Effects of environmental stressors on the black bream *(Acanthopagrus butcheri).*

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

Doctor of Philosophy

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DECLARATION

The contents of this thesis are a result of work undertaken whilst officially enrolled for the degree of Doctor of Philosophy in the School of Applied Sciences, Department of Biotechnology and Environmental Biology of the College of Science, Engineering and Technology at the Royal Melbourne Institute of Technology. All procedures involving vertebrate animals (fish) were conducted in accordance with the RMIT University Animal Ethics Committee, under the approved project AEC 0523.

To the best of my knowledge, all work performed by others, published or unpublished, has been duly acknowledged in this thesis.

This thesis has not been submitted, in whole or in part, to qualify for any other award.

Signed:……

Date:……

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- ***Kathryn Hassell***, Patrick Coutin, Dayanthi Nugegoda. "Climate change: impacts on embryos of the estuarine black bream". Australian Marine Sciences Association (AMSA) Conference, Melbourne, Australia. June, 2007. (ORAL PRESENTATION)

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%S	Percent saturation (of dissolved oxygen in water)
<	Less than
>	Greater than
50X	Fifty times magnification
AEC	Animal Ethics Committee
ALP	Alkaline labile phosphorous
am	Ante meridiem (after midnight)
ANOVA	Analysis of variance
ANZECC	Australian and New Zealand Environment and Conservation Council
ATP	Adenosine triphosphate
B	Blastodisc
b.d.	Below detection
BSA	Bovine serum albumin
C	Chorion
CAC	Controlled atmosphere chamber
CaCl	Calcium chloride
CAF	Central Ageing Facility
CAT	Catalase
CCO	Cytochrome c oxidase
cm	Centimetre
cm^3	Cubic centimetre
CS	Citrate synthase
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CV(%)	Coefficient of variation
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DNH	Did not hatch
DO	Dissolved oxygen
dpf	Days post fertilisation
DPI	Department of Primary Industries
E1	Estrone
E2	17β-estradiol
EDC	Endocrine disrupting chemical
EE2	17α-ethinylestradiol
ELISA	Enzyme linked immunosorbent assay
EPA	Environmental Protection Authority
EPO	Erythropoietin
EROD	Ethoxyresorufin-*O*-deethylase
FL	Fork length

g	Gram
g/l	Grams per litre
GAM-HRP	Goat anti-mouse horseradish peroxidase
GSI	Gonadosomatic index
GtH-1	Gonadotropin hormone 1
h	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	sulphuric acid
HIF-1	Hypoxia inducible factor 1
HIS	Hepatosomatic index
hpf	Hours post fertilisation
i.p.	Intraperitoneal
IAPE	Index of average percent error
IPCC	Intergovernmental Panel on Climate Change
IU	International unit
IU/kg	International units per kilogram
K_2HPO_4	Dipotassium phosphate (dibasic potassium phosphate)
KCl	Potassium chloride
kDa	Kilodaltons
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate (monobasic potassium phosphate)
km	Kilometre
km^2	Square kilometre
l	Litre
l/h	Litres per hour
LC50	50% Lethal effect concentration
LDH	Lactate dehydrogenase
LML	Legal minimum length
LPOX	Lipid peroxidation
LSI	Liver somatic index
LUX	SI unit of illuminance
m	Metre
M	Molarity
MDH	Malate dehydrogenase
mg	Milligrams
mg/l	Milligrams per litre
$MgSO_4$	Magnesium sulphate
min	Minute
ml	Millilitre
Ml	Megalitre

mm	Millimetre
mM	Millimolar
mmHg	Millimetres of mercury
MW	Molecular weight
N	Normality (unit for measuring concentration)
N_2	Nitrogen gas
NaCl	Sodium chloride
$NaHCO_3$	Sodium carbonate
ND-1C8	Monoclonal vitellogenin primary antibody from *Morone saxitilis*
NEG	Negative (control used for ELISA and Western blot)
ng/ml	Nanograms per millilitre
NLWRA	National Land and Water Resources Audit
nm	Nanometre
NSB	Non-specific binding
O_2	Oxygen gas
O_2/l	Oxygen gas per litre
^oC	Degrees Celcius
OECD	Organisation for Economic Co-operation and Development
OG	Oil globule
OMZ	Oxygen minimum zones
OPD	*o*-phenylenediamine dihydrochloride
PAH	Polycyclic aromatic hydrocarbon
PBDE	Polybrominated diphenyl ether
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCDD	Polychlorinated dibenzodioxin
PCDF	Polychlorinated dibenzofuran
pers. comm.	Personal communication
pm	Post meridiem (after midday)
POS	Positive (control used for ELISA and Western blot)
ppt	Parts per thousand
PVDF	Polyvinylidene difluoride
RIA	Radioimmunoassay
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Rainbow trout
RT-VtgEq	Rainbow trout vitellogenin equivalent concentration
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

sec	Seconds
SEM	Standard error of the mean
SOD	Superoxide dismutase
TBT	Tributyltin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TL	Total length
USEPA	United States Environmental Protection Agency
UV	Ultraviolet radiation
V	Volts
v/v	Volume per volume
Vtg	Vitellogenin
w/v	Weight per volume
µg	Micrograms
µg/ml	Micrograms per millilitre
µl	Microlitre
µM	Micromolar
µS/cm	Microseimens per centimetre

SUMMARY

Australia is the world's driest inhabited continent and most Australian rivers have very low environmental flows and are subject to water extraction for agriculture. Many of the 1000 estuaries that account for approximately 40% of coastal waterways are considered modified or degraded. In Victoria, estuarine environments are increasingly subject to a variety of environmental stressors, due to reduced environmental flows, urbanisation, anthropogenic pollution and climate change. This PhD study has investigated the effects of selected environmental stressors on aspects of the reproductive biology in a representative estuarine fish, the native black bream (*Acanthopagrus butcheri*).

Through a series of laboratory-based experiments, and a field study that sampled black bream from five Victorian estuaries, the effects of selected environmental stressors have been described and new techniques have been developed. Firstly, in order to distinguish normal development from abnormal development, the different stages of black bream embryo development were described in detail, and a total of 20 developmental stages were identified. Abnormal development in both embryos and larvae was also described. A series of experiments were then conducted to observe the effects of different environmental conditions on embryo and larval survival.

Hypoxia (low dissolved oxygen – measured as percent saturation %S) was shown to be an important environmental stressor during the early life stages which caused detrimental effects on embryo development and subsequent survival and hatching. Severe hypoxia (30%S) reduced one day survival by 60%, and caused abnormal embryo development

that always resulted in mortality and thus hatch rates of 0%. Exposure to moderate hypoxia (45%S) delayed hatching by 7-8 h, reduced hatch rates by 25% and always produced deformed larvae that died within 24 h of hatching. For embryos exposed to 55%S, time to hatching and hatch rates were not affected, however larval lengths, measured on Day 2 post-hatch were significantly lower, with mean lengths of 2.41 mm as compared to 2.53 mm for those maintained in normoxic (85%S) conditions. In addition, other stressors such as altered salinity and temperature regimes were shown to have interactive effects with dissolved oxygen, such that embryos that were exposed to multiple stressors displayed reduced survival and hatching.

When embryos were exposed to low salinity and low temperature in combination with moderate hypoxia, hatch rates were reduced by 5-35% and the rates of deformities increased. Exposure to low temperature (16^{o}C) delayed hatching by 20 h compared to embryos maintained at 20^{o}C. For embryos exposed to low salinity and high temperature in combination with moderate hypoxia, one day survival was reduced by 5-70% and the interaction between the three variables was significant (p = 0.005), whilst hatch rates were reduced by 5-50%. Time to hatching was more variable (spread over 1-3 days) in embryos exposed to a combination of stressors, and an elevated temperature (26^{o}C) resulted in hatching 12 h earlier than embryos maintained at 22^{o}C. Finally, high salinity (45 ppt) in combination with hypoxia (severe or moderate) significantly affected one day survival, hatch rates and larval survival to Day 2 post-hatch. Exposure to hypoxia also reduced survival of newly hatched larvae. After 4 h exposure to severe hypoxia (20%S), all larvae were exhibiting signs of stress and morbidity, while larvae in moderate hypoxic

(50%) or normoxic (85%) conditions appeared normal. Within 24 h nearly all larvae exposed to severe hypoxia had perished, and by 84 h all larvae exposed to moderate hypoxia had perished, whilst survival of larvae in normoxic conditions was >70%. The combinations of environmental conditions tested were based on water quality characteristics that occur within the Gippsland Lakes, which supports Victoria's largest black bream population. The findings clearly indicate that water quality is a major determinant for early life stage survival, suggesting the degraded environmental conditions within the Gippsland Lakes are a likely cause, or at least a contributing factor to the recruitment variability observed in the Gippsland Lakes black bream population.

In order to test the effects of hypoxia on the early life stages of black bream in the laboratory, a novel chamber system was designed that enabled precise control of dissolved oxygen. The Controlled Atmosphere Chamber, a clear, air tight, Perspex chamber provides a simple, cost-effective means of controlling atmospheric oxygen levels that can be manipulated to control dissolved oxygen levels in small test wells that are placed inside the chamber. This novel chamber system was successfully used to create hypoxic conditions to which black bream embryos and newly hatched larvae were exposed.

In order to measure reproductive condition in mature female black bream, experiments were conducted to measure the egg yolk precursor protein vitellogenin (Vtg), which circulates in the blood plasma of sexually mature female fish. Vitellogenin production was induced in adult black bream and a suitable primary antibody (ND-1C8) was

established by Biosense Laboratories in Norway. Upon establishing a suitable, commercially available primary antibody, two immunological assays for measuring black bream Vtg were developed for the first time. An enzyme-linked immunosorbent assay (ELISA) and Western blot assay were developed and both techniques were optimised then used to measure Vtg in both blood plasma as well as surface mucus. The detection of Vtg in surface mucus of black bream has not been reported previously, and with further development could potentially represent a new method for assessing reproductive condition (and potentially to distinguish females from males) that is both non-destructive and non-invasive. Such a technique has wide application in the fields of both fisheries biology and environmental monitoring. Furthermore, exposure to a potent estrogen (17β-estradiol) stimulated Vtg production in male black bream, indicating that, again with further development the assays could be used to screen for exposure to estrogenic endocrine disrupting chemicals in males.

Following development of the two Vtg assays, the techniques were then validated by measuring Vtg levels in blood plasma of black bream from a range of Victorian estuaries that are subject to different types of environmental stressors. There was one reference site, two sites in close vicinity to a large sewage treatment plant, and two urban sites subject to both industrial effluents and storm water runoff. Vitellogenin was detected in some female fish from all sites, at varying concentrations. The highest concentration of Vtg (measured as rainbow trout Vtg equivalent concentrations) was 59.5 mg/ml RT-VtgEq, in a 245 mm, 302 g, 3 year old fish from the Werribee River. The smallest

female with detectable levels of Vtg (0.73 mg/ml RT-VtgEq) was a 170 mm, 86 g, 2 year old fish from the Yarra River.

Vitellogenin was detected in all female black bream that had vitellogenic or maturing ovaries, whilst females with immature, regressed or spent ovaries did not have detectable levels of Vtg in blood plasma. This is the first time that Vtg has been related to ovarian condition in black bream, and represents a new tool for monitoring reproductive condition in females as well as providing a screening tool for exposure to estrogenic chemicals in males too. There was no evidence of any Vtg induction in male fish from any of the field sites, despite some of them exhibiting a distinct ovotestis that contained ovarian tissue within an otherwise mature testis.

Environmental conditions, both manipulated in the laboratory and measured in different field locations were shown to affect both spawning success (early life stage survival) and reproductive condition in black bream. These findings indicate that black bream are sensitive to environmental stressors, and that wild populations are likely to be affected by environmental degradation, pollution and climate change.

CHAPTER 1: INTRODUCTION

This thesis investigates the effects of selected environmental stressors on spawning success in black bream in the laboratory and the reproductive condition of adult black bream from a range of Victorian estuaries with different environmental conditions. The hypothesis is that natural and anthropogenic factors may affect both survival in the early life stages and reproductive condition in mature adults. The ecological processes underlying reproductive success are often complex and for most species poorly described or unknown. Understanding the causes of reproductive failure, in terms of both adult fitness and mortality in the early life stages are important for fisheries and environmental management because it underpins recruitment variation, and understanding these processes will help to improve monitoring programs, stock assessment models and management strategies for fisheries, habitats and water resources. If natural environmental conditions are contributing to recruitment variation, then it will be useful to predict population trends with 'average' and 'extreme' conditions and management strategies can be adjusted accordingly. If there are anthropogenic factors, such as altered river flows or pollution, then it may be possible to reduce their effects on fish populations by adopting different management strategies. Australia is the driest inhabited continent and is currently experiencing a severe drought. Drought, combined with climate change has the potential to substantially alter environmental conditions within estuarine environments and thus influence fish recruitment.

1.1 Environmental stressors

The term 'environmental stressor' relates to any external factor, of biological, chemical or physical nature that causes a disturbance to the normal physiological state of an organism. Stress physiology is the study of organism responses to factors that extend the physiological state beyond the normal resting state (Yousef, 1985; Barton, 1997).

There are a number of environmental variables that only become stressors when they exist at levels outside the normal range for a particular environment. For example, temperature does not adversely affect the physiological state of salmonid fish between a range of 4-15^{o}C, however above or below this temperature range physiological stress becomes apparent (Iwama *et al.*, 1999). Other examples of environmental variables that have normal 'non-stressful' lower and upper ranges include dissolved oxygen (DO), salinity, water hardness and pH. Other types of environmental stressors include pollutants, contaminants and xenobiotics. Within estuarine environments, a number of environmental stressors may occur, and three physico-chemical factors that play vital roles in determining the ability of an estuary to sustain life are DO, salinity and temperature.

1.1.1 Dissolved oxygen and hypoxia

Oxygen plays an essential role in nearly all living organisms, enabling aerobic cellular respiration to occur to generate metabolic energy in the form of adenosine triphosphate (ATP) (Campbell, 1996). Whilst different organisms have evolved different mechanisms for obtaining and utilising oxygen from the surrounding environment, the need for

oxygen for respiration remains. For aquatic organisms, oxygen is mostly obtained from water in the form of DO, however some species, including certain fishes are capable of obtaining gaseous oxygen by breathing air (Evans and Claiborne, 2006). The amount of oxygen gas that can be dissolved in water depends on the temperature, ambient pressure (altitude) and salinity of the aquatic environment (Colt, 1984). Various units for describing DO concentrations have been developed, and under conditions of 20 parts per thousand (ppt) salinity, 25^{o}C and 1 atmosphere pressure, 1.0 ml O_2/l is equivalent to 1.4 mg/l, 23.9 mmHg, 45.7 mM and 14% saturation (%S) (Diaz and Rosenberg, 1995). Percent saturation (%S) is a measure of the amount of oxygen gas dissolved in water, and is expressed relative to air saturation, which is 100% (Diaz and Rosenberg, 1995).

The term hypoxia describes low DO conditions in aquatic environments, whereby levels of oxygen dissolved in water fall below 2.0 ml O_2/l (~35%S; 2.9 mg/l), whilst oxygen minimum zones (OMZs) are areas that are subject to severe hypoxia (<0.5 ml O_2/l) (Diaz and Rosenberg, 2008), and anoxic environments contain no oxygen (0.0 ml O_2/l) (Diaz and Rosenberg, 1995; Wu, 2002). Recently it has been suggested that the generally accepted threshold level for defining hypoxia (2.0 ml O_2/l) may severely underestimate effects on marine organisms, and a revised threshold of 4.6 mg/l (~62.5%S; 3.2 ml O_2/l), based on mean lethal concentrations for a range of fish and invertebrate species should be considered (Vacquer-Sunyer and Duarte, 2008). Indeed, Breitburg (2002) reported that sensitive fish species show signs of physiological stress, such as avoidance behaviour and reduced growth when DO levels fall below 50%S.

It has been suggested that hypoxia may be the most widespread environmental stressor responsible for adverse effects on estuarine and marine fauna (Diaz and Rosenberg, 1995; Diaz and Rosenberg, 2008). Hypoxia in shallow marine and coastal environments is a global issue that is responsible for a range of environmental problems that result in reduced species abundance and distribution as well as fisheries declines (Diaz and Rosenberg, 1995; Wu, 2002; Breitberg *et al.*, 2003). Vast areas of coastal and shallow marine waters may become devoid of marine life due to hypoxia, and have been termed 'dead zones' (Rabalais *et al.*, 2002). The frequency of hypoxic events, and areas designated 'dead zones' is continually increasing worldwide, largely due to anthropogenic inputs (Diaz and Rosenberg, 1995; Rabalais *et al.,* 2002; Wu, 2002; Diaz and Rosenberg, 2008) (Fig. 1.1).

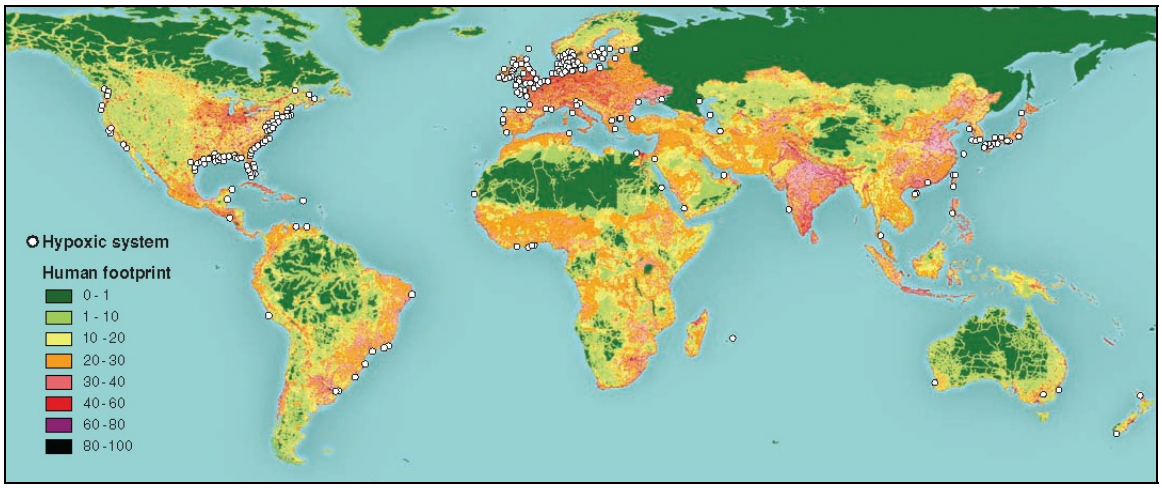


Figure 1.1 Global distribution of 'dead zones' due to hypoxia, and associated degree of human influence (human footprint) on those areas (Source: (adapted from) Diaz and Rosenberg, 2008).

The Australian and New Zealand Guidelines for Fresh and Marine Water Quality have assigned lower and upper guideline values for DO as 80%S and 110%S for estuarine and 90%S and 110%S for marine areas in south-east Australia, whilst for tropical Australia the guideline values are 80%S and 120%S for estuaries with a lower limit of 90%S for marine waters (ANZECC/ARMCANZ, 2000). No data is currently available on the upper limit for DO in tropical marine waters in Australia.

Hypoxia may occur naturally due to processes such as low water flow or limited water-atmosphere gas exchange and may be intensified by the presence of vertical stratification due to haloclines or thermoclines (Diaz and Rosenberg, 1995; Wu, 2002; Breitberg *et al.*, 2003). Hypoxia may also occur when systems become overloaded with organic matter by the process of eutrophication (Wu, 1999; Breitberg, 2002; Wu, 2002). Photosynthetic water plants contribute greatly to oxygen synthesis during daylight hours, however at night time when no photosynthesis occurs, respiration continues, causing a reduction in DO levels. When measuring DO it is important to take diel variations into consideration, because the levels will naturally oscillate throughout a 24 h period due to variations in rates of photosynthesis and respiration.

Prolonged hypoxia can alter water chemistry and thereby increase the toxicity of various compounds (zinc, copper, lead, pentachlorophenol, cyanide, hydrogen sulfide and ammonia) (Diaz and Rosenberg, 1995) and it may also change the redox state at the sediment surface leading to reducing conditions that alter fluxes of nutrients, metals and other compounds (Rosenberg *et al*., 2001). Another consequence of prolonged hypoxia

and anoxia in sediments is the occurrence of sulfur bacteria near the sediment-water interface (rather than deep in the sediments where they are usually found) which results in increases in concentrations of toxic hydrogen sulfide which in turn may exacerbate the effects of hypoxia and alter pH levels (Diaz and Rosenberg, 1995). Thus, it is not only the direct effects of low oxygen availability that cause problems for biota, but it is also the indirect effects of changing water chemistry on the bioavailability of other stressors that contribute to the overall problem of hypoxia.

1.1.1.1 Physiological effects of hypoxia on the early life stages of fishes

The early life stages are considered to be the most sensitive periods of development in fishes (Pickering and Gast, 1972; McKim, 1985), during which time many physiological changes occur that may be affected by environmental stressors. Oxygen is required throughout these stages, enabling cell division and growth, tissue and organ development, enzyme and hormone production and all the other cellular events that take place during normal development. Exposure to hypoxia is thus likely to affect these processes.

Fuda *et al.* (2007) studied the effects of hypoxia and other environmental factors on early life stage rainbow smelt (*Osmerus mordax*), a small anadromous fish found throughout North America. Embryos and yolk-sac larvae were exposed to 4 DO levels (10, 20, 40 and 60%S) and no hatching was observed at 10%S, whilst for all other treatments, 100% hatching occurred and larval survival was high (>90%). In embryos of the dogfish (*Scyliorhinus canicula*), a marine elasmobranch, Diez and Davenport (1990) reported that exposure to 20%S resulted in 100% mortality after three weeks, whilst those exposed to

50%S showed no mortality. Similarly, Keckeis *et al.* (1996) observed increased larval mortality and reduced hatching success in embryos of the freshwater cyprinid, nase (*Chondrostoma nasus*) following exposure to 10%S. Czerkies *et al.* (2001) studied hatching success in embryos of two freshwater salmonids, whitefish (*Coregonus lavaretus*) and vendace (*C. albula*) following exposure to hypoxia. They observed precocious hatching when DO levels were low, and the incidence of precocious hatching increased as the length of exposure to hypoxia increased. Mortality in some eggs was attributable to incomplete hatching, caused by the embryos being too weak to move and disrupt the thin *zona radiata externa* membrane. Hatching involves two processes, the production of proteolytic enzymes which soften the chorion, and tail movements by the larvae, which allow it to break free of the surrounding egg membrane (Yamagami *et al.*, 1992; Czerkies *et al.*, 2001). Failure to hatch following exposure to hypoxia may be due to disruptions in the production of hatching enzymes and/or reduced strength in the tail musculature. Clearly hypoxia affects hatching success in both freshwater and marine fish species and the likely effects of reduced hatching are changes in recruitment and ultimately species abundance and distribution.

In a study of ichthyoplankton distribution in open ocean currents around southern Africa, Ekau and Verheye (2005) found that hypoxia was an important factor in determining species distribution. They reported that the distribution within the water column of eggs and larvae of three pelagic fish species, sardine (*Sardinops sagax*), anchovy (*Engraulis encrasicolus*) and horse mackerel (*Trachurus trachurus capensis*) were significantly

correlated with hypoxia, and no eggs or larvae were collected from areas where the DO levels were below 2.5 mg/l (<20%S).

Another effect that has recently been described is the effect that hypoxia has on early life stage developmental processes that eventually govern sex differentiation (Wu *et al.*, 2003; Shang and Wu, 2004; Shang *et al.*, 2006). Following a 4 month exposure to hypoxia (0.8 mg/l ~ 10%S) throughout both the embryonic and larval stages, Shang *et al.* (2006) reported male-biased populations of the freshwater cyprinid, zebrafish (*Danio rerio*). They reported growth reductions in hypoxia-exposed fish, as well as down regulation of several genes that are involved in the synthesis of sex hormones and an increase in the testosterone/17β-estradiol ratio. A long term change in the sex ratio has the potential for serious impacts on both reproductive success and population sustainability.

The detrimental effects that hypoxia can have on the early life stages of fishes, from reduced hatching success and increased rates of deformities, to higher mortality all translate into negative effects on species abundance and distribution, highlighting the importance of continuing research on this major environmental stressor to the sensitive early life stages. Furthermore, the mobility of the early life stages is much lower than older stages, making them especially susceptible, since they cannot move away from hypoxic conditions easily.

1.1.1.2 Physiological effects of hypoxia on juvenile and adult fishes

The physiological response to hypoxia depends on a number of factors, not simply the DO concentration. Jensen *et al.* (1993) state that the severity of the response to hypoxia depends on the fish species, the level of hypoxia and the duration of exposure, whilst others have reported that the size of the fish is also very important, with larger fish employing anaerobic pathways more frequently than smaller fish (Almeida-Val *et al.*, 2000; Cooper *et al.,* 2002). Cerezo *et al.* (2006) established categories of oxygen levels (optimal, sub-optimal, dangerous and lethal) for a deep water marine sparid species (*Dentex dentex*) based on ventilatory frequency and oxygen consumption in different size ranges and temperatures. They reported that optimal DO levels were >70%S, sub-optimal levels were 35-70%S and anything below 35%S was dangerous.

Wu and Woo (1985) tested hypoxia tolerance and respiratory responses in two marine species, the red grouper (*Epinephelus akaara*) and black seabream (*Mylio macrocephalus*) and observed that both species exhibited tolerance to hypoxia, yet black seabream was more tolerant than red grouper. They reported that the critical tolerance, or threshold levels for mortality were very narrow in both species, and that both were oxygen conformers throughout a wide range of DO levels (0.5-7.0 mg/l ~6-90%S).

In a study of tropical pelagic fishes from both the eastern tropical Pacific (ETP) and Atlantic Oceans, Prince and Goodyear (2006) reported changes in species distribution within the water column due to increasing areas of cold hypoxic water as a result of nutrient upwelling. Distinct stratification resulted in reduced areas of suitable conditions,

leading to habitat compression, as all fishes were forced to move into shallow, well oxygenated waters. Habitat compression may lead to enhanced feeding opportunities due to a concentration of resources in a smaller area, however it also makes the fish more vulnerable to targeted fishing efforts (Prince and Goodyear, 2006) and increased predation by seabirds, sharks and marine mammals. Reductions in suitable habitat due to increasing areas of hypoxia are a major threat to the long term survival of species.

Regulation of the genes that are induced by hypoxia is under the control of a heterodimeric transcription factor called hypoxia-inducible factor 1 (HIF-1) (Nikinmaa, 2002; Wu, 2002). Upon signals from molecular oxygen sensors indicating hypoxia, HIF-1 allows transcription of hypoxia-inducible genes including glucose transporters (Ebert *et al.*, 1995; Nikinmaa, 2002), glycolytic enzymes such as LDH (Cooper *et al.*, 2002) and erythropoietin (EPO) for increasing red blood cell production (Guillemin and Krasnow, 1997; Wu, 2002). In the longjaw mudsucker (*Gillichthys mirabilis*), an estuarine fish capable of breathing air for short periods, down-regulation of genes involved in protein synthesis, up-regulation of genes involved in anaerobic respiration and suppression of cell growth were reported mechanisms for surviving hypoxia (Gracey *et al.*, 2001). Understanding gene regulation and the importance of HIF-1 in coping with hypoxia will assist in the development of novel biomarkers for detecting exposure to hypoxia in fish and other organisms.

In a study of chronic hypoxia exposure, Thomas *et al.* (2006) reported significant impairment of ovarian growth in the estuarine sciaenid fish, Atlantic croaker

(*Micropogonias undulatus*). The gonadosomatic index (GSI), circulating levels of 17β-estradiol (E2) and vitellogenin (Vtg) were all significantly reduced in fish maintained in moderate (38%S) and low (24%S) DO conditions as compared to controls (80%S). Similarly, Landry *et al.* (2007) reported suppression of reproductive capacity in the estuarine Gulf killifish (*Fundulus grandis*), following exposure to prolonged hypoxia (18.3 ± 6.1%S). Both growth and reproduction were significantly reduced. The levels of sex steroids were significantly reduced in females (E2) and males (11-ketotestosterone) by 50% following chronic hypoxia exposure, and furthermore, egg production was significantly reduced and the onset of spawning was delayed in hypoxia-exposed females. However, this study reported no change in plasma Vtg levels between hypoxic and normoxic treatments, suggesting that hypoxia may delay or suppress Vtg uptake by oocytes rather than reducing synthesis of the protein (Landry *et al.*, 2007). Few studies have concentrated on linking exposure to hypoxia with Vtg production in females, yet since oxygen plays such a significant role in normal metabolism and energy production, chronic hypoxia is likely to affect the energy-intensive processes of egg production and thus result in reduced reproductive capacity.

Physiological responses to hypoxia vary greatly across species', depending on a range of factors including behaviour, adaptation, habitat preference, mobility and previous exposure. Ventilation rates may be increased, there may be changes in blood composition and binding capacity, shifts in energy production from aerobic to anaerobic metabolic pathways, reduced movement and altered oxygen consumption, changes in growth rates and feeding efficiency, up or down regulation of a number of hypoxia–

inducible genes and reduced reproductive capacity. Low DO is therefore a very important environmental stressor in fish, and is intrinsically linked to other environmental variables such as salinity and temperature, which affect the solubility of oxygen in water, and contribute to the formation of hypoxic zones below thermoclines and haloclines in stratified environments.

1.1.2 Salinity

Salinity is a measure of the total concentration of inorganic ions (salts) in a solution, including both cations (Na^+, Ca^{2+}, Mg^{2+}, K^+) and anions (SO_4^{2-}, CO_3^{2-}, HCO_3^{2-} , Cl^-). Salinity may be expressed by any of the following units: electrical conductivity (EC – measured as microseimens per centimeter, µS/cm), parts per thousand (ppt), total dissolved solids (TDS), total soluble salts (TSS) or practical salinity units (psu), all of which are equivalent to grams per litre, g/l (ANZECC/ARMCANZ, 2000). The salinity of freshwater environments is usually less than 1000 µS/cm (0.68 g/l) whilst marine environments are approximately 51 000 µS/cm (35 g/l) and estuaries are somewhere in between, depending on factors such as environmental flows and tidal influence.

The salinity of an aquatic environment is very important to the organisms that live within it, since it will strongly influence the movement of ions into and out of the organism, and as such, organisms exhibit physiological differences in osmoregulatory abilities. For example, freshwater fishes experience osmotic water gain across the gills and body surface and compensate by producing large quantities of dilute urine and gaining ions from food and active ion uptake (Evans and Claiborne, 2006). In contrast, marine fishes

experience osmotic water loss across the gills, so compensate by drinking water and actively secreting salt across the gills and body surface (Evans and Claiborne, 2006). Most species that occur in either freshwater or marine environments only are considered stenohaline, exhibiting a narrow salinity tolerance range, whilst many species that inhabit estuaries are considered euryhaline, with a wide salinity tolerance range (Evans and Claiborne, 2006). Furthermore, some species exhibit ontogenetic shifts in salinity tolerances, such as diadromous species (eg. eels, galaxids, salmonids, Australian grayling) that spend much of their life in marine environments before migrating into freshwater environments to spawn, or vice versa (Jobling, 1995; Evans and Claiborne, 2006).

In estuarine environments, salinity is dependent on a number of factors including freshwater (riverine) and tidal flows, evaporation and wind, all of which contribute to the degree of mixing or stratification. Salinity contributes to environmental stress if levels fall below or exceed the normal diurnal or seasonal range in the habitats of the resident species.

1.1.2.1 Physiological effects of salinity on the early life stages of fishes

The effect of salinity on fish eggs and larvae has been studied extensively, and some of the reported effects include: differences in egg size and osmotic pressure; retardation of embryo development; differences in time to hatching; increased mortality at hatching; incomplete hatching; differences in the osmotic pressure of body fluids, leading to differences in size; reduced heart rate; and increased larval mortality (see Holliday, 1969;

Boeuf and Payan, 2001). Haddy and Pankhurst (2000) studied black bream egg survival and hatching under different salinities, and reported significant reductions in low salinities. For salinities > 10 ppt, 24 h egg survival was high, but at 5 ppt survival was much lower and no embryos survived to day 1 at 0 ppt. Low hatching success (<50%) was observed at 5 and 10 ppt, whilst in salinities of 15 to 35 ppt hatching success was much better (>80%). Furthermore, the incidence of larval deformities, such as curvature of the spine and tail flexure was much higher in eggs incubated at salinities of 15 ppt and lower, and all eggs that hatched at 5 ppt were abnormal. Similarly, in Australian bass (*Macquaria novemaculeata*), a percichthyid species that spawns in estuaries, Van der Wal (1985) observed a complete cessation of development within 3 h of fertilisation for embryos held in freshwater (0 ppt). Hatch rates were low at 5 and 10 ppt and increased to a maximum at 25 to 35 ppt. He reported that even though some larvae hatched at 5 ppt, the larvae were of poor quality and all died within 2 d of hatching. In the greenback flounder (*Rhombosolea tapirina*), a coastal, pleuronectid flatfish species, Hart and Purser (1995) reported optimal fertilisation rates between 35-45 ppt, and observed no significant differences in hatch rates for embryos maintained between 15-45 ppt. In another flatfish species, the euryhaline southern flounder (*Platichthys lethostigma*), larval survival, body water percentage and larval osmolality were significantly higher in larvae maintained at 34 ppt compared to larvae maintained at 25 ppt (Moustakas *et al.*, 2004). The authors suggested that the differences may have been due to the fact that the larvae were buoyant at 34 ppt, but non-buoyant at 25 ppt and subsequently, the larvae in the 25 ppt treatment needed to actively maintain their vertical position, resulting in greater energetic costs and therefore lower growth and survival.

Egg buoyancy in marine and estuarine fishes is also affected by salinity. In low salinities eggs become negatively buoyant and sink, which occurs due to osmotic pressure differences between the egg and the surrounding environment, resulting in water movement across the permeable egg membrane into the perivitelline fluid, altering the specific gravity of the egg (Holliday, 1969). Egg buoyancy may also be affected by the lipid and water content, amount of protein in the yolk, size, diameter and egg shape (Craik and Harvey, 1987; Nissling *et al.*, 2003), and all of these factors relate to maternal effects on egg quality (Sakai *et al.*, 1985; Foscarini, 1988; Trippel *et al.*, 1997). When stratification of the water column is weak, egg buoyancy may be lost, causing the eggs to sink into colder water with low DO that may lead to increased mortality (Nissling *et al.*, 2003).

In a study that modeled the effects of hypoxia on eggs of the coastal dwelling bay anchovy (*Anchoa mitchilli*) in the Patuxent River, North America, Breitberg *et al.*, (2003) suggested that sinking eggs were at a much greater risk of increased mortality due to exposure to lethal DO levels. Similarly, in a study of ichthyoplankton ecology in the Hopkins River, Australia, Newton (1996) reported that spawning in two estuarine species, black bream and anchovy (*Engraulis australis*) was linked to both salinity and temperature and that the fish larvae were not found in the surface waters, but rather below the halocline in the salt wedge layer of the estuary. She suggested that due to the presence of a salt wedge, eggs were suspended within the water column above the bottom, out of severely hypoxic water, but below the halocline, which prevented the eggs from being washed out to sea by the out-flowing freshwater surface layer.

The physiological effects of altered salinity on the early life stages of fishes include disrupted embryonic development, increased rates of deformities, incomplete hatching (and thus lower hatch rates) and reduced larval survival. In addition, salinity has other significant influences on the early life stages, being responsible for determining the specific gravity and buoyancy of eggs which then determines where they will develop within the water column. In low salinities, marine and estuarine eggs are likely to lose buoyancy and sink into cold water that may be hypoxic or anoxic, whilst in high salinities the eggs are likely to float on the surface and be exposed to a greater intensity of ultra-violet (UV) radiation, higher temperatures and DO levels, as well as being more vulnerable to predation.

1.1.2.2 Physiological effects of salinity on juvenile and adult fishes

The physiological effects of salinity on juvenile and adult fishes have been described for a variety of freshwater, estuarine and marine fish species and include a range of effects such as altered osmoregulation and growth rates, changes in food consumption and drinking mechanisms, and changes in reproductive condition and spawning output (see Boeuf and Payan, 2001). When juvenile gray snapper (*Lutjanus griseus*), a reef species that utilises estuarine environments as nursery habitat, were grown at a range of different temperatures and salinities, Wuenschel *et al.* (2004) reported decreased feeding rates and growth efficiency as salinity increased. They concluded that the high salinities increased energetic costs, thus resulting in lower growth efficiency. Similarly, in juvenile European sea bass (*Dicentrarchus labrax*), a euryhaline marine species, Saillant *et al.* (2003) observed higher growth rates and survival in fish maintained at 15 ppt compared

to those maintained at 37 ppt. Furthermore, they reported that osmotic stress, such as a change from low to high salinity during the juvenile period, when sex determination occurs, resulted in a significant increase in the numbers of fish that became males.

When juvenile black bream were reared in salinities ranging from 0 to 60 ppt, Partridge and Jenkins (2002) observed changes in growth rates but no mortality. Maximum specific growth rates were observed in fish held at 24 ppt, which were significantly higher than fish held at 60 ppt, but not significantly different to fish in other salinity treatments (12, 36, 48 ppt). Salinity had a significant effect on food conversion ratios and fish maintained at 60 ppt consumed less food than other treatment groups. Osmotic stress in juvenile black bream resulted in lower specific growth rates and lower food consumption, higher plasma osmolality after 3 months exposure and significantly increased water content at the completion of the 6 month trial.

Haddy and Pankhurst (2000) described the effects of salinity on reproductive development and endocrinology in black bream over a 6 month study period. They reported that whilst gonadal maturation was not affected by exposure to low salinities (5, 20 ppt) compared to a control salinity of 35 ppt, levels of the reproductive hormones 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 11-ketotestosterone were lower in males held in low salinities. Following hormonally induced ovulation, females maintained at 5 ppt displayed lower egg production and fewer ovulations compared to females maintained at 20 or 35 ppt. Egg fertility and sperm motility were also significantly reduced in fish maintained at 5 ppt. In a similar study on the euryhaline,

estuarine species, striped mullet (*Mugil cephalus*), Lee *et al.* (1992) reported that gonadal maturation occurred in both full strength seawater (32-34 ppt) and half strength seawater (15-17 ppt) following a 6 month exposure period. Whilst the size and number of eggs produced per female was not affected by the rearing salinity, egg fertility and sperm motility were reduced in the lower salinity treatment. Furthermore, they reported that sperm activation and motility was not directly affected by salinity, but rather was due to the osmotic pressure of the water. Clearly, salinity affects reproductive processes in both male and female fishes.

Physiological responses to salinity in juvenile and adult fishes vary greatly between species, largely due to differences in preferred nursery, adult and spawning habitats. Growth rates and food consumption may be affected by salinity, as well as the timing of spawning, quality of gametes, fertility and spawning capacity. Furthermore, spawning salinity can affect the buoyancy of newly-fertilised eggs, which, as previously mentioned, influences the position of eggs in the water column and the potential exposure to other environmental stressors.

1.1.3 Temperature

Nearly all fish species are considered thermal conformers, meaning that they are unable to regulate heat to any great extent, and thus body temperature will fluctuate with the surrounding environment (Jobling, 1995; Evans and Claiborne, 2006). Temperature affects fish physiology in a number of ways, from regulating metabolic activities and

reaction rates, to controlling the bioavailability of substances within the body, such as oxygen.

In estuarine environments, temperature is influenced by a number of factors including time of day, season, environmental flows and tidal exchange, wind and the degree of stratification. Like salinity, temperature is an important environmental variable that becomes an environmental stressor if it falls below or exceeds the normal range for that aquatic environment. Temperature related stress has been extensively studied in fishes of all life stages, and can cause a range of biological effects.

1.1.3.1 Physiological effects of temperature on the early life stages of fishes

Temperature affects hatch rates and survival of fish eggs in a number of ways, and reduced hatching and survival may occur due to both increased and decreased temperatures above or below the tolerable temperature range for a given species. Elevated temperatures can affect all stages of gamete development (Bromage *et al.*, 1992; Van der Kraak and Pankhurst, 1997), and mature female fishes experiencing thermal stress due to elevated temperatures have been reported to produce smaller eggs with lower fertility (Flett, *et al.*, 1996; King *et al.*, 2003) and lower egg survival (Tveiten and Johnsen, 1999).

In embryos of the Australian bass, Van der Wal (1985) reported reduced time to hatching and increased deformities (eg. curvature of the spine, mouth deformities) when embryos were reared at 24^{o}C as compared to others reared at 16-20^{o}C. Furthermore, at a

temperature of 12°C, time to hatching was prolonged and survival to 7 days post hatch was reduced. In the marine flatfish, turbot (S*cophthalmus maximus*), temperature significantly affected hatch rates. Hatching success was good between 12-18°C, whilst at 9°C and 21°C hatching success was lower (Nissling *et al.*, 2006). In another flatfish, the greenback flounder, Hart and Purser (1995) found that reduced temperatures resulted in lowered or even no hatching and likewise at the elevated temperature of 18°C, very little hatching occurred. Other observations included decreased time to hatching as temperature increased and higher rates of deformity at both low and high temperatures. In the greenback flounder, temperature affected larval length but not survival at the end of the yolk-sac stage. Similarly, in the tropical marine fish, leopard grouper, (*Mycteroperca rosacea*), Gracia-Lopez *et al.* (2004) observed significant effects for a range of variables in eggs and larvae due to thermal stress. Hatch rates were observed to have a parabolic relationship with temperature (inverted U-shape curve), with greatest hatching occurring at 26°C, and lower hatching at both 20°C and 30°C. The time to hatching was inversely proportional to temperature, with the longest time to hatching at 20°C, and the shortest time to hatching at 30°C. Furthermore the length (duration) of the hatching period was also shorter at higher temperatures. Size at hatch was inversely related to temperature, with significantly larger larvae hatching at lower temperatures, whilst larval growth rates were positively related to temperature and there were no significant differences in larval survival for any temperature.

In a study of thermal effects on yolk-sac larvae of the pelagic marine fish, Atlantic mackerel (*Scomber scombrus*), Mendiola *et al.* (2007) reported that temperature was

inversely related to size at hatch, with the largest larvae hatching at low temperatures (11.1^{o}C) and the smallest larvae hatching at higher temperatures (17.8^{o}C). However, once hatched, growth rates increased with increasing temperatures, showing a positive relationship. Yolk utilisation and various developmental processes (eye pigmentation, opening of mouth (gape) and jaw mobility) were also positively related to incubation temperature. In Australia, larvae of the snapper (*Pagrus auratus*), a marine sparid, showed a significant increase in mortality following transfer from 21^{o}C to 27, 30 or 33^{o}C (Fielder *et al.*, 2005). For larvae transferred to water at 24^{o}C, growth was increased relative to larvae at 21^{o}C, whilst larvae transferred to 15^{o}C displayed lower growth. These examples indicate that whilst species specific differences in temperature tolerances do occur, general trends in responses of eggs and larvae to either increased or decreased temperatures are the same.

Another physiological process that is affected by temperature is sex determination and differentiation. Indeed, the use of temperature shock has been widely used as a means of generating monosex cultures of fish in aquaculture for many years (see Baroiller and D'Cotta, 2001 for review). Sex determination refers to the factors (genetic and environmental) that influence sex differentiation, whilst sex differentiation refers to the physiological process of gonad development, resulting in the formation of either ovaries or testes (Yamamoto, 1969; Devlin and Nagahama, 2002; Evans and Claiborne, 2006). When recently hatched larvae are exposed to temperature changes, there may be either feminizing or masculinizing effects, however the direction of the sex determination varies between fish species. For example, elevated temperatures have been shown to cause

female-skewed sex ratios in sockeye salmon (*Oncorhynchus nerka*) (Craig *et al.*, 1996), and male-skewed sex ratios in hirame (*Paralichthys olivaceus*) (Tabata, 1995) and tilapia (*Oreochromis niloticus*) (Baroiller *et al.*, 1999), whilst exposure to low rearing temperatures results in female-skewed sex ratios in European sea bass (Mylonas *et al.*, 2003). In hirame, both elevated and decreased temperatures caused male-skewed sex ratios, yet intermediate temperatures resulted in even male-female sex ratios (Yamamoto, 1999). Whilst controlling sex ratios in fish culture may benefit production, changes in the sex ratios of wild populations of fishes as a result of abrupt temperature changes has the potential to seriously impact both reproductive success and population sustainability.

The physiological effects of altered temperature on the early life stages of fishes include reduced hatch rates, altered time to hatching, increased rates of deformities and differences in larval growth rates. Temperature shows linear relationships (positive or negative) for some variables, whilst for others, inverted U-shape, or parabolic relationships are observed. Furthermore, temperature may also affect sex determination and sex ratios in early life stage fishes.

1.1.3.2 Physiological effects of temperature on juvenile and adult fishes

The physiological effects of temperature on juvenile and adult fishes have been described in detail for a wide range of fish species and some of the reported effects include: changes in growth rates, feed efficiency, metabolism and oxygen consumption, as well as altered reproductive condition, gamete quality and spawning capacity.

In juvenile European sea bass, growth and metabolism were affected by changes in temperature (Person-Le Ruyet *et al.*, 2004). Feed efficiency and specific growth rates displayed a parabolic relationship, with the highest rates recorded at 25^{o}C, and lower rates at 13 and 29^{o}C. Food intake was positively related to increased temperature, as was growth. Following exposure to low temperatures (14^{o}C and less), juvenile gilthead seabream (*Sparus aurata*), another marine sparid species, exhibited reduced growth and weight loss, refusal to feed, as well as changes in the activities of a number of liver enzymes (Ibarz *et al.*, 2007). Weight loss was due to a reduction in the levels of non-polar lipids in muscle tissue, as well as liver weight loss. Decreased growth and metabolism in juvenile fishes can subsequently lead to reduced reproductive fitness in adults.

As fish undertake the transition from juveniles to become sexually mature adults, a series of physiological events take place that are energetically demanding and influenced by several environmental factors such as temperature, photoperiod and nutrition (see section 1.2.3). In female fishes, ovary formation and production of oocytes is very energy intensive, and female sexual maturation is frequently accompanied by reduced somatic growth, as energy is diverted into the production of oocytes and yolk reserves (Jobling, 1995; Evans and Claiborne, 2006). Elevated temperature has been shown to affect steroidogenesis, by reducing E2 levels, in the freshwater salmonid, Atlantic salmon (*Salmo Salar*), resulting in reduced levels of Vtg and lowered fertility and egg survival (King and Pankhurst, 2003; King *et al.*, 2003; Watts *et al.*, 2004). Similar effects have also been observed following exposure to elevated temperatures prior to maturation in the

marine sparid, red seabream (Lim *et al.*, 2003), the coldwater salmonid, Arctic charr (*Salvelinus alpinus*) (Jobling *et al.*, 1995) and the anadromous white sturgeon (*Acipenser transmontanus*) (Webb *et al.*, 1999).

Temperature affects various aspects of juvenile and adult fish physiology, from growth and metabolism to sex determination and gonad development, and therefore all associated reproductive processes. Most importantly, temperature is dynamically linked to DO and salinity, especially in estuarine environments, and therefore all three should be considered together, as well as considering the underlying factor that drives changes in these environmental factors, climate variability.

1.1.4 Climate change

Predictions of climate change suggest that annual average surface temperatures could rise by up to 6^{o}C over the next century (IPCC, 2001). In Australia, CSIRO modelling predicts surface temperature increases of 1-6^{o}C by 2070 and sea level rises of up to 8.0 cm per decade (Hobday *et al.*, 2006). These changes are likely to alter rainfall patterns and tidal movements as well as increasing the frequency of severe weather conditions such as flooding and drought, as well as potentially increasing the intensity of UV radiation. The potential of these climatic changes to dramatically alter estuarine and marine environments is very high.

Climate change is predicted to alter the strength of the two boundary currents that influence ocean temperatures around Australia. The East Australia Current (EAC) flows

southwards from the Coral Sea down the eastern coast of Australia to Tasmania. The Leeuwin Current flows southwards down the western coast of Australia and then continues eastwards through the Great Australian Bight towards Tasmania (Hobday *et al.*, 2006). Climate driven changes in the strength of these currents are likely to result in changes in upwelling, stratification and physico-chemical conditions that will subsequently affect food availability and dispersal patterns in fishes and other organisms.

Upwelling is the process by which cold, nutrient-rich water is drawn towards the sea surface, providing energy resources that encourage phytoplankton growth (primary production) that can subsequently support higher trophic levels (Gross, 1993; Hobday *et al.*, 2006). Whilst increased nutrients may encourage growth, the deep water usually has low DO, and exposure to such conditions for pelagic fish eggs may result in very high embryo mortality, increased rates of deformities and other developmental disruptions that will reduce their overall fitness. Stratification refers to the formation of distinct vertical layers within the water column, with differing salinity and/or temperature and/or DO levels, and mixing between layers is generally limited (Hobday *et al.*, 2006). These oceanographic processes are complex, dynamic processes that are variable with depth, geographical distribution and time, and any changes that affect environmental conditions in estuaries are likely to affect the abundance and distribution of estuarine species.

Climate change has been linked with changes in migratory patterns of various fish species such as the Norwegian herring (*Clupea harengus*) (Sissener and Bjorndal, 2005; Lajus *et al.*, 2007), gilthead seabream (Katselis *et al.*, 2007), three Mugilidae species,

Liza saliens, Liza aurata, Mugil cephalus (Katselis *et al.*, 2007) and European hake (*Merluccius merluccius*) (Abella *et al.*, 2008). In Australia, southward shifts in many species' distributions are also predicted to occur with climate change, threatening major changes to species composition and abundance, which are likely to have follow on effects resulting in changes to overall ecological communities (Hobday *et al.*, 2006). Indeed, such effects have already been reported in Tasmania, where a cold water species, jack mackerel has been replaced by a tropical species, redbait (*Emmelichthys nitidus*) (Welsford and Lyle, 2003).

Climate change already has a major influence on marine and estuarine ecosystems, and in the future these effects are likely to become more pronounced. Understanding the breadth of change in environmental conditions associated with climate variation will assist in predicting the biological effects, and the collection of biological response data will greatly improve such predictions.

1.1.5 Environmental stressors as endocrine disruptors

Endocrine disruption is any significant disturbance to the hormonal (endocrine) system of an organism, and may occur as a result of exposure to chemicals or certain environmental variables (Connell *et al.*, 1999). Endocrine disrupting chemicals (EDCs) interfere with the normal functioning of the endocrine system, and include natural and synthetic hormones such as sex hormones (androgens and estrogens), selected pesticides, heavy metals, polychlorinated biphenyls, dioxins, pharmaceutical products, phytoestrogens and detergent by-products (Kookana *et al.*, 2007). Environmental variables that can affect the

endocrine system, and therefore, strictly speaking, are also endocrine disruptors, include hypoxia, salinity and temperature (see section 1.1). However, the term endocrine disruptor is generally used for environmental contaminants and xenobiotics only. Endocrine disruptors are capable of interfering with many physiological processes and have been implicated in causing detrimental effects, either directly or indirectly, on many different vertebrate systems including the endocrine, reproductive, nervous and immune systems (Birkett and Lester, 2002).

Shang and Wu (2004) have reported that hypoxia acts as an endocrine disruptor during the early life stages of zebrafish by affecting the levels of the two major sex hormones, testosterone and E2. In various fish species, exposure to elevated or decreased temperatures during the early life stages can have feminising or masculinising effects (see section 1.1.3.1), and it is believed that the effects are due to changes in the synthesis or activity of the enzyme cytochrome P450 aromatase, which is responsible for the conversion of testosterone to E2 (see Devlin and Nagahama, 2002). Endocrine disruption in the early life stages of fish can then lead to changes in gonad development and sex ratios which is likely to translate into reduced reproductive capacity, leading to the potential risk of spawning failure, population decline and species extinction.

In adult fish, chronic exposure to hypoxia has also been shown to cause endocrine disrupting effects. In immature adult carp (*Cyprinus carpio*), Wu *et al.* (2003) reported that exposure to hypoxia (1.0 ml O_2/l ~12%S) for a period of 12 weeks resulted in significant reductions in serum levels of the sex hormones testosterone and E2, as well as

the thyroid hormone triiodothyronine (T3). Gonad development was retarded and the gonadosomatic index (GSI) values were reduced in both males and females, whilst spawning, fertilisation and hatching success were also significantly reduced. Similar responses to chronic hypoxia have been observed in Atlantic croaker (Thomas *et al.*, 2006) and Gulf killifish (Landry *et al.*, 2007). Haddy and Pankhurst (2000) have reported alterations in reproductive hormones and subsequent gonad development and spawning success in black bream reared in low salinities, whilst various authors have demonstrated the effects of altered temperatures on steroid synthesis, Vtg production and subsequent egg fertility and survival in fishes (Jobling *et al.*, 1995; Webb *et al.*, 1999; King *et al.*, 2003) (see section 1.1.3.2).

Environmental variables clearly affect endocrine pathways in fish, and continued research is necessary to improve our understanding of how variables such as DO, salinity and temperature affect hormone production and the cascade of events that are controlled by the endocrine system. The continued measurement of plasma or serum concentrations of hormones and other macromolecules (eg. Vtg) that may change in response to environmentally induced endocrine disruption are needed, as is the need to develop techniques for measuring discrete physiological changes. Such research includes identifying genes that control aromatase and Vtg production, the development of methods for quantifying gene expression and transcription, as well as research into the functioning of estrogen receptors and the characterisation of sex-specific and sex-determining genes (Kime, 1999; Denslow *et al.*, 2001; Devlin and Nagahama, 2002; Mills and Chichester, 2005).

The environmental variables DO, salinity and temperature can cause physiological stress and increased mortality in all life stages of fishes, with effects ranging from reduced hatch rates and larval survival, to changes in growth and metabolism, altered sex ratios and reduced reproductive capacity. These variables are intrinsically linked to one another, and naturally fluctuate in estuarine and marine environments due to climate and oceanographic variability. As estuarine and marine environments continue to be impacted by environmental degradation, anthropogenic pollution and climate change, the potential for DO, salinity and temperature to act as environmental stressors is increased and is likely to influence, or in fact drive changes in the abundance and distribution of species that could potentially lead to declines in fish stocks and in severe cases, species extinction.

1.2 Physiological processes that are affected by stress in fish

Stress influences many physiological processes in fish, and the stress response can vary depending on a number of factors including species, life-stage, type of stressor and length of exposure (Pickering, 1990; Barton, 1997; Schreck *et al.*, 2001). Growth suppression and reduced reproductive capacity are two very well recognised responses to physiological stress (Donaldson, 1990; Barton and Iwama, 1991).

1.2.1 Growth

Growth is continuous in fishes, meaning that they get larger with age, and the growth rate is influenced by nutrition, genetics, endocrine factors and importantly, environmental

conditions (Brett, 1979; Boeuf and Payan, 2001; Evans and Claiborne, 2006). Suppression of growth due to adverse environmental conditions has been documented for a variety of fish species, both in captive and wild populations, and temperature, photoperiod and salinity have all been shown to have significant effects (Boeuf and Payan, 2001).

1.2.2 Physiological tissue indices

Physical condition indices can be used to determine the general health status of fish and to establish if they are in a physiologically stressed condition (Morgan and Iwama, 1997). A number of different indices have been developed, based on the ratio of the weight of an organ to the overall weight of the fish, and whilst decreased index values generally indicates physiological stress, some indices vary seasonally and in relation to reproductive condition (Goede and Barton, 1990; Morgan and Iwama, 1997). Furthermore there can be natural, sex-specific differences in some physiological indices. Physiological tissue indices provide information on long term, or chronic stress exposure, since it is only after a prolonged period that changes in the size and gross features of the organs would be observable. Three commonly used physiological tissue indices are the gonadosomatic index (GSI), the liver somatic index (LSI) (also referred to as the hepatosomatic index, HSI) and the condition factor.

1.2.2.1 Gonadosomatic index (GSI)

The gonadosomatic index provides a measure of gonad weight in relation to total body weight (Morgan and Iwama, 1997). The GSI varies seasonally, rising to a peak as the

gonads develop, just prior to the onset of spawning. In females, the maximum GSI value depends on the type of ovary and the style of reproduction (Tyler and Sumpter, 1996). In species that produce a single batch of eggs during the spawning season (synchronous spawners), the normal range for GSI values is 18-25, but with hormonal manipulation GSI values can be increased up to 60 (Tyler and Sumpter, 1996). In species that produce multiple eggs batches throughout the spawning season (asynchronous spawners), the GSI is generally lower (2-14), however since multiple spawnings occur, the overall egg production is generally much higher in batch spawning species. Environmental conditions influence spawning, and stress can delay or disrupt gonad development. Various authors have reported decreased GSI values following exposure to environmental stressors including hypoxia, salinity and temperature (see section 1.1).

1.2.2.2 Liver somatic index (LSI)

The liver somatic index provides a measure of liver weight in relation to total body weight (Morgan and Iwama, 1997). The LSI varies seasonally, and is also strongly influenced by nutritional status and stress. The liver carries out a range of functions in fishes, including glycogen and lipid storage and toxicant detoxification. The LSI may be used as an indicator of nutritional status, as well as an indicator of growth potential (Morgan and Iwama, 1997). During starvation, glycogen and lipid stores within the liver are rapidly consumed, resulting in a decrease in the LSI, whilst at times of food abundance, increased glycogen and lipid stores in the liver may result in higher LSI values. This would indicate that a high LSI is indicative of good health, however, in response to toxicant exposure, the liver often becomes enlarged in order to increase

detoxification capacity (up regulation of the mixed-function oxidase detoxification system). Thus both reduced and elevated LSI values need to be considered possible indicators of physiological stress.

1.2.2.3 Condition factor

The condition factor provides a general measure of fitness and is calculated from length (cm) and weight (g) (Morgan and Iwama, 1997). Length and weight are frequently measured in fisheries related studies, allowing the condition factor to be used as a measure of growth based on observed and expected responses (Goede and Barton, 1990). The condition factor may be reduced due to depleted energy reserves, either as a result of stress, or simply due to seasonal fluctuations in feeding, and it may also be reduced due to other forms of stress, such as exposure to unfavourable environmental conditions (see Goede and Barton, 1990).

1.2.3 Reproduction

Reproduction in fish is very diverse, with some species being capable of continuous spawning, such as zebrafish, whereas others have defined spawning seasons. Some species, such as eels and some salmonids reproduce only once, whilst others, such as sparids reproduce many times within the lifetime. There are also a range of sexual forms, including gonochorism and hermaphroditism and even some that enable reproduction without fertilisation, by the process of parthenogenesis (Hoar, 1969; Jobling, 1995). The initiation of gamete growth and development is commonly associated with environmental cues such as changes in day length and temperature that occur as seasons vary annually

(Bromage *et al.*, 2001). These cues initiate physiological processes (eg. endocrine pathways) that may take several weeks, or even months before final maturation and spawning occurs (Jobling, 1995; Evans and Claiborne, 2006). Fertilisation is the process of fusion between haploid eggs and sperm that results in the formation of a diploid embryo (Kunz, 2004), and in order to understand how environmental stressors can affect spawning success, more information is needed on annual spawning cycles and continuous monitoring of fish populations. Stress influences reproduction in a number of ways, from inhibition of gamete production and maturation, to changes in the timing of spawning, gamete quality and progeny quality (Donaldson, 1990; Pankhurst and Van der Kraak, 1997).

1.2.3.1 Gamete production

Gamete production marks the onset of maturity in fishes, and involves a complex series of events that are under endocrine control. Sperm production (spermatogenesis) and egg production (oogenesis) are critical events in the life history and any external stressors that interfere with or disrupt these processes are likely to impact spawning success and fecundity, which may result in long term changes in fish populations.

Spermatogenesis in fishes involves 5 different stages. Initially, spermatogonia proliferate through a series of mitotic cell divisions, resulting in the formation of primary spermatocytes. Primary spermatocytes go through a growth period, without cell division, and then meiotic cell division occurs, resulting in haploid secondary spermatocytes that further divide to produce spermatids. Finally, spermatids metamorphose into motile

spermatozoa (mature sperm cells) (Jobling, 1995; Rocha and Rocha, 2006). Oogenesis in fishes also involves 5 different stages: the primary growth stage; the cortical alveolus stage; the vitellogenesis stage; the oocyte maturation stage; and finally, ovulation (Tyler and Sumpter, 1996; Lyman-Gingerich and Pelegri, 2007). During the first 2 stages, meiosis and DNA replication occurs, homologous chromosomes condense and thicken and follicle cells first appear. Next, cortical alveoli appear which are important during egg activation and fertilisation. The vitellogenesis stage is marked by a long period of cytoplasmic growth and accumulation of Vtg to provide the yolk reserves for the developing embryo. During this time egg size increases enormously and the ovaries mature, resulting in a 50-100 fold increase in the GSI (Tyler and Sumpter, 1996).

Vitellogenesis is a complex process involving many hormonal interactions (Kime *et al.*, 1999). Gonadotropin-I (GtH-I) stimulates the production of E2 from ovarian follicle cells, which travels to the liver via the blood circulation, where it encounters estrogen receptors that stimulate Vtg synthesis (Tyler *et al.*, 1991; Specker and Sullivan, 1994; Kime *et al.*, 1999). Following vitellogenesis, oocyte maturation occurs, which involves the unpairing of chromosomes and the movement of the nucleus towards the animal pole. Meiosis is resumed and proceeds to the first meiotic metaphase and hydration occurs. The haploid oocyte (egg) is then ready for fertilisation, and is released from the ovary (ovulation).

The presence of hydrated oocytes in the ovary indicates that a fish is actively spawning and is used in fisheries biology to identify spawning periods and the proportion of

spawning fish within a population (Crim and Glebe, 1990; Murua *et al.*, 2003). However, in order to observe ovarian condition, fish are usually sacrificed, which prevents repeated observations on the same fish and furthermore, the removal of large numbers of mature fish from a fishery is often not practical or permissible. Therefore, non-lethal techniques that can measure reproductive condition are needed, and the measurement of Vtg in blood plasma is suitable for this (Specker and Sullivan, 1994; Kokokiris *et al.*, 2001; King and Pankhurst, 2003).

1.2.3.2 Vitellogenin

Vitellogenin is a complex lipophosphoglycoprotein that has a high molecular weight and consists of multiple subunits. Following production in the liver, Vtg is transported to the ovary via blood circulation, where it is incorporated into developing oocytes by the process of micropinocytosis (Droller and Roth, 1966; Jobling, 1995; Kime *et al.*, 1999). In native form, Vtg is a large dimeric protein (300-600 kDa) and in subunit form, the monomers (150-200 kDa) may be the same, or of different size depending on species (Specker and Sullivan, 1994; Denslow *et al.*, 1999; Watts *et al.*, 2003). Since concentrations of Vtg in plasma directly relates to egg synthesis and reproductive processes, it has become a valuable biomarker for measuring reproductive condition in fishes.

In mature female Atlantic salmon, plasma Vtg concentrations of 5-40 mg/ml have been reported during the spawning season (King and Pankhurst, 2003). In the marine sparid, red porgy (*Pagrus pagrus*), peak Vtg concentrations of 405.5 ± 109 μg/ml were observed

during the spawning season, associated with a peak in the GSI (3.1%), whilst the Vtg concentrations declined substantially (<10 μg/ml) during the non-spawning season (Kokokiris *et al.*, 2001). Seasonal changes in Vtg concentrations occur in association with seasonal spawning activity, and this needs to be considered when measuring Vtg concentrations in mature fish. However, low levels of plasma Vtg in reproductive-age female fishes (sampled during the spawning season) may indicate immaturity, poor reproductive condition or reproductive dysfunction, which may be caused by a range of factors including poor nutrition, exposure to environmental stressors, including hypoxia, salinity and temperature (see section 1.1), or exposure to contaminants (Donaldson, 1990; Kime, 1999).

Vitellogenin production is normally only observed in reproductive age females, however the hepatic estrogen receptors that are responsible for Vtg production are found in both female and male fishes. Thus the presence of Vtg in males or immature females provides a good indication of exposure to estrogenic compounds (Sumpter and Jobling, 1995; Denslow *et al.*, 1999; Kime *et al.*, 1999; Moncaut *et al.*, 2003). Subsequently, the use of Vtg as a biomarker for determining reproductive condition and for detecting exposure to estrogenic compounds have become widely accepted tools in both aquaculture and environmental monitoring.

1.2.3.3 Fecundity

Fishes display a number of different reproductive strategies, and the number of eggs produced during a spawning season varies greatly amongst different species.

Synchronous spawners produce one batch of eggs during the spawning season, whilst asynchronous spawners produce several batches of eggs during the spawning season. Extended and multiple spawning periods are characteristic of estuarine fishes and marine sparids, and this reproductive strategy is considered to be a compensatory mechanism for high mortality of eggs and larvae (McEvoy and McEvoy, 1992; Newton, 1996). Many sparids, including black bream, are multiple spawners, producing several batches of eggs over a protracted spawning season of weeks or months. By producing batches of eggs over an extended period, a greater overall number of oocytes can be produced during the spawning season and it also provides the benefit of increasing the likelihood of egg survival, by spreading the risks of predation, starvation and exposure to environmental stressors over a broader time period (McEvoy and McEvoy, 1992).

1.2.3.4 Sex determination and sex ratios

Sex determination, the combination of genetic and environmental factors that influence sex differentiation in fishes is influenced by environmental stressors (see section 1.1.3.1). Hypoxia and temperature have both been observed to alter sex ratios in early life stage fishes, whilst natural sex change processes in adult fishes are known to be related to environmental factors (Devlin and Nagahama, 2002; Frisch, 2004).

1.2.4 Recruitment

Fish populations naturally fluctuate with changes in environmental conditions, and the biomass of a fish population is the result of reproduction, growth and mortality (Jobling, 1995). Environmental conditions determine the carrying capacity, whilst the population

biomass is limited by density-dependent regulation which moderates reproduction, growth or mortality. Since environmental conditions are naturally variable, differences in recruitment are likely to occur from year to year, resulting in either strong year classes or weak year classes. For long term population stability, successive strong year classes are required in order to produce a multi-age fish stock with a high spawning stock biomass, which in turn would produce a large number of individuals to eventually recruit to the fishery. The effects of a poor year class in a multi-age fish stock is moderated by the high spawning stock biomass. In contrast, if a fish population has only few strong year classes, it is likely to have a much lower spawning stock biomass, and subsequently be more susceptible to long term population declines when adverse environmental conditions result in recruitment failure (Jobling, 1995; Trippel *et al.*, 1997).

A range of physiological processes in fish are affected by environmental stressors, and all life stages may be affected. All aspects of reproduction, from gamete production through to the number of fertilised eggs that survive to hatching may be negatively affected, and sex ratios and recruitment may also be impacted. Any environmental factors that can disrupt or adversely affect the life cycle of an organism have the potential to seriously impact population survival and thus need to be identified and where possible managed and improved.

1.3 Environmental stressors in estuarine environments

Estuaries can be broadly defined as semi-enclosed coastal water bodies where the river meets the sea and comes under the influence of the tides, forming a transition zone

between freshwater and seawater (Knox, 1986; NLWRA, 2002; Turner *et al.*, 2004). Estuaries can be classified into different groups based on any one of a number of properties, such as geomorphology, hydrology, circulation, salinity stratification or by the dominant forces (wave action, tidal flow and river flow) that determine their appearance and functionality (Knox, 1986; Turner *et al.*, 2004; Barton *et al.*, 2008).

Salt wedge estuaries are dominated by freshwater flows and deep pools, and they generally have a small tidal range. At the boundary between the freshwater and seawater layers there may be a marked halocline, and these estuaries are often considered to be highly stratified. Partially mixed estuaries are characterised by varying degrees of mixing between the outward flowing freshwater and the inward flowing seawater. There is a sufficient degree of mixing between the two layers such that stratification (like that of salt wedge estuaries) does not occur. Shallow estuaries in which the tidal volume is large compared with the total volume of the estuary basin are often partially mixed. Vertically homogenous estuaries are characterised by gradually decreasing salinity moving upstream towards the estuary head, but without a vertical salinity gradient. The lack of stratification is a result of turbulent mixing, which is characteristic of shallow estuaries with a large tidal range (Knox, 1986).

Australia is the world's driest inhabited continent, covering a land mass of more than 7.6 million km^2, of which 36,700 km is coastline. Of that vast coastline, there are more than 1000 estuaries that make up approximately 40% of all coastal waterways (NLWRA, 2002; Turner *et al.*, 2004). The state of Victoria, in southern Australia covers an area of

227,600 km^2, and supports a population of more than 5 million people (Australian Bureau of Statistics, 2008). It is the second most populous state in Australia, and the 2000 km coastline contains more than 120 bays, inlets and estuaries (Barton *et al.*, 2008). There are a variety of land uses within Victoria, including agriculture, forestry and urban development (Fig. 1.2).

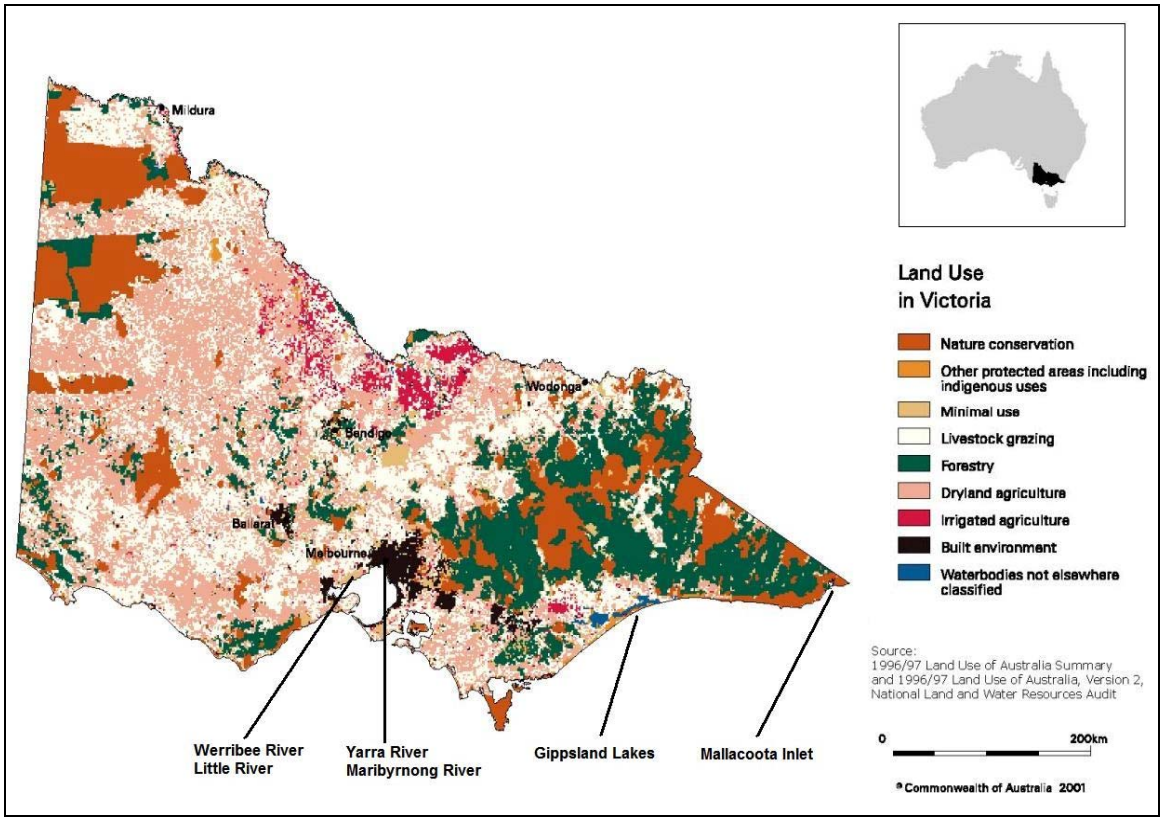


Figure 1.2 Map of land use patterns in Victoria and identification of field collection sites (Source: http://www.nlwra.gov.au/products/er061223).

The Southern Ocean and Bass Strait surround the Victorian coastline, and hence this region is subject to strong ocean currents and is wave-dominated (Turner *et al.*, 2004). Wave-dominated estuaries tend to be poorly flushed, due to wave action at the mouth

leading to sand bar formation, and water quality in such estuaries is a major issue due to pollution and the accumulation of toxicants. Furthermore, estuaries are often sites for major ports, industry, agriculture and urbanisation, and are subject to a variety of environmental stressors that degrade the water quality, including industrial and agricultural discharges, stormwater, increased nutrients, sewage and reduced flows as a result of water extraction.

In 2002, the National Land and Water Resources Audit released the Australian catchment, river and estuary assessment report, which was the first report to provide a comprehensive assessment of Australian estuarine environments (NLWRA, 2002). The condition of estuaries was classified as follows: near-pristine, where >90% of natural vegetation remains, there is limited disturbance to natural conditions and processes, and ecological systems are intact; largely unmodified, where 65-90% of natural vegetation remains, there is limited disturbance to natural conditions and processes, and ecological systems are mostly intact; modified, <65% of natural vegetation remains, there are documented impacts from land use and ecological systems are modified; and severely modified, where <35% of natural vegetation remains, there are documented impacts from land use and ecological systems are degraded (NLWRA, 2002). The classifications used in the NWLRA report are used to describe the Victorian estuaries studied throughout this project, namely Mallacoota Inlet, Gippsland Lakes, Yarra River, Maribyrnong River, Werribee River and Little River (Table 1.1, Fig. 1.2) (see chapter 7).

The Gippsland Lakes are an example of an estuarine environment modified by large dams and rivers, and a history of mining and agricultural contamination. The Gippsland Lakes are a series of shallow coastal lagoons that cover an area of 400 km^2 and are connected to Bass Strait by a permanent opening at Lakes Entrance (37^o 50'S;147^o 40'E) (Bird, 1978). The Gippsland Lakes are classified as a wave-dominated estuary and are considered to be in a severely modified condition (NLWRA, 2002) (Table 1.1). They are a complex system of salt-wedge estuaries arising from five main rivers that are vulnerable to both vertical and horizontal stratification depending on seasonal flow rates, tidal influence, rain fall and evaporation (Knox, 1986; Webster *et al.*, 2001).

For the present study, environmental conditions similar to those found within the Gippsland Lakes were used to examine stress responses in early life stage black bream in the laboratory, whilst the other five estuaries were chosen as field collection sites because they cover a broad geographical area and are examples of environments impacted by different types of environmental stressors.

1.3.1 Recent water quality monitoring in the Gippsland Lakes

Recently a report was released by the Victorian EPA detailing water quality within the Gippsland Lakes over a 12 month period (2006-2007) (EPA, 2008). The median DO levels within Lake Victoria and Lake King (where black bream spawning occurs) were 50-66%S, with a range of 2.5-122%S (Table 1.2). The median salinity values were 21.9-30.6 ppt, with a range of 3.3-38.7 ppt, whilst median temperature values were 16.1-16.4^{o}C with a range of 9.6-24.9^{o}C (Table 1.2). Daily fluctuations were observed in

salinity and DO levels, at a depth of 5 m, and during some periods, severe hypoxia was observed in conjunction with increased salinity (Fig. 1.3).

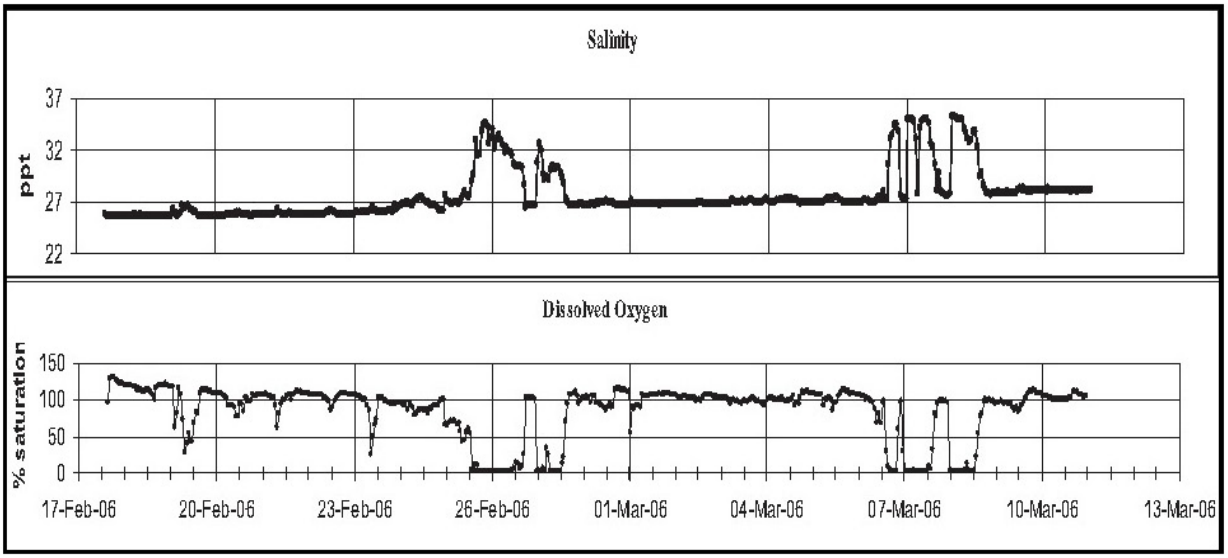


Figure 1.3 Temporal changes in salinity (ppt) and dissolved oxygen (%S) in Lake King north (Gippsland Lakes) (depth 5 m) (source: EPA, 2008).

Table 1.1 Summary of estuary condition and classification for six Victorian estuaries that were used in this study.

Estuary	Condition	Classification*	Sub-class*	Catchment Area (km^2)	Annual Rainfall (mm)
Gippsland Lakes	Extensively modified	Wave-dominated	Wave-dominated Estuary	20, 600	584 - 1350
Mallacoota Inlet	Near-pristine	Wave-dominated	Wave-dominated Estuary	1846	900 - 1000
Maribyrnong	Modified	River-dominated	Wave-dominated Delta	1433	<500 - 1000
Yarra	Modified	River-dominated	Wave-dominated Delta	4078	615 - 1080
Werribee	Modified	River-dominated	Wave-dominated Delta	2700	450 - 1000
Little	Modified	Tide-dominated	Ephemeral	2700	450 - 1000

* based on the National Land and Water Resources Audit (2002)

Table 1.2 Median (range) water quality measurements for dissolved oxygen, salinity and temperature at three locations within the Gippsland Lakes throughout 2006-2007.

Site	DO (%S)	Salinity (ppt)	Temperature (^oC)
Lake Victoria	66 (3.3 - 120)	21.9 (3.3 - 34.7)	16.4 (9.6 - 24.9)
Lake King (south)	60 (8.4 - 118)	30.6 (11.1 - 38.7)	16.2 (10.5 - 22.2)
Lake King (north)	50 (2.5 - 122)	27.2 (5.1 - 37.2)	16.1 (9.6 - 22.6)

1.4 The biology of black bream

The black bream, *Acanthopagrus butcheri* (formerly *Mylio butcheri*), is a native Australian fish belonging to the Sparidae family that was first described in 1949 (Munro, 1949) (Fig. 1.4). The Sparidae family is primarily a marine group consisting of 29 genera and more than 100 species worldwide (Neira *et al.*, 1998). Several sparid fishes are endemic to Australia, and in temperate regions there are 4 closely related species: *A. butcheri* (black bream); *A. australis* (yellowfin bream); *P. auratus* (snapper); and *Rhabdosargus sarba* (tarwhine) (Gomon *et al.*, 1994; Neira *et al.*, 1998). Whilst black bream inhabit estuaries, the other 3 species utilise estuaries for nursery habitat, but are otherwise generally restricted to marine environments.

1.4.1 Distribution

Black bream are a widely distributed species, endemic to the coastal lakes, estuaries and sheltered coastal waters of southern Australia, including Tasmania (Fig. 1.5). They are found in Western Australia as far north as Shark Bay, right around to eastern Victoria (Mallacoota Inlet) and southern New South Wales (Gomon *et al.*, 1994). Separate stocks reside within different estuaries and there is considerable variation in feeding, growth rates and age at maturity amongst stocks (Sarre and Potter, 1999; Norriss *et al.*, 2002). Different stocks may form isolated breeding populations, however the biological characteristics are still considered similar enough to class all groups as the same species (Chaplin *et al.*, 1998; Burridge and Versace, 2007).

Figure 1.4 Adult black bream (*Acanthopagrus butcheri*).

Figure 1.5 The distribution of black bream within Australia (Source: Classon and Booth, 2002).

1.4.2 Black bream habitat and spawning locations

Having such a broad distribution, black bream exist in a range of thermal regimes, where other water quality variables (eg. salinity, temperature and DO), habitat, food availability and predatory interactions are different, indicating that the species is adaptable to prevailing conditions. Black bream are considered an estuarine resident species (Ramm, 1983; Walker and Neira, 2001) that spawn in estuaries, and utilise the salt-wedge to find spawning locations with suitable salinities (15-35 ppt) (Sherwood and Backhouse, 1982; Newton, 1996; Haddy and Pankhurst, 1998; Haddy and Pankhurst, 2000). A study of post-larval and juvenile black bream abundance in the Gippsland Lakes suggested that suitable larval settlement and nursery grounds may be seagrass dominated areas with salinities of 19-22 ppt and a temperature of 21^{o}C (Ramm, 1983). However, the salinity measurements in that study were not very accurate (± 5-6.5 ppt), and furthermore it has been acknowledged that other factors need to be identified that may affect suitable spawning sites, in order to better define the optimal environmental conditions for early life stage survival in black bream (Longmore *et al.*, 1990).

In a study of the reproductive biology of black bream from different estuaries in Western Australia, Sarre and Potter (1999) observed indications of successful spawning events (hydrated oocytes and postovulatory follicles) in fish from two south-western Australian estuaries with salinities as low and high as 3.5 and 45.2 ppt, respectively. The Moore River Estuary is an intermittently open estuary that had salinities in the spring/summer spawning period ranging from 3.5-8.0 ppt, while the Wellstead Estuary that is normally closed had salinities ranging from 40.7-45.2 ppt (Sarre and Potter, 1999). Neither of

these estuaries become stratified due to salt wedge formation. The ability of black bream to successfully reproduce in such a diverse salinity range provides evidence of adaptation to the existing conditions within the Moore River and Wellstead estuaries, and suggests that the usual environmental cues that initiate spawning (ie. salinity, temperature) may be different in black bream populations from these locations (Sarre and Potter, 1999).

1.4.3 Life history

Black bream are a slow growing species that exhibit sex-specific differences in growth rates and can live to an age of at least 29 years (Morison *et al.*, 1998). Black bream eggs are spherical and pelagic, with a diameter of 0.7-0.8 mm and a single oil globule (Butcher, 1945; Partridge *et al.*, 2003). The eggs hatch after 2 days (Haddy and Pankhurst, 2000), and the size at hatching is 1.7 mm. Exogenous feeding begins on Day 2 post hatch, swim bladder inflation occurs on Day 4-12 and larvae reach a size of 10 mm after 22-28 days (Partridge *et al.*, 2003). Notochord flexion occurs at a size of 7.1-7.3 mm and metamorphosis from the larval form to the adult form occurs at a size of 11.7 mm (Neira *et al.*, 1998). Juvenile black bream grow slowly and are capable of growing in salinities ranging from freshwater (0 ppt) to hypersaline water (48 ppt) (Partridge and Jenkins, 2002). Black bream mature after 2 years at a size of 20+ cm (see section 1.4.5), and adults are reported to attain a maximum size of 60 cm (3.5 kg) (Kuiter, 1993).

1.4.4 Growth

Growth rates are highly variable in black bream and can be substantially different between year classes and different estuaries (Sarre and Potter, 2000). Juvenile fish reared at a salinity of 24 ppt (22-24^{o}C) were reported to have specific growth rates of 2.34 ± 0.03% per day (Partridge and Jenkins, 2002), and a comparison in growth of juveniles reared in the same conditions (but from different broodstock) displayed no growth differences (Partridge *et al.*, 2004). This result suggests that it is the differing environmental conditions in different estuaries that causes growth variability rather than inherited genetic differences.

1.4.5 Sexual maturity and hermaphroditism

The size/age at maturity differs in black bream from different estuaries. In an early study of black bream from the Gippsland Lakes, Butcher (1945) reported that they reach maturity at 2+ years of age, or a size of 21.6 cm total length, whilst Walker and Neira (2001) reported the onset of maturity at 3+ years, and Coutin *et al.* (1997) estimated 100% maturity in female black bream at a size of 25 cm total length. In black bream from different estuaries in Western Australia, Sarre and Potter (1999) reported the length at which 50% of females were mature ranged between 15.7-21.8 cm, and the ages at which 50% of females were mature ranged between 1.9-4.3 years. Male black bream show a similar pattern of sexual maturity.

Within the family Sparidae, various forms of sexual functioning exist, including protandry, protogyny and different forms of hermaphroditism (Buxton and Garratt,

1990). In studies of black bream to date, sexual development is not clearly defined. Some authors suggest that they may be a protogynous species, starting as females and later changing to males (Rowland and Snape, 1994), some suggest that black bream are gonochoristic (Sarre, 1999) and others suggest that they may be rudimentary hermaphrodites, with residual ovarian tissue in a fully developed testis, or residual testis in a fully developed ovary (Haddy and Pankhurst, 1998; Sarre and Potter, 1999; Partridge *et al.*, 2003).

Further studies are required to determine the exact sexual development strategy(s) that occur in black bream, in order to understand reproductive processes and how they may be influenced and affected by environmental stressors and fishing pressures. The existing information is sparse, and sometimes contradictory.

1.4.6 Reproduction

Female black bream are highly fecund, pelagic multiple spawners that can produce several million eggs per season (Butcher, 1945; Sarre and Potter, 1999; Partridge *et al.*, 2003). Spawning occurs over a protracted period, which is assumed to be an adaptation to unstable environmental conditions that maximises the chance of at least some successful spawning events per season. They spawn within estuaries, although the exact environmental parameters and habitat requirements are not fixed, and indeed probably differ substantially between different populations. Spawning occurs during spring and early summer (September – February), with some populations spawning earlier than

others depending on geographical location and climatic factors (Newton, 1996; Haddy and Pankhurst, 1998; Sarre and Potter, 1999).

1.4.7 Recruitment

Victorian black bream populations exhibit some of the most variable recruitment patterns of all fish species that are commercially targeted in Australia (Anon, 2006). Year class strength is highly variable and there may be several years between strong year classes, which is attributed to environmental variables, as well as declining stocks of mature (spawning) fish and reduced areas of suitable habitat (MacDonald, 1997; Anon, 2006). In studies of fish from the Gippsland Lakes, marked recruitment variability is observed, and unfavourable environmental conditions and reduced freshwater flows are amongst the suspected causes (Morison *et al.*, 1998; Walker and Neira, 2001; Anon, 2006). The "match-mismatch hypothesis" describes the importance of synchronisation in the time of fish larvae hatching and the presence of planktonic food sources (Cushing, 1972; Cushing, 1990). In a study of ichthyoplankton and zooplankton from the Hopkins Estuary in south-western Victoria, Newton (1996) concluded that black bream conform to the match-mismatch hypothesis, with peak spawning occurring in synchrony with high zooplankton densities. The importance of favourable environmental conditions during the spawning period is thus not only important for black bream survival, but also for the survival of zooplankton that is required as prey. In addition, the survival of larvae is likely to be influenced by factors such as competition and predation, and in some estuaries, survival may be density-dependent.

1.4.8 Commercial and recreational fisheries

The Victorian commercial black bream fishery was worth $0.34 million dollars in 2004/2005, comprising a total of 35 tonnes of fish, and of that, 30 tonnes came from the Gippsland Lakes (Anon, 2006). The Gippsland Lakes supports a large multi-species commercial fishery that was worth $1.3 million dollars in 2004/2005 and comprised a total of 675 tonnes of fish. Both the commercial and recreational fisheries within the Gippsland Lakes have been affected in recent years by highly variable environmental conditions, including both flooding and drought (Anon, 2006). In an effort to protect the Gippsland Lakes black bream fishery, Fisheries Victoria has increased the legal minimum length (LML) from 26 cm to 28 cm for all black bream within the Gippsland Lakes, however no seasonal closures have been introduced. It remains unknown how important large black bream are to recruitment success, in terms of the total numbers of eggs produced, the length of the spawning period, as well as the quality of eggs in larger, older fish compared to smaller, newer recruits. Considering that strong year classes occur infrequently, the importance of these older fish needs to be further investigated.

1.5 Aims

The aim of this PhD study was to investigate the effects of environmental stressors on different life stages of black bream. It was hypothesised that unfavourable environmental conditions adversely affect reproductive success in black bream, by reducing spawning success (poor survival in the early life stages) or reducing reproductive condition in sexually mature adults. Specifically, the aims of this study were:

1. To describe the different stages of embryo development in black bream, in order to identify normal and abnormal development

2. To identify the effects of selected environmental stressors on the early life stages of black bream

3. To develop a vitellogenin assay to measure reproductive condition in adult female black bream

4. To validate the assay by measuring levels of vitellogenin in female black bream from a range of field locations that are subject to different types of environmental stressors

5. To investigate possible endocrine disruption by measuring vitellogenin levels in male black bream collected from a range of field locations that are subject to different types of environmental stressors.

This thesis is laid out as a series of chapters that review the literature and describe new techniques that have been developed and then tested for the purposes of investigating physiological responses to selected environmental stressors in both early life stage and adult black bream.

CHAPTER 2: GENERAL MATERIALS AND METHODS

This section describes the general methods, facilities, equipment and techniques that have been used throughout this study. Detailed descriptions of individual experiments are described in each chapter.

2.1 Animal ethics

All experimental procedures carried out on fish in this study were approved by the RMIT University Animal Ethics Committee on 11th September 2005, approved project number AEC 0523.

2.2 Fish maintenance and handling

2.2.1 Broodstock fish used for egg production

Adult black bream used as broodstock in this study came from two separate stocks that were originally collected from either Lake Tyers (37^{o}28'S; 148^{o}59'E) in East Gippsland, Victoria or Swan Bay (38^{o}14'S; 144^{o}40'E) in Queenscliff, Victoria. Broodstock were fed fresh pilchards and calamari squid daily and had been held in the DPI Queenscliff aquaculture facility for at least 3 years prior to use. The mean weight of fish from Lake Tyers was 590.6 ± 33.6 g and from Swan Bay was 789.0 ± 127.9 g.

2.2.2 Fish used for vitellogenin induction experiments

The first Vtg induction experiment (see chapter 6) was conducted on adult male, Lake Tyers black bream (529.0 ± 31.05 g, *n=6*) that were held in the DPI Queenscliff aquaculture facility. For the second Vtg induction experiment, adult black bream (453.6

± 47.4 g, *n=18*) that were originally captured from the nearby Gippsland Lakes were used. These fish were held in the RMIT Bullock Island aquaculture facility in Lakes Entrance, Victoria (38°41'S; 146°35'E) for at least 12 months prior to use.

2.2.3 Holding tanks

Broodstock fish were maintained in 10 000 l plastic or 5000 l fiberglass tanks supplied with aeration and seawater in a flow-through system (average flow rate 200 l/h). Seawater was sourced via a pipeline from Port Phillip heads, Point Lonsdale, Victoria (38°16 'S; 144°35 'E) that entered the DPI Queenscliff facility and was subjected to sand and carbon filtration prior to entering the aquaculture facility.

Fish that were held in the RMIT Bullock Island aquaculture facility were maintained in 2000 l plastic tanks in a flow-through system (average flow rate 180 l/h). Aeration was provided and seawater was sourced via a pipeline from a nearby channel close to the permanent opening of the Gippsland Lakes (to Bass Strait) and was sand and carbon filtered prior to entering the aquaculture facility. All experimental tanks were cleaned daily.

2.2.4 Anaesthesia

Prior to any handling black bream were anaesthetised using either Benzocaine (Sigma-Aldrich, Australia) at a dose of 20 mg/l or AQUI-S (Crop & Food Research, Lower Hutt, New Zealand) at a dose of 30 mg/l. Anaesthetic baths were prepared in 100 l plastic containers provided with aeration. Following handling procedures, anaesthetised fish

were transferred to a recovery tub (100 l) and allowed to recover for approximately 15 minutes before being transferred back to their holding tanks.

2.2.5 Induction of ovulation in broodstock

Broodstock were anaesthetised as outlined above, then induced to spawn with a single intramuscular injection of the chorionic gonadotropin hormone, Chorulon (Intervet, Australia) at a dose of 500 IU/kg body weight (Fig. 2.1). Chorulon initiates the final maturation of ovarian follicles, and in black bream results in ovulation approximately 48 h after administration. Black bream eggs are externally fertilised, and the male broodstock used in this study did not require hormonal stimulation in order to produce milt. The ratio of males to females was 1:1 throughout the spawning period.

2.2.6 Collection of fertilised eggs (embryos)

Female black bream began releasing eggs approximately 2 d after receiving hormone treatment, and the spawning events usually occurred during the evening and sometimes into the early morning. The variation in time to spawning was probably influenced by environmental factors such as water temperature and light, as well as the time of day that the fish received hormone treatment. To minimize variation, hormone injections were always given in the morning (08:00-11:00 am).

Spawning fish were held in a tank that was fitted with an overflow that led into a 500 µm mesh egg collector (Fig. 2.2). The collector was checked at regular intervals (every 30 min) throughout the spawning period to ensure that only newly fertilised eggs were

collected. The Organisation for Economic Co-operation and Development (OECD) Guidelines for conducting short term toxicity tests on embryo and sac-fry stages states that test commencement must occur prior to the onset of the gastrula stage (OECD, 1998). Any eggs that had entered gastrulation were excluded from experimental procedures. Immediately after collection and determination of cell stage (see chapter 3), the fertilised eggs (embryos) were randomly distributed within test treatments (see chapters 4 and 5).

2.2.7 Egg and larval observations

At various time points throughout each experiment, observations were taken using a Leica Wild M8 dissecting microscope, fitted with an eyepiece graticule. Light was supplied via a 12V Leitz light box, which was illuminated from under the microscope stage. For all measurements the magnification was set to 50X, with a 10X/21B 445111 eyepiece. The resultant magnification was 200 µm per one unit on the eyepiece micrometer. This measurement was verified using a micrometer slide. Photographs were taken at each time point using a Nikon digital camera that was mounted on the microscope to capture images through the eyepiece, allowing for the graticule measurements to be incorporated into all photographs. For all observations, the light box illumination was set at 7.5V, and the time for which the eggs were exposed to the light was kept to a minimum.

Daily observations allowed for the determination of survival rates for both eggs and larvae, and also time to hatching and hatch rates. Each experiment was terminated

between 60 h and 216 h, depending on the availability of live food for the newly hatched larvae.

2.2.8 Methods for capture of wild black bream

All black bream used in the field study (see chapter 7) were captured using rod and line or fishing nets (beach seine or gill nets) with the assistance of both commercial and recreational fishermen.

2.3 Dissection of fishes for tissue analysis

Adult fish used in the laboratory study (chapter 6) were anaesthetised (see section 2.2.4) then humanely sacrificed by cervical transection and dissected. Fish collected for the field study (chapter 7) were either dissected on site (Mallacoota Inlet) or transported live to RMIT Bundoora for processing (Yarra River, Maribyrnong River, Werribee River and Little River). Field collected fish were initially stunned with a blow to the head and then humanely sacrificed by cervical transection.

2.3.1 Tissue collection

Wet weight (g) and fork length (mm) were recorded for all fish. Blood was collected from the caudal vein (Fig. 2.3) using 3 ml heparinised syringes fitted with 20 gauge needles (Terumo, Australia) (see section 2.4.1 for details of heparin preparation). The plasma was separated by centrifugation at 5000 rpm for 10 min at 4^{o}C, then snap frozen in liquid nitrogen. Internal organs were exposed by removing a section of muscle tissue by making a cut from the anal vent forward towards the gills, through the pelvic girdle

and dorsally towards the pectoral fin (Fig. 2.4). Individual tissues (gonads, liver, gills, white muscle) were then removed, weighed and stored for analysis. Carcass weight was also recorded. Gonads were preserved by fixing in Bouin's fixative (see section 2.4.2 for details of preparation) for 1-4 weeks, followed by replacement of the fixative with 70% ethanol. Liver, gills and white muscle were wrapped in aluminium foil, labelled and snap frozen in liquid nitrogen. Bile was extracted from the gall bladder using 1 ml syringes fitted with 26 gauge needles (Terumo, Australia) and transferred to 1.5 ml microcentrifuge tubes then snap frozen in liquid nitrogen. Surface mucus was collected using individual pieces of polyurethane sponge ($2.5cm^3$) (Clark Rubber, Australia), applied with light pressure to the body surface, between the lateral line and dorsal fin base, as described by Schultz *et al.* (2007). Each fish was wiped three times, in an anterior-posterior (frontal-caudal) direction for the entire length of the dorsal fin base. Sponges containing absorbed surface mucus were placed directly into 1.5 ml microcentrifuge tubes and snap frozen in liquid nitrogen. All samples that were initially placed in liquid nitrogen were later transferred to a Sanyo Ultra low -80^oC freezer.

Otoliths were retained from most fish for age determination. Both sagittal otoliths were removed from the head cavity (Fig. 2.5) and then placed into small envelopes and allowed to air dry. The samples were sent to the Central Ageing Facility (CAF) which is located at the Department of Primary Industries Queenscliff Centre, Victoria.

2.3.2 Tissue index calculations

2.3.2.1 Gonadosomatic index (GSI)

[Gonad weight (g) / Total body weight (g)] x 100

2.3.2.2 Liver-somatic index (LSI)

[Liver weight (g) / Total body weight (g)] x 100

2.3.2.3 Condition factor

[Total body weight (g) / Fork length (cm)^3] x 100

2.4 Preparation of solutions for blood collection and gonad preservation

2.4.1 Heparin

All chemicals were purchased from Sigma-Aldrich, Australia. A 500 U/ml heparin solution was prepared by dissolving 100 mg thimerosal (methiolate) and 60 mg (10 000 U) heparin (sodium salt) in 20 ml of acidified saline (working) solution. The acidified saline was prepared as three separate stock solutions A, B and C. Stock solution A contained 5.9 g NaCl, 0.25 g KCl, 0.29 g, $MgSO_4$ and 8.0 g KH_2PO_4 in 100 ml Milli Q water. Stock solution B contained 0.28 g CaCl in 100 ml Milli Q water. Stock solution C contained 2.1 g $NaHCO_3$ in 100 ml Milli Q water. The working solution was prepared by mixing 10 ml of stock solution A, 10 ml stock solution B and 5 ml stock solution C with 75 ml Milli Q water. Once prepared, the heparin solution and remaining stock solutions were stored at 4^{o}C for up to 3 months.

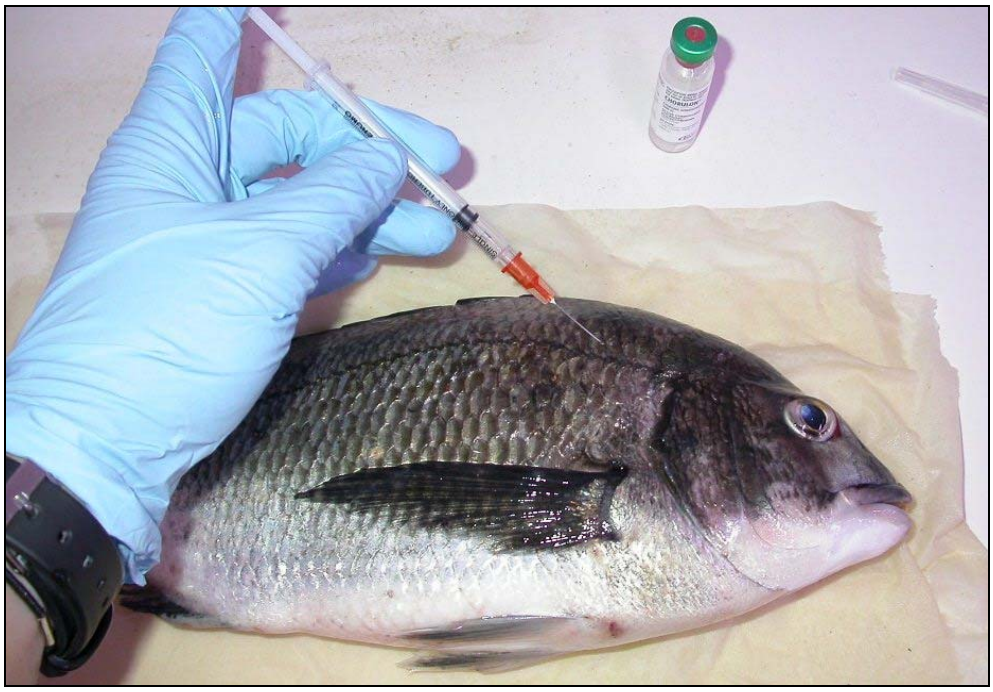


Figure 2.1 Intramuscular administration of the chorionic gonadotropin hormone Chorulon.

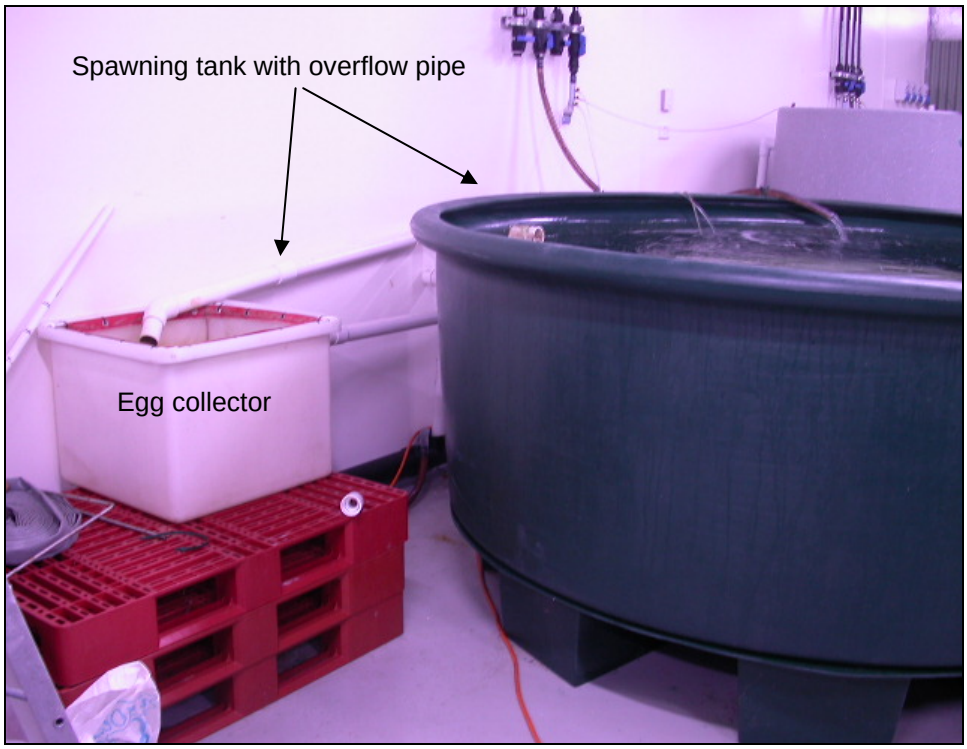


Figure 2.2 Egg collector and spawning tank.

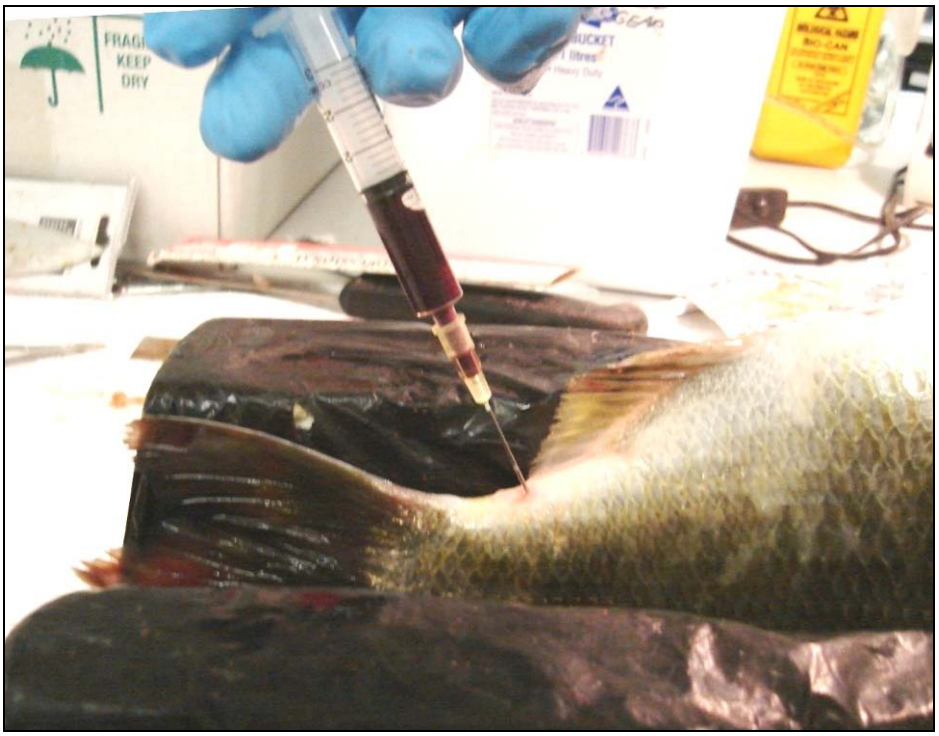


Figure 2.3 Blood collection by caudal puncture.

Figure 2.4 Female black bream dissection with ovaries removed.

Figure 2.5 Removal of sagittal otoliths from the head cavity (inset: black bream otoliths).

2.4.2 Bouin's fixative

Bouin's Fixative was prepared according to Humason (1967), by mixing 250 ml formaldehyde solution (Science Supply Australia), 50 ml glacial acetic acid (Science Supply Australia) and 750 ml picric acid solution (Sigma-Aldrich, Australia).

2.5 Protein determination

The protein content of all tissues used for analysis was determined using the DC Protein Assay Kit (Bio-Rad, Australia) which is a modified version of the Lowry protein assay (Lowry *et al.*, 1951). Protein standard curves were prepared using bovine serum albumin (BSA) (Sigma-Aldrich, Australia) dissolved in Milli Q water. The final concentrations of the standards were 0.0, 0.5, 1.0, 1.5 and 2.0 mg/ml protein (BSA).

2.6 Statistical analyses

Differences between experimental treatments were evaluated using one-way, two-way or three-way analysis of variance (ANOVA) (depending on experimental parameters). Tukey's Honestly Significant Difference or Dunnett's C multiple comparisons tests were used to detect significantly different treatment pairs. Levene's or Bartlett's test was used to assess homogeneity of variance and if the requirements of parametric tests were not satisfied, Kruskal-Wallis and Mann-Whitney tests were used (Zar, 1998). All data were transformed prior to analysis (percentage data were arcsine transformed, length/weight data were $\log_{10}$ transformed). All statistical analyses were performed using SPSS for Windows (version 14.0 or 15.0; SPSS Inc., Chicago, IL USA). The significance level in all tests was 0.05.

CHAPTER 3: EMBRYO DEVELOPMENT IN BLACK BREAM

Sections of this chapter have been submitted for publishing consideration.

Kathryn L. Hassell, Lisa G. Toogood, Patrick C. Coutin, Dayanthi Nugegoda. "Embryo development in the Australian black bream *Acanthopagrus butcheri* (Munro)." *Aquaculture* (submitted September, 2009).

3.1 Introduction

Limited response mechanisms, high surface-volume ratios and limited organ function all contribute to making the early life stages very sensitive to changing physical conditions (Houde, 1987). Consequently, poor environmental conditions are a leading cause of fish recruitment failure. The embryonic period is a critical part of an organism's life history, during which time the basic body plan is determined and cells differentiate into specialised tissues and organs (Slack, 1983). Normal embryo development is a defined sequence of stages that occur in a specific order at specific times (for a given temperature). Abnormal embryo development can be recognised in each of these stages and often results in either direct mortality, or reduced larval fitness, such as reduced swimming ability which may increase the risks of predation or starvation as well as reducing the ability for the larvae to locate suitable nursery and settlement habitat. Based on observations of embryo development, it is possible to predict egg quality, and therefore the likelihood of larval survival and ultimately recruitment (von Westernhagen and Dethlefsen, 1997; Kjorsvik *et al.*, 2003; Radonic *et al.*, 2005). Furthermore, determining the age of the eggs and thus the timing of the spawning event(s) enables

quantitative estimates of daily egg production (Norriss and Jackson, 2002) and is useful in determining the ecological mechanisms that drive recruitment within wild populations.

The morphology of eggs and pattern of embryo development are similar amongst most teleost fishes, whereby the eggs contain a large mass of yolk (telolecithal), may or may not contain oil droplets and undergo meroblastic cleavage in the blastodisc (Kunz, 2004; Falk-Petersen, 2005). A variety of abnormal patterns and deformities have been described for both fish eggs and larvae, and some common ones that occur in many different species are abnormal blastomere shape and cleavage patterns, skeletal deformities, changes in pigmentation, and fin deformities (see Divanach *et al.,* 1996). Normal blastomeres should be symmetrical, have high contact surface area between cells and have an even number of cells. Abnormal blastomere cleavage may result in non-symmetrical cleavage and unequal cell size, incomplete cell margins or low contact surface area between cells, or incorrect cell numbers (Shields *et al.*, 1997; Kjorsvik *et al.*, 2003). Many marine and estuarine fish species have transparent eggs which allow for abnormal cleavage patterns to be easily observed, providing a useful indicator of egg quality.

Recruitment patterns are highly variable in black bream, and it is likely that poor survival in the early life stages due to environmental stressors is a major factor (see section 1.4.7). However, at present there are no published reports detailing the different stages of embryo development in black bream. There are a number of publications that describe black bream eggs and larvae in ecological studies (Newton, 1996; Nicholson *et al.*, 2008;

Walker and Neira, 2001), whilst Partridge *et al.* (2003) have described some aspects of the egg and larval appearance in both black bream and the closely related snapper (*Pagrus auratus*). Neira *et al.* (1998) have described larval development in several sparids from temperate Australia, and the developmental stages of preserved snapper eggs from Shark Bay in Western Australia have been described by Norriss and Jackson (2002).

Embryo development has been described in detail for a number of other sparid species, including: silvery black porgy, *Acanthopagrus cuvieri* (Hussain *et al.*, 1981); silver bream, *Sparus sarba* (Tsukashima and Kitajima, 1982); pink dentex, *Dentex gibbosus* (Fernandez-Palacios *et al.*, 1994); common dentex, *Dentex dentex* (Jug-Dujakovic *et al.*, 1995); and red porgy, *Pagrus pagrus* (Radonic *et al.*, 2005).

This chapter describes the different developmental stages of black bream embryos (at a given temperature, salinity and DO level) for the first time. It was undertaken in order to distinguish normal embryo development from abnormal embryo development for the assessment of egg quality. Furthermore, accurate descriptions of embryo development will enable black bream eggs to be correctly identified and distinguished from eggs of other species with a similar appearance (eg. estuary perch, *Macquaria colonorum*), when mixtures of eggs are collected in field studies.

3.2 Materials and Methods

3.2.1 Collection of fertilised eggs

Spawning was induced in females via injection with chorionic gonadotropin hormones which stimulate ovulation (see section 2.2.5). Induced female black bream began releasing eggs approximately 2 d after receiving hormone treatment. Spawning events occurred during the evening, between the hours of 17:30 pm and 07:00 am. The exact timing of fertilisation was not determined, since the fish were not individually stripped of gametes and no *in vitro* fertilisation was conducted. Instead, spawning fish were held in a tank that was fitted with an overflow that led into a 500 µm mesh egg collector. The collector was checked at regular intervals (every 30 min) throughout the spawning period to ensure that only newly fertilised eggs were collected. Newly fertilised eggs or zygotes (1-cell) were collected and assigned as time 0 (zero).

The general terms used to describe different developmental stages were based on those used by Kunz (2004), whilst the classification of different embryonic stages was based on the detailed descriptions of zebrafish (*Danio rerio*) development by Kimmel *et al.* (1995), as well as published descriptions of embryo development in several sparid species: *Acanthopagrus cuvieri* (Hussain *et al.*, 1981); *Dentex dentex* (Jug-Dujakovic *et al.*, 1995); *Pagrus auratus* (Norriss and Jackson, 2002); and *Pagrus pagrus* (Radonic *et al.*, 2005).

3.2.2 Observations of embryonic development

Newly fertilised eggs were incubated in plastic 6-well plates (Iwama, Australia) containing 10 ml filtered seawater and each well contained 10-20 eggs. The water quality was maintained as follows (mean ± SEM): DO (%S) 87.0 ± 4.19, salinity (ppt) 36.5 ± 0.58, pH 8.01 ± 0.13 and temperature (^{o}C) 21.2 ± 0.45. Every 30 min development was observed using a Leica Wild M8 dissecting microscope and 12V Leitz light box and digital photographs were taken (see section 2.2.7).

3.3 Results

3.3.1 General observations

3.3.1.1 General appearance

Newly fertilised black bream eggs (zygotes) were spherical, transparent and contained one small, round oil globule (Fig. 3.1). The normal size of eggs ranged from 600-800 µm, and the oil globule was 150-250 µm.

3.3.1.2 Egg quality criteria and identification of abnormal embryos

Good quality black bream eggs were positively buoyant in full strength seawater (35 ppt) and had a smooth texture without ridges on the chorion or yolk surface. Eggs that exhibited abnormal development had a non-spherical shape, abnormal colouration, multiple oil globules or were non-buoyant in seawater. In the present study abnormal development often commenced right from the 1-cell stage, resulting in non-symmetrical cleavage patterns and subsequent abnormal blastula formation. Rarely did eggs of this condition survive to proceed through gastrulation.

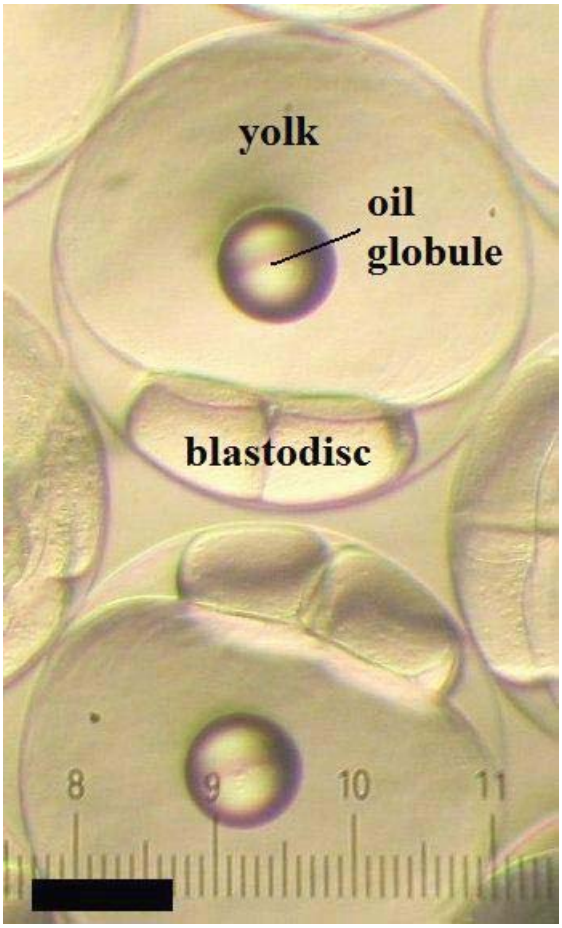


Figure 3.1 Components of a normal black bream embryo. Most of the space inside the chorion is occupied by the large yolk mass. There is a single, spherical oil globule and the blastodisc develops at the animal pole. Bar = 200 µm.

3.3.2 Description of the developmental stages of black bream embryos

A total of 20 separate developmental stages were described for black bream embryos, from spawning through to hatching, based on terminology adapted from Kimmel *et al.* (1995) and Kunz (2004) (Table 3.1).

Table 3.1 Stages of embryonic development in *Acanthopagrus butcheri* eggs, maintained in filtered seawater (mean ± SEM): dissolved oxygen (%S) 87.0 ± 4.19, salinity (ppt) 36.5 ± 0.58, pH 8.01 ± 0.13 and temperature (^{o}C) 21.2 ± 0.45).

Stage	Description	Time (hours)
1	1-cell (zygote)	0
2	2-cell	0.5
3	4-cell	1
4	8-cell	1.5
5	16-cell	2
6	32-cell	2.5
7	Morula	3
8	Blastula	3.5
9	Late blastula	4
10	Gastrula (30% epiboly)	8.5
11	Late gastrula (50% epiboly)	11.5
12	Neurula (90% epiboly)	13
13	Late neurula (100% epiboly)	15
14	Embryo - segmentation begins	16
15	Embryo - seven somites	17
16	Embryo - ten somites	18
17	Embryo - heart beat first detected	27
18	Embryo - tail elongation and erratic movements	28
19	Embryo - immediately prior to hatching	32
20	Newly hatched larva	34

3.3.2.1 Stage 1. One cell. Fertilised egg (zygote)

Newly fertilised eggs were spherical, transparent and contained a single oil globule (Fig. 3.2, 3.8). Cleavage had not yet started and the large yolk mass occupied most of the space within the chorion. It was not possible to distinguish newly fertilised eggs from unfertilised eggs. Stage 1 lasted for approximately 30 min (post fertilisation).

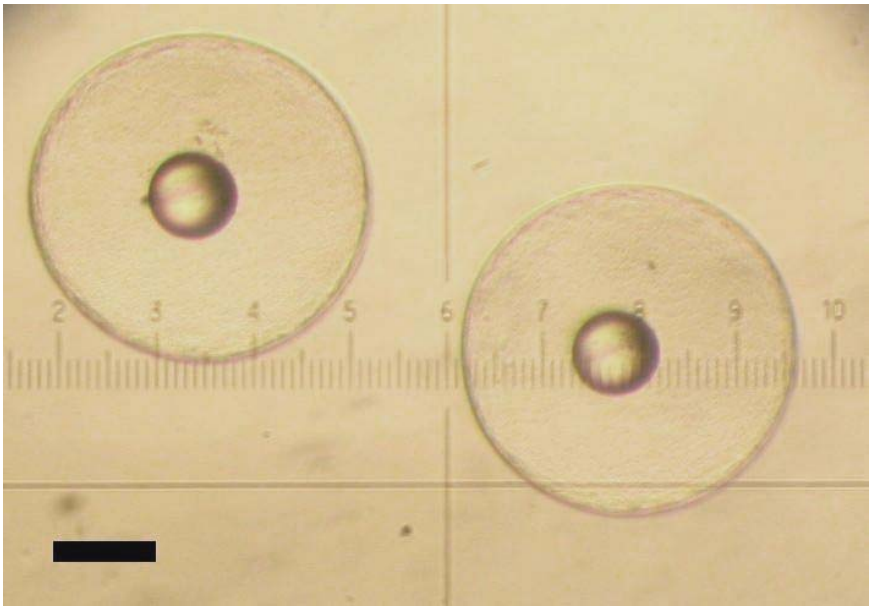


Figure 3.2 *Stage 1.* One cell. Fertilised egg (zygote). Cleavage had not yet started. Bar = 200 µm.

3.3.2.2 Stage 2. Two cells

Thirty min post fertilisation the first cleavage of the blastodisc occurred, resulting in two blastomeres (cells). Cleavage was incomplete (meroblastic), so that blastomeres were only partially separated from each other (Fig. 3.3, 3.8). Stage 2 lasted for approximately 30 min.

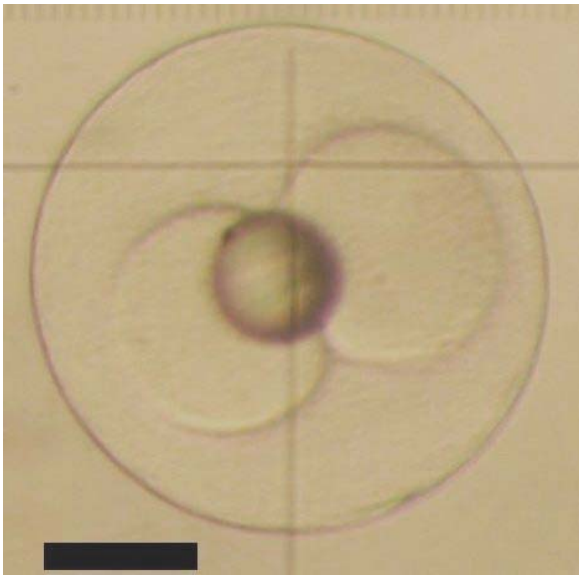


Figure 3.3 *Stage 2.* Two cells. First cleavage of blastodisc occurred and was of a vertical orientation, resulting in two blastomeres (cells). Cleavage was incomplete (meroblastic), so that blastomeres were only partially separated from each other. Bar = 200 µm.

3.3.2.3 Stage 3. Four cells

One h post fertilisation the second cleavage event occurred at right angles to the first cleavage in each blastomere. Stage 3 resulted in four partially separated blastomeres, in a 2x2 array (Fig. 3.4, 3.8). Stage 3 lasted for approximately 30 min.

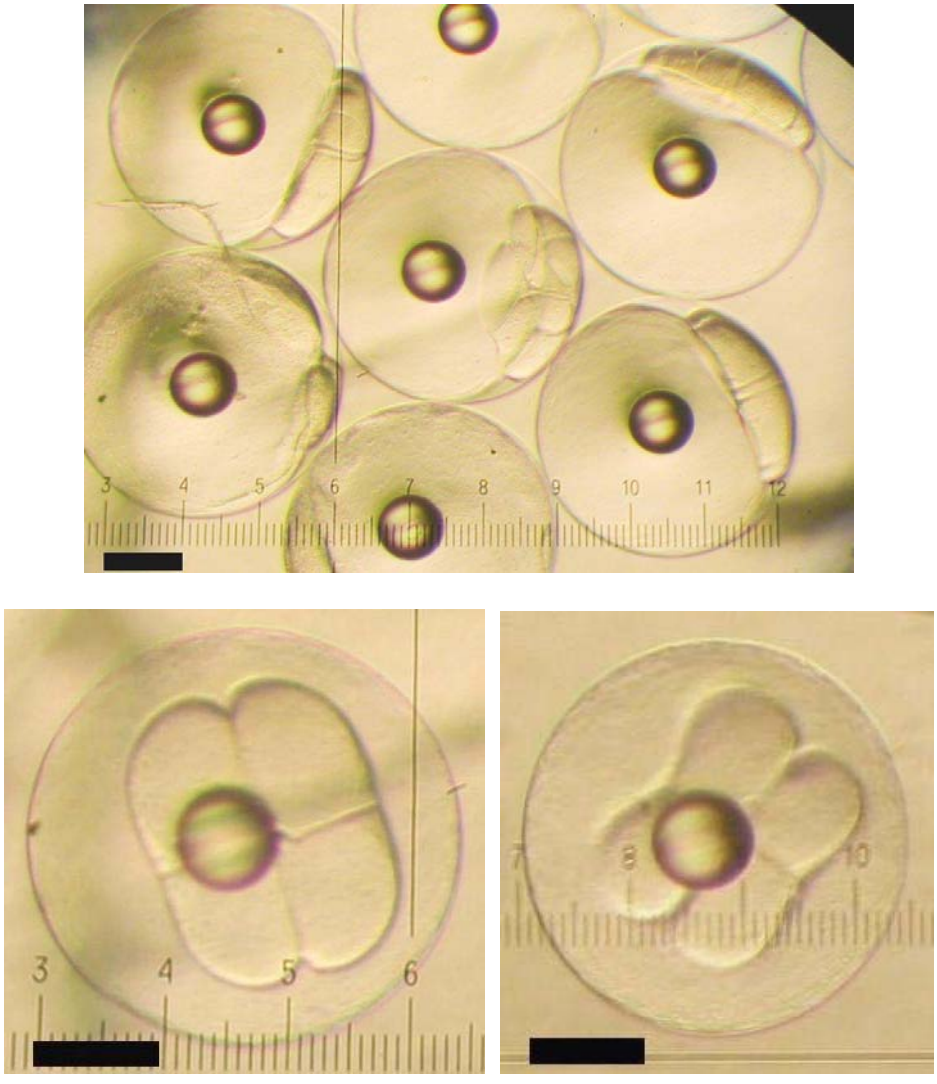


Figure 3.4 *Stage 3.* Four cells. Second cleavage occurred at right angles to the first cleavage in each blastomere, resulting in four partially separated blastomeres. Bar = 200 µm.

3.3.2.4 Stage 4. Eight cells

One h and 30 min post fertilisation the third cleavage occurred at right angles to the second cleavage (parallel or, along the same plane as the first cleavage). Stage 4 resulted in 8 partially separated blastomeres, in a 2x4 array (Fig. 3.5, 3.8). Stage 4 lasted for approximately 30 min.

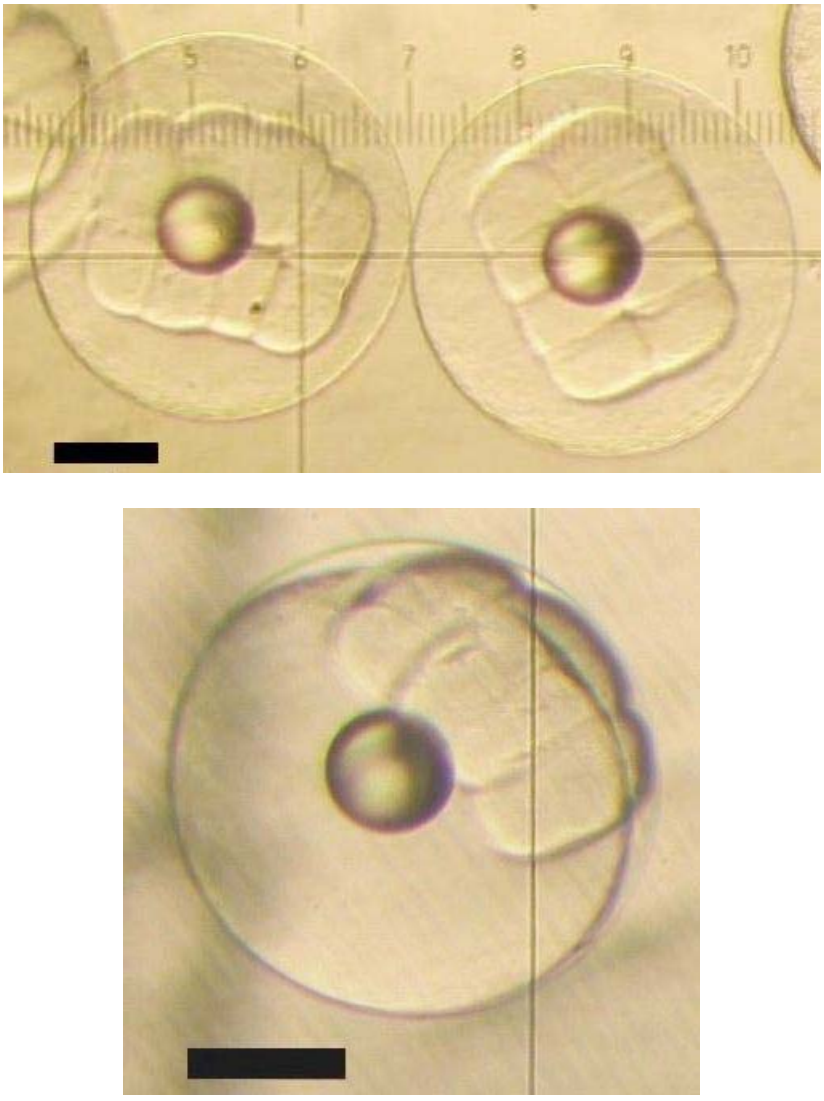


Figure 3.5 *Stage 4.* Eight cells. Third cleavage occurred at right angles to the second cleavage (or, along the same plane as the first cleavage), resulting in 8 partially separated blastomeres. Bar = 200 µm.

3.3.2.5 Stage 5. Sixteen cells

Two h post fertilisation the fourth cleavage occurred at right angles to the third cleavage (parallel or, along the same plane as the second cleavage). Stage 5 resulted in 16 partially separated blastomeres, in a 4x4 array (Fig. 3.6, 3.8). Stage 5 lasted for approximately 30 min.

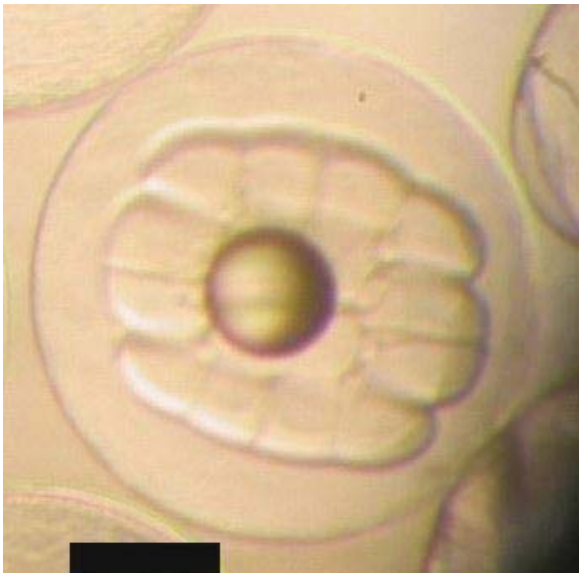


Figure 3.6 *Stage 5.* Sixteen cells. Fourth cleavage occurred at right angles to the third cleavage (or, along the same plane as the second cleavage), resulting in sixteen partially separated blastomeres. Bar = 200 µm.

3.3.2.6 Stage 6. Thirty-two cells

Two h and 30 min post fertilisation the fifth cleavage occurred at right angles to the fourth cleavage (parallel or, along the same plane as the first cleavage). Blastomeres were arranged in a 4x8 array, however individual blastomeres became difficult to see as they were no longer arranged as a single layer (Fig. 3.7, 3.8). Stage 6 lasted for approximately 30 min.

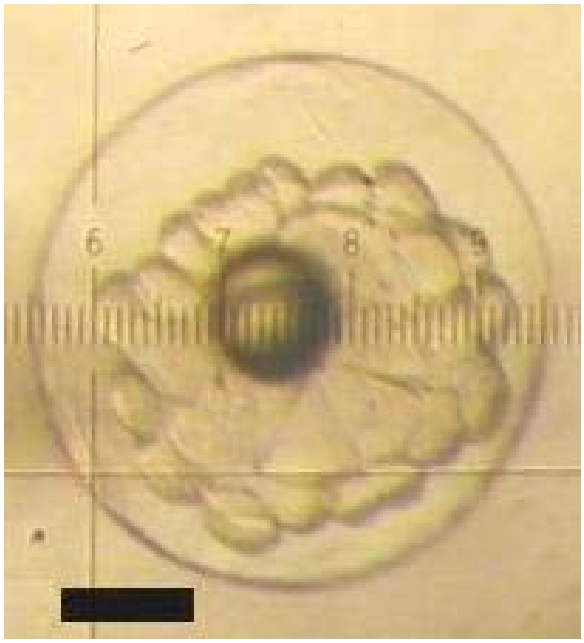
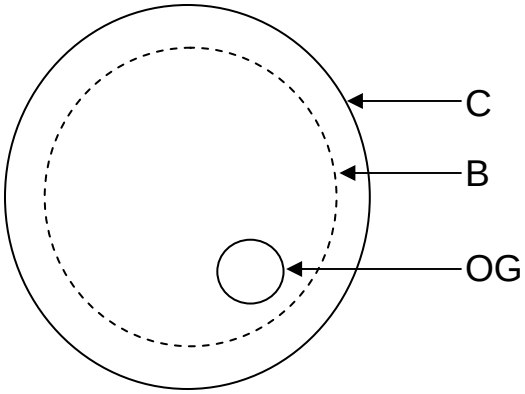
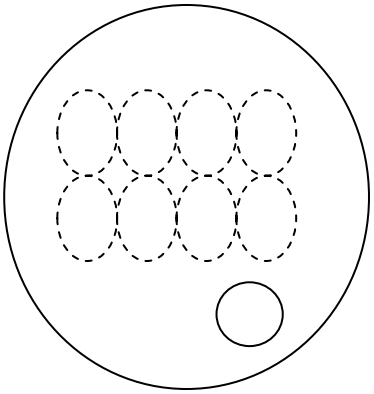


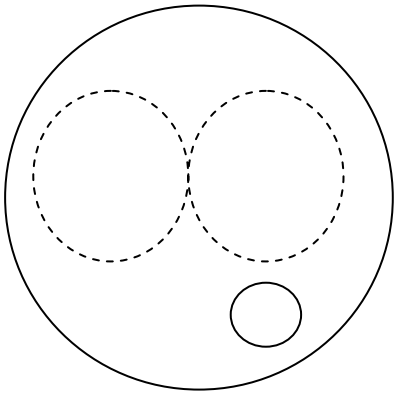
Figure 3.7 *Stage 6.* Thirty-two cells. Fifth cleavage occurred at right angles to the fourth cleavage (or, along the same plane as the first cleavage). Bar = 200 µm.



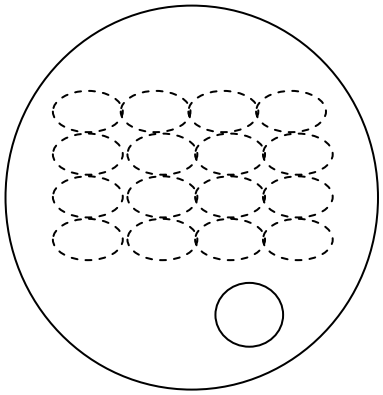
Stage 1. **1-cell.**



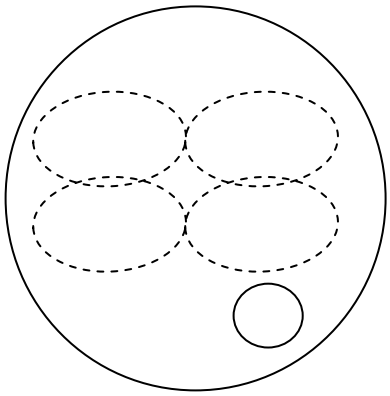
Stage 4. **8-cell.**



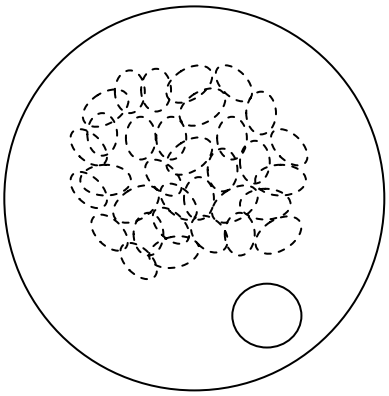
Stage 2. **2-cell.**



Stage 5. **16-cell.**



Stage 3. **4-cell.**



Stage 6. **32-cell.**

Figure 3.8 Diagrammatic view of the first six stages of black bream embryo blastomere cleavage. The orientation of the cleavage events alternated between stages. Stages 2, 4 and 6 were 'vertical', whilst stages 3 and 5 occurred at right angles to stages 2, 4 and 6. C – chorion, B – blastodisc, OG – oil globule.

3.3.2.7 Stage 7. Morula 'mulberry stage'

Three h post fertilisation the blastodisc entered 'mulberry stage', where individual blastomeres were small and no longer visible. The cleavage patterns were not as uniform as previous stages (Fig. 3.9). Stage 7 lasted for approximately 30 min.

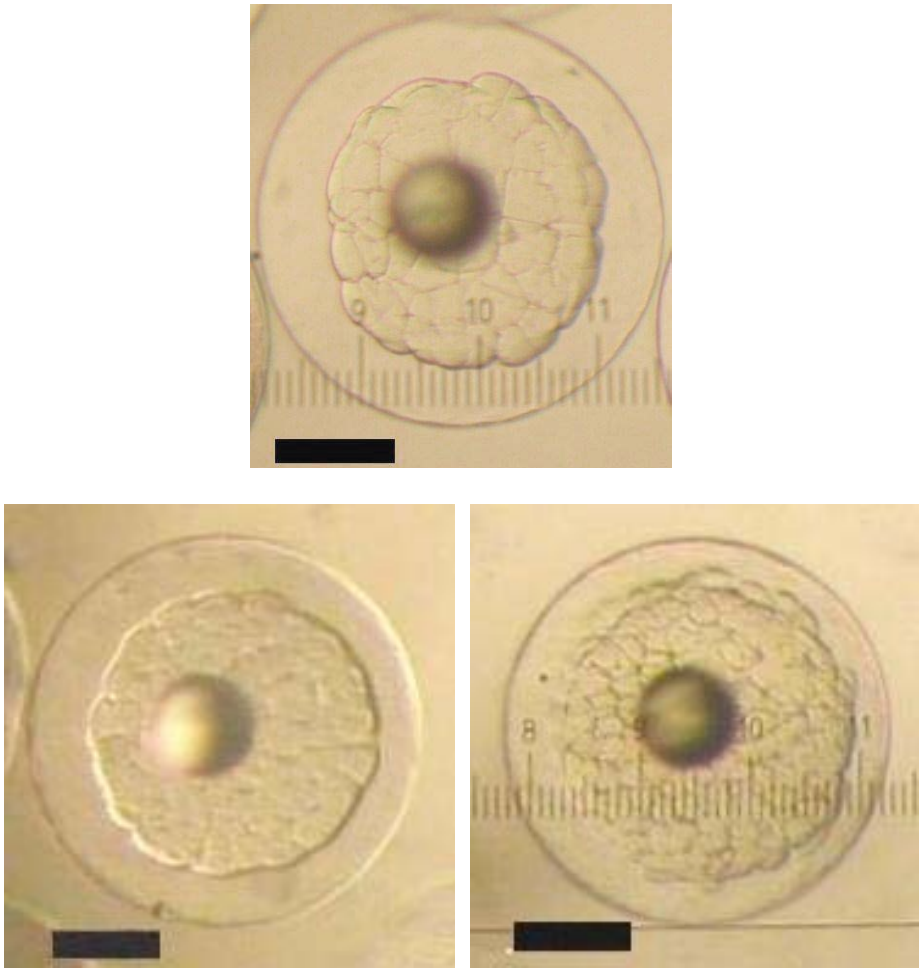


Figure 3.9 *Stage 7.* Morula 'mulberry stage'. Individual blastomeres no longer visible and cleavage patterns are not as uniform as previous stages. Bar = 200 µm.

3.3.2.8 Stage 8. Blastula

Three h and 30 min post fertilisation the blastodisc became disc-like and individual blastomeres could not be distinguished. Cleavage patterns and the arrangement of blastomeres were not uniform (Fig. 3.10). Stage 8 lasted approximately 30 min.

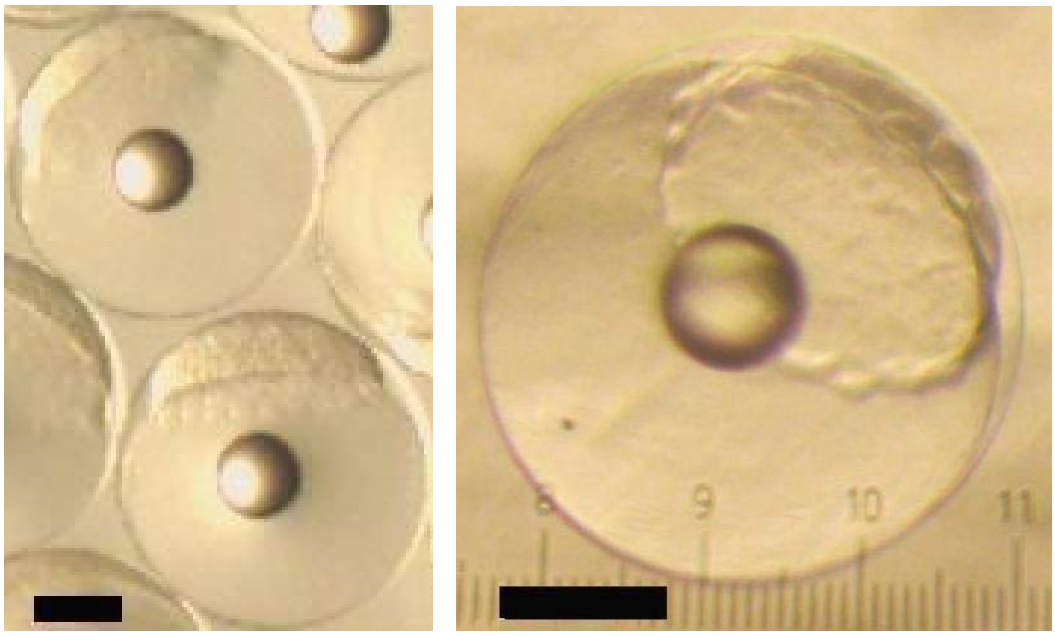


Figure 3.10 *Stage 8.* Blastula. Blastodisc was disc-like and individual blastomeres could not be distinguished. Arrangement of blastomeres was not uniform. Bar = 200 µm.

3.3.2.9 Stage 9. Late blastula

Four h post fertilisation the blastodisc became thinner and started to spread over the surface of the yolk cell (epiboly) (Fig. 3.11). In subsequent stages the blastodisc is referred to as the 'blastoderm'. Stage 9 lasted for approximately 4 h and 30 min.

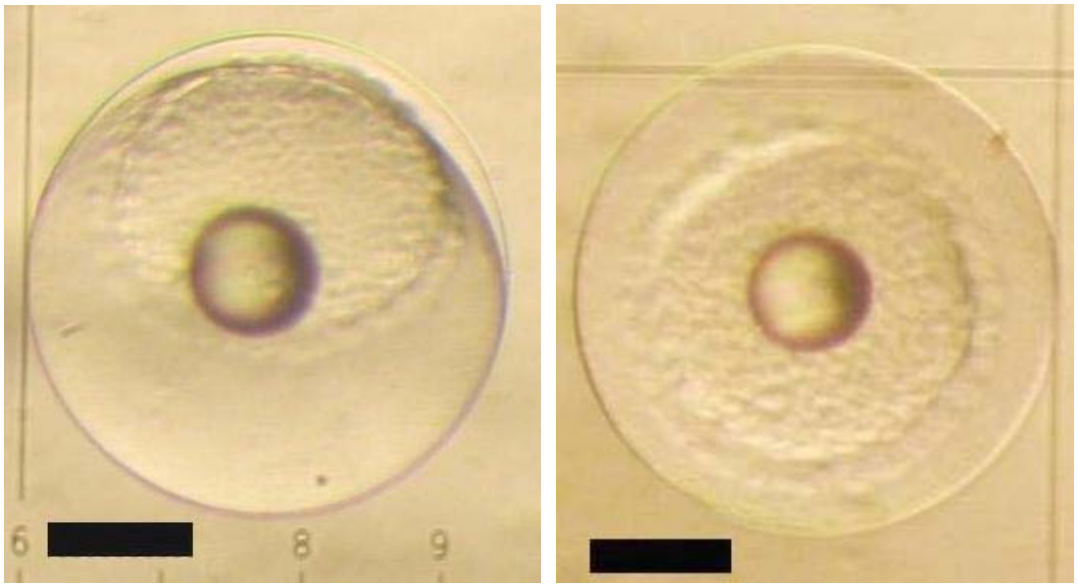


Figure 3.11 *Stage 9.* Late blastula. Blastodisc became thinner and spread over the surface of the yolk cell (epiboly). In subsequent stages the blastodisc is referred to as the 'blastoderm'. Bar = 200 µm.

3.3.2.10 Stage 10. Gastrula

Eight h and 30 min post fertilisation gastrulation (involution) started. The germ ring and embryonic shield form and the blastoderm continued to spread over the surface of the yolk cell (30% epiboly) (Fig. 3.12). Stage 10 lasted for approximately 3 h.

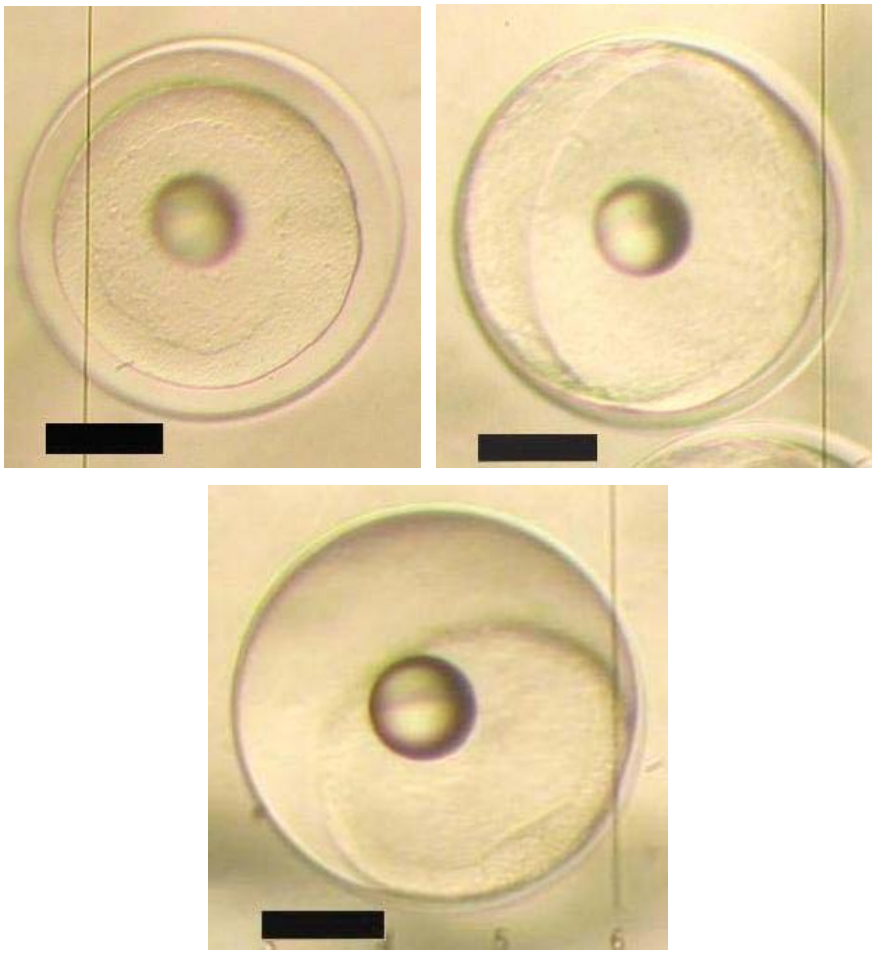


Figure 3.12 *Stage 10.* Gastrula. Gastrulation (involution) started. The germ ring and embryonic shield formed and the blastoderm continued to spread over the surface of the yolk cell (30% epiboly). Bar = 200 µm.

3.3.2.11 Stage 11. Late gastrula

Eleven h and 30 min post fertilisation the blastoderm continued to spread over the surface of the yolk cell, covering half of it (50% epiboly) (Fig. 3.13). Stage 11 lasted for approximately 1 h and 30 min.

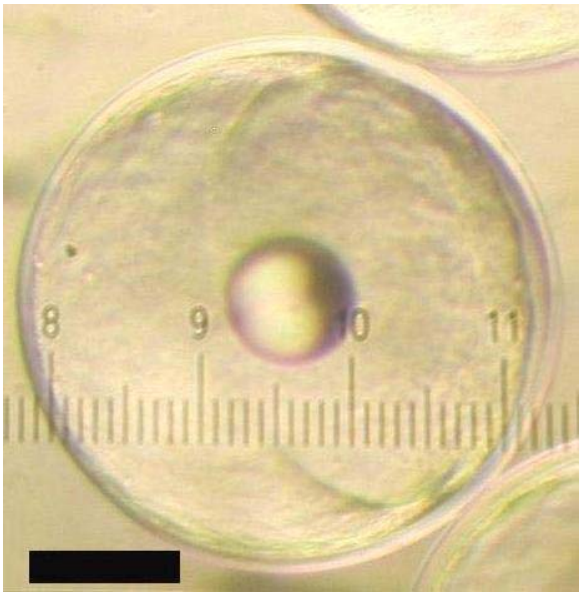


Figure 3.13 *Stage 11*. Late gastrula. Blastoderm continued to spread over the surface of the yolk cell, covering half of it (50% epiboly). Bar = 200 µm.

3.3.2.12 Stage 12. Neurula

Thirteen h post fertilisation the neural plate developed, and the blastoderm covered most of the yolk cell (90% epiboly) (Fig. 3.14). The remaining part of the yolk cell uncovered by the blastoderm is known as the yolk plug. Stage 12 lasted approximately 2 h.

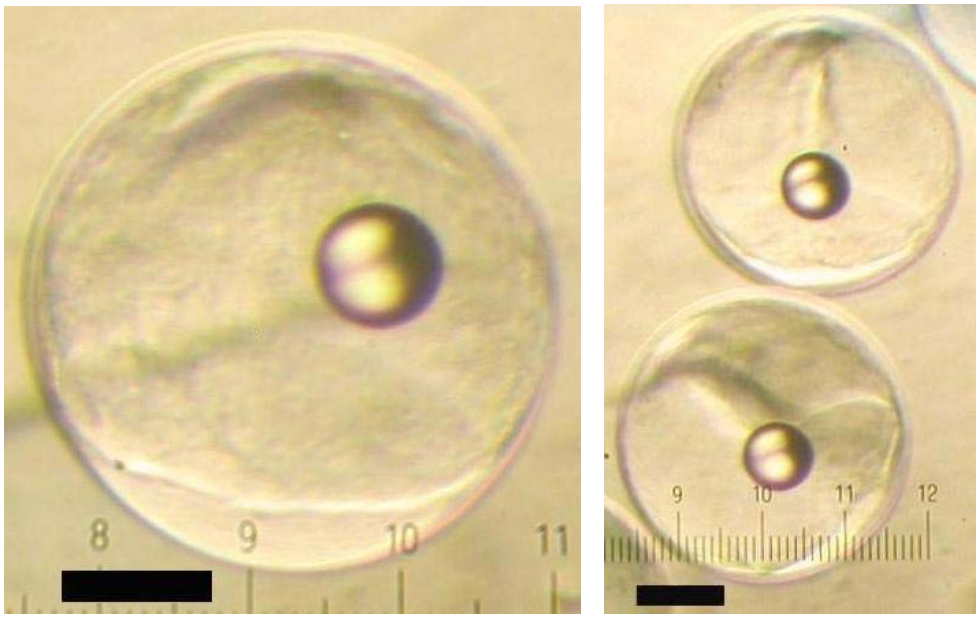


Figure 3.14 *Stage 12.* Neurula. The neural plate developed. Blastoderm covered most of the yolk cell (90% epiboly). The remaining part of the yolk cell uncovered by blastoderm is known as the yolk plug. Bar = 200 µm.

3.3.2.13 Stage 13. Late neurula

Fifteen h post fertilisation the blastoderm completely covered the yolk plug (100% epiboly). During this time the tail bud and optic lobes started to form (Fig. 3.15). Stage 13 lasted approximately 1 h.

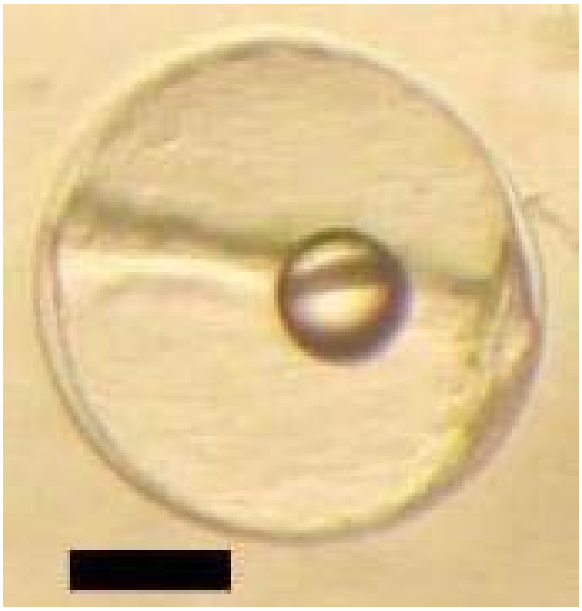


Figure 3.15 *Stage 13.* Late neurula. Blastoderm completely covered the yolk plug (100% epiboly). Tail bud and optic lobes started to form. Bar = 200 µm.

3.3.2.14 Stage 14. Embryo. Segmentation begins

Sixteen h post fertilisation the head region and tail bud could be clearly distinguished and somites became visible. Pigmentation was first observed on the oil globule and the embryo lay along 90-180^{o} of the egg wall (Fig. 3.16). Stage 14 lasted approximately 1 h.

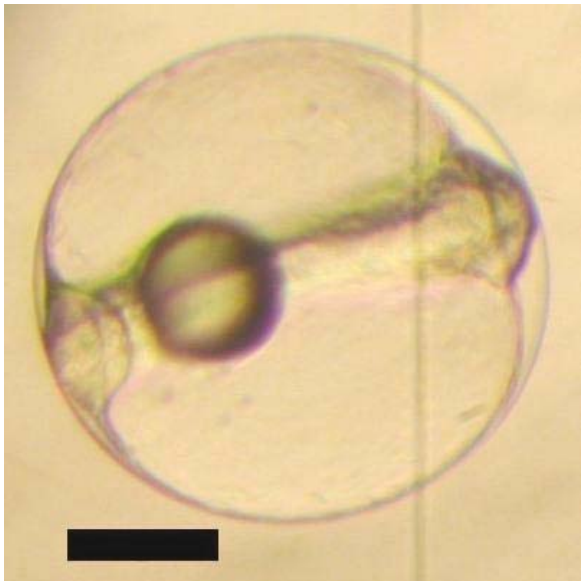


Figure 3.16 *Stage 14.* Embryo. Segmentation started. Somites became visible. Pigmentation on oil globule. Embryo lay along 90-180^{o} of the egg wall. Bar = 200 µm.

3.3.2.15 Stage 15. Embryo. Seven somites

Seventeen h post fertilisation seven somites (precursors to myotomes) were visible on the embryo trunk and the optic vesicles could be seen. Pigmentation occurred on the oil globule and along the embryo, which lay along 90-180^{o} of the egg wall (Fig. 3.17). Stage 15 lasted 1 h.

Figure 3.17 *Stage 15.* Embryo. Seven somites. Pigmentation on oil globule and embryo. Embryo lay along 90-180^{o} of the egg wall. Bar = 200 µm.

3.3.2.16 Stage 16. Embryo. Ten somites

Eighteen h post fertilisation ten somites were visible on the embryo trunk and tail. Pigmentation increased on the oil globule and embryo, which lay along 90-180^{o} of the egg wall (Fig. 3.18). Somites continued to form along the embryo and started developing into V-shaped myotomes. Stage 16 lasted approximately 9 h.

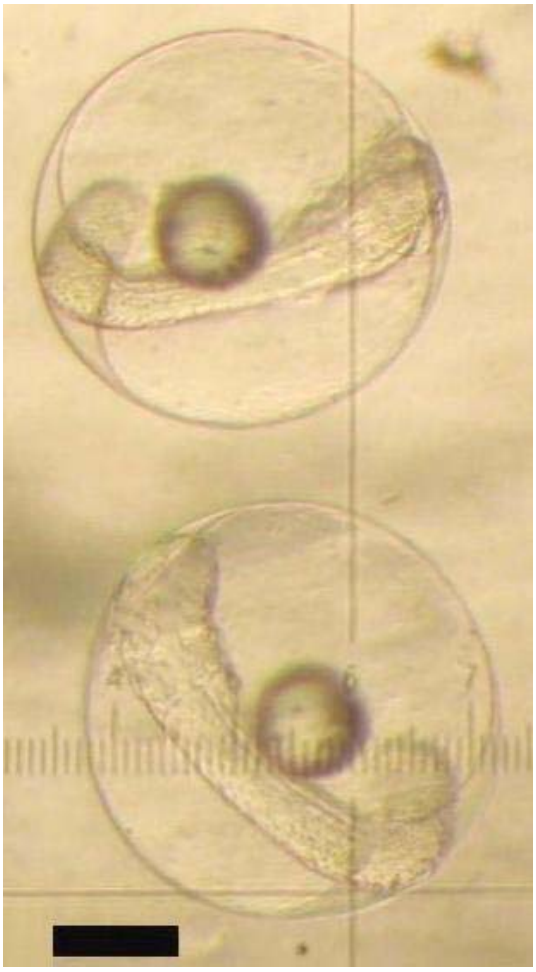


Figure 3.18 *Stage 16.* Embryo. Ten somites. Pigmentation on oil globule and embryo. Embryo lay along 90-180^{o} of the egg wall. Bar = 200 µm.

3.3.2.17 Stage 17. Embryo. Heart beat first detected

Twenty seven h post fertilisation the beating heart was first detected. Myotomes continued to form (resulting in an increase in the embryo length) and pigmentation increased on the embryo, which lay along 180-270^{o} of the egg wall (Fig. 3.19). Stage 17 lasted approximately 1 h.

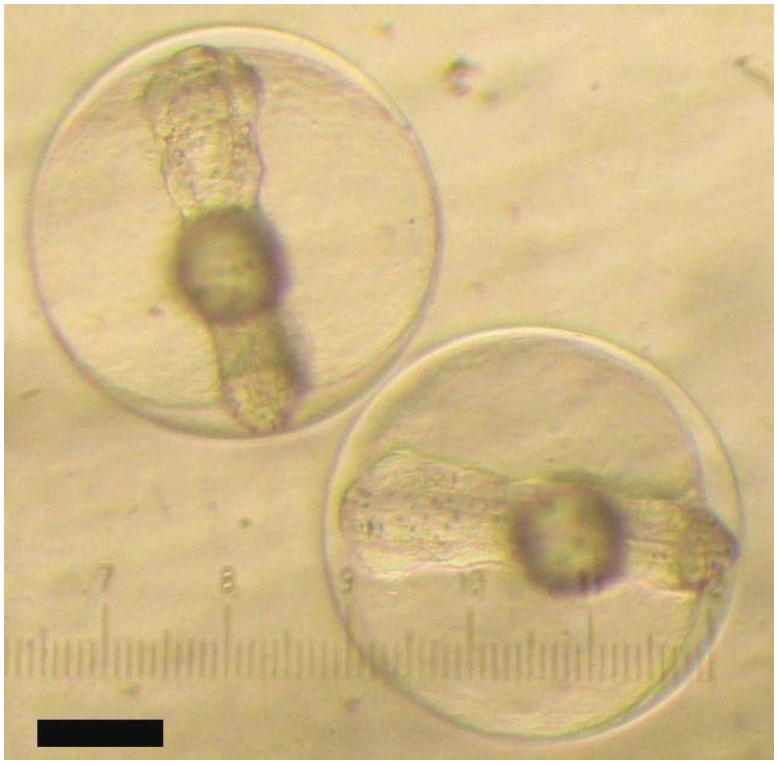


Figure 3.19 *Stage 17.* Embryo. Heart beat first detected. Pigmentation on oil globule. Embryo lay along 180-270^{o} of the egg wall. Bar = 200 µm.

3.3.2.18 Stage 18. Embryo. Tail elongation and erratic movements

Twenty eight h post fertilisation the embryo continued to increase in length and started moving erratically. Otoliths could be observed just posterior to the optic vesicles. Pigmentation increased on the embryo, which lay along 270-360^{o} of the egg wall (Fig. 3.20). Stage 18 lasted for 4 h.

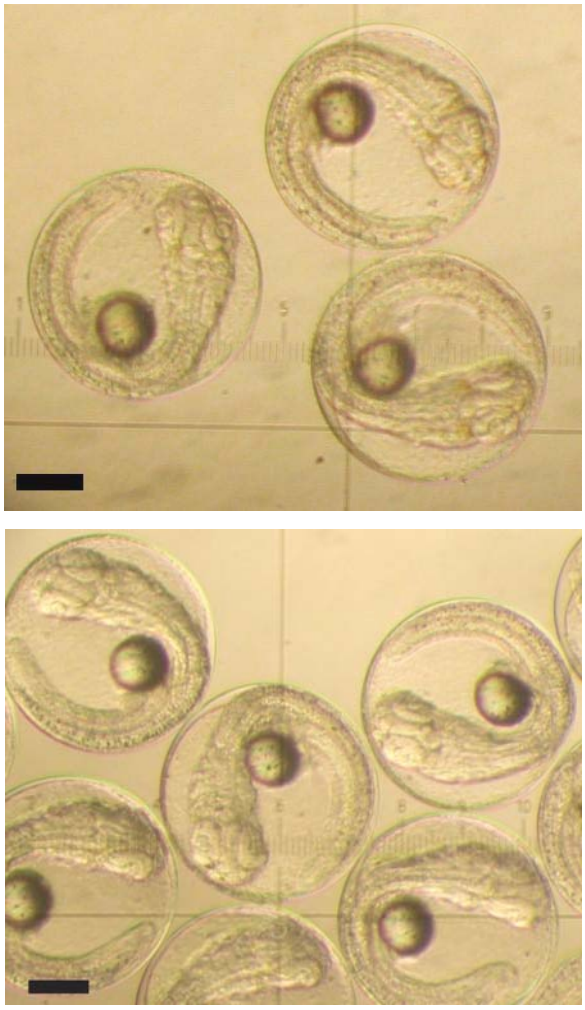


Figure 3.20 *Stage 18.* Embryo. Tail elongation and erratic movements. Pigmentation on oil globule and embryo. Embryo lay along 270-360^{o} of the egg wall. Bar = 200 µm.

3.3.2.19 Stage 19. Immediately prior to hatching

Thirty two h post fertilisation the embryo movements became much more frequent. Pigmentation increased on the embryo, which lay along 270-360^{o} of the egg wall (Fig. 3.21). Stage 19 lasted approximately 2 h.

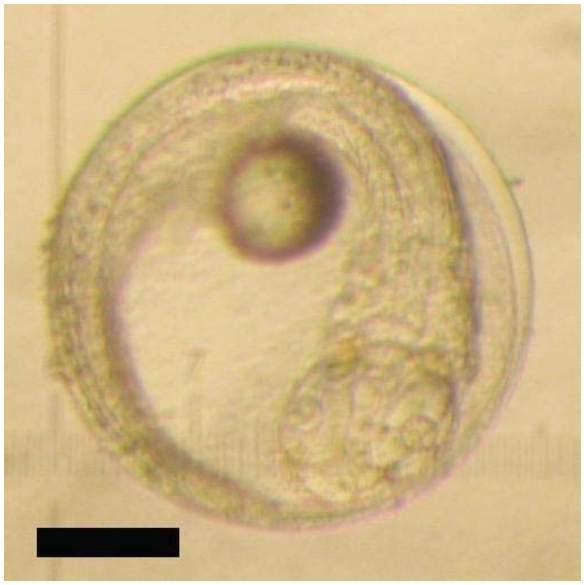


Figure 3.21 *Stage 19.* Immediately prior to hatching. Pigmentation on oil globule and embryo. Embryo lay along 270-360^{o} of the egg wall. Bar = 200 µm.

3.3.2.20 Stage 20. Newly hatched larva

Thirty four h post fertilisation the embryonic period was completed and the larva broke free of the chorion (Fig. 3.22). Rapid tail movements assisted in rupturing the chorion, from which the larva exited tail first.

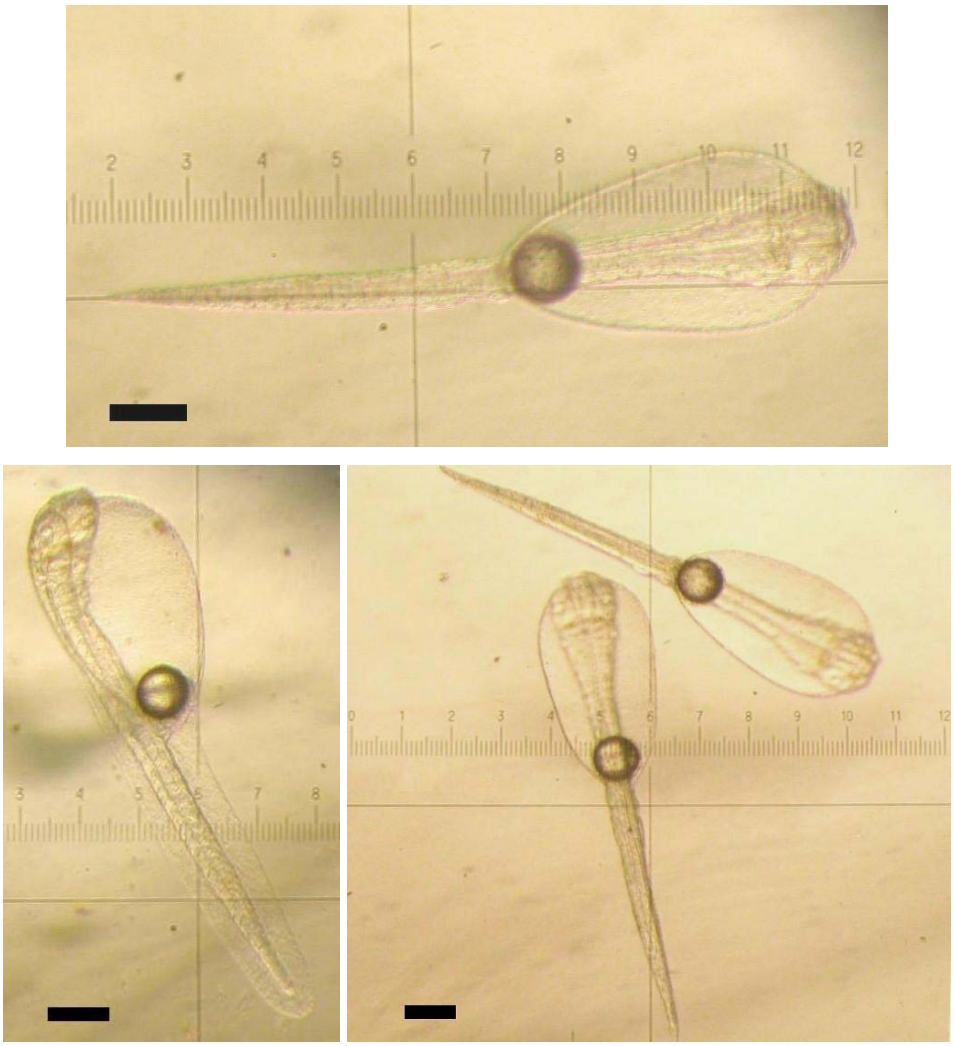


Figure 3.22 ***Stage 20.*** **Newly hatched larva. Embryonic period was completed and the larva broke free of the chorion. Rapid tail movements assisted in rupturing the chorion, from which the larva exited tail first. Bar = 200 µm.**

3.3.3 Description of black bream embryo abnormalities and larval deformities

3.3.3.1 Multiple oil globules

Eggs were or were not spherical and contained 2-4 oil globules (Fig. 3.23). Few eggs with multiple oil globules developed into larvae, and those that did were generally deformed.

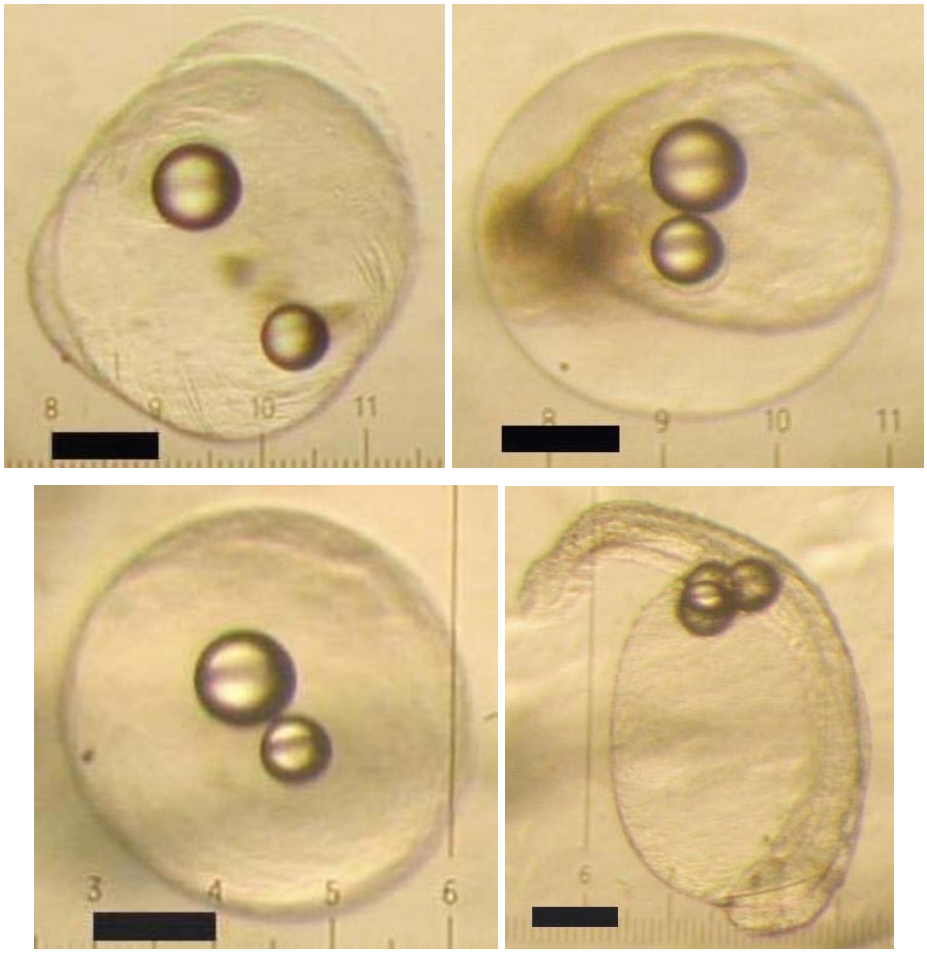


Figure 3.23 Multiple oil globules. Bar = 200 µm.

3.3.3.2 Non spherical chorion

Chorion was a distorted shape and may have or may not have been swollen. Surface may have appeared wrinkled or discoloured (Fig. 3.24).

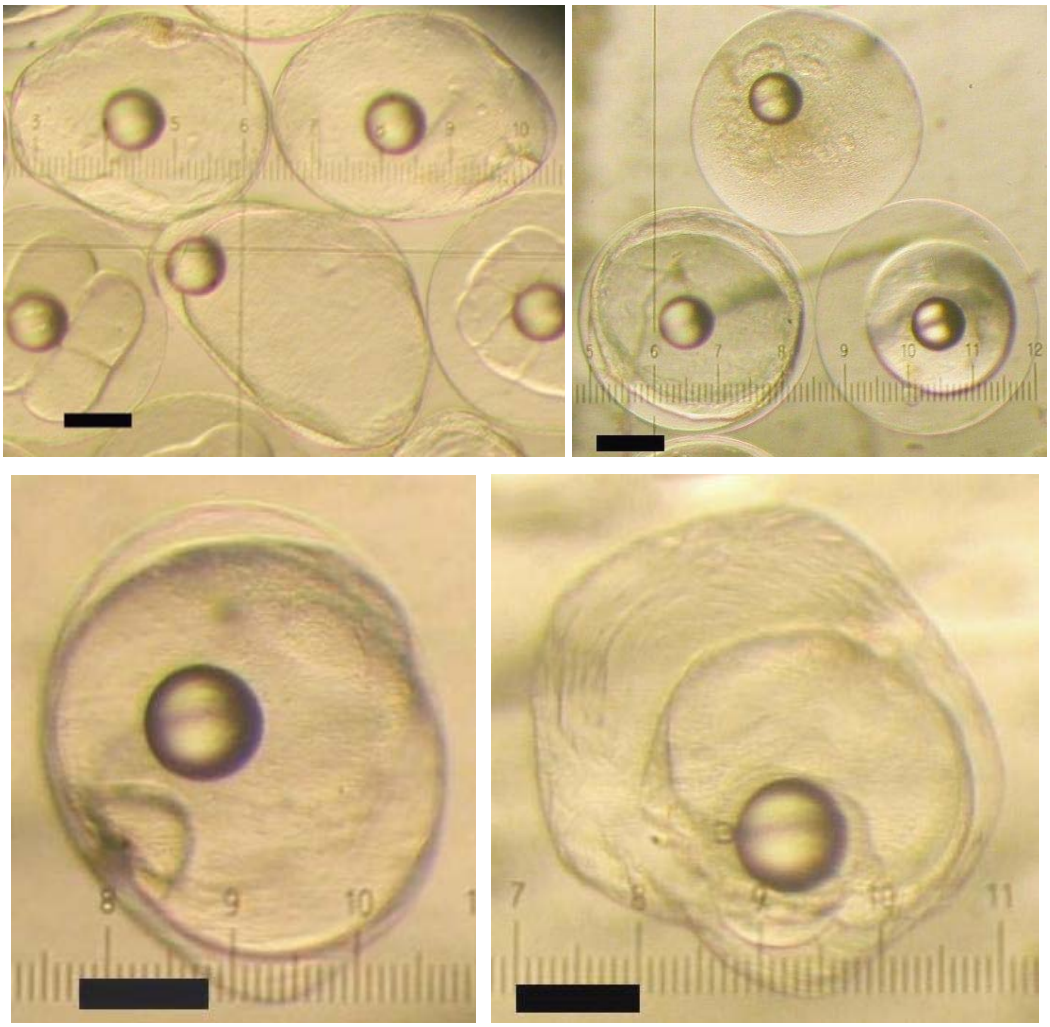


Figure 3.24 Non spherical chorion. Bar = 200 µm.

3.3.3.3 Abnormal blastomere cleavage

Blastomeres may have had non-symmetrical cleavage and unequal cell size, incomplete cell margins or low contact surface area between cells, or incorrect cell numbers (Fig. 3.25).

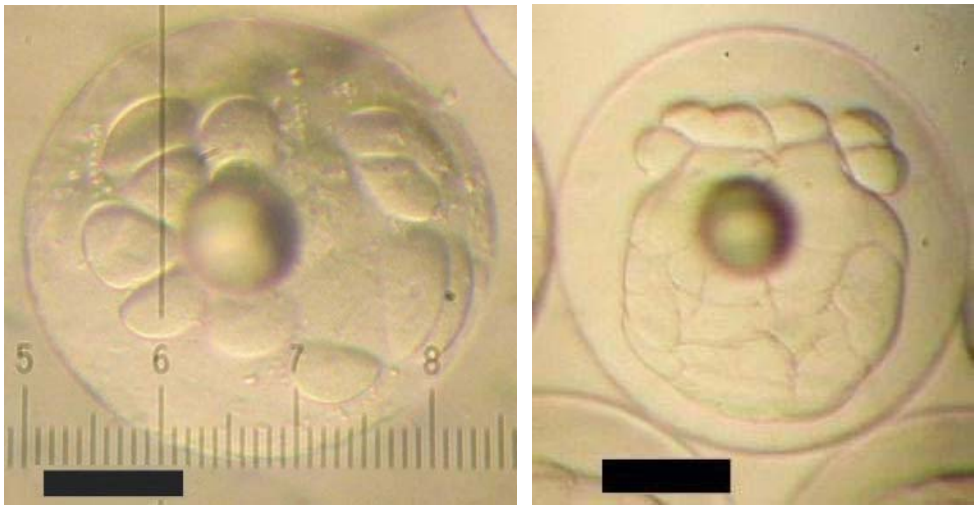


Figure 3.25 Abnormal blastomere cleavage. Bar = 200 µm.

3.3.3.4 Disrupted or retarded development

Blastomere cleavage may have ceased, or abnormal development post-gastrulation may have resulted in abnormal cell growth or complete cessation of development. The developing embryo was observed to be smaller than usual, the head or tail may have been malformed or there may have been a change in the size of the perivitelline space surrounding the embryo (Fig. 3.27).

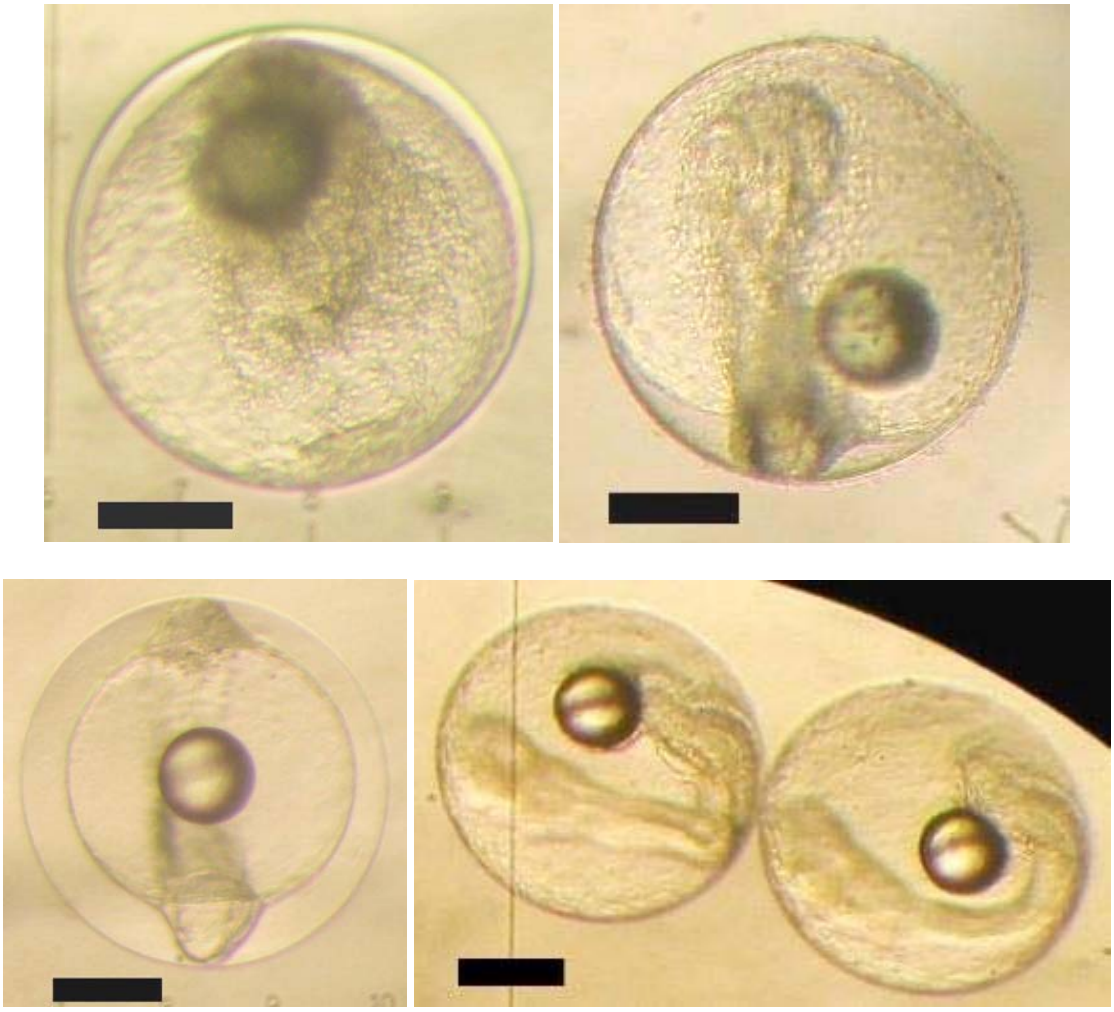


Figure 3.27 Disrupted or retarded development. Bar = 200 µm.

3.3.3.5 Incomplete hatching (partial emergence)

Embryo did not break free completely of the chorion during the hatching process. Larvae exhibited severe spinal deformities and often died within a few hours (Fig. 3.28).

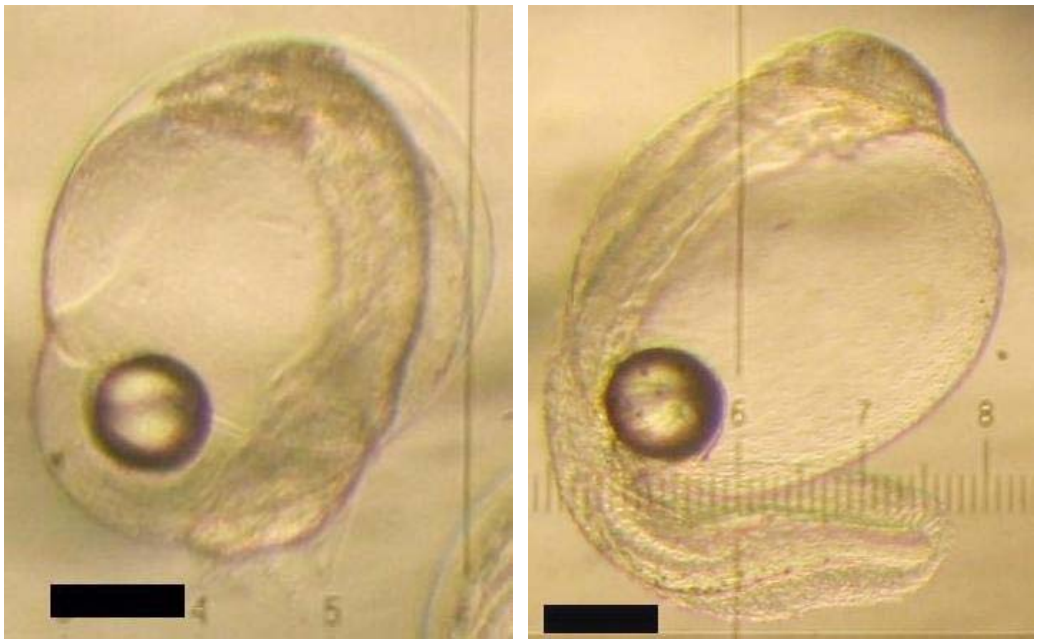


Figure 3.28 Incomplete hatching (partial emergence). Bar = 200 µm.

3.3.3.6 Spinal column deformities

Larvae displayed single or multiple kinks in the spine, or complete curvature such that it 'wrapped' around the yolk-sac. Swimming ability was affected by the severity of the deformity (Fig. 3.29).

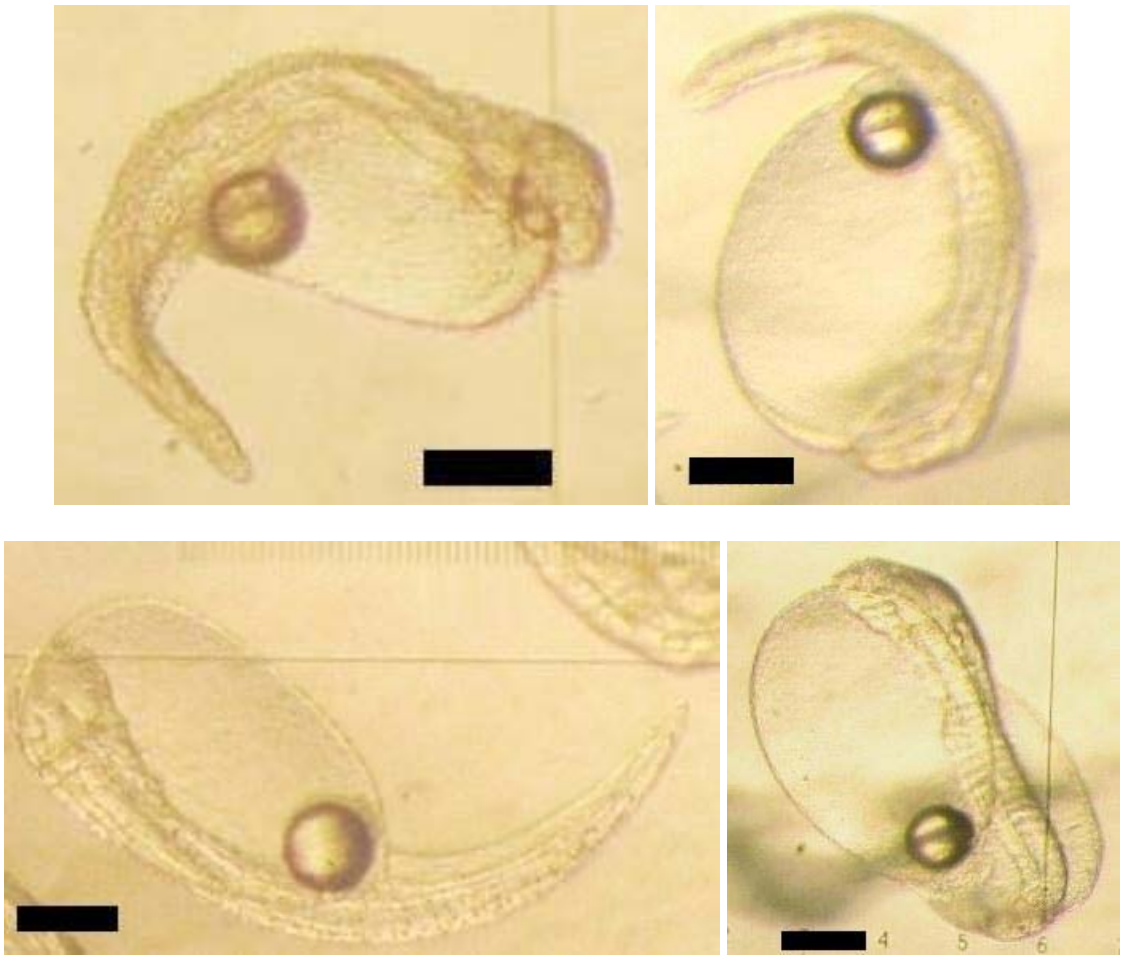


Figure 3.29 Spinal column deformities. Bar = 200 µm.

3.3.3.7 Yolk-sac oedema

Fluid accumulated in the abdominal cavity, resulting in swelling anterior to the yolk-sac (Fig. 3.30).

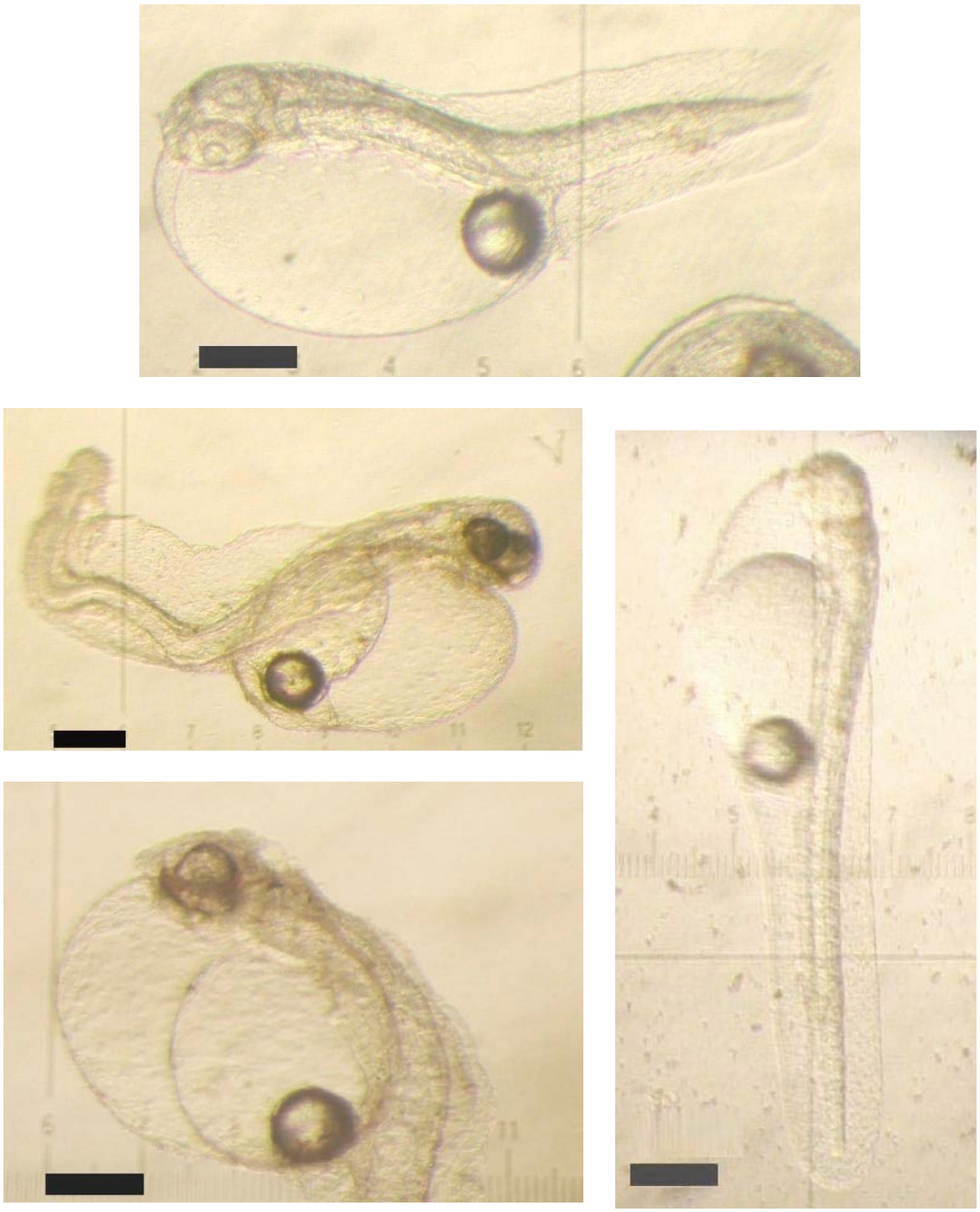


Figure 3.30 Yolk-sac oedema. Bar = 200 µm.

3.3.3.8 Fin deformities

Fins were underdeveloped, non-continuous along the length of the larva or showed signs of necrosis (Fig. 3.31).

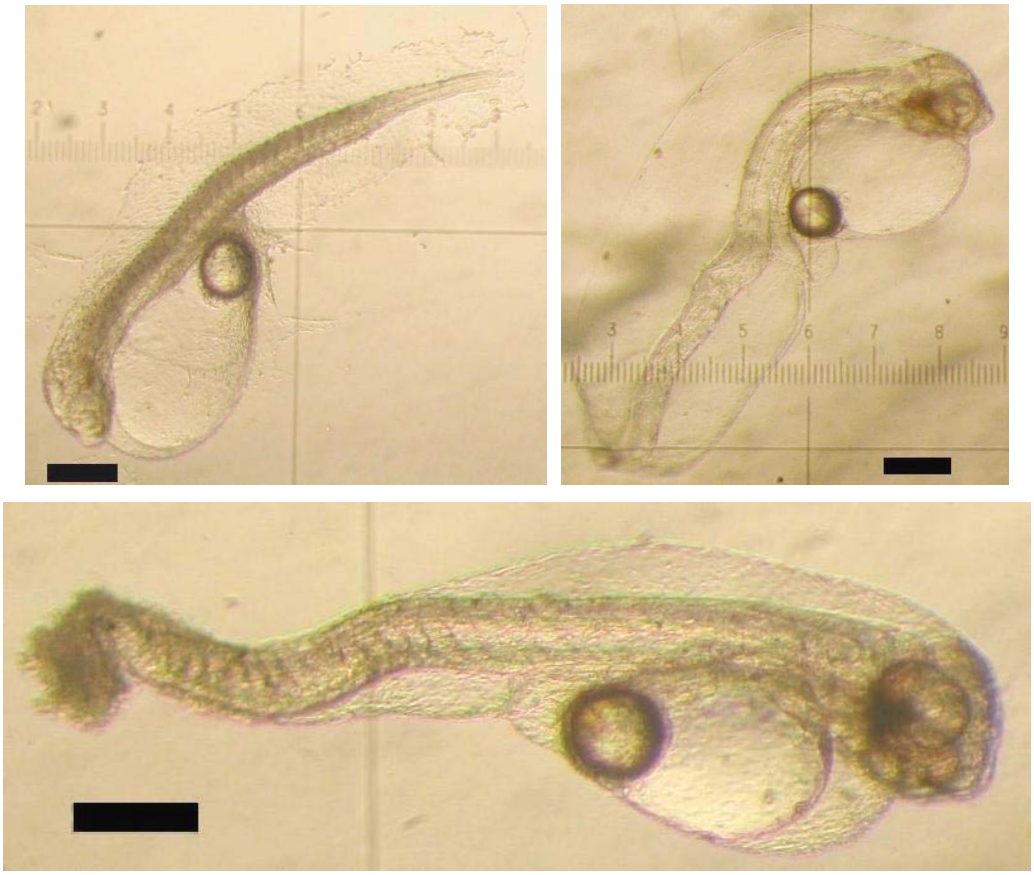


Figure 3.31 Fin deformities. Bar = 200 µm.

3.4 Discussion

The sequence of normal developmental events in black bream embryos, described in this study for the first time, provides a valuable resource for identifying abnormal development in any of the 20 defined stages, from fertilisation through to hatching. The ability to identify abnormal embryo development enables inference to larval survival and the subsequent likelihood of recruitment success or failure. Indeed, when black bream embryos are exposed to different environmental stressors, abnormal embryo development is observed which leads to reduced hatching and lower larval survival (see chapter 5).

Sparids, like many marine fish species, produce large numbers of small, transparent pelagic eggs that hatch within a few days of fertilisation, and the stages of embryo development in black bream are similar to those reported in a range of other sparid species, from both temperate and tropical environments worldwide: silvery black porgy (Hussain *et al.*, 1981); common dentex (Jug-Dujakovic *et al.*, 1995); snapper (Norriss and Jackson, 2002); and red porgy (Radonic *et al.*, 2005). At an incubation temperature of 21.2^{o}C, the embryonic period of black bream was completed by 34 h. In the silvery black porgy the mean time to hatching was 34.5 h at 21^{o}C (Hussain *et al.*, 1981), whilst for red porgy the mean time to hatching was 25.5 h (25^{o}C), 37 h (20^{o}C) and 60 h (15^{o}C) (Radonic *et al.*, 2005), and similarly, at a temperature of 17^{o}C, the mean time to hatching for common dentex was 81 h (Jug-Dujakovic *et al.*, 1995). The short embryonic period in black bream would mean that in the natural environment, the eggs would only experience only one full day of daylight, and two nights. Since predation at night time would be lower than during the day, this may represent a favourable adaptation to

increase the chances of successful reproductive events. In black bream, if the incubation temperature is decreased embryo development time is extended, and if the incubation temperature is increased then the embryo development time is shorter (see chapter 5). In the natural environment, temperature varies diurnally, so determining the exact age of eggs, or time to hatching is difficult. Results from laboratory exposures such as this one, where temperature was controlled closely are a useful guide, but may not reflect the natural rate of development exactly. Furthermore, in nature, the position of the eggs within the water column will also affect the time to hatching, since temperature can be significantly different between shallow and deep waters. In estuaries where stratification is common, both the salinity, which affects egg buoyancy, and the temperature which will be different in the stratified layers are likely to affect time to hatching.

Due to the transparency of the black bream chorion and yolk supply, the developing embryo can be easily observed, which enables close study of the different embryonic stages. This quality facilitates the use of black bream as a species for studying the influence of external factors, such as deteriorating environmental conditions on normal embryo development and larval survival. The small size, transparency and buoyancy of black bream eggs are typical characteristics of pelagic (floating) eggs, which are generally spawned by marine species, whereas freshwater species generally spawn demersal eggs (Kunz, 2004). Pelagic eggs tend to be produced in large numbers and receive no parental care (Kunz, 2004), and natural mortality rates can be as high as 95% (Bromage and Roberts, 1995). Identifying factors that affect embryo development may

improve our understanding of exactly how environmental stressors contribute to natural mortality rates and recruitment success or failure in black bream.

In a field study to measure the abundance and distribution of the early life stages of black bream in two Victorian micro-tidal estuaries, Nicholson *et al.* (2008) reported a strong relationship (R^2 = 0.96) between declining egg abundance and embryo developmental stage. As embryo development progressed, fewer eggs were observed, probably as a result of increased mortality. Low DO levels (<60%S) were suggested as a cause of the stage-specific losses. Similarly, when black bream embryos were reared in low salinities in a laboratory-based experiment, abnormal embryo development was reported, which resulted in a marked increase in the number of larvae that hatched with deformities, or which failed to hatch at all (Haddy and Pankhurst, 2000). Hence, detailed knowledge of the normal sequence of embryo development is valuable in order to understand and predict the likelihood of larval survival, or to identify habitats that might be affected by environmental stressors.

Abnormal development of eggs in sparid fishes has been attributed to several environmental stressors. Three types of abnormal eggs have been described in red seabream (*Pagrus major*): eggs with wrinkled membranes; eggs with small black spots covering the cortex; and eggs with 'bulging' ooplasma (Sakai *et al.*, 1985), and each type of abnormal appearance is associated with lowered embryo survival. Observations of embryo development in two other marine sparids have shown abnormal development following exposure to toxicants (Yamauchi *et al.*, 2006; Oliva *et al.*, 2007). Yamauchi *et*

al. (2006) observed increased larval mortality in red seabream, following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which was associated with under-development of the heart, bradycardia and circulation failure. Oliva *et al.* (2007) reported abnormalities such as reductions in the perivitelline space, irregular chorion shape and smaller head size in gilthead seabream (*Sparus aurata*) exposed to copper. In black bream embryos abnormal development has been observed following exposure to the pesticide metabolite DDE and the heavy metal mercury (Toogood, L., pers. comm.). Although individual species may exhibit some specific environmental preferences due to adaptation, it is reasonable to assume that other sparid fishes with similar early life histories would exhibit similar responses to adverse environmental conditions, including black bream.

Other families of fishes also show abnormal development, particularly near polluted estuaries. In a study of pelagic flatfish eggs, Cameron *et al.* (1992) reported high levels of abnormal development in embryos from different locations within the southern North Sea. They observed species-specific differences in the rates of malformation, as well as differences based on geographical location and season. The highest rates of malformation (44.4%) were observed in dab (*Limanda limanda*) collected during winter from sites that were near-shore and in close proximity to polluted river estuaries. They reported that abnormal development (incomplete or irregular cleavage patterns) was generally restricted to the early developmental stages (prior to closure of the blastopore), citing mortality in abnormal embryos beyond this age as a reason for their absence in samples. Cameron *et al.* (1992) attempted to correlate malformation rates with surface

temperatures, however no consistent relationships were observed, thus they concluded that the malformations may have been caused by something other than physical factors, such as contaminants or low quality broodstock.

Understanding the normal sequence of events, the timing of the developmental stages and the appearance of the embryo at different developmental stages is vital information for the assessment of black bream egg quality (and likely larval survival). This knowledge allows for the identification of abnormal embryo development, which may be used to identify poor quality broodstock, field locations or habitats with adverse environmental conditions, such as contaminated sites or sites with poor water quality (eg. low DO levels), or as a screening tool in aquaculture to identify low quality egg batches.

CHAPTER 4: DEVELOPMENT OF A NEW TECHNIQUE FOR CONTROLLING DISSOLVED OXYGEN LEVELS IN LABORATORY EXPERIMENTS.

Sections of this chapter have been published.

K. L. Hassell, P. C. Coutin and D. Nugegoda (2009) "A novel approach to controlling dissolved oxygen levels in laboratory experiments." *Journal of Experimental Marine Biology and Ecology,* 371, 147-154.

4.1 Introduction

Hypoxia, or low DO (~30%; <2.0 ml/l oxygen) occurs commonly in aquatic environments, and can be caused by a number of factors including diurnal changes in photosynthesis and respiration, tidal and seasonal influences, and increased anthropogenic inputs, leading to eutrophication (Diaz and Rosenberg, 1995; Coiro *et al.*, 2000; Wu, 1999; Wu, 2002). Hypoxia is a worldwide problem, and is deemed responsible for many environmental issues such as periodic fish kills, reduced abundance and distribution of species and reduced fisheries recruitment (Diaz and Rosenberg, 1995; Wu, 2002; Breitberg *et al.*, 2003). Furthermore, evidence suggests that in recent decades, the frequency and severity of hypoxic events has increased (Diaz and Rosenberg, 1995; Wu, 2002; Diaz and Rosenberg, 2008).

The biological effects of hypoxia on biota are numerous and varied, and relate to differences in body size, respiratory mechanisms, mobility and ability to avoid deteriorating environmental conditions, as well as the levels of resistance and tolerance in

each species. Examples of biological responses in fishes include avoidance through migration and altered feeding (Wannamaker and Rice, 2000; Wu *et al*., 2002; Lefrancois *et al*., 2005; McNeil and Closs, 2007), changes in physiological functions (such as energy metabolism) (Taylor and Miller, 2001; Cooper et al., 2002; Cerezo *et al.*, 2006), decreased reproductive activity (Shang *et al.*, 2006; Thomas *et al.*, 2006; Landry *et al.*, 2007) and adverse effects on early life stages (Czerkies *et al.*, 2001; Ciuhandu *et al*., 2005; Hassell *et al.*, 2008a) (see section 1.1.1). Laboratory-based tests provide an effective means of investigating the adverse effects of hypoxia on the early life stages, since all other confounding factors that could not be controlled in field experiments (ie. temperature, salinity or pH fluctuation, predatory-prey interactions) are removed.

Controlling low DO levels accurately and precisely in laboratory situations is challenging, primarily due to difficulties in preventing gas exchange between the water surface and surrounding air. A number of different approaches have been developed to control laboratory DO levels, most often based on the concept of displacing oxygen gas by applying nitrogen gas to the water (Miller *et al.*, 1994; Grecay and Stierhoff, 2002; Wu *et al.*, 2002; Ishibashi *et al.*, 2005; Richmond *et al.*, 2006; Landry *et al.*, 2007; Stecyk *et al.*, 2007). Some systems rely on continual nitrogen gas input which is expensive and wasteful, whilst other approaches rely on manual control that requires frequent monitoring and re-adjustment of nitrogen input. If not monitored properly, this type of control is likely to result in highly variable DO levels, and is very difficult to maintain for long periods (> a few hours). Other systems utilise electronic controls, where DO levels are monitored with an oxygen sensor which relays messages back to a computer

controlled system that can increase or decrease nitrogen and oxygen inputs via solenoid valves (Miller *et al*., 1994; Grecay and Stierhoff, 2002). The requirement for expensive and specialised equipment in order to control low DO precisely is a significant factor that limits research on the environmental effects of hypoxia on aquatic organisms. Maintaining constantly low DO levels is also a major problem for experiments involving small organisms (ie. embryos), since flow through systems would not be suitable, and constant bubbling of nitrogen gas would cause disturbance to the experimental organisms. Thus a static system is required that overcomes the issue of gas exchange and oxygen equilibration, even with very small test volumes.

This chapter describes the development of a novel experimental chamber system that is capable of maintaining low DO levels precisely for a period of up to 4 d with minimal re-adjustment and nitrogen gas requirement. The performance of the chamber design was then validated by conducting a hatchability experiment on black bream eggs.

4.2 Materials and Methods

4.2.1 Maintaining low dissolved oxygen levels (Controlled Atmosphere Chambers)

4.2.1.1 Chamber design

Five airtight containers (45 x 30.2 x 30.5 cm) were constructed from 0.6 mm transparent Perspex, fitted with firmly sealing lids (Resiplex Plastics, Australia). Each controlled atmosphere chamber (CAC) was fitted with two valves, one controlling the influx of nitrogen gas or compressed air (inlet valve), and a second one controlling the release of the nitrogen-air gas mixture from the chamber (outlet valve) (Fig. 4.1, 4.2).

4.2.1.2 Choice of holding vessels

Plastic 6-well plates (10 ml volume per well) were chosen as test vessels, since they are of an appropriate size to hold black bream embryos, they allow for multiple treatments to be conducted on the same plate, and the plates can be washed and re-used if needed. Other experiments on black bream eggs and larvae have used similar test vessels (Haddy and Pankhurst, 2000).

4.2.1.3 Compressed air and nitrogen gas supply

All well plates were placed inside the CACs then with the lid firmly sealed, nitrogen gas (BOC Gases, Australia) was introduced to displace oxygen until the gaseous oxygen reading (% O_2) at the outlet valve stabilised at a level that corresponded with the desired dissolved oxygen levels inside the chamber. At this point, both valves were closed and the CAC became airtight, thus only allowing gas exchange between the internal chamber atmosphere and the water surface of each well.

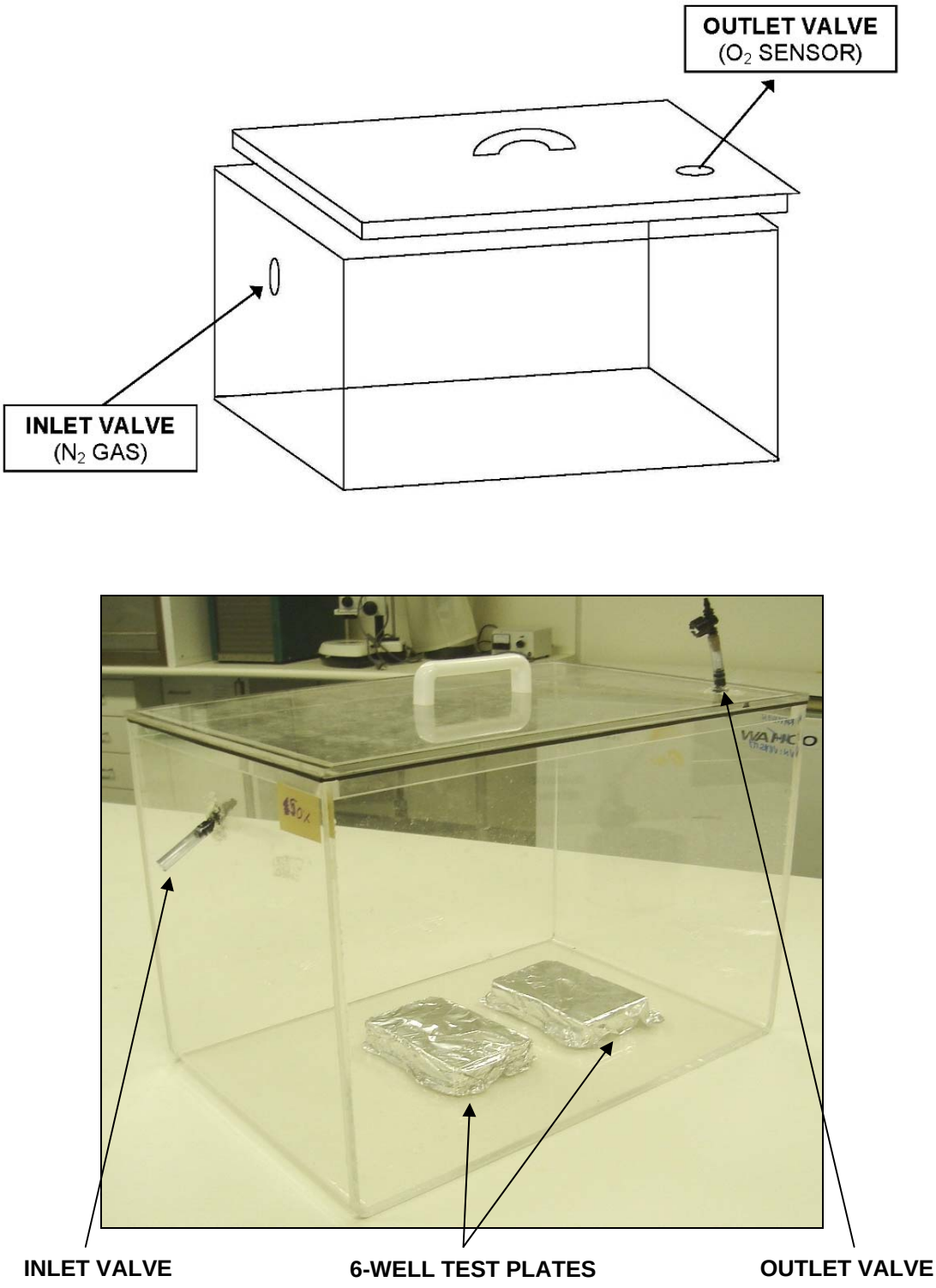


Figure 4.1 Line drawing and photograph of controlled atmosphere chamber design.

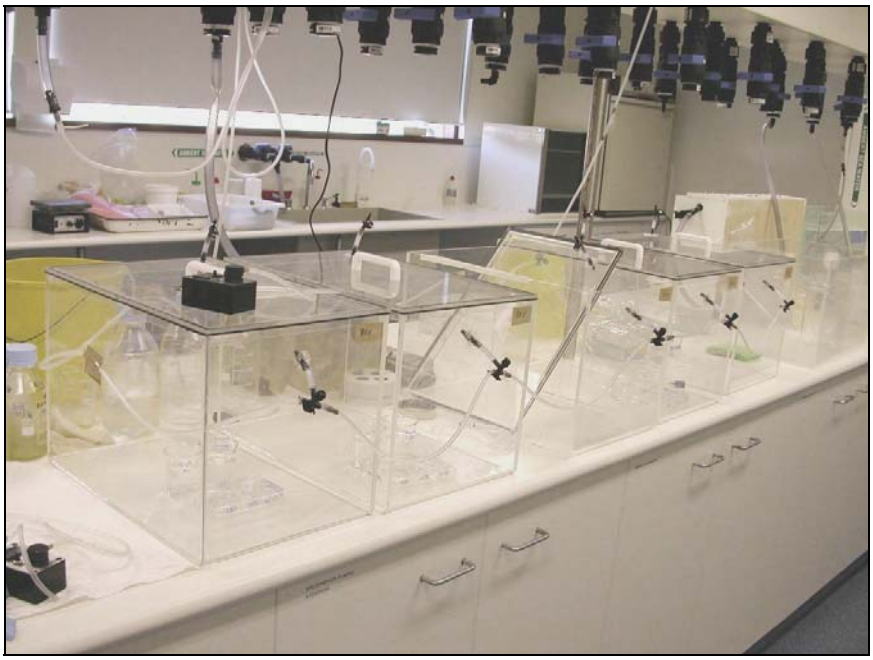


Figure 4.2 Experimental setup of five controlled atmosphere chambers, each set to a different oxygen-atmosphere concentration (and thus dissolved oxygen level).

4.2.1.4 Gas monitoring

The percentage of oxygen (% O_2) in the gas mixture exiting the CAC system via the outlet valve was measured using a DiveTek Monitor oxygen analyser (DiveTek, Brisbane, Australia) calibrated at 21% O_2 with compressed air.

4.2.1.5 Water monitoring

The oxygen levels in water were measured using two types of instruments. An OM-4 Oxygen Meter (with a MI-730 Micro-Oxygen Electrode) (Microelectrodes, Inc, Bedford, USA) was used to measure the percentage of oxygen gas in water (% O_2), and a WP-91 Dissolved Oxygen-pH-mV-Temp Meter (with a YSI Dissolved Oxygen-Temperature Sensor) (TPS Ionode, Springwood, Australia) was used to measure the percent saturation,

or amount of oxygen gas dissolved in water (Fig. 4.2). Normal air contains 21% oxygen, so for pure water that is equilibrated to air (and not influenced by biological activity within the water that may affect oxygen levels), readings on the two instruments would be 21% O_2 (percentage oxygen gas in water) and 100%S (percent saturation of water with oxygen) respectively. All results are expressed as the nominal, or desired DO levels (%S) rather than the measured levels for clarity and consistency.

4.2.1.6 System calibration

The relationship between percent saturation of oxygen in water (%S) and percent of oxygen gas in air (% O_2) needed to be established in order to use the gas reading at the outlet valve to set DO levels inside the CACs. Obviously, once the lid was firmly sealed on the chambers, water quality could no longer be measured, so in order to set the DO at the required levels, the values were based on the gaseous oxygen readings.

To determine this relationship, water samples were prepared with different DO levels (by the addition of nitrogen gas), then measured on the two different DO meters; OM-4 oxygen meter (% O_2) and WP-91 DO meter (%S). The OM-4 oxygen meter measures oxygen (in water) in the same units as the gaseous oxygen meter (DiveTek oxygen analyser) measures oxygen (in air) (% O_2), thus the OM-4 readings could be used to infer the required gaseous oxygen readings that would produce the desired DO levels inside the chambers, measured as percent saturation (%S) (using the WP-91 instrument).

4.2.1.7 Oxygen solubility and gas calculations

Both salinity and temperature influence the solubility of oxygen gas in water, so both of these factors need to be corrected for when reporting DO values (Colt, 1984). The following equation expresses the relationship between DO values (%S and mg/l) and the solubility of oxygen gas in water.

$$\textbf{Percent saturation (\%S)} = \frac{\textbf{dissolved oxygen (mg/l)}}{\textbf{solubility (mg/l)}} \times \textbf{100}$$

For example, the solubility of oxygen in full strength seawater (35 ppt) at 20^{o}C is 7.382 mg/l (Colt, 1984). Therefore the amount of oxygen (mg/L) required to achieve 100%S is:

$$\text{Dissolved oxygen (mg/l)} = \frac{\text{solubility (mg/l) x percent saturation (\%S)}}{100}$$

$$\text{Dissolved oxygen (mg/l)} = \frac{\text{7.382 (mg/l) x 100 (\%S)}}{100}$$

$$= \text{7.382 mg/l}$$

Whereas at 25^{o}C, the solubility of oxygen in full strength seawater is 6.754 mg/l, so, at 100% saturation, the DO level would be 6.754 mg/l. Likewise, the relationships between percent saturation and mg/l change as salinity increases or decreases.

The WP-91 DO meter contains an in-built temperature sensor and is capable of salinity correction. For this reason, all results are presented as %S values, measured using the WP-91 instrument.

4.2.1.8 Chamber Test 1 – Hypoxia generated by gas exchange only

The stability of DO levels were tested over a 48 h period at varying degrees of hypoxia in five separate CACs, each containing 4 replicate wells of 10 ml filtered seawater. The desired DO levels were: 85, 65, 50, 35 and 25%S. At the beginning of the experiment all treatments had the same water quality: DO (92.0%S); salinity (38 ppt); pH (7.81) and temperature (21.0^{o}C). Nitrogen gas was applied to each chamber until the gaseous oxygen reading at the outlet valve reached the value that corresponded to the required DO levels for each treatment.

Every 6-12 h the chambers were opened and the water quality was measured using a WP-91 DO meter and a WP-81 conductivity and pH meter (TPS Ionode, Australia) (Table 4.1). Measurements were taken as quickly as possible to prevent any gas equilibration in air. Once the water quality was measured, the lids were replaced and nitrogen gas was again applied until gaseous oxygen was at the required levels for each treatment.

4.2.1.9 Chamber Test 2 – Hypoxia generated by displacement of oxygen in solution and gas exchange

A second test was conducted to generate hypoxic conditions, however this time each hypoxic treatment was initially generated by bubbling nitrogen gas directly into the

seawater. This allowed for each treatment to start the experiment at the desired DO level, rather than starting out as normoxic and only becoming hypoxic after gas exchange at the air-water interface. The test duration was increased to 54 h for Chamber Test 2, and a 50% water change was conducted every 24 h. Five treatments of hypoxia were tested, with 4 replicate wells each containing 10 ml of hypoxic seawater. The desired DO levels were: 85, 60, 55, 40 and 30%S. Nitrogen gas was applied to each chamber as described above until the gaseous oxygen reading reached the required DO levels for each treatment. In addition, black bream embryos (from Lake Tyers broodstock) were placed into the test wells, to observe their responses to different DO levels. The embryos were observed to be in the morula stage of development when the test started (see chapter 3), and the mean number of embryos per well was 20 (± 1 SEM). One day survival and hatch rates were observed.

Every 4-12 h the chambers were opened briefly (15-30 sec) and the microplates were removed, so that the survival of embryos could be observed and water quality measurements taken. Once all measurements were completed, the lids were replaced and nitrogen gas was again applied until gaseous oxygen was at the required levels for each treatment.

4.2.1.10 Chamber Test 3 – Hypoxia generated by displacement of oxygen in solution and gas exchange

A third test was conducted to compare the generation of hypoxic conditions in the CACs at two different salinities (37 ppt, 45 ppt), since it is known that hyper-saline conditions

can influence oxygen solubility (Colt, 1984). Each hypoxic treatment was initially generated by bubbling nitrogen gas directly into the seawater, (as was done in Chamber Test 2) and the test duration was extended further to 84 h, with a 50% water change every 24 h. Hyper-saline solutions were prepared by dissolving Ocean Nature sea salt (Aquasonic, Wauchope, NSW, Australia), in filtered seawater. Five treatments of hypoxia were tested, with 4 replicate wells each containing 10 ml of hypoxic seawater. Nitrogen gas was applied to each chamber as described above until the gaseous oxygen reading reached the required DO levels for each treatment. The starting water quality for each treatment is listed in Table 4.2.

4.2.1.11 Statistical Analysis

Differences between experimental treatments for one day embryo survival and hatch rates were evaluated using one-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference multiple comparisons tests. All data were transformed prior to analysis (percentage data were arcsine transformed). All statistical analyses were performed using SPSS for Windows (version 15.0; SPSS Inc., Chicago, IL USA). The significance level in all tests was 0.05.

4.3 Results

4.3.1 Controlled atmosphere chambers

The constructed chambers worked well for controlling atmospheric oxygen levels and thus DO levels. Each chamber was set up as an independent chamber, so that up to five different oxygen-atmospheres could be tested simultaneously. The design is light weight and portable, so could easily be moved into controlled temperature rooms.

4.3.2 System calibration

There was a very close relationship (R^2 = 0.9966) observed between the DO levels measured as the percentage of oxygen gas in water (% O_2) compared to the percent saturation, or amount of oxygen gas dissolved in water (%S) (Fig. 4.3). This was expected, since it is a measure of the same variable expressed in different units. The purpose of measuring the percentage of oxygen gas in water (% O_2) was because this unit is the same as the gaseous oxygen units (% O_2) measured at the outlet valve. The equation generated from the linear relationship was used to determine the gaseous oxygen levels required at the CAC outlet valves. This set up the internal chamber atmosphere at a specific oxygen concentration and resulted in equilibration in the well plates within the chambers at the required DO levels.

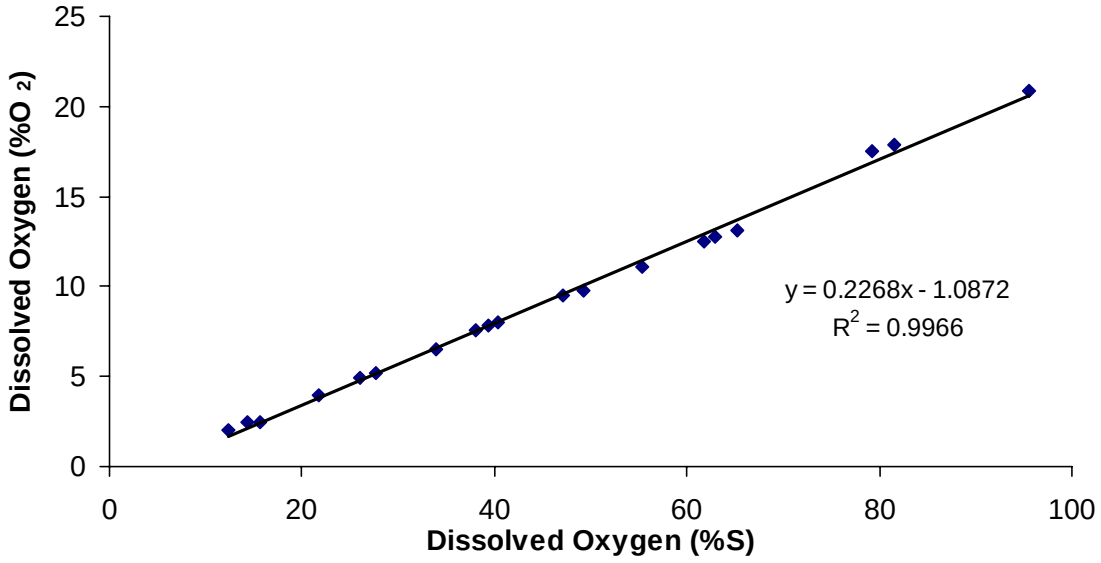


Figure 4.3 Relationship between dissolved oxygen levels in water containing varying amounts of nitrogen gas, measured as the percentage of oxygen gas in water (% O_2) versus the percent saturation or amount of oxygen gas dissolved in water (%S).

4.3.3 Chamber Test 1 – Hypoxia generated by gas exchange only

The chambers maintained the DO levels in the test wells close to the nominal levels consistently over 48 h (Fig. 4.4A). However, it took some time for the required DO levels to be achieved (Fig. 4.4B), thus, the actual exposure would not be one single DO level, but rather a gradually decreasing DO environment, until eventually equilibrium between the chamber atmosphere and water surface is achieved. Temperature and salinity was similar at all DO levels, whereas pH increased slightly (<0.5 units) in the low DO treatments (Table 4.1).

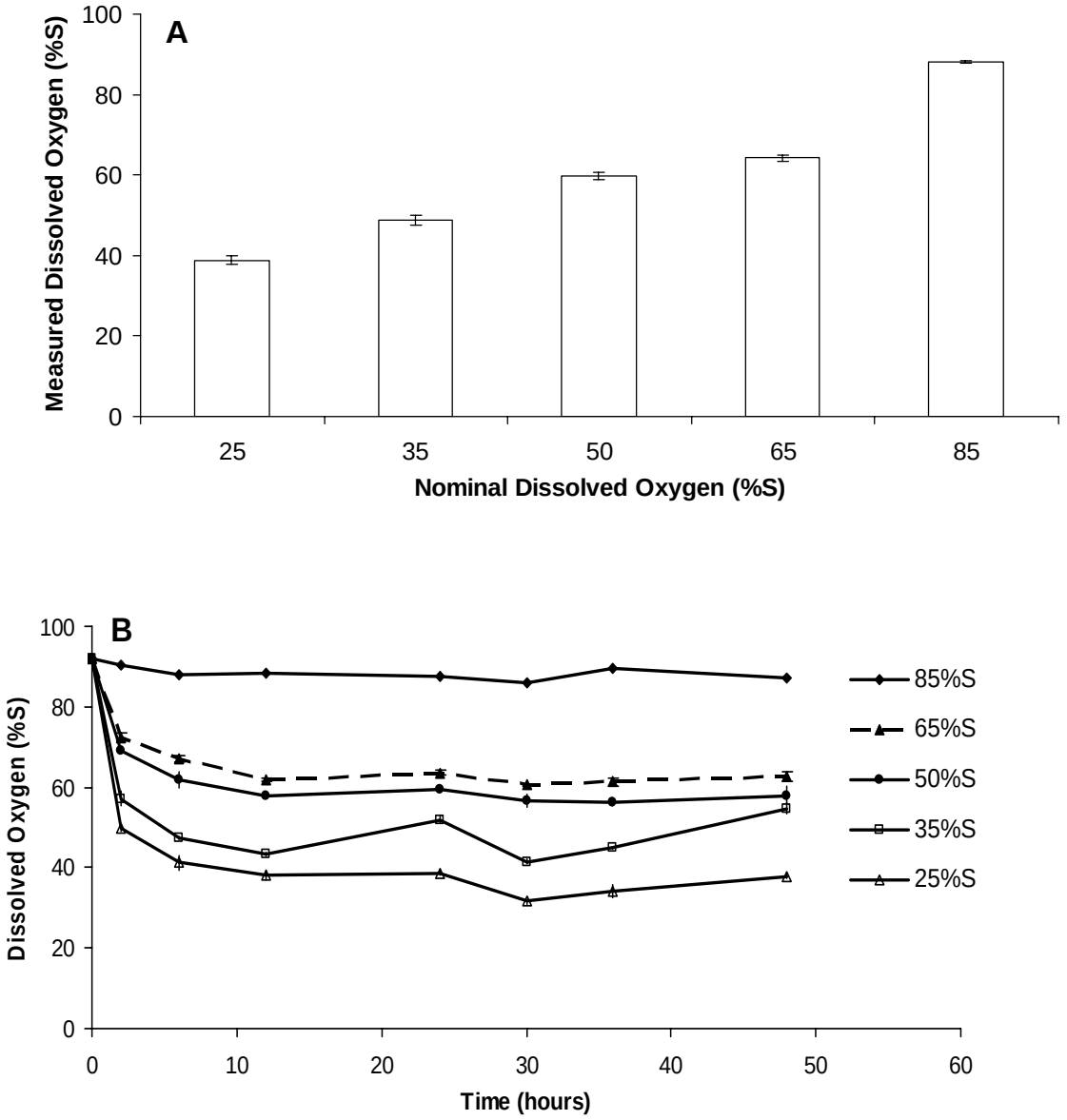


Figure 4.4 Dissolved oxygen levels (%S) in seawater contained in well plates inside controlled atmosphere chambers with different gaseous oxygen levels. Chamber Test 1 - Hypoxia was generated by gas exchange only. A) Mean measured dissolved oxygen levels compared to the nominal dissolved oxygen levels (± SEM) in each treatment over 48 h. B) Mean dissolved oxygen levels (± SEM) of each treatment at each time point.

Table 4.1. Summary of water quality from Chamber Test 1, where hypoxia was generated from gas exchange only.

Nominal Dissolved Oxygen (%S)	Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (^{o}C)	Mean ± SEM pH
85	88.1 ± 0.33	37.6 ± 0.22	20.6 ± 0.07	8.05 ± 0.01
60	64.2 ± 0.79	38.0 ± 0.16	20.5 ± 0.09	8.14 ± 0.02
50	59.8 ± 0.90	37.6 ± 0.20	20.4 ± 0.09	8.18 ± 0.02
35	48.8 ± 1.10	38.3 ± 0.15	20.4 ± 0.08	8.27 ± 0.03
25	38.8 ± 1.08	38.2 ± 0.20	20.5 ± 0.09	8.35 ± 0.03

4.3.4 Chamber Test 2 – Hypoxia generated by displacement of oxygen in solution and gas exchange

Applying nitrogen gas to the seawater prior to placing the test vessels inside the CACs enabled better precision of DO levels from the very beginning of the exposure (Fig. 4.5A, B). There was no delay in reaching the required DO levels, thus a more accurate measure of each treatment was achieved (compared to Chamber Test 1). The pH levels did increase in all treatments throughout the test duration, however the increases were only small (<0.5 units) and unlikely to adversely affect the test outcomes (Table 4.2).

4.3.4.1 24 h embryo survival and hatch rates

Black bream embryo survival to one day post fertilisation was not significantly reduced by low DO ($p = 0.635$) (Fig. 4.6A), however hatch rates were significantly reduced by DO ($p < 0.001$) (Fig. 4.6B). At 30%S, no embryos hatched. This experiment was repeated at a later stage using a greater number of embryos in each treatment as well as recording more observations at each time point (see chapter 5).

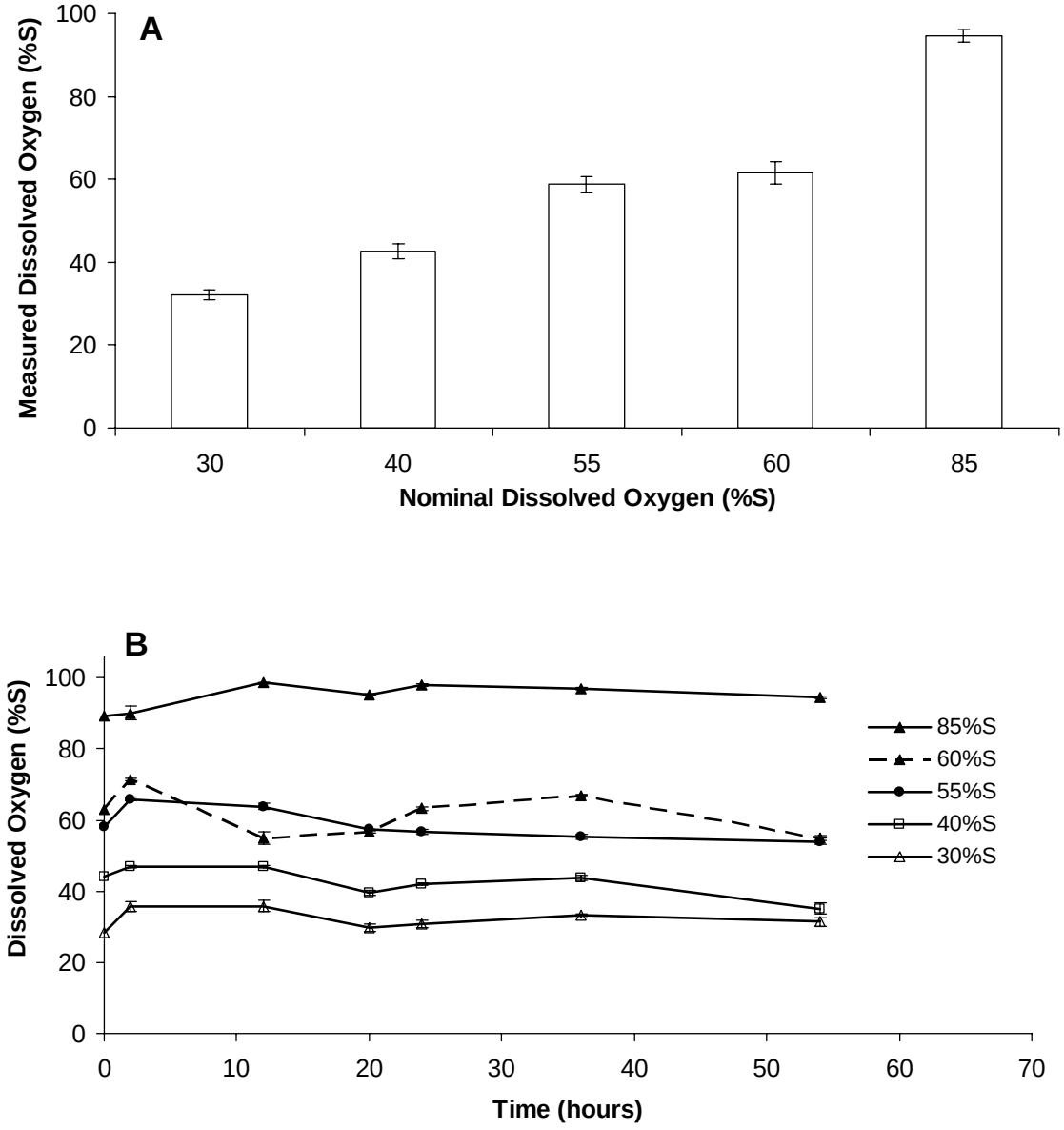


Figure 4.5 Dissolved oxygen levels in seawater contained in well plates inside controlled atmosphere chambers with different gaseous oxygen levels. Chamber Test 2 - Hypoxia was generated by displacement of oxygen in solution and gas exchange. A) Mean measured dissolved oxygen levels compared to the nominal dissolved oxygen levels (± SEM) in each treatment over 54 h. B) Mean dissolved oxygen levels (± SEM) of each treatment at each time point.

Table 4.2. Summary of water quality from Chamber Test 2, where hypoxia was generated from displacement of oxygen in solution and gas exchange.

Nominal Dissolved Oxygen (%S)	Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (^{o}C)	Mean ± SEM pH
85	94.6 ± 1.51	37.4 ± 0.67	21.3 ± 0.55	8.15 ± 0.01
60	61.6 ± 2.59	37.6 ± 0.45	21.4 ± 0.64	8.19 ± 0.01
55	58.7 ± 1.82	37.4 ± 0.67	21.3 ± 0.55	8.18 ± 0.03
40	42.6 ± 1.72	37.4 ± 0.67	21.3 ± 0.55	8.20 ± 0.03
30	32.1 ± 1.16	37.6 ± 0.45	21.3 ± 0.55	8.20 ± 0.01

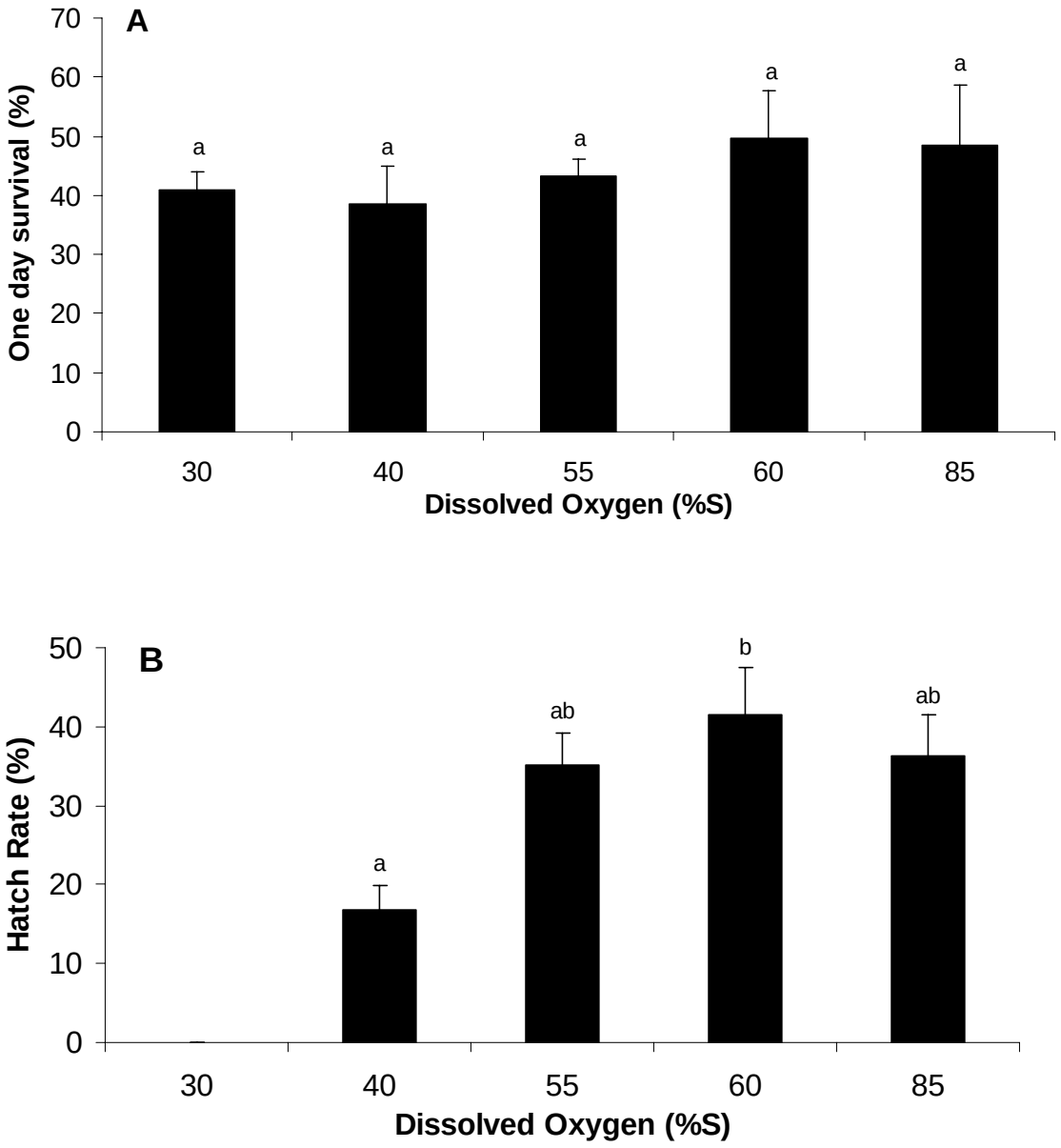


Figure 4.6 Responses of black bream embryos to different levels of dissolved oxygen in seawater (37 ppt, 21°C) contained in well plates inside controlled atmosphere chambers. Chamber Test 2 - Hypoxia was generated by displacement of oxygen in solution and gas exchange. A) One day survival (mean ± SEM) and B) hatch rates (mean ± SEM). Different letters denote significant differences ($p < 0.05$).

4.3.5 Chamber Test 3 – Hypoxia generated by displacement of oxygen in solution and gas exchange, at two different salinities.

Dissolved oxygen levels were successfully maintained close to the nominal levels in both normal seawater (37 ppt) and hypersaline (45 ppt) conditions (Fig. 4.7). Similar to Chamber Test 2, applying nitrogen gas to the seawater prior to placing the test vessels inside the controlled atmosphere chambers enabled precise control of DO levels right from the beginning of the exposure. There was little difference in the DO levels between the two salinity treatments (Table 4.3,Table 4.4, Fig. 4.7), and whilst the pH levels did increase in all treatments throughout the test duration, the increases were only small (<0.5 units).

Table 4.3. Summary of initial (starting) water quality from Chamber Test 3, where hypoxia was generated from displacement of oxygen in solution and gas exchange.

Nominal Dissolved Oxygen (%S)	Dissolved Oxygen (%S)	Salinity (ppt)	Temperature (^{o}C)	pH
85	91.5	37.1	21.5	7.99
65	62.6	37.7	22.0	8.12
50	50.2	37.3	22.0	8.06
35	35.6	37.7	22.0	8.15
25	14.9	37.3	22.0	8.19
85	98.5	44.0	21.0	8.49
65	65.6	44.8	22.0	8.49
50	49.5	44.9	21.5	8.48
35	37.5	45.1	21.5	8.49
25	16.6	44.1	22.0	8.53

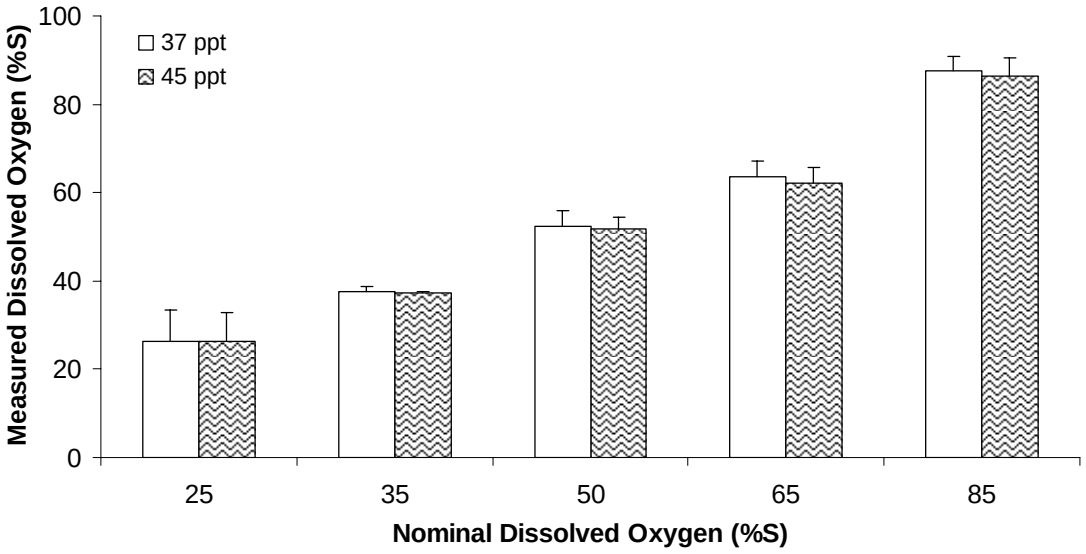


Figure 4.7 Mean measured dissolved oxygen levels compared to the nominal dissolved oxygen levels (± SEM) in ambient (37 ppt) and hyper-saline seawater (45 ppt) contained in well plates inside controlled atmosphere chambers with different gaseous oxygen levels. Hypoxia was generated by displacement of oxygen in solution and gas exchange.

Table 4.4. Summary of water quality from Chamber Test 3, where hypoxia was generated from displacement of oxygen in solution and gas exchange.

Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (^{o}C)	Mean ± SEM pH
87.7 ± 3.06	37.4 ± 0.12	21.2 ± 0.29	8.07 ± 0.07
63.6 ± 3.68	38.0 ± 0.10	21.3 ± 0.34	8.12 ± 0.09
52.5 ± 3.40	37.8 ± 0.19	21.3 ± 0.34	8.08 ± 0.11
37.6 ± 1.25	37.6 ± 0.10	21.3 ± 0.75	8.24 ± 0.09
26.2 ± 7.34	37.1 ± 0.25	21.5 ± 0.50	8.28 ± 0.09
86.3 ± 4.31	44.2 ± 0.33	21.1 ± 0.27	8.33 ± 0.12
62.1 ± 3.48	44.8 ± 0.19	21.2 ± 0.42	8.35 ± 0.12
51.7 ± 2.74	44.8 ± 0.32	21.1 ± 0.37	8.36 ± 0.11
37.3 ± 0.41	44.7 ± 0.45	21.0 ± 0.50	8.43 ± 0.07
26.3 ± 6.53	44.2 ± 0.14	21.3 ± 0.75	8.47 ± 0.06

4.4 Discussion

The new Controlled Atmosphere Chamber described in this study was able to maintain constant DO levels in small test wells over a period of several hours (days). The development of these new CACs is significant because the design is simple and inexpensive. The main advantage is that it is based on manual control with minimal requirement for adjustments once the system has achieved equilibrium. Each controlled atmosphere chamber can be set to a different DO level, hence several different treatments can be tested concurrently. All chambers are supplied from the same nitrogen and compressed air source, removing potential variation in gas quality, and most importantly only small volumes of gas are required so the system is economical.

Since each CAC is a separate unit, and only about the size of a standard 40 l fish tank, the system is portable. This favourable design feature was subsequently applied in a study to investigate the combined effects of hypoxia, low salinity and low temperature in black bream embryos (see chapter 5), where different chambers were set up in controlled temperature rooms (Hassell *et al.*, 2008b). Another potential application of this system's portability is that it could be used in field investigations. The only limitation for field applications is related to the gas supply. However, with this system's low gas requirements, small cylinders of compressed gas would enable short field experiments for a few days. Furthermore, the clear Perspex allows for the observation of organisms within the chamber, and whilst not done in this study (since the well plates were covered in foil), this function could be useful for studying behavioural responses in larger organisms.

Dissolved oxygen (and other water quality) measurements are taken by removing the test wells from the system temporarily. Since the test wells are removed, a single water quality meter can be used for all treatments. Again, this is favourable in terms of measurement consistency and economical considerations. However, a potential drawback of this system is that the DO levels are not monitored continually which is a desirable feature of any DO monitoring system (Diaz and Rosenberg, 1995; Grecay and Stierhoff, 2002). A possible solution to this problem would be to use portable, hand-held water quality meters that are placed inside each chamber for the entire test duration, however in doing so the requirement for multiple meters, and potential variation in readings is reintroduced. As the results show however, DO levels did not vary greatly at each measurement point, indicating that the system equilibrates rapidly (2-6 hours) and the DO levels stabilise. The rate of gas equilibration would be slightly different depending on how much oxygen is already dissolved in the water, with it being more rapid when the water has less oxygen dissolved in it. As long as the chambers are sealed tightly, and only opened to atmospheric air momentarily for test observations and water quality measurements, the DO levels remain very stable.

This system was designed for use with buoyant fish embryos which necessitated a static design, where the embryos would not be washed away (in the case of a flow-through system), or be physically disturbed (in the case of constant bubbling with nitrogen gas). The use of small well plates served this function very well, and allowed for several replicates of several different treatments to be tested simultaneously. Potential issues with evaporation were avoided by regular water changes, which also assisted in

preventing excessive pH changes. Whilst not tested here, this experimental set up could easily be adapted for larger volume test vessels and hence be used for larger aquatic organisms such as juvenile fish, tadpoles, molluscs and crustaceans. The CACs could also be used to test differences in hypoxia tolerance between different species. For example, replicate chambers could be set up, or the same chamber subdivided, to hold different fish species in order to test inter-species tolerances to the exact same levels of hypoxia (and/or other environmental stressors).

Whilst this system was developed for use with constant DO levels, it could potentially be set up to provide fluctuating DO levels that oscillate, for example to represent diurnal or tidal cycles. Different amounts of nitrogen gas and air could be introduced to the chambers at different times of the day to either increase (eg. day, peak photosynthesis) or decrease (eg. evening, no photosynthesis) DO levels. Again, such methods would require optimisation. As well as being useful for studies of constant hypoxia, the CAC concept could be applied equally as successfully in studies of other gases. For example, hyperoxia, or oxygen supersaturation could be tested by pumping oxygen gas into the chambers, or for simulations of climate driven changes in atmospheric conditions, carbon dioxide gas could be pumped into the chambers. This would enable experiments to be conducted under environmental conditions that are expected in the future with climate change.

In summary, a novel, inexpensive and simple chamber design has been developed for use in controlled laboratory exposures to hypoxia. The system works well with fish embryos

and has been used successfully to observe effects on early life-stage black bream. The system offers a number of benefits over existing experimental hypoxia set ups. It is easy to use, portable, low-tech and requires minimal volumes of gas. Controlled atmosphere chambers represent a new tool for testing different types of environmental conditions on biota, including (but not limited to) hypoxia research as well as different environmental scenarios predicted for example, by climate change in the future. It is not possible to test variables such as pH change (in relation to increased carbon dioxide gas) on large oceanic systems, so laboratory-based models are likely to become very important for such research. Furthermore, testing multiple variables simultaneously (eg. DO, salinity, temperature) provide far greater insights towards expected impacts of environmental change compared to single variable experiments. Scientific advice on the potential effects on aquatic ecosystems are needed now in order to provide long term management options for the future in relation to climate change, environmental degradation and pollution. The CAC design fulfils these needs. The development of this novel chamber design and new technique now provides a basis for further experiments to assess potential impacts of deteriorating habitats in aquatic environments on both vertebrate and invertebrate test species.

CHAPTER 5: STRESS RESPONSES IN THE EARLY LIFE-STAGES OF BLACK BREAM.

Sections of this chapter have been published and two manuscripts are being prepared for publishing consideration.

Published articles:

Hassell, K.L., Coutin, P.C. and Nugegoda, D. (2008). Hypoxia, low salinity and lowered temperature reduce embryo survival and hatch rates in black bream (*Acanthopagrus butcheri*, Munro 1949). *Journal of Fish Biology*, 72, 1623-1636.

Hassell, K.L., Coutin, P.C. and Nugegoda, D. (2008). Hypoxia impairs embryo development and survival in black bream (*Acanthopagrus butcheri*). *Marine Pollution Bulletin,* 57, 302-306.

Manuscript details:

Kathryn L. Hassell, Patrick C. Coutin, Dianne Rose and Dayanthi Nugegoda. "Elevated temperatures, hypoxia and low salinity increase rates of deformity and lower hatch rates in black bream (*Acanthopagrus butcheri*, Munro 1949)". To be submitted to the *Journal of Fish Biology*.

Kathryn L. Hassell, Patrick C. Coutin, Dayanthi Nugegoda. "The effects of hypoxia and salinity on egg buoyancy and hatch rates in black bream *Acanthopagrus butcheri* (Munro)". To be submitted to the *Canadian Journal of Fisheries and Aquatic Sciences*.

5.1 Introduction

The early life-stages are considered to be the most sensitive periods of development in fish (Pickering and Gast, 1972; McKim, 1985), and external stressors can have a major influence on the many physiological changes that occur during this time (see chapter 1 for review). Abnormal embryo development and larval deformities are commonly seen in fish eggs and larvae in response to environmental stressors, and are associated with a number of adverse effects such as lower hatch rates, reduced larval fitness, and increased mortality. The consequences of adverse effects in the early life stages are that recruitment is likely to be reduced, potentially reducing the long term sustainability of fish populations (von Westernhagen and Dethlefsen, 1997; Kjorsvik *et al.*, 2003). A thorough description of abnormal development in black bream embryos was given in chapter 3, and the major types of embryo abnormalities and deformities that occur are: abnormal blastomere cleavage, disrupted or retarded embryo development, incomplete hatching, spinal column deformities, yolk-sac oedema and fin deformities.

For species that complete their early life histories in estuarine environments, many environmental stressors are likely to influence embryo development and subsequent survival. Estuaries form the transition area between freshwater and seawater and are very dynamic with tidal, diurnal and seasonal cycles (Turner *et al.*, 2004). Water quality is changeable due to seasonal differences in environmental flows as well as nutrient, sediment and toxicant inputs through industrial and agricultural activities and urbanisation (Turner *et al.*, 2004).

In Victoria, the main recreational black bream fishery is in the Gippsland Lakes, where recruitment is highly variable from year to year (Butcher, 1945; Morison *et al.*, 1998). Since 1990, low recruitment has occurred frequently, and only a few years are considered strong year classes (Morison *et al.*, 1998). An ecological survey of post-larval and juvenile fish in the Gippsland Lakes observed black bream in locations where the salinity was 19-22 ppt and the temperature was 21^{o}C (Ramm, 1983), and a subsequent study of water quality within the Lakes concluded that areas within Lake Victoria and Lake King, with moderate salinities, freshwater flows and seagrass meadows were likely sites for black bream spawning (Longmore, *et al.,* 1990).

The estuarine environmental conditions in the Gippsland Lakes vary seasonally and are influenced by inputs from rivers and tidal exchange through the artificial entrance. Based on Victorian Environmental Protection Authority (EPA) water quality data for the period between 1990-2003, spring/summer averages for DO at three sites (1-5 m depth) within Lake King were 86-99%S, while the minimum recorded was 10%S and the maximum recorded was 240%S. Average salinity levels from the same sites over the same time period were 18-25 ppt, with the lowest recorded as 1 ppt and the highest recorded as 39 ppt. Average temperatures from the same sites over the same time period were 15-21^{o}C with a minimum of 9.6^{o}C and a maximum of 26^{o}C (Anon, 2007).

This chapter describes the effects of selected physico-chemical environmental factors on early life stage black bream which was investigated in an attempt to establish the ecological mechanism that underpins high variation in recruitment and year-class strength in Gippsland Lakes black bream. This was done by evaluating the effects of different DO, salinity and temperature levels on the survival and hatchability of black bream eggs and larvae in controlled laboratory experiments.

5.2 Materials and methods

5.2.1 Broodstock selection

Adult black bream used as broodstock in this study came from two separate stocks that had been held in the DPI Queenscliff aquaculture facility for at least three years (see section 2.2.1). Broodstock were anaesthetised (see section 2.2.4), then induced to spawn with a single intramuscular injection of the chorionic gonadotropin hormone, Chorulon (Intervet, Australia) (see section 2.2.5). All broodstock were fed fresh pilchards and calamari squid daily and the ratio of males to females was 1:1 throughout the spawning period. Both stocks of fish were used to produce eggs for this study, and they were always kept separate. The choice of broodstock used for each experiment was based on the condition of the fish prior to hormonal induction, as well as whether or not those fish had been spawned previously.

5.2.2 Embryo collection

Spawning broodstock fish were held in a tank that was fitted with an overflow that led into a 500 μm mesh egg collector, allowing for the collection of buoyant, fertilised eggs only (see section 2.2.6). Good quality fertilised black bream eggs are positively buoyant in full strength seawater (~35-39 ppt). At the time of each collection, a sub-sample of eggs was examined under the microscope to determine the cell stage (see chapter 3). The Organisation for Economic Co-operation and Development (OECD) Guidelines for conducting short term toxicity tests on embryo and sac-fry stages states that test commencement must occur prior to the onset of the gastrula stage (OECD, 1998).

Immediately after the collection and determination of cell stage, the eggs were randomly distributed to plastic 6-well plates (Iwama, Australia) containing 10 ml of water of the desired DO, salinity and temperature then placed inside the CACs. The eggs were transferred using plastic pipettes.

5.2.3 Comparisons between experiments

An important consideration when comparing the different experiments of this study are that each one used a different batches of eggs from different spawning events, so the egg quality and batch fecundity would be different. Furthermore, two different groups of broodstock fish were used. For these reasons, conclusions have been drawn for specific experiments that used the same eggs only, rather than trying to compare absolute values (eg. hatch rates) across different experiments and thus different batches of eggs.

5.2.4 Controlled atmosphere chambers

Air tight Perspex containers (CACs) were constructed (Resiplex Plastics, Geelong) to control DO levels and each container was fitted with an inlet and outlet valve, as well as an oxygen sensor (see chapter 4). Nitrogen gas was pumped into the chambers to displace oxygen, until the oxygen reading (% O_2) at the outlet gas valve corresponded to pre-determined values that matched the levels of DO within the well plates inside each chamber. Once the required levels were achieved, the nitrogen gas inlet valves and the oxygen outlet valves were closed. The chambers were thus airtight and gas exchange between the chamber atmosphere and the water surface of each well achieved equilibrium

at the desired level of DO. Filtered ambient seawater (35-39 ppt) was either diluted with distilled water, or mixed with Ocean Nature sea salt (Aquasonic, Wauchope, NSW Australia) to achieve different salinities. Experiments were conducted within controlled temperature rooms set at 16 ± 1^{o}C or 26 ± 1^{o}C, or in the laboratory at 21 ± 2^{o}C.

5.2.5 Observations

Observations were taken every 6-12 h to determine the following endpoints: one day embryo survival, time to hatching, hatch rates, larval deformities, larval survival to Day 2 post-hatch and larval length on Day 2 post-hatch. Hatching is defined as the process whereby the embryo develops from a fertilised egg to become a free swimming larva, no longer bound by the surrounding chorion membrane (Kunz, 2004). Upon hatching the larvae were assessed for deformities, and classed as normal if the spine was straight and swimming ability was normal, or deformed if there was any curvature of the spine, incomplete hatching, yolk-sac oedema, irregular swimming patterns or immobility (see chapter 3). Day 2 post-hatch was defined as 48 h after peak hatching, however since this time was different in different experiments, no specific time is associated with Day 2 post-hatch.

5.2.6 List of individual experiments

A total of seven different combinations of DO, salinity and temperature were tested in this study.

EXP. 1: Effects of salinity and light on embryos and yolk-sac larvae

EXP. 2: Effects of hypoxia on embryos and yolk-sac larvae

EXP. 3: Effects of hypoxia, low salinity and low temperature on embryos and yolk-sac larvae

EXP. 4: Effects of hypoxia, low salinity and high temperature on embryos and yolk-sac larvae

EXP. 5: Effects of hypoxia and high salinity on embryos and yolk-sac larvae

EXP. 6: Effects of hypoxia and salinity on egg buoyancy

EXP. 7: Effects of hypoxia on hatched larvae survival (to Day 4 post-hatch)

5.2.7 Water quality

The tested DO, salinity and temperature values were chosen based on spring/summer water quality averages from locations within the Gippsland Lakes that are thought to be spawning sites for black bream (Anon, 2007). Throughout the experiments, water quality measurements were taken regularly. Dissolved oxygen (%S) was measured using a TPS meter (model WP -91), and salinity, pH and temperature were measured using a TPS meter (model WP-81) (TPS Ionode, Springwood, Australia). The ambient salinity of seawater and laboratory temperature did vary slightly due to seasonal differences throughout the study period, so specific values are reported for each individual experiment.

5.2.8 Light exposure

Experiments were conducted in a laboratory fitted with 30 watt fluorescent lights that were approximately 2 m above the bench area that was illuminated. Initial experiments were conducted with constant (24 h) light exposure, however due to low survival, subsequent experiments were conducted in darkness (by wrapping aluminium foil around the well plates).

5.2.9 Statistical analyses

Differences between experimental treatments were evaluated using one-way, two-way or three-way analysis of variance (ANOVA) (depending on experimental parameters). Tukey's Honestly Significant Difference or Dunnett's C multiple comparisons tests were used to detect significantly different treatment pairs. Levene's or Bartlett's test was used to assess homogeneity of variance and if the requirements of parametric tests were not satisfied, Kruskal-Wallis and Mann-Whitney tests were used (Zar, 1998). All data were transformed prior to analysis (percentage data were arcsine transformed, length/weight data were $\log_{10}$ transformed). All statistical analyses were performed using SPSS for Windows (version 14.0 or 15.0; SPSS Inc., Chicago, IL USA). The significance level in all tests was 0.05.

5.3 Individual experimental methods and results

5.3.1 EXP. 1 - Effects of salinity and light on embryos and yolk-sac larvae

Lake Tyers broodstock were used for this experiment and at the time of collection, the eggs were observed to be in the blastula stage (see chapter 3). The eggs were randomly distributed to well plates containing 10 ml of water as follows: DO (>85%S), salinity (0, 5, 15, 23 and 35 ppt) and temperature (20^{o}C). One set of well plates were incubated with a clear plastic lid (light - constant), and the other set were incubated with a clear plastic lid covered in aluminium foil (dark) to limit light exposure. The mean number of embryos in each well for the light treatment was 143 (± 13 SEM), and for the dark treatment the mean number of embryos was 98 (± 7 SEM) and there were four replicates of each treatment. The test duration was 60 h and observations were taken at 12, 18, 24, 36 and 60 h. Results were analysed using three-way ANOVA.

5.3.1.1 Water quality

Dissolved oxygen, salinity, temperature and pH were maintained close to nominal levels throughout the experiment (Table 5.1). Partial water changes (50% replacement) were conducted every 24 h, which helped to maintain the water quality at the required levels.

5.3.1.2 One day embryo survival

Embryo survival to one day post fertilisation was significantly reduced by low (<5 ppt) salinity ($p < 0.001$) and exposure to light ($p = 0.031$). The interaction between salinity and light exposure was not significant ($p = 0.615$) (Fig. 5.1).

Table 5.1 Effects of light exposure and salinity on black bream embryo hatching.

Exposure	Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (^{o}C)	Mean ± SEM pH	Days to hatching (%)			Mean ± SEM Time to Hatch (h)
					1	2	3	
Light - constant	88.5 ± 1.05	2.75 ± 0.99	21.1 ± 0.14	7.97 ± 0.03	0.00	0.00	0.00	-
Light – constant	80.2 ± 3.77	6.00 ± 0.47	21.4 ± 0.28	7.89 ± 0.05	1.50	20.3	78.2	54.5 ± 2.60
Light – constant	86.7 ± 1.98	16.0 ± 0.47	21.3 ± 0.37	8.03 ± 0.01	1.90	44.9	53.2	48.5 ± 5.58
Light – constant	89.3 ± 1.07	23.9 ± 0.36	21.1 ± 0.43	8.08 ± 0.01	4.20	61.1	34.7	43.8 ± 5.56
Light – constant	89.2 ± 0.56	36.5 ± 0.33	21.4 ± 0.14	8.14 ± 0.01	4.00	96.0	0.00	35.5 ± 0.30
Dark	91.5 ± 3.69	2.75 ± 0.55	21.4 ± 0.36	7.92 ± 0.02	0.00	0.00	100	60.0
Dark	91.9 ± 2.32	6.50 ± 0.75	21.3 ± 0.37	7.87 ± 0.03	1.60	24.1	74.4	53.7 ± 0.84
Dark	90.3 ± 2.30	16.8 ± 0.55	21.3 ± 0.17	8.05 ± 0.01	2.00	62.2	35.8	44.3 ± 6.21
Dark	91.2 ± 1.91	23.8 ± 0.55	21.4 ± 0.28	8.07 ± 0.01	0.50	92.9	6.60	37.5 ± 1.85
Dark	92.4 ± 3.15	36.3 ± 0.99	21.0 ± 0.24	8.11 ± 0.01	1.50	96.8	1.70	36.1 ± 0.59

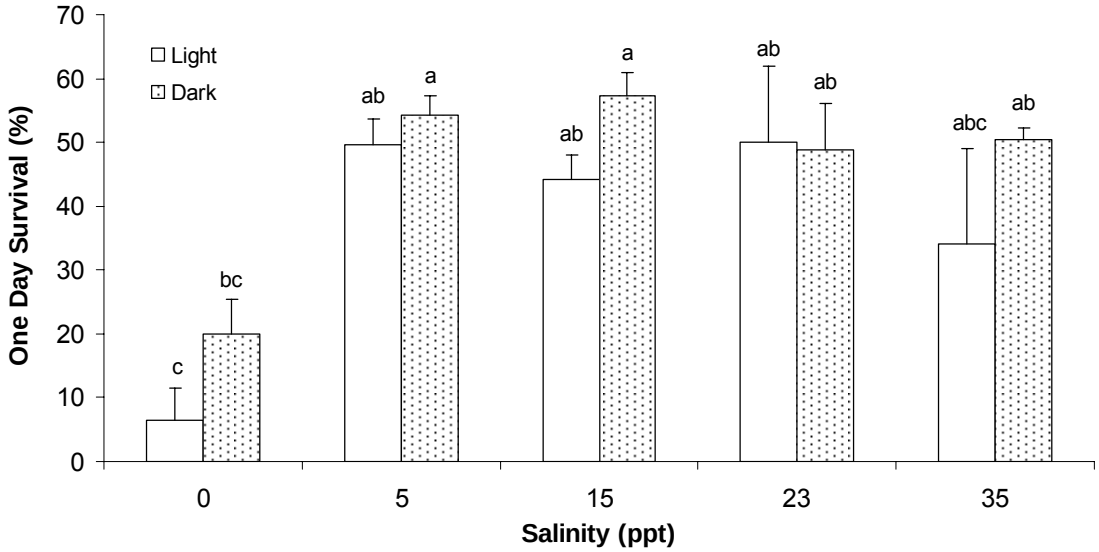


Figure 5.1 One day survival (mean ± SEM) for black bream embryos exposed to five levels of salinity, maintained in either constant light or dark conditions. The dissolved oxygen was maintained at >85%S and the temperature was 21°C. Different letters denote significant differences between mean values ($p < 0.05$).

5.3.1.3 Time to hatching

As salinity was reduced, a significant delay in the days to hatching was observed ($p < 0.001$), such that at 36 ppt (for both light and dark exposures), greater than 98% of larvae had hatched by Day 2, whereas at 6 ppt, only 20-26% had hatched by Day 2. Light exposure did not affect the time to hatching ($p = 0.300$). The mean time to hatching was increased by 18-20 h by exposure to low salinity in both light and dark conditions.

5.3.1.4 Hatch rates

Mean hatch rates in light treatments were all lower than dark treatments at all salinity levels. Hatch rates were significantly affected by salinity ($p = 0.001$), but not light

exposure (p = 0.220), and the interaction between salinity and light exposure was not significant (p = 0.858). When the embryos were held in constant darkness, the mean hatch rates were highest at 35 ppt salinity (36%), whereas when the embryos were held under constant light exposure, the mean hatch rates were highest at 15 ppt salinity (26%) (Fig. 5.2). The mean number of eggs in the light and dark treatments were not significantly different ($p > 0.05$) indicating that stocking density was not a factor that confounded the results of this experiment. However, within each treatment group, there was much variability in results between each replicate, indicated by the large error bars suggesting that something other than salinity or light exposure was influencing the results. Possible causes for this variability include overcrowding (too many eggs) in the test wells, which may reduce DO levels, or excessive time viewed under the light microscope, which could result in thermal shock. These factors were taken into consideration when planning subsequent experiments.

5.3.1.5 Deformities

Embryos maintained in low salinity environments (≤15 ppt) displayed a greater incidence of abnormal development and larval deformities compared to those maintained at 23 or 35 ppt salinity (Fig. 5.3).

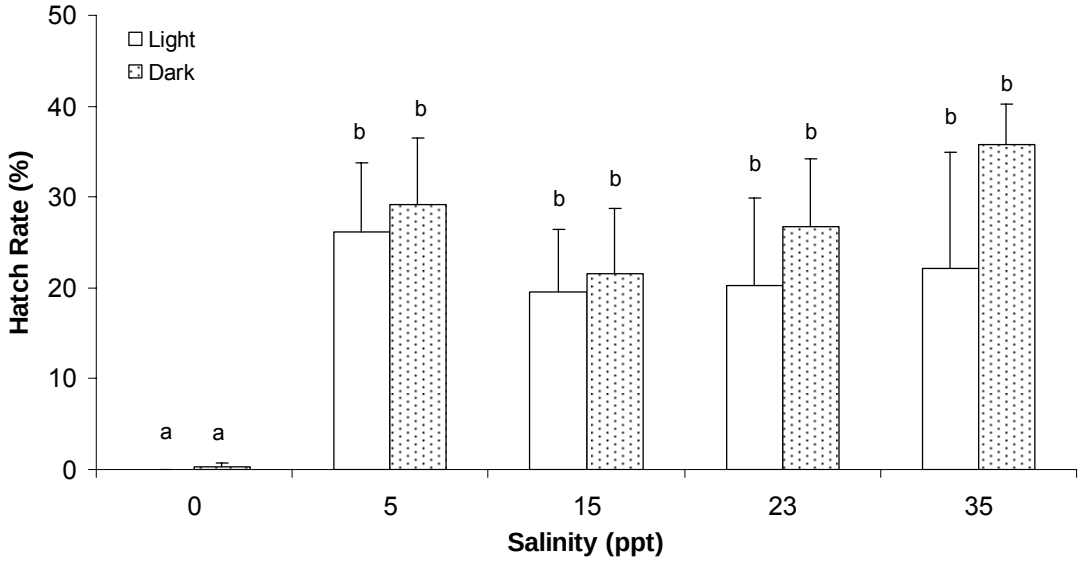


Figure 5.2 Hatch rates (mean ± SEM) for black bream embryos exposed to five levels of salinity, maintained in either constant light or dark conditions. The dissolved oxygen was maintained at >85%S and the temperature was 21°C. Different letters denote significant differences between mean values ($p < 0.05$).

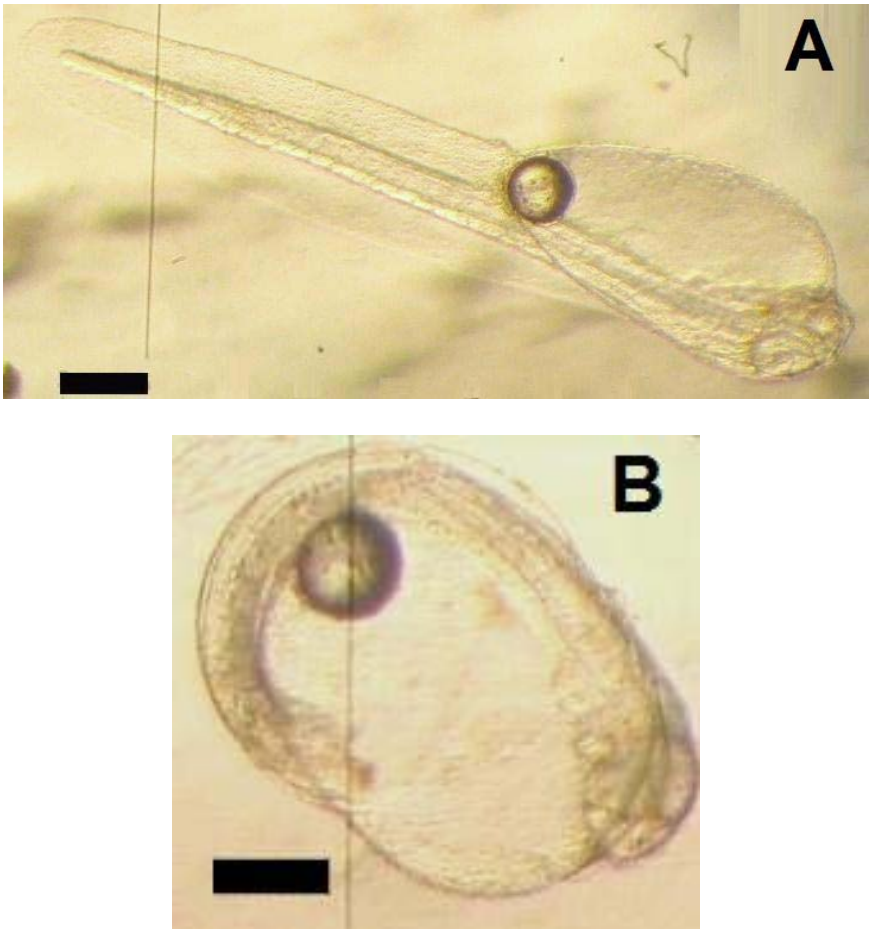


Figure 5.3 Incomplete hatching in black bream following exposure to low salinity: A) normal appearance of newly hatched larva (maintained at 35 ppt); B) abnormal appearance of newly hatched larva (maintained at 5 ppt). The dissolved oxygen was maintained at >85%S and the temperature was 21^{o}C. Bar = 200 µm.

5.3.2 EXP. 2 - Effects of hypoxia on embryos and yolk-sac larvae

Lake Tyers broodstock were used for this experiment and at the time of collection, the eggs were observed to be in the 8-16 cell stage of development (see chapter 3). The eggs were randomly distributed to well plates containing 10 ml of water as follows: DO severely hypoxic (30%S ~ 2.1 mg/l), moderately hypoxic (45, 55, 65%S ~ 3.1 mg/l, 3.8 mg/l and 4.5 mg/l respectively) and normoxic 85%S ~ 5.9 mg/l), salinity (36 ppt) and temperature (23^{o}C). Each treatment consisted of six replicates, containing 70 (± 4 SEM) embryos. The test duration was 72 h and observations were taken at 2, 12, 24, 30, 33, 36, 48 and 72 h. Results were analysed using one-way ANOVA.

5.3.2.1 Water quality

Dissolved oxygen, salinity, temperature and pH were maintained at close to nominal levels throughout the experiment (Table 5.2). Partial water changes (50% replacement) were conducted every 24 h, which helped to maintain the water quality at close to the required levels.

Table 5.2 Effects of hypoxia on black bream embryo hatching and rates of deformity.

Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (^{o}C)	Mean ± SEM pH	Days to hatching (%)			Mean ± SEM Time to Hatch (h)	Mean ± SEM Deformed Larvae (%)
				1	2	3		
86.3 ± 1.54	36.0 ± 0.12	23.2 ± 0.45	8.02 ± 0.05	0.00	99.29	0.71	35.7 ± 0.18	9.61 ± 2.56
66.1 ± 1.23	36.0 ± 0.17	23.2 ± 0.48	8.05 ± 0.07	0.00	100.00	0.00	35.1 ± 0.29	15.2 ± 8.66
55.3 ± 2.61	36.1 ± 0.17	23.2 ± 0.49	8.10 ± 0.07	0.00	100.00	0.00	33.4 ± 0.12	10.9 ± 3.47
46.2 ± 5.80	36.1 ± 0.24	23.1 ± 0.54	8.11 ± 0.12	0.00	100.00	0.00	43.0 ± 0.87	100 ± 0.00
31.9 ± 7.60	36.0 ± 0.35	22.8 ± 0.49	8.18 ± 0.13	-	-	-	-	-

5.3.2.2 One day embryo survival

Embryo survival to one day post fertilisation was significantly reduced ($p < 0.001$) in severely hypoxic conditions (30%S) (Fig. 5.4), however no significant differences were observed between the 45, 55, 65 or 85%S treatments. Associated with the increased mortality of embryos held in 30%S was the observation of disrupted, uncoordinated development, whereby the normal organisation of cells forming the cephalic region during the 'embryo' stages of development did not occur (Fig. 5.5A, 5.5B). Lack of cephalisation represents abnormal development in black bream embryos and always resulted in mortality.

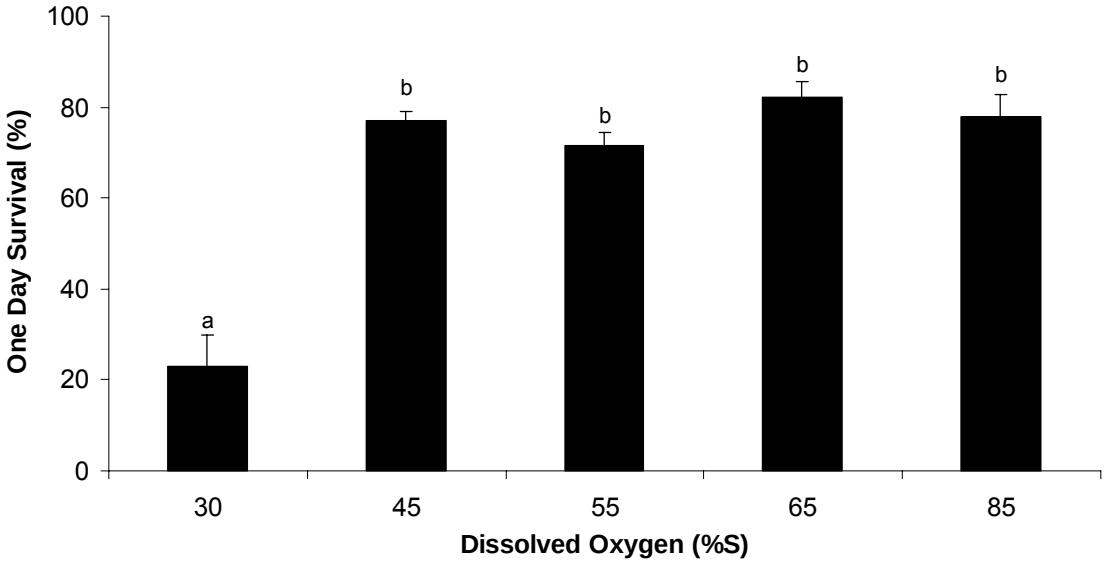


Figure 5.4 One day survival (mean ± SEM) for black bream embryos exposed to five different levels of hypoxia. Ambient salinity was 36 ppt and ambient temperature was 23°C. Different letters denote significant differences between mean values ($p < 0.05$).

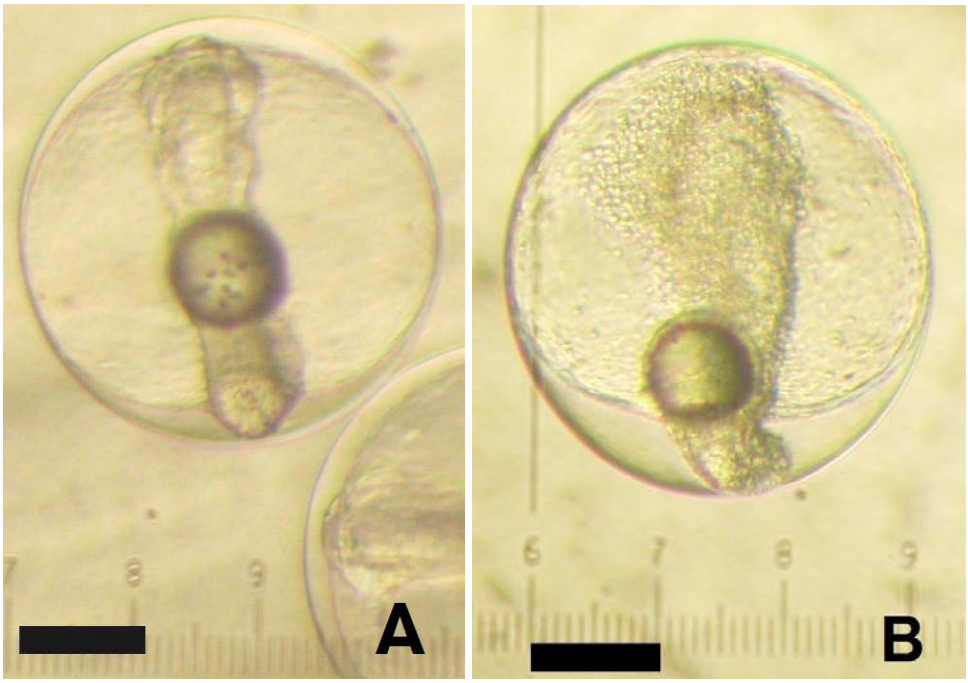


Figure 5.5 Disrupted embryonic development in black bream following exposure to hypoxia: A) normal appearance of a one day old embryo (maintained at 85%S); B) abnormal appearance of a one day old embryo (maintained at 30%S). Ambient salinity was 36 ppt and ambient temperature was 23°C. Bar = 200 µm.

5.3.2.3 Time to hatching

In all DO treatments, greater than 99% of larvae had hatched by Day 2, however the mean time to hatching was significantly different between DO treatments ($p < 0.001$). The mean time to hatching was increased by 7 h following exposure to 45%S, with a mean of 43 h, compared to other treatments which had mean hatching times between 33 and 35 h (Table 5.2).

5.3.2.4 Hatch rates

Hatch rates were significantly affected by DO levels ($p < 0.001$), and no embryos maintained at 30%S hatched. Mean hatch rates at 45%S were 46%, whereas at 55%S mean hatch rates were 60% and at 65%S and 85%S mean hatch rates were >70% (Fig. 5.6).

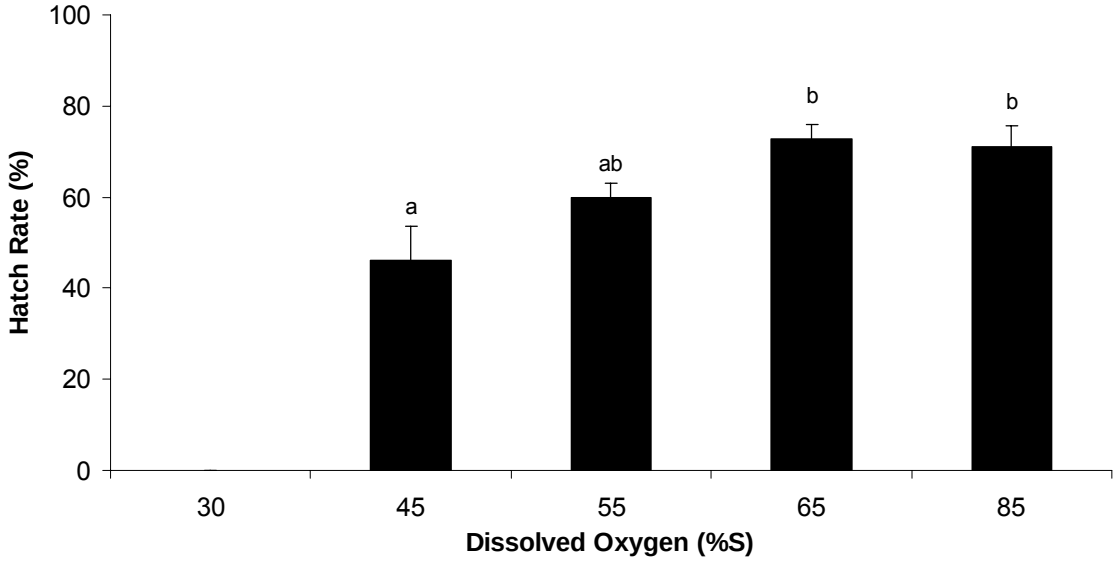


Figure 5.6 Hatch rates (mean ± SEM) for black bream embryos exposed to five different levels of hypoxia. Ambient salinity was 36 ppt and ambient temperature was 23°C. Different letters denote significant differences between mean values ($p < 0.05$).

5.3.2.5 Deformities

Upon hatching larvae were observed to be normal or deformed. Deformed larvae displayed severe curvature of the spine and an inability to swim. Deformed larvae were observed in all treatments that hatched, however the occurrence was significantly higher ($p < 0.001$) at 45%S (100%) compared to all other treatments (9.6-15.2%) (Table 5.2). Furthermore, all embryos that hatched at 45%S died within 24 h.

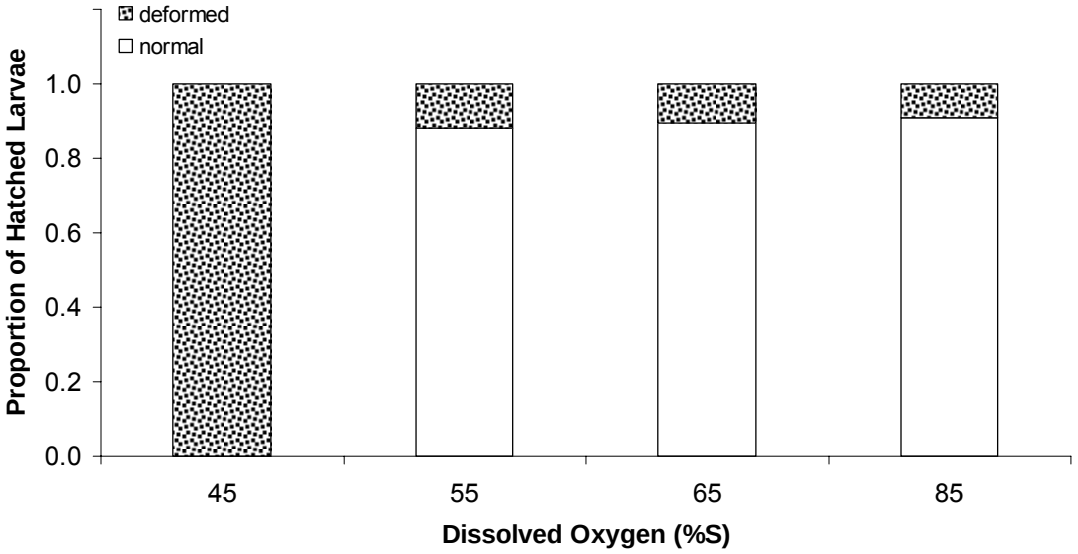


Figure 5.7 Deformities in black bream embryos exposed to five different levels of hypoxia. Ambient salinity was 36 ppt and ambient temperature was 23°C.

5.3.2.6 Larval survival (to Day 2 post-hatch)

No embryos that hatched at 45%S survived to Day 2 post-hatch (Fig. 5.8). There were no significant differences in survival to Day 2 post-hatch for embryos hatched at 55%S, 65%S or 85%S ($p = 0.264$).

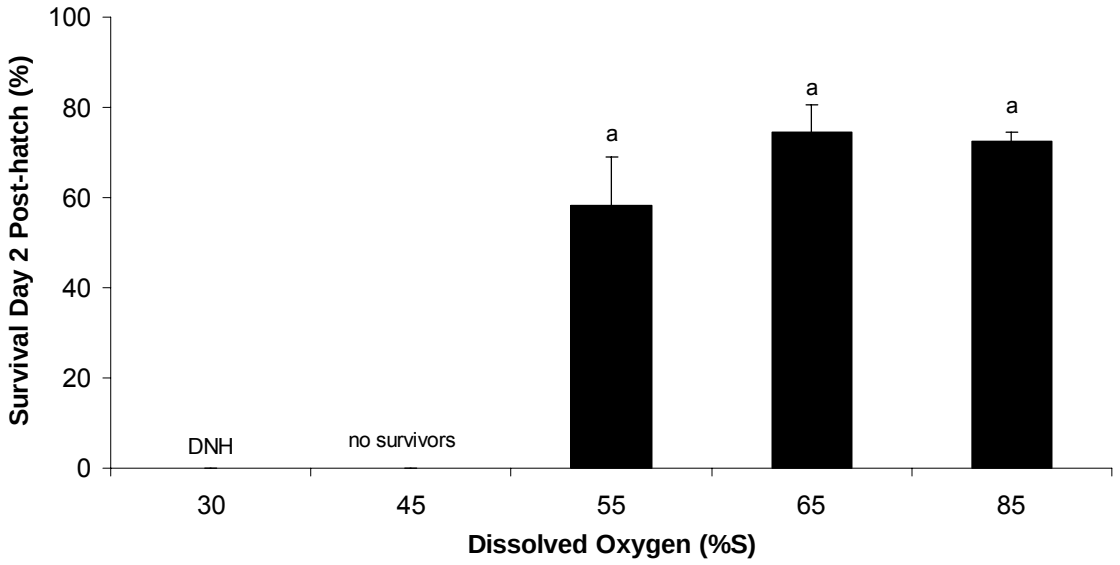


Figure 5.8 Survival to Day 2 post-hatch (mean ± SEM) for black bream embryos exposed to five different levels of hypoxia. Ambient salinity was 36 ppt and ambient temperature was 23°C. Different letters denote significant differences between mean values ($p < 0.05$). DNH – did not hatch.

5.3.2.7 Larval length (on Day 2 post-hatch)

Upon completion of the experiment a sample (*n=50*) of surviving larvae from each treatment were preserved in formalin and then total length (mm) was measured under a dissecting microscope fitted with an eyepiece graticule. Larval length was significantly affected by exposure to hypoxia ($p = 0.001$). At 55%S, a greater percentage of embryos hatched with spinal deformities as compared to the 65%S and 85%S treatments, resulting in shorter mean total lengths and higher variability in length (Fig. 5.9).

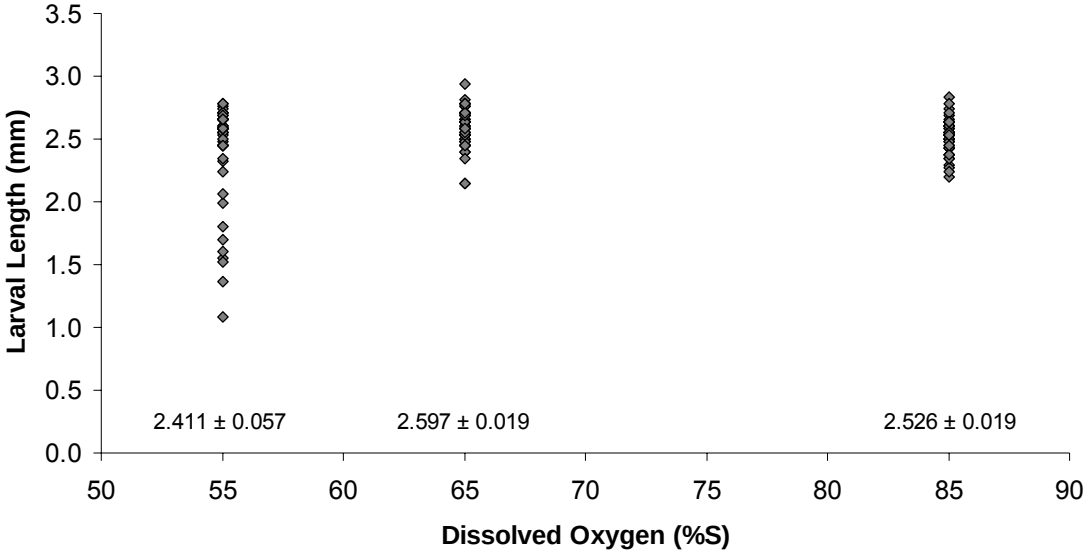


Figure 5.9 Black bream larval lengths (mm) (mean ± SEM) measured on Day 2 post-hatch. Embryos were exposed to five different levels of hypoxia. Ambient salinity was 36 ppt and ambient temperature was 23°C.

5.3.3 EXP. 3 - Effects of hypoxia, low salinity and low temperature on embryos and yolk-sac larvae

Swan Bay broodstock were used for this experiment and at the time of collection, the eggs were observed to be in the blastula stage (see chapter 3). The eggs were randomly distributed to well plates containing 10 ml of water as follows: DO (50%S, >80%S), salinity (15, 23 and 35 ppt) and temperature (16^{o}C, 20^{o}C). The total number of eggs in each well was 72 (± 1 SEM) and there were four replicates of each treatment. The test duration was 72 h and observations were taken at 4, 24, 36, 48 and 72 h. Results were analysed using three-way ANOVA.

5.3.3.1 Water quality

Dissolved oxygen, salinity, temperature and pH were maintained close to nominal levels throughout the experiment (Table 5.3). Partial water changes (50% replacement) were conducted every 24 h, which helped to maintain the water quality at close to the required levels.

Table 5.3 Effects of dissolved oxygen, salinity and temperature on black bream embryo hatching and rates of deformity.

Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (°C)	Mean ± SEM pH	Days to hatching (%)			Mean ± SEM Time to Hatch (h)	Mean ± SEM Deformed Larvae (%)
				1	2	3		
84.9 ± 2.41	15.6 ± 0.22	16.9 ± 0.39	7.92 ± 0.07	0.00	1.52	98.5	73.5 ± 0.63	28.5 ± 17.9
86.8 ± 2.93	23.2 ± 0.34	16.9 ± 0.46	7.91 ± 0.06	0.00	1.33	98.7	73.8 ± 0.29	20.6 ± 13.3
89.3 ± 1.55	35.9 ± 0.44	16.9 ± 0.33	7.95 ± 0.03	0.00	1.22	98.8	73.7 ± 0.36	23.9 ± 8.53
86.0 ± 2.17	15.4 ± 0.22	20.9 ± 0.25	7.92 ± 0.07	2.50	97.5	0.00	53.1 ± 3.96	22.7 ± 4.29
86.7 ± 1.25	22.9 ± 0.37	20.7 ± 0.31	7.95 ± 0.05	0.00	100	0.00	53.7 ± 3.98	36.4 ± 16.0
86.7 ± 1.43	35.6 ± 0.40	20.9 ± 0.25	7.97 ± 0.03	0.00	100	0.00	54.4 ± 4.33	34.4 ± 5.44
54.5 ± 1.02	15.4 ± 0.27	16.8 ± 0.55	8.03 ± 0.03	100	0.00	0.00	24.0	100 ± 0.00
54.8 ± 0.77	23.0 ± 0.00	16.5 ± 0.58	8.14 ± 0.05	-	-	-	-	-
53.2 ± 0.52	35.5 ± 0.37	16.9 ± 0.28	8.15 ± 0.03	1.72	3.45	94.8	72.4 ± 1.84	23.0 ± 11.9
53.2 ± 1.04	15.6 ± 0.27	20.8 ± 0.14	8.08 ± 0.03	0.00	100	0.00	51.3 ± 3.75	50.0 ± 70.7
53.7 ± 1.26	23.5 ± 0.33	20.9 ± 0.14	8.06 ± 0.03	0.00	100	0.00	49.7 ± 2.12	75.0 ± 35.4
50.2 ± 2.03	35.7 ± 0.37	20.8 ± 0.12	8.12 ± 0.04	1.47	98.5	0.00	50.3 ± 2.58	30.3 ± 10.3

5.3.3.2 One day embryo survival

Embryo survival to one day post fertilisation was significantly reduced by low DO ($p < 0.001$). Salinity ($p = 0.299$) and temperature ($p = 0.865$) were not significant factors alone and whilst the interaction between DO, salinity and temperature was not statistically significant, the *p*-value was close to 0.05, suggesting some association/relationship between these factors ($p = 0.053$). At both temperatures the highest survival was observed in normoxic treatments whilst the lowest survival at 20°C was observed at a salinity of 15 ppt, and at 16°C the lowest survival was observed at a salinity of 23 ppt (Fig. 5.10).

5.3.3.3 Time to hatching

The time to hatching was not affected by DO levels or salinity, however lowered temperature delayed hatching by one day ($p < 0.001$) (Table 5.3). In normoxic conditions at all 3 salinities, more than 95% of larvae were hatched by Day 2 at 20°C, whereas at 16°C the same level of hatching (>95%) did not occur until Day 3. Similarly, in hypoxic conditions, more than 95% of larvae in all salinities were hatched by Day 2 at 20°C whilst at 16°C the majority of hatching did not occur until Day 3. The mean time to hatching was delayed by 20 h in low temperature conditions. Only a single larva hatched following exposure to hypoxia, low salinity and low temperature, hatching on Day 1.

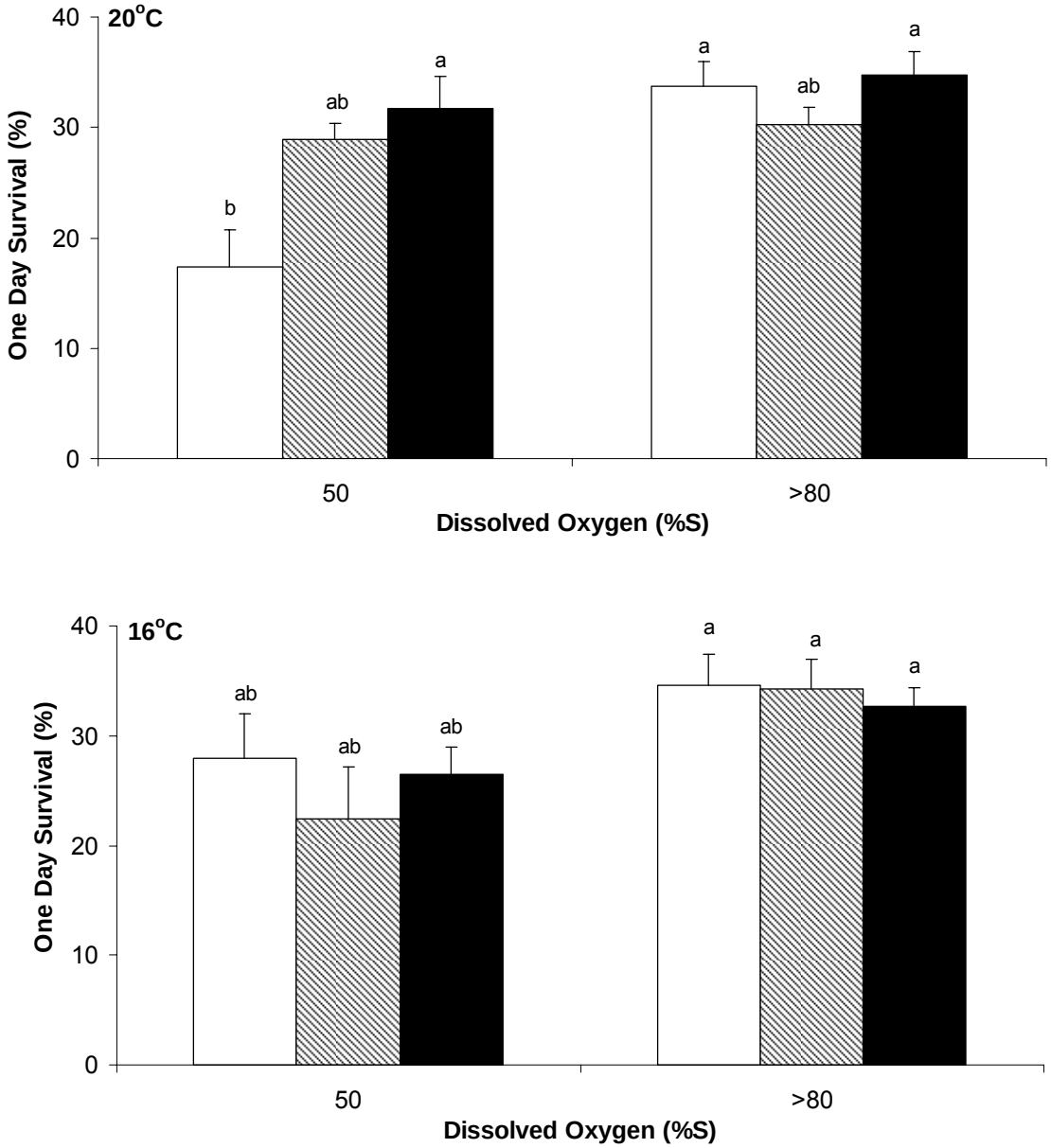


Figure 5.10 One day survival (mean ± SEM) for black bream embryos exposed to three salinities: 15 □ ; 23 ▨ ; and 35 ■ ppt, two levels of dissolved oxygen (%S) and two temperatures (°C). Different letters denote significant differences between mean values ($p < 0.05$).

5.3.3.4 Hatch rates

Hatch rates were significantly affected by DO ($p < 0.001$), salinity ($p < 0.001$) and temperature ($p = 0.029$) (Fig. 5.11). Furthermore the interaction between DO and salinity was highly significant ($p < 0.001$) and whilst the interaction between DO, salinity and temperature was not statistically significant, the *p*-value was close to 0.05, possibly suggesting some association/relationship between these factors ($p = 0.086$). In hypoxic conditions very few eggs hatched (<10%) when salinity was below 35 ppt and at 16^{o}C only a single larva hatched at 15 ppt. In all treatments the mean hatch rates were highest when the salinity was 35 ppt (Table 5.3).

5.3.3.5 Deformities

Larval deformities such as curvature of the spine and incomplete hatching were observed in all test conditions, however the rates of deformity were generally higher in larvae from hypoxic treatments compared to normoxic treatments, and both salinity and temperature influenced the rates (Table 5.3). Very few eggs hatched from hypoxia treatments, and most of them were deformed. For larvae reared in normoxic conditions at 20^{o}C, the percentage of deformities ranged from 22-36%, whereas for larvae reared in hypoxic conditions at 20^{o}C, the percentage of deformities ranged from 30-75%. For larvae reared in normoxic conditions at 16^{o}C, the percentage of deformities ranged from 20-28%, whereas for larvae reared in hypoxic conditions at 16^{o}C, the percentage of deformities ranged from 23-100%.

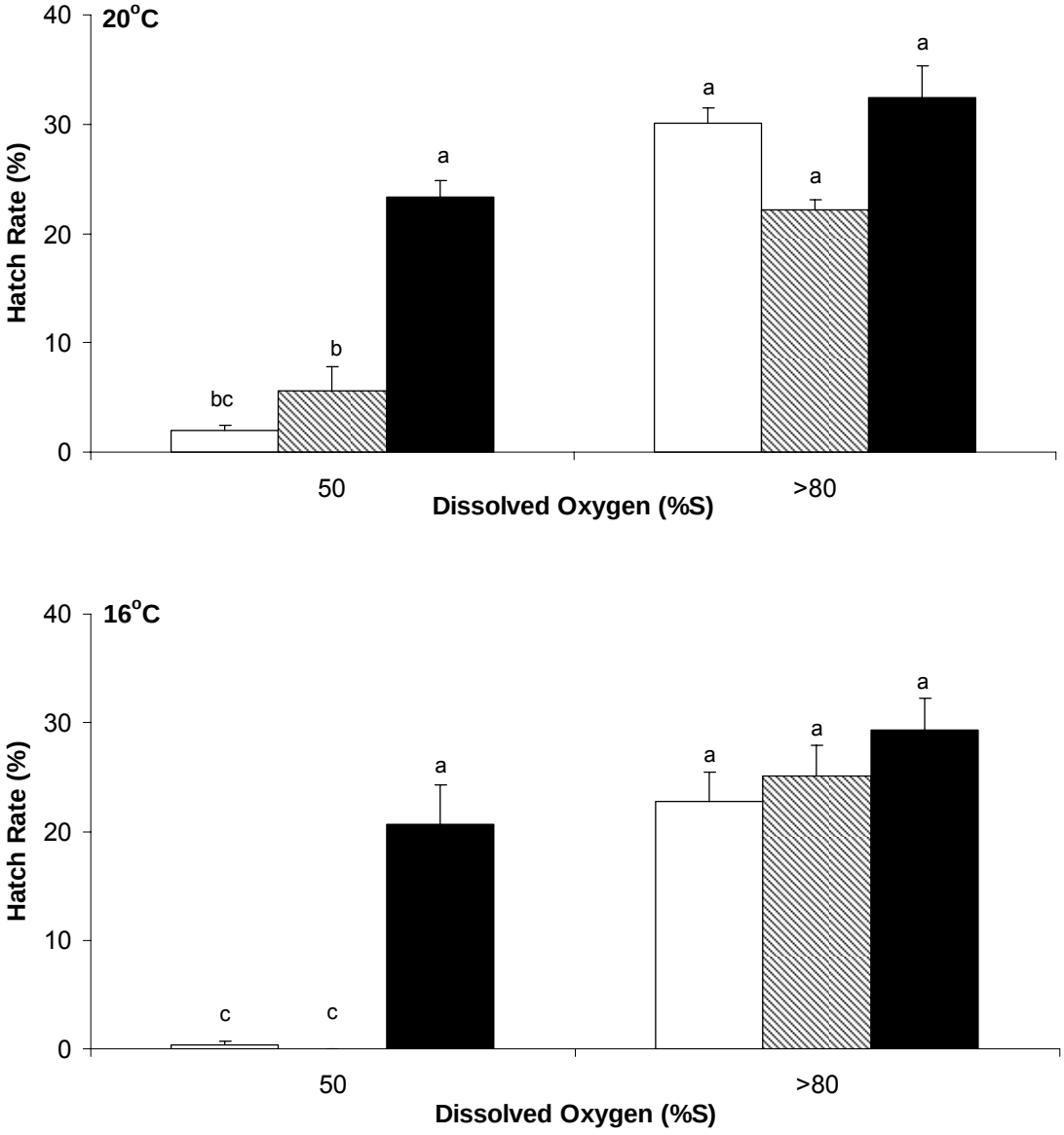


Figure 5.11 Hatch rates (mean ± SEM) for black bream embryos exposed to three salinities: 15 □ ; 23 ▨; and 35 ■ ppt, two levels of dissolved oxygen (%S) and two temperatures (°C). Different letters denote significant differences between mean values ($p < 0.05$).

5.3.4 EXP. 4 - Effects of hypoxia, low salinity and high temperature on embryos and yolk-sac larvae

Lake Tyers broodstock were used for this experiment and at the time of collection, the eggs were observed to be in the morula stage (see chapter 3). The eggs were randomly distributed to well plates containing 10 ml of water as follows: DO (50%S, >80%S), salinity (15, 23 and 35 ppt) and temperature (22^{o}C, 26^{o}C). The total number of eggs in each well was 97 (± 4 SEM) and there were four replicates of each treatment. The test duration was 80 h and observations were taken at 2, 12, 24, 36, 60 and 80 h. Results were analysed using three-way ANOVA.

5.3.4.1 Water quality

Dissolved oxygen, salinity, temperature and pH were maintained close to nominal levels throughout the experiment (Table 5.4). Partial water changes (50% replacement) were conducted every 24 h, which helped in maintaining the water quality close to the required levels.

5.3.4.2 One day embryo survival

Embryo survival to one day post fertilisation was significantly reduced by low DO ($p < 0.001$), salinity ($p < 0.001$) and temperature ($p < 0.001$). Furthermore, the interaction between DO and temperature was highly significant ($p < 0.001$), the interaction between DO and salinity was significant ($p = 0.029$) and the interaction between DO, salinity and temperature was also significant ($p = 0.005$). At both temperatures the highest survival was always observed at 35 ppt salinity (Fig. 5.12).

Table 5.4 Effects of dissolved oxygen, salinity and temperature on black bream embryo hatching and rates of deformity.

Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (^{o}C)	Mean ± SEM pH	Days to hatching (%)			Mean ± SEM Time to Hatch (h)	Mean ± SEM Deformed Larvae (%)
				1	2	3		
87.5 ± 1.59	15.1 ± 0.07	21.8 ± 0.10	7.90 ± 0.03	14.5	73.7	11.8	36.9 ± 0.68	45.3 ± 15.8
87.2 ± 1.63	23.1 ± 0.07	21.8 ± 0.10	7.86 ± 0.03	16.7	79.0	4.30	34.8 ± 1.35	60.7 ± 10.7
86.0 ± 0.96	35.6 ± 0.14	21.9 ± 0.20	7.82 ± 0.06	27.3	72.7	0.00	33.1 ± 2.57	40.6 ± 5.20
87.1 ± 0.92	15.3 ± 0.11	25.7 ± 0.24	8.01 ± 0.03	57.3	26.6	16.1	33.4 ± 2.32	98.1 ± 1.40
86.3 ± 0.92	23.3 ± 0.17	25.7 ± 0.23	7.96 ± 0.04	62.7	37.3	0.00	28.5 ± 2.77	86.2 ± 6.50
84.5 ± 0.79	35.9 ± 0.17	25.6 ± 0.17	7.94 ± 0.05	98.4	1.60	0.00	24.1 ± 0.14	15.0 ± 3.80
55.9 ± 1.42	15.0 ± 0.04	21.8 ± 0.09	7.95 ± 0.02	50.0	50.0	0.00	29.3 ± 4.32	100 ± 0.00
54.0 ± 1.04	22.9 ± 0.13	21.8 ± 0.13	7.95 ± 0.02	33.3	33.3	33.3	37.5 ± 4.62	100 ± 0.00
53.4 ± 0.88	35.5 ± 0.14	21.8 ± 0.09	7.90 ± 0.02	29.7	70.3	0.00	32.6 ± 1.16	34.4 ± 11.3
56.9 ± 1.51	15.2 ± 0.08	25.6 ± 0.27	8.06 ± 0.02	100	0.00	0.00	24.0 ± 0.00	100 ± 0.00
53.7 ± 1.26	23.2 ± 0.13	25.7 ± 0.28	8.05 ± 0.02	83.3	16.7	0.00	25.5 ± 2.12	100 ± 0.00
52.3 ± 0.50	35.9 ± 0.15	25.6 ± 0.22	8.01 ± 0.03	100	0.00	0.00	24.0 ± 0.00	80.2 ± 2.00

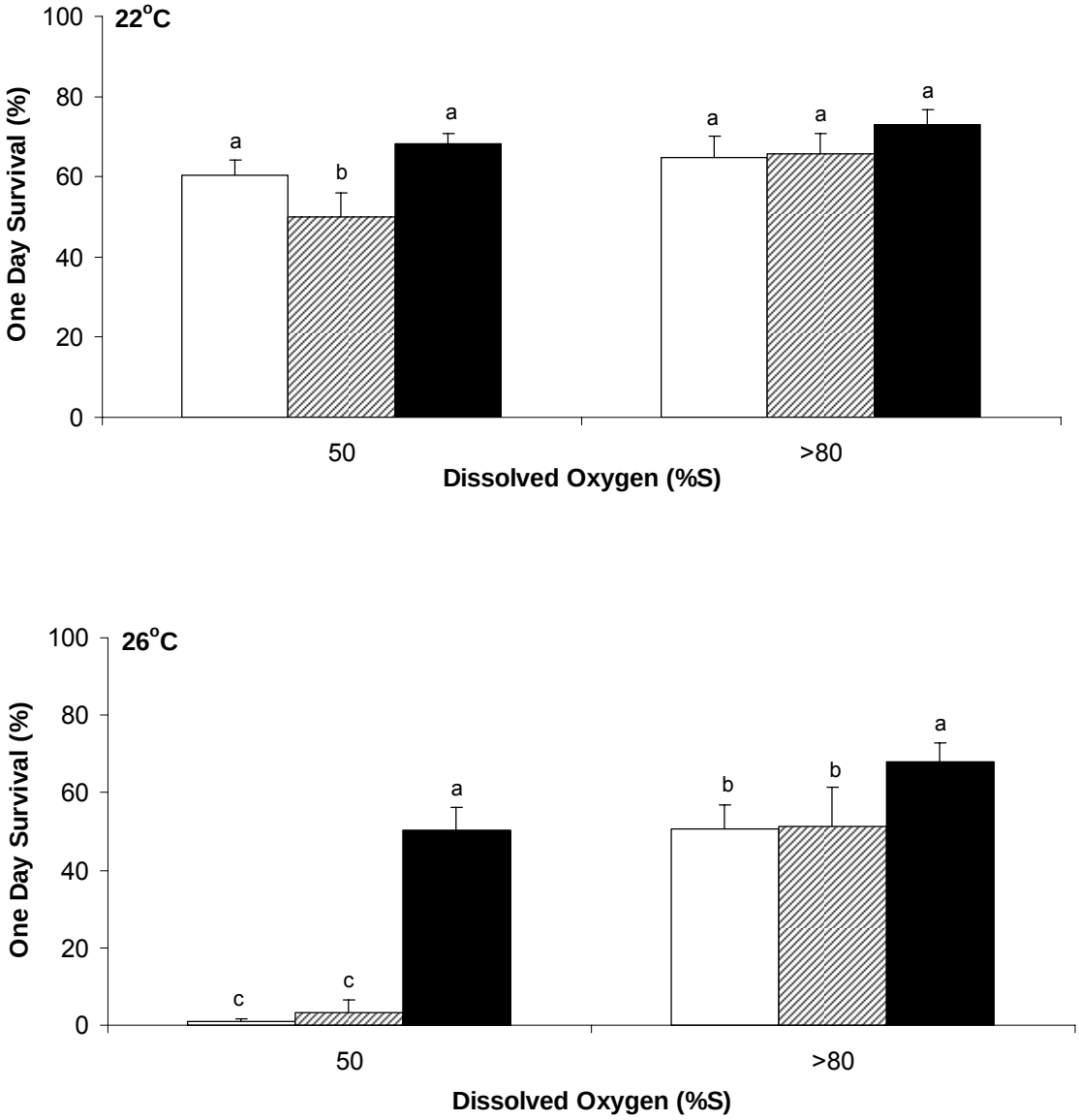


Figure 5.12 One day survival (mean ± SEM) for black bream embryos exposed to three salinities: 15 □ ; 23 ▨ ; and 35 ■ ppt, two levels of dissolved oxygen (%S) and two temperatures (°C). Different letters denote significant differences between mean values ($p < 0.05$).

5.3.4.3 Time to hatching

Time to hatching was significantly affected by DO ($p = 0.006$), temperature ($p < 0.001$), and salinity ($p = 0.05$). The interaction between DO and salinity was highly significant ($p = 0.005$), however the interaction between DO, salinity and temperature was not significant ($p = 0.587$). Elevated temperature (26°C) resulted in the majority of larvae from each treatment group hatching one day earlier than the equivalent treatment group at 22°C (Table 5.4). In normoxic conditions at all 3 salinities, less than 30% of larvae were hatched by Day 1 at 22°C, whereas at 26°C between 57-98% of larvae were hatched by Day 1. Similarly, in hypoxic conditions, less than 50% of larvae in all salinities were hatched by Day 1 at 22°C, whereas at 26°C between 83-100% of larvae were hatched by Day 1. Furthermore, the time to hatching was more variable (spread over 1-3 days) within each group when the embryos were stressed by both low salinity and low DO (Table 5.4).

5.3.4.4 Hatch rates

Hatch rates were significantly affected by DO ($p < 0.001$), salinity ($p < 0.001$) and temperature ($p < 0.001$) (Fig. 5.13). The interaction between DO and salinity was highly significant ($p < 0.001$), however the interaction between DO, salinity and temperature was not significant ($p = 0.222$). When embryos were held in hypoxic conditions with salinities less than 35 ppt, the hatch rates were very low, with only 3.3-5.3% hatching at 22°C and only 1.0-1.4% hatching at 26°C. In all treatments the mean hatch rates were highest when the salinity was 35 ppt (Table 5.4).

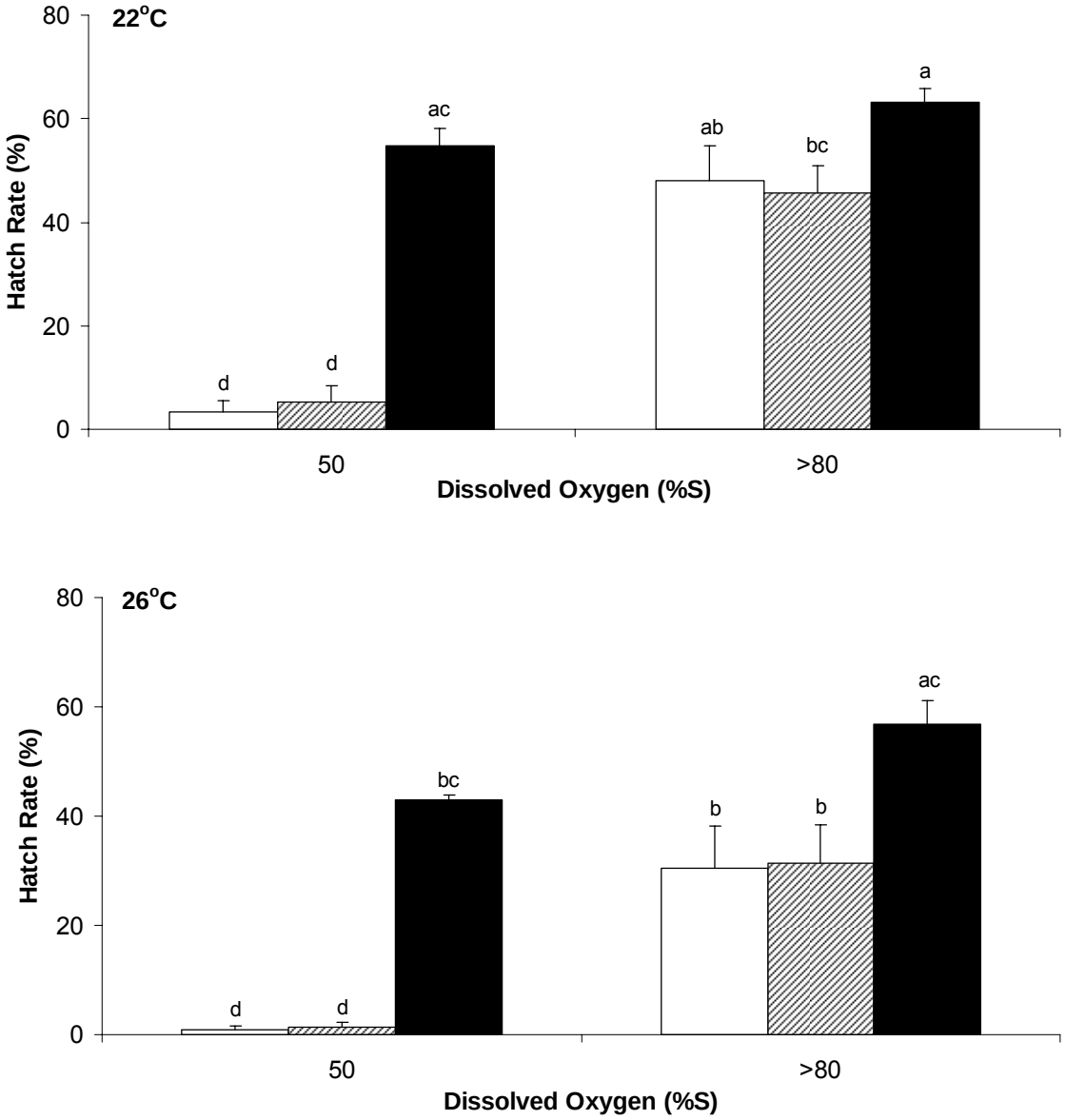


Figure 5.13 Hatch rates (mean ± SEM) for black bream embryos exposed to three salinities: 15 □; 23 ▨; and 35 ■ ppt, two levels of dissolved oxygen (%S) and two temperatures (°C). Different letters denote significant differences between mean values ($p < 0.05$).

5.3.4.5 Deformities

The rates of deformity were much greater for larvae maintained in hypoxic treatments compared to normoxic treatments, and both salinity and temperature influenced the rates as well (Table 5.4). For larvae reared in normoxic conditions at 22°C, the percentage of deformities ranged from 40.6-60.7%, whereas for larvae reared in hypoxic conditions at 22°C, the percentage of deformities ranged from 34.4-100%. Lowered salinity had a greater effect on the rates of deformity when the larvae were already stressed by either low DO or elevated temperature (or both) (Table 5.4). For example, for larvae in normoxic conditions at 22°C, the highest rates of deformities were 60.7 ± 10.7% at 23 ppt salinity, whereas at 26°C, 86.2 ± 6.50% of larvae at 23 ppt salinity were deformed, and this increased to 98.1 ± 1.40% at 15 ppt salinity.

At 26°C, the percentage of deformed larvae in each treatment group was nearly always equal to or greater than that observed for the same treatment groups at 22°C (Table 5.4). The one exception was larvae from the normoxic, 35 ppt salinity treatment group, which had a mean deformity rate of 15.0 ± 3.80% at 26°C and a mean deformity rate of 40.6 ±5.20% at 22°C. The reason for this is unknown, however it may be related to the time to hatching, since the vast majority of embryos at 26°C hatched one day earlier than those at 22°C.

5.3.4.6 Larval survival (to Day 2 post-hatch)

Survival to Day 2 post-hatch was significantly affected by DO ($p < 0.001$) and salinity ($p < 0.001$), but not temperature ($p = 0.335$) (Fig. 5.14). The interaction between DO and salinity was highly significant ($p < 0.001$), and the interaction between DO, salinity and temperature was also significant ($p = 0.005$). Survival in hypoxic treatments was always less than survival in the equivalent normoxic treatment at each salinity and temperature.

5.3.4.7 Larval length (on Day 2 post-hatch)

At the conclusion of the experiment total length (mm) was measured for a sample of larvae from each treatment (*n=2 to 40*). At both temperatures, the mean larval lengths were shorter at salinities of 15 ppt compared to 35 ppt ($p = 0.02$), and the variability between individuals was greater, due to an increased incidence of deformities, resulting in curvature of the spine and hence a shorter total length (Fig. 5.15).

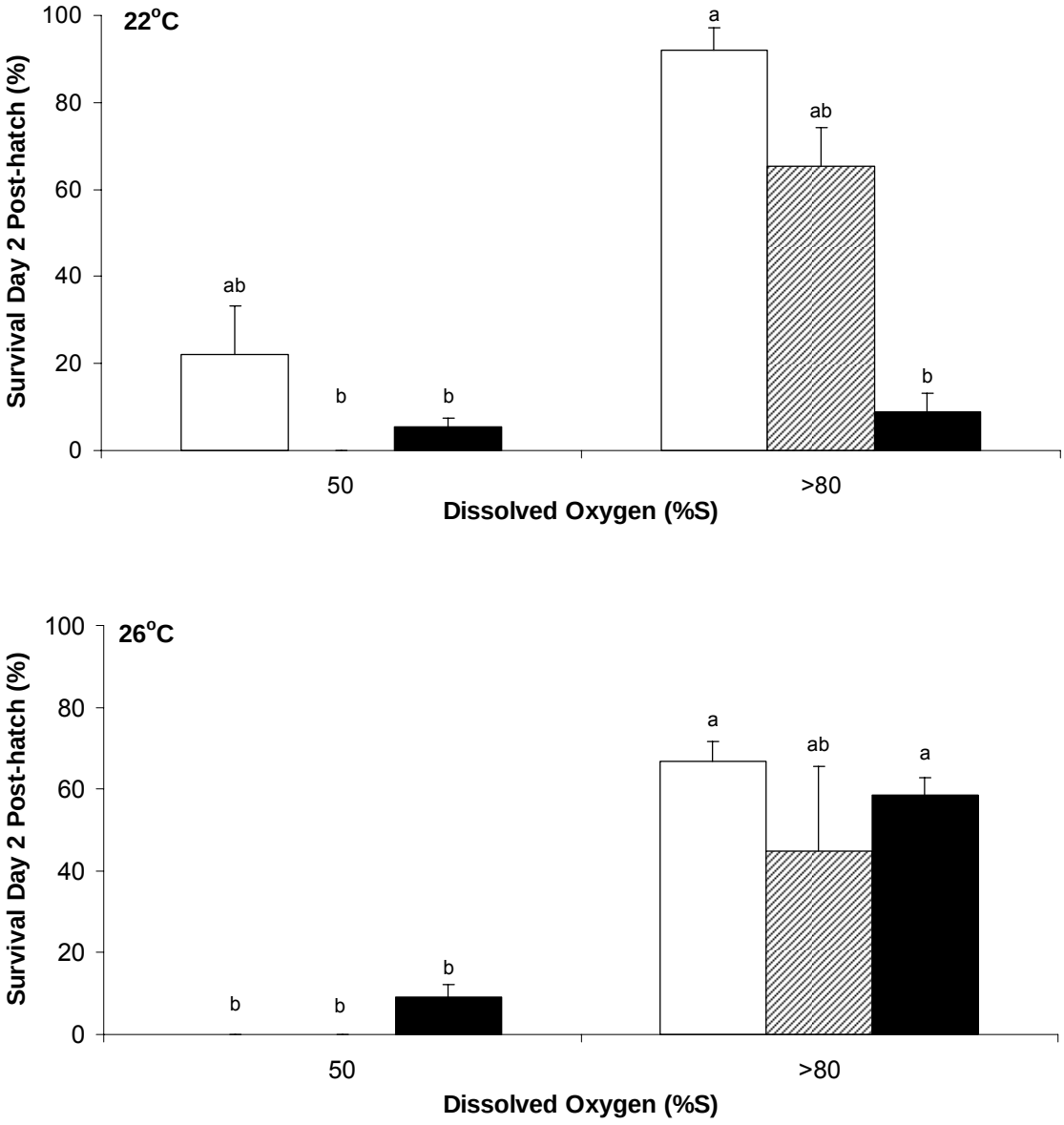


Figure 5.14 Survival to Day 2 post-hatch (mean ± SEM) for black bream embryos at three salinities: 15 ☐; 23 ▨; and 35 ■ ppt, two levels of dissolved oxygen (%S) and two temperatures (°C). Different letters denote significant differences between mean values ($p < 0.05$).

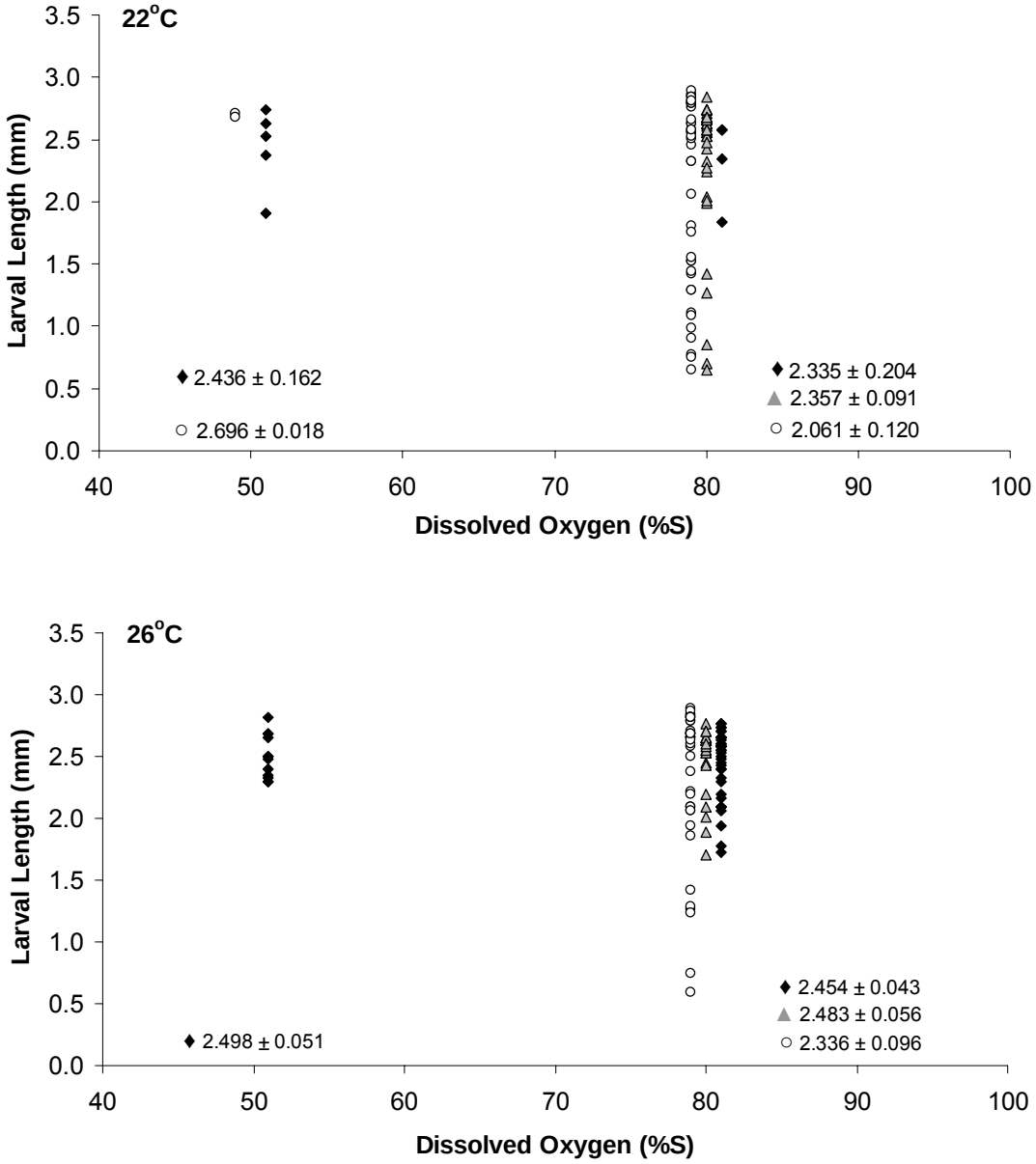


Figure 5.15 Black bream larval lengths (mm) (mean ± SEM) measured on Day 2 post-hatch. Embryos were exposed to three salinities: 15 ○ ; 23 △ ; and 35 ◆ ppt, two levels of dissolved oxygen (%S) and two temperatures (°C).

5.3.5 EXP. 5 - Effects of hypoxia and high salinity on embryos and yolk-sac larvae

Lake Tyers broodstock were used for this experiment and at the time of collection, the eggs were observed to be in the morula stage (see chapter 3). The eggs were randomly distributed to well plates containing 10 ml of water as follows: DO (25, 35, 50, 65, 85%S), salinity (35, 45 ppt) and temperature (21^{o}C). Each treatment comprised 4 replicate samples, containing 56 (± 1 SEM) embryos. The test duration was 84 h and observations were taken at 3, 12, 24, 36, 60 and 84 h. Results were analysed using two-way ANOVA.

5.3.5.1 Water quality

Dissolved oxygen, salinity, temperature and pH were maintained at close to nominal levels throughout the experiment (Table 5.5). Partial water changes (50% replacement) were conducted every 24 h, which helped to maintain the water quality at close to the required levels.

5.3.5.2 One day embryo survival

One day embryo survival was significantly reduced by both hypoxia ($p < 0.001$) and salinity ($p < 0.001$) (Fig. 5.16). At a DO level of 35%S, survival was significantly reduced at 45 ppt compared to 37 ppt. Likewise, at a DO level of 25%S, survival was significantly lower at 45 ppt compared to 37 ppt, and both severely hypoxic salinity treatments displayed significantly lower survival compared to other treatments. The interaction between salinity and DO was highly significant ($p < 0.001$).

Table 5.5 Effects of dissolved oxygen and salinity on black bream embryo hatching and rates of deformity.

Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (°C)	Mean ± SEM pH	Days to hatching (%)			Mean ± SEM Time to Hatch (h)	Mean ± SEM Deformed Larvae (%)
				1	2	3		
87.7 ± 3.06	37.4 ± 0.12	21.2 ± 0.29	8.07 ± 0.07	0.00	100	0.00	36.0 ± 0.00	3.39 ± 1.42
63.6 ± 3.68	38.0 ± 0.10	21.3 ± 0.34	8.12 ± 0.09	6.06	93.9	0.00	35.4 ± 0.53	6.83 ± 2.77
52.5 ± 3.40	37.8 ± 0.19	21.3 ± 0.34	8.08 ± 0.11	0.00	100	0.00	36.0 ± 0.00	21.2 ± 3.87
37.6 ± 1.25	37.6 ± 0.10	21.3 ± 0.75	8.24 ± 0.09	0.00	63.6	36.4	44.0 ± 6.53	100 ± 0.00
26.2 ± 7.34	37.1 ± 0.25	21.5 ± 0.50	8.28 ± 0.09	-	-	-	-	-
86.3 ± 4.31	44.2 ± 0.33	21.1 ± 0.27	8.33 ± 0.12	0.00	100	0.00	36.0 ± 0.00	0.47 ± 0.55
62.1 ± 3.48	44.8 ± 0.19	21.2 ± 0.42	8.35 ± 0.12	0.00	100	0.00	36.0 ± 0.00	28.3 ± 14.3
51.7 ± 2.74	44.8 ± 0.32	21.1 ± 0.37	8.36 ± 0.11	0.00	100	0.00	36.0 ± 0.00	23.4 ± 5.34
37.3 ± 0.41	44.7 ± 0.45	21.0 ± 0.50	8.43 ± 0.07	-	-	-	-	-
26.3 ± 6.53	44.2 ± 0.14	21.3 ± 0.75	8.47 ± 0.06	-	-	-	-	-

5.3.5.3 Time to hatching

Time to hatching was significantly affected by DO (p = 0.021) but not salinity (p = 0.874). For both salinities, all larvae (100%) were hatched by Day 2 when DO levels were equal to or greater than 50%S, however in severely hypoxic conditions (35%S), only 63.6% were hatched by Day 2 at 37 ppt, and no larvae hatched at 45 ppt (Table 5.5). The mean time to hatching was delayed by 8 h by exposure to hypoxia.

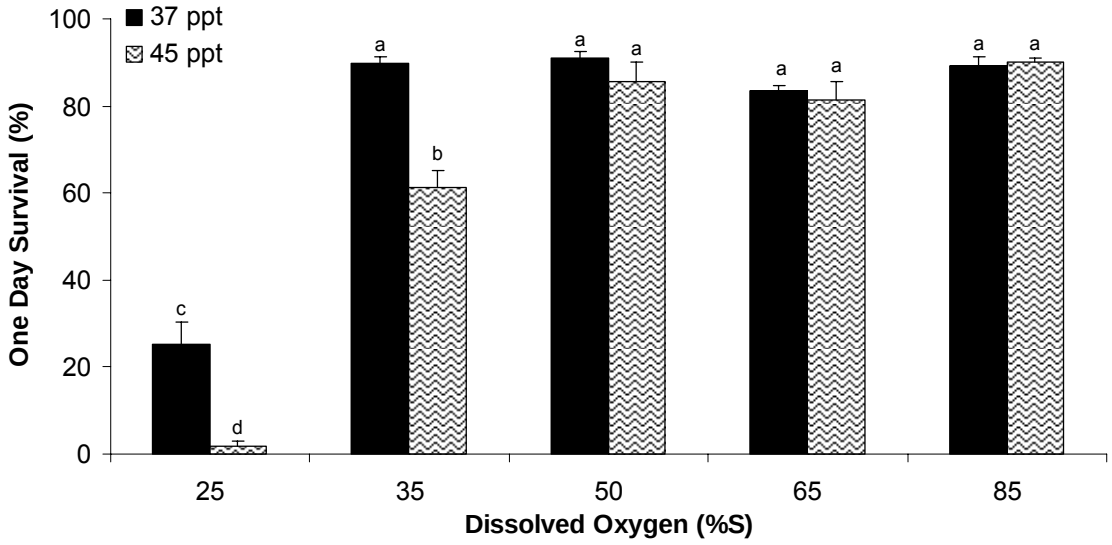


Figure 5.16 One day survival (mean ± SEM) for black bream embryos exposed to two salinities: 35 ■ and 45 ▨ ppt, and five levels of dissolved oxygen (%S). The temperature was maintained at 21°C. Different letters denote significant differences between mean values (p < 0.05).

5.3.5.4 Hatch rates

Hatch rates were significantly reduced by both hypoxia ($p < 0.001$) and salinity ($p = 0.003$) (Fig. 5.17). At DO levels of 50%S and higher, mean hatch rates were greater than 70%, and in all cases hatch rates were greater at 37 ppt than 45 ppt for a given DO level. No hatching was observed at 25%S for either salinity, and at 35%S no hatching occurred at 45 ppt, while less than 10% hatched in the 37 ppt treatment.

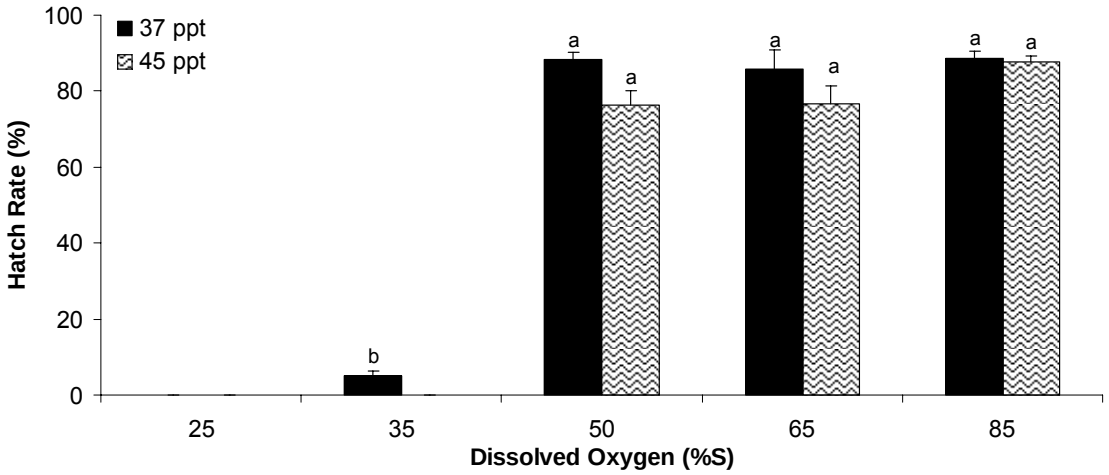


Figure 5.17 Hatch rates (mean ± SEM) for black bream embryos exposed to two salinities: 35 ■ and 45 ▨ ppt, and five levels of dissolved oxygen (%S). The temperature was maintained at 21°C. Different letters denote significant differences between mean values ($p < 0.05$).

5.3.5.5 Deformities

Curvature of the spine and dysfunctional swimming patterns were observed in larvae from all treatments, however the rates of deformity were greatly increased as treatments became more hypoxic (Table 5.5). At 85%S, for both salinities, the mean rates of

deformity were 0.47-3.39%, whereas as treatments became more hypoxic the rates of deformity increased to 6.8-100%.

5.3.5.6 Larval survival (to Day 2 post-hatch)

Survival to Day 2 post-hatch was significantly affected by DO ($p < 0.001$) and salinity ($p = 0.005$) (Fig. 5.18). The interaction between salinity and dissolved oxygen was also significant ($p = 0.005$).

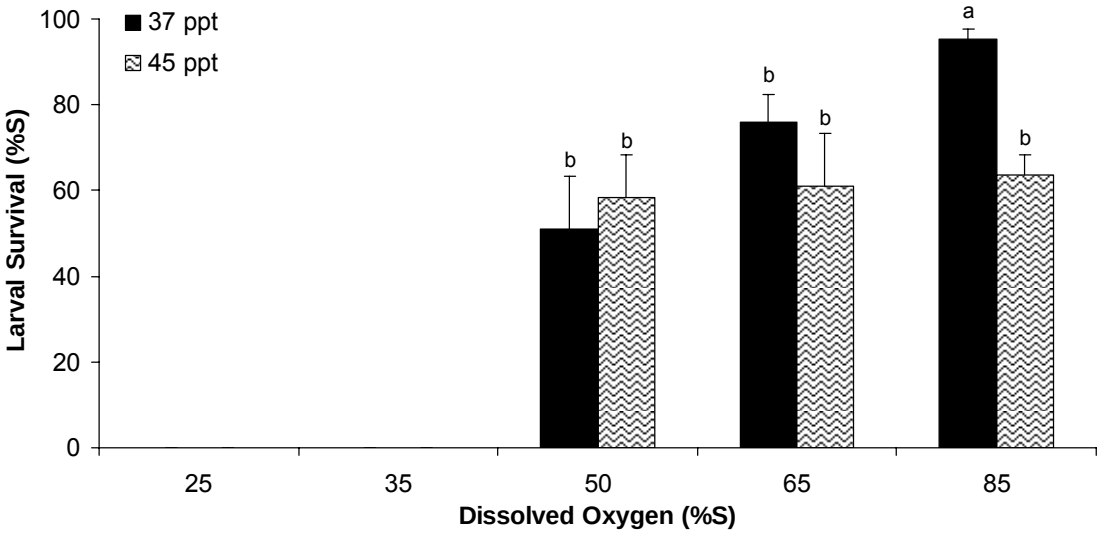


Figure 5.18 Survival to Day 2 post-hatch (mean ± SEM) for black bream embryos exposed to two salinities: 35 ■ and 45 ▨ ppt, and five levels of dissolved oxygen (%S). The temperature was maintained at 21°C. Different letters denote significant differences between mean values ($p < 0.05$).

5.3.5.7 Larval length (on Day 2 post-hatch)

At the conclusion of the experiment total length (mm) was measured for 40 larvae from each treatment (Fig. 5.19, Table 5.5). The DO level significantly affected larval length ($p < 0.001$), while the effect of salinity was not significant ($p = 0.503$). At both salinities, the mean larval lengths became shorter as the level of dissolved oxygen decreased. There was more variability between individuals in hypoxic treatments, due to an increased incidence of deformities which usually resulted in curvature of the spine and hence a shorter total length (Table 5.5).

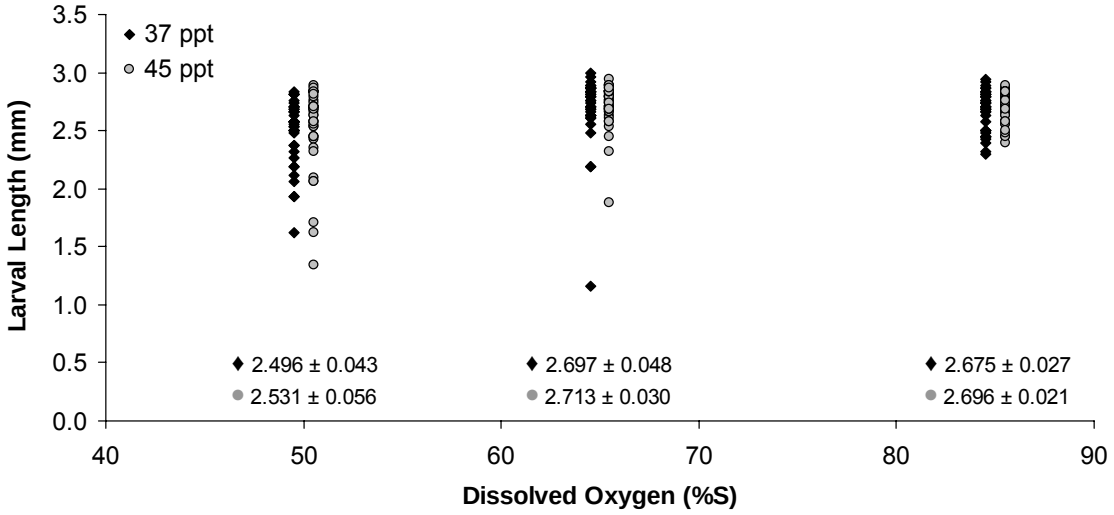


Figure 5.19 Black bream larval lengths (mm) (mean ± SEM) measured on Day 2 post-hatch. Embryos were exposed to two salinities: 37 ◆ and 45 ○ ppt, and five levels of dissolved oxygen (%S). The temperature was maintained at 21°C.

5.3.6 EXP. 6 - Effects of hypoxia and salinity on egg buoyancy

Two tests were conducted to determine at what salinity black bream embryos lose buoyancy, and furthermore to determine if DO affects it. The eggs were transferred to 250 ml Pyrex beakers containing water as follows: DO (variable – see below), salinity (36-37 ppt) and temperature (20-21^{o}C). Distilled water (of the same DO) was slowly added and gently mixed in the beaker until the eggs lost buoyancy. There were approximately 50 eggs in each beaker and there were three replicates of each treatment. Results were analysed using one-way ANOVA.

5.3.6.1 Buoyancy test 1

A mixed sample of Swan Bay black bream eggs was used for this experiment, which contained eggs that were in the gastrula stage (see chapter 3), as well as newly hatched larvae (from a different spawning). The salinity at which the eggs (and larvae) lost buoyancy was determined in both normoxic (>85%S) and hypoxic (50%S) seawater samples. There were no significant differences between the starting salinities of the normoxic and hypoxic treatments (p = 0.184), however the salinity at which eggs lost buoyancy in normoxic conditions (31.7 ppt) was significantly different to the salinity at which eggs lost buoyancy in hypoxic conditions (26.7 ppt) (p = 0.003) (Table 5.6).

5.3.6.2 Buoyancy test 2

A second experiment was conducted using Lake Tyers black bream eggs that were in the blastula stage (see chapter 3). The salinity at which the eggs lost buoyancy was determined at five DO levels: >85, 65, 55, 45 and 30%S. There were no significant

differences between the starting salinities of any of the DO treatments (p = 0.414). In contrast to buoyancy test 1, in this test, the salinities at which the eggs lost buoyancy were not significantly different between any DO treatments (p = 0.114) (Table 5.7).

Table 5.6 Effects of dissolved oxygen and salinity on black bream egg buoyancy (Buoyancy test 1).

Eggs	Mean ± SEM Salinity (ppt)	Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM pH	Mean ± SEM Temperature (^{o}C)
Buoyant	37.0 ± 0.0	91.4 ± 0.49	7.98 ± 0.01	21.0 ± 0.00
	37.7 ± 0.41	53.8 ± 1.56	8.27 ± 0.01	20.2 ± 0.20
Non-buoyant	31.7 ± 0.41	89.0 ± 0.32	8.02 ± 0.01	20.7 ± 0.41
	26.7 ± 0.82	60.2 ± 1.15	8.36 ± 0.02	19.5 ± 0.35

Table 5.7 Effects of dissolved oxygen and salinity on black bream egg buoyancy (Buoyancy test 2).

Eggs	Mean ± SEM Salinity (ppt)	Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM pH	Mean ± SEM Temperature (^{o}C)
Buoyant	36.3 ± 0.00	100 ± 0.00	8.00 ± 0.00	22.3 ± 0.00
	36.1 ± 0.00	62.4 ± 0.00	8.10 ± 0.00	22.4 ± 0.00
	36.4 ± 0.00	49.7 ± 0.00	8.14 ± 0.00	22.3 ± 0.00
	36.7 ± 0.00	33.5 ± 0.00	8.13 ± 0.00	22.7 ± 0.00
	35.7 ± 0.00	10.8 ± 0.00	8.22 ± 0.00	22.8 ± 0.00
Non-buoyant	29.1 ± 0.64	86.0 ± 0.37	8.02 ± 0.01	22.2 ± 0.16
	26.6 ± 0.37	67.4 ± 1.14	8.16 ± 0.00	22.3 ± 0.07
	27.9 ± 0.74	59.8 ± 2.10	8.18 ± 0.01	22.2 ± 0.12
	27.0 ± 0.76	46.4 ± 1.13	8.30 ± 0.01	23.2 ± 0.18
	26.7 ± 1.32	30.6 ± 0.99	8.31 ± 0.01	23.1 ± 0.08

5.3.7 EXP. 7 - Effects of hypoxia on hatched larvae survival (to Day 4 post hatch)

Newly hatched Swan Bay black bream larvae (<8 hours old) were used for this experiment. The larvae were randomly distributed to 100 ml Pyrex beakers containing 50 ml of water as follows: DO (20, 50, 85%S), salinity (38 ppt) and temperature (21^{o}C). Each treatment comprised 4 replicate samples containing 48 (± 3 SEM) healthy larvae. The beakers were placed into CACs and nitrogen gas was added to achieve the required oxygen levels. The test duration was 84 h and observations were taken at 4, 24, 36, 60 and 84 h. Artificial feed was first introduced to the larvae at 60 h. Ideally, live rotifers (*Brachionus plicatilis*) would have been offered, however insufficient quantities were available. Instead, a commercially available micro-encapsulated feed was offered (Golden Pearls, Rotifer Size II, 100-200 μm) (Reef Culture, Ashmore).

5.3.7.1 Water Quality

Dissolved oxygen, salinity, temperature and pH were maintained close to nominal levels throughout the experiment (Table 5.8). Partial water changes (50% replacement) were conducted every 24 h, which helped to maintain the water quality at close to the required levels.

Table 5.8 Water quality measurements for beakers containing newly hatched black bream larvae over a 4 day test period.

Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (^{o}C)	Mean ± SEM pH
20.4 ± 1.93	37.6 ± 0.35	20.7 ± 0.17	8.23 ± 0.01
46.5 ± 7.07	38.1 ± 0.17	21.0 ± 0.21	8.14 ± 0.04
84.4 ± 2.69	38.2 ± 0.23	20.7 ± 0.17	7.97 ± 0.04

5.3.7.2 Larval survival – 4 hours

After 4 h exposure to severe hypoxia (20%S), all larvae were exhibiting signs of stress and morbidity. No larvae were swimming but rather they were resting on the bottom of the beaker not moving. Furthermore, a number of larvae in the 20%S treatment were observed to have yolk-sac oedema or slight curvature of the spine (Fig. 5.20, 5.21). Nearly all larvae that were maintained in moderate hypoxic (50%S) and normoxic (85%S) conditions were swimming actively (Fig. 5.22A).

5.3.7.3 Larval survival – 24 hours

After 24 h, nearly all larvae held in severely hypoxic conditions had perished (Fig. 5.20). The few that remained were lethargic and immobile. Most larvae in moderately hypoxic conditions were swimming normally, however some were observed to be inactive and resting on the bottom of the beaker. All larvae maintained in normoxic conditions were swimming normally.

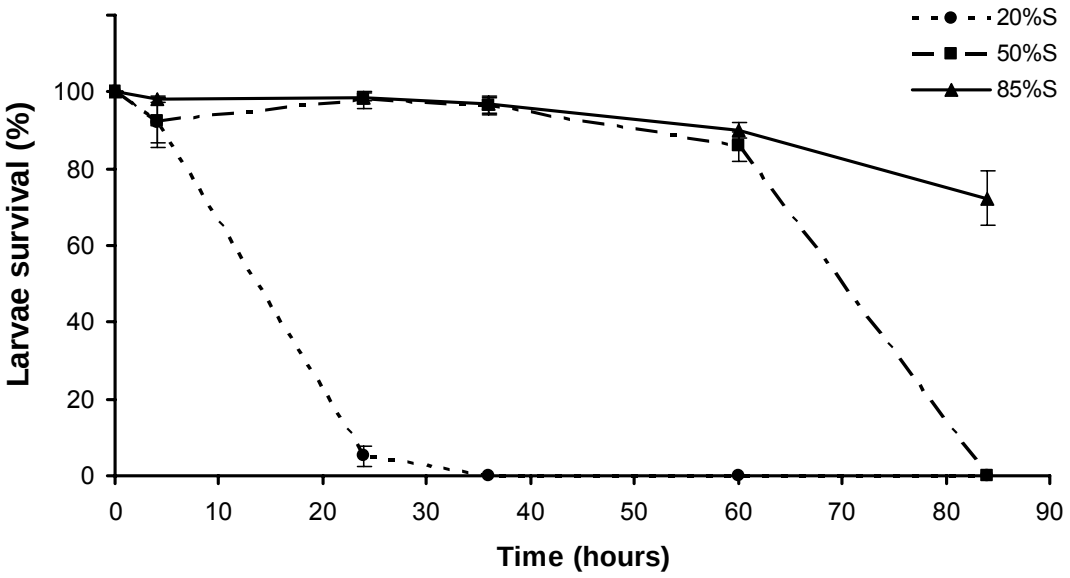


Figure 5.20 Survival (Mean ± SEM) of black bream larvae to Day 4 post-hatch maintained at three different dissolved oxygen levels: severely hypoxic (20%S), moderately hypoxic (50%S) and normoxic (85%S). The salinity was maintained at 38 ppt and the temperature was 21°C.

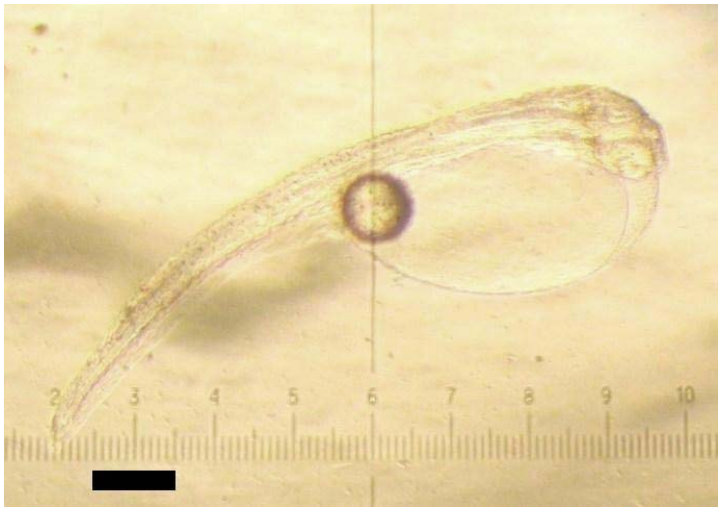


Figure 5.21 Physical appearance of black bream larvae after 4 hours exposure to hypoxia (20%S). The salinity was maintained at 38 ppt and the temperature was 21°C. Lethargy, yolk-sac oedema and slight curvature of the spine were observed. Bar = 200 µm.

5.3.7.4 Larval survival – 36 hours

All larvae in the severely hypoxic treatments were dead after 36 h (Fig. 5.20). Most larvae in the moderately hypoxic and normoxic treatments were swimming normally. At this time all surviving larvae still had a large yolk-sac and were relying on endogenous feeding (Fig. 5.22B).

5.3.7.5 Larval survival – 60 hours

Some mortalities were observed in both moderately hypoxic and normoxic treatments at this time (Fig. 5.20). Artificial feed was first offered at 60 h.

5.3.7.6 Larval survival – 84 hours

After 84 h, all larvae in the moderately hypoxic treatment were dead (Fig. 5.20). Some mortalities were observed in the normoxic treatments, yet the majority of larvae were swimming actively and looked normal (Fig. 5.22C).

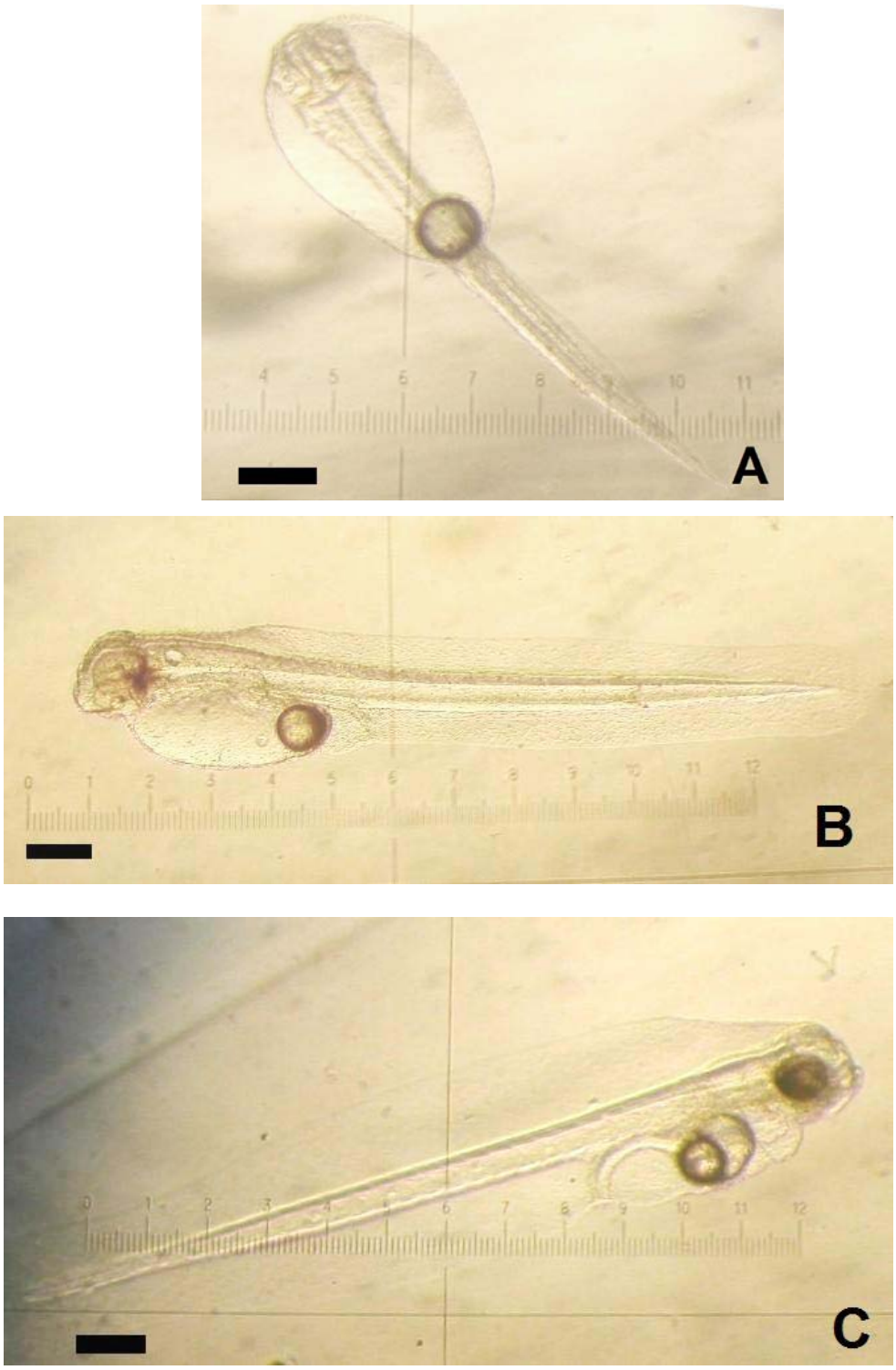


Figure 5.22 Normal black bream larvae at A) 4 h; B) 36 h; C) 84 h, maintained in normoxic (85%S) conditions. The salinity was maintained at 38 ppt and the temperature was 21°C. Bar = 200 µm.

5.4 Discussion

5.4.1 Experimental results

This study demonstrates that black bream eggs and larvae are very sensitive to environmental conditions during the early life-stages, especially to hypoxia. Hypoxia caused abnormal embryo development and larval deformities, reduced hatch rates and lowered larval survival in black bream. Likewise, both low and high salinity resulted in decreased hatch rates, increased rates of deformities and lowered larval survival. Increased and decreased temperatures also had adverse impacts, most notably on the time to hatching, by either delaying (low temperature) or accelerating (high temperature) hatching. Furthermore, interactive effects between DO, salinity and temperature were observed for a number of endpoints, emphasizing the importance of considering multiple factors as determinants of successful reproduction in organisms like the black bream that reproduce in fluctuating environments such as estuaries.

The results of this study show clearly that water quality has profound effects on the early life stages of black bream and whilst no field studies were conducted to confirm these findings, the results may be used to predict habitat conditions that would be suitable for eggs and larvae to develop in the wild. Laboratory experiments allowed the different variables to be controlled with much greater precision than could have been achieved in the field. Laboratory experiments are not always indicative of what takes place in the natural environment, however the paucity of data in relation to the effects of environmental stressors on this species warrants the laboratory approach. Furthermore, in the laboratory confounding factors (ie. predator-prey interactions) are controlled or

removed, so that any effects observed can be attributed directly to the variables that are tested. In a study of fish habitats within the Gippsland Lakes, Gunthorpe (1997) reported that as much as 80% of subsurface waters throughout the lake system would be incapable of sustaining fishery species due to low DO levels, based on the collection of adult fish from commercial fishing (Fig. 5.23). From the laboratory results in the present study, it is clear that black bream eggs and larvae would be unable to survive in large areas of the Gippsland Lakes because of hypoxic conditions and salinity variation.

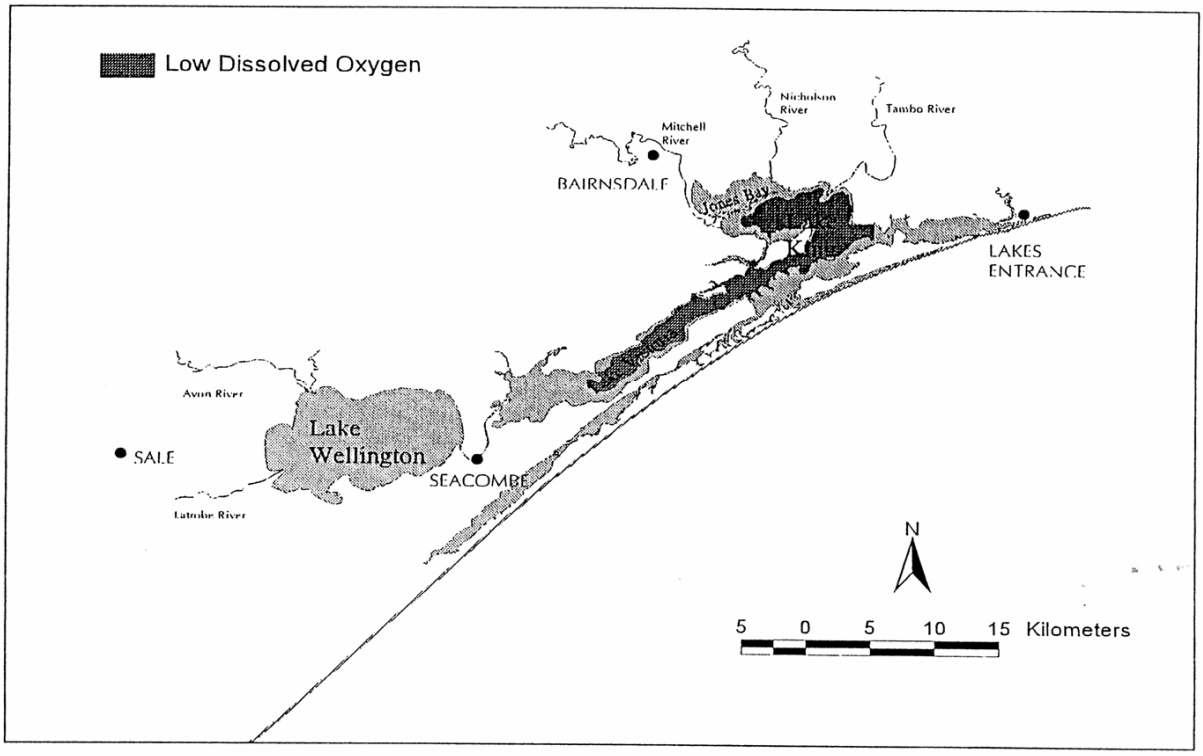


Figure 5.23 Distribution of low dissolved oxygen areas within the Gippsland Lakes, considered incapable of sustaining fishery species (source: Gunthorpe, 1997).

5.4.1.1 Effects of dissolved oxygen

Significant differences were observed in a range of responses as the DO levels decreased, including disruptions and delays to embryo development, altered hatch rates and reduced larval survival. No larvae survived beyond Day 4 post-hatch when exposed to hypoxia (≤ 50%S). These findings indicate that the early life stages of black bream are very sensitive to hypoxia, and the likely consequences of spawning in hypoxic estuaries would be reduced recruitment and lowered population survival. This is an important finding, since severely hypoxic and anoxic conditions are increasingly common in large parts of the Gippsland Lakes and other estuaries in southern Australia due to stratification, high nutrient loadings and agricultural run off (Longmore *et al.*, 1990; Webster *et al.*, 2001; Barton *et al.,* 2008).

Hypoxia is an important, widespread environmental stressor that occurs throughout aquatic environments. This study has demonstrated the significant influences that hypoxia has on the hatching success and early larval survival of an estuarine sparid, and based on these results it is reasonable to assume that hypoxia would subsequently affect other developmental stages too. Furthermore, if hypoxic events continue to occur and expand in geographic extent, and increase in frequency, spawning areas will become smaller and high levels of larval mortality will be reflected in the age structure, size and growth performance of each year class. Other species that utilise estuaries for spawning and share similar early life histories to black bream are also likely to be at risk from hypoxia, which threatens fish stocks and may impact the species composition in fish fauna. Ultimately fisheries productivity will be adversely impacted by deteriorating

conditions in aquatic environments unless catchment management strategies can effectively address the environmental threat from hypoxia.

Results in this chapter showed that in severely hypoxic conditions (30%S) all black bream embryos developed abnormally, resulting in an undifferentiated mass of cells towards the animal pole and a lack of cephalisation. The normal developmental processes that occur during the gastrula and neurula stages (eg. neural plate thickening) were not observed. Shang and Wu (2004) have described similar disruptions in the embryonic development of zebrafish (*Danio rerio*), whereby the apoptotic patterns are altered following exposure to hypoxia, resulting in disruptions to histogenesis and organogenesis processes. Lack of cephalisation in response to hypoxia has not been reported previously in any sparid fishes. However, developmental disturbances have been demonstrated following exposure to toxicants in two marine sparid species (*Pagrus major* and *Sparus aurata*), and observations of embryonic development have shown defects such as reductions in the perivitelline space, irregular chorion shape and smaller head size (Yamauchi *et al.*, 2006; Oliva *et al.*, 2007).

Embryonic development appeared normal at 45%S but was significantly slower than at 55%S, 65%S or 85%S. The embryos required a longer period of time to develop sufficiently to hatch, resulting in an increase in the mean time to hatching. Furthermore, when embryos did hatch at 45%S, all displayed spinal deformities. In severely hypoxic conditions (30%S), embryonic development was abnormal and no hatching occurred.

These results clearly demonstrate that hypoxia interferes with the developmental processes affecting hatching of black bream larvae, and it is thus reasonable to assume that the subsequent growth of those larvae that do survive may also be impaired if embryonic development is abnormal but does not result in death.

Whilst a number of ecological studies have observed changes in community structures and faunal distributions in association with hypoxia (Diaz and Rosenberg, 1995; Breitberg *et al*., 2003; Ekau and Verheye, 2005), only limited information is available on individual level effects, particularly in relation to early life histories. In a study of larval and juvenile fishes from the East and Gulf coasts of the United States of America, (Miller *et al.*, 2002) reported 4-day LC_{50} values ranging from 0.6 – 2.4 mg/l (~10-40%S). Aside from mortality, another effect that has recently been reported is a shift in sex ratios as a result of exposure to hypoxia. Shang *et al.* (2006) reported male-biased populations of zebrafish following a 4-month exposure of embryos and subsequently, larvae to hypoxia, concluding that this would have serious impacts on reproductive success and population sustainability.

There is no literature available on laboratory studies of hypoxia tolerance in black bream eggs or larvae. However a field study conducted in spring/summer 2003 in the Glenelg and Hopkins estuaries of south-western Victoria found the highest proportion of black bream eggs in areas where the DO level was 40%S or less (Nicholson *et al.*, 2008). The eggs were likely to have been transported to the areas by environmental flows, and based

on the results of this study, it is likely that hypoxic conditions would result in very low black bream larval survival as suggested by (Nicholson *et al.*, 2008).

Dissolved oxygen levels are known to influence the hatching process in teleosts (Yamagami *et al.*, 1992; Ciuhandu *et al.*, 2005; Czerkies *et al.*, 2001). For example, if the diffusion rates of oxygen through the chorion become too low to sustain the developing embryo, premature hatching may occur (Jobling, 1995). The process of hatching is initiated by the production of proteolytic enzymes that soften the chorion and allow the embryo to swim free (Yamagami *et al.*, 1992; Czerkies *et al.*, 2001). Czerkies *et al.* (2001) observed precocious hatching in embryos of the freshwater fish vendace (*Coregonus albula*) following 60 or 90 min exposure to hypoxia and reported that some embryos were unable to disrupt the thin residual layer of egg membrane which formed an envelope around the embryo. Ciuhandu *et al.* (2005) observed slower growth as well as both early and delayed hatching in rainbow trout *(Oncorhynchus mykiss)* embryos exposed to hypoxic conditions. Likewise, black bream in the present study displayed both premature and delayed hatching times depending on the severity of the hypoxic stress. Exposure to conditions that may alter the functioning or composition of hatching enzymes are likely to result in unsuccessful hatching. This biological mechanism could account for the inability of larvae to break free of the chorion in hypoxic and low salinity environments observed in this study.

Whilst no physiological mechanisms for unsuccessful hatching were tested in this study, a number of factors are known to influence embryonic and subsequent larval

development in teleost fishes. If the conditions are 'stressful' for the developing embryo, a range of consequences may become apparent including alterations to physiology, morphology or behaviour (Iwama *et al.*, 1997; Johnston, 2006). One process that may be influenced by environmental conditions is myogenesis (Johnston, 2006). Myogenesis is the process of muscle development, and disruptions to normal myogenesis may provide an explanation as to why curvature of the spine and irregular swimming patterns were observed in some larvae in this study. Hypoxia, alone or in combination with altered salinity or temperature resulted in reduced embryo survival and reduced hatch rates which most likely reflects disrupted embryonic development. This finding is in contrast to the suggestion by Johnston (2006) that hypoxia is not likely to influence the development of small pelagic eggs that hatch at an early stage of organogenesis.

Survival to Day 2 post-hatch for embryos exposed to hypoxia was similar in all larvae that survived the hatching process without significant levels of deformity. However, none of the deformed larvae that hatched in 45%S survived to Day 2 post-hatch. Yamauchi *et al.* (2006) observed increased larval mortality in the marine sparid, red seabream (*Pagrus major*) following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), which was associated with under-development of the heart, bradycardia and circulation failure. It is quite possible that hypoxia may cause high mortality from similar malformations in the organs of black bream embryos, but this was not examined in the current study and requires further research.

Clearly, hypoxia plays a significant role in determining the survival of the early life stages of black bream, thus any factors that may increase the severity or duration of hypoxia in estuarine environments that are utilised by black bream for spawning, are likely to have major, negative impacts on populations of this species.

5.4.1.2 Effects of salinity

Significant differences were observed in a range of endpoints as salinity was either decreased or increased, including higher rates of deformities, altered hatch rates and reduced larval survival. At salinities less than 15 ppt, a much greater incidence of abnormal embryo development and larval deformities were observed, whilst at high salinities hatch rates and larval survival were lower than the controls. Incomplete hatching, or partial emergence was commonly observed in black bream embryos in low salinity treatments, a phenomenon that was first described in fish eggs more than 80 years ago (Battle, 1930). Furthermore, at both low and high salinities, black bream embryos were found to be less capable of tolerating hypoxia, indicating that multiple stressors reduce overall fitness and survival.

Haddy and Pankhurst (2000) reported that incubation salinity significantly affected black bream egg survival and hatching when temperature and DO levels were kept constant. Between 10-35 ppt salinity 24 h egg survival was high, but at 5 ppt it was much lower and at 0 ppt no embryos survived to day 1. Hatching success was high in salinities of 15 to 35 ppt, but much lower at 5 and 10 ppt. Furthermore, eggs that hatched at 5 ppt were

all abnormal, showing curvature of the spine and tail flexure. The results of the present study corroborate those findings.

Egg buoyancy is affected by salinity and if buoyancy is lost then the eggs are likely to sink into increasingly hypoxic, cold water. In this study, the salinity at which black bream eggs lost buoyancy was determined and there were some indications that the DO content of the water influences this phenomenon. For eggs that were held in normoxic conditions, the salinity at which buoyancy was lost was 29-31 ppt, whereas for eggs held in hypoxic conditions, the salinity at which buoyancy was lost was 26-27 ppt. These differences may be due to differences in the specific gravity of nitrogen gas (0.967) compared to air (1.00) or differences in the solubility of the two gases in water: i.e. N_2=0.0234 vol/vol, air=0.0292 vol/vol (@1.013 bar pressure; 0^{o}C) (Ebbing, 1996). If more nitrogen gas can dissolve into a given volume of water compared to air, then the density of that liquid would be greater for a given salinity. Hence, the eggs would remain buoyant at lower salinities in water containing nitrogen gas compared to water containing air.

Loss of buoyancy has been associated with increased exposure to hypoxia (Breitburg, 2003), and the importance of salt wedge formation has been acknowledged as a factor affecting survival of black bream and anchovy populations in the Hopkins River estuary, Victoria (Newton, 1996). The results of this study suggest that eggs sinking into water of lower DO (and temperature) would have increased mortality due to delayed hatching, reduced hatch rates and increased deformities, as well as being subject to benthic

predators. These observations highlight the importance of salt-wedge formation as another factor for successful spawning in black bream.

Egg buoyancy is not only related to the environmental salinity that the eggs are in, but also the salinity that the mother is in prior to spawning which affects the neutral buoyancy salinity of the eggs. In spotted seatrout (*Cynoscion nebulsus*), the neutral buoyancy salinity of eggs was affected by the salinity that adults were maintained in prior to spawning. As spawning salinity increased, so too did neutral buoyancy salinity as a result of changes in ovarian fluid osmolarity (Kucera *et al.*, 2002). They hypothesised that fish were adapted to the salinity conditions of the particular estuaries in which they were residents. Throughout this study, black bream broodstock were maintained in full strength seawater (35-39 ppt) and the eggs produced from those fish were positively buoyant at that salinity. This may explain why embryos generally performed best in full strength seawater (compared to other salinities), despite some other studies suggesting that the optimal salinities for black bream embryos are 11-18 ppt (Butcher, 1945) or 19-22 ppt (Ramm, 1983; Longmore *et al.*, 1990). In a manual describing hatchery techniques for the production of black bream, it is recommended that both broodstock and eggs are incubated in full strength seawater (Partridge *et al.,* 2003). Maintaining broodstock in different salinities, then observing the buoyancy and survival of eggs produced from such fish would help explain this hypothesis. Unfortunately, the limited number of broodstock fish available for this study prevented such an experiment being conducted.

Changes in salinity occur commonly in estuarine environments, and with altered environmental flows, as predicted by climate change, salinity and stratification patterns are likely to change. This will affect black bream early life stages directly through reduced hatch rates and lower survival, but also indirectly through the effect of salinity on egg buoyancy and vertical position of eggs in the water column.

5.4.1.3 Effects of temperature

Increased or decreased temperature resulted in significant differences in the time to hatching for black bream embryos, as well as influencing embryo and larval survival, hatch rates and rates of deformities. Low temperatures delayed hatching, and when occurring in combination with hypoxia or low salinity (or both), greatly reduced hatching and larval survival as well as increasing the rates of deformities. By delaying hatching, the potential risk of predation would be increased, as well as the length of time for which eggs would be exposed to unfavourable environmental conditions. At high temperatures accelerated hatching was observed, and in combination with increased salinity or hypoxia (or both) reduced hatch rates and larval survival as well as increased rates of deformities were observed. Again, the interactive effects of temperature, hypoxia and salinity are emphasized.

Temperature is an important factor governing survival of the early life stages and effects may be observed by both increased and decreased levels above and below the optimal temperature range for the species being tested, with effects such as reduced hatch rates, altered hatching times and increased rates of skeletal deformities being reported (Afonso

et al., 2000; Boglione *et al.*, 2003). Van der Wal (1985) reported shorter incubation times (faster hatching) and increased deformities (eg. curvature of the spine, mouth deformities) when Australian bass (*Macquaria novemaculeata*) were hatched at 24^{o}C as compared to larvae hatched at 16-20^{o}C. Furthermore, at a temperature of 12^{o}C, incubation times were prolonged and survival to 7 days post hatch was reduced. In greenback flounder (*Rhombosolea tapirina*), Hart and Purser (1995) found that reduced temperatures resulted in lowered or even no hatching and likewise at the elevated temperature of 18^{o}C, very little hatching occurred. Other observations included decreased time to hatching as temperature increased and higher rates of deformity at both low and high temperatures. In the current study, increased temperatures resulted in lower black bream embryo survival, faster hatching and increased rates of deformity.

Changes in temperature are predicted by climate change, thus estuarine environments are likely to experience even greater variation than currently occurs. Clearly this will have negative impacts on early life stage black bream and probably many other species with similar early life histories.

5.4.1.4 Effects of multiple stressors

It has been clearly demonstrated in this study that black bream embryos are less capable of tolerating non-optimal water quality conditions when they are affected by multiple stressors. When the embryos were exposed to hypoxia only, they were able to hatch successfully at 55%S. However, if the embryos were exposed to both hypoxia and low salinity, the hatch rates were much reduced (relative to controls), and furthermore, if they

were exposed to hypoxia, low salinity and low temperature very low hatch rates (< 10%) were observed. A similar trend was observed when embryos were exposed to hypoxia, low salinity and high temperature, and likewise, when embryos were exposed to both hypoxia and high salinity, hatch rates were always lower at the higher salinity (relative to controls). Thus, it is important to consider multiple environmental stressors when trying to predict effects in aquatic environments, especially in dynamic environments such as estuaries.

Exposure conditions in estuarine environments may also change for species that exhibit diel variation in vertical position in the water column. During daylight hours, species may move down, into deeper, colder water to escape exposure to UV light, and up, into shallower, warmer water at night time for feeding. For embryos, which are non-mobile, no such movement is possible, thus their movement is entirely due to egg buoyancy. Exposure to constant light was reported to increase the time to hatching of red seabream eggs (Foscarini, 1988), whilst in the current study, exposure to constant light affected one day embryo survival, and in combination with low salinity, no eggs hatched under constant light, whilst some did in dark conditions. The negative effects of light on embryos may explain why black bream, like many other marine and estuarine fish species, spawn at dusk when light exposure is low. This finding warrants further investigation.

In conclusion, the results in this chapter have shown that black bream require high DO levels during the early life stages, and exposure to both severe and moderate hypoxia drastically reduces embryo survival and hatching, increases rates of deformity (and hence affects larval size) and reduces survival of yolk-sac larvae. In addition, both salinity and temperature have effects on early life-stage black bream, and both stressors can modify the effects of DO, making the organisms less tolerant to hypoxia when they are already experiencing osmotic or thermal stress.

The variable recruitment observed within the Gippsland Lakes black bream population is probably a reflection of the changing nature of that environment and therefore knowledge of environmental conditions suitable for successful spawning is a key issue to consider in the long term management of this important natural resource.

CHAPTER 6: DEVELOPMENT OF AN ASSAY TO MEASURE THE YOLK SYNTHESIS PROTEIN, VITELLOGENIN IN ADULT BLACK BREAM.

Sections of this chapter have been published.

Published article:

Codi King, S, ***Hassell, K.L.***, Nugegoda D. and Kristiansen, S. I. (2008). The assessment of vitellogenin as a biomarker of exposure to estrogenic compounds in two Australian Perciformes. *Marine Environmental Research*, 66, 116-118.

6.1 Introduction

Vitellogenesis is the major growth phase during egg development in female fishes, whereby yolk proteins are synthesized and incorporated into developing oocytes (Hoar, 1969; Tyler *et al.*, 1991; Jobling, 1995). Vitellogenin (Vtg) is a complex phospholipoglycoprotein, and the size and structure varies amongst different fish species (Specker and Sullivan, 1994; Denslow *et al.*, 1999; Watts *et al.*, 2003). Vitellogenin is produced in the liver in response to increasing estrogen production in the ovaries and provides a useful indicator of reproductive condition in fish (see section 1.2.3.2).

In the previous chapter, it was demonstrated that hypoxia has a major effect on survival in the early life stages of black bream. Recently, hypoxia has also been linked to reduced reproductive capacity, through the influence that low DO has on sex steroid synthesis, gonad development as well as on the production of Vtg (see section 1.1.5). Understanding variability in egg production and vitellogenesis processes is central to

understanding reproductive success and ultimately fish recruitment. A number of factors are known to influence vitellogenesis, in particular environmental cues, nutrition and stress (Jobling, 1995; Iwama *et al.*, 1997; Schreck *et al*., 2001). Thus, measuring the levels of Vtg circulating in the bloodstream provides a useful indicator of reproductive condition in female fishes.

In addition to using Vtg as a biomarker of reproductive condition, it has also been successfully utilised in studies of endocrine disruption. The hepatic estrogen receptors that are responsible for Vtg production are found in both female and male fish, so if Vtg is present in males or immature females, it provides a good indication of exposure to estrogenic compounds (Denslow *et al.*, 1999; Kime *et al.*, 1999; Moncaut *et al*., 2003). The quantification of Vtg levels in plasma has been used as a biomarker of endocrine disruption in several studies (Sumpter and Jobling, 1995; Denslow *et al.*, 1999; Mills and Chichester, 2005).

Various assays for detecting Vtg have been developed, including alkaline-labile phosphorous (ALP) (Emmersen and Petersen, 1976; Kramer *et al.*, 1998), radio-immunoassay (RIA) (Sumpter *et al.*, 1984; Norberg and Haux, 1988; Tyler *et al.*, 1991), enzyme-linked immunosorbent assay (ELISA) (Kishida *et al.*, 1992; Heppell *et al.*, 1995; Denslow *et al.*, 1999), immuno-agglutination (Le Bail and Breton, 1981) and mass spectrometry techniques (Wunschel *et al.*, 2005; Cohen *et al.*, 2006). Of these techniques, the immunoassays are most commonly used, since large numbers of samples can be processed rapidly, and high precision and sensitivity can be achieved. ELISAs are

the preferred immunoassay technique, since they offer similar precision and sensitivity to RIAs, without the complications involved with handling hazardous radioisotopes (Crowther, 1995).

Whilst measuring Vtg levels in blood plasma is an accepted and widely used technique for assessing reproductive condition or endocrine disruption, it is also an invasive technique that may result in stress or discomfort for the fish, as well as potentially introducing pathogens (Blythe *et al.* 1994). A potential alternative biological fluid for measuring Vtg levels is surface mucus. Vitellogenin detection, for the purpose of sex identification was first described in fish mucus by Gordon *et al.* (1984). Fish surface mucus is secreted via mucous cells in the superficial epidermis and functions to assist movement and act as a protective barrier against physical injury and pathogens (Lebedeva, 1999). Mucus can be collected easily and repeatedly from fish with minimal discomfort and rapid handling (Schultz *et al.*, 2007). Furthermore, it does not require the same level of skill as needed to collect blood, and is a less harmful process minimising injury or stress to the fish so for ethical reasons, it is more benign.

In this chapter the development of immunological techniques for measuring black bream Vtg are described for the first time. Specifically, the aim was to utilise a commercially available anti-Vtg primary antibody in order to develop an indirect ELISA and Western blot assay that could be used to measure reproductive condition in females and screen for exposure to estrogenic endocrine disrupting chemicals in males. In order to achieve this, the Vtg protein needed to be identified and Vtg synthesis was confirmed in adult black

bream by exposing fish to a potent Vtg inducer - 17β-estradiol (E2), by intraperitoneal (i.p.) injection. Vitellogenin levels were then measured quantitatively in plasma and semi-quantitatively in surface mucus of both male and female black bream.

6.2 Materials and Methods

Two different immunological techniques were utilised for Vtg assay development and optimisation in this study; indirect enzyme-linked immunosorbent assay (ELISA), and Western blot.

6.2.1 Vitellogenin induction following exposure to 17β-estradiol (E2)

To induce Vtg synthesis, male and female black bream were anaesthetised (see section 2.2.4) then i.p. injected with a high-dose (5 mg/kg body weight) of the potent estrogen, E2, dissolved in corn oil (E2-Treatment). A 10 mg/ml solution of E2 was prepared by dissolving 100 mg E2 (Sigma-Aldrich, Australia) in 10 ml analytical grade corn oil (Sigma Aldrich, Australia). In order for the E2 to dissolve completely, the corn oil was warmed to approximately 40^{o}C. Once dissolved, the prepared solution was wrapped in foil and stored at room temperature. For the procedural controls, male and female black bream were anaesthetised then i.p. injected with an equivalent volume of corn oil only (Control-Treatment).

6.2.1.1 Fish used for vitellogenin assay development (determination of a suitable primary antibody)

Lake Tyers black bream (see section 2.2.2) were used for the first experiment on Vtg induction (to determine a suitable primary antibody) and received two doses of E2 or corn oil, given 7 days apart. Blood was collected at the beginning, after 7 days and after 15 days.

6.2.1.2 Fish used for quantitative vitellogenin measurement in plasma and semi-quantitative vitellogenin measurement in mucus

Gippsland Lakes black bream were used for the second Vtg induction experiment (see section 2.2.2) and received a single dose of E2 or corn oil. Blood was collected at the beginning and after 4 days. When broodstock were selected for this induction experiment, all attempts were made to keep the size ranges similar as well as the numbers of males and females that received either E2 or corn oil. However, since black bream are not sexually dimorphic, some fish that were presumed to be female (based on a lack of milt) turned out to be males, thus affecting the sex ratios used in this experiment.

6.2.2 Blood and mucus collection

Blood was collected from the caudal vein using heparinised syringes (see section 2.4.1). The plasma was separated by centrifugation at 5000 rpm for 10 min at 4^{o}C, then snap frozen in liquid nitrogen, and later stored at -80^{o}C until analysis. Surface mucus was collected from the body surface between the lateral line and dorsal fin base using individual pieces of polyurethane sponge (2.5 cm^{3}) (Clark Rubber, Australia). Sponges containing absorbed surface mucus were placed directly into 1.5 ml microcentrifuge tubes and snap frozen in liquid nitrogen, then later stored at -80^{o}C until analysis. Immediately prior to analysis, mucus absorbed sponges were thawed on ice and centrifuged for 5 min at 2000 rpm. The sponge was then removed and the mucus collected for analysis. The volume of mucus collected from each fish ranged from 20-50 µl.

6.2.3 Vitellogenin primary antibody selection

Plasma samples from six E2-treated male Lake Tyers black bream were shipped on dry ice to Biosense Laboratories in Norway where cross-reactivity was tested against a range of different antibodies at various dilutions. The anti-Vtg primary antibodies tested were: *Morone saxitilis* (Perciformes) (ND-1C8); *Morone saxitilis* (Perciformes) (ND-3G2); *Sparus aurata* (Perciformes) (PO-2); *Gadus morhua* (Gadiformes) (CS-1); *Scophthalmus maximus* (Pleuronectiformes) (CS-2); and *Anarhichas lupus* (Perciformes) (CS-3). Two different *Morone saxitilis* anti-Vtg monoclonal antibodies are commercially available, and each recognizes a different epitope of the Vtg protein. ND-1C8 binds with intact Vtg, whilst ND-3G2 binds with both intact and degraded Vtg, from a range of fish species (Kristiansen, S., pers. comm.). Indirect ELISA and Western blot assays were conducted in Norway according to the standard operating procedure described by Biosense Laboratories (http://www.biosense.com/docs/Biomarker_ELISA_protocol.pdf) (Kristiansen, S. and Eidem, J., pers. comm.).

6.2.4 Indirect ELISA

6.2.4.1 Sample preparation

Initially, samples were thawed on ice and diluted 1:10 (control plasma) or 1:100 (E2-treated plasma) in coating buffer (50 mM carbonate/bicarbonate, pH 9.6) (Sigma-Aldrich) then applied to different types of microplates (see section 6.2.6.2). For all ELISAs, a series of wells were incubated with coating buffer only (no plasma or mucus) and were used to determine the non-specific binding (NSB) for each microplate. In order to determine the optimal plasma dilution, to provide a good titration with low background

absorbance (Crowther, 1995), serial dilutions of plasma were tested (1:100 to 1:1000000). Both positive (POS plasma) and negative (NEG plasma) control samples were prepared by compositing plasma from 5 individual males. The positive controls were plasma samples taken from five male fish after E2-treatment, whilst the negative controls were plasma samples taken from five male fish prior to any treatment. The optimal dilution was determined to be 1:100000, and hence this dilution was used for all subsequent plasma samples.

To determine the optimal mucus dilution, serial dilutions of mucus were tested (1:100 to 1:1000000). Both positive (POS mucus) and negative (NEG mucus) control samples were prepared by compositing mucus from 4 individual males. The positive controls were mucus samples taken from four male fish after E2-treatment, whilst the negative controls were mucus samples taken from four male fish after corn oil treatment. The optimal dilution was determined to be 1:12500, and hence this dilution was used for all subsequent mucus samples.

6.2.4.2 Microplate selection

Ninety-six well, flat-bottomed microplates were used for all ELISA procedures, and three different types were tested for suitability: Sarstedt polystyrene microplates (Sigma-Aldrich, Australia); Nunc MaxiSorp high binding microplates (Sigma-Aldrich, Australia); and Immulon 4HBX high binding microplates (Pathtech, Australia).

Sarstedt polystyrene microplates displayed much higher background, or non-specific binding, compared to Immulon 4HBX high binding microplates (results not shown).

Nunc MaxiSorp high binding microplates displayed generally similar background absorbance to the Immulon 4HBX high binding microplates, however in some samples the background absorbance was much higher. Based on these results, Immulon 4HBX high binding microplates were selected for use in all subsequent ELISAs.

6.2.4.3 Primary and secondary antibody dilutions

Primary antibody (ND-1C8) and secondary antibody (goat anti-mouse IgG horseradish peroxidase conjugate) (GAM-HRP) (BioRad, Australia) dilutions used were based on manufacturers recommendations. The dilutions used were 1:1000 for ND-1C8 and 1:2000 for GAM-HRP. These antibody dilutions were used for all subsequent samples.

6.2.4.4 Standard ELISA procedure

Plasma (1:100000) or mucus (1:12500) samples were diluted in coating buffer (50 mM carbonate/bicarbonate, pH 9.6) (Sigma-Aldrich, Australia) then 100 µl of each diluted sample was applied to a 96-well Immulon 4HBX high binding microplate and incubated overnight at 4^{o}C. The plates were then washed three times with 200 µl PBS-Tween (10 mM phosphate buffered saline, pH 7.4, 150 mM NaCl, 0.05% Tween 20) (Sigma-Aldrich, Australia) then blocked with 200 µl 2% bovine serum albumin (BSA) in PBS (Sigma-Aldrich, Australia) for 1 hour. The plates were then washed again three times with 200 µl PBS-Tween then 100 µl of the primary antibody (ND-1C8) was applied, diluted (plasma - 1:1000, mucus - 1:500) in 1% BSA in PBS. The plates were incubated for 60 min at 37^{o}C then washed again three times with 200 µl PBS-Tween then 100 µl of the secondary antibody (goat anti-mouse IgG horseradish peroxidase conjugate) (GAM-

HRP) (BioRad, Australia), diluted (1:2000) in 1% BSA in PBS was applied to each well and incubated for 60 min at 37^{o}C. The plates were washed a final time with five washes of 200 µl PBS-Tween then developed with 100 µl 0.04% *o*-phenylenediamine dihydrochloride (OPD) in 0.012% hydrogen peroxide (H_2O_2) (Sigma-Aldrich, Australia). The colour reaction was allowed to develop for exactly 12 min before being stopped by the addition of 50 µl 4 N sulphuric acid (H_2SO_4) (Merck, Australia), then the absorbance was measured at 492 nm using a Thermo Multiskan Ascent plate reader (Pathtech, Australia).

6.2.4.5 Vitellogenin standard curve development

In order to obtain quantitative ELISA Vtg results, it was necessary to develop a standard curve and two different purified Vtg standards were tested. Purified Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) Vtg were purchased from Biosense Laboratories and standard curves in the range of 0.2-10 µg/ml were tested. Rainbow trout (RT) Vtg displayed much better binding than Atlantic salmon Vtg, and subsequently, a series of RT Vtg standards were incorporated into each plate using concentrations in the linear range of the curve, which was between 0.2-0.8 µg/ml. All results are presented as RT Vtg equivalent concentrations (RT-VtgEq), in mg/ml (corrected for dilution).

6.2.4.6 Intra-assay and inter-assay variability

Intra-assay variability and inter-assay variability (%CV) was measured from aliquots of positive control plasma (1:100000), measured on a single 96-well plate (*n=6*), or on separate 96-well plates (*n=9*) respectively.

6.2.5 Denaturing electrophoresis

6.2.5.1 Sample preparation

Various dilutions of thawed plasma samples were tested initially to determine appropriate sample concentrations for electrophoresis. The plasma was diluted (neat (1:0) to 1:100) with Milli Q water, then further diluted 1:2 in Laemmli Sample Buffer (62.5 mM Tris-HCl, 25% glycerol, 2% SDS, 0.01% Bromophenol Blue, pH 6.8 containing 2-mercaptoethanol) (BioRad, Australia) and heated for 5 min at 95°C before being loaded onto the stacking gel. The final concentrations of plasma that were used for Coomassie total protein staining were 10 µg protein/lane, whilst for samples that were transferred to membranes (for Western blot) the final concentrations used were 1 µg protein/lane. Surface mucus samples were loaded at a concentration of 10 µg protein/lane for both Coomassie total protein staining and Western blot analyses.

6.2.5.2 Polyacrylamide gel selection

Three different types of pre-cast Tris-HCl polyacrylamide gels (BioRad, Australia) were tested: 5% and 7.5% single percentage gels and 4-15% gradient gels. Gels of these compositions resolve proteins in the range of ~20-250 kDa. Gel selection was based on the expected size of subunit (or denatured) black bream Vtg (100-200 kDa), which was

based on the reported size of subunit Vtg in other sparid fishes: *Pagrus pagrus*, 189 kDa (Kokokiris *et al.*, 2001); *Dentex dentex*, 170 kDa (Pavlidis *et al.*, 2000); and *Sparus aurata*, 180 kDa (Mosconi *et al.*, 1998). The 7.5% single percentage gels resolved the bands most clearly and were thus used for all subsequent polyacrylamide electrophoresis.

6.2.5.3 Electrophoresis conditions

Denatured plasma samples were loaded onto gels and run at 200V for 45 min in electrophoresis buffer (25 mM Tris, 192mM glycine and 0.1% w/v SDS at pH 8.3) (Laemmli, 1970) in a Mini-Protean III Electrophoresis system (BioRad, Australia). The running conditions were subsequently changed to 150V for 60 min to reduce band patterns curving upwards at each side of the gels due to excessive heat production. Following electrophoresis, the resolved gels were either placed in Coomassie blue for total protein staining, or transferred to membranes for Western blot analysis.

6.2.6 Coomassie blue total protein staining

Gels were placed in Coomassie blue total protein staining solution (0.1% Coomassie Brilliant Blue R-250, 25% methanol, 7.5% glacial acetic acid, 67.5% Milli Q water) for 15 min, then transferred to destaining solution (12.5% methanol, 7.5% glacial acetic acid, 80.5% Milli Q water) for 2-3 hours. Unstained molecular weight markers were included on each gel, to allow for the accurate determination of the molecular weight of black bream Vtg. The two different unstained molecular weight markers that were used were Mark12® Unstained Standard (Invitrogen, Australia) and BenchMark® Protein Ladder (Invitrogen, Australia), loaded at a volume of 5 µl. The molecular weight of black bream

Vtg was interpolated from a graph that was generated by measuring the migration distances of eight known molecular weight standards (BenchMark® Protein Ladder) after denaturing electrophoresis. The relative migration distance (Rf value) was plotted against the log molecular weight of the standards, and the molecular weight of black bream Vtg was determined based on the migration distance.

6.2.7 Western blot

6.2.7.1 Western blot membrane selection

Two different types of blotting membranes were tested to determine which one provided the best transfer efficiency: nitrocellulose (0.45 μm) or polyvinylidene difluoride (Immun-Blot PVDF) (0.20 μm) (BioRad, Australia). Immediately prior to use, the membranes were saturated in methanol for 1 min, followed by 5 min in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol). The PVDF membranes transferred samples from electrophoresed gels with better intensity and band resolution compared to nitrocellulose membranes, so all subsequent Western blots were done using PVDF membranes.

6.2.7.2 Western blot transfer conditions

Following electrophoresis, gels were placed in Towbin transfer buffer for at least 5 min to remove salts and other reactive components from the electrophoresis buffer, then resolved proteins (from the gels) were transferred to the blotting membranes at 100V for 60 min in Towbin transfer buffer in a Mini Trans-Blot Electrophoretic transfer cell (BioRad, Australia).

6.2.7.3 Western blot immunodetection

Blotting membranes were developed using the WesternBreeze® Chromogenic immunodetection kit (Invitrogen, Australia), with goat anti-mouse alkaline phosphatase reagents. The blotting membranes were incubated with the same monoclonal antibody (ND-1C8) as used for ELISA at a dilution of 1:2000. A pre-stained molecular weight marker, SeeBlue®Plus2 Pre-Stained Standard (Invitrogen, Australia) was included on each gel, loaded at a volume of 3 µl, to allow for molecular weight estimates and determination of transfer efficiency.

6.2.8 Protein determination

Plasma and mucus total protein levels were determined using the DC Protein Assay Kit (BioRad, Australia) based upon the method of Lowry *et al.* (1951). A protein standard curve was prepared using BSA dissolved in Milli Q water, ranging from 0 to 2.0 mg/ml. Absorbance was measured at 650 nm or 690 nm using a Thermo Ascent Multiskan plate reader.

6.3 Results

6.3.1 Biological data for black bream used for vitellogenin induction experiment

For the Gippsland Lakes black bream used in the second Vtg induction experiment, significant differences were observed in fork length, weight, plasma protein levels and GSI between control and E2-treatment groups ($p < 0.05$), as well as between male and female fish in the control group ($p < 0.05$). The females were larger than the males. For the fish that received E2-treatement, there were no significant differences in any biological characteristics between males and females ($p > 0.05$) (Table 6.1).

Table 6.1 Mean (± SEM) biological characteristics and physiological indices, quantitative plasma vitellogenin (RT-VtgEq-mg/ml), plasma protein (mg/ml) and mucus protein (mg/ml) levels in adult Gippsland Lakes black bream, following i.p. injection of 5 mg/kg body weight 17β-estradiol (E2-treated) or corn oil (Control).

Treatment	*n*=	Fork Length (mm)	Weight (g)	GSI (%)	LSI (%)	K (%)	Age (years)	RT-VtgEq (mg/ml)	Plasma protein (mg/ml)	Mucus protein (mg/ml)
Control										
Female	*3*	326.7 ± 38.9	731.0 ± 255.6	3.92 ± 1.64	1.58 ± 0.06	2.00 ± 0.04	8.7 ± 2.9	7.77 ± 2.75	78.1 ± 28.8	42.30 ± 3.83
Male	*4*	241.3 ± 4.33	335.3 ± 29.9	1.57 ± 0.54	1.68 ± 0.21	2.03 ± 0.09	6.0 ± 0.0	**b.d.*	44.1 ± 14.6	52.53 ± 15.8
E2-treated										
Female	*4*	280.0 ± 12.5	433.3 ± 58.3	3.98 ± 2.83	2.33 ± 0.30	1.95 ± 0.01	6.3 ± 0.7	99.5 ± 3.36	127.6 ± 33.0	48.07 ± 5.29 *(n=3)*
Male	*7*	280.0 ± 6.77	414.0 ± 49.3	2.13 ± 1.19	1.91 ± 0.13	2.09 ± 0.10	6.9 ± 0.6	56.5 ± 11.0	101.9 ± 10.8	38.32 ± 5.71 *(n=5)*

**b.d. = below detection*

6.3.2 Vitellogenin primary antibody selection

Titration curves for black bream plasma Vtg (conducted in Norway by Biosense Laboratories staff) revealed good binding affinity with all the antibodies that were tested, whilst the highest binding affinity to a monoclonal antibody was with ND-1C8, and for a polyclonal antibody was with PO-2 (Fig. 6.1).

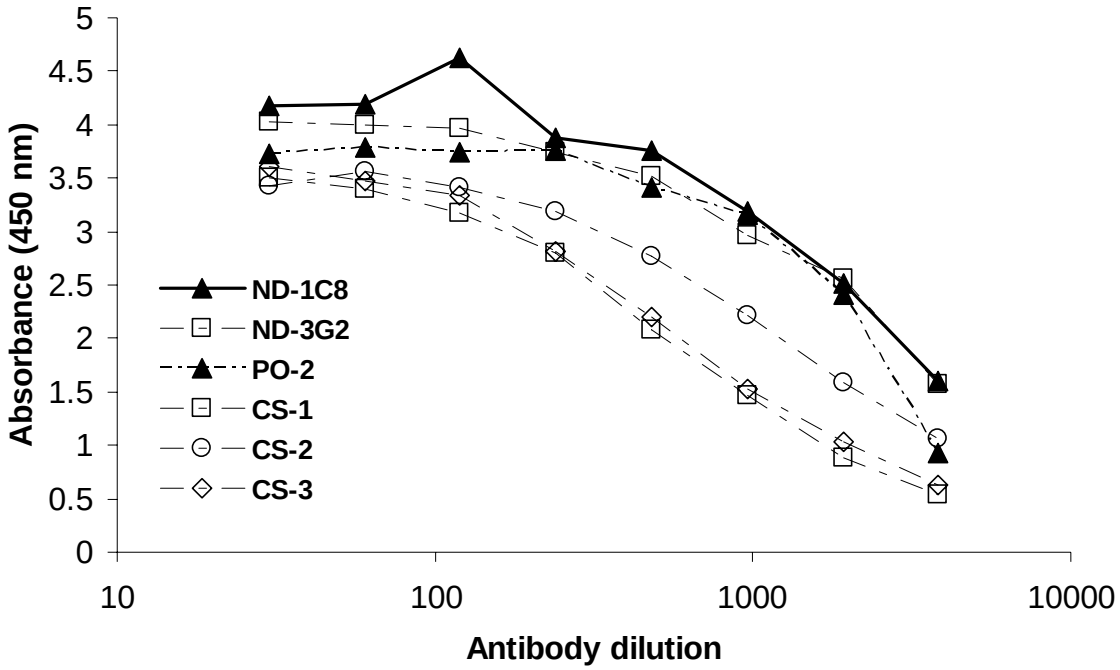


Figure 6.1 Titration curves for E2-treated black bream plasma, tested against different fish species' anti-Vtg primary antibodies using an indirect ELISA. The anti-Vtg primary antibodies tested were (monoclonal): *Morone saxitilis* (ND-1C8); *Morone saxitilis* (ND-3G2) and (polyclonal): *Sparus aurata* (PO-2); *Gadus morhua* (CS-1); *Scophthalmus maximus* (CS-2); and *Anarhichas lupus* (CS-3).

6.3.3 Vitellogenin assay development and optimisation

6.3.3.1 Sample preparation

The optimal dilution of E2-treated black bream plasma (pooled composite sample from 5 individual males), was determined to be 1:100000, which had a relative binding of ~ 60%. At lower dilutions (1:50000 – 1:100) the binding response was not in the linear part of the dilution curve (Fig. 6.2). Plasma samples from individual male and female black bream displayed similar dilution curves, with male plasma and negative control plasma (pooled composite sample of untreated male plasma) showing no binding regardless of sample dilution (Fig 6.3). The optimal dilution of E2-treated black bream mucus (pooled composite sample from 4 individual males), was determined to be 1:12500, which had a relative binding of ~ 70%. At lower dilutions (1:5000 – 1:100) the binding response was not in the linear part of the dilution curve (Fig. 6.4).

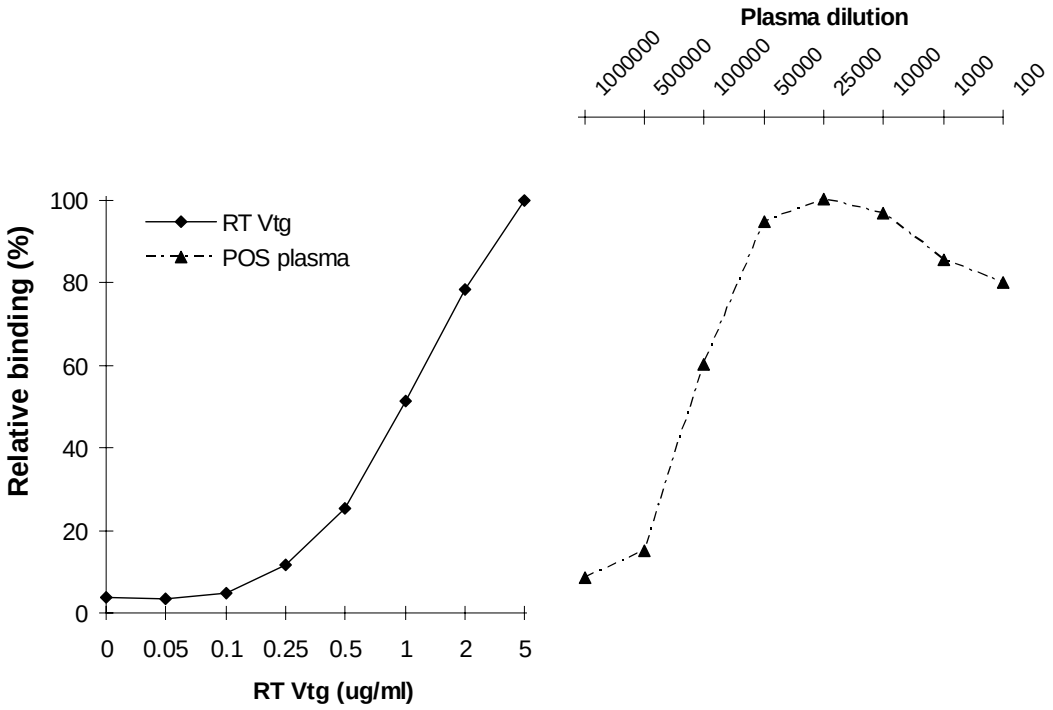


Figure 6.2 Plasma dilution curve for black bream, following i.p. injection of 5 mg/kg 17β-estradiol (POS plasma), compared to rainbow trout Vtg (RT Vtg) curve (µg/ml).

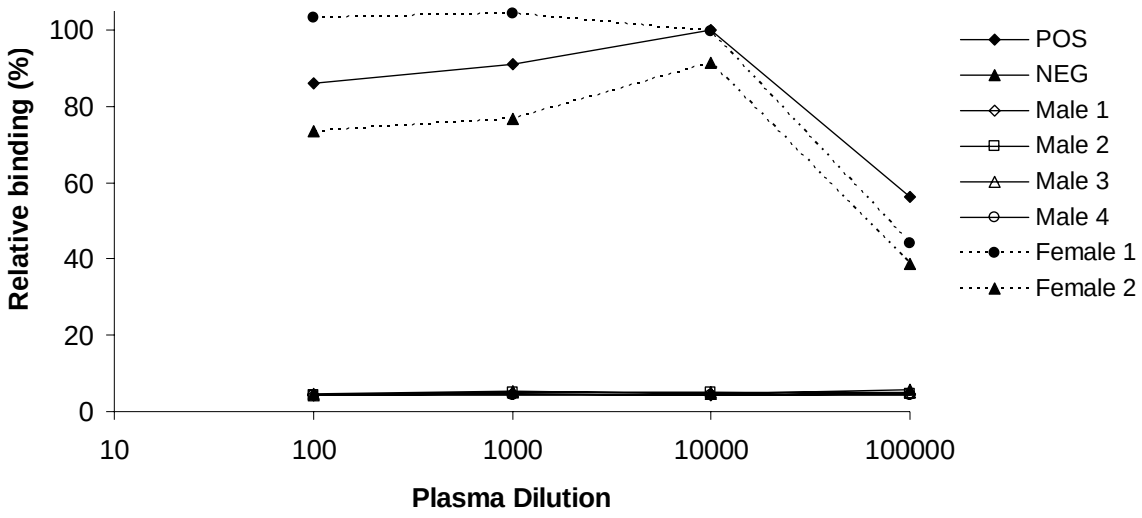


Figure 6.3 Plasma dilution curves for six individual black bream, compared to E2-treated male black bream (POS) plasma and non-treated male black bream (NEG) plasma.

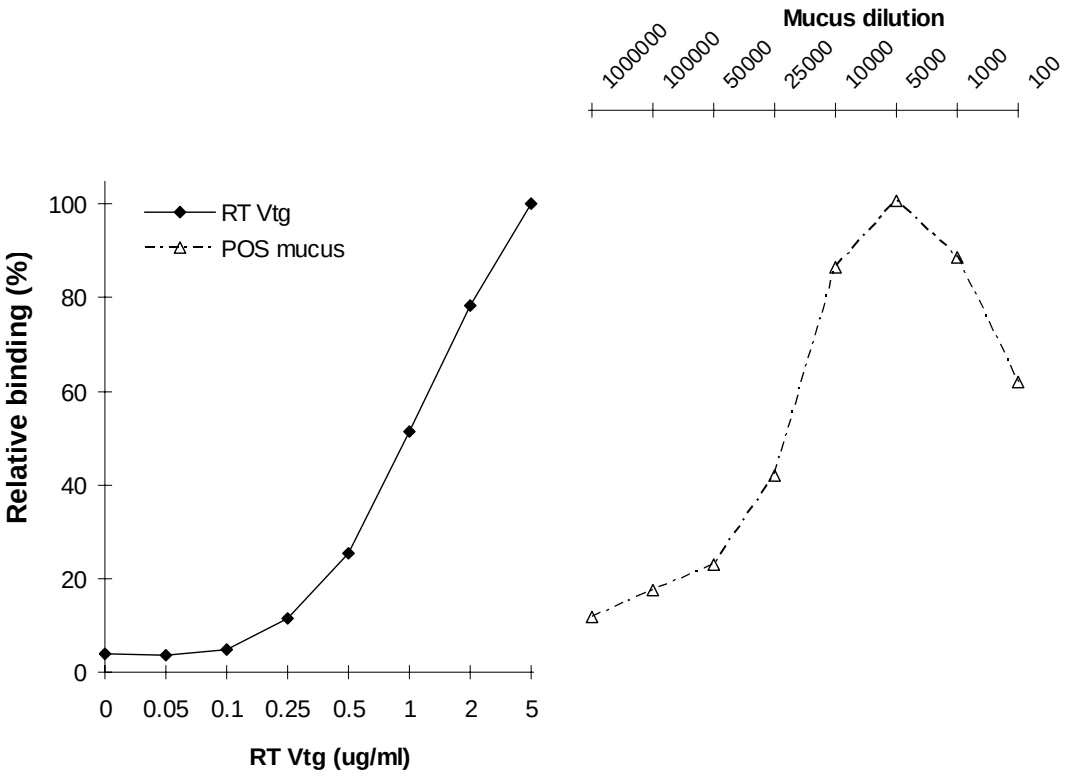


Figure 6.4 Surface mucus dilution curve for black bream, following i.p. injection of 5 mg/kg 17β-estradiol (POS mucus), compared to rainbow trout Vtg (RT Vtg) curve (µg/ml).

6.3.3.2 Vitellogenin standard curve development

The linear range for the four point rainbow trout Vtg (RT Vtg) standard curve was between 0-0.8 µg/ml, and is presented as a rainbow trout Vtg equivalent concentration (RT-VtgEq) (Fig. 6.5). Final concentrations are expressed as mg/ml, to account for dilution. Using linear regression, the goodness of fit could be determined, and is represented by the coefficient of determination (R^2 value). The mean R^2 value for standard curves on 9 separate microplates was 0.988. The range of values was 0.960-0.999.

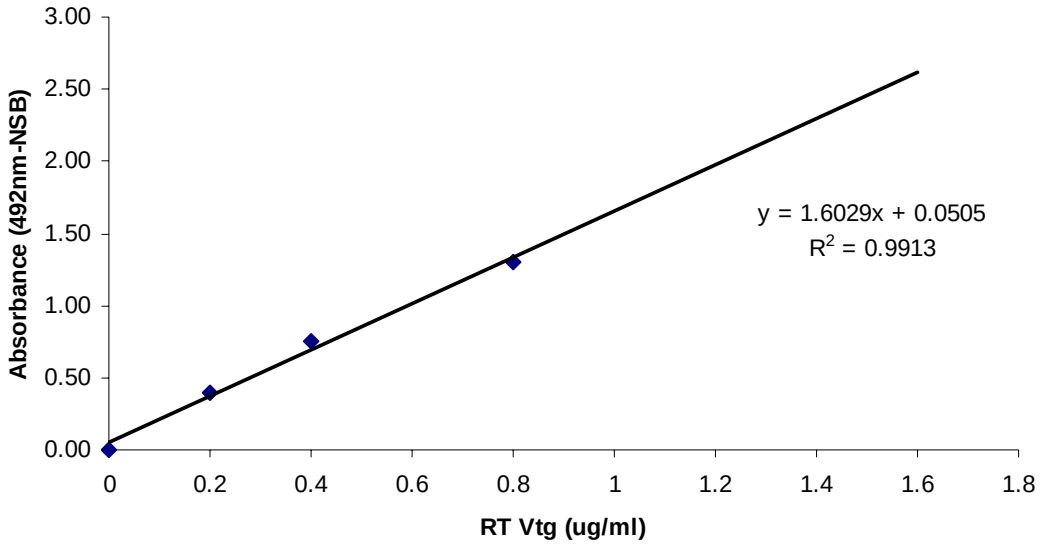


Figure 6.5 Representative standard curve used to determine the Vtg concentration of black bream plasma and surface mucus samples. Rainbow trout Vtg (RT Vtg) was used to generate four standards and are reported as rainbow trout Vtg equivalent concentrations (RT-VtgEq).

6.3.4 Quantitative vitellogenin measurement in black bream plasma and semi-quantitative vitellogenin measurement in black bream mucus

Significant Vtg induction, measured as rainbow trout Vtg equivalent concentrations (RT-VtgEq-mg/ml) in a quantitative ELISA, was observed in all E2-treated fish compared to controls ($p < 0.001$) (Fig. 6.6). Furthermore, there was a significant difference between female and male black bream Vtg induction following E2 treatment ($p = 0.034$), with females exhibiting higher levels than males. For the mucus samples of the same fish, Vtg induction, measured as absorbance (-NSB) in a semi-quantitative ELISA was also detected, and there were no significant differences ($p > 0.05$) between corn oil and E2-treated fish (Fig. 6.7). Low sample volumes (< 50 µl) restricted ELISA work with black

bream mucus, which is why there are no quantitative ELISA results for the mucus samples.

6.3.5 Intra-assay and inter-assay variability

The intra-assay variability ranged between 4.84-10.9% (*n=6*), and the inter-assay variability for nine separate plates was 9.26% (*n=9*).

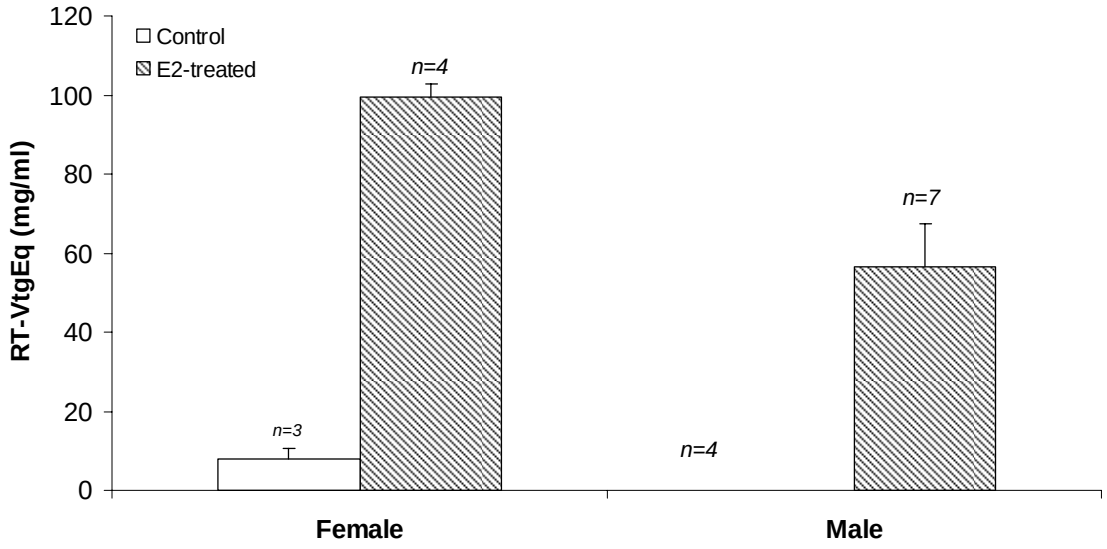


Figure 6.6 Quantitative Vtg levels (mean ± SEM), reported as rainbow trout Vtg equivalent concentrations (RT-VtgEq-mg/ml) in female and male black bream plasma following i.p. injection of corn oil (Control) or 5 mg/kg 17β-estradiol (E2-treated).

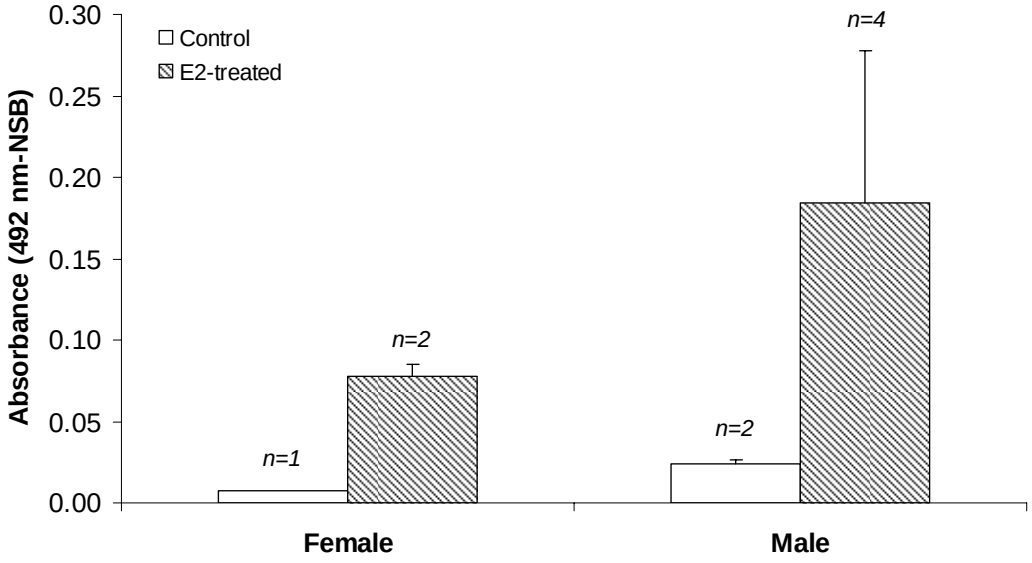


Figure 6.7 Semi-quantitative Vtg levels (mean ± SD), reported as non-specific binding (NSB) corrected absorbance values (492nm) in female and male black bream surface mucus following i.p. injection of corn oil (Control) or 5 mg/kg 17β-estradiol (E2-treated).

6.3.5.1 Denaturing electrophoresis

Four dilutions of both male and female black bream plasma were initially tested and it was determined that a dilution between 1:30-1:50 provided a plasma concentration that was easily observed on the gel (Fig 6.8). For subsequent gels, plasma was loaded based on protein concentration to allow for direct comparisons between samples and for consistency in sample preparation. The final concentrations of plasma used for Coomassie total protein staining were 10 µg protein/lane (Fig 6.9), whilst for samples transferred to membranes (for Western blot) the final concentrations were 1 µg protein/lane (Fig 6.10). Surface mucus samples were loaded at a concentration of 10 µg protein/lane for both Coomassie total protein staining and Western blot analyses.

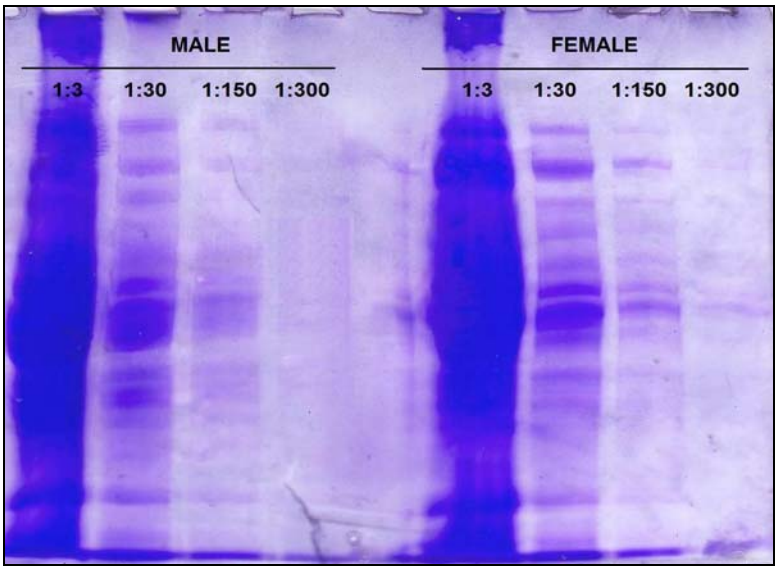


Figure 6.8 Coomassie blue stained polyacrylamide gel showing different dilutions of male and female black bream plasma prior to any injection.

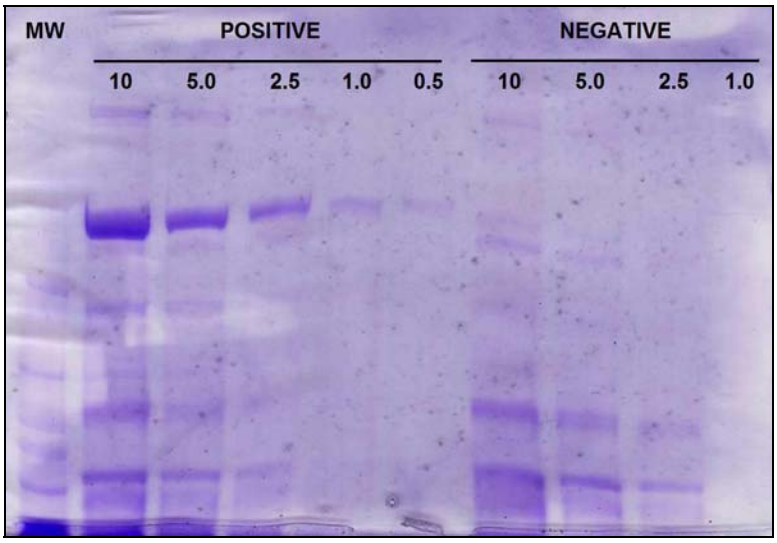


Figure 6.9 Coomassie blue stained polyacrylamide gel showing different concentrations (µg protein/well) of positive (E2-treated) and negative (non-treated) male black bream plasma. BenchMark® Protein Ladder (MW) was used to determine molecular weight.

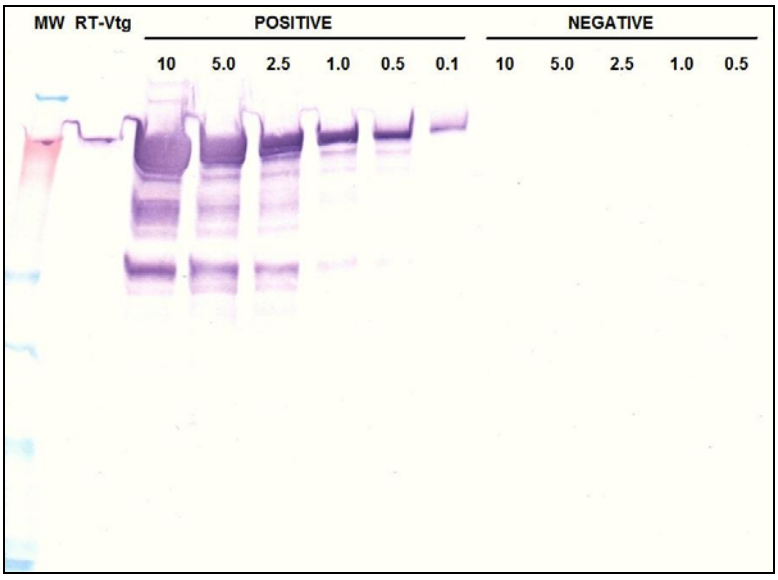
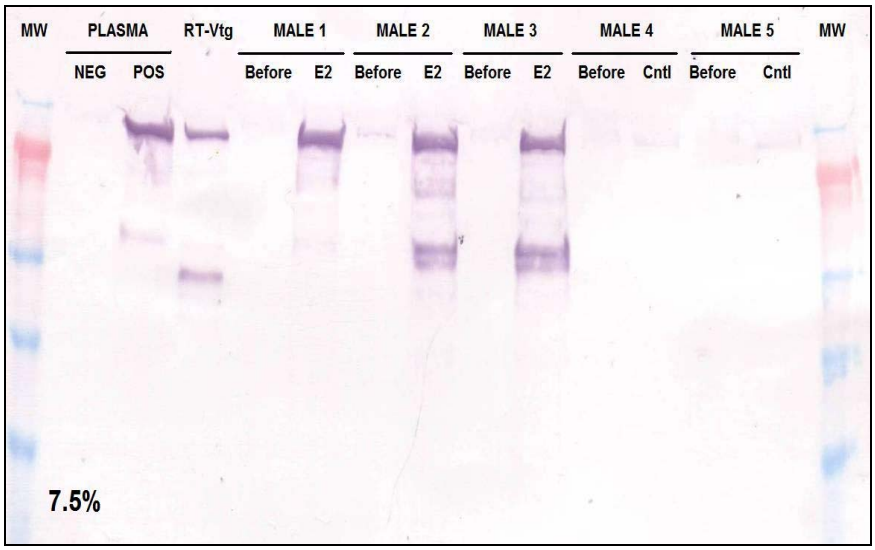


Figure 6.10 Western blot showing different concentrations (µg protein/well) of positive (E2-treated) and negative (non-treated) male black bream plasma. SeeBlue®Plus2 Pre-Stained Standard (MW) was included to allow for molecular weight estimates and determination of transfer efficiency.

6.3.5.2 Polyacrylamide gel selection

The three different types of pre-cast polyacrylamide gels displayed different separation profiles. Both the 7.5% single percentage and 4-15% gradient gels resolved black bream Vtg as neat, clearly distinguishable bands, whilst the 5% single percentage gels did not resolve black bream Vtg well, with diffuse, smudged bands observed (after transfer to PVDF membranes) (Fig. 6.11, 6.12).



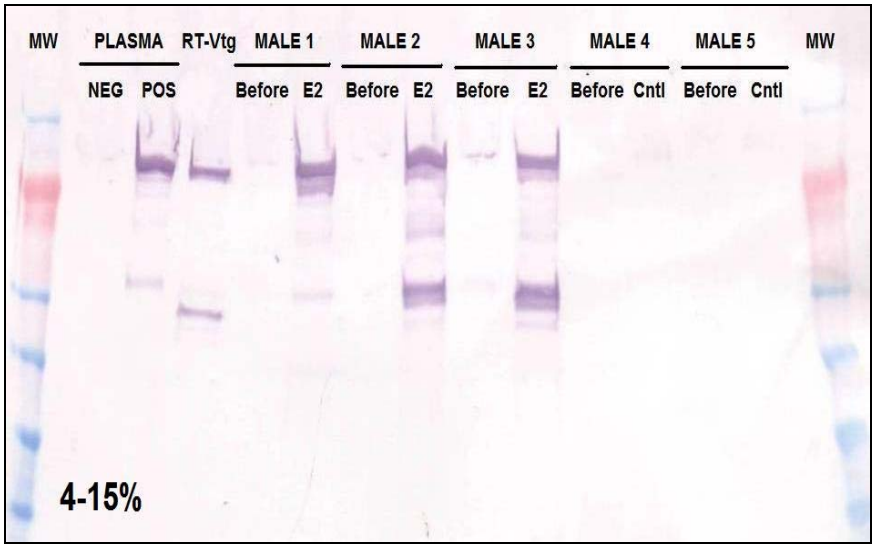


Figure 6.11 Comparison of 7.5% single percentage and 4-15% gradient polyacrylamide gels, transferred to PVDF membranes for Western blot analysis. Plasma from male black bream before injection (Before) and following i.p. injection of corn oil (Cntl) or 17β-estradiol (E2) was used. Positive (POS) and negative (NEG) black bream Vtg controls and rainbow trout Vtg (RT-Vtg) were also included.

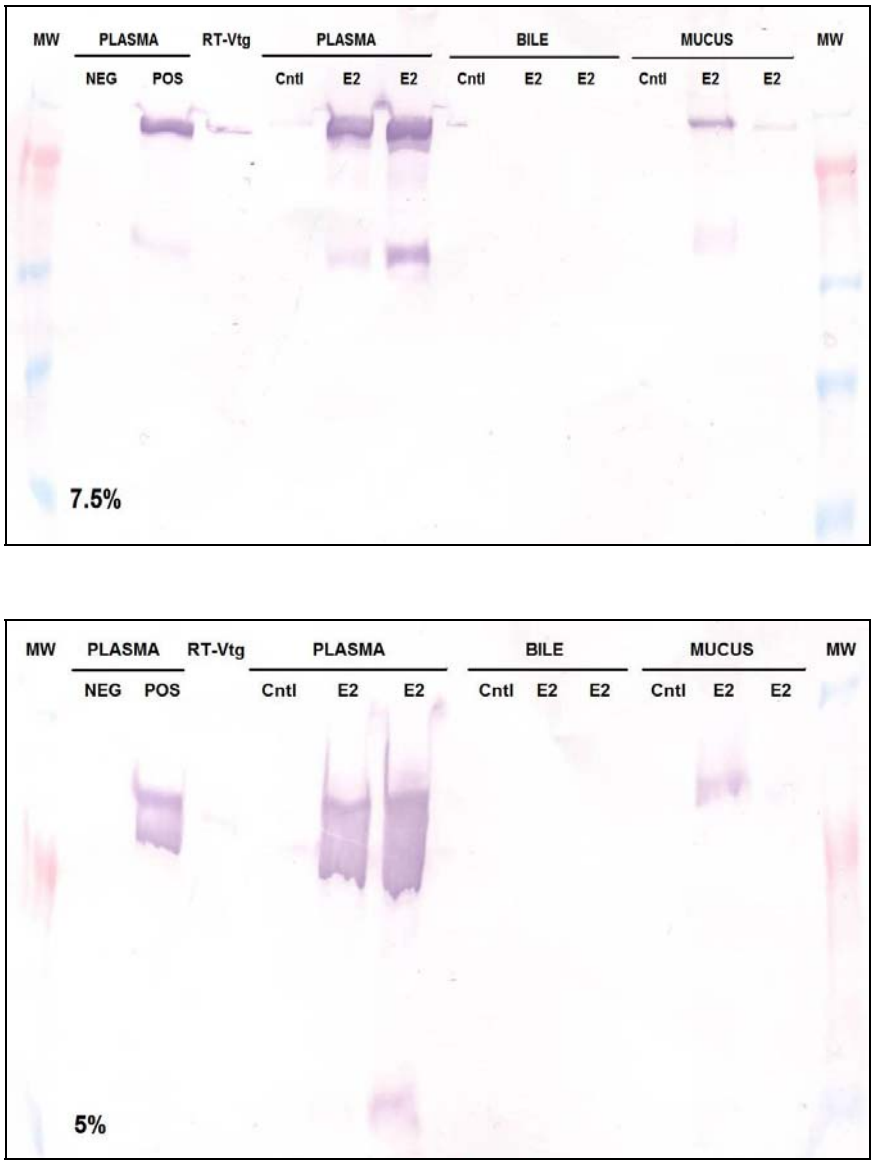


Figure 6.12 Comparison of 7.5% and 5% single percentage polyacrylamide gels, transferred to PVDF membranes for Western blot analysis. Plasma, bile and surface mucus from male black bream following i.p. injection of corn oil (Cntl) or 17β-estradiol (E2) were used. Positive (POS) and negative (NEG) black bream Vtg controls and rainbow trout Vtg (RT-Vtg) were also included.

6.3.5.3 Coomassie blue total protein staining

A prominent band of molecular weight 198 kDa was observed in plasma from E2-treated males and females, and this protein is presumed to be black bream Vtg (Fig. 6.13). The molecular weight of the presumed Vtg was calculated from the graph of relative migration distances (Rf values) plotted against the log molecular weights of eight known molecular weight standards (BenchMark® Protein Ladder) after denaturing electrophoresis (Fig. 6.14). For mucus samples from the E2-treated male and female fish, no prominent bands were observed on Coomassie stained gels, however Western blotting did reveal a band at the same molecular weight (Fig. 6.13). This suggests that the concentration of mucus proteins was too low for detection by Coomassie protein staining.

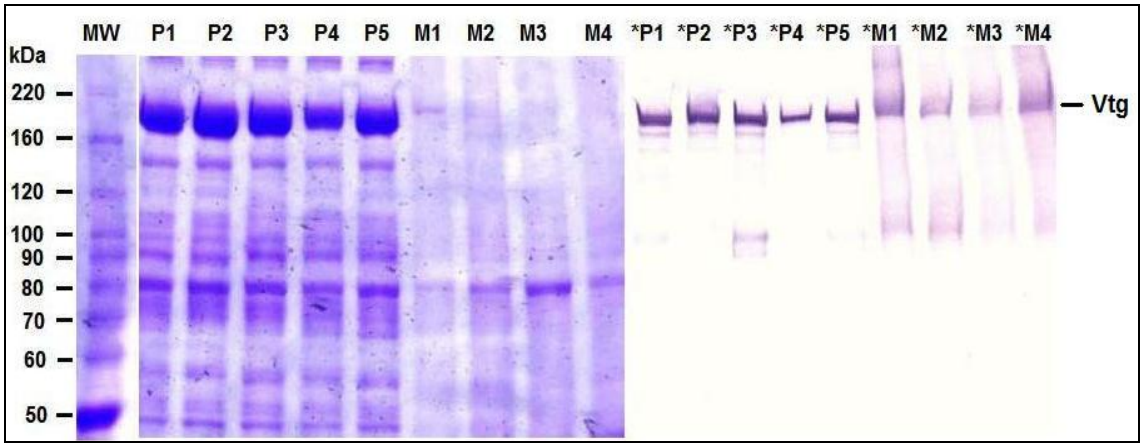


Figure 6.13 Coomassie blue stained polyacrylamide gel and Western blot (*) of male black bream plasma (P) and surface mucus (M) following E2-treatment. Presumed Vtg is indicated on the right hand side (Vtg). The unstained molecular weight standard, BenchMark® Protein Ladder (MW) was used to estimate the molecular weight of Vtg in gels following Coomassie blue total protein staining.

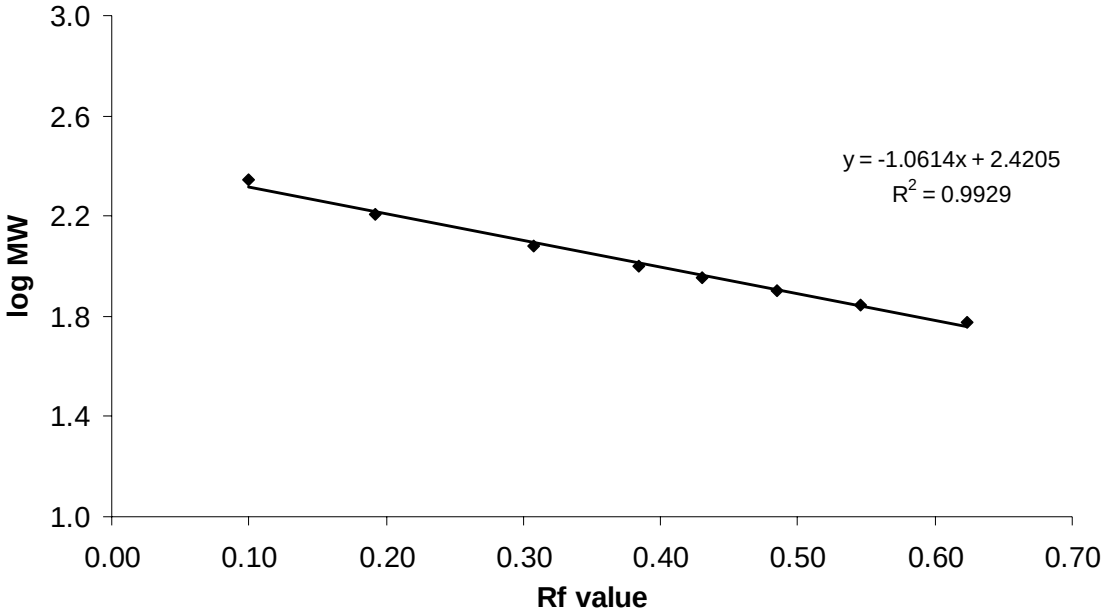


Figure 6.14 Plot of log molecular weight versus the relative migration distance (Rf value) for eight protein standards (BenchMark® Protein Ladder) used to determine the molecular weight of black bream Vtg.

6.3.5.4 Western blot

An immuno-reactive band, considered to be black bream Vtg, was observed in all E2-treated male and female plasma samples that corresponded to the band observed with Coomassie protein staining (Fig. 6.9, 6.10). A corresponding band was observed in mucus samples from the same fish (Fig. 6.13), however it was not observed in negative control male fish (Fig. 6.12, 6.15).

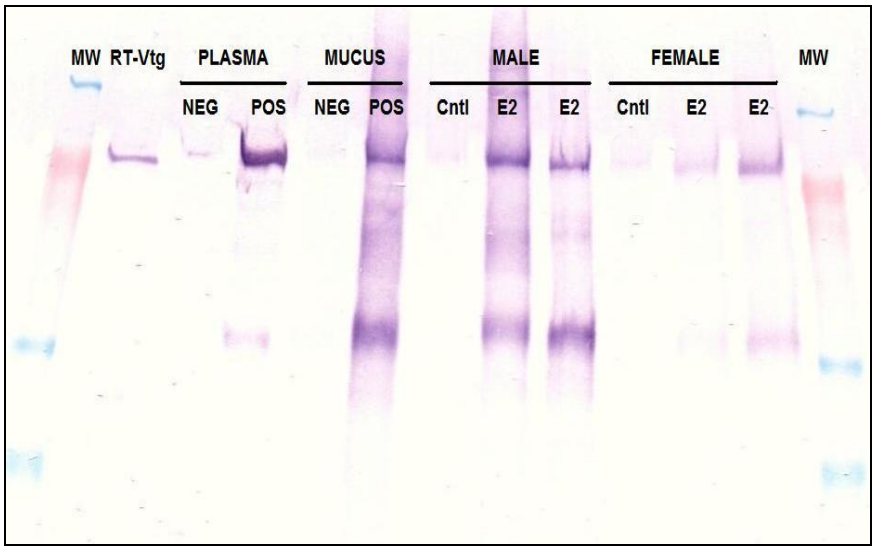


Figure 6.15 Western blot of male and female black bream surface mucus after i.p. injection of 5 mg/kg body weight 17β-estradiol (E2) or corn oil (Cntl). Positive (POS) and negative (NEG) black bream Vtg controls and rainbow trout Vtg (RT-Vtg) were also included.

6.3.6 Protein determination

The protein concentrations of black bream plasma and mucus were calculated based on a linear regression from a five point standard curve (Fig. 6.16). The mean protein concentrations of plasma were between 44 – 78 mg/ml for corn oil treated fish, and there were no significant differences between the sexes ($p > 0.05$). For E2-treated fish the plasma protein concentrations were between 100-128 mg/ml and there were no differences between the sexes ($p > 0.05$). The plasma protein concentrations between corn oil-treated fish and E2-treated fish were significantly different ($p = 0.004$) (Table 6.1). The mean protein concentrations of mucus were between 42 – 52 mg/ml for corn oil treated fish, and there were no significant differences between the sexes ($p > 0.05$) (Table 6.1). For E2-treated fish the mucus protein concentrations were between 38 - 48 mg/ml and there were no differences between the sexes ($p > 0.05$). There were no significant differences ($p > 0.05$) in the mucus protein concentrations between corn oil-treated fish and E2-treated fish.

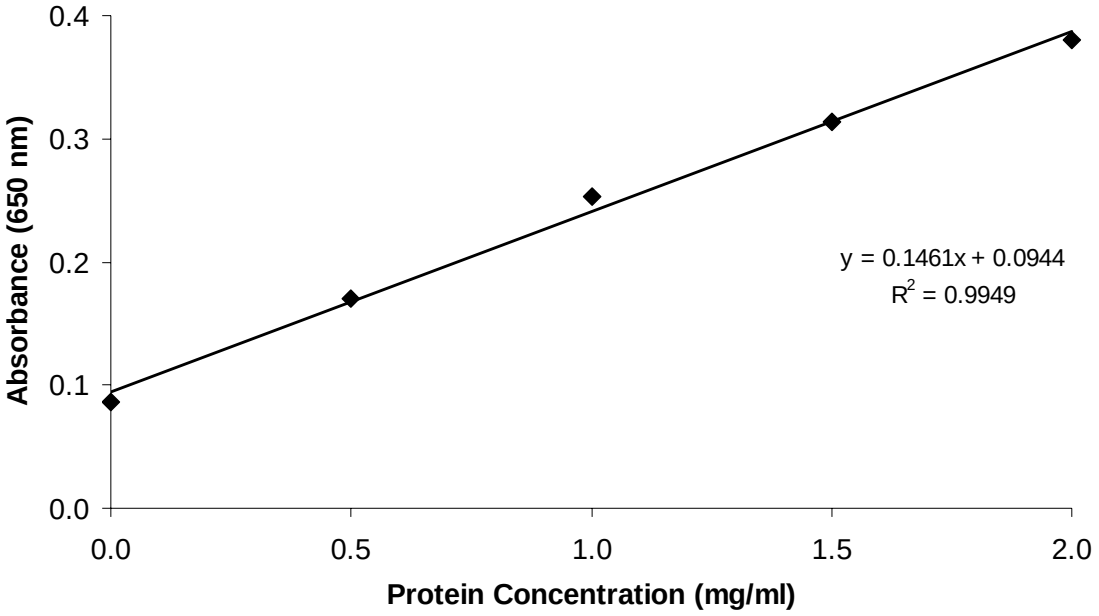


Figure 6.16 Representative standard curve used to determine the protein concentration of black bream plasma and surface mucus samples. Protein concentrations (mg/ml) were measured using the DC Protein Assay Kit (BioRad, Australia), and bovine serum albumin (BSA) was used to generate five protein standards.

6.4 Discussion

Work conducted in Norway and validated in Australia, using the monoclonal antibody ND-1C8 in two different immunological assays (ELISA and Western blot) demonstrates that Vtg is detectable in black bream and is inducible following exposure to E2. Whilst a monoclonal antibody from striped bass (*Morone saxitilis*) was used for this study, black bream Vtg was also found to bind well with a polyclonal antibody (PO-2), from gilthead seabream (*Sparus aurata*). This is the first time that black bream Vtg has been detected in both blood plasma and surface mucus using these techniques.

The molecular weight of denatured (subunit) black bream Vtg was estimated to be 198 kDa, and a second subunit of approximately ~100 kDa was also observed in some samples, which may represent a breakdown product, since teleost Vtg is known to be unstable and can breakdown easily (Specker and Sullivan, 1994; Denslow *et al.*, 1999). The estimated molecular weight of black bream Vtg was similar to reported sizes in other sparid fishes including *Pagrus pagrus*, 189 kDa (Kokokiris *et al.*, 2001), *Dentex dentex*, 170 kDa (Pavlidis *et al.*, 2000), and *Sparus aurata*, 180 kDa (Mosconi *et al.*, 1998). This is the first report of the estimated molecular weight of black bream Vtg.

The use of commercially available rainbow trout Vtg as a standard allowed for the development of a quantitative ELISA, and thus the determination of equivalent Vtg concentrations (RT-VtgEq) for black bream Vtg. However, using a striped bass (*Morone saxitilis* – Perciformes) primary antibody and a rainbow trout (*Oncorhynchus mykiss* - Salmoniformes) Vtg standard to then quantify black bream (*Acanthopagrus butcheri* –

Perciformes) Vtg is not an ideal way to conduct the assay, since it is likely that the different species have different degrees of antigenicity towards the anti-Vtg primary antibody. In a study of Vtg cross-reactivity between three different teleost species, Watts *et al.* (2003) found that even in the closely related rainbow trout and Atlantic salmon, only low levels of cross-reactivity were observed. Thus, in order to successfully utilise Vtg as a biomarker for reproductive condition in black bream, further development of the two immunological assays is needed. The Western blot assay was able to detect Vtg at a total protein concentration of only 0.1 µg/15 µl (6.67 µg/ml), indicating good sensitivity, however the ELISA needs improvement and the development of a quantitative black bream ELISA is necessary. To achieve this, the next step would be to purify and characterise the black bream Vtg molecule, then use the purified protein to generate a black bream specific primary antibody. Upon completion of Vtg purification and primary antibody production, a homologous black bream Vtg ELISA would be achievable. This would provide greater sensitivity which is vital for studies of both the onset of vitellogenesis in maturing females, as well as for assessment of Vtg induction as a result of exposure to estrogenic endocrine disrupting chemicals. Unfortunately this was not achievable within the timeframe and scope of the present study.

The initial step of the ELISA is to coat the microplate with diluted plasma (antigen). The adsorption of proteins to microplates is due to interactions with nonpolar (hydrophobic) portions of the protein, so different proteins will have different binding affinities (Crowther, 1995). For this reason it is important that the concentration of protein that is applied to each well is similar, and hence a single dilution was used for all plasma

(1:100000) and mucus (1:12500) samples. If the concentration of antigen is too high, it may actually reduce binding with antibodies, due to steric inhibition (Crowther, 1995). This was observed in the plasma dilution curve between 1:10000-1:100, where the absorbance was actually lower than at 1:25000, despite the sample being more concentrated. Likewise, the mucus dilution curve displayed a similar effect, where the absorbance was lower at 1:1000 and 1:100, compared to 1:5000. These findings highlight the importance of conducting serial dilutions of samples to determine the optimal dilution for a titration.

In addition to reporting Vtg detection in blood plasma, this study also demonstrates for the first time that Vtg can be detected in the surface mucus of black bream. Vitellogenin was detected in the surface mucus of both male and female black bream following exposure to E2, whilst it was not detected in control fish of either sex. This is an important result, since it represents a potential non-invasive, minimal-stress technique for determining both sexual maturity and exposure to estrogenic endocrine disrupting chemicals in black bream. However, as mentioned above, in order to achieve this, the Vtg assay needs to be refined to improve sensitivity.

The repeated collection of surface mucus from the same individuals has been reported as a suitable technique for determining Vtg levels over time, without apparent injury to the fish (Schultz *et al.*, 2007). This technique would be very appropriate and beneficial for captive breeding programs where regular sampling may be required in order to maximise the effectiveness of expensive hormonal treatments or to determine suitable timing for

induced spawning and artificial fertilisation. Whilst the use of mucus to measure Vtg in black bream has great potential for the above mentioned reasons, collection techniques, especially for external fluids need to be standardized to maintain integrity of the sample since mucus samples may be easily contaminated by water on the surface of the skin or scales. For this reason it is important to determine protein concentrations of mucus samples (Schultz *et al.*, 2007). In this study, the protein concentrations of different individuals were all of a similar range (~10's mg/ml), indicating consistency in sample collection.

Since Vtg is only produced in females normally, with further development of quantitative black bream Vtg assays, it may eventually be possible to use blood plasma or surface mucus assays as tools for sex identification. However, this could only be useful in situations where exposure to estrogenic contaminants could be strictly ruled out since such exposure in males could lead to incorrect sex identification. The induction of Vtg is already widely used as an indicator of exposure to estrogenic endocrine disrupting chemicals in male fish (Heppell *et al.*, 1995; Sumpter and Jobling, 1995; Jobling *et al.*, 1998). Male fish are capable of producing Vtg, however circulating estrogen levels in male blood is normally too low to stimulate Vtg production (Specker and Sullivan, 1994). With improved sensitivity of black bream Vtg assays, it may become possible to detect environmentally induced changes in the endocrine pathways of male fish.

The fish used in the Vtg induction experiment displayed some differences in size, weight and gonad condition, however these differences are not considered to have been a result

of the E2-treatment, since the induction experiment was conducted over only a short time period, so large changes in size and condition were unlikely. Like many other sparids, black bream are not sexually dimorphic, so it was not until the fish were sacrificed and dissected that the sex could be confirmed by gonad observation. Indeed a number of the fish that were thought to be females (based on the absence of milt), turned out to be males. Since the fish were not in spawning condition at the time of sampling, the lack of milt in some males was not unexpected. Being unable to determine the sex of black bream if eggs and milt are not present with light pressure on the abdomen (which only occurs during spawning season), is a hindrance to hatchery management protocols. This also becomes an issue with field sampling, if non-destructive sampling is required, yet determining the sex of the fish is important. The development of techniques to determine sex-specific differences in non-sexually dimorphic fishes is needed, and with further development to improve sensitivity, the Vtg assay may fulfill these needs.

In order to establish a reliable test for sex identification and sexual maturity, it is essential to determine baseline and seasonal levels of Vtg in both blood plasma and surface mucus of male and female black bream. It has been reported that Vtg in blood plasma can rise several-fold during vitellogenesis (Kokokiris *et al.*, 2001; King and Pankhurst, 2003) then return to very low 'basal' levels outside the breeding season. Determining exactly how low Vtg concentrations are in the non-spawning season, as well as the investigation of how environmental stressors (ie. hypoxia and endocrine disrupting chemicals) affect Vtg levels in both male and female black bream would substantially improve our understanding of why recruitment success varies so much in this species.

Another important application for minimally invasive, non-destructive tests that can determine sexual maturity, sex identification or exposure to environmental stressors is that large numbers of fish can be tested. For statistically robust data analyses, large sample numbers of fish are required, but for many fish such as protected and large species, the collection of large numbers from the wild is no longer practical or permitted. In order to meet ethical standards, there is an increasing requirement to reduce the number of fish killed for research purposes, thus there are significant practical and economic advantages of non-lethal sampling as the fish can be returned to the water and retained by the fishery.

In conclusion, an indirect, quantitative ELISA has been developed for black bream Vtg, and successfully used to determine the Vtg concentrations (in RT-VtgEq units) in both female and male adult black bream following exposure to a potent estrogen. Secondly, a Western blot technique has also been developed and used to confirm the results of the ELISA. A number of different factors were tested to optimise both assays, which has resulted in the establishment of preferred protocols for both ELISA and Western blot assays to measure black bream Vtg in both blood plasma and surface mucus. Since there is a growing need to develop non-destructive and minimally invasive sampling techniques, the detection of Vtg in surface mucus is a very significant finding. With further development to improve assay sensitivity, surface mucus potentially represents a non-invasive, non-destructive method for determining the reproductive condition of black bream, and could also provide a minimally invasive biomarker for exposure to EDCs and for identifying the sex of non-dimorphic fishes.

CHAPTER 7: APPLICATION OF THE VITELLOGENIN ASSAY TO EVALUATE REPRODUCTIVE CONDITION IN FIELD COLLECTED BLACK BREAM

Sections of this chapter are being prepared for publishing consideration.

Manuscript details

Kathryn L. Hassell, Susan Codi King, Vincent Pettigrove, Patrick C. Coutin and Dayanthi Nugegoda. "Vitellogenin detection in the blood plasma and surface mucus of black bream *(Acanthopagrus butcheri)* from southern Australia". To be submitted to *Australasian Journal of Ecotoxicology*.

7.1 Introduction

Victoria supports a population of more than 5 million people (Australian Bureau of Statistics, 2008), and the capital city, Melbourne, is situated upon the largest bay in Victoria, Port Phillip Bay, which covers an area of 1950 km^2. Victorian land use patterns, urbanisation and climate change all contribute to the increasing load of environmental stressors that degrade estuarine water quality, including industrial and agricultural discharges, stormwater, nutrients, sewage and reduced flows as a result of water extraction. Recently, the Australian catchment, river and estuary assessment report series was released, which provided a comprehensive assessment of Australian estuarine environments (NLWRA, 2002) (see section 1.3). Of the 60 Victorian estuaries that were included in the study, 7% were considered extensively modified, 38% were considered modified, 35% were considered largely unmodified and 20% were considered to be in a near pristine condition (NLWRA, 2002). For the present study, five Victorian estuaries

were selected as collection sites for black bream, with the locations chosen to represent sites with different types of environmental conditions. The five sites were: Mallacoota Inlet, Yarra River, Maribyrnong River, Werribee River and Little River.

Mallacoota Inlet (37^{o} 57'S;149^{o} 76'E), in far eastern Victoria is a wave-dominated estuary and is considered to be in a near-pristine condition (NLWRA, 2002). It is Victoria's 5^{th} largest estuary, covering an area of 25 km^2 in far eastern Victoria, 525 km from Melbourne (MacDonald *et al.*, 1997). The inlet comprises two lakes (top and bottom) that are joined by a deep, narrow channel and the inlet is surrounded by 86, 000 hectares of land, the majority of which is part of the Croajingalong National Park (DPI, 2006). Mallacoota Inlet is a popular fishing location, important for both recreational and commercial fishing efforts. Sources of environmental degradation that have been identified for Mallacoota Inlet are mainly related to erosion and sediment deposition due to inappropriate land use and forestry practices, and management plans are currently in place to minimise impacts (DPI, 2006). Mallacoota Inlet has extensive areas of seagrass cover and is listed as a wetland of national importance (Murray *et al.*, 2006), and was selected as the reference site for the present study because of its near-pristine condition

The Yarra River (37^{o} 85'S;144^{o} 91'E) flows from the Great Dividing Range near Warburton, 76 km north-east of Melbourne in a south-westerly direction where it drains into the northern end of Port Phillip Bay (Melbourne Water, 2008a). It is classified as a wave-dominated delta and is considered to be in a modified condition (NLWRA, 2002). The river is 245 km in length, and more than 1.5 million people live within the 4,000 km^2

Yarra catchment. The upper reaches of the river are mostly unmodified and surrounded by a rural catchment, of which approximately 70% of Melbourne's drinking water is sourced from. The lower, estuarine reaches are turbid and heavily urbanised.

The Maribyrnong River ($37^{o}46$'S; $144^{o}53$'E) arises from Deep Creek and Jackson's Creek north-west of Melbourne, and flows into the Yarra River before entering Port Phillip Bay (Melbourne Water, 2008b). The Maribyrnong River estuary is classified as a wave-dominated delta and is considered to be in a modified condition (NLWRA, 2002). The river is approximately 25 km in length and is surrounded completely by urban and industrial development. The Maribyrnong catchment is small (1433 km^2) and is subject to extended periods of low flow, which combined with tidal influences can result in the intrusion of salt water as far as 15 km upstream, to a ford at Avondale Heights which creates a natural barrier.

In 2005, an environmental audit was conducted by the Victorian Environmental Protection Authority (EPA), to assess the condition of the lower Maribyrnong and Yarra estuaries, which both have long histories of urban and industrial activity (EPA, 2007). The study incorporated analyses of water, sediments and fish samples. Black bream were one of the test species used in that study, since they are commonly found in the two estuaries and are a popular species targeted by recreational fishing. Skin-free muscle samples were analysed for both organic chemicals and heavy metals, and a number of contaminants were detected. All fish samples contained polychlorinated biphenyls (PCBs), and the total PCB content exceeded USEPA Guideline values (EPA, 2007). In

addition to PCBs, a number of dioxins (PCDD/F congeners) as well as two dichlorodiphenyltrichloroethane (DDT) metabolites were detected (*pp*-DDE, *pp*-DDD), however all were reported at concentrations below accepted guideline values (EPA, 2007). Arsenic, copper, mercury, selenium and zinc were detected in all black bream samples, whilst lead and tributyl tin (TBT) were detected in some samples. Based on these findings, the EPA has issued recommendations to limit the consumption of fish from both the Yarra River and Maribyrnong River. For the present study, these two rivers were selected as examples of rivers under the influence of environmental stress from urban development and industrial activities.

The Werribee River (37°98'S; 144°69'E) flows from the Wombat State Forest, approximately 40 km south-west of Melbourne, in a south-easterly direction where it flows past the Western Treatment Plant (Melbourne's main sewage treatment facility), and drains into Port Phillip Bay (Melbourne Water, 2008c). It is classified as a wave-dominated delta and is considered to be in a modified condition (NLWRA, 2002). The river is 93 km in length, and is surrounded by agriculture and market gardens. The Werribee catchment is 2700 km^2 and occurs in a low rainfall area. The mouth of the estuary is approximately 50 km from Melbourne, and the shallow Werribee River estuary is 8.25 km in length and upstream flows are limited by a man made ford which was constructed in the 1860s (Sherwood, *et al.*, 1995).

The Little River (38°01'S; 144°59'E) flows from the Brisbane Ranges National Park, approximately 100 km south-west of Melbourne, in a south-easterly direction where it

flows through the Western Treatment Plant, before draining into Port Phillip Bay (Melbourne Water, 2008c). It is classified as a tide-dominated, ephemeral estuary and is considered to be in a modified condition (NLWRA, 2002). The mouth of the estuary is approximately 65 km from Melbourne, and the Little River is part of the Werribee catchment. During summer months the Little River estuary dries up completely. Both the Werribee River and Little River are in close vicinity to the Western Treatment Plant, which is Victoria's largest sewage treatment facility, treating approximately 52% of Melbourne's sewage every day (Melbourne Water, 2007). Sewage is treated in a series of modern lagoons, before being discharged into Port Phillip Bay, and in addition to treating domestic sewage, the Western Treatment Plant also processes 70% of Melbourne's industrial and commercial (trade) wastes, which account for 15% of the total sewage flow. Both the Werribee River and Little River are impacted by low flows, which cause the estuarine sections to be very saline and vertically stratified (Sherwood *et al.*, 1995). Furthermore, algal blooms due to excessive nutrients have been identified and contribute to an increasing incidence of hypoxic/anoxic conditions in both estuaries. The Werribee River and Little River were chosen for the present study as examples of rivers under the influence of environmental stress from agriculture and a large sewage treatment facility.

Black bream are estuarine residents and based on genetic research of black bream from 10 different areas in Western Australia, it appears that there are distinct populations that do not often mix (Chaplin *et al.*, 1998). Subsequently, it may be expected that growth rates, reproduction and age/size at maturity will differ between sub-populations of black bream, possibly due to differences in local environmental conditions. Indeed, in Western

Australia such phenomena have been observed, where the age composition and growth rates are markedly different in black bream from different estuaries (Sarre and Potter, 2000; Partridge *et al*., 2004).

In this chapter the reproductive condition of five separate black bream populations from Victorian estuaries was investigated, utilising the Vtg assay that was developed (see chapter 6). The five different estuaries occur in areas with different land use patterns and environmental conditions and are subject to different types of environmental stressors. The aim was to validate the Vtg assay, and try to determine how different environmental stressors may affect reproductive condition in female black bream, as well as to screen male black bream for evidence of exposure to estrogenic endocrine disrupting chemicals that may induce Vtg synthesis.

7.2 Materials and Methods

7.2.1 Fish collection

Black bream were captured using rod and line, seine nets or gill nets with the assistance of both commercial and recreational fishermen.

7.2.1.1 Mallacoota Inlet

Fish were collected from Mallacoota Inlet during December 2006 and February 2007 (summer) using both a seine net (3/4 inch, 50 m length) and a gill net (5 inch, 30 m length). Different types of nets were required to target different sized fish. Fishing was conducted with the assistance of Mr. John McGuckin (Streamline Research) and Dr. Vincent Pettigrove (Melbourne Water). A second collection of fish from Mallacoota Inlet was undertaken in November 2007 (spring) using rod and line (no. 4 or 6 hooks and frozen prawns for bait) with the assistance of Mr. John Wood. Fish were captured from a number of sties within this estuary, from Snapper Point in the northern end of the bottom lake, which is 4.6 km from the estuary mouth, to Gipsy Point in the northern end of the top lake, which is 13 km from the estuary mouth where the Genoa River and Wallagaraugh River feed into the Inlet (see Appendix 1 for map).

7.2.1.2 Yarra River and Maribyrnong River

Fish were collected from the Yarra River during September, October, November and December 2007 (spring-summer) and May 2008 (autumn), and from the Maribyrnong River during August (winter), October, November, December 2007 (spring-summer), February 2008 (summer) and May 2008 (autumn) using rod and line. Fishing was

conducted with the assistance of Mr. Anthony Hocking, using no. 4 or 6 hooks and live shrimp and earthworms for bait. Fish were captured from a number of sites within the Yarra estuary, from the Docklands, near the estuary mouth, to Herring Island (Richmond), approximately 13 km upstream of the estuary mouth. Fish were captured from a number of sites within the Maribyrnong estuary, from Lynch's Bridge (Footscray) which is 3.05 km upstream from the mouth of the Yarra River, to Solomon's Ford (Avondale Heights), which is 15.3 km upstream from the mouth of the Yarra River (see Appendix 1 for map).

7.2.1.3 Werribee River and Little River

Fish were collected from the Werribee River during October 2007 (spring) and the Little River during December 2007 (summer). Fishing was conducted with the assistance of Mr. Philip McAdam (Vancouver Bait Supply) using a beach seine (1/2 inch, 100 m length) and a gill net (4 inch, 300 m length). All fish were captured from an area approximately 500 m from the Werribee River estuary mouth and from an area approximately 300 m from the Little River estuary mouth (see Appendix 1 for map).

7.2.2 Biological information

Fish were humanely sacrificed and dissected as described in section 2.3. Length, weight, age, growth, macroscopic gonad appearance, tissue indices, sex ratios and incidence of ovotestis were all recorded. Tissue indices were calculated as described in section 2.3.2. Blood plasma, gonads, liver, bile, gills, white muscle, otoliths and fin clips were retained for analysis.

7.2.3 Vitellogenin detection

7.2.3.1 Quantitative ELISA

ELISA procedures were carried out as described in section 6.2.4. Briefly, plasma (1:100000) samples were diluted in coating buffer then applied to microplates and incubated overnight at 4^{o}C. Following sequential washing and blocking steps, incubation with primary antibody (ND-1C8) and then secondary antibody (GAM-HRP) was done, and then the plates were developed with OPD-H_2O_2. The colour reaction was developed for exactly 12 min then stopped by the addition of 4 N H_2SO_4. Absorbance was measured at 492 nm using a Thermo Ascent Multiskan plate reader.

7.2.3.2 Denaturing electrophoresis, Coomassie blue total protein staining and Western blot

Plasma samples from five individual males and females from each field site were randomly selected and electrophoresis was conducted as described in section 6.2.5. A molecular weight standard (MW), positive black bream Vtg control (POS), negative black bream Vtg control (NEG) and purified rainbow trout Vtg control (RT-Vtg) were included on each gel. Following electrophoresis, the gels were stained in Coomassie blue as described in section 6.2.6, or transferred to 0.20 µm PVDF membranes and developed as described in section 6.2.7. Plasma protein levels were determined as described in section 6.2.8.

7.3 Results

Throughout this project, a total of 258 black bream were collected between December 2006 and May 2008, from five separate locations: Mallacoota Inlet (77), Yarra River (51), Maribyrnong River (46), Werribee River (42) and Little River (42). Fishing efforts occurred over a broad time period that included the pre-spawning, spawning and post-spawning seasons. Since biological measures can change seasonally and especially in relation to reproductive activities, only fish that were captured during the black bream spawning season, between September (spring) and December (summer) were included in tissue index and Vtg analyses. A total of 82 females and 71 males were analysed for Vtg.

7.3.1 Water quality at field collection sites

Water quality was measured at each site during each fishing trip (Table 7.1). Dissolved oxygen (%S) was measured using a TPS meter (model WP -91), and salinity, pH and temperature were measured using a TPS meter (model WP-81) (TPS Ionode, Springwood, Australia). One litre water samples were collected from some sites for the measurement of estrogenic activity, which was carried out by the Ecotoxicology unit at the Department of Primary Industries, Queenscliff. They measured total estrogenic activity using a commercially available Total Estrogens ELISA test kit (Table 7.1).

Table 7.1 Water quality (mean ± SEM) in the five estuaries sampled in this study.

Site	Mean ± SEM Dissolved Oxygen (%S)	Mean ± SEM Salinity (ppt)	Mean ± SEM Temperature (^{o}C)	Mean ± SEM pH	Total Estrogens (ng/l)
Mallacoota Inlet	89.6 ± 3.61	32.0 ± 0.92	23.2 ± 1.06	7.72 ± 0.07	-
Yarra River	53.1 ± 9.92	14.2 ± 3.15	20.8 ± 0.24	7.28 ± 0.19	1.41
Maribyrnong River	85.6 ± 10.6	20.5 ± 3.40	21.0 ± 3.44	7.74 ± 0.22	1.83
Werribee River	78.3 ± 3.32	34.9 ± 0.99	12.5 ± 0.71	7.97 ± 0.04	-
Little River	78.0 ± 2.19	36.4 ± 0.64	18.0 ± 1.41	7.70 ± 0.02	3.56

7.3.2 Biological information

Since fishing was conducted over a broad time period and collections from different sites did not always utilise the same fishing gear or occur during the same season, no comparisons will be made between sites, but rather each site will be reported separately.

7.3.2.1 Length, weight and age

In order to estimate age, each otolith was weighed to the nearest 0.001 g then one from each fish was sectioned (0.30 mm thickness) and mounted in polyester resin, according to the method of Anderson *et al.* (1992). An experienced reader then estimated age by counting increments from the otolith primordia out to the edge (Fig. 7.1). Counts were repeated by the same reader to allow determination of intra-reader variability, described as the index of average percent error (IAPE) (Beamish and Fournier, 1981). The IAPE for repeated readings in this instance was 0.3846%. The estimated ages of black bream collected during this study ranged from 1-19 years old, with the most frequent age (mode) being 2 years old. Two year old black bream ranged in size from 135 mm to 237 mm (FL), and the smallest two year olds were from the Yarra River, whilst the largest two year olds came from the Werribee River. For each age class, the smallest fish were

generally from the Yarra River or the Maribyrnong River, whilst the largest fish were from the Werribee River or Little River.

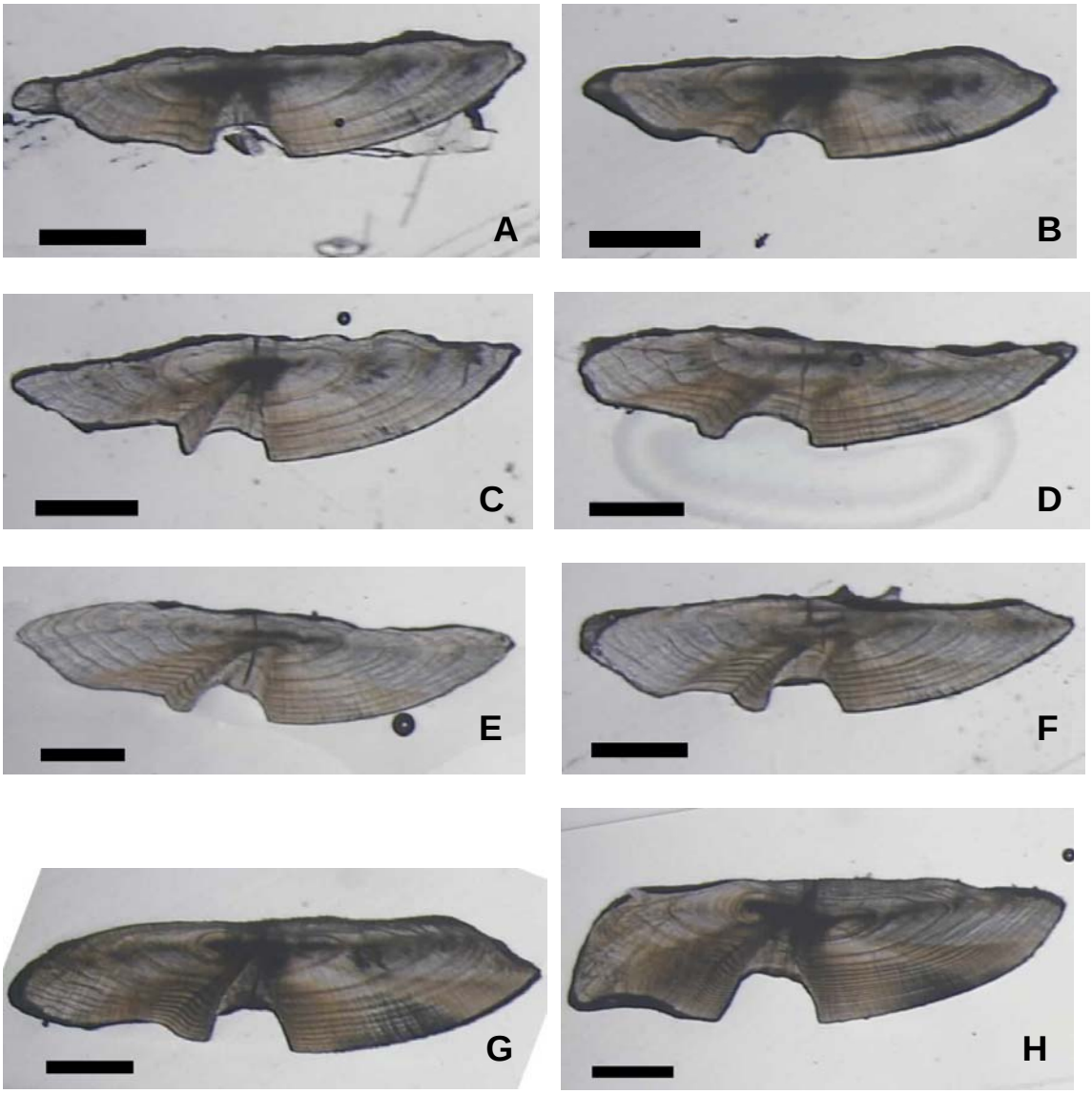


Figure 7.1 Examples of sectioned otoliths and age estimates (bar = 1.0 mm). A) 2 yrs; B) 3 yrs; C) 4 yrs; D) 6 yrs; E) 7 yrs; F) 8 yrs; G) 10 yrs; H) 19 yrs.

The largest fish caught during this study was a male caught in Little River, measuring 41 cm in length and weighing 1340 g. This fish was also the oldest and was estimated to be 19 years old. There were no sex-specific differences in length, weight or age for black bream from any site.

Fish collected from Mallacoota Inlet ranged in size from 54-360 mm (Table 7.2, Appendix 2), and there were no significant differences in the fork length between female and male black bream ($p = 0.724$). The weight of these fish ranged from 5-971 g (Table 7.2, Appendix 2), and again there were no significant differences between sexes ($p = 0.678$). Due to limited time and sample storage availability at Mallacoota Inlet, otoliths were only retained from a small number of female ($n=4$) and male ($n=5$) fish. The youngest fish was estimated to be 2 years old, whilst the oldest fish was estimated to be 8 years old (Table 7.2, Appendix 2) and there were no significant differences between sexes ($p = 0.891$).

Fish collected from Yarra River ranged in size from 135-310 mm (Table 7.2, Appendix 2), and there were no significant differences in the fork length between female and male black bream ($p = 0.600$). The weight of these fish ranged from 47-553 g (Table 7.2, Appendix 2), and again there were no significant differences between sexes ($p = 0.505$). Otoliths were retained from most ($n=43$) fish, and the youngest fish was estimated to be 2 years old, whilst the oldest fish was estimated to be 9 years old (Table 7.2, Appendix 2) and there were no significant differences between sexes ($p = 0.684$).

Fish collected from Maribyrnong River ranged in size from 138-280 mm (Table 7.2, Appendix 2), and there were no significant differences in the fork length between female and male black bream (p = 0.421). The weight of these fish ranged from 53-468 g (Table 7.2, Appendix 2), and again there were no significant differences between sexes (p = 0.354). Otoliths were retained from most (*n=38*) fish, and the youngest fish was estimated to be 1 year old, whilst the oldest fish was estimated to be 9 years old (Table 7.2, Appendix 2) and there were no significant differences between sexes (p = 0.153).

Fish collected from Werribee River ranged in size from 49-360 mm (Table 7.2, Appendix 2), and there were no significant differences in the fork length between female and male black bream (p = 0.237). The weight of these fish ranged from 2-1200 g (Table 7.2, Appendix 2), and again there were no significant differences between sexes (p = 0.351). Otoliths were retained from most (*n=38*) fish, and the youngest fish was estimated to be 2 years old, whilst the oldest fish was estimated to be 10 years old (Table 7.2, Appendix 2) and there were no significant differences between sexes (p = 0.116).

Fish collected from Little River ranged in size from 105-410 mm (Table 7.2, Appendix 2), and there were no significant differences in the fork length between female and male black bream (p = 0.589). The weight of these fish ranged from 21-1340 g (Table 7.2, Appendix 2), and again there were no significant differences between sexes (p = 0.635). Otoliths were retained from most (*n=40*) fish, and the youngest fish was estimated to be 1 year old, whilst the oldest fish was estimated to be 19 years old (Table 7.2, Appendix 2) and there were no significant differences between sexes (p = 0.994).

Table 7.2 Mean (± SEM) length (mm), weight (g) and age (years) for black bream captured from five different Victorian estuaries between December 2006 and May 2008.

SITE	*n*=	Fork Length (mm)	Weight (g)	Age (years)
MALLACOOTA				
Female	*45*	193.6 ± 11.3	212.4 ± 34.6	4.4 ± 1.3
Male	*20*	195.3 ± 13.6	190.0 ± 34.3	4.0 ± 0.6
YARRA				
Female	*32*	194.7 ± 7.52	138.1 ± 19.69	3.5 ± 0.4
Male	*19*	186.9 ± 8.14	166.8 ± 21.38	3.2 ± 0.5
MARIBYRNONG				
Female	*22*	189.7 ± 8.82	152.7 ± 24.87	2.9 ± 0.5
*Male	*20*	196.3 ± 6.19	158.3 ± 14.19	3.3 ± 0.3
WERRIBEE				
Female	*22*	271.1 ± 9.95	459.1 ± 56.42	5.4 ± 0.6
*Male	*19*	253.9 ± 7.96	377.1 ± 44.28	4.2 ± 0.5
LITTLE				
Female	*17*	267.6 ± 11.6	432.9 ± 53.85	4.9 ± 0.6
*Male	*24*	276.0 ± 10.0	468.2 ± 52.61	5.3 ± 0.8

*fish with ovotestes were classified as males since all produced milt.

7.3.2.2 Macroscopic appearance of gonads and tissue indices

Descriptions of the macroscopic appearance of black bream gonads used in this study are based on the classifications described by Haddy and Pankhurst (1998) (Table 7.3; Fig. 7.2, Fig. 7.3). Ovaries of all stages were observed in this study, with the majority being in the regressed stage. The smallest fish observed to have vitellogenic ovaries was captured in the Yarra River, measuring 190 mm, 143 g and 3 years old (GSI - 4.20%). The youngest fish observed to have vitellogenic ovaries was captured in the Werribee River measuring 205 mm, 176 g and 2 years old (GSI - 2.27%). Hydrated and ovulated ovaries were observed in some fish, all of which measured greater than 195 mm. The

largest ovary observed was a Stage 4 gonad weighing 121 g, from a fish captured in the Werribee River measuring 360 mm, 1200 g and 10 years old (GSI - 10.1%).

Table 7.3 Macroscopic classification of black bream gonads (from Haddy and Pankhurst, 1998).

Stage	Classification	Macroscopic appearance	Histological characteristics
Female			
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 epithelium	Final oocyte maturation and hydration
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
Male			
1	immature	Testis white threads	Spermatogonia and a few previtellogenic oocytes[A]
2	spermatogenic	Testis firm and ivory white	Secondary spermatocytes, spermatozoa
3	partially spermiated	Testis firm and ivory white with viscous milt in sperm duct	Spermatozoa predominate
4	fully spermiated	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

[A]Oocytes in dorsal section of gonad in all male stages.

Milt was detected in a number of males with gonads weighing less than 1 g, and the smallest male fish that expressed milt was captured in the Yarra River, measuring 136 mm, 49 g and 2 years old (GSI < 2.04%). The youngest male that expressed milt was captured in the Little River, measuring 180 mm, 106 g, 1 year old (GSI - 3.77%). The largest testis observed was a Stage 4 gonad weighing 121 g, from a fish captured in the Werribee River measuring 310 mm, 785 g and 9 years old (GSI - 15.4%).

The gonads of fish from Mallacoota Inlet were mostly in Stage 1 or Stage 2 for both females and males, with immature or regressed ovaries, or immature or spermatogenic

testes, respectively. Some immature gonads could not be distinguished (macroscopically) as either female or male, and so these were classified as juveniles. Some females had vitellogenic ovaries (Stage 3) (smallest 260 mm, 5 years old, GSI - 4.99%) and some males expressed milt (Stage 3 or Stage 4) (smallest 195 mm, 2 years old, GSI < 0.67%).

The gonads of fish from the Yarra River were mostly in Stage 2 or Stage 3 in females (smallest 190 mm, 3 years old, GSI - 4.20%) and Stage 3 or Stage 4 in males (smallest 136 mm, 2 years old, GSI < 2.04%). No females had hydrated (Stage 4) or ovulated (Stage 5) ovaries.

The gonads of fish from the Maribyrnong River were mostly in Stage 2, whilst some were spent (Stage 6) and some were hydrated or ovulated (smallest 255 mm, 9 years old, GSI - 2.52%). Most males were in Stage 2, and some expressed milt (Stage 3 or Stage 4) (smallest 160 mm, 2 years old, GSI < 1.35%).

The gonads of fish from the Werribee River were mostly in Stage 3 for females (smallest 205 mm, 2 years old, GSI - 2.27%), and Stage 3 or Stage 4 for males (smallest 200 mm, 2 years old, GSI - 2.63%). Some females had hydrated (Stage 4) or ovulated (Stage 5) ovaries.

The gonads of fish from the Little River were mostly in Stage 3 for females (smallest 225 mm, 3 years old, GSI - 8.16%), and Stage 3 or Stage 4 for males (smallest 180 mm, 1

year old, GSI - 3.77%). Some females had hydrated (Stage 4) or ovulated (Stage 5) ovaries, some had spent (Stage 6) ovaries, and others were regressed (Stage 2).

An interesting finding in some male fish was the observation of a distinct ovotestis, consisting of both ovarian and testicular tissue (Fig. 7.4). Ovotestes, in the form of residual ovarian tissue in a fully developed testis, or residual testis in a fully developed ovary has been reported previously in black bream (Haddy and Pankhurst, 1998; Sarre and Potter, 1999; Partridge *et al.*, 2003), and is common in the closely related yellowfin bream (*Acanthopagrus australis*) (Pollock, 1985). In the present study, 13 fish from a total sample of 240 fish had a macroscopic ovotestis. All ovotestes observed in the present study consisted of distinct ovarian tissue (Stage 2 appearance) in the dorsal, anterior portion of a mature testis (Fig. 7.4), and all of these fish produced milt.

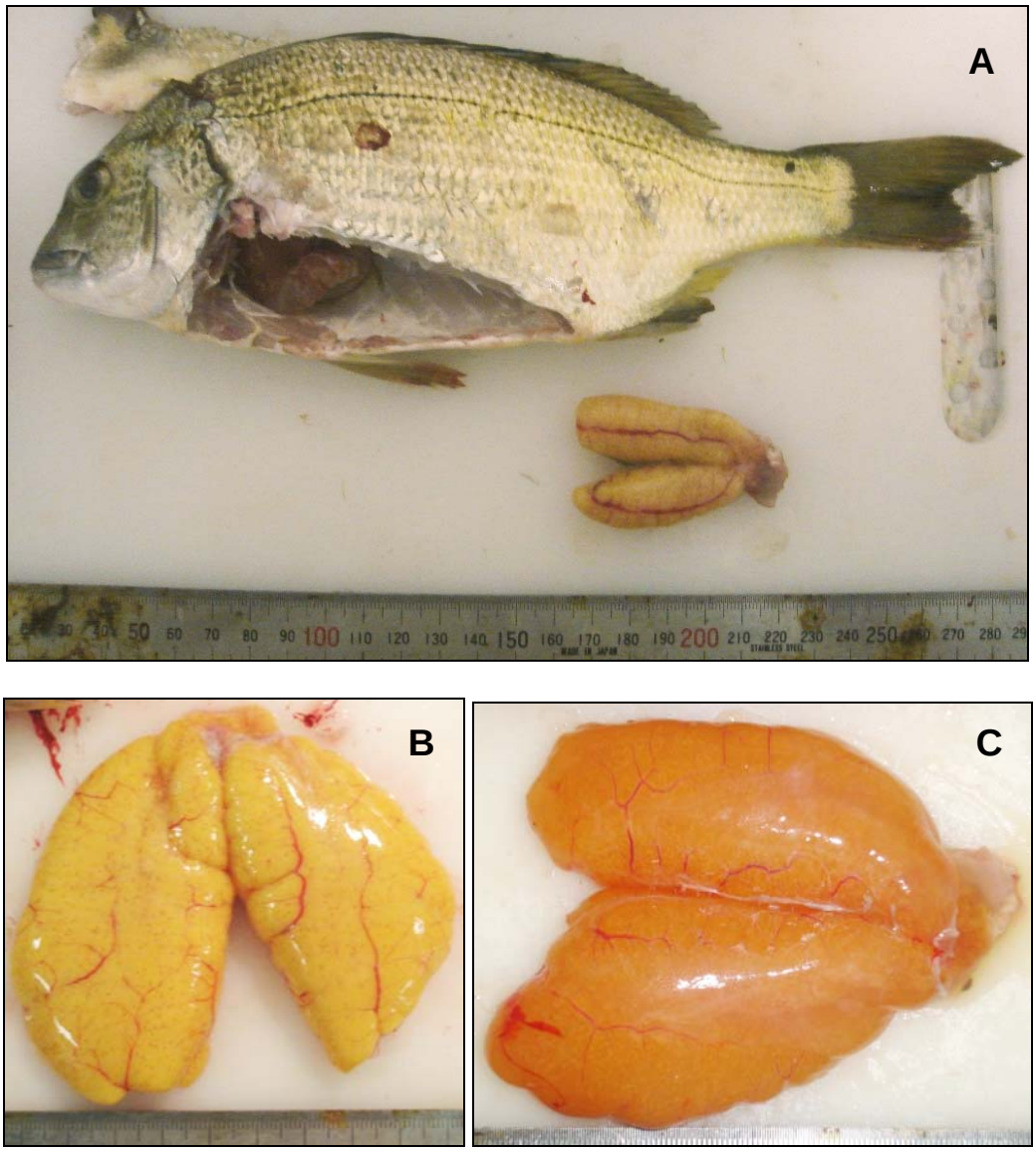


Figure 7.2 A) Female black bream with ovaries removed; B) vitellogenic ovaries – opaque and orange; C) hydrated ovaries – orange, hydrated oocytes visible and eggs are easily extruded from oviduct.

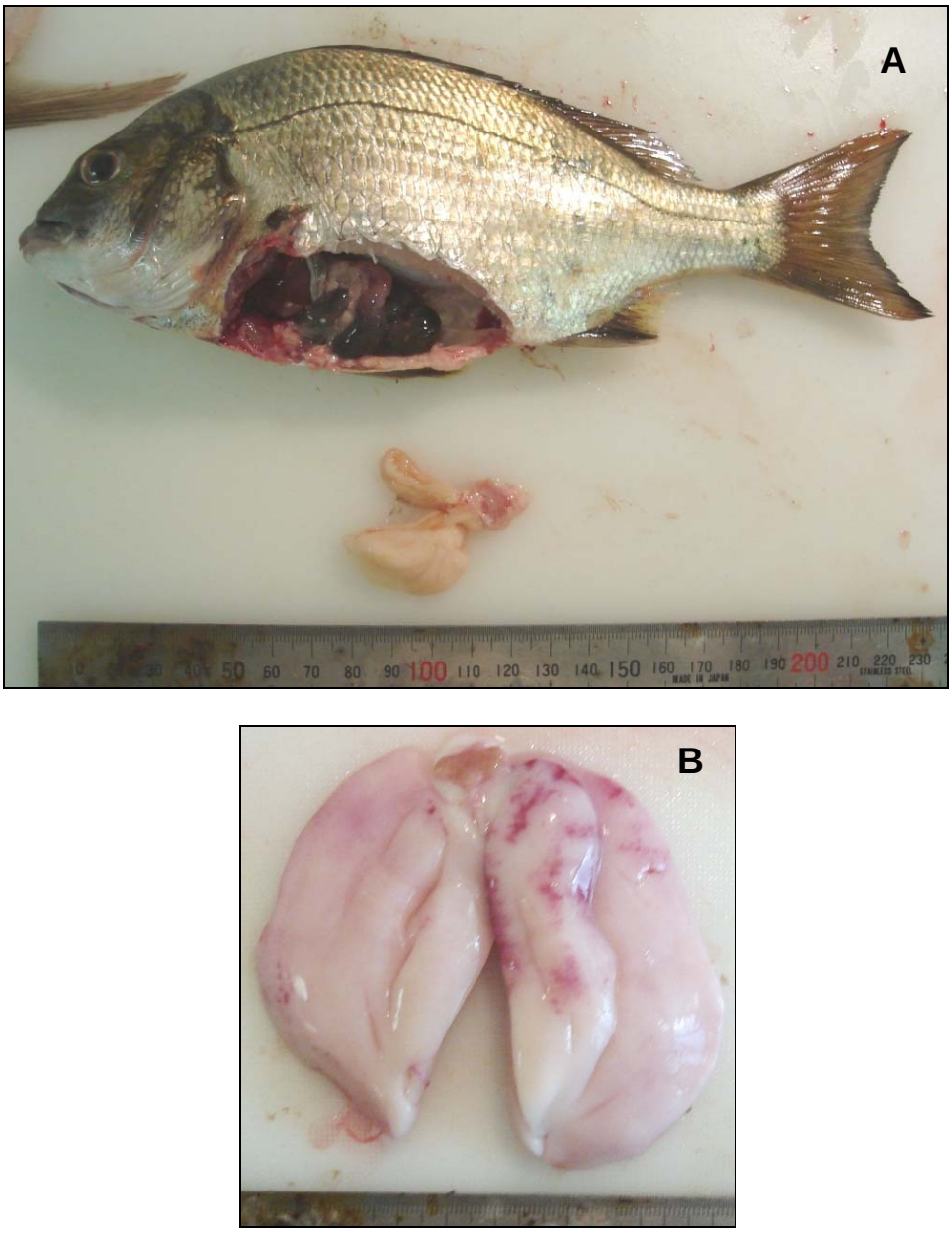


Figure 7.3 A) Male black bream with testis removed; B) mature testis – large, firm, creamy white and milt is easily extruded from the sperm duct.

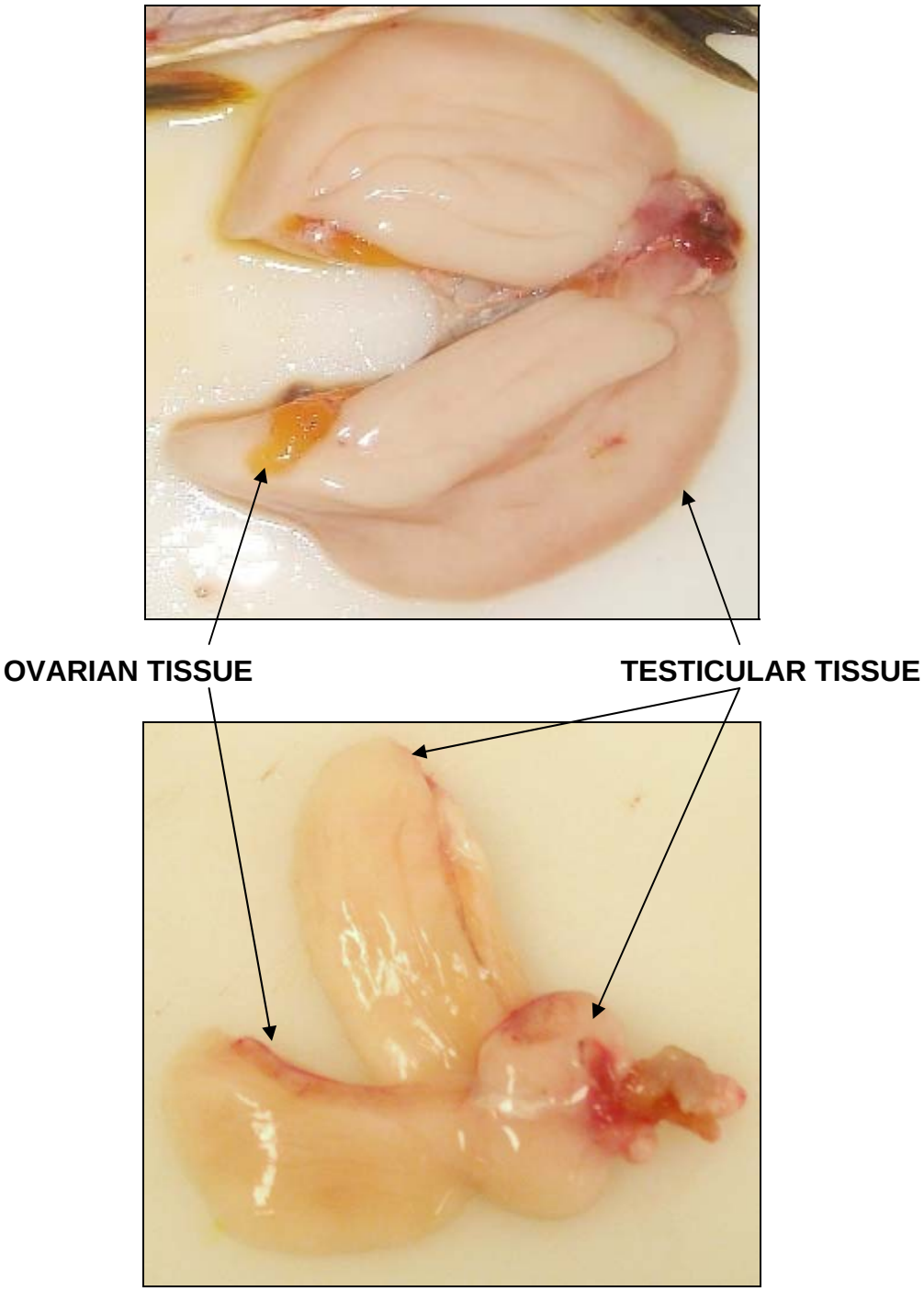


Figure 7.4 Black bream ovotestis, with distinct ovarian tissue (orange colour) in the anterior portion of a mature testis (creamy white colour, milt present).

The highest GSI values for fish collected from Mallacoota Inlet (December 2006, November 2007) were 5.66 (female) and 7.76 (male) and there were no significant differences between sexes (p = 0.880) (Table 7.4). In both sexes the GSI was not related to fork length (R^2 < 0.500) (Appendix 3). The LSI values for female fish ranged from 0.72-1.80 and from 0.64-2.65 in males, and there were no significant differences between the sexes (p = 0.992) (Table 7.4). In both sexes the LSI was not related to fork length (R^2 < 0.500), however in females, the LSI values did appear to increase slightly with increasing fork length (Appendix 3). Condition factor values ranged from 1.74-2.15 in females and 1.90-2.29 in males, and there were no significant differences between the sexes (p = 0.410) (Table 7.4). In both sexes the condition factor was not related to fork length (R^2 < 0.500) (Appendix 3).

The highest GSI values for fish collected from the Yarra River (September - December 2007) were 4.92 (female) and 6.17 (male) and there were differences between sexes, with males having significantly higher GSI values than females (p = 0.003) (Table 7.4). In both sexes the GSI was not related to fork length (R^2 < 0.500) (Appendix 3). The LSI values for female fish ranged from 0.85-2.60 and from 0.66-1.94 in males, and there were no significant differences between the sexes (p = 0.168) (Table 7.4). In both sexes the LSI was not related to fork length (R^2 < 0.500) (Appendix 3). Condition factor values ranged from 1.75-2.47 in females and 1.44-2.10 in males, and there were no significant differences between the sexes (p = 0.221) (Table 7.4). In both sexes the condition factor was not related to fork length (R^2 < 0.500) (Appendix 3).

Many of the smaller fish (< 175 mm) that were caught from the Maribyrnong River (October - December 2007) had very small gonads (<1.0 g) so the GSI was only calculated for 4 females and 3 males. The highest GSI values were 2.52 (female) and 3.95 (male) and there were differences between sexes, with males having significantly higher GSI values than females (p = 0.045) (Table 7.4). The LSI values for female fish ranged from 1.20-1.92 and from 1.19-2.60 in males, and there were no significant differences between the sexes (p = 0.538) (Table 7.4). In both sexes the LSI was not related to fork length (R^2 < 0.500) (Appendix 3). Condition factor values ranged from 1.73-2.03 in females and 1.80-2.05 in males, and there were no significant differences between the sexes (p = 0.434) (Table 7.4). In both sexes the condition factor was not related to fork length (R^2 < 0.500) (Appendix 3).

The highest GSI values for fish collected from the Werribee River (October 2007) were 12.4 (female) and 15.4 (male) and there were no significant differences between sexes (p = 0.521) (Table 7.4). In both sexes the GSI was positively related to fork length (R^2 > 0.500), with the GSI increasing rapidly as fork length increased (Appendix 3). The LSI values for female fish ranged from 0.72-3.08 and from 0.90-1.74 in males, and there were no significant differences between the sexes (p = 0.800) (Table 7.4). In both sexes the LSI was not related to fork length (R^2 < 0.500), however in females, the LSI values did appear to increase slightly with increasing fork length (Appendix 3). Condition factor values ranged from 1.80-2.57 in females and 1.56-2.64 in males, and there were no significant differences between the sexes (p = 0.545) (Table 7.4). In both sexes the condition factor was not related to fork length (R^2 < 0.500) (Appendix 3).

The highest GSI values for fish collected from the Little River (December 2007) were 12.5 (female) and 7.03 (male) and there were no significant differences between sexes (p = 0.187) (Table 7.4). In both sexes the GSI was not related to fork length ($R^2 < 0.500$), however the values did appear to increase slightly with increasing fork length (Appendix 3). The LSI values for female fish ranged from 0.35-2.72 and from 0.80-2.96 in males, and there were differences between the sexes (p = 0.028), with females having significantly higher LSI values than males (Table 7.4). In both sexes the LSI was not related to fork length ($R^2 < 0.500$) (Appendix 3). Condition factor values ranged from 1.79-2.46 in females and 1.82-2.29 in males, and there were no significant differences between the sexes (p = 0.538) (Table 7.4). In both sexes the condition factor was not related to fork length ($R^2 < 0.500$) (Appendix 3).

Table 7.4 Mean (± SEM) values for tissue indices, quantitative plasma vitellogenin levels (RT-VtgEq-mg/ml) and plasma protein levels (mg/ml) in black bream captured from five different Victorian estuaries between September and December.

SITE	*n=*	GSI (%)	LSI (%)	K (%)	Vitellogenin RT-VtgEq (mg/ml)	Plasma protein (mg/ml)
MALLACOOTA						
Female	*13*	2.32 ± 0.61	1.10 ± 0.10	1.98 ± 0.03	10.2 ± 3.55 *(n=3)*	48.98 ± 7.37
Male	*13*	2.46 ± 0.76	1.10 ± 0.19	2.02 ± 0.03	**b.d.*	50.05 ± 5.31
YARRA						
Female	*22*	2.23 ± 0.47	1.51 ± 0.09	1.94 ± 0.03	6.98 ± 2.38 *(n=12)*	40.57 ± 2.50
Male	*15*	5.11 ± 0.82	1.30 ± 0.12	1.88 ± 0.04	**b.d.*	37.51 ± 3.54
MARIBYRNONG						
Female	*10*	1.29 ± 0.82 *(n=4)*	1.78 ± 0.09	1.89 ± 0.03	12.7 *(n=1)*	36.04 ± 2.45
Male	*5*	2.98 ± 0.22 *(n=3)*	1.58 ± 0.24	1.94 ± 0.05	**b.d.*	36.78 ± 3.87
WERRIBEE						
Female	*22*	6.85 ± 0.81	1.83 ± 0.13	2.07 ± 0.05	28.7 ± 3.17 *(n=20)*	59.99 ± 4.89
Male	*17*	7.29 ± 1.10	1.21 ± 0.06	2.12 ± 0.06	*b.d.*	48.11 ± 5.10
LITTLE						
Female	*15*	6.58 ± 0.87	1.82 ± 0.13	2.07 ± 0.04	26.6 ± 3.35 *(n=15)*	58.35 ± 5.03
Male	*21*	5.58 ± 0.37	1.38 ± 0.11	2.04 ± 0.03	*b.d.*	42.58 ± 2.62

b.d – below detection
(n=) number of fish used for the analysis when not all could be used.

7.3.3 Vitellogenin detection

7.3.3.1 Quantitative ELISA

Vitellogenin was detected in female black bream from all field sites, but not detected in any males at all, even those that had an ovotestis (Fig. 7.5-7.9). The concentrations were variable which is probably due to the differences in sampling times (seasonal variability) as well as differences in the size, age and reproductive condition of the fish at the different field sites (Table 7.3, 7.4). Despite only including fish that were sampled between September and December (the black bream spawning season), a number of females from Mallacoota Inlet (10 out of a total of 45), Yarra River (10 out of a total of

32) and Maribyrnong River (9 out of a total of 22) had Vtg levels below detection, resulting in low *n* values for these sites (Table 7.4).

The smallest female from Mallacoota Inlet with a detectable concentration of Vtg in blood plasma was 260 mm, 361 g, 5 years old, and had a concentration of 6.82 RT-VtgEq-mg/ml. The ovaries of this fish were vitellogenic (Stage 3). The largest fish with a detectable concentration of Vtg in blood plasma was 360 mm, 971 g, and had a concentration of 15.9 RT-VtgEq-mg/ml. The age of this fish was not determined, however based on the size in comparison to other females from Mallacoota Inlet, it is estimated that it would have been at least 7 years old. This fish also had the highest Vtg concentration of all Mallacoota Inlet females, and the ovaries were vitellogenic (Stage 3). Plasma Vtg levels in fish from Mallacoota Inlet were not related to GSI, LSI or condition factor ($R^2 < 0.500$) (Appendix 4).

The smallest female from the Yarra River with a detectable concentration of Vtg in blood plasma was 170 mm, 86 g, 2 years old, and had a concentration of 0.73 RT-VtgEq-mg/ml. The ovaries of this fish were vitellogenic (Stage 3). The largest fish with a detectable concentration of Vtg in blood plasma was 310 mm, 553 g, 9 years old and had a concentration of 15.7 RT-VtgEq-mg/ml. The ovaries of this fish were vitellogenic (Stage 3). The highest Vtg concentration observed in a female from the Yarra River was 24.7 RT-VtgEq-mg/ml, and the fish was 260 mm, 366 g and 8 years old, with vitellogenic (Stage 3) ovaries. Plasma Vtg levels in fish from the Yarra River were not related to GSI, LSI or condition factor ($R^2 < 0.500$) (Appendix 4).

Vitellogenin was only detected in one fish from the Maribyrnong River, a female measuring 255 mm, 318 g and 9 years old. The concentration was 12.7 RT-VtgEq-mg/ml, and this was the largest fish collected from the Maribyrnong River between September and December. The ovaries were observed to be in a hydrated state (Stage 4). Plasma Vtg levels in fish from the Maribyrnong River were not related to GSI, LSI or condition factor ($R^2 < 0.500$) (Appendix 4).

The smallest female from the Werribee River with a detectable concentration of Vtg in blood plasma was 205 mm, 176 g and had a concentration of 14.5 RT-VtgEq-mg/ml. The age of this fish was not determined, however based on the size in comparison to other females from the Werribee River, it is estimated that it would have been 2-3 years old. The ovaries of this fish were vitellogenic (Stage 3). The largest fish with a detectable concentration of Vtg in blood plasma was 360 mm, 1200 g, 10 years old and had a concentration of 33.2 RT-VtgEq-mg/ml. The ovaries of this fish were vitellogenic (Stage 3). The highest Vtg concentration observed in a female from the Werribee River was 59.5 RT-VtgEq-mg/ml, and the fish was 245 mm, 302 g and estimated to be 3 years old, with ovulated (Stage 5) ovaries. Plasma Vtg levels in fish from the Werribee River were not related to GSI, LSI or condition factor ($R^2 < 0.500$) (Appendix 4).

The smallest female from the Little River with a detectable concentration of Vtg in blood plasma was 225 mm, 245 g, 3 years old, and had a concentration of 21.6 RT-VtgEq-mg/ml. The ovaries of this fish were hydrated (Stage 4). The largest fish with a detectable concentration of Vtg in blood plasma was 345 mm, 783 g, 8 years old and had

a concentration of 44.5 RT-VtgEq-mg/ml. The ovaries of this fish were ovulated (Stage 5). The highest Vtg concentration observed in a female from the Little River was 46.9 RT-VtgEq-mg/ml, and the fish was 230 mm, 237 g and 5 years old, with vitellogenic (Stage 3) ovaries. Plasma Vtg levels in fish from the Little River were not related to GSI, LSI or condition factor ($R^2 < 0.500$) (Appendix 4).

7.3.3.2 Coomassie blue total protein staining

The mean protein concentrations in plasma from field collected black bream ranged between 36-60 mg/ml (Table 7.4). Total protein staining of polyacrylamide gels following electrophoresis showed a similar general protein profile between males and females from all field sites, and in some females a prominent band was observed at ~ 200 kDa (Fig. 7.5-7.9). This female-specific band appeared at the same position on the gel as the positive control (POS) sample appeared, suggesting the band was Vtg.

7.3.3.3 Western blot

Western blot analysis confirmed the Coomassie blue stained protein band observed in some female samples was Vtg (Fig. 7.5-7.9). Furthermore, as was observed in the ELISA, Vtg was detected in some, but not all of the 5 randomly selected female plasma samples tested from each field site. Based on the absence of any bands on Western blot, Vtg was not detected in males from any of the field sites (Fig. 7.5-7.9).

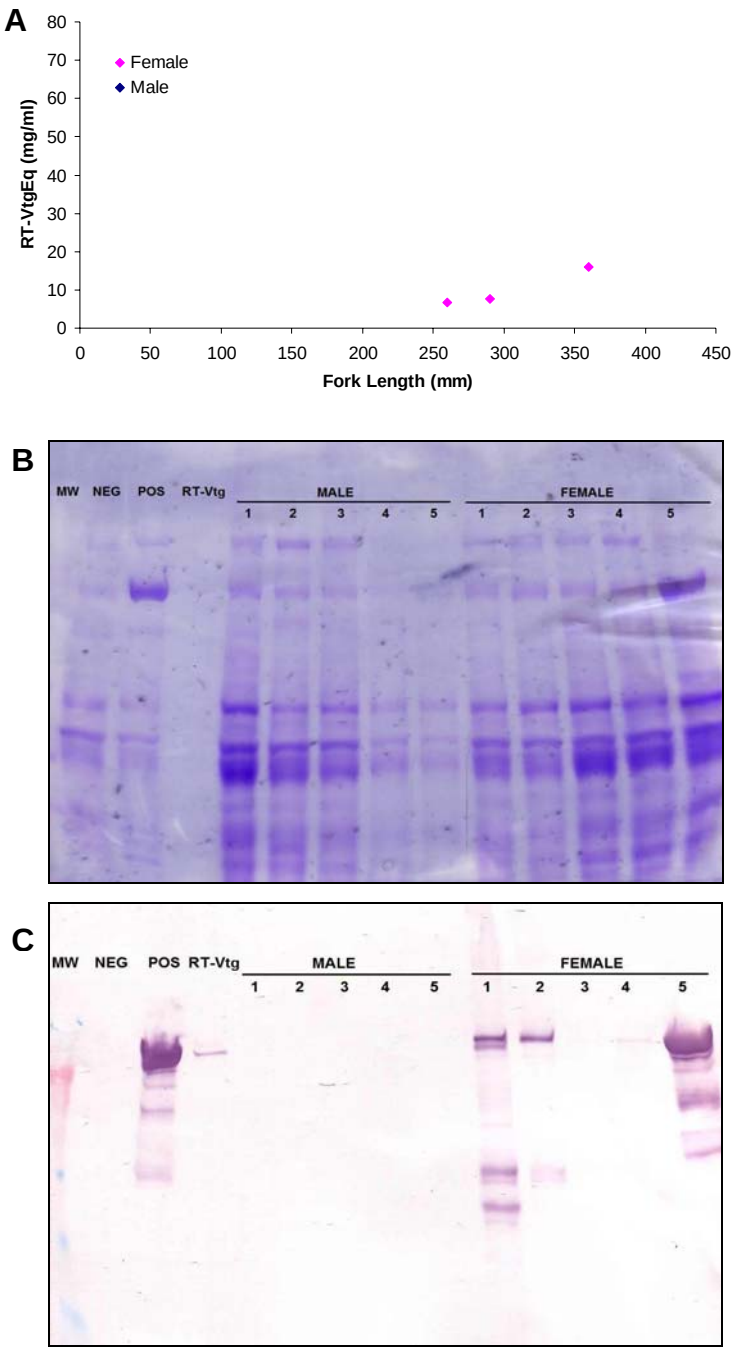


Figure 7.5 Vtg detection in male and female black bream captured from Mallacoota Inlet during December 2006 and November 2007 A) quantitative ELISA concentration (RT-VtgEq-mg/ml); B) Coomassie blue stained gel for total protein; and C) Western blot. Positive (POS) and negative (NEG) black bream and rainbow trout Vtg (RT-Vtg) controls also shown.

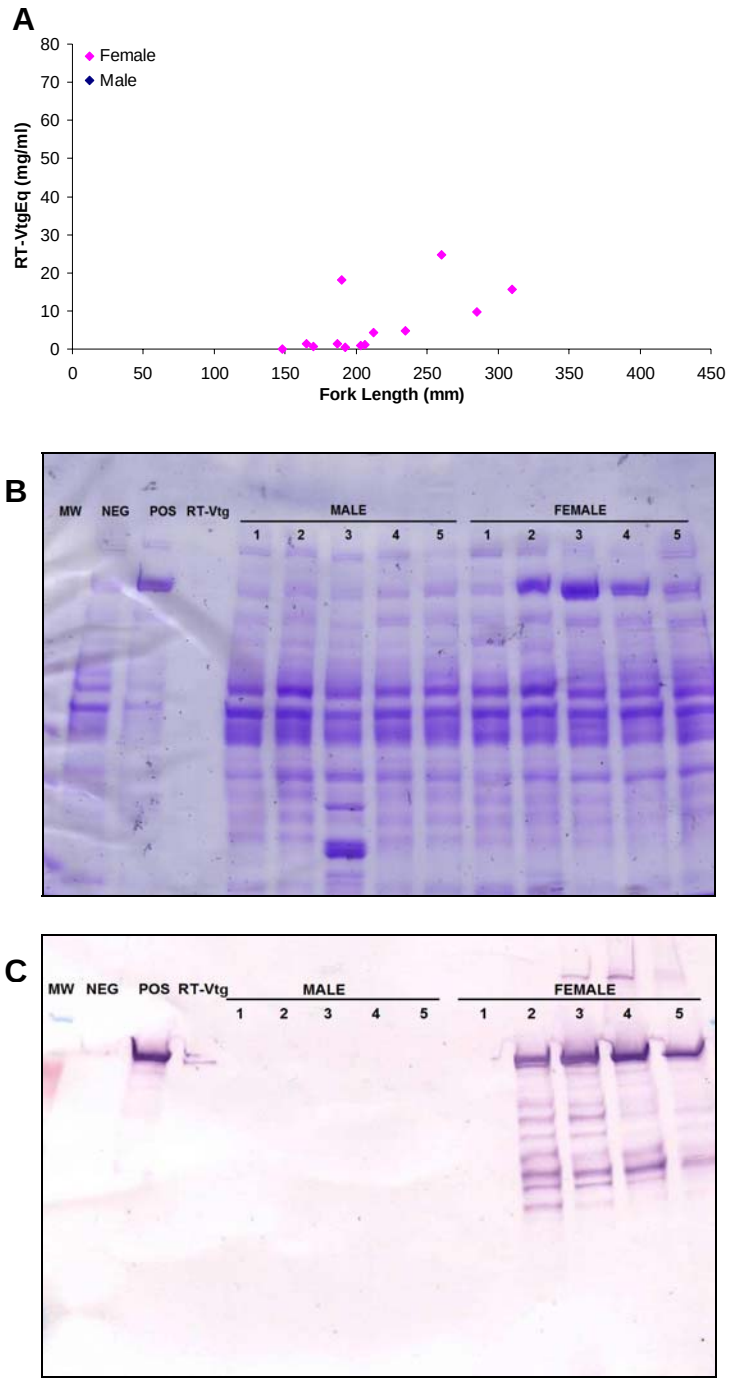


Figure 7.6 Vtg detection in male and female black bream captured from Yarra River between September and December 2007 A) quantitative ELISA concentration (RT-VtgEq-mg/ml); B) Coomassie blue stained gel for total protein; and C) Western blot. Positive (POS) and negative (NEG) black bream and rainbow trout Vtg (RT-Vtg) controls also shown.

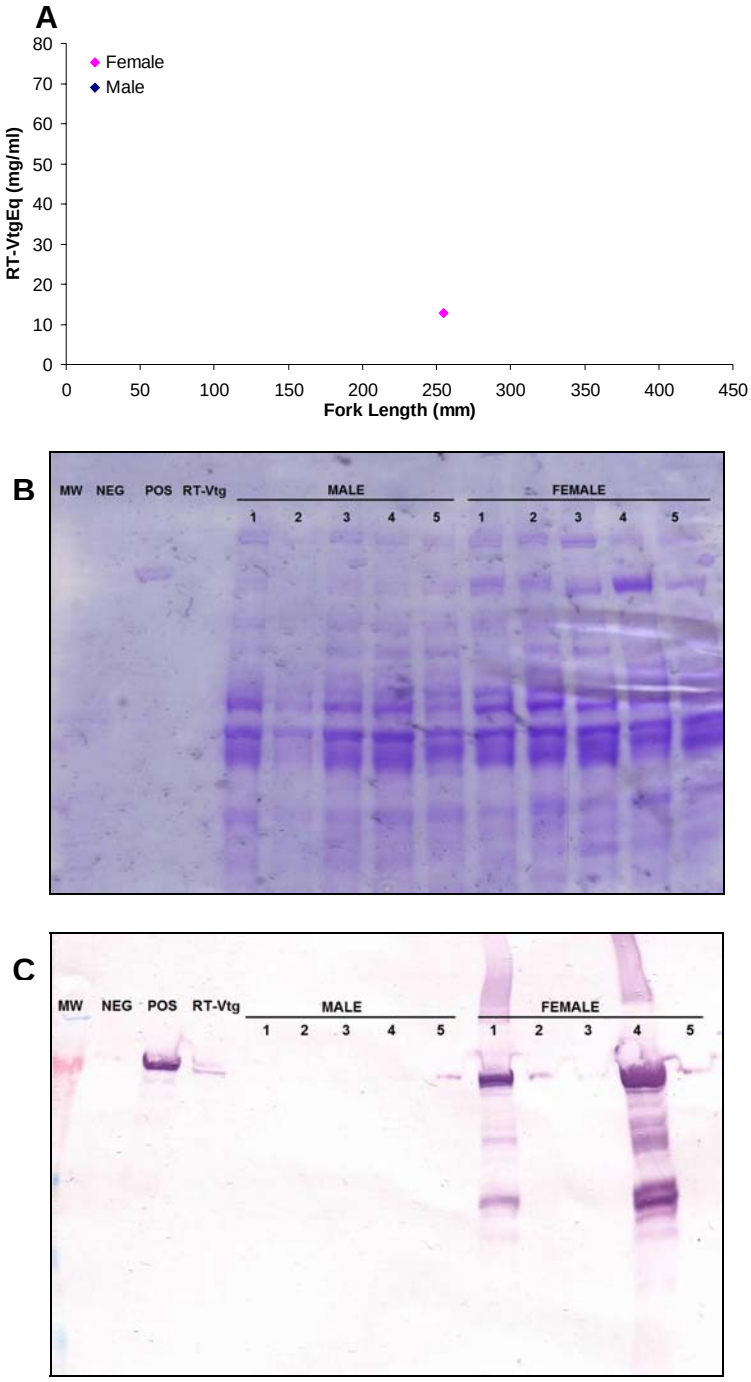


Figure 7.7 Vtg detection in male and female black bream captured from Maribyrnong River between October and December 2007 A) quantitative ELISA concentration (RT-VtgEq-mg/ml); B) Coomassie blue stained gel for total protein; and C) Western blot. Positive (POS) and negative (NEG) black bream and rainbow trout Vtg (RT-Vtg) controls also shown.

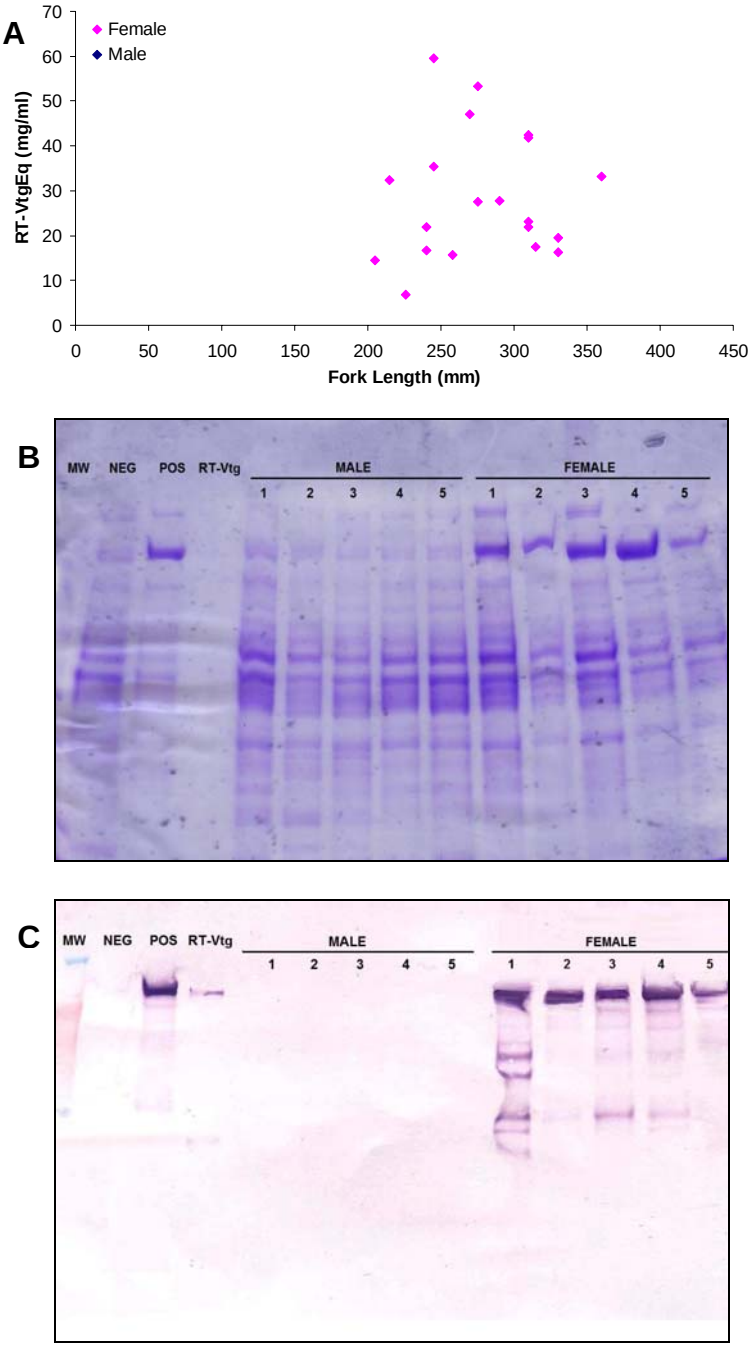


Figure 7.8 Vtg detection in male and female black bream captured from Werribee River during October 2007 A) quantitative ELISA concentration (RT-VtgEq-mg/ml); B) Coomassie blue stained gel for total protein; and C) Western blot. Positive (POS) and negative (NEG) black bream and rainbow trout Vtg (RT-Vtg) controls also shown.

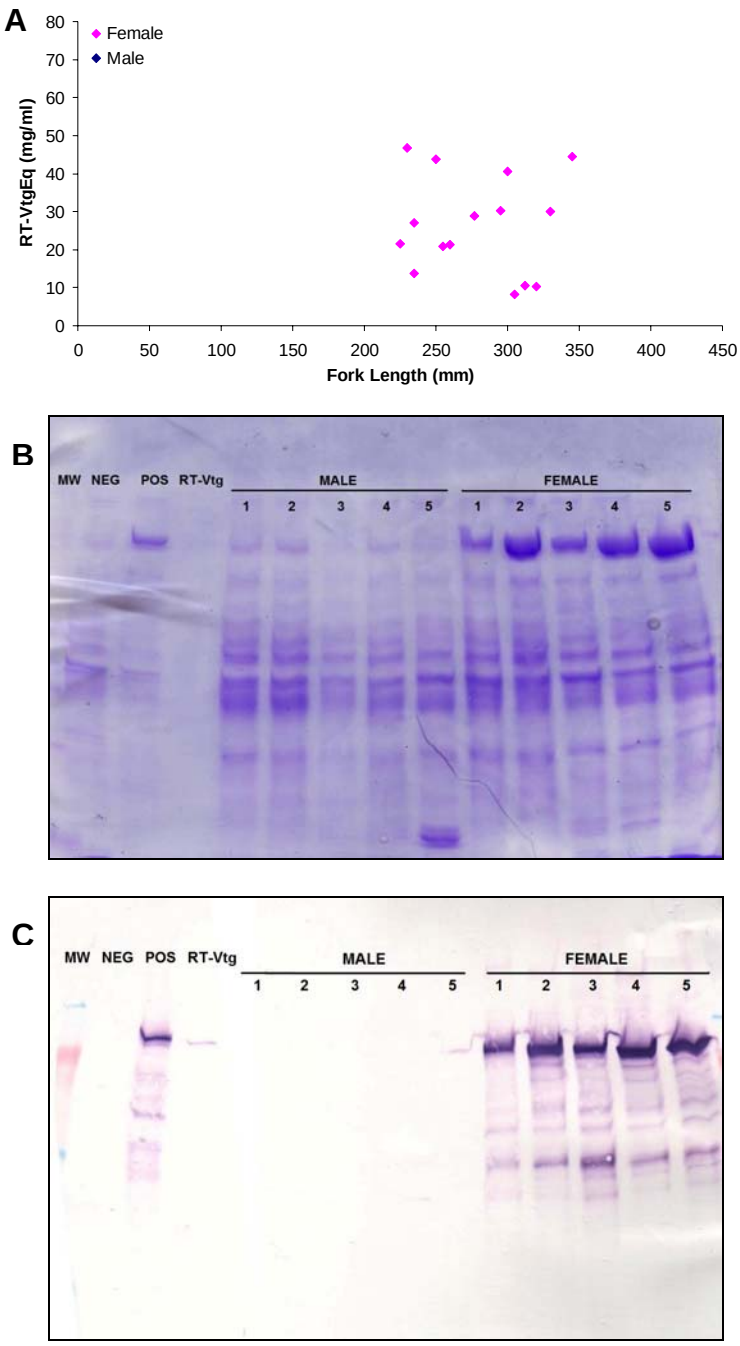


Figure 7.9 Vtg detection in male and female black bream captured from Little River during December 2007 A) quantitative ELISA concentration (RT-VtgEq-mg/ml); B) Coomassie blue stained gel for total protein; and C) Western blot. Positive (POS) and negative (NEG) black bream and rainbow trout Vtg (RT-Vtg) controls also shown.

7.4 Discussion

This study was successful in measuring Vtg concentrations in black bream collected from a variety of Victorian field sites that are subject to different types of environmental stressors. Sampling was restricted because of limitations of time, personnel and fishing permit restrictions. Consequently, the data set was collected over a protracted period, which encompassed spawning and non-spawning seasons and unfortunately, comparable samples between sites for each season were not attained. For these reasons physiological analyses were restricted to fish collected during the spawning season (September-December) only, which reduced sample sizes to between 15-39 fish per field site.

7.4.1 Field sites

Water quality at the 5 field sites was substantially different, with DO levels ranging from 53-89%S, salinity 14.2-36.4 ppt and temperature 12.5-23.2^{o}C. Despite this, sexually mature fish (ie. Stage 4 gonads) were observed at 3 of the sites, namely the Maribyrnong River, the Werribee River and the Little River. The average DO level in the Yarra River was only 53%S, the salinity was 14.2 ppt and the temperature was 20^{o}C, and none of the 22 females that were captured between September and December had hydrated or ovulated ovaries, and very few (4) had detectable concentrations of Vtg in blood plasma. This finding may suggest that fish from the Yarra River are experiencing hypoxic stress, resulting in lowered reproductive condition. Indeed, chronic exposure to hypoxia has been shown to cause significant reductions in serum levels of the sex hormones testosterone and E2 in carp (Wu *et al.* 2003); Vtg levels in Atlantic croaker (Thomas *et al.*, 2006); and sex hormones in Gulf killifish (Landry *et al.*, 2007).

Unlike the Yarra River, the water quality at Mallacoota Inlet was very good (89.6%S, 32.0 ppt, 23.2^{o}C), so the lack of hydrated or ovulated ovaries in any of the 13 females sampled may indicate that they had already spawned. Butcher (1945) reported that black bream populations east of the Gippsland Lakes (eg. Mallacoota Inlet) spawn earlier than fish within the Gippsland Lakes, and spawn progressively later westwards. Fish were captured in Mallacoota Inlet in December 2006 or November 2007, and the average water temperature was 3^{o}C warmer than the Yarra River. The timing of spawning is known to be linked with both temperature and salinity in black bream, with populations in high salinity, high temperature conditions spawning earlier than fish in low salinity and low temperature conditions (Haddy and Pankhurst, 1998; Sarre and Potter, 1999). Therefore it is likely that the fish captured in Mallacoota Inlet had already spawned at the time of collection, which is further supported by the Vtg concentrations in plasma which were low, or below detection for female fish from Mallacoota Inlet. Another potential cause of low Vtg in the blood plasma of fish collected during the spawning season could be physiological stress associated with capture. Capture stress has been reported to cause elevated cortisol levels and reduced E2 and testosterone in black bream (Haddy and Pankhurst, 1998). Reduced steroidogenesis could affect Vtg levels, however, it is unlikely that capture stress would affect gonad condition at sampling.

7.4.2 Biological characteristics

7.4.2.1 Size, age and condition

Otolith analysis revealed differences in the age of black bream sampled in this study. The oldest fish were caught in the Werribee River and Little River, and the age, length

and weight distributions were similar for males and females from each site. There were no sex-specific differences in size but there were differences in the length of fish of the same age in different estuaries. Black bream from the Yarra River and Maribyrnong River were generally smaller than black bream from the Werribee River and Little River for the same age, and fish from Mallacoota Inlet were in between. Potential differences in growth could be related to environmental stressors, and field studies with black bream have demonstrated that there can be substantial differences in both the size-at-age and size-at-maturity for black bream from estuaries with different environmental conditions (Morison *et al.*, 1998; Sarre and Potter, 2000).

Differences were observed in the three physiological tissue indices and in some cases sex-specific differences were noted. In fish from some sites, the GSI appeared to increase with fork length, although the correlation was weak ($R^2 < 0.500$). For fish captured in the Maribyrnong River and Yarra River, the GSI was significantly higher in males than females. For the other sites, Mallacoota Inlet, Werribee River and Little River, there were no significant differences in the GSI between males and females.

No sex-specific differences were observed for the LSI. The lowest LSI values were observed in fish from Mallacoota Inlet (1.10%), and this supports the suggestion that fish from this site had already spawned when they were collected. The LSI can act as an indicator of nutritional status, since the liver is a major site for lipid and glycogen storage. High LSI values may indicate that the fish has good stores of lipids and carbohydrates for growth, whilst low LSI values may indicate poor stores of lipids and

carbohydrates. Since the liver is the site of Vtg production in female fish, enlargement is normal when active vitellogenesis is taking place. In a study of black bream from a range of estuaries in Western Australia (Webb and Gagnon, 2002) reported increases in the LSI of reproductively active females, whilst Haddy and Pankhurst (1998) observed a peak in the LSI of female black bream from Tasmania prior to spawning, followed by a gradual decrease to the lowest levels at the same time as GSI values were at the lowest.

The relationship between LSI values and fork length were different between field sites, with some showing a slightly positive relationship with fork length and others showing a negative relationship with fork length, although none were strongly correlated ($R^2 < 0.500$). Aside from those captured in the Little River, all females showed a slight increase in LSI with increasing fork length. The ovaries in a number of females from the Little River were observed to be hydrated, ovulated or spent, and it was the larger females that were in these stages, indicating that spawning was actively occurring, or had recently occurred in these fish. The apparent decrease in LSI with size may be due to this, in that the larger females were spawning or had already spawned, and hence a decrease in the LSI would be expected.

In males, the LSI values showed a slight decrease with increasing fork length at all sites except for the Maribyrnong River. Again, this may be due to the stage of gonad development, since most males from all sites apart from the Maribyrnong River were observed to be producing milt, and thus the energy reserves in the liver would be depleted. The condition factor displayed no sex-specific differences, was not correlated

with fork length ($R^2 < 0.500$), and was highest in fish from the Werribee River and Little River.

7.4.2.2 Macroscopic gonad appearance, sex ratios and ovotestis

In female black bream, vitellogenic (Stage 3) ovaries were first observed at 190 mm, and maturing females, with hydrated (Stage 4) or ovulated (Stage 5) ovaries were first observed at a size of 195 mm, whilst in males, milt was first observed in fish measuring 136 mm. Maturing gonads were observed in fish that were captured from sites with varying environmental conditions, indicating that adults are tolerant of a range of conditions. Similar findings have been reported in other studies of sexual maturation in black bream. Haddy and Pankhurst (1998) reported reproductively active black bream (size not indicated) in the Meredith River and Swan River in Tasmania that were collected over a wide range of environmental conditions: DO - 4.2-13.6 mg/l (~50-150%S), salinity - 13.9-35.0 ppt, and temperature - 15.5-26.2^{o}C. Likewise, Sarre and Potter (1999) reported successful spawning in black bream from two Western Australian estuaries, the Moore River estuary with salinities as low as 3.5-8.0 ppt, and the Wellstead Estuary, with salinities ranging from 40.7-45.2 ppt. The fish in these two estuaries attained maturity at a significantly smaller size (~129-159 mm) compared to fish in the other estuaries studied (~158-218 mm) (Sarre and Potter, 1999), which may be a compensatory response to salinity stress. Thus, it appears that black bream can produce gametes in highly variable environmental conditions, indicating that it might be the subsequent survival of gametes and eventually embryos that are sensitive to environmental conditions. Indeed this was observed when early life stage black bream

were challenged with various environmental conditions in laboratory experiments (see chapter 5).

The sex ratios of black bream in this study were generally female biased, with the percentage of females ranging from 42-69%, however the sex ratios showed no difference with age. Rowland and Snape (1994) reported a female bias in small black bream and a male bias in larger black bream collected from two locations in southern New South Wales, yet found no such bias in fish collected from the Gippsland Lakes in Victoria. In contrast, Sarre (1999) reported no bias in sex ratios of black bream collected from the Swan River, Western Australia, except during the spawning season, when a male bias was observed, which was attributed to the aggregation of males during this time. Further studies are required to understand sex ratios in Victorian black bream, since the present study was limited in both timing of sampling and fishing gears used for collection. Standardised sampling, using the same fishing gear, in order to collect a similar size distribution of fish, conducted during the same season, and preferably across multiple seasons would greatly improve our understanding of sex ratios.

All gonads were preserved for histological examination, and are currently being processed by Dr. Susan Jobling of Brunel University in the United Kingdom. Preliminary results suggest that intersex males (containing oocytes within otherwise normal testes) occur at all of the field sites, however the incidence and severity of intersex is lower at Mallacoota Inlet compared to all other sites (Jobling, S. pers. comm.). There doesn't appear to be any trends with age and there is no indication of degenerative

gonadal tissue, which would be expected if the gonad was undergoing sex change (ie. protandry or protogyny). This work is on-going and further samples from all field sites are being collected, to provide comparable samples between sites for both time of collection (season) and in size distribution. Water samples are also being collected from all sites to be tested for total estrogen levels.

With continued sampling of Victorian black bream populations, spawning aggregations, potential sex change processes and the influence of environmental stressors on sex determination, sex differentiation and gonad development could be elucidated, and this information could be used to improve the management of black bream stocks.

7.4.2.3 Vtg levels

Vitellogenin was detected in some female black bream from all field sites which was expected since sampling occurred during the spawning season when circulating Vtg levels would be high due to egg production. Vtg concentrations were highest in the larger females (Stage 3 – Stage 5 ovaries) from the Werribee River and Little River, with all concentrations greater than 10 RT-VtgEq-mg/ml, and the highest concentration being in a 245 mm, 3 year old fish with ovulated ovaries from the Werribee River at 59.5 RT-VtgEq-mg/ml. Female black bream from the other 3 sites had lower concentrations of Vtg in their plasma (1-25 RT-VtgEq-mg/ml) whilst a number of females had levels below detection. These results indicate that the Vtg assay works well and can quantitatively detect concentrations of Vtg in blood plasma from field collected fish. The use of Vtg as a measure of reproductive condition in females may be possible, since all females that

had detectable concentrations of Vtg were observed to have ovaries that were in at least Stage 3 (vitellogenic) of development and the concentrations increased as the ovaries matured to become hydrated and eventually ovulated. However, interpretation of elevated Vtg levels needs to be carefully considered, since other factors may also influence Vtg levels in fish, such as agonistic or antagonistic effects of different xenobiotics on different stages of Vtg production and regulation (Kime *et al.*, 1999).

In the present study, Vtg was not detected in males from any field sites, even those with a distinct ovotestis. Western blot confirmed this, with no male samples having a protein detected at ~200 kDa, whilst for females the protein band was observed in some, but not all plasma samples. This observation could indicate two different things. Firstly, it may indicate that black bream are rudimentary hermaphrodites, and that the gonads initially differentiate into ovaries, and then later a proportion of them change to testes and the ovarian tissue regresses and does not produce eggs. Secondly, the Vtg levels in males with ovotestes may be below the level of detection in the ELISA. In all cases the ovarian section of the ovotestis did resemble a regressed (Stage 2) ovary. Histological examination of the gonads will assist in determining whether or not the ovarian tissue in fish with ovotestes contains developing (vitellogenic) oocytes. Indeed, the total estrogen concentrations measured for some of the field sites in this study were very low (1.4-3.6 ng/l). These values are lower than levels reported to induce Vtg in juvenile and adult life stages of a range of teleost test species, which are generally greater than 20 ng/l for waterborne exposures to E2 (Kramer *et al.*, 1998; Routledge *et al.*, 1998; Van den Belt *et*

al., 2003). Vitellogenin induction, as a dose-dependent response to estrogenic compounds was not tested in this study.

Vitellogenin has been detected and quantified in a number of other sparid species, in both field studies to determine 'natural' concentrations, as well as controlled laboratory experiments to determine Vtg induction. In the common Dentex (*Dentex dentex*) Vtg was not detectable in 8-20 month old fish, but increased to a peak of 1.3 mg/ml in 22 month old fish (Pavlidis *et al.*, 2000). In the red porgy (*Pagrus pagrus*), Vtg levels have been reported at peak concentrations of 405.5 µg/ml during the spawning season (Kokokiris *et al.*, 2001). In the Gilthead seabream (*Sparus aurata*), Vtg levels of 1.5 mg/ml were detected following i.p. administration of 5 mg/kg E2 dissolved in cocoa butter (Mosconi *et al.*, 1998) and Vtg production (12 mg/ml) was observed in black porgy (*Acanthopagrus schlegeli*) following exposure to E2 in feed (4 mg/kg) over a 4 month period (Chang *et al.*, 1996). Conducting dose-response experiments in both juvenile and adult black bream would assist in determing what concentrations of estrogenic chemicals induce Vtg synthesis, whilst repeated sampling over a number of months would help determine how high Vtg concentrations get in naturally maturing female black bream. Knowledge of both of these aspects of Vtg production in black bream would then enable the development of testing procedures to screen both reproductive condition in females, and for evidence of exposure to estrogenic EDCs in males.

In the present study, all large (> 215 mm) females with Stage 3+ gonads had detectable concentrations of Vtg in blood plasma, as indicated by ELISA and Western blot, whilst in

males no Vtg was detected. The absence of Vtg in males may represent a potential biomarker that could be used to identify sex in adult fish, assuming that exposure to any compounds that could induce Vtg synthesis in males (ie. exposure to estrogenic EDCs) could be categorically ruled out.

Understanding sex ratios and sex-specific differences in growth are important considerations for fish biologists (Evans and Claiborne, 2006). Several lethal and non-lethal techniques have been used for sex identification and assessment of maturity in fish. The most common and effective methods for assessing gonad condition are lethal and involve dissection of the gonads for histological examination (Hoar, 1969; Chang and Yueh, 1990; Sarre and Potter, 1999). Whilst direct gonad observations can be conducted for sex determination of individuals, the fact that they are lethal procedures prevents the continued monitoring of the same individual over time. A number of non-lethal techniques have been developed, including both invasive and non-invasive techniques. Invasive methods include endoscopy, biopsy, cannulation and blood sampling and non-invasive methods include techniques such as ultrasound and mucus collection (Crim and Glebe, 1990). Urogenital cannulation and biopsy, which involve taking samples of gametes and gonadal tissue from live fish are commonly used (Kynard and Kieffer, 2002; Partridge *et al.*, 2003), and whilst useful, can damage the gonads and increase stress, so repeat sampling of the same fish is inappropriate for aquaculture and other studies. Blood sampling provides a less-invasive method and has been widely used for the assessment of circulating sex steroid and Vtg levels (Scott *et al.*, 1983; Pankhurst and Kime, 1991; Haddy and Pankhurst, 1998; Kokokiris *et al.*, 2001; King and Pankhurst,

2003). Few non-lethal, non-invasive methods have been developed, and whilst methods such as ultrasound provide high sexing accuracy and a good indication of maturation status in fish (Blythe *et al.*, 1994; Evans *et al.*, 2004; Newman *et al.*, 2008), the requirement for expensive equipment and interpretation of images by specialists imposes a major limitation for this technique.

The use of non-lethal tools for sex identification and assessment of reproductive condition are becoming an increasingly important part of fisheries management and aquaculture. Routine monitoring of the sex ratio in fish catches is not common practice, and yet it is likely that there will be selective fishing pressure on males or females if there are differences in the growth rates. Furthermore, alterations in the distribution and schooling behaviour during spawning can lead to increased vulnerability of fish stocks to fishing and may result in different mortality rates for spawning males or females. Stock assessments and fishery management would be greatly improved if there was better information on the proportion of spawning females and sex ratio in catches. For black bream, more information on sex ratios, spawning condition and reproductive success is needed since recruitment is highly variable and has been at historically low levels for many years in some fisheries (Morison *et al.*, 1998). In the present study the Vtg assay was successfully utilised in blood plasma and was also detected in surface mucus in the laboratory study (see chapter 6), indicating that the assay is useful and worthy of further development.

In conclusion this study has shown that there are physiological differences in black bream from different Victorian estuaries with different prevailing environmental conditions. Vitellogenin was detected at varying concentrations in females, however it was not detected in any males, even those with a distinct ovotestis. Now that a suitable assay has been developed and validated for measuring Vtg in black bream, further research is possible, including the study of seasonal variability in Vtg levels, effects of environmental stressors on reproductive condition, and determination of what concentrations of estrogenic EDCs can induce Vtg synthesis in male black bream.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

This study has contributed new biological information on aspects of black bream reproduction, as well as describing new techniques for use in fisheries research and environmental studies. Information on the early life stage development of black bream has been described in detail (Chapter 3), a new method for testing hypoxia has been developed (Chapter 4) and the effects that environmental stressors have on early life stage black bream have been tested (Chapter 5). Furthermore, a method for measuring reproductive condition in adult black bream has been developed (Chapter 6) and validated with a field study which contributes new information on the biological characteristics of 5 separate Victorian black bream populations (Chapter 7). The hypothesis that environmental stressors may affect spawning success by reducing survival in the early life stages has been confirmed by the laboratory studies, indicating that this is a possible explanation for the variable recruitment observed in Victorian black bream populations. Furthermore, differences in the reproductive condition of black bream sampled from field sites with different environmental conditions suggest that environmental stressors may affect reproductive output by lowering reproductive condition or preventing maturation in adult fish. Therefore, this study has shown that both spawning success and reproductive condition are indeed affected by environmental stressors.

Physiological stress, due to environmental variables is known to influence both the timing of reproductive events, as well as the quality of the gametes produced (see Schreck *et al.* (2001) for review). If a fish is under chronic stress, the allocation of

energy for reproduction may be reduced in order to sustain sufficient energy for somatic growth and survival (Schreck *et al.*, 2001). Thus, fecundity may be reduced and fewer, smaller eggs would be produced. Generally, egg size is positively correlated with early larval survival, whereby larger eggs result is larger, fitter larvae that exhibit better survival compared to smaller eggs (Tyler and Sumpter, 1996; Trippel *et al.*, 1997; Schreck *et al.*, 2001). In the present study, the quality of gametes produced from adults exposed to environmental stressors was not tested, however, for gametes produced from healthy broodstock, exposure to environmental stressors clearly affected embryo and larvae survival. Based on these findings, it can be stated that environmental stressors, acting on the early life stages are likely to play a critical role in determining recruitment success in black bream.

Hypoxia is an important environmental stressor during the early life stages, and it can have detrimental effects on embryo development and subsequent survival and hatching. For the first time in black bream, exposure to severe hypoxia (30%S) was shown to cause disruption to the normal process of cell differentiation, resulting in a lack of cephalisation which always resulted in mortality. This finding is similar to that reported by Shang and Wu (2004) in zebrafish embryos, where exposure to hypoxia caused a disruption to the apoptotic pattern resulting in increased embryo malformation. In the present study DO levels below 55%S significantly affected one day embryo survival, hatch rates, rates of deformity and larval survival, and in addition there was more variability in larval lengths at Day 2 post-hatch. These findings indicate that if DO levels are below 55%S at the time of spawning in black bream, then reduced spawning success is likely. A similar

level of hypoxia (50%S) has been reported to cause adverse effects on the growth and behaviour of sensitive estuarine fish species (Breitburg, 2002). In addition, other stressors such as altered salinity and temperature have interactive effects with DO, such that embryos experiencing multiple stressors have a lower tolerance and are less likely to survive to hatching. This highlights the importance of considering multiple variables when trying to determine suitable spawning locations for black bream. The results show that eggs can develop and hatch normally at a range of salinities and at 3 different temperatures if the DO levels are high, yet for the same range of salinities and temperatures, very low hatch rates and increased rates of deformities were observed when the DO levels were low. The combinations of environmental conditions tested in this study were based on water quality within the Gippsland Lakes during the black bream spawning season, and hence it is suggested that water quality is a likely cause of recruitment variability and failure in the Gippsland Lakes black bream population (Victoria's largest black bream population). The development of predictive models for hatching success that incorporate DO, salinity and temperature would be very useful in determining suitable spawning locations as well as predicting recruitment success in black bream.

The early life stages are a sensitive period in the fish life history, and clearly environmental conditions affect the success of early development, hatching and survival in black bream. However, what remains unknown are the long term effects of exposure to environmental stressors on post-larval survival, metamorphosis and eventually recruitment to the fishery. In juvenile turbot, exposure to hypoxia has been shown to

lower growth rates (Pichavant *et al.*, 2000), whilst in spot croaker hypoxia has been shown to affect the activity of various metabolic enzymes (Cooper *et al.*, 2002). Salinity and temperature are also know to influence juveniles by affecting growth (Partridge and Jenkins, 2002; Person-Le Ruyet *et al.*, 2004; Wuenschel *et al.*, 2004) and sex differentiation (Saillant *et al.*, 2003). Laboratory experiments to expose the early life stages to adverse environmental conditions, followed by rearing to the juvenile stage for example, would provide valuable knowledge on growth rates, rates of deformities and most importantly survival. Information like this would greatly assist in predictions of recruitment success and year class strength, which could be utilised by fisheries managers to ensure protection of this species. Furthermore, the early life history of black bream is similar to many other species that inhabit coastal and estuarine environments throughout temperate Australia (Neira *et al.*, 1998), so based on experimental results for black bream, predictions could be made for the fitness and survival of other species too.

In addition to the existing complexity of estuarine environments, areas that provide suitable habitats for successful spawning of black bream are also likely to change with alterations in climatic conditions. Climate change is an increasingly important factor to consider for the future management of estuaries, since it has been predicted that global warming may lead to an increase in annual average surface temperatures by up to 6^{o}C over the next century (IPCC, 2001). Associated with increased temperatures are changes in rainfall patterns, which may lead to an increase in the frequency of severe weather conditions such as floods and droughts. Obviously these changes will affect environmental conditions within estuaries, including DO, salinity and temperature. The

effects on the early life stages of black bream may be direct, however there may also be indirect effects on other species (such as plankton prey) that could influence black bream survival. A major theory that has been developed to explain fish recruitment variability is Cushing's 'match-mismatch' hypothesis, which states that larval hatching needs to be synchronous with peaks in plankton prey so that larvae have adequate food sources to ensure growth and survival (Cushing, 1972). Climate driven changes in the environmental conditions within estuaries has the potential to impact on the timing of reproduction in both fish and invertebrates and is thus likely to alter recruitment patterns.

Another indirect consequence of climate change in Victorian estuaries is a shift in species distributions which could potentially alter various ecological processes. A closely related species to black bream is the yellowfin bream (*Acanthopagrus australis*), which is distributed throughout eastern Australia from northern Queensland to Eastern Victoria (Edgar, 2000). Yellowfin bream share similar biological characteristics to black bream, inhabiting estuaries and producing large quantities of small pelagic eggs over an extended spawning period (Pollock, 1985). Yellowfin bream spawn during winter months (June-August), near the entrances to estuaries, where the larvae are transported to coastal areas, before returning to estuaries as juveniles (Pollock, 1985; Edgar, 2000). Hybridisation between yellowfin and black bream is possible, and since the range of both species overlaps in eastern Victoria, there is potential for mixed populations to co-exist in some estuaries. A consequence of climate change may be a southward shift in the distribution of yellowfin bream, which would increase the numbers of mixed populations of the two species and increase the possibility of hybrids being produced.

Climate change is a complex process that occurs on a global scale. Studies that measure the responses of biota to altered environmental conditions as a result of climate change are relatively straight forward, however studies to examine the causes of climate change are much more challenging. Climate change is largely due to anthropogenic increases in the levels of greenhouse gases (carbon dioxide, methane, nitrous oxide) entering the atmosphere, which have an insulating effect and therefore increase the temperature near the Earth's surface (Gross, 1993). Increased surface temperatures then affect a range of atmospheric processes such as rainfall, atmospheric pressure and oceanic processes, thus affecting climate. The scale of these processes is vast, and cannot be logistically studied in the natural environment. Thus future modeling is likely to be based on studies that come from smaller scale experiments, such as those that can be conducted in laboratory settings. In the present study, a new method was developed to test hypoxia in the laboratory (the Controlled Atmosphere Chamber), which was based on the manipulation and control of gas exchange between the chamber atmosphere and the water surface. Whilst nitrogen gas was used in this study to control DO levels, it could easily be applied to research involving other gases, such as greenhouse gases. The CAC provides a very suitable means for testing changes in atmospheric levels of carbon dioxide in a laboratory environment where other factors can be controlled for.

The novel CAC design was successfully developed and implemented in the testing of a range of hypoxia scenarios for black bream embryos and newly hatched larvae. The system's simplicity, low cost and small size make it a very useful new tool for laboratory-based investigations of hypoxia. In addition, CACs could be used to set up microcosm

experiments that simultaneously test the effects of hypoxia on an assortment of organisms, in order to understand interactions between species. Breitburg *et al.* (2003) created a model for survival of bay anchovy eggs and larvae in the hypoxic Patuxent River, and reported changes in mortality due to differences in predation rates and predator-prey interactions as DO levels changed. At present, very limited information is available on the hypoxia tolerances of estuarine vertebrate and invertebrate species in temperate Australia, and the CAC design provides a suitable method of exposure that is simple and could be readily implemented in research facilities without the need for expensive, specialised or complicated equipment.

Measuring reproductive condition, as well as developing suitable means for testing it, has been a major focus of fisheries research for decades. The importance of understanding the relationships between maternal and paternal health and gonad condition on subsequent gamete quality have long been recognised (Hoar, 1969; Crim and Glebe, 1990). Subsequently, several non-lethal methods for assessing reproductive condition have been developed, including invasive techniques such as cannulation and biopsy of gonadal tissue, less invasive techniques such as blood collection for analysis of sex hormones and egg yolk proteins, and non-invasive techniques such as ultrasound for examining the gonads, and collection of surface mucus for analysing maturity-related proteins. Until now, methods for examining reproductive condition in black bream have been restricted to cannulation and biopsy of gonads (Partridge *et al.*, 2003), examination of mature gametes (ie. sperm motility, egg viability) and measurement of sex steroids in blood plasma (Haddy and Pankhurst, 1998; Haddy and Pankhurst, 2000).

In the present study an assay was developed to measure the major egg-yolk precursor protein, Vtg in blood plasma. Vitellogenin proteins are species-specific, and prior to this research, no immunological techniques for measuring black bream Vtg had been published. Vitellogenin production was induced in adult black bream in the laboratory and screened for cross reactivity with commercially available antibodies by Biosense Laboratories in Norway. Having access to a commercially available primary antibody with good cross reactivity to black bream Vtg enabled the development of two immunological techniques, ELISA and Western blot. Standard procedures for both techniques were optimised and utilised in a subsequent laboratory study in adult black bream, in which Vtg was measured in both blood plasma as well as surface mucus. Blood plasma is an excellent biological fluid for measuring reproductive condition, since it can be collected relatively easily, and importantly does not require any direct contact with the gonads (which could potentially damage them). However, the collection of blood is still invasive, and if conducted incorrectly may cause significant stress. Therefore, the detection of Vtg in surface mucus is a very significant finding, which has great potential to be developed as a non-invasive, minimal stress method for measuring Vtg levels in black bream. Furthermore, this method could be similarly developed for use with other native species, especially for those in which understanding reproductive condition is particularly important for ensuring species survival, such as endangered or vulnerable species.

Upon developing suitable methods for measuring black bream Vtg, a field study was undertaken, to measure and compare Vtg levels in fish subject to different types of

environmental stressors. The field sites consisted of one reference site, two sites surrounded by agriculture and in close vicinity to a large sewage treatment plant, and two urban sites subject to both industrial effluents and storm water runoff. Vitellogenin was detected in female black bream from all sites, at varying concentrations, whilst there was no evidence of any Vtg induction in male black bream from any of the field sites, even for those males that had distinct ovotestes. Differences in the size of the fish collected, as well as the timing of sampling (in relation to the spawning season) are likely to have contributed to differences in Vtg concentrations in fish from the different locations.

Fish were sampled during the pre-spawning, spawning and post-spawning seasons, however analyses were restricted to fish captured between September and December, which encompasses the black bream spawning season. Vitellogenin was first detected in Stage 3 (vitellogenic) ovaries, and was also detected in Stage 4 (hydrated) and Stage 5 (ovulated) ovaries. The highest concentration of Vtg (66.9 RT-VtgEq-mg/ml) was recorded in a 245 mm, 3 year old female from the Werribee River that was observed to have ovulated (Stage 5) ovaries. This concentration is within a similar range to those recorded in mature female Atlantic salmon (*Salmo salar*), of 5-40 mg/ml (King and Pankhurst, 2003) and in mature female fathead minnows (*Pimephales promelas*) of 15-60 mg/ml (Jensen and Ankley, 2006). Whilst the Vtg assay was able to detect different concentrations of Vtg in female black bream, no Vtg was detected in any males, even those from the Maribyrnong River, Werribee River and Little River that had ovotestis.

The development of innovative testing techniques for measuring reproductive condition in black bream in this study now enables the study of maternal health, by monitoring Vtg levels during both the spawning and non-spawning seasons. Such studies would provide valuable information on physiological aspects of egg production, such as when vitellogenesis begins seasonally, as well as how high the circulating concentrations can get to, and how the levels change throughout the extended black bream spawning season. Developing an understanding of the relationship between Vtg levels in plasma and possibly surface mucus with egg quality would provide significant benefits to the aquaculture industry as well as providing a new tool for assisting fisheries research and management that is non-destructive. Associated with such research would be the study of juvenile black bream, to determine at exactly what size/age sex differentiation occurs and vitellogenesis first begins, as well as how long it takes fish to mature (ie. produce sufficient Vtg to enable successful egg production). Fish could be raised in different environmental conditions to determine the effects of chronic, sub-lethal exposure to environmental stressors on vitellogenesis processes. Predictive modeling could then be used with such data to assist fisheries management, in particular for the setting of legal minimum lengths, to ensure that fish have the opportunity to reproduce before being removed from the fishery.

A potential application of the black bream Vtg work is that the assay could be developed as a tool to accurately identify the sex of large, mature black bream during the spawning season. Since Vtg was not detected in any males, but was present in all large (> 215 mm) females that were mature or maturing, the presence of Vtg could be developed as a

simple positive/negative test for sex identification. Identifying the sex in species that display no sexual dimorphism in mature adults has application in aquaculture, fisheries research and environmental monitoring situations.

Another application of the Vtg assay would be to use it in studies of fecundity and senescence in larger, older fish. It is generally assumed that large fish have higher fecundity than small fish, and hence are valued as important contributors to spawning stocks. However, senescence, the period of old age in fish, is marked by reduced growth rates and reduced production of gametes (Murua *et al.*, 2003), and thus, such fish would contribute less to the reproductive capacity of the spawning stock biomass. By examining fish of varying size, the peak Vtg levels during the spawning season could be measured and related to egg production, to determine if there is a peak in fecundity and then a decrease with increasing size (and age). Again, such information would be valuable to fisheries managers for calculating the reproductive output and spawning stock biomass of fish populations.

Perhaps the most important benefit of developing a Vtg assay for the assessment of reproductive condition, sex identification or exposure to estrogenic EDCs is that levels can be measured non-destructively, and as such, no individuals that are sampled need to be removed from the fishery, which has previously been an issue when analysis is based on invasive or even lethal procedures.

In order to successfully utilise Vtg as a biomarker for reproductive condition in black bream, further development of the two immunological assays are needed. Whilst both the ELISA and Western blot procedures successfully detected black bream Vtg, and quantitative results were obtained with the ELISA, the fact that the assays rely on primary antibodies and Vtg standards from different fish species' is a limiting factor which needs to be addressed. The sensitivity of the assays, and the confidence that the measurements are in fact Vtg and not another protein need to be validated. The next step in the development of a quantitative black bream ELISA would be to purify and characterise the black bream Vtg molecule, then use the purified protein to generate a black bream-specific primary antibody. Once the Vtg purification and primary antibody production are complete, a homologous/homogenous black bream Vtg ELISA would be achievable. This would provide greater sensitivity which is very important for studies of both the onset of vitellogenesis in maturing females, as well as for assessment of Vtg induction as a result of exposure to estrogenic endocrine disrupting chemicals.

The use of Vtg as a biomarker of exposure to endocrine disruptors is well established for a number of teleost test species. In Australia, information on the effects of endocrine disruptors to aquatic biota is very limited, so new techniques and tools for monitoring Australian waterways are much needed. Analytical chemistry-based measurements of environmental stressors, in particular toxicants is an important part of environmental monitoring, however the information generated does not provide any information on the effects of exposure. In contrast, biological monitoring tools enable the detection of an environmental stressor, as well as providing information on the biological or

physiological effects of such exposure. In the case of endocrine disruptors, which often occur at very low concentrations in the environment, a sensitive biological assay may detect effects at concentrations that may not be detectable using analytical chemistry techniques. Furthermore, biological monitoring tools can be developed for specific toxicant exposures, such as the EROD assay for exposure to organic trace pollutants, or metallothioneins for exposure to metals. In order for Vtg induction in black bream to become a useful biomonitoring tool, dose-response experiments need to be conducted to establish what concentrations of estrogenic chemicals (either single chemicals, or mixtures) will induce Vtg production in males. This study has shown that Vtg is inducible following administration of a high dose of estrogen, however it is not known how sensitive black bream are, or what the threshold concentrations are at which Vtg production is first induced.

In conclusion, this thesis presents original research that shows that both early life stage and adult black bream are sensitive to environmental stressors. Environmental stressors affect a number of physiological processes, including early life stage survival, and growth and reproductive condition in adults. In the early life stages, environmental stressors affect embryo development, embryo survival and hatching, hatch rates, rates of deformity and early larval survival. Hence, it is concluded that environmental stressors play a critical role in determining recruitment success in black bream by affecting early life stage survival. For adult fish, an assay for measuring reproductive condition was developed and applied in a field study, and similarly indicates that environmental stressors affect reproductive condition. The Vtg assay provides a tool for monitoring

reproductive condition in female black bream, as well as offering a screening tool for exposure to estrogenic chemicals in male black bream, and potentially, as an indicator of sex in mature fish as well.

This study provides valuable baseline information on the native black bream. Normal embryo development has been described in detail for the first time, and can be used to assess spawning success. Multiple experiments conducted in the controlled laboratory environment confirmed that the early life stages are sensitive to hypoxia (<55%S), high (>35 ppt) and low (<35 ppt) salinity and high (26^{o}C) and low (16^{o}C) temperature. Furthermore, combinations of multiple stressors have a greater effect than single stressors alone. This knowledge can now be applied to field studies in order to identify normal and abnormal development in black bream embryos that could be related to different types of environmental stressors. This would assist in determining suitable locations for successful spawning and early life stage survival in this species. Similarly, for the first time Vtg levels were reported in black bream from 5 Victorian field sites, and related to macroscopic gonad condition, length, weight, age and tissue indices (GSI, LSI and condition factor). This information also provides valuable baseline data that can be applied to field studies and in particular be used to reassess and reconsider current management strategies for the Victorian black bream fishery.

The black bream is a long lived, widely distributed, iconic native species of major importance in recreational and commercial fisheries that has great potential as a biomonitoring species for estuaries throughout Southern Australia. As this study

demonstrates, black bream are a suitable test species for both laboratory- and field-based research and they are sensitive to environmental stressors. With the tools that have been developed during this PhD, research involving black bream can now be further developed to assist in understanding the effects of environmental stressors on biota within estuaries due to changing environmental conditions brought about by processes such as climate change, changes in land use patterns and increasing levels of pollution.

CHAPTER 9: REFERENCES

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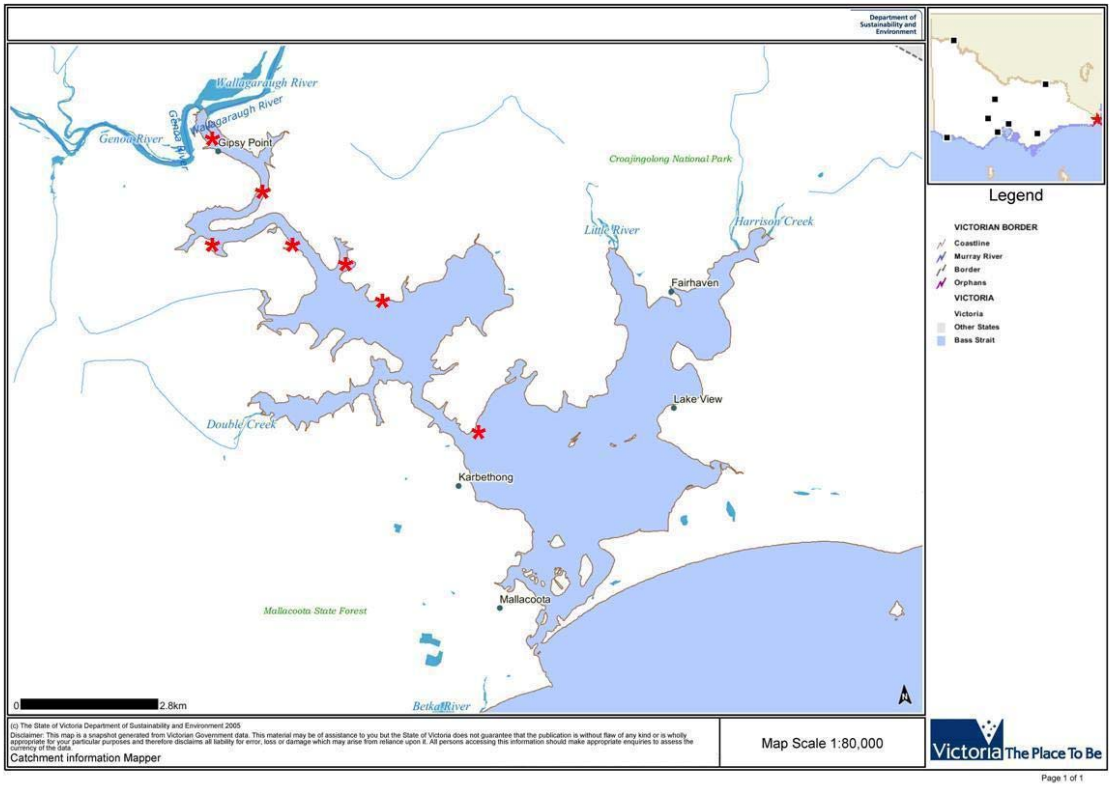
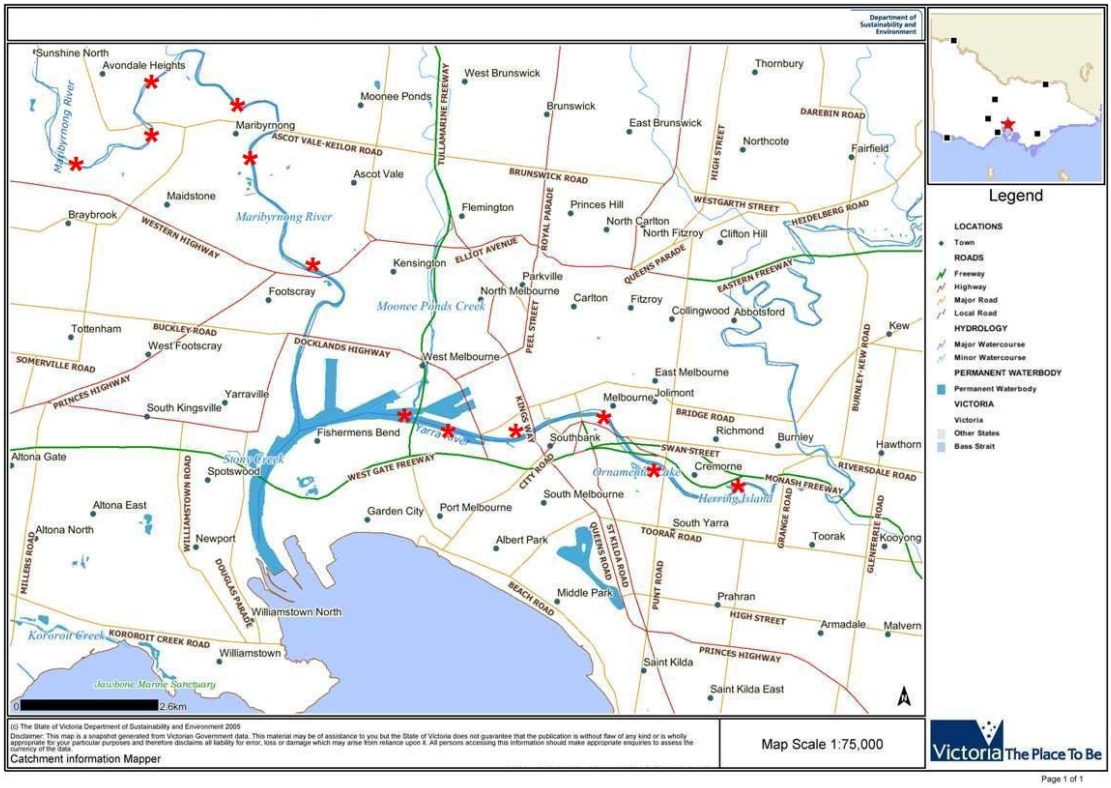
APPENDICES

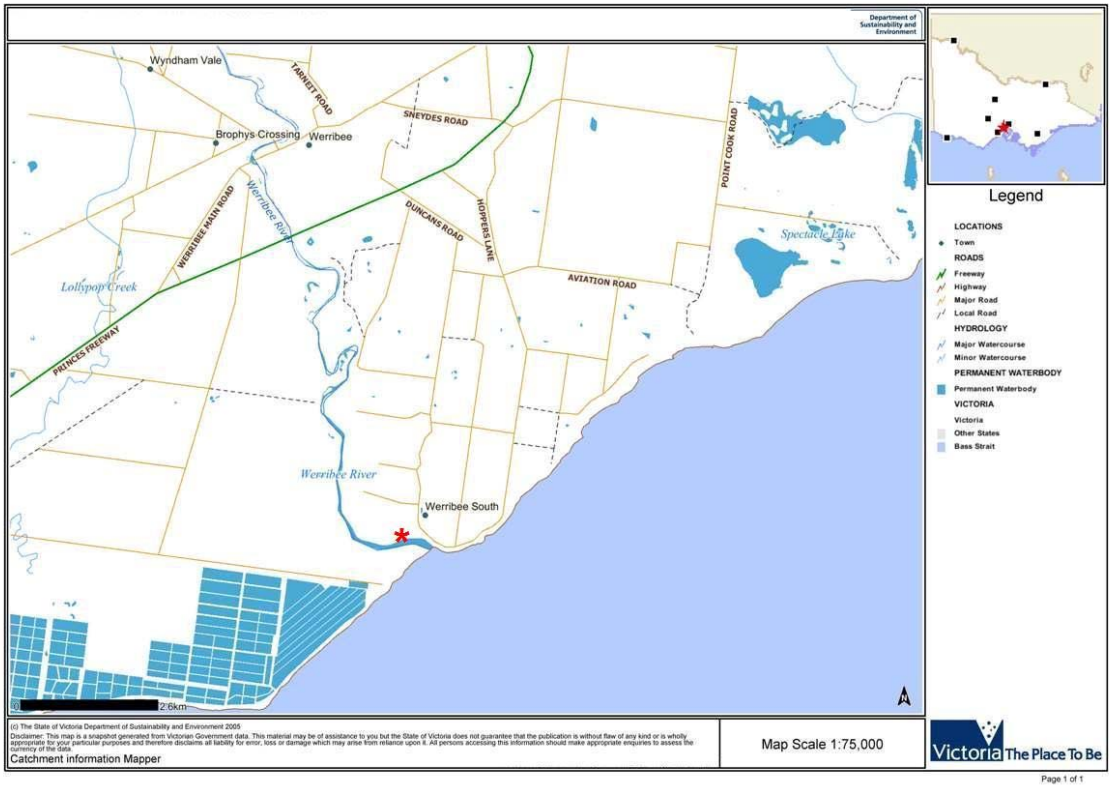
Appendix 1: Maps indicating the locations of fish collection for the field study.



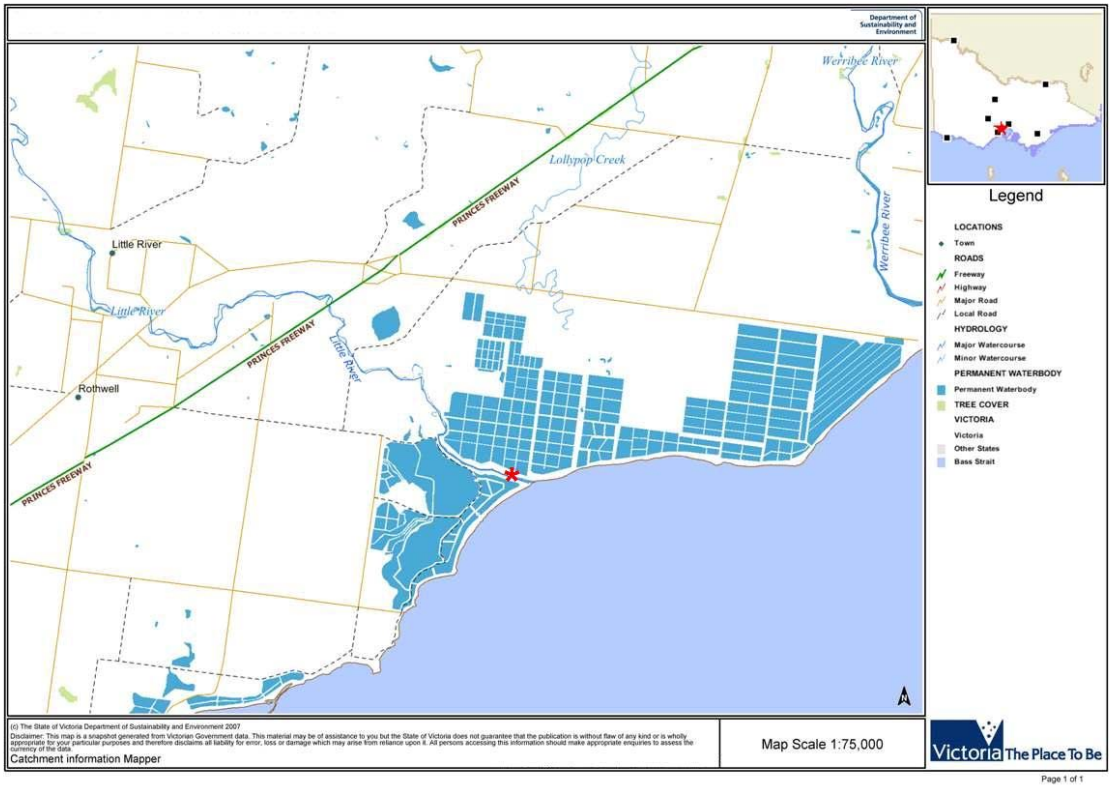
Mallacoota Inlet, a near-pristine estuary in eastern Victoria was utilised as a reference site in this study. Red asterisks (*) mark locations where black bream were captured.



The Yarra River and Maribyrnong River, two modified estuaries in Melbourne were utilised as urban sites in this study. Red asterisks (*) mark locations where black bream were captured.

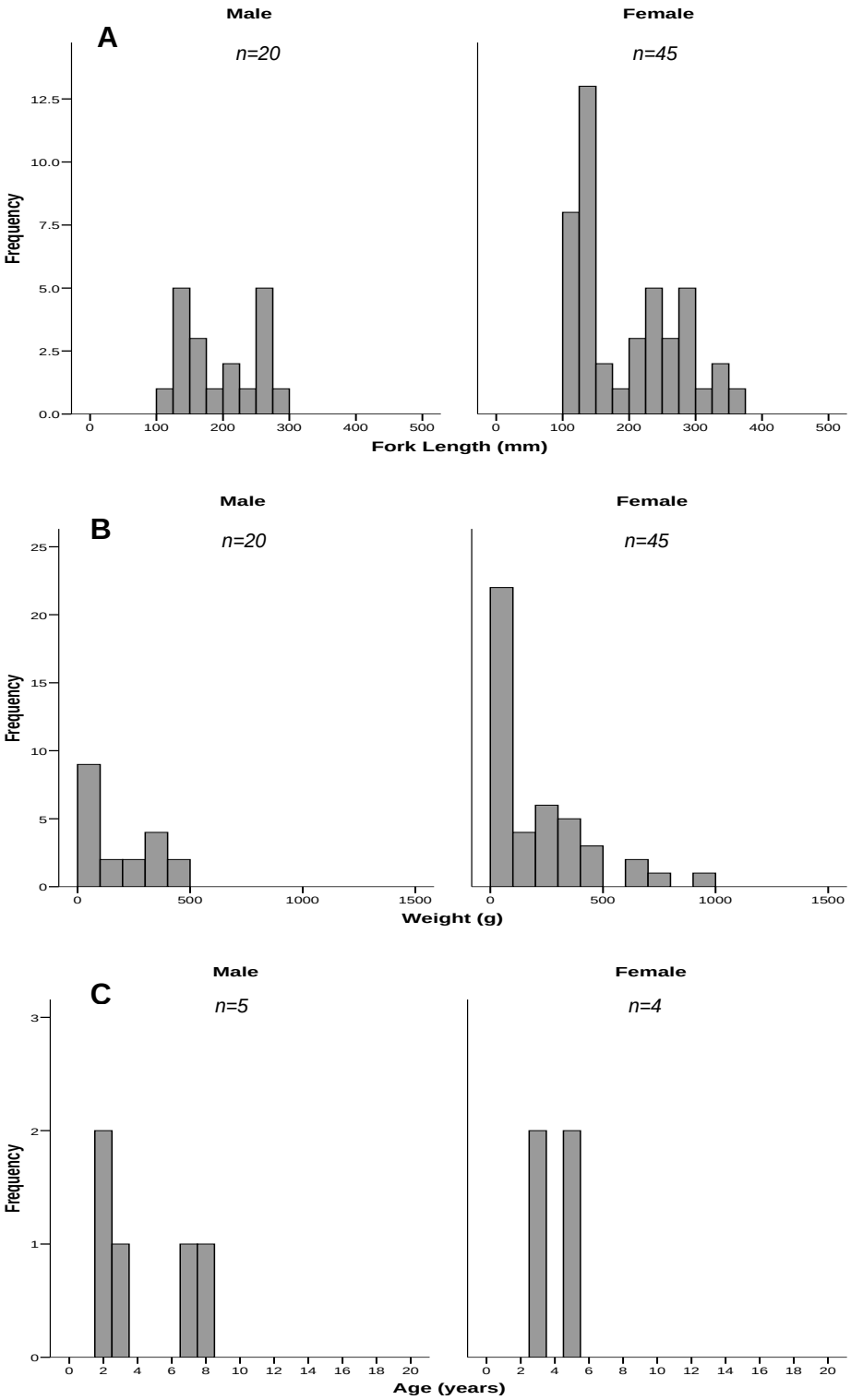


Werribee River is a modified estuary south-west of Melbourne that was utilised as an agriculture/sewage treatment facility site in this study. The Western Treatment Plant is represented by the blue grids on the map. A red asterisk (*) marks the location where black bream were captured.

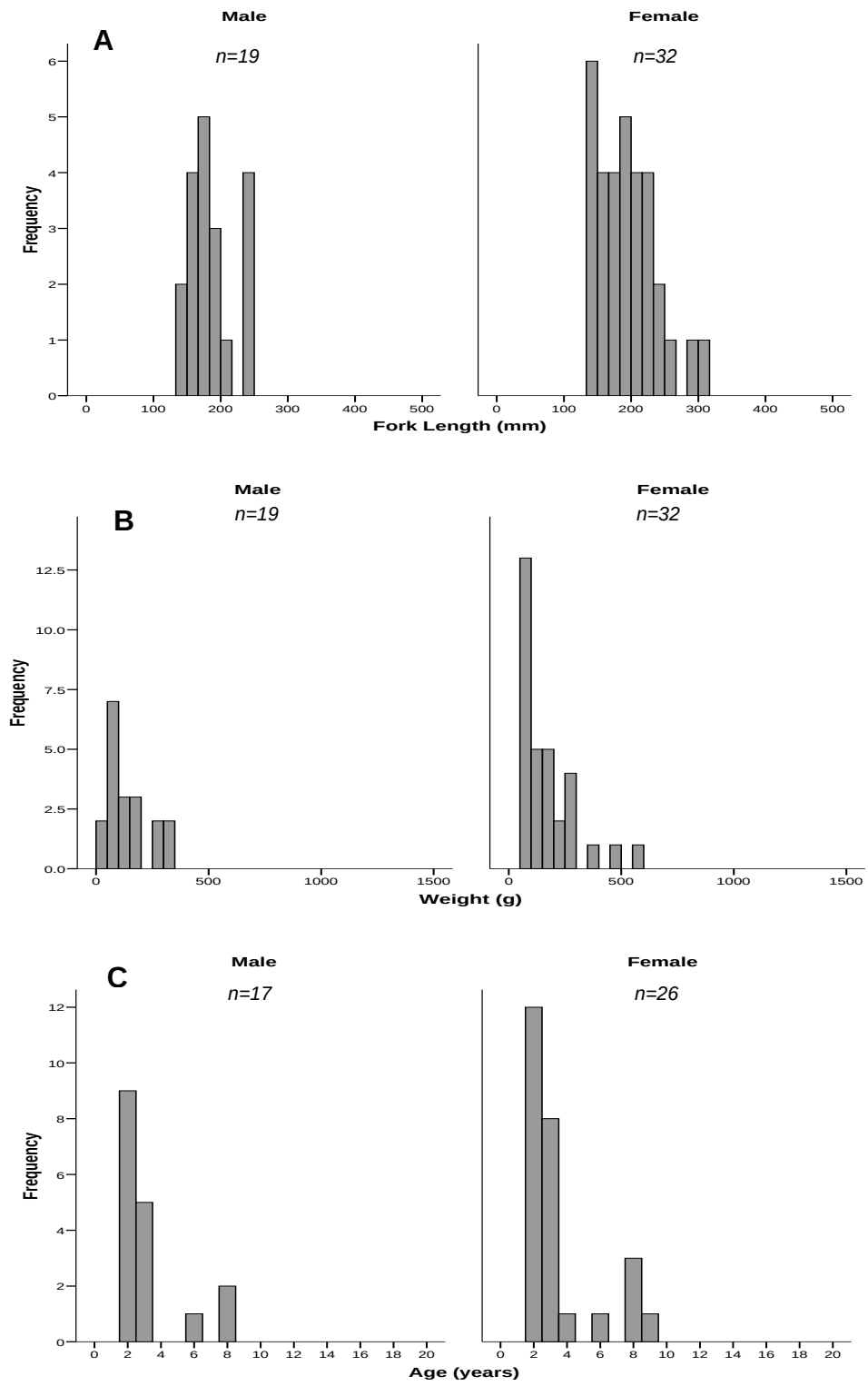


Little River is a modified estuary south-west of Melbourne that was utilised as an agriculture/sewage treatment facility site in this study. The Western Treatment Plant is represented by the blue grids on the map. A red asterisk (*) marks the location where black bream were captured.

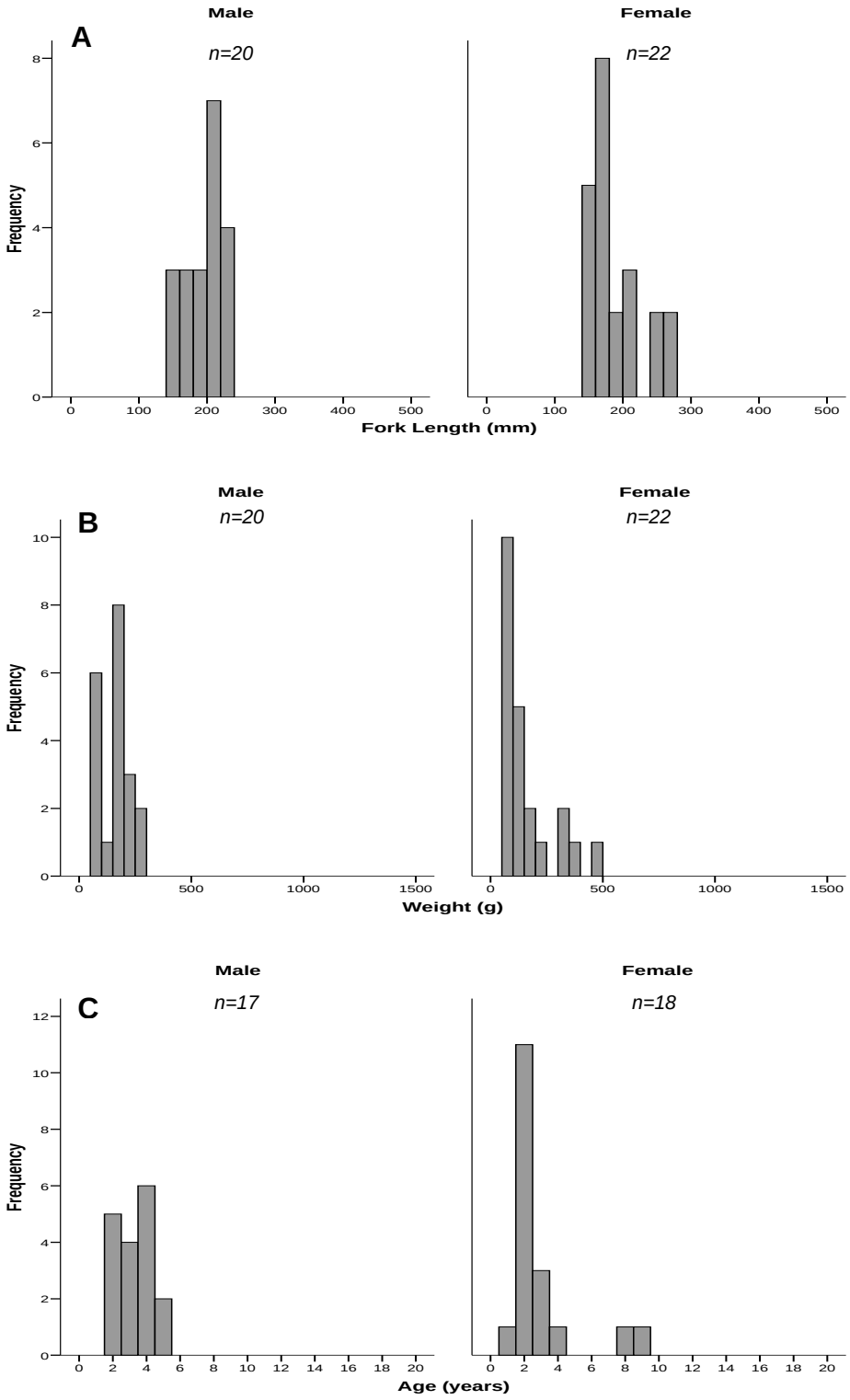
Appendix 2: Frequency distributions of length, weight and age for field collected black bream.



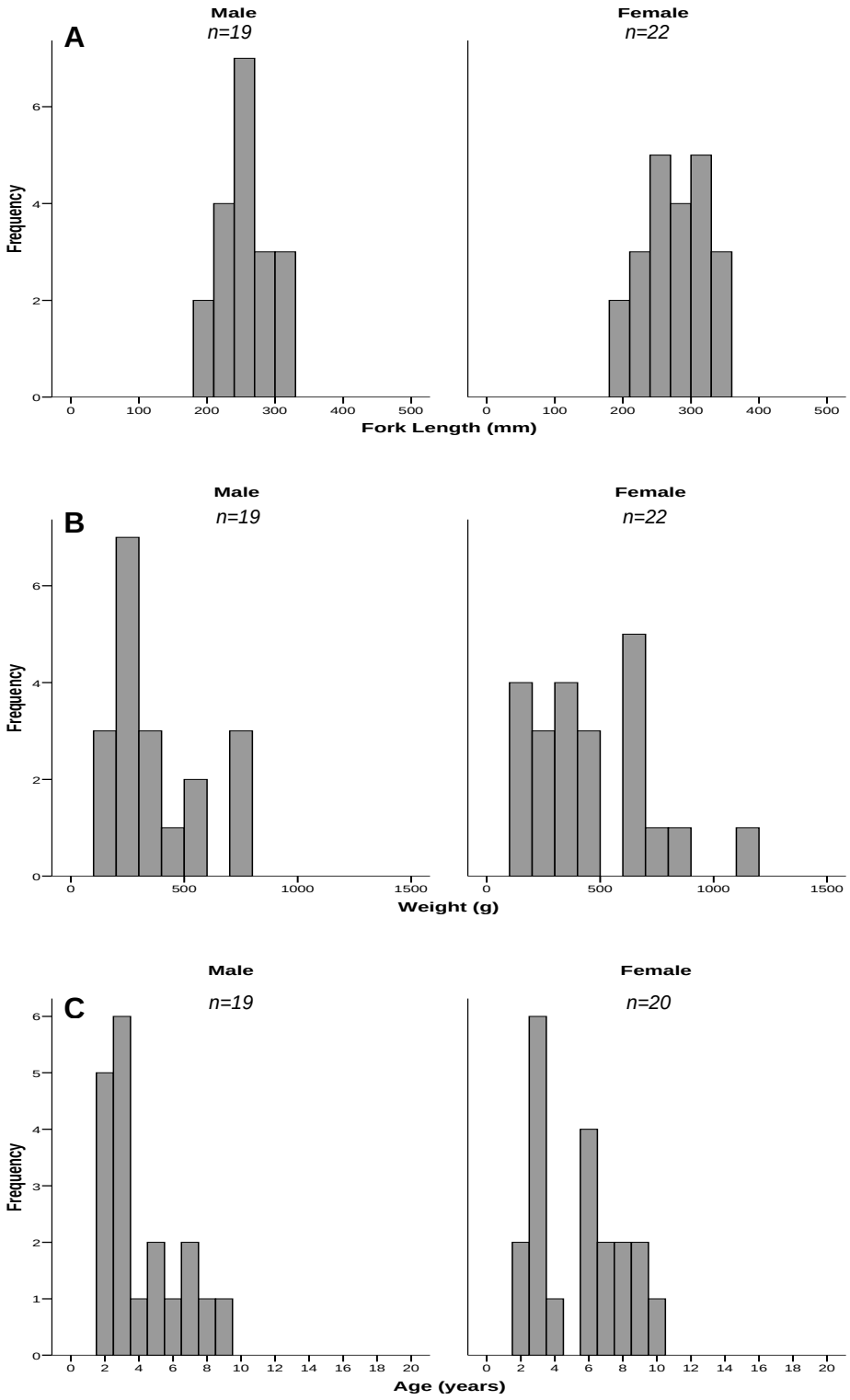
Frequency distributions for A) Length; B) Weight; and C) Age of black bream captured from Mallacoota Inlet between December 2006 and November 2007.



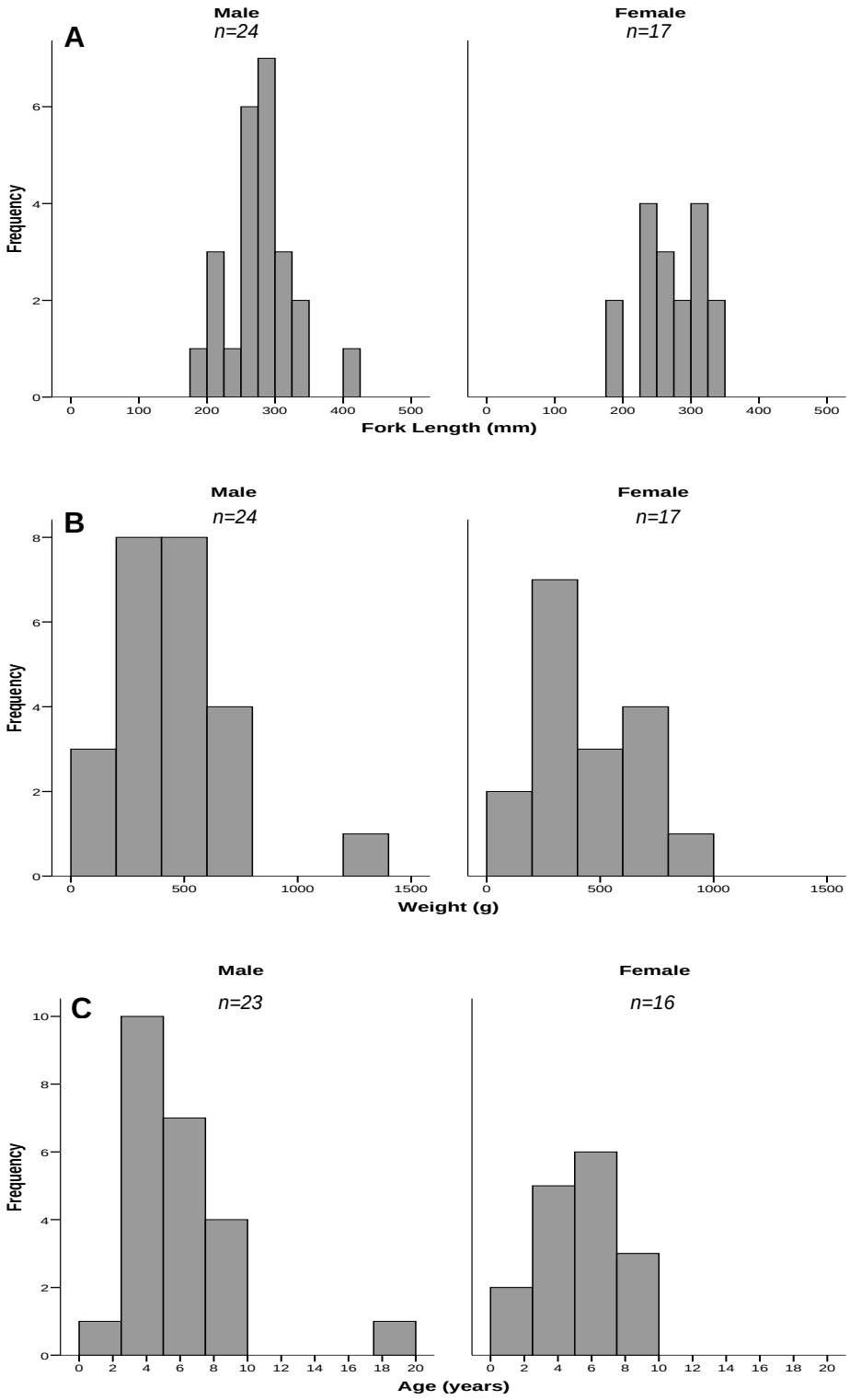
Frequency distributions for A) Length; B) Weight; and C) Age of black bream captured from the Yarra River between September 2007 and May 2008.



Frequency distributions for A) Length; B) Weight; and C) Age of black bream captured from the Maribyrnong River between August 2007 and May 2008.

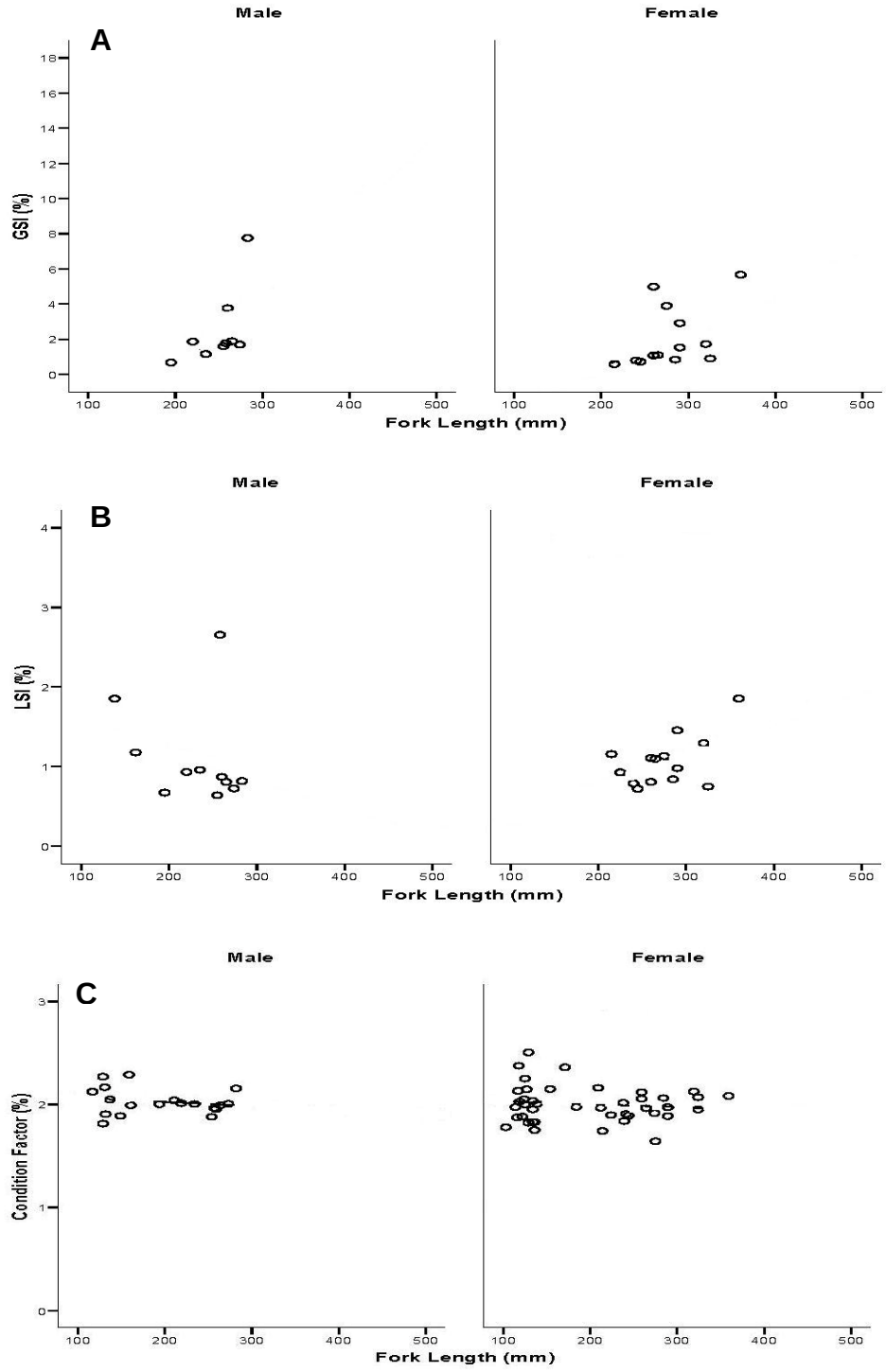


Frequency distributions for A) Length; B) Weight; and C) Age of black bream captured from the Werribee River during October 2007.

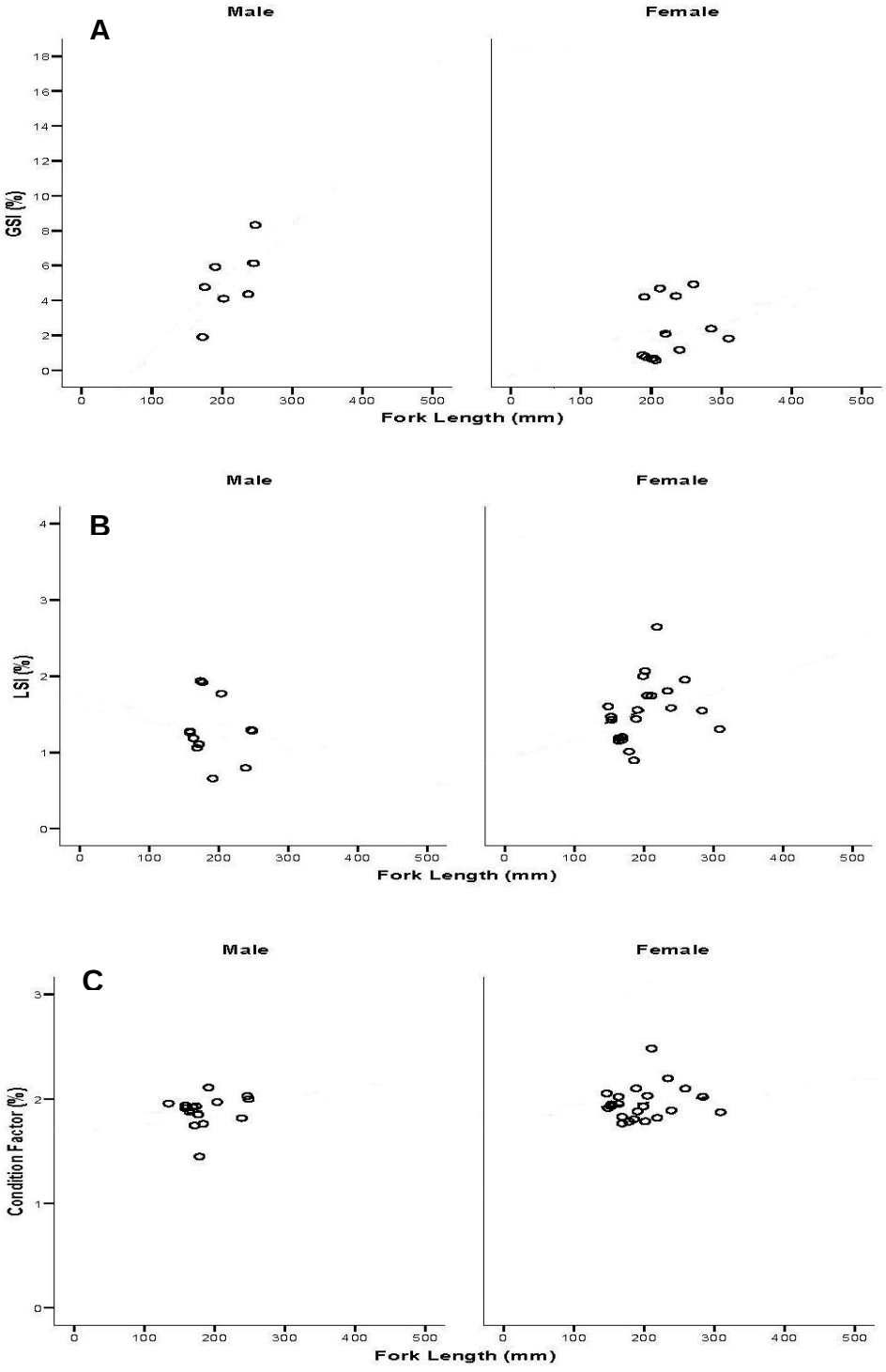


Frequency distributions for A) Length; B) Weight; and C) Age of black bream captured from the Little River during December 2007.

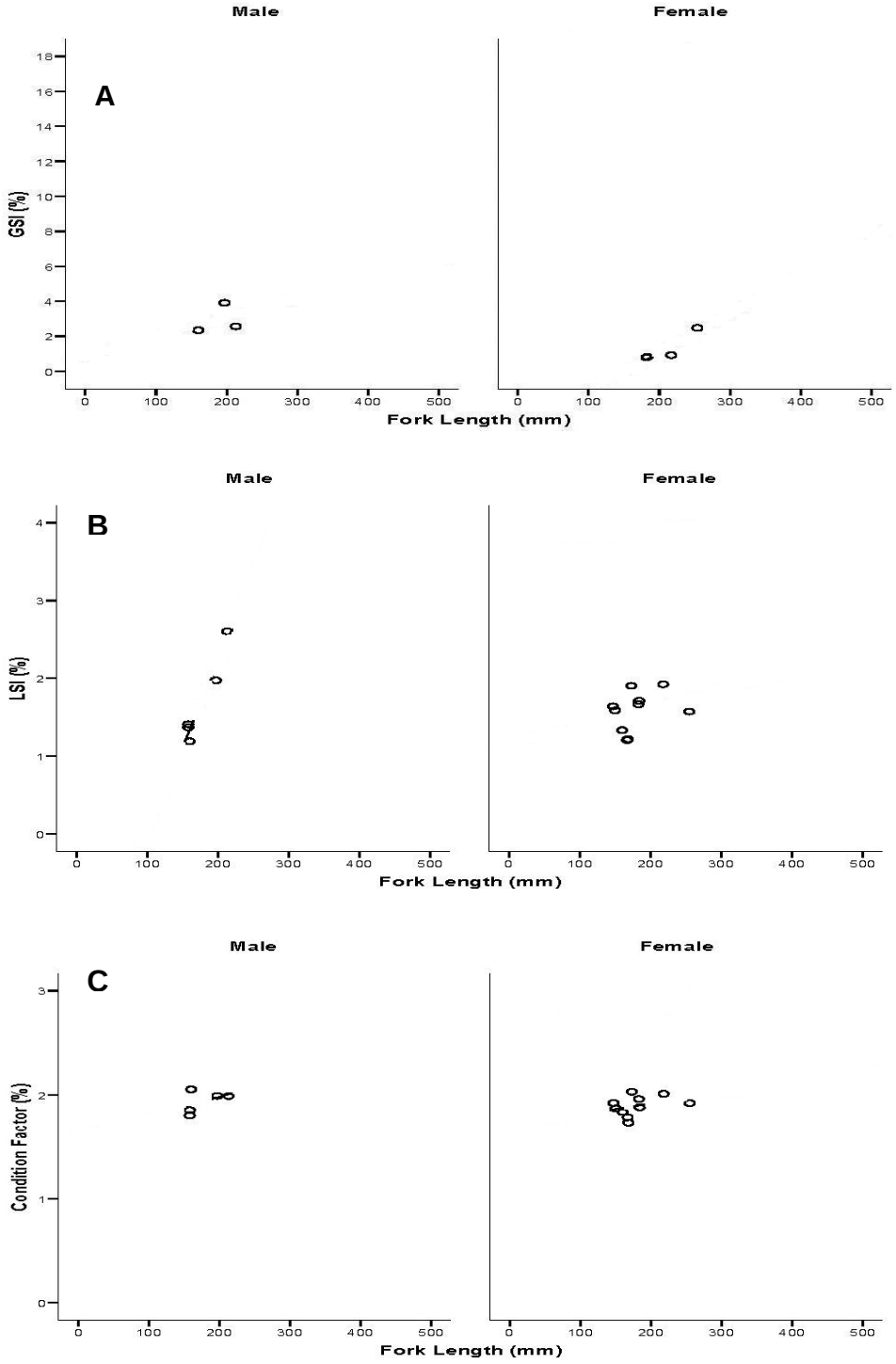
Appendix 3: Scatterplots indicating relationships between fork length and GSI, LSI or condition factor for field collected black bream.



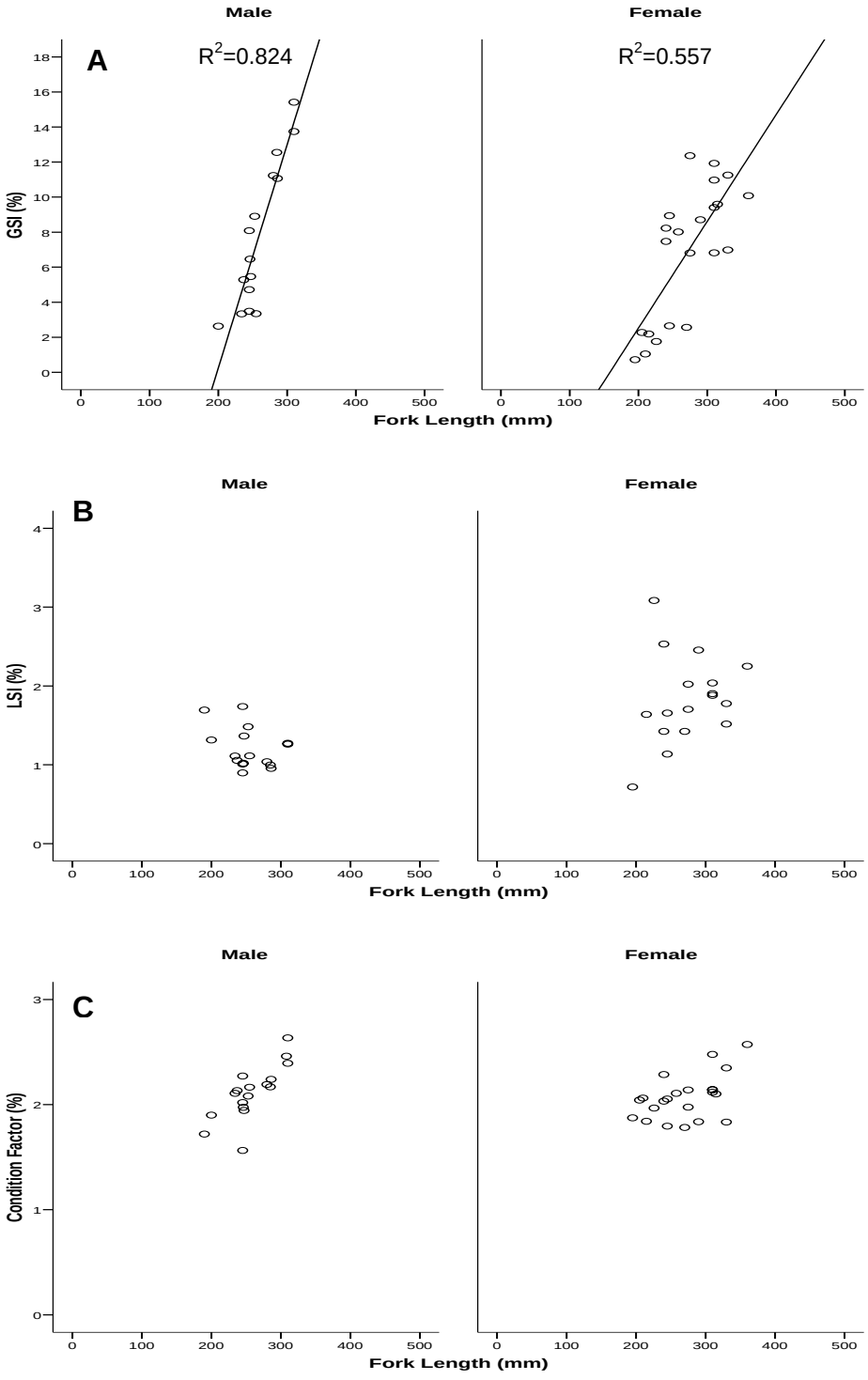
Relationship between fork length (mm) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from Mallacoota Inlet during December 2006 and November 2007.



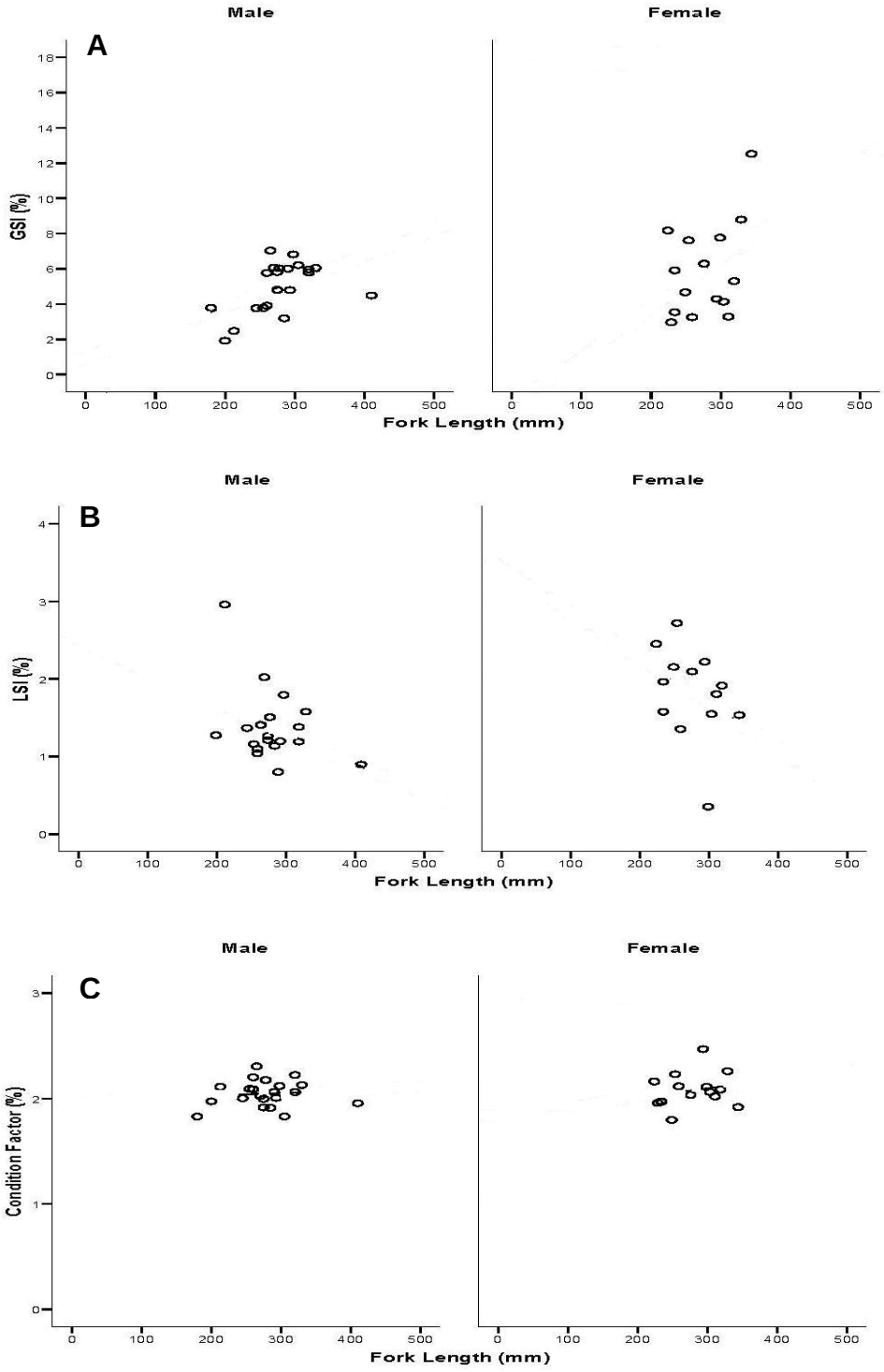
Relationship between fork length (mm) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from the Yarra River between September and December 2007.



Relationship between fork length (mm) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from the Maribyrnong River between October and December 2007.

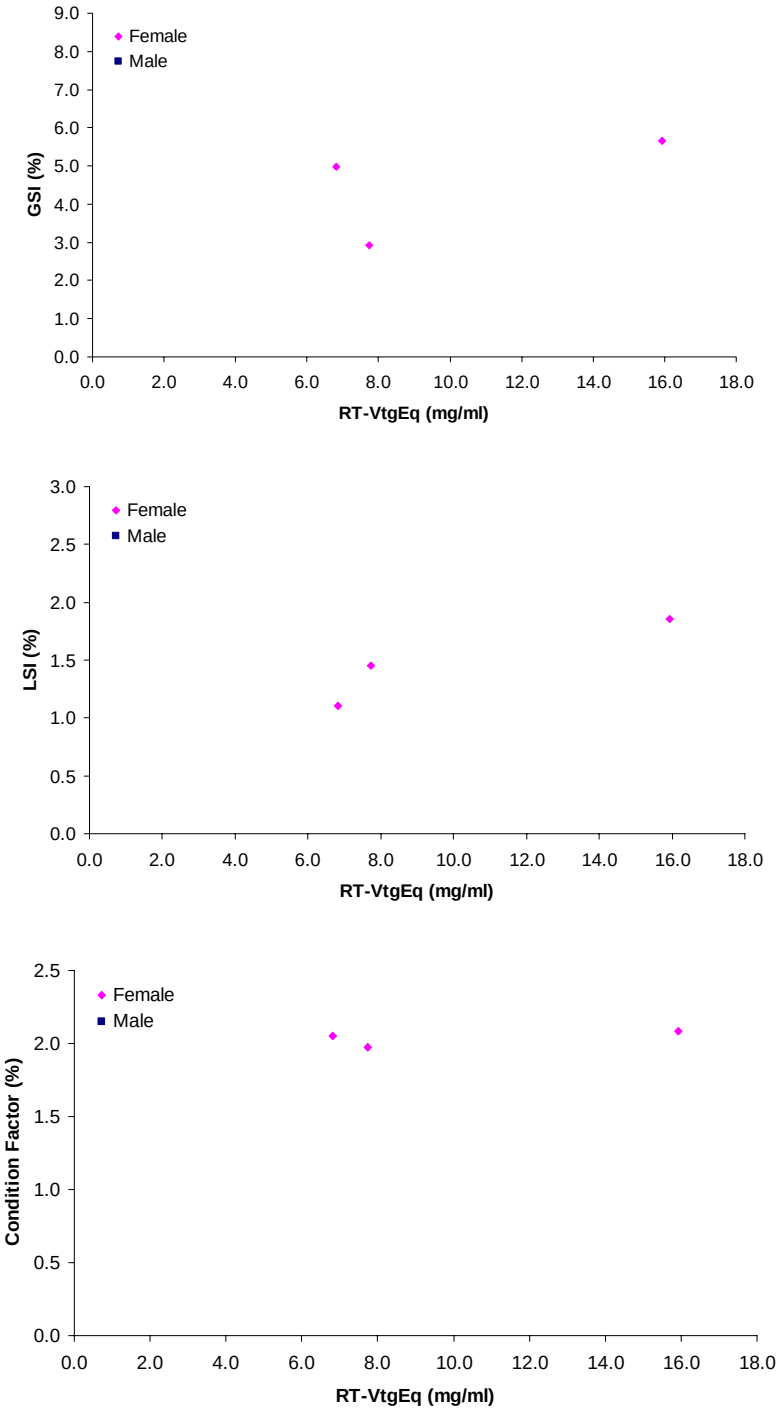


Relationship between fork length (mm) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from the Werribee River during October 2007.

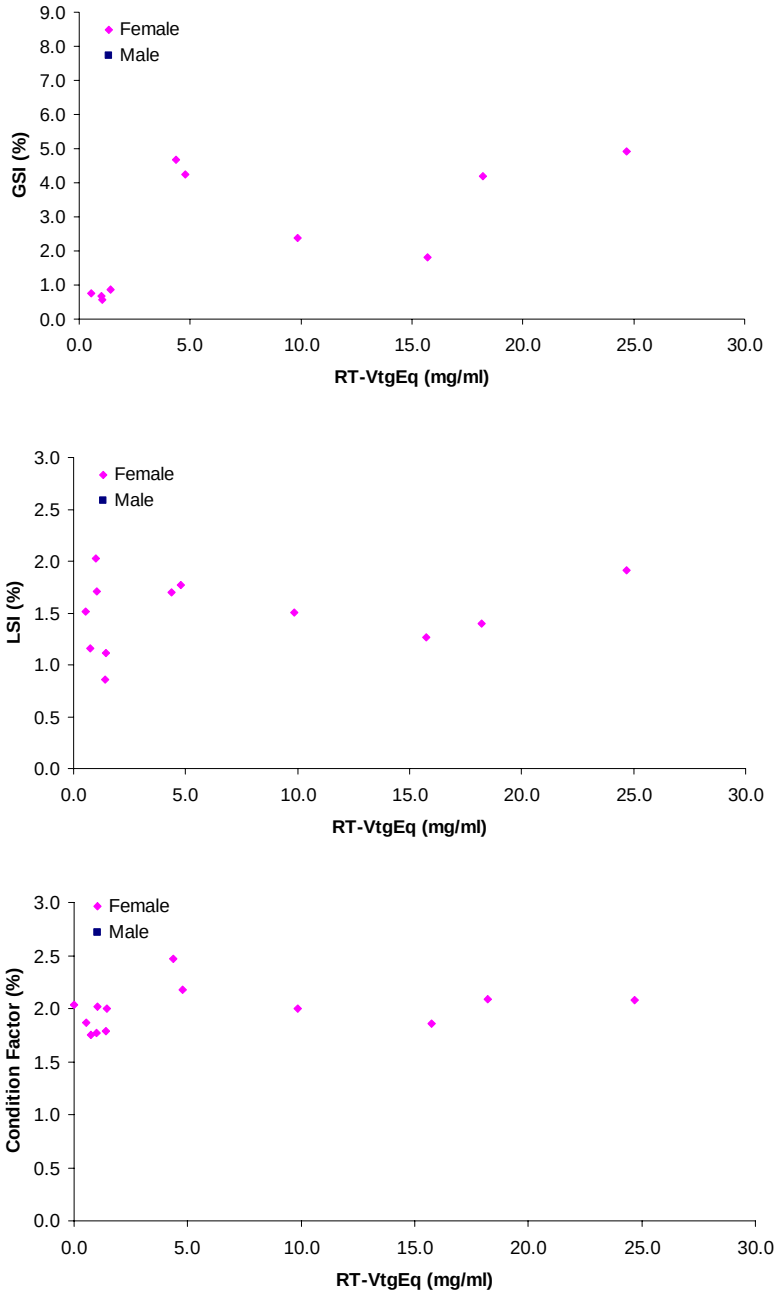


Relationship between fork length (mm) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from the Little River during December 2007.

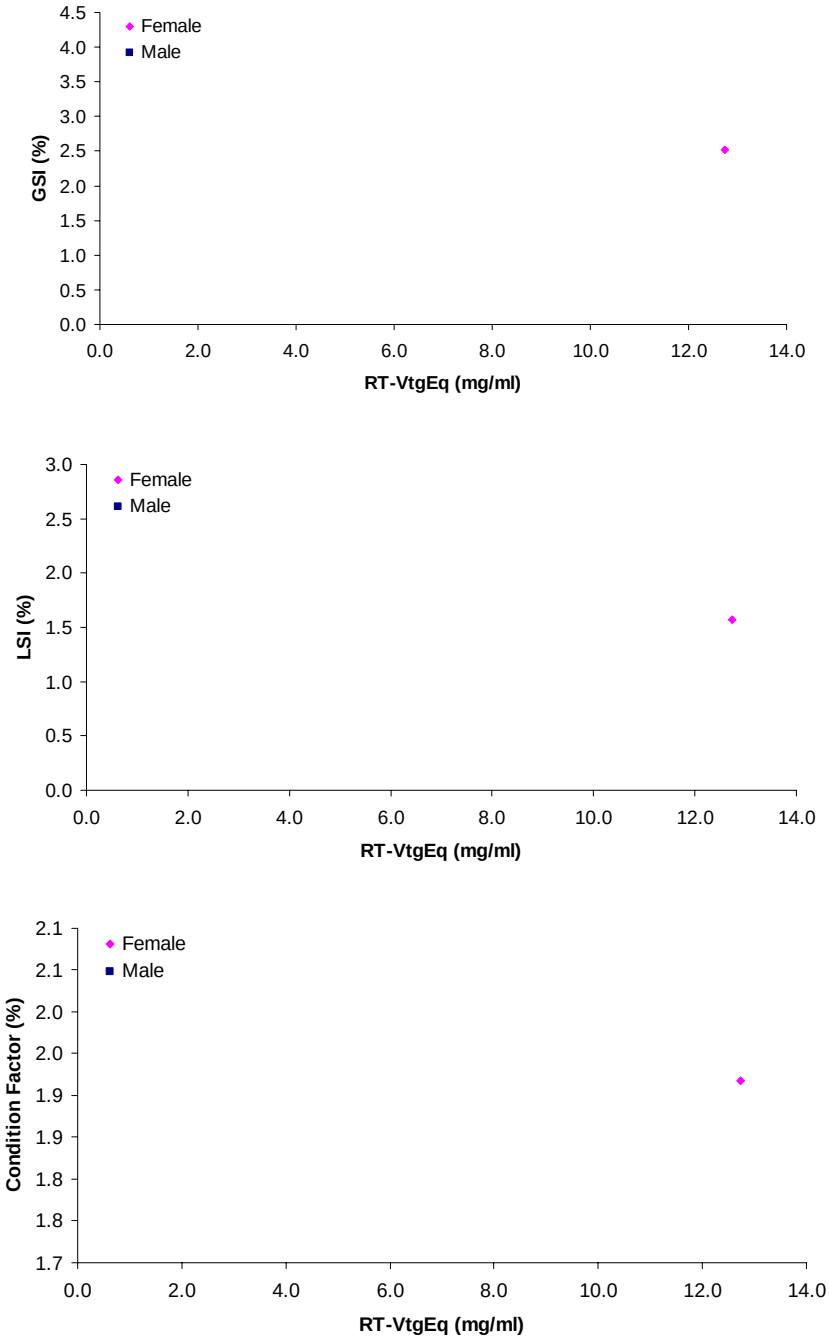
Appendix 4: Scatterplots indicating relationships between plasma vitellogenin and GSI, LSI or condition factor for field collected black bream.



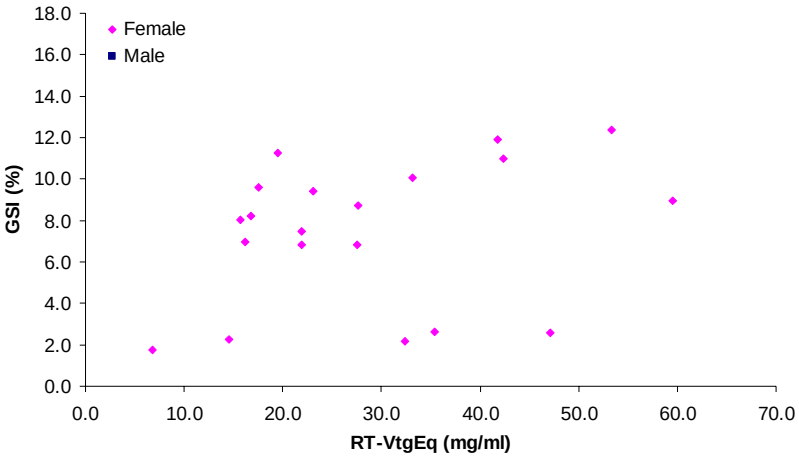
Relationship between plasma vitellogenin (RT-VtgEq-mg/ml) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from Mallacoota Inlet during December 2006 and November 2007.

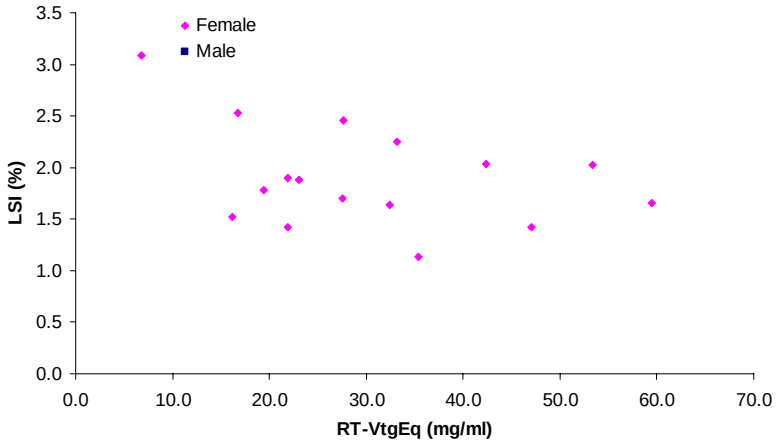


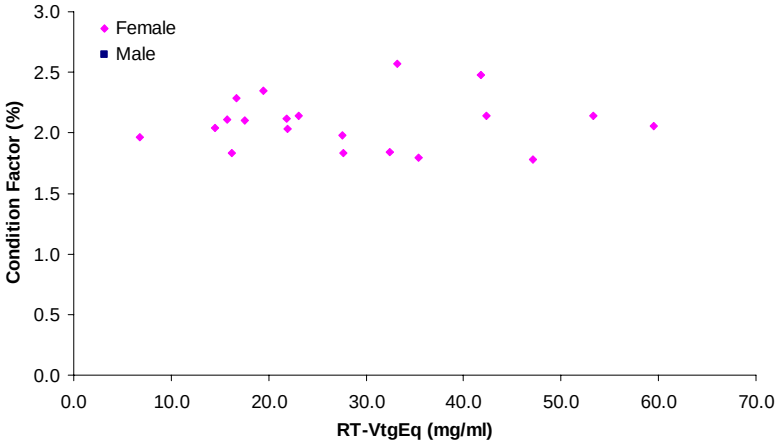
Relationship between plasma vitellogenin (RT-VtgEq-mg/ml) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from the Yarra River between September and December 2007.



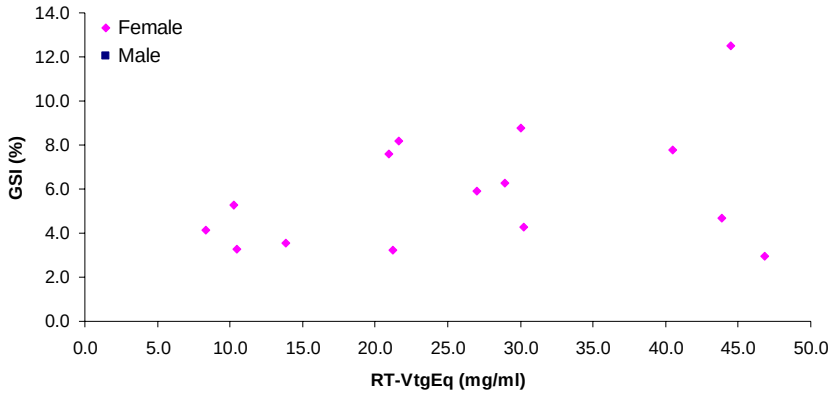
Relationship between plasma vitellogenin (RT-VtgEq-mg/ml) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from the Maribyrnong River between October and December 2007.

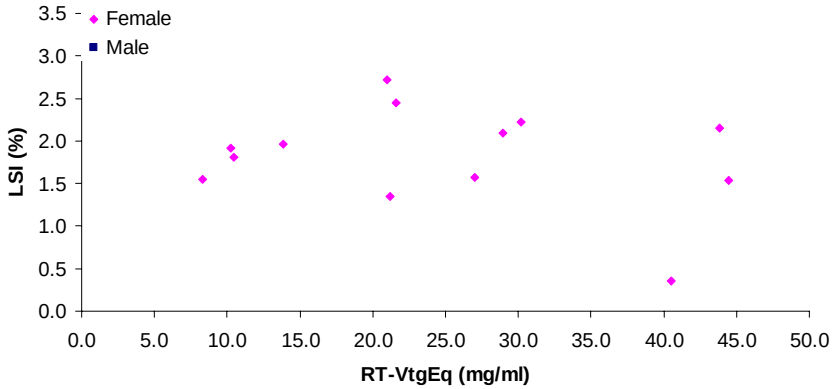


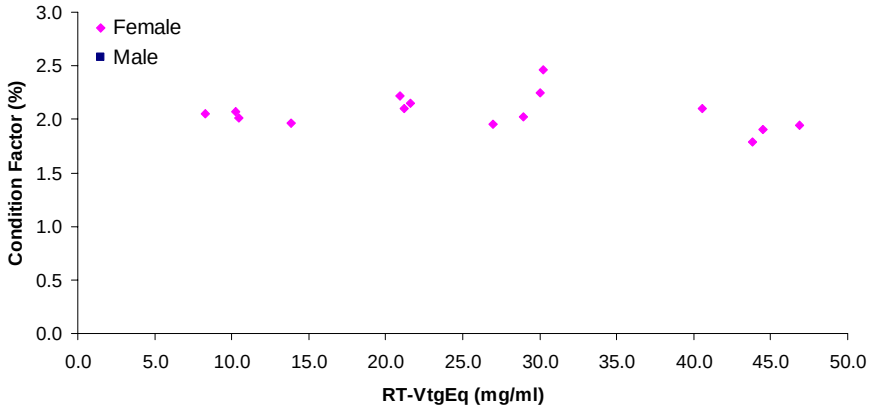




Relationship between plasma vitellogenin (RT-VtgEq-mg/ml) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from the Werribee River during October 2007.







Relationship between plasma vitellogenin (RT-VtgEq-mg/ml) with A) gonadosomatic index (GSI %); B) liver somatic index (LSI %); and C) Condition factor (%) for black bream captured from the Little River during December 2007.