.1	85 \pm 12
Bull trout <i>Salvelinus confluentus</i>	8.1 \pm 1.2	90 \pm 11
Brown trout <i>Salmo trutta</i>	1.0 \pm 0.3	94 \pm 11
Lake trout <i>Salvelinus namaycush</i>	2.8 \pm 0.4	129 \pm 11
Walleye <i>Stizostedion vitreum</i>	11 \pm 4.4	229 \pm 16

2-2 Methods

2-2-1 Part One: Recovery Time from a Standard Stressor Event

2-2-1-1 Fish

Undersize black bream (19.81 cm mean fork length; 168.9 g mean wet weight) were obtained from a commercial-operated seine net from the “Nowa Nowa Arm”, north of the “Mud Islands”; and from “Fishermans Landing Arm” in Lake Tyers (Figure 2-1) and transported to

the RMIT research facility at Lakes Entrance. All 56 fish received at least 3 weeks acclimatisation in six 1.58 m diameter, circular 2000 L flow-through tanks, each receiving approximately 5-8 L/min of $12 \pm 3^{\circ}\text{C}$ filtered and aerated water pumped from the “Cunningham Arm” region of the Gippsland Lakes. Fish were about equally divided amongst the tanks, with two tanks containing 11 fish each, one tank with 10 fish, and three tanks with 8 fish each. A natural photoperiod was maintained during the acclimatisation period, light entering the facility through skylights. It was decided not to cover tanks during the acclimation period in case fish habituated to this process. Given the close proximity and positioning of all experimental tanks, there was very little risk of differential light treatment. Fish were fed to satiation every second day on live sandworm *Australonereis ehlersi* (Nereidae).

2-2-1-2 Apparatus

After Sigismundi and Weber (1988), behavioural experiments were undertaken in three wooden troughs, each having four channels 0.3 m wide, 0.8 m long, and 0.54 m deep (Figure 2-2). Each trough was partially submerged to a depth of 10cm in a 2 t tank identical to those utilised for acclimatisation. A number of 8.0 mm diameter holes in the floor of each channel allowed the passage of aerated tank water into and out from the apparatus.

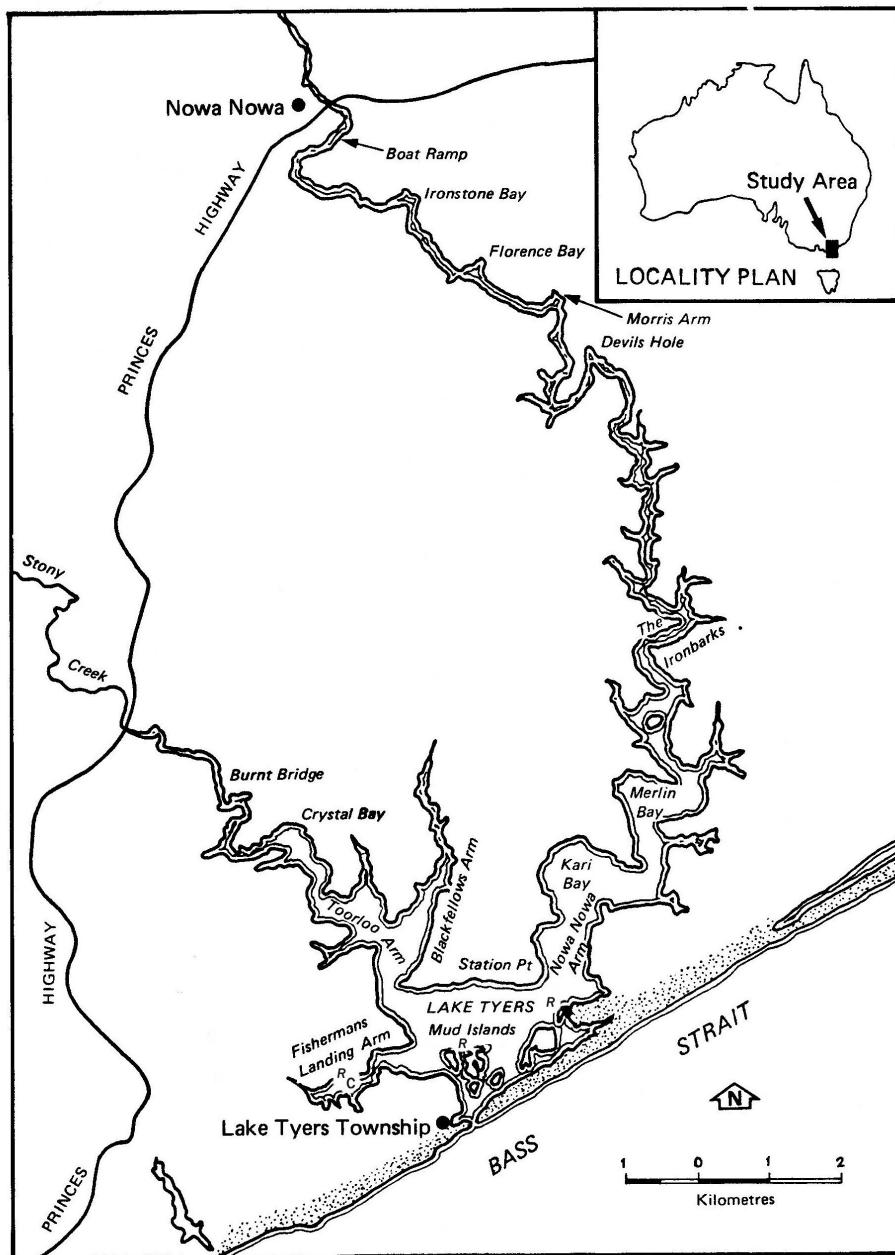


Figure 2-1: Map of Lake Tyers. Undersize black bream caught by a commercially operated seine net for: (i) the recovery experiment (R) were obtained from the “Nowa Nowa Arm” north of the “Mud Islands”; and “Fishermans Landing Arm” and (ii) the cumulative effects study (C) were obtained from “Fishermans Landing Arm” only.

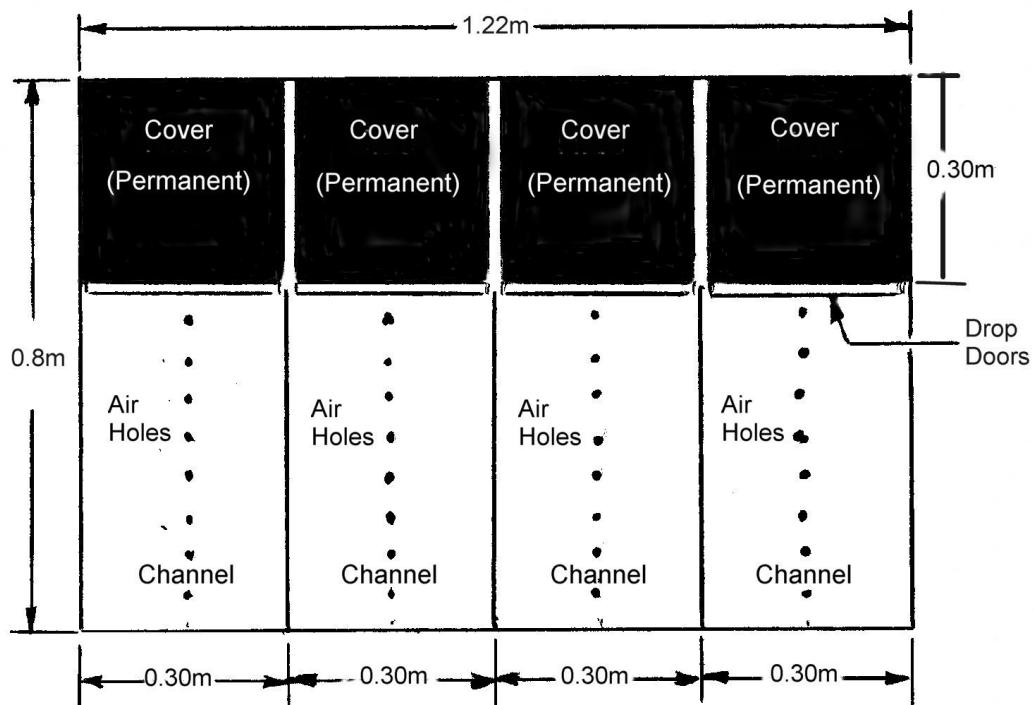


Figure 2-2: Diagram of channel apparatus used to test response times of undersize black bream when exposed to a startle stimulus, namely an overhead light, removal of a protective cover and opening of an escape gate.

Aeration within each channel was further supported by air stones centrally controlled by a manifold. Each channel was divided by a vertically operated gate, which when raised allowed unrestricted access to the complete length of a channel. A permanent 30 cm-long black cover was secured at one end of each channel; remaining compartments were covered with removable black plastic sheets. Tanks containing the test apparatus were illuminated with 400 W ceiling-mounted lights located 4 m above each tank. Water quality was monitored daily in all tanks containing fish and apparatus.

2-2-1-3 Response time

The response time was measured as the time it took for a fish to swim under cover following removal of plastic sheets whilst the apparatus was illuminated by overhead lights (after Sigismondi and Weber, 1988). Preliminary experiments determined the apparatus design, appropriate light source, number of fish to use in each channel and location and degree of conspicuousness of people around the apparatus. It was found that having a single fish in each channel offered the best opportunity to measure the responses to a “treatment”. With more than one fish, preliminary investigations found that there were fish-to-fish interactions on some occasions.

2-2-1-4 The stressor

Each “treatment” fish was held in the air with a dip net above the apparatus for 30 s; controls were fish similarly placed in the apparatus but which did not receive this additional handling and air exposure (Barton *et al.*, 1986; Sigismondi and Weber, 1988). The behavioural responses of the fish were then measured immediately or after a recovery period of 6 or 12 h (after Sigismondi and Weber, 1988).

2-2-1-5 The behavioural test

With the gates closed, the dark hide was not available to the test fish. Individual fish were placed in the non-hide compartment of each channel. Fish holding tanks were close to the test apparatus and so it took approximately 3-5 s to transfer fish to the apparatus once netted. Channels were then covered with the black plastic sheets to provide a dark shelter for the fish, and fish were left to adjust to the apparatus for approximately 16 hours before a behavioural test was commenced. For each experimental run, those tanks containing the apparatus were randomly selected for each of the three behavioural tests (i.e. recovery after 0 h, 6 h and 12 h post stressor event). The randomisation was achieved by drawing identical cards labelled from 1 to 3 from a hat and sequentially matching the outcome to a list of the three behavioural tests to be performed. In a similar fashion, the location of the “control” channel out of the four available in each apparatus was determined by “drawing from a hat” one card from four that matched each channel. Those remaining channels were assigned for treatments.

After the acclimation period of 16 h, and at the appropriate times, those fish labelled for treatment were captured in the channel to which they had been assigned with a dip-net (approximately 3-5 s) and exposed to the 30 s air exposure stressor, after which they were returned to the apparatus and covered until the appropriate recovery time (0 h, 6 h, 12 h) had elapsed. Controls were left untouched (after Sigismondi and Weber, 1988).

Following the allotted “recovery times,” avoidance response was measured by initiating a startle reflex, and recording the time taken for the fish to swim under the permanent cover. The startle stimulus was the removal of the covering sheet over the channel, thus exposing the fish to the overhead light, while simultaneously opening the gate to the covered end of the channel, thus providing access to the darkened refuge. Aeration was stopped individually in

each channel so that observations of fish behaviour were unobstructed during each trial. The dissolved oxygen for each apparatus was recorded on a daily basis. A trial ended when the fish swam under the cover, or alternatively after 30 min. In a similar fashion to Sigismondi and Weber (1988), aspects of behaviour during the trials were also recorded, namely:

- if a fish swam out from under the hide,
- if a fish explored a channel,
- if a fish remained motionless, and
- if motionless, a fish's position in the water column.

This procedure was repeated for all channels in each apparatus. To determine if there was any diurnal influence on fish recovery, the three behavioural tests (0 h, 6 h, 12 h post-stressor) were each performed at three time periods: early morning, late morning and afternoon. Trials were run sequentially over 5 days for each recovery period and control, with one-three fish of each treatment per day (total of 13-15 fish per treatment), enabling analysis for diurnal influence. Separate fish were used for each treatment and recovery period to satisfy independence.

2-2-1-6 Statistics

An exact calculation of mean and standard error could not be undertaken because some fish did not respond within the assigned time period of 30 min. This meant that the ultimate time could not be recorded. Therefore, the response time was categorised as: 1: 0-5 min, 2: >5<=10 min, 3: >10<=15 min, 4: >15<=20 min, 5:>20<=25 min, 6: >25<=30 min, 7:>30 min. Categorical data were tested for normality and analysed for differences in response time between air exposure treatments using Analysis of Variance (ANOVA) with post-hoc Tukey's family error tests and linear regression using the statistical program Minitab

(www.minitab.com). Categorical data were also analysed by the non-parametric Mood's Median test. Linear regression was also used to test the effect of recovery time on air-exposed fish only. Significance was recorded at $p<0.05$.

2-2-2 Part Two: Cumulative Effects

2-2-2-1 Fish

Undersize black bream were obtained from a commercially operated seine net. For this study, the same commercial operator supplied fish from one site in Lake Tyers, namely "Fishermans Landing Arm" (Figure 3-1) on a single date.

2-2-2-2 Transportation

The bunt of the commercial seine net was left in the water and black bream were removed from the bunt of the net with 10 L plastic buckets and placed into two of four 75 L covered aerated plastic tubs located in a 3.6 m aluminium boat moored or located close to the retrieved net. Each tub was previously prepared with water obtained from that location (the water in the two remaining 75 L tubs was required for later use in the laboratory; see 3-2-12 below). Fish were then transported (15–20 min) to prepared tanks at the Lakes Entrance research facility. Stocking densities of fish in the transport tubs never exceeded 100 g L⁻¹.

2-2-2-3 Tanks

Four adjacent circular tanks were used in this study. The receiving tanks had been cleaned and desiccated prior to the experiment, before being refilled with water. Each of these 2000 L, 1.58 m diameter flow-through tanks received approximately 5-8 L min⁻¹ of filtered and then aerated water at $12 \pm 3^\circ\text{C}$ pumped from the Cunningham Arm.

2-2-2-4 The cumulative effect test

On arrival at the facility, eight fish from each of the two tubs were removed with 10 L plastic buckets and placed into a single tank. This “control” tank ($n=16$) was then left undisturbed for the duration of the experiment. After Barton *et al.*, (1986), treatment fish were subjected to three degrees of stress:

- (a) single 30 s air exposure as in Section 2-2-5 ($n=18$)
- (b) two such air exposures separated by a 3 h interval ($n=17$), or
- (c) three such air exposures, each 3 h apart ($n=17$).

For the single handling stressor experiment (a), approximately equal numbers of fish were removed from both transport tubs with the dip-net, and transferred to a holding tank after a 30 s air exposure. For the experiment involving two handlings, experiment (b), fish were again removed from the first set of two tubs with the dip-net and provided with 30 s of air exposure, but were then transferred into a second pair of aerated tubs containing water from the field collection point. After 3 hours, the dip-net was again used to transfer a number of fish to a holding tank, allowing a second period of 30 s of air exposure during the transfer. For three handlings of fish (c), a similar procedure was followed, but with three air exposures, until the fish ended up in their specific holding tank.

No further handling of fish was required after this and tanks were then only minimally disturbed for activities such as removing a dead fish, feeding or monitoring of water quality. Each tank was kept under the natural photoperiod offered by the facility, with fish observed on a daily basis for the next 3 months, with recording of mortalities and any behavioural events. During this study, all fish were fed to satiation with live sandworm *Australonereis ehlersi* (Nereidae).

2-2-2-5 Necropsy of fish

A modified necropsy method (after Goede and Barton, 1990; Adams *et al.*, 1993; Goede, 1993; Haddy and Pankhurst, 1998) was performed on each dead fish. Dead fish were collected from the tanks, weighed and fork length measured. External and internal organs were classified by appearance (after Goede and Barton, 1990; Adams *et al.*, 1993; Goede, 1993) of: thymus, fins, skin, eyes, gills, pseudobranchs, spleen, kidney, liver, gall bladder, hindgut and presence or absence of parasites. Hepatosomatic and gonadosomatic indices were also obtained. A macroscopic staging of gonads (Table 2-4) was also undertaken (after Haddy and Pankhurst, 1998). Muscle tissue pH was also measured.

In order to identify possible influential health factors predisposing individual animals to mortality, at the end of the experiment four randomly-selected control fish were collected, killed and autopsied in the same manner.

2-2-2-6 Statistics

Categorical data were tested for normality and analysed for differences in proportion of mortalities between air exposure treatments using Analysis of Variance (ANOVA) with post-hoc Tukey's family error tests and Mood's Median tests as in Part One. Linear regression was also used to test the effect of recovery time on air-exposed fish only as before. Significance was recorded at p<0.05.

Table 2-4: Criteria for macroscopic classification of black bream gonads (Haddy and Pankhurst 1998).

Stage	Classification	Macroscopic appearance	Histological characteristics
<i>Female</i>			
1	Immature	Ovary small clear threads	Previtellogenic oocytes
2	Regressed	Ovary small clear and orange	Cortical alveoli-stage oocytes appear
3	Vitellogenic	Ovary orange with opaque oocytes visible through epithelium	Oocytes in exogenous vitellogenesis
4	Hydrated	Ovary orange with hydrated oocytes visible through hydration epithelium	Final oocyte maturation and
5	Ovulated	Eggs in the oviduct can be extruded with gentle pressure	Hydrated oocytes in the oviduct, post-ovulatory follicles present
6	Spent	Ovary flaccid and bloody	Atretic vitellogenic oocytes but predominantly previtellogenic oocytes present
<i>Male</i>			
1	Immature	Testis white threads	Spennatogonia and a few previtellogenic oocytes ^A
2	Spermatogenic	Testis firm and ivory white	Secondary spermatocytes, spermatozoa
3	Partially spermated	Testis firm and ivory white	Spermatozoa predominate
4	Fully spermated	Testis firm and ivory white with free-flowing milt in sperm duct	Spermatozoa predominate
5	Spent	Testis grey to bloody and flaccid	Residual spermatozoa, reduced spermatocytes and increased connective tissue

^AOocytes in dorsal section of gonad in all male stages.

2-3 Results

2-3-1 Part One: Recovery time from a standard stressor event

There were significant differences in categorical data with treatment (ANOVA, $F=52.34$, $p<0001$; Mood's median test, $\chi^2=28.47$, $p<0.001$). Post-hoc Tukey's family error tests showed that each treatment was significantly different from the others (Figure 2-3). The least median response time was for the control treatment and median response time decreased with recovery time; regression analysis showed that the response category = $6.73 - 0.276$ recovery time (h) ($R^2=50.7\%$; ANOVA, $F=41.16$, $p<0001$). By contrast, there was no significant effects of day or time of day on response time (Day: ANOVA, $F=0.06$, $p=0.994$, Mood's median test, $\chi^2=0.47$, $p=0.976$; Time of day: ANOVA, $F=0.33$, $p=0.721$, Mood's median test, $\chi^2=1.67$, $p=0.435$) Calculations and outputs of statistical tests for recovery times are in Appendix 2(a).

There was a large range of response times for the treatment fish ($6.31 - >30$ min) (Table 2-5). Most of those fish tested immediately after air exposure did not move or made little attempt to seek shelter. When tested at 6 or 12 hours after air exposure, response times were again highly variable Although significantly quicker at seeking shelter than treatment fish, controls also displayed a range of response times (0.80-9.72 min.) and differing behaviours. There was greater variability in response times with 6-12 h recovery time than with no recovery time or with the control treatment (Figure 2-4).

Individual fish behaved in a variety of ways to the startle stimulus, ranging from an early response to seek cover to remaining motionless with only slight operculum or ventral fin movement (Table 2-5). Fish provided with no recovery period following air exposure typically remained in one place near

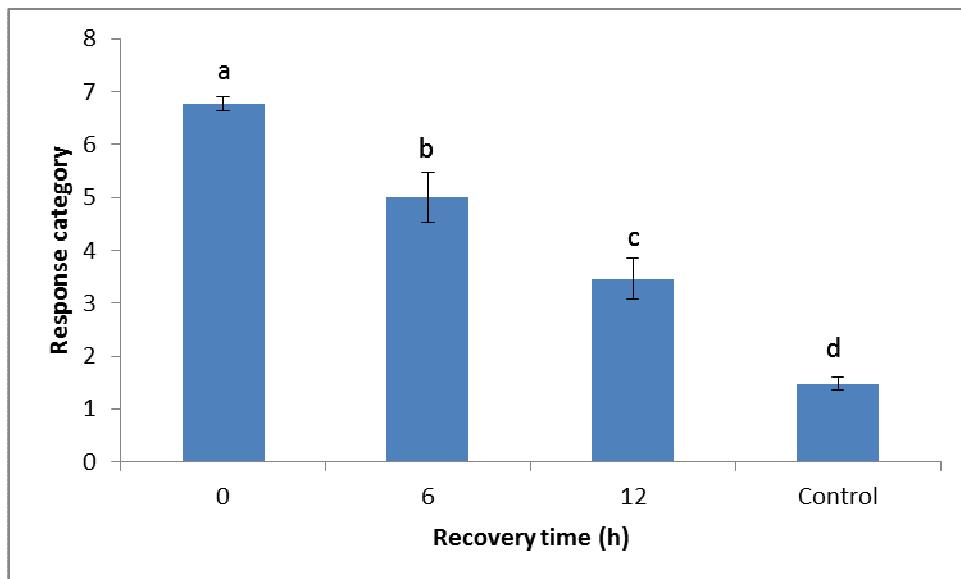


Figure 2-3: Black bream median response time (categories) to swim under cover when exposed to overhead light after allowing 0, 6 or 12 h recovery following air exposure for 30 s. Values that do not share a letter are significantly different from post-hoc Tukey's family error test at $p<0.05$ following ANOVA ($F=52.34$, $p<0.001$). Data are means \pm SE. Categories: 1: 0-5 min, 2: $>5\leq 10$ min, 3: $>10\leq 15$ min, 4: $>15\leq 20$ min, 5: $>20\leq 25$ min, 6: $>25\leq 30$ min, 7: >30 min ($n=13-15$ per treatment).

Table 2-5: Individual black bream response times (min) to seek cover under a hide when suddenly encountering an overhead light after being subjected to a simple stressor (30 s air exposure in a dip-net); fish were allowed differing periods of time to recover after the air exposure (0 h, 6 h and 12 h), and no trial exceeded 30 min. Controls were not subjected to the 30 s air exposure in a dip-net. Trials were conducted in both morning and afternoon.

^EEarly morning, ^LLate morning, ^AAfternoon.

Recovery time (h)	Median response time (min)					
	Day 1	Day 2	Day 3	Day 4	Day 5	Median
Control	5.10 ^L	3.28 ^A	5.35 ^E	1.82 ^A	6.00 ^L	4.28
	9.72 ^E	0.80 ^A	3.15 ^L	6.17 ^A	1.02 ^E	
	4.28 ^A	1.39 ^E	7.40 ^A	2.12 ^L	5.01 ^A	
0	escaped	>30 ^A	>30 ^E	>30 ^A	>30 ^L	30
	>30 ^L	>30 ^A	>30 ^E	29.13 ^A	>30 ^L	
	27.00 ^L	>30 ^A	24.72 ^E	>30 ^A	-	
6	12.80 ^E	26.04 ^A	16.25 ^L	17.55 ^A	9.04 ^E	22.19
	18.34 ^E	30+ ^A	30+ ^L	16.04 ^A	>30 ^E	
	>30 ^E	13.45 ^A	26.38 ^L	28.36 ^A	-	
12	7.24 ^A	6.31 ^E	10.55 ^A	22.54 ^L	18.35 ^A	10.55
	21.28 ^A	8.72 ^E	20.62 ^A	8.72 ^L	-	
	21.26 ^A	10.01 ^E	9.40 ^A	24.30 ^L	-	

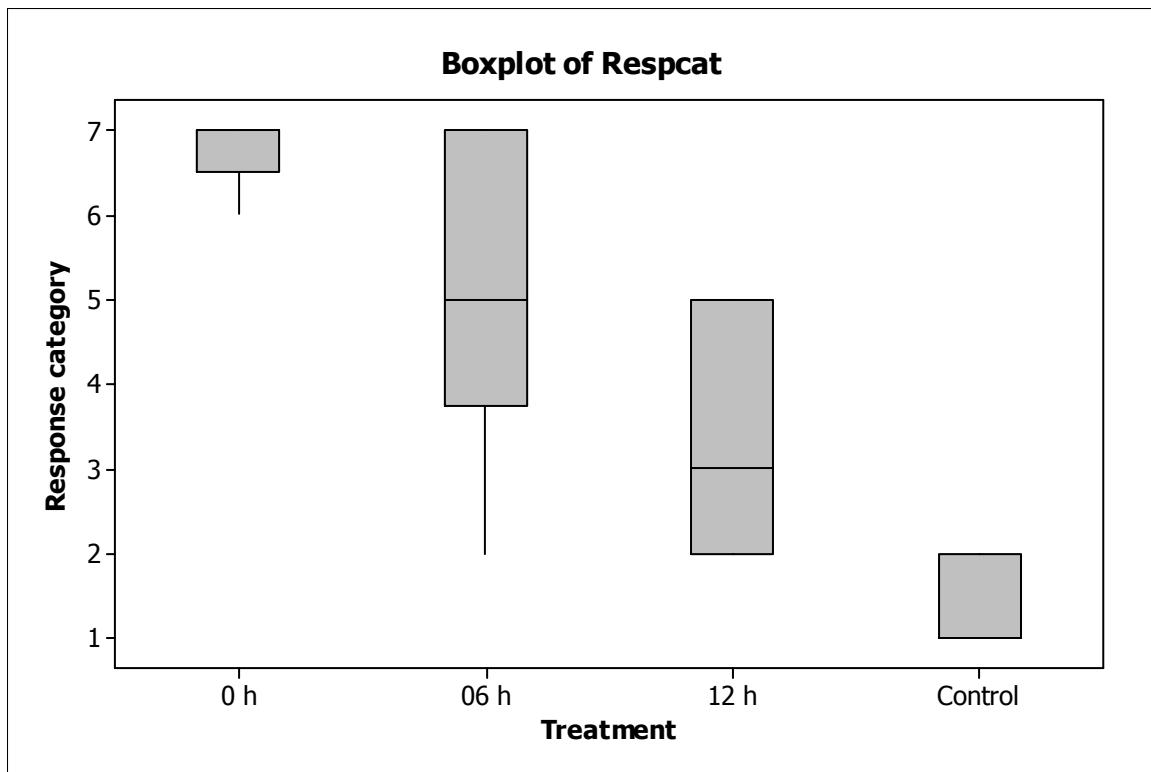


Figure 2-4. Boxplot showing variability of data for each treatment. Boxes extend from the bottom quartile (bottom limit) to the top quartile (top limit), means are marked by the horizontal lines within the boxes and whiskers extend to the greatest data value within the upper limit. $[Q3 + 1.5 (Q3 - Q1)]$ or least value within the lower limit $[Q1 - 1.5 (Q3 - Q1)]$.

the bottom of the water column at that location where they were released from the dip-net after application of the stressor for most for the 30 min duration of the behavioural test. Fish provided with recovery times of 6 h or 12 h before the startle stimulus showed a contrasting range of behaviour, from remaining motionless to that of a steady movement toward the hide. All fish preferred the bottom of the water column. Preliminary experiments indicated that stressed fish when challenged ignored additional stimuli. For example, placing a stick into the water immediately after the air exposure stressor elicited no avoidance response. However, after 6 h or 12 h of recovery time, the appearance or movement of a stick within sight of the fish at times elicited a response – usually a rapid swimming away from the vicinity of the stick – and not always toward the hide. This behaviour was similar to that seen in the

unstressed control fish. No fish displayed disorientation to the point of being inverted in the water column.

Stopping the airflow during the experiment reduced the dissolved oxygen level from 87% to 46% within the first 5 min and it reached a plateau of about 36-40% within the first 10 min (Figure 2-5).

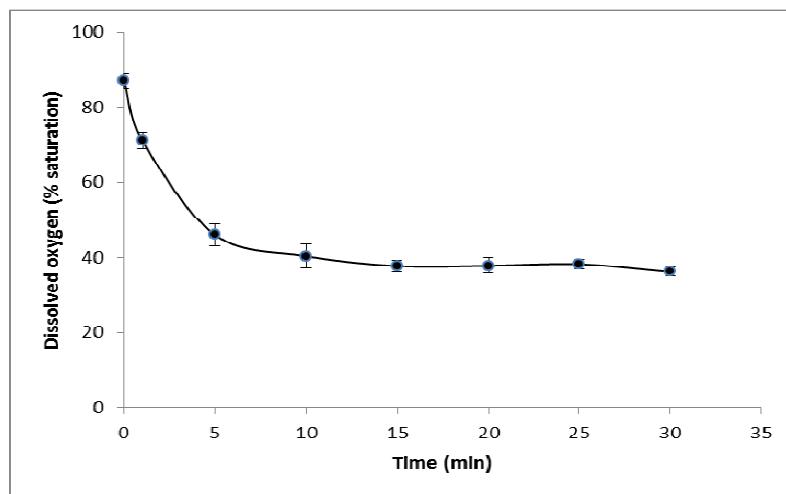


Figure 2-5: Reduction in dissolved oxygen levels during behavioural trials (30 min duration) after cessation of aeration in holding channels. Values are averages \pm standard deviations for each trial ($n = 132$).

2-3-2 Part Two: Cumulative Effects

Healthy black bream in all tanks had an overall light colouration and preferred to remain on or close to the bottom of the water column. By contrast, unhealthy/stressed animals developed a dark colouration and a head-down orientation in the water column (Figure 2-6) and were found progressively higher in the water column of the tank. The dark colouration was only apparent when fish were in the water. A small number of fish, including controls, displayed dark snouts 72 h post-capture, but this disappeared over the next 5-7 days. This was possibly due to physical contact with hard objects during the capturing process. All fish in the control tank were actively feeding by 48 –96 h post-capture. Fish dispersed in the water column of treatment tanks actively sought food; those animals located at, or near the surface did not.

Mortalities began at day 5 and continued to day 62 after air exposure (Figure 2-7). Increasing stressor application resulted in an increasing percentage of fish mortalities (Figure 2-8), with a significant difference between treatments in chi-square tests on contingency tables ($\chi^2=11.372$, $p=0.01$). The cumulative impact of one, two and three sequential air-exposures in a dip-net (the applied stressor) was significant (correlation coefficient $r = 0.956$, $p=0.044$). The linear regression for % mortality = $12.9 + 18.0 \times$ Number of stressors (air exposures), $R^2 = 87.0\%$, (ANOVA , $F=21.14$, $p=0.044$). The control treatment had significantly fewer mortalities than the single exposure treatment, which had fewer mortalities than the double and triple exposures, which were not significantly different from one another. Calculations and outputs of statistical tests for cumulative effects can be found in Appendix 2(b).

When released, those animals subjected to repeated air exposure generally floated to the bottom of a tank with very little movement or activity. Control fish released into their

holding tank actively swam to the bottom. Within the first 24 h, there were no other discernable differences in either appearance or behaviour between treatment fish and



Figure 2-6: Preliminary investigation illustrating behaviour of undersize (< 28cm) black bream (*Acanthopagrus butcheri*) in a tank ~72 h after capture and transportation. Healthy fish are those of light appearance at the bottom of the tank. Unhealthy animals were disorientated, of dark appearance which was apparent only while fish were in the water, and physically located higher in the water column (arrows). Note also the light colouration of healthy fish matching their surrounding environment.

controls. However, after 72 h, 3-4 fish in those tanks subjected to two and three air exposures were of a dark appearance and had physically separated from healthy cohorts. The most severely stressed of these animals were higher in the water column with a head-down orientation. By comparison, only one fish in the “one stressor” tank moved away from the bottom, but was still of light appearance. At the same time, controls were all of a light appearance and swimming close to the bottom of the tank.

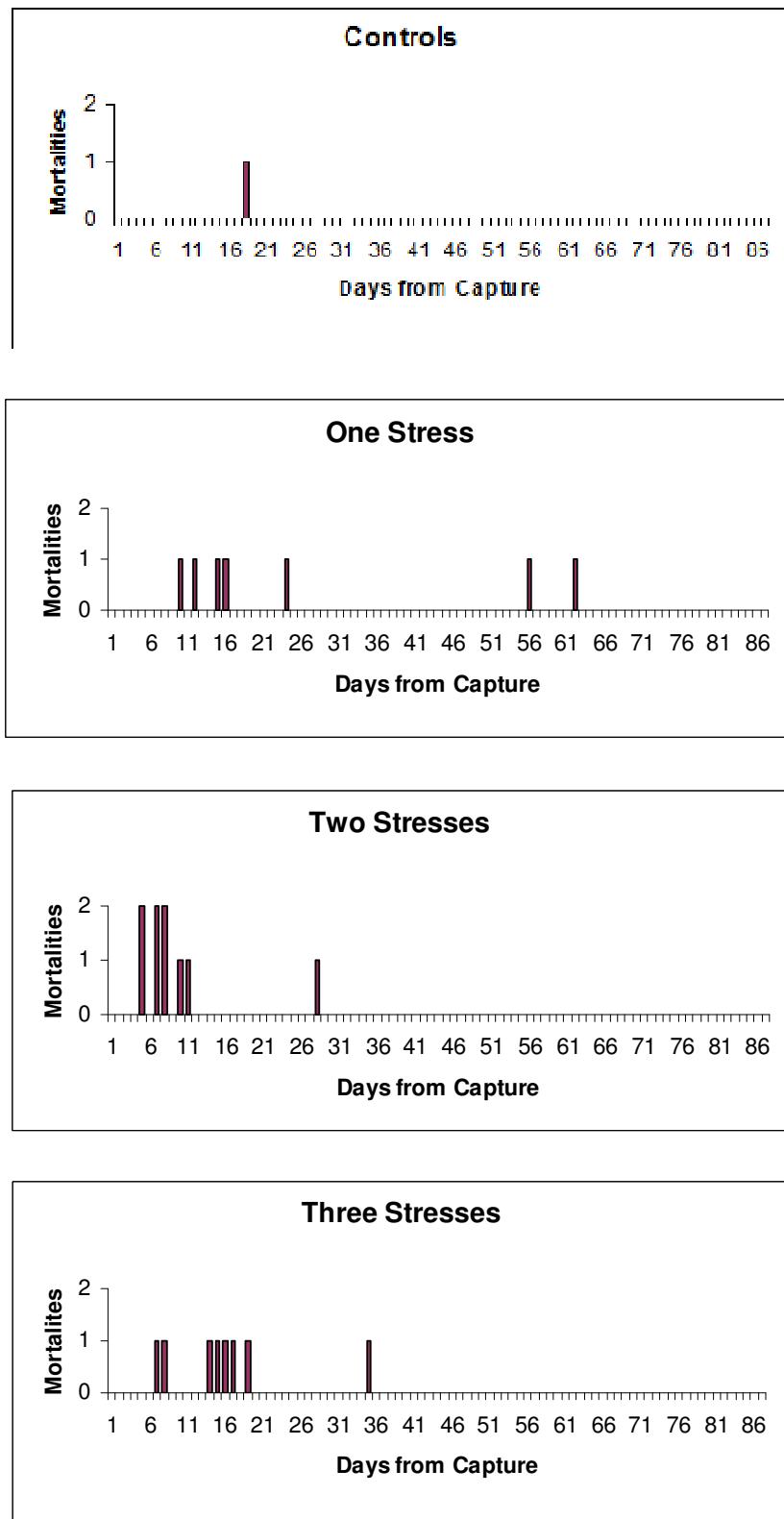


Figure 2-7: Incidence of mortality for each stress event from day of capture and 0, 1, 2, or 3 treatments (39-s air exposure stresses) of 30-s air-exposure in a dip-net with a 3-h recovery between treatments. Controls had no air exposure..

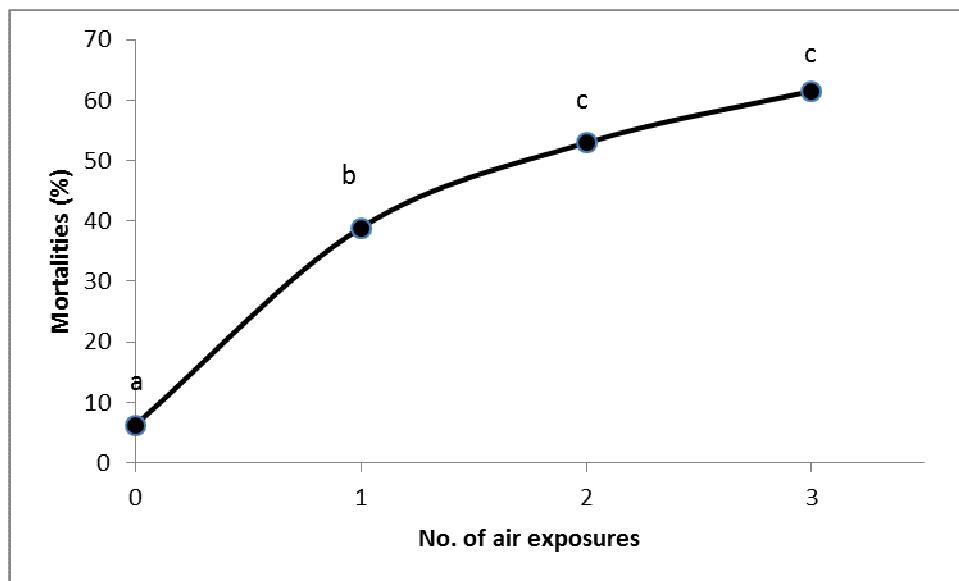


Figure 2-8: Cumulative mortality in response to air exposure for black bream. Each “treatment” (applied stressor number), was a 30 s air-exposure in a dip-net; for multiple “treatments” (2 or 3 stressor events) there was a recovery period of 3 h between each application. Controls (0 stressor number) were fish that had no air exposure. All mortalities occurred over a 62-d period. A correlation coefficient (r) = 0.956 ($p=0.044$) for percentage mortality versus stressor number. Chi-square (contingency table) analysis $\chi^2=11.372$, $p=0.01$). Linear regression: % mortality = $12.9 + 18.0 \times$ Number of stressors (air exposures), $R^2 = 87.0\%$ (ANOVA , $F=21.14$, $p=0.044$).

A progressive deterioration was observed in the stressed individuals, which led to further displacement away from the bottom of tanks. It appeared that these animals were physically unable to swim to the bottom of the tank, although some tried to when attempting to feed. Eventually, the stressed fish ended up in a moribund state on the surface, with the first mortality observed at 120 h after treatment (Figure 2-6). Although mortality in individual tanks increased progressively but differentially over the next 62 days (Figure 2-6), all fish about to die went through the same physical deterioration over a period of 48-72 h before dying. Most appeared normal up to the time of the physical rapid deterioration and death.

Necropsy. Extensive erosion of fins and tail was only evident in animals that had died, along with often extensive necrotic lesions in the vicinity of the caudal peduncle. Eyes were generally clear, with little evidence of any pathological condition. The gills, by contrast, displayed erosion of gill lamellae and some clubbing at margins along with a light colouration on some specimens, and excess mucus was universally present. Excess mucus was not evident in control fish. Gill pseudobranchs of mortalities displayed no observable differences from control specimens. The thymus usually appeared normal, with only a mild haemorrhaging in some mortalities. All observed mortalities except one were male, and along with sacrificed controls, had undeveloped gonads identified as a stage 1 according to Haddy and Pankhurst (1999). No observable abnormalities were evident in either spleen or kidney. Some inflammation (swollen and red) was observed in the hindgut of animals that had died. Full gall-bladders, containing dark-green to green bile, were characteristic of mortalities (Index value of 3). Control animals, however, displayed either empty or only partially full gall bladders, with a clear bile present (Index value of 0). Without exception, all mortalities had inflated swim-bladders. This was not evident in controls. No mesenteric fat was observed in any fish. Some mild post-mortem changes were evident on fish that had died. There was gill discolouration and expression of moisture from flesh when gently

pressed on several specimens. Lenses of eyes on all samples were clear, and only one specimen displayed a low pH (6.6) of muscle tissue, indicating a post-rigor condition.

2-4 Discussion

Both the behavioural and survival tests showed the significant impact of air exposure on black bream in terms of slower responses and greater mortality. This is the first time that a behavioural test has been used to detect stress of the type that could reduce the survival of fish released after capture by commercial and recreational fisheries in the presence of predators such as sea birds. This is also the first time that repeated air exposures, such as could be experienced by undersized black bream during heavy fishing, has been shown to significantly increase mortality to up to 50% of fish, compared with only one such exposure. These results have important consequences for the catch-and-release policies currently in place for undersized fish.

2-4-1 Preliminary Investigations

(a) *Operation of apparatus:* Preliminary studies found that if more than one fish were placed in each channel of the apparatus there was a risk of social interactions between fish that would confound results. For example, at times it was observed that one animal would prevent or delay another from entering a hide. Under such circumstances, it was difficult to know whether the recorded response times were the result of the external stimulus or the consequence of fish-fish interactions. It was therefore decided that all behavioural investigations when utilising the apparatus would be undertaken with a single fish in each channel. With the difficulty in obtaining sufficient numbers of this fish from the wild, this approach was also added insurance against apparatus failure – in such an event only a minimal number of naïve fish would be lost to the experiment.

(b) *Infrastructure requirements and orientations:* Preliminary investigations demonstrated the importance of minimal operator activity around the apparatus during trials – all investigations and observations were carried out from behind a hide. The selection of appropriate lighting also required several preparatory trials using a number of differing light sources. A source was required that would not “blind” the fish, but stimulate it. Daylight was found to be the most appropriate, but unfortunately this source reached the apparatus in tanks via “skylights” (poly–methyl methacrylate sheeting located in the roof), resulting in shadows being cast at different locations within channels at different times of the day, or alternatively, a much diminished illumination on overcast days. The decision was to use overhead fluorescent lighting already in place for normal illumination in the building and to locate each of the three testing apparatuses under that lighting in order to ensure a consistent illumination across each, with enough intensity to stimulate fish.

(c) *Minimising duration of stress:* There is always a potential problem when undertaking behavioural experiments to induce stressors other than those associated with the “treatment.” In these experiments, fish had to be transferred to the test apparatus. In addition, when repeatedly catching animals out of the same container, cohorts remaining behind would have been stressed by the capture attempts. In the recovery study, an attempt was made to minimise this, by locating holding tanks as close as possible to each apparatus, and by allowing each fish approximately 16 h to recover and acclimate to its new surroundings before experimentation. In this situation, control animals underwent the same preparatory stresses as the treatment animals, but it was intended that they would be less stressed than animals receiving the “treatment” (30 s air exposure). Thus, all fish were handled the same during transfer, with the only difference being the “treatment”. In the cumulative response investigation, handling and transfer of fish was designed into the experimental protocols. Any additional stress experienced by cohorts remaining in tubs for a longer time as part of the

treatment process would be expected to have a cumulative enhancing effect rather than a confounding effect. If present, these additional stressors might have contributed to the magnitude of the results obtained.

2-4-2 Behavioural Response after Air Exposure

This study shows for the first time that the application of a simple common handling stressor, a 30 s air exposure, influenced black bream response to a stimulus (sudden bright light), the response measured being a delay in the time for this fish to swim to seek cover under a hide. This sluggish behaviour would severely reduce a fish's chance of survival if released after catch-and-release, as it is common for birds to frequent fishing boats and anglers. Similar delays in recovery after air exposure have been reported in other studies, such as in juvenile Chinook salmon (Sigismondi and Weber 1988; Mesa 1994), where lethargic behaviour, disorientation and occasional injury were reported after single air exposures. It is also similar to the problems of maintaining equilibrium and lack of motion after release noted in exhaustively-angled bonefish (*Albula* spp.) after exposure to air for 30 s. Ferguson and Tufts (1992) identified 60 s air exposure as a significant additional stressor after rainbow trout (*Oncorhynchus mykiss*) were exhaustively exercised. Cooke *et al.* (2001) found that rock bass (*Ambloplites rupestris*), after 30 s simulated angling and 30 s or 180 s air exposure, experienced bradycardia, and identified air exposure as a significant additional stressor.

2-4-3 Recovery: Acclimation Periods

Observation during preliminary investigations for this study and other research undertaken at the Lakes Entrance facility indicated that black bream (originating from both the Gippsland Lakes and Lake Tyers) required approximately 3 weeks acclimation prior to any experimentation in order to recover from stressors associated with the capture from

commercial seine nets and the transportation process back to the research facility. If there were to be fish mortalities associated with the capture operation, they would generally occur within this period. This was similar to studies on other fish, which have found considerable differences in acclimation times even for a single species. Artigas *et al.* (2005) reviewed acclimation times and estimated that cod (*Gadus morhua*) required as little as 15 min or, alternatively, several weeks. Similarly, brown trout (*Salmo trutta*) had acclimating periods ranging from 3 days to more than 2 weeks. Influencing factors included degree of handling stress, individual species' sensitivity to handling, physiological status, temperature, photoperiod, age and sex of fish (Artigas *et al.*, 2005).

2-4-4 Recovery: Variable Experimental Results

Fish provided with a recovery time of 6 h or 12 h after the air exposure stressor showed considerable variability in response times, implying that some black bream were better able to cope with the stressor than others. However, this variability is often encountered in stress studies and can be influenced by the intensity and duration of the stressor (Strange *et al.*, 1978; Barton *et al.*, 1980; Foo and Lam, 1993 Barton,1997; Olla *et al.*, 1998); differences between strains of fish (Barton *et al.*, 1986; Pickering and Pottinger, 1989 and Pottinger and Moran, 1993; differences between discrete stocks (Iwama *et al.*, 1992); differences between hybrids (Williamson and Carmichael, 1986; Noga *et al.*, 1994). Given the fishing pressure on black bream in the Gippsland Lakes and Lake Tyers from both the commercial and recreational sectors and the homogeneity of the fish stocks, the variability is probably influenced by prior encounters and experience with fishing gear, in particular dip nets, which are commonly used to collect fish from the cod-end of a net or to retrieve a fish caught on a line at the side of the boat (Schreck *et al.*, 1997; Gray *et al.*, 2001 and Meka and McCormick, 2005).

An early recovery (lower response time) from a stressor would offer a selective advantage to animals in the natural environment, reducing risks from predators and other hazards (Sigismondi and Weber 1988). In the Gippsland Lakes there is significant predation on fish by birds, particularly from the great cormorant (*Phalacrocorax carbo*) (Coutin *et al.*, 1997; Reside and Coutin, 2001 and Cowx, 2003). Survival of undersize black bream would be enhanced after fishing capture and release if recovery from the stressor was rapid. In these experiments, fish receiving the stressor of air exposure often remained stationary for some time on release. This would make these fish vulnerable to a predator such as the great cormorant.

2-4-5 Cumulative Response in Black Bream

This investigation demonstrated for the first time that multiple application of a simple common stressor, when sequentially applied, resulted in a progressive deterioration in response time in black bream that this was indicative of fish mortality in the longer-term study. The only partially comparable studies in black bream are those of Grixti *et al.* (2008), who showed that in black bream caught in the Glenelg River, SE Victoria, mortality varied from 11-89% after 3 days incubation in tanks, but there was only one mortality in the 266 seine net-caught control fish (0.4%) and so most of the deaths were attributed to injuries from angling. By contrast, in this study there were no deaths in seine net-caught control fish within the first 3 days and only one death over 62 days, and most of the deaths were attributed to the air-exposure(s) acute stressor.

Other studies have also shown that multiple stressors (the same or differing stressors applied a number of times, or different stressors applied at the same time) can evoke cumulative behavioural and physiological responses (Barton, 1986; Pickering and Pottinger, 1987; Maule *et al.*, 1988; Peters *et al.*, 1988; Sigismondi and Weber, 1988; Mesa and Schreck, 1989; Jarvi,

1990 and Mesa, 1994; Waring *et al.*, 1997; Mugnier *et al.*, 1998; Pickering, 1998; Sharpe *et al.*, 1998; Ortuno, *et al.*, 2002) and, in extreme cases, result in mortality (Wood *et al.*, 1983; Kwak and Henry, 1995; Winckler and Fidhiany, 1996; Wagner *et al.*, 1997; Davis, 2002).

The large discrepancies in response times in fish from the air exposure treatments may have been due to elevated cortisol levels. Haddy and Pankhurst (1999) and Hobby *et al.* (2000) compared cortisol levels (as a chemical indicator of stress) in black bream at different times after capture and consequential air exposure. Haddy and Pankhurst collected two blood samples from each fish, one as soon as possible (5 min) after capture by rod and line and the second after 15 min-24 h of tank confinement. There was little plasma cortisol at capture and levels were similar to those of snapper using an underwater sampling technique to avoid air exposure (Pankhurst and Sharples, 1992). Cortisol levels in black bream then rose rapidly within the first 15 min -1 h of confinement followed by a slow decline over 24 h (Haddy and Pankhurst, 1999). Similarly, Hobby *et al.* (2000) reported cortisol concentrations of >200 ng/ml in blood samples taken from black bream after capture, handling and transportation. In order to remove the hook, collect the first blood sample (within 5 min of capture) and transfer individual fish to a confinement tank, each animal must have experienced not only air exposure but also trauma similar to that reported by Grixti *et al.* (2008) and so part of the initial rise in confinement was probably due to angling as well as simple air exposure. If the acclimatised fish in the short-term behavioural studies behaved as did the caught fish in these studies, their cortisol level would have soared within the time in the channel apparatus and this may explain why fish that did not react within the first 5-10 min frequently took longer than 30 min to seek shelter. A factor exacerbating this torpor was the halving in dissolved oxygen concentration in the channel, from 87% to 40%, within the first 10 min and plateauing at this reduced level up to 30 min; fish that did not react within the first 5 min had reduced capacity to do so.

In this study, the undersize black bream were asymptomatic for gross morphological appearance for at least 48 h after the air exposure stressor. After that time, deaths occurred in fish receiving multiple air exposures. A time delay of this magnitude would hide the existence of such an impact on undersize black bream in any catch-and-release or discarding operations from either recreational or commercial sectors – all these fish would have appeared healthy when released.

There was a cumulative mortality of black bream following the multiple application of the air exposure stressor. Immunosuppression cannot easily account for the delayed onset of mortalities over a 62 day period. Other factors could be influential here. For example, on an individual basis, wild fish introduced into a tank environment may have been disadvantaged through natural selection (Sigismondi and Weber, 1988) to cope with confinement and/or air exposure treatments, and social interactions within each tank may have adversely affected subordinate animals (Ejike and Schreck, 1980; Pottinger and Pickering, 1992). These “other” stressors, negatively impacting on fish as individual stressors would each be sublethal, but when acting synergistically may have induced a cumulative response that at some point (within that 62 day period) exceeded the ability of the fish to compensate (stage of exhaustion) and so the animal died (Selye, 1950, 1973; Schreck, 1982, 2000; Roberts and Rodger, 2001). Conversely, previous encounters with nets and/or confinement may have habituated individual fish to such procedures and enhanced their chances of survival (Schreck *et al.*, 1997; Gray *et al.*, 2001 and Meka and McCormick, 2005).

All necropsies indicated that feeding had not occurred for an extended period prior to death. No material was found in the gut of animals that had died, no significant mesenteric (visceral) fat deposits were observed, gall bladders were full and bile was of a dark-green appearance (index value of 3). The lack of material in the gut was self-evident as an indicator of absence of recent feeding behaviour. The absence of readily observable mesenteric fat suggests that

non-feeding was a long-term behaviour (Goede and Barton, 1990) and control fish at a later date displayed a similar appearance. This may be explained by prolonged drought conditions at the time of sampling in the Gippsland Lakes and Lake Tyers catchments adversely affecting fish in these estuaries. Alternatively, the diet of only live sandworm supplied to captive fish may have been inadequate. In order to better understand the impact of such a diet, further investigation would be required to establish seasonal and inter-estuarine diet profiles for this teleost in estuaries of south-eastern Australia (Sarre, *et al.*, 2000; Partridge *et al.*, 2004). The best insight into the feeding history was obtained from the gall bladder and its contents. A full gall bladder highlighted that feeding had probably not occurred for several hours, but the dark green colour of the bile suggested that the animal had not fed for over a much more extended period, possibly for 3 – 4 days (Goede and Barton, 1990). By comparison, two control animals had empty but clear gall bladders, the other controls displaying half-full gall bladders with clear contents, both indicating recent feeding.

Common to all morbid black bream observed in this study was their dark appearance. This was a good indicator of the health-status of this fish – a familiar characteristic shared with many fishes (Ferguson, 1988; Roberts and Rodger, 2001). Healthy black bream displayed a light greenish appearance that matched that of the tank environment. This was consistent with observations made by Weng (1971), who reported black bream matching the colour of their habitat, and an ability to change that colour (i.e. “Colour is an unstable character in the bream and varies with the turbidity of the water...” Weng, 1971, p.9.). It was the consistent dark appearance of individual fish amid healthy light-coloured cohorts that was the first physiological difference observed in “treatment” tanks. If colour change to match the environment is a predator avoidance strategy in black bream, then the development of a dark or black colouration in stressed individuals might handicap this strategy in the presence of visually cued predators. More significantly, given the progressive number of mortalities over

an extended period, on many occasions there was a batch of unhealthy animals displaying a range of symptoms, from mild darkening in colour to very dark moribund fish on the surface.

The regular occurrence in the natural environment of visual clues to future mortalities might keep predators in the vicinity of stressed and weakening fish.

In the Gippsland Lakes, as in other southern Australian estuarine environments, the diets of black bream undergo ontogenetic changes (Rigby, 1982, 1984; Sarre *et al.*, 2000). Juvenile (undersize) black bream in the Gippsland Lakes are more prevalent in shallow seagrass habitats where they seek out suitable prey (and shelter), while larger bream obtain suitable food supplies from un-vegetated substrates in deeper water (Rigby, 1982, 1984). If a demarcation of feeding habitats based on size of black bream exists, any factor (e.g., release of stressed undersize fish) that can expose (sign post) in their natural environment (e.g., sea grass beds) the exact location of this category of animal for an extended period post-release to excessive predation (e.g., ongoing appearance of unhealthy fish on or near the water surface) will at the same time expose all black bream at those locales (e.g. hiding places in seagrass beds) to a risk of predation. Furthermore, what would be the significance of this scenario under different rates of fishing pressure and release of undersized catch? Would a low frequency but high number of released undersize fish occurring in restricted locations result in different predation rates, compared to a high frequency but low number release of undersize fish with no restriction on location?

Low classifications in this study for the gonadosomatic indices (and no readily observable mesenteric fat), suggest that energy partitioning favoured maintenance rather than reproduction during the 3 week acclimatisation period, or that holding time was insufficient for maturation. Consequently, the degree of maturation could not have been contributing to observed mortalities in this experiment. Low gonadosomatic index values recorded in black bream from June to September in this study correspond with similar observations made

previously in black bream sampled from the Gippsland Lakes at the same time of the year (Coutin *et al.*, 1997). It has been noted (Haddy and Pankhurst, 1999), that stressors can inhibit gonadal steroidogenesis. Such an impact and its possible effect on spawning success for black bream in the Gippsland Lakes, requires further research under conditions suitable for induction of maturation.

2-4-6 The Question of Realism: Sampling Method

To support a realistic approach, fish were sought from commercial nets so that experimental animals in this study experienced the same stressors as other black bream encountering the commercial sector in the Gippsland Lakes and Lake Tyers. Unfortunately, when following this approach, there is a risk that the presence of an observer on location during fishing operations modifies the behaviour (handling of fish) of commercial operators (Gray *et al.*, 2001; 2003). To minimise this (which was not always possible), arrival at the fishing ground was timed to occur when fish were being sorted out of the net. Similarly, by only targeting undersize fish for the study, there was no interference with the commercial catch and the commercial incentive to harvest as quickly and efficiently as possible. Nonetheless, there was only one fish mortality at 18 days after capture, occurring in the “control” tank on the study of cumulative response. This suggests that stress associated with capture, post-capture handling and transportation was sub-lethal in this study.

2-4-7 The Question of Realism: Captive vs Wild Environment

In this study, predation did not occur in the tank environment. Those fish that were unable to control vertical position in the water column would have been at risk of avian predation, changes in the water temperature and salinity that were depth-related, feed availability, ectoparasite infestation and ultraviolet radiation (Sayer, 1998). However, many of those parameters supporting the tank environment reflected those of the surrounding estuarine

environment. Water for the flow-through system in the tanks was directly supplied from Cunningham Arm via filtration. Therefore, many water quality parameters (e.g. temperature, salinity etc.) were those from this region of the Gippsland Lakes. Also, pathogenicity experienced by fish in this study reflected natural flora present on this animal or that present in the surrounding water that it naturally inhabited – many recreational anglers sought black bream from surrounding jetties at the site of the water inlet-pipe that maintained supply to those tanks located in this research facility.

2-4-8 Concluding Comments

Legally-undersize black bream displayed altered behaviour for approximately 12 h when provided with a startle stimulus following a single exposure to a commonly encountered stressor following fishing capture and release (air exposure for 30 s). This altered behaviour may predispose released stressed fish to increased susceptibility to predation or harm in their natural environment. The behavioural response will be dependent on other acute and chronic stressors impinging on individual fish in that environment, because wild-caught under-size black bream responded cumulatively to multiple stressor exposure in these experiments. Also, a positive correlation was evident between increased serial stressor exposure and increasing percentages of fish mortality. Those fish dying late in the study (e.g. after 20 days) were asymptomatic until a few days prior to their death. Given the similarity in symptoms displayed in all mortalities, proliferation of opportunistic pathogens naturally present could have been the cause, due to a suppressed immune system of the fish, this possibly being the result of sustained elevated corticosteroid levels, previously identified to occur in stressed black bream.

The predictive ability of the cumulative response for the delayed and sustained mortalities of released undersize black bream, should be of interest to all stakeholders involved in the

management of such a fishery. All mortalities observed in this study were from undersize black bream that would have appeared healthy if they had been immediately released to the wild after capture. For wild black bream, it is likely that every aspect of any encounter that it perceives as stressful will have a cumulative effect. Its survival will depend on whether the sum of those stressors exceeds its ability to compensate. For those animals that do survive, the cost of such compensation may alter their physiological and behavioural performance. As a code of practice, care must be undertaken to minimise, and if possible, eliminate air exposure to those wild black bream destined to be returned to the water. Air exposure is a significant additional stressor to this fish, having the potential to create delayed mortality even in healthy-looking animals. For the Gippsland Lakes, there should be some investigation of the relationship between bird predation and the release of undersize black bream. Sustained mortalities of returned fish over a period of time might act as an ongoing “sign-post”, with moribund animals continuously surfacing. This could lead predators to those regions. Many fish catch-and-releases of undersized fish will occur in the same nursery areas where they had been previously collected by other recreational or commercial fishers, thus causing multiple stresses of the type investigated. The practice, therefore, might increase the predator density in those nursery areas, thus increasing the rate of predation on all fish in the region, including those which have escaped the initial fishing event. Further research is required in this area.

Chapter 3. Chronic Stressors

3-1 Introduction

3-1-1 Black Bream and its Estuary Existence

Black bream naturally inhabits estuaries of southern Australia and can complete its life cycle within its natal estuary (Butcher, 1945; Sherwood and Backhouse, 1982; Chaplin *et al.* 1998; Sarre *et al.* 2000; Williams *et al.* 2012). On rare occasions, post-larval stage black bream can be observed at sea, but this is usually attributed to adverse conditions such as periods of high river discharge (Lenanton, 1977) or anoxic conditions within an estuary (Burridge *et al.* 2004). In Victoria, black bream are most abundant in the Gippsland Lakes which Victoria's largest estuary (MacDonald, 1997b) and throughout the 20th century, commercial catches of black bream in the Gippsland Lakes have fluctuated considerably (Butcher, 1945; Jenkins *et al.*, 2010; Williams *et al.* 2012). In the last decade, however, there has been a steady decline in annual catch rates from between c. 90 and 400 t to between c. 26 and 140 t. with 2002-2003 (26 t) being the lowest on record (Kemp *et al.* 2011; Williams *et al.* 2012).

3-1-2 Multiple Stressors in the Environment

In their natural environments, fish encounter multiple stressors, such as fluctuations in dissolved oxygen, temperature and sediment loads, along with changes in food and habitat availability, conspecific social interactions, and predation (Adams, 1990, Adams *et al.* 1993; Heath, 1995; Adams and Greeley, 2000). Many of these stressors on fish are experienced at a low level, but are continuous or of an episodic nature that may extend over periods of weeks to years and are endured by fish as chronic stress (Adams, 1990, Adams *et al.* 1993). In recent decades, many aquatic systems have experienced increasing anthropogenic stress,

usually as increased nutrient loads, exposure to contaminants, and diversion of traditional freshwater inflows (Adams and Greeley, 2000; Williams *et al.* 2012). In the Gippsland Lakes, the nutrient status of Lake Wellington is eutrophic by OECD standards (Bek and Bruton, 1979; Robinson, 1995; Harris *et al.* 1998); similarly, mercury concentrations in black bream (organochlorines are also present) caught from the Gippsland Lakes have increased by at least 58% from the late 1970's to 1999 (Fabris *et. al.* 1999) and the present loading is unknown. The decline in black bream catches corresponds with a 10-year trend in decreasing rainfall, leading to a reduction in freshwater inflow into the Gippsland Lakes (Jenkins *et al.*, 2010); this is compounded by anthropogenic pressures such as diverting, extracting and damming freshwater flow for agriculture (e.g. The Glenmaggie Dam to support the dairy industry of the Macalister Irrigation District), industry and human consumption (e.g. Electricity generation in the La Trobe Valley and the Thomson River Dam for Melbourne's water supply) (Williams *et al.* 2012).

In contrast, within the Lake Tyers catchment is the “Aboriginal Trust Estate” land, which ranges along the western shore of the Nowa Nowa Arm and along the northern shore of the main lake (MacDonald, 1997a). Over half of this land is still forest, with the rest mainly cleared for pasture (Hall, 1984). With about 80% of East Gippsland classified as public land and the Lake Tyers catchment being in this region, the catchment is little changed from its form of 200 years ago (EGCMA, 2005).

3-1-3 Chronic Stress effects on Fish

Chronic stress, depending on its severity, may load physiological systems, reduce growth, impair reproduction, predispose organisms to disease and reduce the capacity of fish to tolerate subsequent stress (Adams, 1990; Barton and Iwama, 1991; Pickering, 1993b; Pankhurst and Van Der Kraak, 1997; Wendelaar Bonga, 1997).

Chronic stress in black bream, as in other fish, may be influenced by adverse environmental conditions (Adams 1990). Environmental conditions of many estuaries supporting black bream populations vary significantly (Sarre and Potter, 2000; Sarre *et al.* 2000; Partridge, 2004). In Western Australian studies, black bream was a hardy fish (Holt, 1978; Sarre *et al.* 1999; Partridge *et al.* 2002). However, biological characteristics, such as growth-rate, size and age at first maturity, have varied with differing environmental conditions found in each estuary (Sarre and Potter, 2000; Sarre *et al.* 2000; Partridge, 2004).

3-1-4 Tertiary Responses and Performance Tests

In the Gippsland Lakes, Morrison *et al.* (1998) found that black bream were experiencing slow growth and episodic recruitment. Coutin *et al.* (1997) indicated that black bream originating from both the Gippsland Lakes and Lake Tyers displayed variable growth but black bream from Lake Tyers were generally larger fish than black bream of the same age originating from the Gippsland Lakes (Coutin, 2000). In black bream, growth rate was proposed to be influenced significantly by environmental surroundings rather than by genetic differences (Partridge *et al.* 2004).

A tertiary (or whole animal) response such as changes in health, metabolic rate, growth, behaviour, reproductive success, and survival can indicate that unfavourable environmental conditions have exceeded the tolerance limits of fish (Wedemeyer *et al.* 1990). Performance tests can measure the capacity of fish to carry out essential life processes such as mount an immune response, resist disease, swim, avoid predators and respond physiologically to another stressful situation (Schreck, 1990). Performance tests incorporate several levels of biological organisation and are therefore integrative in nature (Schreck, 1990). However, the effect of stress on performance is polymorphic and depends on the fish's present environment, developmental stage, genetic constitution and prior history (Schreck, 1990).

As a performance test, behaviour (Schreck, 1990) can offer the advantage over other methodologies of being more readily interpreted within an ecological context (Rand *et al.* 1985; Schreck, 1997), thereby increasing the efficacy of extrapolating laboratory results to the natural environment (Olla *et al.* 1980; Sherer, 1992; Schreck, *et al.* 1997). In a natural habitat such as the Gippsland Lakes, black bream prefer those locations that provide shelter and places to hide, namely regions of seagrass, jetties, snags etc. (Hobday and Moran, 1983; MacDonald, 1997b). Therefore, an investigation of behaviour such as shelter-seeking (that incorporates hides) exploits this trait. The natural response to hide may therefore be affected in fish with different histories of stressor loading. Inappropriate behaviour resulting from such stressor loading may increase the vulnerability to risks such as predation (Kruzynski *et al.* 1994).

3-1-5Aims

The intention of this inter-estuarine study was to investigate and compare black bream behaviour and physiological responses to chronic stress.

3-2 Methods

3-2-1 Capture and Acclimation Periods

Fish –Live black bream of various fork lengths (18-32 cm) were obtained from commercially-operated seine nets from “Medusa Point”, “Mason Bay”, “Fraser Island” and “Cunningham Arm” of the Gippsland Lakes over a 10-month period (May-September 2002) (**Figure 3-2**). These sites were along a 50 km continuum, ranging from brackish to marine waters. Undersize black bream (<26 cm TL) were obtained from the “Nowa Nowa Arm”,

“Mud Islands” and “Fishermans Landing Arm” of Lake Tyers from commercially-operated seine nets over a 5-month period (May-September 2002) when that estuary was open to commercial fishing (Figure 3-1).

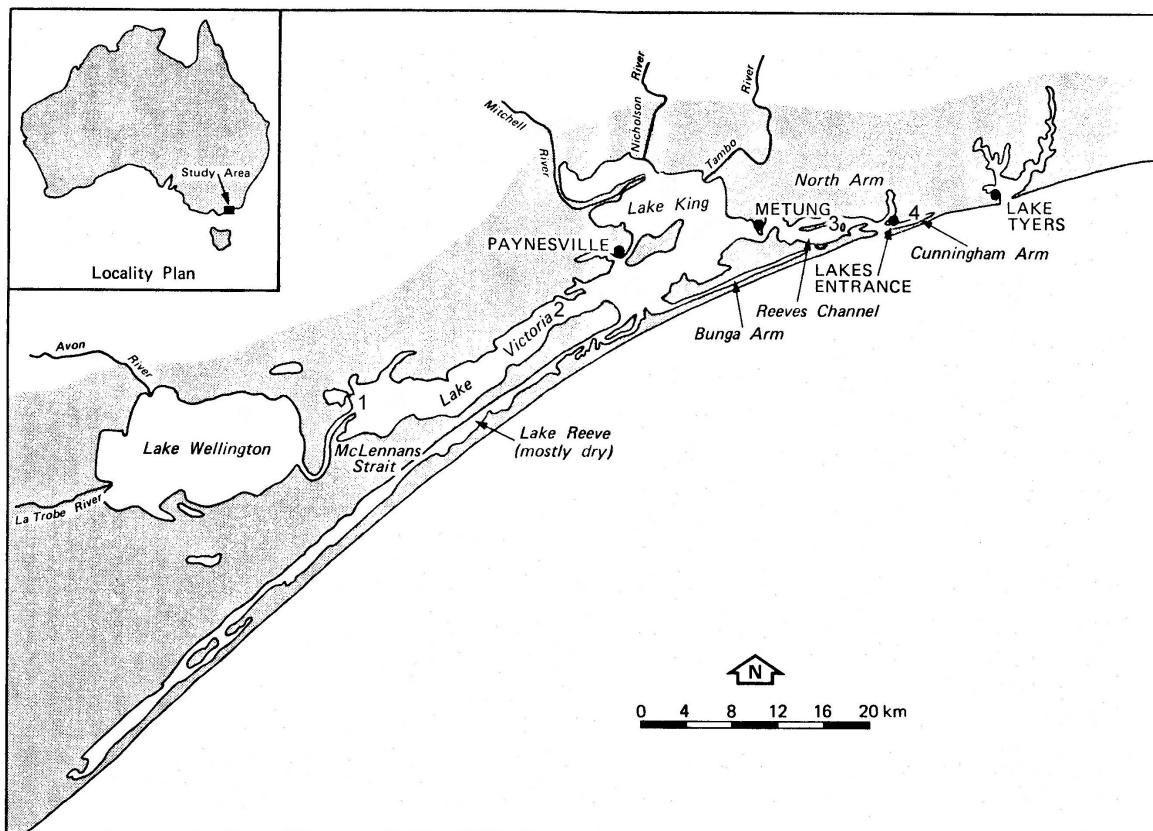


Figure 3-1: Sampling sites in Gippsland Lakes (1. Medusa Point, 2. Mason Bay, 3. Fraser Island, 4. Cunningham Arm) and Lake Tyers (5. Nowa Nowa Arm, 6. Mud Islands, 7. Fishermans Landing Arm).

All fish were transported to the RMIT research facility at Lakes Entrance and placed in six circular 2000 L flow-through tanks (four tanks for Lake Tyers fish and two for Gippsland Lakes fish), 1.58 m diameter, each receiving approximately $5\text{-}7 \text{ L min}^{-1}$ of filtered and then aerated water with an annual temperature range of $8\text{-}24^\circ\text{C}$ pumped from the “Cunningham Arm” region of the Gippsland Lakes. A natural photoperiod was maintained during the acclimation period. Fish were fed to satiation twice a week on live sandworm *Australonereis*

ehlersi (Neridae), but due to financial constraints and the long acclimation period, this was later changed to chopped pilchard *Sardinops neopilchardus* (Clupeidae). Fish from the Gippsland Lakes and Lake Tyers remained separated throughout this investigation. A 10-month capturing period was required to obtain sufficient numbers of undersized black bream from the Gippsland Lakes.

All fish received at least 12 months acclimation to the tank environment (from approximately 21st January 2002 to 5th November 2003) in order to minimise any impact of handling stress due to their capture or the disturbances around holding tanks when introducing new fish. During this period, no other fish were introduced, or in contact with tanks or infrastructure associated with this investigation. The reason for the unusually long acclimation period was that the relevant government authority gave notice that commercial fishing in Lake Tyers would cease shortly and so considerable effort was expended to capture sufficient fish for this experiment while possible, though the experimental design and equipment were not quite ready. While not ideal, the permits for capture and holding of live fish had limits and there were indications that the commercial fishery might never re-open.

3-2-2 Cage Confinement

The experiment was designed to test if variation in a chronic stressor (density) induced changes in black bream from Gippsland Lakes and Lake Tyers. The ultimate experiment was intended to be factorial with two variables:

- source of fish (two levels: Gippsland Lakes and Lake Tyers)
- confinement density (=stocking density) (three levels: control, low and high).

The experimental groups were as shown in **Table 3-1**. Fish were subjected to different confinement densities, which were manipulated by enclosing groups of fish inside cylindrical mesh cages within tanks (**Figure 3-2**). As only five tanks were available at this time (due to competing requirements), it was decided to run a preliminary trial to see if fish were affected by: stocking density (three tanks with caged fish and two with uncaged controls) and fish source (three tanks with Lake Tyers fish and two tanks with Gippsland Lakes fish).

Table 3-1: Experimental treatments, tanks and fish. **Codes for treatments are in red.**

Fish source	Density No. tanks (no. fish)			
	Control (0.001-0.003 kg m ⁻³)		Low (14 kg m ⁻³)	High (32 kg m ⁻³)
Gippsland Lakes	1	1 (21) GL(C)	2	1 (6)* GL(LSD)
Lake Tyers	4	1 (30) LT(C)	5	1 (10,10) LT(LSD) No. 1 LT(LSD) No. 2
Totals		2 (51)		3 (26)
				3 (30)

*These treatments shared a common tank.

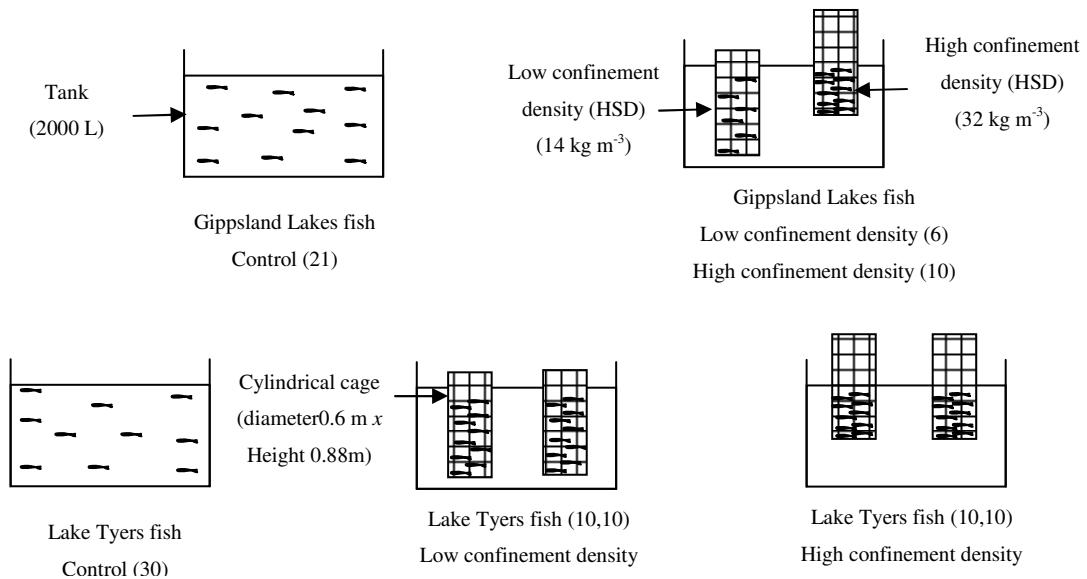


Figure 3-2: Arrangement of tanks, cages and fish for confinement density experiment (not to scale).

The following methods were used to assess the effects of the treatments:

1. the behavioural test devised in Chapter 2
2. opercular response
3. health assessment index (HAI)
4. condition factor and organosomatic indices

Fish transfer – Water levels in acclimation tanks were reduced by siphon over 2 days so that fish from both estuaries could be restrained underwater with a landing net to avoid exposure to air. In each tank, individual fish were randomly selected and removed, submerged, in a 10 L plastic bucket.

Weight of Fish – The water level of a 10 L plastic bucket containing a submerged fish was reduced to a 3 L mark previously scribed onto the side of the bucket. The bucket and its contents were weighed via mechanical hanging scales. The fish in the bucket was transferred to its respective cage. The empty bucket was refilled to the 3 L mark and re-weighed. The

approximate weight of the fish was obtained by difference (Appendix 3). The scale was accurate to 20 g and so final individual fish weights were accurate to ± 20 g. The tanks were adjusted to have weights as close as possible to one another by re-randomisation as necessary (**Table 3-2**).

Confinement Density – Density was adjusted by raising or lowering cylindrical cages (0.6 m diameter x 0.88 m height) with a mesh base and sides in the water in the tank (**Figure 3-2**). A preliminary investigation on fish behaviour in cages assisted in determining appropriate volumes to minimise physical trauma, while offering a stressful environment due to physical confinement.

High confinement density was set at 32 kg m^{-3} and low confinement density at 14 kg m^{-3} for individual cages. With the approximate total weight of fish for each experimental group known, water volumes of each cage were adjusted to establish the prescribed confinement density (Table 3-3). In the event of fish mortality, cage volumes were re-adjusted so as to maintain the initial confinement density. Fish were maintained in their respective cages and tanks for 1 month. Control fish from each fish group were similarly weighed and were transferred to identical tanks with identical conditions but without cages.

Apparatus – Two confinement cages plus their feeding trays (described in detail in Appendix 3) were placed in a single 2 t holding tank. Three tanks held cages, while two contained control fish, one from each estuary. To minimise social stressor impact, existing hierarchies were maintained by not mixing cages containing fish from differing estuaries (Figure 3-2).

Table 3-2: Numbers and mean weights of black bream in tanks and cages. Fish sourced from the Gippsland Lakes have the prefix GL; similarly, Lake Tyers is LT. The treatments for each group of fish were: high confinement density (HSD), low confinement density (LSD) and control (C). For example, Gippsland Lakes low confinement treatment was GL (LSD). (See Appendix 3 for the weights of individual fish from each experimental group).

Group	No. fish per		Mean fish weight (g) per	
	Tank	Cage	Tank	Cage
GL(C)	11		5020 ± 440 (tank)	
GL(LSD)		6		2760 ± 240
GL(HSD)		10		4140 ± 400
LT(C)	10		2640 ± 400 (tank)	
LT(LSD) No. 1		10		3200 ± 400
LT(LSD) No. 2		10		2800 ± 400
LT(HSD) No. 1		10		2980 ± 400
LT(HSD) No. 2		10		2860 ± 400

Table 3-3: Required cage depths to maintain confinement densities of 32 kg m^{-3} and 14 kg m^{-3} . GL – Gippsland Lakes; LT – Lake Tyers; HSD – High confinement density (high stocking density); LSD – Low confinement density (low stocking density); and C – Controls.

Group	Cage depth (cm)	Density (kg m^{-3})
GL[C]	N/A	0.003 (tank)
GL(LSD)	70	14
GL(HSD)	50	32
LT[C]	N/A	0.001 (tank)
LT(LSD) No.1	81	14
LT(LSD) No.2	71	14
LT(HSD) No.1	40	32
LT(HSD) No.2	40	32

Necropsy of mortalities – A modified necropsy method (based on Goede and Barton, 1990; Adams *et al.*, 1993; Goede, 1993; Haddy and Pankhurst, 1998) was performed on each dead fish. Any dead fish were collected from the cages, weighed and fork length measured. External and internal organs were classified by appearance of: thymus, fins, skin, eyes, gills, pseudobranchs, spleen, kidney, liver, gall bladder and hindgut and by presence or absence of parasites. Hepatosomatic and gonadosomatic indices were also obtained. A macroscopic staging of gonads was also undertaken (Haddy and Pankhurst, 1998).

3-2-3 Acute stress

3-2-3(a) Behavioural Response

The behavioural test– After the month's acclimation in different confinement densities, a subset of a maximum of eight fish was taken from each replicate and exposed to air for 30 seconds (as described previously in Chapter 2) before being randomly placed in one of the

twelve available non-hide compartments of each channel. The remaining channels out of the twelve available were allocated to control fish of the same density treatment collected directly from their holding tanks without air exposure. As in the recovery experiment (Chapter 2), channels containing fish were covered with black plastic sheets and fish were left to adjust to the apparatus for approximately 16 hours before a behavioural test was commenced.

As for Chapter 2, the avoidance/hiding response was recorded by a hidden video camera and by measuring the time taken for each fish to swim under the permanent cover. Removal of the covering sheet from one end of each channel was used to initiate a response, but the other end was left under cover as a refuge. Aeration was stopped individually in each channel so that fish behaviour could be observed during each trial. Any decline in dissolved oxygen for each apparatus was recorded on a daily basis. A trial ended when the fish swam under the cover, or alternatively after 30 minutes. This procedure was repeated for all channels in each apparatus. All behavioural tests were commenced at the same time each day.

Statistics – The exact mean could not be calculated because some fish in raceways did not respond within the assigned time period of 30 minutes and so medians were calculated and responses were also categorised as <1 min and >1 min (as if fish did not react within 1 min of a threat, it was likely to be fatal. Data were analysed with non-parametric Kruskal-Wallis tests and χ^2 . Significance was recorded at $p<0.05$. Pooling of data was validated by heterogeneity testing of contingency tables (Zar. 1984).

3-2-3(b) Opercular Response

Opercular rates – Opercular counts were obtained from a video recording of each fish whilst held in the channel apparatus. Associated time intervals were measured with a stop-watch.

Statistics – The intensity of association between opercular rate and stimulus-response response-time was measured by calculating a correlation coefficient. The significance of the correlation coefficient was tested for $H_0: \rho = 0$ against $\rho \neq 0$ (Zar 1984).

3-2-3(c) Health Assessment Index (HAI)

At the completion of the behavioural investigation, fish were sacrificed. The health assessment index followed the procedures of Goede and Barton (1990), Adams *et al.* (1993) and Goede (1993). For each fish, length and weight were measured, the condition of various body parts were assigned values (e.g. for fins, eye, liver etc.) and a Health Assessment Index (HAI) calculated (as described in Chapter 2) for comparison of treatments, with high HAI values indicating poor health and low HAI values indicating good health (Adams *et al.* 1993).

Statistics – The mean, standard deviation and coefficient of variation were calculated for each experimental group. Differing experimental groups were compared by a single factor analysis of variance (Zar, 1984). Any differences between groups were identified by the Tukey test (Zar, 1984).

3-2-3(d) Condition Factor and Organosomatic Indices

Condition factor – The condition factor (K) was established for each fish and was determined by:

$$K = \frac{\text{wet weight of fish (g)} \times 100}{[(\text{fork length}) (\text{cm})]^3} \quad (\text{Ricker, 1975; Bagenal and Tesch, 1978})$$

Splenosomatic index – The splenosomatic index (SSI) was established for each fish and was determined by:

$$\text{SSI} = \frac{\text{spleen weight}}{\text{gutted weight of fish}} \times 100 \quad (\text{Goede and Barton, 1990})$$

Liversomatic (Hepatosomatic) index – The liver somatic index was established for each fish and was determined by:

$$\text{HSI} = \frac{\text{Liver weight}}{\text{gutted weight of fish}} \times 100 \quad (\text{Bulow et al. 1978}).$$

Gonadosomatic index – The gonadosomatic index (GSI) was established for each fish and was determined by:

$$\text{GSI} = \frac{\text{gonad weight}}{\text{gutted weight of fish}} \times 100 \quad (\text{Crim and Glebe, 1990})$$

Statistics Condition factor and organosomatic indices for differing treatments were compared by a single factor analysis of variance using an arcsine or other transformation to normalise data (Zar, 1984). Any differences between groups were identified by the Tukey family error test at P=0.05 (Zar, 1984). Fish mortalities were not included in the analyses.

3-3 Results

3-3-1 Acclimation Period

The acclimation period (from approximately 21st January 2002 to 5th November 2003) resulted in black bream sourced from both the Gippsland Lakes and Lake Tyers being exposed to minimal human contact for at least 12 months, successfully weaned off live food and displaying a mortality-free status for over 12 months.

Unfortunately, overnight on 21st-22nd May 2003, due to a failure in security at the Lakes Entrance research facility, some or all acclimating animals were possibly contaminated by aerial deposition of automotive spray-paint and its associated solvents. Whilst it is likely that any effects had equal impact on all fish, this possible contamination needs to be borne in mind in considering these results.

3-3-2 Cage Confinement – Fish Colour and Mortality

All caged fish developed a characteristic darkening in colouration (as in Chapter 2) 4-7 days after placement in the cages; the degree to which this developed varied amongst fish in each cage. Animals held in at low confinement density were first to display a lighter colouration, followed by those fish in higher confinement treatments. The most extreme darkening occurred in cages where fish in high-density confinement. This dark appearance of fish in cages continued for approximately a week and then they reverted to a lighter colouration. There were no observable differences in darkening between fish sources (Gippsland Lakes or Lake Tyers). Control animals that were in the 2000 L tanks without cages remained of light colouration throughout this period.

In the Gippsland Lakes high confinement treatment, two fish were seen swimming higher in the water column and they died on days 9 and 10 after placement into the cage (**Figure 3-3**). There were no other mortalities during this experiment. Contingency table and Fisher exact test analysis indicated that observed mortalities were not significantly ($p>0.05$) related to the treatment they had received or their original location (Appendix 3a).



Figure 3-3: Moribund fish in Gippsland Lakes high confinement density cage. Pictured is one of two fish that died during this investigation; both animals were from this cage. Note the dark appearance of several other fish in the cage.

Results of necropsy – Extensive erosion of fins and tail were evident in both animals that had died (**Figure 3-3, Figure 3-4**). Necrotic lesions were also evident on the sides of these fish. The eyes were clear, with little evidence of any pathological condition. The gills on both fish displayed erosion of gill lamellae, some clubbing at margins, a light colouration and the presence of excess mucus. Gill pseudobranchs of both fish were swollen. The thymus of one

animal indicated haemorrhaging. Some inflammation (swollen and red) was observed in the hindgut of both animals. Both fish had full gall-bladders and the bile was dark-green in appearance. Swim bladders of both fish were inflated. Livers of both fish appeared normal, but hepatosomatic index (HSI) values of 2.5 and 2.7 indicated possible enlargement. The appearance of gonads indicated that both animals were male and in a ripe condition with gonadosomatic index (GSI) values of 11.2 and 7.7. The spleen appeared enlarged, but splenosomatic indices (SSI) of 0.14 and 0.21 did not indicate this. No mesenteric fat was observed in either animal.



Figure 3-4: Fish mortalities occurring in at days 9 and 10 after placement into Gippsland Lakes high confinement density cage. A, C) Fish that died on day 9, B,D) Fish that died on day 10.

3-3-3 Acute response

3-3-3(a) Behavioural Response

Individual fish behaviour to the stimulus-response in the experimental channel varied, from an immediate response with the fish colliding with the walls of the apparatus, to the fish remaining motionless.

Median response time to a stimulus-response varied from 0.03 min to >30 min (**Figure 3-5**, Appendix Table A3-1). Median response time did not indicate a significant difference (at $p \leq 0.05$) between fish sourced from the Gippsland Lakes or Lake Tyers (Kruskal-Wallis, $H=3.00$, $p=0.083$) or between caged fish and controls (Kruskal-Wallis, $H=1.33$, $p=0.248$). This applied also to category means (Kruskal-Wallis, $H=0.33$, $p=0.564$ for fish source, $H=3.00$, $p=0.083$ for caging).

The two critical times in fish response are <1 min, which means that a fish has reacted quickly enough to hide from threats (**Figure 3-6a,b**), and >30 min, which means that a fish has become essentially unresponsive to threats (**Figure 3-6c,d**). The proportions of fish in these categories were different with fish source but not with caging by χ^2 tests.

The proportion of fish that sought shelter in <1 min was greater in Lake Tyers fish than in Gippsland Lakes fish ($\chi^2=8.389$, $p=0.004$) (**Figure 3-6a**) but there was no significant effect of caging ($\chi^2=22.046$, $p=0.153$) (**Figure 3-6b**). This difference in response time with fish source applied to both control ($\chi^2=4.126$, $p=0.042$) and caged fish ($\chi^2=3.857$, $p=0.050$). Caging density in cages made no significant difference to this category of response time ($\chi^2=0.738$, $p=0.390$).

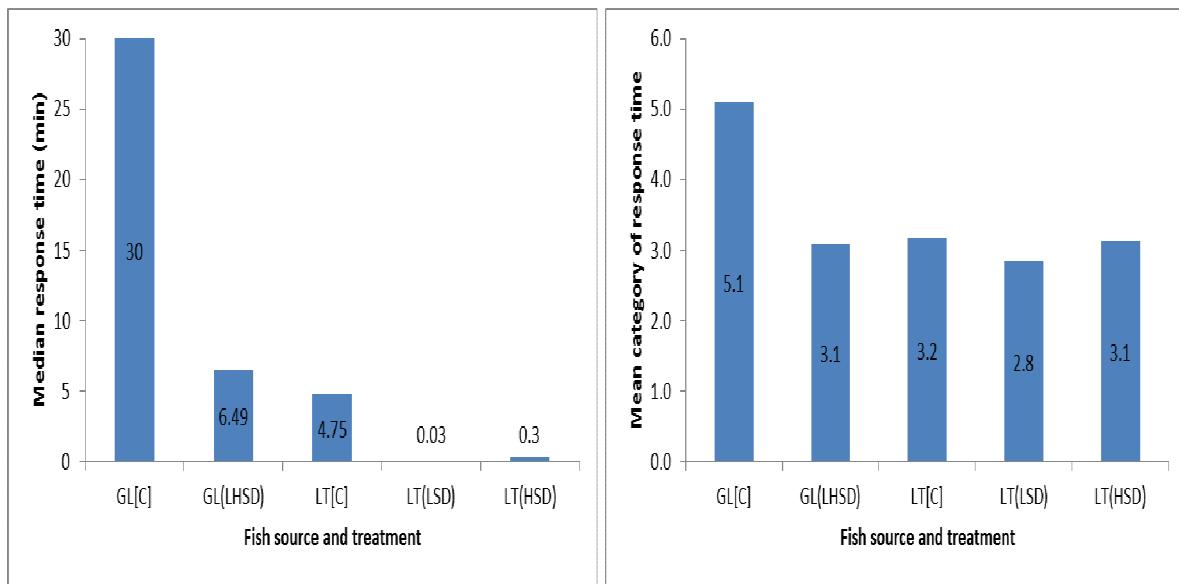


Figure 3-5: (a) Effect of fish source and treatment on time to seek shelter in the stimulus-response test. (a) Median response time, (b) mean time category.

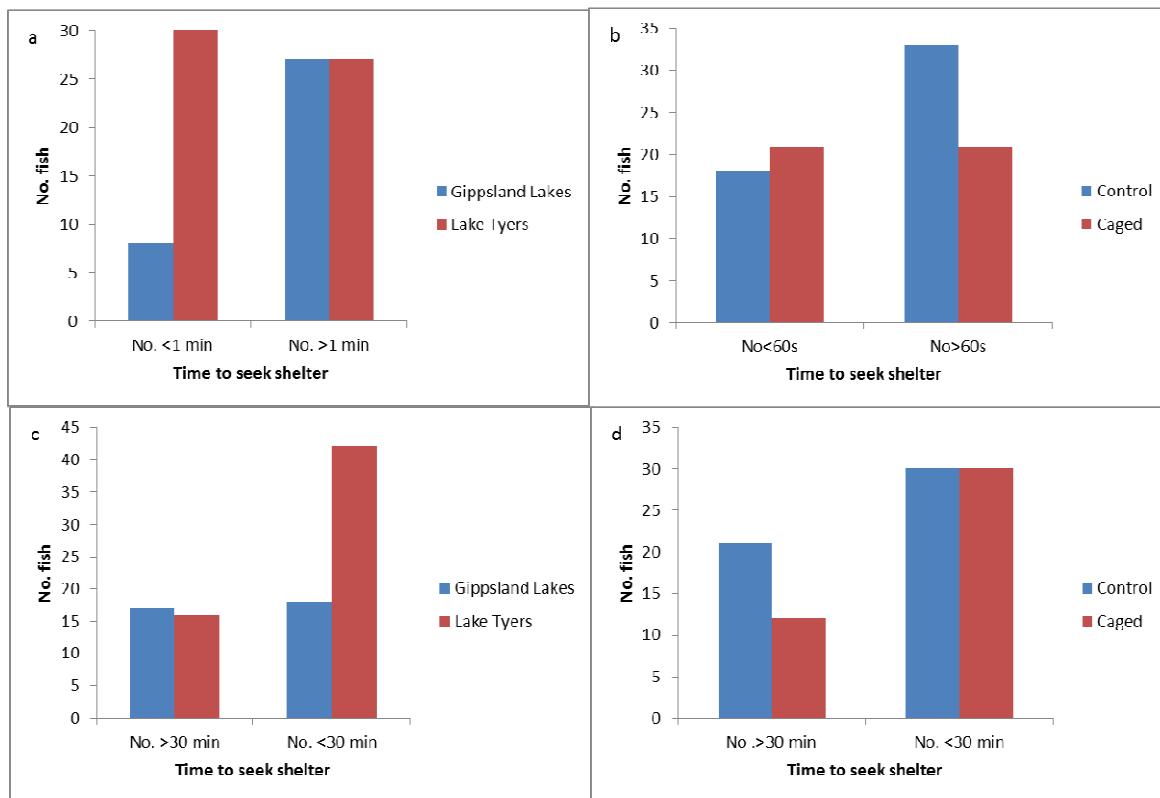


Figure 3-6. Effect of (a,c) fish source and (b,d) cage confinement on time to seek shelter in the stimulus-response test.

Similarly, the proportion of fish that had not sought shelter after 30 min was greater in Gippsland Lakes fish than Lake Tyers fish ($\chi^2=4.199$, $p= 0.040$) (**Figure 3-6c**) and there was again no significant effect of caging ($\chi^2=1.599$, $p= 0.206$) (**Figure 3-6d**). This difference in response time with fish source again applied to both control ($\chi^2=6.333$, $p= 0.012$) and caged fish ($\chi^2=0.000$, $p=1.000$) and again there was no significant difference with caging density ($\chi^2=0.057$, $p=0.811$).

3-3-3(b) Opercular rates

For Gippsland Lakes fish, no significant correlation existed between opercular rate and response time to seek cover under a hide for Gippsland Lakes control fish ($r=-0.522$, $p=0.122$) but opercular rate declined with increasing response time for caged fish ($r=-0.522$, $p=0.040$) (**Table 3-4**, Appendix Table A3-2). For Lake Tyers fish, a significant positive correlation existed between opercular rate and response time to enter a hide in one low-confinement cage ($r = 0.942$, $p<0.001$). No other correlation existed for Lake Tyers black bream.

Table 3-4: Correlation of mean opercular response and stimulus-response response times for Gippsland Lakes (GL) black bream and Lake Tyers black bream (LT), at high confinement density (HSD), low confinement density (LSD), or as controls [C]. * indicates a significant correlation ($p<0.05$).

Day	Treatment	Response time (min)	Mean opercular rate (beats s ⁻¹)	Correlation coefficient
1	GL[C]	17.616	1.03	0.246
		30	0.9692	
		30	1.113	
		30	1.11	
	GL(HSD)	0.26	1.297	-0.576
		6.533	1.33	
		0.2	0.964	
		2.316	1.2	
		30	0.846	
		30	0.96	
		30	1.13	
		11.266	1.12	
2	GL[C]	30	1.152	-0.368
		0.1	1.24	
		30	1.222	
		30	1.04	
		8.083	1.80	
		0.05	1.163	
	GL(LSD)	6.45	1.37	-0.580
		0.133	1.27	
		0.2	1.33	
		8.716	1.37	
		2.033	1.68	
		30	1.134	
3	LT[C]	0.02	1.726	0.107
		2.883	1.192	
		30	1.55	
	LT(LSD) No. 1	0.02	1.215	0.942
		30	1.45	
		30	1.54	
		0.02	1.261	
		0.383	1.31	
4	LT[C]	30	0.971	-0.894
		30	1.31	
		11	1.496	
		0.283	1.70	
	LT(LSD) No. 2	0.02	1.44	-0.622

		0.233	1.397	
		0.02	1.236	
		0	1.57	
		30	1.16	
		0.02	1.27	
		30	1.16	
		0.02	1.23	
5	LT[C]	7.3	1.25	-0.451
		0.03	1.32	
		22.25	1.29	
		0.27	1.331	
	LT(HSD) No.1	0.3	1.45	-0.034
		0.2	1.314	
		1.083	1.372	
		30	1.49	
		21.883	1.164	
	LT[C]	30	1.24	-0.401
		4.75	1.13	
		0.03	1.34	
		0.216	1.55	
	LT(HSD)No. 2	0.02	1.49	-0.101
		0.466	1.59	
		0.04	1.27	
		0.04	1.45	
		0.06	1.38	
		30	1.37	
		0.04	1.62	
		30	1.51	

3-3-3(c) Health Assessment Index

The HAI value ranged from 2.7 (Gippsland Lakes and Lake Tyers controls) to 30 (Gippsland Lakes caged fish) (**Table 3-5, Figure 3-7**, Appendix Table A3-3). Some fish in all treatments had an enlarged spleen (**Figure 3-8**) but caged Gippsland Lakes fish also had two fish displaying a focal discolouration of the liver and one fish had lost an eye, apart from the two early mortalities. No other fish showed any other defect (Table 3-6). There was no significant difference in HAI value with fish source (ANOVA, $F=0.66$, $p=0.475$) or caging (ANOVA, $F=1.15$, $p=0.363$) alone.

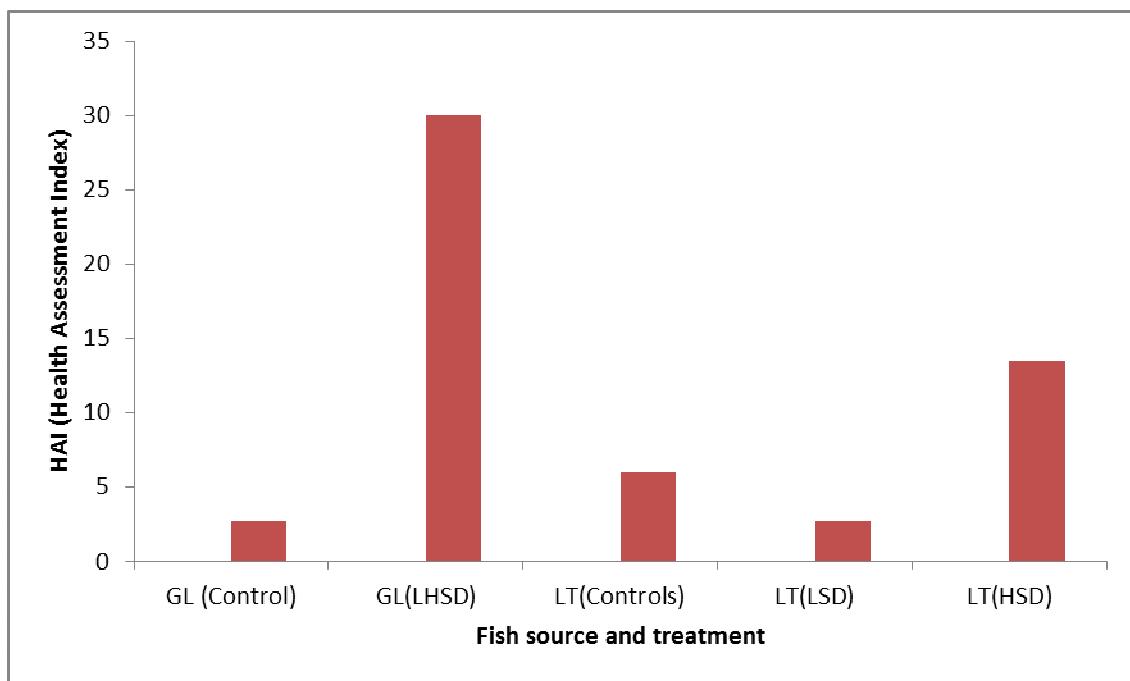


Figure 3-7: Effect of fish source and treatment on Health Assessment Index (HAI) values of all groups at the conclusion of the chronic stress investigation. A large (HAI) value indicates more observed abnormalities and more unhealthy fish in that experimental group while a small HAI value indicates few observed abnormalities and more healthy fish in that experimental group. Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatment: LSD=low confinement density, HSD=high confinement density, LHSD= low/high confinement density.

Table 3-5: Mean values \pm SE for Health Assessment Index (HAI) of Gippsland Lakes (GL) and Lake Tyers (LT) black bream experimental groups following cage confinement. Refer to Appendix Table A3-3 for details.

Treatment	Liver	Eye	Skin	Gills	Fin	Pseudobranch	Thymus	Spleen	Hindgut	Kidney	Parasites	HAI
GL (Control)	0	0	0	0	0	0	0	2.7	0	0	0	2.7
GL(LHSD)	4.3	2.1	0	0	0	0	0	23.6	0	0	0	30.0
LT(Controls)	0	0	0	0	0	0	0	6.0	0	0	0	6.0
LT(LSD)	0	0	0	0	0	0	0	2.7	0	0	0	2.7
LT(HSD)	0	0	0	0	0	0	0	13.5	0	0	0	13.5

A score of 30 due to an enlarged spleen was present in 9-79% of fish (**Figure 3-8, Figure 3-9**). There was no significant effect on this proportion of fish source ($\chi^2=1.970$, $p=0.160$) or caging ($\chi^2=2.447$, $p=0.118$) alone. However, the proportions of fish displaying enlarged spleens were significantly different among treatments ($\chi^2=23.938$, $p< 0.001$), between controls and caged Gippsland Lakes fish ($\chi^2= 11.914$, $p<0.001$), among controls and caged Lake Tyers fish ($\chi^2=7.370$, $p=0.025$) and between low confinement density and high confinement density LT fish ($\chi^2=6.988$, $p=0.008$) but not between controls ($\chi^2= 0.509$, $p=0.476$). The greatest proportion of fish with an enlarged spleen was shown by the Gippsland Lakes caged fish and the least by the Gippsland Lakes control fish and the Lake Tyers LSD and control fish, with the Lake Tyers HSD fish intermediate.



Figure 3-8: An enlarged spleen of black bream.

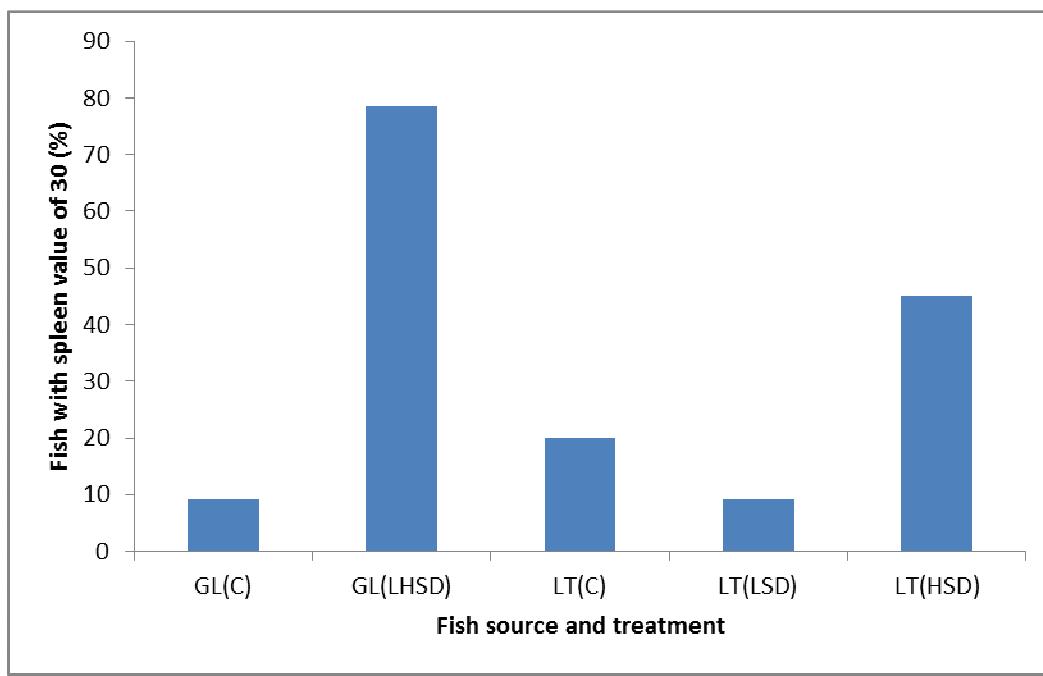


Figure 3-9: Effect of fish source and treatment on the percentage of fish with a score of 30 due to an enlarged spleen. Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatment: LSD=low confinement density, HSD=high confinement density, LHSD= low/high confinement density.

3-3-3(d) Condition Factor & Organosomatic Indices

Condition factor

The wet weight of fish ranged from 228.3-385.1 g (**Figure 3-10**, Appendix Table A3-4); Gippsland Lakes fish were significantly heavier than Lake Tyers fish (ANOVA, $F=771.93$, $p<0.001$) but there was no significant difference between control and caged fish (ANOVA, $F=0.10$, $p=0.768$) (**Figure 3-10a**).

The fork length of fish ranged from 222-265 cm; Gippsland Lakes fish were significantly longer than Lake Tyers fish (ANOVA, $F=132.91$, $p<0.001$) but there was no significant difference between control and caged fish (ANOVA, $F=0.12$, $p=0.756$) (**Figure 3-10b**).

The condition factor (K) ranged from 1.95-2.26; there was no significant difference between Gippsland Lakes and Lake Tyers fish (ANOVA, $F=1.11$, $p=0.369$) or between control and caged fish (ANOVA, $F=0.00$, $p=0.994$) (**Figure 3-10c**).

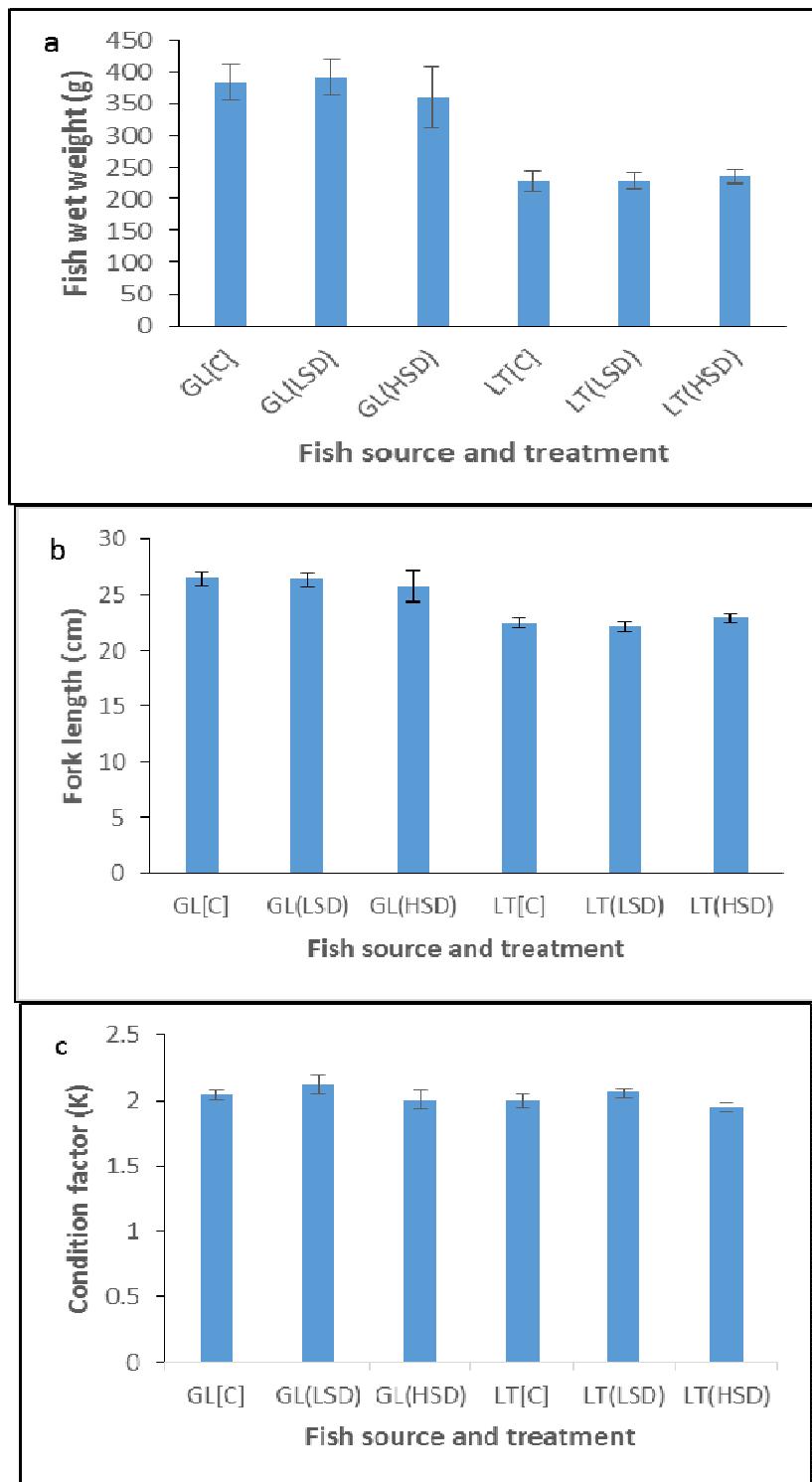


Figure 3-10: Effect of fish source and caging treatment on fish (a) wet weight, (b) fork length and (c) condition factor (K). Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatment: LSD=low confinement density, HSD=high confinement density, LHSD= low/high confinement density.

Splenosomatic Index (SSI)

The spleen weight ranged from 0.31-0.60 g (**Figure 3-11**, Appendix Table A3-5); Gippsland Lakes fish had significantly heavier spleens than Lake Tyers fish (ANOVA, $F=17.95$, $p=0.024$) but there was no significant difference between control and caged fish (ANOVA, $F=0.05$, $p=0.840$) (**Figure 3-11a**).

The gutted fish wet weight ranged from 203.69-354.98 g; Gippsland Lakes gutted fish were significantly heavier than Lake Tyers fish (ANOVA, $F=197.7$, $p=0.001$) but there was no significant difference between control and caged fish (ANOVA, $F=0.0.14$, $p=0.737$) (**Figure 3-11b**).

The splenosomatic index (SSI) ranged from 0.144-0.183; there was no significant difference between Gippsland Lakes and Lake Tyers fish (ANOVA, $F=0.00$, $p=0.952$) but there was a significant difference between control and caged fish (ANOVA, $F=9.82$, $p=0.052$) (**Figure 3-11c**).

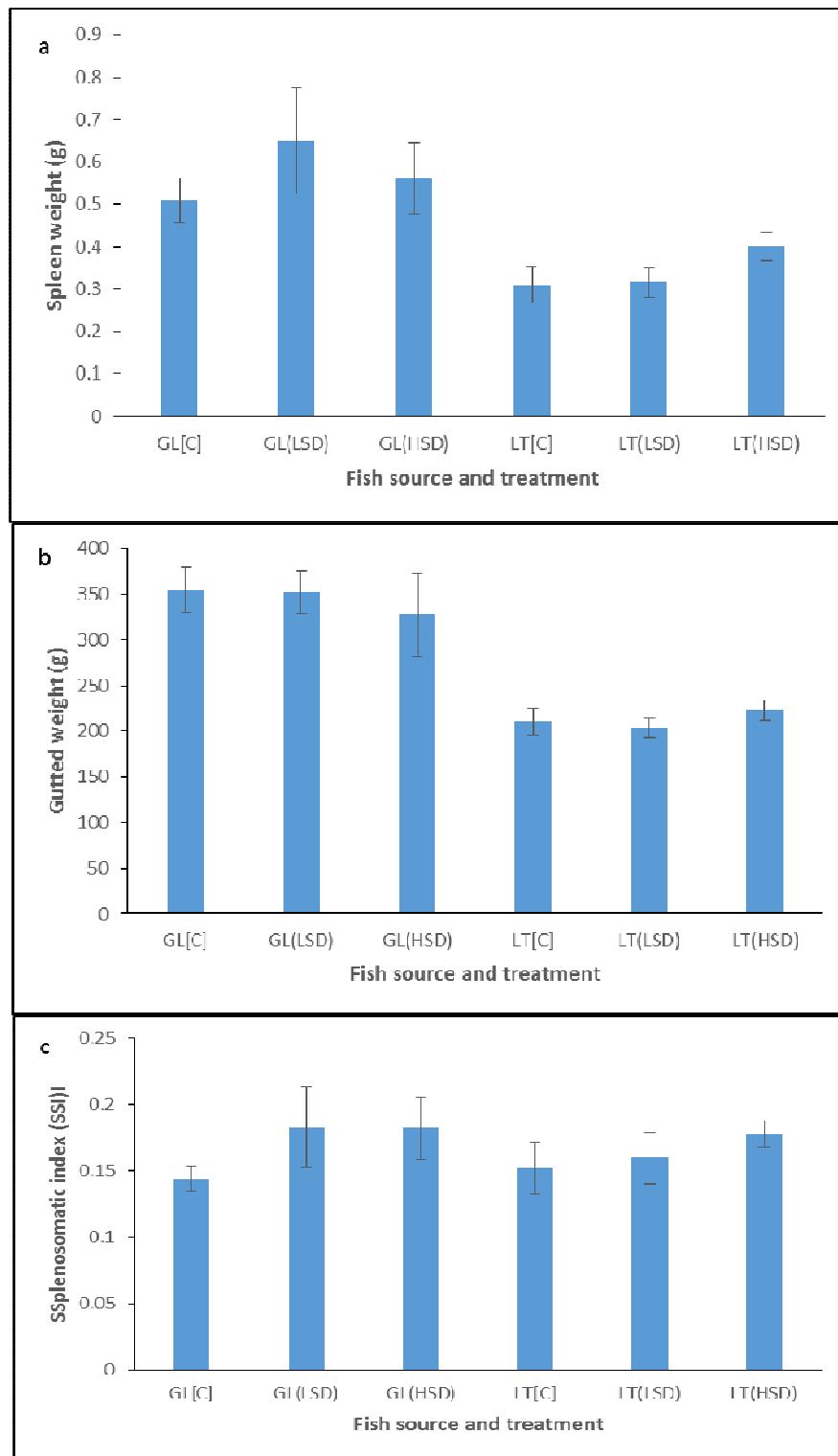


Figure 3-11: Effect of fish source and caging treatment on (a) spleen wet weight, (b) gutted fish wet weight and (c) splenosomatic index (SSI). Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatment: LSD=low confinement density, HSD=high confinement density, LHSD= low/high confinement density.

Hepatosomatic (Liversomatic) Index (HSI)

The liver weight ranged from 2.18-5.24 g (**Figure 3-12**, Appendix Table A3-6); Gippsland Lakes fish had significantly heavier livers than Lake Tyers fish (ANOVA, $F=703.5$, $p<0.001$) but there was no significant difference between control and caged fish (ANOVA, $F=0.15$, $p=0.725$) (**Figure 3-12a**).

As for the SSI, the gutted fish wet weight ranged from 203.69-354.98 g; Gippsland Lakes gutted fish were significantly heavier than Lake Tyers fish (ANOVA, $F=197.7$, $p=0.001$) but there was no significant difference between control and caged fish (ANOVA, $F=0.14$, $p=0.737$) (**Figure 3-12b**).

The hepatosomatic index (HSI) ranged from 1.04-1.45; Gippsland Lakes fish had a greater HSI than Lake Tyers fish (ANOVA, $F=114.70$, $p=0.002$) but there was no significant difference between control and caged fish (ANOVA, $F=0.022$, $p=0.674$) (**Figure 3-12c**).

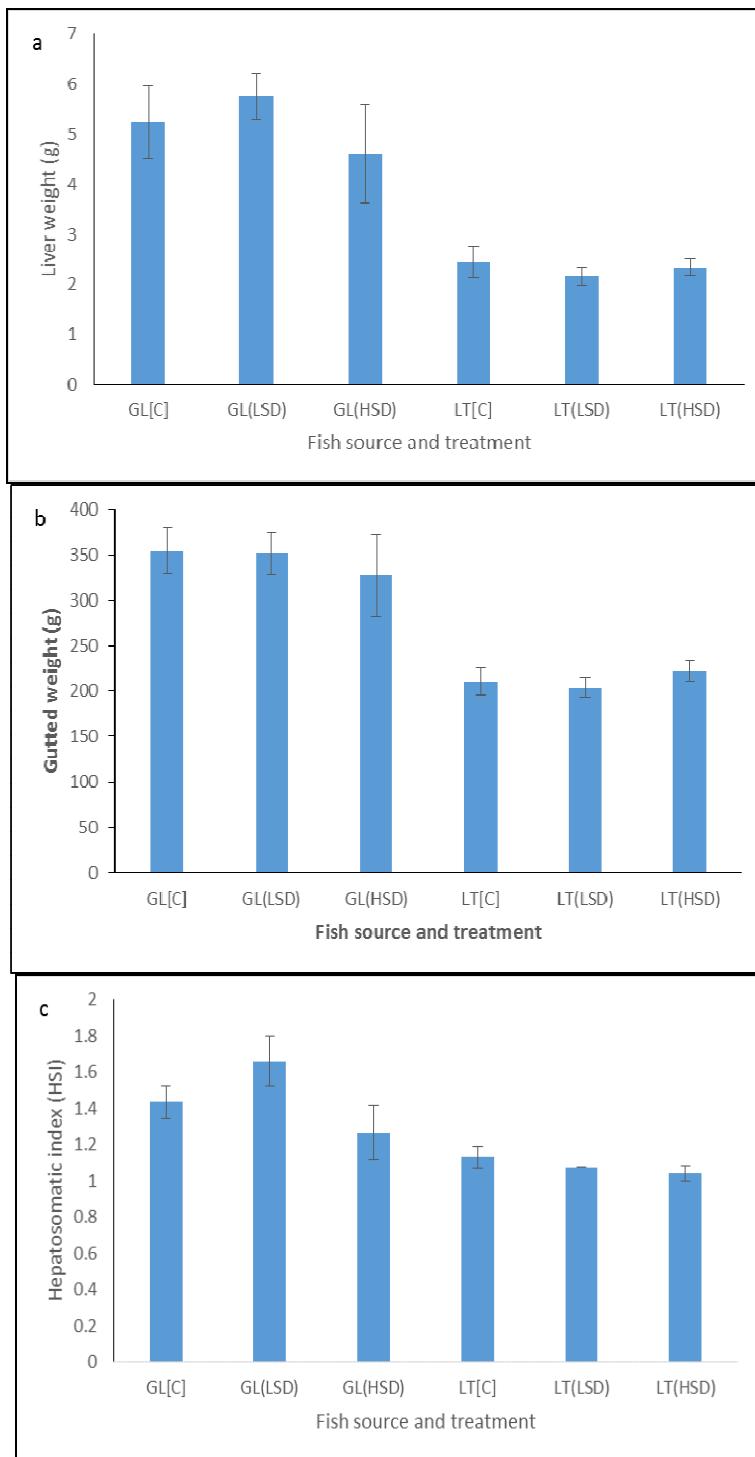


Figure 3-12: Effect of fish source and caging treatment on (a) liver wet weight, (b) gutted fish wet weight and (c) hepatosomatic (liversomatic) index (HSI). Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatment: LSD=low confinement density, HSD=high confinement density, LHSD= low/high confinement density.

Gonadosomatic Index

The wet weight of gonads ranged from 4.23-20.21 Gippsland Lakes fish had significantly heavier gonad weights than Lake Tyers fish (ANOVA, $F=73.49$, $p<0.001$) but there was no significant difference between control and caged fish (ANOVA, $F=0.00$, $p=0.990$) (Figure 3-12a).

Similar to the SSI and HSI, the gutted fish wet weight ranged from 206.75-354.98 g (**Figure 3-13**, Appendix Table A3-7); Gippsland Lakes gutted fish were significantly heavier than Lake Tyers fish (ANOVA, $F=293.38$, $p<0.001$) but there was no significant difference between control and caged fish (ANOVA, $F=0.14$, $p=0.735$) (**Figure 3-13b**).

The gonadosomatic index (GSI) ranged from 1.98-5.95; Gippsland Lakes fish had a greater GSI than Lake Tyers fish (ANOVA, $F=23.16$, $p=0.017$) but there was no significant difference between control and caged fish (ANOVA, $F=0.02$, $p=0.888$) (**Figure 3-13c**).

Ovotestes were observed in 26% of Gippsland Lakes black bream and 25.5% of Lake Tyers fish; these fish were categorised as transitional (**Table 3-6**). There was no significant effect on the percentage of male fish with fish source (ANOVA, $F=0.06$, $p=0.828$) or caging (ANOVA, $F=0.29$, $p=0.629$) alone.

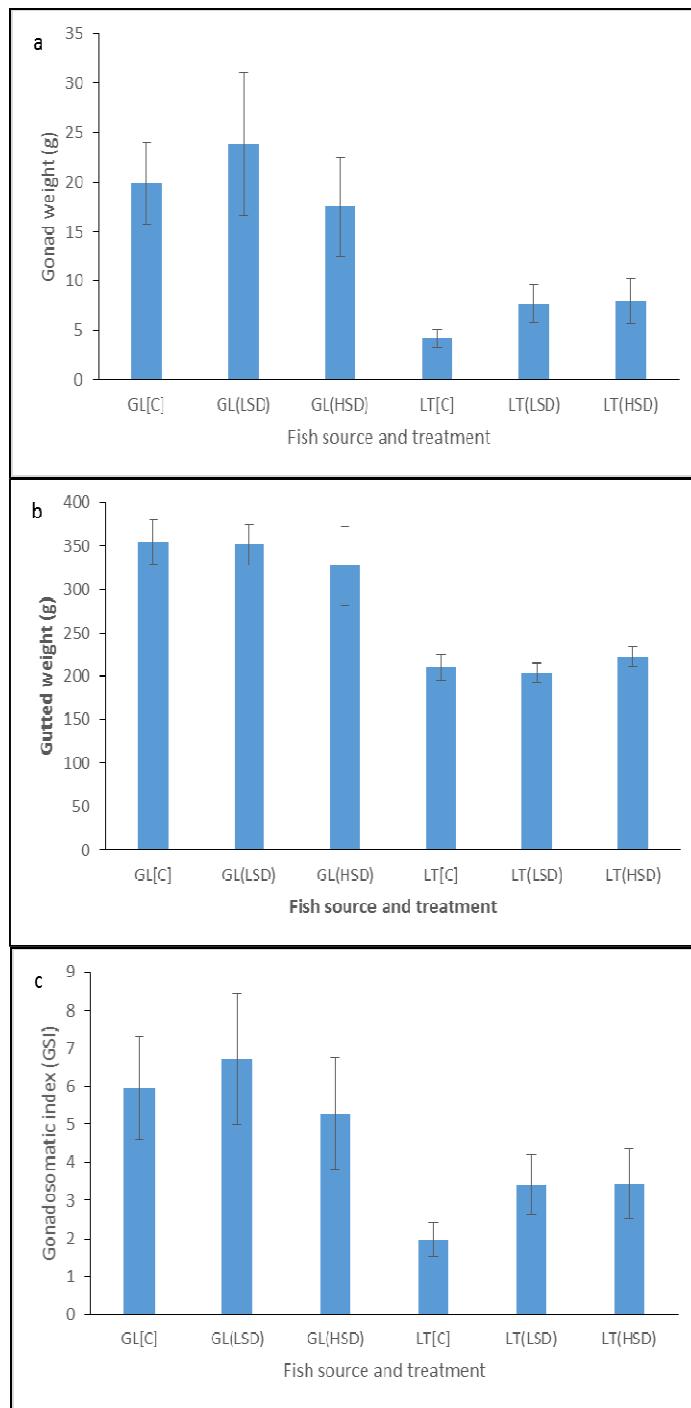


Figure 3-13: Effect of fish source and caging treatment on (a) gonad wet weight, (b) gutted fish wet weight and (c) gonadosomatic index (GSI). Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatment: LSD=low confinement density, HSD=high confinement density, LHSD= low/high confinement density.

Table 3-6: Sex ratios [male (M): transitional (T): female (F)] of Gippsland Lakes (GL) and Lake Tyers (LT) caged black bream at high confinement density (HSD), low confinement density (LSD), LHSD= low/high confinement density or uncaged controls (C) at termination of experiment.

Group	Sex ratio (M:T:F)	% male	% transitional	% female
GL[C]	2:2:6	0.30	0.30	0.40
GL(LHSD)	4:7:6	0.36	0.29	0.36
LT[C]	5:2:3	0.50	0.20	0.30
LT(LSD)	9:5:5	0.47	0.24	0.29
LT(HSD)	4:5:9	0.13	0.33	0.53

The GSI and HSI were significantly correlated ($r=0.515$, $p<0.001$) (**Figure 3-14**). Ovotestes only occurred in fish with a large ratio of GSI to HSI (**Figure 3-14A**). These fish occurred in all fish sources and treatments. Regression analysis showed that the GSI was predictable from the equation (with defaults of transitional sex and Lake Tyers controls):

$$GSI = 6.69^* - 0.12 HSI^{NS} - 6.19 (\text{female})^* - 4.63 (\text{male})^* + 3.00 (\text{GL})^* + 0.489 (\text{Caged})^{NS}$$

$$(R^2=64.0\% \text{: ANOVA, } F=24.09, p<0.001)$$

where $^*P \leq 0.05$, $^{NS}=\text{not significant at } P=0.05$

This shows that both males and females had significantly less GSI values than transitional sex fish and that neither HSI nor caging had a significant effect on GSI.

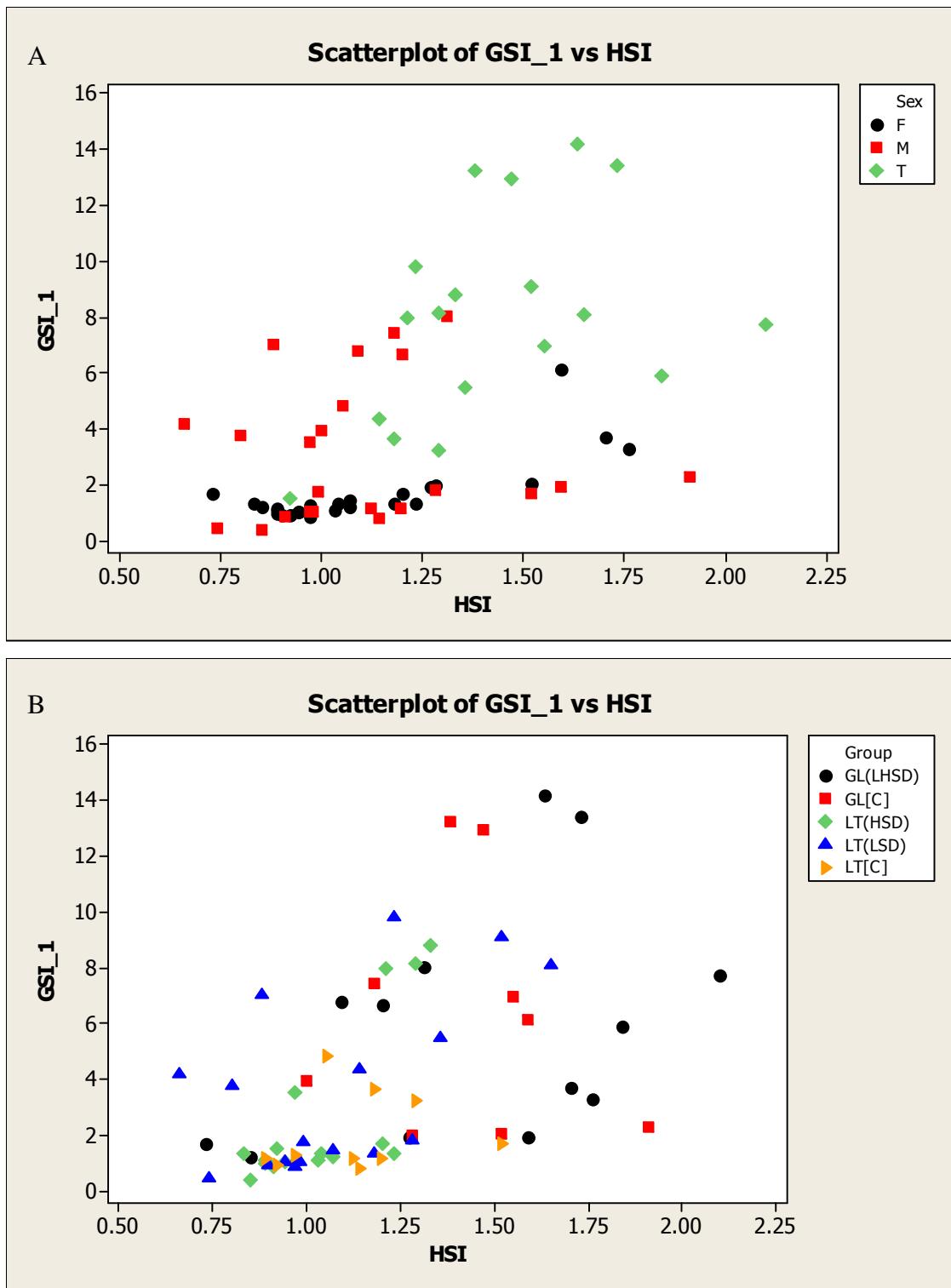


Figure 3-14: Effect of sex on GSI to HSI ratio for black bream. A) Grouped by sex: F=female, M=male, T-transitional (ovotestes present). B) Grouped by fish source and treatment: fish source: GL=Gippsland Lakes, LT=Lake Tyers; treatment: LSD=low confinement density, HSD=high confinement density, LHSD=mixed confinement density.

3-3-4 Chronic stress

In the stimulus-response test, four Lake Tyers fish from Treatment 5 had a median response time of 19 min, compared with 4.75 min for the corresponding control, 0.03 min for the low confinement density treatment and 0.3 min for the high confinement density treatment (**Table 3-7**, Appendix Table A3-8).

Table 3-7: Effect of handling stressor (blood sampling) 12 h after air exposure in the stimulus-response test.

Treatment	Median response time (min)
LT(C)	4.75
LT(LSD)	0.03
LT(HSD)	0.3
LT(B)	19

3-3-5 Summary

In summary, 9/14 parameters showed that Gippsland Lakes black bream were significantly different from Lake Tyers black bream but that only 1/14 parameters (SSI) showed any difference between caged and control fish) (**Table 3-8**).

The Gippsland Lakes fish were longer and heavier than Lake Tyers fish and had correspondingly greater weights of spleen, liver and gonads. Despite this, there was no significant difference with fish source in HAI, K, SSI or HSI though there was with GSI.

There was no effect of caging on response time, HAI, K, HSI, GSI or sex ratio and the effect on SSI was of marginal significance ($p=0.052$).

Table 3-8: Summary of results and statistical tests for fish from Gippsland Lakes (GL) and Lake Tyers (LT) in control (C) and caged treatments at low confinement density (LSD) or high confinement density (HSD) or a mixture (LHSD).

Treatment	Response time (min)	HAI	% enlarged spleen	Fish wet wt	Fork length	K	Spleen wt	Gutted fish wt	SSI	Liver wt	HSI	Gonad wt	GSI	Sex ratio (M:T:F)
GL[C]	6.49	2.7	9.1	385.1	26.5	2.04	0.51	354.98	0.144	5.24	1.44	19.93	5.948	0.30:0.30:0.40
GL(LHSD)	30.00	30	78.6	373.8	26.1	2.05	0.60	337.94	0.183	5.15	1.45	20.21	5.891	0.36:0.29:0.36
LT[C]	4.75	6	20.0	228.3	22.4	2.00	0.31	210.76	0.148	2.45	1.13	4.23	1.983	0.50:0.20:0.30
LT(LSD)	0.03	2.7	9.1	228.5	22.2	2.06	0.32	203.69	0.161	2.18	1.07	7.73	3.415	0.47:0.24:0.29
LT(HSD)	0.30	13.5	45.0	236.1	22.9	1.95	0.40	222.59	0.178	2.34	1.04	7.98	3.425	0.13:0.33:0.53
P value for														
Fish source	0.00-0.04	0.475	0.160	<0.001	<0.001	0.369	0.024	0.001	0.952	<0.001	0.002	<0.001	0.017	0.828
Caging	0.15-0.21	0.363	0.118	0.768	0.756	0.994	0.840	0.737	0.052	0.725	0.674	0.990	0.888	0.629
All			<0.001											
GL(C) vs LT(C)			0.476											
GL (C vs LHSD)			<0.001											
LT all			0.025											
LT (LSD vs HSD)			0.008											
Statistic	χ^2	ANOVA	χ^2	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA	ANOVA

3-4 Discussion

The greater time taken for Gippsland Lakes than Lake Tyers black bream to seek shelter in the stimulus-response behavioural test suggests that (1) the Gippsland Lakes fish suffered from a greater level of stress and (2) the stimulus test was a useful short-term non-destructive test for chronic stress that avoids sacrificing fish for destructive tests like HAI, K, SSI, HSI and GSI.

The lack of significant effects of caging on any of these latter variables (except GSI) suggests that caging for only a month did not change the level of chronic stress suffered by fish from different estuaries even after acclimation under identical conditions for a year. The differences between fish from different estuaries may relate to difference in (a) fish size, (b) previous environmental conditions or (c) genetics. These are discussed in more detail below.

3-4-1 Cage Confinement– Fish Colour and Mortality

Two types of response occurred before the stimulus tests: transient darkening in colour of caged fish and mortalities in the caged Gippsland Lakes fish.

Darkening in colour

There was no obvious difference in the degree of dark colouration that first appeared 4–7 days after placement and remained for 5 – 14 days depending on how dark individual fish were. The darkening and its duration showed no difference between the Gippsland Lakes and Lake Tyers fish, suggesting that their ability to cope with confinement as a stressor was not markedly different. Fish of dark colouration eventually recovered their light appearance and position at the bottom of the cage, suggesting cage confinement was less stressful than capture and air exposure for black bream. Haddy and Pankhurst (1999) have also suggested

that tank confinement might be less stressful than the initial capture and handling for black bream after observing a decline in cortisol levels following 24 h of confinement. Similarly, Rotllant and Tort (1997) found that cortisol was less in red porgy (*Pagrus pagrus*) following 3 weeks confinement than in acutely stressed fish.

In other species, darkening of the skin occurred in cortisol-treated Atlantic salmon (*Salmo salar*) and carp (*Cyprinus carpio*) (Langdon *et al.* 1984; Iger 1992; Iger *et al.* 1995) and was identified as a sign of submission in territorial disputes (O'Conner *et al.* 1999). The disappearance of iridocytes also occurred in carp exposed to cadmium, and in trout (*Oncorhynchus mykiss*) exposed to raised temperatures; in either case elevated cortisol levels were reported not to be a prerequisite (Iger *et al.* 1994a, Iger *et al.*, 1994b, Balm *et al.* 1994a, Iger 1995; Balm, 1997; Iwama *et al.* 1997).

Cage mortalities

The occurrence of two mortalities in the Gippsland Lakes caged treatment could not be ascribed to the caging because of the lack of replication and hence power in the analysis. Several factors compromised this experiment, rendering the results of interest but only of use in a preliminary sense prior to planning a further experiment with adequate replication. Four factors may have made these fish more vulnerable than the rest: fish size, reproductive state, previous environment and genetic differences.

(i) *Fish Size* – Dominance hierarchies in each cage may have also increased with increasing discrepancies in fish size (Wedemeyer, 1997; Pankhurst, 1998). The largest range in fish size was 130 mm for the Gippsland Lakes high confinement density cage, whereas the largest range in Lake Tyers fish was 62 mm, in the high confinement density No. 2 cage. This may have made the Gippsland Lakes dominance hierarchy stronger than in other cages and, coupled with sexual maturity, increased the level of stress in the fish.

(ii) - Reproductive State – Both fish that died were male with GSI values of 11-7.7, less than the mean value of 19-20 for Gippsland Lakes fish. Limited energy reserves due to their reproductive condition (mesenteric fat was absent) may have made both animals more vulnerable than their peers to additional stress associated with caging and therefore being unable to compensate. Both fish were possibly at stages 3 and 4 of the Haddy and Pankhurst (1998) macroscopic classification. Death could have resulted from exhaustion (Selye, 1973; Barton, 1997; Schreck, 2000) or the immunosuppressive effects of the cortisol response to the acute stress of handling and caging (Broadhurst *et al* 1999; Roberts and Rodger, 2001).

(ii) - Previous environment - The enlarged livers of both dead fish (HSI values of 2.5 and 2.7) suggest exposure to a toxicant (Fletcher *et al.* 1982; Buckley *et al.* 1985; Goede and Barton, 1990) and are due to hyperplasia or hypertrophy as an adaptive response that increases the capacity of the liver to detoxify foreign compounds via the mixed-function-oxidase system (MFO) (Goede and Barton, 1990). Gippsland Lakes black bream were obtained from a variety of locations and, consequently, this suggests that some animals had encountered contaminated sites and developed enlarged livers as a result (Fabris *et al.* 1999).

(iii) Genetic differences – Mortalities in Gippsland Lakes fish may be the consequence of a population of black bream more stress-sensitive than fish from Lake Tyers. However, Burridge *et al.* (2004) found that Gippsland Lakes and Lake Tyers black bream were genetically similar. Also, if there was a more stress-sensitive strain, then mortalities would have probably occurred during the initial capture and handling of fish prior to and during their acclimation.

3-4-2 Behavioural Response –A Stimulus Test

Gippsland Lakes fish were slower and less likely to seek shelter than Lake Tyers fish after an acute stressor (air exposure), suggesting that the Gippsland Lakes fish suffered from a greater degree of stress. As caged fish showed no difference in response time from control fish, there was no evidence that cage confinement altered fish behaviour due to an allostatic load as proposed by Schreck (2000).

This was despite a large variation in response time. No overt signs of stress were evident, suggesting that surviving fish were compensating for the confinement. However, the rate of re-establishing homeostasis may not have been the same for all fish. If behavioural experiments were performed before all fish had fully compensated for confinement, then many fish would experience differing levels of stress. Response times to a stimulus in the behavioural apparatus would then reflect this variation in stress levels. Variability in response is often encountered in stress studies and can be influenced by the intensity and duration of the stressor (Strange *et al.* 1978; Barton *et al.* 1980; Foo and Lam, 1993 Barton, 1997; Olla *et al.* 1998), differences between strains of fish (Barton *et al.* 1986; Pickering and Pottinger, 1989 and Pottinger and Moran, 1993), differences between discrete stocks (Iwama *et al.* 1992) and differences in social interactions (Pottinger and Pickering, 1992; Alvarenga and Volpato; 1995; Lowe and Wells, 1996; Barnett and Pankhurst, 1998; Pickering, 1998; Makinen *et al.* 2000). Prior encounters and experience with fishing operations may have also resulted in a positive or negative “conditioning” or “psychological hardening” (e.g. Laird *et al.* 1972; Schreck *et al.* 1995, Schreck *et al.* 1997) influencing fish behaviour when subjected to tank and cage confinement.

Fish size

The sensitivity of this study may have been affected by fish size when individual fish were relocated from a tank or confinement cage to the channel apparatus. The larger fish from the Gippsland Lakes may have experienced greater confinement stress than the smaller fish from Lake Tyers and so recorded slower responses. In ideal conditions, all fish from each estuary would be within one standard deviation of that of the other, but as the captive populations were wild-caught over several months, that was not within the control of the experiment.

Previous treatment

The confinement of the behaviour apparatus may also have cumulatively affected individual fish arriving from a tank or low confinement density treatment more than those fish arriving from a high confinement density treatment. The 16 h acclimation period in the behaviour apparatus prior to experimentation was greater than the 12 h required for recovery from the acute stressor in Chapter 2. With physiological mechanisms responsible for initiating and maintaining behavioural sequences (Schreck *et al.* 1997), a 16-hour delay before a behavioural test would be expected to minimise any acute stress response associated with relocation to the behavioural apparatus. Therefore, individual fish from high confinement density may have experienced a greater degree of recovery than fish from low confinement density experiencing greater confinement.

Genetic differences

Genetic differences may have affected the sensitivity of fish from the Gippsland Lakes and Lake Tyers to stressors and therefore response times. Genetic differences seem unlikely from the work of Farrington *et al.* (2000) and Burridge *et al.* (2004), which described a gene flow in black bream between adjacent estuaries of south-eastern Australia that is consistent with a

one-dimensional stepping-stone model and more recent studies (see also Chapter 6). The Gippsland Lakes and Lake Tyers are adjacent estuarine systems with their entrances less than 15 km apart and so genetic similarity would be expected for black bream, because black bream from the Gippsland Lakes and a more distant estuary, Sydenham Inlet (**Figure 3-15**), showed no genetic differences (C. Burridge, pers. comm.). Genetic isolation could, however, have occurred if historically Lake Tyers had a greater frequency of isolation than that of the Gippsland Lakes and Sydenham Inlet (C. Burridge, pers. comm.). The ocean entrance to Lake Tyers has been closed more often than that of the Gippsland Lakes since the permanent opening of the latter in 1889 (Bird, 1961). However, Sydenham Inlet is similar to Lake Tyers in that it is only open intermittently to the ocean (Ramm, 1983; Hall, 1984; NREC, 1991; MacDonald, 1997; EGCMA, 2006). Fish from the Gippsland Lakes and Sydenham Inlet have been sampled previously for genetic studies (see Chapter 6), but those from Lake Tyers have not and it is possible that frequent long-term closure of the Lake Tyers estuary has isolated the black bream population genetically.

Social interaction

Social interaction may have been different in Gippsland Lakes and Lake Tyers fish. Fish of healthy appearance aligned together on or near the bottoms of tanks or cages during the day but were more dispersed throughout the volume of a holding tank when light intensity surrounding that tank was diminished and when fish were observed under red light. This could explain the occurrence of physical injuries such as a loss of an eye observed on individual fish in some cages, as Rigby (1984) reported that black bream were active in their natural environment at night. With social interactions, stress levels are variable, in particular

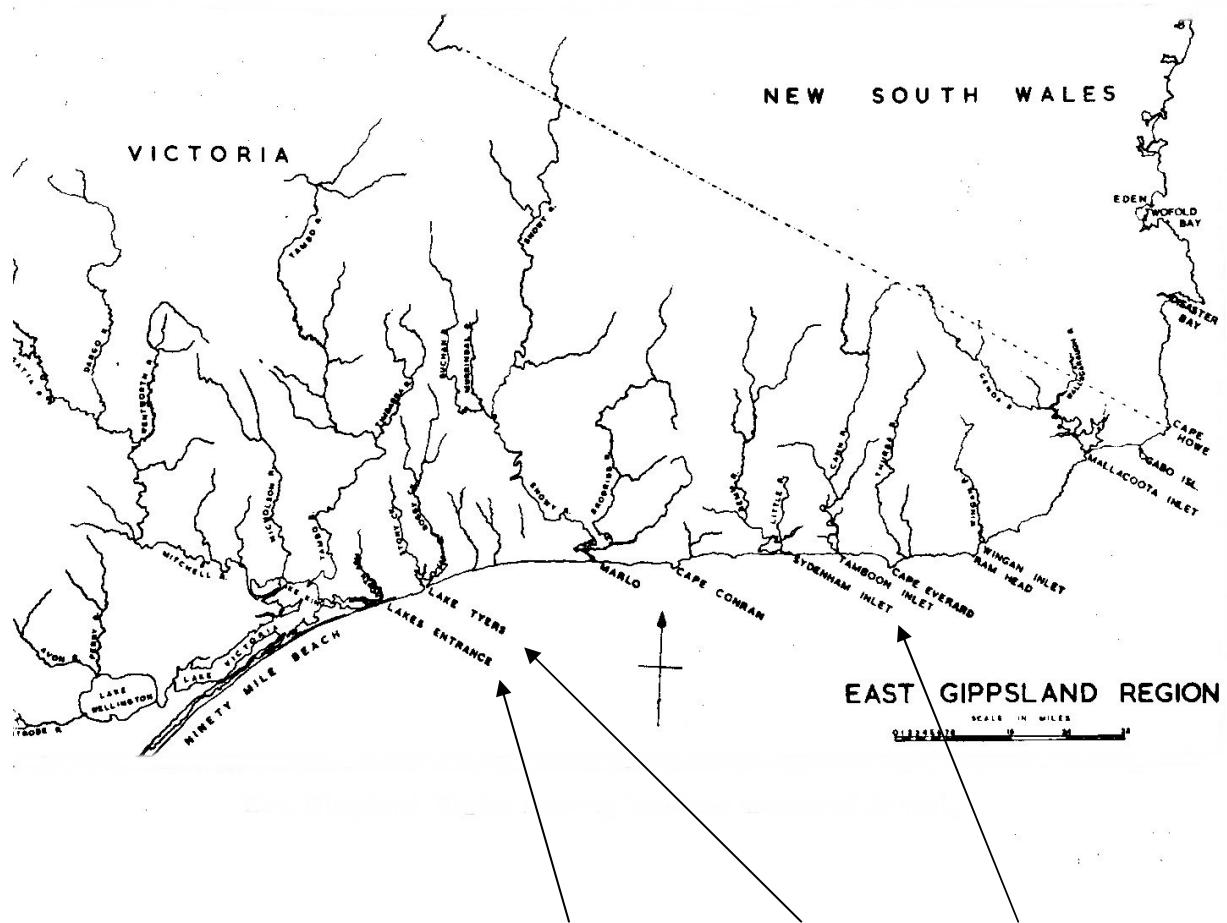


Figure 3-15: Locations of Gippsland Lakes, Lake Tyers and Sydenham Inlet.

between the primary dominant and subordinate fish (Schreck *et al.*, 1997; Barnett and Pankhurst, 1998). In other studies, undertaken at a similar time in the year, spawning behaviour of black bream in tanks involved groups of males chasing receptive females (Pankhurst, 1998), which would tend to increase chronic stress. These might tend to be greater in the larger Gippsland Lakes fish than in the smaller Lake Tyers fish.

Social interactions in fish can also be influenced by exposure to contaminants. An increased level of aggression was observed in bluegill (*Lepomis macrochirus*) when exposed to a sublethal mixture of metals (Henry and Atchison, 1979) and pesticides (Henry and Atchison, 1984; Fairchild and Little, 1999). Black bream in the Gippsland Lakes are also exposed to

contaminants such as heavy metals and pesticides (Fabris *et al.* 1999). Contaminant exposure of black bream in Lake Tyers has yet to be investigated but would be expected to be less than in the Gippsland Lakes because of the less developed nature of the catchment.

Pre-capture experience

Past experience may have conditioned or psychologically hardened (Schreck *et al.* 1995, 1997) some fish in each experimental group. At the time of this study, Gippsland Lakes and Lake Tyers, black bream were exposed to commercial and recreational fishing operations. Therefore, some fish from each estuary may have previously experienced confinement and handling whilst others may not, but any previous influences would have to have persisted through a year of acclimation. With identical treatment, it is doubtful that such behavioural modification would have occurred during the tank acclimation period.

The significant difference in response between fish from the two estuaries may reflect different environmental stressor loading, as this is unlikely to be the same between Gippsland Lakes and Lake Tyers because of the different states of development in their catchments. Chronic stress loading in fish may be identified by reduced growth (Adams, 1990). Black bream in the Gippsland Lakes experience low growth rates compared to black bream in Lake Tyers (Coutin, 2000) and throughout the species range (MacDonald, 1997b). Furthermore, growth rates in black bream are more influenced by environmental factors than genetic differences (Partridge *et al.* 2004). If Gippsland Lakes black bream are slower in their response to an external stimulus due to stressor loading, then this may retard necessary avoidance responses in their natural environment and may predispose caught-and-released black bream to an increased risk from predation.

Indirectly, a difference in response times may be a consequence of environmental loading. Anthropogenic exposure to organochlorines alters behaviour in fish (Henry and Atchison,

1984; Brown *et al.* 1997; Kruzynski and Birtwell, 1994), as does exposure to heavy metals (Peterson, 1976; Henry and Atchison, 1979). Swimming behaviour such as that associated with predator avoidance is affected by metals and organochlorine contaminants (Little, 2002). Kruzynski and Birtwell (1994) reported a reduction in “ecological fitness” that was measured by an increase in bird predation after exposure to contaminants had altered fish behaviour. Black bream in the Gippsland Lakes are exposed to organochlorine insecticides and mercury (Fabris *et al.* 1999). Furthermore, predation by the Great Cormorant (*Phalacrocorax carbo*) has already had an impact on mortality levels of black bream in the Gippsland Lakes (Reside and Coutin, 2001).

Replication

A significant handicap facing this study was the shortage of Gippsland Lakes undersized black bream available during the sampling period. When comparing control groups from each estuary, a sample size comparable to that of Lake Tyers fish would have better supported this comparison. This indicates a serious limitation to the behavioural approach. A large variation in stress response to the same stressor is to be expected. This necessitates large sample sizes. Therefore, a readily available supply of black bream is required. If there is a limitation to access to live fish, then an alternative method needs to be utilised.

A further problem was the shortage of available tanks in which to conduct the experiments to avoid the problem of pseudoreplication. It was hoped that the subsequent relocation of the tanks to the new facility at Bullock Island in Lakes Entrance would alleviate this but that was not the case. To repeat this experiment entirely with adequate replication and known variability, it was estimated that a total of 36 tanks and 360 undersized fish would be required [3 treatments (control, HSD and LSD) x 6 tanks per treatment x 2 estuaries x 10 fish per

estuary] and this seemed impractical, as the total number of tanks available was 18 and the permit was limited to 300 fish.

3-4-3 Physiological Responses

Opercular Rate

There was a significant negative correlation between opercular rate and response times to seek cover under a hide for Gippsland Lakes fish. For Lake Tyers, a single positive correlation existed ($r = 0.942$) for fish in a low-confinement cage. The significant negative correlation in this study is as predicted from previous work by others. In fish, a positive correlation generally exists between opercular beat rate and metabolic demands (van Rooij and Videler, 1996; Adams *et al.* 2001; Dalla Valle *et al.* 2003; Artigas *et al.* 2005) and opercular beat rate also increases when fish perceive a situation as stressful (Dalla Valle *et al.* 2003; Woodely and Peterson, 2003). Therefore fish that perceive a situation as stressful are more likely to have a high opercular rate and respond quickly, whereas fish not perceiving a situation as stressful are more likely have an unchanged opercular rate and to take a long time to respond, producing a negative correlation.

Health Assessment Index (HAI)

The item of major influence on HAI values in all experimental groups was the presence of an enlarged spleen. An enlarged spleen has been primarily associated with three causes: infection/disease, spawning and fish age. The spleen serves a hemopoietic function and its enlargement condition in this organ could indicate bacterial infection or disease (Adams *et al.* 1993; Adams *et al.* 1999; Roberts and Rodger, 2001). Therefore, the appearance of the same symptoms in differing experimental groups may indicate an opportunistic infection by pathogens already present in all tanks as part of the natural flora on/in black bream

(Broadhurst *et al.* 1999; Roberts and Rodger, 2001). Splenomegaly can also be observed in some fish species at spawning time (Ferguson, 1988). Many black bream were in a reproductively mature condition at the time of the HAI, making splenomegaly from this cause possible. However, this cannot explain the greater proportion of fish with an enlarged spleen in Gippsland Lakes caged than control fish. Morphological alterations in hematopoietic tissues can occur in subordinate fish as a consequence of stress due to social hierarchy (Rice and Arkoosh, 2002), but that was unlikely to be the case in this study, as the proportion of fish with enlarged spleens did not relate to fish size range. The age of fish can also contribute to enlarged spleens (Roberts and Rodger, 2001) but again, does not explain the difference between caged and control Gippsland Lakes fish. This is discussed further under SSI.

The Gippsland Lakes caged fish was the only treatment that also displayed focal discolouration of the liver, which can be due to focal necrosis caused by bacterial infection (Adams *et al.* 1993). Liver discolouration can also be a consequence of contaminant exposure and, in particular, PCB toxicity (Lipsky *et al.* 1978; Teh, *et al.* 1997; Adams *et al.* 1999). This treatment also produced the only two mortalities of this study. These observations are consistent with a pollutant-mediated susceptibility to infection (Vethaak *et al.* 1993; Arkoosh *et al.* 1994; and Dunier *et al.* 1994) but the lack of replication makes these conclusions only tentative.

A change in fish health is a tertiary stress response (Barton *et al.* 2002), which has incorporated several lower levels of biological organization (i.e. primary and secondary levels) (Schreck, 1990; Wedemeyer *et al.* 1990; Barton *et al.* 2002). However, primary and secondary responses can vary depending on their early life history and nutritional status, which in turn depend on their environment, and their genetic differences (Barton *et al.* 2002).

The early life history for black bream in this study was those locations these fish inhabited prior to their capture. Therefore, black bream from the Gippsland Lakes may have come into the study with a greater chronic stress loading (Adams *et al.* 1993) than those from Lake Tyers, but there was no difference in the proportion of fish with enlarged spleens between Gippsland Lakes and Lake Tyers controls. This may be because the Lake Tyers fish are exposed to widely fluctuating environmental conditions associated with seasonal closure of estuaries (Hall, 1984; NREC, 1991; MacDonald, 1997a).

Condition factor (K)

The lack of significant difference in condition factor (K) or “well-being” (Busacker *et al.* 1990; Morgan and Iwama, 1997) was as expected, since during the study all fish fed to satiation, apart from the two mortalities, and there was no indication of differences in feeding behaviour due to stress effects (Brown *et al.* 1987). This contrasts with the increase in cortisol and the decrease in Fulton’s condition factor in carp (*Cyprinus carpio*) during attack by cormorants (Kortan *et al.*, 2011). However, within this, there were significant increases in SSI (with caging) and in HSI and GSI (with fish source).

Splenosomatic Index (SSI)

The SSI indicated no significant difference with fish source, despite the differences in frequency of enlarged spleens among treatments. The SSI is a relatively gross measure of splenic function and there may have been internal damage similar to the increases in peroxisomes, enlargement of lysosomes and cell debris in the spleen of Cardinal fish (Apogonidae) at a polluted site (Fishelson, 2006).

The difference between control and caged treatments in the SSI was, however, marginally significant ($p=0.052$) despite the difference in size between Gippsland Lakes and Lake Tyers

fish. This was different from the lack of effect of stocking density on SSI in the African catfish (*Clarias gariepinus*) (Wang *et al.*, 2013) and the opposite of the reduction in SSI (from 0.27 to 0.20) with increase in stocking density in Amur sturgeon (*Acipenser schrenckii*) (Ni *et al.*, 2014). Both of these studies also noted no effect of stocking density on serum cortisol level. The stocking densities used in this study were less than those used in African catfish but greater than those used in Amur sturgeon and both studies lasted almost twice as long as this one. Both acute and chronic stress also led to immunosuppression in other species, such as jundiá (*Rhamdia quelen* and *Gairnard pimelodidae*) (Barcellos, 2004), Senegalese sole (*Solea senegalensis*) (Salas-Leiton, 2010) and Atlantic salmon (*Salmo salmar*) (Fast, 2008). This is consistent with the down-regulation of the HPI axis as a result of negative feedback by cortisol in chronic stress (Barton *et al.* 2002).

As discussed earlier, the spleen is vital to the immune system in fish by producing IgMb mature B cells and clearing blood-borne antigens (Barton, 2002; Ni *et al.*, 2014). An increase in SSI indicates an increase in challenge to the immune system, which could be caused by stress-stimulated increase in pathogens or parasites. For example, in rainbow trout (*Oncorhynchus mykiss*) elevated levels of serum cortisol were provoked by increases in the numbers of the bacterium *Vibrio anguillarum* in the spleen during transport (Tacchi *et al.*, 2015). In aquacultured carp (*Cyprinus carpio*), SSI and serum cortisol also increased significantly during attack by cormorants (Kortan *et al.*, 2011). Increases in spleen size and SSI in Atlantic salmon fed genetically modified soybean meal were thought to indicate depression of immune function (Hemre *et al.*, 2005; Sagstad *et al.*, 2008). Enlarged spleens were also thought to indicate immune stress in the three-spined stickleback (*Gasterosteus aculeatus*) (Barber *et al.*, 2001).

As noted by Barton (2002), the immune responses in fish vary widely and the marginally significant increase in the SSI observed here requires confirmation in this or other species of *Acanthopagrus*.

A technical problem may have contributed to lack of sensitivity in SSI. There was not a sufficiently sensitive balance available at the time of processing fish to resolve differences of less than 0.01 g in spleen weight. This should not have caused a problem, since the least difference in spleen weights between control and corresponding caged treatments was 0.09 g and this was $\geq 18\%$ of the spleen weight.

Hepatosomatic (Liversomatic) Index(HSI)

The HSI was significantly greater in Gippsland Lakes than Lake Tyers black bream. Liver enlargement can be a consequence of exposure to pollution and specific toxicants (Fletcher *et al.* 1982; Buckley *et al.* 1985; Goede and Barton 1990) and increased lipid storage can be the result of organochlorine exposure (Adams *et al.* 1996). Therefore, these fish from the Gippsland Lakes may have been exposed to toxicants not encountered by those from Lake Tyers. This increase in HSI is consistent with identified pesticide and mercury loading in Gippsland Lakes black bream by Fabris *et al.* (1999). The level and impact of toxicant exposure of black bream in Lake Tyers has yet to be investigated.

There was no evidence for a difference in HSI with increased stocking rate, similar to the lack of effect in African catfish (Wang *et al.*, 2013) but unlike the decline in HSI observed in Amur sturgeon (Ni *et al.*, 2014).

Gonadosomatic Index (GSI) and Sex Ratio

GSI values for Gippsland Lakes black bream were greater than those of Lake Tyers fish, as expected from the Gippsland Lakes fish being longer and heavier than those from Lake Tyers. There was no difference in the GSI with caging for the relatively short time of 30 days.

The GSI is an indication of sexual maturity and a GSI of 5.9 for Gippsland Lakes fish is equivalent to the greatest level reached during the spawning season (September-December) in wild-caught black bream from Tasmania (Haddy and Pankhurst, 1998). By contrast, the GSI of 1.9-3.4 for Lake Tyers fish is not much greater than the minimum level observed out of the breeding season by Haddy and Pankhurst (1998), though it is greater than the GSI of 1.23-1.57 for field-collected black bream from Western Australia (Gagnon and Hodson, 2012). The smaller size of the Lake Tyers fish may be the explanation, as they may not yet have fully matured.

Black bream is a multiple-repeat spawner probably on a daily basis (Haddy and Pankhurst, 1998; Hobby *et al.*, 2000).

The incidence of fish with ovotestes in both estuaries was 26% and only occurred in males with elevated GSI and mid-high HSI values. A suppression of GSI and elevation of HSI values was observed after exposure to contaminants such as estrogenic compounds in sewage (e.g. Sheahan *et al.* 2002; Noaksson *et al.* 2005), which is the opposite of the conditions in the acclimation tanks. As the same proportion of experimental fish from both estuaries developed ovotestes after over a year in tanks and feeding to satiation, the equal development of ovotestes may be an artefact of the artificial conditions. In the Gippsland Lakes, Rowland and Snape (1994) found a sex ratio close to 1:1 and no evidence of ovotestes in 52 black bream but later Norriss *et al.* (2002) found ovotestes in all 40 black bream sampled.

Sex inversion is strongly controlled by social conditions in many fish species (Pankhurst, 1998). From observations made in Hoyers Lake (New South Wales), Rowland and Snape (1994) suggested that protogynous hermaphroditism is labile in black bream and is induced by environmental conditions or intense fishing pressure. Fish may have already had ovotestes on capture and could be responding to changes in both commercial and recreational fishing sectors in both estuaries (Rowland and Snape, 1994). Commercial fishing started in the Gippsland Lakes in 1878 but has changed in response to quotas; meanwhile, the amount of recreational angling has increased in both estuaries. Further research is needed to identify what factors are affecting the development of ovotestes in black bream in these two estuaries.

3-4-4 Concluding Comments

The intention of this inter-estuarine study was to investigate and compare black bream behaviour and physiological responses to chronic stress. Unfortunately, pseudoreplication had occurred in the caged experiments, thus rendering treatments with no replication.

It was however, found that Gippsland Lakes black bream were significantly different from Lake Tyers black bream in 9/14 parameters tested, but only 1/14 parameters (SSI) displayed a significant difference between treatments and their controls. Of the physiological indices, GSI was significantly different between estuaries, with Gippsland Lakes fish displaying greater values, this difference may have been influenced by salinity and temperature profiles of water also sourced from the Gippsland Lakes for experimental tanks.

The difficulties encountered in trying to work with live fish and restrictions on access to fish and tanks made planning further experiments such as this problematic and so different approaches were used to compare chronic stress in Gippsland Lakes and Lake Tyers fish and the reasons for any difference found.

Chapter 4. Histology

4-1 Introduction

4-1-1 Gippsland Lakes Situation

Gippsland Lakes black bream must cope with and react to a multi-stressor environment. There are increasing pollution loads (Fabris *et al.* 1999), significant bird predation (Reside & Coutin 2001), declining freshwater inflows (due to water abstraction and diversion from tributary rivers for irrigation and consumption in Melbourne) (Williams *et al.* 2012, 2013), ongoing stressors associated with catch-and-release fishing and increasing environmental challenges associated with a changing climate (Jenkins *et al.* 2010; Gillanders *et al.* 2011; Booth *et al.* 2011; Williams *et al.* 2013). Multiple stresses may result in a reduction in growth, impaired reproduction, susceptibility to disease, and a reduced capacity to tolerate subsequent stress (Adams *et al.* 1989; Adams 1990; Wendelaar Bonga, 1997; Hontela, 1998). Previous studies on the wild-caught Gippsland Lakes black bream fishery report the occurrence of variable growth, episodic recruitment, and a decline in annual catch rates (Coutin *et al.* 1997; Williams *et al.* 2012, 2013). Furthermore, stressors associated with angling, an activity endemic to the Gippsland Lakes, have been found to inhibit reproduction in wild-caught Tasmanian black bream (Haddy and Pankhurst, 1999). Chronic stress loading impacting on Gippsland Lakes black bream from such multiple stressors has long been suspected but has not been investigated.

Black bream in the Gippsland Lakes supports a major commercial fishery (MacDonald, 1997b; Williams *et al.* 2012), and up until 2003 this fishery also included black bream caught from Lake Tyers an adjacent but dissimilar estuarine system as discussed in chapter one.

Each of these estuarine systems imposes multiple stressors on fish peculiar to that system (Gillanders *et al.* 2011). Historically, black bream commercially caught from Lake Tyers often obtained higher prices because they were in “superior condition” when compared to Gippsland Lakes bream (Hall, 1984). Previous studies indicated that Lake Tyers black bream were larger than Gippsland Lakes fish of the same age (Coutin , 2000). Retardation of growth in a fish population is a recognised chronic stress indicator (Adams 1990; Wendelaar Bonga, 1997; Hontela, 1998; Mishra and Mohanty, 2009).

4-1-2 Fish condition and feeding

One of the factors in attaining a high growth rate and “superior condition” is the abundance of adequate food and its consumption by fish. As discussed previously, the condition factor is a generalised indicator of the overall health of a fish and can reflect the integrated effect of both nutritional states and metabolic stress (Adams *et al.* 1992; Adams *et al.* 1996), but is relatively insensitive to environmental stressors (Barton *et al.* 2002).

Recent feeding history and nutritional status of fish can be obtained from appearance of the gall bladder and its contents (Goede and Barton, 1990). Bile in the gall bladder can display a colour range from clear or straw-yellow (feeding within the last 1-2 days) to blue-green. The blue-green colouration is caused by oxidation of bilirubin pigments to biliverdin, indicating feeding in the last couple of days to not eating for a week or longer (Goede and Barton, 1990). An empty gall bladder indicates that the fish have probably eaten within the last few hours; an empty blue-green gall bladder indicates recently resumed feeding after a period of starvation (Goede and Barton, 1990). To identify feeding history, Goede and Barton (1990) created an index ranging from 0-2.

4-1-3 Kidney and Stress

In teleost fish, cellular changes in the spleen and kidney have been used to identify chronic stress (Wedemeyer *et al.* 1990). The kidney, along with the gills and intestine, is responsible for lymphoid, haemopoietic, osmoregulatory, excretion and the maintenance of homeostasis of body fluids (Hinton *et al.*, 1992; Evans, 1993) but is also one of the main organs of the endocrine system. The fish kidney consists of an anterior kidney (or head) kidney and a posterior kidney (or body or trunk kidney) (Silva and Martinez, 2007). The anterior kidney is integrated into the endocrine system and is important in the response to stress (Silva and Martinez, 2007). The anterior kidney is mediated by the hypothalamus–pituitary–interrenal cell (HPI) axis and by the hypothalamus–sympathetic nervous system–chromaffin tissue (HSC) axis (Donaldson, 1981; Wendelaar Bonga, 1997; Silva and Martinez, 2007); of these, it is the HPI axis, a homologue of the mammalian hypothalamo–pituitary–adrenal (HPA) axis, that is crucial for the ability of fish to cope with stressors (Mishra and Mohanty, 2009b). Interrenal cells in the anterior kidney secrete corticosteroids, chiefly cortisol (the stress hormone); a rise in plasma cortisol levels in response to some stressor stimulus implies an increase in interrenal cell activity (Donaldson, 1981; Silva and Martinez, 2007).

4-1-4 Continuous Stressor: Chronic Stress & Cell Appearance

A continuous response to a stressor implies continued activity of the HPI axis and synthetic and secretory activity of the interrenal tissue. As a consequence, there are changes in interrenal cells: hypertrophy (increase in cell and nuclear size) and hyperplasia (increase in cell abundance) (Robertson and Wexler. 1959; Nandi, 1962; Ball and Olivereau. 1965; Fagerlund *et al.* 1968; Fagerlund and McBride, 1969; Clarke and Nagahama, 1977; Barton and Iwama, 1991; Barton *et al.* 2002; Silva and Martinez, 2007; Mishra and Mohanty, 2008,

2009a,b). By counting and measuring interrenal cells after standard tissue histological preparation, the impact of chronic stress on fish can be quantified (Barton *et al.* 2002).

Changes in interrenal cells, in terms of biological organisation, are medium-term responses to sub-lethal stressors (Bernet *et al.*, 1999; Silva and Martinez, 2007). Quantifying histological changes of interrenal cells represents a rapid method to detect a definite biological end-point of historical chronic stress exposure in fish (Donaldson, 1981; Teh, *et al.* 1997; Hontela, 1998; Bernet *et al.* 1999; Steniford *et al.* 2003; Silva and Martinez, 2007).

Changes in interrenal cells appear as medium-term responses to sub-lethal stressors (Bernet *et al.*, 1999; Silva and Martinez, 2007). In laboratory-based studies, the effect of toxic sanitary landfill leachate on rainbow trout produced a transitory effect on plasma cortisol after one hour of exposure while the effect on interrenal nuclear diameter (hypertrophy) was only apparent in trout exposed for 7 days (McBride *et al.*, 1979; Donaldson 1981). In acid-stressed brook trout (*Salvelinus fontinalis*), interrenal nuclear hypertrophy and cell hyperplasia occurred respectively on days 4 and 15 (Tam *et al.* 1988). Chinook salmon (*Oncorhynchus tshawytscha*) when exposed to non-lethal biologically treated bleached Kraft mill effluent for periods of 60 and 144 days resulted in increased interrenal nuclear diameters (hypertrophy) (Servizi, *et.al.* 1993). Low temperature was also suspected as a stressor when control fish in the Servizi, *et.al.* (1993) study also developed interrenal nuclear hypertrophy during the 60-day period. Wild-caught eel (*Anguilla anguilla*) sampled from an oil spill site exhibited interrenal hypertrophy characterized by increased nuclear diameter for 8 months after the spill (Hontella, 1997).

Exposure to metals produced cellular atrophy. After a three-month exposure to mercuric chloride and methyl mercury, interrenal cell atrophy and a decrease in plasma cortisol response were observed in *Clarias batrachus* (Kirubagaran and Joy, 1991). In another

laboratory study, the freshwater fish *Chana punctatus*, chronically exposed to hexavalent chromium [Cr(VI)] for one month, similarly produced interrenal cellular atrophy (Mishra and Mohanty, 2009). Historically, Gippsland Lakes black bream have recorded mercury loads (Fabris *et al.* 1999) but heavy metal contamination has never been investigated in Lake Tyers black bream.

4-1-5 Aims

The intention of this inter-estuarine investigation was therefore (1) to identify any histological differences in interrenal tissue sourced from Gippsland Lakes and Lake Tyers black bream and thereby (2) to identify a difference in chronic stress loading as perceived by black bream in each estuarine system.

4-2 Methods

4-2-1 Sample collection and preparation

4-2-1-1 Collection

Gippsland Lakes fish were caught on 18-2-2013 and Lake Tyers fish on 30-1-2013. It was considered important that black bream were captured from the two estuaries within a short time of one another. Multiple attempts to catch fish from the tributary rivers of from the Gippsland Lakes with a rod and line by the Gippsland Lakes Angling Club failed over a two-week sampling period. Gippsland Lakes black bream (n=10) were therefore obtained from a single commercially-operated seine net shot at the eastern end of Lake Victoria. Lake Tyers black bream (n=10) were caught from two locations in "Black Snake Bite" at the southern

end of the Nowa Nowa Arm over a 4 h period by a research angler (John Harrison) using multiple lines and baited hooks from a small boat.

4-2-1-2 Field recording and measurement

After capture, each fish was weighed and then humanely sacrificed by cervical transection and the head kidney (**Figure 4-1**) immediately removed (by punching out a section of the backbone together with the attached kidney) and fixed in Bouin's solution (9% formaldehyde:5% acetic acid: 0.9% picric acid:85.1% water) (Sigma HT10132). Fixed tissue was stored at 4°C until later histological examination (Norris *et al.* 1997).

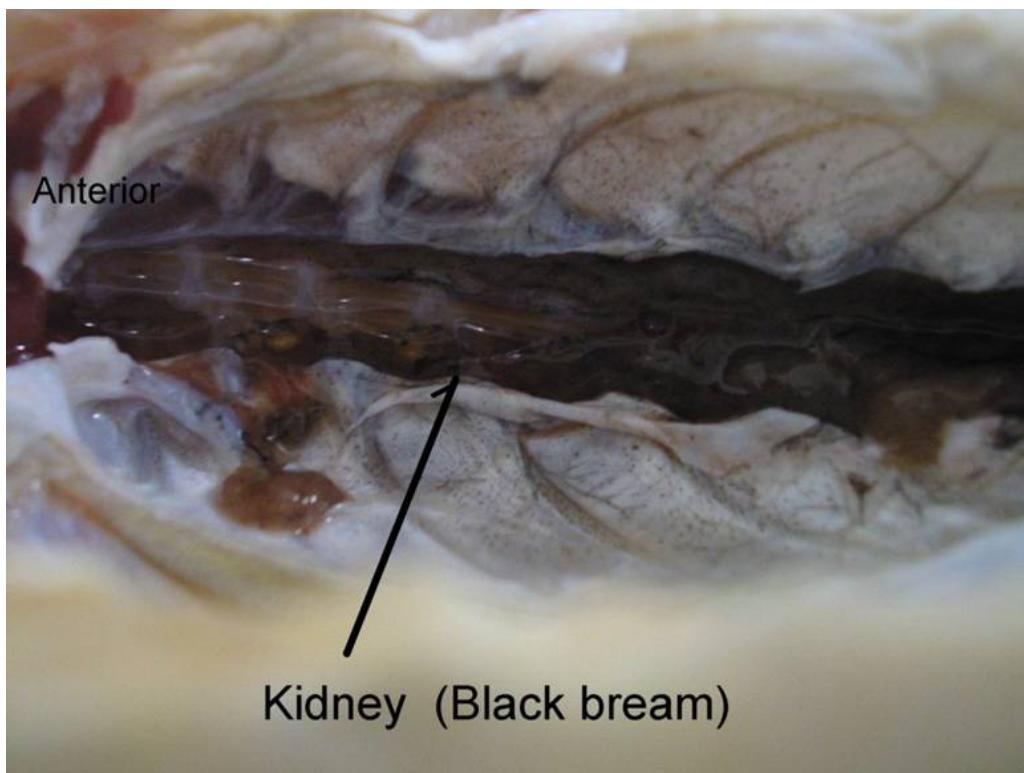


Figure 4-1: Position of kidney in black bream. The anterior (head) kidney was removed.

Nutritional status and feeding history for each fish was determined utilising a bile index (Goede and Barton, 1990).

0 = “straw-yellow” coloured bile and gall bladder partially full or empty

1 = “yellow” coloured bile and full gall bladder

2 = “grass-green” coloured bile and full gall bladder

3 = “dark-green” to “blue-green” coloured bile and full gall bladder.

An empty gall bladder indicates recent feeding within the last couple of hours, whereas an empty blue-green gall bladder indicates resumption of feeding following a period of starvation; the colour of the bile ranges from clear to straw-yellow where feeding has occurred in the last couple of days to blue-green where there has been starvation for over a week (Goede and Barton, 1990).

Sex and macroscopic gonad condition were recorded and wet weight and fork-length measured. Water temperature ($^{\circ}\text{C}$) and salinity (ppt) were also recorded at each site.

4-2-1-3 Histological processing

Head kidney sample was excised from the backbone and placed in cassettes for processing in a Shandon Histocentre 3 (Thermo Electron Corporation). The schedule used was as shown in **Table 4-1.**

Table 4-1: Processing used for kidney samples of black bream in Shandon Histocentre 3.

Solution	Time (min)	Temperature (°C)	Drain time (min)
70% ethanol	60	22	2
90% ethanol	60	22	2
95% ethanol	60	22	2
Absolute ethanol (I)	60	22	2
Absolute ethanol (II)	60	22	2
Absolute ethanol (III)	60	22	2
Xylene (I)	55	22	2
Xylene (II)	55	22	2
Xylene (III)	55	22	140
Paraffin wax (I)	75	62	140
Paraffin wax (II)	75	62	140
Paraffin wax (III)	75	62	140

The sample was embedded in molten paraffin wax and left to set at ambient temperature and then stored at 4°C for sectioning on a Leica Model RM2235 Rotary Microtome at 3 µm thick. Sections were stretched on a Labmaster FBC Flotation Bath at 40°C and picked up on glass slides (Seil Brand JIA 7105WT). At least ten sections were cut and mounted from each kidney.

Slides were placed in an oven for 45 min at 60°C to dewax and seal the sections on to the slides. Sections were stained with Mayer's haematoxylin and counter-stained with eosin according to the schedule shown in **Table 4-2**.

Table 4-2: Schedule for staining sections with haematoxylin and eosin.

Stage	Solution	Exposure
Dewaxing -> aqueous	Xylene (I)	5 min
	Xylene (II)	0.5
	Absolute ethanol	1
	70% ethanol	Rinse
	Tap water	2 min
Haematoxylin staining	Mayers's haematoxylin*	3 min
	Tap water	30 s
	Scott's tap water#	1 min
	Tap water	2 min
Eosin counter-staining	1% aqueous eosin	90 s
	Tap water	30 s
Dehydration	70% ethanol	5-10 dips
	Absolute ethanol (I)	5-10 dips
	Absolute ethanol (II)	5-10 dips
	Xylene (I)	1 min
	Xylene (II)	2 min

*Mayer's haematoxylin: 1 g haematoxylin, 0.2 g sodium iodate, 50 g potassium aluminium sulphate, 1 L deionised water; dissolved overnight, then 50 g chloral hydrate and 1 g citric acid added.

#Scott's tapwater: 2 g potassium bicarbonate, 20 g magnesium sulphate, 1 L distilled water.

Following staining, sections were mounted in DPX under coverslips and left for 48 h to polymerise at ambient temperature. This staining was expected to stain nuclei and other nucleic acid-containing structures, e.g. ribosomes, mitochondria, blue-black and cytoplasm and other proteinaceous content pink-red.

4-2-3 Microscopic Analysis

Two sample-based measures of the interrenal axis were used to assess the state of inter-estuarine black bream chronic stress-response. Measurements were made at 100x or 400x magnification with a Nikon Eclipse E200" microscope using an ocular graticule inserted into the right eye-piece and calibrated against a micrometre slide.

- i) *Interrenal cell and nuclear hypertrophy*: Interrenal cell and nuclear diameters were measured to identify hypertrophy (Ball and Olivereau, 1965; Clarke and Nagahama, 1977; Fagerlund and McBride, 1969; Fagerlund et al, 1968; Norris *et al.* 1997). For cells, the 10 largest were measured in representative interrenal sections from each of ten slides of serial sections through the head kidney for each of ten fish. For nuclei, the ten largest nuclei were measured from each of ten slides of serial sections through the head kidney for each of ten fish
- ii) *Interrenal hyperplasia* was determined by counting the number of cells impinging on a 100 µm transect on the scale of the ocular graticule in ten serial sections of the head kidney for each of ten fish.

4-2-4 Statistical analysis

Fish condition factors were compared between estuaries using a two-tailed, two-sample t test.

Mean values for measurements were compared between fish from the Gippsland Lakes and Lake Tyers using one-way analysis of variance (ANOVA) followed by Tukey's family error test at P=0.05 (Zar, 1984).

4-3 Results

4-3-1 Field Measurements

Lake Tyers fish were heavier (ANOVA, $F=11.20$, $p=0.004$) and longer (ANOVA, 5.27, $p=0.034$) than those caught in the Gippsland Lakes (**Table 4.3**, Appendix Table A4-1). The condition factor (K) was greater for Lake Tyers fish than those from the Gippsland Lakes (ANOVA, $F=8.94$, $p=0.008$) (**Table 4.3**).

At sampling sites, Lake Tyers surface water temperature and salinity were greater those for the Gippsland Lakes.

Table 4-3. Mean weight, fork length and condition factors for Gippsland Lakes and Lake Tyers black bream.

Location	No. of fish	Mean±SE	Mean±SE	Mean±SE	Surface water	
		wet body weight (g)	fork length (cm)	condition factor (K) ¹	Temperature (°C)	Salinity (ppt)
Gippsland Lakes	10	380.8±34.3	25.0±0.64	2.38±0.07	20.0	32.0
Lake Tyers	10	668.0±78.7	28.5±1.37	2.79±0.07	22.7	35.9

¹Condition factor (K) = $100 \times \text{weight (g)} / [\text{length (cm)}]^3$

Most Gippsland Lakes fish had a bile ranking of 2 and therefore had not fed for several days, whereas all ten Lake Tyers fish displayed an empty gall bladder and a bile ranking of 0, indicating that fish were actively feeding prior to capture (**Table 4.4**).

Table 4-4: Nutritional status and feeding history.

Location	No. of fish	Recorded bile ranking¹		Proportion with bile ranking=0
		0	2	
Gippsland Lakes	10	3	7	30
Lake Tyers	10	10	0	100

¹Bile ranking : 0 = “straw”-yellow coloured bile with gall bladder partially full or empty; 1= yellow coloured bile and the bladder fully distended; 2= light green bile and full gall bladder; 3 = dark green or “blue-green” bile and full gall bladder (Goede and Barton, 1990).

4-3-2 Microscopic Analysis

The head kidney sample of Gippsland Lakes and Lake Tyers fish differed greatly in the abundance of interrenal cells (**Figure 4-2**, **Figure 4-3**). In sections of Gippsland Lakes fish, there were large numbers of interrenal cells between functional kidney structures (glomeruli and tubules) (**Figure 4-2**), whereas there were small numbers in Lake Tyers fish (**Figure 4-3**).

4-3-2-1 Interrenal Cell and Nuclear Hypertrophy

With 400x magnification, there was no difference in the diameter of interrenal cells or nuclei in fish from either Lake Tyers or the Gippsland Lakes (**Table 4.5**). For all cells, diameters were measured at between 7.0 – 8.0 µm. For all nuclei, diameters were measured at between 2.0 – 3.0 µm respectively.

Table 4-5: Cell and nuclear diameter for interrenal cells in Gippsland Lakes and Lake Tyers black bream head kidney.

Location	No. of fish	Mean ± SE for cell diameter (μm)	Mean ± SE for nuclear diameter (μm)
Gippsland Lakes	10	7.45±0.08	2.44±0.08
Lake Tyers	10	7.55±0.07	2.53±0.08

4-3-2-2 Interrenal Hyperplasia

Gippsland Lakes fish had a significantly greater interrenal cell number impinging a 100 μm transect than Lake Tyers fish (ANOVA, $F=107.57$, $p<0.001$; Kruskal-Wallis, $H=14.30$, $p<0.001$) (**Figure 4.4, Table 4-6**, Appendix Table A4-2). ANOVA explained 85% of the variation in the data.

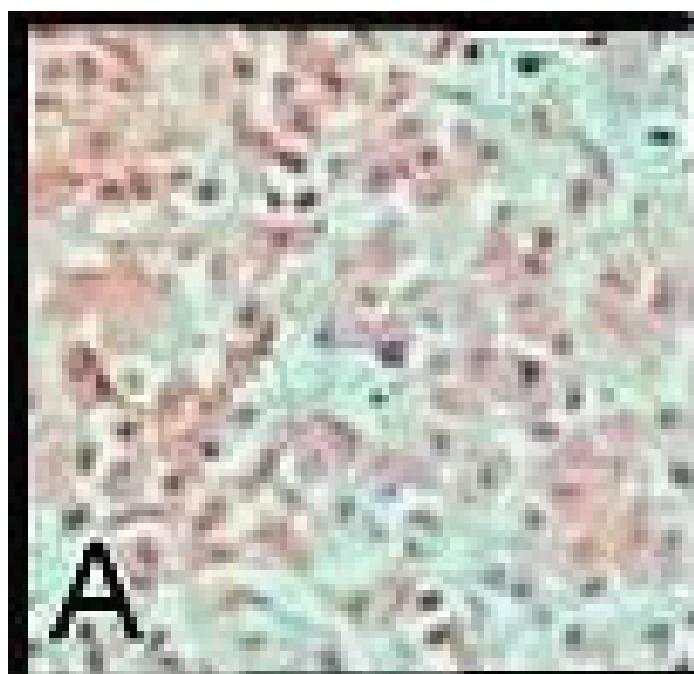
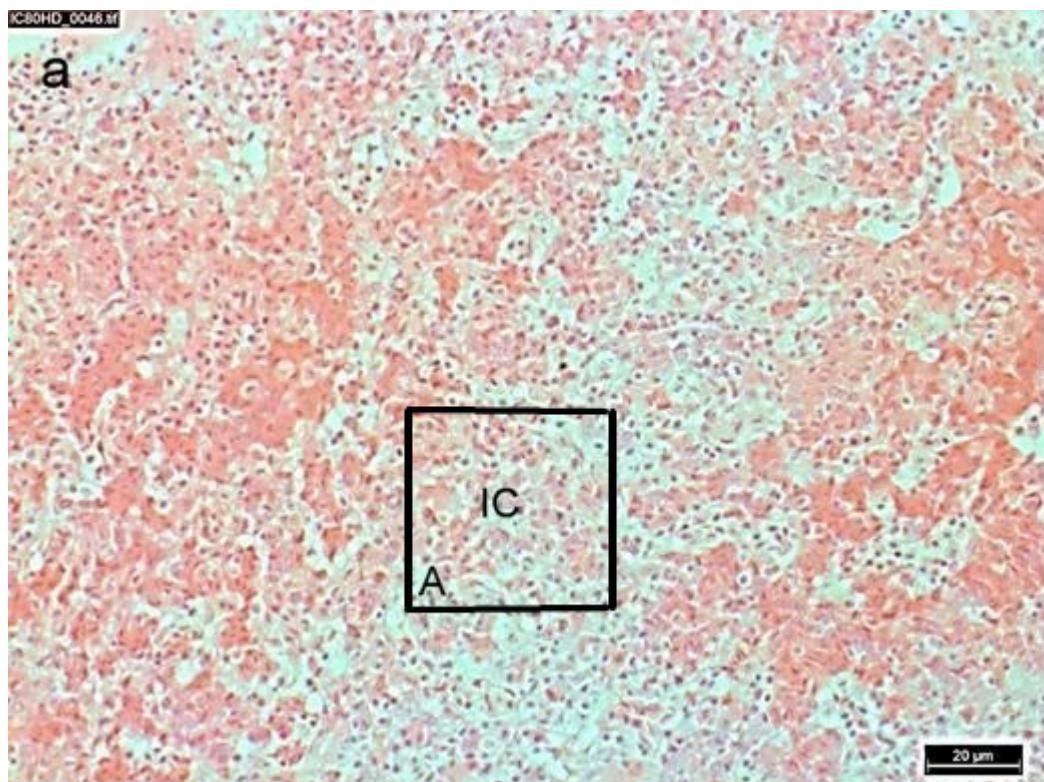


Figure 4-2: Sections through head kidney of Gippsland Lakes fish (slide GL35-2). a) Low power view showing large masses of interrenal cells, A) Enlarged section showing details of interrenal cells.

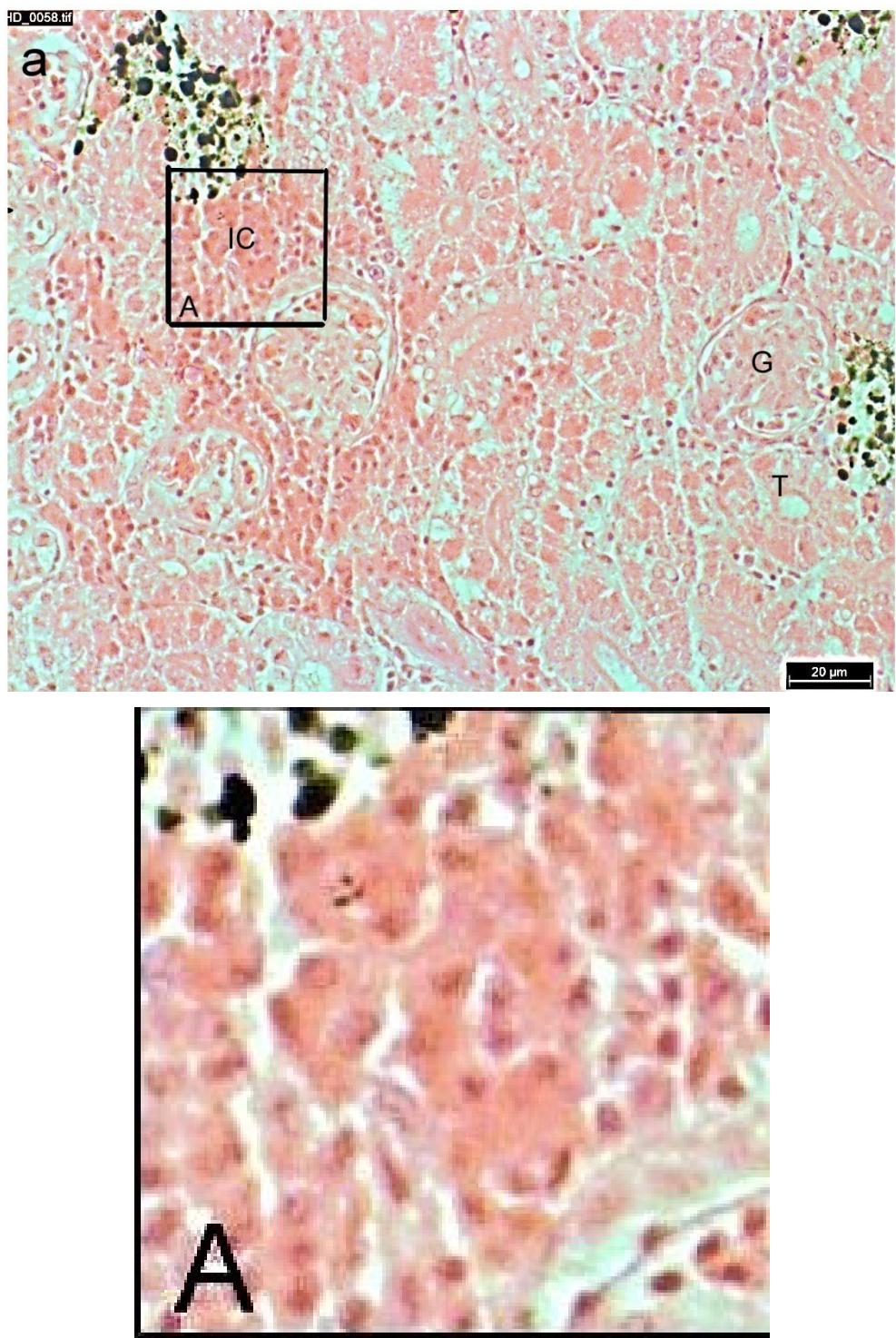


Figure 4-3: Sections through head kidney of Lake Tyers fish (slide LT12-5). a) Low power view showing details of interrenal cells. IC=interrenal cells, T=tubule, G=glomerulus.
A) Enlarged section showing details of interrenal cells.

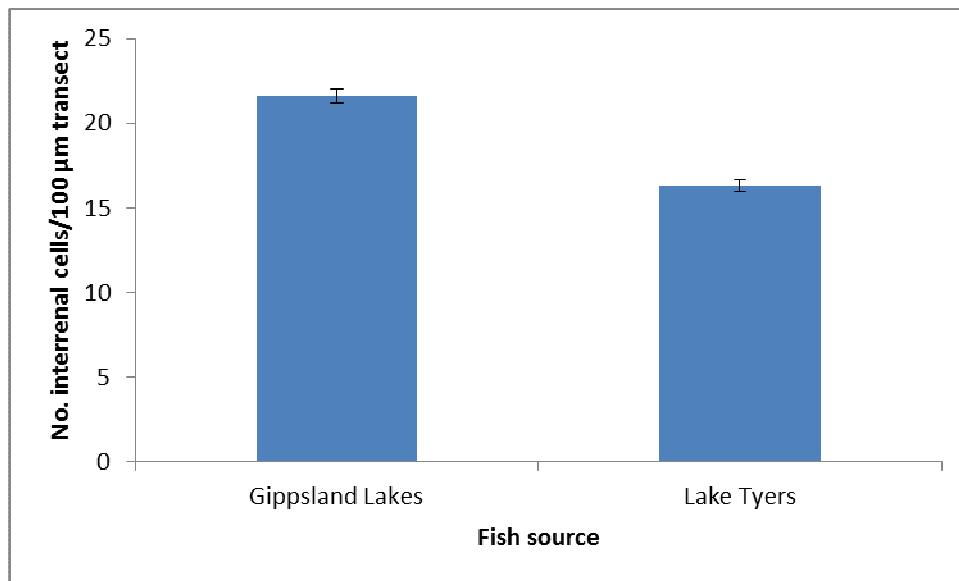


Figure 4-4: Effect of fish source on mean number of interrenal cells impinging a 100 μm transect of anterior (head) kidney.

Table 4-6: Summary of Analysis of Variance for number of interrenal cells per 100 μm transect in black bream from adjacent estuaries, the Gippsland Lakes and Lake Tyers.

Source of variation	df	SS	MS	F	p
Fish source	1	142.4	142.4	107.57	<0.001
Error	18	23.83	1.32		
Total	19	166.23			

S = 1.151 R-Sq = 85.67% R-Sq(adj) = 84.87%

Gonads had developed in 4/10 Gippsland Lakes fish and 5/10 Lake Tyers fish. As gonad development is known to increase interrenal cells in fish, its effect was tested. There was no significant difference in interrenal cell counts between fish with developed or undeveloped gonads when counts from fish from both estuaries were combined and compared (ANOVA, F=0.11, p=0.748; Kruskal-Wallis, H=0.07, p=0.787) (Table 4-7, Appendix Table A4-3).

Table 4-7: Summary of Analysis of Variance for number of interrenal cells per 100 μm transect in black bream with developed or undeveloped gonads from the Gippsland Lakes and Lake Tyers combined.

Source of variation	DF	SS	MS	F	P
Gonad development	1	0.98	0.98	0.11	0.748
Error	18	165.25	9.18		
Total	19	166.23			

S = 3.030 R-Sq = 0.59% R-Sq(adj) = 0.00%

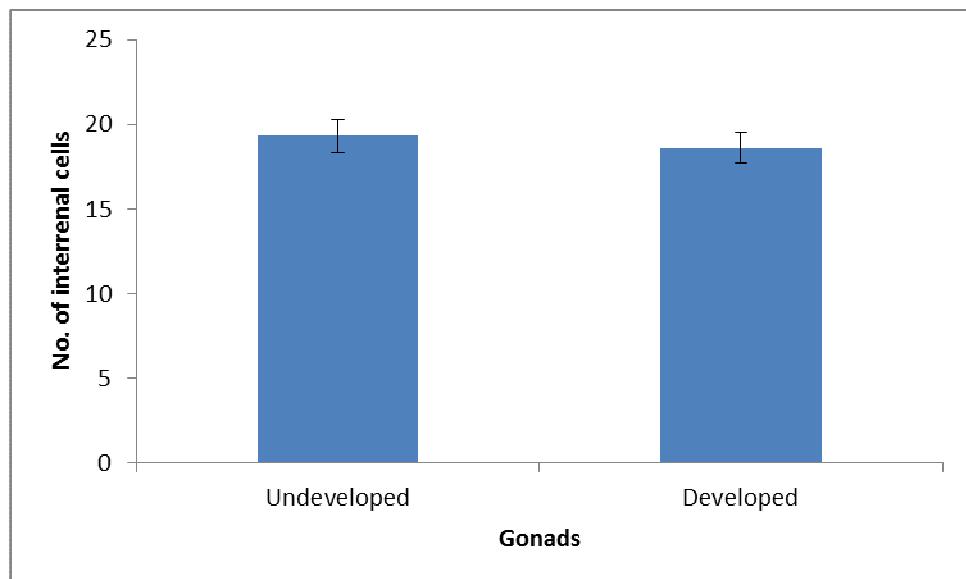


Figure 4-5: Effect of gonad development on mean number of interrenal cells impinging a 100 μm transect.

Re-analysis of the data by Generalised Linear Modelling (GLM) with fish source as the independent variable and gonad development as a covariate confirmed that gonad development did not have a significant effect on cell number in the transect (GLM, F=112.82, p<0.001 for fish source; F=1.72, p=0.207 for gonad development) (**Table 4-8**).

Table 4-8: Summary of Generalised Linear Modelling on number of interrenal cells per 100 μm transect in black bream from adjacent estuaries, the Gippsland Lakes and Lake Tyers, with gonad development as a covariate (with SS adjusted for tests).

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Fish source	1	143.612	143.612	143.612	112.82	<0.001
Gonad development	1	0.976	2.188	2.188	1.72	0.207
Error	17	21.64	21.64	1.273		
Total	19	166.228				

S = 1.12825 R-Sq = 86.98% R-Sq(adj) = 85.45%

4-4 Discussion

Several data suggest that Gippsland Lakes black bream may have been suffering greater chronic stress than Lake Tyers black bream at the time of capture. The measurement of interrenal cells has potential to identify and detect a history of chronic stress in black bream, but does require further investigation. Gippsland Lakes black bream interrenal tissue was hyperplastic compared with that of Lake Tyers black bream, indicating a history of prolonged cortisol synthesis, which correlated with the lesser condition factor in the Gippsland Lakes fish and the appearance and contents of fish gall bladders, indicating that Lake Tyers fish were feeding at the time of sampling, whereas most Gippsland Lakes fish were not. The source of the differences could be a consequence of the sampling methods used, more stressful environmental conditions in the Gippsland Lakes or that the Gippsland Lakes fish were genetically more predisposed to stress responses than those from Lake Tyers.

4-4-1 Sampling

The need to sample using two different fishing methods may have created a bias. Angling may select a certain behavioral type of fish and not a subset of the entire population (Wilson *et al.* 2015). Vulnerability to angling has been demonstrated to be a heritable trait (Philipp *et al.* 2009) and has been linked to various personality traits, such as boldness (Kleftho *et al.* 2013; Wilson *et al.* 2015) and aggression (Cooke *et al.*, 2007;. Kekäläinen *et al.* 2014); where, surprisingly, body size was not always a predictor of these traits (Wilson *et al.* 2015). Conversely, Wilson *et al.* (2011) found that angling, using simple baited hooks, in the wild appeared to target timid rather than bold juvenile bluegill sunfish (*Lepomis macrochirus*). With this evidence, Wilson *et al.* (2011) suggested that personality-based vulnerability to angling might vary by life history stage, habitat, species and particular angling technique/gear being used. Seine netting is not without sampling bias (Bertelli and Unsworth, 2013)

where mesh size (Ceni and Vieira, 2013), length of hauling ropes (Lombardi *et al.* 2014), fishing in daylight versus night allowing fast swimming fish to escape the net (Guest *et al.* 2003); Bertelli and Unsworth, 2013; Ceni and Vieira, 2013); can all influence the type of fish caught. Despite this, seine netting is a preferred method to sample fish (Bertelli and Unsworth, 2013; Guest *et al.* 2003) where a customised seine can target certain fish. For example, Warren *et al.* (2010) indicate that an appropriately set seine when seeking juvenile cod (*Gadus morhua* and *G.ogac*) had a capture efficiency of 95%. How angling in Lake Tyers and the commercial seine in the Gippsland Lakes selected the type of fish caught was unknown and requires further research, in particular, if that selectivity distinguished between hybrid and indigenous *Acanthopagrus* present.

4-4-2Interrenal Response

The point prior to capture at which Gippsland Lakes black bream perceived their surrounding environment as stressful, and therefore a prolonged cortisol response was stimulated, is unknown but is likely to be weeks or months rather than days. Changes in interrenal cell hyperplasia typically appeared after 15 days in acid-stressed brook trout and followed nuclear hypertrophy (Tam *et al.*, 1988).

The lack of nuclear or cellular hypertrophy along with the cellular hyperplasia is puzzling, as hypertrophy is more commonly reported and nuclear hypertrophy appeared before hyperplasia in brook trout (Tam *et al.*, 1988). For example, nuclear hypertrophy was reported in 4 days as a response to acid stress in brook trout (Tam *et al.* 1988), in 7 days as a response to sewage in rainbow trout (McBride *et al.*, 1979; Donaldson 1981) and to persist for 5 months as a response to bleached Kraft mill effluent and low temperature in Chinook salmon (Servizi, *et.al.* 1993) and 8 months after an oil spill in wild-caught eel (Hontella, 1997). Perhaps black bream do not respond to stress events by nuclear or cellular hypertrophy,

though it seems unlikely, or perhaps the response is only transient while the hyperplasia response is long-lasting; a laboratory study in black bream with some of the common contaminants in the Gippsland lakes could elucidate this.

No interrenal cell atrophy, which is typical of fish exposure to toxic levels of mercury (Kirubagaran and Joy, 1991) and hexavalent chromium (Mishra and Mohanty, 2009) was observed, though there were occasional pyknotic nuclei. Historically, Gippsland Lakes black bream have recorded mercury loads (Fabris *et al.* 1999) but Lake Tyers black bream have never been investigated for heavy metal contamination. In this study, cellular atrophy was not apparent in interrenal tissue from fish sourced from either estuarine system, suggesting that any metal stress encountered has not been of such a duration or severity as to reach a stage of exhaustion, where cells may have displayed degeneration.

4-4-3 Fish condition and feeding

Lake Tyers black bream were caught by angling and so fish were clearly feeding, whereas multiple attempts to catch Gippsland Lakes black bream from the tributary rivers of the Gippsland Lakes failed for 2 weeks. This is the reason why fish were ultimately obtained from the main lakes (commercial netting is not permitted in tributary rivers) by a commercially operated seine net. Fortunately, the 18-day time gap between the Gippsland Lakes and Lake Tyers collections was not affected by any significant weather event that might have contributed to an extraordinary inter-estuarine difference.

There are two possible explanations of why angling failed to capture Gippsland Lakes black bream: Gippsland Lakes black bream may have been present in rivers at locations not fished, or they were at those locations, but were not feeding. All Lake Tyers black bream had empty gall bladders, an index value of 0, and were obviously feeding prior to capture. Three Gippsland Lakes bream had been feeding (index value of 0) but seven fish with an index

value of 2 had not been feeding for a couple of days, and so the most likely explanation of the failure to capture Gippsland Lakes fish by angling is that they were not feeding.

In this study, Fulton's condition factor (K) for black bream originating from Lake Tyers was significantly greater than black bream from the Gippsland Lakes. Such a difference may be the consequence of gonad development, abundance of food, quality of food, or feeding frequency (Barton *et al.* 2002). There was no significant difference in inter-estuarine black bream gonad development. There may have been a difference in the abundance or quality of the food between the estuaries, but given the much larger area of the Gippsland Lakes, and the ability of black bream to move extensively throughout the main lakes and their tributary rivers (Hindell *et al.* 2008), fish would have been expected to relocate to more favourable regions to overcome these deficiencies. Furthermore, given the close proximity of both catchments, any seasonal factor would have had a similar impact on each estuarine system.

This leaves, as a possible reason for the difference in hyperplasia in the interrenal cells, the abundance and quality of food affecting feeding frequency, which was also different between fish from the Gippsland Lakes and Lake Tyers. When caught, all Lake Tyers bream had been feeding prior to capture, whereas 70% of the Gippsland Lakes fish had not. Some factor(s) was preventing many of the Gippsland Lakes black bream from feeding prior to sampling. The low condition factor observed in Gippsland Lakes black bream supports this: it indicates that they had decreased energy reserves (Goede and Barton 1990) than Lake Tyers bream.

4-4-4Gonad development

Gonad development in fish can confound the interpretation of cortisol and interrenal tissue response by elevating levels and promoting cellular hypertrophy, particularly in several salmonid species (Robertson and Wexler, 1960; Schmidt and Idler, 1962; Donaldson and Fagerlund, 1968, 1970; McBride and van Overbeeke, 1969; Fagerlund and Donaldson, 1970;

van Overbeeke and McBride, 1971). In other studies, method of capture, season, time of day and sex can also all influence plasma cortisol levels (Barton and Iwama, 1991; Pankhurst and Sharples, 1992; Foo and lam, 1993; Sumpter, 1997; Haddy and Pankhurst, 1999). However, in a study of Tasmanian wild-caught black bream, Haddy and Pankhurst (1999) found that such factors did not influence plasma cortisol concentrations in black bream. Such a response makes the study of interrenal cells, the site of cortisol synthesis, an attractive method to investigate the possible impact of chronic stress loading on wild-caught black bream, provided that the stage of gonad development had no effect, which it did not. Consequently, an factor or factors other than reproductive condition is likely to be the cause of the hyperplasia in Gippsland Lakes black bream. This could be either an external difference in the environment of each estuary to which the fish are exposed or an .intrinsic genetic difference in the sensitivity of the fish in each estuary.

4-4-5 Environmental stressors

Estuarine systems are often classified as stressful environments; however, for the indigenous biota this so-called stressed state is normal and it has the ability and resilience to adapt to the highly variable conditions (Elliott and Quintino, 2007). Furthermore, in multi-stressor environments such as estuarine systems, one stressor may increase a fish's resistance to another stressor (Basu *et al.* 2002; Web and Gagnon, 2008). Therefore, it would be reasonable to expect black bream indigenous to the Gippsland Lakes, and recognised as possessing wide environmental tolerances with respect to temperature, salinity and dissolved oxygen concentration (Norriess *et al.* 2002; Burridge *et al.* 2004) to acclimate to normal, but variable, estuarine conditions and have high resistance to stressors they encounter. The consequence of this up-regulation of the HPI axis activity may be the hyperplasia of interrenal tissue in Gippsland Lakes black bream compared with Lake Tyers black bream as a

physiological consequence of extended cortisol synthesis following stimuli in the Gippsland Lakes. The observation of inter-estuarine differences in condition factor and fish feeding history support this.

4-4-6 Fish movement between estuaries

Black bream prefer to spend their entire life cycle within estuaries (Burridge *et al.* 2004; Williams *et al.* 2013). However, during periods of high river discharge or estuarine anoxia post-larval stage individuals can be found at sea (Burridge *et al.* 2004). Tagging studies of juvenile and adult black bream indicate infrequent dispersal between estuaries greater than 50 km apart with only 6/149 tag returns from Victorian coastal waters (Burridge *et al.* 2004). In contradiction to this, genetic studies indicated homogeneity among south-east Australian estuaries with contemporary but infrequent gene flow occurring mostly between adjacent estuaries, consistent with a one-dimensional stepping stone model (Farrington *et al.* 2000; Burridge *et al.* 2004, 2006). As Gippsland Lakes and Lake Tyers are adjacent systems with entrances to the sea less than 15 km apart, it would therefore be reasonable to expect black bream to move between these systems given appropriate seasonal conditions. In this situation, it is unlikely that the inter-estuarial difference observed in chronic stress loading, condition factors and feeding frequency are influenced by genetic difference. However, the more recent suggestion of hybridisation between estuarine black bream (*A. butcheri*) and marine yellow fin bream (*A. australis*) in this region, which includes the Gippsland Lakes and Lake Tyers (Roberts *et al.* 2011), may have led to genetic differences because of their different frequency of estuarine opening (and consequently their different frequency of opportunities for cross-breeding between species. The most recent study in Victorian black bream used fin clippings from fish caught in 2000 and it is possible that changes in the populations of both estuaries

have led to genetic differences, though a historical survey suggested that the populations in Victoria had remained genetically stable from 1941-2000 (Roberts *et al.*, 2011b).

4-4-7 Multiple Stressors in the Environments

Utilising acoustic telemetry, Hindell *et al.* (2008) found that Gippsland Lakes black bream move extensively throughout the lakes, with an average movement of 8.7 km.d^{-1} and up to 2600 km in a year; in their tributary rivers 30 km.d^{-1} , and during the year there is also relocation to and from rivers, river entrances and lakes. Given this degree of movement, to avoid any adverse environmental condition in a region, fish could simply relocate to a more favourable environment. For sampled fish to be displaying chronic stress there must have been large areas of the lakes stressful to them or, alternatively, the stressor was consistently following or with the fish and so produced an extended period of cortisol synthesis and the consequential interrenal hyperplasia. There is no corresponding information about the degree of movement of Lake Tyers black bream.

4-4-7-1 Contaminants

There was no histological evidence, such as an enhanced number of pycnotic nuclei in interrenal cells, of heavy metal contaminant exposure in fish from either estuary despite the fact that Gippsland Lakes black bream are known to carry both mercury and organochlorine insecticides, (Fabris *et al.* 1999). Any contaminant loading carried by Lake Tyers black bream is unknown.

4-4-7-2 Fishing

Fishing is a frequent activity in both estuaries, though commercial fishing is now allowed only in the Gippsland Lakes, and could be a contributing factor to the interrenal hyperplasia and poorer condition and smaller weight of Gippsland Lakes fish than Lake Tyers fish. Black

bream under a total length of 28 cm in both the commercial and recreational fishery must be released (Grixiti *et al.* 2008, 2010) and though short-term survival (72 h) after release from a gill-net in the commercial fishery was 74-95% (Grixiti *et al.* 2008; Grixiti *et al.* 2010) such capture and release of undersized fish is a possible cause of chronic stress. Chapter 2 also identified delayed but ongoing mortality over a 62 d period from black bream obtained from a commercial seine net, a method that has minimal impact on fish (Hontela, 1997), and exposed to multiple air-exposures. Individual dark and surface-bound moribund fish that were observed have the potential to attract visual opportunistic predators such as birds and as a consequence leading to ongoing harassment and stressing of healthy fish in the vicinity.

4-4-7-3 Predation by birds

In the Gippsland Lakes, bird predation on black bream, in particular by the great cormorant (*Phalacrocorax carbo*) is significant: at a roosting site, black bream otoliths were found in 94% of regurgitated pellets over a summer period and 81% during the winter with a mean value of 2.6 fish per pellet (Reside and Coutin 2001). The preponderance of black bream in the diet was probably due to the successful spawning periods of 1994 and 1996 for black bream and the subsequent abundance of this fish in the Gippsland Lakes at that time (Reside and Coutin 2001). Great cormorants predate by relocating and changing their behaviour to take advantage of feeding opportunities by hunting co-operatively and by driving and restraining shoals of fish in shallow water (Reside and Coutin, 2001). In Japan, the great cormorant has been observed to alter its feeding areas to correlate with mass stocking of ayu (*Plecoglossus altivelis*) and also the catching of ayu in cast nets (Kumada *et al.* 2013). The great cormorant has been classified as a generalist feeder with its diet reflecting the composition of the fish community in a given body of water (Reside and Coutin 2001; Kameda *et al.* 2002; Lorentsen *et al.* 2004; Casaux *et al.* 2009; Kumada *et al.* 2013). The

significance of the great cormorant as a predator is known and it is likely that its attacks contribute to chronic stress in black bream. These may be greater in the Gippsland Lakes than in Lake Tyers because of the presence of commercial fishing boats and netting retrieval in the Gippsland Lakes, but there is no information on bird numbers in each estuary.

4-4-7-4 Climate change

Tributary river inflows into estuarine systems such as the Gippsland Lakes are affected by water abstraction and precipitation, and also by evapotranspiration, which in turn is affected by air temperature, CO₂ changes, net radiation, vapour pressure and wind speed, all of which may change with a changing climate (Donohue *et al.* 2010). Annual rainfall has declined and mean temperature has increased over the last century, but especially so during the last 40 years (**Figure 4-7**). Precipitation in southern Australia is also predicted to decrease and to reduce water quality (Christensen *et al.* 2007; Perkins and Pitman 2009; Gillanders *et al.* 2011), which might be expected to produce chronic stress in estuarine fish.

There has been a steady decline in annual Gippsland Lakes black bream commercial catch rates with 2002-2003 producing 26 t, the lowest on record (Kemp *et al.* 2011; Williams *et al.* 2012); this decline corresponds with a 10 year trend in decreasing rainfall and consequently a reduction in freshwater inflow into the Gippsland Lakes (Jenkins *et al.*, 2010; Williams *et al.* 2012). Since then, the annual catch has risen but the proportion of older black bream has declined, consistent with ‘faster growing cohorts entering the fishery at a younger age in recent years’ and productivity was expected to remain low due to reduced freshwater inflows (Kemp *et al.*, 2013).

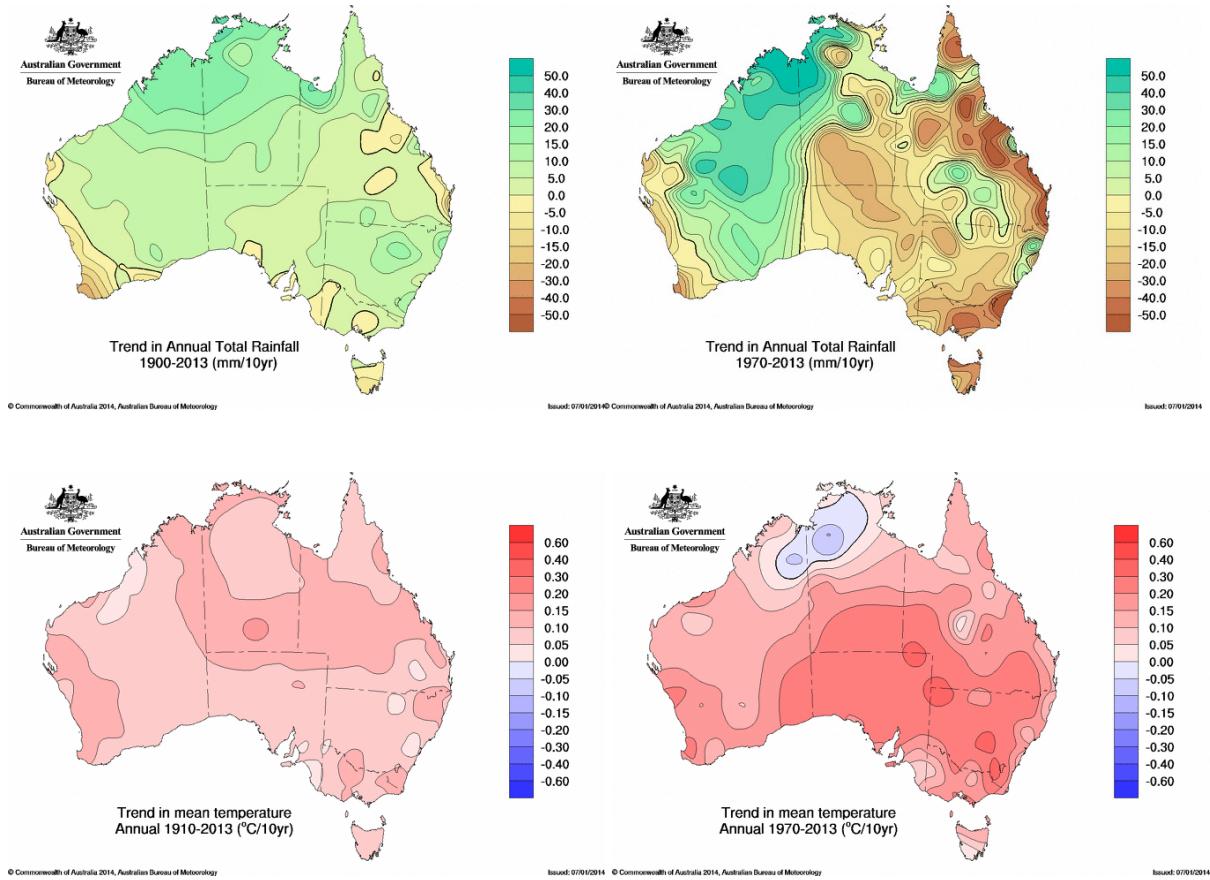


Figure 4-6: Historic trends in rainfall and temperature in Australia. A, B) rainfall; C,D) temperature. A,C) last 100-110 years; B,D) last 43 years. (www.bom.gov.au).

Stratification developed from fresh water inflows may be necessary for providing suitable environmental conditions for successful black bream spawning and larval development in this system (Jenkins *et al.* 2010; Williams *et al.* 2012, 2013). Decreased water inflows are environmental stressors that may have already cumulatively impacted on black bream, but occurring over an extended period of time and given the potential resilience to acclimate to such highly variable conditions (Elliott and Quintino, 2007), it is unlikely to be observed as interrenal hyperplasia, but to be evident at a higher level of biological organisation such as the decreased growth or reproduction already observed (Adams and Greeley, 2000). Any decrease in freshwater inflows will allow further saltwater intrusion into the Gippsland Lakes and its tributary rivers via the permanent opening at Lakes Entrance transforming the system

to that of a more marine environment (Bird, 1978). This permanent channel to the sea will also allow an ongoing passage for the introduction of opportunistic marine predators into regions of the Gippsland Lakes. For example, a number of an unknown seal species were observed approx. 20 km into the Mitchell River at the time of field sampling and may have contributed to the difficulty of obtaining black bream from the Gippsland Lakes. At this time, Lake Tyers was closed to the sea and its fish population was not encountering marine predators to the same level.

4-4-8 Concluding Comments

This study has identified a histological difference in black bream interrenal tissue sourced from fish in adjacent estuaries, the Gippsland Lakes and Lake Tyers, though such a difference requires confirmation with more closely matched samples of fish in size and development. Identification of such a difference suggests a difference in extended cortisol synthesis, and therefore, for the first time, differences in how black bream were responding to their surrounding environment in each estuary prior to capture. Angling as a catching method may have selected a subset of less stressed fish from Lake Tyers. Further research therefore into any angling selectivity is required for both the Gippsland Lakes and Lake Tyers, in particular, if any selectivity has a genetic basis. Also unknown, and requiring further research, are any influences of contaminants entering the estuary, fishing pressure, predation and decreases in freshwater inflows on the *Acanthopagrus* present.

Chapter 5. Otoliths: Movement & Environment

5-1 Introduction

Black bream that inhabit the Gippsland Lakes and the adjacent estuarine system, Lake Tyers are a demersal long-lived (c.29 years) estuarine species (MacDonald, 1997b; Morison *et al.* 1998; Sarre and Potter, 1999; Norriss *et al.* 2002) that prefer to remain in their natal estuary throughout their life cycle (Sarre and Potter, 1999; Williams *et al.* 2013). On occasions, post-larval stage black bream have been observed at sea, but these observations have been attributed to adverse conditions that have occurred within an estuary, such as periods of high river discharge (Lenanton, 1977) or anoxic estuarine water (Burridge *et al.* 2004). Movement of black bream between these adjacent estuaries has long been suspected, but never shown.

5-1-1 Adjacent Estuaries but a Different History

The Gippsland Lakes and Lake Tyers each have entrances to the sea less than 15 km apart, but despite their close proximity, have a different history of formation as discussed in chapter 1.. Today, as a consequence of such climatic changes, geomorphology and recent anthropogenic involvement, hydrological differences exist between the systems (Bird, 1961, 1978; Hall, 1984; Longmore, 1988; NREC, 1991; MacDonald, 1997a). For example, since 1889, water of the Gippsland Lakes has been modified by a man-made permanent opening to the sea (Bird, 1961, 1978); by contrast, Lake Tyers still encounters periodic closure to the sea by sandbars (Hall, 1984; NREC, 1991; MacDonald, 1997a). Lake Tyers is supported by a small (470 km^2) feeder catchment (Hall, 1984; Hall and MacDonald, 1985b) little changed from its form of 200 years ago (EGCMA, 2005), as a consequence, water conditions within Lake Tyers display wide fluctuations in salinity, dissolved oxygen concentrations,

temperature and turbidity (Hall, 1984; NREC, 1991; MacDonald, 1997a). The Gippsland Lakes, supported by a large 20,000 km² much modified catchment that naturally drains 10% of freshwater flows from the State of Victoria (Bird, 1978; Fabris et al. 1999; Gillanders *et al.* 2011), has also in its catchment various forms of agriculture and heavy industry such as brown coal mining for electricity generation in the Latrobe Valley. Water conditions within the Gippsland Lakes are very much dependent on highly variable annual river freshwater flows that determine salinity levels and therefore stratification and degree of hypoxic conditions within the lakes; tributary rivers also deliver and determine anthropogenically-driven suspended sediments, nutrients and contaminants (CSIRO, 2001; Jenkins *et al.* 2010; Williams *et al.* 2012, 2013). Therefore, black bream that inhabit these two systems may also carry a unique chemical signature that they have accrued from their environment.

5-1-2 Otolith Structure and Chemical Signature

Otoliths are paired structures found within the inner ear of all teleost fish to assist in balance and hearing (Campana, 1999). They are composed of biogenic calcium carbonate, typically in the form of aragonite deposited on a protein matrix (Campana, 1999; Popper and Lu, 2000; Elsdon *et al.* 2008; Webb *et al.* 2012), where new crystalline and protein materials are daily accreted onto the exterior surface of each otolith from the surrounding endolymph (Campana and Neilson, 1985; Campana, 1999). Along with the major constituents of aragonite (C, O and Ca), some 31 other elements at either minor (>100 ppm) or trace (<100 ppm) concentrations have been detected within these accreted layers (Campana and Neilson, 1985; Campana, 1999).

Incorporation of certain elements into otoliths can be influenced by environmental variables such as temperature, salinity and elements in the surrounding water (Hamer *et al.* 2006). Once in the metabolically inert otolith, the accreted layers provide a chemical chronology

providing information on environmental and movement histories over the life of a fish (Campana, 1999; Campana and Thorrold, 2001; Elsdon *et al.* 2008). Individual fish migration histories can therefore be constructed from otoliths by coupling chronologies of otolith chemistry with knowledge of spatial and temporal variation in the environmental parameters known to influence otolith chemistry (Hamer *et al.* 2006; Elsdon *et al.* 2008; Morales-Nin *et al.*, 2014; Stocks *et al.*, 2014).

Those elements substituting calcium in interstitial crystal sites such as Sr, Ba and O, are likely to reflect environmental parameters (Elsdon *et al.* 2008) and are best for determining fish movement (Kennedy *et al.* 1997; Bath *et al.* 2000; Limburg *et al.* 2003; Elsdon and Gillanders, 2004; Dorval *et al.* 2007; Kerr *et al.* 2007; Elsdon *et al.* 2008). In elemental ratios, it is the calcium concentration that determines the degree of alternate elemental incorporation into the otolith (Elsdon *et al.* 2008). Physiological processes within the fish can also reflect the occurrence of certain elements such as N, K, Cl, Zn, and Cu; however, these elements are less likely to reflect environmental parameters (Hamer *et al.* 2006; Elsdon *et al.* 2008); they are also unstable (Kalish, 1989; Proctor and Thresher, 1998; Rooker *et al.* 2001; Miller *et al.* 2006; Elsdon *et al.* 2008).

Elemental composition of otoliths has been studied by UV laser ablation connected to a HR-ICP-MS (High Resolution-Inductively Coupled Plasma-Mass Spectrometer) (Elsdon and Gillanders, 2003) or a plasma mass spectrometer (LA-ICPMS) (Morales-Nin *et al.*, 2014; Stocks *et al.*, 2014). The use of LA-ICPMS with otoliths of Chum salmon (*Oncorhynchus keta*) differentiated between the initial freshwater phase in estuaries and the later marine phase (Kang *et al.*, 2014). Line scanning from the core to the edge of otolith sections showed that Sr was greater at the core and the edges (thought to represent the maternal and marine components) than the area between (thought to represent the freshwater phase). However, these methods have some disadvantages, primarily that the preparation involves sonication in

water, leading to selective leaching of some elements, e.g. Na, S, P, N, K and Cl (Campana, 1999; Elsdon *et al.* 2008). In addition, existing methods are destructive, meaning that otoliths can only be used once and not for any other purpose, e.g. age determination afterwards, and necessitating regular collection. A non-destructive method that could detect elements with adequate resolution and precision would be highly desirable.

5-1-3 Aims

To identify any similarities or differences at an elemental chemical level that may distinguish black bream inhabiting different estuarine systems.

To identify any inter-estuarine movement between adjacent estuaries, Lake Tyers and Gippsland Lakes, by obtaining ad hoc otolith elemental chemical signatures of resident black bream.

5-2 Methods

5-2-1 Sample collection and preparation

Black bream otolith samples originating from the Gippsland Lakes and Lake Tyers were sourced from (i) archived Department of Primary Industries (DPI) Victoria - Queenscliff Centre (courtesy of Lauren Brown) ($n = 37$) and from (ii) fish utilised in other research associated with this study ($n = 61$). The years when the fish were sampled were: 2003, 2009 and 2013.

Otoliths originating from this study were individually wiped by a tissue to remove most extraneous material remaining on their surfaces at the time of dissection from the fish but those from DPI were not. Otoliths were subsequently placed in labelled paper envelopes singly or in pairs, representing individual black bream. Samples were stored dry in accordance with Morison *et al.* (1999).

5-2-2 Sample analysis

Otoliths that had been stored dry in paper envelopes were mounted on carbon-impregnated double-sided sticky tabs attached to aluminium stubs (Figure 6-1). Most otoliths were mounted singly and inner (concave) side up, but some were mounted in pairs to compare signals from both otoliths from one fish. For quality control tests, some otoliths were examined on both outer and inner (concave) surfaces. To examine if the signals from the convex side of otoliths were typical of those from inner regions, some otoliths were also broken in half, mounted with the broken surface uppermost and the outer and inner signals compared.

Otoliths were examined uncoated on a FET Quanta 200 Environmental Scanning Electron Microscope (ESEM) fitted with a Oxford X-MaxN 20 EDXS (electron-dispersive X-ray spectroscopy) detector located at RMIT University city campus with settings of 30 kV, tilt 0 or 36.5°, take-off of 26.8 or 52.4, detector UTW+, resolution 127, Tc 100, FS 1109 or 4678 and 100 µS. Ten elements (C, N, O, Na, P, S, Cl, K, Ca and Sr) were detected in initial scans and were selected for detection in each otolith. Most samples were analysed as whole otoliths at 100x magnification, when the otolith image filled the whole screen, with the inner (concave) surface uppermost.

To identify a difference in otolith surface appearance, a colour index was developed where: 1= white; 2 = white, small yellow regions; 3 = white and yellow regions; 4 = white and brown, white and greater than 50% yellow; and 5 = yellow and lots of debris.

5-2-3 Statistical analysis

The non-parametric Kruskal-Wallis test was used to compare atom% for each element and its ratio with atom% using the statistical application Minitab Version 16 (www.minitab.com). In some cases, data were transformed to normality and re-tested using ANOVA or regression analysis.

Atom% data and weight% readings were compared for each element separately by correlation analysis.

Significance was taken as $P \leq 0.05$.

5-3 Results

5-3-1 Elemental analysis of atom% data

Otoliths as viewed on the ESEM were similar but had various irregularities in shape, even between pairs of otoliths from a single fish. For each otolith, a micrograph, a spectrum and a table of atom% and weight% for each element was produced (e.g. **Figure 5-1**) and these were compared (**Figure 5-2**). The main elements detected were C, N, O and (in much lesser amounts) Na, ‘P’, K, Ca and Sr. The P K_{α} peak coincided exactly with a minor K peak for Ca, which was one of the major elements, and it was calculated from the heights of the other peaks for Ca that this was mostly the minor peak for Ca rather than the K_{α} peak for P, hence the inverted commas for ‘P’.

The atom% data distributions were non-parametric apart from atom% for O, P and Sr and so only non-parametric Kruskal-Wallis analyses are displayed in **Table 5-1**. There was no significant difference in atom% for 8/10 elements selected for analysis (i.e. C, N, O, Na, P, K, Ca and Sr) but for S and Cl, the Lake Tyers fish otoliths had greater atom% than those from Gippsland Lakes fish. Na, N, S and Cl were not detected in some Gippsland Lakes black bream otolith samples. Na and P were similarly not detected in some Lake Tyers samples. In each inter-estuarine comparison, the Fisher exact test (Zar, 1984) indicated that the failure to detect the presence of an element was not significant.

Otolith samples, in particular archived specimens, varied in colour and surface appearance, some displaying possible remnants of tissue (**Figure 5-2**). Those that were most coloured also recorded the greatest atom% C (**Figure 5-3**), which distorted the atom% data. Linear regression of atom% C against colour index was significant, displaying a coefficient of determination $r^2 = 0.3663$, and with a straight line of Atom% C = 6.116 x Colour index +.

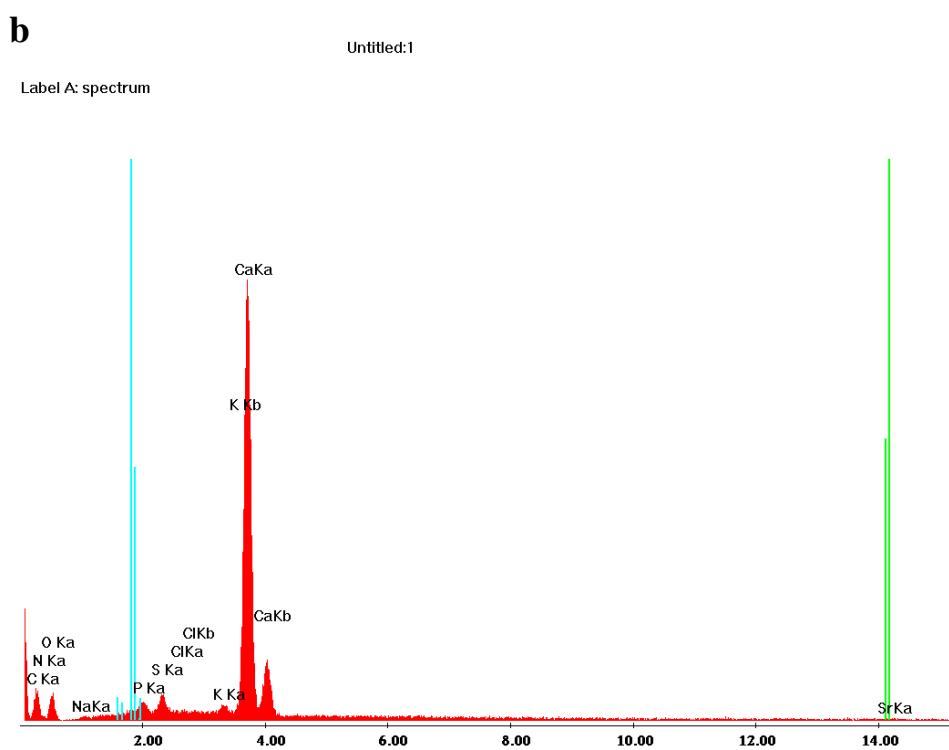


Figure 5-1: Otolith from black bream GL12F-1 (Gippsland Lakes, 12 cm fork length, female) as viewed by ESEM. (a) appearance, (b) EDXS spectrum.

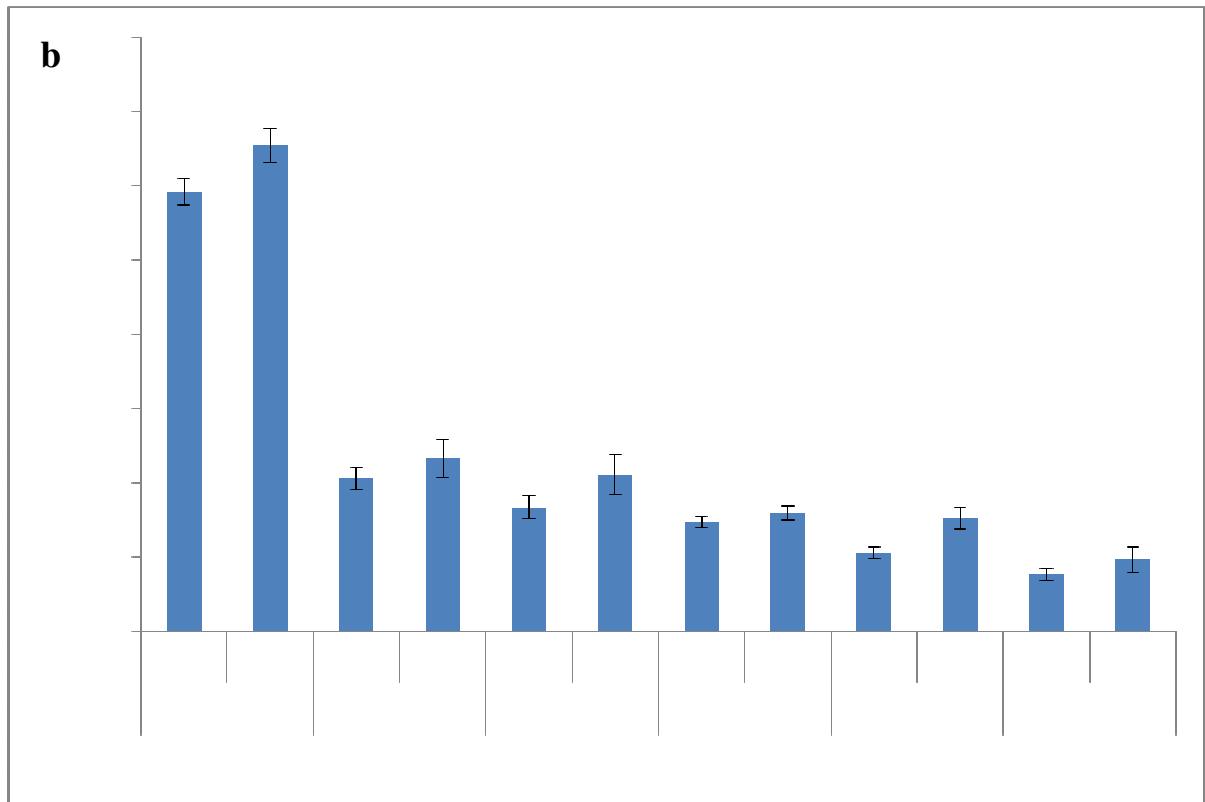
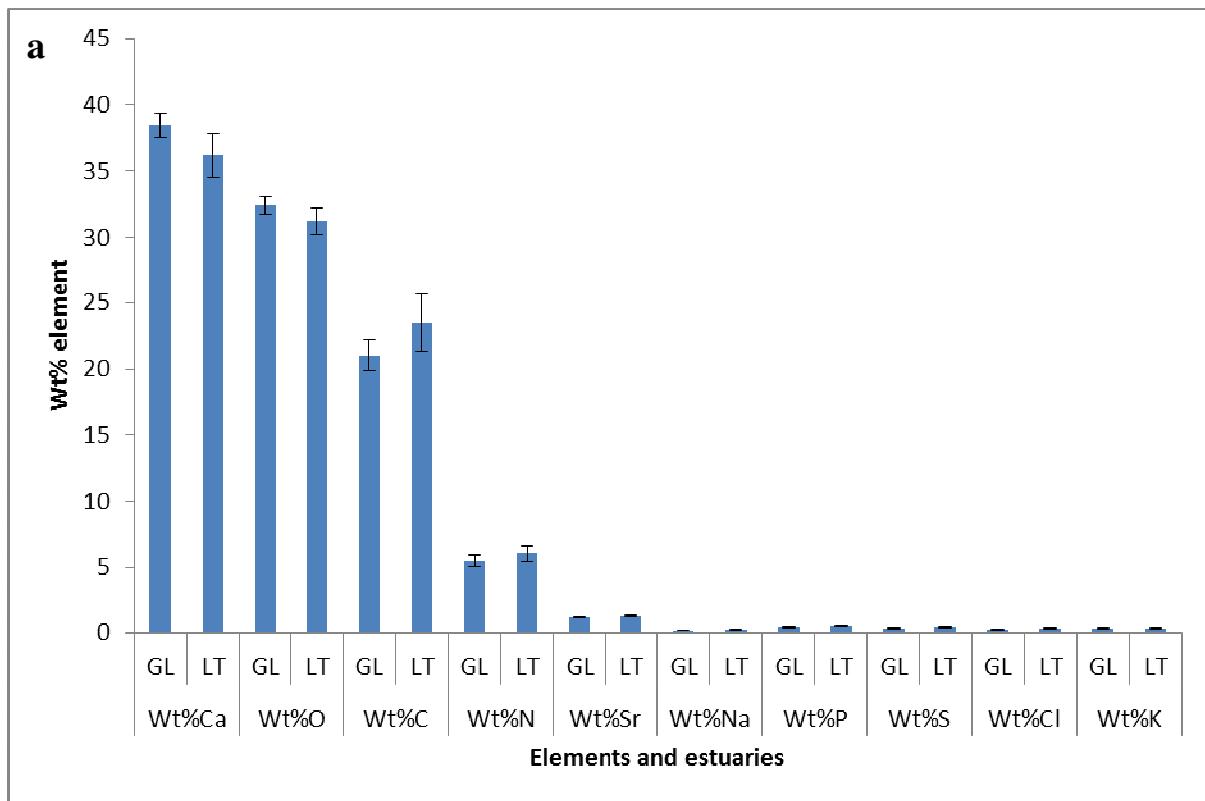


Figure 5-2: Variation in weight% composition for ten elements in black bream otoliths from the Gippsland Lakes (GL) and Lake Tyers (LT) analysed by EDXS on an ESEM. (a) All elements, (b) elements with smaller weight%. Data are mean \pm SE.

Table 5-1: Summary of non-parametric Kruskal-Wallis analysis of elemental composition (atom%) in otoliths from fish from the Gippsland Lakes and Lake Tyers (n=92; 64 Gippsland Lakes:28 Lake Tyers; fork length 5-61 cm; 21 male:2 indeterminate:32 female). Not all samples had all information.

Factor	Component analysed																		
	Atom%									Atom% element/atom% Ca									
	C	N	O	Na	P	S	Cl	K	Ca	Sr	C/Ca	N/Ca	O/Ca	Na/Ca	P/Ca	S/Ca	Cl/Ca	K/Ca	Sr/Ca
Location																			
H	0.65	2.31	1.11	0.70	1.62	4.71	10.74	1.37	1.33	0.55	0.92	1.56	0.43	0.26	1.62	3.45	9.78	4.11	7.12
p	0.420	0.129	0.293	0.403	0.203	0.030	0.001	0.242	0.248	0.457	0.338	0.212	0.514	0.609	0.203	0.453	0.002	0.043	0.008
						LT>GL	LT>GL										LT>GL	LT>GL	LT>GL
Fork length																			
H											50.71	45.10	25.49	21.59	45.52	42.54	34.35	39.02	36.69
p											0.019	0.062	0.786	0.918	0.057	0.101	0.356	0.183	0.260
Sex																			
H											0.71	2.48	2.34	2.49	3.74	1.82	1.03	0.79	3.84
p											0.699	0.290	0.310	0.288	0.154	0.403	0.598	0.674	0.147

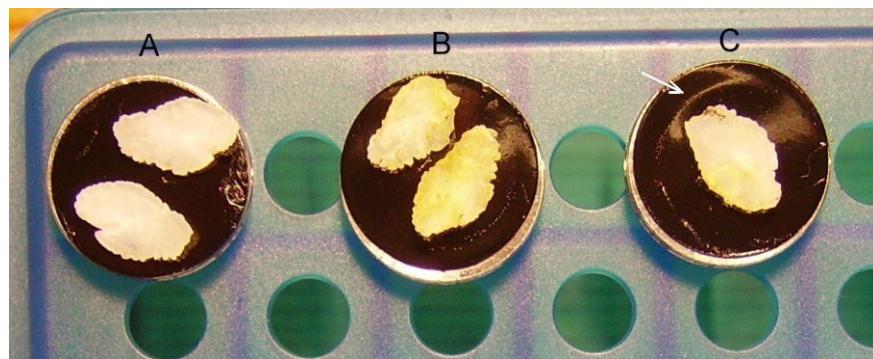


Figure 5-3: Black bream otoliths mounted on aluminium stubs for EDXS analysis on ESEM and displaying differing colouration: (A) white (category 1), (B) yellow (category 5), and (C) region of yellow organic debris (arrow) (category 3).

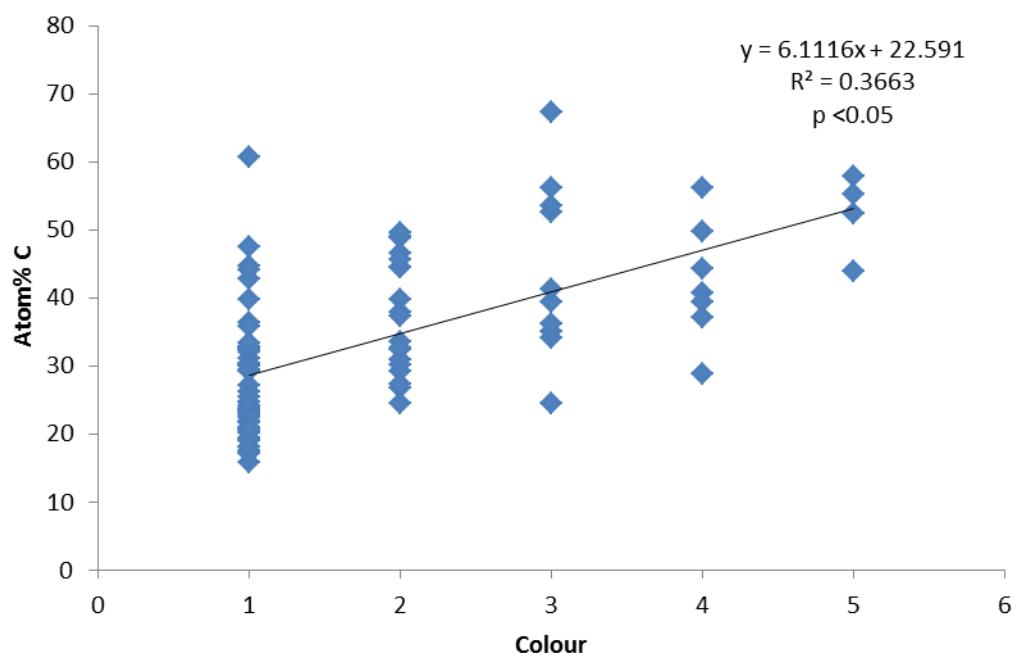


Figure: 5-4: Effect of colour on atom% C detected by EDXS analysis in 92 otoliths of black bream collected from Gippsland Lakes and Lake Tyers, East Gippsland, Victoria in 2009-2013.

22.591 (**Figure 5-3**). It was therefore decided only to proceed with data in the form of atom% element/atom% Ca to stabilise the data for the elements relative to that of Ca, which had the greatest atom% for all samples

Atom% data were highly correlated with weight% data for each element ($r=0.402-0.794$, $p<0.001-0.003$) and so it was decided to proceed with analysis of atom% data only.

Analysis of the atom% element/atom% Ca data showed that fish source did not affect the ratio for 7/10 elements (C, N, O, Na, P and S) but that for Cl, K and Sr, the ratio in otoliths from Lake Tyers fish was greater than that in Gippsland Lakes fish (**Table 5-1**).

Fork length did not significantly affect the atom% ratio for O, Na, S, Cl, K and Sr but did for C and was close to significance for N and P but a scatterplot of C/Ca against fork showed no obvious trend of increase in the C/Ca ratio with increase or decrease in fork length (**Figure 5-5a**). When data were normalised with a \log_{10} transformation, the same lack of trend was seen (**Figure 5-5b**) and regression analysis showed no significant variation with fork length (ANOVA for regression line, $F=1.56$, $p=0.216$). There was, however, a tendency for the C/Ca ratio to be greater in small (<15 cm) and mid-sized (15-35 cm) than large (≥ 35 cm) fish.

There was no effect of sex on the atom% ratio for any of the ten elements (**Table 5-1**).

Cluster analysis of atom% data for all elements together using complete linkage and squared Euclidean distance showed three main groups: a small group on the right that were yellow, had lots of what appeared to be membrane remnants on their surface and were high in %C and a group to the left/centre comprising two main divisions, each containing samples from both estuaries (**Figure 5-6**). Principal components analysis using a correlation matrix showed similarly that samples from both estuaries were intermixed (**Figure 5-7**). A subsequent examination of scree and loading plots and a Tukey multiple comparison test showed that the

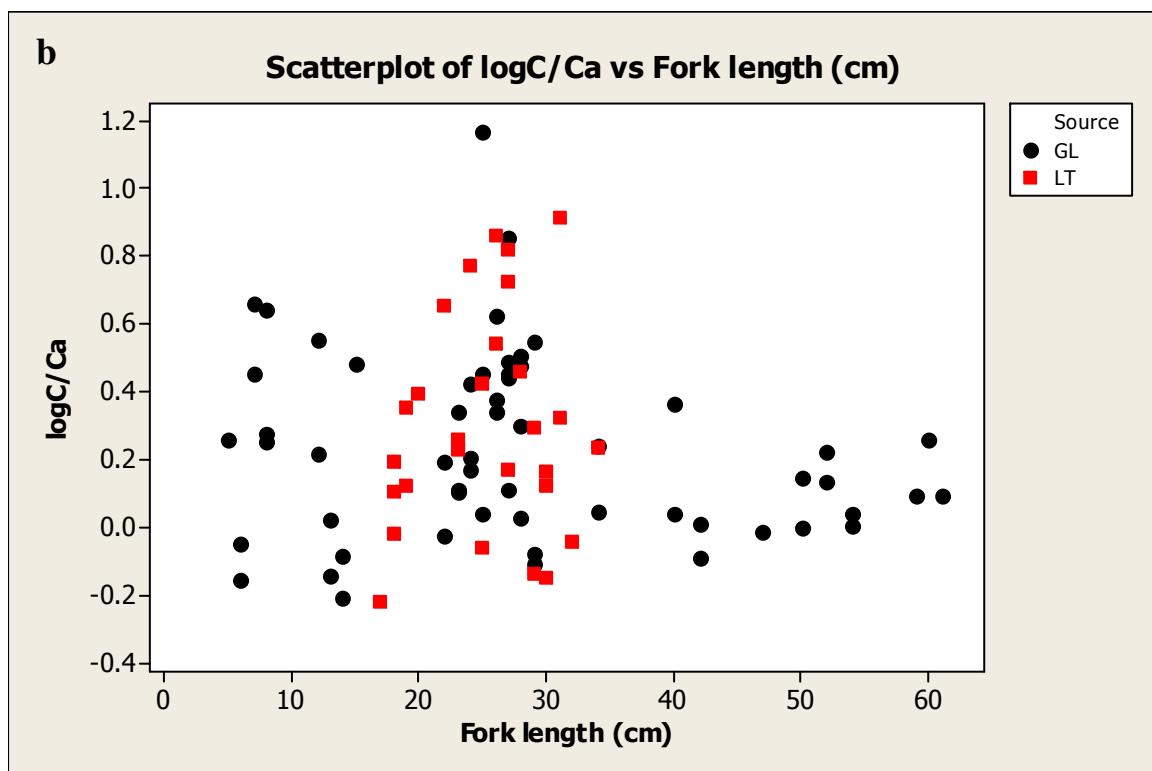
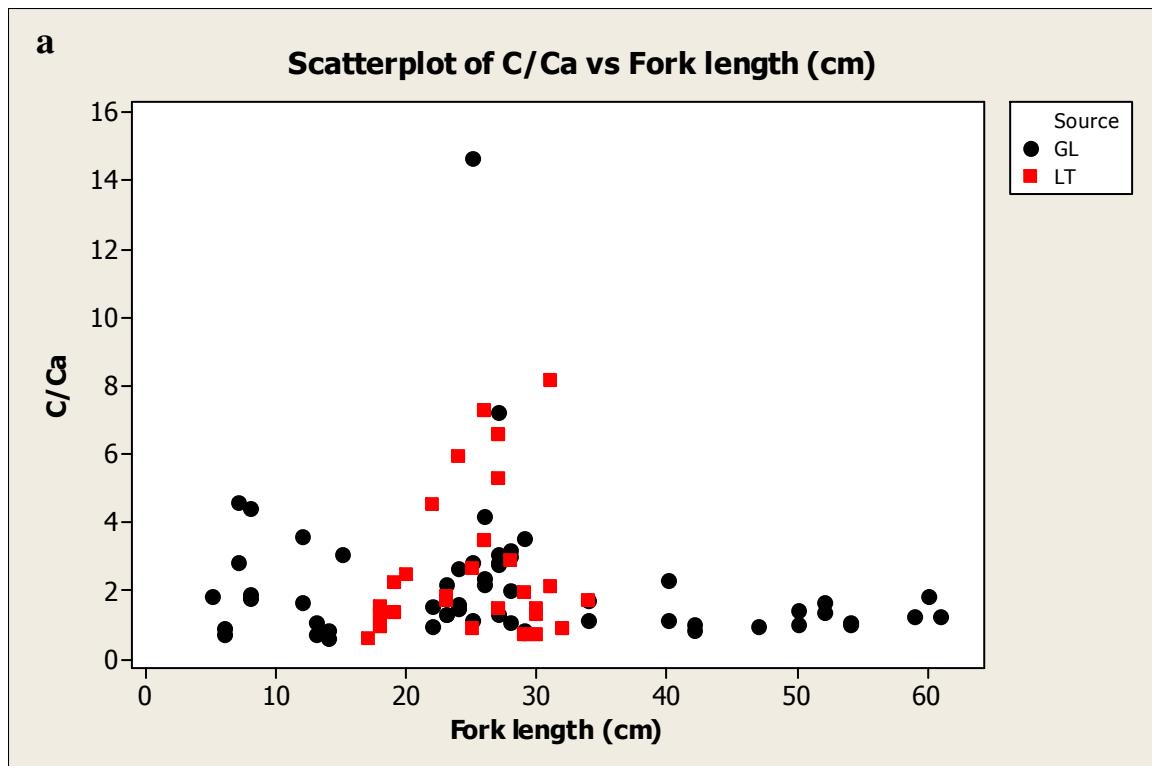


Figure 5-5: Variation in atom% C/Ca ratio with fork length and fish source in otoliths of black bream from the Gippsland Lakes (GL) and Lake Tyers (LT).

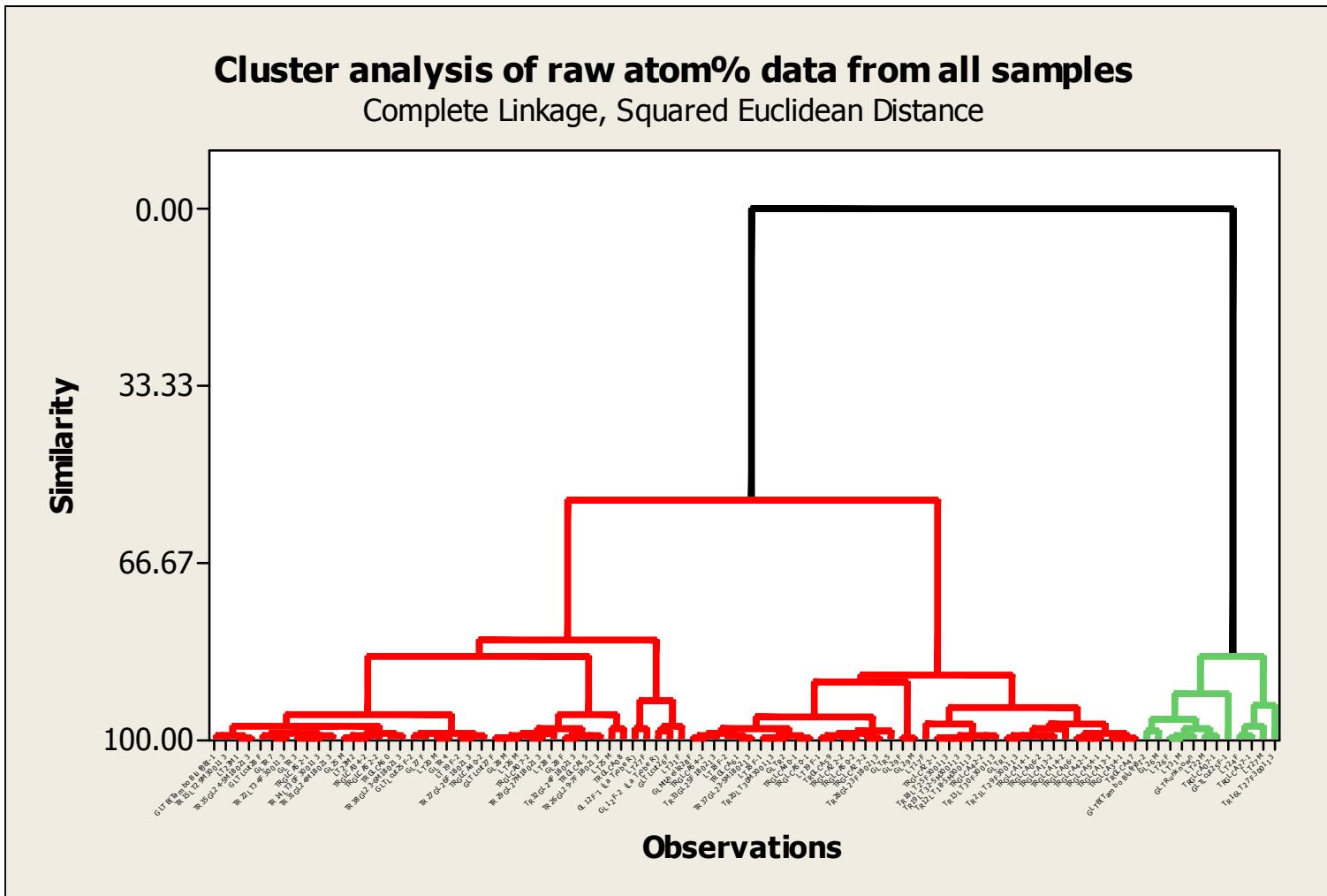


Figure 5-6: Dendrogram from hierarchical cluster analysis using complete linkage and squared Euclidean distance of raw percentage data for ten elements detected in otoliths of black bream in EDXS analysis of uncoated specimens on an ESEM. Red circles=Gippsland Lakes fish, blue triangles=Lake Tyersfish.

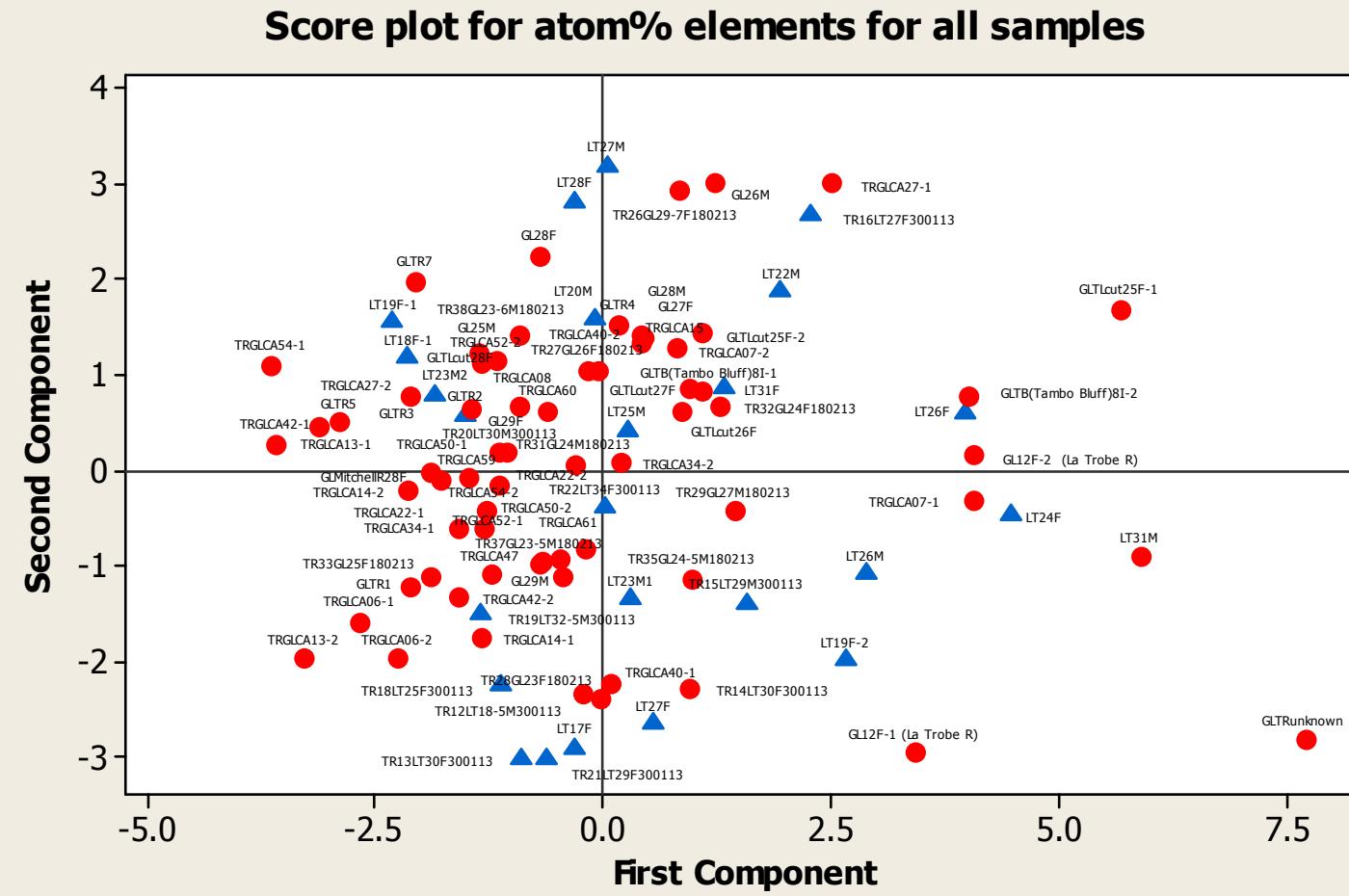


Figure 5-7: Score plot from principal components analysis of raw percentage data for ten elements detected in otoliths of black bream in EDXS analysis of uncoated specimens on an ESEM. . Red circles=Gippsland Lakes fish, blue triangles=Lake Tyers fish.

atom% carbon strongly influenced the grouping. When subjected to a two-tailed runs test (Zar, 1984), the final arrangement of samples originating from both estuaries was found to be sequentially random ($P > 0.05$) in the dendrogram. This indicates that whatever was influencing the character of the dendrogram was equally affecting otolith samples from both estuaries. This again validated the preferential use of the atom% element/atom% Ca for analyses.

Cluster analysis of atom% element/atom% Ca data for all elements together showed a small group of three samples and a larger group subdivided ultimately into six smaller groups, all of which were >70% similar; all groups contained samples from both estuaries (**Figure 5-11**). Principal components analysis showed similarly that samples from both estuaries were tightly intermixed apart a few samples from both estuaries, which were outliers in different directions (**Figure 5-12**).

As time may have been a factor in the composition of otoliths, data from collections for this thesis in 2013 were analysed separately. Both cluster analysis and principal components analysis of atom% data (**Figure 5-13**, **Figure 5-14**) and atom% element/atom% Ca data (**Figure 5-15**, **Figure 5-16**) confirmed the previous patterns.

5-3-2 Use of ESEM and EDXS to analyse otoliths

5-3-2-1 Variation between successive analyses

Replicate readings of the same otolith without removing it from the ESEM and replacing resulted in a coefficient of variation (CV) varying from 8-64% (**Table 5-2**). CV values for C, O, K, Ca and Sr were <20% but those for N, 'P', S and Cl were <40% and that for Na was 64%.

5-3-2-2 Variation with magnification

Replicate readings of the same otolith without removing from the ESEM and replacing it resulted in a CV of 6-49% (**Table 5-2**). CV values for C, O, K, Ca and Sr were <20% while those for ‘P’, S and Cl were 20-40% and those for N and Na were >40%.

5-3-2-3 Variation between convex and concave surfaces

Variation in CV between readings of the same otolith on convex and concave surfaces was 6-48% (**Table 5-2**). CV values for C, N, O, K, Ca and Sr were <20% while those for ‘P’, S and Cl were 20-40% and those for N and Na were >40%.

5-3-2-4 Variation between inner and outer areas of same otolith

Otoliths broken in half and mounted with the broken surface facing upwards (**Figure 5-8**) had a crystalline structure, with crystals radiating out from the centre to the edge. At intervals, new crystals began and radiated out with those pre-existing to the edge. There was no indication of any regular rings where crystals started simultaneously around the otolith.

There was no significant difference in the atom% of any element between the central and edge areas of otoliths (**Figure 5-9**).

The CV of readings of central and outer areas of broken otoliths varied from 5-75% (**Table 5-2**). CV values for C, O, K, Ca and Sr were <20% but those for N, ‘P’, S and Cl were ≤40% and that for Na was 51%.

5-3-2-5 Variation between otoliths from individual fish

The composition of pairs of otoliths from individual fish did not vary significantly (**Table 5-4, Figure 5-10**). The mean CV of pairs of otoliths was 7-51% (**Table 5-2**). CV values for C, O, K, Ca and Sr were <20% but those for N, ‘P’, S and Cl were ≤40% and that for Na was 51%.

Table 5-2: Variation due to various aspects of otolith analysis by EDXS on an ESEM.

Source of variation	n	Coefficient of variation (CV) (%)									
		C	N	O	Na	'P'	S	Cl	K	Ca	Sr
Successive scans	10	13.4	37.2	8.0	64.4	28.3	30.2	29.8	13.9	10.3	11.1
Different magnification	8	16.3	44.5	4.9	49.0	26.7	34.9	31.8	12.6	6.3	17.4
Convex and concave surfaces	5	10.3	16.3	6.0	46.9	48.0	36.2	54.1	17.4	17.1	12.9
Centre and edge of same otolith	12	10.77	30.80	7.27	75.31	26.08	32.46	17.76	7.07	5.43	21.84
Different otoliths from same fish	12	13.9	39.8	5.0	50.9	31.4	32.8	32.9	14.4	7.2	12.2
Mean		12.9	33.7	6.2	57.3	32.1	33.3	33.3	13.1	9.3	15.1
SE		1.1	4.9	0.6	5.4	4.1	1.0	5.9	1.7	2.1	2.0

Table 5-3: Summary of non-parametric Kruskal-Wallis analysis of differences in elemental composition (atom%) between the centre and the edge of single otoliths of single black bream from the Gippsland Lakes and Lake Tyers.

Statistic	Atom% element									
	C	N	O	Na	'P'	S	Cl	K	Ca	Sr
H	0.00	0.00	0.60	0.00	0.60	2.40	2.40	1.50	2.40	2.40
p	1.000	1.000	0.439	1.000	0.439	0.121	0.121	0.221	0.121	0.121

Table 5-4: Summary of non-parametric Kruskal-Wallis analysis of differences in elemental composition (atom%) between pairs of otoliths from single black bream from the Gippsland Lakes and Lake Tyers.

Statistic	Atom% element									
	C	N	O	Na	'P'	S	Cl	K	Ca	Sr
H	0.00	0.01	0.16	0.01	0.12	0.27	0.48	0.91	0.75	0.56
p	1.000	0.908	0.686	0.905	0.729	0.603	0.488	0.339	0.386	0.453

The mean CV varied from 6.2-57.3% (**Table 5-2**). It was least ($\leq 15\%$) for C, O, K, Ca and Sr, intermediate (30-35%) for N, 'P', S and Cl, and greatest (57%) for Na.

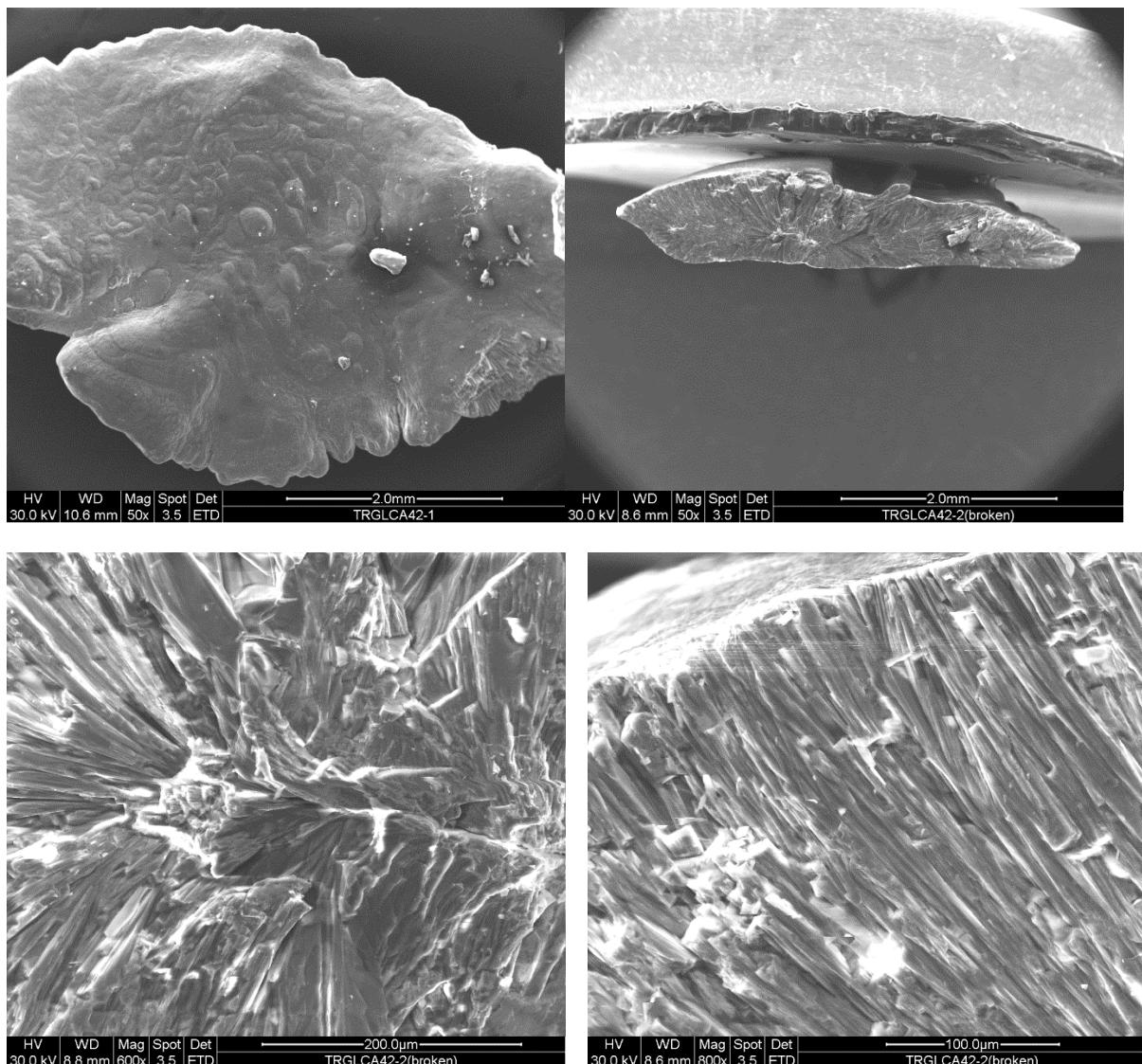


Figure 5-8: ESEM examination of different parts of otolith from black bream (fish TRGLCA42 – Gippsland Lakes, Cunningham Arm, undersized). (a) Whole otolith – convex surface, (b) broken surface mounted for examination, (c) centre of otolith, (d) edge of otolith.

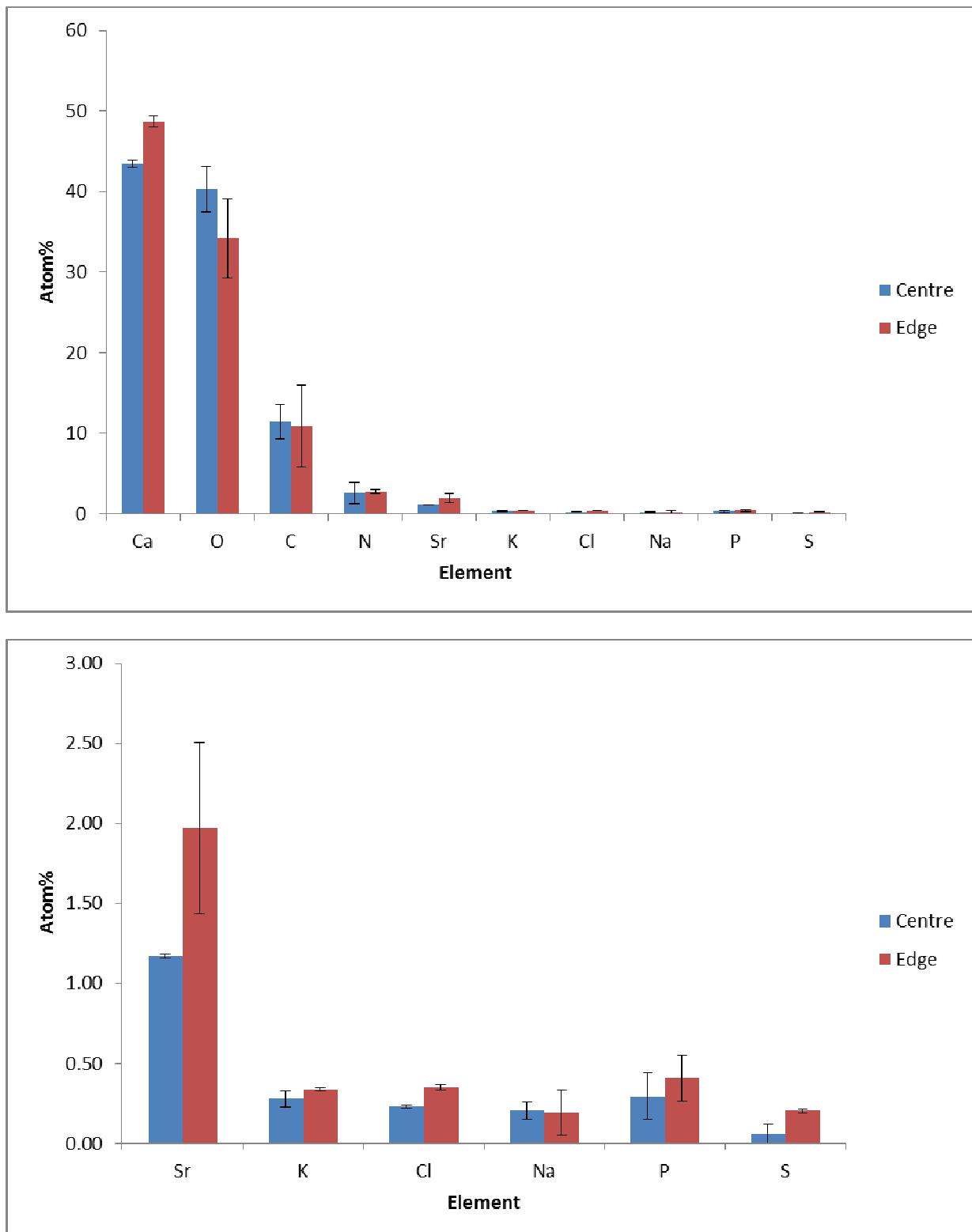


Figure 5-9: Atom% elements in readings from centre and edge in paired black bream otoliths from the Gippsland Lakes and Lake Tyers analysed by EDXS on an ESEM. . (a) All elements, (b) elements with smaller atom%. Data are mean \pm SE

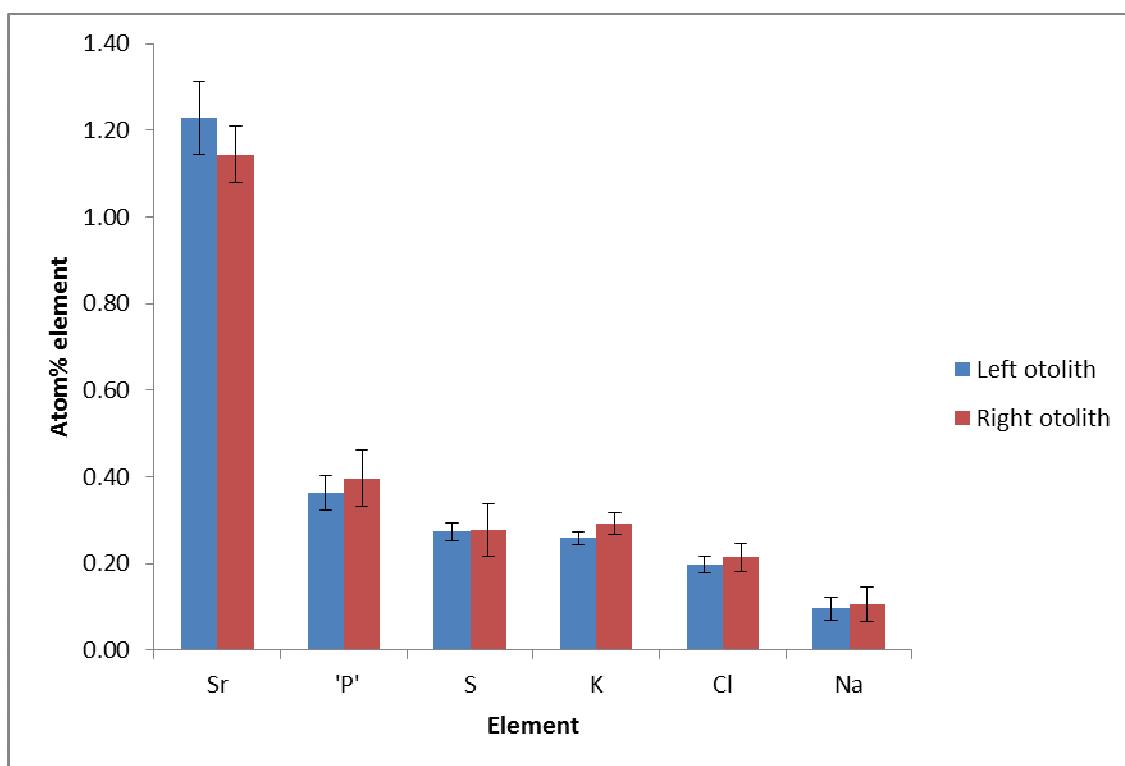
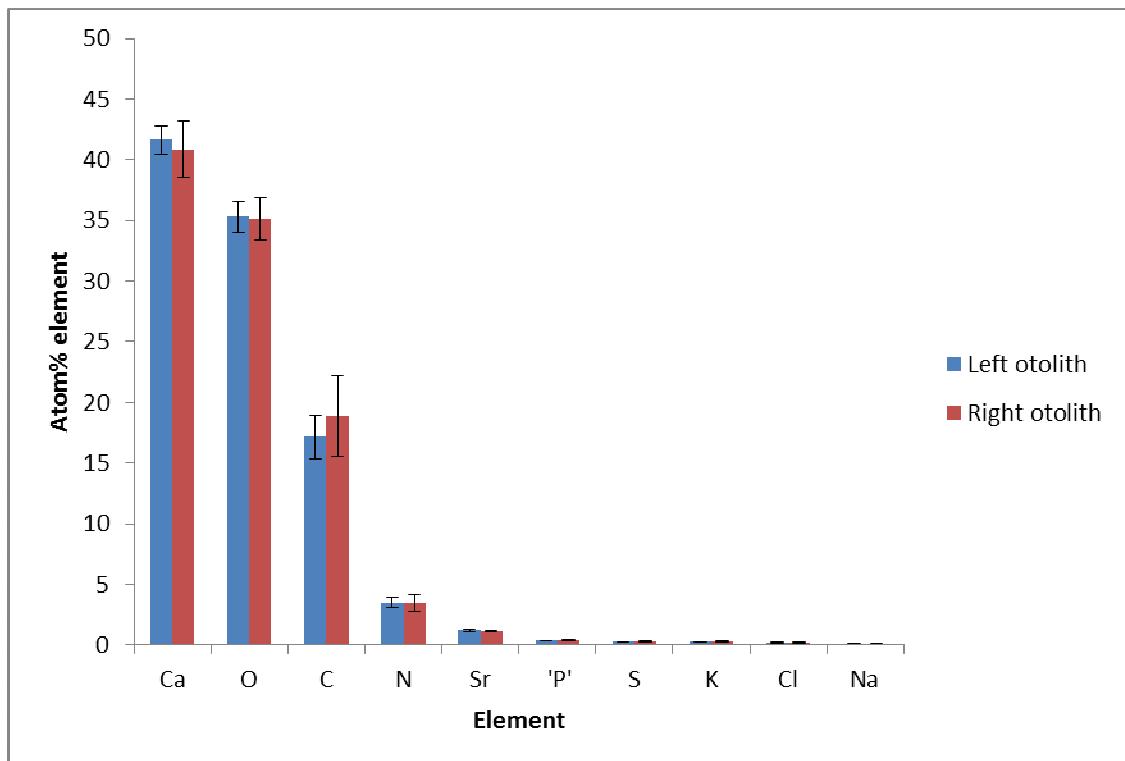


Figure 5-10 Variation in atom% composition for ten elements in paired black bream otoliths from the Gippsland Lakes and Lake Tyers analysed by EDXS on an ESEM. (a) All elements, (b) elements with smaller atom%. Data are mean \pm SE.

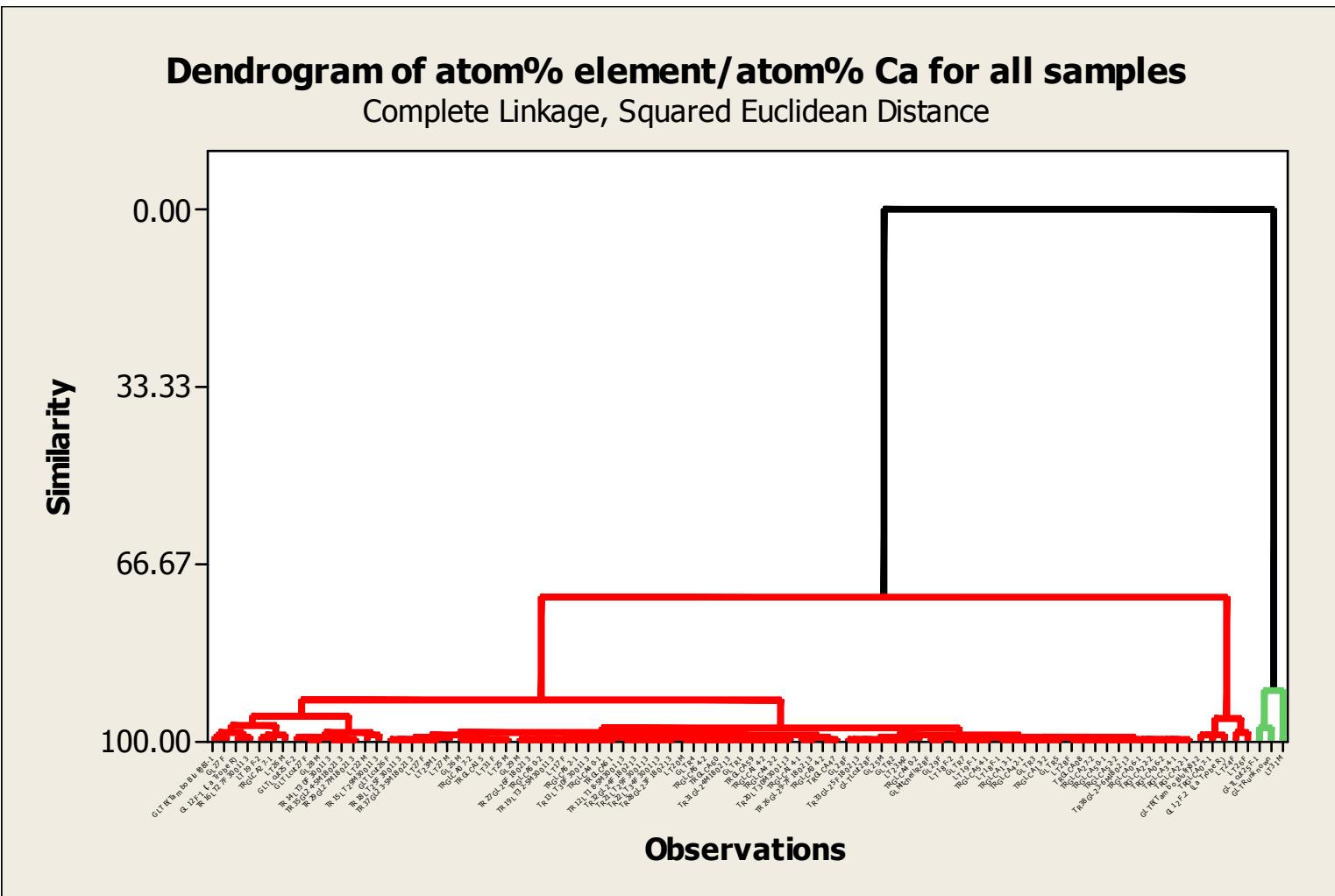


Figure 5-11: Dendrogram from hierarchical cluster analysis using complete linkage and squared Euclidean distance of ratio of %element:%Ca for ten elements detected in otoliths of black bream in EDXS analysis of uncoated specimens on an ESEM. . Red circles=Gippsland Lakes fish, blue triangles=Lake Tyers fish.

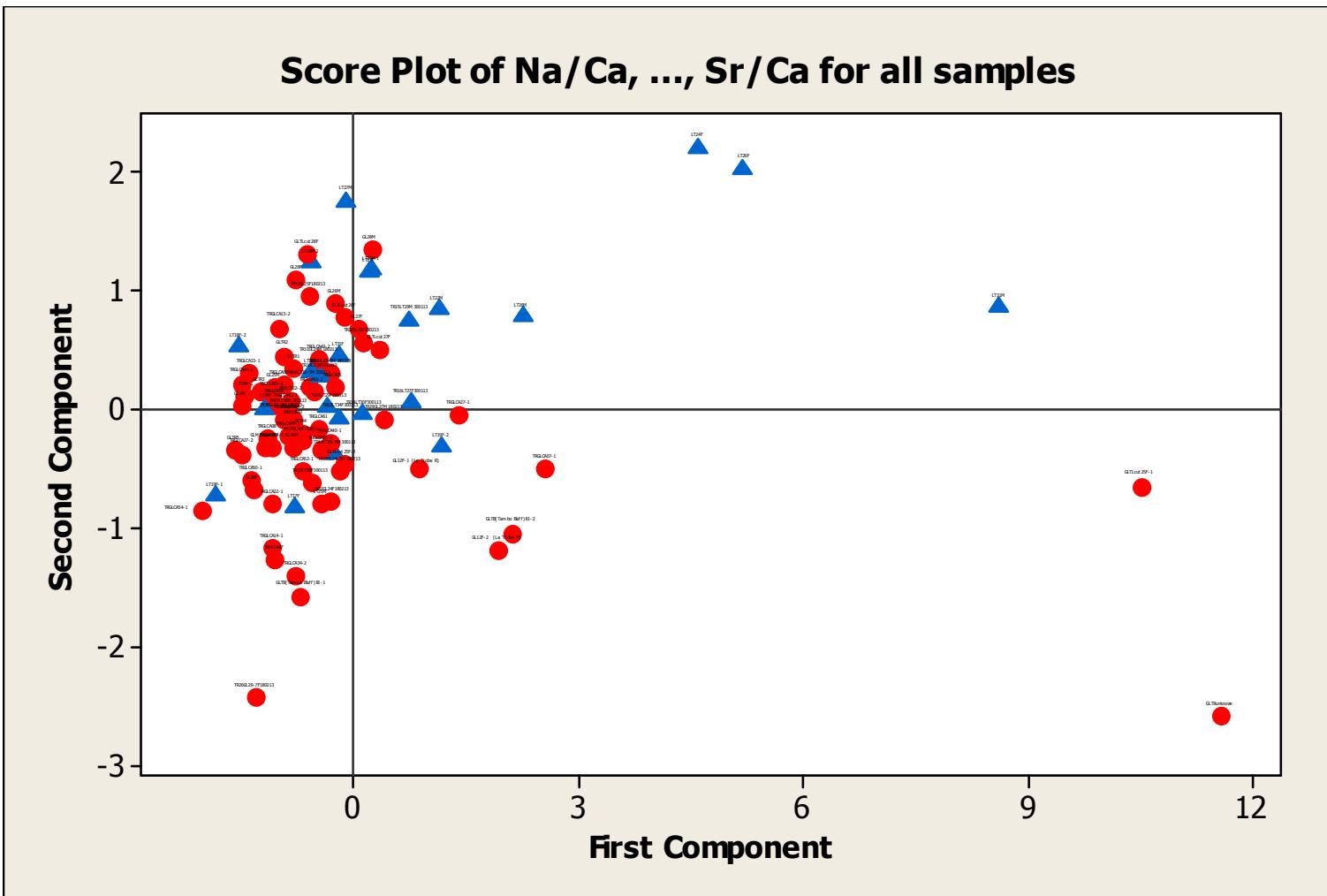


Figure 5-12: Score plot from principal components analysis of ratio of %element:%Ca for ten elements detected in otoliths of black bream analysis of uncoated specimens by EDXS on an ESEM . Red circles=Gippsland Lakes fish, blue triangles=Lake Tyersfish.

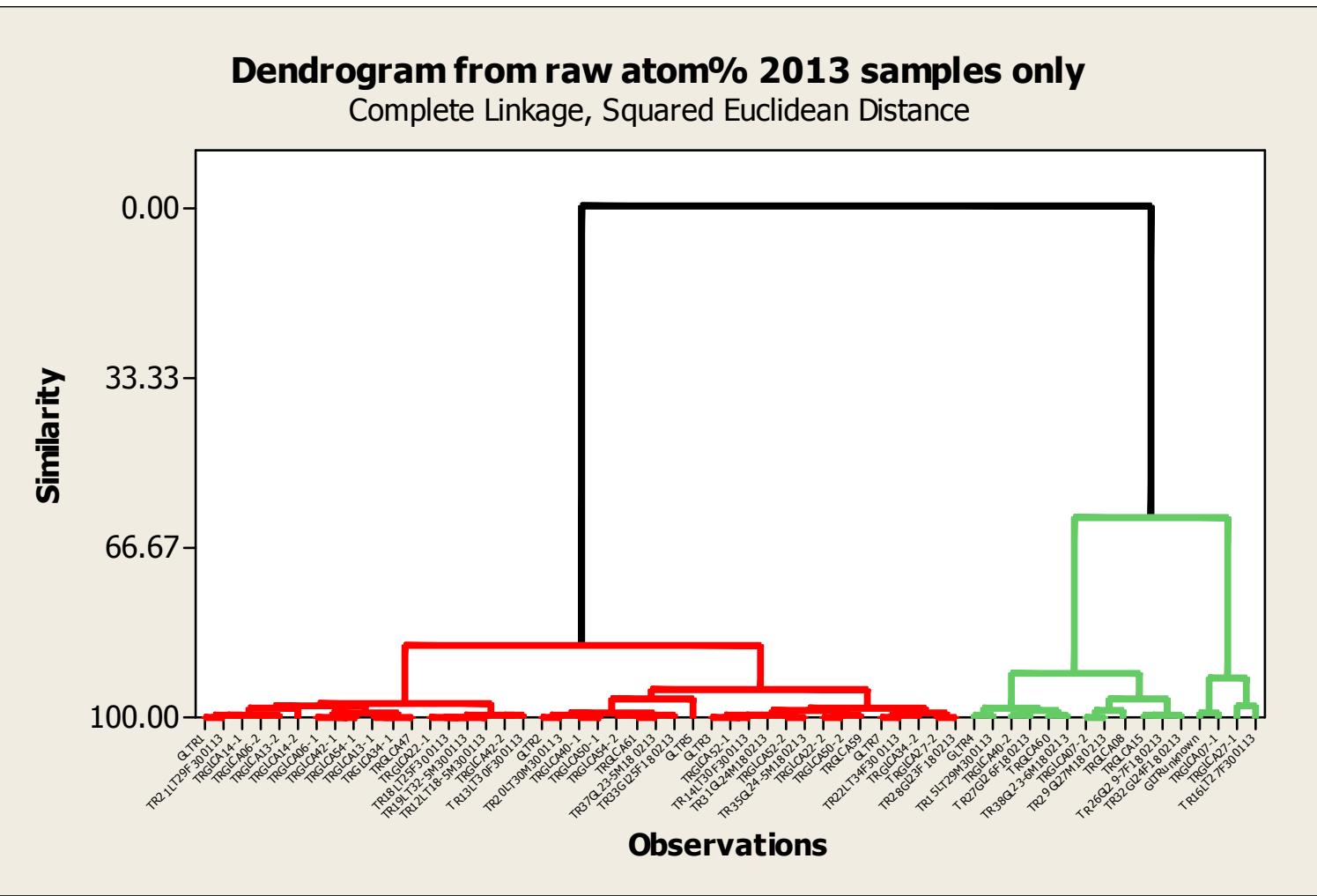


Figure 5-13: Dendrogram from hierarchical cluster analysis using complete linkage and squared Euclidean distance of raw percentage data for ten elements detected in 2013 otoliths of black bream in EDXS analysis of 2013 uncoated specimens on an ESEM. . Red circles=Gippsland Lakes fish, blue triangles=Lake Tyersfish.

Score Plot of C, ..., Sr from raw atom% 2013 samples only

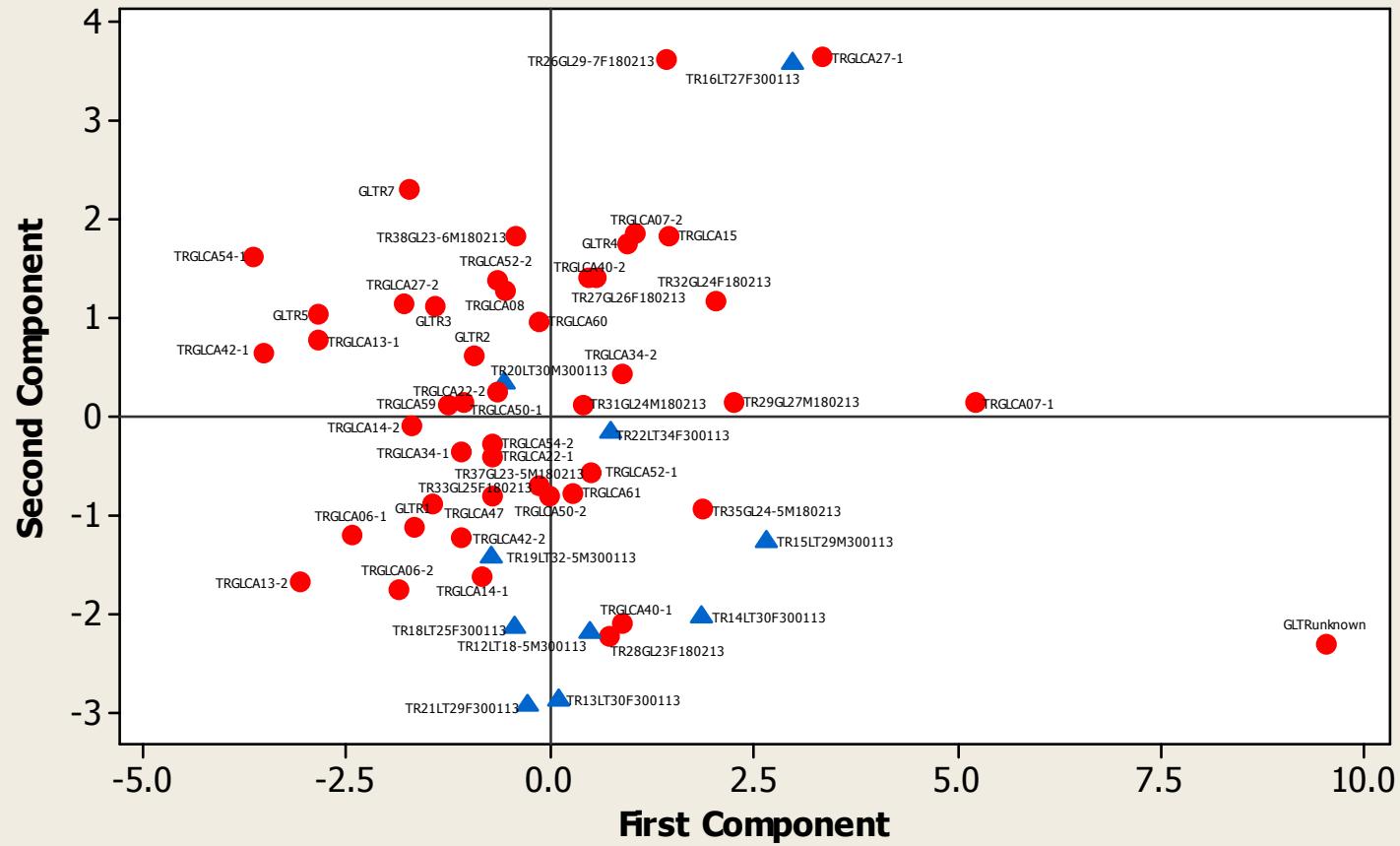


Figure 5-14: Score plot from principal components analysis of raw percentage data for ten elements detected in otoliths of black bream in EDXS analysis of 2013 uncoated specimens on an ESEM. . Red circles=Gippsland Lakes fish, blue triangles=Lake Tyersfish.

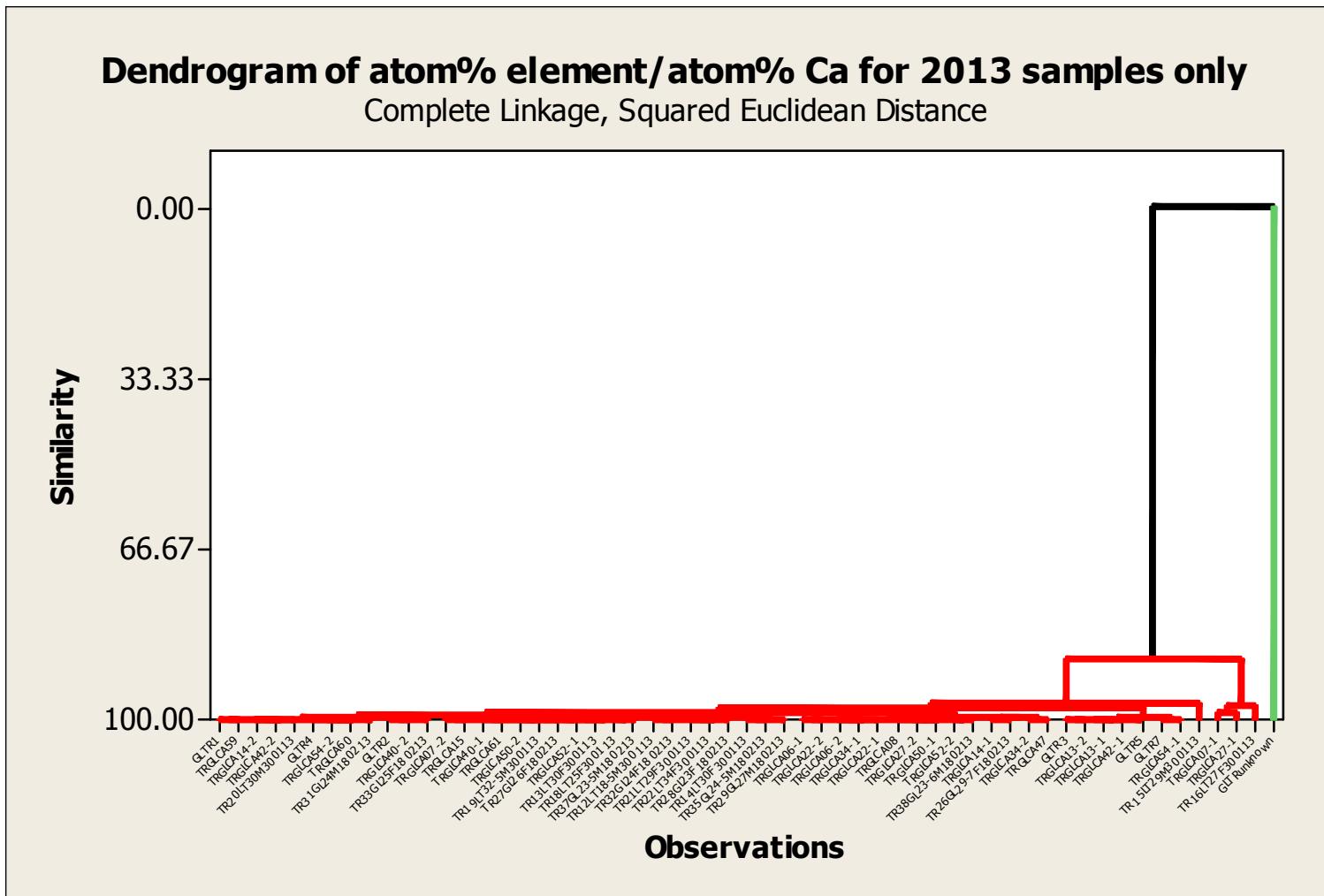


Figure 5-15: Dendrogram from hierarchical cluster analysis using complete linkage and squared Euclidean distance ratio of %element:%Ca for ten elements detected in otoliths of black bream in EDXS analysis of 2013 uncoated specimens on an ESEM. . Red circles=Gippsland Lakes fish, blue triangles=Lake Tyersfish.

Score Plot of Na/Ca, ..., Sr/Ca for 2013 samples only

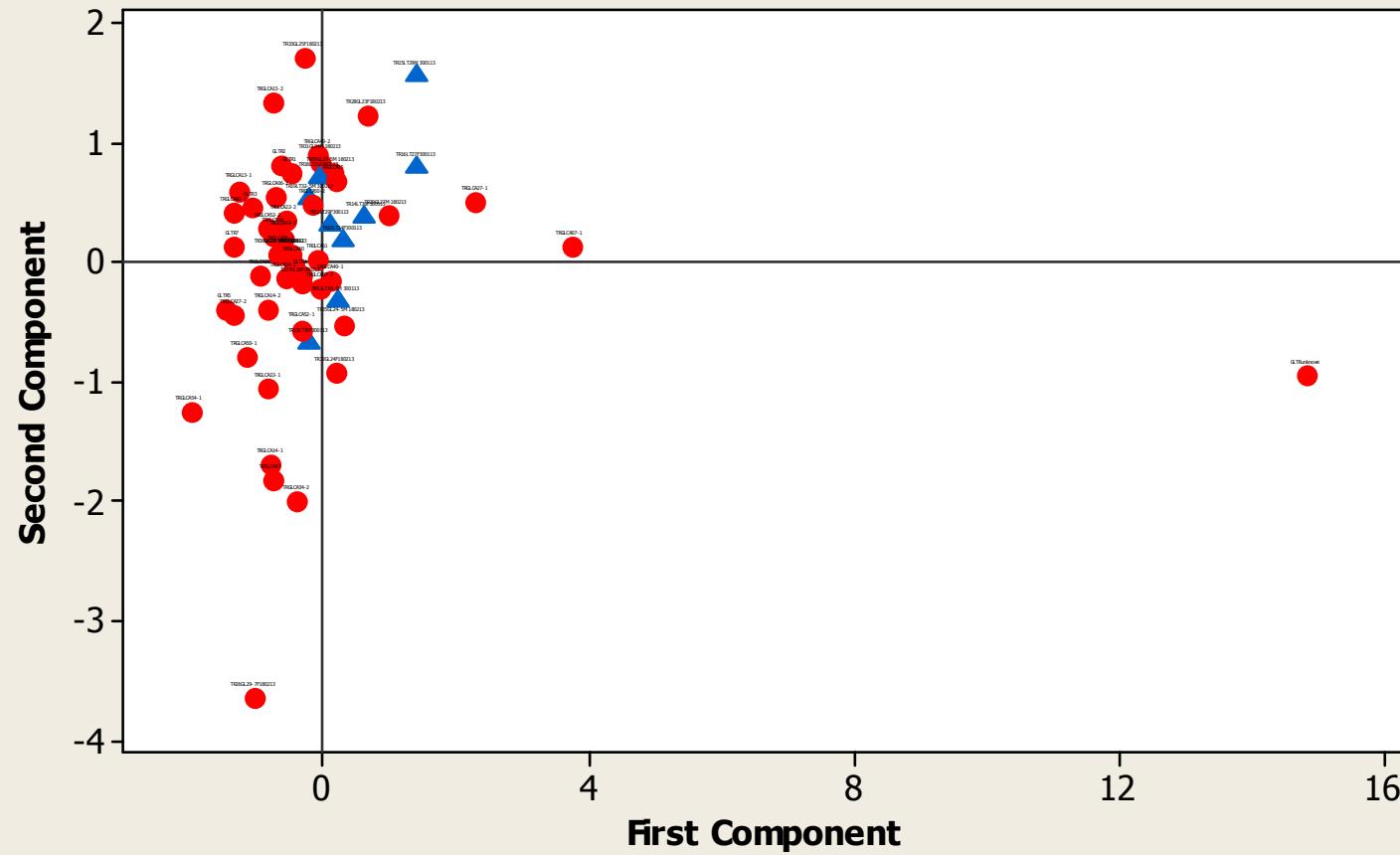


Figure 5-16: Score plot from principal components analysis of %element:%Ca for ten elements detected in otoliths of black bream in EDXS analysis of 2013 uncoated specimens on an ESEM.

5-4 Discussion

This study found that for the elements C, N, O, Na, ‘P’, S and Ca, and their ratios N:Ca, O:Ca, Na:Ca, ‘P’, Ca, S:Ca and K:Ca there were no differences between otoliths of black bream from the Gippsland Lakes and Lake Tyers by EDXS, suggesting either that the estuaries shared many features or that fish moved between them. An inter-estuarine difference did, however, exist for Cl and Sr and the ratios Cl:Ca, Sr:Ca and K:Ca (though that for K was marginal). EDXS detected a significantly greater concentration of Cl, Sr and K in otoliths originating from Lake Tyers than Gippsland Lakes black bream. Probable organic residue (high C and N atom%) found on individual otolith surfaces determined the carbon atom% in all samples irrespective of estuary of origin and this necessitated the use of the ratio of atom% of the element to atom% Ca to compare samples. These results suggest that EDXS efficiently analysed the otoliths by a non-destructive method but that the coefficients of variation were high, particularly for Na. These aspects are discussed in more detail below.

5-4-1 Use of ESEM and EDXS to analyse otoliths

Elements selected for ESEM analysis were major elements detected in preliminary EDXS scans and were mainly those that make up calcium carbonate Ca, O, and C (CaCO_3) and minor elements (>100 ppm) Na, Sr, N, K, S, Cl, and P found previously in otoliths (Campana, 1999). Those elements incorporated into the otolith via substitution for Ca (Sr, C and O) were relatively stable once incorporated into the otolith (Elsdon *et al.* 2008). However, elements under physiological regulation were not and may under certain conditions leach out of otoliths: Na, S, P, N, K and Cl (Campana, 1999; Elsdon *et al.* 2008), which could explain their large coefficients of variation. The large coefficient of variation for N could also be because of variable amounts of dried soft tissue on the outside of the otoliths. EDXS is a non-destructive technique that required minimal preparation [e.g. no water sonification of

the otolith prior to analysis that would displace Cl (Campana (1999)] and so physiologically controlled elements could in theory be quantified *in situ*.

The non-destructive EDXS technique did not find any differences in historical accretion of elements in the otoliths even when cross sections of otoliths were also examined. Black bream can have an otolith accretion rate of c. $5 \mu\text{m day}^{-1}$ (Elsdon and Gillanders, 2006). The ESEM electron beam penetration depth is $3\mu\text{m}$ (Phil Francis, RMIT Microscopy and Microanalysis Facility.) and so records primarily are of the outer layer of whole otoliths. Therefore, all whole otolith samples record the surrounding environmental history and any movement for approximately the last 24 h prior to capture and sacrifice. The lack of significant difference between the centre and the edge scans of a small sample of otoliths suggests that otolith composition was relatively stable.

There were no significant differences in elemental composition between the left and right otoliths, as found previously (Elsdon and Gillanders, 2003) and so scanning of one otolith per fish should be adequate, though further testing is needed to validate this.

The presence of variable amounts of what appeared to be organic debris on the surface of the otoliths was probably the cause of the variation in colour and hence the need to use the ratio of the elements to one expected to be large and stable in the otoliths – Ca, as used previously with measurements of otolith composition by laser ablation (Elsdon and Gillanders, 2003). Samples should also preferably be wiped briefly to remove soft tissue and the coefficients of variation may reduce, particularly for C, N and O, which it would be expected to contain in large amounts.

Relative concentrations of elements in the otoliths measured here using the ratio with Ca are similar to those recorded by others in black bream (Elsdon and Gillanders, 2003) using laser

ablation, suggesting that EDXS was a valid technique that had the advantage of being non-destructive. Further testing is needed to validate this, but EDXS looks promising.

5-4-2 Differences between estuaries

5-4-2-1 Strontium

The greater concentration of Sr in Lake Tyers than Gippsland Lakes otoliths was unexpected, as Sr has been used as a marker for pollution and some of the 2009 Tambo River (Gippsland Lakes) samples had noticeably high levels of Sr. Otolith Sr has been linked to salinity, temperature and fish diet – with obvious significance for an inter-estuarine study.

In black bream, ambient water was the primary contributor to otolith Sr (59-84%), with a secondary dietary influence also possible (Webb *et al.* 2012). Webb *et al.* (2012) found a significant interaction of temperature and salinity on water contributions to otolith Sr; this was most evident at high temperatures (optimum $23.79 \pm 0.14^\circ\text{C}$) and water contributions of Sr decreased with increasing salinity (10.9 ± 0.12 to $32.4 \pm 0.34\%$). Therefore, for the days of sampling in 2003, 2009 and 2013, Lake Tyers black bream may have been exposed to greater temperatures and lesser salinities compared with black bream from the Gippsland Lakes. This is as might be expected from a mostly land-locked aquatic system dominated by fresh water flows, by comparison with the lower temperatures and greater salinities in the Gippsland Lakes (Harris *et al.*, 1998; BMT WBM Pty Ltd, 2010; Ladson and Tilleard, 2011).

Both Lake Tyers and the Gippsland Lakes are subjected to water stratification as a result of freshwater inflows (Bird, 1978; Bek and Bruton, 1979; Hall, 1984; Longmore, 1988; Longmore *et al.* 1990; NREC, 1991; MacDonald, 1997a; Fabris *et al.* 1999), which influences black bream, in particular with regard to spawning and recruitment (Jenkins *et al.* 2010; Williams *et al.*, 2012; Williams *et al.*, 2013). This longitudinal study by EDXS over

2003, 2009 and 2013 has also identified otoliths in black bream as a bioindicator of Sr and so of salinity and temperature history in both the Gippsland Lakes and Lake Tyers.

5-4-2-2 Chlorine

Under physiological regulation, chlorine is not a stable element within an otolith (Campana, 1999). Therefore, the inter-estuarine difference observed for chlorine may be a consequence of two possible factors: a result of a physiological change prior to or during capture or elemental leaching out of the otolith post-mortem.

The pathway of inorganic Cl into the otolith is from the water into the blood plasma via the gills or intestine, then into the endolymph fluid surrounding the otolith, occurring with little impediment at each stage (Campana, 1999). Otoliths often display micro-channel architecture and the poorly bound Cl is found as an elemental inclusion within these interstitial regions and can be severely affected by exposure to fluids (Campana, 1999).

A stressor such as capture for this study would have evoked strenuous activity in fish such as a burst of swimming in an attempt to escape netting. As a consequence, within some fish, this would have created an osmoregulatory imbalance resulting from anaerobic activity (following aerobic exhaustion and accumulation of lactate in muscle and blood); associated with this imbalance would have been an increase in plasma chloride solute (Donaldson et al. 2014).

If during the death of a fish, the composition of endolymph fluid surrounding the otolith changes, then leaching of Cl out of otolith via micro-channels is quite possible (Campana, 1999). A subset of samples may have had a delay in otolith removal for a considerable time period after fish were caught, as they are normally collected as anglers bring their catches for counting and weighing, allowing for leaching to occur. This was unintentional, because

archived otolith samples DPI supplied for this study were originally collected not for elemental analysis but for an annual age determination survey and samples collected in 2013 were also collected when anglers volunteered their fish. In future, it would be desirable for otoliths to be collected immediately black bream are landed.

5-4-2-3 Potassium

Potassium is also a relatively mobile ion and relatively easily leached out of otoliths by a delay in extraction or washing (Campana, 1999; Elsdon *et al.* 2008). The large coefficient of variation coupled with the marginal significance of the difference between otoliths from black bream from the two estuaries renders any attempts to explain differences only tentative at this stage.

5-4-3 Conclusion

The ESEM technique identified differences in the Sr:Ca ratio in otoliths from black bream from the Gippsland Lakes and Lake Tyers in archived samples 2009-2013 and thereby the salinity and temperature exposure profile for black bream at those sampling times. An inter-estuarine difference in Cl:Ca was also identified, and as a consequence, a possible physiological stress response to capture and handling or post-mortem modulation of otolith samples. The conclusion was that black bream from the Gippsland Lakes and Lake Tyers differed in their otolith composition and so it is unlikely that they moved frequently between the estuaries. Thus the difference in chronic stress noted in Chapters 3 and 4 could be linked to their differing environments.

Chapter 6. Genetic polymorphism

6-1 Introduction

Black bream (*Acanthopagrus butcheri* Munro, Family Sparidae) is a sought after recreational and commercial fish species (Rowland, 1984; Lenanton and Potter, 1987; Kailola *et al.* 1993; Norriss *et al.* 2002; Ferguson and Ye, 2008; Gardner *et al.* 2013). It is found in southern Australian river mouths and estuaries ranging from Myall Lakes in New South Wales around the southern part of the Australian Continent to the Murchison River in Western Australia; it is also found in Tasmanian rivers (Norriss *et al.* 2002). The lack of suitable habitat across the Great Australian Bight makes black bream rare in this region of Australia (Kailola *et al.* 1993), as a consequence isolating Western Australian black bream populations from those of south-eastern Australia (Burridge *et al.* 2004).

6-1-1 Genetic differences

Genetic differences between black bream populations were first studied by allozymes (isozymes), a technique now accepted to be inferior to modern genetic analysis of DNA. In Western Australia, allozyme studies were the first to indicate that black bream in differing estuaries were reproductively isolated (Chaplin *et al.*, 1998) and confined to individual estuaries (Potter and Hyndes, 1999), as expected from a fish usually completing its life cycle in its natal estuary (Potter and Hyndes, 1999; Sarre and Potter, 1999; Potter *et al.*, 2008). The development of primers that discriminated at several loci (Yap *et al.*, 2000) allowed the examination of genetic variation using DNA and showed that black bream in Western Australia had relatively low amounts of genetic polymorphism. This also showed that black bream from nine water bodies in Western Australia were genetically different from those from Gippsland Lakes in Victoria, south-eastern Australia. More recent studies using DNA

have confirmed that, in Western Australia, black bream has a genetic composition that differs among estuaries (Chaplin *et al.*, 1998; Gardner *et al.*, 2013).

In south-eastern Australia, until recently it was thought, based on allozyme studies, that there was genetic homogeneity in black bream from differing estuaries (Farrington *et al.* 2000), but more recent studies based on DNA have shown that the pattern is more complex. Burridge *et al.* (2004), through data from mitochondrial DNA as well as from allozymes and a wider geographic sampling range, proposed that there was a low-level gene flow restricted to adjacent estuaries, consistent with a one-dimensional stepping stone model.

DNA analysis by microsatellite primers (Yap *et al.*, 2000; Jeong *et al.*, 2003; Jeong *et al.*, 2007) as well as mitochondrial primers (Jean *et al.*, 1995) allowed Roberts *et al.* (2009; 2010a,b; 2011a,b) to identify hybridisation between marine yellowfin bream (*Acanthopagrus australis*) and estuarine black bream (*A. butcheri*) in many estuaries of south-eastern Australia. In the Gippsland Lakes, originally thought to be beyond the range of sympatry (southern New South Wales) for these two species, Roberts *et al.* (2011b) found that the *Acanthopagrus* population in estuaries was genotypically complex, but genotype frequencies had been surprisingly stable for the presence of an introgressed fish, indicating that hybrid swarms had persisted for a considerable time. Recent samples had a genetic diversity of 8.2-9.2 alleles per locus and expected heterozygosity (H_e) ranging from 0.66-0.70, through producing little allele differentiation ($F_{ST} = 0.003$) across sampling times of 69, 67, 14 and 10 years (Roberts *et al.* 2011). In New South Wales, 95% of ‘black bream’ in estuaries surveyed were genetically either *A. australis* or hybrids with *A. butcheri* (Roberts *et al.*, 2010a).

In south-eastern Australia, the formation of stable hybrid swarms within the region of sympatry were particularly dominant in estuaries characterised by prolonged entrance closure, which was thought to trap the usually transient *A. australis* within such estuaries and

therefore enable the two species to spawn simultaneously (Rowland, 1984; Roberts *et al.* 2010, 2011; Ochwada-Doyle *et al.* 2012). The Gippsland Lakes, with its permanent entrance, is beyond the normal region of sympatry but contains stable hybrid swarms (Roberts *et al.* 2011); the adjacent Lake Tyers, with a seasonally opened entrance, contains *Acanthopagrus* species of unknown genotype, but with less frequent access to the ocean and so the genetic composition of its population might be expected to be different. Juvenile hybrids were more genetically similar to pure *A. butcheri* in estuaries mostly closed whereas they were more genetically similar to pure *A. australis* in estuaries permanently open in NSW (Roberts *et al.*, 2011b).

6-1-2 Genetic differences and stress response

In fish, genetic differences may affect the cortisol response between and within taxa when exposed to the same stressor, thus creating uncertainty as to whether there is an equal capacity to respond to stress (Barton *et al.* 2002). Such differences occur not only among fish species (Barton and Iwama, 1991; Vijayan and Moon, 1994, Ruane *et al.*, 1999; Barton 2000; Barton *et al.* 2002) and strains or stock within a species (Woodward and Strange, 1987; Iwama *et al.* 1992; Barton *et al.* 2002) but also within the same population (Pottinger *et al.* 1992; Barton *et al.* 2002). Genetic variation between populations (genetic distance) and within a population (genetic diversity) can be changed by natural processes over extended periods of time (natural selection) (Theodorakis and Wirgin, 2002). Such changes in genetic diversity or structure can also be changed over short time periods (decades, years or days) through failure to reproduce or mortality mortality of sensitive individuals when impacted on by anthropogenic factors such as habitat alteration and fragmentation; changes in physical characteristics of the water (e.g. temperature), and changes in chemical composition of the

water (e.g. increased nutrient loads or the presence of xenobiotic chemicals) (Bickham and Smolen, 1994; Theodorakis and Wirgin, 2002).

6-1-3 Differences between the Gippsland Lakes and Lake Tyers

The Gippsland Lakes are permanently open to the sea, whereas Lake Tyers is only open intermittently. The Gippsland Lakes are fed by a catchment that contains industrial (e.g. power stations) as well as horticultural (e.g. sweetcorn) and agricultural development (e.g. grazing) and various known pollution-related chemicals and events have been recorded in them, e.g. blue-green ‘algal’ blooms. Levels of mercury (probably from old gold-mining operations) and selenium (probably from power stations) in fish in the Gippsland Lakes were just under WHO (World Health Organisation guidelines (Harris *et al.*, 1998). Recent analyses of water quality in the Gippsland Lakes for chlorophyll a, dissolved oxygen, pH, salinity, total nitrogen and total phosphorus at five EPA monitoring sites showed that water quality only met guidelines 60-80% of the time (BMT WBM Pty Ltd, 2010; Ladson and Tillyard, 2011). Lake Tyers, by contrast, is fed by a catchment that is relatively undeveloped and comprises mostly forest. It might therefore be expected that the Gippsland Lakes black bream would be susceptible to more rapid genetic change than Lake Tyers black bream.

6-1-4 Aims

The intention of this study was to compare the genetic composition of black bream inhabiting the permanently open Gippsland Lakes with that inhabiting the adjacent seasonally open Lake Tyers, to find if differences could explain the differences in chronic stress observed between them.

6-2 Methods

6-2-1 Fish samples

Samples of 50 fish from both the Gippsland Lakes and Lake Tyers were collected from anglers during 2012 (**Table 6-1**). The Gippsland Lakes samples were collected in 2012 by donations from the Gippsland Lakes angling club but were mostly collected during a fishing competition (run by the Gippsland Lakes angling club) in August. The Lake Tyers samples were collected shortly afterwards at one time by a research angler (John Harrison) from the start of the Nowa Nowa Arm and Black Snake Bight (map at http://www.gippslandports.vic.gov.au/pdfs/boating/gippslandport_4_1.pdf). Samples of muscle or fin clippings were stored frozen at -20°C in 2 mL microcentrifuge tubes until DNA was extracted in 2013.

6-2-2 DNA extraction

Samples of either muscle or fin clippings were removed individually from the tubes and allowed to thaw briefly on absorbent paper towel. Muscle was used in preference to skin scraped off fin clippings where possible. Aliquots of ~25 mg were used for DNA extraction using a Qiagen DNeasy Blood and Tissue Kit according to the manufacturer's instructions, except that the sample was first ground to a fine powder in liquid nitrogen and the final elution was into two aliquots of 100 µL for fin clippings instead of two aliquots of 200 µL for samples of muscle. The full protocol is detailed below.

The sample (25 mg) was chopped into small pieces and ground to a fine powder in liquid nitrogen using a mortar and pestle before being scraped into a 1.5 mL microcentrifuge tube. Before this thawed, 180µ 1 Buffer ATL and 20 µL proteinase K were added and mixed

Table 6-1: Black bream samples used for DNA extraction. * m=muscle, f=fin clippings.

Sample*	Location (Gippsland Lakes)	Collection date	Sample*	Location (Lake Tyers)	Collection date
GL1m	Mitchell River	25/02/2012	LT1m	Lake Tyers	21/07/2012
GL2m	Gippsland Lakes	25/02/2012	LT2m	Lake Tyers	21/07/2012
GL3m	Mitchell River	25/02/2012	LT3m	Lake Tyers	21/07/2012
GL4m	Mitchell River	25/02/2012	LT4m	Lake Tyers	21/07/2012
GL5m	Mitchell River	25/02/2012	LT5m	Lake Tyers	21/07/2012
GL6m	Mitchell River	25/02/2012	LT6m	Lake Tyers	21/07/2012
GL7m	Mitchell River	25/02/2012	LT7m	Lake Tyers	21/07/2012
GL8m	Mitchell River	25/02/2012	LT8m	Lake Tyers	21/07/2012
GL9m	Mitchell River	25/02/2012	LT9m	Lake Tyers	21/07/2012
GL10m	Mitchell River	25/02/2012	LT10m	Lake Tyers	21/07/2012
GL11f	Mitchell River	25/02/2012	LT11m	Lake Tyers	21/07/2012
GL12m	Mitchell River	25/02/2012	LT12m	Lake Tyers	21/07/2012
GL13m	Mitchell River	25/02/2012	LT13m	Lake Tyers	21/07/2012
GL14f	Tambo River	25/02/2012	LT14m	Lake Tyers	21/07/2012
GL15m	Tambo River	25/02/2012	LT15m	Lake Tyers	21/07/2012
GL16m	Tambo River	25/02/2012	LT16m	Lake Tyers	21/07/2012
GL17m	Tambo River	25/02/2012	LT17m	Lake Tyers	21/07/2012
GL18m	Tambo River	25/02/2012	LT18m	Lake Tyers	21/07/2012
GL19f	Metung	8/04/2012	LT19m	Lake Tyers	21/07/2012
GL20f	Mitchell River	21/07/2012	LT20m	Lake Tyers	21/07/2012
GL21f	Mitchell River	21/07/2012	LT21m	Lake Tyers	21/07/2012
GL22f	Mitchell River	21/07/2012	LT22m	Lake Tyers	21/07/2012
GL23f	Mitchell River	21/07/2012	LT23m	Lake Tyers	21/07/2012
GL24f	Mitchell River	21/07/2012	LT24m	Lake Tyers	21/07/2012
GL25f	Mitchell River	21/07/2012	LT25m	Lake Tyers	21/07/2012

Sample	Location (Gippsland Lakes)	Date	Sample	Location (Lake Tyers)	Date
GL36f	Tambo River	21/07/2012	LT26m	Lake Tyers	21/07/2012
GL26f	Tambo River	21/07/2012	LT27m	Lake Tyers	21/07/2012
GL43f	Tambo River	21/07/2012	LT28m	Lake Tyers	21/07/2012
GL57f	Mitchell River	21/07/2012	LT29m	Lake Tyers	21/07/2012
GL58f	Mitchell River	21/07/2012	LT30m	Lake Tyers	21/07/2012
GL59f	Mitchell River	21/07/2012	LT31m	Lake Tyers	21/07/2012
GL63f	Tambo River	21/07/2012	LT32m	Lake Tyers	21/07/2012
GL65f	Newlands Arm	21/07/2012	LT33m	Lake Tyers	21/07/2012
GL66f	Newlands Arm	21/07/2012	LT34m	Lake Tyers	21/07/2012
GL83f	Tambo River	21/07/2012	LT35m	Lake Tyers	21/07/2012
GL93f	Nicholson River	21/07/2012	LT36m	Lake Tyers	21/07/2012
GL94f	Nicholson River	21/07/2012	LT37m	Lake Tyers	21/07/2012
GL98f	Eagle Point	21/07/2012	LT38m	Lake Tyers	21/07/2012
GL99f	Eagle Point	21/07/2012	LT39m	Lake Tyers	21/07/2012
GL100f	Eagle Point	21/07/2012	LT40m	Lake Tyers	21/07/2012
GL101f	Eagle Point	21/07/2012	LT41m	Lake Tyers	21/07/2012
GL102f	Eagle Point	21/07/2012	LT42m	Lake Tyers	21/07/2012
GL103f	Eagle Point	21/07/2012	LT43m	Lake Tyers	21/07/2012
GL108f	Tambo River	21/07/2012	LT44m	Lake Tyers	21/07/2012
GL118f	Tambo River	21/07/2012	LT45m	Lake Tyers	21/07/2012
GL120f	Nicholson River	21/07/2012	LT46m	Lake Tyers	21/07/2012
GL127f	Gippsland Lakes	21/07/2012	LT47m	Lake Tyers	21/07/2012
GL128f	Gippsland Lakes	21/07/2012	LT48m	Lake Tyers	21/07/2012
GL129f	Gippsland Lakes	21/07/2012	LT49m	Lake Tyers	21/07/2012
GL130f	Gippsland Lakes	21/07/2012	LT50m	Lake Tyers	21/07/2012

by vortexing, followed by overnight incubation at 56°C, which usually resulted in the tissue being completely lysed. The samples were vortexed for 15 s before adding 200 µL Buffer AL, mixed thoroughly by vortexing and incubated at 56°C for 10 min. at 56°C for 10 min. Then 200 µL ethanol (96–100%) was added, mixed thoroughly by vortexing and the samples pipetted into a DNeasy Mini spin column placed in a 2 mL collection tube and centrifuged at 6000 x g for 1 min in a Hettich microcentrifuge to bind the DNA to the column. The flow-through and the collection tube were discarded. The spin column was placed in a new 2 mL collection tube, 500 µL Buffer AW1 added and the spin column was centrifuged for 1 min at 6000 x g. The flow-through and collection tube were discarded. The spin column was placed in a new 2 ml collection tube, 500 µL Buffer AW2 added and centrifuged for 3 min at 20,000 x g. The flow-through and collection tube were discarded. The spin column was transferred to a new 1.5 mL microcentrifuge tube. DNA was eluted by adding 200 µL (100 µL for fin and skin samples) Buffer AE to the centre of the spin column, incubating for 1 min at ambient temperature (22°C) and centrifuging for 1 min at 6000 x g. The elution step was repeated to give 400 µL extracts from muscle and 200 µL from fin clippings.

DNA quantity and quality were assessed by loading 10 µL of each extract with 2 µL of loading dye into a 1.4% agarose gel in 1x TBE [Tris-borate-EDTA: 10.8 g L⁻¹ Trisma base, 5.5 g L⁻¹ boric acid, 4 mL 0.5 M ethylene diamine tetra-acetic acid (EDTA), pH 8.0] at 80–100 V alongside a GeneRuler™ 100 bp Plus ladder, stained with ethidium bromide and imaged using a Bio-Rad Gel Doc system with Quantity One software.

Of the 50 DNA samples extracted from individual fish from each location, 47 were chosen for ISSR using microsatellite primers on the basis of having greater quantity and quality of DNA. Samples GL19, GL24, GL102, LT35, LT36 and LT39 were excluded.

6-2-3 Microsatellite primers

Diversity among 47/50 DNA samples from each location was analysed by PCR with sets of forward and reverse primers used previously for fish, in particular black bream (Table 6-3).

For PCR, Each 25 µL reaction contained: 12.5 µL Promega GoTaq Green Master Mix, 9.5 µL nuclease-free water, 1 µL microsatellite primer (25 µM for pAb2B7, Acs1, Acs16 and Acs21; 12.5 µM for pAb2A5 and pAb2D11) and 2 µL containing 2-5 ng of genomic DNA or sterile nuclease-free water. A G-Storm thermocycler was programmed for each pair of primers as shown in Table 6-3. The entire PCR products were separated by electrophoresis on 1.4% agarose gel in 1x TAE buffer at 80-100 V alongside a GeneRuler™ 100 bp Plus ladder and products recorded as before.

The presence or absence of each amplicon was entered into a binary matrix in the statistical application Minitab Version 16 (www.minitab.com) and samples were grouped by similarity using multivariate analysis (principal components analysis and hierarchical cluster analysis with complete linkage and squared Euclidean distance at similarity=0.05).

Table 6-2: Primer pairs used for microsatellites analysis of DNA from black bream.

Primer pair	Core repeat sequence	Sequences (5' -> 3')	Reference
pAb2A5 For pAb2A5 Rev	(TG) ₁₉	AGTTACTTCTCCAGAGTGGCGC GGCAACAGATAAGCACTGAGCATA	Yap <i>et al.</i> (2000)
pAb2B7 For pAb2B7 Rev	(TG) ₂₄	GGTGCCTGCATTGTTAACGTGT GATCTGCTTCCTTGACTCAGC	Yap <i>et al.</i> (2000)
pAb2D11 For pAb2D11 Rev	(TG) ₁₅	CGGTCCAGTTTCACTCTGATGTT AACTGCCGTCATGCCCTGTT	Yap <i>et al.</i> (2000)
Acs1 For Acs1 Rev	(CA) ₁₃	TTGCAGCAGATGGGTTCAGA <u>GGTCATCTGTATCGACGACT</u>	Jeong <i>et al.</i> (2003)
Acs16 For Acs16 Rev	(CA) ₂₈	ACCAAGGACCCTTGTGAATG GCATGTCTGCAGCAACAGCA	Jeong <i>et al.</i> (2007)
Acs21 For Acs21 Rev	(CA) ₁₂	CGGAAAGGAAGCAGCTTTG AACTGTTAGCCAGGGTCAGC	Jeong <i>et al.</i> (2007)

Table 6-3: Primer pairs and thermocycling conditions used for microsatellites analysis of DNA from black bream.

Primer pair	Tm (°C)	Initial denaturation	No. of cycles	Cycling parameters			Final extension
				Denaturation	Annealing	Extension	
pAb2A5 For pAb2A5 Rev	56.9 55.6	95°C (15 min)	35	95°C (30 s)	54°C (30 s)	72°C (1 min)	72°C (10 min)
pAb2B7 For pAb2B7 Rev	52.9 55.2	95°C (10 min)	35*	94°C (30 s)	61->53°C*(30 s)	72°C (1 min)	72°C (10 min)
pAb2D11 For pAb2D11 Rev	55.6 56.2	95°C (10 min)	35	94°C (30 s)	54°C (30 s)	72°C (1 min)	72°C (10 min)
Acs1 For Acs1 Rev	51.7 51.7	95°C (10 min)	35#	94°C (30 s)	60->52°C# (30 s)	72°C (1 min)	72°C (10 min)
Acs16 For Acs16 Rev	51.7 53.7	95°C (10 min)	35	94°C (30 s)	51°C (30 s)	72°C (1 min)	72°C (10 min)
Acs21 For Acs21 Rev	53.7 53.7	95°C (10 min)	35^	94°C (30 s)	62->54°C^ (30 s)	72°C (1 min)	72°C (10 min)

The annealing temperatures and cycles were modified from those used by the original authors according to the melting temperature (Tm) supplied by Micromon) and the touchdown cycles detailed in the supplement to Roberts *et al.* (2008).

*Touchdown: 5 cycles at 61°C, 59°C, 57°C, 55°C and 53°C followed by 10 cycles at 53°C.

#Touchdown: 5 cycles at 60°C, 58°C, 56°C, 55°C and 52°C followed by 10 cycles at 52°C.

^ Touchdown: 5 cycles at 62°C, 60°C, 58°C, 56°C and 54°C followed by 10 cycles at 54°C.

6-2-4 Mitochondrial primers

Diversity in the mitochondrial DNA among the same 47/50 DNA samples from each location was analysed by PCR-RFLP (restriction fragment length polymorphism). Firstly ~400 bp of the D-loop region was amplified using the primers PW (5'-CAGGGACAAAAATTGTGGGG-3') and PX (5'- TGCAGAAGTGTAGTGATCCC-3') (Jean *et al.*, 1995; Roberts *et al.*, 2009). Each 25 µL reaction contained: 12.5 µL Promega GoTaq Green Master Mix, 9.5 µL nuclease-free water, 1 µL primer PW (10 µM), 1 µL primer PX (10 µM) and 2 µL containing 2-5 ng of genomic DNA or sterile nuclease-free water. A G-Storm thermocycler was programmed for cycling parameters modified from Jean *et al.* (1995) and Roberts *et al.* (2009) by reducing the annealing temperature. The cycling parameters were: initial denaturation at 94°C for 10 min; followed by 35 cycles of: 94°C for 45 s, 52°C for 30 s, 72°C for 1 min 30 s; and final extension at 72°C for 10 min. Five microlitres of the PCR products were separated by electrophoresis on 1.4% agarose gel in 1x TAE (40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA) buffer at 80-100 V alongside a GeneRuler™ 100 bp Plus ladder and products recorded as before.

RFLP analysis was used to discriminate among the PW/PX PCR products from fish from the two estuaries. Two endonucleases, *Alu*I (Fermentas) and *Dde*I (New England Biolabs), were used separately to digest the PW/PX PCR product as in Roberts *et al.* (2009 – online supplement). These endonucleases distinguished between PW/PX amplicons from *A. butcheri* and *A. australis* (yellowfin bream) (Table 6-3, Table 6-4). Each 7 µL digest contained 0.5 µL enzyme, 0.5 µL nuclease-free water, 1 µL 10 x matching buffer and 5 µL PCR product and was digested for 2 h at 37°C. The entire digests were separated by electrophoresis on 1.5% agarose gel in 1x TAE buffer at 80-100 V alongside a GeneRuler™ 100 bp Plus ladder and products recorded as before.

Table 6-4: DNA sequences of mitochondrial control regions for *Acanthopagrus* species from NCBI used to plan RFLP analysis.

Accession No.	Length (bases)	Species	Authors	Title	Date
AF406647	1016	<i>A. australis</i>	Murphy, N.P., Hurt, A.C. and Austin, C.M.	Molecular genetic evidence for a new species of bream of the genus <i>Acanthopagrus</i>	2001
AF406646	978	<i>A. butcheri</i>	Murphy, N.P., Hurt, A.C. and Austin, C.M.	Molecular genetic evidence for a new species of bream of the genus <i>Acanthopagrus</i>	2001
AF410879	299	<i>A. butcheri</i>	Yap, E.S. and Chaplin, J.A.	Population genetic structure of the black bream, <i>Acanthopagrus butcheri</i> , in Western Australia	2002

Table 6-5: Some endonucleases that discriminate between DNA sequences of mitochondrial control regions for *Acanthopagrus* species from NCBI according to Restriction Mapper v3.

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions	
					AF40664 <i>A. australis</i>	AF406646 <i>A. butcheri</i>
<i>AluI</i>	AGCT	4	Blunt	5	261, 554, 958, 982, 1005	-
<i>DdeI</i>	CTNAG	4	Five prime	1	596	-

6-3 Results

6-3-1 DNA extraction

A high molecular weight band was visible in almost all DNA extracts, although bands for some were faint (**Figure 6-1**). Extracts from muscle usually had only a single band whereas those from skin scraped from fin clippings usually also showed a smear (**Figure 6-1, Figure 6-2**).

6-3-2 PCR

The Bio-Rad Gel Doc had a fault at the time Figs 6-3 to 6-5 gels were recorded and the UV illumination is not even, especially at the corners of the gels, but all bands were clearly visible when examined at greater magnification for scoring.

DNA from all samples reacted with all primer pairs, producing 3-13 bands, of which 1-11 were polymorphic (**Figure 6-3, Figure 6-4, Figure 6-5**). The Acs primers produced more bands, and more polymorphic bands, than the pAb2xxx primers.

Both methods of multivariate analysis grouped the DNA samples into three clusters by location of fish collection (**Figure 6-6**): one cluster with only Gippsland Lakes fish, a second with only Lake Tyers fish and a third of mostly Gippsland Lakes and a few Lake Tyers fish. Within the Gippsland Lakes cluster, there was no particular pattern of clustering by the location where the fish were caught.

Gippsland Lakes

Lake Tyers

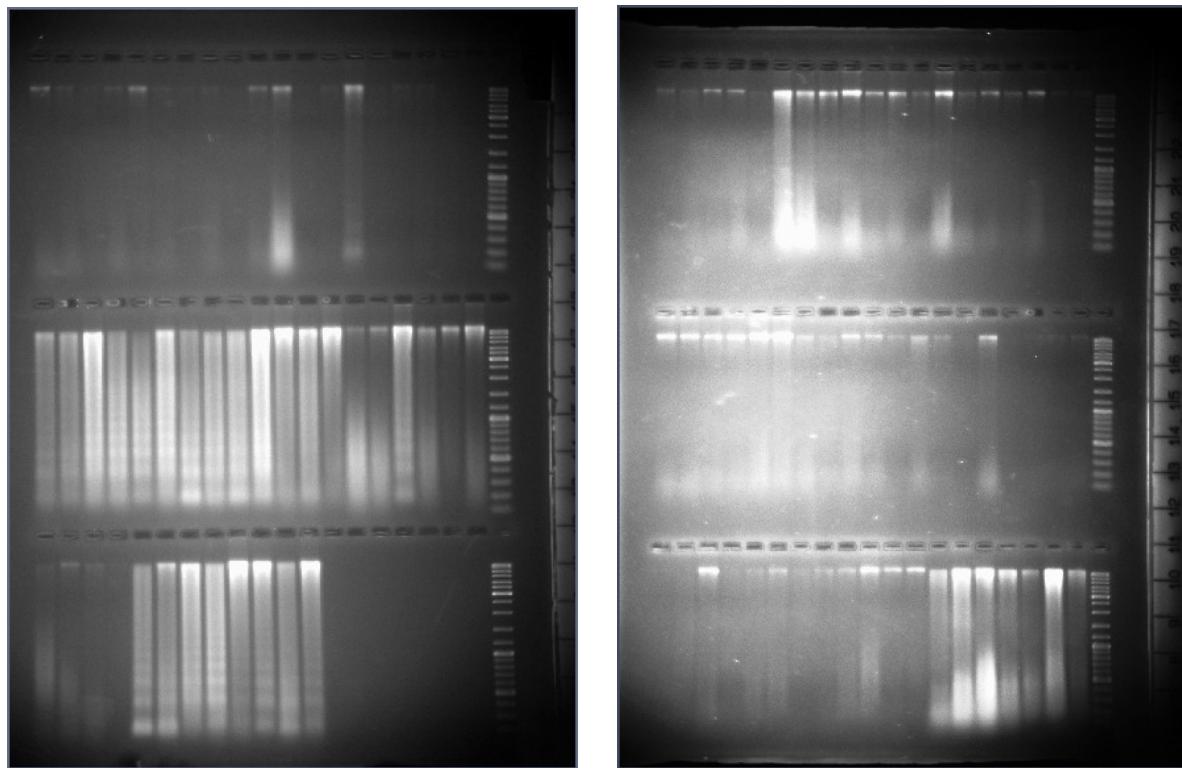


Figure 6-1: Gel electrophoresis of DNA extracts of black bream from (left) Gippsland Lakes and (right) Lake Tyers. Last lane on the right in each row of each gel = molecular weight marker (GeneRuler™ 100 bp Plus ladder), extra bright bands at (top to bottom): 10,000 bp, 3,000 bp, 1,000 bp, 500 bp. Gippsland Lakes samples. Lanes are: top row, left to right: GL1-19; middle row, left to right: GL20-26, 36, 43, 57-59, 63, 65-66, 83, 93-94, 98; bottom row, left to right: GL99-103, 108, 118, 120, 127,-130. Lake Tyers samples. Lanes are: top row, left to right: LT1-19; middle row, left to right: LT20-38; bottom row, left to right: LT39-50, LT9-10, 45-49.

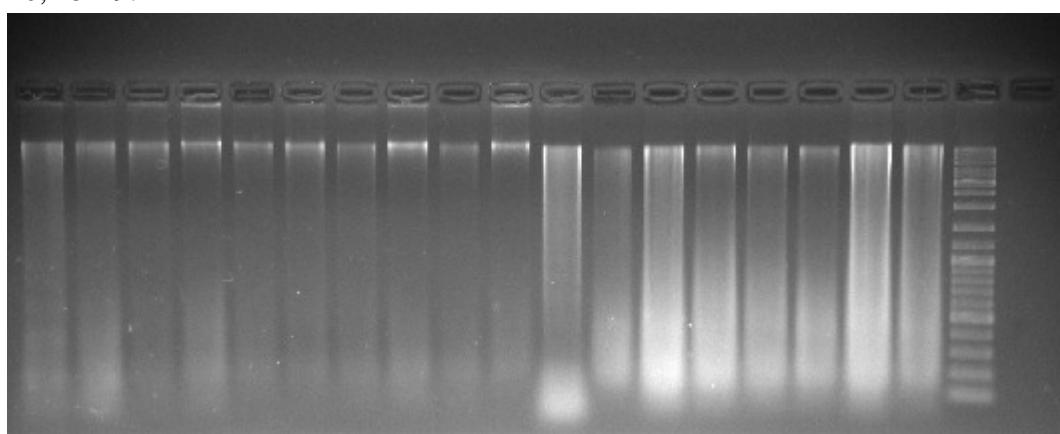
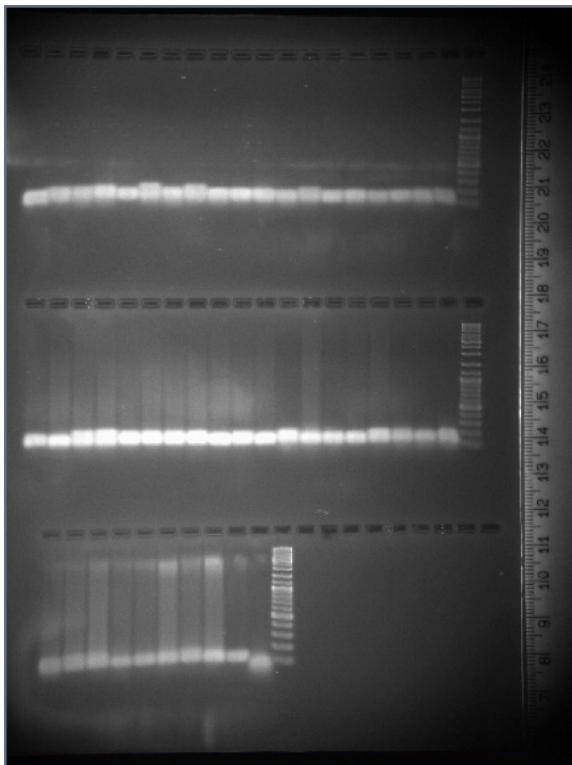


Figure 6-2: Gel electrophoresis of DNA extracts from muscle and fin clippings of black bream. Lanes are (left to right): Lake Tyers (LT 6-15) from muscle, Gippsland Lakes samples (GL8-10, 45-49), molecular weight marker ((GeneRuler™ 100 bp Plus ladder), extra bright bands at (top to bottom): 10,000 bp, 3,000 bp, 1,000 bp, 500 bp.

Gippsland Lakes



Lake Tyers

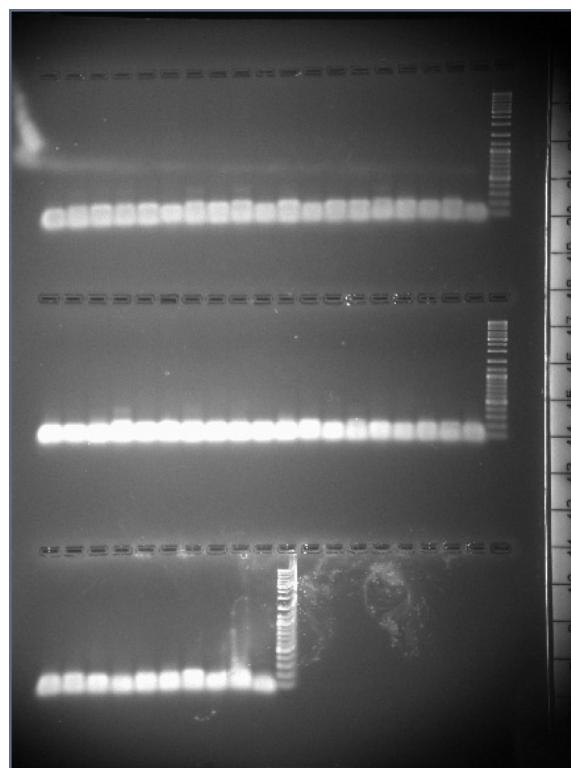
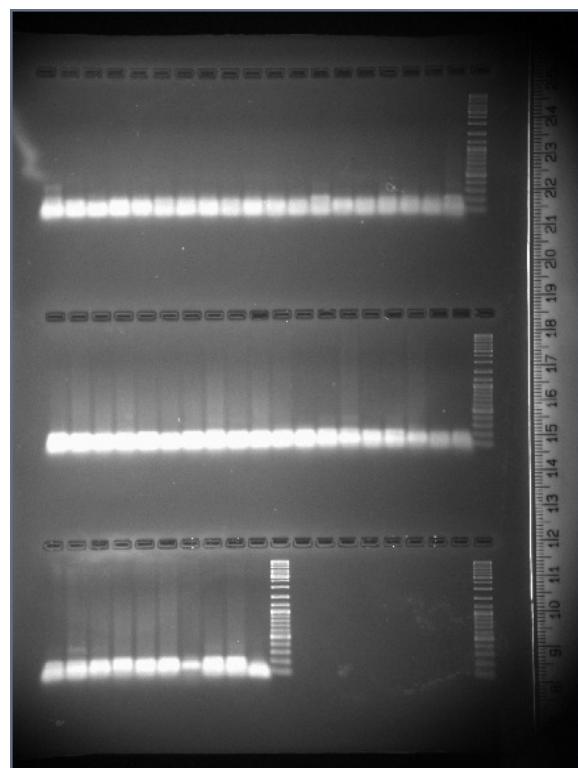
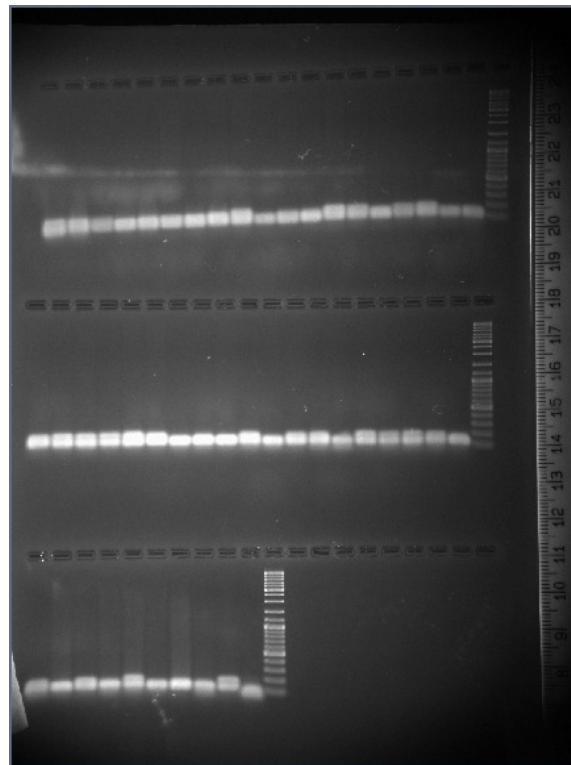
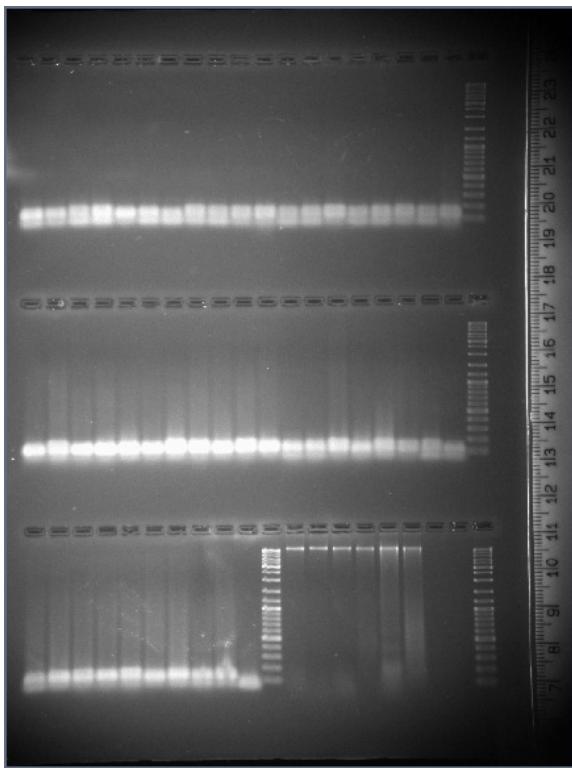


Figure 6-3: Gel electrophoresis of PCR products from (top) primer pair pAb2A5, (bottom) primer pair pAb2B7. Rightmost lane in each row = GeneRuler™ 100 bp Plus ladder. Gippsland Lakes samples (left to right): top row: GL1-18, 20; middle row: GL21-23, 25-26, 36, 43, 57-59, 63, 65-66, 83, 93-94, 98-100; bottom row: GL101, 103, 108, 118, 120, 127-130, blank (no DNA). Lake Tyers samples (left to right): top row: LT1-19; middle row: LT20-34, 37-38, 40-41; bottom row: LT42-50, blank (no DNA).

Gippsland Lakes



Lake Tyers

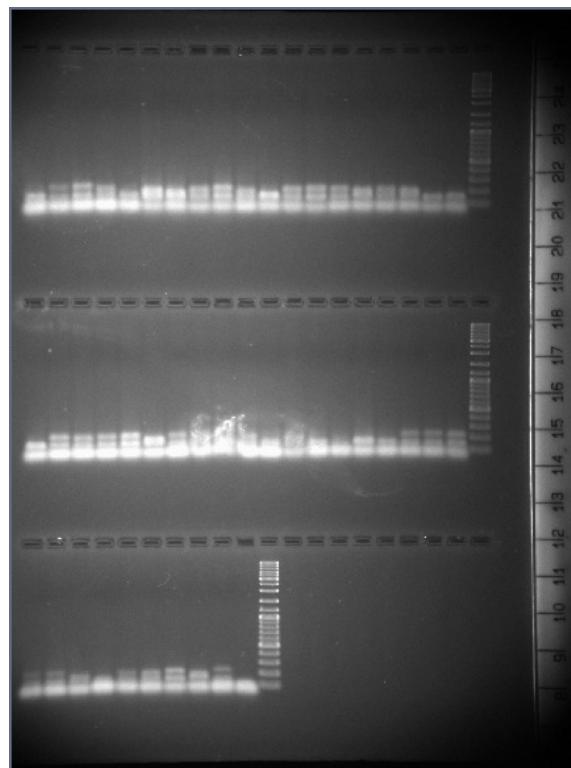
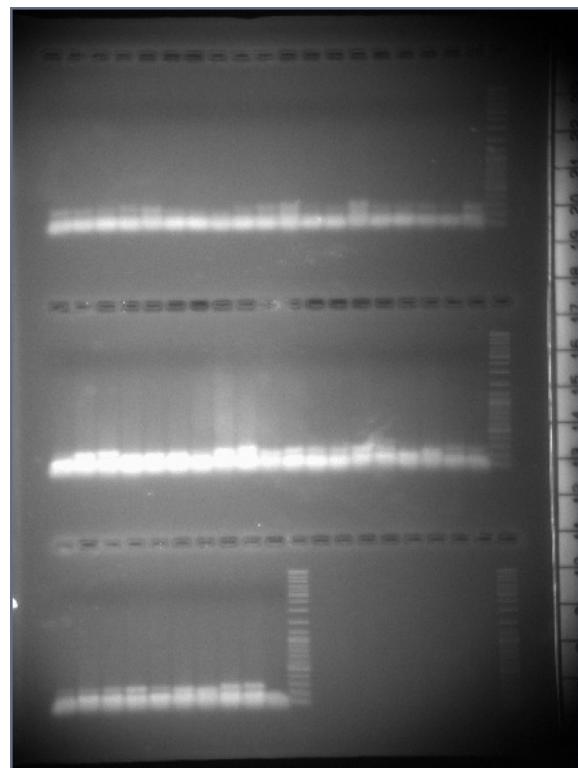
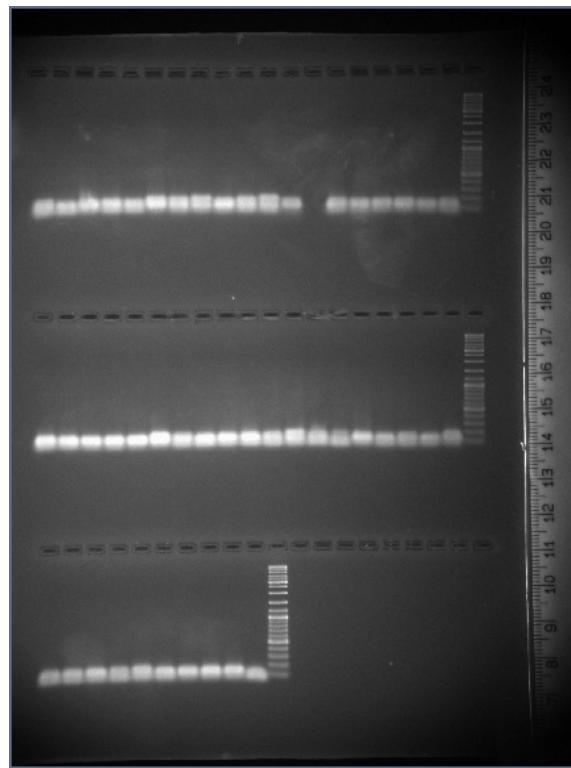
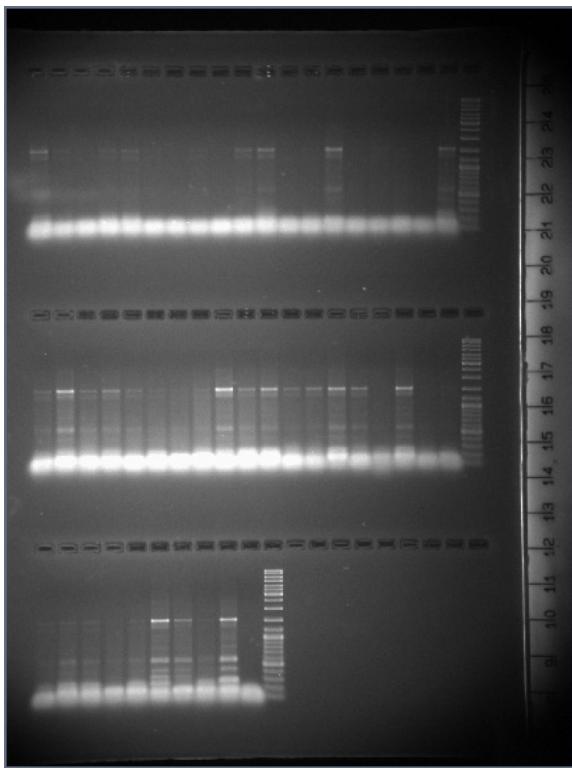


Figure 6-4: Gel electrophoresis of PCR products from (top) primer pair pAb2D11, (bottom) primer pair Acs1. Rightmost lane in each row = GeneRuler™ 100 bp Plus ladder. Gippsland Lakes samples (left to right): top row: GL1-18, 20; middle row: GL21-23, 25-26, 36, 43, 57-59, 63, 65-66, 83, 93-94, 98-100; bottom row: GL101, 103, 108, 118, 120, 127-130, blank (no DNA). Lake Tyers samples (left to right): top row: LT1-19; middle row: LT20-34, 37-38, 40-41; bottom row: LT42-50, blank (no DNA).

Gippsland Lakes



Lake Tyers

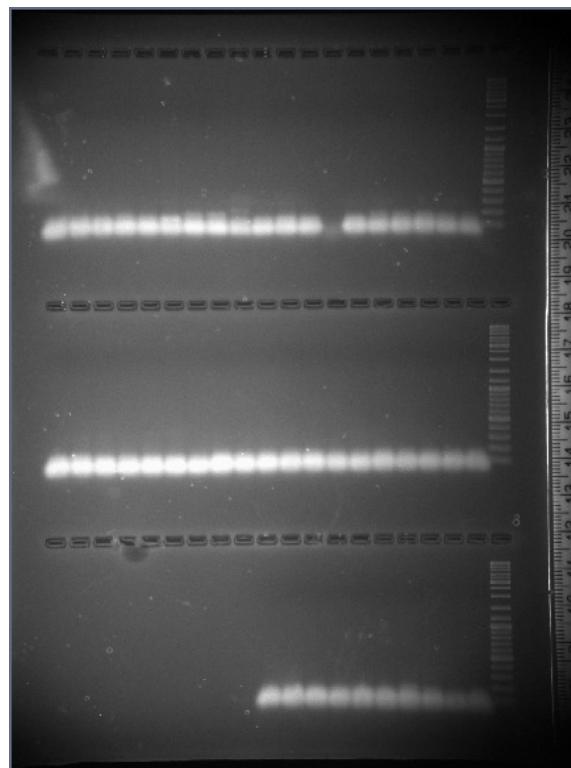
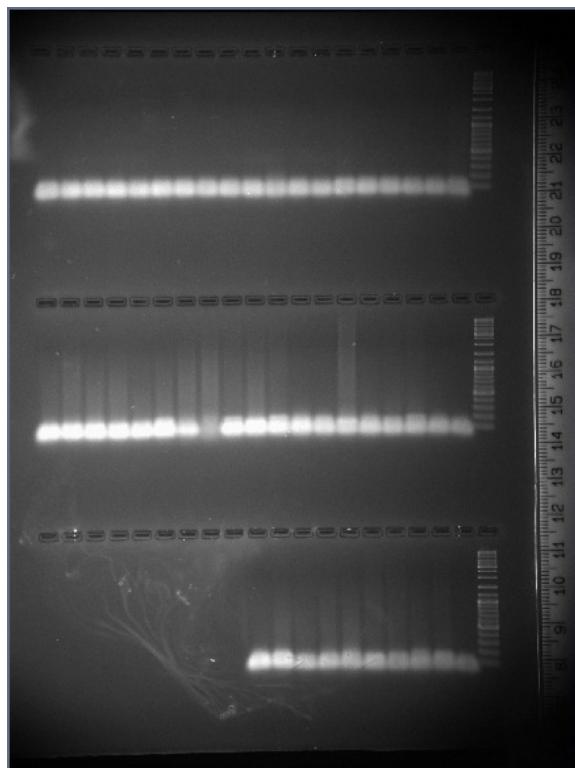
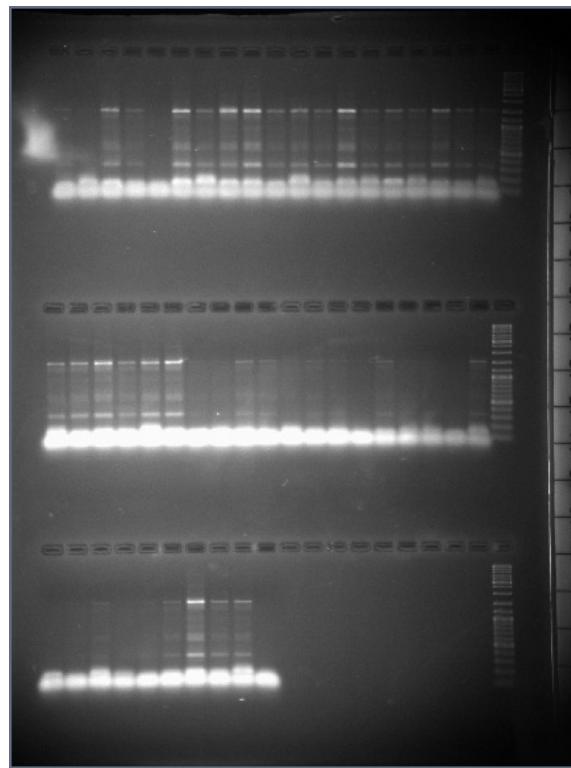


Figure 6-5: Gel electrophoresis of PCR products from (top) primer pair Acs16, (bottom) primer pair Acs21. Rightmost lane in each row = GeneRuler™ 100 bp Plus ladder. Gippsland Lakes samples (left to right): top row: GL1-18, 20; middle row: GL21-23, 25-26, 36, 43, 57-59, 63, 65-66, 83, 93-94, 98-100; bottom row: GL101, 103, 108, 118, 120, 127-130, blank (no DNA). Lake Tyers samples (left to right): top row: LT1-19; middle row: LT20-34, 37-38, 40-41; bottom row: LT42-50, blank (no DNA).

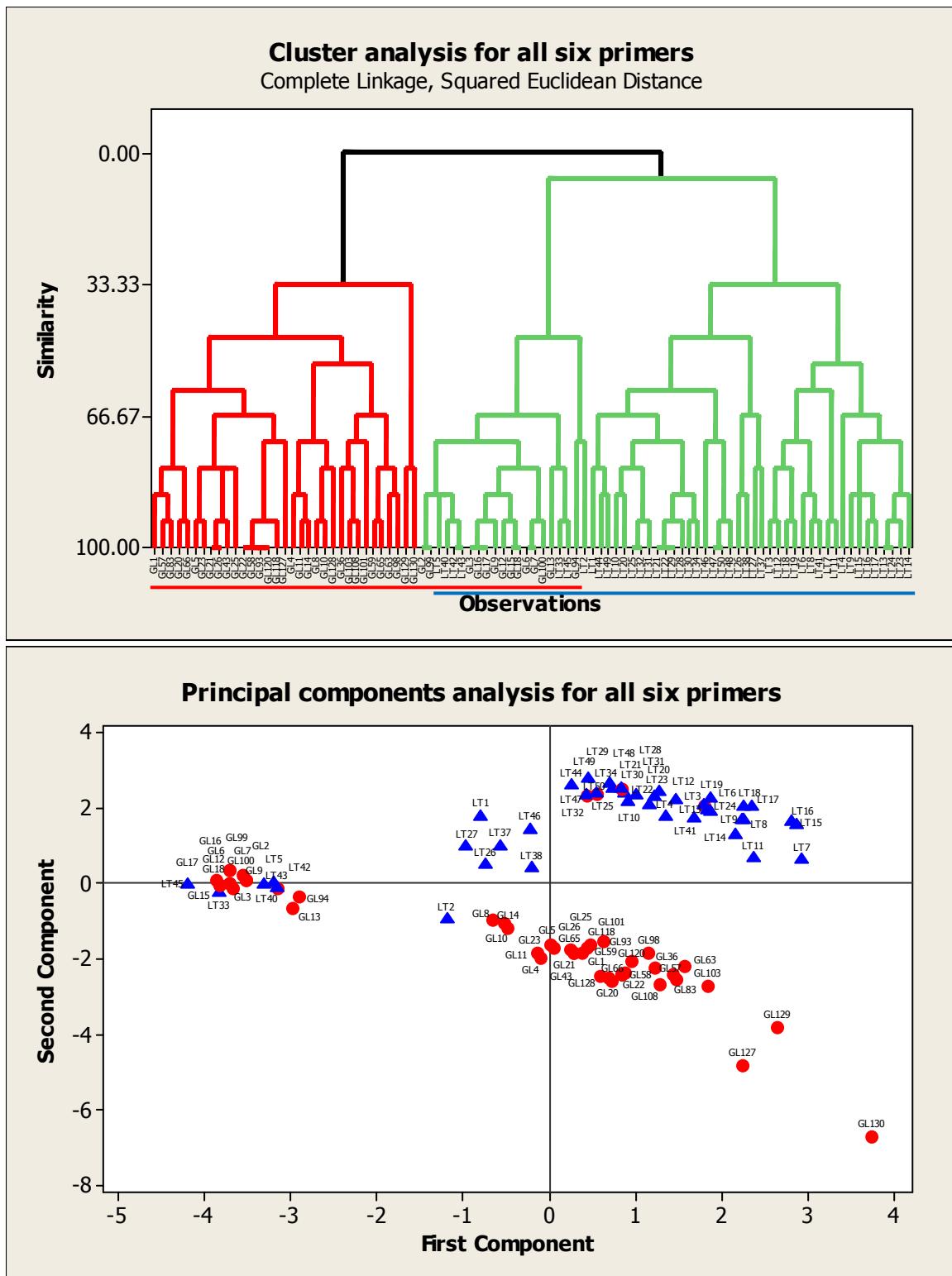


Figure 6-6: Relationships among DNA samples from black bream as shown by multivariate analysis in Minitab™16. Top: hierarchical cluster analysis at similarity=0.05 with complete linkage and squared Euclidean distance. Bottom: principal components analysis using correlation matrix analysis. Main clusters in cluster analysis are shown by horizontal lines under the dendrogram that match the colours of the symbols in the principal components analysis.

6-3-3 Mitochondrial analysis

DNA from all samples reacted with the PW/PX primer pair, producing a single band of ~480 bp (**Figure 6-7**).

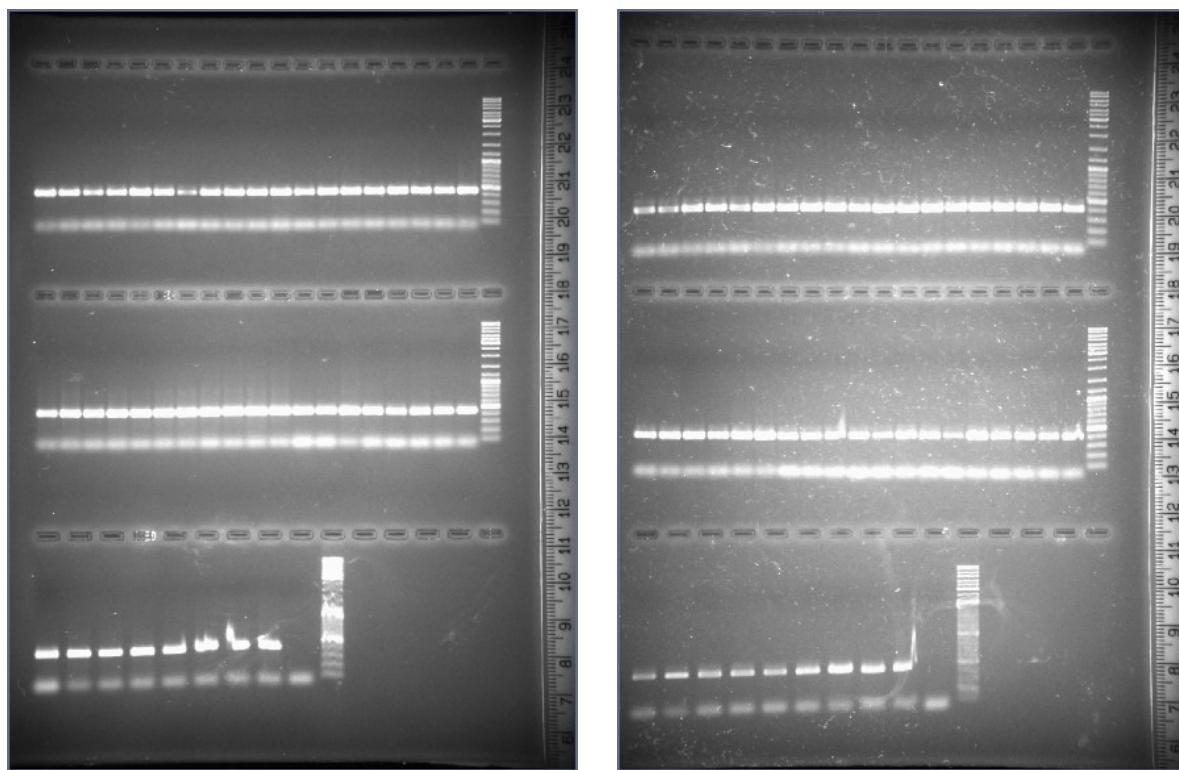


Figure 6-7: Gel electrophoresis of PCR products from black bream with primer pair PW/PX. Left gels: Gippsland Lakes; right gels: Lake Tyers. Rightmost lane in each row = GeneRuler™ 100 bp Plus ladder. Gippsland Lakes samples (left to right): top row: GL1-18, 20; middle row: GL21-23, 25-26, 36, 43, 57-59, 63, 65-66, 83, 93-94, 98-100; bottom row: GL101, 103, 108, 118, 120, 127-130, blank (no DNA). Lake Tyers samples (left to right): top row: LT1-19; middle row: LT20-34, 37-38, 40-41; bottom row: LT42-50, blank (no DNA).

*Alu*I did not digest any of the PW/PX PCR products in fish from either Gippsland Lakes or Lake Tyers (**Figure 6-8**).

*Dde*I digested 38/47 Gippsland Lakes PW/PX products into two fragments (~300 bp and ~180 bp) but did not digest those from the remaining 9/47 (GL6, GL21, GL58, GL65, GL83,

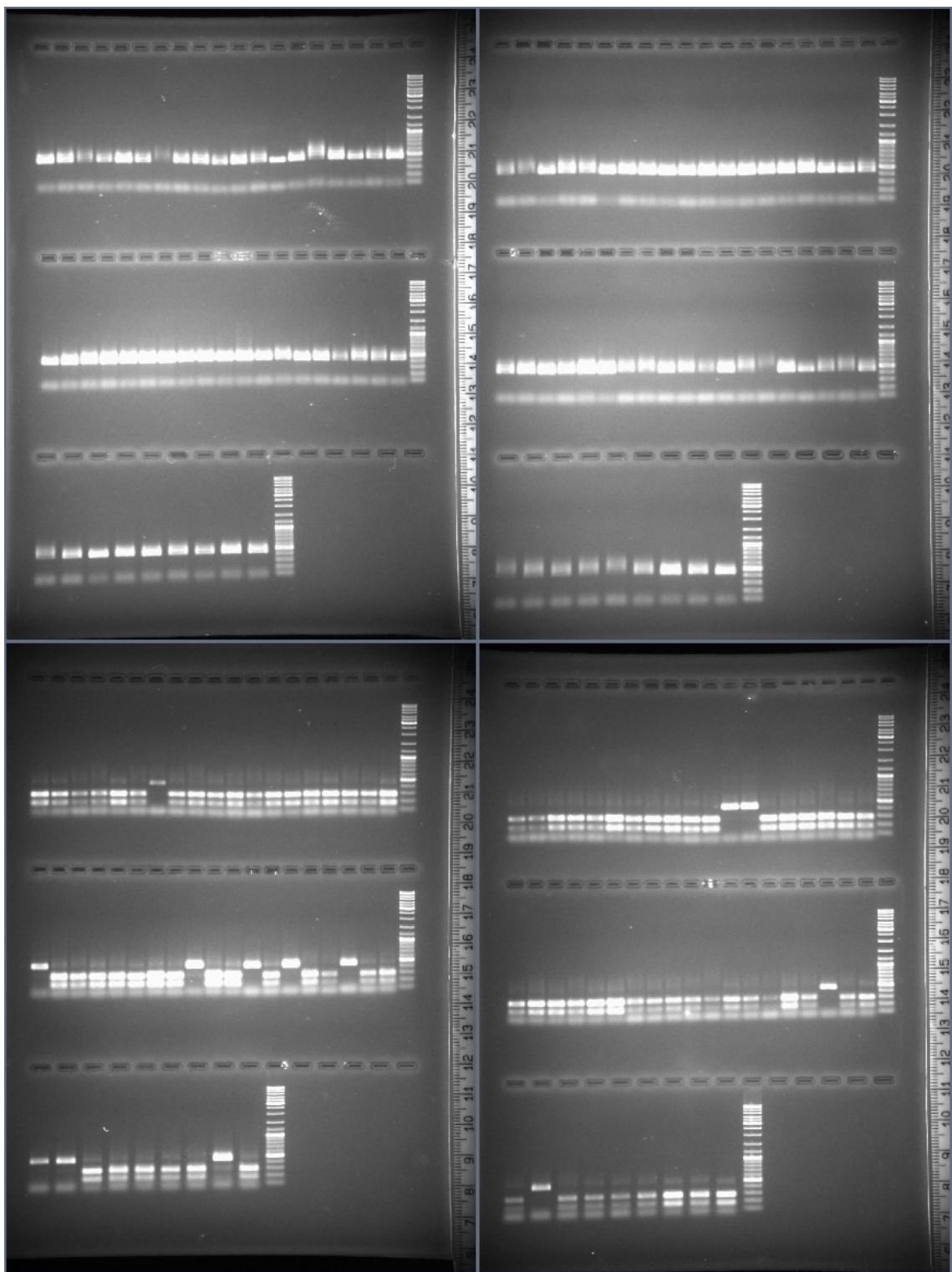


Figure 6-8: Gel electrophoresis of RFLP of PW/PX PCR products from black bream with (top) *Alu*I and (bottom) *Dde*I.. Left gels: Gippsland Lakes; right gels: Lake Tyers. Rightmost lane in each row = GeneRuler™ 100 bp Plus ladder. Gippsland Lakes samples (left to right): top row: GL1-18, 20; middle row: GL21-23, 25-26, 36, 43, 57-59, 63, 65-66, 83, 93-94, 98-100; bottom row: GL101, 103, 108, 118, 120, 127-130. Lake Tyers samples (left to right): top row: LT1-19; middle row: LT20-34, 37-38, 40-41; bottom row: LT42-50.

GL98, GL101, GL103, GL129) (**Figure 6-8**). Eight of the nine Gippsland Lakes fish PCR products that were not digested were all from the Lake King area (listed nearest to furthest from the permanent opening): 1/2 Newlands Arm, 3/6 Eagle Point, 3/21 Mitchell River, 1/12 Tambo River, and no further information was available for the remaining sample.

Likewise, *DdeI* digested 43/47 Lake Tyers PW/PX products into two fragments but did not digest those from the remaining 4/47 (LT12, LT13, LT38, LT43). Samples with lower numbers came from the start of the eastern Nowa Nowa Arm and samples with higher numbers from Black Snake Bight. The number and proportion of undigested PCR products from Lake Tyers fish were half that from Gippsland Lakes fish but there was no significant difference in proportions between fish sources ($\chi^2 = 1.528$, $p=0.216$).

The GL and LT samples in **Figure 6-8** showing lack of *DdeI* digestion were mostly clustered within the main groups of Gippsland Lakes and Lake Tyers samples respectively in the microsatellite analysis (**Figure 6-6**).

6-4 Discussion

In the microsatellite analysis, the differences in ‘black bream’ between those caught in the Gippsland Lakes and those caught in Lake Tyers suggests that genetic differences could have contributed to the difference in chronic stress observed between fish from these systems. In the mitochondrial analysis, the lack of digestion of only 12/94 PW/PX amplicons with *DdeI* suggests that relatively few ‘black bream’ have maternal mitochondrial sequences characteristic of *A. butcheri* and that most are part of a hybrid swarm with *A. australis*.

6-4-1 Genetic mixing of black bream populations

The genetic differences between Gippsland Lakes and Lake Tyers ‘black bream’ are consistent with what would be expected when comparing a permanently open versus a seasonally open estuary. Any coastal movement of black bream (and consequently chances for interbreeding between these estuaries) would occur only in wet seasons in the seasonally opened estuary and would not occur on every occasion. However, migrating fish trapped by prolonged entrance closure in Lake Tyers may have extended periods of spawning in the estuary compared with an open estuary (Roberts *et al.*, 2009; Roberts *et al.*, 2010a). This could explain the separate main clusters containing Gippsland Lakes and Lake Tyers fish respectively. The cluster containing a mix of Gippsland Lakes and Lake Tyers fish is possibly a consequence of a relatively recent flooding event in either or both the Gippsland Lakes and Lake Tyers, when both entrances were open simultaneously, allowing passage of fish and consequent interbreeding by a fraction of the stock in each estuary. Finally, fish movement as a consequence of feeding or avoiding predators offers an explanation for there being no pattern of clustering within Gippsland Lakes fish sourced from different locations within the lakes.

The Gippsland Lakes offers the largest area in Victoria of suitable habitat for black bream (MacDonald, 1997b), where fish distribution closely relates to seasonal fluctuations in amount of freshwater inflow, and the physical characteristics of the estuarine system (Coutin *et al.*, 1997). For example, during low rainfall periods resulting in low river flows and high salinity levels, black bream are found throughout the lakes and their tributary rivers (Coutin *et al.*, 1997). Alternatively, during periods of flooding, black bream are found around Reeves Channel and the Cunningham Arm at the extreme eastern end of the system, and are located near the permanent entrance to the sea (Coutin *et al.*, 1997). In a telemetric study by Hindell *et al.* (2008), black bream were found to move on average 30 km.d⁻¹ in their tributary rivers and 8.7 km d⁻¹ within the lakes - up to 2600 km in a year. During the year, there was also relocation to and from rivers, river entrances and the lakes (Hindell *et al.*, 2008). Therefore black bream were amply able for to swim the 15 km between the entrances to the Gippsland Lakes and Lake Tyers and the limiting factor is likely to be the incidence of sufficiently high rainfall to open the entrance to Lake Tyers and flush fish out of the Gippsland Lakes. This in itself is unlikely to lead to interbreeding, as recruitment in black bream is most frequent during periods of low rainfall (Harris *et al.*, 1998; Ladson and Tilleard, 2011) and may explain why the incidence of hybridisation with *A. australis* is greater in seasonally closed than open estuaries, as discussed below.

6-4-2 Hybridisation with *Acanthopagrus australis*

The infrequent occurrence (8.5%) of *DdeI* digestion of the PW/PX amplicon of the mitochondrial D-loop from 'black bream' suggests that pure black bream with the characteristic maternal mitochondrial sequence were relatively rare. The lack of the expected cleavage of the *A. australis* amplicons by *AluI* is most likely to be lack of activity in the enzyme and the RFLP should be repeated. The scarcity of the black bream mitochondrial

type is similar to that seen in five NSW lakes, where estimates of pure *A. butcheri* based on eight microsatellite loci ranged from 1-21% at a q level of 0.05, to 1-51% at a q value of 0.20 (Roberts *et al.*, 2010a). This fits with the proposal that, in intermittently closed and open estuaries of south-eastern Australia and within the region of sympatry of *A. butcheri* and *A. australis* there is hybridisation of the two species, forming stable hybrid swarms (Roberts *et al.*, 2010a; Roberts *et al.*, 2010b; Roberts *et al.*, 2011b; Ochwada-Doyle *et al.*, 2012).

Furthermore, the estimate of pure *A. australis* was 0-1% in the seasonally open lagoons but 66-87% in lagoons permanently open to the sea, suggesting that hybrids were much more common in lagoons normally closed (such as Lake Tyers), though this difference was not seen in this study. Roberts *et al.* (2010a) suggested that hybrid swarms are particularly dominant in estuaries characterised by prolonged entrance closure, as is Lake Tyers, possibly trapping the usually transient *A. australis* and enabling the two species to spawn simultaneously. By contrast, the open entrance of the Gippsland Lakes would allow more *A. australis* to enter but also more to leave and so minimise the occurrence of simultaneous spawning and hybridisation (Roberts *et al.*, 2010a). As the gametes from both species are compatible (Rowland, 1984; Roberts *et al.*, 2010b), this would produce first-generation hybrids, which by back-crossing, particularly with the (at least initially) majority *A. butcheri*, would lead to later-generation hybrid swarms similar to those seen in lakes in NSW (Roberts *et al.*, 2010a; Ochwada-Doyle *et al.*, 2012).

It is possible that the infrequent appearance 250 km south of its normal range by *A. australis* may on occasions coincide with the seasonal opening of Lake Tyers, allowing its entrance and subsequent entrapment. By contrast, since 1889, the permanent channel into the Gippsland Lakes has allowed unimpeded access for any *A. australis*, resulting in the domination by hybrid swarms as in three of the five NSW lagoons (Roberts *et al.* 2011). Due to the close proximity of the Gippsland Lakes and Lake Tyers catchments, any seasonal

weather event would simultaneously impact on both, creating an exit into the sea at Lake Tyers and a flushing effect out to sea in the Gippsland Lakes, allowing movement of fish between both systems. Accordingly, hybrid fish from the Gippsland Lakes would have access to Lake Tyers and subsequently be trapped following its closure. In this situation, the Gippsland Lakes may act as a permanent collecting station for the vagrant *A. australis* and then dispersing hybrids to adjacent estuaries during periods of high rainfall. Here, any inter-estuarine genetic differences would be influenced by the frequency of entrance opening.

The relative scarcity of the mitochondrial type of *A. butcheri* suggests dominance by hybrids of male *A. butcheri* with female *A. australis* or their female offspring. In theory, mitochondria should be inherited from the maternal parent, which would be exclusively *A. butcheri* if the invading *A. australis* were male, *A. australis* if the invading *A. australis* were female, or a mixture proportional to the sex of the invading *A. australis*. From the low frequency of *A. butcheri* mitochondrial loci, logically most of the invading *A. australis* should have been female, or the ensuing male hybrids preferably back-crossed with *A. australis* females, leading to a dominance of *A. australis* mitochondrial types in the population, or the female first-generation hybrids from *A. butcheri* were sterile, which seems unlikely (Rowland, 1984; Roberts *et al.*, 2010b). Over several generations, the mitochondrial type of *A. butcheri* would decline but stabilise at a low level. In this regard, Roberts *et al.* (2010a) found a dominance of *A. australis* mitochondrial DNA in ‘black bream’ in three NSW lakes more open to the sea and a dominance of *A. butcheri* mitochondrial DNA in two lakes mostly closed, suggesting the possibility of genetic drift depending on the environment. This does not seem to have occurred in the Gippsland Lakes and Lake Tyers, where mitochondrial types were almost 90% in favour of *A. australis*. This could, in theory, lead to the extinction of pure *A. butcheri*, as suggested by Roberts *et al.* (2011a). The genetics of ‘black bream’ in other estuaries should be similarly studied to see if there exists a pure form of *A. butcheri* and what, if any,

steps should be taken to conserve the species. The alternative is to consider that *A. butcheri* and *A. australis* are one interbreeding population (i.e. a species) for all conservation purposes and to conserve them as ‘one cohesive hybrid complex’ (Ochwada-Doyle *et al.*, 2012).

6-4-3 Sex Inversion

In genetic studies, the use of the maternally inherited mitochondrial DNA to trace ancestry may be confounded if there is frequent sex inversion. Rowland & Snape (1994) studied the incidence of ovotestes in black bream and found that 8% of their specimens displayed ovotestes but that the ovarian component was non-functional. They sampled 52 black bream from the Gippsland Lakes and found no evidence of ovotestes. By contrast, Norriss *et al.* (2002) sampled 40 Gippsland Lakes black bream and found ovotestes in all samples, but ovotestis functionality was not indicated. In Chapter 3, 26% of the black bream sourced from both the Gippsland Lakes and Lake Tyers had ovotestes, though their functionality was not tested. Further research is required to explain the appearance of ovotestes in Gippsland Lakes black bream.

Sex reversal has been identified in fish exposed to organochlorine pesticides, polychlorinated biphenyls (PCBs), heavy metals, pharmaceuticals and surfactants (Hinck *et al.* 2009). Contaminants found in the axial muscle and livers of Gippsland Lakes *Acanthopagrus* species include mercury and insecticides, in particular organochlorine compounds (DDE - a by-product of DDT decomposition - was most frequently detected) and compounds of the cyclodiene group (aldrin, dieldrin, heptachlor, heptachlor epoxide, and endosulfan) (Fabris *et al.*, 1999). There is a possibility, therefore, that mitochondrial DNA analysis of black bream may be confounded by sex inversion when fish are sampled from the Gippsland Lakes, in particular, at those locations adjoining significant urban development, though this would be unlikely to apply in fish from Lake Tyers.

Conclusion

This study has shown for the first time that the population of ‘black bream’ that inhabits the Gippsland Lakes is genetically different from that inhabiting Lake Tyers. This genetic difference is likely to affect fish cortisol response to certain stressors and ultimately lead to other indicators of chronic stress. Several contaminants are known to load *Acanthopagrus* species in the Gippsland Lakes and this potentially creates an inter-estuarine difference that may also affect fish genetics. Contaminants also directly affect the cortisol response in fish. The demonstration that the ‘black bream’ population in both estuaries is dominated by a mitochondrial type characteristic of yellowfin bream suggests that hybridisation with *A. australis* has produced a hybrid swarm in which pure *A. butcheri* is relatively rare, but that this is the same in both estuaries. The presence of hybrids is not correlated with and does not negate the microsatellite differences between ‘black bream’ from the two estuaries, which could contribute to the differences in chronic stress between fish from the Gippsland Lakes and Lake Tyers.

Chapter 7. Overview and Conclusion

The main intention of this study was to identify the suitability of black bream (*Acanthopagrus butcheri*) to be a valued ecological monitoring tool (bioindicator) to monitor the environmental health of the Gippsland Lakes. An enabling intention was to find if black bream showed symptoms of chronic stress in the Gippsland Lakes relative to Lake Tyers and to find if environmental or genetic factors could explain the differences. These two estuarine lakes were chosen for their contrasting character: the Gippsland Lakes are open to the sea at all times and their catchment includes well developed industry and agriculture, whereas Lake Tyers is only open intermittently to the sea and its catchment is mostly undeveloped forest.

A custom-made channel apparatus was devised for a stimulus-response behavioural test for acute stress that provided accurate predictions of proportional mortality in black bream over a longer period. The apparatus was easy and cheap to make and simple and low-technology to use, making it suitable for use in undeveloped as well as developed countries. This leads to the following recommendations:

Recommendation 1: that the stimulus--response test in the channel apparatus be tested with other fish species to compare their responses to acute stress caused by simulated catch-and-release (air exposures) and that serum cortisol level be measured in parallel.

This study showed for the first time that repeated 30 s air exposures, such as could be experienced by undersized black bream during heavy fishing, significantly increased response time in the stimulus-response test and accurately predicted mortality of up to 50% of fish for up to 2 months after release. For fisheries management, this shows that the catch-and-release policy needs more long-term research than previously in order to predict accurately its effects on black bream. This leads to the following recommendation:

Recommendation 3: that the impact of catch-and-release on black bream be evaluated short-term by the stimulus-response test and long-term by tagging fish before release and monitoring their long-term survival and health.

In the stimulus-response test, black bream from the Gippsland Lakes had a lesser proportion of fish that took <1 min to seek cover and a greater proportion of fish that took >30 min to do so, suggesting that the Gippsland Lakes fish suffered greater chronic stress than those from Lake Tyers, though indices such as the HAI, SSI, HSI and GSI, and sex ratio showed no difference between estuaries after fish had been acclimated for over a year. All fish suffered to an extent from enlarged spleens but the proportion with enlarged spleens from the Gippsland Lakes was greater than that from Lake Tyers, again suggesting greater chronic stress. A quarter of fish were of transitional sex, with ovotestes, suggesting that sex reversal was common. Caging fish to change their stocking density had no impact on response time or any index, apart from the SSI at a marginal level of significance. The conclusions were tentative because of lack of adequate replication and the long acclimation time. This leads to the following recommendations:

Recommendation 4: that the stimulus-response test be repeated without caging and with adequate tank replication with black bream from both estuaries acclimated only for a few weeks to see if these results are repeatable and that serum cortisol level be measured in parallel.

Recommendation 5: that the health assessment index be monitored at regular intervals in samples of black bream from the two estuaries so as to compile data on, and then to compare the long-term effects of their environments.

Recommendation 6: that the incidence of ovotestes in black bream be assessed and monitored regularly in samples of black bream from the two estuaries to find if it has changed from the most recent publication in 2000.

Microscopic comparison of hyperplasia in interrenal tissue from anterior kidneys of black bream identified greater chronic stress in black bream from the Gippsland Lakes than Lake Tyers. This was supported by the lesser condition factor and a green bile colour in the gall bladder of the Gippsland Lakes fish, which indicated that at the time of sampling all Lake Tyers black bream were feeding, whereas 70% of Gippsland Lakes black bream were not and this led to their lesser condition. This long-term bioindicator supported the results from the stimulus-response test in concluding for the first time that black bream caught in the Gippsland Lakes were under greater chronic stress than those caught in Lake Tyers. This leads to the following recommendation:

Recommendation 7: that head kidney hyperplasia be monitored at regular intervals in black bream from the two estuaries as a bioindicator of chronic stress and that serum cortisol level be measured in parallel.

Differences in chronic stress between fish caught in the Gippsland Lakes and Lake Tyers suggests that fish reflect the stressors in their environment or that fish from each estuary are genetically different in their perception of, and responses to, stressors and stress events. This rests on the assumption that black bream movement between estuaries is rare. This assumption was tested by analysing otolith composition and genetic polymorphism in black bream from the two estuaries.

Elemental analysis by EDSX in an ESEM was used for the first time to see if black bream from the Gippsland Lakes and Lake Tyers differed in their trace element composition sufficiently to be able to assess their fidelity to their natal estuary. Elements found and their

ratios were very similar, as might be expected from adjacent estuaries in water bodies in the same geological formation, but the greater Sr:Ca ratios in otoliths from Lake Tyers fish suggested greater variability in Lake Tyers than the Gippsland Lakes water quality, as might be expected in a freshwater system that varies widely in the degree of filling and is open to the sea only intermittently, resulting in salt penetration. This variability emphasises the resilience of estuarine species to naturally occurring variability in estuarine environments (Elliott and Quintino, 2007) and is a significant natural stressor impacting on black bream. There was also an inter-estuarine difference in Cl:Ca, with higher ratio values being found in Lake Tyers fish which may be linked to the greater exposure of Gippsland Lakes fish to saline seawater, although it may also be due to physiological stress prior to capture or post-mortem changes leaching Cl from some otolith samples (Campana, 1999). The conclusion was that it was unlikely that fish moved between estuaries. This leads to the following recommendation:

Recommendation8: that regular otolith sampling by DPI continue in order to have these available as an easily stored biomonitor of estuarine water quality.

This study identified for the first time a genetic difference between Gippsland Lakes and Lake Tyers black bream. Therefore, their differing genetics may modulate the chronic stress response, in that Lake Tyers fish may be less sensitive to stressors. Hybridisation of estuarine black bream with coastal yellowfin bream had already been noted in the Gippsland Lakes, with its permanent channel to the sea, but this is the first time that hybrids have been found in Lake Tyers, an estuary mostly closed to the sea. The scarcity of the black bream mitochondrial type in both estuaries suggests that pure black bream is relatively rare and may become endangered in future as it is swamped by hybrids.

Recommendation 9: that the responsible authorities conduct regular monitoring of the genetic polymorphism in *Acanthopagrus* species in both estuaries and that appropriate action be taken to ensure that the frequency of black bream genetics in the population does not decline further.

During this study, observations were made that brought to focus a number of topics associated with black bream and the Gippsland Lakes that require further investigation. The evidence above has indicated that black bream is a good bioindicator: it has responded quantitatively, despite genetic differences, to both acute and chronic stressors that are likely to be encountered within the Gippsland Lakes, and has brought to light topics that require further research. Black bream is therefore a good candidate as a valuable ecological component to assist in the identification and assessment of multiple stressors impacting on the Gippsland Lakes, Lake Tyers and their environs.

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Appendices

Appendix 2

Appendix 2(a): Part One

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Welcome to Minitab, press F1 for help.

Retrieving project from file: 'D:\ANN\TEACHING - PGRES\POSTGRADS\TREVOR RONALDS\TR REVISED THESIS\CH2ACUTESTRESS\TR CH3 EXP1.MPJ'

Data were categorised as: 1: 0-5 min, 2: >5<=10 min, 3: >10<=15 min, 4: >15<=20 min, 5:>20<=25 min, 6: >25<=30 min, 7:>30 min in column Respcat.

Results for: TR Ch3 Exp1 worksheet.XLS

Probability Plot of Respcat

Kolmogorov-Smirnov test for normality p<0.010, so data distribution not normal

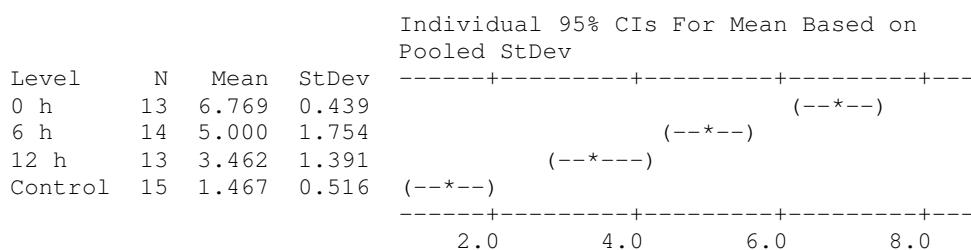
Histogram of Respcat

Too many 1 and 7 categories for normality

One-way ANOVA: Respcat versus Treatment

Source	DF	SS	MS	F	P
Treatment	3	213.27	71.09	52.34	<0.001
Error	51	69.27	1.36		
Total	54	282.55			

S = 1.165 R-Sq = 75.48% R-Sq(adj) = 74.04%



Pooled StDev = 1.165

CIs are Confidence Intervals

Grouping Information Using Tukey Method

Treatment	N	Mean	Grouping
0 h	13	6.769	A
6 h	14	5.000	B
12 h	13	3.462	C
Control	15	1.467	D

Means that do not share a letter are significantly different.

Probability Plot of RESI1

Kolmogorov-Smirnov test p=0.037 not far away from normality

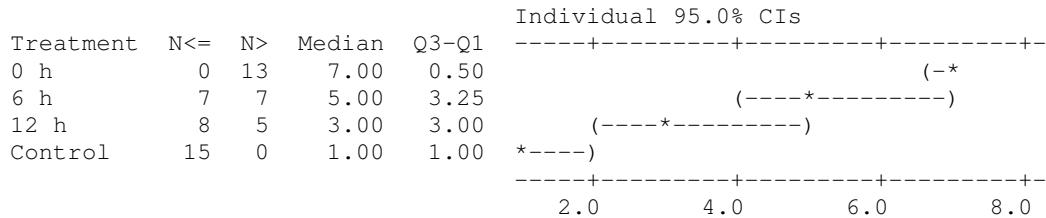
Anderson-Darling test p=0.999 OK for normality
 Ryan-Joiner test p>0.100 OK for normality
 So residuals are acceptably normally distributed and so ANOVA is valid.

Histogram of RESI1

Looks OK for normality except for slightly heavy top tail.

Mood Median Test: Respcat versus Treatment

Mood median test for Respcat
 Chi-Square = 28.47 DF = 3 P = <0.001

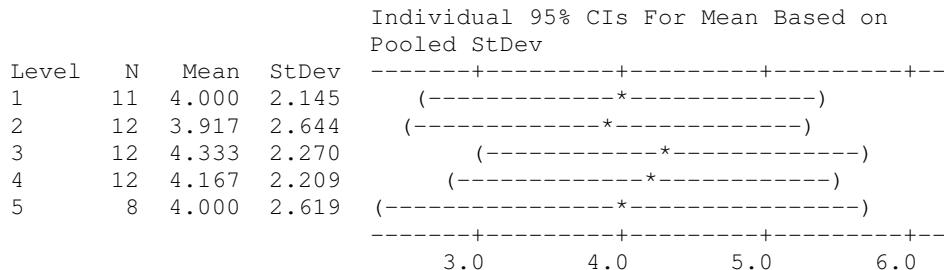


Overall median = 4.00

One-way ANOVA: Respcat versus Day tested

Source	DF	SS	MS	F	P
Day tested	4	1.30	0.32	0.06	0.994
Error	50	281.25	5.63		
Total	54	282.55			

S = 2.372 R-Sq = 0.46% R-Sq(adj) = 0.00%



Pooled StDev = 2.372

Grouping Information Using Tukey Method

Day tested	N	Mean	Grouping
3	12	4.333	A
4	12	4.167	A
5	8	4.000	A
1	11	4.000	A
2	12	3.917	A

Means that do not share a letter are significantly different.
 No significant differences among days.

Mood Median Test: Respcat versus Day tested

Mood median test for Respcat
Chi-Square = 0.47 DF = 4 P = 0.976

Day tested	Individual 95.0% CIs				
	N<=	N>	Median	Q3-Q1	
1	6	5	4.00	4.00	(-----*-----)
2	7	5	3.00	5.75	(-----*-----)
3	6	6	4.50	4.75	(-----*-----)
4	6	6	4.50	4.00	(-----*-----)
5	5	3	3.00	5.00	(-----*-----)
					-----+-----+-----+-----
					1.6 3.2 4.8 6.4

Overall median = 4.00
No significant differences among days.

One-way ANOVA: Respcat versus Time of day tested

Source	DF	SS	MS	F	P
Time of day tested	2	3.53	1.76	0.33	0.721
Error	52	279.02	5.37		
Total	54	282.55			

S = 2.316 R-Sq = 1.25% R-Sq(adj) = 0.00%

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev			
				-----+-----+-----+-----+--	(-----*-----)	(-----*-----)	(-----*-----)
Afternoon	26	4.115	2.251				
Early morning	15	3.733	2.374				
Late morning	14	4.429	2.377				
				-----+-----+-----+-----+--	3.20	4.00	4.80
							5.60

Pooled StDev = 2.316

Grouping Information Using Tukey Method

Time of day tested	N	Mean	Grouping
Late morning	14	4.429	A
Afternoon	26	4.115	A
Early morning	15	3.733	A

Means that do not share a letter are significantly different.
No significant differences among times of day.

Mood Median Test: Respcat versus Time of day tested

Mood median test for Respcat
Chi-Square = 1.67 DF = 2 P = 0.435

Time of day tested	N<=	N>	Median	Q3-Q1	Individual 95.0% CIs		
					-----+-----+-----+-----+--	(-----*-----)	(-----*-----)
Afternoon	14	12	4.00	4.25			
Early morning	10	5	3.00	5.00			
Late morning	6	8	5.00	5.00			
					-----+-----+-----+-----+--	3.0	4.5
							6.0

Overall median = 4.00
No significant differences among times of day.

=====

Repeat ANOVA but with only air-exposure data

One-way ANOVA: Respcat_1 versus Recovery time (h)_1

Source	DF	SS	MS	F	P
Recovery time (h)_1	2	71.24	35.62	20.11	<0.001
Error	37	65.54	1.77		
Total	39	136.77			

S = 1.331 R-Sq = 52.08% R-Sq(adj) = 49.49%

Individual 95% CIs For Mean Based on Pooled StDev					
Level	N	Mean	StDev	-----*	-----*
0	13	6.769	0.439	(-----*)	(-----*)
6	14	5.000	1.754	(-----*)	(-----*)
12	13	3.462	1.391	(-----*)	(-----*)
				3.0	4.5
				6.0	7.5

Pooled StDev = 1.331

Grouping Information Using Tukey Method

Recovery time (h)_1	N	Mean	Grouping
0	13	6.769	A
6	14	5.000	B
12	13	3.462	C

Means that do not share a letter are significantly different.

Probability Plot of RESI3

KS test p=0.039

AD test p=0.067

RJ test p>0.100

So residuals are acceptably normally distributed and so ANOVA is valid.

Regression Analysis: Respcat_1 versus Recovery time (h)_1

The regression equation is
Respcat_1 = 6.73 - 0.276 Recovery time (h)_1

40 cases used, 1 cases contain missing values

Predictor	Coef	SE Coef	T	P
Constant	6.7288	0.3311	20.32	<0.001
Recovery time (h)_1	-0.27564	0.04297	-6.42	<0.001

S = 1.31449 R-Sq = 52.0% R-Sq(adj) = 50.7%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	71.115	71.115	41.16	<0.001
Residual Error	38	65.660	1.728		
Total	39	136.775			

Unusual Observations

Obs	time (h)_1	Repcat_1	Fit	SE Fit	Residual	St Resid
27	6.0	2.000	5.075	0.208	-3.075	-2.37R

R denotes an observation with a large standardized residual.

Probability Plot of RESI5

KS test p=0.017

AD test p=0.051

RJ test p>0.100

So residuals are acceptably normal.

Appendix 2(b): Part Two

16/06/2014 3:18:55 PM

Welcome to Minitab, press F1 for help.

Retrieving project from file: 'D:\ANN\TEACHING - PGRES\POSTGRADS\TREVOR RONALDS\TR REVISED THESIS\CH2ACUTESTRESS\TR CH2 EXP2.MPJ'

Scatterplot of % mortality vs Number of exposures

Regression Analysis: % mortality versus Number of exposures

The regression equation is

% mortality = 12.9 + 18.0 Number of exposures

Predictor	Coef	SE Coef	T	P
Constant	12.917	7.321	1.76	0.220
Number of exposures	17.992	3.913	4.60	0.044

S = 8.74990 R-Sq = 91.4% R-Sq(adj) = 87.0%

Analysis of Variance

Source	DF	SS	MS	F	P
Regression	1	1618.5	1618.5	21.14	0.044
Residual Error	2	153.1	76.6		
Total	3	1771.6			

$$LSD(0.05) = t(0.05, df) * \sqrt{2 * MSE/n}$$

where df=df(error), MSE=mean square error, n=no. replicates/treatment

$$LSD(0.05) = t(0.05, 2) * \sqrt{2 * 76.6 / 15}$$

$$= 4.303 * \sqrt{10.213}$$

$$= 4.303 * 3.196$$

$$= 13.752$$

So data points have to be 13.752 units apart to be significantly different.

So control (0 exposure) is significantly different from all other treatments.

Single exposure is significantly different from 3 exposures but not double exposure.
 Double exposure is not significantly different from either single or triple exposure.
 So if control is a, single exposure is b and double and triple exposure are c.

Probability Plot of RESI4

KS test p>0.150 so residuals normally distributed and regression is valid.

Chi-Square Test: Mortalities, Live

Expected counts are printed below observed counts
 Chi-Square contributions are printed below expected counts

	Mortalities	Live	Total
1	1	15	16
	6.25	9.75	
	4.410	2.827	
2	7	11	18
	7.03	10.97	
	0.000	0.000	
3	9	8	17
	6.64	10.36	
	0.838	0.537	
4	8	5	13
	5.08	7.92	
	1.681	1.078	
Total	25	39	64

Chi-Sq = 11.372, DF = 3, P-Value = 0.010

Correlations: % mortality, Number of exposures

Pearson correlation of % mortality and Number of exposures = 0.956
 P-Value = 0.044

Appendix 3

Table A3-1: Raw data from stimulus-response experiment. Caging treatments: C=control (no caging), LSD=low confinement density, HSD=high confinement density.

Code	Fish source	Treatment	Tank	Cage	Response time (min)
GL(C)	Gippsland Lakes	Control	1	-	0.05
GL(C)	Gippsland Lakes	Control	1	-	0.1
GL(C)	Gippsland Lakes	Control	1	-	8.08
GL(C)	Gippsland Lakes	Control	1	-	17.62
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	17.61
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	0.1
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	30
GL(C)	Gippsland Lakes	Control	1	-	8.08
GL(C)	Gippsland Lakes	Control	1	-	0.05
GL(LSD)	Gippsland Lakes	LSD			6.45
GL(LSD)	Gippsland Lakes	LSD			0.13
GL(LSD)	Gippsland Lakes	LSD			0.2
GL(LSD)	Gippsland Lakes	LSD			8.72
GL(LSD)	Gippsland Lakes	LSD			2.03

GL(LSD)	Gippsland Lakes	LSD			30
GL(HSD)	Gippsland Lakes	HSD	2	2	0.05
GL(HSD)	Gippsland Lakes	HSD			0.05
GL(HSD)	Gippsland Lakes	HSD			6.53
GL(HSD)	Gippsland Lakes	HSD			2.32
GL(HSD)	Gippsland Lakes	HSD			30
GL(HSD)	Gippsland Lakes	HSD			30
GL(HSD)	Gippsland Lakes	HSD			30
GL(HSD)	Gippsland Lakes	HSD			11.27
LT(C)	Lake Tyers	Control			22.25
LT(C)	Lake Tyers	Lake Tyers			7.3
LT(C)	Lake Tyers	Lake Tyers			0.05
LT(C)	Lake Tyers	Lake Tyers			0.05
LT(C)	Lake Tyers	Lake Tyers			30
LT(C)	Lake Tyers	Lake Tyers			0.05
LT(C)	Lake Tyers	Lake Tyers			4.75
LT(C)	Lake Tyers	Lake Tyers			0.22
LT(C)	Lake Tyers	Lake Tyers			0.03
LT(C)	Lake Tyers	Lake Tyers			21.88
LT(C)	Lake Tyers	Lake Tyers			30
LT(C)	Lake Tyers	Lake Tyers			30
LT(C)	Lake Tyers	Lake Tyers			30
LT(C)	Lake Tyers	Lake Tyers			0.18
LT(C)	Lake Tyers	Lake Tyers			0.28
LT(C)	Lake Tyers	Lake Tyers			0.03
LT(C)	Lake Tyers	Lake Tyers			21.88
LT(C)	Lake Tyers	Lake Tyers			30
LT(C)	Lake Tyers	Lake Tyers			30
LT(C)	Lake Tyers	Lake Tyers			30
LT(C)	Lake Tyers	Lake Tyers			0.18
LT(C)	Lake Tyers	Lake Tyers			0.28
LT(C)	Lake Tyers	Lake Tyers			22.25

LT(C)	Lake Tyers	Lake Tyers			7.3
LT(C)	Lake Tyers	Lake Tyers			0.05
LT(C)	Lake Tyers	Lake Tyers			0.05
LT(C)	Lake Tyers	Lake Tyers			30
LT(C)	Lake Tyers	Lake Tyers			0.05
LT(C)	Lake Tyers	Lake Tyers			4.75
LT(C)	Lake Tyers	Lake Tyers			0.21
LT(LSD)No.1	Lake Tyers	Lake Tyers			0.03
LT(LSD)No.1	Lake Tyers	Lake Tyers			30
LT(LSD)No.1	Lake Tyers	Lake Tyers			30
LT(LSD)No.1	Lake Tyers	Lake Tyers			0.03
LT(LSD)No.1	Lake Tyers	Lake Tyers			0.38
LT(LSD)No.2	Lake Tyers	Lake Tyers			0.03
LT(LSD)No.2	Lake Tyers	Lake Tyers			0.23
LT(LSD)No.2	Lake Tyers	Lake Tyers			0.03
LT(LSD)No.2	Lake Tyers	Lake Tyers			0.03
LT(LSD)No.2	Lake Tyers	Lake Tyers			30
LT(LSD)No.2	Lake Tyers	Lake Tyers			0.03
LT(LSD)No.2	Lake Tyers	Lake Tyers			30
LT(HSD)No.1	Lake Tyers	Lake Tyers			0.3
LT(HSD)No.1	Lake Tyers	Lake Tyers			0.03
LT(HSD)No.1	Lake Tyers	Lake Tyers			1.08
LT(HSD)No.1	Lake Tyers	Lake Tyers			30
LT(HSD)No.1	Lake Tyers	Lake Tyers			30
LT(HSD)No.1	Lake Tyers	Lake Tyers			21.88
LT(HSD)No.1	Lake Tyers	Lake Tyers			22.22
LT(HSD)No.2	Lake Tyers	Lake Tyers			0.05
LT(HSD)No.2	Lake Tyers	Lake Tyers			0.03
LT(HSD)No.2	Lake Tyers	Lake Tyers			0.06
LT(HSD)No.2	Lake Tyers	Lake Tyers			0.06
LT(HSD)No.2	Lake Tyers	Lake Tyers			0.1

LT(HSD)No.2	Lake Tyers	Lake Tyers			30
LT(HSD)No.2	Lake Tyers	Lake Tyers			0.06
LT(HSD)No.2	Lake Tyers	Lake Tyers			30

Table A3-2: Raw data from observations of opercular rate with response time in stimulus experiment. Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatments: C=control (no caging), LSD=low confinement density, HSD=high confinement density.

Day	Treatment	Response time (min)	Mean opercular rate (beats s-1)	Correlation coefficient
1	GL[C]	17.616	1.03	0.246
		30	0.9692	
		30	1.113	
		30	1.11	
	GL(HSD)	0.26	1.297	-0.576
		6.533	1.33	
		0.2	0.964	
		2.316	1.2	
		30	0.846	
		30	0.96	
		30	1.13	
		11.266	1.12	
2	GL[C]	30	1.152	-0.368
		0.1	1.24	
		30	1.222	
		30	1.04	
	GL(LSD)	8.083	1.80	
		0.05	1.163	
		6.45	1.37	-0.580
		0.133	1.27	
		0.2	1.33	
		8.716	1.37	
		2.033	1.68	
		30	1.134	
3	LT[C]	0.02	1.726	0.107

		2.883	1.192	
		30	1.55	
	LT(LSD) No. 1	0.02	1.215	0.942
		30	1.45	
		30	1.54	
		0.02	1.261	
		0.383	1.31	
4	LT[C]	30	0.971	-0.894
		30	1.31	
		11	1.496	
		0.283	1.70	
	LT(LSD) No. 2	0.02	1.44	-0.622
		0.233	1.397	
		0.02	1.236	
		0	1.57	
		30	1.16	
		0.02	1.27	
		30	1.16	
		0.02	1.23	
5	LT[C]	7.3	1.25	-0.451
		0.03	1.32	
		22.25	1.29	
		0.27	1.331	
	LT(HSD) No.1	0.3	1.45	-0.034
		0.2	1.314	
		1.083	1.372	
		30	1.49	
		21.883	1.164	
	LT[C]	30	1.24	-0.401
		4.75	1.13	

		0.03	1.34	
		0.216	1.55	
	LT(HSD)No. 2	0.02	1.49	-0.101
		0.466	1.59	
		0.04	1.27	
		0.04	1.45	
		0.06	1.38	
		30	1.37	
		0.04	1.62	
		30	1.51	

Table A3-3: Raw data parameters and HAI (Health Assessment Index) calculation. Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatments: C=control (no caging), LSD=low confinement density, HSD=high confinement density.

Fish No.	Liver	Eye	Skin	Gills	Fin	Pseudobranch	Thymus	Spleen	Hindgut	Kidney	Parasites	Index Value
GL(C)												
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	30	0	0	0	30
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0
GL(LSD)												
1	0	0	0	0	0	0	0	30	0	0	0	30
2	0	0	0	0	0	0	0	30	0	0	0	30
3	0	0	0	0	0	0	0	0	0	0	0	0

4	0	0	0	0	0	0	0	30	0	0	0	30
5	0	0	0	0	0	0	0	30	0	0	0	30
6	0	0	0	0	0	0	0	30	0	0	0	30
GL(HSD)												
1	0	0	0	0	0	0	0	30	0	0	0	30
2	0	0	0	0	0	0	0	30	0	0	0	30
3	30	0	0	0	0	0	0	30	0	0	0	60
4	0	0	0	0	0	0	0	0	0	0	0	0
5	30	0	0	0	0	0	0	30	0	0	0	60
6	0	0	0	0	0	0	0	30	0	0	0	30
7	0	30	0	0	0	0	0	30	0	0	0	60
8	0	0	0	0	0	0	0	0	0	0	0	0
LT(C)												
1	0	0	0	0	0	0	0	30	0	0	0	30
2	0	0	0	0	0	0	0	30	0	0	0	30
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0

9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
LT(LSD)												
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	30	0	0	0	30
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	30	0	0	0	30
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0

7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
LT(HSD)												
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	30	0	0	0	30
4	0	0	0	0	0	0	0	30	0	0	0	30
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	30	0	0	0	30
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	30	0	0	0	30
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	30	0	0	0	30
6	0	0	0	0	0	0	0	30	0	0	0	30

7	0	0	0	0	0	0	0	30	0	0	0	30
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	30	0	0	0	30
10	0	0	0	0	0	0	0	30	0	0	0	30

Table A3-4: Raw data parameters and K (Fuller's condition factor) calculation. Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatments: C=control (no caging), LSD=low confinement density, HSD=high confinement density.

Group	Wet weight (g)	Fork length (cm)	K
GL(C)	403.9	27.5	1.942
	568.9	30	2.107
	325.1	25	2.0806
	364	25.5	2.1952
	322.7	24.6	2.1677
	455	28.2	2.0289
	377.4	27	1.9174
	290.9	24.8	1.9072
	357.7	26	2.0352
	395	27	2.0068
GL(LSD)	481.8	27	2.4478
	422.4	27.8	1.966
	422.2	27	2.145
	281.8	23.5	2.1714
	349.5	26	1.9885
GL(HSD)	362.8	27.8	1.6886
	401.4	26.7	2.1088
	520.6	28.5	2.2489
	376.4	27	1.9123
	253.7	23.5	1.9549
LT[C]	553.2	31.5	1.7699
	137.6	18.5	2.1732
	274.3	23.3	2.1685
	336.1	25	2.151
	183.5	21	1.9814
LT[HSD]	213.1	22.3	1.9216
	215.2	22	2.021

	291	24	2.105
	217.2	21.5	2.1855
	204.8	21.5	2.0607
	211.1	22.5	1.8533
	183.1	22	1.7196
LT(LSD)	282.6	23.6	2.15
No.1	276.9	24	2.003
	294.5	23.5	2.2692
	142.8	18.5	2.2553
	140.1	18.7	2.1425
	226	22.5	1.9841
	263.7	24	1.9076
	154	19.2	2.1758
	312.8	25	2.0019
LT(LSD)	246.2	22.7	2.1048
No.2	180.3	21.3	1.8658
	241.3	22.3	2.1759
	202.1	22	1.898
	253.7	23.2	2.0317
	263.4	24	1.9054
	266.1	23.3	2.1037
	166.4	19.7	2.1765
	199.9	21.7	1.9563
LT(HSD)	222.4	22	2.0887
No.1	201.2	21	2.1726
	228.2	22.5	2.0034
	292.5	25	1.872
	314.7	25	2.0141
	180.7	21	1.9512
	209.6	22	1.9684
	321.3	25.3	1.984
	235.6	22.5	2.0684

	217.3	22.6	1.8825
LT(HSD)	200.7	22	1.8849
No.2	278.7	27.2	1.3849
	259.3	24	1.8757
	181.9	21	1.9642
	227.1	23	1.8665
	312.7	25	2.0013
	225.5	21.5	2.269
	215.3	22.5	1.8902
	213.4	22	2.0041
	183	21.5	1.8413

Table A3-5: Raw data parameters and SSI calculation. Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatments: C=control (no caging), LSD=low confinement density, HSD=high confinement density.

Group	Spleen wt (g)	Gutted wt (g)	SSI
GL(C)	0.5	362.3	0.14
	0.9	531.8	0.17
	0.5	294.2	0.17
	0.4	311.9	0.13
	0.6	302.5	0.2
	0.5	428.3	0.12
	0.6	392.5	0.15
	0.4	353.3	0.11
	0.4	269.2	0.15
	0.3	303.8	0.1
GL(LSD)	0.4	355.8	0.11
	0.6	404	0.15
	1.2	396.2	0.3
	0.5	379.1	0.13
	0.4	251.9	0.16
	0.8	323.2	0.25
GL(HSD)	0.6	324.6	0.18
	0.6	341.6	0.18
	0.9	481.1	0.19
	0.3	334.4	0.09
	0.5	229.5	0.22
	0.9	520	0.17
	0.4	129.5	0.31
LT(C)	0.3	260.2	0.12
	0.6	314	0.19
	0.4	173.4	0.23
	0.3	201.6	0.15
	0.3	204.7	0.15
	0.3	270.9	0.11

	0.2	201.8	0.1
	0.3	180.7	0.17
	0.1	202	0.05
	0.4	184.1	0.22
	0.2	174.4	0.11
LT(LSD)	0.4	249.7	0.16
No.1	0.2	264.3	0.08
	0.5	253.9	0.2
	0.3	135.5	0.22
	0.2	133.7	0.15
	0.2	151.7	0.13
	0.5	215.3	0.23
	0.4	255.6	0.16
	0.2	140.2	0.14
LT(LSD)	0.2	225.7	0.09
No.2	0.7	171.7	0.41
	0.2	211.3	0.09
	0.1	190.6	0.05
	0.3	235.7	0.13
	0.4	244.2	0.16
	0.2	237.9	0.08
	0.4	158.6	0.25
	0.3	190.9	0.16
LT(HSD)	0.4	202.5	0.2
No.1	0.5	278.4	0.18
	0.5	300	0.17
	0.2	167.4	0.12
	0.5	199.8	0.25
	0.5	306.6	0.16
	0.5	223.6	0.22
	0.3	208.3	0.14
LT(HSD)	0.3	189.8	0.16

No.2	0.4	246.7	0.16
	0.2	172.6	0.12
	0.4	215.3	0.19
	0.7	279.9	0.25
	0.3	200.4	0.15
	0.4	194.2	0.21
	0.3	175.9	0.17

Table A3-6: Raw data parameters and HSI calculation. Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatments: C=control (no caging), LSD=low confinement density, HSD=high confinement density.

Groups	Liver wt (g)	Gutted wt (g)	HSI
GL(C)	4.3	362.3	1.19
	10.2	531.8	1.92
	4.7	294.2	1.6
	4.6	311.9	1.47
	3.9	302.5	1.29
	6.5	428.3	1.52
	6.1	392.5	1.55
	2.7	269.2	1
	4.2	303.8	1.38
	3.9	355.8	1.1
GL(LSD)	6.6	404	1.63
	6.3	396.2	1.59
	7	379.1	1.85
	5.3	251.9	2.1
	5.5	323.2	1.7
	3.9	324.6	1.2
GL(HSD)	5.9	341.6	1.73
	8.5	481.1	1.77
	4.4	334.4	1.32
	6.6	520	1.27
	1.1	129.5	0.85
	1.9	260.2	0.73
LT(C)	4.8	314	1.53
	1.6	173.4	0.92
	2.3	201.6	1.14
	2.3	204.7	1.12
	3.5	270.9	1.29
	2.4	201.8	1.19

	1.9	180.7	1.05
	1.8	202	0.89
	2.2	184.1	1.2
	1.7	174.4	0.97
LT(LSD)	3.8	249.7	1.52
No.1	2.4	264.3	0.91
	4.2	253.9	1.65
	1.6	135.5	1.18
	1.3	133.7	0.97
	1.5	151.7	0.99
	2.1	215.3	0.98
	1.9	255.6	0.74
	1.9	140.2	1.36
	1.5	225.7	0.66
	2.2	171.7	1.28
LT(LSD)	2.6	211.3	1.23
No.2	1.9	190.6	1
	1.9	235.7	0.81
	2.8	244.2	1.15
	2.1	237.9	0.88
	1.7	158.6	1.07
	1.8	190.9	0.94
LT(HSD)	2.7	202.5	1.33
No.1	3	278.4	1.08
	3.7	300	1.23
	1.4	167.4	0.84
	2.4	199.8	1.2
	2.9	306.6	0.95
	2.3	223.6	1.03
	1.9	208.3	0.91
LT(HSD)	1.7	189.8	0.9
No.2	2.2	246.7	0.89

	1.8	172.6	1.04
	2	215.3	0.93
	3.4	279.9	1.21
	2.6	200.4	1.3
	1.9	194.2	0.98
	1.5	175.9	0.85

Table A3-7: Raw data parameters and HSI calculation. Fish source: GL=Gippsland Lakes, LT=Lake Tyers. Caging treatments: C=control (no caging), LSD=low confinement density, HSD=high confinement density.

Group	Sex	Gonad wt (g)	Gutted wt (g)	GSI
GL[C]	M	27.06	362.30	7.470
	M	12.18	531.80	2.290
	F	18.09	294.20	6.150
	T	40.49	311.90	12.983
	F	6.08	302.50	2.010
	F	8.69	428.30	2.030
	T	27.40	392.50	6.980
	F	8.48	353.30	2.400
	M	10.61	269.20	3.940
	T	40.19	303.80	13.230
GL(LSD)	M	24.09	355.80	6.770
	T	57.40	404.00	14.207
	M	7.77	396.20	1.960
	T	22.37	379.10	5.900
	T	19.50	251.90	7.740
	F	11.99	323.20	3.710
GL(HSD)	M	21.59	324.60	6.650
	T	45.80	341.60	13.407
	F	15.78	481.10	3.280
	M	26.80	334.40	8.014
	M	13.68	229.50	5.960
	F	10.19	520.00	1.960
	F	1.59	129.50	1.230
	F	4.40	260.20	1.690
LT[C]	M	5.28	314.00	1.680
	F	1.60	173.40	0.920
	M	1.59	201.60	0.790
	M	2.39	204.70	1.170
	T	8.70	270.90	3.210

	T	7.39	201.80	3.660
	M	8.69	180.70	4.810
	F	2.30	202.00	1.138
	M	2.19	184.10	1.190
	F	2.20	174.40	1.260
LT(LSD)	T	22.70	249.70	9.090
No.1	F	2.48	264.30	0.940
	T	20.49	253.90	8.070
	F	1.79	135.50	1.320
	F	1.19	133.70	0.890
	M	2.26	215.30	1.050
	M	2.71	255.60	1.060
	M	0.60	140.20	0.430
LT(LSD)	M	9.50	225.70	4.210
No.2	M	3.09	171.70	1.800
	T	20.69	211.30	9.790
	M	3.39	190.60	1.780
	M	8.89	235.70	3.770
	T	10.60	244.20	4.340
	M	16.70	237.90	7.020
	F	2.30	158.60	1.450
	F	2.00	190.90	1.047
LT(HSD)	T	22.07	202.50	10.900
No.1	T	26.37	300.00	8.790
	F	2.04	167.40	1.220
	F	2.66	199.80	1.330
	F	4.02	306.60	1.310
	F	3.80	223.60	1.700
	F	2.23	208.30	1.070
	F	1.90	189.80	1.000
LT(HSD)	F	2.79	246.70	1.130
No.2	F	2.30	172.60	1.330

	T	3.29	215.30	1.530
	T	22.39	279.90	8.000
	T	16.29	200.40	8.130
	M	6.89	194.20	3.550
	M	0.69	175.90	0.390

Table A3-8: Raw data from stimulus experiment on fish sampled for cortisol. Fish source: LT=Lake Tyers.

Group	Selection	Response time (min)
LT(B)*	Blood test	0.03
		8
		30
		30

Appendix 4

Table A4-2: Variation in number of interrenal cells with source of fish.

Fish No.	Gippsland Lakes												Mean	Lake Tyers												Mean
1	23	22	15	21	19	22	19	21	18	22		20.2	15	16	15	15	14	17	14	16	17	14		15.3		
2	22	23	22	23	21	17	23	22	22	21		21.6	15	14	18	17	18	16	14	14	18	18		16.2		
3	21	21	20	23	22	22	24	23	23	24		22.3	14	15	13	15	14	16	15	15	14	17		14.8		
4	20	20	18	23	19	18	21	20	22	20		20.1	17	14	19	16	19	18	17	17	21	N/A ¹		17.6		
5	22	24	20	19	22	21	21	23	20	19		21.1	11	19	16	17	21	18	20	11	18	N/A ¹		16.8		
6	22	24	25	24	24	25	21	24	24	24		23.7	20	19	18	18	18	19	15	16	20	20		18.3		
7	23	20	24	20	22	21	22	24	22	20		21.8	16	14	16	17	16	15	19	16	17	15		16.1		
8	22	25	28	22	20	15	22	24	24	22		22.4	13	18	16	14	16	16	14	17	15	17		15.6		
9	22	23	20	17	19	21	18	20	19	22		20.1	19	17	16	14	15	12	17	17	14	17		15.8		
10	20	19	24	22	24	23	24	25	24	24		22.9	17	16	17	15	17	17	15	17	15	18		16.4		

Table A4-3: Gonad development versus interrenal cell counts.

Fish source	Fish no.	No. cells	Gonads
GL	1	20.2	Developed
GL	2	21.6	Developed
GL	3	22.3	Developed
GL	4	20.1	Undeveloped
GL	5	21.1	Undeveloped
GL	6	23.7	Undeveloped
GL	7	21.8	Developed
GL	8	22.4	Undeveloped
GL	9	20.1	Developed
GL	10	22.9	Undeveloped
LT	1	15.3	Undeveloped
LT	2	16.2	Developed
LT	3	14.8	Developed
LT	4	17.6	Undeveloped
LT	5	16.8	Developed
LT	6	18.3	Undeveloped
LT	7	16.1	Undeveloped
LT	8	15.6	Undeveloped
LT	9	15.8	Undeveloped

Table A4-1: Fish weight and length.

Sample	Fork length (cm)	Wet weight (g)	Condition factor (K)
GL26	29.7	632.8	2.42
GL27	26.0	440.1	2.50
GL28	23.0	347.9	2.86
GL29	27.0	476.2	2.42
GL31	24.0	323.6	2.34
GL32	24.0	354.7	2.57
GL33	25.0	325.9	2.09
GL35	24.5	348.6	2.37
GL37	23.5	267.1	2.06
GL38	23.6	291.4	2.22
LT12	18.5	229.9	3.63
LT13	30.0	725.1	2.69
LT14	30.0	849.9	3.15
LT15	29.0	601.0	2.46
LT16	27.0	500.0	2.54
LT18	25.0	450.0	2.88
LT19	32.5	900.0	2.62
LT20	30.0	724.0	2.68
LT21	29.0	600.1	2.46
LT22	34.0	1100.0	2.80

Appendix 5

Table A5-1. Specimens examined with EDXS on ESEM. Source: GL=Gippsland Lakes, LT=Lake Tyers; CA=Cunningham Arm, MR=Mitchell River. Colour: 1=white, 2=white with small yellow bits, 3=white with medium yellow, 4=white with lots of yellow or brown, 5=yellow

Date collected	ESEM label	Surface examined	Colour (1-5)	Atom%									
				C	N	O	Na	P	S	Cl	K	Ca	Sr
2/12/2009	GLTB(Tambo Bluff)8I-1	Inner	1	31.07	12.42	38.01	0.16	0.45	0.24	0.06	0.12	17.32	0.14
2/12/2009	GLTB(Tambo Bluff)8I-2	Outer	2	46.59	14.53	26.58	0.24	0.55	0.50	0.09	0.16	10.62	0.15
3/12/2009	GL12F-1 (La Trobe R)	Inner	2	32.39	16.17	29.49	0.18	0.72	0.46	0.17	0.30	19.82	0.30
3/12/2009	GL12F-2 (La Trobe R)	Outer	2	39.76	18.98	28.47	0.24	0.39	0.53	0.08	0.24	11.16	0.15
17/07/2009	GLTLCut25F-1	Outer	5	57.93	18.60	17.92	0.29	0.40	0.42	0.21	0.17	3.95	0.10
17/07/2009	GLTLCut25F-2	Outer	3	39.42	10.54	35.17	0.21	0.13	0.27	0.09	0.14	13.85	0.18
15/07/2009	GLTLCut26F	Inner	4	40.62	15.07	26.08	0.08	0.29	0.15	0.13	0.16	17.11	0.32
18/07/2009	GL27F	Inner	4	39.44	8.18	37.23	0.16	0.39	0.26	0.00	0.10	13.98	0.26
18/07/2009	GLTLCut27F	Outer	2	44.51	7.11	32.79	0.25	0.15	0.18	0.19	0.13	14.45	0.25
18/07/2009	GL28F	Outer	1	47.42	5.46	30.56	0.08	0.09	0.09	0.06	0.10	15.84	0.30
17/07/2009	GLTLCut28F	Inner	1	31.94	8.99	42.21	0.11	0.03	0.10	0.09	0.11	16.09	0.32
4/08/2009	GLMitchellIR28F	Outer	1	23.31	5.79	48.06	0.14	0.10	0.08	0.13	0.12	22.01	0.27
18/07/2009	GL29F	Inner	1	19.31	17.08	39.56	0.10	0.15	0.06	0.12	0.14	23.22	0.25
18/07/2009	GL25M	Inner	2	33.45	6.13	41.12	0.16	0.06	0.06	0.08	0.11	18.58	0.27

18/07/2009	GL26M	Inner	4	49.73	16.72	20.87	0.00	0.22	0.15	0.07	0.10	11.91	0.23
18/07/2009	GL28M	Outer	2	44.43	6.75	33.75	0.22	0.15	0.19	0.11	0.10	13.91	0.29
18/07/2009	GL29M			18.62	15.13	41.12	0.11	0.37	0.22	0.15	0.13	23.85	0.31
17/12/2009	LT17F	Inner	1	17.13	8.84	43.81	0.17	0.44	0.26	0.22	0.19	28.63	0.31
18/11/2009	LT18F-1	Outer	1	26.26	10.22	42.33	0.00	0.10	0.06	0.06	0.10	20.58	0.29
18/11/2009	LT18F-2	Inner	1	27.09	7.44	47.60	0.07	0.00	0.05	0.00	0.07	17.41	0.27
13/11/2009	LT19F-1	Outer	2	30.11	6.00	40.75	0.00	0.09	0.04	0.02	0.12	22.63	0.23
13/11/2009	LT19F-2	Inner	2	37.88	5.81	37.39	0.25	0.54	0.50	0.26	0.17	16.95	0.26
24/10/2009	LT24F	Outer	3	56.23	8.66	23.65	0.35	0.37	0.51	0.28	0.17	9.50	0.28
16/11/2009	LT26F	Outer	3	52.65	11.06	27.33	0.25	0.38	0.50	0.18	0.16	7.27	0.22
30/10/2009	LT27F	Outer	3	34.14	8.74	32.20	0.10	0.37	0.35	0.21	0.23	23.19	0.48
24/11/2009	LT28F	Inner	1	42.83	10.97	30.78	0.00	0.15	0.11	0.04	0.09	14.82	0.21
18/07/2009	LT31F	Inner	3	35.14	22.45	24.49	0.00	0.40	0.21	0.15	0.11	16.75	0.29
13/11/2009	LT20M	Outer	4	37.09	10.67	36.33	0.10	0.17	0.16	0.08	0.11	15.06	0.24
23/10/2009	LT22M	Outer	2	48.83	9.73	29.44	0.29	0.15	0.27	0.14	0.10	10.84	0.21
16/11/2009	LT23M1	Inner	1	32.72	10.27	36.10	0.11	0.33	0.24	0.22	0.16	19.44	0.40
17/11/2009	LT23M2	Inner	1	32.49	6.33	42.33	0.20	0.09	0.05	0.08	0.12	17.96	0.35
30/10/2009	LT25M	Outer	4	44.36	0.00	37.71	0.00	0.36	0.24	0.16	0.15	16.83	0.20
16/11/2009	LT26M	Inner	1	44.70	8.19	32.42	0.33	0.44	0.25	0.29	0.18	12.91	0.27
24/11/2009	LT27M	Inner	4	56.19	3.17	29.35	0.07	0.12	0.09	0.07	0.08	10.62	0.24

18/11/2009	LT31M	Inner	3	53.48	10.56	27.13	0.49	0.61	0.52	0.24	0.21	6.56	0.19
	GLTR1	I	1	19.11	4.42	51.89	0.13	0.30	0.19	0.09	0.12	23.38	0.37
	GLTR2	I	1	24.71	9.77	44.19	0.23	0.10	0.13	0.06	0.09	20.40	0.32
	GLTR3	I	1	30.17	7.36	40.69	0.00	0.22	0.09	0.03	0.13	20.99	0.31
	GLTR4	I	1	36.33	11.26	35.17	0.14	0.21	0.18	0.08	0.10	16.31	0.22
	GLTR5	I	1	29.94	0.00	44.80	0.00	0.28	0.00	0.01	0.13	24.53	0.30
	GLTR6												
	GLTR7	I	1	30.37	6.96	43.50	0.14	0.04	0.02	0.00	0.09	18.60	0.25
	GLTRunknown	I	5	55.15	9.69	26.67	0.41	0.62	0.72	0.31	0.39	5.93	0.12
Date died	in tanks												
13/09/2003	TRGLCA06-1	I	1	21.58	0.00	53.13	0.00	0.27	0.14	0.12	0.17	24.22	0.37
13/09/2003	TRGLCA06-2	I	1	17.54	2.25	53.95	0.08	0.24	0.20	0.13	0.18	25.07	0.35
13/09/2003	TRGLCA07-1	I	5	52.43	7.48	26.81	0.32	0.50	0.41	0.18	0.22	11.46	0.19
13/09/2003	TRGLCA07-2	I	5	43.98	5.44	34.11	0.07	0.32	0.19	0.10	0.12	15.45	0.21
13/09/2003	TRGLCA08	I	3	41.28	2.87	33.07	0.00	0.19	0.19	0.06	0.17	21.88	0.29
13/09/2003	TRGLCA13-1	I	1	22.47	4.40	51.14	0.00	0.11	0.05	0.05	0.11	21.36	0.32
13/09/2003	TRGLCA13-2	O	1	19.08	1.53	51.71	0.00	0.20	0.13	0.09	0.19	26.61	0.46
13/09/2003	TRGLCA14-1	I	1	15.73	4.80	52.66	0.18	0.27	0.17	0.17	0.17	25.60	0.23
13/09/2003	TRGLCA14-2	I	1	17.18	5.74	55.27	0.16	0.20	0.11	0.09	0.10	20.89	0.26

15/09/2003	TRGLCA15	I	2	49.08	6.08	27.59	0.09	0.32	0.20	0.09	0.14	16.15	0.26
17/09/2003	TRGLCA22-1	I	1	20.99	7.61	48.23	0.05	0.28	0.15	0.13	0.15	22.16	0.24
17/09/2003	TRGLCA22-2	I	1	32.67	4.82	40.61	0.00	0.23	0.18	0.13	0.15	20.91	0.31
17/09/2003	TRGLCA27-1	I	4	60.69	7.02	22.85	0.20	0.22	0.23	0.11	0.11	8.44	0.14
17/09/2003	TRGLCA27-2	I	1	28.86	4.84	43.29	0.00	0.15	0.12	0.05	0.12	22.31	0.27
17/09/2003	TRGLCA34-1	I	1	23.11	4.45	50.49	0.08	0.24	0.17	0.12	0.14	20.92	0.28
17/09/2003	TRGLCA34-2	I	1	32.33	5.90	41.97	0.07	0.32	0.23	0.17	0.14	18.71	0.16
19/09/2003	TRGLCA40-1	I	1	25.41	7.28	42.64	0.12	0.41	0.27	0.22	0.19	23.14	0.32
19/09/2003	TRGLCA40-2	I	1	39.74	6.96	35.03	0.23	0.09	0.18	0.09	0.13	17.29	0.28
19/09/2003	TRGLCA42-1	I	1	24.03	0.00	51.74	0.00	0.13	0.05	0.06	0.09	23.55	0.34
19/09/2003	TRGLCA42-2	I	1	20.57	6.76	46.05	0.21	0.22	0.16	0.10	0.16	25.41	0.36
19/09/2003	TRGLCA47	I	1	21.90	2.84	51.60	0.00	0.37	0.20	0.19	0.14	22.58	0.20
21/09/2003	TRGLCA50-1	I	1	23.74	7.58	43.91	0.00	0.22	0.14	0.11	0.13	23.90	0.27
21/09/2003	TRGLCA50-2	I	2	30.85	4.47	41.18	0.14	0.32	0.21	0.14	0.15	22.21	0.34
21/09/2003	TRGLCA52-1	I	1	29.48	7.86	39.79	0.08	0.32	0.20	0.17	0.17	21.68	0.27
21/09/2003	TRGLCA52-2	I	1	32.77	7.63	39.18	0.08	0.14	0.17	0.09	0.08	19.59	0.28
21/09/2003	TRGLCA54-1	I	1	23.48	0.00	52.81	0.00	0.03	0.00	0.00	0.13	23.34	0.22
21/09/2003	TRGLCA54-2	I	1	22.75	7.15	48.17	0.17	0.25	0.14	0.10	0.13	20.84	0.29
22/09/2003	TRGLCA59	I	2	29.29	5.47	40.29	0.13	0.32	0.14	0.06	0.11	23.85	0.34
22/09/2003	TRGLCA60	I	1	35.88	4.20	39.15	0.18	0.25	0.17	0.08	0.11	19.72	0.27

24/09/2003	TRGLCA61	I	2	24.45	8.34	46.19	0.10	0.29	0.24	0.15	0.16	19.79	0.28
Histology fish													
30/01/2013	TR12LT18-5M300113	I	1	20.87	6.01	49.74	0.24	0.27	0.22	0.22	0.21	21.93	0.29
30/01/2013	TR13LT30F300113	I	1	18.10	6.10	48.85	0.11	0.34	0.24	0.24	0.23	25.48	0.31
30/01/2013	TR14LT30F300113	I	1	30.23	8.07	39.45	0.14	0.30	0.35	0.22	0.25	20.67	0.32
30/01/2013	TR15LT29M300113	I	1	33.42	11.92	36.08	0.32	0.00	0.34	0.27	0.22	17.11	0.32
30/01/2013	TR16LT27F300113	I	3	67.36	3.05	18.25	0.00	0.35	0.26	0.11	0.14	10.30	0.18
30/01/2013	TR18LT25F300113	I	1	20.07	6.70	48.83	0.06	0.30	0.25	0.19	0.19	23.04	0.37
30/01/2013	TR19LT32-5M300113	I	1	20.61	6.55	48.91	0.12	0.34	0.19	0.15	0.14	22.65	0.35
30/01/2013	TR20LT30M300113	I	2	26.69	8.35	43.79	0.14	0.20	0.11	0.14	0.10	20.21	0.28
30/01/2013	TR21LT29F300113	I	1	18.09	3.96	51.59	0.21	0.39	0.26	0.18	0.20	24.75	0.37
30/01/2013	TR22LT34F300113	I	1	30.19	7.06	43.80	0.20	0.28	0.19	0.15	0.15	17.72	0.26
18/02/2013	TR26GL29-7F180213	I	2	49.49	5.53	30.32	0.10	0.17	0.09	0.10	0.12	14.04	0.05
18/02/2013	TR27GL26F180213	I	2	37.33	6.83	37.78	0.09	0.27	0.18	0.10	0.11	17.08	0.23
18/02/2013	TR28GL23F180213	I	2	27.26	6.14	43.70	0.19	0.34	0.23	0.23	0.20	21.33	0.38
18/02/2013	TR29GL27M180213	I	1	44.09	5.44	33.17	0.18	0.21	0.25	0.18	0.24	15.99	0.25
18/02/2013	TR31GL24M180213	I	1	29.23	10.52	39.30	0.24	0.28	0.17	0.09	0.11	19.74	0.32
18/02/2013	TR32GL24F180213	I	2	45.68	6.00	29.76	0.17	0.27	0.22	0.16	0.16	17.17	0.20
18/02/2013	TR33GL25F180213	I	3	24.44	6.00	46.28	0.16	0.12	0.09	0.13	0.17	22.20	0.41

18/02/2013	TR35GL24-5M180213	I	2	32.55	8.57	37.21	0.16	0.30	0.33	0.21	0.18	20.24	0.26
18/02/2013	TR37GL23-5M180213	I	1	24.73	7.62	47.29	0.13	0.26	0.19	0.17	0.15	19.15	0.31
18/02/2013	TR38GL23-6M180213	I	3	36.20	5.15	41.27	0.05	0.14	0.17	0.09	0.10	16.61	0.23

Appendix 6

DNA sequences and predicted RFLP outcomes.

LOCUS AF410879 299 bp DNA linear VRT 02-SEP-2002
DEFINITION *Acanthopagrus butcheri* mitochondrial control region, partial sequence.
ACCESSION AF410879
VERSION AF410879.1 GI:22652224
KEYWORDS .
SOURCE mitochondrion *Acanthopagrus butcheri* (southern black bream)
ORGANISM *Acanthopagrus butcheri*
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Actinopterygii; Neopterygii; Teleostei; Neoteleostei;
Acanthomorphata; Eupercaria; Spariformes; Sparidae; Acanthopagrus.
REFERENCE 1 (bases 1 to 299)
AUTHORS Yap,E.S., Chaplin,J.A. and Potter,I.C.
TITLE Population genetic structure of the black bream, *Acanthopagrus butcheri*, in Western Australia
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 299)
AUTHORS Yap,E.S. and Chaplin,J.A.
TITLE Direct Submission
JOURNAL Submitted (14-AUG-2001) School of Biological Sciences and Biotechnology, Murdoch University, South Street, Murdoch, Western Australia 6150, Australia
FEATURES Location/Qualifiers
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61 gagaaaaatgt tctattcaat atttaatgac aattgtatgt ttatactcat tctattaatc
121 caaaaataaat taaaatctag caaattaatc acgttaacag tttatgcgca ggcataaca
181 tactttgctt ctacagaaaa ttacccaata agaaccgacc aacttattat atcttaatgc
241 attcgtttat tgaaggtagg gggcaaaagt tgtgggggtc acacaatctg ctttattcc

Name:AF410879 *Acanthopagrus butcheri* mitochondrial control region, partial sequence

Conformation:linear

Overhang:five_prime, three_prime, blunt

Minimum Site Length:4 bases

Maximum Number of Cuts:all

Included:all commercial, prototypes only

Noncutters:AarI, AatII, AbsI, AccI, AciI, AclI, AcyI, AflIII, AgeI, AjuI, AlfI, AloI, AluI, AlwNI, ApaI, ApaLI, ApoI, ArsI, AscI, AsuII, AvaI, AvaII, AvrII, BaeI, Ball, BamHI, BarI, BbvI, BbvCI, BccI, BcgI, BciVI, BclI, BdaI, BfiI, BglII, BisI, BplI, Bpu10I, BsaAI, BsaBI, BsaXI, BseMII, BsePI, BseRI, BseSI, BseYI, BsgI, BsmAI, Bsp1407I, BspHI, BspMI, BsrI, BsrBI, BsrDI, BstEII, BstXI, BtgZI, BtrI, BtsI, CfrI, Cfr10I, Clal, CviJI, DdeI, DpnI, DraII, DraIII, DrdI, Eam1105I, EciI, Eco31I, Eco47III, Eco57I, Eco57MI, EcoNI, EcoP15I, EcoRI, EcoRII, EcoRV, Esp3I, FaiI, FauI, Fnu4HI, FokI, FseI, FspAI, GsuI, HaeII, HaeIII, HaeIV, HgaI, Hin4I, HindIII, Hinfl, HpaII, Hpy99I, Hpy188I, KpnI, MauBI, MboI, MboII, MluI, MmeI, MsII, MwoI, NaeI, NarI, NcoI, NdeI, NheI, NlaIV, NmeAIII, NotI, NruI, NspI, OliI, PacI, PasI, PflMI, PfoI, PleI, PmaCI, PmeI, PpuMI, PshAI, PsiI, PI-PspI, PspXI, PsrI, PstI, PvuI, PvuII, RsaI, RsrII, SacI, SacII, Sall, SanDI, SapI, Scal, PI-SceI, ScrFI, SduI, SexAI, SfiI, SgfI, SgrAI, SgrDI, SmaI, SmlI, SnabI, SpeI, SphI, SrfI, Sse8387I, StuI, StyI, SwaI, TaqI, TaqII, TatI, TauI, TfiI, TseI, TsoI, TspDTI, TspGWI, TspRI, TstI, Tth111I, XbaI, XcmI, XhoI, XhoII

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
AgsI	TTSAA	5	three_prime	2	77, 252
BsmI	GAATGC	6	three_prime	1	239
Cac8I	GCNNNGC	4	blunt	1	170
CspCI	CAANNNNNGTGG	7	three_prime	2	252, 287
FaiI	YATR	4	blunt	14	3, 5, 19, 21, 23, 28, 33, 37, 45, 97, 103, 164, 180, 229
GlaI	GCGC	4	blunt	1	167
HhaI	GCGC	4	three_prime	1	168
HindII	GTYRAC	6	blunt	1	155
HpaI	GTAAAC	6	blunt	1	155
HphI	GGTGA	5	three_prime	2	41, 267

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
MaeI	CTAG	4	five_prime	1	137
MaeII	ACGT	4	five_prime	1	151
MaeIII	GTNAC	4	five_prime	1	277
MfeI	CAATTG	6	five_prime	1	90
MnlI	CCTC	4	three_prime	1	251
MseI	TTAA	4	five_prime	6	83, 115, 130, 145, 154, 234
NlaIII	CATG	4	three_prime	1	35
PpiI	GAACNNNNCTC	7	three_prime	2	52, 84
SetI	ASST	4	three_prime	2	154, 257
SfaNI	GCATC	5	five_prime	1	181
SspI	AATATT	6	blunt	1	80
Tsp45I	GTSAC	5	five_prime	1	277
TspEI	AATT	4	five_prime	4	90, 127, 142, 198
VspI	ATTAAT	6	five_prime	2	115, 145
XmnI	GAANNNTTC	6	blunt	1	67

LOCUS AF406646 978 bp DNA linear VRT 30-AUG-2001
DEFINITION *Acanthopagrus butcheri* mitochondrial control region, partial sequence.
ACCESSION AF406646
VERSION AF406646.1 GI:15384261
KEYWORDS .
SOURCE mitochondrion *Acanthopagrus butcheri* (southern black bream)
ORGANISM *Acanthopagrus butcheri*
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Actinopterygii;
Neopterygii; Teleostei; Neoteleostei; Acanthomorphata; Eupercaria; Spariformes; Sparidae;
Acanthopagrus.
REFERENCE 1 (bases 1 to 978)
AUTHORS Murphy,N.P., Hurt,A.C. and Austin,C.M.
TITLE Molecular genetic evidence for a new species of bream of the genus *Acanthopagrus*
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 978)
AUTHORS Murphy,N.P., Hurt,A.C. and Austin,C.M.
TITLE Direct Submission
JOURNAL Submitted (06-AUG-2001) School of Ecology and Environment, Deakin University, PO Box
423, Warrnambool, Vic 3280, Australia
FEATURES Location/Qualifiers
source 1..978
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121 ctattcaata ttgtatgaca attgtatgtt catagtattt cttatattcc aaaataaatt
181 aaaatcttagc aaattaatca tgtcaacaat ttatgcgcaa gcatcaacat acttgctcc
241 tacagaaaaat taccataaa gaaccgacca acttattata tcttaataca ttgttatt
301 gaaggtgagg ggcaaaagtt gtgggggtca cacgatctgc ttatccctg gcatctggtt
361 cctatttcag ggcataaaac agnttgattt cattaacttt taatggcgct tgataagtt
421 aatggtggca accagatggc gagataactc cccatgccga gcgttctc cacaggtgcc
481 aaagttctt tttttttt atcccttcgt ggtcatttc cagtcacgc tagagtccta
541 taatcaaggt ggtattttt cctgcaaaca gtaataatc tgaggattt aaagacttt
601 acttatgaat tgcatataattt atatcaggag cataatatgt tttaacc cagaatttat
661 atattcgacc ccccttgagg gttttggc gttaaacccc cccccccccc taaactccag
721 ggatcactaa cacttcgca aacccctcta aaacagaaat tcttggccccc tagagtaagg
781 gggaaattata tccaaaatgc atcttttaa tatattaaaaa taatgatttt taactaaatt
841 cctaatttc cccaaaggaag cctattctc agttaaaat tttaaatata accagttcac
901 gtagcttaat taaagcataa cactgaagct gttaaagatgg accctaaaaa gccccatgaa
961 cacaagggtt tggtcctg

Name: AF406646 *Acanthopagrus butcheri* mitochondrial control region, partial sequence

Conformation: linear

Overhang: five_prime, three_prime, blunt

Minimum Site Length: 4 bases

Maximum Number of Cuts: all

Included: all commercial, prototypes only

Noncutters: AarI, AatII, AbsI, AccI, AciI, AcII, AcylI, AflIII, AflIII, AgeI, AjuI, AlfI, AloI, AluI, AlwNI, ApaI, ApaLI, ApoI, ArsI, AscI, AsuII, AvaI, AvaII, AvrII, BaeI, BalI, BamHI, BarI, BbvI, BbvCI, BccI, BcgI, BciVI, BclI, BdaI, BfiI, BglI, BglII, BisI, BplI, Bpu10I, BsaAI, BsaBI, BsaXI, BseMII, BsePI, BseRI, BseSI, BseYI, BsgI, BsmAI, Bsp1407I, BspHI, BspMI, BsrI, BsrBI, BsrDI, BstEII, BstXI, BtgZI, BtrI, BtsI, CfrI, Cfr10I, ClaI, CviJI, DdeI, DpnI, DraII, DraIII, DrdI, Eam1105I, EciI, Eco31I, Eco47III, Eco57I, Eco57MI, EcoNI, EcoP15I, EcoRI, EcoRII, EcoRV, Esp3I, FalI, FauI, Fnu4HI, FokI, FseI, FspAI, GsuI, HaeII, HaeIII, HaeIV, HgaI, Hin4I, HindIII, Hinfl, HpaII, Hpy99I, Hpy188I, KpnI, MauBI, MboI, MboII, MluI, MmeI, MsI, MwoI, NaeI, NarI, NcoI, NdeI, NheI, NlaIV, NmeAIII, NotI, NruI, NspI, OliI, PacI, PasI, PflMI, PfoI, PleI, PmaCI, PmeI, PpuMI, PshAI, PsiI, PI-PspI, PspXI, PsrI, PstI, PvuI, PvuII, RsaI, RsrII, SacI, SacII, SalI, SanDI, SapI, ScaI, PI-SceI, ScrFI, SduI, SexAI, SfiI, SgfI, SgrAI, SgrDI, SmaI, SmlI, SnaBI, SpeI, SphI, SrfI, Sse8387I, StuI, StyI, SwaI, TaqI, TaqII, TatI, TauI, TfiI, TseI, TsoI, TspDTI, TspGWI, TspRI, TstI, Tth111I, XbaI, XcmI, XhoI, XhoII

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
AgsI	TTSAA	5	three_prime	2	77, 252
BsmI	GAATGC	6	three_prime	1	239

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
Cac8I	GCNNNGC	4	blunt	1	170
CspCI	CAANNNNNGTGG	7	three_prime	2	252, 287
FaiI	YATR	4	blunt	14	3, 5, 19, 21, 23, 28, 33, 37, 45, 97, 103, 164, 180, 229
GlaI	GCGC	4	blunt	1	167
HhaI	GCGC	4	three_prime	1	168
HindII	GTYRAC	6	blunt	1	155
HpaI	GTAAAC	6	blunt	1	155
HphI	GGTGA	5	three_prime	2	41, 267
MaeI	CTAG	4	five_prime	1	137
MaeII	ACGT	4	five_prime	1	151
MaeIII	GTNAC	4	five_prime	1	277
MfeI	CAATTG	6	five_prime	1	90
MnlI	CCTC	4	three_prime	1	251
MseI	TTAA	4	five_prime	6	83, 115, 130, 145, 154, 234
NlaIII	CATG	4	three_prime	1	35
PpiI	GAACNNNNCTC	7	three_prime	2	52, 84
SetI	ASST	4	three_prime	2	154, 257
SfaNI	GCATC	5	five_prime	1	181
SspI	AATATT	6	blunt	1	80
Tsp45I	GTSAC	5	five_prime	1	277
TspEI	AATT	4	five_prime	4	90, 127, 142, 198
VspI	ATTAAT	6	five_prime	2	115, 145
XmnI	GAANNNTTC	6	blunt	1	67

LOCUS AF406647 1016 bp DNA linear VRT 30-AUG-2001

DEFINITION *Acanthopagrus australis* mitochondrial control region, partial sequence.

ACCESSION AF406647

VERSION AF406647.1 GI:15384262

KEYWORDS .

SOURCE mitochondrion *Acanthopagrus australis* (surf bream)

ORGANISM *Acanthopagrus australis*

Eukaryota; Metazoa; Chordata; Craniata; Vertebrata;

Euteleostomi;

Actinopterygii; Neopterygii; Teleostei; Neoteleostei;

Acanthomorphata; Eupercaria; Spariformes; Sparidae;

Acanthopagrus.

REFERENCE 1 (bases 1 to 1016)

AUTHORS Murphy,N.P., Hurt,A.C. and Austin,C.M.

TITLE Molecular genetic evidence for a new species of bream of the genus *Acanthopagrus*

JOURNAL Unpublished

REFERENCE 2 (bases 1 to 1016)

AUTHORS Murphy,N.P., Hurt,A.C. and Austin,C.M.

TITLE Direct Submission

JOURNAL Submitted (06-AUG-2001) School of Ecology and Environment, Deakin

University, PO Box 423, Warrnambool, Vic 3280, Australia

FEATURES Location/Qualifiers

source 1..1016

/organism="*Acanthopagrus australis*"

/organelle="mitochondrion"

/mol_type="genomic DNA"

/db_xref="taxon:[160963](#)"

misc_feature <1..>1016

/note="control region"

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121 atatatggtt taaaatgcat acatctatag taaccaatca aggaatataa catatgtaat
181 gtctaacaac agtttatgtt ctaaagcact cttctcatta tatgcaaatt aaggtacagc
241 aagatcttc acctaaccaa gctgtgtaca agtatcaaca caccttatat ctccagaata
301 ttgtccaata agaaccgatc aacctattat atcttaatgc attcgttat tgaaggtag
361 gggcaaaaat cgtgggggtc acacaatatg ttttattcct ggcattctgg tcctattca
421 gggccataaa tagattaatt ccattaacgt ttatcgacgc ttgcataagt taatggtggt
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601 gggggaatct cccccctgcgc gcacgtaaca atgatgagac attcaaagac tttctattta
661 taatggcata aacttattca tgagcataat atgggtttt ttcccctaaa tcttctatat
721 cgacccccc ttgagggttt taggtttaa accccccc cccctaaac tccaggaatc
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841 ttatataccaa aatgcgtttt ttaatataat taaaataatg ctttttaatt aaattcctt
901 ttcccccaag gaaatctgtc tctcaattta aaattcata tatgaccagt tcacgttagct
961 taattaaagc ataacactga agctgttaag atggccgta aaaagctcca tgagca

Name: AF406647 *Acanthopagrus australis* mitochondrial control region, partial sequence

Conformation: linear

Overhang: five_prime, three_prime, blunt

Minimum Site Length: 4 bases

Maximum Number of Cuts: all

Included: all commercial, prototypes only

Noncutters: AarI, AatII, AbsI, AccI, AciI, AcyI, AflII, AflIII, AgeI, AjuI, AlfI, AloI, AlwNI, ApaI, ApaLI, ArsI, AscI, AsuII, AvaI, AvrII, BaeI, Ball, BamHI, BarI, BbvI, BbvCI, BcgI, BciVI, BclI, BdaI, BfiI, BglI, BisI, BplI, Bpu10I, BsaBI, BsaXI, BseMII, BseRI, BseYI, BsgI, BspMI, BsrBI, BsrDI, BstEII, BstXI, BtgZI, BtrI, BtsI, CfrI, Cfr10I, ClaI, DraIII, DrdI, Eam1105I, EcI, Eco31I, Eco47III, EcoP15I, EcoRI, EcoRV, Esp3I, FalI, FauI, Fnu4HI, FseI, FspAI, HaeII, HaeIV, Hin4I, HindII, HindIII, HpaI, KpnI, MauBI, MfeI, MluI, MmeI, NaeI, NarI, NcoI, NheI, NmeAIII, NotI, NruI, NspI, OliI, PasI, PflMI, PleI, PmeI, PpiI, PshAI, PI-PspI, PspXI, PsrI, PstI, PvuI, PvuII, RsrII, SacI, SacII, SalI, SanDI, SapI, ScaI, PI-SceI, SexAI, SfiI, SgfI, SgrAI, SgrDI, SmaI, SnaBI, SpeI, SphI, SrfI, Sse8387I, StuI, SwaI, TaqII, TauI, TseI, TspGWI, TstI, Tth111I, XbaI, XcmI, XhoI, Xmni

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
AclI	AACGTT	6	five_prime	1	447
Agsl	TTSAA	5	three_prime	2	352, 644
Alul	AGCT	4	blunt	5	261, 554, 958, 982, 1005
Apol	RAATTY	6	five_prime	2	891, 931
Avall	GGWCC	5	five_prime	1	821
Bccl	CCATC	5	five_prime	2	480, 984
BglII	AGATCT	6	five_prime	1	242
BsaAI	YACGTR	6	blunt	3	580, 624, 954
BsePI	GCGCGC	6	five_prime	1	617
BseSI	GKGCMC	6	three_prime	1	579
BsmAI	GTCTC	5	five_prime	2	630, 923
Bsml	GAATGC	6	three_prime	1	339

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
Bsp1407I	TGTACA	6	five_prime	3	20, 40, 265
BspHI	TCATGA	6	five_prime	1	678
Bsrl	ACTGG	5	three_prime	1	946
Cac8I	GCNNNGC	4	blunt	2	461, 619
CspCI	CAANNNNNTGG	7	three_prime	2	352, 387
CviJI	RGCY	4	blunt	8	261, 423, 554, 587, 958, 982, 995, 1005
Ddel	CTNAG	4	five_prime	1	596
DpnI	GATC	4	blunt	2	244, 318
Drall	RGGNCCY	6	five_prime	1	821
Eco57I	CTGAAG	6	three_prime	2	276, 998
Eco57MI	CTGRAG	6	three_prime	3	276, 755, 998
EcoNI	CCTNNNNNAGG	6	five_prime	1	416
EcoRII	CCWGG	5	five_prime	3	397, 529, 771
Fail	YATR	4	blunt	51	19, 25, 27, 29, 31, 33, 35, 37, 39, 51, 57, 64, 66, 68, 75, 100, 104, 106, 108, 115, 117, 121, 123, 125, 139, 147, 167, 172, 174, 196, 220, 222, 287, 329, 388, 426, 465, 505, 660, 668, 680, 686, 691, 717, 843, 867, 938, 940, 942, 971, 1010
FokI	GGATG	5	five_prime	1	22
GlaI	GCGC	4	blunt	2	618, 620
Gsul	CTGGAG	6	three_prime	1	755
HaeIII	GGCC	4	blunt	2	423, 995
Hgal	GACGC	5	five_prime	2	465, 843
Hhal	GCGC	4	three_prime	2	619, 621
HinfI	GANTC	4	five_prime	3	605, 776, 813
HpaII	CCGG	4	five_prime	1	508
HphI	GGTGA	5	three_prime	2	241, 367
Hpy188I	TCNGA	4	three_prime	1	295
Hpy99I	CGWCG	5	three_prime	1	459
Mael	CTAG	4	five_prime	1	825

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
Maell	ACGT	4	five_prime	4	447, 579, 623, 953
Maelli	GTNAC	4	five_prime	3	149, 377, 624
Mbcl	GATC	4	five_prime	2	242, 316
Mbcll	GAAGA	5	three_prime	3	202, 282, 703
Mnll	CCTC	4	three_prime	5	13, 25, 351, 726, 810
Mscl	TTAA	4	five_prime	16	91, 130, 229, 334, 435, 444, 470, 747, 862, 870, 885, 889, 928, 960, 964, 986
Mscll	CAYNNNNNRTG	6	blunt	1	48
Mwcl	GCNNNNNNNGC	4	three_prime	1	1011
Ndcl	CATATG	6	five_prime	1	172
NlalII	CATG	4	three_prime	5	21, 53, 507, 682, 1012
NlalIV	GGNNCC	4	blunt	3	410, 528, 822
Pacl	TTAATTAA	8	three_prime	2	889, 964
Pfol	TCCNGGA	6	five_prime	1	771
Pmacl	CACGTG	6	blunt	1	580
PpuMI	RGGWCCY	7	five_prime	1	821
Psil	TTATAA	6	blunt	1	660
Rsal	GTAC	4	blunt	5	22, 42, 111, 235, 267
ScrFI	CCNGG	4	five_prime	4	399, 509, 531, 773
Sdul	GDGCHC	6	three_prime	1	579
Setl	ASST	4	three_prime	16	235, 254, 263, 285, 325, 357, 450, 528, 556, 582, 626, 745, 956, 960, 984, 1007
SfaNI	GCATC	5	five_prime	1	411
Smcl	CTYRAG	6	five_prime	1	730
Sspl	AATATT	6	blunt	1	299
Styl	CCWWGG	6	five_prime	1	906
Taql	TCGA	4	five_prime	2	454, 720
Tatl	WGTACW	6	five_prime	4	20, 40, 109, 265
Tfil	GAWTC	5	five_prime	3	605, 776, 813
Tscl	TARCCA	6	three_prime	2	167, 270

Name	Sequence	Site Length	Overhang	Frequency	Cut Positions
Tsp45I	GTSAC	5	five_prime	1	377
TspDTI	ATGAA	5	three_prime	4	66, 83, 667, 925
TspEI	AATT	4	five_prime	8	226, 436, 838, 886, 891, 924, 931, 961
TspRI	CASTG	5	three_prime	1	981
Vspl	ATTAAT	6	five_prime	1	435
XholI	RGATCY	6	five_prime	1	242

Table A6-1: Score sheet for amplicons from microsatellite primers.

Primer					Acs16										Acs1		2Ab2A5		2Ab2B7		2Ab2D11		Acs21	
Band (bp)	Sample	Location	Date	Acs16-2000	Acs16-1700	Acs16-1000	Acs16-800	Acs16-650	Acs16-600	Acs16-450	Acs16-380	Acs16-330	Acs16-250	Acs16-180	Acs1-280	Acs1-220	Acs1-180	2Ab2A5-230	2Ab2A5-150	2Ab2B7-380	2Ab2B7-250	2Ab2D11-200	Acs21-250	
1	GL1	Mitchell River	25/02/2012	1	1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	1	1	1	0	0
2	GL2	Gippsland Lakes	25/02/2012	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0	0
3	GL3	Mitchell River	25/02/2012	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0	0
4	GL4	Mitchell River	25/02/2012	1	1	0	0	0	1	1	0	0	1	0	0	1	1	1	1	0	0	1	0	0
5	GL5	Mitchell River	25/02/2012	1	1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	0	0	0	0	0
6	GL6	Mitchell River	25/02/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
7	GL7	Mitchell River	25/02/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0
8	GL8	Mitchell River	25/02/2012	1	1	0	0	0	1	1	0	0	0	0	0	0	0	1	1	1	0	0	1	0
9	GL9	Mitchell River	25/02/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	1	0
10	GL10	Mitchell River	8/04/2012	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	1	0
11	GL11	Mitchell River	8/04/2012	1	1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	1	0	0	1	0
12	GL12	Mitchell River	8/04/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	1	0
13	GL13	Mitchell River	8/04/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	1	1	0
14	GL14	Tambo River	8/04/2012	1	1	0	0	0	1	1	0	0	0	0	0	0	1	1	0	1	0	0	1	0
15	GL15	Tambo River	8/04/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1	0
16	GL16	Tambo River	8/04/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0
17	GL17	Tambo River	8/04/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	1	0
18	GL18	Tambo River	8/04/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	1
19	GL20	Mitchell River	21/07/2012	1	1	0	0	0	0	1	1	0	0	1	0	0	1	1	1	1	1	1	1	0
1	GL21	Mitchell River	21/07/2012	1	1	0	0	0	0	1	1	0	0	1	0	0	0	1	0	1	0	1	0	0
2	GL22	Mitchell River	21/07/2012	1	1	0	0	0	0	1	1	0	0	1	1	0	1	1	0	1	0	1	1	0
3	GL23	Mitchell River	21/07/2012	1	1	0	0	0	0	1	1	0	0	1	0	0	1	1	1	1	0	0	0	0

4	GL25	Mitchell River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	0	1	1	1	0	0	0	0
5	GL26	Tambo River	21/07/2012	1	1	0	0	0	1	1	0	0	1	0	0	0	1	0	1	0	1	0	0
6	GL36	Tambo River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	0	1	1	1	0	1	1	1
7	GL43	Tambo River	21/07/2012	1	1	0	0	0	1	1	0	0	1	0	0	0	1	1	1	0	1	0	0
8	GL57	Mitchell River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	1	1	1	0	0	0
9	GL58	Mitchell River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	0	1	0	1	1	0
10	GL59	Mitchell River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	1	1	0	1	0	0
11	GL63	Tambo River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	0	1	0	1	0	1
12	GL65	Newlands Arm	21/07/2012	1	1	0	0	0	1	1	0	0	1	0	0	1	1	1	1	0	1	0	0
13	GL66	Newlands Arm	21/07/2012	1	1	0	0	0	1	1	0	0	1	0	0	1	1	0	1	1	1	1	0
14	GL83	Tambo River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	0	1	1	1	0	0
15	GL93	Nicholson River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	0	1	0	1	1	0
16	GL94	Nicholson River	21/07/2012	0	0	0	0	0	0	0	0	0	1	0	0	0	1	1	1	0	1	1	0
17	GL98	Eagle Point	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	1	1	0	0	0	1
18	GL99	Eagle Point	21/07/2012	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
19	GL100	Eagle Point	21/07/2012	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
1	GL101	Eagle Point	21/07/2012	1	1	0	0	0	1	1	0	0	0	1	0	1	0	1	1	0	1	1	1
2	GL103	Eagle Point	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	1	1	1	1	1	1
3	GL108	Tambo River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	1	1	1	1	1	0
4	GL118	Tambo River	21/07/2012	1	1	0	0	0	1	1	0	0	0	1	0	1	1	0	1	0	1	1	0
5	GL120	Nicholson River	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	0	1	1	0	1	0	1	1	0
6	GL127	Gippsland Lakes	21/07/2012	1	1	0	1	0	1	1	0	1	1	0	1	1	0	1	0	1	0	1	0
7	GL128	Gippsland Lakes	21/07/2012	1	1	0	0	0	1	1	0	1	0	1	0	1	1	1	1	0	0	1	0
8	GL129	Gippsland Lakes	21/07/2012	1	1	0	0	0	1	1	0	0	1	1	1	0	1	0	1	1	1	0	1
9	GL130	Gippsland Lakes	21/07/2012	1	1	0	1	0	1	1	0	1	1	1	1	0	1	0	1	1	1	1	1
1	LT1	Lake Tyers	21/07/2012	1	1	1	0	0	0	0	1	0	0	0	0	0	1	1	1	0	0	1	0
2	LT2	Lake Tyers	21/07/2012	1	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1	0	1	0	0
3	LT3	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	0	1	0	1	0	0
4	LT4	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	1	0	0	1	1	1	0	0

5	LT5	Lake Tyers	21/07/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	1	0	0
6	LT6	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	0	1	1	0	0	1
7	LT7	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	1	1	0	0	1	0	1	1	1	1	1
8	LT8	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	0	1	0	1	1	1
9	LT9	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	1	1	1	1	0	0
10	LT10	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	0	1	0	1	0	0	1	0
11	LT11	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	1	1	0	0	1	0	1	1	1	1	0
12	LT12	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	0	1	0	0	0	0
13	LT13	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	1	1	0	1	0	0
14	LT14	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	1	1	0	1	1	1	1	0	1	0	0
15	LT15	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	0	1	1	1	0	1
16	LT16	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	1	1	1	1	0	1
17	LT17	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	1	1	1	0	1	0
18	LT18	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	0	1	0	1	0	1	0	1
19	LT19	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	0	1	0	1	0	0	0	1
1	LT20	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	0	1	1	1	1	0	0	0
2	LT21	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	0	1	1	1	1	0	0	0
3	LT22	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	0	0
4	LT23	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	1	0	0
5	LT24	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	1	1	0	1	0	0
6	LT25	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	0	1	1	1	1	0	0	1
7	LT26	Lake Tyers	21/07/2012	1	1	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	1	1	0
8	LT27	Lake Tyers	21/07/2012	1	1	0	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	0	0
9	LT28	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	1	1	0	1	0	0	0	0
10	LT29	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	1	1	1	1	0	0	0	0
11	LT30	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	1	0	1	0	0	0	0	0
12	LT31	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	0	1	1	1	0	1	1	0
13	LT32	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	0	1	1	1	0	0	1	0
14	LT33	Lake Tyers	21/07/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0

15	LT34	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	0	0	0	1	1	1	0	0	0	0
16	LT37	Lake Tyers	21/07/2012	1	1	0	0	0	0	0	1	0	0	1	0	0	1	1	1	0	0	0	0
17	LT38	Lake Tyers	21/07/2012	1	1	0	0	0	0	0	1	0	0	1	0	1	1	1	1	0	1	1	0
18	LT40	Lake Tyers	21/07/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0
19	LT41	Lake Tyers	21/07/2012	1	1	1	0	1	1	0	1	0	0	1	0	1	1	0	1	0	1	1	0
1	LT42	Lake Tyers	21/07/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0
2	LT43	Lake Tyers	21/07/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	1	0
3	LT44	Lake Tyers	21/07/2012	1	1	1	0	1	0	0	1	0	0	0	0	0	1	1	1	0	1	0	0
4	LT45	Lake Tyers	21/07/2012	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0
5	LT46	Lake Tyers	21/07/2012	1	1	1	0	1	0	0	0	0	0	0	0	0	1	1	1	1	0	1	0
6	LT47	Lake Tyers	21/07/2012	1	1	1	0	1	0	0	1	0	0	0	0	0	1	1	0	1	0	1	0
7	LT48	Lake Tyers	21/07/2012	1	1	1	0	1	0	0	1	0	0	0	0	0	1	1	0	1	0	1	1
8	LT49	Lake Tyers	21/07/2012	1	1	1	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	1
9	LT50	Lake Tyers	21/07/2012	1	1	1	0	1	0	0	1	0	0	0	0	0	1	1	0	1	0	1	0

