UNIVERSITY OF TASMANIA

The Effect of Stress on Reproduction in Snapper (*Pagrus auratus*).

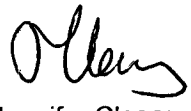
Jennifer Jane Cleary, BSc (Hons)

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy.
Department of Aquaculture
University of Tasmania, December 1997

Declaration

This Thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the Thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

signed



Jennifer Cleary

Authority of Access

This thesis may be made available for loan and limited copying in accordance with the Copyright Act, 1968.

Signed

Jennifer Cleary

Abstract

Stress inhibits reproduction in most fish and can be exerted through normal hatchery practices in aquaculture. Previous research with wild New Zealand snapper, has shown inhibitory effects of stress on reproduction, however, there is no information on the effect of stress in captive or domesticated stocks. This study examines the role of stress in the reproduction of snapper, by determining the effect of capture and confinement on plasma steroid levels, gonadal condition, the ovulatory response to exogenous hormone treatment and *in vitro* ovarian steroidogenesis.

The effects of stress were investigated in wild fish caught by trap, 5-year-old fish, caught as juveniles and on-grown in captivity and 2- and 3-year-old hatchery-reared snapper. Fish were held post capture for up to 168h. Blood was sampled at 0h and after the confinement period, the fish were sacrificed and the gonads preserved for histology. Plasma levels of cortisol and the gonadal steroids, 17β–estradiol (E_2), testosterone (T), 11-ketotestosterone (11KT) and 17,20β dihydroxy-4-pregnen-3-one (17,20βP) were determined by radioimmunoassay (RIA). Stress resulted in an increase in plasma cortisol and concomitant decreases in E_2, and T in females, and plasma T and 11KT in males. In addition there was an increase in the incidence of ovarian atresia in females, and in the proportion of spermatozoa in the testes of males. These results confirm that snapper are highly susceptible to stress-induced impairment of reproduction, and this response is still present in hatchery-reared fish.

Induced ovulation is a common requirement in aquaculture, but stress effects associated with handling may affect the efficacy of exogenous hormone treatments. This was tested by treating hatchery-reared female snapper, 0h (unstressed) or 24h after capture (stressed), with either luteinising hormone releasing hormone analogue (LHRHa), human chorionic gonadotropin (hCG), or 17α–hydroxyprogesterone (17P). Blood was sampled prior to treatment and again after 168 h and fish were checked periodically for ovulation. In unstressed fish, hCG gave the best ovulatory response, followed by LHRHa, in terms of numbers of ovulators, egg volumes, egg quality and percent fertilisation. A delay in injection resulted in significantly lower E_2 and T at injection, smaller egg volumes, and poorer egg quality, confirming that treatment at first capture, yields a better ovulatory response than treatment after exposure to capture and handling stress.

Studies on plasma levels of gonadal steroids suggest that stress effects of the type found in snapper might result from impairment of ovarian steroidogenesis. Accordingly 3-year-old female hatchery-reared snapper were stressed for up to 168h and isolated ovarian follicles were incubated with hCG, 17P, or hCG plus 17P. 17P was most effective at stimulating production of E_2 and T, and there was no further benefit in co-stimulation with hCG. Stress markedly reduced the capacity of ovarian follicles to convert 17P to T. E_2 production was unaffected, suggesting that aromatase-mediated conversion of T to E_2 is not affected by stress. This indicates that decreases in E_2 concentrations evident in the plasma following stress are possibly the result of stress-induced reduction in substrate availability and not a reduction in aromatase activity.

Acknowledgments

This study was carried out as a joint project between NSW Fisheries and the Department of Aquaculture, University of Tasmania (UTAS), and was funded by the Co-operative Research Centre (CRC) for Aquaculture. I am very grateful to the CRC for Aquaculture for the provision of my scholarship and generous financial support of the research project, and to NSW Fisheries for substantial in kind contributions, particularly with the provision of captive fish stocks and research facilities.

Thanks to my supervisors, Ned Pankhurst (Department of Aquaculture, UTAS) and Stephen Battaglene (formerly at the NSW Fisheries, Port Stephens Research Centre: PSRC), both of whose input has been invaluable. Thank you both for your encouragement, guidance and commitment, despite your own busy schedules. I deeply appreciate your efforts.

Thanks must also go to Stuart Fielder, Paul Beevers and Bill Bardsley of PSRC, and David Barker of Fisheries Research Institute (FRI), without whose help it would have been physically impossible to conduct the experiments. Thanks for getting up repeatedly for fishing at 4 AM; helping on field trips; caring for fish; and helping with all manner of other tasks. Thank you all for your constant enthusiasm and your friendship and for going the extra mile.

I also gratefully acknowledge Ian Walker for generously providing live trap-caught fish for two seasons; all the staff at PSRC who assisted in many and various ways; and Nino Quatararo, Andrew West and Brett Rankin (all previously of FRI) for maintaining fish stocks, helping with experiments and hospitality during our visits to FRI. Thanks to Barabara Nowak (Department of Aquaculture, UTAS) for generously providing accommodation during my extended visits to Launceston and friendship and encouragement throughout my studies.

To my fellow PhDers (Vera, Tiina, Phil, Mark, Jo, Ellen, Caroline, Brad, Andy): thanks for company on the dog-watch, little stats chats, diversional therapy, eating my cashews and most of all for making the 1st floor such a great place to work. Thanks to my phone and email buddies (The Big B, Christine, Jen, Gary and Margaret) and "Lonnie" friends (Uncle Dick, Rooy, Kristina, Doug, Marcia, Tim and Cherida, Bernard and Ree, and Anne and Andrew) for dragging my feet back to earth from time to time. Matt and Conor, you had the unenviable task of being my flatmates while I was writing up. Thanks for your friendship, patience and prayers. And Max, thanks for your constant little distractions, your grubby paws on my papers and your company at uni into the wee small hours.

And finally and most importantly I would like to thank my parents. Dad, thank you for proof reading chapters, all those motivational chats, late night emails, and statistical help. Mum thanks for endless hours on the phone, wisdom and encouragement. To both of you: thanks for your practical support, love, constant prayers and timely reminders to keep perspective! You were right; it was only a Ph.D.!

Table of Contents

List of abbreviations

11KT	11-ketotestosterone
17P	17α- hydroxyprogesterone
17,20βP	17,20β dihydroxy-4-pregnen-3-one
α-MSH	α-melanocyte stimulating hormone
ACTH	adrenocorticotrophic hormone
ANOVA	analysis of variance
c.v.	coefficient of variation
DAH	days after hatch
E_2	17β-estradiol
FL	fork length
FOM	final oocyte maturation
FRI	NSW Fisheries Research Institute
GnRH	Gonadotropin Releasing Hormone
GnRHa	Gonadotropin Releasing Hormone analogue
GtH	Gonadotropin
hCG	human chorionic gonadotropin
GSI	gonadosomatic index
GW	gonad weight
Hct	haematocrit
HSI	hepatosomatic index
LHRHa	Luteinising Hormone Releasing Hormone analogue
LW	liver weight
pc	post-capture
pi	post-injection
PSRC	NSW Fisheries, Port Stephens Research Centre
RIA	radioimmunoassay
SE	standard error
T	testosterone
µm	micrometer
VTG	plasma vitellogenin

List of Figures

List of Tables

Chapter 1: General Introduction

1.1 Aquaculture: global perspective

Many factors have led to a global increase in the demand for fish products. The most notable include world population growth, increased awareness of health advantages of fish (particularly ω-3 fatty acids), improved fish processing and more user-friendly products, and increased consumer confidence in product quality (Kearney, 1996). Demand for fish for human consumption in the year 2010 has been estimated to reach between 110 and 120 million tonnes (FAO, 1996). Total production of food fish in 1994, however, was only 76.6 million tonnes, 40 million tonnes short of the predicted consumption in 2010 (FAO, 1996). Of the world's top 200 marine fishery resources, the percentage of stocks requiring management has increased from virtually zero in 1950, to 60% in 1994, demonstrating the rapid increase in fishing pressure in recent decades (FAO, 1996). Although continued improved management of fisheries resources should increase or at least maintain the current wild fisheries production, it is clear that there is much room for expansion of the aquaculture sector. Indeed, the increase in total production of food fish from 1993 to 1994 (some 3.4 million tonnes) was almost entirely due to an increase in aquaculture production (FAO, 1996). Not surprisingly, the aquaculture sector is considered to be one of the fastest growing food production activities in the world. Continued expansion of the aquaculture sector is essential if fish production is to keep pace with increasing consumer demand.

1.2 Aquaculture: Australian perspective

Although Australia has the world's third largest 200 mile economic zone, its fish resources are sparse due to nutrient-poor waters, and consequent low productivity (Williams and Stewart, 1993). Australia's total fisheries production is less than 200,000 tonnes per year (i.e. 2 % of fisheries production in China). Currently more than 50 % of seafood consumed in Australia is imported (Kearney, 1996), and exploitation of fish stocks is resulting in reduced catch rates in many species (Kailola *et al*, 1993). Clearly, Australia's wild fishery has little capacity to match increasing consumer demand. Therefore, the increasing demand for growth in the aquaculture sector from business, environmental and public interest groups in Australia is not surprising.

Although Australian aquaculture is small by world standards, its importance in Australia's fisheries sector is increasing. Between 1990 and 1995, the value of aquaculture production in Australia increased by 77 % to over 396 million dollars, and the current contribution of aquaculture to national fisheries production is approximately 25 % (ABARE, 1996). In Australia, commercial production of finfish is virtually limited to Atlantic salmon (*Salmo salar*) in Tasmania, southern bluefin tuna (*Thunnus maccoyii)* in South Australia and barramundi *(Lates calcarifer)* in Queensland, South Australia and New South Wales. In order to keep up

with demand, compete with overseas markets and protect industry against potentially crippling disease outbreaks and environmental change, there is a need to diversify our marine finfish culture portfolio. In keeping with this necessity, a variety of finfish species is currently being investigated throughout Australia (Table 1.1; Battaglene and Fielder, 1997).

In order to establish culture of new species, an understanding of basic biology, nutritional requirements, farming techniques, and market profiles and projections is required. Furthermore, although in its infancy an aquaculture operation may be able to be supported by collection of wild fry, the long term success of a fish culture operation will generally depend on the ability to control the entire life cycle (Lam, 1982). Southern bluefin tuna culture in South Australia has been a notable exception, but this industry is now under increasing pressure from environmental lobbyists to close the life cycle. At present, relatively little is known about the control of reproduction for the majority of farmed finfish, nor of the nutrient, metabolic and husbandry requirements of the broodstock, or the ways in which they might be managed to optimise seed production, and improve the quality of eggs and larvae. Therefore, an understanding of reproduction, the factors that affect it and hence appropriate broodstock management, is integral to the development of new culture species. Furthermore, sound understanding of reproduction enables sophisticated control and thereby enables the use of more advanced technologies, such as advancement of spawning, hybridisation, triploidy, control of sex differentiation, and phasing of synchrony of ovulation/spermiation to meet production schedules (reviewed in Donaldson and Devlin, 1996). Snapper

Pagrus auratus (snapper), is a sparid considered to have potential as a temperate marine aquaculture species in Australia. It has an extended spawning season in spring, asynchronous ovarian development and a daily spawning rhythm (Scott *et al*, 1993). Ovulation is synchronised to occur at midday, and spawning follows in the early evening (Matsuyama *et al*, 1988; Scott *et al*, 1993). Snapper occur off the coasts of the southern part of Australia, and New Zealand. There is also a Northern Hemisphere population focused around China and Japan (Hutchins and Swainston, 1986). Previously the northern and southern populations were considered to be separate species, and were classified as *Pagrus major* (red seabream) and *Chrysophrys auratus* (snapper) respectively. Recently, however, the two populations have been reclassified as reproductively isolated populations of the same species, *Pagrus auratus* (Paulin, 1990). To distinguish between the different populations and research done on each, in the present volume, the Northern population will be referred to by its common name, red seabream, while the Southern populations will be referred to as New Zealand or Australian snapper as appropriate.

Table 1.1: Australian marine finfish under investigation or produced for commercial farming or enhancement programmes.

Common Name	Scientific Name	Climate	State or Territory
Atlantic Salmon [a]	*Salmo salar*	Coldwater	TAS
Rainbow Trout [a]	*Oncorhynchus mykiss*	Coldwater	TAS
Greenback Flounder [b]	*Rhombosolea tapirina*	Coldwater	TAS
Long-snout Flounder	*Ammotretis rostratus*	Coldwater	TAS
Striped Trumpeter	*Latris lineata*	Coldwater	TAS
Banded Morwong	*Chelidodactylus spectabilis*	Coldwater	TAS
Yellowtail Kingfish	*Seriola lalandi*	Temp/Cold	TAS
Black Bream	*Acanthopagrus butcheri*	Temp/Cold	TAS, WA
Yellowfin Bream	*Acanthopagrus australis*	Temperate	QLD
Southern Blue-finTuna [a]	*Thunnus maccoyii*	Temperate	SA
Australian Bass [a]	*Macquaria novemaculeata*	Temperate	NSW, QLD
Snapper [b]	*Pagrus auratus*	Temperate	NSW, SA,
Mulloway [b]	*Argyrosomus hololepidotus*	Temperate	NSW, SA
Sand Whiting	*Sillago ciliata*	Temperate	NSW, QLD
Trumpeter Whiting	*Silago maculeata*	Temperate	NSW
Dhufish	*Glaucosoma hebriacum*	Temperate	WA
Dolphin Fish	*Coryphaena hippurus*	Trop/Temp	QLD
Pikey Bream	*Acanthopagrus berda*	Tropical	QLD
Barramundi [a]	*Lates calcarifer*	Tropical	QLD, SA,
Coral Trout	*Plectropomus leopardus*	Tropical	QLD
Golden Snapper	*Lutjanus johnii*	Tropical	NT, QLD
Mangrove Jack	*Lutjanus argentimaculatus*	Tropical	QLD
Grouper	*Epinephelus* spp	Tropical	QLD, WA

Modified from Battaglene and Fielder (1997). [a] Produced commercially. [b] Experimental growout

Red seabream have been farmed intensively for almost 30 years (Foscarini 1988). In 1990, 40,000 tonnes were produced in Japan (Davey, 1990). In 1985, the total revenue from culturing red seabream was 10% more than the yield from the wild fisheries catch (Foscarini, 1988). The southern population has not yet been commercially produced on a large scale, however, it is considered to have high potential as a temperate marine aquaculture species in Australia (Battaglene and Bell, 1991). Snapper are highly prized both commercially and recreationally (Bell *et al*, 1991; Francis, 1994), fetching up to $20 per kilogram. The total annual catch in Australia is approximately 2,000 tonnes, however, in NSW catches have declined from 1,000 tonnes in 1980 to 440 tonnes in 1990 (Kailola *et al*, 1993). In addition, there is large body of knowledge already available, relating to the culture and biology of red seabream, due to the experience of Japanese researchers (reviewed in Foscarini, 1988; Fukusho, 1989). There is also considerable information on general biology of New Zealand snapper (e.g. Scott and Pankhurst, 1992; Scott *et al*, 1993; Francis, 1994). Recently in Australia, research has been conducted into developing techniques for farming snapper. This includes larval rearing (Battaglene and Talbot, 1992; Battaglene *et al*, 1993; Battaglene and Allan, 1994), grow out of juveniles (Bell *et al*, 1991, Quartararo, 1996), pilot scale seacage culture (Quartararo, 1996), feed development (Quartararo *et al*, 1992; Allan and Quartararo, 1996) and growth rates (Bell et al, 1991, Quartararo, 1996). The successful culture of the closely related gilthead seabream (*Sparus aurata*) in the Mediterranean further supplements this growing body of knowledge (Sweetman, 1992).

There is growing interest in snapper culture in Australia, with the first commercial farms already established in Jurian Bay, Western Australia and Spencer Gulf, South Australia, together with a number of other interested parties. Despite this interest, and despite recent technical advances, an unreliable supply of good quality eggs remains a major impediment to the establishment of a viable Australian snapper farming industry (Battaglene and Talbot, 1992; Battaglene, 1995). Red seabream culture in the Northern Hemisphere initially relied on wild-caught fry and hormonal induction of ovulation of wild-caught mature adult fish. Hormonal induction of ovulation, however, usually resulted in poor egg quality and low fertilisation (reviewed in Foscarini, 1988). Furthermore, the supply of adult fish was unreliable, and often wild fish were immature or difficult to acclimate to culture conditions (Foscarini, 1988). The problem of seed supply has largely been overcome by domestication of red seabream broodstock. There are now fully domesticated red seabream broodstock, which have been propagated for nearly 30 years (Foscarini, 1988). These stocks spawn "naturally" (i.e. without the use of hormones) in tanks, even at high densities, so much so, that they are considered to be particularly favourable to reproduction under culture conditions (Foscarini, 1988). Due to the lack of fully domesticated broodstock in Australia, culture of snapper has until recently, relied totally on the use fish treated with exogenous hormones. Although in the long term it is likely that solution to the problem of egg quality and supply from Australian snapper will be to domesticate broodstock, it will be some time

before a degree of domestication similar to the current status of the red seabream is achieved. To this end, recent attempts at domestication have been successful, with first-generation hatchery-reared snapper spawning and fertilising "naturally" under photo-therm stimulation (G. Allan and S. Fielder, NSW Fisheries, Port Stephens Research Centre, pers com; G. Jenkins, Fremantle Aquaculture Centre, Fremantle pers. comm.). Wild-caught broodstock, despite being held for two successive seasons in identical conditions to the first-generation fish, have not yet spawned in captivity. Despite early success with first-generation stocks, hormonal induction of ovulation will remain a necessary component of snapper culture (at least in the short term) due to requirements for broodstock replacement, maintenance of genetic diversity, and establishment of new farming ventures. Ovulation has been induced in wild-caught snapper in New Zealand with human chorionic gonadotropin (Pankhurst and Pankhurst, 1989; Pankhurst and Carragher, 1992) and in Australia with luteinising hormone releasing hormone analogue and ovaprim (Battaglene and Talbot, 1992; Battaglene and Allan, 1994; Battaglene, 1995). The results however have been disappointing, with a low proportion of mature fish responding to the treatments, and the eggs being of poor quality, with low fertilisation and subsequent survival (Battaglene and Talbot, 1992; Pankhurst and Carragher, 1992; Battaglene and Allan, 1994).

Snapper are known to be highly sensitive to capture and handling stress, exhibiting a rapid increase in plasma cortisol, and a concomitant decrease in plasma gonadal steroid levels (Carragher and Pankhurst, 1991). It is likely, therefore, that reproductive problems may be associated with the stress of capture and handling, prior to hormone treatment (Battaglene and Talbot, 1992). Therefore, an understanding of reproduction in snapper and how stress affects natural reproductive processes is a priority in the development of a snapper farming industry. For this reason, this thesis expands the examination of the effect of stress on reproduction in snapper (*Pagrus auratus*).

1.3 Endocrinology of stress and reproduction

Problems with reproduction in captive fish are common. Many fish, despite apparent acclimation, fail to complete vitellogenesis, oocyte maturation, ovulation, spermiation or spawning (Lam, 1982; Pankhurst, 1998). Several factors are implicated in captivity-impaired reproduction, including poor nutrition, absence of environmental cues or spawning substrate, poor water quality and inadequate tank size (Pankhurst, 1998). The specific requirements will be expected to vary from species to species. In addition to these factors, stress has been found to have a profound effect on reproduction in a variety of species. Stress, which is virtually unavoidable in aquaculture practice, can result from poor water quality, capture, handling, sorting, grading, transport, confinement, and even routine tank maintenance (Barton and Iwama, 1991).

Although the concept of stress may appear to be simple, there has been considerable debate over a working definition of stress. In this thesis the term stress will be used to describe the state of an organism, caused by a stress factor, or stressor, that deviates from the normal resting state (Barton and Iwama, 1991). The stress response refers to the physiological or behavioural modifications designed to re-establish homeostasis. These responses can be measured to give an indication of the degree of stress (Barton, 1997). Stress can be either chronic or acute in nature. An acute stress episode terminates before the stress response is complete (e.g. capture and handling), while chronic stress results from long term factors (e.g. overcrowding, confinement, and poor water quality; Pickering *et al*, 1982). Although in the short term, the stress response is designed to aid the organism to respond and overcome the stressor (i.e. it is adaptive), the stress response can also be maladaptive, if the stress exceeds the capacity of the organism to adjust and so maintain equilibrium (Barton and Iwama, 1991; Barton, 1997). The response to stress has been classified into three categories: primary, secondary, and tertiary according to the physiological level of effect (Mazeaud *et al*, 1977; Sumpter, 1997). The primary or hormonal response includes corticosteroids (primarily cortisol), catecholamines (primarily adrenaline and noradrenaline) and a range of other hormones including adrenocorticotrophic hormone, α–melanocyte stimulating hormone, and endorphins (Sumpter *et al*, 1993). The catecholaminergic response occurs rapidly, with the release of adrenaline and nor-adrenaline from the chromaffin tissue in the kidney, occurring within seconds of the imposition of a stressor. In contrast, the release of corticosteroids from the interrenal occurs within minutes (reviewed by Sumpter, 1997). Stress also elicits a physiological or secondary response, affecting metabolic (plasma glucose, plasma lactic acid), haematological (haematocrit, erythrocytes, leucocytes), hydromineral (plasma protein, chloride, sodium) and structural (interrenal cell size) condition. The tertiary response involves the "whole animal". It includes changes in growth rate, condition factor, disease resistance and reproductive capacity (Barton and Iwama, 1991). Although in proposing this multi-level stress response, Mazeaud *et al* (1977) implied a causal relationship between the different levels, Barton and Iwama (1991), point out that the fact that the different responses co-vary, does not necessarily imply a causal relationship.

That stress has a detrimental effect on reproduction has long been recognised (e.g. De Montalembert *et al*, 1978). The majority of early work addressed the effects of pollutants, low oxygen and reduced pH (reviewed in Barton and Iwama, 1991). More recently, the effect of culture-induced stress on reproduction has been studied in various species by a number of investigators (e.g., Pickering *et al*, 1987; Safford and Thomas, 1987; Sumpter *et al*, 1987). The studies have been directed at a range of levels in the reproductive cascade, and have reported changes in morphology and histology of gonads (Pickering *et al*, 1987; Carragher *et al*, 1989; Carragher and Pankhurst, 1991; Clearwater and Pankhurst, 1997), changes in plasma and pituitary gonadotropin (Pickering *et al*, 1987; Sumpter *et al*, 1987), a decrease in plasma levels of gonadal steroids (e.g. Pickering *et al.*, 1987; Sumpter *et al.*, 1987; Safford and Thomas, 1987; Clearwater and Pankhurst, 1997), reduced egg production (De Montalembert *et al*, 1978), reduced quality of gametes (Campbell *et al.*, 1994), and an increase in incidence of abnormal embryos (Wilson *et al*, 1995).

1.4 Scope and aims of this project

The overall goal of the research described in this volume was to address the problem of egg quality and supply from Australian snapper. This was done by investigating the effect of stress on reproduction, with a view to extending current knowledge of the underlying endocrinology and in so doing, identifying ways of ameliorating stress effects on egg production. The particular stress selected for examination was capture, handling and confinement, due to its relevance to the aquaculture situation, and its simple and repeatable mode of application. The study comprised three main experiments, which are described in Chapters 3 to 5.

Chapter 3 reports the effect of capture and culture history on the stress response in snapper. The response of wild-caught, lab-acclimated and hatchery-reared fish to capture, handling and confinement stress were compared, in terms of changes in plasma cortisol and plasma gonadal steroids, and gonadal condition.

Stress-reduced plasma gonadal steroid levels and gonadal condition have been described for a number of species, however, the effect that these have on subsequent ovulatory events is largely undescribed. It is of practical concern to fish culturists, whether capture, handling and other routine husbandry practices detrimentally affect subsequent ovulatory events, gamete quality, hatchability or larval survival (Barton and Iwama, 1991). Furthermore, this research can indicate means of improving broodstock management. Therefore in Chapter 4, the effect of stress on the ovulatory and endocrine response to exogenous hormone therapy was examined in both wild and hatchery-reared fish.

Although stress is known to alter at least some reproductive processes in all fish so far studied, the mechanism by which stress affects reproduction is unknown. Due to the consistent stress-induced elevation in plasma cortisol levels (and inverse relationship with plasma gonadal steroid levels) and suppression of plasma gonadal steroid levels by exogenous cortisol, cortisol has been considered the most likely candidate for direct involvement. The effect of cortisol on *in vitro* ovarian steroidogenesis by cortisol, however, is inconsistent, bringing into question the theory that cortisol is directly involved in stress induced impairment of reproduction. Since cortisol and stress are not synonymous, in Chapter 5, the effect of capture and handling stress (rather than cortisol alone) on *in vitro* ovarian steroidogenesis was investigated, in order to determine if reduced plasma gonadal steroid levels following stress may (at least in part) be the result of suppressed gonadal steroidogenesis.

In summary, the effect of stress on reproduction in snapper was examined in terms of plasma reproductive steroids, gonadal condition, ovulatory response to hormone treatment and ovarian steroidogenesis, and provides a sound framework for successful management of snapper and other stress-sensitive species.

Chapter 2: General Materials and Methods

2.1 Fish Capture and Husbandry

A variety of snapper, *Pagrus auratus,* with different capture and culture histories was used in the experiments: wild fish caught by trap or line; captive fish caught as juveniles and on-grown in pools; and hatchery-reared fish on-grown in seacages. Since snapper are serial spawners, with a daily spawning rhythm, initial capture of all fish was restricted to between 0600 and 1200 h, in order to minimise variation due to natural diel change (Carragher and Pankhurst, 1993).

Wild Fish

Wild Fish were caught at depths of 20 to 60 m from the waters surrounding Port Stephens (32° 38' S, 152° 15' E), New South Wales between September and November 1994 and 1995 (Chapters 3 and 4). Fish were caught by handline by NSW Fisheries staff (Line-Caught) and in baited traps by commercial fishermen (Trap-Caught). Traps (2 x 1 x 1 m) were set at depths between 19 and 80 m, for 3 to 168 h. Upon landing, the traps were emptied onto the deck of the commercial fishing vessel. Snapper were sorted from the by-catch and transferred at sea, in buckets partially filled with seawater, to the NSW Fisheries Research Vessel, *Pagrus*. Immediately after landing, swim bladders of both line and trap-caught fish were deflated using a 25 gauge needle and the fish transported to the Port Stephens Research Centre (PSRC) by boat in a 375 L, covered and oxygenated tank with regular water exchange (Boat Transporter).

Captive Fish

Captive Fish were collected as juveniles in 1989, and held in two pens (each 10 x 7.5 x 2.4 m) in the Marine Research Pool (30 x 15 x 2.4 m), New South Wales Fisheries Research Institute (FRI), Cronulla (34° 47' S, 151° 8' E). These fish were held for a growth experiment comparing pellet and fresh meat diets. Details can be found in Bell *et al.* (1991), which describes fish, growth and holding conditions.

Prior to the current experiment all fish were fed to satiation (0.5 - 2.5 % of wet body weight) on a commercially produced pellet (Barramundi Grower, Kinta Feeds, Yarrawonga, Victoria), supplemented by flesh (e.g. pilchard, squid, prawn). They were held at ambient seawater temperatures, ranging from 23 °C in January to 14 °C in July. The water in the pool was mixed and aerated by airlift pumps. Water exchange was approximately 1200 $L.min^{-1}$. Dissolved oxygen was approximately 70-90 %. Fish for use in the experiments were caught from the pen by handline or by crowding in a shortened net and removing with a handnet as logistically appropriate (outlined in individual experiments). Fish were then allocated to tanks as described in individual experiments. Captive Fish were used in experiments in 1994 (Chapter 3).

Hatchery-reared Fish

Two different groups of Hatchery Fish were used:

The first group was hatchery reared from eggs stripped from wild fish caught in October 1992 at PSRC. They were transferred to 4000 L flow-through tanks at 54 days after hatch (DAH) (30-35 mm total length) and held for 5 months, after which they were stocked into floating sea-cages in Botany Bay (5 x 5.5 x 3.5 m), at Kurnell (34° 47' S, 151° 8' E) (Quartararo, 1996). Fish from this source were used in experiments in September 1994 when they were 2-year-old fish (Chapter 3), and in September 1995 when they were 3-year-old fish (Chapter 3 and 4).

The second group was hatched in November 1993 transferred directly to the seacage on day 54 DAH (30-35 mm total length) and reared as per the first group. These fish were transferred to a pen (10 x 7.5 x 2.4 m) in the Marine Research Pool, FRI in November 1995, when they were 2 years old. They were maintained in the pool until September 1996 when they were used in an experiment as 3-year-old fish (Chapter 5).

Hatchery Fish were reared and held by NSW Fisheries as a pilot scale study on rearing snapper in seacages. For details see Quartararo (1996), but in brief, Hatchery Fish were fed to satiation daily (0.5 - 2.5 % of wet body weight) on commercially prepared pelleted snapper diet from Kinta Feeds, Yarrawonga, Victoria, supplemented by flesh as for captive fish. They were kept at ambient seawater temperature, which ranged from 22 °C in February to 15 °C in August. Nets surrounding the cages were changed bimonthly to control biofouling. In order to minimize disturbance to maturing fish, net changes were avoided from August until the time of sampling each year.

Hatchery Fish were captured from the cage by handline and from the pool either by handline or crowding in a shortened net and individuals removed by handnet. After capture from the seacage they were held in 400 L covered and oxygenated polyurethane tanks (max. 20 fish/tank) onboard a punt alongside the cages (Onboard Holding Tanks). They were transferred by bucket to 750 L insulated and oxygenated tanks on a truck (Truck Transporter; max. 20 fish/tank) for transport to FRI. At FRI, fish were transferred by bucket to experimental tanks as described in Chapters 3 and 4.

2.2 General Experimental Procedures

Fish Handling

Fish were either anaesthetised with 0.01 % 2-phenoxyethanol (Sigma) in seawater until cessation of locomotory movement or not anaesthetised, as required by experimental design. For ease of handling, all fish were restrained ventral side up in a slit in a dense foam block for blood sampling and other procedures. The slit was lined with plastic and thoroughly wetted with seawater to prevent abrasion damage during sampling. Fish were handled with gloved hands to further reduce abrasion damage. At the time of capture, fish were marked for identification by Hallmark® t-bar tags, or fin clips. Fish were processed according to the requirements of each experiment immediately after capture.

During the experiments fish were held in a range of different tanks for up to a week (see Chapters 3 to 5). Tank disturbance was kept to a minimum during this time. To this end, fish were not fed during the experiments in order to reduce the need for tank cleaning. The tanks were siphoned to remove sediment as required only at designated sampling times.

Gonadal Biopsy

At first capture, sex and reproductive stage were determined by inserting silicon tubing (1 mm internal diameter) into the gonadal duct and removing a gonadal sample with gentle back pressure (Gonadal Biopsy). Reproductively mature females and males were identified according to criteria described in Tables 2.1 and 2.2.

Blood Sampling

Assembled 22 gauge 1.5" needles and 2 ml syringes were preheparinised to prevent blood clotting by aspirating and expelling heparin solution (5 $mg.ml^{-1}$ methiolate + 500 $U.ml^{-1}$ heparin in acidified saline). Heparinised syringes were stored on ice, or in the refrigerator until used. Blood was taken from the caudal vein (1-2 ml) at each sampling. After collection, blood samples were kept on ice. Plasma was separated by centrifuging at 6400 rpm for 2 minutes within 6 h of collection and stored frozen until assay.

Haematocrit

Whole blood haematocrit (Hct) was measured using non-heparinised glass capillary tubes (50 x 1.5 -1.6 mm) filled with blood by capillary action and centrifuged at 6400 rpm for 2 minutes. Hct was calculated as the length of the red blood cell portion as a percentage of the total length.

Biological Data

At the conclusion of experiments, fish were sacrificed by brainspiking, and fork length (FL; mm), whole body (g), liver (LW; g) and gonad weights (GW; g) were determined. Gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated as GW or LW as a percentage of body weight minus GW or LW respectively.

Macroscopic Gonad Staging

Gonads from freshly killed fish were macroscopically staged according to criteria adapted from Scott and Pankhurst (1992) (Tables 2.1 and 2.2) in order to confirm initial biopsy staging.

Table 2.1. Biopsy, macroscopic and histological criteria used to stage snapper ovaries.

Stage	Classification	Biopsy Criteria	Macroscopic Criteria*	Histological Criteria*
I	Immature	• no sample	• ovary small and transparent	• previtellogenic oocytes only
II	Previtellogenic	• cream or pink sample	• ovary small and cream or pale pink • some small oocytes visible by eye	• cortical alveoli stage oocytes present
III	Vitellogenic	• opaque oocytes visible against light	• proliferation of small visible oocytes • ovary pale pink to creamy yellow	• vitellogenic oocytes present
IV	Maturing	• large transparent oocytes visible	• hydrated oocytes present	• hydrated oocytes present • possibly oocytes undergoing germinal vesicle migration
V	Ovulated	• eggs flow with light pressure to abdomen • no biopsy required	• ovulated eggs free in the oviduct	• hydrated oocytes present • post ovulatory follicles present
VI	Spent	• no sample	• ovary flaccid and 'bloody' • residual eggs sometimes present in the oviduct	• primarily previtellogenic oocytes present • post-ovulatory follicles present • increased vascularisation • follicular atresia

* Adapted from Scott and Pankhurst (1992)

Table 2.2. Biopsy, macroscopic and histological criteria used to stage snapper testes.

Stage	Cassification	Biopsy Criteria	Macroscopic Criteria*	Histological Critera*
I	Immature or Regressed	• no sample	• testis threadlike	• spermatagonia and primary spermatocytes predominate
II	Spermatogenic	• no sample	• testis increased in size • testis ivory white in colour • no expressible milt	• secondary spermatocytes predominate • spermatids present • spermatozoa present in larger gonads
III	Partially Spermiated	• opaque ivory white sample	• viscous milt expressible	• spermatozoa predominate
IV	Fully Spermiated	• milt flows freely with light pressure to the abdomen • no biopsy required	• milt released into sperm duct and flowing freely	• spermatozoa predominate
V	Spent	• no sample	• testis 'bloody' or grey • flaccid • no milt	• residual spermatozoa • spermatogonia present towards testis margin

* Adapted from Scott and Pankhurst (1992)

Histology

A small piece of gonad (approximately 1-1.5 cm^3) from each fish was fixed in Bouin's fixative (glacial acetic acid : picric acid : formalin; 1:15:5 v/v) for 24 to 72 h and then transferred to 70 % ethanol for storage. The tissue was later dehydrated in an ethanol series, embedded in paraffin and sectioned transversely at 4 µm prior to staining with haematoxylin and eosin (H&E). Sections were scored with the aid of a microscope.

Histological Gonad Staging

Gonads were staged according to histological criteria adapted from Scott and Pankhurst (1992) (Tables 2.1 and 2.2). Ovarian development was assessed by the most mature oocyte stage present (West, 1990). Testis development was assessed according to the predominant cell type.

Histological Scoring

Ovary

Ovarian sections were examined by scoring the stage, occurrence and condition (ie. normal or atretic) of each oocyte that lay along the axis line of an eye piece micrometer, at each unit on the vernier scale. Normal oocyte stages were identified using criteria adapted from Nagahama (1983) and Matsuyama *et al.* (1988) (Table 2.3). Atretic oocytes were identified by the disintegration of the zona radiata and granulosa layer, and from a breakdown of regular structure within the cell (Clearwater and Pankhurst, 1997; Nagahama, 1983). Micrographs of each oocyte type are presented in Chapter 3 (Fig. 3.3). One hundred vitellogenic oocytes were counted from each section. Previtellogenic oocytes were scored as encountered while counting the vitellogenic oocytes. Numbers of vitellogenic and previtellogenic oocyte types were presented as a percentage of total vitellogenic or previtellogenic oocytes counted, respectively.

Testis

Sections of testes were examined by scoring the occurrence of each cell type, blood vessels, and interstitial space at each intersection point on a 10 by 10 eyepiece graticule after Pankhurst *et al.* (1987) (Table 2.4). Two hundred intersections were scored per fish. The grid was placed on the perimeter of the section to avoid the central areas where sperm ducts dominated the field. Micrographs of each cell type are presented in Chapter 3 (Fig. 3.12).

Table 2.3. Criteria for identifying normal and atretic forms of oocyte stages found in cross sections of snapper ovaries stained with haematoxylin and eosin. Shaded area indicates oocyte types that were counted as part of the first 100 vitellogenic oocytes.

	Oocyte Classification	Size (µm)	Normal	Atretic
1	Primary	15 - 125	• cytoplasm stains deeply with haematoxylin. • acidophilic nucleoli (red) in the nucleus	
2	Cortical Alveoli	125 - 165	• stained with haematoxylin (purple) • proliferation of lipid bodies and cortical alveoli (white)	• breakdown of cortical alveoli • disintegration of zona radiata and granulosa layers
3	Early Vitellogenic	215 - 295	• first appearance of acidophilic yolk globules (red), increasing in size and number	• breakdown of cortical alveoli and yolk globules • disintegration of zona radiata and granulosa layers
4	Late Vitellogenic	315 - 490	• acidophilic yolk globules predominant, increasing in size and number	• breakdown of cortical alveoli and yolk globules • disintegration of zona radiata and granulosa layers
5	Migratory Nucleus	440 - 540	• migrating nucleus • lipid droplets and yolk globules coalesce	• breakdown of cortical alveoli and yolk globules • disintegration of zona radiata and granulosa layers
6	Maturing	490 - 590	• very large acidophilic yolk globules • lipid droplets coalesce to form large lipid body	
7	Hydrated	540 - 785	• yolk globules and lipid droplets completely fused • oocyte becomes pale pink in colour and very large	
8	Post-Ovulatory		• infolded follicular layer • irregular cellular mass • stained lightly with haematoxylin	

Adapted from Matsuyama *et al* (1988) and Nagahama (1983).

Table 2.4. Histological criteria used to identify cell types in cross sections of snapper testes stained with haematoxylin and eosin.

	Cell Classification	Size (µm)	Description*
1	Spermatogonia	4 - 8	• large cell with pale staining cytoplasm • dark staining granular perimeter
2	Primary Spermatocyte	3 - 5	• large medium staining cytoplasm • large dark staining nucleus • granular appearance • tightly packed
3	Secondary Spermatocyte	2 - 3	• dark staining cytoplasm • tightly packed
4	Spermatid	1 - 2	• small cell • dark staining nucleus • clear cytoplasm
5	Spermatozoa	1 - 2	• small dense staining heads • light staining tails visible

* Adapted from Nagahama (1983).

Steroid Measurement

Plasma

Two hundred µl of plasma were extracted with 1 ml ethyl acetate. Replicate 100 µl (17β-estradiol, E_2; testosterone, T; 11-Ketotestosterone, 11KT; 17α,20β dihydroxy-4-pregnen-3-one, 17,20βP) or 50 µl (cortisol) aliquots were dried down in assay tubes and redissolved in 200 µl of assay buffer (0.05 M phosphate buffer containing 1 % gelatine). This was analysed for concentrations of E_2, T, 17,20βP, 11KT and/or cortisol by radioimmunoassay (RIA) as described below.

Incubation Media

Incubation media from *in vitro* experiments were thawed and centrifuged at 1200 rpm for 3 min at 4 °C to remove any follicular debris. Replicate 150 µl aliquots of the media were added to 50 µl of assay buffer and then E_2 and T were measured by RIA as described below.

Radioimmunoassay

Assays were conducted using the reagents and protocol given in Pankhurst and Carragher (1992). Briefly, 200 µl of radiolabelled steroid and 200 µl of appropriate antibody were added to the assay tubes prepared above (plasma and incubation and media samples). The assay tubes were incubated overnight at room temperature. After being chilled on ice, 200 µl of cold dextran coated charcoal suspension were added, the tubes vortexed, left to stand for a further 10 minutes, and centrifuged at 3000 rpm for 10 minutes at 4 °C. The supernatant was decanted and 4 ml of scintillation cocktail (12 g PPO and 0.6 g POPOP in toluene: Triton X-100 (2:1 v/v), Crown Scientific) or Ecolite (Imbros) added.

Assay Quality Control

Extraction efficiency was determined by recovery of labelled steroid from plasma and assay values corrected accordingly. Interassay variability within each experiment was measured using aliquots of pooled standards. Detection limits for each experiment were calculated as the mean of the detection limit for each assay.

Extraction efficiency for plasma from the experiment described in Chapter 3 was on average 82.8 %, 95.3 %, 99.4 % and 99.5 % (n=8) respectively for E_2, T, 17,20βP and cortisol. Extraction efficiency was not determined for 11KT, but it was assumed to be 100 % based on previous assays (N.W. Pankhurst, University of Tasmania, pers com 1995). Interassay variation was (%c.v.(n)) 16.9(10), 20.3(14), 23.9(10), 22.0(5) and 18.9(13) respectively for E_2, T, 17,20βP, 11KT and cortisol. Detection limits (plasma equivalents) were 0.17, 0.15, 0.15, 0.15 and 0.60 $ng.ml^{-1}$ respectively for E_2, T, 17,20βP, 11KT and cortisol (n=10, 14, 10, 5, 13 respectively).

Plasma from the experiment described in Chapter 4 was analysed together with plasma from another experiment, not described in this volume. Extraction efficiency was 75.9 % (n=5), 91.2 % (n=5), 96.7 % (n=5) and 97.1 % (n=7) for E_2, T, 17,20βP and cortisol respectively. Interassay variation was (%c.v.(n)) 12.7(6), 20.9(6), 18.0(6) and 17.4(7) for E_2, T, 17,20βP and cortisol respectively. Assay detection limits (plasma equivalents) were 0.20, 0.17, 0.16 and 0.62 $ng.ml^{-1}$ for E_2, T, 17,20βP and cortisol (n=6, 6, 6, 7 respectively).

Plasma for the experiment described in Chapter 5 were analysed in one assay per steroid. Extraction efficiencies were 78.5 %, 86.4 % and 96.5 % for E_2, T and cortisol respectively. Assay detection limits (plasma equivalents) were 0.19 $ng.ml^{-1}$ for E_2, 0.17 $ng.ml^{-1}$for T and 0.62 $ng.ml^{-1}$ for cortisol. Interassay variability for media samples was (%c.v. (n)) 16.2 (9) and 25.2 (9) for E_2 and T respectively. Assay detection limits (media equivalents per 500 follicles) were 16.6 $pg.ml^{-1}$ for both E_2 and T (n=9).

Statistics

Data were tested for homogeneity of variance (Bartlett's test) prior to performing parametric analyses. Analysis of Variance (ANOVA) of data with heterogeneous variances increases the probability of a Type 1 error (Underwood, 1981). Therefore data sets with heterogeneous variances were transformed by logarithmic, square root or arc-sin (for proportional data) transformations before further analysis. Where transforms failed to correct heterogeneity of variance, data were still analysed, however α was set at 0.01 for these analyses. Comparisons of means following ANOVA were done using the Tukey-Kramer HSD or SNK procedures. All analyses were run on the JMP statistical package (version 3.1.6.2) with α set at 0.05 except in the situation described above. All means are expressed as mean + SE.

Chapter 3: The effect of capture and handling stress on plasma steroid levels and gonadal condition in wild and captive snapper.

3.1 Introduction

The response of an organism to stress can be both adaptive and maladaptive (Barton and Iwama, 1991; Barton, 1997). The primary stress response involves the release of corticosteroids (e.g. cortisol) and catecholamines (e.g. adrenaline and noradrenaline) into the blood stream, and is designed to mobilise energy reserves and maximise respiratory capacity to enable the organism to overcome the stressor (Mazeaud *et al*, 1977). In the longer term, however, stress can have maladaptive effects on a range of functions, including reproduction (Barton and Iwama, 1991). Stress has been shown to affect reproduction in a number of teleost species, and at various levels in the reproductive-endocrine cascade, including steroidogenesis (reviewed in Pankhurst and Van Der Kraak, 1997). Assessment of the effects of stress on reproduction requires some understanding of endocrine regulation of reproduction in the target species.

In female teleosts, 17β-estradiol (E_2) is synthesised by the oocyte follicular layer from testosterone (T) via aromatase activity (Sundararaj *et al*, 1982; Young *et al*, 1982a; b). E_2 stimulates the production of vitellogenin by the liver (Ng and Idler, 1983; Tyler, 1991) which is taken up by the developing oocytes in the ovary (reviewed in Specker and Sullivan, 1994). T may also be involved in feedback via the pituitary to stimulate GtH synthesis and secretion (Trudeau *et al*, 1993). Plasma E_2 and T levels are generally high in teleosts, with maximum levels commonly ranging from 50 to 160 $ng.ml^{-1}$ (Wingfield and Grimm, 1977). In snapper, and other sparids, however, plasma levels of E_2 and T are somewhat lower. Plasma E_2 and T levels measured in New Zealand snapper (*Pagrus auratus*) are typically less than 3 and 1 $ng.ml^{-1}$ respectively (Hobby and Pankhurst, 1997a). Both plasma E_2 and T levels were highest in wild-caught New Zealand snapper undergoing ovarian recrudescence and ovulation (Carragher and Pankhurst, 1993) and increased significantly following treatment with human chorionic gonadotropin (hCG), concurrent with increasing oocyte diameter (Pankhurst and Carragher, 1992). Therefore plasma E_2 and T are considered useful plasma indicators of vitellogenesis and ovarian recrudescence (Pankhurst and Carragher, 1991). 17α, 20β-dihydroxy 4-pregnen 3-one (17,20βP) has been demonstrated to be a potent inducer of final oocyte maturation (FOM) in females of a variety of species (Scott and Canario, 1982; 1987). In New Zealand snapper treated with hCG, plasma 17,20βP levels peaked during FOM (Pankhurst and Carragher, 1992), however, in naturally spawning

wild-caught fish, plasma 17,20βP levels remained low throughout the year, regardless of reproductive stage (Carragher and Pankhurst, 1993). Ventling and Pankhurst (1995), however, demonstrated that 17,20βP was the most effective of a range of C21 steroids, at inducing FOM in New Zealand snapper. In addition, 17,20βP has been identified as the most likely FOM inducing steroid in red seabream (*Pagrus auratus*; Kagawa *et al*, 1991).

In male teleosts, T and 11-ketotestosterone (11KT) are the predominant plasma androgens during testicular development. The Leydig cells are the major sites of synthesis of gonadal steroids in males, however, spermatozoa and the epithelial cells around the vas deferens are also steroidogenic (Fostier *et al*, 1987). T and 11KT are involved in testis growth, the development of secondary sexual characteristics (Liley *et al*, 1987; Cardwell and Liley, 1991), various stages of spermatogenesis (Fostier *et al*, 1987) and feedback control (Trudeau *et al*, 1991). In New Zealand snapper, plasma T and 11KT levels increased with testicular recrudescence, lending support to their being involved in initiating and maintaining testicular development (Carragher and Pankhurst, 1993). 17,20βP is believed to play a role in stimulation of milt hydration in male teleosts generally (reviewed in Pankhurst, 1994), and is elevated in spawning males of a number of species (Scott *et al*, 1984; Liley and Kroon, 1995; Pankhurst, 1995). Plasma 17,20βP levels in New Zealand snapper caught from the wild, were highest in spermiating fish, supporting its suggested role in controlling spermiation in male teleosts (Carragher and Pankhurst, 1993), and treatment of New Zealand snapper with 17,20βP increased milt volume (Pankhurst, 1994).

Capture and handling stress has been found to have a profound effect on circulating levels of gonadal steroids in a variety of teleosts. For example, E_2 and T levels have been shown to decrease rapidly following stress of capture, handling and confinement in female red gurnard (*Chelidonichthys kumu*; Clearwater and Pankhurst, 1997), spotted seatrout (*Cynoscion nebulosus;* Safford and Thomas, 1987), rainbow trout (*Oncorhynchus mykiss;* Pankhurst and Dedual, 1994) and New Zealand snapper (Carragher and Pankhurst, 1991). Plasma 11KT and T levels in male brown trout, *Salmo trutta* decreased following chronic confinement for one month, and also following acute handling stress for 1h (Pickering *et al*, 1987). In contrast to plasma levels of androgens and estrogens, plasma 17,20βP levels have been found to increase, or remain unchanged following stress. Plasma 17,20βP levels in wild-caught female snapper increased significantly, following 6 h of confinement (Pankhurst and Carragher, 1992). A similar result was found for wild-caught snapper exposed to capture, confinement and daily handling stress, with significantly higher plasma 17,20βP levels after 5 days (Carragher and Pankhurst, 1991). In contrast, no change in plasma 17,20βP levels were detected following capture and handling of wild-caught female rainbow trout (Pankhurst and Dedual, 1994).

Stress is also accompanied by degenerative changes in the ovary. Oocyte atresia is a degenerative process, which eliminates developing follicles before ovulation, through apoptosis (Tilley *et al*, 1991; Janz and Van Der Kraak, 1997). It is detected by irregular structure, or breakdown of the zona radiata and loss of regular structure of cell contents (Nagahama, 1983; Clearwater and Pankhurst, 1997). It has been identified in the ovaries of wild-caught fish throughout the spawning period, but is generally more marked at the end of the spawning season, as unspawned vitellogenic oocytes are resorbed (Hunter and Macewicz, 1985). Atresia has also been observed in fish subjected to capture stress and is thought to be irreversible in that season (De Montalembert *et al* 1978; Barton and Iwama, 1991; Clearwater and Pankhurst, 1997). Atresia is generally more prevalent in captive than wild fish (Hunter and Macewicz, 1985; Barton and Iwama, 1991). Capture and confinement of wild-caught red gurnard resulted in an increase in the incidence of oocyte atresia, preceded by a decrease in plasma E_2 (Clearwater and Pankhurst, 1997). Janz and Van Der Kraak (1997) showed that this may be due to the modulating effect that E_2 has on apoptosis. Although the effects of stress on ovarian condition have received a deal of attention, it is still not clear what the effects of stress on the testes may be. Campbell *et al* (1992), however, did report a detrimental effect of repeated acute stress on spermatocrit, and possibly sperm viability of male trout.

The effect of stress varies according to the genetic background of the animal. Differences in the stress response have been reported for different strains within species, different early rearing environments and even within individuals (Pickering and Pottinger, 1997). Pickering and Pottinger (1989) found differences in the magnitude of the cortisol response among 5 strains of hatchery-reared rainbow trout, while Pottinger and Moran (1993) reported differences in plasma cortisol and cortisone dynamics between strains of rainbow trout. Differences have also been detected between wild and hatchery-reared fish. Wild rainbow trout showed a more extreme response to a variety of stressors in terms of plasma cortisol and glucose levels than hatchery reared trout (Woodward and Strange, 1987). Salonius and Iwama (1993) found that wild coho (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tschawytscha*) had significantly higher plasma cortisol levels following handling than their hatchery-reared counterparts. This demonstrated the importance of environmental conditions under which the fish are reared, as both groups of fish were from the same genetic stock. Through research on the heritability of the stress response, it has been found that individuals can vary in their response to stress. Pottinger *et al* (1992) reported that only 30% of individuals showed a repeatable response to similar stress exerted on separate occasions. Further work has, however, confirmed that the stress response is at least to some degree heritable (Fevolden *et al*, 1991; Heath *et al*, 1993; Pottinger *et al*, 1994). This leads to the possibility that Australian snapper, *Pagrus auratus*, may show variation in the stress response between wild, captive and hatchery-reared stocks. In addition it also possible that the stress effects are different to those of New Zealand snapper.

Haematocrit (Hct) measures the packed cell volume in the blood (Blaxhall, 1972) and is used as an indicator of blood oxygen-carrying capacity (Houston, 1990). It is altered by changes in erythrocyte or plasma volume (Härdig and Höglund, 1984) and splenic release of erythrocytes into the circulation (Pearson *et al*, 1992). Hct has been shown to increase following stress episodes in a variety of teleosts, as erythrocyte volume and number increase (e.g. Fletcher, 1975; Ling and Wells, 1985; Fuchs and Albers, 1988; Iwama *et al*, 1989; Ryan, 1992; Pankhurst *et al,* 1992). This effect has been shown to be under adrenergic (Ling and Wells, 1985; Fuchs and Albers, 1988; Pearson *et al*, 1992), rather than corticosteroid control (Johansson-Sjobeck *et al*, 1978), as part of a suite of catecholaminergic effects designed to rapidly increase respiratory capacity following stress (Gamperl *et al*, 1994). Hct therefore may be an indirect indicator of the catecholamine response to stress in some species.

The objective of this study was to examine the effect of capture and culture history on the stress response in Australian snapper. Three groups of snapper were used: wild fish caught by trap; captive fish caught as juveniles and held in captivity for 5 years; and 2- and 3-year-old fish, hatchery-reared from wild broodstock. Access to these groups of fish afforded a unique opportunity to assess the capacity of snapper to acclimate to an artificial environment and the effect of domestication on the stress response. The stress response after capture and handling was determined by measuring plasma levels of cortisol and gonadal steroids. In addition, the effect of stress on the gonads was also examined by determining the incidence of ovarian atresia and the proportion of various cell-types in the testes. The effect of stress on the testes has not been examined for any species. Hct was also measured to determine its usefulness as an indicator of the catecholamine response to stress in snapper.

3.2 Materials and Methods

To determine the effect of capture and handling stress on plasma steroid levels and gonadal condition, snapper were captured, sampled and then confined for 0, 1, 6, 24, 48 or 168 h. This research was conducted on Captive, 2-year-old Hatchery, and Trap-Caught fish in 1994. An attempt was made to complete the experiment on line-caught fish as well, however, insufficient mature fish were caught by handline. In order to confirm findings with more reproductively mature fish additional Hatchery fish were sampled 12 months later (in 1995) when the fish were 3 years old.

Captive Fish

Sixty-four fish were caught from the Marine Research Pool by handline, with each fish being processed, before the next was caught. Immediately after capture, a gonadal biopsy was performed and reproductively mature males and females (stage III and IV) retained. The mature fish were tagged or fin clipped for identification, and bled. Each fish was placed into a holding tank until 1, 6, 24 or 48 h pc. For logistical reasons fish were allocated to the 4 treatments, a group at a time, in the order of 1, 6, 24 and then 48 h (16 fish per treatment). Sixteen further fish were caught by net crowding, handled as before and placed into holding tanks until 168 h pc. Initial capture of Captive fish occurred over three days in September 1994, between 0730 and 1200 h. Surface water temperature in the Marine Research Pool at the time of capture was 16.5 °C.

A range of holding tanks was used, depending on the length of confinement and logistics. Fish confined for 24, 48 and 168 h were placed in covered and aerated 4000 L tanks with constant water exchange (1 treatment/tank). Floating pens (1050 x 550 x 600 mm with 15 mm mesh sides: 2 fish/pen) placed in the Marine Research Pool were used for 1 and 6 h treatments. After the designated time period, the fish were removed from the tank, a second blood sample taken and the fish killed. Fork length, body, liver and gonad weight were measured from each fish and the gonads macroscopically staged. A gonad sample was collected and prepared for histology. Hct was measured and plasma cortisol, T, and 17,20βP levels were determined for both males and females by radioimmunoassay (RIA). In addition, plasma of females was assayed for E_2 and males for 11KT.

An identification problem resulted in ambiguous matching of some fish to corresponding plasma and other data. Data in question were ignored. This resulted in low numbers of males in the 48 h treatment, and females in the 168 h treatment. Data sets for captive males at 48 h were not used in the analysis. Data sets for females at 168 h were retained, however, where samples sizes were less than 3 this group was excluded from statistical analyses.

2-Year-Old Hatchery Fish

Hatchery fish were treated in a similar way to the Captive fish, except that all fish were caught from the seacage by handline, and due to the small size of the 2-year-old Hatchery fish, no attempt was made to ascertain sex or reproductive maturity prior to treatment allocation. Instead, 14 fish were allocated to each treatment, again by filling a group at a time, with 168 followed by 48, 24, 6 and finally 1 h treatments. Surface water temperature at the time of initial capture was 16.0 °C. Fish were caught between 0820 and 1330 h on 22nd September 1994.

After initial capture and handling, the fish were held in onboard holding tanks in a punt, next to the sea cages. Fish in the 1 h treatment group remained in these for the duration of the experiment. Fish in the 6 h treatment group were transferred to the Truck Transporter for transport to FRI, but remained in the Transporter for the remainder of the experiment. All other groups (24, 48 and 168 h) were transported back to FRI in the Truck Transporter and then transferred to covered and aerated 4000 L tanks with constant water exchange at FRI (1 treatment/tank).

A sex ratio in favour of females meant that insufficient males for statistical analyses were allocated to 3 of the 5 treatments. Therefore male Hatchery Fish were excluded from analyses.

Trap-Caught Fish

Trap-Caught fish were treated in a similar way to Captive fish, except that fish sampled at 1 h remained in the Boat Transporter for the duration of the experiment. All other fish were transferred to floating pens moored in a sheltered bay (1-6 fish/pen), 200 L polyurethane tanks (1-3 fish/tank), 4000 L tanks (1-5 fish/tank) or 10 000 L tanks (1-5 fish/tank) as deemed logistically appropriate for the remainder of the experiment. Different treatment groups were kept in separate tanks whenever logistically possible. All tanks were aerated, covered and had constant water exchange. Mean ocean surface water temperature at the time of capture of Trap-Caught fish was 18.3 °C. Due to difficulties in catching sufficient mature females, only 2 were allocated to the 48 h treatment, so this treatment was omitted from the analysis.

3-Year-Old Hatchery Fish

Three-year-old Hatchery fish were treated in a similar way to the 2-year-old Hatchery fish, however, only 24 and 168 h treatments (n=10) were used and only stage III or IV females were allocated to treatments. Twenty-four hour fish followed by 168 h fish were caught by handline between 0650 and 0800 h on 5th September, 1995. After capture fish were transferred to covered and aerated 4000 L tanks at FRI as before (1 treatment/tank). Ten more females were caught by handline and killed immediately. Their gonads were dissected for histology for "zero" hour comparisons. Surface water temperature at time of capture was 16 °C.

Statistics

One way Analysis of variance (ANOVA) among times for steroid and Hct data were used to determine if there were any differences among treatments in first bleeds. Biological (fork length, FL; whole body weight; gonadosomatic index, GSI; and hepatosomatic index, HSI) and histological data sets were analysed by one way ANOVA among treatments. For 3-year-old Hatchery fish, with the exception of histology data sets, t-tests were used instead of one-way ANOVAs as there were only 2 groups in the comparison.

Separate paired T-tests were used to test for differences between first bleeds and corresponding second bleeds for steroid and Hct data. A one-tailed test was used for E_2, T, and 11KT data to determine if there was a decrease in each of these responses following stress. A similar one-tailed test was used for cortisol, however, an increase following stress was expected. Two-tailed tests were used for both 17,20βP and Hct, due to their variable response to stress. Where n values for first and second bleeds were different due to missing samples, paired t-tests were run on paired data only, although the complete data sets are presented in the graphs. Groups with sample sizes < 3 were excluded from all statistical analyses, and analyses were conducted on remaining groups.

3.3 Results

Females

Trap-Caught Fish

Mean plasma cortisol levels in first bleeds for Trap-Caught fish were less than 11 $ng.ml^{-1}$ for all treatments, and there was no significant difference among first bleeds (Fig. 3.1a). Cortisol levels increased significantly in 1, 6 and 24 h groups by the second bleed ($P<0.05$, $t= -2.571$ (d.f. = 5), -4.404 (d.f. = 6), -2.062 (d.f. = 7), respectively). The maximum mean plasma cortisol level was 35 $ng.ml^{-1}$ at 6 h pc.

Mean plasma E_2 ranged from 0.17 to 0.39 $ng.ml^{-1}$ in first bleeds and there was no significant difference among treatments. By the second bleed, plasma E_2 had decreased to non-detectable or slightly above non-detectable in all treatments. This decrease was significant in the 1h treatment ($P<0.05$, $t = 2.095$ (d.f. = 5); Fig. 3.1b).

Mean plasma T was detectable in first bleeds in only 1 and 168 h treatments (Fig. 3.1c). At 1 h pc, plasma T in the second bleeds was similar to initial levels. By 6 h pc and for all succeeding times, T was non-detectable in the second bleeds.

Figure 3.1. Plasma concentrations of a) cortisol, b) E_2, c) T and d) 17,20βP in female Trap-Caught fish at first capture (white bars) or after confinement for 1, 6, 24, or 168 h (grey bars). Values are means + SE. Numbers indicate n values (number of fish). There were no significant differences among first bleeds. * denotes significant difference between first and corresponding second bleeds at each time ($P<0.05$).

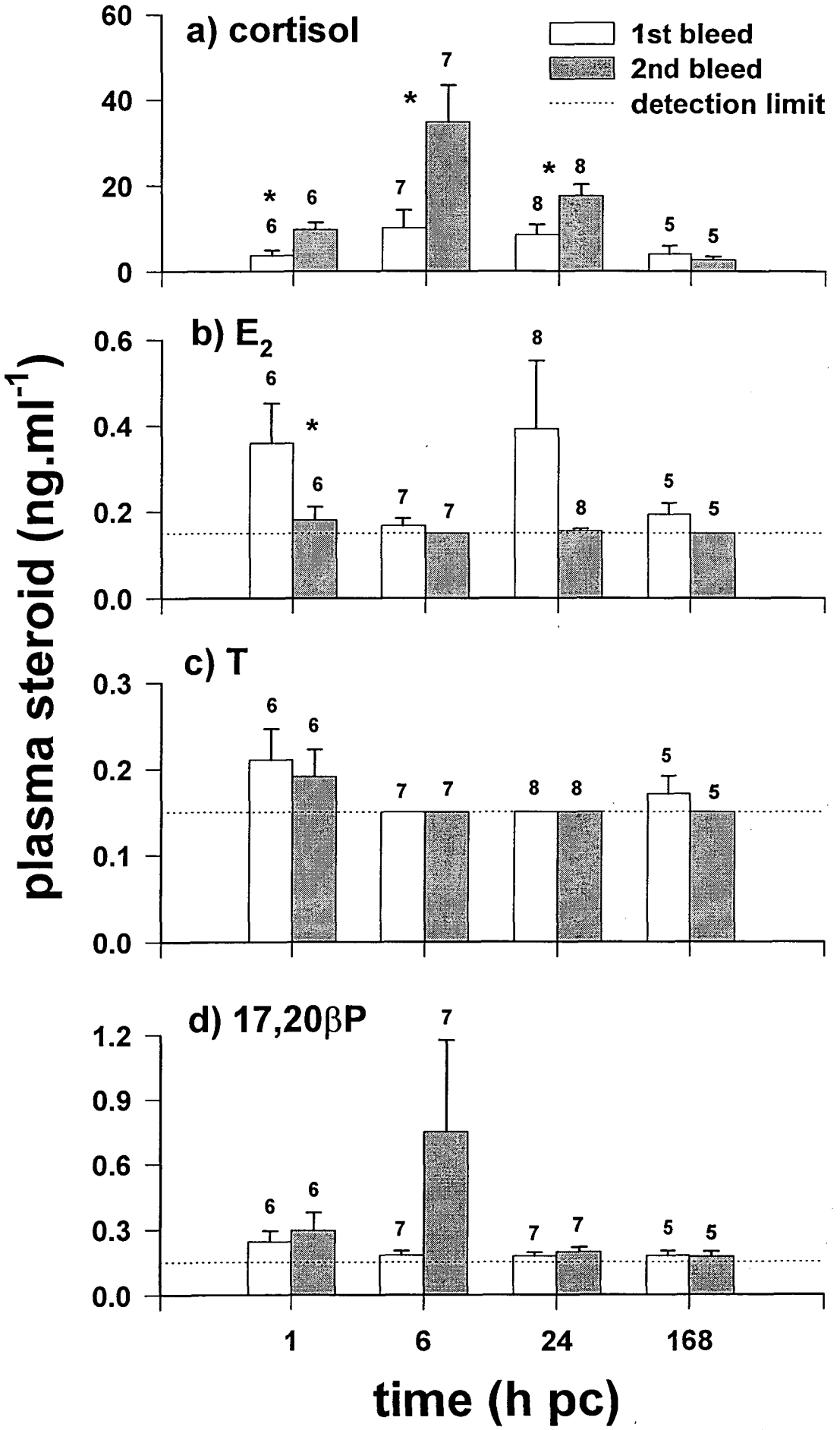

a) cortisol
1st bleed
2nd bleed
detection limit
b) E2
c) T
d) 17,20βP
plasma steroid (ng.ml-1)
time (h pc)
1
6
24
168

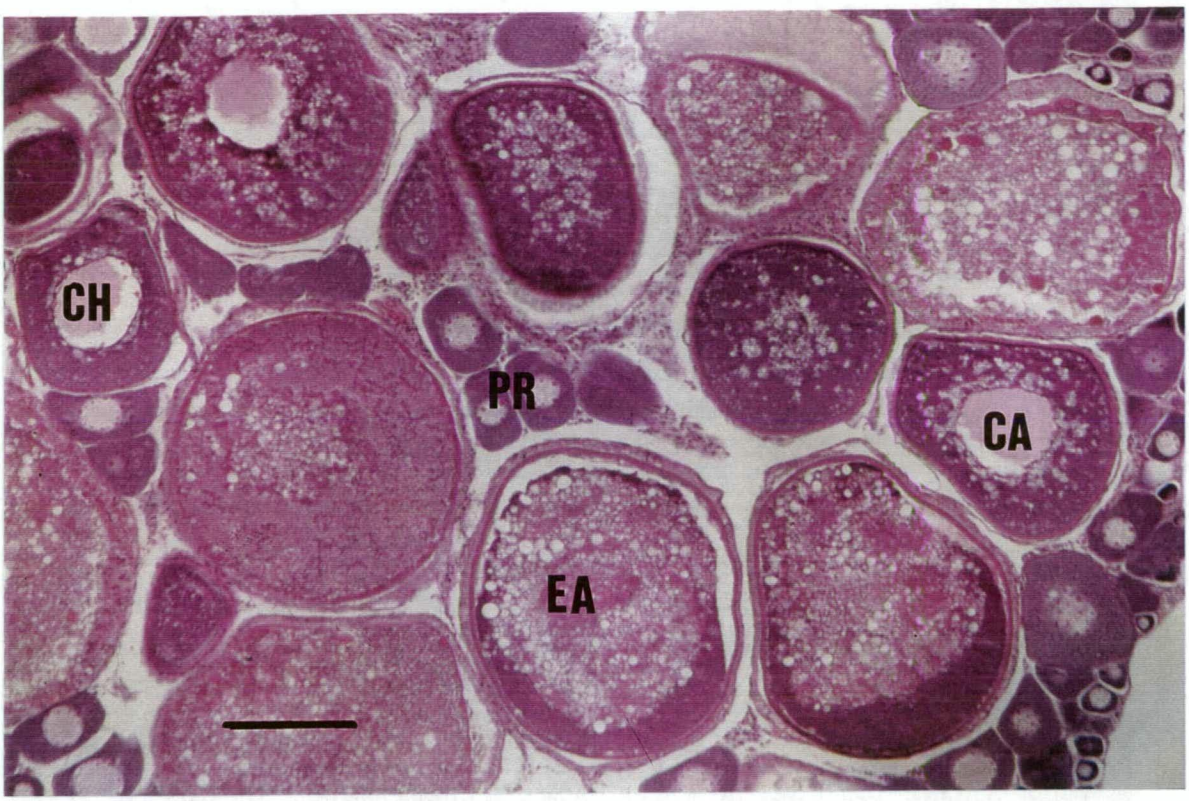
Table 3.1: Hct (%) (mean ± SE) for female and male Trap-Caught, Captive, 2- and 3-year-old Hatchery fish.

			Time of Confinement				
		Bleed no.	1 h pc	6 h pc	24 h pc	48 h pc	168 h pc
♀	**Trap-Caught**	1	29.2 ± 2.2	28.3 ± 1.6	32.7 ± 1.2		31.0 ± 0.7
		2	23.7 ± 1.6	25.3 ± 2.2	25.8 ± 2.3		27.0 ± 1.1
	Captive	1	41.5 ± 1.2	40.5 ± 7.0	41.9 ± 1.8	42.6 ± 5.6	43.7 ± 0.2
		2	43.3 ± 1.5	35.4 ± 1.3	34.7 ± 2.8	35.8 ± 1.1	32.3 ± 1.6
	2-year-old	1	42.3 ± 1.3	37.9 ± 1.8	37.2 ± 1.1	39.6 ± 1.8	36.6 ± 3.4
	Hatchery	2	33.8 ± 0.9	32.6 ± 2.2	32.0 ± 1.6	31.8 ± 2.3	30.2 ± 2.6
	3-year-old	1			45.8 ± 0.7		45.7 ± 0.8
	Hatchery	2			36.7 ± 1.6		40.0 ± 2.8
♂	**Trap-Caught**	1	34.7 ± 2.8	31.0 ± 2.8	27.1 ± 0.9	36.8 ± 1.6	29.2 ± 2.2
		2	23.8 ± 2.8	23.9 ± 2.1	28.4 ± 5.8	29.2 ± 2.0	27.3 ± 1.0
	Captive	1	41.0 ± 2.3	41.8 ± 1.4	44.5 ± 1.6		40.0 ± 3.7
		2	40.2 ± 3.4	34.7 ± 1.1	41.1 ± 4.5		31.3 ± 5.0

Shaded cells indicate a significant difference between first and second bleeds ($P<0.05$).

Figure 3.2. Micrographs of transverse sections from the ovaries of fish showing various oocyte stages and condition: CA, atretic cortical alveoli; CH, normal cortical alveoli; EA, atretic early vitellogenic; EH, normal early vitellogenic; LA, atretic late vitellogenic; LH, normal late vitellogenic; PR, primary. Scale bar = 100 μm.

a)



b)

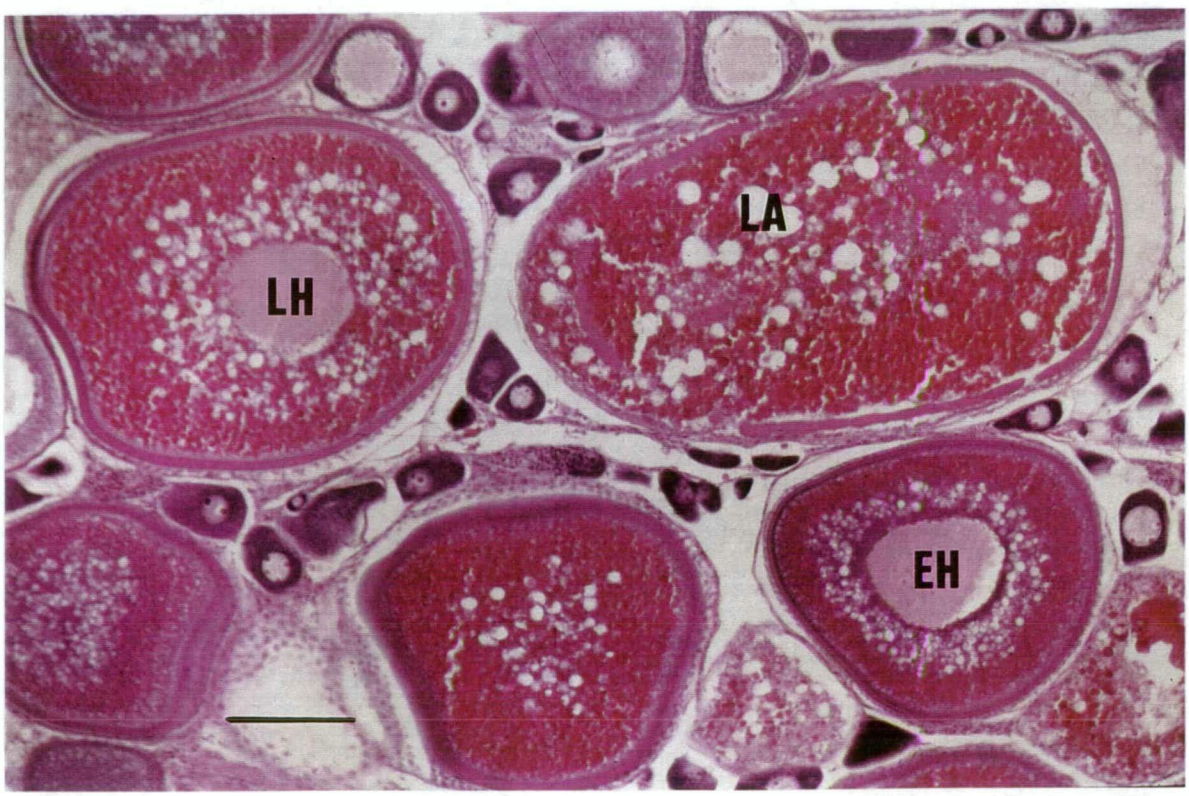
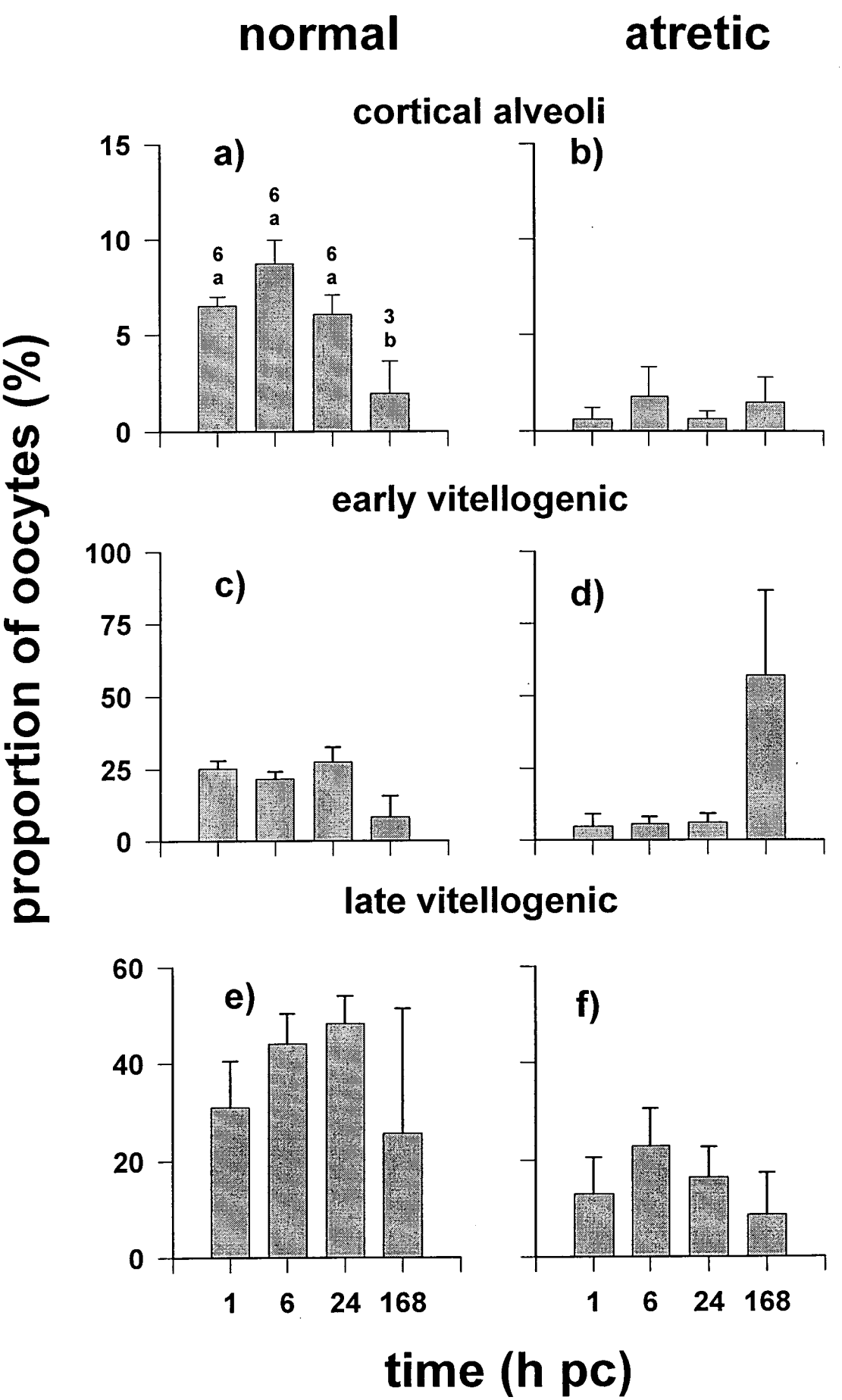


Figure 3.3. Proportion (%) of a) normal and b) atretic cortical alveoli oocytes, c) normal and d) atretic early vitellogenic oocytes and e) normal and f) atretic late vitellogenic oocytes in histological cross-sections of ovaries from female Trap-Caught fish confined for 1, 6, 24 or 168 h. Counts are expressed as a percentage of total previtellogenic oocytes (cortical alveoli) or of 100 vitellogenic (early and late vitellogenic) (mean + SE). Numbers indicate n values (number of fish). Different letters show significantly different Tukey groupings among treatments for any oocyte type. No letter indicates same Tukey grouping ($P<0.05$).


normal
atretic
cortical alveoli
a)
b)
15
10
5
0
6 a
6 a
6 a
3 b
early vitellogenic
c)
d)
100
75
50
25
0
late vitellogenic
e)
f)
60
40
20
0
1
6
24
168
proportion of oocytes (%)
time (h pc)

Mean plasma 17,20βP levels ranged from non-detectable to 0.24 $ng.ml^{-1}$ in first bleeds and there was no significant difference among treatments (Fig. 3.1d). There was no significant difference in mean plasma 17,20βP between first and second bleeds for any treatments. In fish sampled at 1 h pc, 17,20βP levels increased in 3 out of 6 fish, while in fish sampled at 6 h pc, 17,20βP increased in 5 out of 7 fish. Plasma 17,20βP in 1 of these fish increased from 0.25 to 3.26 $ng.ml^{-1}$ contributing to the large mean and SE at 6 h pc.

Mean Hct in first bleeds ranged from 28 to 35 % (Table 3.1). Hct decreased in 1 and 24 h groups by the second bleed ($P<0.05$, $t=5.152$ (d.f. = 5), 2.246 (d.f. = 7) respectively).

Eight distinct oocyte stages were observed in the histology of ovaries from trap fish: primary, cortical alveoli, early vitellogenic, late vitellogenic, migratory nucleus, maturing and hydrated oocytes and post-ovulatory follicles (Fig. 3.2). Of these, atretic forms were observed for cortical alveoli, early vitellogenic and late vitellogenic oocytes. Primary oocytes comprised 89 to 97 % of all previtellogenic oocytes. The proportion of normal cortical alveoli oocytes ranged from 2 to 9 % of all previtellogenic oocytes (Fig. 3.3a). Fish confined for 168 h had significantly fewer normal cortical alveoli oocytes than 1, 6 and 24 h fish ($P<0.05$, $F=5.114$ (d.f. = 20)). The proportion of atretic cortical alveoli oocytes ranged from 0.6 to 1.8% and there were no significant differences among treatments (Fig. 3.3b).

The proportion of normal early vitellogenic oocytes ranged from 8 to 27 % of all vitellogenic oocytes, while atretic early vitellogenic oocytes ranged from 5 to 57 % (Fig. 3.3c,d). There was no significant difference in the proportion of normal or atretic early vitellogenic oocytes among treatments, however, there was a tendency for more atretic and fewer normal early vitellogenic oocytes in 168 h than 1 h treatments. Normal late vitellogenic oocytes comprised 26 to 48 % of all vitellogenic oocytes, while atretic late vitellogenic oocytes comprised 9 to 23 % and there was no significant difference among treatments (Fig. 3.3e,f). Migratory nucleus stage oocytes comprised less than 0.3 % of vitellogenic oocytes in all groups. No maturing oocytes were observed. The proportion of hydrated oocytes ranged from 0 to 25 % and post-ovulatory follicles comprised less than 2 % of vitellogenic oocytes for all groups of fish.

Mean FL ranged from 319 ± 8 to 396 ± 42 mm for treatment groups, while mean whole body weight ranged from 739 ± 43 to 1472 ± 410 g. Mean GSI ranged from 1.44 ± 0.35 to 2.24 ± 0.41 %, while HSI ranged from 1.12 ± 0.09 to 1.50 ± 0.16 %. There were no significant differences among treatments for FL, weight, GSI or HSI consistent with absence of bias in allocation of fish to treatments (see Appendix A).

Captive Fish

Mean cortisol levels in first bleeds were less than 5 $ng.ml^{-1}$ in female captive snapper for all treatments and there was no significant difference among treatments (Fig. 3.4a). Cortisol levels tended to increase by the second bleed in all treatments, except 48 h. The highest mean cortisol was 28 $ng.ml^{-1}$ at 24 h pc and this was significantly higher than its corresponding first bleed ($P<0.05$, $t=-3.004$ (d.f. = 4)).

Mean plasma levels of E_2 in first bleeds ranged from 0.18 to 0.40 $ng.ml^{-1}$. There was a significant difference among treatments, with fish allocated to 1 h group having significantly higher E_2 in first bleeds than both 24 and 48 h groups ($P<0.05$, $F=5.625$ (d.f. = 13); Fig. 3.4b). There were no significant differences between first and second bleeds in any of the treatments. By 1 and 6 h pc, plasma E_2 had decreased in 2 of 3 fish. After 24 h pc plasma E_2 levels had decreased to non-detectable in all fish except one at 168 h pc.

Mean plasma T in first bleeds followed a similar pattern to E_2 with levels tending to decrease from 1 h through to 168 h treatments, although there were no statistical differences among treatments (Fig. 3.4c). Plasma T in fish allocated to 48 and 168 h treatments was non-detectable in first bleeds. There were no significant differences between first and second bleeds in any of the treatments. By 1 h pc plasma T was detectable in 1 out of 3 fish but was non-detectable in any fish from 6 to 168 h pc.

Mean plasma 17,20βP in first bleeds ranged from non-detectable to 0.59 $ng.ml^{-1}$. There was no significant difference among first bleeds (Fig. 3.4d). Mean plasma 17,20βP was higher at 24 h pc than in first bleeds ($P<0.05$, $t=-3.004$ (d.f. = 4)). At 6 h pc, 2 out of 3 fish had higher levels of 17,20βP than corresponding initial bleeds.

In first bleeds mean Hct ranged from 40 to 44 % (Table 3.1). There was no significant difference in Hct in first bleeds among treatments. Hct tended to decrease in second bleeds and was significantly lower at 24 h pc ($P<0.05$, $t=3.807$ (d.f. = 2)).

Primary oocytes comprised 81 to 90 % of all previtellogenic oocytes. Normal cortical alveoli oocytes comprised 5 to 11 % of all previtellogenic oocytes, while atretic cortical alveoli oocytes comprised 1.5 to 9 %. There were no significant differences among treatments in the occurrence of normal or atretic cortical alveoli oocytes (Fig. 3.5a,b).

Normal early vitellogenic oocytes comprised 16 to 30 % of all vitellogenic oocytes, while atretic early vitellogenic oocytes comprised 8 to 79 % (Fig. 3.5c,d). There was no significant difference among treatments for normal early vitellogenic oocytes, but the 6 h treatment had significantly fewer atretic early vitellogenic oocytes than the 168 h treatment ($P<0.05$, $F=3.542$ (d.f. =17)). Normal late vitellogenic oocytes comprised 35 to 45 % of all

Figure 3.4. Plasma concentrations of a) cortisol, b) E_2, c) T and d) 17,20βP in female Captive fish at first capture (white bars) or after confinement for 1, 6, 24, 48 or 168 h (grey bars). Values are means + SE. Numbers indicate n values (number of fish). Letters indicate significantly different Tukey groupings for comparisons of first bleeds. No letter indicates same Tukey grouping. * denotes significant difference between first and corresponding second bleeds at each time ($P<0.05$). 168 h data were excluded from statistical analyses because of small sample sizes.

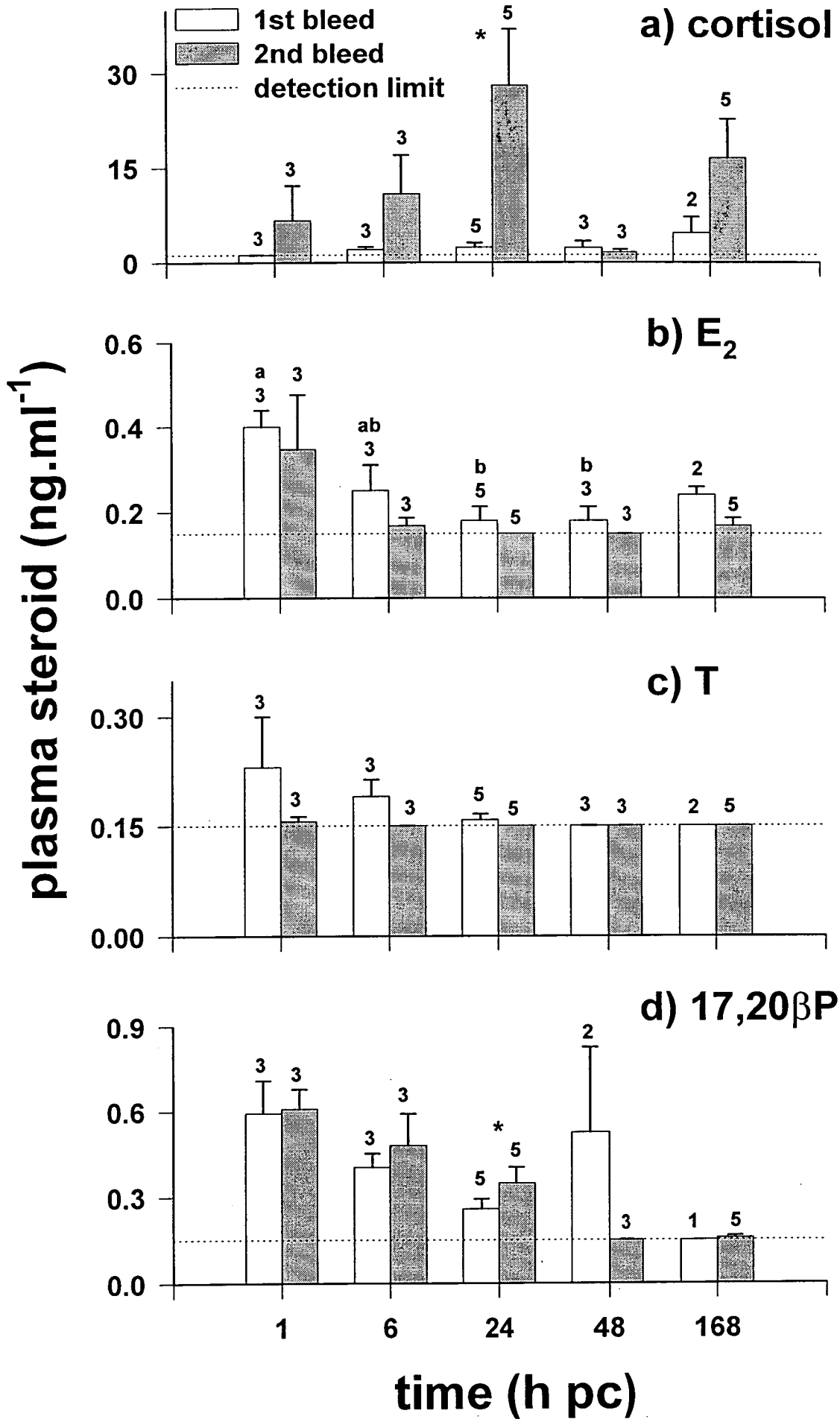
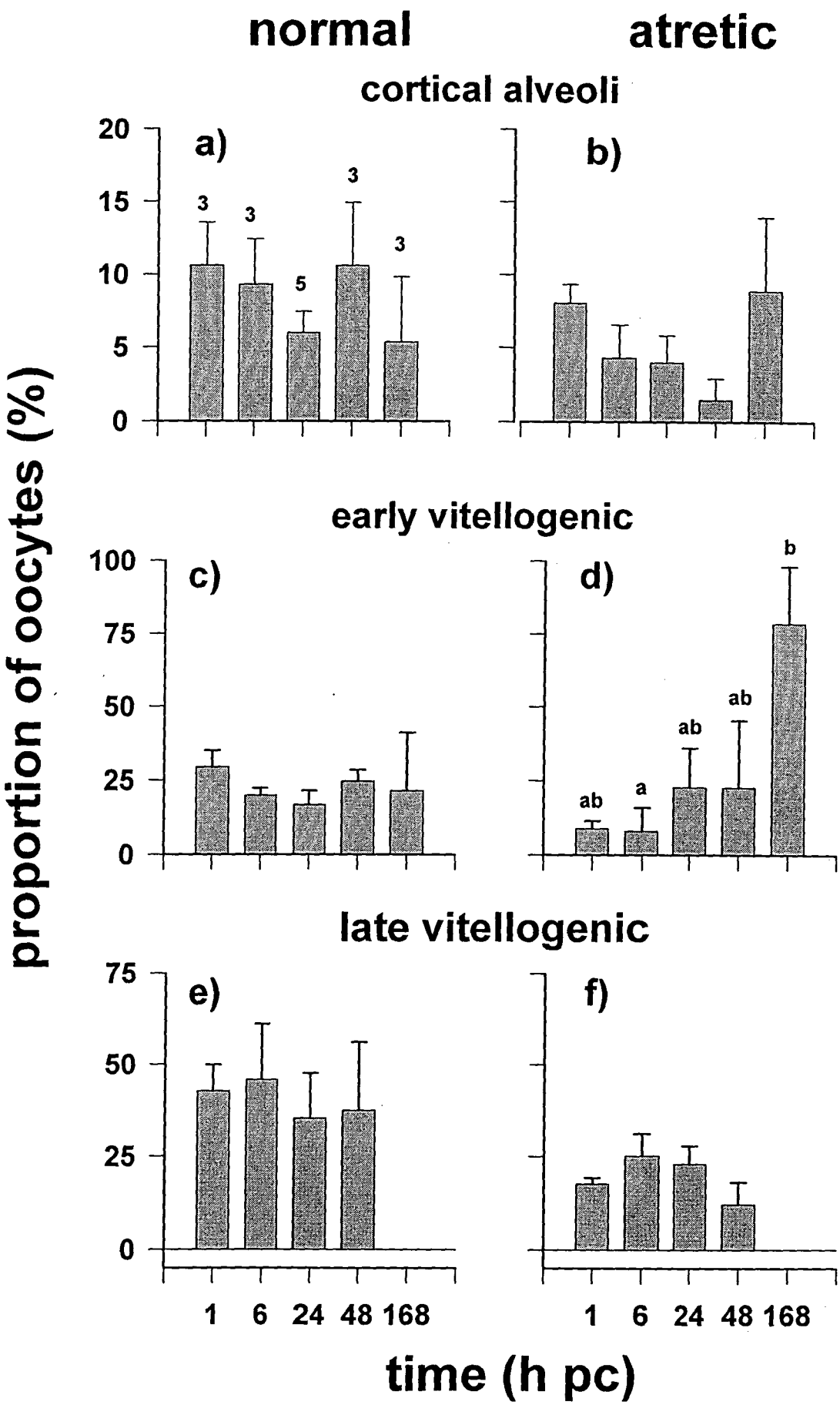

1st bleed
2nd bleed
detection limit
a) cortisol
b) E2
c) T
d) 17,20βP
plasma steroid (ng.ml-1)
time (h pc)
1
6
24
48
168

Figure 3.5. Proportion of a) normal and b) atretic cortical alveoli oocytes, c) normal and d) atretic early vitellogenic oocytes and e) normal and f) atretic late vitellogenic oocytes in histological cross-sections of ovaries from female Captive fish confined for 1, 6, 24, 48 or 168 h. Counts are expressed as a percentage of total previtellogenic oocytes (cortical alveoli) or of 100 vitellogenic (early and late vitellogenic) (mean + SE). Numbers indicate n values (number of fish). Different letters show significantly different Tukey groupings among treatments for any oocyte type. No letter indicates same Tukey grouping ($P<0.05$).


normal
atretic
cortical alveoli
a)
b)
early vitellogenic
c)
d)
late vitellogenic
e)
f)
proportion of oocytes (%)
time (h pc)
1 6 24 48 168
3 3 5 3 3
ab a ab ab b

vitellogenic oocytes for all groups and atretic late vitellogenic oocytes comprised 12 to 25 %, with the exception of 168 h which had no late vitellogenic oocytes at all (Fig. 3.5e,f). There was no significant difference among treatments for normal or atretic late vitellogenic oocytes (excluding the 168 h treatment). No migratory nucleus, maturing or hydrated oocytes were observed. Post-ovulatory follicles comprised less than 3.3 % of all vitellogenic oocytes.

Mean FL ranged from 388 ± 12 to 467 ± 8 mm; mean whole body weight ranged from 1272 ± 112 to 2227 ± 121 g; mean GSI ranged from 2.18 ± 0.57 to 4.16 ±1.12 % and mean HSI ranged from 1.15 ± 0.05 to 1.30 ± 0.19 %. There were no significant differences among the treatments for FL, whole body weight, GSI or HSI, consistent with an absence of bias in allocation of fish to treatments (see Appendix A).

2-Year-Old Hatchery Fish

Mean plasma cortisol in first bleeds ranged from 1.4 to 9 $ng.ml^{-1}$ and there was a significant difference between 6 and 24 h treatments (Fig. 3.6a). Cortisol tended to increase at second bleeds and at 6 and 168 h pc mean plasma cortisol was significantly higher than in first bleeds ($P<0.05$, $t = -4.617$ (d.f. = 8), -3.371 (d.f. = 4) respectively). The maximum mean plasma cortisol level was 20 $ng.ml^{-1}$ in 6 h fish.

Mean plasma E_2 in first bleeds ranged from 0.2 to 0.8 $ng.ml^{-1}$ and there was no significant difference among treatments (Fig. 3.6b). There was, however, a tendency for E_2 in first bleeds to increase from 1 h through to 168 h treatments. Mean plasma E_2 decreased by the second bleed, being significantly lower at 1, 6, 24 and 48 h pc than in first bleeds ($P<0.05$, $t = 1.999$ (d.f. = 9), 3.497 (d.f. = 8), 2.762 (d.f. = 7), 5.035 (d.f. = 4) respectively). By 168 h pc, however, 3 out of 5 fish had detectable levels of E_2.

In first bleeds, mean plasma T was non-detectable in all treatments except at 48 h in which the mean was 0.22 $ng.ml^{-1}$ (Fig. 3.6c). This decreased to 0.16 $ng.ml^{-1}$ by the second bleed. In all other groups of fish T remained non-detectable for the second bleed.

Mean plasma 17,20βP in first bleeds ranged from 0.2 to 0.5 $ng.ml^{-1}$ and there was no difference among treatments (Fig. 3.6d). Plasma 17,20βP tended to decrease in 4 of the 5 treatments, but only significantly in the 1 h treatment ($P<0.05$, $t = 2.576$ (d.f. = 9)).

Mean Hct in first bleeds ranged from 36 to 43 % and there was no difference among treatments (Table 3.1). By the second bleed Hct had decreased significantly in 1, 24 and 48 h treatments ($P<0.05$, $t = 5.243$ (d.f. = 9), 4.643 (d.f. = 7), 2.920 (d.f. = 6) respectively).

Figure 3.6. Plasma concentrations of a) cortisol, b) E_2, c) T and d) 17,20βP in 2-year-old female Hatchery fish at first capture (white bars) or after confinement for 1, 6, 24, 48 or 168 h (grey bars). Values are means + SE. Numbers indicate n values (number of fish). Different letters show significantly different Tukey groupings among first bleeds. No letter indicates same Tukey grouping. * denotes significant difference between first and corresponding second bleeds at each time ($P<0.05$).

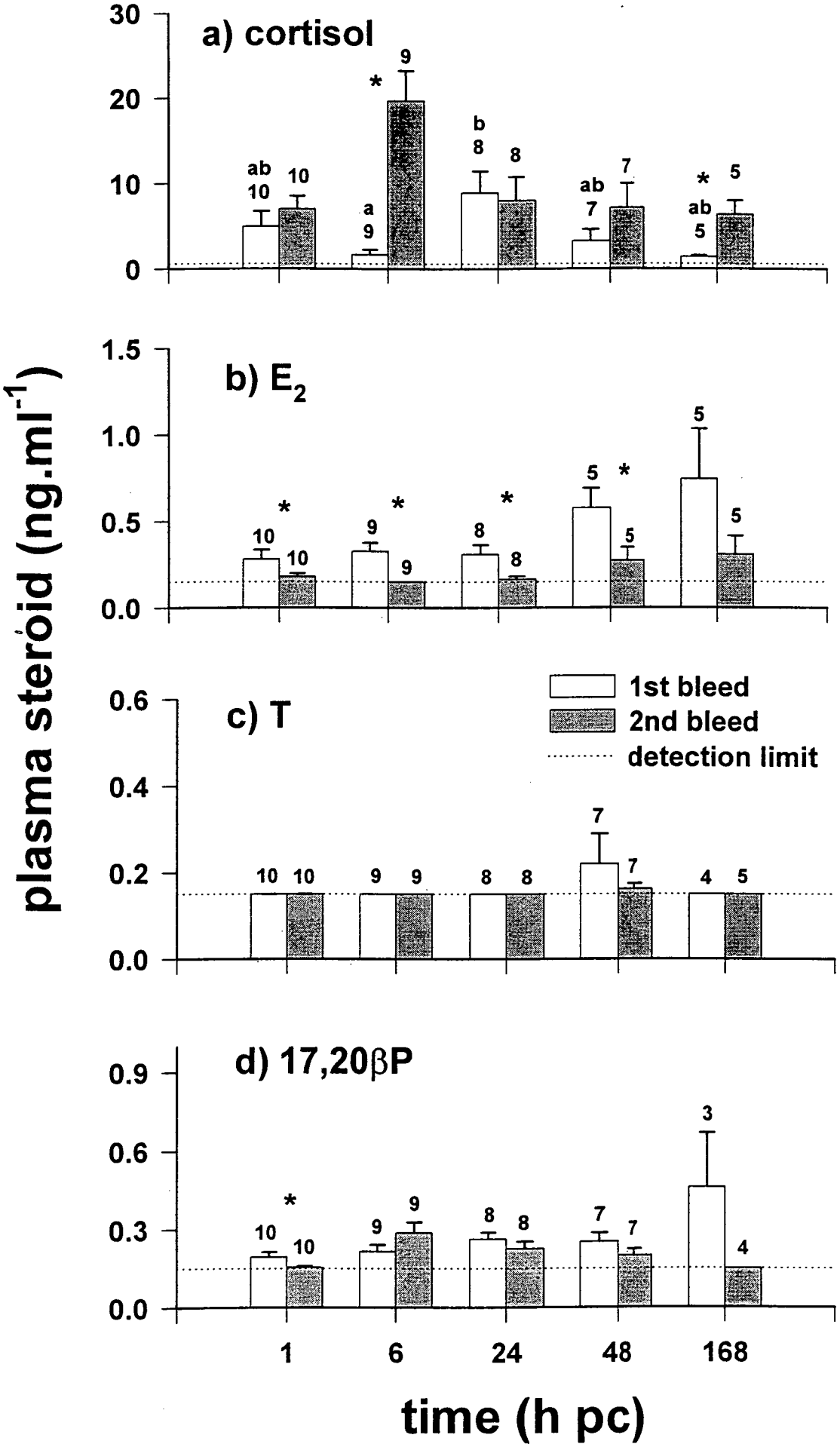
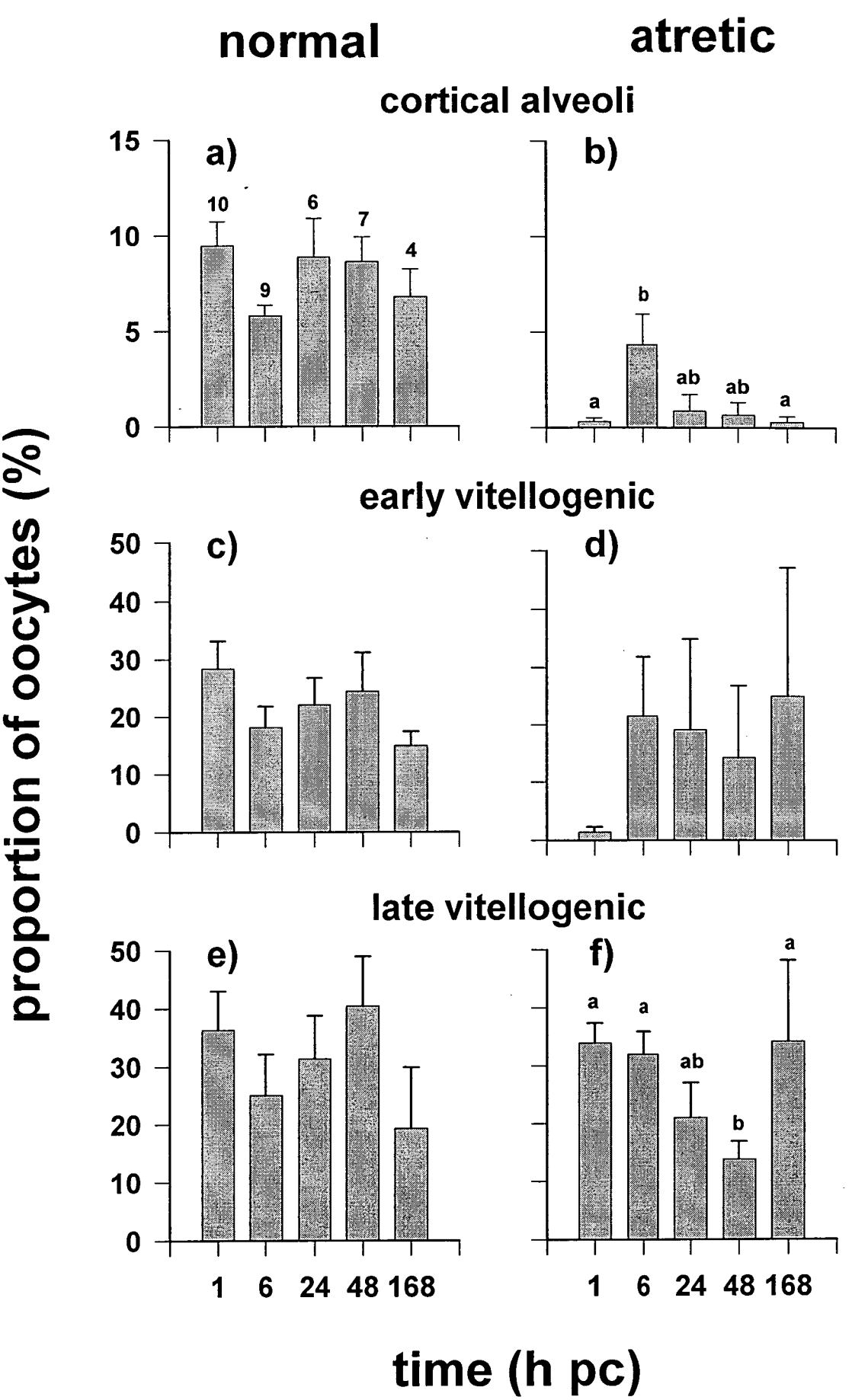

a) cortisol
b) E_2
c) T
d) 17,20βP
plasma steroid (ng.ml^{-1})
1st bleed
2nd bleed
detection limit
time (h pc)
1
6
24
48
168

Figure 3.7. Proportion of a) normal and b) atretic cortical alveoli oocytes, c) normal and d) atretic early vitellogenic oocytes and e) normal and f) atretic late vitellogenic oocytes in histological cross-sections of ovaries of 2-year-old female Hatchery fish, confined for 1, 6, 24, 48 or 168 h. Counts are expressed as a percentage of total previtellogenic oocytes (cortical alveoli) or of 100 vitellogenic (early and late vitellogenic) (mean + SE). Numbers indicate n values (number of fish). Different letters show significantly different Tukey (b) or SNK (f) groupings among treatments for any oocyte type. No letter indicates same Tukey grouping ($P<0.05$).


normal
atretic
cortical alveoli
early vitellogenic
late vitellogenic
a)
b)
c)
d)
e)
f)
proportion of oocytes (%)
time (h pc)
1
6
24
48
168
10
9
6
7
4
a
b
ab
ab
a
a
a
ab
b
a

Primary oocytes comprised from 89 to 93 % of all previtellogenic oocytes. The proportion of normal cortical alveoli oocytes constituted between 5 and 10 % of all previtellogenic oocytes, while atretic cortical alveoli oocytes constituted between 0.3 and 5 % (Fig. 3.7a,b). There was no significant difference in the proportion normal cortical alveoli oocytes among treatments, however, there were more atretic cortical alveoli oocytes in 6 h fish than in both 1 and 168 h ($P<0.05$, $F=4.073$ (d.f. = 35)).

The proportion of normal early vitellogenic oocytes was 15 to 29 % and atretic early vitellogenic oocytes was 1 to 25 % of all vitellogenic oocytes (Fig. 3.7 c,d). Although not significantly different, 1 h fish appeared to have more normal and less atretic oocytes than 168 h fish. Normal late vitellogenic oocytes comprised 19 to 41 % while atretic late vitellogenic oocytes comprised 13 to 34 % of all vitellogenic oocytes (Fig. 3.7e,f). There was no significant difference among treatments for normal late vitellogenic oocytes, however, there was a significant difference among treatments for atretic late vitellogenic oocytes. Tukey-Kramer HSD failed to separate the means, however, SNK showed that there were less atretic late vitellogenic oocytes in the 48 h treatment than 1, 6 and 168 h treatments. No migratory nucleus or hydrated oocytes were observed. Maturing oocytes accounted for less than 0.5 % of all vitellogenic oocytes and post-ovulatory follicles accounted for 0 to 7 % in all treatments.

Mean FL ranged from 249 ± 4 to 262 ± 4 mm; whole body weight ranged from 357 ± 15 to 424 ± 22 g; GSI ranged from 0.66 ± 0.15 to 0.99 ± 0.20 % and HSI ranged from 1.08 ± 0.14 to 1.40 ± 0.16 %. There was no significant difference among treatments for any of these measures, consistent with lack of bias in allocation of fish to treatment (see Appendix A).

3-year-old Hatchery Fish

Mean plasma cortisol levels in first bleeds were significantly higher in 24 h (5.7 $ng.ml^{-1}$) than 168 h treatments (2.4 $ng.ml^{-1}$) ($P<0.05$, $t=3.240$ (d.f. = 18); Fig. 3.8a). At 24 and 168 h pc, mean plasma cortisol was similar to the respective first bleeds.

Mean plasma E_2 levels in first bleeds were significantly higher in 24 than 168 h treatments at 2.4 and 1.4 $ng.ml^{-1}$ respectively ($P<0.05$, $t=2.147$ (d.f. = 18); Fig. 3.8b). These decreased significantly to 0.2 and 0.5 $ng.ml^{-1}$ by 24 and 168 h pc ($P<0.05$, $t=6.248$ (d.f. = 9), 4.125 (d.f. = 9) respectively). By 168 h pc, 8 out of 10 fish had detectable levels of E_2.

In first bleeds, mean plasma T levels were 1 and 0.7 $ng.ml^{-1}$ for 24 and 168 h treatments, and there was no significant difference between treatments (Fig. 3.8c). These levels decreased significantly to be virtually non-detectable in both groups ($P<0.05$, $t=6.955$ (d.f. = 9), 5.641 (d.f. = 9)).

Figure 3.8. Plasma concentrations of a) cortisol, b) E_2, c) T and d) 17,20βP in 3-year-old Hatchery fish at first capture (white bars) or after confinement 24 or 168 h (grey bars). Values are means + SE (n=10). Different letters show significantly different Tukey groupings between first bleeds. No letter indicates same Tukey grouping. * denotes significant difference between first and corresponding second bleeds at each time ($P<0.05$).

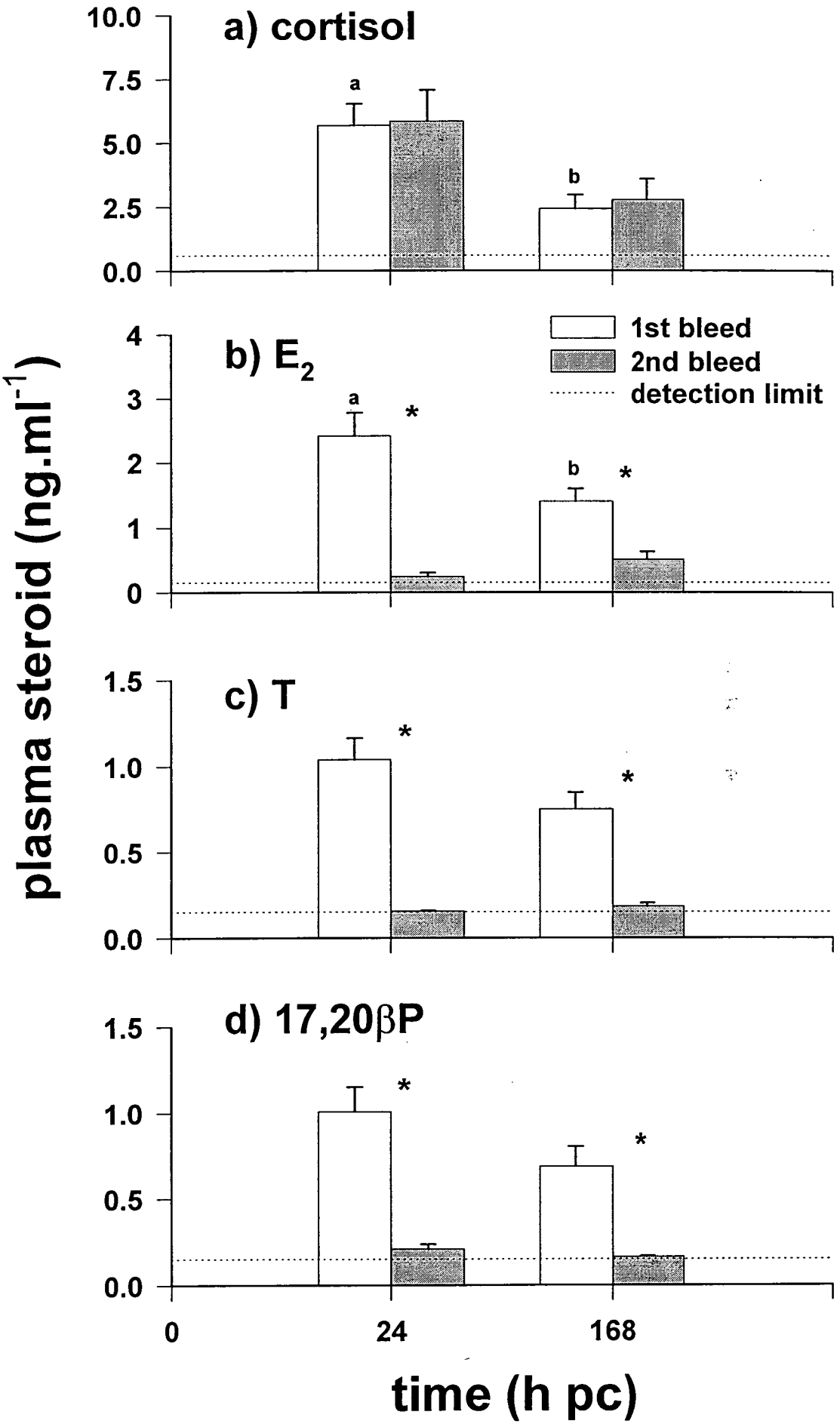
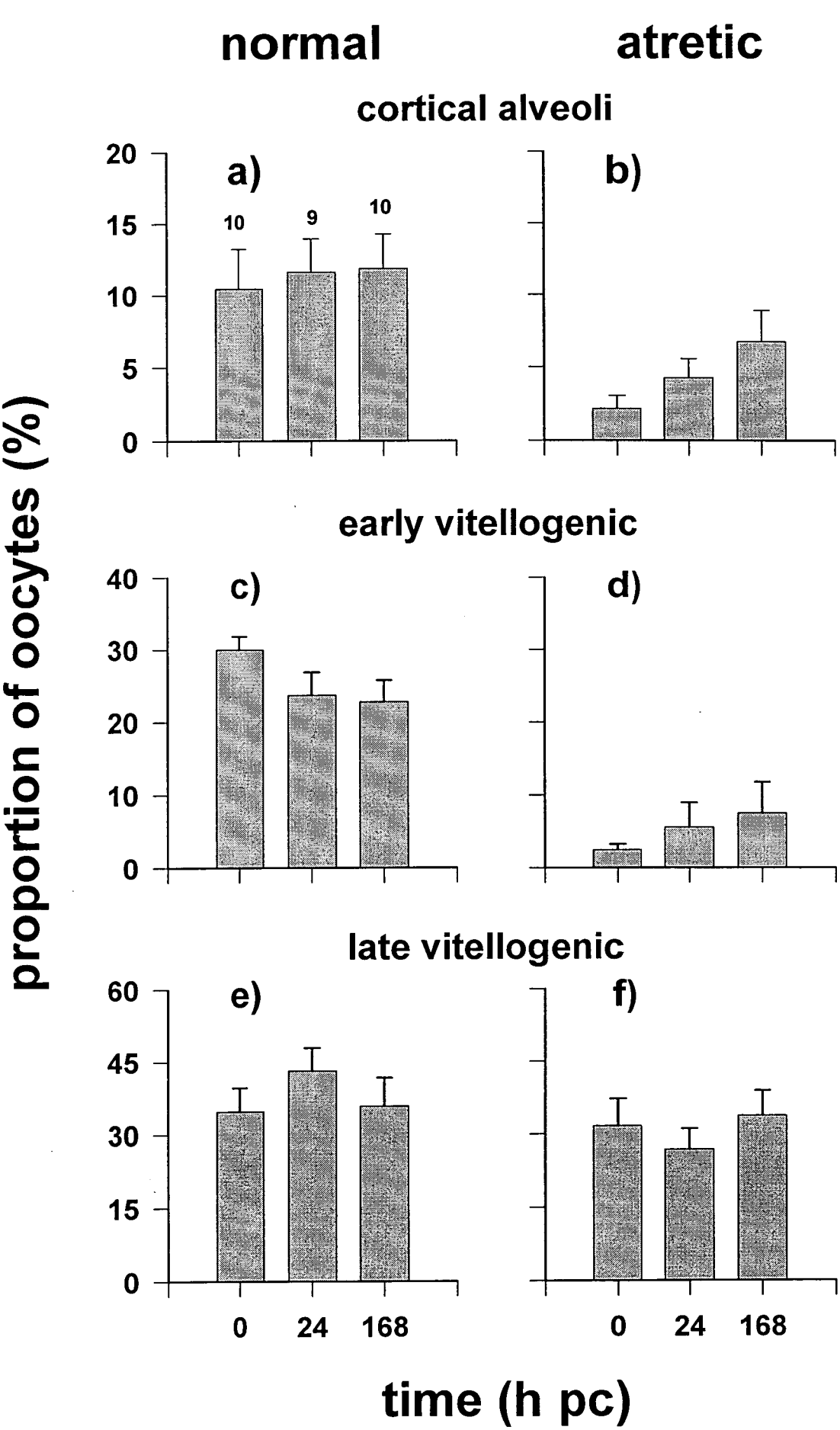

a) cortisol
b) E_2
c) T
d) 17,20βP
1st bleed
2nd bleed
detection limit
plasma steroid (ng.ml^{-1})
time (h pc)
0
24
168
a
b
*

Figure 3.9. Proportion of a) normal and b) atretic cortical alveoli oocytes, c) normal and d) atretic early vitellogenic oocytes and e) normal and f) atretic late vitellogenic oocytes in histological cross-sections of ovaries of 3-year-old female Hatchery fish, confined for 0, 24 or 168 h. Counts are expressed as a percentage of total previtellogenic oocytes (cortical alveoli) or of 100 vitellogenic (early and late vitellogenic) (mean + SE). Numbers indicate n values (number of fish). There were no significantly different Tukey groupings ($P<0.05$).


normal
atretic
cortical alveoli
a)
b)
10
9
10
early vitellogenic
c)
d)
late vitellogenic
e)
f)
proportion of oocytes (%)
0
5
10
15
20
30
40
45
60
0
24
168
time (h pc)

Mean plasma 17,20βP levels in first bleeds were 1 and 0.7 ng.ml^{-1} for 24 and 168 h treatments respectively and there was no difference between them (Fig. 3.8d). These decreased significantly to virtually non-detectable by both 24 and 168 h pc ($P<0.05$, $t=5.415$ (d.f. = 9), 4.423 (d.f. = 9) respectively).

Mean Hct in first bleeds was 45 % for both groups of fish (Table 3.1). These decreased significantly by the second bleed in both 24 and 168 h treatments ($P<0.05$, $t=5.749$ (d.f. = 9), 2.537 (d.f. = 9) respectively).

Primary oocytes constituted 81 to 87 % of all previtellogenic oocytes in all treatments. Normal cortical alveoli oocytes constituted 10 to 12 % of all previtellogenic oocytes, while atretic cortical alveoli oocytes constituted 2 to 7 % (Fig. 3.9a,b). Although not significant, there was a tendency for more atretic cortical alveoli oocytes at 168 h than at 0 h.

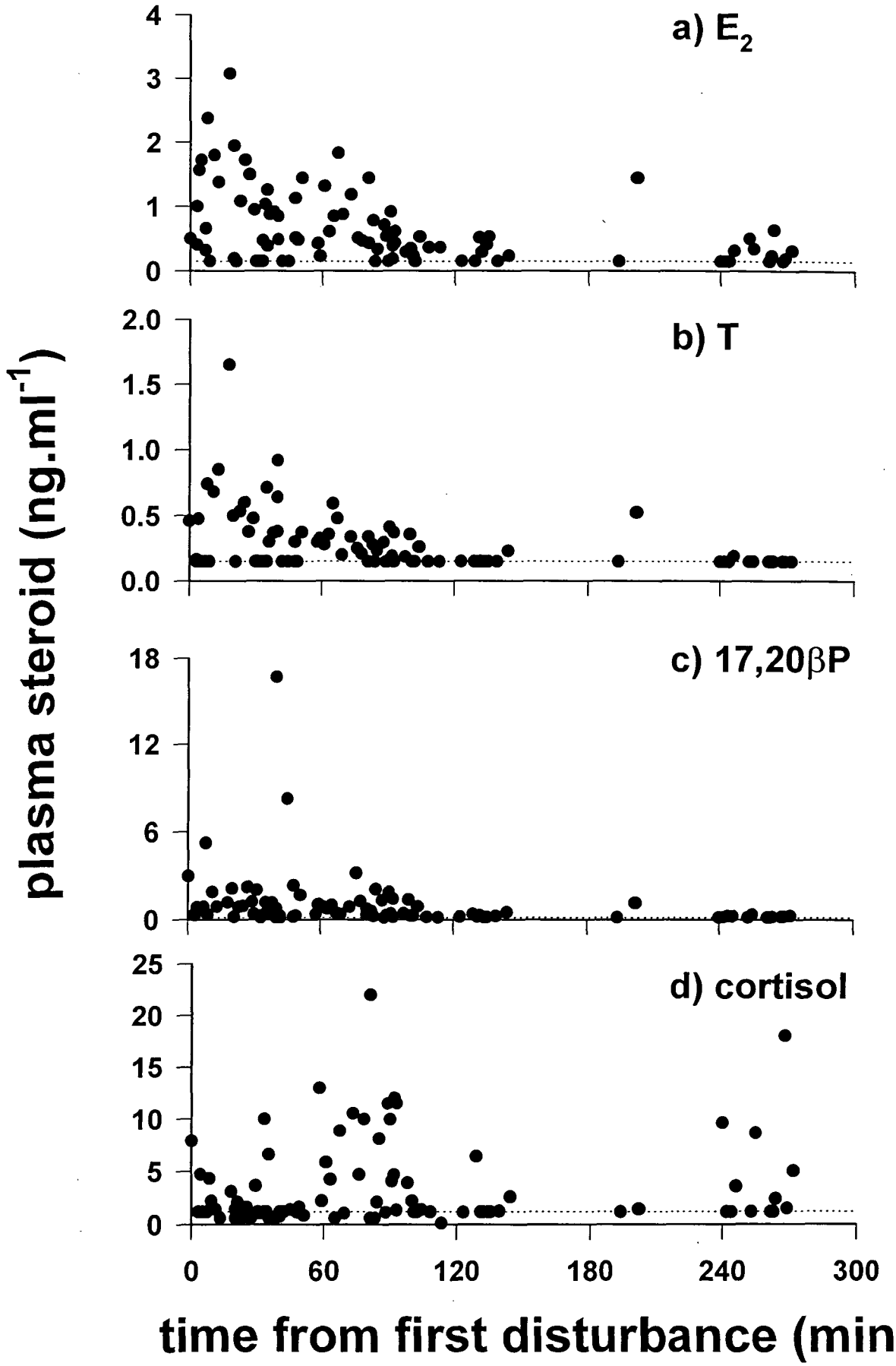
Normal early vitellogenic oocytes constituted 23 to 30 % of all vitellogenic oocytes, while atretic early vitellogenic oocytes constituted 2 to 7 % (Fig. 3.9c,d). Although the difference was not significant, there appeared to be more normal and less atretic early vitellogenic oocytes at 0 h pc than at 24 and 168 h pc. Normal late vitellogenic oocytes comprised 35 to 43 % of all vitellogenic oocytes, while atretic late vitellogenic oocytes comprised 27 to 34 % and there was no significant difference among treatments (Fig. 3.9e,f). Migratory nucleus and hydrated oocytes comprised less than 1 and 1.5 % of all vitellogenic oocytes respectively, post-ovulatory follicles constituted less than 0.3 %. No maturing oocytes were observed.

Mean FL ranged from 330 ± 4 to 333 ± 4 mm; whole body weight ranged from 834 ± 42 to 911 ± 45 g; GSI ranged from 4.05 ± 0.42 to 4.98 ± 0.49 % and HSI ranged from 1.31 ± 0.04 to 1.45 ± 0.09 %. There was no significant difference among treatments for FL, weight, GSI or HSI, consistent with absence of bias in allocation of fish to treatments (see Appendix A).

Steroid Levels at First Bleed

The envelope for plasma E_2 in first bleeds decreased from 0.15 - 3 ng.ml^{-1} at first disturbance to 0.15 - 0.5 ng.ml^{-1} by 120 min (Fig. 3.10a). A similar tendency is evident for plasma T, with a decrease in the envelope from 0.15 - 1.6 to <0.15 ng.ml^{-1} by 120 min (Fig. 3.10b). There was no obvious pattern for 17,20βP or cortisol (Fig. 3.10c,d). Data presented in Figure 3.10 were steroid levels for first bleeds of all mature female Hatchery and Captive fish used in this experiment, combined with another experiment conducted on Hatchery fish in 1995 (see Chapter 4).

Figure 3.10. The relationship between time from first pool-side disturbance until sampling and plasma a) E_2, b) T, c) 17,20βP, and d) cortisol levels in first bleeds of female Captive and Hatchery fish (1994 and 1995).


a) E_2
b) T
c) 17,20βP
d) cortisol
plasma steroid (ng.ml^{-1})
time from first disturbance (min)
0
60
120
180
240
300

Males

Trap-Caught Fish

Mean plasma cortisol levels in first bleeds ranged from 1.5 to 8.1 $ng.ml^{-1}$ and there was no difference among treatments (Fig. 3.11a). Cortisol significantly increased in 1 and 6 h treatments by the second bleed ($P<0.05$, $t = -2.366$ (d.f. = 3), -5.654 (d.f. = 5)). Second bleeds ranged from 7 to 40 $ng.ml^{-1}$ with a maximum at 6 h pc.

There was no significant difference in mean plasma T in first bleeds among treatments (Fig. 3.11b). Mean plasma T was non-detectable in 1 and 24 h fish in first bleeds. Other treatments ranged between 0.23 and 0.47 $ng.ml^{-1}$. By the second bleed, plasma T was non-detectable in all treatments, except for 6 h fish which had only 0.17 $ng.ml^{-1}$. The decrease in T in 48 and 168 h fish was significant ($P<0.05$, $t = 2.450$ (d.f. = 6), 2.479 (d.f. = 6) respectively).

Mean plasma 11KT in first bleeds ranged between 0.19 and 0.68 $ng.ml^{-1}$ (Fig. 3.11c). Plasma 11KT decreased to non-detectable for all but the 168 h treatment, which remained similar to levels in first bleed.

In first bleeds, mean plasma 17,20βP ranged between 0.22 and 0.56 $ng.ml^{-1}$ and there was no significant difference among treatments (Fig. 3.11d). There were no significant differences between first and second bleeds for any of the treatments.

Mean Hct ranged between 27 and 37 % in first bleeds and there was no difference among treatments (Table 3.1). Mean Hct decreased significantly by 1, 6 and 48 h pc ($P<0.05$, $t = 7.948$ (d.f. = 3), 2.627 (d.f. = 5), 3.380 (d.f. = 5) respectively).

Five distinct cell types were observed in the histology of the testes: spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa (Fig. 3.12). Spermatogonia comprised 7 to 12 % of all categories scored and there were no significant differences among treatments (Fig. 3.13a). Primary spermatocytes comprised 3 to 16 %, with a significant increase in the proportion of primary spermatocytes over time, with 1 and 6 h treatments having significantly less primary spermatocytes than the 168 h treatment ($P<0.05$, $F=6.543$ (d.f. = 23); Fig. 3.13b). The proportion of secondary spermatocytes ranged from 5 to 18%, with a statistically significant sequential decrease over time ($P<0.05$, $F=2.993$ (d.f. = 23); Fig. 3.13c). Similarly, the proportion of spermatids decreased significantly over time, being almost absent after 168 h ($P<0.05$, $F=10.348$ (d.f. = 23); Fig. 3.14d). The proportion of spermatozoa ranged from 38 to 67 %, with a statistically non significant tendency for proportions to increase with time (Fig. 3.13e). Blood vessels comprised less than 0.3 %; connective tissue, less than 3.3 % and interstitial space, less than 6 %.

Figure 3.11. Plasma concentrations of a) cortisol, b) T, c) 11KT and d) 17,20βP in male Trap Caught fish at first capture (white bars) or after confinement for 1, 6, 24, 48 or 168 h (grey bars). Values are means + SE. Numbers indicate n values (number of fish). There were no significantly different Tukey groupings among first bleeds. * denotes significant difference between first and corresponding second bleeds at each time ($P<0.05$).

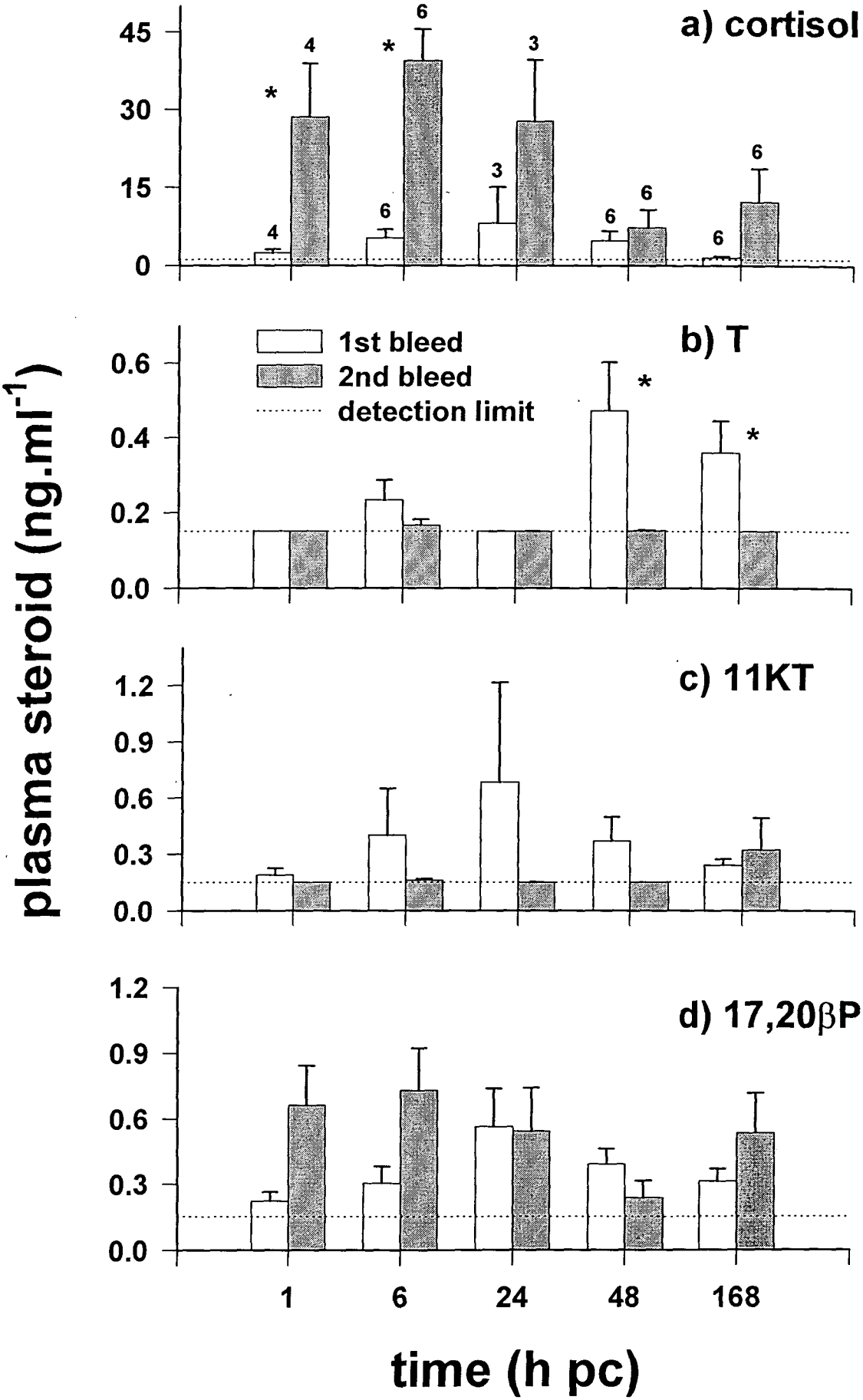

a) cortisol
b) T
c) 11KT
d) 17,20βP
1st bleed
2nd bleed
detection limit
plasma steroid (ng.ml-1)
time (h pc)
1
6
24
48
168

Figure 3.12. Micrographs of transverse sections from the testes of fish showing different stages of maturation: PS, primary spermatocyte; SD, spermatid; SG, spermatagonium; SS, secondary spermatocyte; SZ, spermatozoa. Scale bar = 25 µm.

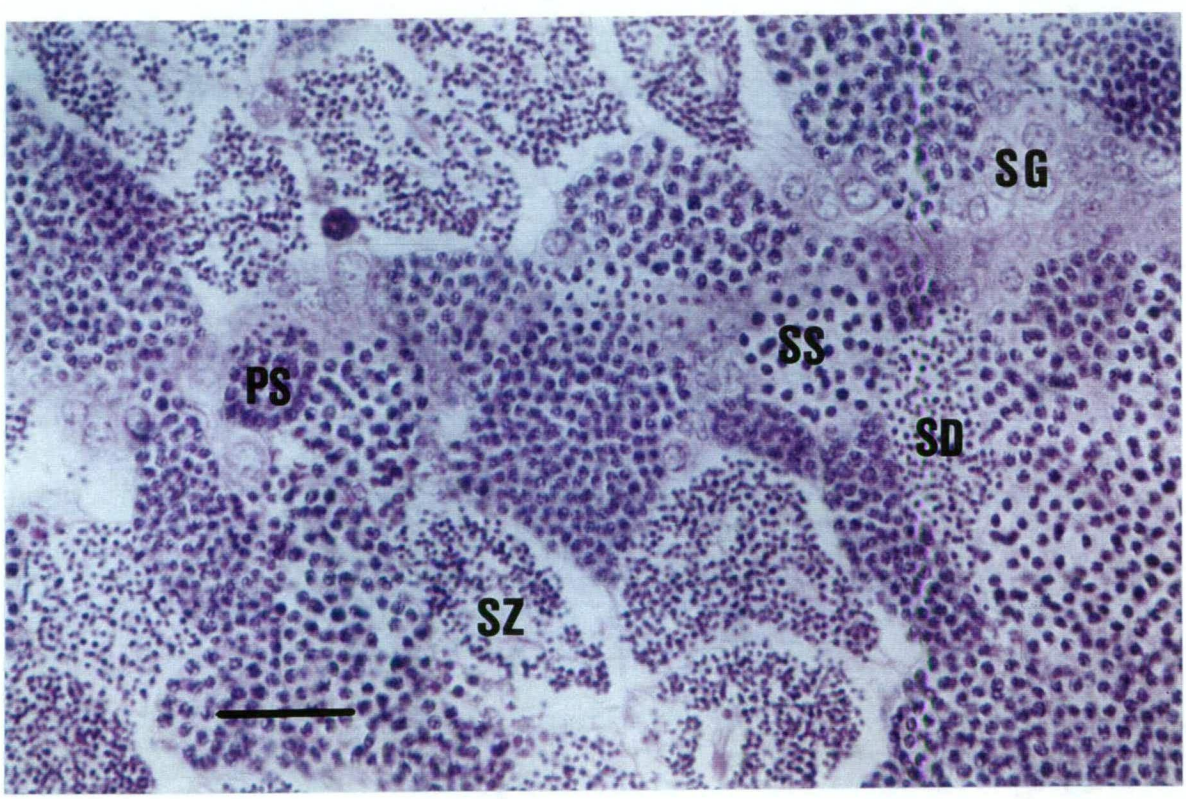
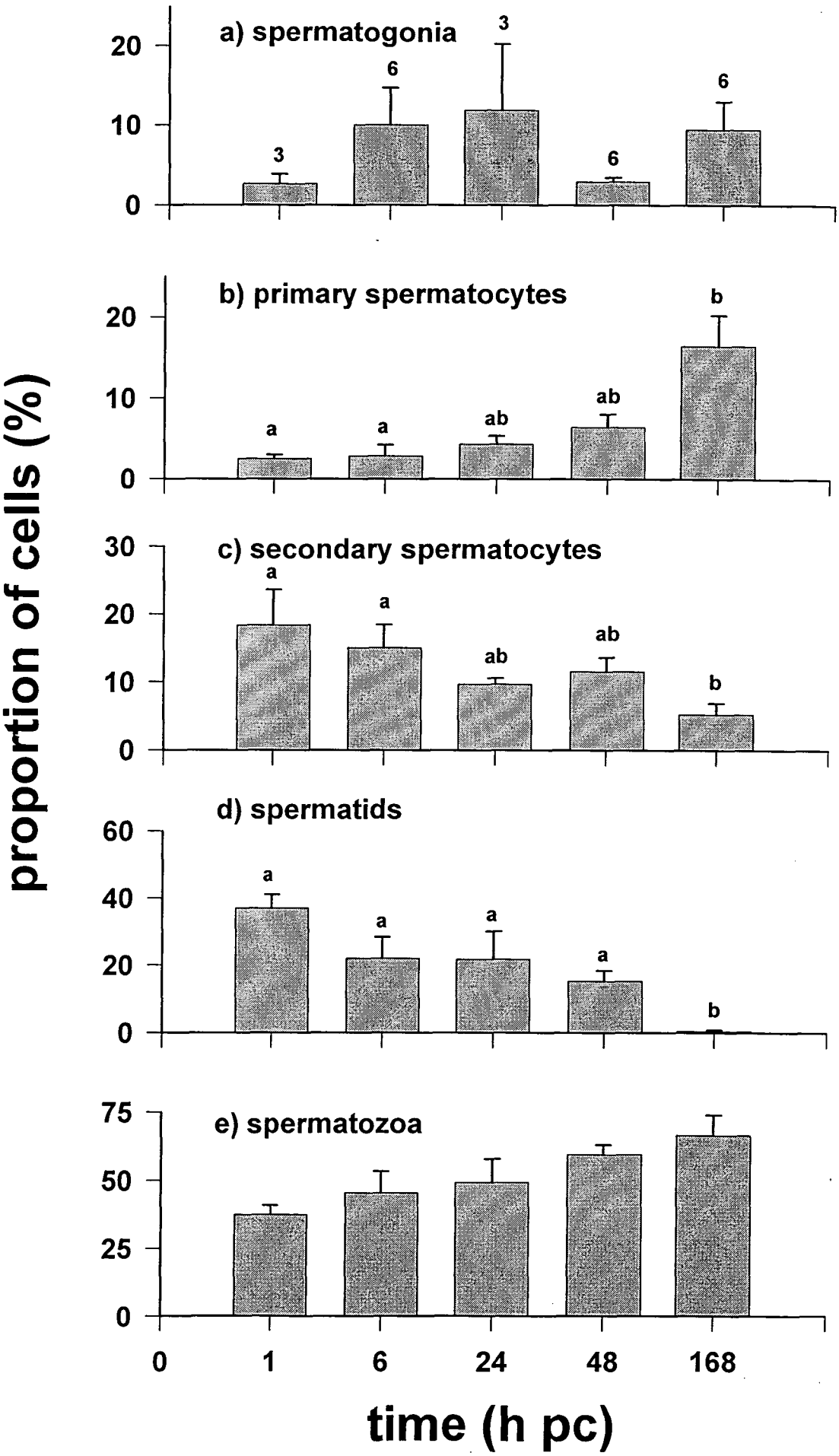

SG
SS
PS
SD
SZ

Figure 3.13. Proportion of a) spermatogonia, b) primary spermatocytes, c) secondary spermatocytes, d) spermatids and e) spermatozoa in histological cross-sections of testes from male Trap-Caught fish confined for 1, 6, 24, 48 or 168 h. Values are means + SE. Numbers indicate n values (number of fish). Different letters show significantly different Tukey (b,d) or SNK (c) groupings among treatments for each oocyte type. No letter indicates same grouping ($P<0.05$).


a) spermatogonia
b) primary spermatocytes
c) secondary spermatocytes
d) spermatids
e) spermatozoa
proportion of cells (%)
time (h pc)
0
1
6
24
48
168

Mean FL of males ranged from 279 ± 25 to 361 ± 26 mm, and whole body weight ranged from 509 ± 135 to 1224 ± 478 g. Mean GSI ranged from 1.15 ± 0.20 to 2.0 ± 0.84 % and HSI ranged from 0.77 ± 0.06 to 1.08 ± 0.04 %. There was no significant difference among treatments for FL, whole body weight, GSI or HSI, consistent with the absence of any accidental bias in allocation of fish to treatments (see Appendix A).

Captive Fish

Mean plasma cortisol was less than 6 $ng.ml^{-1}$ in first bleeds for all treatments and there was no significant difference between treatments ($P>0.05$), although cortisol tended to increase with allocation to successive treatments until 24 h (Fig. 3.14a). Mean cortisol appeared to increase in all treatments by the second bleed, but only significantly in the 24 h treatment ($P<0.05$, $t = -5.478$ (d.f. = 5)).

Plasma T levels in first bleeds were less than 0.2 $ng.ml^{-1}$ for all treatments and there was no significant difference among treatments (Fig. 3.14b). By the second bleed, plasma T was less than 0.16 $ng.ml^{-1}$ in all groups.

There was no significant difference among first bleeds for 11KT, with all levels being less than 0.18 $ng.ml^{-1}$ (Fig. 3.14c). Confinement had no detectable effect on 11KT levels.

Plasma 17,20βP ranged from 0.16 to 0.29 $ng.ml^{-1}$ in first bleeds and there was no significant difference among treatments (Fig. 3.14d). There were no significant differences between first and second bleeds.

Mean Hct in first bleeds ranged from 40 to 45 % (Table 3.1). Mean Hct decreased significantly by 6 h pc ($P<0.05$, $t = 3.454$ (d.f. = 12)).

Spermatogonia constituted 2 to 11 % of all categories scored, and primary spermatocytes constituted 4 to 5 % (Fig. 3.15a,b). There was no significant difference among treatments. Secondary spermatocytes comprised 2 to 11 % (Fig. 3.15c). There was a tendency for the proportion of secondary spermatocytes to decrease over time, which was almost significant ($P=0.06$, $F=2.719$ (d.f. = 32)). Spermatids comprised 3 to 26 % of categories counted (Fig. 3.15d). There was a significant difference in the proportion of spermatids between 6 and 168 h ($P<0.05$, $F = 3.185$ (d.f. = 32)). Spermatozoa were the most abundant cell type, comprising 58 to 87 % of all categories scored (Fig. 3.15e). There were significantly more spermatozoa in 168 h fish than 1 and 6 h fish ($P<0.05$, $F=4.803$ (d.f. = 32)). Blood vessels comprised only 0 to 0.3 %; connective tissue, 1 to 3.3 % and space 1 to 6 % of categories scored.

Figure 3.14. Plasma concentrations of a) cortisol, b) T c) 11KT and d) 17,20βP in male Captive fish at first capture (white bars) and after confinement for 1, 6, 24, 48 or 168 h (grey bars). Values are means + SE. Numbers indicate n values (number of fish). There were no significantly different Tukey groupings among first bleeds. * denotes significant difference between first and corresponding second bleeds at each time ($P<0.05$).

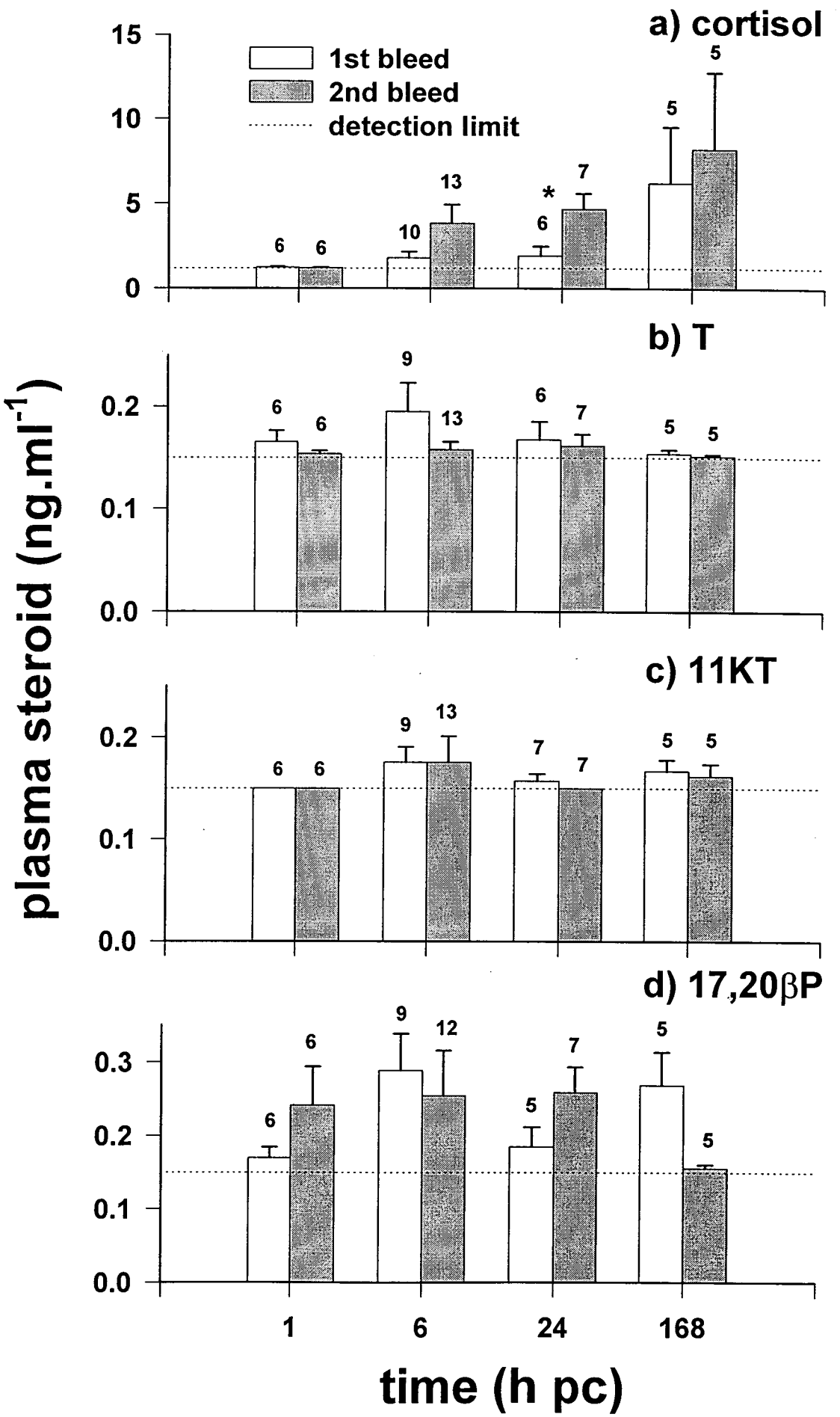

a) cortisol
1st bleed
2nd bleed
detection limit
15
10
5
0
6
6
10
13
*
6
7
5
5
b) T
0.2
0.1
0.0
6
6
9
13
6
7
5
5
c) 11KT
0.2
0.1
0.0
6
6
9
13
7
7
5
5
d) 17,20βP
0.3
0.2
0.1
0.0
6
6
9
12
5
7
5
5
1
6
24
168
time (h pc)
plasma steroid (ng.ml-1)

Figure 3.15. Proportion of a) spermatogonia, b) primary spermatocytes, c) secondary spermatocytes, d) spermatids and e) spermatozoa in histological cross-sections of testes from male Captive fish confined for 1, 6, 24, or 168 h. Values are means + SE. Numbers indicate n values (number of fish). Different letters show significantly different Tukey groupings among treatments for each oocyte type. No letter indicates same Tukey grouping ($P<0.05$).

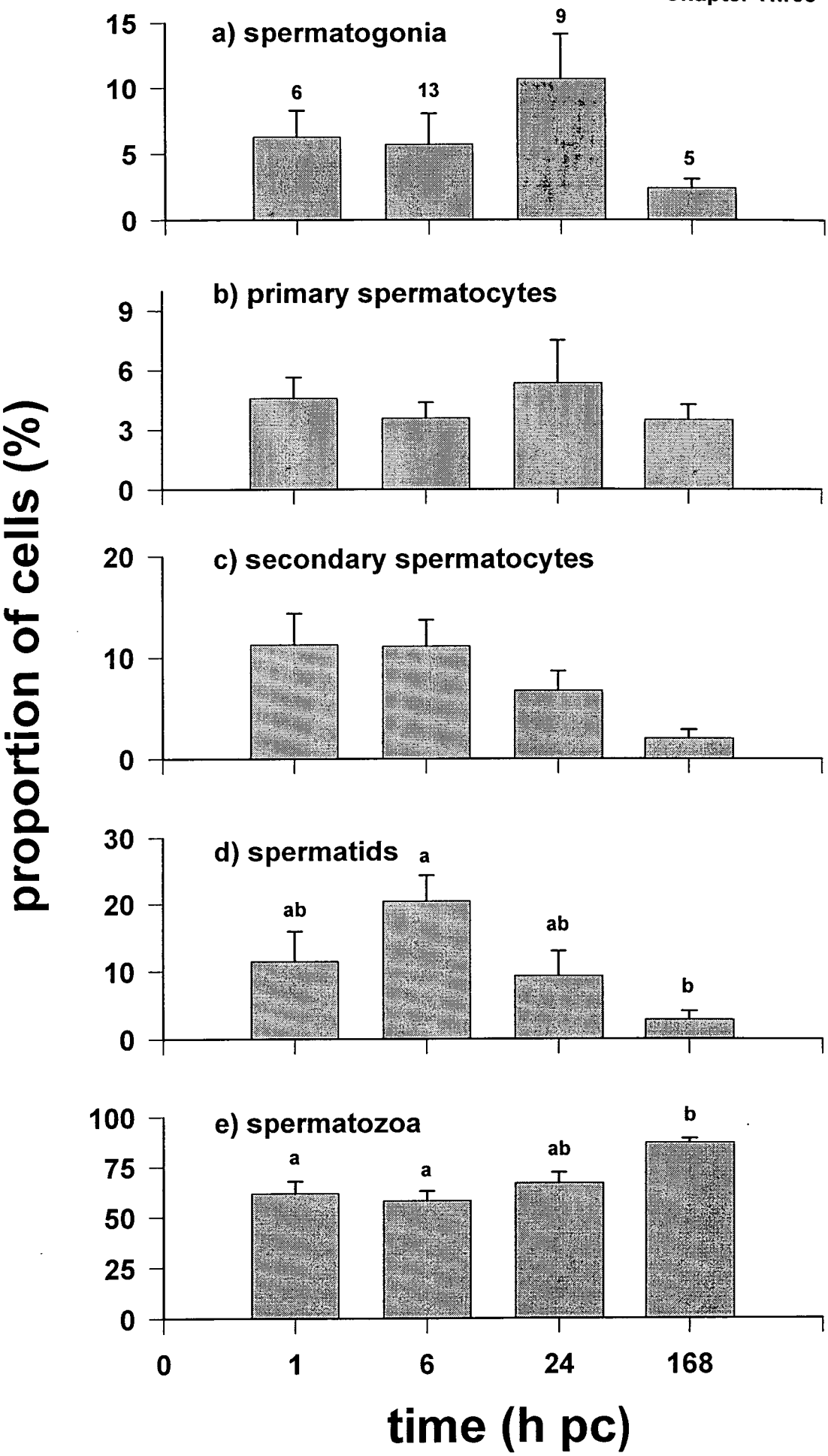
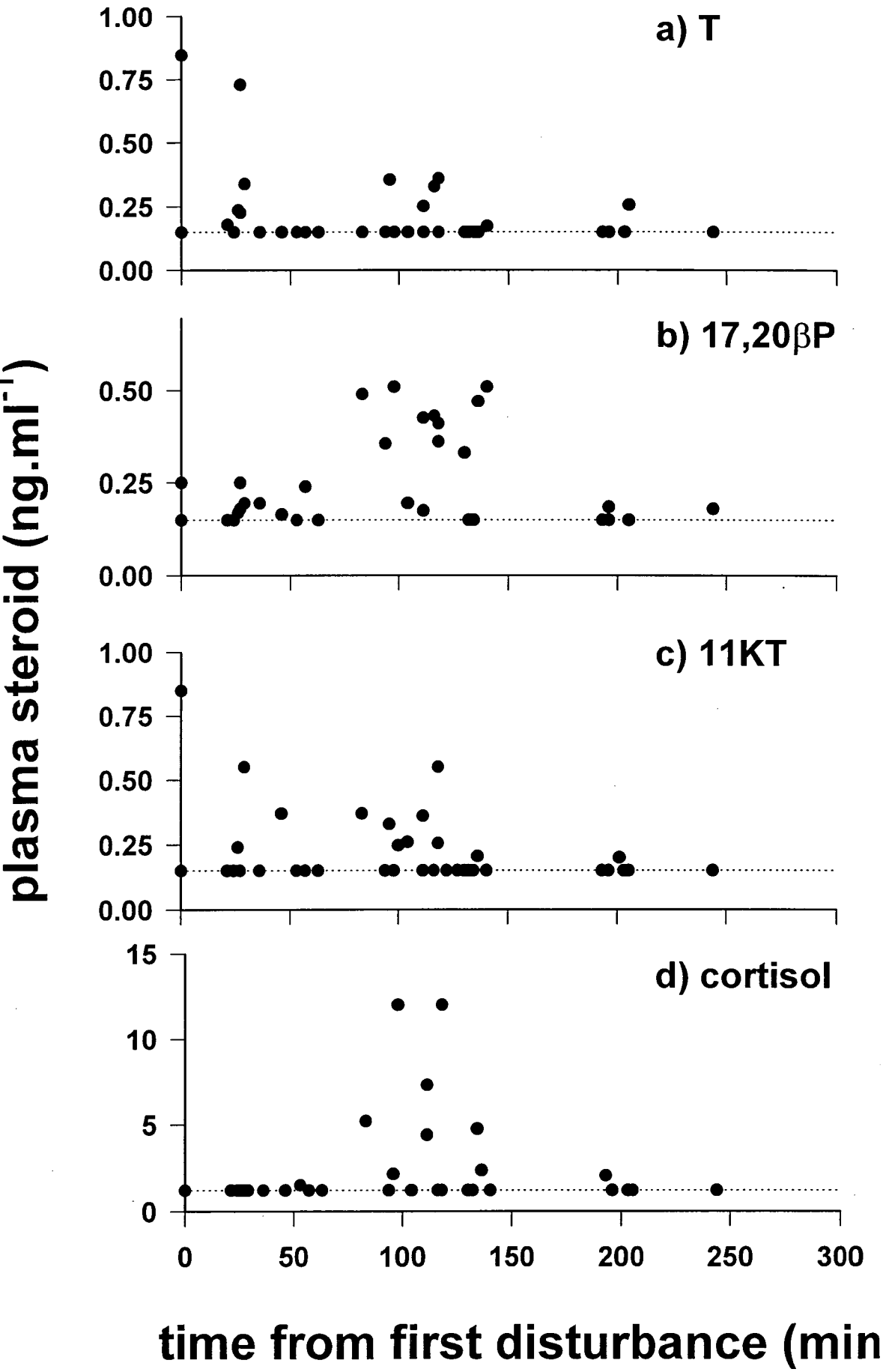

a) spermatogonia
b) primary spermatocytes
c) secondary spermatocytes
d) spermatids
e) spermatozoa
proportion of cells (%)
time (h pc)
0
1
6
24
168
6
13
9
5
ab
a
ab
b
a
a
ab
b

Figure 3.16. The relationship between time from first pool-side disturbance until sampling and plasma a) E_2, b) T, c) 17,20βP, and d) cortisol levels in first bleeds of male Captive and 2-year-old Hatchery fish.


a) T
b) 17,20βP
c) 11KT
d) cortisol
plasma steroid (ng.ml-1)
time from first disturbance (min)
1.00
0.75
0.50
0.25
0.00
15
10
5
0
0
50
100
150
200
250
300

Mean FL of captive males ranged from 412 ± 15 to 474 ±11 mm; whole body weight ranged from 1492 ± 6 to 2249 ± 141 g. There was a significant difference in FL and whole body weight among treatments, with 24 h fish being heavier than both 1 and 168 h fish ($P<0.05$, $F=3.031$ (d.f. = 30), 6.403 (d.f. = 30) respectively). Tukey's mean comparison did not detect where this difference lay for FL. Mean GSI ranged from 2.58 ± 0.42 to 4.29 ± 1.16 %, and HSI ranged from 1.04 ± 0.08 to 1.15 ± 0.03 %, however, there was no significant difference among treatments. Therefore, there is some indication that there was an accidental bias in allocation of fish to treatment groups, with larger fish in the 24 h treatment. However, even under conditions of completely random allocation, such differences will occasionally occur. In any case , the larger fish at 24 h did not appear to be reproductively more mature (see Appendix A).

Steroid Levels at First Bleed

The envelope for both plasma T and 11KT in first bleeds decreased from approximately 0.15 - 0.8 $ng.ml^{-1}$ to 0.15 - 0.25 $ng.ml^{-1}$ by 130 min after first disturbance (Fig. 3.16a,b). Plasma 17,20βP and cortisol did not show this tendency (Fig. 3.16c,d). The envelope for plasma 17,20βP increased from 0.15 - 0.25 $ng.ml^{-1}$ at first disturbance to 0.15 - 0.5 $ng.ml^{-1}$ by 80 min post disturbance, and decreased by 200 min to less than 0.2 $ng.ml^{-1}$. The envelope for cortisol increased from <0.6 $ng.ml^{-1}$ in first bleeds to 0.6 - 12 $ng.ml^{-1}$ at 80 min, and had decreased by 200 min to less than 2 $ng.ml^{-1}$. The data presented in Figure 3.16 were steroid levels of first bleeds for all mature male Hatchery fish and Captive fish used in this experiment.

3.4 Discussion.

Plasma Cortisol Levels

Determination and comparison of basal plasma cortisol (i.e. levels in normally active fish) is difficult, as a wide range of parameters influences measurements. These include sex and sexual maturity (Campbell *et al* , 1976; Cook *et al*, 1980; Bry, 1985; Bradford and Taylor, 1987), time of day (Redgate, 1974; Kühn *et al*, 1986), developmental stage (Barton *et al*, 1985), feeding (Bry 1982), species (Pickering and Pottinger, 1989), and strains within species (Heath *et al*, 1993). Most importantly, pre-sampling history markedly affects what are thought to be basal levels of plasma cortisol (Bollard *et al*, 1993; Pankhurst and Sharples, 1992). Therefore measurements of "basal levels" of plasma cortisol can vary broadly. However, in studies where attention has been paid to minimising pre-sampling stress, initial levels are generally less than 20 $ng.ml^{-1}$ (e.g. Pickering, 1984; Pickering et al, 1982; Tomasso et al,

1981; Waring et al, 1992). The best estimate of basal levels of plasma cortisol in snapper come from wild New Zealand snapper sampled underwater. These had plasma cortisol concentrations of 1.7 to 8 $ng.ml^{-1}$ (Pankhurst and Sharples, 1992). In the present study, initial mean plasma levels of cortisol in females and males were less than 11 $ng.ml^{-1}$ in all groups of fish, and are therefore similar to those of normally active snapper in the wild.

The low cortisol levels in fish in captivity (Captive and Hatchery) at first capture, in the present study indicate that the culture conditions have not led to detectable chronic elevations in cortisol levels. Bollard *et al* (1993) found that different maintenance techniques led to different resting levels of plasma cortisol, with snapper in high traffic areas having approximately 60 $ng.ml^{-1}$ compared to only 2.1 $ng.ml^{-1}$ in fish in a more secluded area. Fish in the present study may still be suffering from chronic stress, however, as sometimes plasma cortisol levels can return to basal levels despite the continued presence of the stressor (Pickering and Stewart, 1984). Also, even slight chronic elevations of plasma cortisol can impact on the fish. For example, Pickering and Pottinger (1985a), found that chronic artificial elevation of plasma cortisol of only a few $ng.ml^{-1}$ significantly affected the immune response of brown trout.

The low initial levels of plasma cortisol found in Trap-caught fish is surprising, as the presence of abrasions and other injuries indicate that trapping is a stressful event. External damage is not, however, always correlated with plasma cortisol levels (Pankhurst and Sharples, 1992). Pankhurst and Sharples (1992) found that plasma cortisol in New Zealand snapper began to elevate within 30 min of confinement in nets underwater and were significantly elevated after 60 min of confinement. Therefore it would be expected that snapper confined in the trap for 30 min or more would have elevated cortisol. This suggests three possibilities. Firstly, that most of the fish were in the trap for less than 30 minutes. It is not known how long individual fish were in the trap prior to landing and sampling. However, as the traps were set for between 3 and 168 h it seems unlikely that most fish caught had been in the trap for less than 30 minutes. Secondly, trap-confinement may not be as stressful as the net-confinement used in Pankhurst and Sharples (1992) and did not cause an elevation in plasma cortisol. The net-confined fish in that study were herded and caught by handnet before confinement, whereas the trap-confined fish, in the present study, entered the trap freely in search of food. Barton and Peter (1982) found that initial capture and handling of fingerling rainbow trout was the most stressful component of transportation, not confinement and transport. The stress of trap capture may therefore be mainly due to hauling the trap to the surface rather than the actual confinement within the trap. Finally, plasma cortisol may have initially increased following entrapment, however, by the time the trap was pulled, the fish had physiologically acclimated to the trap environment, and plasma cortisol had returned to resting levels (e.g. Pickering and Stewart, 1984; Pickering and Pottinger, 1985b). Recovery could occur in the traps, provided the trap environment was not continuously stressful and the fish were in the trap for long enough. Return to resting levels

of cortisol after a stress episode make take days or even weeks in many species (Schreck, 1981; Pickering and Stewart, 1984). Recovery of wild-caught New Zealand snapper in the laboratory took up to 48 h (Pankhurst and Sharples, 1992) and in the present study took at least 24 h. However, as the cortisol response is affected by the type and duration of the stressor (Barton *et al*,, 1980), it is possible that recovery rates in fish confined in traps were more rapid than in the laboratory (following transport and handling). Therefore the most plausible account of these data is that cortisol levels in these Trap-Caught fish increased initially, but had recovered by the time of capture.

With the exception of Captive males, capture and handling of snapper in the present study tended to increase plasma cortisol levels. The peak mean cortisol level occurred at either 6 or 24 h pc for all groups of fish, with the exception of Captive males, indicating that maximal interrenal stimulation probably occurred somewhere between these two sampling times. Maximum plasma cortisol levels in captive males occurred at 168 h. This is probably of little biological significance as plasma cortisol means for all treatments in captive males were less than 10 $ng.ml^{-1}$ (i.e. still near basal levels). Either the sampling regime did not have the temporal sensitivity to detect an increase in plasma cortisol or none was elicited. Interestingly, Captive females showed a more typical response with an increase in plasma cortisol levels to a maximum by 6 h. The reason for the difference between sexes is not known, especially as males and females were confined in the same tank. Spawning females, however, are often reported as having higher plasma cortisol than males. Pickering and Christie (1981) found that hatchery-reared mature female brown trout showed a four-fold increase in plasma cortisol levels compared to males at the onset of spawning. Migrating and spawning female Pacific salmon (*Oncorhynchus tschawytscha)* were found to have more active adrenocortical tissue than male counterparts (Hane *et al,* 1966).

The highest mean plasma cortisol in second bleeds among all the groups of fish occurred in Trap-Caught females and males, with 35 and 40 $ng.ml^{-1}$ respectively. These are somewhat lower than those found in other studies with other species, where cortisol levels often reach hundreds of $ng.ml^{-1}$ after stress (e.g. Redgate, 1974; Zelnik and Goldspink, 1981). Maximum plasma cortisol levels in snapper, however, are generally lower than for many other teleosts. Pankhurst and Sharples (1992) reported that the highest plasma cortisol level measured in that study was 76 $ng.ml^{-1}$ in a New Zealand snapper caught from the wild by long-line and transported to the laboratory. The maximum level detected in this study was 62 $ng.ml^{-1}$ in a Trap-Caught fish after transport and confinement for 6 h. Therefore the cortisol response in Australian and New Zealand snapper appears similar in magnitude, assuming that the stressors are equivalent.

Hct

Resting Hct levels have been reported for a large number of teleosts. These range from 15 % in Atlantic salmon (*Salmo salar*) (Nichols and Weisbart, 1984) to 52 % in *Colisa fasciastus* (Mishra and Srivastava, 1979). The broad range in values is due to the large number of factors that may affect Hct. Hct is affected by water temperature (Woo, 1990), sample storage time prior to measurement (Fletcher, 1975), species (Blaxhall, 1972) and pre-sampling history (Fletcher, 1975). Haematocrit values reported for *Pagrus auratus* range from 21 (Woo, 1990) to 54 % (red seabream: Foscarini, 1988). In the present study mean Hct for each of Trap-Caught males and females, Captive males and females and 2-and 3-year-old Hatchery females ranged from 31 to 44 %. Therefore values in this study are within the range of others reported in the literature for snapper.

In the present study Hct levels were found to decrease rather than increase following stress. Other workers have also reported this phenomenon. Mishra and Srivastava (1979) reported a decrease in Hct with exposure to zinc in the freshwater teleost, *Colisa fasciatus* Soivio *et al* (1974) found that *in vitro* incubation of erythrocytes in an hypoxic environment resulted in a 10-30 % increase in Hct and in an aerobic environment resulted in a 10 % decrease. Other workers, however, have attributed decreased Hct to repeat sampling induced anaemia (Iwama *et al*, 1989; Pankhurst *et al*, 1992). As the fish in this study were also bled twice, it is possible that the decrease in Hct was also due to sampling-induced anaemia. This, however, does not preclude the occurrence of erythrocyte swelling or the release of erythrocytes into the blood stream. By measuring mean corpuscular haemoglobin content (MCHC) Pankhurst *et al* (1992) demonstrated that a decrease in Hct in serially sampled blue mao mao, *Scorpia violaceus*, was not accompanied by erythrocyte swelling. MCHC was not measured in this study, so it is possible that an increase in Hct due to erythrocyte swelling and splenic erythrocyte release was overshadowed by a decrease in Hct due to anaemia. Therefore Hct does not appear to be useful as an indicator of acute stress in snapper.

Plasma E_2 and T: females

Plasma E_2 and T levels in female snapper are generally less than 3 and 1 $ng.ml^{-1}$ respectively (e.g. Carragher and Pankhurst, 1993; Hobby and Pankhurst, 1997a). These are somewhat lower than levels found in other species. For example, levels as high as 162 and 62 $ng.ml^{-1}$ for E_2 and T have been measured in unstressed adult female plaice, *Pleuronectes platessa* L (Wingfield and Grimm, 1977). In this study the highest levels of E_2 and T were measured in 3-year-old Hatchery females, which had a maximum mean of approximately 2.5 and 1 $ng.ml^{-1}$ respectively. Trap-Caught, 2-year-old Hatchery and Captive females all had lower levels of E_2 and T at first capture, with levels being less than 0.75 and 0.25 $ng.ml^{-1}$ for E_2 and T respectively.

In Trap-Caught females, (assuming the wild population is reproductively healthy) it appears that the capture technique had already compromised the levels of gonadal steroids by the time of blood sampling. It appears that while plasma cortisol levels had recovered by the time of sampling, plasma E_2 and T in contrast, remained suppressed. This is in accordance with the results of Carragher and Pankhurst (1991) who demonstrated that plasma E_2 and T levels do not recover following a stress episode in wild New Zealand snapper. It must be noted, however, that GSI's for Trap-Caught fish were at least half that of both Captive and 3-year-old Hatchery fish. It is likely that this also contributed to the low levels of plasma E_2 and T measured in Trap-Caught fish. Two-year-old Hatchery females were from the same stock as 3-year-old Hatchery females and so have a similar genetic propensity for stress tolerance. They were also held in similar rearing conditions prior to the experiment. This suggests that the low level of plasma E_2 in 2-year-old Hatchery females is due to 1994 being their first breeding season (with small gonads) rather than differential stress effects in 1994 and 1995. The large numbers of females in 1994 with non-detectable levels of T supports this, suggesting that T was being rapidly metabolised into E_2. Captive females were 5 years old and would be expected to have high levels of E_2 and T if they were reproductively healthy. As they did not show convincing evidence of vitellogenesis and maturation, it appears that chronic stress has prevented normal gonadal recrudescence.

Plasma E_2 and T levels decreased following confinement in all groups of females in the present study, with plasma levels of both steroids being virtually non-detectable by 6 h pc. A similarly rapid effect was demonstrated for New Zealand snapper, where chronic stress significantly reduced plasma E_2 levels by 1 h pc and plasma T by 6 h pc (Carragher and Pankhurst, 1991). Capture and confinement of red gurnard resulted in a decrease in plasma E_2 and T by 24 h pc (Clearwater and Pankhurst, 1997), however, the coarseness of the temporal sensitivity of the sampling regime in that study prevented detection of possible more rapid changes. Wild-caught rainbow trout subjected to capture and handling had significantly reduced plasma E_2 and T levels 24 h after initial capture, although there were some indications of a reduction after only 1 h (Pankhurst and Dedual, 1994). In the present study, E_2 and T levels remained non-detectable in Trap-Caught and Captive fish for the remainder of the experiment. Plasma E_2 and T in confined red gurnard and New Zealand snapper also remained low for 96 and 120 h. Interestingly in the present study, by 168 h pc, 2 out of five 2-year-old Hatchery females and 8 out of 10 3-year-old Hatchery females had detectable levels of E_2. This indicates either preservation in steroidogenic capacity following the stress episode in some fish, or a recovery by 168 h. This finding is in contrast to all other studies on plasma E_2 and T levels in wild fish, in which there was no documented recovery of plasma E_2 levels (Carragher and Pankhurst, 1991; Pankhurst and Dedual, 1994; Clearwater and Pankhurst, 1997).

Further evidence for the extreme sensitivity to disturbance of plasma E_2 and T levels was provided in the present study by the decrease in E_2 and T levels with time from first disturbance for Captive and Hatchery fish combined. The disturbance here was due only to pool-side or cage-side activity, resulting from capture of other fish by handline. This seemingly low level of disturbance had a demonstrable depressive effect on E_2 and T levels, with virtually all E_2 and T being less than 0.5 $ng.ml^{-1}$ within 2 h. Rainbow trout, caught from a common tank by dipnet, showed increasing plasma cortisol with time from first disturbance (Laidley and Leatherland, 1988), however, serial disturbance has not previously been reported to have an effect on gonadal steroids. In the present study, E_2 and T for Captive and Hatchery fish decreased to virtually non-detectable following capture and 1 h of confinement, with the exception of E_2 in Captive fish, which took until 6 h. It appears that pool-/cage-side disturbance induced reductions in plasma E_2 and T, although not as rapid as that resulting from capture and handling, are still rapid and noticeable effect. The snapper in the present study showed a more dramatic response to capture and 1 h confinement than that demonstrated by Carragher and Pankhurst (1991) for New Zealand snapper, where T did not approach 0.15 $ng.ml^{-1}$ (the detection limit in both studies). In the light of the sensitivity of snapper to pool-/cage-side disturbance fish in future experiments were allocated to treatments in cyclic order.

Plasma T and 11KT: males

Plasma T and 11KT levels in first bleeds of Trap-Caught males were less than 0.5 $ng.ml^{-1}$, and levels for Captive fish were lower still, with the maximum mean being 0.175 $ng.ml^{-1}$. These values are lower than plasma levels described for New Zealand snapper of 1.1 and 2.8 $ng.ml^{-1}$ for T and 11KT respectively (Carragher and Pankhurst, 1993). The values found in the present study for Trap-Caught males are more similar to those described for wild-caught New Zealand snapper 1 day after capture (Pankhurst, 1994). It appears, therefore, that plasma levels of T and 11KT in Trap-Caught males (as for E_2 and T in females) have already been compromised due to the capture technique. Similarly, the low plasma levels of T and 11KT in Captive fish suggest the presence of chronic stress, and reflect their long-term failure to acclimate to the holding environment.

In the present study stress affected plasma levels of T and 11KT levels by reducing them to low or non detectable levels after as little as 1 h. Plasma levels of T and 11KT in male spotted seatrout were found to decrease significantly within 1 h of capture by gill-net or handline and subsequent handling (Safford and Thomas, 1987). Chronic confinement of brown and rainbow trout for 1 month resulted in plasma T and 11KT levels decreasing by approximately 50 % (Pickering *et al*, 1987). In a parallel study, acute handling stress for 1 h resulted in a significant reduction in T and 11KT by 1 and 4 h respectively (Pickering *et al*, 1987). Pool-/cage-side disturbance of fish in the present study, resulted in a decrease in the gonadal steroids, T and 11KT, again emphasising the sensitivity of snapper to stress induced impairment of reproduction, similarly to E_2 and T for females. The decrease, however, took longer than that following capture, handling and 1 h confinement.

Plasma 17,20βP

Plasma 17,20βP levels in snapper are typically low, with values generally being less than 1.5 $ng.ml^{-1}$ (Kagawa *et al*, 1991; Pankhurst and Carragher, 1992; Carragher and Pankhurst, 1993; Pankhurst, 1994; Hobby and Pankhurst, 1997a). Initial plasma levels of 17,20βP for females in the present study were similarly low, ranging from virtually non-detectable to 1 $ng.ml^{-1}$. Most marine species appear to produce only small amounts of 17,20βP (Carragher and Pankhurst, 1991). Plasma levels of 17,20βP, however, have been measured as high as 350 $ng.ml^{-1}$ 4 days prior to ovulation in rainbow trout (Scott *et al*, 1983). Low levels of plasma 17,20βP in New Zealand snapper were initially thought to be in conflict with its apparent role as the maturation-inducing steroid. Hobby and Pankhurst (1997a), however, demonstrated the need for fine-scale sampling to detect changes in plasma 17,20βP levels, since the changes can be of short duration. This is consistent with reports from other species that 17,20βP is rapidly metabolised or conjugated within the ovary (Canario and Scott, 1989; Scott *et al*, 1990; Scott and Canario, 1992), and plasma levels seldom become significantly elevated. In addition, Ventling and Pankhurst (1995) demonstrated 17,20βP to be the most effective of a range of C21 steroids at inducing FOM in New Zealand snapper oocytes *in vitro*.

In contrast to E2 and T, plasma 17,20βP levels in the present study did not exhibit a stress-induced reduction in plasma levels until 48 h pc, when plasma 17,20βP levels were reduced to low or non-detectable in all fish. Prior to 48 h pc plasma levels of 17,20βP either decreased, increased or showed no change. Plasma 17,20βP levels were also less sensitive to pool-/cage-side disturbance. The response of plasma 17,20βP to stress in other studies is similarly inconsistent. Pankhurst and Dedual (1994) found no change in 17,20βP of rainbow trout following capture, handling and confinement for 24 h and Carragher and Pankhurst (1991) found that plasma 17,20βP levels increased in stressed snapper. Pankhurst and Van Der Kraak (1997) suggest that the observed conservation or increases in plasma 17,20βP levels may be due to resilience of 20β-hydroxy-steroid dehydrogenase activity (converting 17P to 17,20βP) to stress episodes. If stress impedes conversion of 17P to T and E_2 (see Chapter 5), the resultant increased substrate pool of 17P may generate the possibility of stress-induced increases in 17,20βP in stressed fish. The possibility of stress-induced increases in 17,20βP further emphasises the need for stress management, if accurate assessment of reproductive endocrine condition is to be possible.

Gonadal Histology

No atresia was detected in primary oocytes, and only a low level in cortical alveoli stage oocytes in the ovaries of Captive, Hatchery and Trap-Caught snapper in the present study. Although high variation in the occurrence of atresia and small sample sizes has led to a lack of power to detect significant differences, early vitellogenic oocytes of snapper in the present study showed a general increase in the proportion of atresia, with increasing confinement up to 168 h in all 4 groups of fish. Late vitellogenic oocytes, however, did not show a convincing effect. It appears that early vitellogenic oocytes in snapper are more susceptible to stress induced atresia than late vitellogenic oocytes. An increase in the degree of oocyte atresia has also been found with increasing confinement time in red gurnard (Clearwater and Pankhurst, 1997), and Northern pike (*Esox lucius:* De Montalembert *et al*, 1978), however, differential effects of stress on early and late vitellogenic oocytes were not examined in these studies. Mature post-vitellogenic follicles did not show any detectable signs of atresia in the present study. This is consistent with the fact that these mature follicles can undergo FOM and ovulation despite the imposition of stress (Pankhurst, 1998).

Stress has a differential effect on Trap-Caught, Captive and Hatchery fish, in terms of the incidence of atresia. At 168 h pc, Captive and Trap-caught fish both had greater than 50 % atretic early vitellogenic oocytes by 168 h pc, while Hatchery fish had less than 25 %. This is a further indication that the Captive fish have not acclimated to the artificial environment, since they are at least as sensitive to stress as their wild counterparts.

Janz and Van Der Kraak (1997) found that E_2 was important in maintaining follicular integrity in developing trout oocytes by preventing apoptosis. Matsuyama *et al* (1988) found that red seabream with low levels of atresia had higher levels of plasma E_2 than those with high levels of atresia. Furthermore, estrogens delayed atresia caused by hypophysectomy (Sundararaj and Goswami, 1968; Anand and Sundararaj, 1974). Therefore it appears that E_2 and GtH may be involved in preventing atresia in the developing oocyte. Clearwater and Pankhurst (1997) suggest that atresia may result from the inhibitory action of cortisol on GtH and hepatic vitellogenesis, resulting in interference with the synthesis of vitellogenin or vitelline envelope proteins. Atretic oocytes are probably not steroidogenic (Clearwater and Pankhurst, 1997), therefore once atresia has begun, plasma levels of gonadal steroids would be expected to decrease, and would be unlikely to recover until the next clutch of oocytes is recruited (Clearwater and Pankhurst, 1997).

Confinement stress did appear to change the proportions of gamete stages in males. In Trap-Caught males the proportion of primary spermatocytes increased and there was some indication of an increase in the proportions of spermatozoa, whereas the proportion of secondary spermatocytes, and spermatids decreased. Captive fish showed a less marked response, but with similar trends. It appears that the increase in spermatozoa by 168 h is due to an increase in the rate of development of secondary spermatocytes and spermatids

into spermatozoa. The high levels of primary spermatocytes, however, indicate that this increase in rate has not affected primary to secondary spermatocyte development. Spermiation is regulated by 17,20βP in snapper and other species (Pankhurst, 1994). As discussed previously, stress may result in an increase in plasma levels of 17,20βP. This paradoxical effect of stress in males may actually advance sperm maturation via stress induced increases in 17,20βP.

Comparison of Groups

Despite having been held in captivity for 5 years, Captive fish did not show evidence of acclimation to the artificial environment in terms of plasma cortisol and gonadal steroid levels or ovarian condition. Similarly Salonius and Iwama (1993) found that after 7 months in the hatchery, the cortisol response to handling stress of wild-caught coho salmon was still higher than hatchery-reared fish. Although still sensitive to stress, the stress response of Hatchery fish in the present study was less profound than for both Trap-Caught and Captive fish. This is demonstrated by reduced cortisol response, incidence of ovarian atresia and indications of post-stress recovery of plasma gonadal steroid levels. Therefore the decreased cortisol response observed in this study may be due to inadvertent selection for stress tolerant fish during hatchery-rearing, rather than acclimation (Barton *et al*, 1987; Schreck *et al*, 1995; Pottinger and Pickering, 1997). Presumably, highly stress sensitive fish in an aquaculture environment would succumb quickly to diseases and so be removed, leaving only the more stress tolerant individuals. Alternatively, Pottinger and Pickering (1997) cite evidence to suggest that there may be a critical developmental period, during which stress exerted may permanently down-regulate the stress-response. If this is the case for snapper, this must be in the larval or juvenile stages, as the pool fish were caught as young-of-the-year.

Several studies have examined the heritability of the stress response and the use of selective markers (mainly cortisol) which can be used to identify stress tolerant individuals (e.g. Refstie, 1982; 1986). Fevolden *et al,* (1991) demonstrated a genetic component and hence heritability to the stress response in Atlantic salmon and rainbow trout, when they succeeded in selecting for fish with high and low cortisol responses. In Japan, 30 years of domestication appears to have produced a highly stress tolerant strain of red seabream (Foscarini, 1988). It is attractive to consider the possibility of fast-tracking the domestication process of snapper in Australia by selection for individuals with a low cortisol response to stress. However, it must be remembered that cortisol is not a synonym for stress (Pickering and Pottinger, 1987), nor do low levels of plasma cortisol necessarily indicate a lack of stress (Pickering and Pottinger, 1987) or of other stress-related effects on growth, immunocompetence or reproduction. In addition, although the stress response can have negative impacts on the fish, it is essential for mobilising energy reserves to overcome stressors (Pickering, 1993). Therefore, selection for a low cortisol response may actually also mean concomitant selection for poor adaptability to environmental fluctuations. This suggests that inadvertent

selection, or selection based on a wide-range of parameters is likely to yield better results. Indeed, Pottinger and Pickering (1997) suggest that selection based on the behavioural response to stress may be more convenient and reliable than the use of physiological parameters such as plasma levels of cortisol.

Conclusion

In summary, both male and female Australian snapper are highly susceptible to stress-induced impairment of reproduction. The response to stress was found to be similar to that of New Zealand snapper, with a rapid increase in plasma cortisol and a concomitant decrease in plasma E_2 and T in females, and T and 11KT in males. The extreme sensitivity of these steroids to disturbance was emphasised by the rapid decrease in plasma levels after minimal pool-/cage-side disturbance. Only Hatchery fish showed any signs of recovery following the stress episode after 1 week, suggesting improvement with domestication after only one generation. This, however, is inconclusive and further work needs to be done to confirm this, using larger sample sizes and perhaps examining steroid levels over a longer period of time. This study raises several implications for broodstock management. Firstly, wild-caught fish are unlikely to acclimate, even when caught as juveniles and maintained in captivity until maturity. Secondly, while Hatchery fish are more robust, they are still highly sensitive to stress, and care should be taken to ensure that the fish are held in locations with minimal disturbance. It is likely that other stress sensitive species require similar treatment.

Chapter 4: The effect of capture and handling stress on the endocrine and ovulatory response to exogenous hormone treatment.

4.1 Introduction

In any aquaculture venture, there are two main sources of broodstock: wild-caught or captive/domesticated populations. For logistical reasons, fish caught from the wild are often treated with exogenous hormones some time after capture. For example, Pankhurst and Carragher (1992) treated New Zealand snapper, *Pagrus auratus,* with hCG on the day following capture, while wild-caught snapper at the NSW Fisheries Port Stephens Research Centre (PSRC) are normally not treated until return to the hatchery, which may be 6 or more hours following capture (Battaglene, 1995). In addition, captive/domesticated stocks are regularly exposed to disturbance such as net changes, feeding, tank maintenance and periodic sampling, which generates increases in plasma cortisol above those found in wild fish (Bollard *et al*, 1993; Wedemeyer, 1997). The extreme sensitivity of snapper to disturbance was demonstrated in Chapter 3 (this volume). Capture, handling and confinement of both Hatchery and Trap-caught snapper resulted in a decrease in plasma levels of gonadal steroids by 6h pc, and a concomitant increase in the incidence of oocyte atresia. In addition, plasma gonadal steroids also decreased markedly following capture of other fish from the same enclosure by hand-line. It might be expected therefore, that both wild-caught and captive/domesticated fish will already have stress-modified plasma levels of reproductive hormones at the time of exogenous hormone treatment. Although the effect of exogenous hormones on subsequent ovulatory events has been examined in detail in many species (reviewed by Donaldson and Devlin, 1996), the effect of stress-reduced plasma levels of gonadal steroids on the response to the treatment, in terms of subsequent ovulatory events, is not described for any species. This chapter addresses whether the stress history affects the ability of fish to respond to exogenous hormone treatment, by comparing the response of snapper treated with exogenous hormones immediately after capture ("unstressed") and 24h after capture ("stressed"). In particular, it examines whether stress impairment renders attempts to induce ovulation in wild-caught fish futile; whether hatchery fish are equally susceptible to stress-induced impairment of reproduction; or whether the process of partial domestication has overcome these effects. Should stress compromise the effects of exogenous hormones, these results will offer an approach to optimising treatment protocols for production of viable ova from wild and domesticated fish.

In naturally spawning female teleosts, gonadotropin releasing hormone (GnRH), produced by the hypothalamus, stimulates the pituitary to secrete 2 forms of gonadotropin (GtH I and II) into the circulation. GtH I is present during the early stages of reproductive development, while GtH II is present during oocyte maturation (Swanson, 1991). GtH has its primary effect through binding to specific membrane-bound receptors in the ovary. This binding initiates signal transduction by activation of protein kinase A via cyclic AMP, which both activates existing steroid synthesising enzymes and initiates synthesis of new ones (reviewed in Van Der Kraak and Wade, 1993). Steroid synthesising enzymes stimulate the step-wise production of gonadal steroids from the steroid precursor, cholesterol (reviewed in Peter and Yu, 1997). Maturation inducing steroids (MIS) are produced by post-vitellogenic follicles, which in turn stimulate final oocyte maturation (FOM). In most species, including snapper, the MIS is 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) (see Chapter 3, this volume), however in some species it is 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S; Thomas, 1994). Once FOM is complete, ovulation is stimulated by release of F-series prostaglandins under the influence of GtH (reviewed in Pankhurst, 1998).

When fish are caught or held in captivity, natural ovulation may not occur. It is therefore often necessary to induce ovulation with exogenous hormone treatment. There are 2 main approaches to inducing ovulation: treatment with GtH preparations or analogues of GnRH (GnRHa). GtH preparations mimic the action of natural gonadotropin, secreted by the pituitary, in stimulating steroidogenesis, FOM and ovulation. GnRHas act directly on the pituitary and stimulate secretion of natural GtH by mimicking endogenous GnRH. In some species, GnRHa may need to be administered in conjunction with a dopamine antagonist (e.g. domperidone or pimozide), since the release of GtH in many teleosts is regulated by the inhibitory tone maintained by dopaminergic hypothalamic neurones (reviewed by Peter and Yu, 1997).

In this study, wild and hatchery-reared vitellogenic snapper with different stress histories were treated with exogenous GtH (human chorionic gonadotropin; hCG) or GnRHa (luteinising hormone releasing hormone analogue; LHRHa). HCG is commonly used to induce ovulation in a wide range of marine and freshwater teleosts. Other studies have demonstrated the efficacy of this gonadotropin in a variety of species, including New Zealand snapper (Pankhurst and Carragher, 1992), gilthead seabream *(Sparus aurata;* Zohar and Gordin, 1979), mulloway (*Argyrosomus hololepidotus;* Battaglene and Talbot, 1994), red drum (*Sciaenops ocellatus;* Colura, 1990), Atlantic croaker (*Micropogonias undulatus;* Middaugh and Yoakum, 1974), and Australian snapper (Battaglene and Allan, 1994). GnRHa is also highly effective at inducing gonadal maturation and ovulation. Matsuyama *et al* (1995) found that sustained administration of LHRHa to red seabream induced gonadal development and maturation via substantial release of GtH from the gonadotropes. LHRHa has also been shown to stimulate oocyte maturation and ovulation in other species of fish, including goldfish (Lam *et al*, 1975), Atlantic croaker (Gwo et al, 1993), gilthead seabream (Zohar *et al*, 1988),

red seabream (Matsuyama *et al*, 1995), salmonids (reviewed in Donaldson and Devlin, 1996) and Australian snapper (Battaglene, 1995). Fish in the present study were also treated with 17α-hydroxyprogesterone (17P); an intermediate in the steroid synthetic pathway which can be converted by maturing follicles to 17,20βP, which in turn mediates FOM in snapper (Ventling and Pankhurst, 1995). 17P and steroid precursors have been used in combination with GnRHa or GtH to stimulate FOM in some induced ovulation protocols in other species (De Montalembert *et al*, 1978; King and Young, 1995; Ohta *et al*, 1996). Treatment of Japanese eels (*Anguilla japonica*) with pituitary extract, followed by 17,20βP stimulates ovulation (Ohta *et al*, 1996), while De Montalembert *et al* (1978) found that treatment of Northern pike (*Esox lucius*) with partially purified salmon gonadotropin (PPSG), followed by 17P one day later induced maturation and partial ovulations, compared with PPSG alone which induced only maturation, and not ovulation. Furthermore, treatment of male Atlantic salmon with 17P, in combination with GnRHa, increased milt volume, over that in response to GnRHa alone (King and Young, 1995). In the present study, 17P was used to test whether the effects of stress are generated by a reduction of steroid precursor. The ovulatory response of injected fish was assessed in terms of the numbers of ovulators, the volume of eggs produced, and the quality of the eggs (as determined by fertilisation and egg morphology). A broad range of ovulation parameters was used since treatments can still generate ovulation of compromised eggs (Campbell *et al*, 1994). The steroidogenic response of treated fish to handling, and the respective hormone treatments was assessed by measuring plasma levels of 17β-estradiol (E_2), testosterone (T), 17α,20β-dihydroxy 4-pregnen 3-one (17,20βP) and cortisol.

4.2 Methods

Source of Fish

This experiment was conducted in 1995 on 3 separate groups of female snapper: 3-year-old Hatchery, Trap-Caught and Line-Caught fish.

Experimental Protocol

Hatchery Fish

Fish were caught from the seacages by handline between 0700 and 1100 h in September 1995. Surface water temperature was 18 °C. Immediately after capture a gonadal biopsy was conducted, stage III and IV females were retained and a blood sample was taken. Gonadal biopsy samples were stored on ice in the cannula tubing until the diameters of the 10 largest oocytes were measured.

The fish were allocated to one of 8 groups in a 2 x 4 factorial design as follows:

Fish ("unstressed") were allocated cyclically as they were caught to 4 treatments (n=10) and injected with the appropriate hormone and dose as below. Fish were given intraperitoneal injections of either:

1) acidified saline (saline) injected at 0.5 $ml.kg^{-1}$;

2) 17α-hydroxyprogesterone (17P) injected at 0.5 $ml.kg^{-1}$ of a 1 $mg.ml^{-1}$ suspension in ethanol:saline (1:9 v/v) to give a dose of 0.5 $mg.kg^{-1}$;

3) Des-Gly^{10},(D-Ala^{6})-luteinising hormone releasing hormone ethylamide (LHRHa) injected at 0.5 $ml.kg^{-1}$ of a 200 $\mu g.ml^{-1}$ solution in saline to give a dose of 100 $\mu g.kg^{-1}$;

4) Human chorionic gonadotropin (hCG) injected at 0.5 $ml.kg^{-1}$ of 2000 $U.ml^{-1}$ solution in saline to give a final dose of 1000 $U.kg^{-1}$.

All hormones were purchased from Sigma. Injection dose was determined by measuring fork length (FL) and converting this to whole body weight using a length weight relationship (Y= 8.1313x - 1790.22: r^2 = 0.9581: n=68) calculated from data collected from wild fish captured in 1994.

The next 40 fish ("stressed") were similarly captured, tagged, measured, bled and transported to the NSW Fisheries, Fisheries Research Institute, Cronulla (FRI), however, they were not injected until 24 h pc (described below).

At FRI, all fish were held in covered and aerated 4000 L tanks with constant water exchange for the remainder of the experiment (20 fish/tank). The fish were maintained in tanks fitted with internal standpipes and 500 µm screens covering the water outlets to retain any eggs released in the tank. Water was at ambient temperature (14.9 ± 0.3 °C). Although this reduced temperature may have affected the ovulatory response, it was not logistically possible to heat the water during the experiment. A fine mesh dipnet was used daily to collect any eggs in the water column. The volume of these eggs was measured.

At 24 h pc, "stressed" fish were recaptured, anaesthetised and a blood sample taken. Ovulated fish were rinsed with freshwater to remove anaesthetic residue, dried and manually stripped with gentle abdominal pressure. Ovulation volume, fertility and egg morphology was assessed, as described below. The fish were then injected as for "unstressed" fish and released back into the tanks. At 24, 48 and 72 h post injection (pi) all fish were anaesthetised, checked for ovulation and any eggs stripped. Egg volume and fertility were measured and egg morphology assessed. Finally, at 168 h pi, the fish were recaptured, anaesthetised, bled, stripped, and egg volume and morphology assessed (see below). Fertility was not assessed at 168 h pi due to a lack of spermiating males. The fish were

killed, measured and the body, gonad and liver weighed. Gonads were macroscopically staged. Ovarian samples were collected and prepared for histology, and the proportion of normal and healthy oocytes for each oocyte stage was counted. Plasma was analysed by RIA for E_2, T, and 17,20βP and cortisol as described in Chapter 2.

Assessment of Egg Quality

In order to assess fertility, approximately 100 µl of sperm from 2 freshly stripped males were mixed with a subsample of eggs from any ovulated fish. A small volume of sea water was added to activate the sperm, and the eggs were incubated at room temperature in plastic petri dishes. After 3 h, fertility was determined by counting the proportion of eggs undergoing cell division. The males had been captured from the seacage at the same time as the females, and transported to FRI where they were held in floating pens (10 x 7.5 x 2.4 m; 2 fish/pen). Sperm from the same pair of males was used each day. Egg morphology was assessed according to shape (round or misshapen), opacity (clear or opaque), and buoyancy (buoyant or sinking) under a dissecting microscope, and the percentages of each egg type recorded. Three arbitrary categories were assigned to eggs on the basis of the percentage of round clear and buoyant eggs: 80-100 % (Grade 1), 20-80 % (Grade 2) and 0-20 % (Grade 3).

Wild Fish

A similar experiment was conducted on wild fish (caught by trap and by line), however, due to difficulties in catching sufficient numbers of wild fish a reduced protocol was used. All fish were treated at first capture. Therefore the experiment had only 1 factor (hormone treatment) with 3 levels (saline, LHRHa and 17P).

After initial handling, Trap- and Line-Caught fish were transported to PSRC and placed in covered and aerated 10,000 L tanks with constant water exchange for the remainder of the experiment. Tanks were fitted with internal standpipes and outflow screens. Fish caught on each fishing day were held in the same tank. Water temperatures ranged from 19.3 ± 0.2 to 22.5 ± 0.2 °C (mean daily min./max. ± SE). Mean surface water temperature at capture was 18.7 ± 0.3 °C. Sperm used to fertilise eggs was obtained from fish from the same source as the females (i.e. Line-Caught or Trap-Caught).

Statistics

The experiment with the hatchery fish had 2 factors. The first factor, stress had 2 levels: unstressed (treated at first capture) and stressed (treated at 24 h pc). The second factor, hormone treatment had 4 levels: saline, 17P, LHRHa and hCG. To test for differences between unstressed and stressed fish, biological, histological and plasma steroid data sets were analysed using separate 2-way ANOVAs with stress and hormone treatment as the 2 factors at each of the three sampling times (0 h pc, 0 h pi and 168 h pi). Comparisons of

means, following the 2-way ANOVA were made using Tukey-Kramer HSD after Underwood (1981). When there was no significant interaction effect, and only one factor was significant, the data set was pooled for the non-significant factor and comparison of means (of the significant factor) performed on pooled data. Results of pooled means comparisons are indicated on insert graphs within the figures. When there was a significant interaction effect, and/or both factors were significant, all treatment means were compared without pooling. In this latter case the results of the comparisons are indicated on the main figure. Due to the highly variable number of fish ovulating at each stripping, statistical comparison of egg volumes was made only on LHRHa- and hCG-treated fish, on data pooled across all stripping times, and analysed by 2-way ANOVA, followed by Tukey-Kramer HSD mean comparison, as described above.

The wild fish formed a single factor design with 3 levels (saline, hCG, LHRHa). However, the data sets from wild fish were not analysed statistically due to the large number of sample sizes of less than 3.

4.3 Results

Hatchery Fish

Effects of hormone treatments on ovulation

In stressed fish, 30 % of 17P-treated fish, and 10 % of each of saline-, LHRHa- and hCG-treated fish had ovulated by the time of injection (24 h pc) (data not shown). Due to logistical constraints, unstressed fish were not checked for ovulation prior to injection. Proportions of fish treated with hCG or LHRHa that ovulated were similar and high, irrespective of stress status, with between 70 and 90 % of fish in these groups ovulating by 168 h pi (Fig. 4.1). The proportion of 17P- and saline-treated fish ovulating was similar and low in both unstressed and stressed fish with between 10 and 33 % of fish in these groups ovulating by 168 h pi. Fish treated with LHRHa or hCG often ovulated more than once and were stripped up to 4 times by 168 h pi. (Fig. 4.2). Those treated with 17P and saline rarely ovulated more than once, with only 2 fish (stressed, 17P-treated fish) ovulating twice.

Immediately prior to injection (24 h pc), ovulating stressed fish produced from 1 to 17 ml of eggs (data not shown). Unstressed fish produced a maximum mean egg volume of 33 ml at 48 h pi with a normal distribution around that point (Fig. 4.3 a). Mean egg volumes for stressed fish were less than 14 ml at each stripping, and no eggs were produced at 48 h pi

(Fig. 4.3 b). There were significant effects for stress and hormone and stress by hormone interaction ($P<0.05$) for mean egg volumes (data pooled across stripping times) produced by unstressed fish and stressed hCG- and LHRHa-treated fish. Comparison of means indicated that egg volumes from unstressed hCG-treated fish were significantly higher than the other

three groups. Saline- and 17P-treated fish from both stressed and unstressed groups produced less than 10 ml of eggs at each stripping, however no statistical comparison was made. A maximum of 15 ml of released eggs was collected from each tank on any one day (Table 4.1). On each occasion, a tank containing released eggs also contained fish that were known ovulators. Thus the volumes of eggs measured at each stripping are an underestimation of the total number of eggs ovulated. As each tank held fish from all treatments, it was not possible to determine whether any particular treatment contributed more to eggs released in the water.

Ovulations of the best quality eggs, in terms of egg morphology, came from unstressed fish, with 21 Grade 1 ovulations compared to only 4 Grade 1 ovulations in stressed fish (Table 4.2). Treatment of unstressed fish with hCG yielded the best quality eggs, with 13 Grade 1 ovulations. There were 7 Grade 1 ovulations from LHRHa-treated fish. The greatest number of Grade 1 ovulations was obtained at 72 h pi, with 15 of the 25 Grade 1 ovulations occurring at this time (Table 4.3). There were no Grade 1 ovulations from 17P-treated fish, and only 1 from a saline-treated fish (24 h pi).

Apart from a single spontaneous ovulation with high fertilisation (70 %) from a saline injected fish at 24 h pi, all fertilised eggs came from either hCG- or LHRHa-treated fish (Fig. 3 c,d). The best fertilisation resulted from stripping at 72 h pi for both unstressed and stressed fish. Unstressed fish stripped at 72 h from both hCG and LHRHa treatments appeared to give better fertilisation than corresponding stressed fish. Unstressed hCG treated fish stripped at 72 h pi overwhelmingly produced the greatest volume of fertilised eggs, with approximately 3 times more being produced than at all other times and for all other hormone treatments (Fig 4.3 e,f)

For unstressed and stressed fish respectively mean (± SE) gonadosomatic index (GSI) was 4.3 ± 0.2 % and 4.5 ± 0.23 %; mean hepatosomatic index (HSI) was 1.6 ± 0.2% and 1.8 ± 0.1 %; and mean oocyte diameters were 563 ± 19 µm and 556 ± 19 µm. There was no significant difference in GSI among hormone levels or between stress levels, nor was there an interaction effect. HSI was significantly lower in unstressed than stressed fish ($P<0.05$), however, there was no significant hormone or interaction effect. There was no significant difference in oocyte diameters among hormone levels or between stress levels, nor was there an interaction effect. Mean FL was 331.5 ± 2.7 mm and 326.9 ± 3.8 mm and mean whole body weight was 802.2 ± 22.6 g and 823.7 ± 9.3 g for unstressed and stressed fish respectively. There was no significant difference in FL or whole body weight among treatments, as would be expected if there was no bias in allocation of fish to different treatments (see Appendix B).

Figure 4.1. Cumulative percentage of a) unstressed and b) stressed Hatchery snapper which ovulated by 24, 48, 72, and 168 h pi with saline, 17P, LHRHa or hCG (n=9-10). Superscripts are numbers of fish actually ovulating at that time.

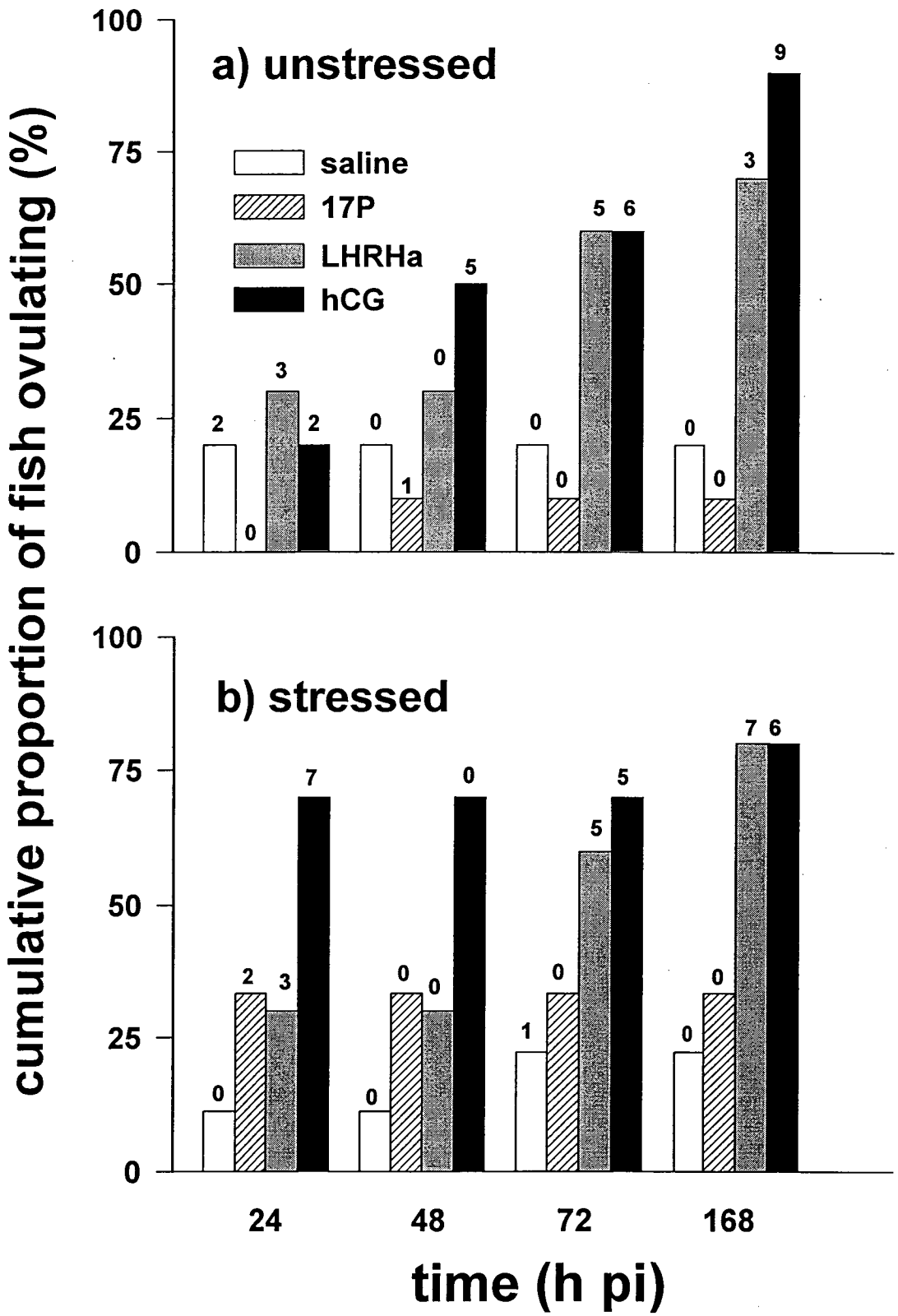

a) unstressed
b) stressed
saline
17P
LHRHa
hCG
cumulative proportion of fish ovulating (%)
100
75
50
25
0
24
48
72
168
time (h pi)

Figure 4.2. Percentage of a) unstressed and b) stressed Hatchery snapper ovulating 0, 1, 2, 3, or 4 times after injection with saline, 17P, LHRHa or hCG.

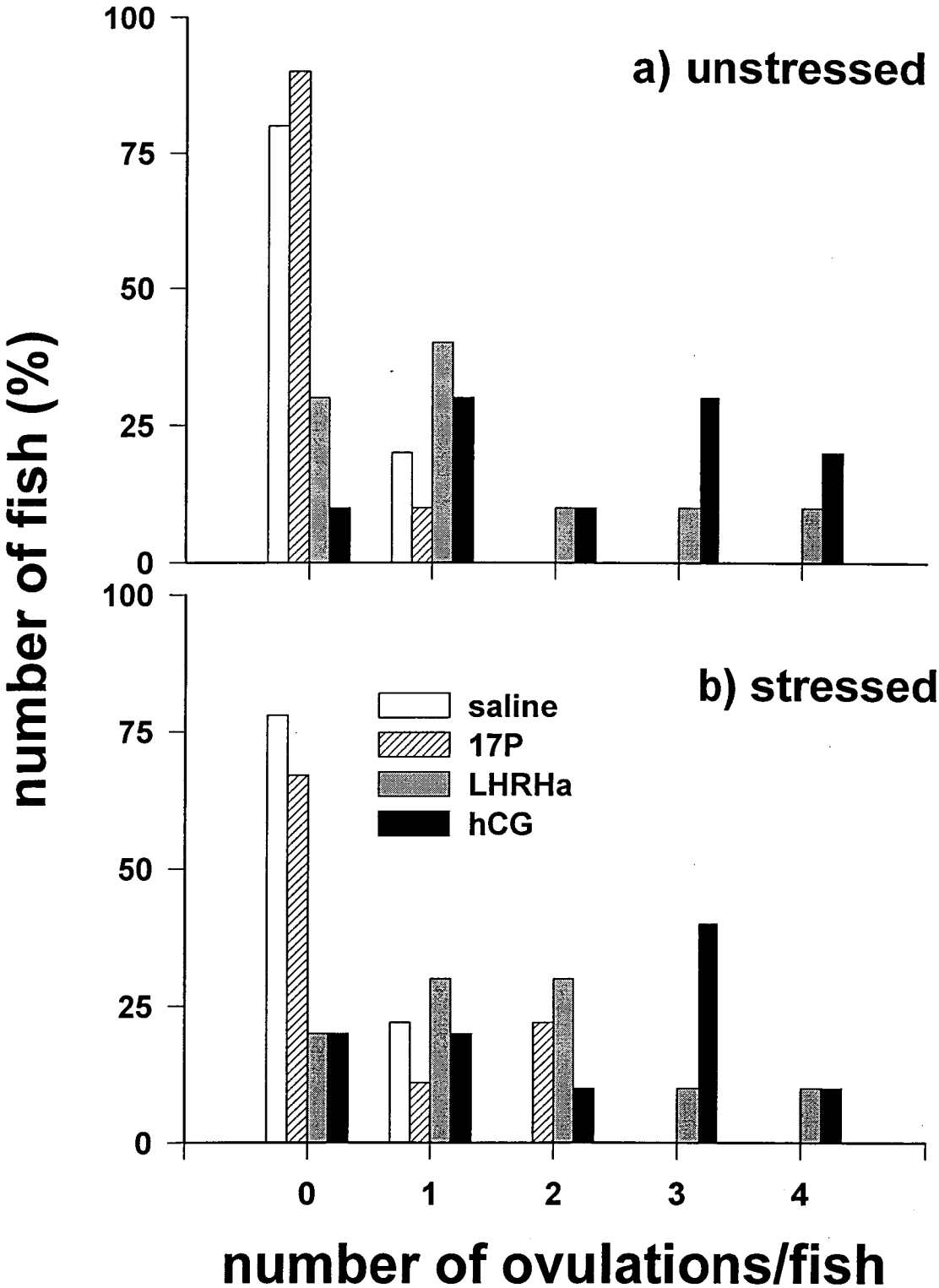

a) unstressed
b) stressed
saline
17P
LHRHa
hCG
number of fish (%)
number of ovulations/fish
100
75
50
25
0
0
1
2
3
4

Figure 4.3.

Mean volume of eggs stripped from a) unstressed and b) stressed Hatchery snapper at 24, 48, 72, and 168 h pi with saline, 17P, LHRHa or hCG. Values are means + SE. Superscripts show numbers of ovulating fish at that time. Insert shows means for LHRHa- and hCG-treated unstressed and stressed fish, pooled across all stripping times. There was a significant hormone, stress and interaction effect. * indicates mean is significantly different from the other three means according to Tukey-Kramer HSD comparison of means($P<0.05$).

Percentage fertilisation of eggs stripped from c) unstressed and d) stressed Hatchery snapper at 24, 48 and 72 h pi with saline, 17P, LHRHa or hCG.

Volume of fertilised eggs from e) unstressed and f) stressed Hatchery snapper stripped at 24, 48 and 72 h pi with saline, 17P, LHRHa or hCG.

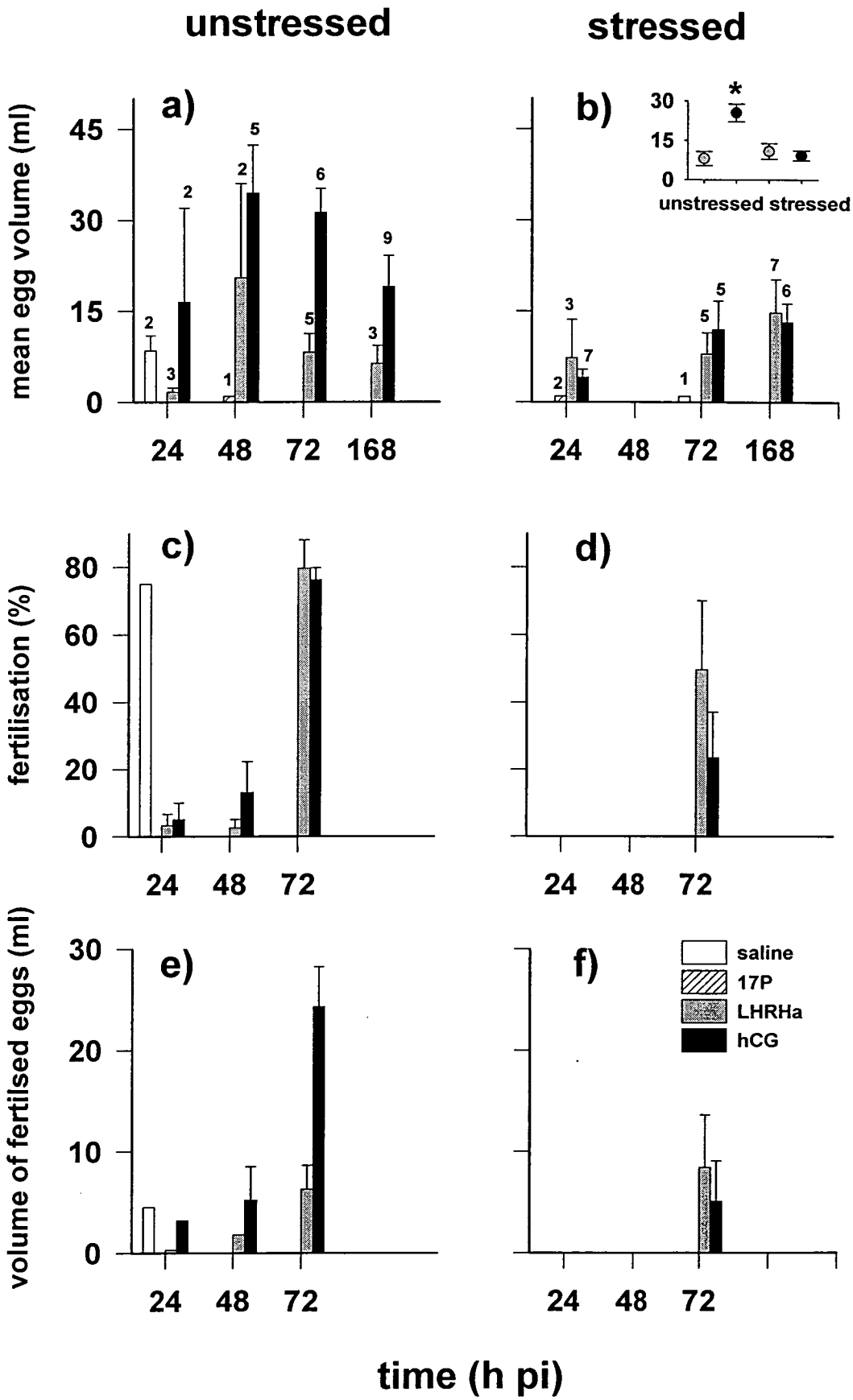

unstressed
stressed
a)
b)
c)
d)
e)
f)
mean egg volume (ml)
fertilisation (%)
volume of fertilised eggs (ml)
45
30
15
0
80
60
40
20
0
30
20
10
0
24
48
72
168
*
unstressed stressed
saline
17P
LHRHa
hCG
time (h pi)

Table 4.1: Volume of eggs collected from tanks holding Hatchery snapper.

	Egg Volume (ml)			
	Unstressed		Stressed	
Time (h pc)	Tank A	Tank B	Tank C	Tank D
24	0	0	0	0
48	0	0	0	0
72	0	0	0	0
96	0	0	0	0
120	15	10	7	1
144	7	2	7	5
168	na	na	15	12

Table 4.2: Egg morphology grade of ovulated eggs from Hatchery snapper injected with saline, 17P, LHRHa or hCG at 0 h pc (unstressed) or 24 h pc (stressed).

		Egg Morphology		
		Grade 1	**Grade 2**	**Grade 3**
Unstressed	**Saline**	1	1	0
	17P	0	0	1
	LHRHa	7	1	5
	hCG	13	6	3
Stressed	**Saline**	0	0	2
	17P	0	0	5
	LHRHa	2	5	9
	hCG	2	9	8

Proportion (%) of round, clear, buoyant eggs present (n=100). Grade 1 = 80-100%; Grade 2 =20-80%; Grade 3=0-20%.

Table 4.3: The number of "Grade 1" ovulations at each stripping from Hatchery snapper injected with saline, 17P, LHRHa or hCG at 0 h pc (unstressed) or 24 h pc (stressed).

		Time of stripping			
		24 h pi	**48 h pi**	**72 h pi**	**168 h pi**
Unstressed	**Saline**	1	0	0	0
	17P	0	0	0	0
	LHRHa	1	1	5	0
	hCG	0	4	6	3
Stressed	**Saline**	0	0	0	0
	17P	0	0	0	0
	LHRHa	0	0	2	0
	hCG	0	0	2	0

Ovarian Histology

Generally, hormone treatment had a stronger effect on the proportion of each oocyte type than stress status. Primary oocytes comprised over 75 % of the total number of previtellogenic oocytes in all treatment groups and no atretic primary oocytes were observed. Cortical alveoli stage oocytes constituted approximately 15 to 20 % of all previtellogenic oocytes. Hormone treatment had a significant affect on the incidence of normal cortical alveoli oocytes, with hCG- and LHRHa-treated fish having significantly more than saline-treated fish ($P<0.05$; Fig. 4.4a). There was no significant stress or interaction effect. Atretic cortical alveoli oocytes comprised from 2 to 12 % of all previtellogenic oocytes (Fig. 4.4b). There was a significant effect of hormone treatment on the incidence of atretic cortical alveoli oocytes, with saline treatments yielding more atretic cortical alveoli oocytes than LHRHa and hCG treatments ($P<0.05$). There was no stress or interaction effect.

There was no difference in the occurrence of normal early vitellogenic oocytes between unstressed and stressed fish. However, amongst treatments there was a significant difference, with hCG- and LHRHa-treated fish having more normal early vitellogenic oocytes than saline-treated fish ($P<0.05$; Fig. 4.4c). Low levels of atresia were evident in early vitellogenic oocytes and there were more atretic early vitellogenic oocytes in stressed than unstressed fish ($P<0.05$) (Fig. 4.4d). Hormone treatment had a significant effect with saline-treated fish having more atretic early vitellogenic oocytes than hCG-treated fish ($P<0.05$). There was no interaction effect.

Normal late vitellogenic oocytes showed a trend similar to that of the normal early vitellogenic oocytes with a significant hormone effect ($P<0.05$), and no stress or interaction effect (Fig. 4.4e). There were fewer normal late vitellogenic oocytes in saline than hCG and LHRHa treatments. Late vitellogenic oocytes had the highest levels of atresia of all oocyte stages scored, with 15 to 70 % atresia in the various treatments (Fig 4.4f). There was a significant hormone and interaction effect ($P<0.05$). Tukey mean comparison of all 8 means shows that in unstressed fish hCG-treated fish had less atretic oocytes than saline-, 17P- and LHRHa-treated unstressed fish. In stressed fish, both LHRHa- and hCG-treated fish had significantly fewer atretic oocytes than saline-treated, but not 17P-treated fish. Migratory nucleus, maturing, hydrated oocytes and post-ovulatory follicles comprised less then 10 % of all vitellogenic oocytes. No atresia was detected in these oocyte stages.

Plasma Steroids

In general, stress status had a stronger effect on plasma steroid levels than did hormone treatment at all sampling times. However, at 168 h pi, hormone treatment affected levels of E_2 and T in unstressed fish. Unstressed fish had higher plasma levels of E_2 and T than stressed fish at first capture ($P<0.05$) and immediately prior to injection (0 h pi) ($P<0.05$)

Figure 4.4. Mean proportion of oocyte types in histological cross-sections of ovaries from unstressed and stressed Hatchery snapper 1 week after injection with saline, 17P, LHRHa or hCG (mean + SE; n=9-10).

a) Proportion of normal cortical alveoli oocytes expressed as a percentage of total previtellogenic oocytes. There was a significant difference among hormone treatments ($P<0.05$), but not between stress levels, nor was there an interaction effect. Therefore, stress-levels were pooled for comparison of means. Insert shows means for each hormone treatment (± SE) with unstressed and stressed treatments pooled. Different superscripts indicate significantly different Tukey-Kramer HSD groupings among hormone treatments.

b) Proportion of atretic cortical alveoli oocytes. Other details as for a).

c) Proportion of normal early vitellogenic oocytes expressed as a percentage of all vitellogenic oocytes. Other details as for a).

d) Proportion of atretic early vitellogenic oocytes expressed as a percentage of all vitellogenic oocytes. There was a significant difference between unstressed and stressed treatments ($P<0.05$) and among hormone treatments ($P<0.05$), therefore all means were compared to each other. Different subscripts indicate significantly different Tukey-Kramer HSD groupings among all 8 treatments.

e) Proportion of normal late vitellogenic oocytes expressed as a percentage of all vitellogenic oocytes. Other details as for a)

f) Proportion of atretic late vitellogenic oocytes. Significant difference among hormones and a significant interaction effect ($P<0.05$), therefore all means were compared to each other. Different superscripts indicate significantly Tukey-Kramer HSD groupings among all 8 treatments.

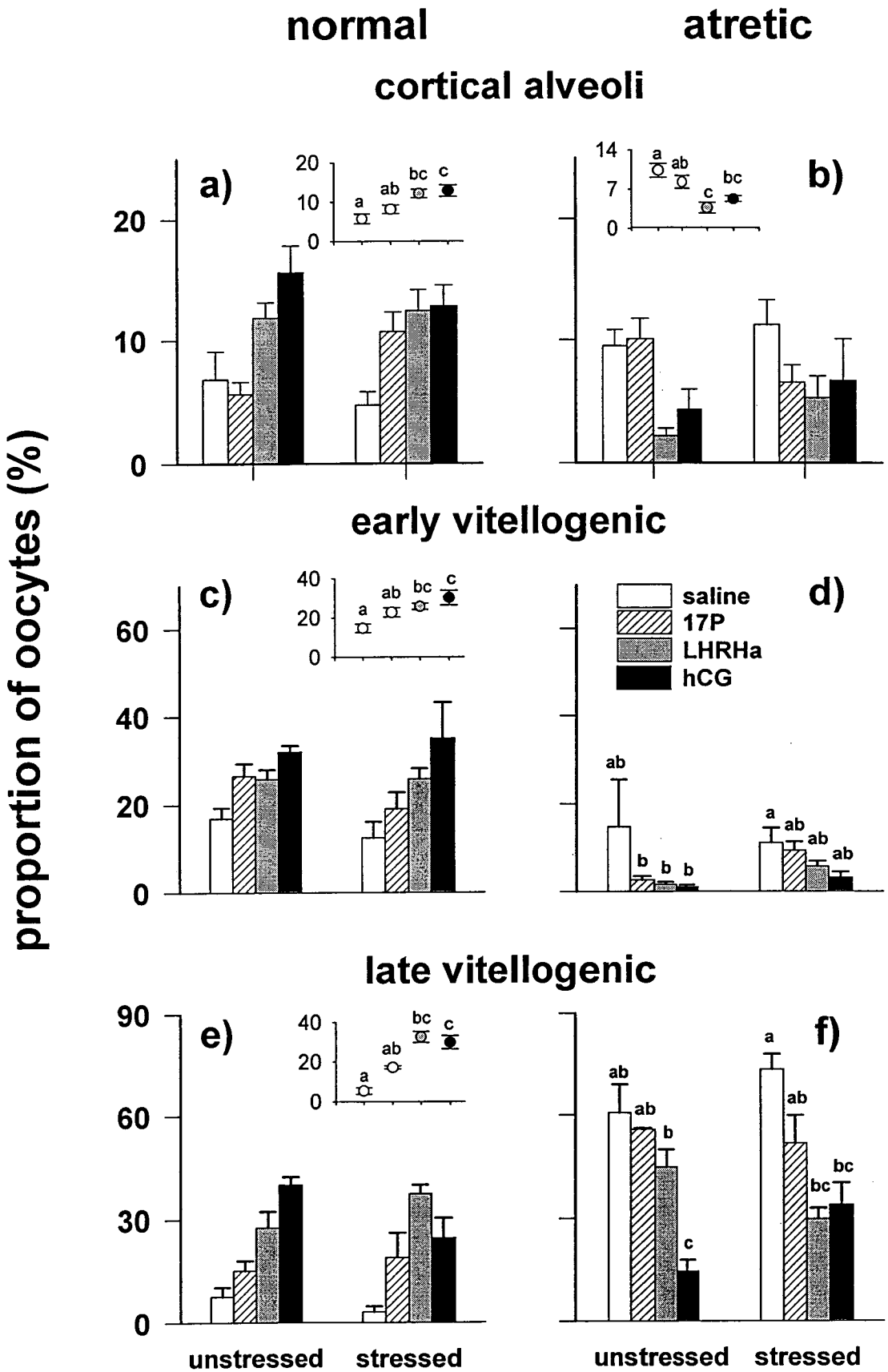

normal
atretic
cortical alveoli
early vitellogenic
late vitellogenic
proportion of oocytes (%)
a)
b)
c)
d)
e)
f)
saline
17P
LHRHa
hCG
unstressed
stressed
unstressed
stressed

Figure 4.5.

Mean plasma concentration of E_2 in a) unstressed and b) stressed Hatchery snapper at first capture (0*; stressed fish only), pre-injection (0) and 168 h pi with saline, 17P, LHRHa or hCG. Values are mean + SE (n=9-10). Insert graph shows significant difference between unstressed and stressed groups at 0 h pc and 0 h pi, indicated by #. There was no significant difference between stressed and unstressed groups at 168 h pc, therefore hormone treatment means were compared within stressed and unstressed groups. * indicates that hCG-treated unstressed group is significantly different to other unstressed groups at 168 h pi. $P < 0.04$.

Mean plasma concentration of T in c) unstressed and d) stressed Hatchery snapper at first capture (0*; stressed fish only), pre-injection (0) and 168 h pi with saline, 17P, LHRHa or hCG. Values are mean + SE (n=9-10). Insert graph shows significant difference between unstressed and stressed groups at 0 h pc, 0 h pi and 168 h pi, indicated by *. There was a significant hormone treatment and interaction effect, therefore treatment means were compared to each other within each time. # indicates that hCG-treated unstressed group is significantly different to all other groups at 168 h pi. $P < 0.05$.

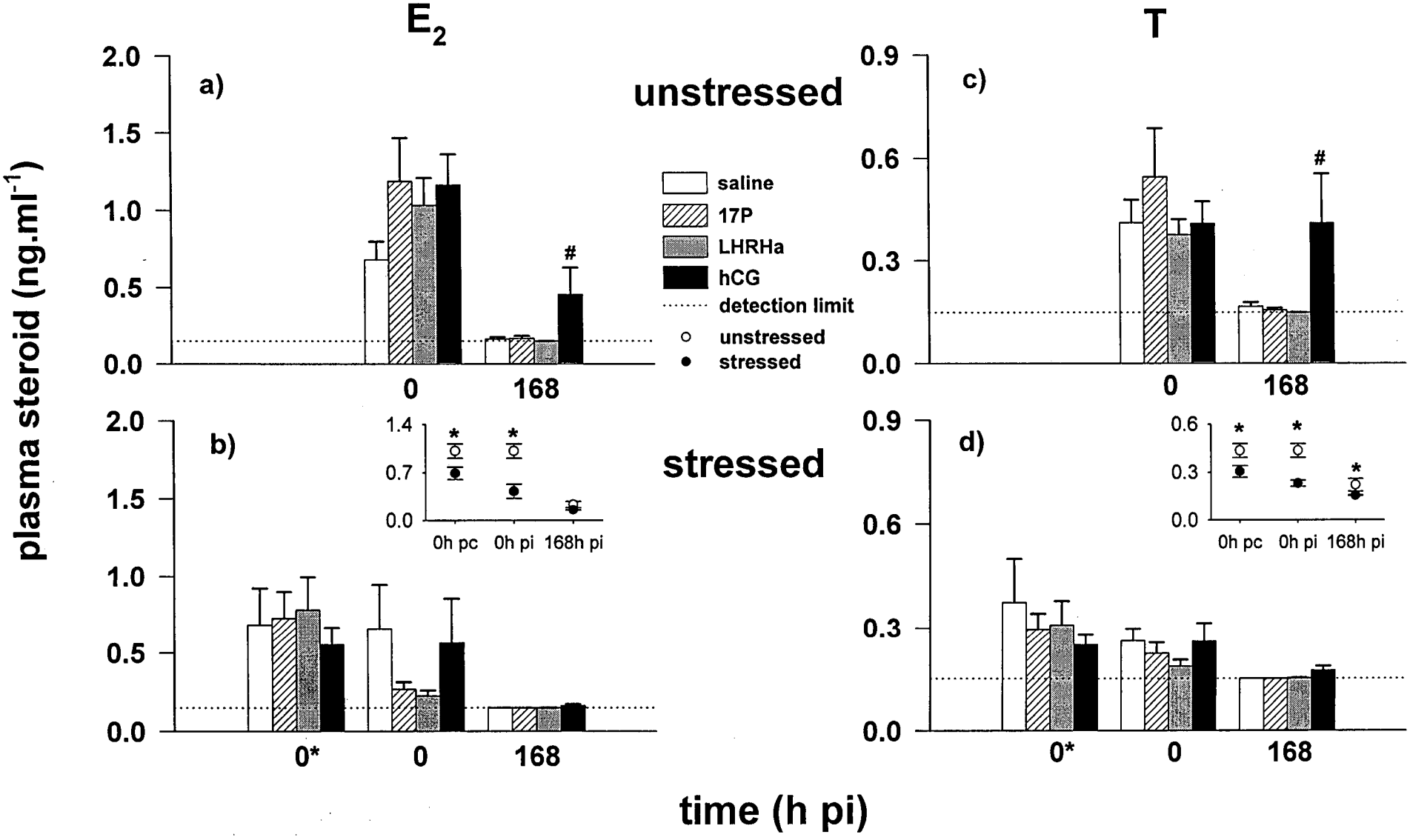
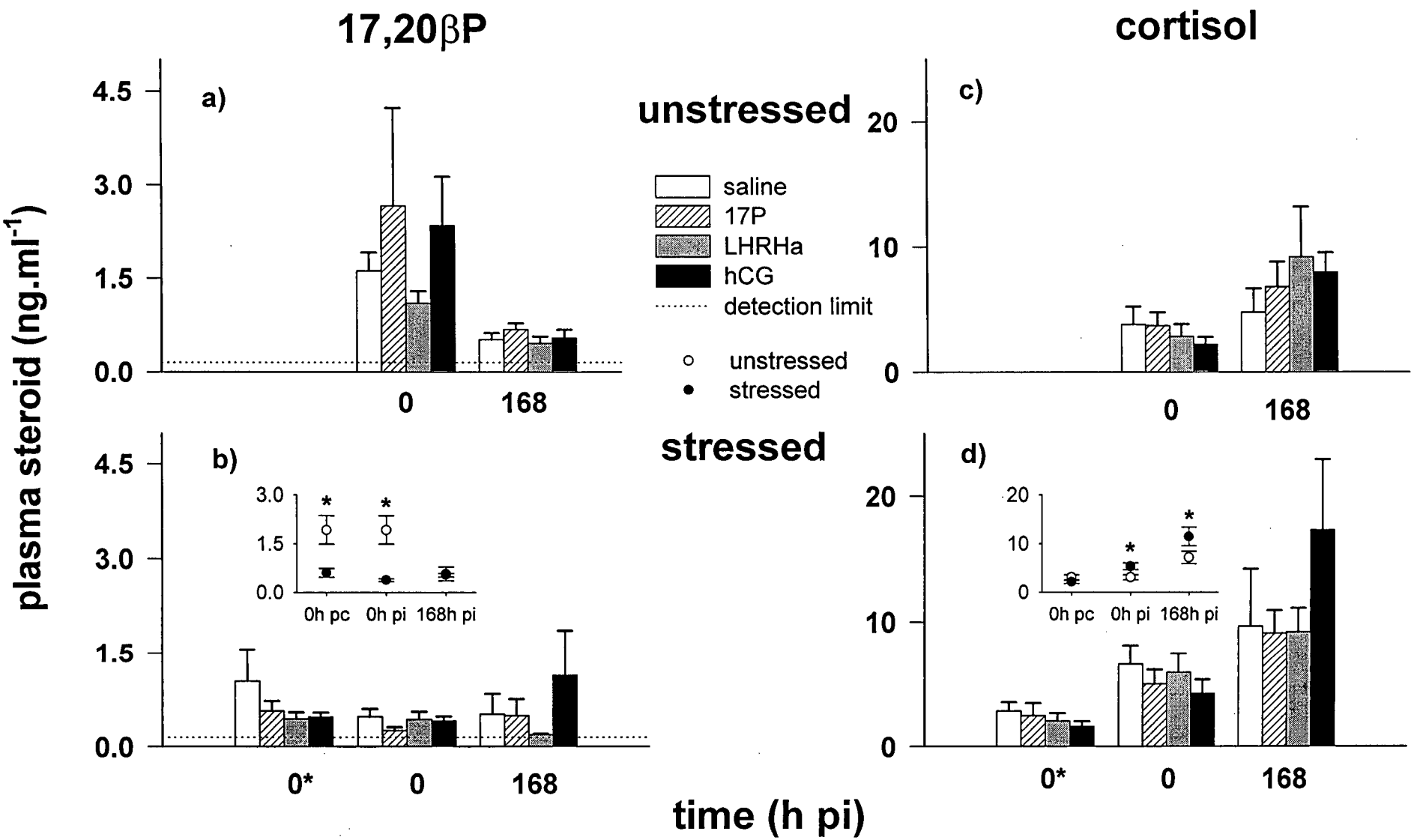

E2
T
unstressed
stressed
a)
b)
c)
d)
plasma steroid (ng.ml-1)
time (h pi)
saline
17P
LHRHa
hCG
detection limit
unstressed
stressed
0h pc
0h pi
168h pi
0*
0
168
#
*

Figure 4.6. Mean plasma concentration of 17,20βP in a) unstressed and b) stressed Hatchery snapper and cortisol in c) unstressed and d) stressed Hatchery snapper at first capture (0*; stressed fish only), pre-injection (0) and 168 h pi with saline, 17P, LHRHa or hCG. Values are mean + SE (n=9-10). Significant differences between unstressed and stressed groups at each sampling time indicated by * on insert graph.


17,20βP
cortisol
a)
b)
c)
d)
unstressed
stressed
saline
17P
LHRHa
hCG
detection limit
unstressed
stressed
plasma steroid (ng.ml-1)
time (h pi)
0h pc
0h pi
168h pi
0*
0
168

(Fig. 4.5). There was no hormone or stress by hormone interaction effect for E_2 or T at first capture or at 0 h pi. By 168 h pi mean plasma E_2 and T had decreased to be virtually non-detectable in all groups of fish, except unstressed hCG-treated fish. Both stress status and hormone treatment had a significant effect on plasma levels of E_2 ($P<0.05$) and T ($P<0.05$) at 168 h pi. There was also a significant interaction effect for T ($P<0.05$). Tukey-Kramer means comparison showed that plasma E_2 and T levels in unstressed hCG-treated fish were significantly higher than other unstressed groups at 168 h pi for E_2, and than all other groups at 168 h pi for T.

Mean plasma levels of 17,20βP were higher in unstressed than stressed fish at first capture ($P<0.05$) and at 0 h pi ($P<0.05$) (Fig. 4.6 a,b). There were no significant hormone or stress effects at first capture or 0 h pi. By 168h pi, plasma 17,20βP in unstressed fish had decreased, while levels in stressed fish remained similar to levels at 0 h pi. At 168 h pi there were no significant hormone, stress or interaction effects. At first capture, unstressed fish had similar levels of cortisol to stressed fish, with all means being <7 $ng.ml^{-1}$, but by 0 h pi, cortisol levels were higher in stressed than unstressed fish ($P<0.05$) (Fig. 4.6 c,d). There was no significant hormone or interaction effect at first capture or 0 h pi. By 168 h pi, cortisol had increased in both stressed and unstressed fish, but plasma cortisol levels were significantly higher in the stressed than the unstressed group ($P<0.05$).

Wild Fish

Sample sizes of wild fish were low and varied in this experiment. This was due to difficulties in catching snapper, caused in part by fluctuating water temperatures resulting in the capture of low numbers of reproductively mature females. Fishing occurred on approximately 20 days, with an average fishing time of 6 h. In all, approximately 160 fish with a FL of greater than 240 mm were caught. Of these 19 % were immature males, 24 % were mature males, 43 % were immature females, and 13 % were vitellogenic females. This represents a catch rate of 1 vitellogenic female per 5 to 6 fishing hours. The aberrant nature of snapper spawning dynamics that year is indicated in the catch on the 16th October, 1995 (i.e. the middle of the usual spawning season): 18 snapper (FL > 240 mm) were caught. Six of these were more than 350 mm in FL, yet there were only 2 mature males, and no vitellogenic females. Surface water temperature on that day was 19 °C. Although the usual peak spawning season is from September to November, in the 1995 season, commercial fishermen reported the presence of large numbers of females with hydrated oocytes in January 1996. Unfortunately it was not possible to sample in this period. In addition to low catch rates, some fish died due to damage sustained while in the traps, compounded with handling damage during successive strippings. This was particularly a problem with fish that produced large volumes of eggs. Twenty one fish were treated with hormones. Of these 2 were euthanased at 48 h pc; a further 3 were euthanased at 72 h pc. Therefore only 16 vitellogenic wild-caught fish survived the duration of the experiment.

Effect of hormone treatments on ovulation

The majority of ovulations came from Trap-Caught fish, with 6 out of 11 fish ovulating, compared to 2 out of 10 Line-Caught fish (Table 4.4). Treatment with hCG yielded the most ovulations with 4 out of 7 fish ovulating. Of the Line-Caught fish, only 2 hCG-treated fish ovulated (at 168 h pi); whereas in Trap-Caught fish, 2 LHRHa-, 2 saline- and 2 hCG-treated fish ovulated. There were no repeat ovulations from Line-Caught fish, but 3 Trap-Caught fish (one each from saline, LHRHa and hCG treatments) ovulated more than once (Table 4.5). Two hCG-treated fish were euthanased at 48 h pi. These fish both produced large volumes of eggs (80 and 178 ml) at 48 h pi and were deemed unlikely to survive to completion of the experiment. Had these fish survived it is possible that the number of repeat ovulations from hCG-treated fish would have been higher.

Trap-caught fish treated with hCG produced the greatest mean ovulation volume at 48 h pi (129 ml), followed by 24 h pi (84 ml) (Fig. 4.7). The next greatest volume came from Trap-Caught fish treated with LHRHa and stripped at 48 h pi. The volume of eggs produced declined at each successive stripping. Saline-treated Trap-Caught fish gave the smallest ovulations with volumes of only 20 to 30 ml of eggs. These ovulations occurred at 24 and 48 h pi. The only ovulations (mean volume of approximately 20 ml) from Line-Caught fish occurred at 168 h pi in 2 hCG-treated fish. A maximum of 5 ml of released eggs was collected from a tank on one day, indicating that egg volumes are slightly underestimated, but it was not possible to determine if a particular treatment contributed more than any other to the number of released eggs in the tank.

Only one ovulation (hCG-treated Trap-Caught fish at 24 h pi) was Grade 1 (Table 4.6). Nine of the 11 ovulations were Grade 3, while 1 (LHRHa, Trap-Caught) was Grade 2. No fertilisation occurred in ovulations from Line-Caught fish, while fertilisation was highly variable in ovulations Trap-Caught fish. Fertilisation occurred in only 3 ovulations from Trap-Caught fish. One of these (saline-treated fish at 48 h pi) was only 1 %. The other two (both LHRHa-treated fish at 72 h pi) were 72 and 25 % respectively. The volume of fertilised eggs produced from each of these 3 ovulations was <1, 22 and 12 ml respectively.

Mean GSI was 1.97 ± 0.44 % for Trap-Caught fish and 1.18 ± 0.23 % for Line-Caught fish; mean HSI for Trap-Caught fish was 1.48 ± 0.08 % and for Line-Caught fish was 1.14 ± 0.10 %; and mean oocyte diameter for Trap-Caught fish was 399 ± 43 µm and for Line-Caught fish 280 ± 32 µm. Mean FL was 427 ± 19.23 mm and 330 ± 22.72 mm for trap and Line-Caught fish respectively; mean whole body weight for Trap-Caught fish was 1759.44 ± 245.64 g and for Line-Caught fish was 692 ± 91.35 g. Of the fish used in this experiment, the method for allocating fish to treatments resulted in the heaviest 2 fish being both treated with hCG. These also had the 2 highest GSI and HSI values. This bias in treatment allocation means that the data must be interpreted with caution (Appendix B).

Table 4.4: Number of Line- and Trap-Caught fish ovulating (numerator) over the number of experimental fish (denominator) during 1 week following treatment with saline, LHRHa or hCG.

	Line	Trap	total
Saline	0/4	2/3	2/7
LHRHa	0/3	2/4	2/7
hCG	2/3	2/4	4/7
Total	2/10	6/11	8/21

Table 4.5: The number of successive ovulations per fish from Line- and Trap-Caught snapper during the week following treatment with saline, LHRHa, or hCG.

		No. of ovulations per fish			
		0	**1**	**2**	**3**
Line	**Saline**	4	0	0	0
	LHRHa	3	0	0	0
	hCG	1	2	0	0
Trap	**Saline**	1	1	1	0
	LHRHa	2	1	0	1
	hCG	2	1	1	0

Figure 4.7. Mean volume of eggs stripped from a) Line- and b) Trap- Caught snapper at 24, 48, 72 and 168 h after injection with saline, LHRHa or hCG at first capture. Superscripts show numbers of ovulating fish. No subscript = 0. Values are means + SE.

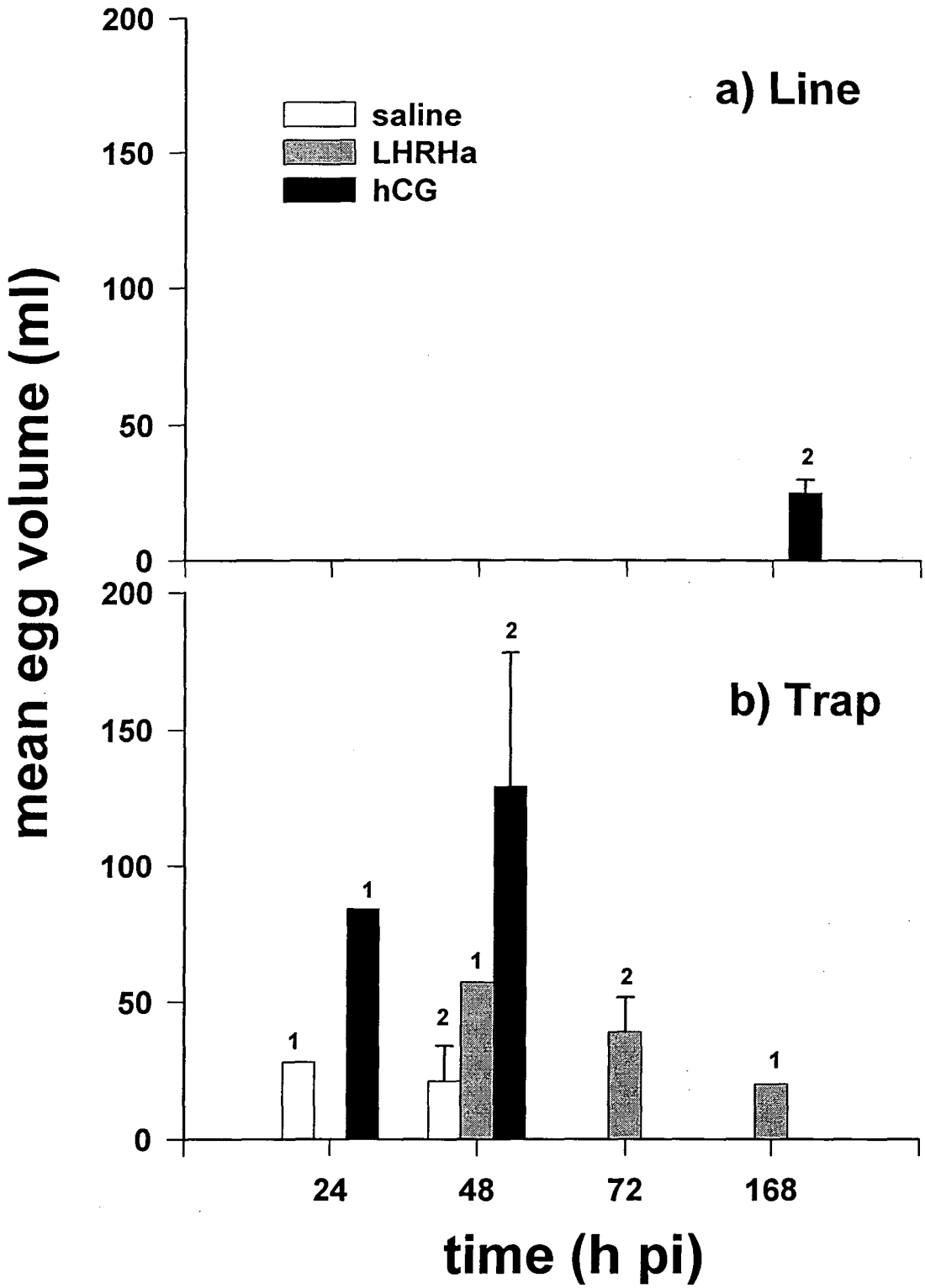

a) Line
saline
LHRHa
hCG
200
150
100
50
0
2
b) Trap
200
150
100
50
0
1
1
2
1
2
2
1
24
48
72
168
mean egg volume (ml)
time (h pi)

Table 4.6: Number of ovulations with a given morphology, from Line- and Trap-Caught snapper injected with saline, 17P, LHRHa or hCG.

		Egg Morphology		
		Grade 1	**Grade 2**	**Grade 3**
Line	**Saline**	0	0	0
	LHRHa	0	0	0
	hCG	0	0	2
Trap	**Saline**	0	0	3
	LHRHa	0	1	2
	hCG	1	0	2

Proportion (%) of round, clear, buoyant eggs present (n=100). Grade 1 = 80-100%; Grade 2 =20-80%; Grade 3=0-20%.

Figure 4.8. Mean proportion of a) normal and b) atretic cortical alveoli, c) normal and d) atretic early vitellogenic and e) normal and f) atretic late vitellogenic stage oocytes in histological cross-sections of ovaries from Line- and Trap-Caught snapper 1 week after injection with saline, LHRHa or hCG. Counts for cortical alveoli stage oocytes are a percentage of total previtellogenic oocytes, while early and late vitellogenic oocytes are a percentage of total vitellogenic oocytes (mean + SE; n = 9-10). Numbers indicate n values

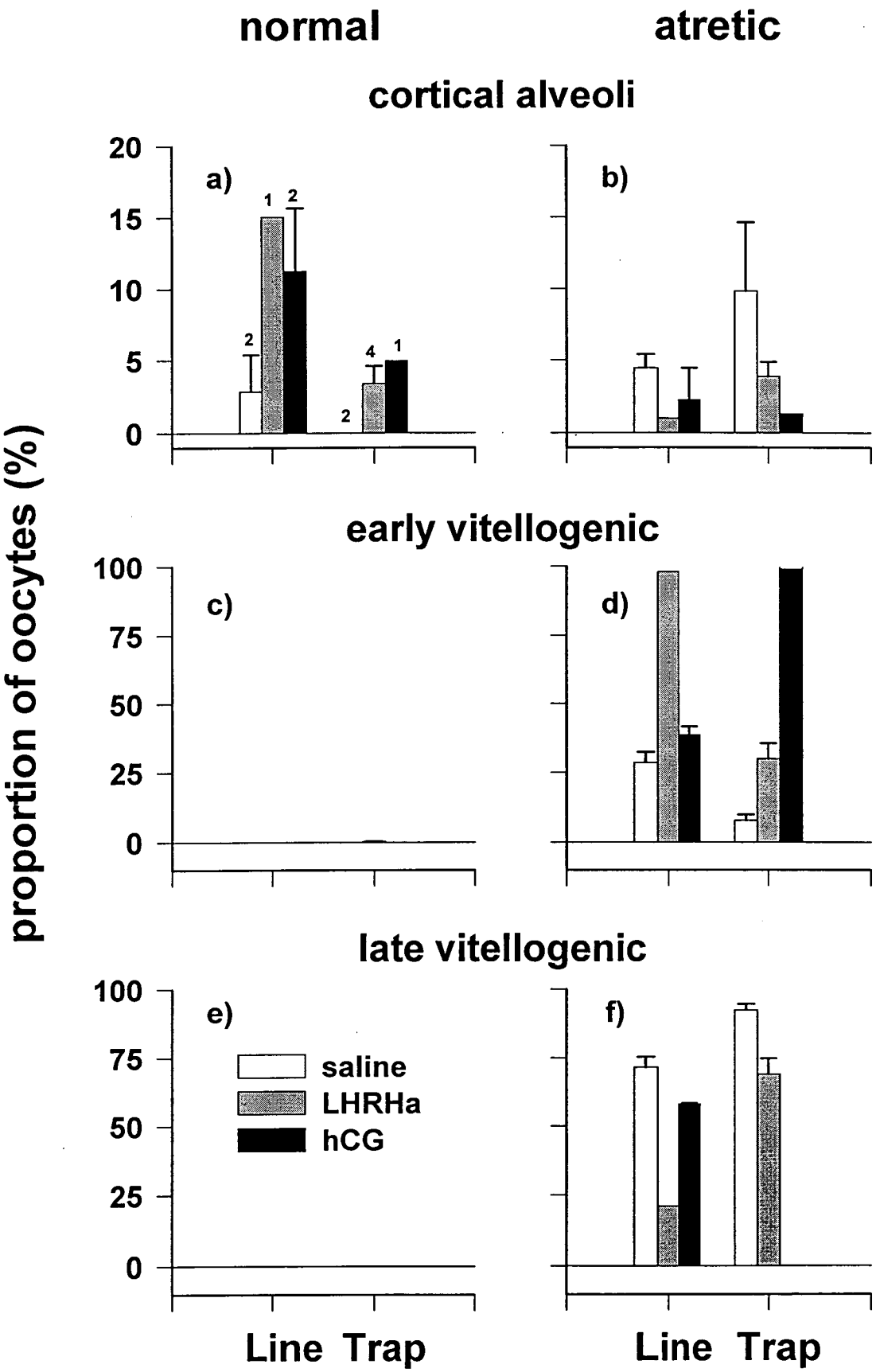

normal
atretic
cortical alveoli
early vitellogenic
late vitellogenic
proportion of oocytes (%)
a)
b)
c)
d)
e)
f)
20
15
10
5
0
100
75
50
25
0
1
2
2
4
1
2
saline
LHRHa
hCG
Line Trap
Line Trap

Figure 4.9. Mean plasma concentration of E_2 in a) Line- and b) Trap-Caught snapper and T in c) Line- and d) Trap-Caught snapper at first capture (0 h pi) and at 168 h pi with saline, LHRHa or hCG. Values are mean + SE. Numbers indicate n values.

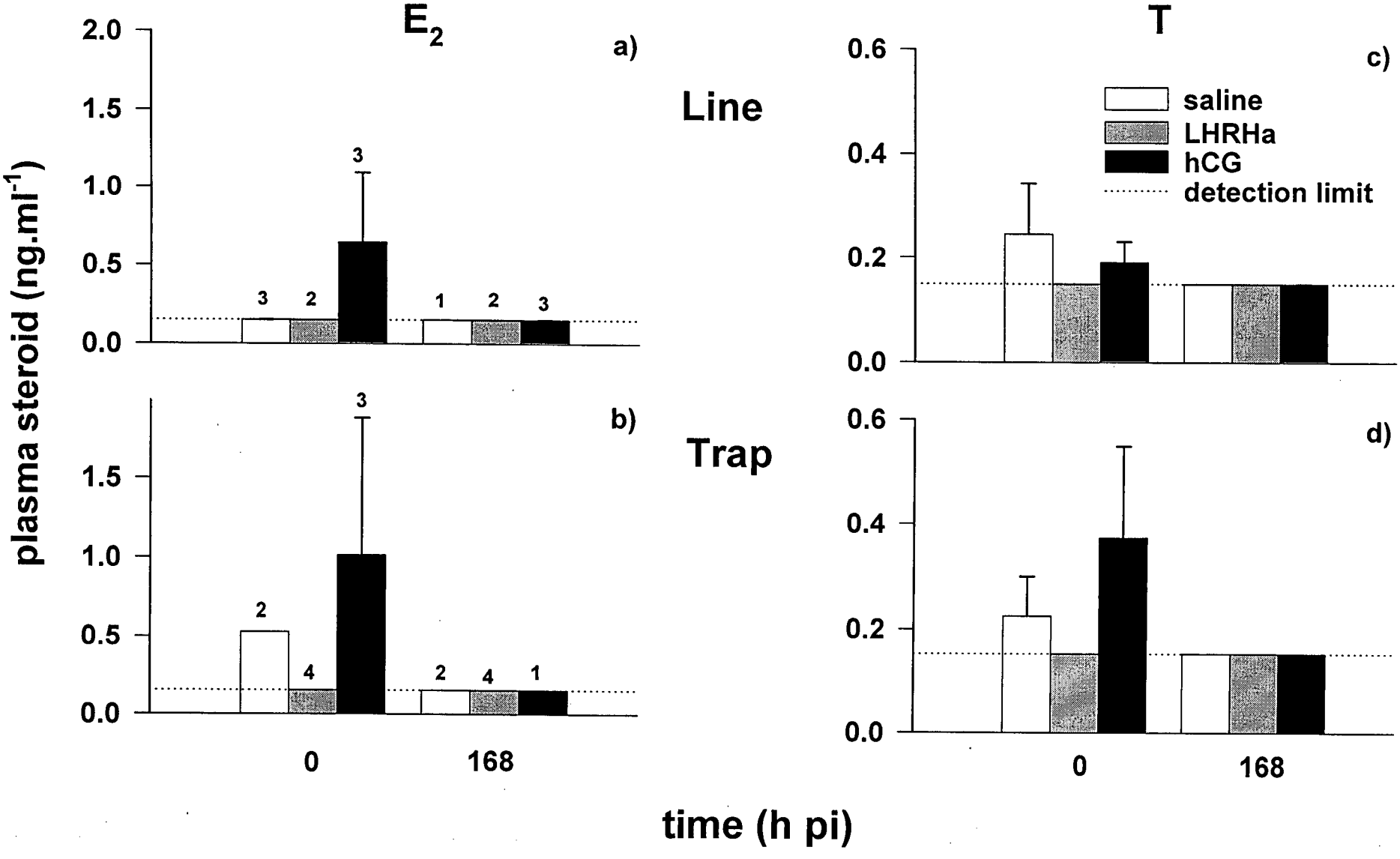
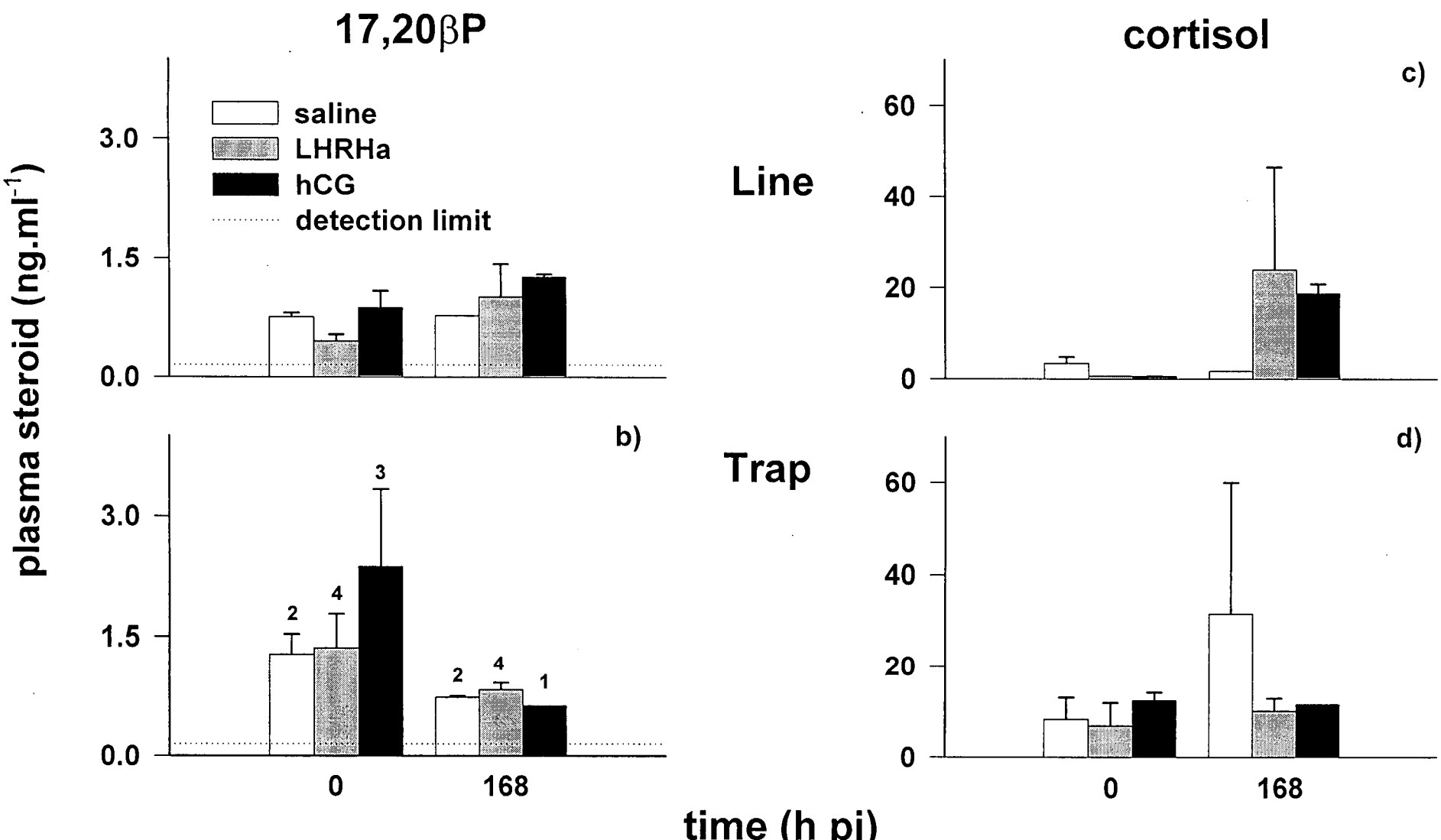

E2
T
a)
b)
c)
d)
Line
Trap
plasma steroid (ng.ml-1)
2.0
1.5
1.0
0.5
0.0
0.6
0.4
0.2
0.0
3
2
1
2
3
4
4
2
4
1
saline
LHRHa
hCG
detection limit
0
168
time (h pi)

Figure 4.10. Mean plasma concentration of 17,20βP in a) Line- and b) Trap-Caught snapper and cortisol in c) Line- and d) Trap-Caught snapper at first capture (0 h pi) and at 168 h pi with saline, LHRHa or hCG. Values are mean + SE. Numbers indicate n values.


17,20βP
cortisol
saline
LHRHa
hCG
detection limit
plasma steroid (ng.ml-1)
Line
Trap
b)
c)
d)
3.0
1.5
0.0
60
40
20
0
2
4
3
2
4
1
0
168
time (h pi)

Ovarian Histology

Due to the small sample sizes, ovarian histology data were compared qualitatively. Primary oocytes comprised at least 84 % of all previtellogenic oocytes. For both Line and Trap-Caught groups, LHRHa- and hCG-treated fish had more normal and fewer atretic cortical alveoli oocytes than saline-treated fish (Fig. 4.8 a,b). Virtually all vitellogenic oocytes were atretic in both Line- and Trap-Caught fish and there were fewer atretic early vitellogenic oocytes in saline-treated fish than hCG-treated fish (Fig. 4.8 c,d). The converse was true for late vitellogenic oocytes, with saline-treated fish having more atretic late vitellogenic oocytes than both LHRHa and hCG-treated fish (Fig. 4.8 e,f). Very low numbers (< 5 % in total) of more mature follicles were observed and there was no evidence of atresia in these.

Plasma Steroids

At 0h pi, mean plasma E_2 and T were low in both line and Trap-Caught fish with maximum levels of 1 and 0.4 $ng.ml^{-1}$ respectively, and any detectable plasma E_2 at first capture decreased to non-detectable by 168 h pi (Fig. 4.9). The 2 hCG-treated Trap-Caught fish that were euthanased at 48 h pi had E_2 levels of 0.15 and 0.85 $ng.ml^{-1}$ and T levels of 0.15 and 0.22 $ng.ml^{-1}$ at 48 h pi. Trap-caught fish appeared to have higher levels of 17,20βP than Line-Caught fish at 0 h pi (Fig. 4.10 a,b). At 168 h pi, mean 17,20βP levels in Line-Caught fish were similar to 0 h pi levels. In Trap-Caught fish, mean 17,20βP levels decreased in all treatments to at least half their original level, while in hCG treatments levels remained similar. Mean cortisol levels were very low in Line-Caught fish with all mean values being less than 3.5 $ng.ml^{-1}$ (Fig. 4.10 c,d). Mean plasma cortisol in Line-Caught fish increased in LHRHa and hCG treatments from 18.5 to 24 $ng.ml^{-1}$ by 168 h pi, but levels in saline treatments remained similar to 0 h pi levels. At 0 h pi, Trap-Caught fish had higher mean cortisol levels than Line-Caught fish (6 - 12 $ng.ml^{-1}$). Mean plasma cortisol in Trap-Caught fish rose to between 10 and 31 $ng.ml^{-1}$ by 168 h pi.

4.4 Discussion

Exposure of snapper to capture and handling stress in the present study resulted in reduced capacity of fish to respond to exogenous hormone treatment, in terms of decreased egg volumes and egg quality (egg morphology and fertilisation). However, pre-treatment stress did not affect the number of fish responding to the various hormone treatments, nor the number of ovulations per fish. Pankhurst and Carragher (1992) reported that 64% of wild-caught female New Zealand snapper treated with hCG 1 day after capture, ovulated between 6 and 24 h pi. Similarly 44 to 66 % of wild-caught New Zealand snapper injected with LHRHa

ovulated (Pankhurst and Pankhurst, 1989) and 61 % of trapped Australian snapper ovulated following hCG treatment in the present study. The number of stressed Hatchery fish (i.e. those injected at 24 h pc) ovulating subsequent to treatment with hCG or LHRHa in the present study was similarly 70% by 24h pi, but increased to 90 % by 168 h, and 43 % of hCG- or LHRHa-treated trap- and Line-Caught fish in the present study ovulated. Therefore the proportion of snapper responding to hormone treatment within 24 h pi is generally consistent among studies.

Despite the fact that snapper are serial spawners with a daily spawning rhythm (Scott *et al*, 1993), Carragher and Pankhurst (1991) found that the percentage of untreated wild-caught New Zealand snapper ovulating on 2 successive days after capture was approximately 60 %, and those ovulating on three successive days was 25 %. In contrast, in the present study, no control fish ovulated more than once. The reason for this difference is not certain, however, it is unlikely to be merely a reflection of differences between Australian and New Zealand snapper, since Pankhurst and Carragher (1992) reported anecdotally that wild-caught New Zealand snapper, even when treated with hCG spawned only once. It is more likely that the differences are due to differences in maturity at the time of capture (since the untreated fish were clearly ovulating at capture). In the present study, however, over half the hatchery fish treated with LHRHa and hCG ovulated more than once. Similarly, in a study of another sparid, gilthead seabream (*Sparus aurata),* 43 % of the fish treated with hCG ovulated on successive days (Zohar and Gordin, 1979). In contrast, only 14 % of wild-caught hCG- and LHRHa-treated fish in the present study ovulated more then once. The higher number of repeat ovulating hatchery fish, relative to wild-caught fish may be due to differences between hatchery and wild fish (see Chapter 3, this volume). However, Battaglene (1995) found that trapped or long-lined wild-caught snapper could be successfully stripped on up to 5 consecutive days following hCG treatment at an identical dose to the present study. It is possible that the differences are due to differences in husbandry practices. For example, the New Zealand snapper were treated on the day following capture (Pankhurst and Carragher, 1992), while the Australian snapper (Battaglene, 1995) were treated on the day of capture. However, in the present study, a delay in treatment of 24 h did not markedly affect the numbers of repeat ovulators, making this scenario unlikely. It is therefore most likely that in the present study, the difference is again due to differences in maturity between the wild and hatchery fish. This is supported by the fact that in general GSI and oocyte diameters of the wild-caught fish were lower than for hatchery fish. Furthermore, only 7 out of 21 wild-caught fish had GSIs of greater than 1.9 % (the smallest for repeat ovulating fish). Of these, 3 fish were repeat ovulators, and one was euthanased following a huge ovulation (and presumably would have ovulated on successive days if it had survived). Similarly, 6 fish had oocyte diameters of 370 :m (smallest for a repeat ovulator), and 4 of these were the same fish just mentioned.

Gilthead seabream show a much greater response to exogenous hormones, than both New Zealand or Australian snapper, with most fish ovulating on between 10 and 15 successive days, and some fish ovulating on more than 30 successive days in response to injection with [D-Ala6-Pro9-NET]-LHRH (Zohar, 1988). It appears that gilthead seabream are more amenable to hormonal induction of ovulation in captivity, which may be a reflection of higher stress tolerance. Interestingly, Zohar (1988) found that gilthead seabream had a daily rhythm of gonadal responsiveness to GnRH treatment, with all fish treated at 2200 h spawning daily for an average of 30 days each, compared to 7 days each when fish were treated at 1400 h. In the present study, all fish were treated between 0600 and 1200 h, in order to minimise any variation due to a possible diurnal rhythm of responsiveness. The effect of treatment at different times clearly warrants further investigation.

Pre-treatment stress dramatically reduced the volume of eggs produced in response to exogenous hormones in hatchery fish, in the present study. A similar effect has been found in other species, in response to exposure to a range of stressors. Starving or underfed salmonids (Scott, 1962; Bagenal, 1969) and roach *(Rutalis rutalis) (*Kuznetsov and Khalitou 1978) produced fewer eggs compared to controls, and chronically acid-stressed brook trout, (*Salvelinus fontinalis)* produced smaller egg volumes than control fish (Tam and Payson, 1986; Mount *et al*, 1988). Since the volume of eggs produced by females is closely correlated to female body weight, both these authors concluded that the decrease in egg volume was a consequence of smaller body weight due to reduced growth. Both these studies were conducted over several months, thereby allowing growth rate variations. The present study, however, was conducted over only 1 week, and there was no significant difference in FL or whole body weight between stressed and unstressed fish. Therefore the observed decrease in egg volumes is unlikely to be the result of impaired growth, but rather differential ability of stressed and unstressed fish to respond to hormone treatments. Similarly De Montalembert *et al* (1978) found that the ovulation rate (ratio of ovulated oocyte weight to initial ovary weight) declined from 96 % in Northern pike (*Esox lucius*) treated with exogenous hormones at first capture, to 40 % in those treated three days later. The volume of eggs produced from Trap-Caught fish in the present-study was much higher than from hatchery and Line-Caught fish, with the maximum volume for both these groups (25 ml) being the minimum volume in Trap-Caught fish. Indeed, the maximum mean volume from Trap-Caught fish, treated with hCG was 125 ml. This, however, is likely to be due to the fact that the mean whole body weight of Trap-Caught fish is more than twice that of hatchery fish and FL was approximately 100 mm greater in trap than hatchery fish. The relationship between body size and batch fecundity has not been measured in Australian snapper, but Scott (1991) demonstrated a log-log relationship between FL and batch fecundity in wild-caught New Zealand snapper.

Egg quality, as determined by morphology and fertilisation, was greatly reduced in stressed, compared with unstressed hatchery fish. Since percentage fertilisation is considered to be a predictor of subsequent egg and fry performance (Springate *et al*, 1984; Campbell *et al*, 1994), we can assume that larval survival would also be similarly affected. Survival of eggs to eyed stage from female brown (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) exposed to 2 weeks of confinement stress, and fertilised with milt from similarly stressed males was 15 times less than that for unstressed females and males (Campbell *et al*, 1994). The authors, however, were uncertain whether this mortality was due to failure at fertilisation or death at an early stage of embryonic development. In contrast, exposure of white sucker (*Catostomus commersoni*) to bleached kraft mill effluent disrupted plasma steroids but had no effect on fertilisation, larval development or survival rate (Munkittrick *et al*, 1991) and De Montalembert *et al* (1978) found that eggs from wild-caught female Northern pike held in captivity for 3 days prior to hormone treatment did not show a decrease in fertilisation, despite a reduction in ovulation volume. Similarly, Wilson *et al* (1995) reported no effect of chasing, capture and confinement on fertilisation and hatching in naturally spawning Atlantic cod (*Gadus morhua)*, however, stress did result in an increase in the number of abnormal embryos. Kjesbu (1989), however, found that an unspecified stress imposed on Atlantic cod resulted in both low fertilisation and an increase in the number of abnormal embryos. These differences in the effect of stress on egg quality and fertilisation and subsequent larval survival may be due to differences in the type of stressor. Equally they could be due to differences in the timing of application of the stressor, since Contreras-Sanchez *et al* (1995), showed that the effect of stress on egg quality and progeny of rainbow trout varies depending on when the female is stressed during maturation. Clearly, however, stress of various kinds can affect fertilisation and subsequent larval survival in some fish, including Australian snapper.

The effects of stress on egg quality in the present study may be due to stress impacting directly on egg quality, as reported by Campbell *et al* (1994) for chronically stressed rainbow trout (see above). Alternatively, the effect of stress on egg quality may result from a change in the time to ovulation, following hormone treatment. All externally fertilising species have a window of post-ovulatory viability after which there is a time-dependent decline in egg viability (reviewed in Hobby and Pankhurst, 1997b). In snapper, the period of peak fertility only extends for about 4 h after ovulation (Scott *et al*, 1993; Hobby and Pankhurst, 1997b). Therefore, even a small change in the time to ovulation may result in eggs being stripped outside of the window of post-ovulatory viability. It is not known exactly when the fish in the present study ovulated, with the result that differential egg quality in stressed versus unstressed fish could have arisen from undetected differences in the time of ovulation. It is also possible that stress could change the length of the window of post-ovulatory viability.

The relative importance of direct stress-induced effects on egg quality and indirect effects resulting from possible shifts in the timing of ovulation, or the window of post-ovulatory viability is not known. Despite the large volumes of eggs produced by Trap-Caught fish, egg morphology and subsequent fertilisation of ovulations was extremely poor, with only 1 out 8 ovulations being Grade 1, and reasonable fertilisation occurring in only 1 ovulation. In the light of the effect of pre-treatment stress on egg quality in hatchery fish in the present study, it is possible that this poor egg quality and fertilisation in Trap-Caught fish is stress-induced as a consequence of the time spent in the trap prior to treatment. However, it also possible that the poor egg quality and fertilisation may be a consequence of the smaller oocyte diameter at injection of the wild fish relative to their hatchery-reared counterparts (approx. 400 μm c.f. 550 μm).

Only the effect of stress on the maternal component (i.e. egg quality) was considered in the present study. Although reproductive failure is often assessed in terms of female competence, it should also be remembered that stress also affects male reproductive processes (Pickering *et al*, 1987). Campbell *et al* (1992) found that rainbow trout repeatedly exposed to 5 min emersion stress at random intervals during the 9 months of reproductive development had reduced sperm counts. In Chapter 3 (this volume), capture and handling of snapper resulted in reduced plasma gonadal steroid levels in males, and an increase in the proportion of spermatocytes in the testes. Thus, the possible confounding effect of stress in males cannot be ignored, since in the present study, males, like females, were subjected to capture, handling, and confinement (although not bled or serially stripped).

The measure of ovulation quality of most interest to aquaculturalists is that of the volume of fertilised eggs produced, as this gives the only true indication of the relative usefulness of an ovulation. In addition, unfertilised dead eggs can be reservoirs for disease. Since there are approximately 1.6×10^3 snapper eggs per ml (S.C. Battaglene, ICLARM, Coastal Aquaculture Centre, pers com), the maximum mean volume of fertilised eggs produced by hatchery fish in this study (25 ml), translates to 40 000 fertilised eggs. In comparison, the two most successful ovulations from Trap-Caught fish produced 35 200 and 19 200 fertilised eggs. Therefore, despite the larger ovulatory volumes of wild-caught fish, practical fertilisation success was similar, if not lower for Trap-Caught fish compared with hatchery fish. In the present study, the Trap-Caught fish which yielded these 2 ovulations, weighed 4329 and 1914 g. In contrast, the hatchery fish were less than 1000 g (i.e. less than half the size of the wild fish). Since ovulation volume is correlated to the size of the female (Scott, 1991), given the same fertility, the hatchery fish would be expected to produce increasingly greater volumes of fertile eggs relative to their wild counterparts in future years, as their body size continues to increase.

The capture of vitellogenic wild fish proved extremely difficult in the present study, with 5-6 hrs of fishing required to catch 1 vitellogenic female. Battaglene (1995) reported that "erratic and relatively small numbers of mature wild females" were caught when fishing in the same area as the present study, in 1993. He found that of the 85 fish caught in that season, 24 % were vitellogenic or ovulated females. In the present study, the percentage of vitellogenic females caught was almost half that caught in 1993. Availability of vitellogenic females depends on water temperature which fluctuates with the East Australian Current (Cresswell, 1994). In addition, unpredictable sea conditions, and strong currents often made it difficult to set, pull or find traps and even to fish by line. The difficulty in obtaining vitellogenic females from the wild, combined with the fact that first generation hatchery snapper in the present study, injected at first capture, yielded equivalent if not better volumes of fertilised eggs, and the potential for improvement with increasing size indicates the necessity to domesticate snapper broodstock, as has been done for red seabream in Japan (Foscarini, 1988; Fukusho *et al*, 1986). Further generations of domestication are also likely to improve the yields. In Japan, hormonal induction of ovulation of domesticated broodstock still results in a poorer ovulatory response compared with fish which spawn unaided in tanks (Foscarini, 1988). Therefore the long-term solution to the problem of egg quality and supply in Australian snapper is likely to be domestication of broodstock, which spawn spontaneously in captivity. To this end, recent success has been achieved in environmental induction of spawning of first generation hatchery-produced Australian snapper (G. Allan and S. Fielder, NSW Fisheries, Port Stephens Research Centre, pers com).

Capture and handling of fish prior to treatment in the present study, affected plasma steroid levels in hatchery fish in a similar fashion to those for 3 year-old hatchery snapper described in Chapter 3 (this volume). By the time of injection, plasma cortisol levels had significantly increased relative to unstressed fish and plasma levels of E_2, T and 17,20βP had decreased. This indicates that the "stressed" fish in the present study, were indeed suffering stress-induced impairment of at least some reproductive processes at the time of treatment. Plasma E_2, T and 17,20βP levels in stressed fish were also lower than in unstressed fish at first capture. Due to the extreme sensitivity of plasma E_2 and T levels to cage-side disturbance demonstrated in Chapter 3, it is likely that this reflects the sampling strategy, as unstressed fish were caught prior to stressed fish, rather than differences in initial reproductive condition. Plasma 17,20βP levels in 3-year-old stressed hatchery fish described in Chapter 3 (this volume), did not show the same sensitivity to cage-side disturbance as E_2 and T. Therefore it not known why levels of 17,20βP were lower in stressed than unstressed fish at first capture in this part of the study.

In unstressed hatchery fish, LHRHa and hCG treatments were approximately equal in terms of the number of ovulators, repeat ovulations and fertilisation, however, hCG treatment yielded greater egg volumes and volume of fertilised eggs. This indicates that, in snapper, direct administration of a GtH substitute is more effective than stimulating a natural GtH surge via administration of a GnRH analogue. In some species of teleosts, however, hCG treatment can cause an unusually large percentage of oocytes to hydrate and ovulate, resulting in swelling of the ovary and subsequent death (Prentice and Colura, 1984; Gwo *et al*, 1993). It appears that hCG may cause generalised hydration of all eggs, while LHRHa causes selective hydration of eggs at a specific developmental stage (Gwo *et al*, 1993). This phenomenon has led some workers to conclude that LHRHa is more reliable at inducing ovulation, despite similarities in fertilisation rates (Gwo *et al*, 1993), since LHRHa has not been found to over-stimulate the ovary (Prentice and Thomas, 1987). The present study provides only limited anecdotal evidence for a similar problem in Australian snapper. One hCG-treated hatchery fish had a blocked oviduct at 168h pi and a consequent highly distended and compacted abdomen. Furthermore, two hCG-treated Trap-Caught fish were euthanased following large ovulations, however, in this case it is not certain whether their moribund state was due to the volume of the ovulation or the extra handling required to strip all the eggs.

The reason for the difference in response to hCG and LHRHa in unstressed fish is likely be due to differences in the mode of action of the respective hormones and endogenous control factors. GtH secretion in goldfish is controlled by a neuro-hormonal system in which GnRH stimulates GtH secretion, while dopamine inhibits the action of GnRH and the subsequent release of GtH (reviewed in Peter and Yu, 1997). A role for dopamine in the control of GnRH has now been confirmed for a range of species including catfish (De Leeuw *et al*, 1985), and a variety of cyprinids (reviewed in Peter *et al*, 1988). The presence of dopamine inhibition in many teleosts is uncertain, however it appears that it is quite widespread (Zohar, 1988). Therefore, the efficiency of exogenous GnRHa treatment in many species may be improved through the removal of the inhibitory effect of dopamine, using dopamine antagonists such as pimozide or domperidone. In some species, such as the goldfish (Chang and Peter, 1983), GnRHa is impotent without co-treatment with a dopamine antagonist. Other species, however, such as Atlantic croaker (Thomas *et al*, 1995) and gilthead seabream (Zohar *et al*, 1987) showed a lack of enhancement of GnRHa effectiveness with the application of a dopamine antagonist, hence casting doubt on the role of dopamine in GnRH inhibition in these species. The role of dopamine in snapper has not been specifically addressed, however since the action of hCG would short-circuit dopamine control and given that in the present study, hCG gave a better response than LHRHa, it is possible that the action of GnRH in snapper is under dopaminergic control. Co-treatment with LHRHa and a dopamine antagonist remains to be explored in snapper.

The bioactivity of LHRHa and other GNRHas, is short lived, and that of the natural peptide is even shorter (Goren *et al*, 1987; Zohar *et al*, 1989). Zohar (1988) found that plasma GtH levels in female gilthead seabream following injection with GnRHa ([D-Ala^6 Pro^{9}NEt] LHRH ethylamide) had returned to baseline levels by 48 to 72 h pi. *In vitro* studies on gilthead seabream have shown that enzymatic cleavage rapidly terminates bioactivity of GnRH (Goren *et al*, 1987; Zohar *et al*, 1989). Therefore, the lower activity of LHRHa than hCG in the present study could have been due to rapid clearance following injection. This can be overcome by sustained administration of LHRHa by slow release pellets (Matsuyama *et al*, 1993), serial injections (Lam, 1982), osmotic pumps (Matsuyama *et al*, 1995) or in the diet (Thomas *et al*, 1995). All of these vehicles have all been used successfully to promote FOM, ovulation and spawning in various species, and in some cases to promote out of season spawning. Therefore, it is possible that administration of LHRHa in a slow-release pellet, or through another sustained release mechanism, may also improve the response to LHRHa in snapper, relative to hCG.

The relationship between the response to hCG and LHRHa treatments was different in stressed compared to unstressed fish. This is most evident in the egg volumes, egg morphology, fertilisation, volume of fertilised eggs and the incidence of atresia in late vitellogenic oocytes. With egg volumes and late vitellogenic atresia, the superiority of hCG over LHRHa observed in unstressed fish was not evident, and the responses to hCG and LHRHa were not significantly different. For fertilisation and the volume of fertilised eggs, LHRHa-treatment gave a better response than hCG treatment in stressed fish, however this was not tested statistically. Clearly, stress affected the ability of fish to respond to hCG treatment more than to LHRHa treatment. The reason for this is not clear.

In the present study, all females were identified as being vitellogenic (stage III or IV) prior to treatment by ovarian biopsy, and this was later confirmed by histological staging. Nevertheless there was large variation in the mean oocyte diameters among the different groups of fish. Carolsfeld *et al* (1988) found that the response of pacific herring *(Clupea harengus)* to GnRHa treatment was dependent on the stage of sexual maturity and that early vitellogenic fish (i.e. early stage III) did not respond to the treatment. This was not an issue for hatchery fish in the present study, since all oocytes were between 500 and 600 µm in diameter. However, wild-caught fish showed broad variation in initial oocyte diameters, ranging between 145 µm for LHRHa-treated Line-Caught fish and 477 µm in hCG-treated Trap-Caught fish. In fact, all Line-Caught fish had lower oocyte diameters than fish in corresponding Trap-Caught treatments. This is likely to have contributed to the poor ovulatory response in Line-, compared to Trap-Caught fish. Carolsfeld *et al* (1988) suggest that longer exposure of GnRH, at higher doses may be required to stimulate maturation in early vitellogenic fish, and Battaglene (1995) found that snapper with oocyte diameters less than 550 µm did not respond to hCG treatment. In the present study, the 2 Line-Caught hCG-treated fish which ovulated (oocyte diameters = 296 and 342 µm) both had ovulated by 168 h pi with poor quality eggs. Clearly, these fish had ovulated sometime after 72 h pi and before 168 h pi, indicating that the hCG treatment had taken longer to take effect in these fish with small oocytes. Similarly, the best ovulations in wild-caught fish came from hCG-treated Trap-Caught fish which had the largest oocyte diameters.

In contrast to both hCG and LHRHa, treatment with 17P had little effect on the ovulatory response, the incidence of atresia or on plasma gonadal steroid levels in unstressed or stressed fish in the present study. It appears, therefore, that substrate limitation is not the primary impediment to successful FOM, ovulation and spawning in unstressed or stressed fish. As described above, GtH stimulation, either directly through administering hCG, or indirectly through LHRHa, results in enhancement of the ovulatory response compared to control treatments. This suggests that there is sufficient endogenous substrate available for GtH-mediated stimulation of steroidogenesis, and provision of extra substrate did not, on its own, stimulate an ovulatory response. Therefore, the rate-limiting step in the reproductive endocrine cascade appears to be the release of endogenous GtH. 17P (or other substrate) may be useful but probably only in conjunction with GtH or GnRHa.

Decreases in ovulation volumes in stressed fish have been reported to correspond to increases in the incidence of atresia, suggesting that some developing oocytes fail to mature and become atretic (De Montalembert *et al*, 1978; Zohar, 1988). This is in agreement with the present study, where treatment of Hatchery fish with hCG or LHRHa both increased ovulation volumes and decreased the incidence of ovarian atresia, relative to saline and 17P treatments. In Trap- and Line-Caught fish, all vitellogenic oocytes were atretic. Therefore variations in the incidence of atresia among treatments in Trap- and Line-Caught fish only reflect differences in proportions of all cortical alveoli, early and late vitellogenic oocytes. The high proportion of atresia in Trap- and Line-Caught fish, relative to the Hatchery fish, is likely to be a reflection of the capture and culture histories of the 2 groups. As Hatchery fish were not subjected to ovarian biopsy, while Trap-Caught and Captive fish were, it may also be argued that this fact rather than the capture method determined the magnitude of the stress. However, it is difficult to believe that ovarian biopsy would increase stress levels beyond those already exerted by capture, and subsequent inversion into a foam block for bloodsampling. This is especially so considering that once a fish is in the foam block an ovarian biopsy usually takes less than 5 seconds, and was often performed concurrently with bloodsampling by a second operator. Therefore it more likely that differences between Hatchery fish and Line- and Trap-Caught fish are a reflection of capture and culture histories. As discussed in Chapter 3 (this volume), even after only 1 generation of hatchery-rearing, the Hatchery fish show a decreased sensitivity to stress in terms of reduced cortisol response, incidence of ovarian atresia and indications of post-stress recovery of plasma gonadal steroids.

In the present study, hCG, and to a lesser extent LHRHa reduced the incidence of atresia in hatchery fish relative to controls. In 3-year-old hatchery fish described in Chapter 3 (this volume), approximately 30 % of early and late vitellogenic oocytes combined were atretic in fish killed at first capture. A similar incidence of atresia was found in the present study following treatment with hCG, even after 1 week of confinement, in both unstressed and stressed groups. Atresia in LHRHa-treated groups was also approximately 30 % in stressed groups, but was slightly higher in unstressed groups. Clearly, both exogenous and

endogenous GtH suppresses the onset of stress-induced atresia. This observation is in agreement with Janz and Van Der Kraak (1997) who found that partially purified salmon GtH suppressed apoptosis *in vitro* in hatchery rainbow trout. This may be via action on steroidogenesis since E_2 also modulates apoptosis (Janz and Van Der Kraak, 1997; see Chapter 3, this volume). The fact that in the present study, GtH preparations, but not 17P, prevent atresia, indicates that either 17P alone is insufficient to protect follicles, since GtH action is still required to stimulate steroid conversion; or GtH has some direct role, in addition to steroid production, in the maintenance of follicular integrity.

In contrast to other treatment groups, plasma E_2 and T in unstressed, hCG-treated fish in the present study, remained detectable until 168 h pi. The higher levels of E_2 and T in unstressed hCG-treated fish is possibly a reflection of the steroidogenic activity of developing oocytes stimulated by hCG treatment. This is possibly due to the continued effect of residual hCG, or alternatively stimulation by endogenous GtH. It is not clear at this stage which mechanism is involved. Endogenous GtH is more strongly implicated, however, as exogenous hCG is expected to have cleared from the circulation by 168 h pi. Although the clearance rate of hCG in snapper is unknown, plasma levels of exogenous salmon GtH was reduced to approximately 10 % of its original concentration by 120 min post-treatment in maturing female goldfish (Cook and Peter, 1980). This indicates that clearance of hCG in the present study may be extremely rapid. Therefore it is likely that hCG treatment has stimulated endogenous GtH production. The most likely mechanism is by steroid positive feedback (Peter and Yu, 1997). Pankhurst and Carragher (1992) found that plasma E_2 and T were elevated by 24 h pi to approximately 1.25 and 0.75 $ng.ml^{-1}$ respectively in New Zealand snapper successfully induced to ovulate with hCG. In the present study, plasma E_2 and T were somewhat lower with both being approximately 0.5 ng/ml. Although it is possible that this is due to variation between New Zealand and Australian populations, it is more likely that it reflects the difference in timing post-injection of the blood sample (24 c.f. 168 h pi). As data for goldfish suggest (Cook and Peter, 1980), the clearance rate of hCG is rapid. Therefore the lower concentration of E_2 and T at 168 h pi is likely to be due to the difference between exogenous hCG-stimulated steroidogenesis (at 24 h pi), compared with endogenous GtH-stimulated steroidogenesis (at 168 h pi).

The non-detectable levels of E_2 and T in LHRHa, 17P and saline groups (both stressed and unstressed) and the stressed hCG group in the present study, indicate an absence of steroidogenically active follicles. The absence of elevated E_2 and T in stressed hCG-treated fish, compared with unstressed hCG-treated fish indicates that stress affects more than just the availability of endogenous GtH, possibly exerting its effects on GtH receptor binding, steroidogenic enzyme activity and/or substrate availability. The low plasma steroid levels in all groups, except the unstressed hCG-treated fish, are similar to those measured for all groups of female snapper (in the absence of exogenous hormone treatment), 168 h after capture, as described in Chapter 3 (this volume). Plasma levels of 17,20βP, in the present study were also low (although still detectable) by 168 h pi in all groups. This may indicate a

lack of maturing follicles in the ovary. The absence of elevated levels of plasma 17,20βP in hCG-treated unstressed fish is surprising, since E_2 and T levels indicate that ovarian development is occurring. However, as discussed in Chapter 3 (this volume), the maturational surge of plasma 17,20βP is of short duration in snapper (Hobby and Pankhurst, 1997a). Therefore it is possible that the sampling regime was too coarse in the present study to detect it.

By 168 h pi, plasma cortisol in the present study was between 5 and 10 $ng.ml^{-1}$ in unstressed Hatchery fish, and 10 and 15 $ng.ml^{-1}$ in stressed hatchery fish. These are somewhat higher than those previously measured at 168 h pc in 3-year-old Hatchery fish, which had a mean plasma concentration of 2.5 $ng.ml^{-1}$ (Chapter 3, this volume). This difference is likely to be a reflection of the impact of repeated handling when stripping the fish. Unstressed fish had significantly lower cortisol at 168 h pi than stressed fish. The reason for this is possibly due to the extra occasion that stressed fish were stripped. Equally, it is possibly due to a tank effect, particularly as the unstressed fish were kept separately from the stressed fish, and were culled one day prior to the unstressed fish. Mean plasma cortisol in line and trap fish ranged from 1.7 to 32 $ng.ml^{-1}$. With the exception of saline-treated Line-Caught fish (based on only 1 measurement), all groups were substantially higher than those measured for wild (Trap-Caught) Australian snapper confined for 168 h (Chapter 3, this volume). Again, this likely to be due to the effect of repeated stripping.

Conclusions

The capture of wild-caught fish proved to be highly unreliable and inefficient, and the results of hormonal induction of ovulation were variable and generally poor. Therefore industry reliance on hormonal induction of ovulation of wild-caught broodstock is not a viable option. In contrast, hatchery fish showed more reliable results from hormone treatment, after only 1 generation of domestication, and hold potential for continued improvement with further generations and increasing age (hence size) of the broodstock. With hatchery fish, there is potential for pre-treatment history to be managed and also for environmental induction of ovulation. Therefore domestication of broodstock should be a priority.

Hormonal induction of ovulation of wild-caught fish will, however, continue to be necessary, either when hatchery fish are not available (e.g. when establishing a new aquaculture venture) or in maintaining genetic diversity. When hormone treatment is required, both wild and hatchery fish should be treated immediately after capture, since stress impairs the response to exogenous hormone treatment. The effect of stress is multidimensional, affecting plasma gonadal steroid levels, egg volumes, egg morphology and the volume of fertilised eggs (Fig. 4.11). A synthesis of broodstock sources, optimum treatments and potential outcomes is shown in Figure 4.12. It is likely that these will be general considerations for broodstock management of all new marine aquaculture species, particularly stress-sensitive ones.

Figure 4.11. Generalised summary of the possible effects of stress on production of viable ova. Solid lines indicate known effects. Dotted lines indicate potential, but unexplored effects.

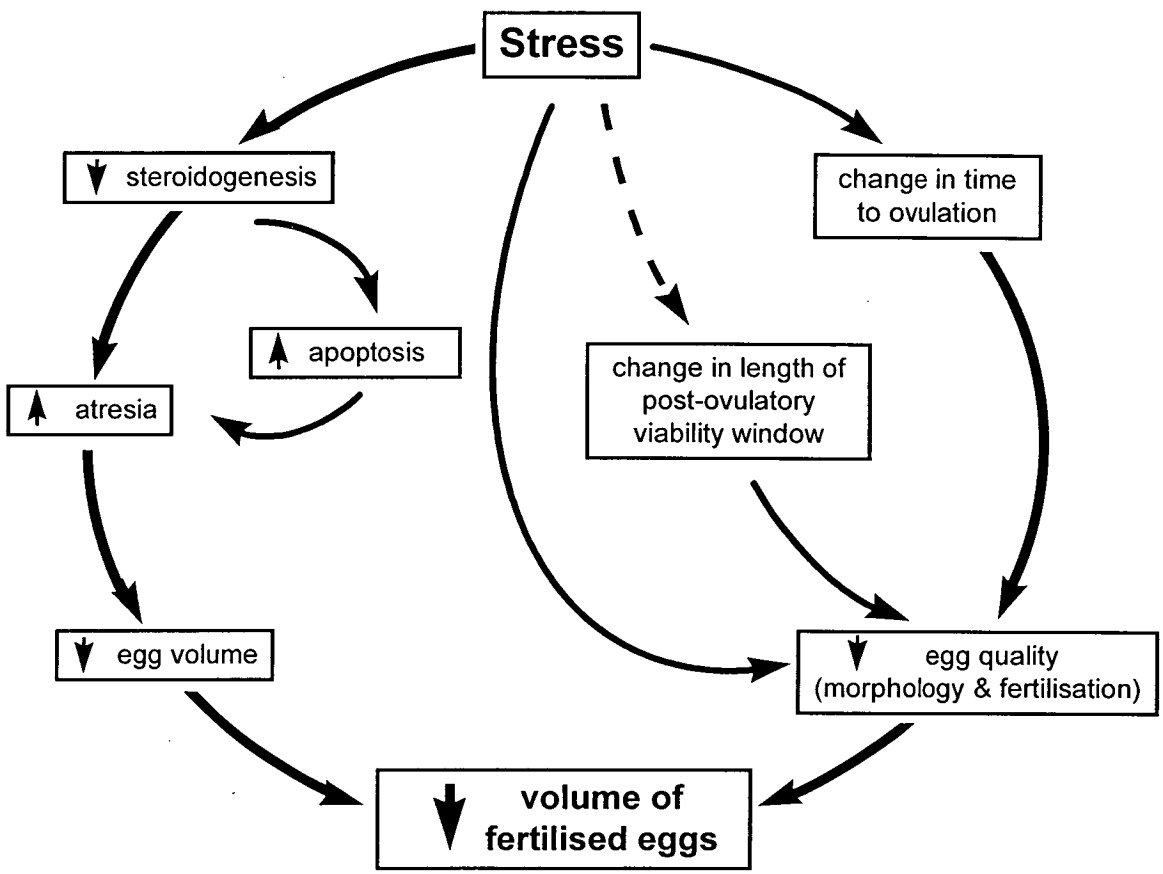
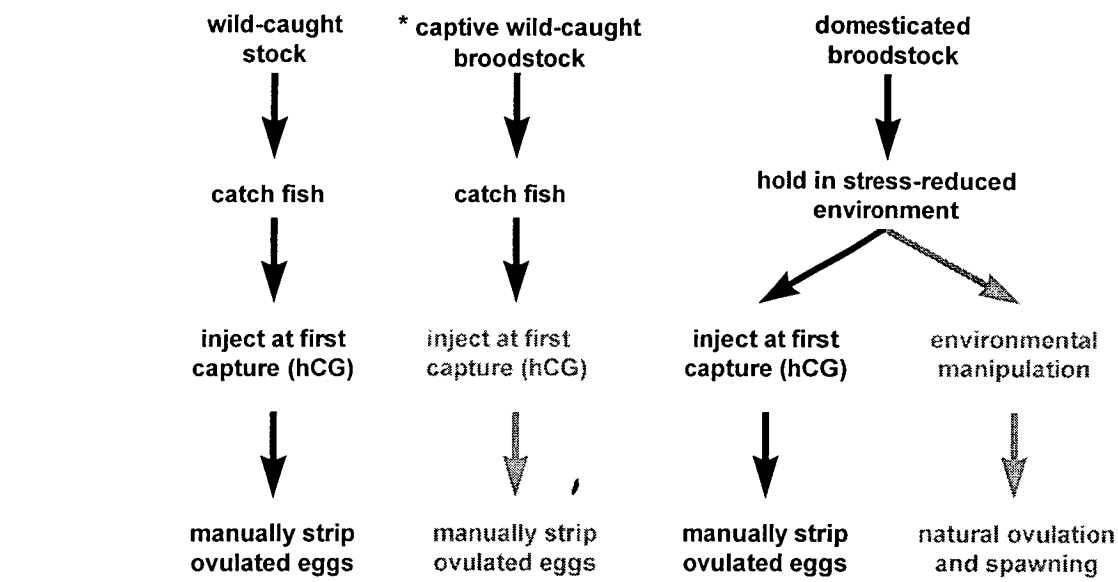
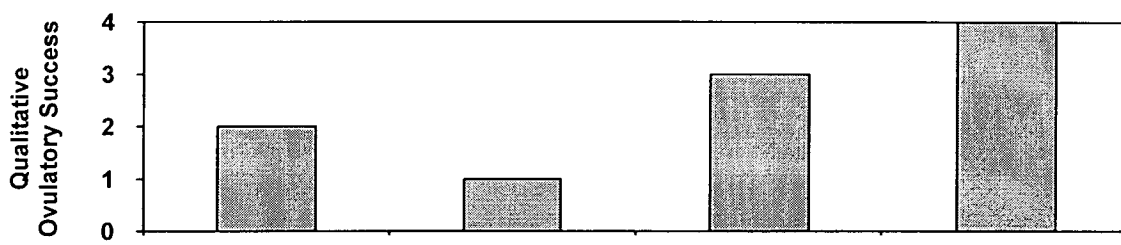

Stress
steroidogenesis
change in time to ovulation
apoptosis
atresia
change in length of post-ovulatory viability window
egg volume
egg quality (morphology & fertilisation)
volume of fertilised eggs

Figure 4.12. Summary of potential snapper broodstock sources and predicted optimum treatment for egg production. Graph indicates the predicted ovulatory success of hormonal and environmental induction of ovulation of wild, captive and domesticated fish. Grey text indicates areas not specifically addressed in this chapter. * captive broodstock = wild-caught-laboratory-acclimated broodstock.


wild-caught stock
* captive wild-caught broodstock
domesticated broodstock
catch fish
catch fish
hold in stress-reduced environment
inject at first capture (hCG)
inject at first capture (hCG)
inject at first capture (hCG)
environmental manipulation
manually strip ovulated eggs
manually strip ovulated eggs
manually strip ovulated eggs
natural ovulation and spawning


Qualitative Ovulatory Success
4
3
2
1
0

Chapter 5: The effect of capture and handling stress on *in vitro* ovarian steroidogenesis.

3.1 Introduction

Despite the fact that stress is known to have a profound and multidimensional effect on a broad range of reproductive parameters in a variety of species (reviewed by Pankhurst and Van Der Kraak, 1997), including Australian snapper (Chapter 3 and 4, this volume), the mechanism by which stress exerts its effect is poorly understood. Stress could exert its effect on any level of the hypothalamus-pituitary-gonadal axis, including secretion of gonadotropin releasing hormone (GnRH) or gonadotropin (GtH), GtH-receptor binding sites, subsequent signal transduction or gonadal synthesis of steroids. The present study was undertaken to examine the biosynthetic capacity of ovarian follicles taken from fish with different stress histories, to determine if reduced plasma levels of reproductive steroids following stress result, in part, from impairment of ovarian steroidogenesis.

Stress typically results in elevated plasma cortisol, concomitant with a decrease in plasma levels of reproductive steroids (Sumpter *et al*, 1987), and a causal relationship has sometimes been assumed. In addition, cortisol implants have been found to mimic some aspects of the effects of stress on reproduction (Carragher *et al*, 1989; Pottinger *et al*, 1991; Foo and Lam, 1993a,b). Foo and Lam (1993a) found that cortisol implants decreased plasma testosterone (T) levels in tilapia (*Oreochromis mossambicus)*. Moreover, using cortisol implants, Carragher *et al* (1989) demonstrated that prolonged elevation of plasma cortisol can affect a wide range of reproductive parameters such as gonad size, plasma gonadal steroid levels, gonadotropin (GtH), and vitellogenin in brown (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*). Carragher and Sumpter (1990) examined the effect of cortisol on ovarian steroidogenesis *in vitro* in female rainbow trout. They found that cortisol was able to suppress basal secretion of T and 17β-estradiol (E_2) by ovarian follicles, however, the effect was variable. The authors suggested that this may be due to undocumented individual differences in stress histories of the fish. Sumpter *et al* (1987) found that cortisol inhibited both basal and GtH-mediated *in vitro* secretion of E_2 in rainbow trout follicles. These studies have led to the suggestion that the effect of stress on plasma concentrations of reproductive steroids may be mediated, at least in part, by a direct action of cortisol on the gonads (Sumpter *et al*, 1987; Carragher and Sumpter, 1990).

This view, however, is not universally held. Pankhurst *et al* (1995a) re-examined the effect of cortisol on ovarian steroidogenesis in rainbow trout, but found that although cortisol could inhibit E_2 production, the effect was not consistent and inhibition was less frequent than for

previous workers. Jalabert and Fostier (1984) found that in rainbow trout follicles, cortisol actually increased GtH-stimulated production of 17,20βP (17α,20β dihydroxy-4-pregnen-3-one) *in vitro*. Furthermore, Pankhurst *et al* (1995b) examined the effect of cortisol on ovarian steroidogenesis *in vitro* in goldfish (*Carassius auratus*), carp (Cyprinus carpio) and New Zealand snapper (*Pagrus auratus).* They found that there was no evidence of inhibition of ovarian production of E_2 or T by cortisol in any of these 3 species, and in some cases, cortisol actually had a stimulatory effect. The lack of evidence of cortisol inhibition in non-salmonids, and the inconsistent effects in salmonids has led to the conclusion that the inhibitory effects of stress on reproduction are not mediated directly by cortisol, at least in non-salmonid species, or that if cortisol is involved it is not at the level of steroidogenesis. Therefore, the mechanism by which stress exerts its effect remains unclear. Stress generates a suite of endocrine changes of which cortisol is but one (Sumpter, 1997), therefore stress and cortisol are not synonymous. The effect of these other parameters on reproduction is not known (Sumpter, 1997). It remains possible that stress does affect ovarian steroidogenesis, but via a combination of endocrine responses, of which elevated cortisol levels may be only a part.

To date, there are no published data on the effect of stress (as opposed to cortisol) on ovarian steroidogenesis. Therefore, this study investigates the effect of capture and handling, followed by differential periods of confinement on subsequent steroidogenesis of E_2 and T by isolated ovarian follicles. The capture and handling stress employed in the present study was similar to that found to reduce plasma gonadal steroid levels and inhibit egg production in similar hatchery-reared fish in previous studies (Chapters 3 and 4, this volume). Follicles were incubated with human chorionic gonadotropin (hCG) or 17α–hydroxyprogesterone (17P) to investigate the capacity of follicles to respond to GtH stimulation, and the activity of steroid synthesizing enzymes downstream of 17P respectively. In addition, the effect of co-stimulation of hCG plus 17P was examined in order to determine if steroid synthesis following provision of sufficient substrate (17P) could be further enhanced by GtH stimulation.

3.2 Methods

Five fish were caught by handline from the Marine Research Pool (0 h treatment) between 0850 and 1015 h on 7th September 1996. Immediately after capture, sex and reproductive stage were determined by gonadal biopsy. Stage III females were selected, tagged, and a blood sample taken before each fish was killed. A further 28 fish were collected by crowding, between 1020 and 1145 h. Stage III females were selected, tagged and a blood sample taken. Fish were then cyclically allocated, to each of the 4 treatments (holding for 24, 48, 72 or 168 h: n=4-5). For each treatment, fish were held in a separate 4000 L aerated tank with

constant water exchange. At the designated time (i.e. at 24, 48, 72 or 168 h pc), a second blood sample was taken and the fish killed. Immediately after death, the ovaries were dissected and placed in ice-cooled Leibovitz (L-15) medium (Sigma), adjusted to pH 7.6 with 0.1 M hydrochloric acid. The length of each fish was measured and the body, gonad and liver were weighed. Only macroscopically confirmed stage III females were used in the *in vitro* assays. Water temperature in the pool at the time of capture was 15.9 °C.

In vitro *Incubation Protocol*

Ovarian follicles were separated from the connective tissue using forceps, and dispersed further by repeated aspiration with a pipette. The original L-15 was decanted and fresh L-15 was added to make a volume of approximately 1 part follicles to 2 parts L-15. Using a magnetic stirrer to keep the follicles in suspension, 200 µl aliquots were pipetted into each of 24 wells on flat bottom polypropylene tissue culture plates (Corning, New York) (Pankhurst *et al,* 1995). The stirrer speed was constant for all fish, and the aliquots were taken from the same position and depth in the suspension, in order to minimise variation between aliquots. Six more 200 µl aliquots were taken for counting to determine the average number of vitellogenic follicles in each 200 µl aliquot, and the diameters of 30 oocytes were measured. Four hormone treatments, with 6 replicates per treatment were set up as follows: 1) L-15 alone (control); 2) L-15 with 100 $U.ml^{-1}$ of hCG (added as 100 µl of a 1000 $U.ml^{-1}$ solution in acidified saline); 3) L-15 with 100 $ng.ml^{-1}$ of 17P (added as 10 µl of a 10 $\mu g.ml^{-1}$ solution in ethanol); 4) L-15 with 100 µl of hCG plus 10 µl of 17P (solutions as above), in a final volume of 1000 µl. Non-steroid treatments received 10 µl of ethanol. The assays were incubated overnight on the bench top, at room temperature (min.= 12-13.5 °C, max.= 20-21°C). The following day, the supernatant was aspirated by pipette into 2 ml polypropylene tubes and frozen for storage.

Ovarian samples from each fish used were prepared for histology as described in Chapter 2, and the proportion of each oocyte type, and its condition (i.e. normal or atretic) was scored. Plasma concentrations of E_2, T, and cortisol and media concentrations of E_2 and T were measured by radioimmunoassay as described in Chapter 2. Assay values for media samples were expressed as pg steroid.500 $follicles^{-1}$.

Statistics

The data for each fish consisted of 6 replicate determinations of E_2 and T *in vitro* concentrations within each of the 4 hormone treatment conditions (control, hCG, 17P, hCG plus 17P). There were 5 groups of fish characterised by 5 different confinement times following capture (0, 24, 28, 72, 168 h pc). The effects of hormone treatments on E_2 and T *in vitro* concentrations were analysed using a separate one-way ANOVA for each of the 21 fish with the 6 replicates in each treatment providing the "Within Groups" data. Each ANOVA was followed as appropriate by Tukey-Kramer HSD tests of the gaps between means. The

effect of duration of confinement (time) on E_2 and T yields, and on the ratio E_2:T was examined separately for each hormone treatment. The mean of the 6 replications of E_2, T and the E_2:T ratio for each fish were averaged across all fish with the same confinement time. The differences between these means for the 5 confinement times were analysed using a separate one-way ANOVA for each hormone treatment. The means of the 6 replicates for each fish provided the "Within Groups" data in each case. Each ANOVA was followed as appropriate by Tukey-Kramer HSD tests of the gaps between means. Plasma steroid levels at first capture, as well as biological and histological data were analysed using a one-way ANOVA examining differences between the means of the 5 confinement times.

3.3 Results

In vitro *E_2 and T production*

For each fish the overall Between Hormone Treatments F-test was significant (in each case $p<.0001$). Therefore, Tukey-Kramer HSD tests were carried out for each fish on the mean E_2 levels for the 4 hormone treatments. The groupings of the means are shown in Figures 5.1-5.5

In unstressed fish (0 h pc), ovarian follicles in control treatments had detectable levels of E_2 (Fig. 5.1). Follicles treated with hCG produced more E_2 than did the control treatment in all five fish. For follicles treated with 17P, E_2 levels were higher than in the control treatment for all fish, and higher than in the hCG treatment for 3 fish. Follicles treated with hCG plus 17P had similar amounts of E_2 to those treated with 17P in all fish, and higher amounts of E_2 than for the hCG treatment in 3 fish.

In all fish confined for 24 h, there were detectable levels of E_2 following the control treatment. E_2 levels were higher following the hCG treatment than the control treatment, (Fig. 5.2). The 17P treatment produced higher levels of E_2 than did the control treatment in all fish, while in 3 fish 17P treatments produced higher levels of E_2 than did hCG treatments. For follicles treated with hCG plus 17P, E_2 levels were similar to 17P treatments, with all four fish giving significantly higher levels of E_2 than following either control or hCG treatments.

In fish confined for 48 h, E_2 was detectable in all control treatments. E_2 was significantly higher following hCG than the control treatment (Fig. 5.3). Levels of E_2 were also higher after 17P treatment than after the control treatment in all 48 h-fish, and higher than the hCG treatment in 1 fish. Follicles treated with hCG plus 17P had higher levels of E_2 than did those receiving the control treatment in all 48 h-fish. In 2 fish, E_2 levels were higher following the combined hCG plus 17P treatment than was the case following hCG alone. Levels of E_2 were similar in the 17P, and the combined hCG plus 17P treatment in all 48 h fish.

Figure 5.1. E_2 production by ovarian follicles from unstressed snapper (0 h). Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P<0.05$). 1 - 5 = 5 separate fish.

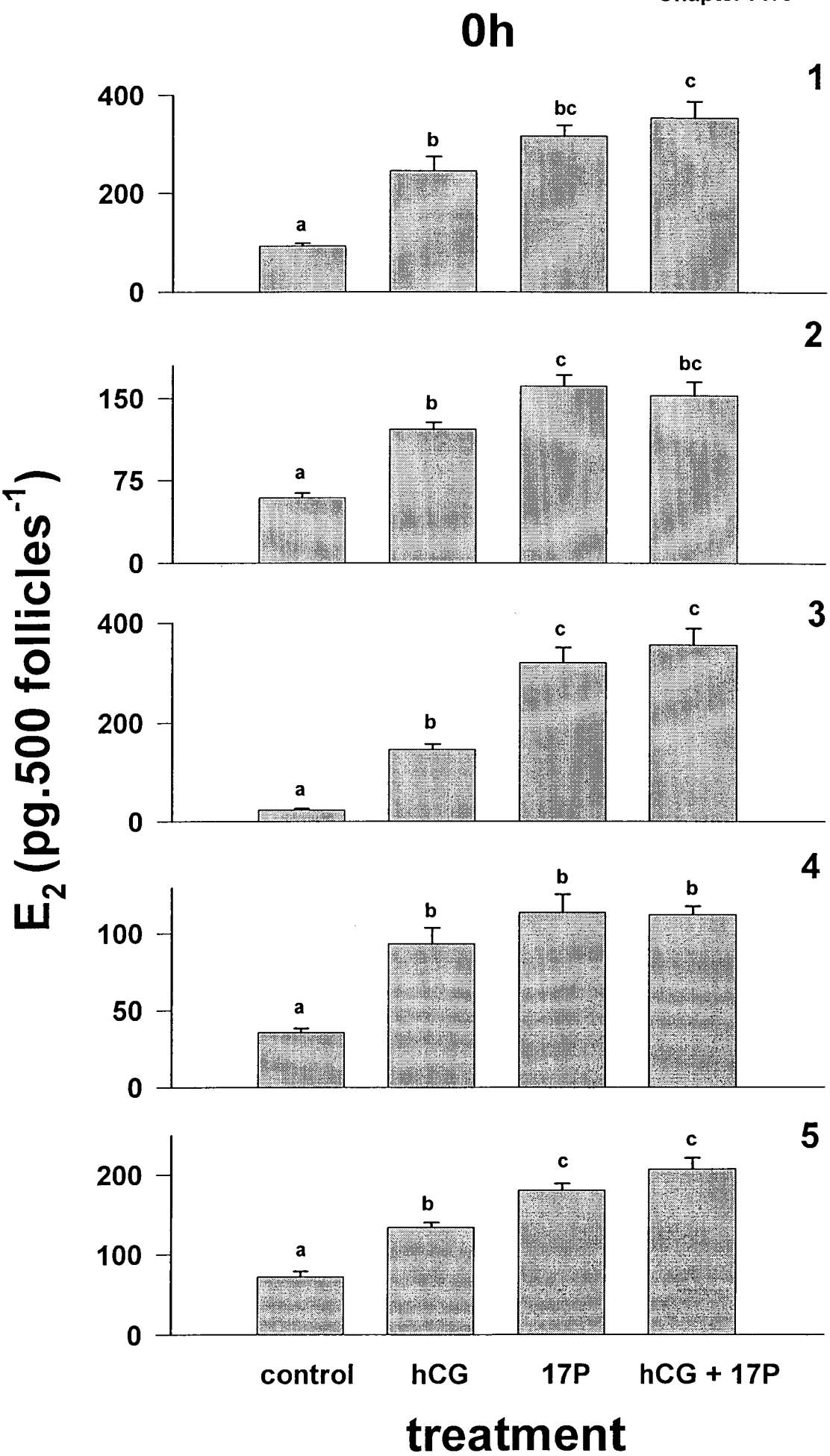

0h
1
2
3
4
5
400
200
0
150
75
100
50
a
b
bc
c
E_2 (pg.500 follicles^{-1})
control
hCG
17P
hCG + 17P
treatment

Figure 5.2. E_2 production by ovarian follicles from snapper confined for 24 h. Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P < 0.05$). 1 - 4 = 4 separate fish.

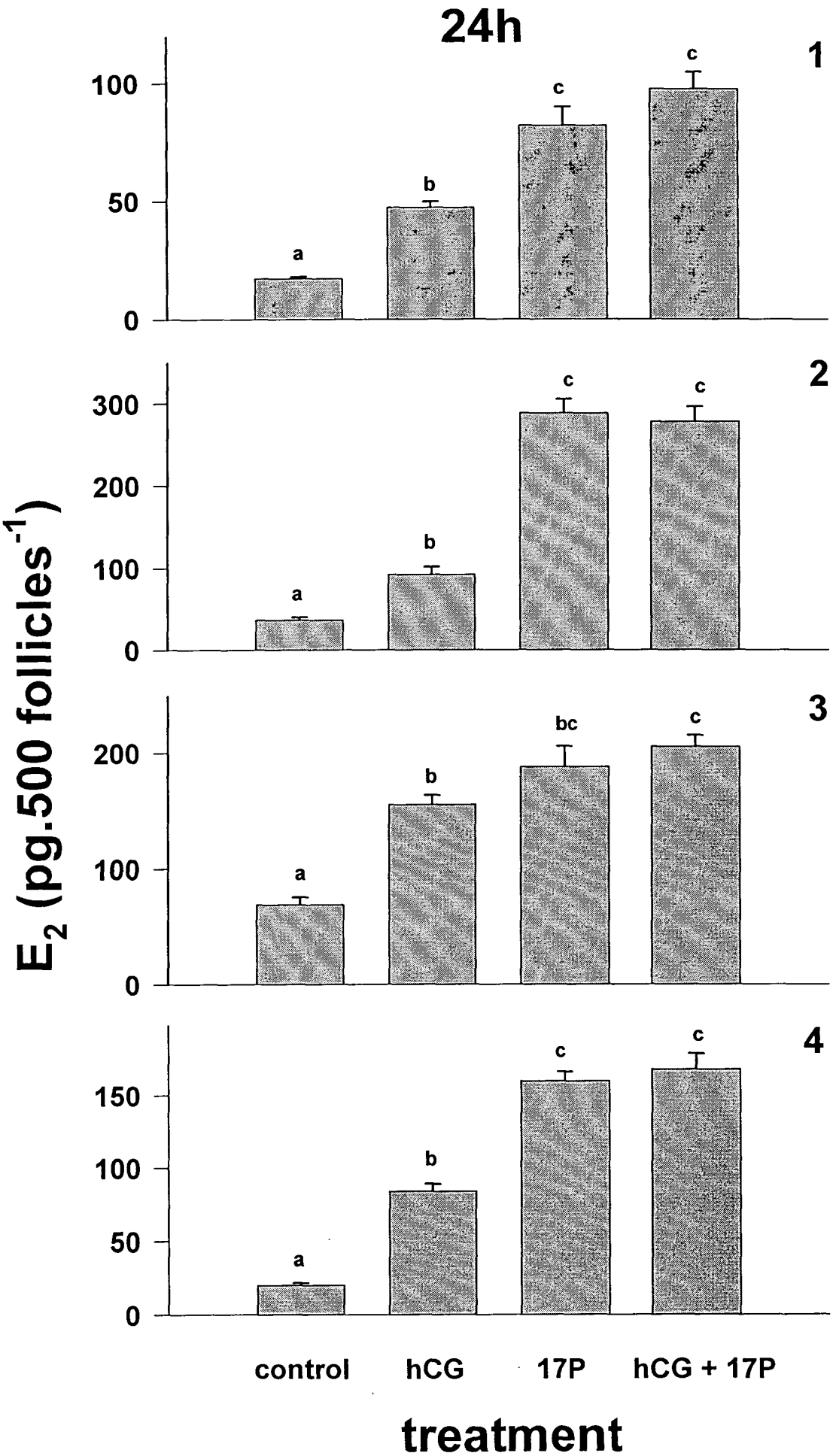

24h
1
2
3
4
100
50
0
300
200
100
0
200
100
0
150
100
50
0
a
b
c
c
a
b
c
c
a
b
bc
c
a
b
c
c
E_2 (pg.500 follicles^{-1})
control
hCG
17P
hCG + 17P
treatment

Figure 5.3. E_2 production by ovarian follicles from snapper confined for 48 h. Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P < 0.05$). 1 - 4 = 4 separate fish.

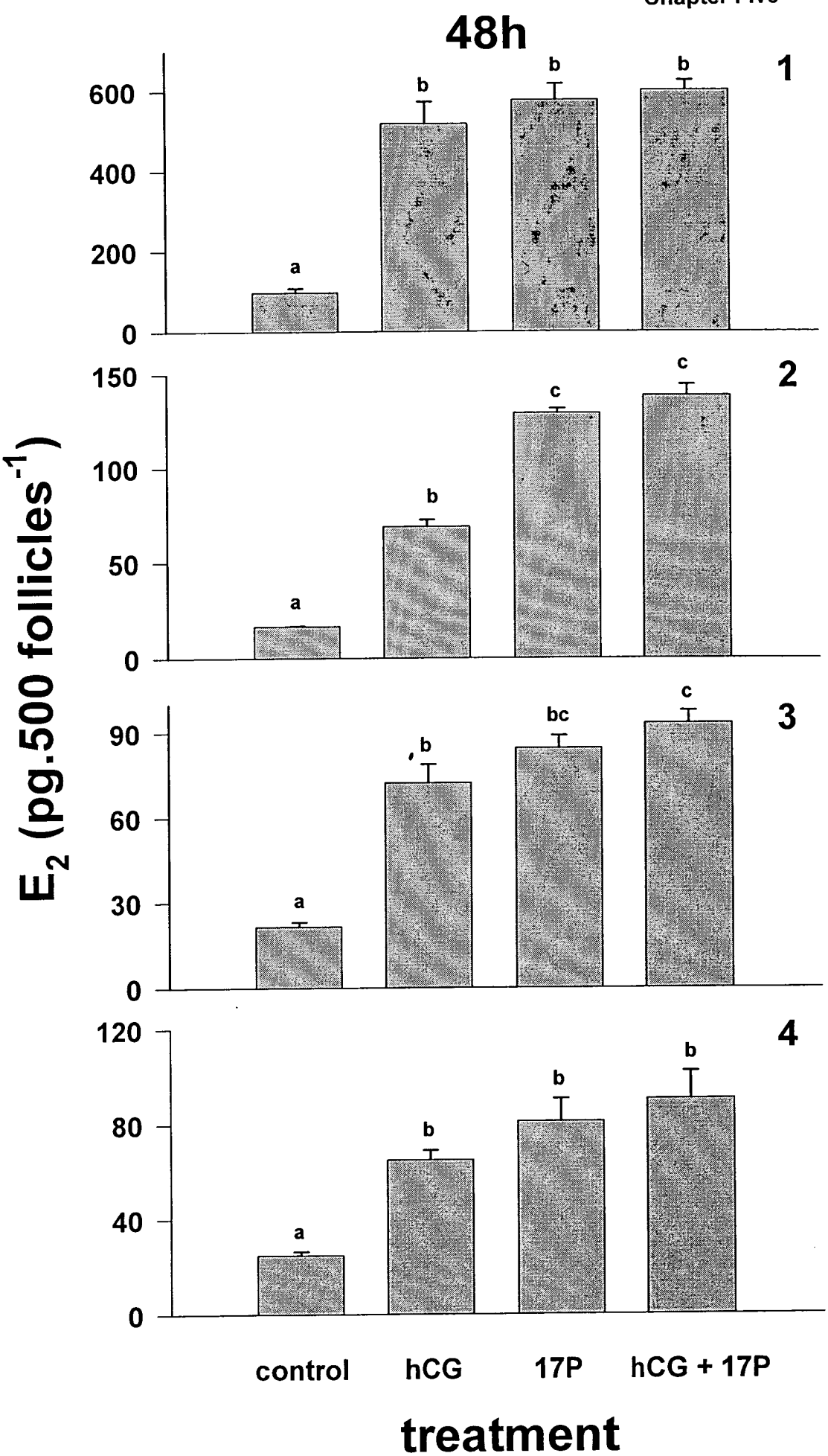

48h
1
2
3
4
600
400
200
0
150
100
50
0
90
60
30
0
120
80
40
0
a
b
b
b
a
b
c
c
a
b
bc
c
a
b
b
b
E_2 (pg.500 follicles^{-1})
control
hCG
17P
hCG + 17P
treatment

Figure 5.4. E_2 production by ovarian follicles from snapper confined for 72 h. Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P < 0.05$). 1 - 4=4 separate fish.

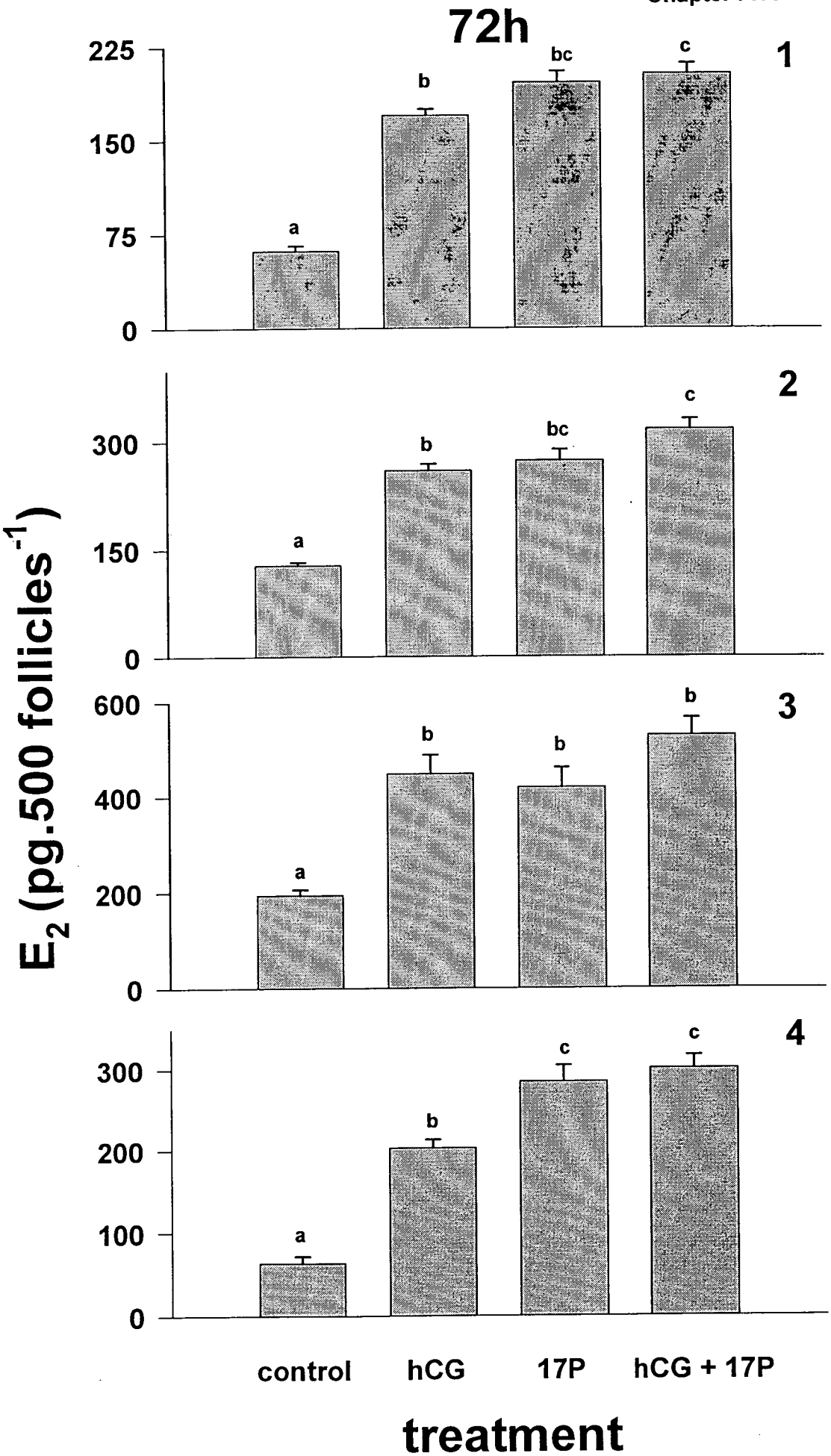
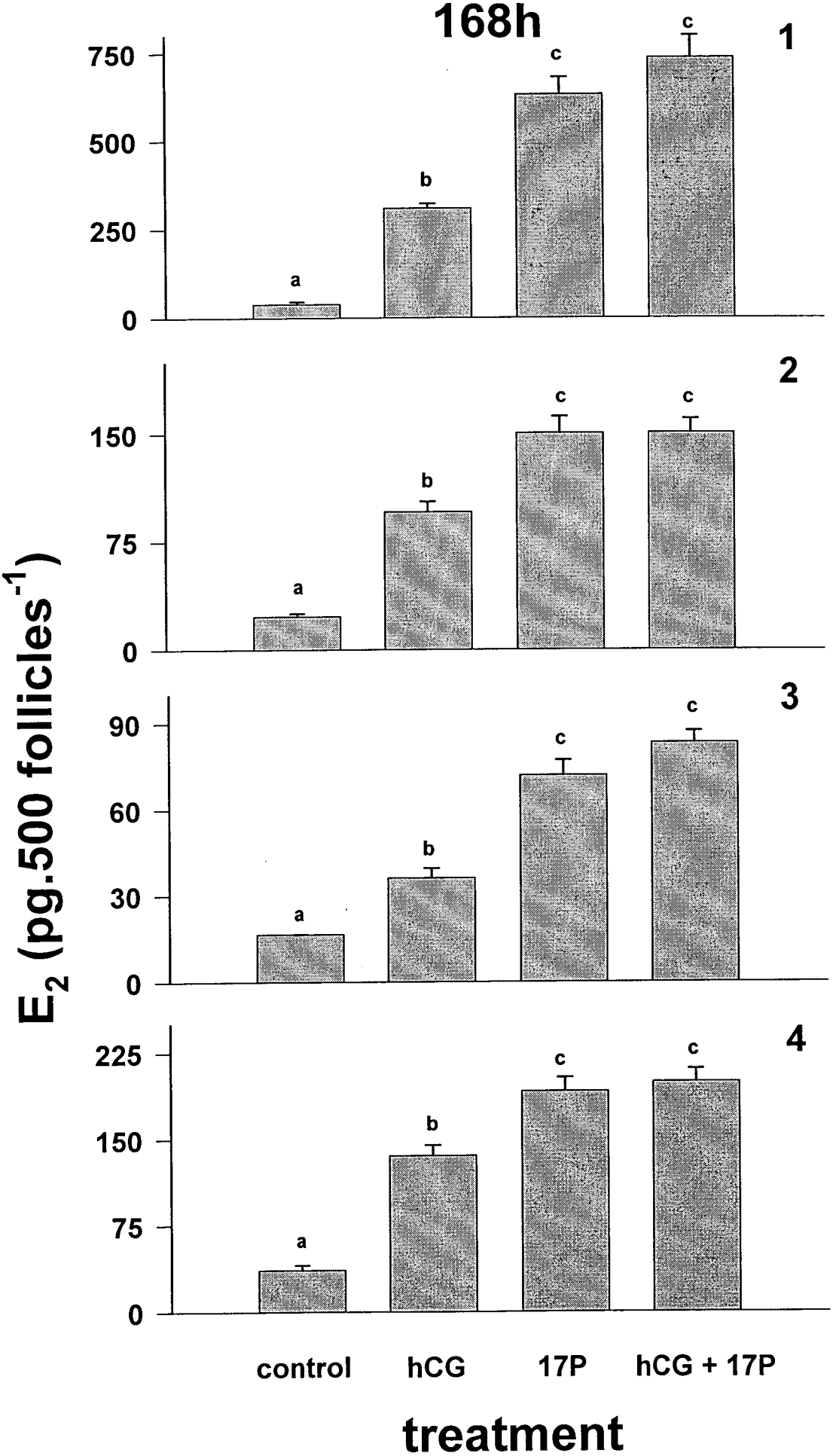

72h
1
2
3
4
225
150
75
0
300
150
0
600
400
200
0
300
200
100
0
a
b
bc
c
E_2 (pg.500 follicles^{-1})
control
hCG
17P
hCG + 17P
treatment

Figure 5.5. E_2 production by ovarian follicles from snapper confined for 168 h. Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer groupings ($P < 0.05$). 1 - 4= 4 separate fish.


168h
1
2
3
4
750
500
250
0
150
75
90
60
30
225
a
b
c
E_2 (pg.500 follicles^{-1})
control
hCG
17P
hCG + 17P
treatment

In all fish confined for 72 h, E_2 was detectable following the control treatment. The hCG treatment produced higher levels of E_2 than did the control treatment (Fig. 5.4). In the 17P treatment, all four 72 h-fish showed higher levels of E_2 than was the case with the control, while only 1 fish showed a significantly higher level of E_2 after 17P than the hCG treatment. In follicles treated with hCG plus 17P, there was no difference in E_2 concentrations from those treated with 17P alone. However, in three 72 h-fish, the combined hCG plus 17P treatment produced significantly higher E_2 concentrations than did the hCG treatment, and all the concentrations were higher than for the control treatment.

In follicles from fish confined for 168 h, detectable levels of E_2 were produced by the control treatment and significantly higher levels were obtained after hCG than the control treatment in all fish (Fig. 5.5). Levels of E_2 following both the 17P and hCG plus 17P treatments were similar in all 168 h-fish and these were significantly higher than hCG and control treatments.

Each overall Between Hormone Treatments F test was significant (in each case $p<.0001$), so for each fish Tukey-Kramer HSD tests were carried out on the mean T levels for the 4 hormone treatment conditions. The groupings of these means are shown in Figures 5.6-5.10. No T was detectable for the control treatment for any of the fish for any confinement time. Detection limits varied between fish due to differences in the mean number of follicles per well. In the absence of detectable T in the control condition, the actual detection limit for each fish was entered as the data for that fish (Fig. 5.6 - 5.10). For comparisons between confinement times which involved averaging across several fish, however, detection limits were set as the mean detection limit of all 21 fish (Fig. 5.12- 5.14).

In 3 unstressed fish, levels of T following treatment with hCG were significantly higher than control levels (Fig. 5.6). Concentrations of T after 17P and combined hCG plus 17P treatments were similar, and were significantly higher than following both control and hCG treatments for all unstressed fish (0 h confinement).

In fish confined for 24 h, hCG produced a significantly higher level of T than did the control treatment in only 1 fish (Fig. 5.7). Treatment with 17P gave similar levels of T to the combined hCG plus 17P, and both these treatments produced higher levels than did the control and hCG treatment in all 24 h-fish.

In fish confined for 48 h, treatment with hCG produced significantly higher levels of T than did the control treatment in 2 fish (Fig. 5.8). Treatment with 17P gave higher levels of T than did both the control and hCG treatments. Treatment with 17P and with hCG plus 17P gave similar levels of T in all but one 48 h-fish (where treatment with 17P plus hCG, resulted in a lower concentration of T than following treatment with 17P alone). Treatment with hCG plus 17P gave higher levels of T than did both control and hCG treatments in all 48 h-fish.

Figure 5.6. T production by ovarian follicles from unstressed snapper (0 h). Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P < 0.05$). 1 - 5= 5 separate fish

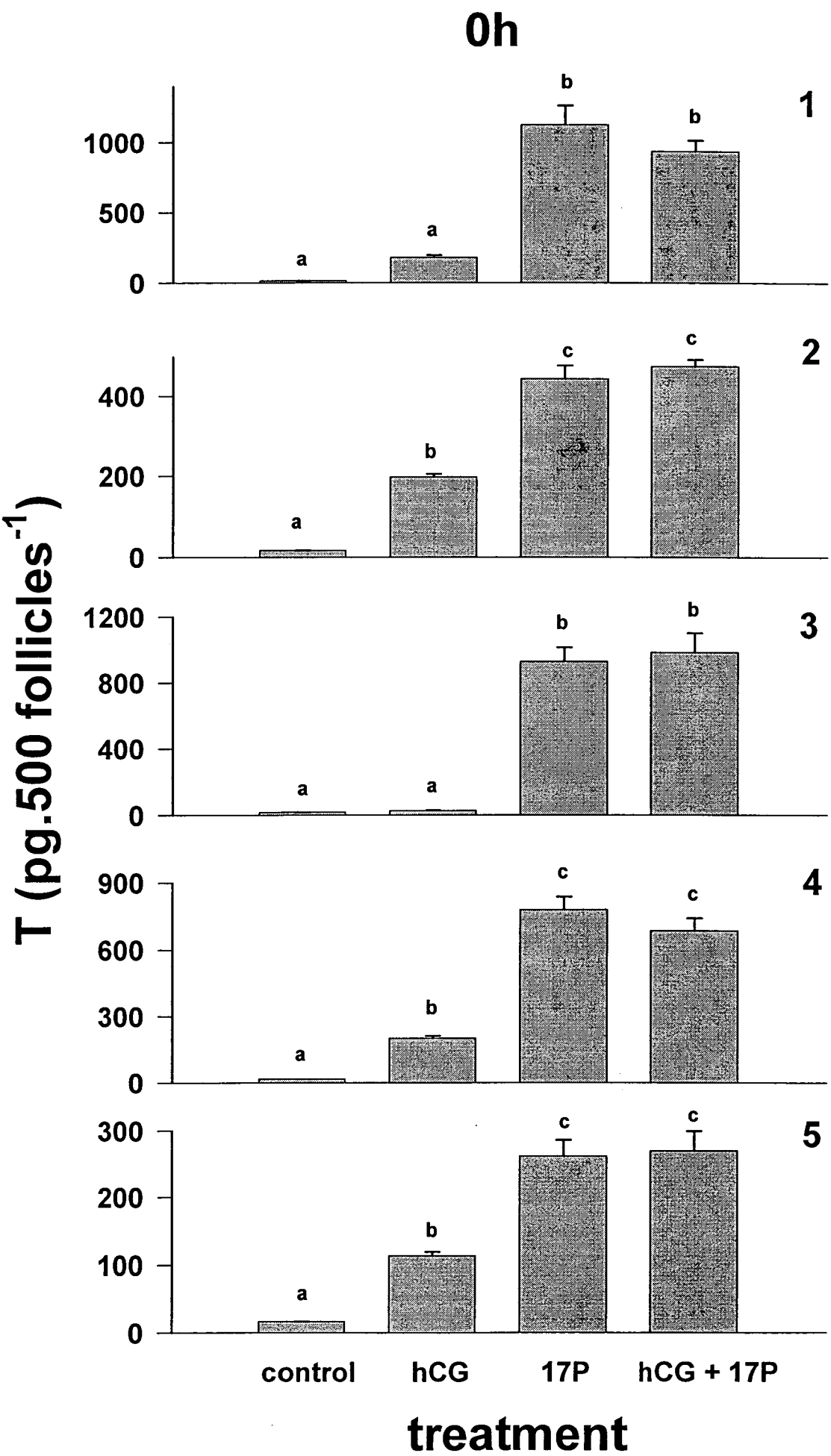

0h
1
2
3
4
5
T (pg.500 follicles-1)
1000
500
0
400
200
1200
800
900
600
300
100
a
b
c
control
hCG
17P
hCG + 17P
treatment

Figure 5.7. T production by ovarian follicles from snapper confined for 24 h. Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P < 0.05$). 1 - 4 = 4 separate fish.

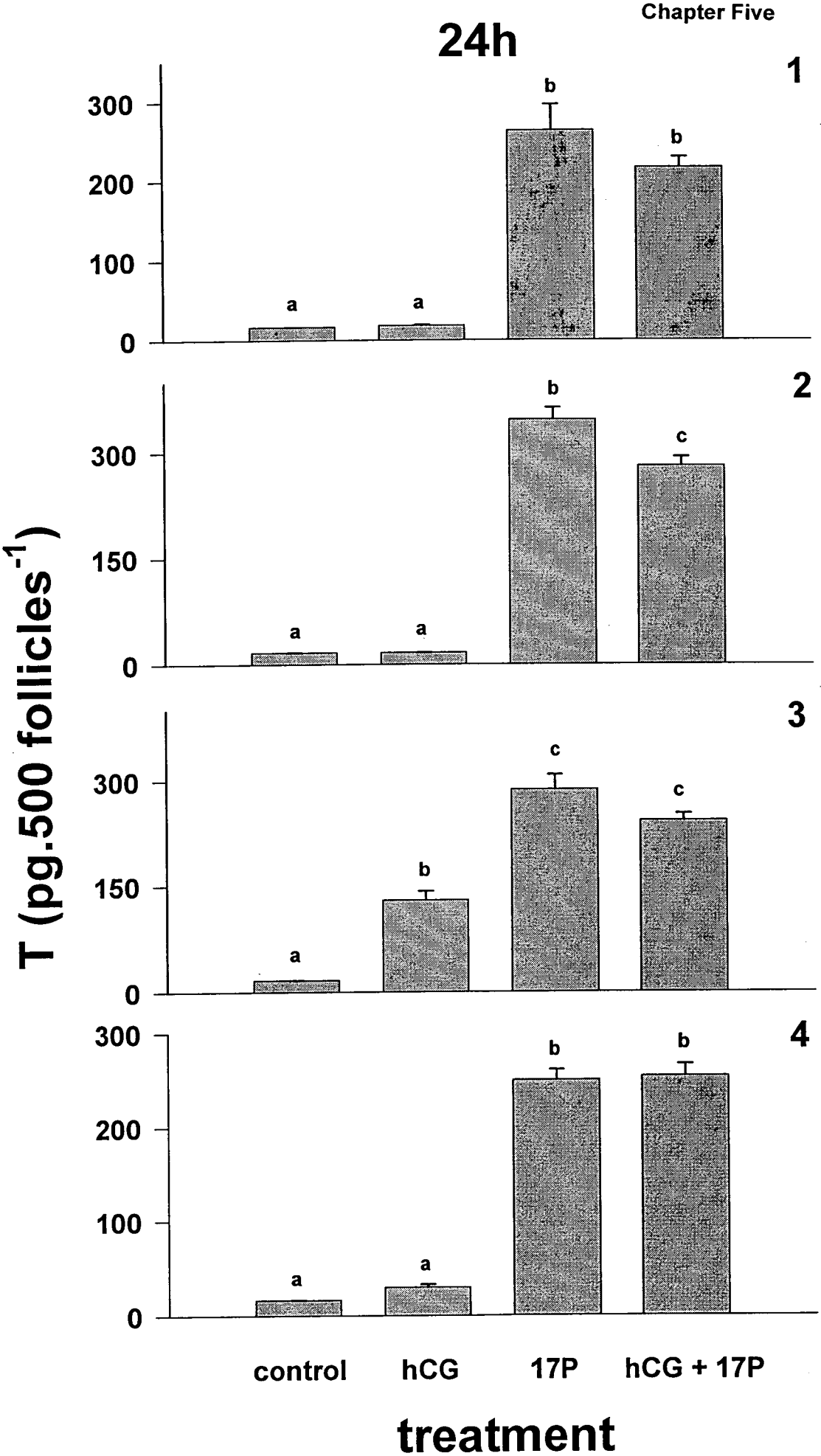

24h
1
2
3
4
300
200
100
0
150
a
b
c
T (pg.500 follicles-1)
control
hCG
17P
hCG + 17P
treatment

Figure 5.8. T production by ovarian follicles from snapper confined for 48 h. Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P < 0.05$). 1 - 4 = 4 separate fish.

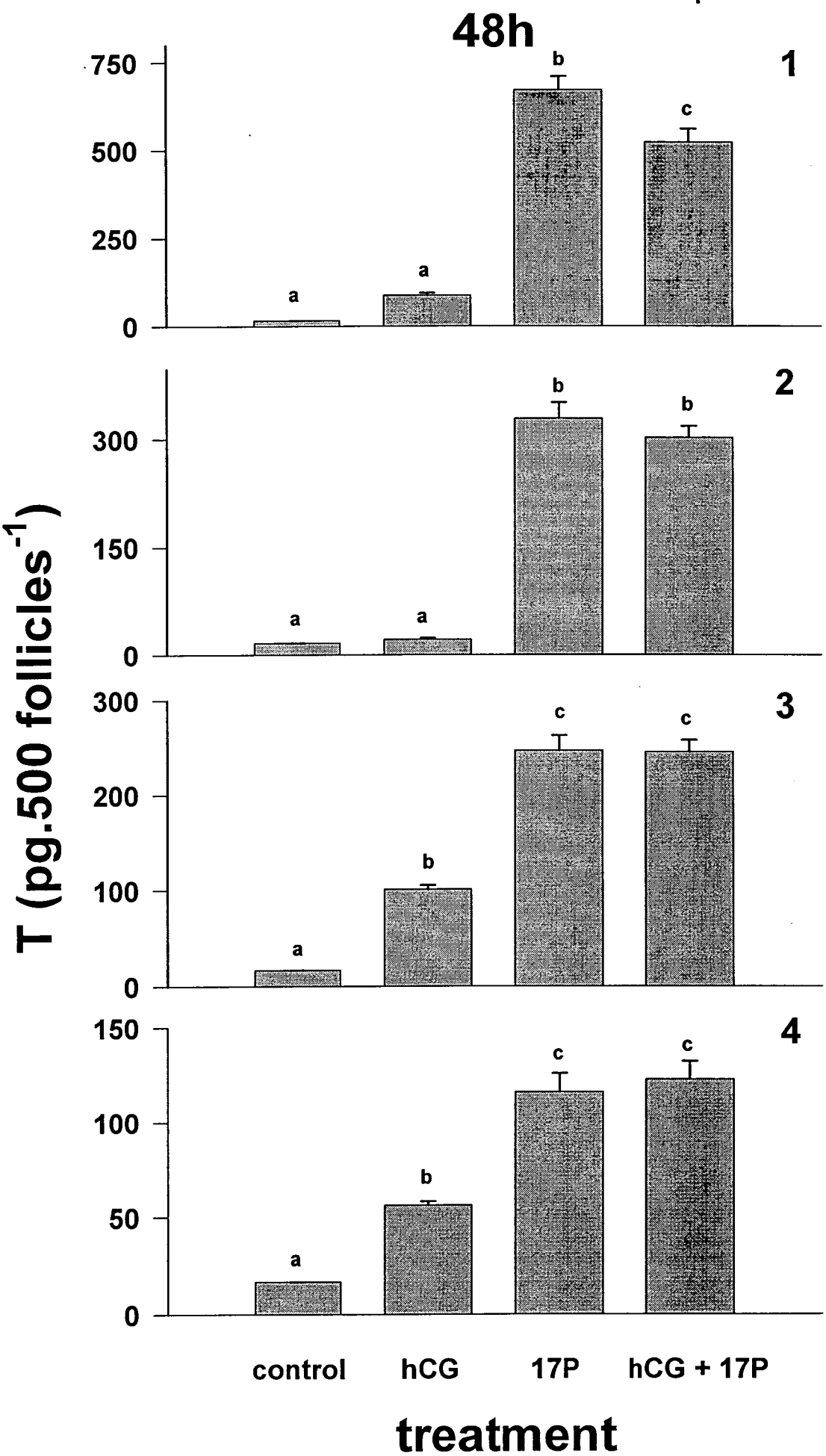

48h
1
2
3
4
750
500
250
0
300
150
200
100
50
a
b
c
T (pg.500 follicles-1)
control
hCG
17P
hCG + 17P
treatment

Figure 5.9. T production by ovarian follicles from snapper confined for 72 h. Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P < 0.05$). 1 - 4 = 4 separate fish.

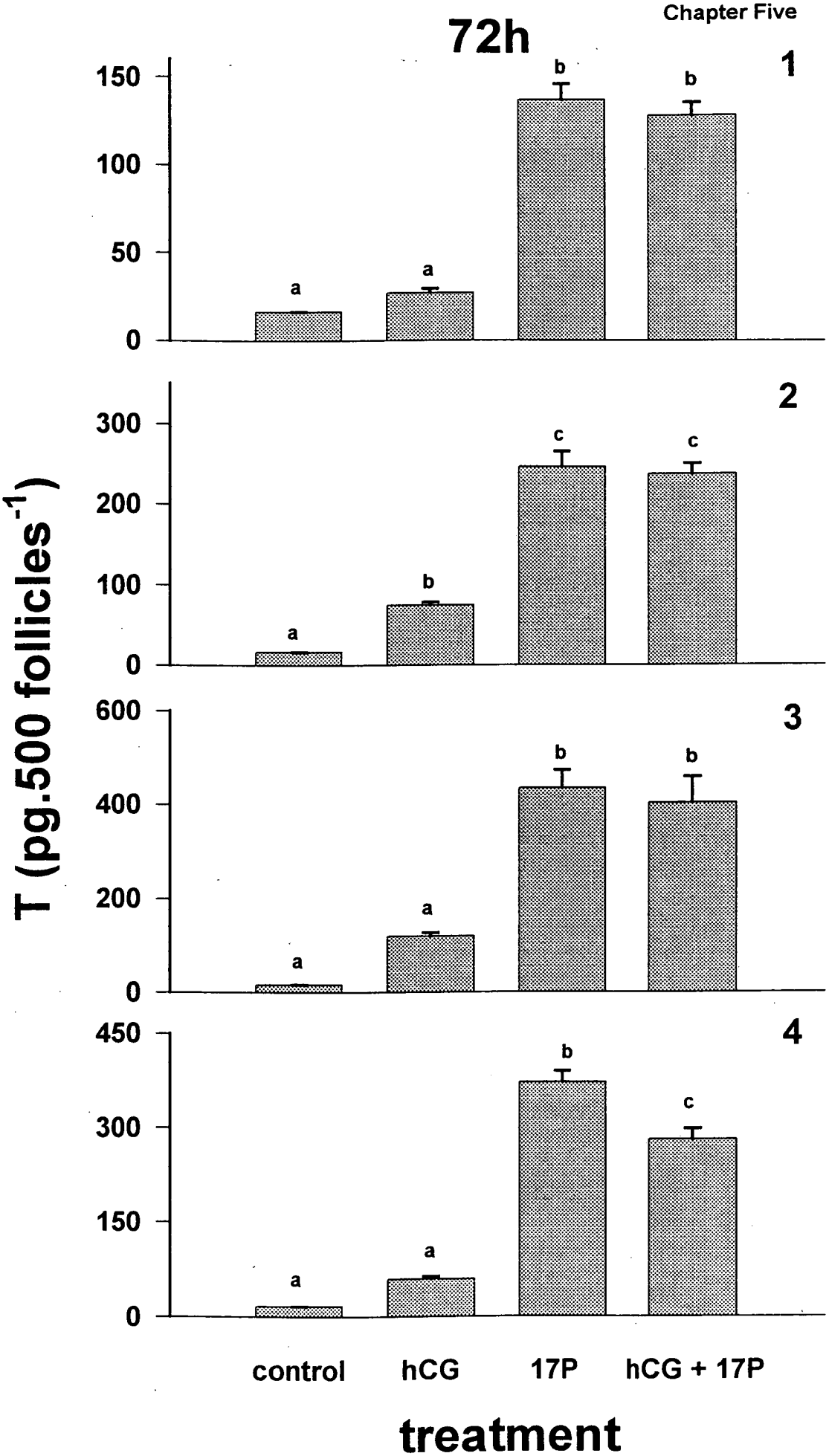
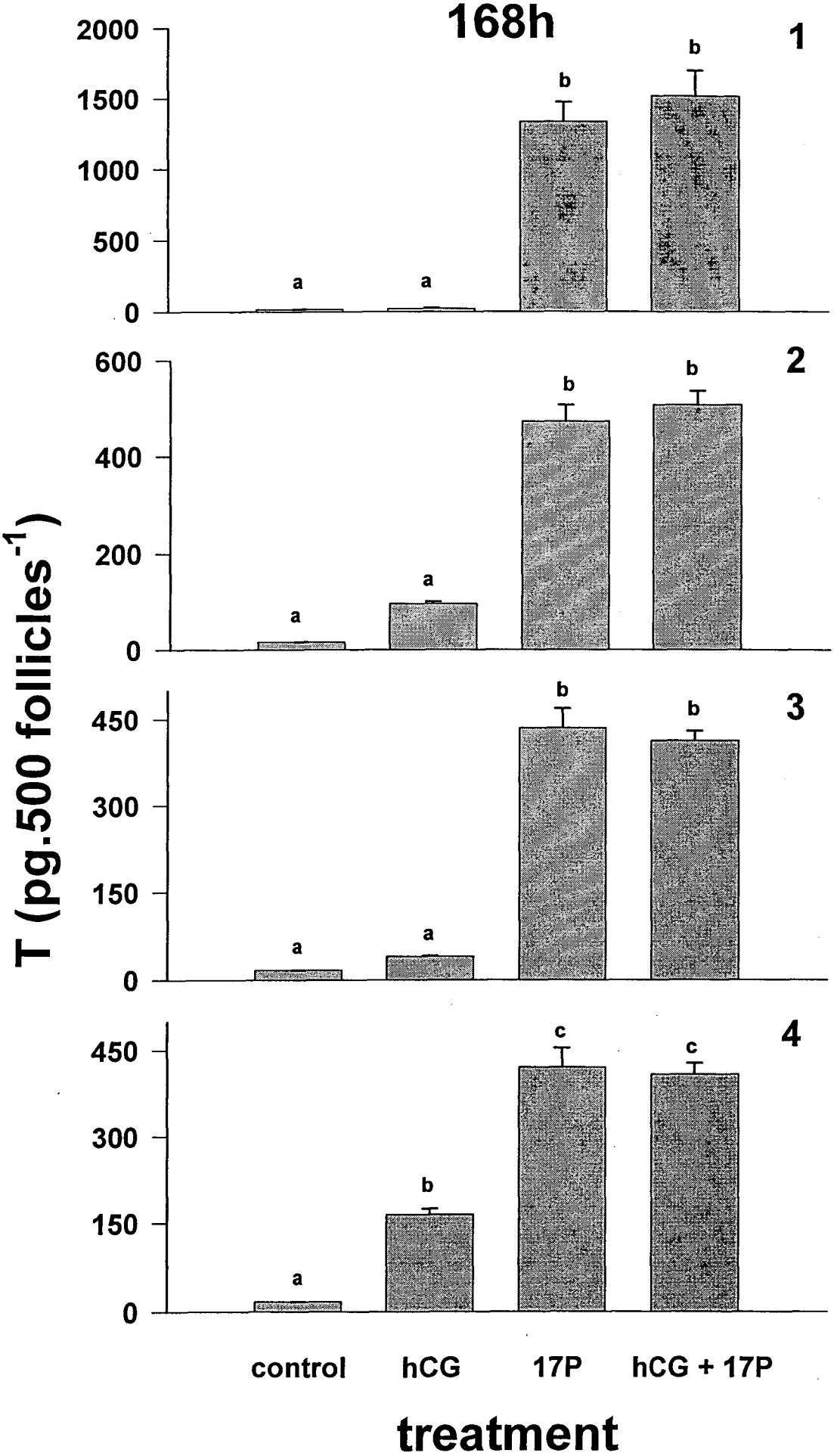

72h
1
2
3
4
150
100
50
0
300
200
100
0
600
400
200
0
450
300
150
0
a
a
b
b
a
b
c
c
a
a
b
b
a
a
b
c
T (pg.500 follicles-1)
control
hCG
17P
hCG + 17P
treatment

Figure 5.10. T production by ovarian follicles from snapper confined for 168 h. Follicles were incubated at room temperature for 24 h in L-15 medium alone, or with the addition of hCG (100 $U.ml^{-1}$), 17P (100 $ng.ml^{-1}$) or both hCG and 17P (doses as before). Values are means ± SE (n=6), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P < 0.05$). 1 - 4 = 4 separate fish.


168h
1
2
3
4
2000
1500
1000
500
0
600
400
200
450
300
150
a
b
c
T (pg.500 follicles-1)
control
hCG
17P
hCG + 17P
treatment

In fish confined for 72 h, treatment with hCG gave higher levels of T than did the control treatment in 1 fish, and treatment with 17P gave higher levels of T than did both control and hCG treatments in all fish (Fig. 5.9). Treatment with 17P and with hCG plus 17P gave similar levels of T in all but 1 of the 72 h-fish (when 17P levels were higher than with hCG plus 17P).

In fish confined for 168 h, 1 fish had higher T following hCG than after the control treatment (Fig. 5.10). The effects of 17P and hCG plus 17P on T levels were similar and greater than the effects of both the control and hCG treatment.

Oocyte diameters ranged from 0.3 to 0.6 mm, while the number of follicles per aliquot ranged from 500 to 800, with the exception of fish # 1 at 48 h and fish # 1 at 168 h which had 270.7 ± 7.2 and 234.5 ± 9.0 follicles respectively (Fig. 5.11). Due to the low nature of these follicle counts, the consequent extremely high steroid levels per 500 follicles and the unknown effect that widely varying counts might have on yields per follicle, these data were excluded from comparisons between confinement times. However, since the relative yields, for the different steroid treatments, of E_2 and T were similar to those of other fish, the anomalous fish were retained in the steroid comparisons described above.

Comparisons of the effects of different confinement times were based on the mean steroid levels of the individual fish (excluding the anomalous data) averaged across all fish subject to each of the 5 confinement durations. The data were examined separately for each of the hormone treatment conditions (See Fig. 5.12). In control and hCG treatments, there was no significant difference between E_2 levels at 0 h and at any other time, although E_2 levels at other times did differ among themselves viz. at 72 h pc E_2 levels were significantly higher than at 24, 48 and 168 h pc ($P<0.01$ for both; Fig. 5.12a,b). In 17P and the hCG plus 17P treatments, the pattern of the E_2 levels is similar to that in the control and hCG treatment, with the maximum at 72 h. However, none of the differences among times was statistically significant (Fig. 5.12c,d).

There was no significant difference in T levels between confinement times for control or hCG treatments (Fig. 5.13a,b), but there was for 17P, and for hCG plus 17P ($P<0.05$; Fig. 5.13c,d). Levels of T in controls were non-detectable at all times. At 48 h pc, T levels following the 17P treatment were less than at 0 h pc. At all other times T levels were similar to those at 0 h pc. At 24, 48 and 72 h pc, T levels following the hCG plus 17P treatment were less than those at 0 h pc, however by 168 h pc, T levels were not significantly different from those at 0 h pc.

Figure 5.11. Mean a) oocyte diameter and b) number of follicles per aliquot for *in vitro* experiments. Values are means ± SE (n=30 or 6 for a and b respectively). * indicates anomalous low follicle counts.

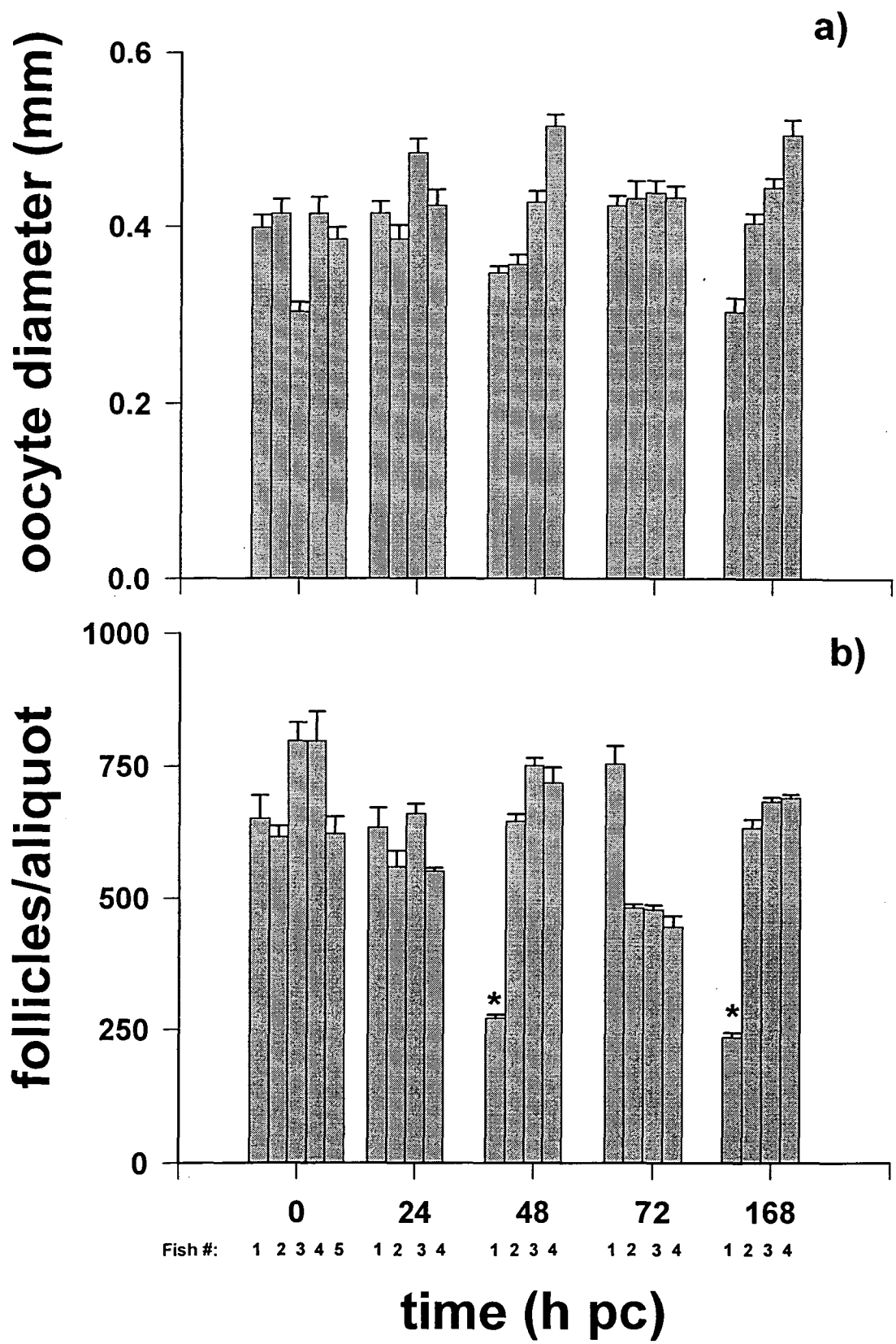

a)
oocyte diameter (mm)
0.6
0.4
0.2
0.0
b)
follicles/aliquot
1000
750
500
250
0
*
*
0
24
48
72
168
Fish #: 1 2 3 4 5 1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
time (h pc)

Figure 5.12. Comparison of E_2 production by ovarian follicles from snapper after confinement times of 0, 24, 48, 72 or 168 h pc. Follicles were incubated at room temperature for 24 h in L-15 incubation media and a) ethanol (10 µl), b) hCG (100 $U.ml^{-1}$), c) 17P (100 $ng.ml^{-1}$) or d) both hCG and 17P (doses as before). Values are means across all fish with the same duration of confinement ± SE (n = 3-5), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P<0.05$). No superscript indicates that means are not significantly different.

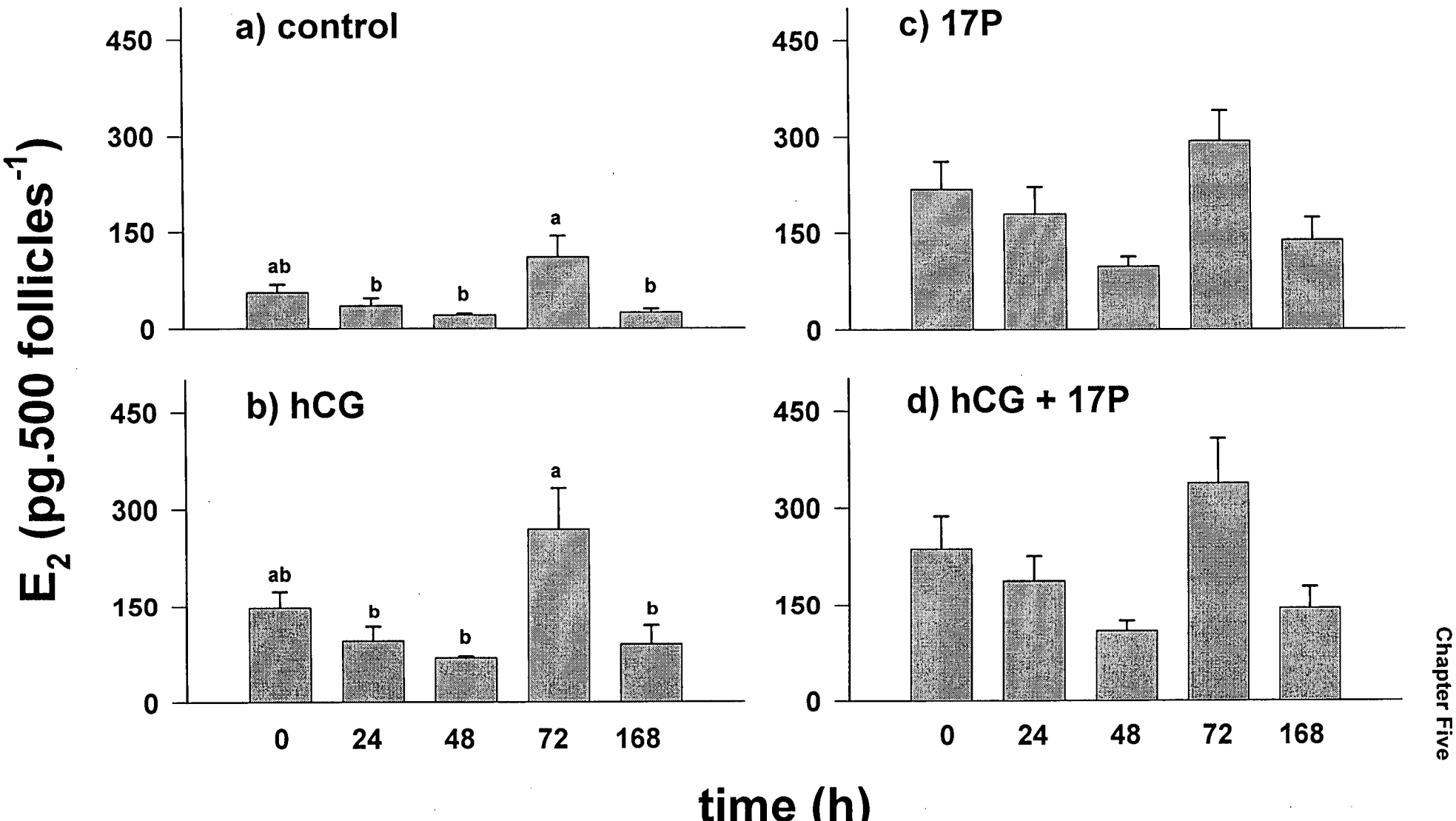

a) control
b) hCG
c) 17P
d) hCG + 17P
E_2 (pg.500 follicles^{-1})
450
300
150
0
ab
b
b
a
b
0
24
48
72
168
time (h)

Figure 5.13. Comparison of T production by ovarian follicles from snapper after confinement times of 0, 24, 48, 72 or 168 h pc. Follicles were incubated at room temperature for 24 h in L-15 incubation media and a) ethanol (10 µl), b) hCG (100 $U.ml^{-1}$), c) 17P (100 $ng.ml^{-1}$) or d) both hCG and 17P (doses as before). Values are means across all fish with the same duration of confinement ± SE (n = 3-5), expressed as $pg.500\ follicles^{-1}$. Different superscript letters indicate significantly different Tukey-Kramer HSD groupings ($P<0.05$). No superscript indicates means are not significantly different.

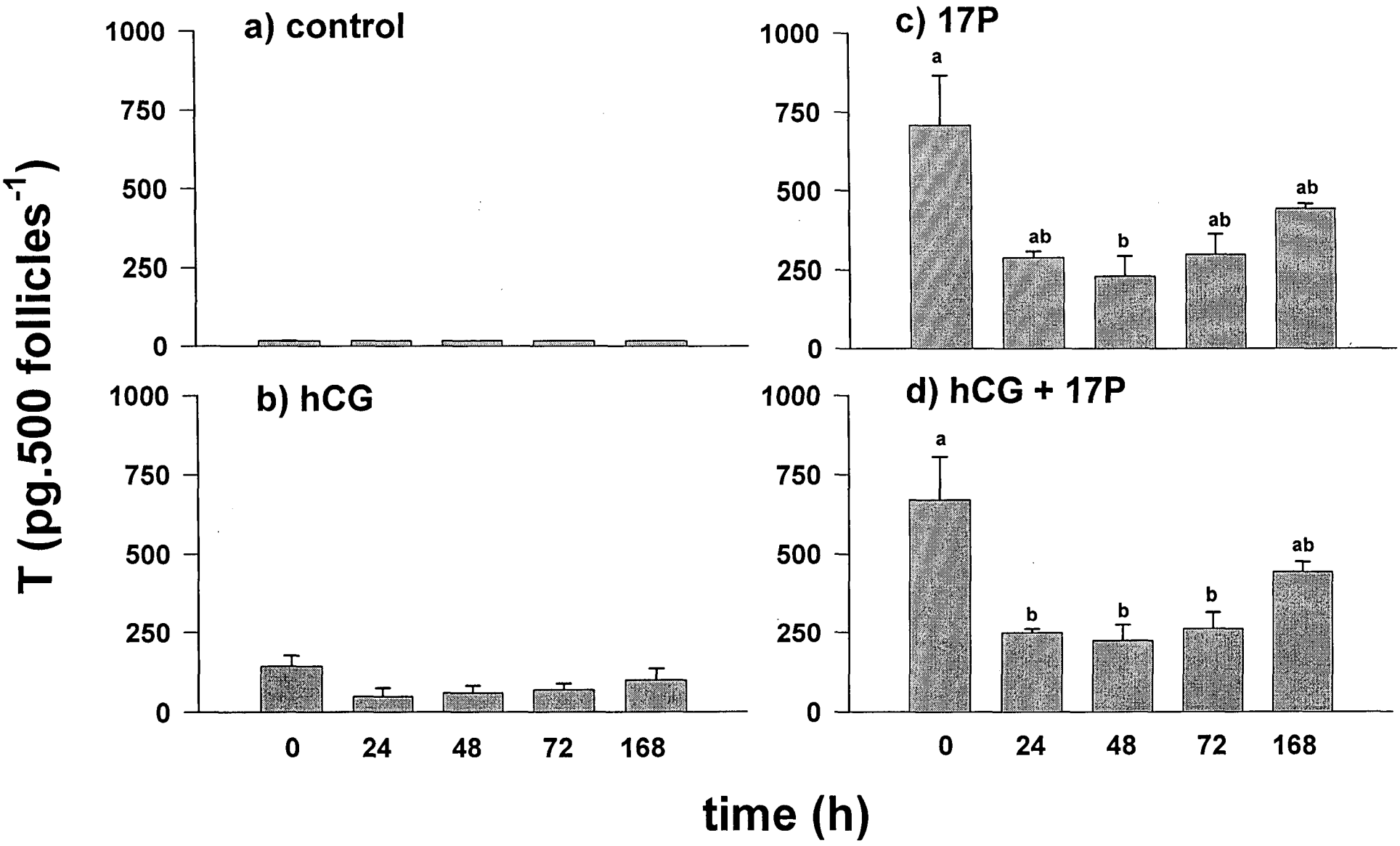
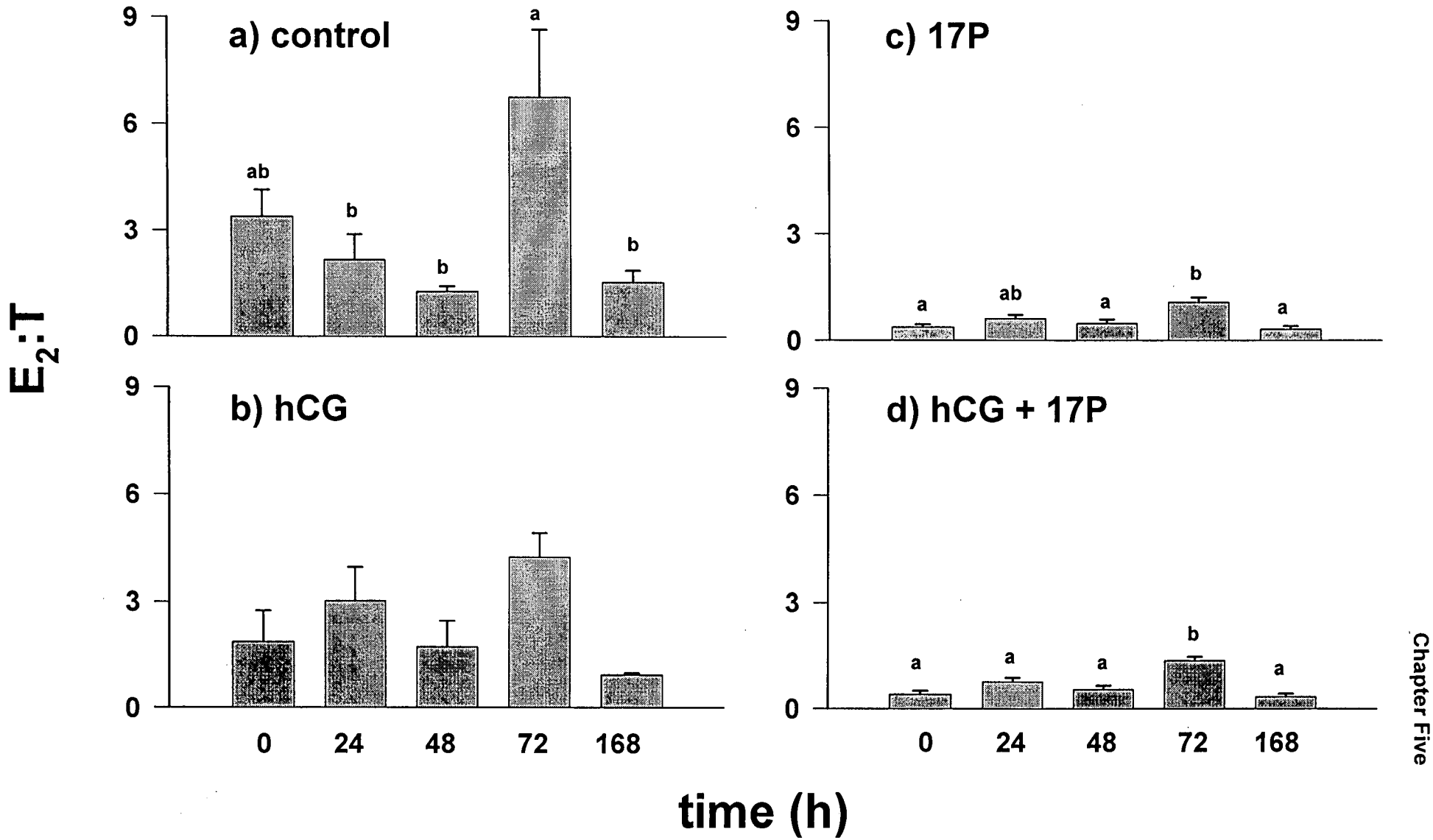

a) control
b) hCG
c) 17P
d) hCG + 17P
T (pg.500 follicles-1)
1000
750
500
250
0
a
ab
b
ab
ab
a
b
b
b
ab
0
24
48
72
168
time (h)

Figure 5.14. Comparison of the ratio of E_2 to T production by ovarian follicles from snapper after confinement times of 0, 24, 48, 72 or 168 h pc. Follicles were incubated at room temperature for 24 h in L-15 incubation media and a) ethanol (10 µl), b) hCG (100 $U.ml^{-1}$), c) 17P (100 $ng.ml^{-1}$) or d) both hCG and 17P (doses as before). Values are means across all fish with the same duration of confinement ± SE (n = 3-5). Different letters indicate significantly different Tukey-Kramer HSD groupings ($P<0.05$). No superscript indicates means are not significantly different.


a) control
b) hCG
c) 17P
d) hCG + 17P
E2:T
time (h)
0
24
48
72
168
3
6
9
ab
b
a

The mean E_2:T ratio ranged from 1.5 to 6.7 for the control condition, with E_2:T at 72h, being significantly higher than at 24, 48 and 168 h pc (P=0.009; Fig. 5.14a). E_2:T for the hCG treatment ranged from 0.90 to 4.22, and there was no significant difference among times (Fig. 5.14b). The ratio of E_2:T for the 17P treatment ranged from 0.31 and 1.01, and was significantly higher at 72 h pc, than at 0, 48, and 168 h pc (P=0.002; Fig. 5.14c). In the hCG plus 17P treatment, E_2:T ranged from 0.3 to 1.3, and was significantly higher at 72 h pc than at all other times (P<0.0001; Fig. 5.14d).

Mean gonadosomatic Index (GSI) (± SE) of fish used in the experiment was 1.28 ± 0.19 %; hepatosomatic index (HSI) was 1.61 ± 0.08 %; fork length (FL) was 321 ± 5 mm; and whole body weight was 735 ± 33 g. There was no significant difference among treatments for GSI, HSI, FL or whole body weight, consistent with absence of bias in allocation of fish to treatments (Appendix E).

Plasma steroids

Plasma levels of E_2 and T at first capture were very low with the majority being non-detectable. Plasma E_2 at first capture was only detectable in the 0, 24 and 168 h groups, and these were all less than 0.25 $ng.ml^{-1}$ (Fig. 5.15a,b). There was no significant change in levels of E_2 in blood samples. T was at or near detection limit for all samples. Mean plasma cortisol levels at first capture ranged from 0.6 to 5.2 $ng.ml^{-1}$ and there was no significant difference among times or between first and corresponding second blood samples in any group (Fig. 5.15c).

Histology

Primary oocytes comprised between 83 and 91 % of all previtellogenic oocytes, while normal cortical alveoli oocytes comprised between 8 and 14 % (Fig. 5.16a). There was no difference between confinement times for primary oocytes (data not shown) nor for normal cortical alveoli oocytes. The proportion of atretic cortical alveoli oocytes was between 0.3 and 5.2 % of previtellogenic oocytes with 168 h fish having significantly more atretic cortical alveoli oocytes than 1, 24 and 72 h fish (P=0.001) (Fig. 5.16b,c). Normal early vitellogenic oocytes constituted 14 to 37 % of all vitellogenic oocytes, while atretic early vitellogenic oocytes constituted 3 to 33 % (Fig. 5.15d). There appeared to be more normal and fewer atretic early vitellogenic oocytes in 0 h fish and fewer normal and more atretic early vitellogenic oocytes in 168 h fish but the trend was not significant (P=0.061, (d.f.=4); P=0.214, (d.f.=4) respectively). The proportion of normal late vitellogenic oocytes ranged from 19 to 46 % of all vitellogenic oocytes, while atretic late vitellogenic oocytes ranged from 22 to 52 % (Fig. 5.15e,f). There was no difference among times for normal or atretic late vitellogenic oocytes.

Figure 5.15. Plasma concentrations of a) E_2, b) T and c) cortisol in fish used for *in vitro* experiments after confinement for 0, 24, 48, 72 or 168 h (grey bars). Values are means + SE (n= 4-5). White bars show first capture values for fish which were subsequently confined. There were no significant differences among first bleeds, or between first and corresponding second bleeds at each time ($P<0.05$).

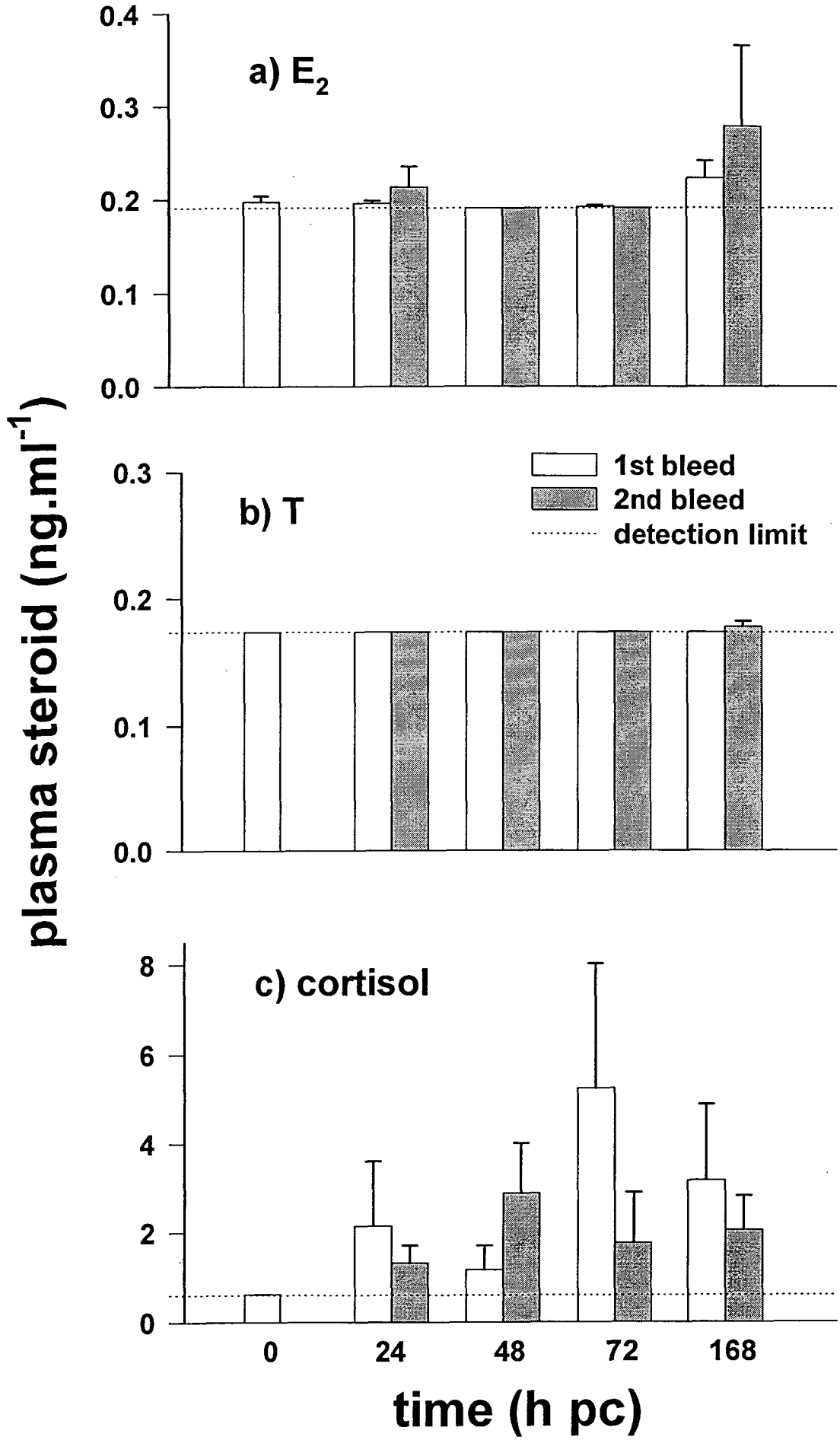
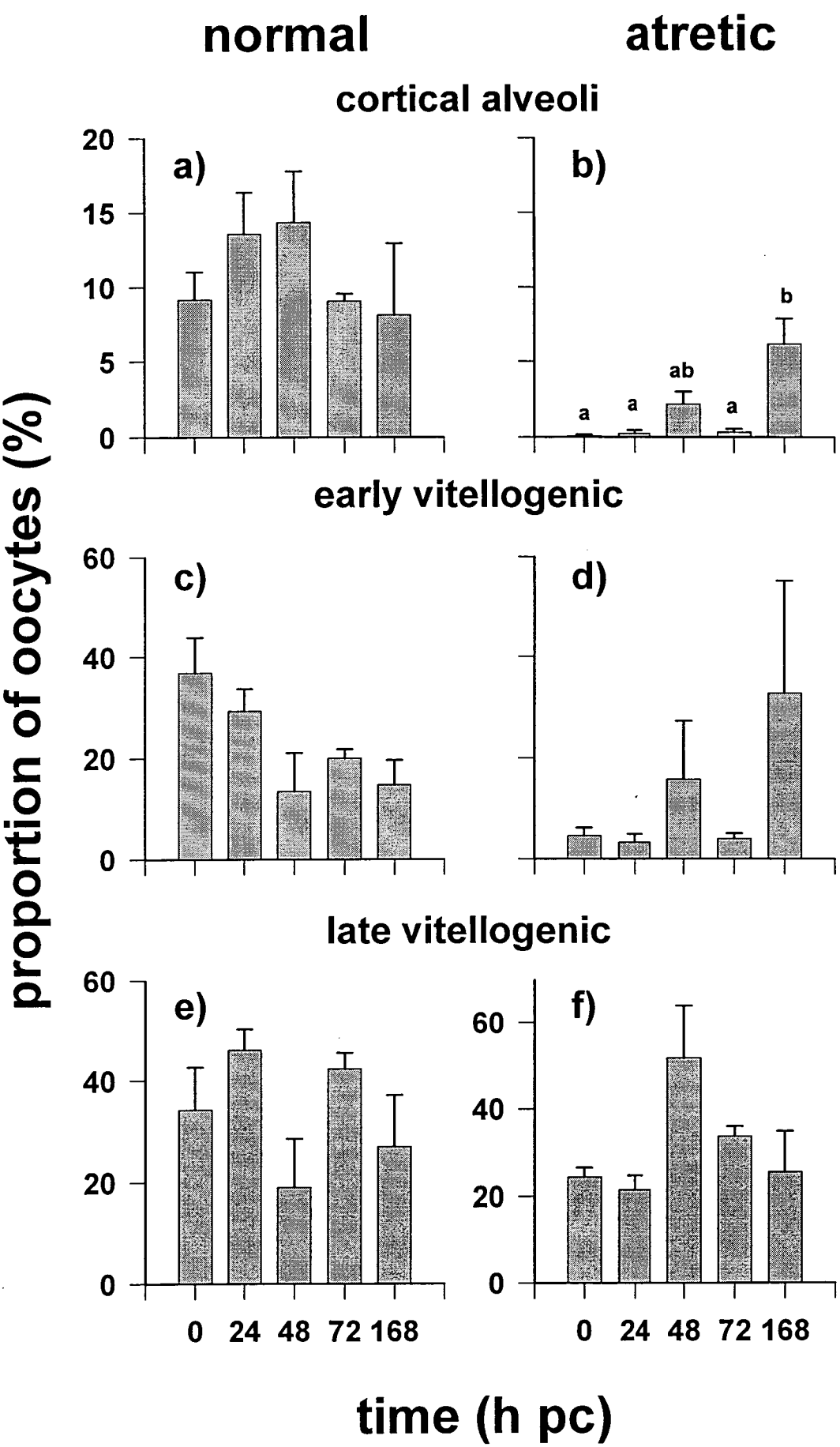

a) E_2
b) T
c) cortisol
1st bleed
2nd bleed
detection limit
plasma steroid (ng.ml^{-1})
time (h pc)
0.4
0.3
0.2
0.1
0.0
8
6
4
2
0
0
24
48
72
168

Figure 5.16. Proportion of a) normal and b) atretic cortical alveoli oocytes, c) normal and d) atretic early vitellogenic and e) normal and f) atretic late vitellogenic oocytes in histological cross-sections of ovaries from female snapper confined for 0, 24, 48, 72 or 168 h. Counts are expressed as a percentage of total previtellogenic oocytes (cortical alveoli) or of 100 vitellogenic (early and late vitellogenic) (mean ± SE; n = 4- 5). Different letters show significantly different Tukey-Kramer HSD groupings between treatments for each oocyte type ($P < 0.05$). No superscript indicates means are not significantly different.


normal
atretic
cortical alveoli
early vitellogenic
late vitellogenic
proportion of oocytes (%)
a)
b)
c)
d)
e)
f)
20
15
10
5
0
60
40
20
a
a
ab
a
b
0 24 48 72 168
time (h pc)

3.4 Discussion

HCG at a concentration of 10 $U.ml^{-1}$ failed to significantly stimulate E_2 production from vitellogenic New Zealand snapper follicles, however, T production was stimulated (Pankhurst *et al*, 1995b). In the present study, Australian snapper follicles were found to be sensitive to hCG at the higher concentration of 100 $U.ml^{-1}$, with increased production of E_2 and to a lesser extent T for all fish, regardless of confinement times. This is consistent with effects *in vivo*, since Pankhurst and Carragher (1992) found that hCG significantly increased plasma T and E_2 by 6 and 24 h post-injection respectively. The ability of the snapper follicles to respond to hCG-stimulation in the present study, despite capture, handling and various confinement periods, demonstrates that GtH binding with follicle cells and subsequent signal transduction processes are active, and that activation of steroid converting enzymes and mobilisation of substrate can occur even following stress episodes.

HCG has variable potency among teleosts. Incubation of vitellogenic goldfish follicles in hCG at 1 $U.ml^{-1}$ stimulated E_2 production (but not T) (Kagawa *et al*, 1984), however, at 10 $U.ml^{-1}$ both E_2 and T production were stimulated (Kagawa *et al*, 1984; Pankhurst *et al*, 1995b). At 10 $U.ml^{-1}$, hCG stimulated E_2 (but not T) production in amago salmon (*Oncorhynchus rhodurus)* follicles (Kagawa *et al*, 1982), while T (but not E_2) production was stimulated in striped trumpeter *(Latris lineata)* follicles (Pankhurst, 1997). In contrast hCG at concentrations of 10 $U.ml^{-1}$ did not stimulate either E_2 or T production in New Zealand long-finned eel *(Anguilla dieffenbachii;* Lokman and Young, 1995) or carp (Pankhurst *et al*, 1995b) follicles. HCG at a dose as high as 100 $U.ml^{-1}$ was required to stimulate E_2 production (but not T) in rainbow trout (Haddy and Pankhurst, 1997) and in amago salmon (Young *et al*, 1983). Clearly, there is broad variation in sensitivity to hCG stimulation among species. It must be noted, however, that there is also considerable variation with stage of development of the oocyte. Kagawa *et al* (1984) found greater sensitivity to hCG (in terms of E_2 production) in mid-vitellogenic follicles, than early and late vitellogenic follicles. Therefore the above species comparison may be clouded by unidentified differences in oocyte stage, as most researchers classified follicles as only "vitellogenic" not "early-, mid-, or late-vitellogenic". This could explain the apparent disparity in hCG-sensitivity between the two studies on amago salmon listed above (Kagawa *et al*, 1982; Young *et al*, 1983). The sensitivity to a particular GtH appears to depend on the degree of similarity to native GtH (reviewed in Pankhurst, 1997). It is possible that a piscine GtH, if used in the present study may have increased the follicular response relative to hCG, depending on the similarity of the piscine GtH in question to native snapper GtH. The relative efficacy of hCG and piscine GtHs in stimulating steroidogenesis in snapper follicles has not been determined.

In the present study, both basal and hCG-stimulated production of T was lower than respective E_2 production: basal T levels were undetectable in all fish; hCG stimulated an increase in T above basal levels in only 38 % of fish (compared with 100 % for E_2); and E_2:T in control and hCG-treated follicles ranged between 1 and 7. The low T levels indicate that T was converted to E_2 as rapidly as it was produced. Similarly, fragments of early vitellogenic winter flounder (*Pseudopleuronectes americanus)* ovary spontaneously produced at least four times as much E_2 as T, while homologous pituitary extract (PE) significantly increased *in vitro* yields of E_2 and to a lesser extent, T (Nagler and Idler, 1992). In ovaries from late vitellogenic winter flounder, however, both basal and PE-stimulated T production was higher than E_2 production (Nagler and Idler, 1992). Previtellogenic follicles of striped trumpeter produced T levels which were more than 4 times lower than that of basal E_2, and hCG-stimulated previtellogenic follicles had T levels which were 3 to 8 times lower than E_2 levels (Pankhurst, 1997). In contrast, however, in vitellogenic oocytes of striped trumpeter, basal and hCG-stimulated T production was either similar to or higher than E_2 production (Pankhurst, 1997). A similar occurrence to the present study has been reported for female New Zealand snapper (Pankhurst *et al*, 1995b) and rainbow trout (Carragher and Sumpter, 1990) where media containing small oocytes did not spontaneously accumulate T, however larger oocytes produced detectable E_2 and T. It is likely that the non-detectable T levels in the present study are due to the fact that these fish were quite early in vitellogenesis as demonstrated by the small follicle sizes (300 to 500 :m), low GSI (0.4 to 2.6) and low plasma T concentrations at first capture (<0.25 $ng.ml^{-1}$). Therefore production of T is a limiting step in the production of E_2 in early vitellogenic snapper.

17P stimulated both E_2 and T production in all fish in the present study. 17P-stimulated production of E_2 was significantly higher than hCG-stimulated production in 57 % of fish, while T was higher in all fish. This indicates that the enzymes necessary for the conversion of 17P to T to E_2 are present in the unstimulated follicle, despite capture, handling and various confinement periods. Similar effects have been reported for conversion of T to E_2 for New Zealand snapper (Pankhurst *et al*, 1995b), T to E_2, 17P to T for vitellogenic striped trumpeter follicles (Pankhurst, 1997), 25-hydroxycholesterol to T and E_2 (Pankhurst *et al*, 1995b) and T to E_2 (Kagawa *et al*, 1984) in goldfish. Although as acknowledged above, a piscine GtH may have a better stimulatory response than hCG, the greater steroidogenic response to 17P than hCG appears to indicate that production of E_2 and T *in vitro* is more strongly limited by substrate availability, and less by rates of enzymatic conversion of steroid precursors. The low and similar E_2:T ratios in 17P treatments, in the present study, further support this, since adding substrate (17P) did not result in an increase in E_2 production relative to T (i.e. an increase in E_2:T ratio), but rather a 3-fold decrease. The added 17P saturated aromatase activity, resulting in maximal E_2 production and an increase in accumulation of T, and hence a lowering of the E_2:T ratio. Therefore E_2 production is less sensitive than T production to substrate availability, indicating that aromatase (converting T to E_2) is saturated at lower substrate concentration than 17,20-lyase and/or 17α-hydroxylase.

HCG did not augment conversion of 17P to E_2 or T in the present study. Similar lack of augmentation by hCG has been reported for conversion of T to E_2 for New Zealand snapper and goldfish (Pankhurst *et al*, 1995b), T to E_2 and 17P to T for striped trumpeter (Pankhurst, 1997) and conversion of 25-hydroxycholesterol, pregnenolone and progesterone to T and E_2 in killifish (Petrino *et al*, 1989a,b; 1990). In contrast, conversion of T to E_2 by early-vitellogenic goldfish follicles was augmented by hCG, but not in mid- or late- vitellogenic follicles (Kagawa *et al*, 1984). Similarly Nagahama *et al* (1991) found that pregnant mare serum gonadotropin (PMS) augmented conversion of T to E_2 in early vitellogenic follicles of medaka (*Oryzias latipes*). These authors suggest that this may be due to the direct action of hCG on aromatase activity. Similarly Petrino *et al* (1989a,b) suggested that GtH, in addition to acting directly on mobilisation of cholesterol, may possibly increase aromatase activity. The present study shows no evidence of increased aromatase activity from hCG stimulation, since the E_2:T ratio in hCG treatments was similar if not slightly lower than control treatments, and E_2:T ratio in hCG plus 17P treatment was the same as for 17P alone. This indicates that the primary action of GtH is to increase substrate availability (Yaron *et al*, 1995). The lack of augmentation in the present study (and in others) is likely to be due to the large volumes of substrate used, swamping any GtH-induced increase in substrate availability.

Stress has been found to decease plasma E_2 levels in females of a variety of species including red gurnard *Chelidonichthys kumu* (Clearwater and Pankhurst, 1997), spotted seatrout *Cynoscion nebulosus* (Safford and Thomas, 1987), rainbow trout (Pankhurst and Dedual, 1994), New Zealand snapper (Carragher and Pankhurst, 1991) and Australian snapper (Chapter 3, this volume). The mechanism by which stress has its effect is unknown, however Carragher and Sumpter (1990) found that cortisol could suppress E_2 production from rainbow trout follicles. The concentration of cortisol required to elicit an effect, however, was sometimes as high as 1000 $ng.ml^{-1}$ (10-fold higher than physiological levels). The authors postulated that since E_2 and T were both suppressed by cortisol, that cortisol may have its affect either by inhibiting synthesis of a precursor in the early part of the steroidogenic pathway, or by generally slowing enzyme activity. In the present study, however, capture and handling stress did not decrease production of E_2 *in vitro*, as was expected from the stress-induced reduction in plasma steroid levels previously observed (Chapter 3, this volume). Instead, at all times E_2 levels were similar to those at 0 h pc in all treatments. Therefore, the effect of stress on plasma reproductive steroid levels is not the result of impaired aromatase activity. Further, this indicates that the action of stress is possibly to inhibit synthesis of precursor for T and E_2 synthesis. It is unlikely that stress is acting by generally slowing the conversion rates of steroidogenic enzymes, since the activity of aromatase was maintained throughout the stress episode.

Capture and handling stress markedly decreased the accumulation of T *in vitro* by 24 and 48 h pc in response to treatment with hCG plus 17P, and 17P respectively. This indicates that the conversion of 17P to T (by 17α-hydroxylase and 17,20-lyase), unlike conversion of T to E_2 (by aromatase) is affected by stress. The decrease in T concentrations, but the maintenance of E_2, in 17P and hCG plus 17P treatments in the present study, suggests that aromatisation of T to E_2 occurs more rapidly than T synthesis in stressed fish, resulting in reduced T concentrations. This could occur via reduced synthesis of T and/or increased conversion of T to E_2. Increased conversion of T to E_2, is unlikely, since this would have resulted in an increase in E_2 concentrations and hence the E_2:T ratio, which was not found in the present study. Therefore a reduction in the rate of synthesis of T is more likely. Such a reduction could occur both through a decrease in conversion of 17P to T (i.e. reduced activity of 17α-hydroxylase and/or 17,20-lyase), or through an increase in conversion of 17P to 17,20βP (i.e. increased activity of 20β-hydroxysteroid dehydrogenase; 20β-HSD), thus reducing available 17P for conversion to T. When the large amount of 17P available in the 17P and hCG plus 17P treatments is considered, it seems unlikely that the latter mechanism is operating *in vitro* since even a large increase in the activity of 20β-HSD would be unlikely to deplete 17P. However, 17,20βP was not measured in the present study (due to media limitations and the early stage of vitellogenesis of the fish), but *in vivo,* plasma 17,20βP tends to increase in the initial stages following stress (Chapter 3, this volume). Therefore, the possibility of increased 20β-HSD activity decreasing 17P levels cannot be totally discounted. A more likely scenario, however, is a decrease in the rate of conversion of 17P to T. The finding by Carragher and Sumpter (1990) that cortisol decreased *in vitro* production of T in rainbow trout follicles lends general support for this, since this could have occurred via a decrease in conversion of 17P to T. However it must also be acknowledged that disruptions further upstream in the conversion pathway could also result in a similar effect. It appears therefore, that the effect of stress is unlikely to be due to a general slowing down of enzymatic conversion but rather, varying effects on different parts of the steroidogenic pathway. This effect is consistent with the action of stress being mediated, at least in part, by the effect of cortisol since differential effects of cortisol on specific hormones have been described previously. Carragher *et al* (1989) found that cortisol implantation in maturing male brown trout decreased plasma T levels, but not 11KT. They suggested that enzymes of the steroidogenic pathway may have different sensitivities to cortisol.

Given that in all species so far studied, including snapper (Chapter 3, this volume), stress results in decreases in plasma levels of both T and E_2, the apparent maintenance of aromatase activity and hence E_2 synthesis would seem paradoxical. As described above, 17P is limiting *in vitro*. If *in vitro* experiments reflect the situation *in vivo*, and therefore 17P is also limited *in vivo*, then inhibition of either 17α-hydroxylase and/or 17,20-lyase activity by stress, would lead to a decrease in plasma T and then E_2 *in vivo* as substrate was exhausted. *In vitro*, a decrease in E_2 was presumably not observed because T production was still adequate to support conversion to E_2 due to the adequate supply of 17P.

In the light of the possible direct action of cortisol on ovarian steroidogenesis in salmonids (Sumpter *et al*, 1987; Carragher and Sumpter, 1990), Pankhurst *et al* (1995b) investigated the effect of cortisol on ovarian steroidogenesis in goldfish, carp and snapper, and found no evidence of direct inhibition of ovarian production of E_2 or T by cortisol in any of the three species. In New Zealand snapper, cortisol did not inhibit hCG or T stimulated E_2 or T production, and actually enhanced production of E_2 stimulated by hCG plus T. Although in the present study capture and handling stress did not result in an increase in E_2, the lack of inhibition is consistent between studies. In vitellogenic rainbow trout follicles treated with 5 $ng.ml^{-1}$ of cortisol there was also a significant increase in E_2, concomitant with a significant decrease in T (Carragher and Sumpter, 1990). These data are also consistent with the effect of stress described in the present study, in that T showed the greatest inhibition, and E_2 levels were not inhibited. Cortisol at higher concentrations of 25-3125 $ng.ml^{-1}$, did however, inhibit both T and E_2 production in rainbow trout follicles (Carragher and Sumpter 1990). Therefore although it seems that cortisol can mimic the effects of stress to some extent on some occasions, it would seem unlikely that the effect of stress is mediated by simple direct inhibition by cortisol of enzymatic activity. This does not discount the possibility of indirect action by cortisol or alternatively, the involvement of different stress factors. The possibility of other stress hormones, such as catecholamines, adrenocorticotrophic hormone (ACTH), α–melanocyte stimulating hormone (α-MSH), and endorphins having a role in inhibition of reproductive function has not been widely addressed (Pankhurst and Van Der Kraak, 1997). Sumpter *et al* (1987) found that ACTH had no effect on basal or GtH-stimulated E_2 production by rainbow trout follicles and preliminary observations indicate that mammalian α-MSH and β-endorphin have no effect on *in vitro* E_2 production by rainbow trout follicles (N.W.Pankhurst unpublished data). It is of course possible that the effect of stress on ovarian steroidogenesis may be mediated by a combination of different stress factors, acting on different components of the steroidogenic pathway.

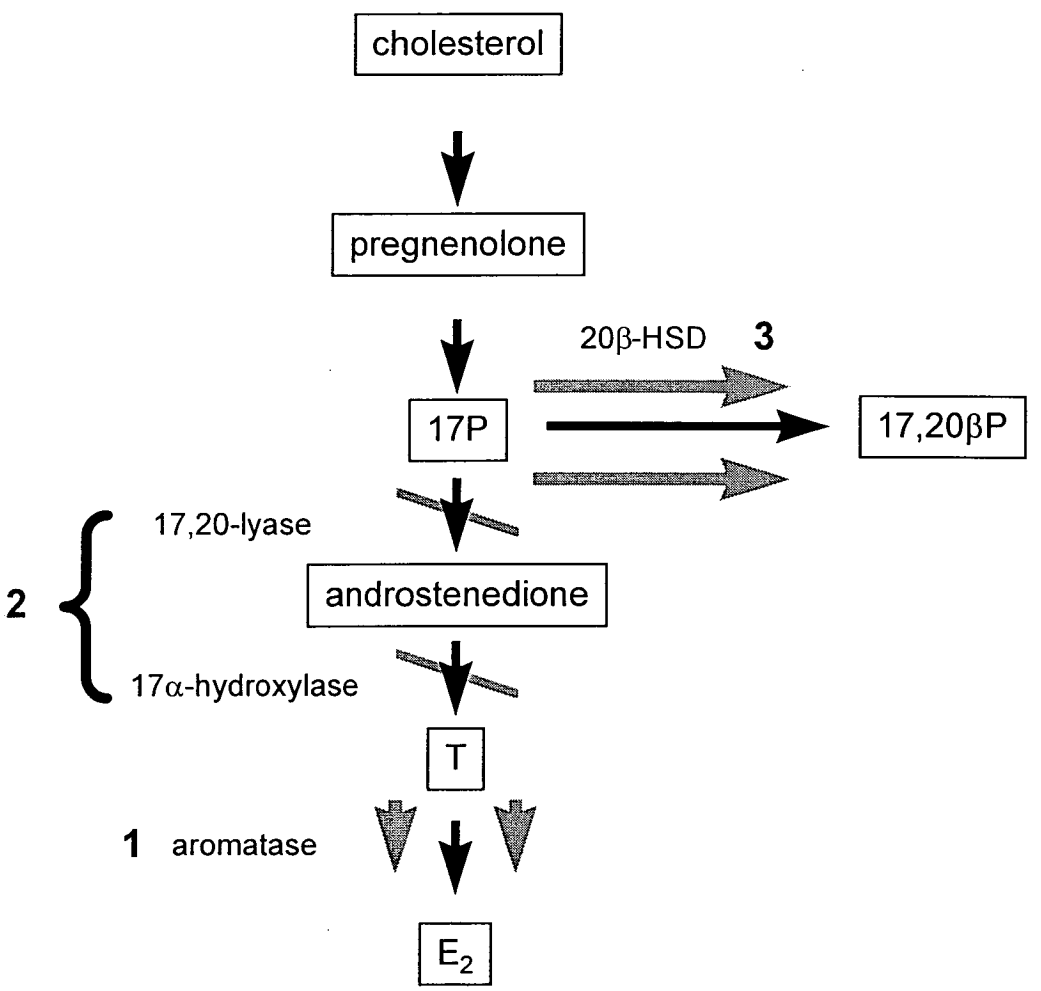
Plasma levels of E_2 and T in the present study were extremely low, with maximum E_2 and T levels being less than 0.23 $ng.ml^{-1}$. In similar 3-year-old hatchery fish sampled in 1995, initial mean plasma E_2 and T levels were approximately 2.5 and 1 $ng.ml^{-1}$ respectively (Chapter 3). Although the fish in the present study were 3 years old, their steroid profile more closely resembles that of the 2-year-old hatchery fish described in Chapter 3, which had maximum E_2 and T of approximately 0.6 and 0.2 $ng.ml^{-1}$ respectively. There are two possible explanations for this: that the fish were already suffering from stress-impairment of reproduction due to some unknown factor, or that the fish used in the present study sexually matured later than the 3-year-old fish described in Chapter 3, and were still in the early stages of vitellogenesis. It is more plausible that the later scenario applies, since GSI for fish in the present study were approximately 4 times less than for the 3-year-old fish described in Chapter 3, and more closely resembled those of the 2-year-old fish. This may have implications for the interpretation of the data described in the present chapter. However, given that E_2 and T were both produced *in vitro* in the present study, and assuming that the pathway of steroidogenesis of E_2 and T in fish in the early stages of maturity are similar to those in later stages, it is reasonable, although not necessarily optimal, that these ovarian follicles be used to measure the effects of stress on steroidogenic pathways.

Plasma cortisol levels were low (<10 $ng.ml^{-1}$) throughout the experiment. This is similar to both 2- and 3-year-old hatchery fish described in Chapter 3, where all mean cortisol levels at 0, 24, 48 and 168 h of confinement were less than 10 $ng.ml^{-1}$. The low plasma cortisol levels measured in the present study are indicative of the fish having recovered to some extent in the laboratory environment by 24 h pc. Based on results described in Chapter 3 (this volume), where changes in plasma cortisol were measured on a finer scale (at 1 and 6 h pc, in addition to sampling times used in the present study), and were found to increase above 10 $ng.ml^{-1}$ between 6 and 24 h pc, it is likely that the sampling regime in the present study was too coarse to detect early changes in cortisol following capture and handling stress. Therefore it can be assumed that plasma cortisol did in fact increase initially, since, it is highly unlikely that the capture and handling protocol employed in this study was any less stressful than the same protocol imposed on the fish in Chapter 3.

In the present study, the proportion of atretic cortical alveoli oocytes was significantly higher in fish confined for 168 h than in unstressed (0 h) fish, with approximately 6 % of all previtellogenic oocytes being atretic cortical alveoli stage by 168 h pc. This is similar to the proportions observed in pool, 2-year-old and 3-year-old hatchery females described in Chapter 3 (this volume). Of the groups of fish described in Chapter 3 (this volume), only 3-year-old hatchery fish showed a similar trend of an increase in the incidence of atretic cortical alveoli stage oocytes with confinement time, although this was not significant. In the present study, early vitellogenic oocytes showed some evidence of a trend of increasing atresia with confinement time. Similar trends were observed for female pool, 2-year-old and 3-year-old hatchery fish described in Chapter 3 (this volume). These results lend further support to the stressful nature of the capture, handling and confinement imposed on the fish in the present study. As discussed in Chapter 3 and 4 (this volume), apoptosis is modulated by GtH and E_2 (Janz and Van Der Kraak, 1997). Therefore the observed increase in atresia in cortical alveoli and early vitellogenic oocytes in the present study is evidence that follicular integrity is compromised, presumably via stress-induced changes to reproductive processes.

Capture and handling stress does affect ovarian steroidogenesis in snapper with a marked decrease in 17P-stimulated T production. This suggests that at least part of the observed stress-induced decrease in plasma reproductive steroid levels are probably due to the direct effect of stress on the ovary (Fig. 5.17). The mechanism by which this occurs is unknown, however, it would appear that it is unlikely to be via the direct action of cortisol. In order to elucidate the mechanism(s) involved in stress-induced impairment of ovarian steroidogenesis, further *in vitro* studies are required, however the focus must be broadened to include other stress factors. The observed decrease in plasma steroid levels are likely to be affected not only by ovarian steroidogenesis, but also potentially by rates of steroid metabolism, conjugation and/or protein binding dynamics.

Figure 5.17. Possible mechanisms producing differential effects of stress on accumulation of E_2 and T in *in vitro* incubations of isolated ovarian follicles: 1) increase in aromatase activity; 2) decrease in 17α-hydroxylase and/or 17,20-lyase activity; and/or 3) increase in 20β-HSD. See text for abbreviations. Gray lines indicate proposed effect of stress on enzymatic activity.


cholesterol
pregnenolone
20β-HSD
3
17P
17,20βP
17,20-lyase
2
androstenedione
17α-hydroxylase
T
1
aromatase
E2

Chapter 6: General Discussion.

Successful propagation of fish requires collection of viable gametes from freshly-caught mature wild fish, from acclimated wild fish, or from hatchery-reared fish. In order to obtain fertilised eggs, it is necessary that the fish have matured, ovulated/spermiated, and spawned. Under culture conditions, it is common that normal maturation through to spawning does not occur. This problem appears to be greatest in females, and hence females have received most research. Because of this concern, and because this thesis focuses mainly on female reproduction, the following discussion reflects this bias. Culture-induced interruptions to normal male reproduction, however, have been documented. For example, plasma testosterone and 11-ketotestosterone (11KT) decreased following both acute and chronic stress in rainbow trout (Pickering *et al*, 1987) and Australian snapper (Chapter 3, this volume). The fact that successful spermiation is more common than successful ovulation in captivity, may be related to the fact that following stress, plasma 17,20β dihydroxy-4-pregnen-3-one (17,20βP) levels commonly temporarily increase (e.g. Australian snapper; Chapter 3 this volume). Since 17,20βP advances sperm maturation (reviewed in Pankhurst, 1994), sperm production is consequently increased. In Chapter 3 (this volume) it was found that the proportion of spermatozoa in the testes increased with increasing periods of confinement. Although Campbell *et al* (1992), found that spermatocrit was significantly reduced in stressed male trout and that larvae resulting from stressed males and females suffered significantly higher larval mortality up to 28 days post-hatch, the quality (i.e. ability to fertilise) of sperm produced by stressed males has not been specifically examined.

Bottlenecks in egg production

There are three main bottlenecks which prevent successful reproduction in captivity: failure to complete vitellogenesis; failure to undergo final oocyte maturation (FOM) and failure to spawn (reviewed in Pankhurst, 1998). Each of these problems implies a specific cause, and hence management solution.

Failure to Complete Vitellogenesis:

Failure to complete vitellogenesis may be caused primarily by culture-induced stress, or inappropriate environmental cues. Completion of vitellogenesis depends on normal functioning of vitellogenic machinery including synthesis of gonadotropin releasing hormone (GnRH) and gonadotropin (GtH), GnRH and GtH receptors, ovarian steroidogenesis, and consequent synthesis of vitellogenin. Failure to complete FOM is common during attempts to acclimate wild-caught fish (Pankhurst, 1998). Fish can appear to have acclimated, in terms resumption of feeding, normal behavior patterns and good growth rates, however, still fail to complete vitellogenesis. This syndrome is characterised by falling plasma gonadal steroid levels and an increased incidence of oocyte atresia (De Montalembert *et al*, 1978; Stacey *et al*, 1984; Carragher and Pankhurst, 1991; Pankhurst and Carragher, 1992; Clearwater and Pankhurst, 1997).

Captive stocks of wild-caught Australian snapper described in Chapter 3 (this volume) are a classic example of incomplete vitellogenesis. Although fish caught as juveniles have not only survived, but also exhibited good growth rates and hence are apparently adjusted to the artificial environment (Bell *et al*, 1991), they have failed to complete vitellogenesis, despite 5 years in which to acclimate (Chapter 3, this volume). Plasma gonadal steroid levels were more than 5 times lower in these undisturbed 5-year-old captive fish, than in 3-year-old hatchery-reared fish. Furthermore, under photo-therm manipulation, captive wild fish (caught as adults) have not completed vitellogenesis, despite being held in optimal conditions with minimal disturbance for 2 consecutive seasons (G.L. Allan and S. Fielder, NSW Fisheries, Port Stephens Research Centre, pers com). New Zealand snapper and red seabream are also reported to show problems in reproductive acclimation of wild caught fish (Foscarini, 1988; Pankhurst and Carragher, 1992; Bollard *et al*, 1993). Wild-caught snapper, however, have been observed to spawn in captivity in large public aquaria (Smith, 1986; N.W. Pankhurst, University of Tasmania, pers com). The size of the tank required, however, precludes their use in farming situations. Therefore, for practical purposes, wild-caught snapper do not acclimate to the culture environment, even when caught as juveniles, and held for several years under optimum conditions with minimal disturbance.

Where failure to complete vitellogenesis is due to culture-induced stress, it can only be overcome by domestication of fish stocks. Attempts to hormonally induce ovulation using human chorionic gonadotropin (hCG), Luteinising Hormone Releasing Hormone analogue (LHRHa) and ovaprim in wild-juvenile-caught snapper, held in captivity for 5 years, generally resulted in a poor ovulatory response (Battaglene, 1995). In contrast, domestication can act to reduce the sensitivity of the fish to the culture environment, thereby reducing the incidence of atresia and hence increasing egg production. For example, wild salmonids show greater sensitivity to stress than domesticated counterparts, in terms of the magnitude and duration of stress-induced elevations of plasma cortisol, despite 7 months of acclimation (Salonius and Iwama, 1993). Improvement with successive generations of domestication have also been reported for red seabream, with fully domesticated fish spawning naturally in tanks, even when held at high densities (Foscarini, 1988). Domestication has also shown potential to improve the robustness of Australian snapper to stress, since plasma gonadal steroid levels in first generation snapper, showed a capacity to recover following stress and the ovulatory response to exogenous hormone therapy was greater than for wild-caught fish.

Failure to undergo final oocyte maturation:

Stress typically results in an increase in plasma 17,20βP and cortisol levels (e.g. Carragher and Pankhurst, 1991; Chapter 3, this volume). FOM is induced by 17,20βP in many species, including snapper (Scott and Canario, 1982; 1987; Kagawa *et al*, 1991; Ventling and Pankhurst, 1995). Since stress commonly elevates plasma 17,20βP levels it is unlikely that failure to undergo FOM is a consequence of stress. Furthermore, in wild salmon, spawning normally occurs when cortisol levels are at their highest, and hence fish are maximally

stressed, indicating that stress has not prevented FOM (Pankhurst and Dedual, 1994). It is more likely that failure to undergo FOM is due to inappropriate environmental conditions and/or absence of environmental cues (Pankhurst, 1998), such as rising temperature (Cyprinids, Stacey, 1984), changing day-length (damselfish; Goulet, 1995), water flow or level (tropical freshwater species; Moyle and Cech, 1996), or social interactions (African catfish, Baikal sculpin, zebra fish; reviewed in Stacey *et al*, 1994: goldfish; Stacey, 1991).

Failure to undergo FOM can be overcome in two ways: provision of necessary environmental conditions, or the use of exogenous hormones. The use of hormones, provides the fish with either exogenous GtH (e.g. hCG) to stimulate steroidogenesis, or GnRH (e.g. luteinising hormone releasing hormone analogue; LHRHa) to stimulate secretion of endogenous GtH and hence FOM. GnRH analogue (GnRHa) administered either by injection or in a slow release pellet, is commonly used to induce ovulation in fish that have completed vitellogenesis, but have failed to undergo FOM (Peter and Yu, 1997). In Chapter 4 (this volume), hatchery fish were successfully induced to spawn using both LHRHa and hCG injections and yielded better results than captive fish (Battaglene, 1995) and wild fish (Chapter 4, this volume). Provision of appropriate environmental conditions/changes allows natural cues to stimulate FOM. Having overcome the barrier of failure to complete vitellogenesis by domestication, first generation hatchery-reared Australian snapper, have been stimulated to undergo FOM, and spawning under photo-therm manipulation. Wild-caught fish held in identical conditions, although failing to undergo FOM, were suffering primarily from the failure to complete the preceding step of vitellogenesis (G.L. Allan and S. Fielder, NSW Fisheries, PSRC, pers com). Manipulation of environmental factors, rather than the use of hormones, provides the added advantage of a "hands free" management approach, thereby minimising further stress related reproductive problems.

Failure to spawn:

Spawning is not guaranteed following completion of oocyte development and ovulation. In captivity, spawning is largely triggered by the provision of, or imitation of critical environmental factors, in addition to the correct ratio of males to females. In some species, such as the spiny damselfish (*Acanthochromis polyacanthus;* Thresher, 1983), and cyprinids (Stacey, 1984), provision of suitable substratum such as real (or imitation) aquatic vegetation or caves may also be required to initiate the behavioral act of spawning. For other species, holding volume is critical. For example, in the gilthead seabream *Sparus aurata,* spawning occurs in holding volumes of greater than $4m^3$ (Zohar *et al*, 1995), while sea bass *Dicentrarchus labrax* require $10m^3$ or more (Carrillo *et al*, 1995). It is likely that holding volumes (and tank depth) are critical for natural spawning of snapper, since typical spawning behaviour involves chasing on the tank floor, followed by rush to the surface, where gamete release occurs (Smith, 1986). It is thought that the rapid change in pressure aids gamete expulsion (Smith, 1986).

In snapper, failure to spawn is not a problem for domesticated fish being held in 20000 L tanks under photo-therm manipulation. Both domesticated and wild fish, however, rarely spawn in tanks following exogenous hormone treatment, despite successful ovulation (Chapter 4, this volume). On all occasions, the treated fish were held in 4000 or 10000 L tanks following treatment; 2 to 4 times smaller than the photo-therm tanks. The small holding volumes of the hormone treated fish used in Chapter 4 (this volume), may have contributed to the lack of natural spawning and should be investigated further. When natural spawning fails to occur, the fish require manual stripping, which is labour intensive. Furthermore, manual stripping tends to result in eggs of poorer quality (Foscarini, 1988), since the chance of stripping outside of the period of peak viability is high. This chance is particularly high in fish with a brief period of post-ovulatory viability, such as snapper, in which the window is only 4 h (Scott *et al*, 1993; Hobby and Pankhurst, 1997b). In Chapter 4 (this volume), the possibility of stress altering the window of post-ovulatory viability and/or the time to ovulation post-injection was raised. This superimposes another level difficulty in choosing the appropriate stripping time. Therefore it is desirable that every effort be made to encourage natural spawning.

Stress Mechanisms

Despite considerable research effort, the mechanism by which stress exerts its affect on reproduction is still poorly understood. Until now, the role of cortisol has been the primary subject of investigation, with particular focus on the direct role of cortisol on ovarian steroidogenesis (Sumpter *et al*, 1987; Carragher and Sumpter, 1990; Pankhurst *et al*, 1995a;b). The results from this work have not been conclusive (see discussion Chapter 5, this volume). Therefore in Chapter 5 (this volume), the effect of stress from capture and handling of the whole fish (rather than just a single stress hormone) on ovarian steroidogenesis *in vitro* was examined. It was found that in contrast to cortisol alone (Pankhurst *et al*, 1995b), capture and handling stress did impair ovarian steroidogenesis in snapper, resulting in a decrease in accumulation of testosterone (T) in the media. It appears, therefore, that at least part of the effect of stress on plasma reproductive steroids is mediated via impairment of ovarian steroidogenesis. Furthermore, due to the difference between the effect of stress on *in vitro* ovarian steroidogenesis and the effect of cortisol (Pankhurst *et al*, 1995b), it is unlikely that stress-induced impairment of reproduction is mediated entirely by cortisol. Therefore, other stress hormones, such as α-melanocyte stimulating hormone (α-MSH), endorphins and adrenocorticotrophic hormone (ACTH) are implicated. These possibilities have not been fully assessed to date, however, clearly are areas worthy of further research, in order to elucidate the mechanism(s) involved in stress-induced impairment of reproduction. The focus must be broadened to include other stress factors.

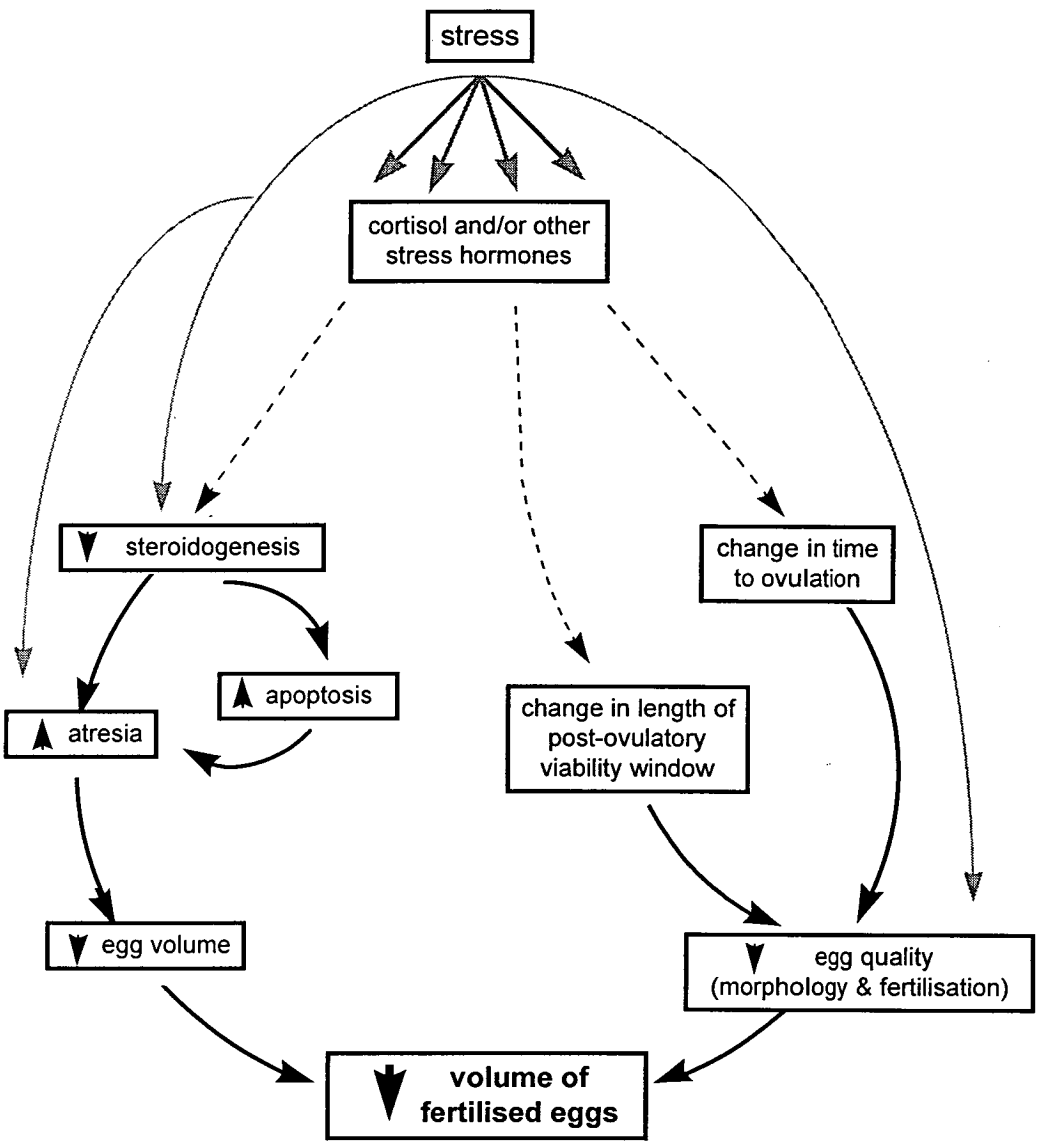
In Chapter 4 (this volume), further light was shed on the mechanism by which stress affects reproduction, and in particular egg production. It was proposed that two separate processes could result in a poor supply of good quality eggs: diminished egg volume and diminished egg quality. It is suggested that stress inhibits ovarian steroidogenesis (however, as discussed above, the exact hormones involved are unknown) and so increases the incidence of oocyte atresia (by increasing apoptosis; Janz and Van Der Kraak, 1997). Increased atresia, results in a reduced number of eggs proceeding to ovulation. Concurrently, stress affects egg quality. Reduced egg quality is most likely the result of a change in time to ovulation, or the length of the post-ovulatory viability window. Both of these changes result in an increased chance that manual stripping, and hence *in vitro* fertilisation could unwittingly be done outside of the period of optimum viability, resulting in reduced fertilisation. The mechanism by which stress may change time to ovulation and/or the length of the post-ovulatory viability window is unknown. Further understanding of how stress affects these factors, however, may result in the development of management tools to circumvent these affects (Fig. 6.1).

Conclusions

Stress has a profound effect on reproduction in Australian snapper, resulting in a decrease in plasma reproductive steroid levels, an increase in the incidence of ovarian atresia, changes in gonadal histology and a diminished response to exogenous hormone therapy. Stress management should be an integral component of broodstock management protocols. Wild-caught broodstock held in captivity respond to stress in a similar manner to freshly caught wild fish, making the use of acclimated broodstock for egg production non-viable. Domestication, however, shows potential in decreasing the degree of sensitivity to stress, since post-stress plasma gonadal steroid levels in hatchery-reared fish show signs of recovery by 168 h pc and hatchery-reared fish outperform wild counterparts following hormonal induction of ovulation. These results with hatchery-reared fish highlight the importance of focusing attention on domestication of broodstock over successive generations. At least part of the effect of stress on reproduction is due to impairment of ovarian steroidogenesis. Although cortisol may be involved in this impairment, it is unlikely that the effects of stress are due solely to the action of cortisol.

Successful reproduction in captivity requires an understanding and provision of the particular requirements of individual species. Since chronic and acute stress can so dramatically affect reproduction, stress management should be a priority in husbandry practices, particularly for those that are stress-sensitive. Further understanding of stress and how it affects reproduction is imperative in designing management protocols that minimise reproductive impairment due to husbandry practices.

Figure 6.1. Generalised summary of the possible effects of stress on production of viable ova. Solid grey lines indicate known effects, but do not imply a mechanism. Solid black lines indicate known mechanisms. Dotted lines indicate potential, but unexplored mechanisms.


stress
cortisol and/or other stress hormones
steroidogenesis
change in time to ovulation
apoptosis
atresia
change in length of post-ovulatory viability window
egg volume
egg quality (morphology & fertilisation)
volume of fertilised eggs

Summary.

1. Capture and handling stress profoundly affects reproductive processes in Australian snapper, resulting in:
    - Impaired ovarian steroidogenesis (Chapter 5)
    - Reduced plasma gonadal steroid levels (Chapter 3 & 4)
    - Changes in gonadal histology
        - Females: increased incidence of ovarian atresia (Chapter 3, 4 & 5).
        - Males: increased proportion of spermatozoa (Chapter 3)
    - Reduced egg production (Chapter 4)
    - Reduced egg quality (Chapter 4)

2. Capture and culture history affects the stress response:
    - Both captive (laboratory acclimated) and wild-caught snapper are equally stress-sensitive (Chapter 3)
    - Wild snapper do not completely acclimate to the captive environment (unless holding facilities are very large), even after long periods of acclimation, capture as juveniles or being held under a minimal disturbance management regime with photo-therm manipulation (Chapter 3 & 4).
    - Domesticated snapper are more robust to stress, and so have greatest potential for use as broodstock and for hormonal induction of ovulation. Therefore domestication of broodstock should be a priority, and these broodstock should be preferentially used for egg production (Chapter 3 & 4).

3. To maximise production of good quality eggs from exogenous hormone therapy regardless of source of fish:
    - Exogenous hormone treatment should be carried out immediately following capture (i.e. on unstressed fish) (Chapter 4).
    - Treat fish with human chorionic gonadotropin (hCG). However as discussed in Chapter 4, Luteinising Hormone Releasing Hormone analogue (LHRHa) administered by a slow release vehicle such as a pellet remains to be compared with hCG in Australian snapper (Chapter 4).

4. Capture and handling stress inhibited ovarian production of testosterone (T), but not 17β-estradiol (E_2) (Chapter 5).

    - This inhibition *in vitro* was in contrast to the lack of inhibition of *in vitro* ovarian steroidogenesis by cortisol previously reported (Pankhurst *et al*, 1995b). Therefore it is unlikely that the effect of stress on ovarian steroidogenesis is the result of direct action of cortisol, but rather involves other stress hormones (Chapter 5).
    - The focus of research must be broadened to include other stress hormones, rather than cortisol alone.

5. Stress-induced reproductive impairment is common among other species. Therefore, results of the present study are expected to be widely applicable in addressing egg production problems in other species.

Appendix A: Fork length, whole body weight, GSI and HSI (mean ± SE) for male and female Trap-Caught and Captive snapper and female 2-and 3-year-old Hatchery snapper used in experiments described in Chapter 3

			Treatment					1-way ANOVA		
			1	6	24	48	168	f	df	P
♀	trap	FL(mm)	349 ± 33	396 ± 42	326 ± 18		347 ± 33	0.956	25	0.4308
		W (g)	1015 ± 254	1472 ± 410	763 ± 160		929 ± 269	1.2290	25	0.3229
		GSI (%)	2.24 ± 0.41	1.99 ± 0.63	1.78 ± 0.42		1.44 ± 0.35	0.4106	25	0.7469
		HSI (%)	1.12 ± 0.09	1.28 ± 0.12	1.50 ± 0.16		1.28 ± 0.12	1.4219	25	0.2631
	captive	FL(mm)	388 ± 12	436 ± 3	467 ± 8	432 ± 16	426 ± 29	2.1859	18	0.1236
		W (g)	1272 ± 112	1738 ± 139	2227 ± 121	1813 ± 202	1619 ± 336	2.2323	18	0.1179
		GSI (%)	3.67 ± 0.45	4.16 ± 1.12	4.16 ± 0.70	3.72 ± 0.82	2.18 ± 0.57	1.4840	18	0.2598
		HSI (%)	1.30 ± 0.19	1.13 ± 0.13	1.11 ± 0.09	1.15 ± 0.12	1.15 ± 0.05	2.3170	18	0.1081
	2-year-old hatchery	FL(mm)	255 ± 4	249 ± 4	262 ± 4	2.56 ± 4	260 ± 4	1.8500	38	0.1420
		W (g)	404 ± 13	357 ± 15	424 ± 22	376 ± 29	404 ± 29	1.7852	38	0.1546
		GSI (%)	0.89 ± 0.12	0.82 ± 0.13	0.95 ± 0.16	0.99 ± 0.20	0.66 ± 0.15	0.5458	38	0.7032
		HSI (%)	1.08 ± 0.14	1.17 ± 0.10	1.26 ± 0.04	1.37 ± 0.09	1.40 ± 0.16	1.1267	37	0.3608
	3-year-old hatchery	FL(mm)	331 ± 5		333 ± 4		330 ± 4	0.2016	28	0.8187
		W (g)	911 ± 45		896 ± 33		834 ± 42	1.0047	29	0.3794
		GSI (%)	4.05 ± 0.42		4.98 ± 0.489		4.63 ± 0.44	1.0805	29	0.3537
		HSI (%)	1.45 ± 0.09		1.31 ± 0.04		1.34 ± 0.08	1.1416	29	0.3342
♂	trap	FL(mm)	335 ± 62	361 ± 26	279 ± 25	353 ± 20	347 ± 21	0.7885	24	0.5462
		W (g)	1224 ± 478	1109 ± 222	509 ± 135	979 ± 176	930 ± 162	0.8392	24	0.5165
		GSI (%)	2.02 ± 0.84	1.27 ± 0.94	1.92 ± 1.02	1.84 ± 0.31	1.15 ± 0.20	0.6555	24	0.6299
		HSI (%)	1.08 ± 0.04	0.87 ± 0.15	0.92 ± 0.10	1.02 ± 0.06	0.77 ± 0.06	1.5272	24	0.2323
	captive	FL(mm)	412 ± 15	465 ± 15	474 ± 11		437 ± 10	3.0305	30	0.0465
		W (g)	1492 ± 136	2000 ± 96	2249 ± 141		1702 ± 107	6.4030	30	0.0020
		GSI (%)	2.58 ± 0.42	4.21 ± 0.60	2.68 ± 0.62		4.29 ± 1.16	1.7059	30	0.1894
		HSI (%)	1.04 ± 0.08	1.14 ± 0.02	1.15 ± 0.03		1.09 ± 0.09	0.9117	30	0.4484

Shaded cells indicate significant difference among treatments (P<0.05).

Appendix B: Fork length (FL), whole body weight (W), GSI, HSI and oocyte diameter (OD)(mean ± SE) for female 3-year-old hatchery and wild-caught snapper used in experiment described in Chapter 4.

			treatment unstressed saline	17P	LHRHa	hCG	2-way ANOVA stress			hormone			interaction		
							F	df	P	F	df	P	F	df	P
3-year-old Hatchery	FL (mm)	unstressed	330 ± 6	325 ± 5	329 ± 5	342 ± 5	1.08	1	0.30	0.61	3	0.61	2.16	3	0.10
		stressed	327 ± 5	324 ± 7	337 ± 9	319 ± 7									
	W (g)	unstressed	854 ± 46	797 ± 40	838 ± 46	959 ± 38	1.28	1	0.26	0.98	3	0.40	2.68	3	0.05
		stressed	808 ± 44	797 ± 52	916 ± 76	766 ± 48									
	GSI (%)	unstressed	3.47 ± 0.40	3.85 ± 0.57	4.51 ± 0.29	5.27 ± 0.26	0.29	1	0.59	2.01	3	0.12	1.41	3	0.25
		stressed	4.48 ± 0.30	4.25 ± 0.53	4.47 ± 0.31	4.56 ± 0.63									
	HSI (%)	unstressed	1.61 ± 0.05	1.48 ± 0.05	1.63 ± 0.07	1.64 ± 0.07	21.88	1	0	1.84	3	0.15	0.16	3	0.92
		stressed	1.85 ± 0.10	1.74 ± 0.09	1.94 ± 0.09	1.84 ± 0.07									
	OD (µm)	unstressed	564 ± 59	549 ± 22	544 ± 23	593 ± 44	0.05	1	0.82	12773	3	0.83	3707	3	0.97
		stressed	559 ± 39	559 ± 31	544 ± 15	564 ± 61									
Trap-Caught	FL (mm)		413 ± 3		397 ± 15	480 ± 71									
	W (g)		1482 ± 7		1295 ± 177	2492 ± 939									
	GSI (%)		1.85 ± 0.06		1.86 ± 0.17	3.56 ± 1.74									
	HSI(%)		1.58 ± 0.03		1.68 ± 0.08	1.81 ± 0.23									
	OD (µm)		381 ± 125		335 ± 15	477 ± 85									
Line-Caught	FL (mm)		363 ± 48		324 ± 14	369 ± 73									
	W (g)		985 ± 338		707 ± 77	638 ± 195									
	GSI (%)		2.26 ± 0.77		0.55 ± 0.15	1.22 ± 0.39									
	HSI (%)		1.00 ± 0.30		1.25 ± 0.28	1.41 ± 0.07									
	OD (µm)		366 ± 35		145 ± 23	303 ± 21									

Shaded cells indicate significant difference among treatments (P<0.05).

Appendix C: Hct (mean ± SE) for female 3-year-old Hatchery and Wild-Caught snapper used in experiment described in Chapter 4, and statistical comparisons for Hatchery snapper.

			time			2-way ANOVA								
			0 h pc	0 h pi	168 h pi	time	stress			hormone			interaction		
							f	d.f	P	f	d.f.	P	f	d.f.	P
3-year-old Hatchery	unstressed	saline		42.5 ± 1.4	31.8 ± 1.8	0 h pc	9.79	1	0.0026	0.19	3	0.90	0.29	3	0.83
		17P		44.2 ± 1.8	32.5 ± 1.1										
		hCG		41.5 ± 2.5	30.1 ± 1.1	0 h pi	42.11	1	<0.0001	0.39	3	0.76	0.20	3	0.89
		LHRHa		42.6 ± 1.0	30.2 ± 1.8										
						168 h pi	20.11	1	<0.0001	0.82	3	0.49	0.93	3	0.43
	stressed	saline	38.4 ± 2.0	35.9 ± 2.0	28.8 ± 1.3										
		17P	38.9 ± 0.8	35.2 ± 1.3	25.3 ± 1.5										
		hCG	39.4 ± 1.1	34.5 ± 1.1	27.7 ± 1.5										
		LHRHa	39.0 ± 2.1	34.7 ± 1.5	24.7 ± 1.7										
Wild	Trap-Caught	saline		32.0 ± 3.8	24.9 ± 4.9										
		hCG		35.4 ± 8.1	21.3 ± 5.4										
		LHRHa		35.6 ± 3.1	24.3 ± 2.0										
	Line-Caught	saline		40.5 ± 5.1	20.2 ± 8.9										
		hCG		40.2 ± 2.3	21.4 ± 2.8										
		LHRHa		37.5 ± 2.6	20.4 ± 1.8										

Shaded cells indicate significant difference among treatments, for each time (0 h pc, O h pi and 168 h pi) ($P < 0.05$)

Appendix D: F ratio, degrees of freedom (d.f.) and probability from 1-way ANOVAs for each fish, among *in vitro* treatments described in Chapter 5.

time	fish no.	E_2			T		
		F	d.f	P	F	d.f	P
0	1	32.267	3	< 0.001	47.493	3	< 0.001
	2	26.582	3	< 0.001	124.396	3	< 0.001
	3	93.707	3	< 0.001	56.404	3	< 0.001
	4	25.543	3	< 0.001	83.045	3	< 0.001
	5	39.869	3	< 0.001	40.248	3	< 0.001
24	1	134.060	3	< 0.001	54.025	3	< 0.001
	2	131.593	3	< 0.001	265.741	3	< 0.001
	3	27.169	3	< 0.001	84.572	3	< 0.001
	4	168.092	3	< 0.001	240.160	3	< 0.001
48	1	66.817	3	< 0.001	152.674	3	< 0.001
	2	730.348	3	< 0.001	152.471	3	< 0.001
	3	48.791	3	< 0.001	117.106	3	< 0.001
	4	40.233	3	< 0.001	54.012	3	< 0.001
72	1	90.622	3	< 0.001	117.522	3	< 0.001
	2	50.030	3	< 0.001	99.157	3	< 0.001
	3	90.622	3	< 0.001	36.991	3	< 0.001
	4	55.516	3	< 0.001	191.465	3	< 0.001
168	1	129.885	3	< 0.001	51.792	3	< 0.001
	2	90.093	3	< 0.001	123.1793	3	< 0.001
	3	69.546	3	< 0.001	146.251	3	< 0.001
	4	61.157	3	< 0.001	95.667	3	< 0.001

Appendix E: Fork length, whole body weight, GSI and HSI (mean ± SE) for female 3-year-old Hatchery fish used in experiments described in Chapter 5

			Treatment 0	24	48	72	168	1-way ANOVA F	d.f.	P
♀	3-year-old	FL (mm)	315 ± 9.35	337.25 ± 9.36	318.0 ± 12.7	323.75 ± 10.28	311.25 ± 9.47	0.9495	4	0.4612
	Hatchery	W (g)	655.8 ± 45.7	829.4 ± 84.6	718.2 ± 95.9	795.3 ± 93.6	698.4 ± 43.52	0.9645	4	0.4537
		GSI (%)	0.60 ± 0.21	1.60 ± 0.19	1.59 (1 only)	1.79 ± 0.29	1.31 ± 0.86	3.4181	3	0.0608
		HSI (%)	1.48 ± 0.21	1.66 ± 0.07	1.71 ± 0.10	1.83 ± 0.17	1.42 ± 0.13	0.7937	4	0.5463

ABARE (Australian Bureau of Agricultural and Resource Economics) (1996). Australian Fisheries Statistics, 1996. ABARE, Canberra, ACT, Australia.

Allan, G.L. and Quartararo, N. (1996). Developing diets for snapper. In *Proceedings of the Marine Finfish Farming Workshop*. (ed. N. Quartararo). NSW Fisheries Research Institute, Cronulla, 23rd June, 1995. pp 71-92

Anand, T.C. and Sundararaj, B.I. (1974). Ovarian maintenance in the hypophysectomised catfish, *Heteropneustes fossilis* (Bloch), with mammalian hypophyseal and placental hormones, and gonadal and adrenocortical steroids. *General and Comparative Endocrinology* 22: 154-168.

Bagenal, T.B. (1969). The relationship between food supply and fecundity in brown trout *Salmo trutta* L.. *Journal of Fish Biology* 1:167-182

Barton, B.A. (1997). Stress in finfish: past, present and future-a historical perspective. In *Fish Stress and Health in Aquaculture*, (eds G.K. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck) Cambridge University Press, Cambridge. pp 1-33

Barton. B.A. and Iwama, G.K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* 1: 3-26

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

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

Barton, B.A., Schreck, C.B., Ewing, R.D., Hemmingsen, A.R. and Patino, R. (1985). Changes in plasma cortisol during stress and smoltification in coho salmon, *Oncorhynchus kisutch. General and Comparative Endocrinology* 59: 468-471.

Barton, B.A., Schreck, C.B. and Barton, L.D. (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. *Diseases of Aquatic Organisms. 2: 173-85*

Battaglene, S.C. (1995). Induced ovulation and larval rearing of four species of Australian marine fish. *Ph.D. thesis*, University of Tasmania. 215pp

Battaglene, S.C. and Allan, G.L. (1994). Development of hatchery techniques for snapper, *Pagrus auratus*, in Australia. *Third Asian Fisheries Forum (*eds L.M. Chou, A.D. Munro, T.J. Lam, T.W. Chen, L.K. Cheong, J.K. Ding, K.K. Hooi, H.W. Khoo, V.P.E. Phang, K.F. Shim and C.H. Tam). Asian Fisheries Society, Manila, Philippines pp 108-111

Battaglene, S.C. and Bell, J.D. (1991). Aquaculture prospects for marine fish in New South Wales. *Fishnote* DF/6, NSW Agriculture and Fisheries, Sydney.

Battaglene, S.C. and Fielder, S. (1997). The status of marine larval rearing technology in Australia. *Hydrobiologia* in press

Battaglene, S.C. and Talbot, R.B. (1992). Induced spawning and larval rearing of snapper, *Pagrus auratus* (Pisces: Sparidae), from Australian waters. *New Zealand Journal of Marine and Freshwater Research* 26: 179-183.

Battaglene, S.C. and Talbot, R.B. (1994). Hormone induction of ovulation and larval rearing of mulloway, *Argyrosomus hololepidotus* (Pisces: Sciaenidae). *Aquaculture* 126: 73-81

Battaglene, S.C., Talbot, R.B., Taylor, J.J. (1993). Advances in hatchery production of snapper, *Pagrus auratus*. Australian Society for Fish Biology. 20th Annual Conference, Sorrento, Western Australia. 27-28 August 1993 (Abstract)

Bell, J.D., Quartararo, N. and Henry, G.W. (1991). Growth of snapper, *Pagrus auratus*, from South-eastern Australia in captivity. *New Zealand Journal of Marine and Freshwater Research* 25:117-121

Blaxhall, P.C. (1972). The haematological assessment of the health of freshwater fish. A review of selected literature. *Journal of Fish Biology* 4: 593 - 604

Bollard, B.A., Pankhurst, N.W. and Wells, R.M.G. (1993). Effects of artificially elevated plasma cortisol levels on blood parameters in the teleost fish *Pagrus auratus* (Sparidae). *Comparative Biochemistry and Physiology* 106A: 157-162.

Bradford, C.S. and Taylor, M.H. (1987). Semilunar changes in estradiol and cortisol coincident with gonadal maturation and spawning in the killifish *Fundulus heteroclitus*. *General and Comparative Endocrinology* 66: 71-78

Bry, C. (1982). Daily variations in plasma cortisol levels of individual female rainbow trout *Salmo gairdneri*: Evidence for a post-feeding peak in well-adapted fish. *General and Comparative Endocrinology* 48: 462- 468

Bry, C. (1985). Plasma cortisol levels of female rainbow trout (*Salmo gairdneri*) at the end of the reproductive cycle: relationship with oocyte stages. *General and Comparative Endocrinology* 57:47-52

Campbell, P.M., Pottinger, T.G. and Sumpter, J.P. (1992). Stress reduces the quality of gametes produced by the rainbow trout. *Biology of Reproduction* 47: 1140-1150

Campbell, P.M., Pottinger, T.G. and Sumpter, J.P. (1994). Preliminary evidence that chronic confinement stress reduces quality of gametes produced by brown and rainbow trout. *Aquaculture* 120: 151-169.

Campbell, C.M., Walsh, J.M. and Idler, D.R. (1976). Steroids in the plasma of the winter flounder *(Pseudopleuronectes americanus* Walbaum). A seasonal study and investigation of steroid involvement in oocyte maturation. *General and Comparative Endocrinology* 29: 14-20.

Canario, A.V.M. and Scott, A.P. (1989). Conjugates of ovarian steroids, including 17α,20β-dihydroxy-4-pregnen-3-one (maturation-inducing steroid), accumulate in the urine of a marine teleost (plaice; *Pleuronectes platessa*). *Journal of Endocrinology 123: R1-R4*

Cardwell, J.R. and Liley, N.R. (1991). Androgen control of social status in males of a wild population of stoplight parrotfish *Sparisoma viride* (Scaridae*). Hormones and Behaviour* 25: 1-18

Carolsfeld, J., Sherwood, N.M., Kreiberg, H. and Sower, S.A. (1988). Induced sexual maturation of herring using GnRH 'quick release' cholesterol pellets. *Aquaculture* 70: 169-181.

Carragher, J.F. and Pankhurst, N.W. (1991). Stress and reproduction in a commercially important marine fish, *Pagrus auratus* (Sparidae). In *Reproductive Physiology of Fish, 1991* (eds A.P. Scott, J.P. Sumpter, D.E. Kime, M.S. Rolfe). pp 253-255.

Carragher, J.F. and Pankhurst, N.W. (1993). Plasma levels of sex steroids during sexual maturation of snapper, *Pagrus auratus* (Sparidae), caught from the wild. *Aquaculture* 109: 375-388.

Carragher, J.F. and Sumpter, J.P. (1990). The effect of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. *General and Comparative Endocrinology* 77:403-407

Carragher, J.F., Sumpter, J.P., Pottinger, T.G. and Pickering, A.D. (1989). The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. *General and Comparative Endocrinology* 67:310-321

Carrillo, M., Zanuy, S., Prat, F., Cerda, J., Ramos, J., Mananos, E. and Bromage, N. (1995). Sea bass (*Dicentrarchus labrax*). In *Broodstock Management and Egg and Larval Quality* (eds N.R. Bromage and R.J. Roberts), Blackwell Science, Oxford. pp 138-168

Chang, J.P. and Peter, R.E. (1983). Effects of pimozide and Des Gly^{10},[D-Ala^{6}] luteinising hormone -releasing hormone ethylamide on serum gonadotropin concentrations, germinal vesicle migration, and ovulation in female goldfish, *Carassius auratus. General and Comparative Endocrinology* 52: 30-37

Clearwater, S.J. and Pankhurst, N.W. (1997). The response to capture and confinement stress of plasma cortisol sex steroids and vitellogenic oocytes in the marine teleost, red gurnard (*Chelidonichthys kumu*) (Triglidae). Journal of Fish Biology 50: 429-441

Colura, R.L. (1990). Hormone induced strip-spawning of red drum. In *Red Drum Aquaculture. Proceedings of a symposium on the culture of red drum and other warm water fishes* (eds. G.W. Chamberlain, R.J. Miget and M.G. Haby). August 1990 pp 33-34.

Contreras-Sanchez, W.M., Schreck, C.B. and Fitzpatrick, M.S. (1995). Effect of stress on the reproductive physiology of rainbow trout, *Oncorhynchus mykiss.* In *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish*, University of Texas, Austin, U.S.A., 2-8 July 1995 p 183

Cook, A.F. and Peter, R.E. (1980). Plasma clearance of gonadotropin in goldfish, *Carassius auratus,* during the annual reproductive cycle. *General and Comparative Endocrinology* 42: 76-90

Cook, A.F., Stacey, N.E. and Peter, R.E. (1980). Periovulatory changes in serum cortisol levels in the goldfish *Carassius auratus. General and Comparative Endocrinology* 40: 507-510

Creswell, G. (1994). Nutrient enrichment of the Sydney continental shelf. *Australian Journal of Marine and Freshwater Research* 45:677-691

Davy, F.B. (1990). Mariculture in Japan. *World Aquaculture* 21: 36-47

De Leeuw, R., Smit-Van Dijk, W., Zigterman, J.W.J., Van Der Loo, J.C.M., Lambert, J.G.D. and Goos, H.J.Th. (1985). Aromatase, estrogen 2-hydroxylase, and catechol-O-methyltransferase activity in isolated, cultures gonadotropic cells of mature African catfish, *Clarias gariepinus* (Burchell). *General and Comparative Endocrinology* 60: 171-177

De Montalembert, G., Jalabert, B. and Bry, C. (1978). Precocious induction of maturation and ovulation in northern pike *(Esox lucius). Ann. Biol. Anim. Bioch. Biophys.* 18: 969-975

Donaldson, E.M. and Devlin, R.H. (1996). Uses of biotechnology to enhance production. In *Developments in Aquaculture and fisheries science. Principles of salmonid culture.* (eds W. Pennel and B.A. Barton). Elsevier, Amsterdam. pp 969-1021

FAO (Food and Agricultural Organisation) (1996). The state of the world fisheries and aquaculture, 1996 (SOFIA). 125pp

Fevolden, S.E., Refstie, T. and Røed, K. H. (1991). Selection for high and low cortisol stress response in Atlantic salmon (*Salmo salar)* and rainbow trout (*Oncorhynchus mykiss). Aquaculture*, 95:53-65

Fletcher, G.L. (1975). The effects of capture, "stress", and storage of whole blood on the red blood cells, plasma proteins, glucose, and electrolytes of the winter flounder *(Pseudopleuronectes americanus). Canadian Journal of Zoology* 53: 197-206.

Foo, J.T.W. and Lam, T.J. (1993a). Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone levels in the tilapia, *Oreochromis mossambicus. Aquaculture* 115: 133-143.

Foo, J.T.W. and Lam, T.J. (1993b). Retardation of ovarian growth and depression of serum steroid levels in the tilapia, *Oreochromis mossambicus,* by cortisol implantation. *Aquaculture* 115: 1145-158.

Foscarini, R. (1988). A review: Intensive farming procedure for red seas bream *(Pagrus major)* in Japan. *Aquaculture* 73: 357-372.

Fostier, A., Le Gac, F. and Loir, M. (1987). Steroids in male reproduction. *In Proceedings of the Third International Symposium on the Reproductive Physiology of Fish,* Memorial University of Newfoundland, Newfoundland, Canada, 2-7 August 1987. pp 239-245

Francis, M.P. (1994). Growth of juvenile snapper, *Pagrus auratus. New Zealand Journal of Marine and Freshwater Research* 28:201-218

Fuchs, D.A. and Albers, C. (1988). Effect of adrenaline and blood gas conditions on red cell volume and intra-erythrocytic electrolytes in the carp, *Cyprinus carpio. Journal of Experimental Biology* 137: 457-477.

Fukusho, K, Fujimura, T. and Yamamoto, T. (1986). Broodstock and advanced spawning of the red seabream in an indoor tank with manipulation of water temperature. *The Aquiculture* 34: 70-73

Gamperl, A.K., Vijayan, M.M. and Boutlier, R.G. (1994). Experimental control of stress hormone levels in fishes: techniques and applications. *Reviews in Fish Biology and Fisheries* 4: 215-255.

Goren, A., Zohar, Y., Koch, Y. and Fridkin, M. (1987). Degradation of GnRH and analogs in the gilthead seabream *Sparus aurata*: an *in vitro* study. In *Proceedings of the Third International Symposium on the Reproductive Physiology of Fish*, Memorial University of Newfoundland, Newfoundland, Canada, 2-7 August 1987. pp 96

Goulet, D. (1995). Temporal patterns of reproduction in the Red Sea damselfish *Amblyglyphidodon leucogaster. Bulletin of Marine Science* 57: 582-595

Gwo, J.C., Strawn, K. and Arnold, C.R. (1993). Induced ovulation in Atlantic croaker (sciaenidae) using hCG and an LHRH analog: a preliminary study. *Theriogenology* 3: 353-361

Haddy, J.A. and Pankhurst, N.W. (1997). The dynamics of *in vitro* 17β-estradiol secretion by isolated ovarian follicles of the rainbow trout *(Oncorhynchus mykiss)*. in press

Hane, S., Robertson, O.H., Wexler, B.C. and Krupp, M.A. (1966). Adrenocortical response to stress and ACTH in Pacific salmon *(Oncorhynchus tschawytscha)* and steelhead trout *(Salmo gairdneri)* at successive stages in the sexual cycle. *Endocrinology* 78: 791-800.

Härdig, J. and Höglund, B. (1984). Seasonal variation in blood components of reared Baltic salmon, *Salmo salar* L. *Journal of Fish Biology* 24: 565 - 579

Heath, D.D., Bernier, N.J., Heath, J.W. and Iwama, G.K. (1993). Genetic, environmental, and interaction effects on growth and stress response of chinook salmon *(Onchorhynchus tshawytscha)* fry. *Canadian Journal of Fisheries and Aquatic Sciences*, 50: 435-42

Hobby, A.C. and Pankhurst, N.W. (1997a). The relationship between plasma and ovarian levels of gonadal steroids in the repeat spawning marine fishes *Pagrus auratus* (Sparidae) and *Chromis dispilus* (Pomacentridae). *Fish Physiology and Biochemistry*. 16: 65-75

Hobby, A.C. and Pankhurst, N.W. (1997b). Post-ovulatory egg viability in the snapper *Pagrus auratus* (Sparidae). *Marine and Freshwater Research* 48: 385-9

Houston, A.H. (1990). Blood and circulation. In *Methods for Fish Biology* (eds C.B. Schreck and P.B. Moyle). American Fisheries Society, Bethesda, Maryland, USA. pp 273-333.

Hunter, J.R. and Macewicz, B.J. (1985). Measurement of spawning frequency in multiple spawning fishes. In *An Egg production method for estimating spawning biomass of pelagic fish: application to the Northern anchovy, Engraulis mordax.* (ed. R. Lasker). NOAA Technical Report NMFS 36. pp 79-94

Hutchins, B. and Swainston. R. (1986). Sea Fishes of Southern Australia: Complete field guide for anglers and divers. Swainston Publishing, Perth 1986

Iwama, G.K, McGeer, J.C. and Pawluk, M.P. (1989). The effects of five fish anaesthetics on acid-base balance, haematocrit, blood gases, cortisol, and adrenaline in rainbow trout. *Canadian Journal of Zoology* 67: 2065-2073.

Jalabert, B. and Fostier, A. (1984). The modulatory effect *in vitro* of oestradiol-17β, testosterone or cortisol on the output of 17α-hydroxy-20β-dihydroprogesterone by rainbow trout *(Salmo gairdneri)* ovarian follicles stimulated by the maturational gonadotropin s-GtH. *Reprod. Nutr. Dévelop.* 24: 127-136

Janz, D.M. and Van Der Kraak, G. (1997). Suppression and apoptosis by gonadotropin, 17β-estradiol, and epidermal growth factor in rainbow trout preovulatory ovarian follicles. *General and Comparative Endocrinology* 105: 186-193

Johansson-Sjobeck, M, Goran, D., Larsson, A, Lewander, K. and Lidman, U. (1978). Hematological effects of cortisol in the European eel, *Anguilla anguilla* L. *Comparative Biochemistry and Physiology* 60A: 165-168.

Kagawa, H., Tanaka, H., Okuzawa, K., Matsuyama, M. and Hirose, K. (1991). Diurnal changes in plasma 17a, 20bP- dihydroxy-4-pregnen-3-one levels during spawning season in the red sea bream, *Pagrus major. Nippon Suisan Gakkaishi.* 57: 769

Kagawa, H., Young, G. and Nagahama, Y (1982). Estradiol-17β production in isolated amago salmon *(Oncorhynchus rhodurus)* ovarian follicles and its stimulation by gonadotropins. *General and Comparative Endocrinology* 47: 361-365

Kagawa, H., Young, G. and Nagahama, Y. (1984). *In vitro* estradiol-17β and testosterone production by ovarian follicles of the goldfish, *Carassius auratus. General and Comparative Endocrinology* 54: 139-143

Kailola, P.J., Williams, M.J., Stewart, P.C., Reichelt, R.E., McNee, A. and Grieve, C. (1993). Australian fisheries resources. Department of Primary Industries and Energy, Canberra (Australia). 422 pp

Kearney, R. (1996). Prospects for Aquaculture. *In Proceedings of the Marine Finfish Farming Workshop.* (ed. N. Quartararo). NSW Fisheries Research Institute, Cronulla, 23rd June, 1995. pp 1-7

King, H. and Young, G. (1995). Increased milt production by gonadotropin releasing hormone analog (GnRHa(-treated Atlantic salmon (*Salmo salar*) after injection of 17α-hydroxyprogesterone. In *Reproductive Physiology of Fish, 1995 (*eds F.W. Goetz and P. Thomas). Fish Symposium '95. Austin. p 95

Kjesbu, O.S. (1989). The spawning activity of cod, *Gadus morhua* L. *Journal of Fish Biology* 34: 195-206

Kühn, E.R., Corneillie, S. and Ollevier, F. (1986). Circadian variations in plasma osmolality, electrolytes, glucose, and cortisol in carp *(Cyprinus carpio). General and Comparative Endocrinology* 61: 459- 468.

Kuznetsov, V.A. and Khalitou, N.K. (1978). Alterations in the fecundity and egg quality of the roach, *Rutilis rutilis*, in connection with different feeding conditions. *Journal of Ichthyology* 18: 63-70

Laidley, C.W. and Leatherland, J.F. (1988). Cohort sampling, anaesthesia and stocking density effects on plasma cortisol, thyroid hormone, metabolite and ion levels in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* 33: 73-88

Lam, T.J. (1982). Applications of endocrinology to fish culture. *Canadian Journal of Fisheries and Aquatic Science* 39: 111-137

Lam, T.J., Pandey, S. and Hoar, W.S. (1975). Induction of ovulation in goldfish by synthetic luteinising hormone-release hormone (LH-RH). *Canadian Journal of Zoology* 53: 1189-1192

Liley, N.R., Cardwell, J.R. and Rouger, Y. (1987). Current status of hormones and sexual behaviour in fish. In *Proceedings of the Third International Symposium on the Reproductive Physiology of Fish*, Memorial University of Newfoundland, Newfoundland, Canada, 2-7 August 1987. pp 142-149

Liley, N.R. and Kroon, F.J. (1995). Male dominance, plasma hormone concentrations, and availability of milt in male rainbow trout *(Oncorhynchus mykiss). Canadian Journal of Zoology* 73: 826-836

Ling, N. and Wells, R.M.G. (1985). Plasma catecholamines and erythrocyte swelling following capture stress in a marine teleost. *Comparative Biochemistry and Physiology* 82C: 231-234.

Lokman, P.M. and Young, G. (1995). *In vitro* biosynthesis of oestradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one by vitellogenic ovarian follicles from migrating New Zealand longfinned eels *(Anguilla dieffenbachii). Aquaculture* 135: 17-26

Matsuyama, M., Adachi, S., Nagahama, Y. and Matsuura, S. (1988). Diurnal rhythm of oocyte development and plasma steroid hormone levels in the female red sea bream, *Pagrus major,* during the spawning season. *Aquaculture* 73: 357-372.

Matsuyama, M., Hamada, M., Ashitani, T., Kashiwagi, M., Iwai, T, Okuzawa, K. Tanaka, H, and Kagawa, H. (1993). Development of LHRH-a copolymer pellet polymerized by ultraviolet and its application for maturation in red sea bream *Pagrus major* during the non-spawning season. *Nippon Suisan Gakkaishi* 59: 1361-1369

Matsuyama, M., Takeuchi, H., Kashiwagi, M., Hirose, K and Kagawa, H. (1995). Induced gonadal development and spawning of immature red sea bream *Pagrus major* with LHRHa administration in different ways during winter season. *Fisheries Science* 61: 472-477.

Mazeaud, M.M, Mazeaud, F. and Donaldson, E.M. (1977). Primary and secondary effects of stress in fish: some new data with a general review. *Transactions of the American Fisheries Society* 106: 201-209

Middaugh, D.P. and Yoakum, R.L. (1974). The use of chorionic gonadotropin to induce laboratory spawning of Atlantic croaker, *Micropogonius undulatus,* with notes on subsequent embryonic development. *Chesapeake Science* 15: 110-123

Mishra, S. and Srivastava, A.K. (1979). Hematology as index of sublethal toxicity of zinc in a freshwater teleost. *Bulletin of Environmental Contamination and Toxicology* 22: 695-698

Mount, D.R., Ingersoll, C.G., Gulley, D.D., Fernendez, J.D., LaPoint, T.W. and Bergman, H.L. (1988). Effect of long-term exposure to acid, aluminium, and low calcium on adult brook trout *(Salvelinus fontinalis).* 1. survival, growth, fecundity, and progeny survival. *Canadian Journal of Fisheries and Aquatic Sciences* 45: 1623-1632

Moyle, P.B. and Cech, J.J. (1996). Fishes: An Introduction to Ichthyology, Prentice Hall, Upper Saddle River, New Jersey.

Munkittrick, K.R., Van der Kraak, G.J., Mc Master, M.E. and Portt, C.B. (1991). Bleached kraft pulpmill effluent (BKME) alters steroid production, regulation and metabolism in white sucker *(Catostomus commersoni).* In *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish,* University of East Anglia, Norwich, U.K., 7-12 July 1991. p 284

Nagahama, Y. (1983). The Functional Morphology of Teleost Gonads. In *Fish Physiology, Vol. IXA.* Academic Press. pp 223-275

Nagahama, Y., Matsuhisa, A., Iwamatsu, T., Sakai, N. and Fukada, S. (1991). A mechanism for the action for the action of pregnant mare serum gonadotropin on aromatase activity in the ovarian follicle of the medaka, *Oryzias latipes. Journal of Experimental Zoology.* 259: 53-58

Nagler, J.J. and Idler, D.R. (1992). *In vitro* ovarian estradiol-17β and testosterone responses to pituitary extract and corresponding serum levels during the prespawning to vitellogenic phases of the reproductive cycle in winter flounder *(Pseudopleuronectes americanus). Comparative Biochemistry and Physiology* 101A: 69-75

Ng, T.B. and Idler, D.R. (1983). Yolk formation and differentiation in teleost fishes. *Fish Physiology, Vol. IXA* (eds W.S. Hoar, D.J. Randall and E.M. Donaldson). Academic Press, New York. pp 373-404.

Nichols, D.J. and Weisbart, M. (1984). Plasma cortisol concentrations in Atlantic salmon, *Salmo salar:* episodic variations, diurnal change, and short term response to adrenocorticotrophic hormone. *General and Comparative Endocrinology* 56: 169-176

Ohta, H., Kagawa, H., Tanaka, H., Okuzawa, K. and Hirose, K. (1996). Changes in fertilisation and hatching rates with time after ovulation induced by 17,20β-dihydroxy-4-pregnen-3-one in the Japanese eel, *Anguilla japonica. Aquaculture* 139:291-301

Pankhurst, N.W. (1994). Effects of gonadotropin releasing hormone analogue, human chorionic gonadotropin and gonadal steroids on milt volume in the New Zealand snapper, *Pagrus auratus* (Sparidae). *Aquaculture* 125: 185-197.

Pankhurst, N.W. (1995). Hormones and reproductive behaviour in male damselfish. *Bulletin of Marine Science*, 57: 569-581

Pankhurst, N.W. (1997). *In vitro* steroid production by isolated ovarian follicles of the striped trumpeter. *Journal of Fish Biology* 50: in press

Pankhurst, N.W. (1998). Chapter 1: Reproduction. In *Biology of Farmed Fish* (eds. K. Black and A.D. Pickering). Sheffield Academic Press, Sheffield. In press.

Pankhurst, N.W. and Carragher, J.F. (1991). Seasonal endocrine cycles in marine teleosts. In *Reproductive Physiology of Fish 1991* (eds A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe). FishSymp 91. pp 131-135

Pankhurst, N.W. and Carragher, J.F. (1992). Oocyte maturation and changes in plasma steroid levels in snapper *Pagrus (=Chrysophrys) auratus* (Sparidae) following treatment with human chorionic gonadotropin. *Aquaculture* 101: 337-347.

Pankhurst, N.W. and Dedual, M. (1994). Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout, *Oncorhynchus mykiss. Journal of Fish Biology* 45: 1013-1025

Pankhurst, N.W., McMillan , P.J. and Tracey, D.M. (1987). Seasonal reproductive cycles in three commercially exploited fished from the slope waters off New Zealand. *Journal of Fish Biology* 30: 193-211

Pankhurst, N.W. and Pankhurst, P.M. (1989). Induced spawning of snapper *Chrysophyrus auratus*. Prospects for aquaculture. In *Proceedings of Aquanz '88.* New Zealand Fisheries Occasional Publication No 4: 31-34

Pankhurst, N.W and Sharples, D.F. (1992). Effects of capture and confinement on plasma cortisol concentrations in the snapper, *Pagrus auratus. Australian Journal of Marine and Freshwater Research* 43: 345-56

Pankhurst, N.W. and Van Der Kraak, G. (1997). Effects of stress on reproduction and growth of fish. In *Fish Stress and Health in Aquaculture, Society for Experimental Biology Seminar Series 62* (eds G.K. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck). Cambridge University Press, Cambridge. pp 73-93

Pankhurst, N.W., Van Der Kraak, G. and Peters, R.E. (1995a). A reassessment of the inhibitory effects of cortisol on ovarian steroidogenesis. In *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish,* University of Texas, Austin, U.S.A., 2-8 July 1995. pp 74-93

Pankhurst, N.W., Van Der Kraak, G. and Peters, R.E. (1995b). Evidence that the inhibitory effects of stress on reproduction in teleost fish are not mediated by the action of cortisol on ovarian steroidogenesis. *General and Comparative Endocrinology* 99: 249-257.

Pankhurst, N.W., Wells, R.M.G. and Carragher, J.F. (1992). Effects of stress on plasma cortisol levels and blood viscosity in blue mao mao, *Scorpis violaceus* (Hutton), a marine teleost. *Comparative Biochemistry and Physiology* 101A: 335-339.

Paulin, C.D. (1990). *Pagrus auratus*, a new combination for the species known as "snapper" in Australasian waters (Pisces: Sparidae). *New Zealand Journal of Marine and Freshwater Research* 24: 259-265

Pearson, M., Van Der Kraak, G. and Stevens, E.D. (1992). *In vivo* pharmacology of spleen contraction in rainbow trout. *Canadian Journal of Zoology* 70: 625-627

Peter, R.E., Lin, H. and Van Der Kraak, G. (1988). Induced ovulation and spawning of cultures freshwater fish in China: advances in application of GnRH analogues and dopamine antagonists. *Aquaculture* 74: 1-10

Peter, R.E. and Yu, K.L. (1997). Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. *Reviews in Fish Biology and Fisheries* 7: 173-197

Petrino, T.R., Greely, M.S.Jr, Selman, K., Lin, Y.-W.P. and Wallace, R.A. (1989a). Steroidogenesis in *Fundulus heteroclitus*. II. Production of 17α-hydroxy-20β-dihydroprogesterone, testosterone, and 17β-estradiol by various components of the ovarian follicle. *General and Comparative Endocrinology* 76: 230-240

Petrino, T.R., Hoch, K.L., Lin, Y.-W. and Wallace, R.A. (1990). Steroidogenesis in *Fundulus heteroclitus*: III. Evidence for involvement of cAMP and protein synthesis in the gonadotropic modulation of ovarian steroid production and aromatase activity. *Journal of Experimental Zoology* 253: 177-185

Petrino, T.R., Lin, Y.W.P and Wallace, R.A. (1989b). Steroidogenesis in *Fundulus heteroclitus*. I. Production of 17α-hydroxy,20β-dihydroprogesterone, testosterone and 17β-estradiol by prematurational follicles *in vitro*. *General and Comparative Endocrinology* 73: 147-156

Pickering, A.D. (1984). Cortisol-induced lymphocytopenia in brown trout, *Salmo trutta* L. *General and Comparative Endocrinology* 53: 252-259.

Pickering, A.D. (1993). Growth and stress in fish production. *Aquaculture* 111: 51-63.

Pickering, A.D. and Christie, P. (1981). Changes in the concentrations of plasma cortisol and thyroxine during sexual maturation of the hatchery-reared brown trout, *Salmo trutta*. L.. *General and Comparative Endocrinology* 44: 487-496

Pickering, A.D. and Pottinger, T.G. (1985a). Cortisol can increase the susceptibility of brown trout, *Salmo trutta* L., to disease without reducing the white blood cell count. *Journal of Fish Biology* 27: 611-619.

Pickering, A.D. and Pottinger, T.G. (1985b). Factors influencing blood cortisol levels of brown trout under intensive culture conditions. *In Current Trends in Comparative Endocrinology* (eds. B. Loftus and W.N. Holmes). Hong Kong, Hong Kong University Press pp 1239-1242

Pickering, A.D. and Pottinger, T.G. (1987). Poor water quality suppresses the cortisol response of salmonid fish to handling and confinement. *Journal of Fish Biology* 30: 363-374

Pickering, A.D. and Pottinger, T.G. (1989). Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. *Fish Physiology and Biochemistry,* 7: 253-8

Pickering, A.D., Pottinger, T.G. and Carragher, J.F. (1989). Differences in sensitivity of brown trout, *Salmo trutta* L., and rainbow trout, *Salmo gairdneri* Richardson, to physiological doses of cortisol. *Journal of Fish Biology* 34: 757-768.

Pickering, A.D., Pottinger, T.G., Carragher, J. and Sumpter, J.P. (1987). The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, *Salmo trutta* L. *General and Comparative Endocrinology* 68: 249-259.

Pickering, A.D., Pottinger, T.G. and Christie, P (1982). Recovery of brown trout, *Salmo trutta* L., from acute handling stress: a time course study. *Journal of Fish Biology* 20:229-244.

Pickering, A.D. and Stewart, A. (1984). Acclimation of the interrenal tissue of the brown trout, *Salmo* truttta L., to chronic crowding stress. Journal *of Fish Biology* 24: 731-740.

Pottinger, T.G., Campbell, P.M. and Sumpter, J.P (1991). Stress-induced disruption of the salmonid liver-gonad axis. In *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish*, University of East Anglia, Norwich, U.K., 7-12 July 1991. pp 114-116.

Pottinger, T.G. and Moran, T.A. (1993). Differences in plasma cortisol and cortisone dynamics during stress in two strains of rainbow trout *(Oncorhynchus mykiss). Journal of Fish biology,* 43: 121-130

Pottinger, T.G., Moran, T.A. and Morgan, J.A.W. (1994). Primary and secondary indices of stress in the progeny of rainbow trout *(Oncorhynchus mykiss)* selected for high and low responsiveness to stress. *Journal of Fish Biology* 44: 149-163

Pottinger, T.G. and Pickering, A.D. (1997). Genetic basis to the stress response: selective breeding for stress-tolerant fish. In *Fish Stress and Health in Aquaculture, Society for Experimental Biology Seminar Series 62* (eds G.K. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck). Cambridge University Press, Cambridge. pp 171-193

Pottinger, T.G., Prunet, P., Pickering, A.D. (1992). The effects of confinement stress on circulating prolactin levels in rainbow trout *(Oncorhynchus mykiss)* in fresh water. *General and Comparative Endocrinology* 88: 454-460

Prentice, J.A. and Colura, R.L (1984). Preliminary observations of orangemouth corvina spawn inducement using photoperiod, temperature and salinity cycles. *Journal of the World Mariculture Society* 15: 162-171

Prentice, J.A. and Thomas, P. (1987). Successful spawning of orangemouth corvina following injection with des-Gly10[D-Ala6]-luteinising hormone-releasing hormone (1-9) ethylamide and pimozide. *Progressive Fish Culturist* 49: 66-69

Quartararo, N. (1996). Grow-out of snapper and mulloway in seacages. *In Proceedings of the Marine Finfish Farming Workshop* (ed. N. Quartararo). NSW Fisheries Research Institute, Cronulla, 23rd June, 1995. pp 37-70

Quartararo, N., Allen, G.L. and Bell, J.D. (192). Fish meal substitution in a diet for snapper, *Pagrus auratus*. In *Proceedings of the aquaculture nutrition workshop* (eds G.L. Allan and W. Dall). NSW Fisheries Brackish Water Fish Culture Research Station, Salamander Bay, Australia. pp125-126

Redgate, E.S. (1974). Neural control of pituitary adrenal activity in *Cyprinus carpio*. *General and Comparative Endocrinology* 22: 35-41

Refstie, T. (1982). Preliminary results: differences between rainbow trout families in resistance against vibriosis and stress. *Developmental and Comparative Immunology Supplement,* 2: 205-9

Refstie, T. (1986). Genetic differences in stress response in Atlantic salmon and rainbow trout. *Aquaculture* 52: 374

Ryan, S. (1992). The dynamics of MS-222 anaesthesia in a marine teleost *(Pagrus auratus). Comparative Biochemistry and Physiology* 101C: 593-600

Safford, S.E. and Thomas, P. (1987). Effects of capture and handling on circulating levels of gonadal steroids and cortisol in the spotted seatrout, *Cynoscion nebulosus.* In *Proceedings of the Third International Symposium on Reproductive Physiology of Fish.* August 2-7, 1987. p 312

Salonius, K. and Iwama, G.K. (1993). Effects of early rearing environment on stress response, immune function, and disease resistance in juvenile coho *(Oncorhynchus kisutch)* and chinook salmon *(O. tschawytscha). Canadian Journal of Fisheries and Aquatic Science* 50: 759-766

Schreck, C.B. (1981). Stress and compensation in teleostan fishes: response to social and physical factors. In *Stress and Fish.* (ed A.D. Pickering). Academic Press, London. pp 295-321

Schreck, C.B., Jonsson, L., Feist, G. and Reno, P. (1995). Conditioning improves performance of juvenile chinook salmon, *Oncorhynchus tschawytscha*, to transportation stress. *Aquaculture.* 135: 99-110

Scott, D.P. (1962). Effect of food quantity on fecundity of rainbow trout, *Salmo gairdneri. Journal of the Fisheries Research Board Canada* 19: 715-730

Scott, A.P. and Canario, V.M. (1987). Status of oocyte maturation-inducing steroids in teleosts. In *Proceedings of the Third International Symposium on the Reproductive Physiology of Fish*, Memorial University of Newfoundland, Newfoundland, Canada, 2-7 August 1987. pp 224-234

Scott, A.P. and Canario, A.V. (1992). 17α,20β-dihydroxy-4-pregnen-3-one 20-sulphate: A major new metabolite of the teleost oocyte maturation-inducing steroid. *General and Comparative Endocrinology* 85: 91-100.

Scott, A.P., Canario, A.V. and Prat, F. (1990). Radioimmunoassay of ovarian steroids in plasmas of ovulating female sea bass *(Dicentrarchus labrax)*. *General and Comparative Endocrinology* 78: 299-302

Scott, A.P., MacKenzie, S and Stacey, N.E. (1984). Endocrine changes during natural spawning in the white sucker *Catostomus commersoni:* II. Steroid hormones. *General and Comparative Endocrinology* 56: 349- 359.

Scott, A.P., Sumpter, J.P. and Hardiman, P.A. (1983). Hormone changes during ovulation in the rainbow trout (*Salmo gairdneri* Richardson). *General and Comparative Endocrinology* 49:128-134

Scott, S.G. (1991).The reproductive biology of the New Zealand snapper, *Pagrus auratus. Masters thesis*, University of Auckland 49pp

Scott, S.G. and Pankhurst, N.W. (1992). Interannual variation in the reproductive cycle of the New Zealand snapper *Pagrus auratus* (Bloch and Schneider) (Sparidae*). Journal of Fish Biology* 41: 685-696

Scott, S.G., Zeldis, J.R. and Pankhurst, N.W. (1993). Evidence of daily spawning in natural populations of the New Zealand snapper *Pagrus auratus* (Sparidae*). Environmental Biology of Fishes* 36:149-156

Smith, P.J. (1986). Spawning behaviour of snapper, *Chrysophrys auratus,* in captivity (Note). *New Zealand Journal of Marine and Freshwater Research* 20: 513-515.

Soivio, A., Westman, K and Nyholm, K. (1974). Changes in haematocrit values in blood samples treated with and without oxygen: a comparative study with four salmonid species. *Journal of Fish Biology* 6: 763-769

Specker, J.L. and Sullivan, C.V. (1994). Vitellogenesis in fishes: status and perspectives. In *XII International Conference in Comparative Endocrinology*, Toronto, Ontario, Canada. 16-21 May 1993. pp 304-315

Springate, J.R.C., Bromage, N.R., Elliott, J.A.K. and Hudson, D.L. (1984). The timing of ovulation and stripping and their effects on the rates of fertilisation and survival to eying, hatch and swim-up in the rainbow trout *(Salmo gairdneri* R.*). Aquaculture* 43: 313-322

Stacey, N.E. (1984). Control of the timing of ovulation by exogenous and endogenous factors. In *Fish Reproduction: Strategies and Tactics* (eds G.W. Potts and R.J. Wootton), Academic Press, New York and London. pp 207-222

Stacey, N.E. (1991). Hormonal pheromones ib fish. status and prospects. *In Reproductive Physiology of Fish 1991* (eds A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe), FishSymp 91, Sheffield, pp 177-181

Stacey, N.E., Cardwell, J.R., Liley, N.R., Scott, A.P. and Sorenson, P.W. (1994). Hormones as sex pheremones in fish. In *Perspectives in Comparative Endocrinology* (eds. K.G. Davey, R.E. Peter and S.S. Tobe). National Research Council of Canada, Ottawa. pp 438-448

Stacey, N.E., MacKenzie, D.S., Marchant, T.A., Kyle, A.L. and Peter, R.E. (1984). Endocrine changes during natural spawning in the white sucker, *Catostomus commersoni. General and Comparative Endocrinology* 56: 333 - 348.

Sumpter, J.P (1997). The endocrinology of stress. In *Fish Stress and Health in Aquaculture, Society for Experimental Biology Seminar Series 62* (eds. G.K. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck). Cambridge University Press, Cambridge. pp 95-118

Sumpter, J.P., Carragher, J., Pottinger, T.G. and Pickering, A.D. (1987). The interaction of stress and reproduction in trout. In *Proceedings of the Third International Symposium on the Reproductive Physiology of Fish,* Memorial University of Newfoundland, Newfoundland, Canada, 2-7 August 1987. pp 299-302

Sumpter, J.P., Pottinger, T.G., Rand-Weaver, M., Campbell, P.M. (1993). The wide-ranging effects of stress on fish. In *XII International Conference in Comparative Endocrinology,* Toronto, Ontario, Canada. 16-21 May 1993. pp 535-58

Sundararaj, B.I. and Goswami, S.V. (1968). Effect of short- and long-term hypophysectomy on the ovary and interrenal of catfish, *Heteropneustes fossilis* (Bloch). *Journal of Experimental Zoology* 168: 85-104

Sundararaj, B.I., Goswami, S.V. and Lamba, V.J. (1982). Role of testosterone, estradiol-17β and cortisol during vitellogenin synthesis in the catfish, *Heteropneustes fossilis* (Bloch). *General and Comparative Endocrinology* 48: 390-397

Swanson, P. (1991). Salmon gonadotropins: reconciling old and new ideas. *In Reproductive Physiology of Fish 1991* (eds A.P. Scott, J.P. Sumpter, D.E. Kime and M.S. Rolfe). FishSymp 91, Sheffield. pp 2-7

Sweetman, J.W. (1992). Larviculture of mediterranean marine fish species: current status and future trends. *Journal of the World Aquaculture Society* 23: 330-337

Tam, W.H. and Payson, P.D. (1986). Effects of chronic exposure to sublethal pH on growth, egg production, and ovulation in brook trout, *Salvelinus fontinalis. Canadian Journal of Fisheries and Aquatic Science* 43: 275-280

Thomas, P. (1994). Hormonal control of final oocyte maturation in sciaenid fishes. In *Perspectives in Comparative Endocrinology* (eds K.G. Davey, R.E. Peter and S.S Tobe). National Research Council, Canada, Ottawa. pp 619-625

Thomas, P., Arnold, C.R. and Holt, G.J. (1995). Sciaeinid Fishes. In *Broodstock Management and Egg and Larval Quality.* Ed. N. Bromage and Robert, R.J. Blackwell Scientific Publishing.

Thresher, R.E. (1983). Habitat effects on reproductive success in the coral reef fish, *Acanthochromis polyacanthus* (Pomacentridae). *Ecology* 64: 1184-1199

Tilly, J.L., Kowalski, K.I., Johnson, A.L. and Hsueh, J.W. (1991). Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* 129: 2799-2801

Tomasso, J.R., Davis, K.B. and Parker, N.C. (1981). Plasma corticosteroid dynamics in channel catfish, *Ictalurus punctatus* (Rafiñesque), during and after oxygen depletion. *Journal of Fish Biology* 18: 519-526.

Trudeau, V.L., Lin, H.R. and Peter, R.E. (1991). Testosterone potentiates the serum gonadotropin response to gonadotropin-releasing hromone in the common carp *(Cyprinus carpiio)* and Chinese loach *(Paramisgurnus dabryanus). Canadian Journal of Zoology* 69: 2480-2484

Trudeau, V.L.; Murthy, C.K., Habibi, H.R., Sloley, B.D. and Peter, R.E. (1993). Effects of sex steroid treatment on gonadotropin-releasing hormone-stimulated gonadotropin secretion from the goldfish pituitary. *Biology of Fish Reproduction* 48: 300-307

Tyler, C. (1991). Vitellogenesis in salmon. In *Proceedings of the Fourth International Symposium on the Reproductive Physiology of Fish,* University of East Anglia, Norwich, U.K., 7-12 July 1991. pp 295-299

Underwood, A.J. (1981). Techniques of analysis of variance in experimental marine biology and ecology. *Oceanography and Marine Biology Annual Review* 19: 513-605.

Van Der Kraak, G. and Wade, M.G. (1993). A comparison of signal transduction pathways mediating gonadotropin actions in vertebrates. In *XII International Conference in Comparative Endocrinology*, Toronto, Ontario, Canada. 16-21 May 1993. pp 59-63

Ventling, A.R. and Pankhurst, N.W.(1995). Effects of gonadal steroids and human chorionic gonadotropin on final oocyte maturation *in vitro* in the New Zealand snapper *Pagrus auratus* (Sparidae). *Marine and Freshwater Research* 46: 467-73.

Waring, C.P., Stagg, R.M. and Poxton, M.G. (1992). The effects of handling on flounder *(Plactichthys flesus* L.) and Atlantic salmon (*Salmo salar* L.). *Journal of Fish Biology* 41: 131-144.

Wedemeyer, G.A. (1997). Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In *Fish Stress and Health in Aquaculture* (eds. G.K. Iwama, A.D. Pickering, J.P. Sumpter and C.B. Schreck). Cambridge University Press. pp 35-71

West, G. (1990). Methods of assessing ovarian development in fishes: a review. *Australian Journal of Freshwater Research* 41: 199-222

Williams, M.J. and Stewart, P.C. (1993). Australia's fisheries. In *Australian Fisheries Resources*, (eds P.J. Kailola and M.J. Williams), Bureau of Resource Sciences and the Fishing Research and Development Coorporation. Canberra, Australia. pp 1-2

Wilson, C.E., Crim, L.W. and Morgan, M.J. (1995). The effects of stress on spawning performance and larval development in the Atlantic cod, *Gadus morhua* L. In *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish*, University of Texas, Austin, U.S.A., 2-8 July 1995. p 198

Wingfield, J.C. and Grimm, A.S. (1977). Seasonal changes in plasma cortisol, testosterone and oestradiol-17β in the plaice, *Pleuronectes platessa* L. *General and Comparative Endocrinology* 31: 1-11

Woo, N.Y.S. (1990). Metabolic and osmoregulatory changes during temperature acclimation in the red sea bream, *Chrysophrys major:* Implications for its culture in the subtropics. *Aquaculture* 87: 197-208

Woodward, C.C. and Strange, R.J. (1987). Physiological stress responses in wild and hatchery-reared rainbow trout. *Transactions of the American Fisheries Society* 116: 574-579

Yaron, Z., Gur, G., Melamed, P., Levavi-Sivan, B., Gissis, A., Bayer, D., Elizur, A., Holland, C., Zohar, Y., Schreibman, M.P. (1995). Blocks along the hypothalamo-hypophyseal-gonadal axis in immature black carp, *Mylopharyngodon piceus.* In *Proceedings of the Fifth International Symposium on the Reproductive Physiology of Fish*, University of Texas, Austin, U.S.A., 2-8 July 1995. pp 22-24.

Young, G., Kagawa, H., Nagahama, Y. (1982a). Inhibitory effect of cyanoketone on salmon gonadotropin-induced estradiol-17b production by ovarian follicles of the amago salmon *(Oncorhynchus rhodurus) in vitro. General and Comparative Endocrinology* 47: 357-360

Young, G., Kagawa, H., Nagahama, Y. (1982b). Evidence for a decrease in aromatase activity in the ovarian granulosa cells of amago salmon *(Oncorhynchus rhodurus)* associated with final oocyte maturation. *Biology of Reproduction* 29: 310-315

Young, G., Ueda, H. and Nagahama, Y. (1983). Estradiol-17β and 17α,20β-dihydroxy-4-pregnen-3-one production by isolated ovarian follicles of amago salmon *(Oncorhynchus rhodurus)* in response to mammalian pituitary and placental hormones and salmon gonadotropin. *General and Comparative Endocrinology* 52: 329-335

Zelnik, P.R. and Goldspink, G. (1981). The effect of exercise on plasma cortisol and blood sugar levels in the rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* 19: 37-43.

Zohar, Y. (1988). Gonadotropin releasing hormone in spawning induction in teleosts: basic and applied considerations. In *Reproduction in Fish - Basic and applied aspects in endocrinology and genetics,* Tel-Aviv, Israel, 10-12 November 1986. pp 48-62

Zohar, Y. and Gordin, H, (1979). Spawning kinetics in the gilthead sea-bream, *Sparus aurata* L. after low doses of human chorionic gonadotropin. *Journal of Fish Biology* 15: 665-670

Zohar, Y., Goren, A., Tosky, M., Pagelson, G., Leibovitz, D. and Koch, Y. (1989). The bioactivity of gonadotropin releasing hormones and its regulation in the gilthead seabream, *Sparus aurata*: *in vivo* and *in vitro* studies. *Fish Physiology and Biochemistry* 7: 59-67

Zohar, Y., Harel, M., Hassin, S. and Tandler, A. (1995). Gilt-head sea bream (*Sparus aurata*). In *Broodstock Management and Egg and Larval Quality* (eds N.R. Bromage and R.J. Roberts). Blackwell Science, Oxford. pp 94-117

Zohar, Y., Pagelson, G. and Tosky, M. (1988). Daily changes in reproductive hormone levels in the female gilthead seabream *Sparus aurata* at the spawning period. In *Reproduction in Fish - Basic and applied aspects in endocrinology and genetics.* Tel-Aviv, Israel, 10-12 November 1986. pp 119-125

Zohar, Y., Pagelson, G., Tosky, M. and Finkelman, Y. (1987). GnRH control of gonadotropin secretion, ovulation and spawning in the gilthead seabream, *Sparus aurata.* In *Proceedings of the Third International Symposium on the Reproductive Physiology of Fish*, Memorial University of Newfoundland, Newfoundland, Canada, 2-7 August 1987. p 106