Reproductive biology and endocrinology of black bream *Acanthopagrus butcheri*

by

James Andrew Haddy B.Appl.Sci. (Hons)

UNIVERSITY OF TASMANIA

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

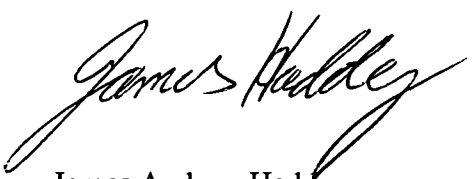
University of Tasmania
at Launceston

May 2000

DECLARATION AND AUTHORITY OF ACCESS

I hereby declare that the material in this thesis is original except where due acknowledgement is given, and the material has not been accepted for the award of any other degree or diploma.

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



James Andrew Haddy

Abstract

This study provides baseline information on the annual reproductive activity of wild black bream, the effect of capture and confinement on plasma steroid levels, suitable induced ovulation protocols and the effect of salinity on reproductive development, induced ovulation and egg fertility and development.

The annual change in reproductive condition and plasma levels of sex steroids in black bream, was investigated by measuring changes in gonadosomatic index (GSI), hepatosomatic index (HSI), gonad stage and plasma concentrations of sex steroids. Black bream have an annual reproductive cycle with a 3 month spawning season in spring / early summer with daily cycles of gonadal maturation and plasma steroid levels. Elevated levels of plasma estradiol-17β (E_2), testosterone (T) and 11-ketotestosterone (11KT) were associated with gonadal recrudescence, and elevated plasma 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) levels were associated with final oocyte maturation and spermiation in female and male fish respectively.

The stress-induced changes in concentrations of plasma sex steroids in black bream were investigated by blood sampling at capture and in fish confined for 15, 30 minutes, 1, 3, 6, 12 or 24 hours. Confinement resulted in significantly elevated plasma cortisol levels, reduced plasma levels of E_2 and T within 1 h in females, and suppressed plasma levels of T and 11KT after 30 min and 6 h respectively in males. Plasma levels of 17,20βP increased decreased or remained unchanged. This study indicates that stress exerts a rapid inhibitory effect on gonadal steroidogenesis in black bream.

The effect of hormone therapy to induce ovulation was assessed by injecting mature female black bream with saline, human chorionic gonadotropin (hCG) or luteinizing hormone releasing hormone analogue (LHRHa) at capture, or 24 hrs post capture. Treatment with LHRHa or hCG resulted in fish ovulating throughout the experiment, with LHRHa treatment at capture resulting in the best ovulatory response. Injection with hCG or LHRHa at capture resulted in the short term elevation of plasma E_2 and T, whereas, injection of LHRHa 24 hrs post-capture did not elevate plasma E_2 or T levels over controls. Plasma levels of cortisol and 17,20βP were unaffected by hormone treatment. These

results show that capture and handling stress reduces the responsiveness of fish to exogenous hormone treatment and that best results are obtained if hormonal treatment is administered at the time of capture.

The effects of salinity (5, 20 or 35‰) on seasonal reproductive development, plasma steroid levels, the efficacy of LHRHa to stimulate ovulation, sperm motility, and egg fertility and development to hatching were investigated. Gonadal maturation and seasonal plasma steroid levels were essentially unaffected by salinity in both sexes. Hormone therapy resulted in the typical endocrine and ovulatory response in all three salinities, however, egg production was reduced in fish held at 5 ‰. Both fertilisation and sperm motility were significantly reduced at 5‰. Egg development was best over a salinity range of 20-35‰.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Ned Pankhurst for his assistance, support, valuable criticism of this manuscript, and for proposing a PhD topic which involved a high degree fishing with a rod and line to obtain samples.

Thanks are extended to the property owners of the Swan, and Meredith rivers for allowing access at all hours of the day and night. Special thanks must go to Sandy Shaw of Redbanks farm who provided riverside accomodation at the infamous "Slack Shack" and local information on river conditions and hot fishing spots throughout the study period. I'd also like to thank Mick and Lisa Hannon for the 5 star accomodation at nine mile beach during the extended sampling trips in the first year.

Thanks go to the fishing skills of the many people who have assisted me during field sampling. These people are duly acknowledged in each relevant chapter of this thesis. However, special thanks to Mick Attard who, dare I say it, introduced me to bream fishing and taught me the art of finding and hooking fish even when they were being finicky. Paul Hannon, Jamie Welsford and Allan Shepherd are also deeply thanked for their regular appearances as fishing buddies even when the weather was cold, wet and miserable and for fishing at ungodly hours of the night.

To my colleages and friends at the University of Tasmania my sincere thanks for all of your assistance. I thank the secretaries, Jan Daniel and Annabel Tyson because without their knowledge and skills the whole department would be in mayhem. I am also indebted to Andy Hobby, Philip Thomas, Carolyn Barnett, Jenny Cleary, Jo Sadler, Polly Hilder, Mark Hilder and Tish Pankhurst for their assistance and ideas throughout this study.

Thanks go to Peter Monatgue, Jim Baker and Trevor Anderson of the CRC for Aquaculture, for their enthusiastic approach to all student projects and their financial support which allowed me to attend a number of international conferences.

A big thank you must go to my family, Mum Dad, Mark, Kaye and Paul and my partners family Robin, Barbara, Natalie, James and Chantal for their constant and unconditional support both emotionally and financially.

Finally to my partner Belinda Wood, I thank you for being with me and supporting me in both the good and bad times. For without your support this PhD would not have been completed.

Table of Contents page

List of Figures **Page**

List of Figures **Page**

List of Figures **Page**

List of Figures **Page**

List of Tables **Page**

CHAPTER 1

General introduction.

1. General introduction

1.1 Aquaculture; a rapidly growing industry

Australia has the third largest fishing zone in the world, but due to its nutrient-poor waters, productivity is not high with fisheries production being ranked at 53 in the world for 1996 (FAO 1996). Similarly, Australian aquaculture is also small by world standards, however, its share of the total value of Australian fisheries production has steadily increased in recent years to around 25% in 1996 (Brown et al., 1997). Australia's total landed weight of fisheries products is not expected to expand much beyond its present level and with improving technology and increase in fishing pressure, some fisheries are becoming over-exploited (Williams and Stewart 1993). Public demand for fish products continues to increase and natural resources will not be able to meet this demand. Therefore, it is clear that expansion of the aquaculture sector is essential if local fish production is to satisfy increasing consumer demand.

In 1995-96 the volume of Australian aquaculture production was dominated by Atlantic salmon (*Salmo salar*; 7647 t), followed by rainbow trout (*Oncorhynchus mykiss*; 2498 t), southern bluefin tuna (*Thunnus maccoyii*; 2013 t), and barramundi (*Lates calcarifer*; 529 t) (Brown et al., 1997). In order to keep up with increasing local demand and also to establish and compete with international export markets, there is a need to develop additional finfish species which can be cultured within Australia. Diversification of the Australian finfish portfolio is considered necessary to protect industry against potentially crippling disease outbreaks in existing species, reduce imports of fish products into Australia, and expand the regions in Australia in which marine fin fish farming can be undertaken (Williams and Stewart 1993).

Before a species can be commercially cultured it must first have market acceptability and be biologically manageable. Market profiles are easily determined, but the biological suitability of a species can only be determined through understanding its reproductive biology, nutritional and growth requirements and susceptibility to health problems in the culture environment (Pankhurst 1998a). Gaining this knowledge is one of the key components of research and development in establishing sustainable aquaculture. Australia is yet to develop a significant marine fish farming industry of non-salmonid species (Brown et al., 1997), with new species

development being slow and problematic (Pankhurst 1998a). One of the major constraints in the development of a marine fish farming industry has been the absence of suitable technology for Australian species (Brown et al., 1997). However, the transfer of technology and techniques developed for marine fish species in other countries has initiated the development of various marine farm operations and research facilities across Australia (Pankhurst 1998a). This is particularly so for members of the sparid family, which form significant aquaculture fisheries throughout the world, and the methods used are directly transferable between species (Foscarini 1988; Battaglene 1995; Cowden 1995). Snapper *Pagrus auratus*, has been intensively farmed in Japan for over 30 years (reviewed by Foscarini 1988), however, in Australia the first recorded induced spawning and larval rearing of snapper occurred in 1992 (Battaglene and Talbot 1992). Since then, research into snapper has developed at an accelerated rate due to the thorough understanding of its biology (Pankhurst and Carragher 1992; Carragher and Pankhurst 1993; Scott and Pankhurst 1993; Scott et al., 1993), and commercial hatcheries and grow out farms have already been established in Western Australia, South Australia and New South Wales (Cleary 1998). The major bottleneck for the development of the snapper farming industry has been a unreliable supply of high quality eggs for hatchery production of juveniles (Battaglene 1995; Cleary 1998) However, with the recent improvements in controlled spawning, larval rearing, and the domestication of broodstock, the future for snapper farming in Australia looks promising (Battaglene 1995; Cleary 1998; Fielder et al., 1999).

Another constraint in the development and expansion of marine finfish farming is the lack of suitable sites, with the majority of appropriate sites having already been occupied and the remainder of Australias unpolluted coastline being relatively exposed (Williams and Stewart 1993; Brown et al., 1997). The alternative is to develop shore based coastal sites or inland sites using saline groundwater. Australia has huge resources of inland saline water, including natural saline lakes, shallow aquifers and deep aquifers (Nulsen 1999) . There are numerous natural saline lakes across Australia, but these tend to be ephemeral and often have high ecological importance. As a consequence, the use of these resources for aquaculture appears to be limited with the exception of the stocking of saline lakes that do not dry up

seasonally with euryhaline species for establishing put and take fisheries (Jenkins 1999).

Saline ground water is generally considered more in terms of its treat potential for agriculture than as a potential aquaculture resource. The replacement of deep rooted perennial vegetation with shallow rooted agricultural crops combined with the irrigation of these crops is responsible for rising saline water tables and increasing salination and waterlogging of agricultural land (Blackwell 1999; Nulsen 1999). Saline ground waters do not need to be highly saline to cause problems for agriculture, with salinities as low as 3‰ quickly accumulating to toxic levels in the plant root zone (Nulsen 1999). Current practice to reduce the height of rising water tables involves the pumping of water into evaporation basins. To date these ponds are not exploited but there is considerable interest in using the resource for inland mariculture of a variety of fish species (Allan and Fielder 1999; Gooley et al., 1999; Hutchinson 1999; Jenkins 1999; Paust 1999). The use of this resource for the production of aquaculture products would help to offset saline water management costs and reduce capital costs in developing an aquaculture venture, as the engineering infrastructure is often already in place (Pankhurst 1999).

Deep saline aquifers generally have very favourable characteristics for aquaculture as they typically contain very low bacterial and viral counts and temperature is stable throughout the year. However, deep extraction does not contribute to ground water management and could even exacerbate the salinisation of ground water. Conflict between the requirements of ground water management programs and the desire to have good quality water for aquaculture does not preclude use of the resource. The availability of high quality water is one of the key requirements of the hatchery production of marine finfish and saline water from deep aquifers is the most desirable for this purpose. In this case, special water management will be needed, such as the reinjection of saline water into deep bores after use in order to meet the requirements for both land management and aquaculture (Ogburn 1999; Trendall et al., 1999).

Examples exist elsewhere in the world where fish mariculture has developed in arid areas using ground water as a resource. For example, tilapia (*Oreochromis* spp) are intensively farmed using brackish water from an aquifer in an Israeli desert

(Pruginin et al., 1988), and red drum have been shown to survive and grow well in saline (5-15‰) ground waters in Texas (Forsberg et al., 1996). Therefore, the development of inland saline aquaculture in Australia will be assisted by using the knowledge and technology developed in other countries. Forsberg et al. (1996) suggested that the salinity and specific-ion concentrations were the best guidelines for measuring the potential of saline ground water for red drum culture. Therefore, characterisation of the composition of saline waters in Australia is a priority for research.

1.2 Black bream

The black bream *Acanthopagrus butcheri*, is an euryhaline sparid endemic to the estuarine waters of southern Australia and forms important recreational and commercial fisheries (Stewart and Grieve 1993). It is thought to spend its entire life cycle in the estuarine environment, and there is little evidence of movement of fish between estuaries. In consequence, there are distinct genetic differences between black bream populations that are geographically isolated (Chaplin et al., 1998). Therefore care must be taken when generalising about black bream populations. This is particularly so in respect of the timing of reproductive events, as the environmental conditions between estuaries that black bream inhabit are extremely variable (Sarre and Potter 1999).

Black bream is reported to reach a maximum size of 60 cm and 4 kg (Stewart and Greive 1993) and is long lived, reaching a maximum reported age of 29 years (Morison et al., 1998). It is a deep bodied fish with colouration ranging from a dark bronze or olive green to bright silver. The species favours snaggy and rocky areas of habitat where sufficient cover is provided, and is an opportunistic feeder on crustaceans, molluscs, polychaetes, small fish, and may consume large amounts of algae (Sarre et al., 2000). As a truly euryhaline species, black bream has the ability to withstand a wide range of environmental conditions.

Black bream stocks across Australia are in danger of being over-exploited, which has resulted in the initiation of several studies on stock assessment, reproduction, fingerling production and their use in stock enhancement (Morison et al., 1998; Jenkins et al., 1999; Lenanton et al., 1999; Sarre and Potter 1999; Sarre et al., 2000).

In addition, the ability of black bream to withstand a wide range of environmental conditions has promoted considerable interest in its aquaculture potential for stocking into brackish and saline waters of inland Australia (Ingram et al., 1996; Jenkins 1999; Maguire and Sarre 1999). Under experimental conditions, growth and survival of juvenile black bream is not affected over a salinity range of 12-48 ‰ (Jenkins et al., 1999). Juvenile black bream have now been stocked in numerous water ways across Australia (Jenkins 1999; Jenkins et al., 1999), however, it is currently not known whether these fish will become reproductively active in these saline waters. To date, no information is available on the reproductive physiology of black bream or the effect of salinity and common aquacultural practices on the reproductive activity of black bream. It is clear that black bream can undergo sexual maturation over salinity ranges of 3-45‰ (Sarre and Potter 1999) but there is little information on either the specifics of reproductive management or larval survival. This information is essential for the successful development of restocking programs, the establishment of new black bream fisheries and the development of commercial black bream aquaculture in Australia.

1.3 Endocrine control of reproduction

Except for the capture of juvenile fish for on-growing to market size, the successful development of marine fish farming is dependent on the availability of viable gametes. This can be achieved by several routes including the collection of naturally spawned eggs from the wild, the stripping of gametes from mature wild caught fish, acclamation of wild fish as broodstock and finally the ongrowing of hatchery reared fish to sexual maturity (Pankhurst 1998b). The effective management of reproduction in aquaculture relies on a thorough understanding of the pattern of gamete development, spawning duration and frequency and the associated endocrine changes. With this knowledge, potential bottlenecks can be identified and husbandry procedures optimised.

Reproductive processes in fish start when the germinal tissue in the developing gonad differentiates into spermatogonia and oogonia in the testis and ovary respectively (reviewed in Nagahama 1983). In females oogonia develop into previtellogenic oocytes and at the time of sexual maturation these oocytes begin to

sequester yolk proteins synthesised in the liver. This process is known as vitellogenesis and is responsible for the majority of gonad growth (Mommsen and Walsh 1983; Tyler 1991; Specker and Sullivan 1994; Tyler and Sumpter 1996). Final oocyte maturation (FOM) begins with the migration of the oocyte nucleus (germinal vesicle) to the animal pole, the coalescence of yolk and lipids and in most marine species a large increase in oocyte size due to hydration (Wallace and Selman 1981; Nagahama 1983). Oocytes that have completed FOM are released from the ovary by rupture on the ovarian follicle and at this stage the fish is ready for the behavioural act of spawning (Pankhurst 1998b).

There are three recognised modes of ovarian growth - synchrony, group synchrony or multiple group synchrony (reviewed by Wallace and Selman 1981; Wallace et al., 1987; Pankhurst 1998b). Fish that display a synchronous mode of reproduction posses a single clutch of maturing oocytes in the ovary. Such an example is the pacific salmon (*Onhorhynchus* spp) that spawn only once before dying. Group synchronous species, such as rainbow trout, spawn more than once in their life time but typically only once per season. The ovaries in these types of fish contain a batch of previtellogenic oocytes and a maturing clutch of oocytes for spawning in the current season. Multiple group synchrony describes ovarian development where there are multiple clutches of oocytes produced within a spawning season, and is the most common among the teleosts. In extreme cases, such as in snapper, FOM, ovulation and spawning occur on a daily basis over an extended spawning season of several months (Scott et al., 1993). This pattern is sometimes termed asynchronous (Wallace and Selman 1981; Wallace et al., 1987).

In males, spermatogonia mitotically divide to produce primary spermatocytes before meiotic division to form secondary spermatocytes and subsequently spermatids (reviewed by Grier 1981; Nagahama 1983; Pankhurst 1998b). This process is termed spermatogenesis. Once complete the process of spermiogenesis begins and spermatids divide again to produce spermatozoa. Spermatozoa are then released into the sperm ducts via the process of spermiation and there is an increase in water content of the seminal fluid to produce milt (Pankhurst 1994). The mode of gamete development in males essentially mirrors the patterns described for females.

The initiation of reproductive development in fish is controlled by exogenous (environmental) and endogenous (endocrine) factors. Environmental cues, such as photoperiod and temperature, regulate endocrine activity by exerting their effect on the hypothalamic-pituitary-gonad axis (reviewed by Peter 1983; Peter and Yu 1997; Pankhurst 1998b). In response to environmental cues the hypothalamus produces gonadotropin releasing hormone (GnRH) and dopamine (DA). GnRH and DA act in a stimulatory and inhibitory fashion respectively on the release of gonadotropins (GtHs) from the pituitary. Once released into circulation GtHs act by binding to specific membrane-bound receptors in the ovary and testis, which subsequently initiates steroid biosynthesis. Two forms of GtHs have been identified GtH-I and GtH-II (Suzuki et al., 1988; Swanson 1991; Van Der Kraak et al., 1992). Both GtHs have similar actions in stimulating gonadal DNA synthesis and steroid biosynthesis, but have temporally separated actions. Plasma levels of GtH-I tend to be highest during gonad growth and GtH-II is associated with final maturation (reviewed by Swanson 1991; Peter and Yu 1997).

Within the ovary, oocytes are surrounded by the ovarian follicle comprised of two cell layers, thecal (outer), and granulosa (inner). The outer thecal layers contribute to steroid production by synthesising steroid precursors such as 17α-hydroxyprogesterone (17P) and testosterone (T) (Kagawa et al., 1982; Kagawa et al., 1983). During vitellogenesis the inner granulosa cells then aromatise T to 17β-estradiol (E_2) which is subsequently released into the blood stream and stimulates the liver to synthesise yolk proteins (reviewed by Specker and Sullivan 1994). At the completion of vitellogenesis, there is a shift in the steroidogenic activity of the granulosa cells. This marks the beginning of FOM where E_2 is no longer produced and 17P is converted to the maturation inducing steroid 17,20β-dihydroxy-4-pregnen-3-one (17,20βP), or in some species 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β21P) (reviewed in Nagahama et al., 1983; Thomas 1994; Nagahama 1995; Pankhurst 1998b) Ovulation follows FOM but is generally steroid-independent, with F-series prostaglandins initiating the rupture and expulsion of the mature oocyte (Goetz 1983; Pankhurst 1998b).

The leydig cells are the major sites of gonadal steroids in males, however, spermatozoa and the epithelial cells around the vas deferens are also steroidogenic

(reviewed by Fostier et al., 1983; Fostier et al., 1987). Males differ from females in that aromatase activity is low or absent, and that T and its derivative 11-ketotestosterone (11KT) are the dominant steroids during testicular development. These two steroids control the initiation and maintenance of spermatogenesis, the development of secondary sexual characteristics, and may also modulate various aspects of reproductive behaviour. 17,20βP is produced later in the reproductive cycle of males and is believed to regulate spermiation and milt hydration. (reviewed Fostier et al., 1987; Pankhurst 1994; Pankhurst 1998b)

1.4 Stress and Reproduction

It is now well established that stress has the capacity to inhibit reproductive processes at every level of endocrine control so far examined (reviewed by Pankhurst and Van Der Kraak 1997). Stress results in two types of endocrine response, the adrenergic response which results in the rapid (within seconds) release of adrenaline and noradrenaline, and the hypothalamo-pituitary-interrenal (HPI) response (reviewed by Mazeaud and Mazeaud 1981; Barton and Iwama 1991; Sumpter 1997). Adrenaline and noradrenaline promote changes that increase oxygen uptake and blood glucose levels, providing an immediate energy source to deal with the stressor. Activation of the HPI axis stimulates the hypothalamic neurones to secrete corticotropin releasing factor which acts on the pituitary to release adrenocorticotrophic hormone (ACTH). ACTH subsequently stimulates the interrenal tissue to synthesise cortisol, which acts in releasing further energy reserves by the stimulation of gluconeogenesis. The effect of stress and / or cortisol are also know to elicit several secondary responses affecting metabolic (plasma glucose, liver and muscle glycogen and adenylate levels and lactic acid dissociation to $lactate^-$ and H^+), hematological (haematocrit, leucorit, erythrocyte and leucocyte numbers), hydromineral (plasma chloride, sodium, potassium, protein and osmolarity) and structural (interrennal cell size and condition factor) condition factors (reviewed by Barton and Iwama 1981; Barton 1997) In the natural environment the effect of stress responses are thought to be short lived, however, in the aquacultural environment, the stressor may be prolonged (Barton and Iwama 1981). Long term exposure to stress generates tertiary responses that can compromise immunocompetence, growth rates and reproductive capacity. Stress and / or cortisol is

known to affect plasma and pituitary gonadotropin levels, steroidogenesis, plasma vitellogenin levels, gamete development and quality, and subsequent egg and larval survival and development (reviewed by Pankhurst and Van Der Kraak 1997). These effects can have severe consequences for the management of captive or farmed fish and therefore stress reduction must be a priority of fish husbandry.

1.5 Artificial maturation

Although the failure to undergo vitellogenesis, FOM and / or spawning can occur in captive fish, there are several endocrine tools that can be used to address these problems (reviewed by Donaldson and Devlin 1996; Peter and Yu 1997; Pankhurst 1998b). These typically involve administration of exogenous hormones to artificially stimulate or prime the reproductive endocrine system. Synthetic analogues of GnRH (GnRHa) are used to stimulate the production of native GTHs. In some cases GnRHa is co-administered with a DA antagonist to block the inhibitory effects of DA. Synthetic GTHs are not available and piscine GTHs are difficult and expensive to acquire. This has resulted in mammalian GTHs being used, with the most common of these being human chorionic gonadotropin (hCG). Treatment of fish with hCG or GnRHa is usually achieved either by injection or the use of a slow release pellet. The best choice of hormone, method, dose and timing of treatment varies within individual species. Therefore the investigation of these factors is a key component in the development of new species for aquaculture.

1.6 Scope and aims of this study

This study aimed to provide information on the reproductive biology and endocrinology of black bream, investigated ways of obtaining good quality gametes, and assessed the effects of salinity on reproductive activity. The approach taken was to assess: a) the seasonal reproductive activity of black bream, b) the effect of capture and confinement on plasma steroid levels, c) the effect of hormone therapy and timing of treatment to induce ovulation, and d) the effect of salinity on plasma steroid levels, hormone induced ovulation, and egg fertility and development. The objective of this was to provide baseline information on the reproductive biology and endocrinology of black bream, to optimise hormone therapy protocols used to induce ovulation, to

increase the understanding of how salinity may affect reproductive success in black bream and to contribute to the growing understanding of the reproductive physiology of non-salmonid fishes.

Chapters 2-5 are presented in the form that they been submitted for publication (See below). This has resulted in some planned overlap of introduction and methods sections. The chapters are either in press or published as described below.

Chapter 2; Haddy, J.A., Pankhurst, N.W., 1998. Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). Mar. Freshwat. Res. 49, 389-397.

Chapter 3; Haddy, J.A., Pankhurst, N.W., 1999. Stress-induced changes in concentrations of plasma sex steroids in black bream. J. Fish Biol. 55, 1304-1316.

Chapter 4; Haddy, J.A., Pankhurst, N.W., 2000. The efficacy of exogenous hormones in stimulating changes in plasma steroids and ovulation in wild black bream *Acanthopagrus butcheri* is improved by treatment at capture. Aquaculture, in press.

Chapter 5; Haddy, J.A., Pankhurst, N.W., 2000. The effects of salinity on reproductive development, plasma steroid levels, fertilisation and egg survival in black bream *Acanthopagrus butcheri.* Aquaculture, in press.

Ethical clearance for the work conducted throughout this study was provided under the permit A0005451 University of Tasmania, Tasmanian Animal Experimentation Ethics Committee.

1.7 References

Allan, G.L., Fielder, D.S., 1999. Inland saline aquaculture activities in NSW. In: Smith, B., Barlow, C., (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceeding 83, Canberra. pp. 14-15.

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

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann. Rev. Fish Dis. 1, 3-26.

Battaglene, S.C., 1995. Induced ovulation and larval rearing of Australian marine fish. Unpublished PhD Thesis, University of Tasmania. 213p.

Battaglene, S.C., Talbot, R.B., 1992. Induced spawning and larval rearing of snapper, *Pagrus auratus* (Pisces: Sparidae), from Australian waters. NZ J. Mar. Freshwat. Res. 26, 179-183.

Blackwell, J., 1999. Using serial biological concentration to combine irrigation and saline aquaculture in Australia. In: Smith, B., Barlow, C. (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 83, Canberra. pp. 26-29.

Brown, D., Van Landeghem, K., Schuele, M., 1997. Australian Aquaculture; industry profiles for selected species. Australian Bureau of Agricultural and Resource Economics (ABARE) Research Report 97.3., Canberra. 102p.

Blackwell, J., Towensend, J., 1999. Irrigated agriculture's major problem, a possible springboard for an Australian aquaculture industry. In: The Annual International Conference and Exposition of the World Aquaculture Society, Book of Abstracts, World Aquaculture 99. p. 78.

Carragher, J.F., 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.

Chaplin, J.A., Baudains, G.A., Gill, H.S., McCulloch, R., Potter, I.C., 1998. Are assemblages of black bream (*Acanthopagrus butcheri*) in different estuaries genetically distinct? Int. J. Salt lake Res. 6, 303-321.

Cleary, J.J., 1998. The effects of stress on reproduction in snapper (*Pagrus auratus*) Unpublished PhD Thesis, University of Tasmania. 162p.

Cowden, K.L., 1995. Induced spawning and culture of yellowfin bream, *Acanthopagrus australis* (Günther, 1859) and mangrove jack, *Lutjanus argentimaculatus* (Forsskal, 1775). Unpublished PhD Thesis, James Cook University, Townsville, Australia. 270p.

Donaldson, E.M., Devlin, R.H., 1996. Uses of biotechnology to enhance production. In: Pennell, W., Barton, B.A. (Eds.), Developments in Aquaculture and Fisheries Science, Volume 29; Principles of Salmonid Culture. Elsevier Science B.V. Amsterdam. pp. 969-1020.

FAO (Food and Agricultural Organisation), 1996. Fisheries statistics; capture and production. vol 82.

Fielder, D.S., Bardsley, W.J., Allan, G.L., 1999. Culture of snapper *Pagrus auratus* in saline groundwater from western NSW, Australia. In: The Annual International Conference and Exposition of the World Aquaculture Society, Book of Abstracts, World Aquaculture 99. p. 78.

Forsberg, J.A., Dorsett, P.W., Neill, W.H., 1996. Survival and growth of red drum *Sciaenops ocellatus* in saline groundwaters of west Texas, USA. J. World Aquaculture Soc. 27, 462-474.

Foscarini, R., 1988. A review: Intensive farming procedure for red sea bream (*Pagrus major*) in Japan. Aquaculture 72, 191-246.

Fostier, A., Jalabert, B., Billard, R., Breton, B., Zohar, Y., 1983. The gonadal steroids. In: Hoar, W.S., Randall, D.J., Donaldson, E.M., (Eds.), Fish Physiology, Vol. 9, Part A. Academic Press, London. pp 277-347.

Fostier, A., Le Gac, F., and Loir, M. (1987). Steroids in male reproduction. In: Idler, D.R., Crim, L.W., Walsh, J.M., (Eds.), Reproductive Physiology of Fish 1987, Memorial University of Newfoundland, St John's. pp. 239-245.

Goetz, F.W., 1983. Hormonal control of oocyte final maturation and ovulation in fishes. In: Hoar, W.S., Randall, D.J., Donaldson, E.M., (Eds.), Fish Physiology, Vol. 9, Part B. Academic Press, London. pp. 117-170.

Gooley, G., Ingram, B., McKinnon, L., 1999. Inland saline aquaculture - a Victorian perspective. In: Smith, B., Barlow, C. (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 83, Canberra. pp. 16-19.

Grier, H.J., 1981. Cellular organisation of the testis and spermatogenesis in fishes. Am. Zool. 21, 345-357.

Hutchinson, W., 1999. Inland saline aquaculture in South Australia. In: Smith, B., Barlow, C. (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 83, Canberra. pp. 20-23.

Ingram, B., Gooley, G., McKinnon, L., 1996. Potential for inland mariculture in Victorian saline groundwater evaporation basins. Austasia Aquaculture 10, 61-63.

Jenkins, G.I., 1999. Potential for inland saline aquaculture of fishes. In :Smith, B., Barlow, C., (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceeding 83, Canberra. pp. 42-46.

Jenkins, G.I., Frankish, K.R., Partridge, G.J., 1999. Manual for the hatchery production of black bream (*Acanthopagrus butcheri*). Aquaculture Development Unit, Fremantle Maritime Centre South Metropolitan College of TAFE, Fremantle, Western Australia. 125p.

Kagawa, H., Young, G., Adachi, S., Nagahama, Y., 1982. Estradiol-17β production in amago salmon (*Oncorhynchus rhodurus*) ovarian follicles: role of the thecal and granulosa cells. Gen. Comp. Endocrinol. 47, 440-448.

Kagawa, H., Young, G., Adachi, S., Nagahama, Y., 1983. Estrogen synthesis in the teleost ovarian follicle: The two-cell type model in salmonids. In: Iwamoto, R.N., Sower, S., (Eds.), Salmonid Reproduction, Washington Sea Grant Program, University of Washington, Seattle. pp. 20-25.

Lenanton, R.C., Ayvazian, S.G., Dibden, C, Jenkins, G., Sarre, G., 1999. The use of stock enhancement to improve the catch rates of black bream, *Acanthopagrus butcheri* (Munro) for western Australian recreational fishers. In: Howell, B.R., Moksness, E., Svasand, T., (Eds.), Stock Enhancement and Sea Ranching, Fishing News Books, London. pp. 219-230.

Mazeaud, M.M., Mazeaud, F., 1981. Adrenergic responses to stress in fish. In: Pickering, A.D., (Ed.), Stress and Fish, Academic Press, London. pp. 49-75.

Maguire, G., Sarre, G., 1999. Farming black bream. Aquaculture WA, No 10, Fisheries Western Australia. 7p.

Mommsen, T.P., Walsh, P.J., 1983. Vitellogenesis and oocyte assembly. In: Hoar, W.S., Randall, D.J., Donaldson, E.M., (Eds.), Fish Physiology, Vol. 9, Part A. Academic Press, London. pp. 347-406.

Morison, A.K., Coutin, P.C., Robertson, S.G., 1998. Age determination of black bream, *Acanthopagrus butcheri* (Sparidae), from the Gippsland lakes of south-eastern Australia indicates slow growth and episodic recruitment. Mar. Freshwat. Res. 49, 491-498.

Nagahama Y., 1983. The functional morphology of teleost gonads. In: Hoar, W.S., Randall, D.J., Donaldson, E.M., (Eds.), Fish Physiology, Vol. 9, Part A. Academic Press, London. pp. 222-275.

Nagahama, Y., Young, G., Ueda, H., Kagawa, H. and Adachi, S., 1983. Endocrine control of final maturation in salmonids. In: Iwamoto, R.N., Sower, S., (Eds.), Salmonid Reproduction, Washington Sea Grant Program, University of Washington, Seattle. pp 8-19.

Nagahama, Y., Yoshikuni, M., Yamashita, M., Tanaka, M., 1995. Regulation of oocyte maturation in fish. In: Sherwood, N.M., Hew, C.L., (Eds.), Fish Physiology, Vol. 13. Academic Press, New York. pp 393-439.

Nulsen, B., 1999. Inland saline waters in Australia. In: Smith, B., Barlow, C. (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 83, Canberra. pp. 6-11.

Ogburn, D.M., 1999. Environmental considerations in the use and management of inland saline water bodies for aquaculture. In: Smith, B., Barlow, C. (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 83, Canberra. pp. 32-34.

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, 185-97.

Pankhurst, N.W., 1998a. Aquaculture: an international perspective. In: Maori and the Business of Aquaculture, Kia Ora Promotions, Auckland. pp.1-11.

Pankhurst, N.W., 1998b. Reproduction. In: Black, K.D., Pickering, A.D. (Eds.), Biology of Farmed Fish. Sheffield Academic Press, Sheffield. pp. 1-26.

Pankhurst, N.W., 1999. Summation of outcomes of day 1. In: Smith, B., Barlow, C. (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 83, Canberra. p 47.

Pankhurst, N.W., Carragher, J.F., 1991. Seasonal endocrine cycles in marine teleosts. In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolfe,. M.S., (Eds.), Reproductive Physiology of Fish 1991, FishSymp 91, Sheffield. pp. 131-5

Pankhurst, N.W., 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, 337-47.

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

Paust, G., 1999. Inland saline aquaculture in Western Australia. In: Smith, B., Barlow, C. (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 83, Canberra. pp. 24-25.

Peter, R.E., 1983. The brain and neurohormones in teleost reproduction. In: Hoar, W.S., Randall, D.J., Donaldson, E.M., (Eds.), Fish Physiology, Vol. 9, Part A. Academic Press, London. pp. 97-127.

Peter, R.E., Yu, K.L., 1997. Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. Rev. Fish Biol. Fish. 7, 173-197.

Pruginin, Y., Fishelson, L., Koren, A., 1988. Intensive tilapia farming in brackish water from an Israeli desert aquifer. In: Pullin, R.S.V., Bhukaswan, T., Tonguthai, K., Maclean, J.L., (Eds.), The Second International Symposium on Tilapia in Aquaculture. ICLARM 15, Bangkok, Thailand. pp. 75-81.

Sarre, G.A., Potter, I.C., 1999. Comparisons between the reproductive biology of black bream *Acanthopagrus butcheri* (Teleostei: Sparidae) in four estuaries with widely differing characteristics. Int. J. Salt Lake Res. 8, 179-210.

Sarre, G.A., Platell, M.E., Potter, I.C., 2000. Do the dietary compositions of *Acanthopagrus butcheri* (Sparidae) in four estuaries and a coastal saline lake vary with body size and within and amongst these water bodies? J. Fish Biol. 56, 103-122.

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

Scott, S.G., Zeldis J.R., Pankhurst, N.W., 1993. Evidence of daily spawning in natural populations of the New Zealand snapper *Pagrus auratus* (Sparidae). Env. Biol. Fish. 36, 149-56.

Specker, J.L., Sullivan, C.V., 1994. Vitellogenesis in fishes: Status and persectives. In: Davey, K.G., Peter, R.E., Tobe,. S.S., (Eds.), Perspectives in Comparative Endocrinology, National Science and Engineering Research Council of Canada, Ottawa. pp. 304-315.

Stewart, P.C., Greive, C., 1993. Bream, *Acanthopagrus* species. In: Kailola, P.J., Williams, M.J., Stewart, P.C., Reichelt, R.E., McNee, A., Grieve, C., (Eds.), Australian Fisheries Resources, Bureau of Resource Sciences and Fisheries Research and Development Corporation, Canberra. pp. 311-314.

Sumpter, J.P., 1997. The endocrinology of stress. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B., (Eds.), Fish, Stress and Health in Aquaculture, Cambridge University Press, Cambridge. pp. 95-118.

Suzuki, K., Nagahama, Y., Kawauchi, H., 1988. Steroidogenic activities of two distinct salmon gonadotrophins. Gen. Comp. Endocrinol. 71, 452-458.

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

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

Trendall, J., Alder, J., Lymbery, A., 1999. A national environmental management policy for land based fish farming. In: Smith, B., Barlow, C. (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 83, Canberra. pp. 30-31.

Tyler, C.R., 1991. Vitellogenesis in salmonids In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolf, M.S., (Eds.), Reproductive Physiology of Fish 1991. FishSymp 91, Sheffield. pp. 295-299.

Tyler, C.R., Sumpter, J.P., 1996. Oocyte growth and development in teleosts. Rev. Fish Biol. Fish. 6, 287-318.

Van Der Kraak, G., Suzuki, K., Peter, R.E., Itoh, H., Kawauchi, H., 1992. Properties of common carp gonadotropin I and gonadotropin II. Gen. Comp. Endocrinol. 85, 217-229.

Wallace, R.A., Selman, K. 1981. Cellular and dynamic aspects of oocyte growth in teleosts. Am. Zool. 21, 325-343.

Wallace, R.A., Selman, K., Greely, M.S. Begovac, P.C., Lin, Y-W. P., Mcpherson, R., Petrino, T.R., 1987. Current status of oocyte growth. In: Idler, D.R., Crim, L.W., Walsh, J.M., (Eds.), Reproductive Physiology of Fish 1987, Memorial University of Newfoundland, St John's. pp. 167-177.

Williams, M.J., Stewart, P.C., 1993. Australia's Fisheries. In: Kailola, P.J., Williams, M.J., Stewart, P.C., Reichelt, R.E., McNee, A., Grieve, C., (Eds.), Australian Fisheries Resources, Bureau of Resource Sciences and Fisheries Research and Development Corporation, Canberra. pp. 1-21.

CHAPTER 2

Annual change in reproductive condition and plasma levels of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae)

2. Annual change in reproductive condition and plasma levels of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae)

2.1 Summary

Changes in gonadosomatic index (GSI), hepatosomatic index (HSI), gonad stage and plasma concentrations of sex steroids were studied over one year in black bream (*Acanthopagrus butcheri*). Black bream have an annual reproductive cycle with a 3 month spawning season in spring / early summer. GSI and HSI values were highest in October and May respectively. Plasma concentrations of estradiol (E_2), testosterone (T) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) were highest in females in October. Plasma concentrations of E_2 and T were highest in ovulated fish. Concentrations of 17,20βP were higher in fish undergoing final oocyte maturation (FOM) than in fish with regressed gonads. In males, plasma concentrations of T and 11-ketotestosterone (11KT) increased in September and remained elevated until January, but concentrations of 17,20βP did not change with season. However, 17,20βP concentrations in spermiated fish were higher than in non-spermiated fish. Daily changes in gonad condition indicated that females undergo daily cycles of ovarian maturation with ovulation occurring after midday. Plasma T and 17,20βP concentrations of females were elevated at midday in association with FOM, but E_2 showed no diel change. In males, partially spermiated fish were dominant in the early morning and fully spermiated fish at midday. Plasma T, 11KT and 17,20βP concentrations were low at midnight and reached maximal levels at 6 am.

2.2 Introduction

Black bream (family Sparidae) are endemic to the estuaries of southern Australia, with approximately 200-500 t being harvested commercially per annum (Stewart and Grieve 1993). Sparids form the basis of many major fisheries and aquaculture projects around the world (Foscarini 1988). Consequently, there is an increasing interest in their reproductive physiology and biology. Both seasonal and-short term cycles of plasma concentrations of sex steroids and/or seasonal gonad development have been reported for many sparids, including snapper *Pagrus auratus* (reviewed in Carragher and Pankhurst 1993), yellowfin bream *Acanthopagrus australis* (Pollock 1982; Pollock 1985), yellowfin porgy *Acanthopagrus latus* (Abu-Hakima 1984) and the black porgy *Acanthopagrus schlegeli* (Chang and Yueh 1990). However, there is little information on the reproductive biology of euryhaline sparids and no information on the reproductive physiology of black bream.

Sparids typically show an annual reproductive cycle with asynchronous gonad development and a daily spawning pattern occurring over a period of 2-5 months. Anecdotal reports indicate that black bream in Tasmania spawn between spring and early summer in the upper to middle reaches of estuaries. The objective of this study was to describe reproductive development and gonadal cycling in black bream, and correlate reproductive development with changes in endocrine characteristics. Seasonal and daily changes in reproductive condition were assessed against changes in plasma concentrations of testosterone (T) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) in both sexes and estradiol (E_2) and 11-ketotestosterone (11KT) in female and male fish respectively. These hormones were chosen because of their roles in regulation of vitellogenesis, ovarian recrudescence (E_2 and T) and final oocyte maturation (17,20βP) in females, and spermatogenesis (T and 11KT) and spermiation (17,20βP) in males (reviewed in Pankhurst 1998). Because capture stress can elevate plasma cortisol concentrations, and in many species this is associated with depression of plasma concentrations of reproductive steroids (reviewed in Pankhurst and Van Der Kraak 1997), plasma cortisol concentrations were measured to assess the possible impact of sampling stress on blood hormone concentrations.

Since photoperiod and temperature influence reproductive activity in temperate teleosts (Bye 1987), and additional triggers of spawning in black bream might include

salinity and the level of dissolved oxygen (DO)(Sherwood and Backhouse 1982), we monitored temperature, salinity and dissolved oxygen of the spawning areas at the time when fish were caught. In addition to allowing a comparison of the pattern of reproduction in an estuarine sparid with that of stenohaline sparids, this study provides the baseline for the investigation of reproduction in black bream and the possible controlling effects that salinity may exert on this process.

2.3 Materials and Methods

Sampling

Black bream were captured from April 1996 to May 1997 by rod and line from the Meredith (148°7'S, 42°4'E) and Swan Rivers (148°4'S, 42°4'E) at Swansea, Tasmania. Data from fish from both estuaries have been combined for presentation. Where differences between estuaries occurred, this is noted in the text. Diurnal sampling was conducted from 18 to 22 October 1996 from the Meredith River. Fish were caught throughout the 24-h period and allocated to 4 sampling blocks of 6 h each according to the time of capture. The times indicated in the text and figures are the mid points of each sampling block. All other fish were caught between 0530 and 2300 hours. Blood was sampled by caudal puncture using heparinized syringes within 5 min of hooking. Fish were then killed by spinal transection, fin-clipped for identification and placed on ice. Blood was stored on ice, plasma obtained by centrifugation, then frozen and stored at -18°C until required for assay. Fork length, body, liver and gonad weights, sex and macroscopic gonad condition were recorded from each fish. Criteria for macroscopic staging of gonads are given in Table 1 and were verified by histological examination according to the histological characteristics outlined in Scott and Pankhurst (1992). Gonadosomatic (GSI) and hepatosomatic (HSI) indices were calculated as gonad/gonad free body weight) x 100 and (liver/liver free body weight) x 100 respectively. Fish were provisionally aged by counting presumptive annual rings in otoliths and scales under a dissecting microscope. Temperature, salinity and DO were measured at 1-m intervals with a submersible multi probe sensor (Perstorp Water Analyser), at locations when and where fish were caught, throughout the study period.

Table 2.1. Criteria for macroscopic classification of bream gonads (modified from Scott and Pankhurst 1992)

Stage	Classification	Macroscopic appearance	Histological characteristics
		female	
1	immature	Ovary small clear threads	Previtellogenic oocytes
2	regressed	Ovary small clear and orange	Cortical alveoli stage oocytes appear
3	vitellogenic	Ovary orange with opaque oocytes visible through epithelium	Oocytes in exogenous vitellogenesis
4	hydrated	Ovary orange with hydrated oocytes visible through epithelium	Final oocyte maturation and hydration
5	ovulated	Eggs in the oviduct which can be extruded with gentle pressure	Hydrated oocytes in the oviduct and post-ovulatory follicles present
6	spent	Ovary flaccid and bloody	Atretic vitellogenic oocytes but predominantly previtellogenic oocytes present
		male	
1	immature	Testis white threads	Spermatogonia and a few previtellogenic oocytes*
2	spermatogenic	Testis firm and ivory white	Secondary spermatocytes, spermatozoa
3	partially spermiated	Testis firm and ivory white with viscous milt in sperm duct	Spermatozoa predominate
4	fully spermiated	Testis firm and ivory white with free flowing milt in sperm duct	Spermatozoa predominate
5	spent	Testis grey to bloody and flaccid	Residual spermatozoa, reduced spermatocytes and increased connective tissue

* Oocytes located in dorsal section of gonad in all male stages.

Steroid measurement

Plasma steroid concentrations were measured by radioimmunoassay (RIA), using the reagents and protocols given in Pankhurst and Carragher (1992) for E_2, T, 17,20βP and cortisol, and Pankhurst and Kime (1991) for 11KT. Extraction efficiency was determined by recovery of [^{3}H]-labelled steroid extracted with plasma, and assay values were corrected accordingly. Assay detection limits in plasma were 0.15 ng mL^{-1}; for 11KT, E_2, T and 17,20βP and 0.3 ng mL^{-1} for cortisol. Interassay variability

(%CV) measured using aliquots of a pooled standard was as follows: 11KT = 17.4% (n=8); E_2 = 9.9% (n=8); T = 7.8% (n=8); 17,20βP = 23.0% (n=8) and cortisol = 12.1% (n=8).

Statistics

Kruskal-Wallis, One way ANOVA and Tukey's multiple comparison of means tests were performed using the SPSS statistical package. Analysis of steroid data was performed on raw or log transformed data to satisfy homogeneity of variance. Percentage data were arcsin transformed. In some instances variances were still heterogeneous after transformation; however, the data were also assessed by Kruskal-Wallis one way ANOVA, and in all cases the outcomes were unchanged. We chose to present ANOVA results because of the utility of means comparison tests. Sample values ≤ 3 were not included in the statistical analyses.

2.4. Results

Seasonal changes in GSI, HSI and gonad stage

The GSI of female fish was low (below 2%) throughout autumn and winter, increased rapidly in September and remained high until January (Fig. 2.1a). The HSI was high during the winter and early spring, before gradually decreasing to low levels in March, then increased to highest levels in May (Fig. 2.1c). In males, the mean GSI was also low in autumn and winter, began to rise in August, and peaked at the end of October (Fig. 2.1b). The seasonal increase in male GSI was not as rapid as in females. Male HSI gradually decreased throughout winter, spring and summer, reaching its lowest level in January, then increased to the highest level in May (Fig. 2.1d).

From March to July, all female fish had regressed ovaries (stage 2) (Fig. 2.2). Vitellogenic fish (stage 3) first appeared in August, one month before the main increase in GSI, and remained present until January. Fish with hydrated oocytes (stage 4) were associated with the first GSI peak and also remained present until January. Very few ovulated (stage 5) fish were caught; however, they were present from the end of October to the end of December. Low numbers of spent fish were caught towards the end of February when fish at stages 3-5 were no longer present.

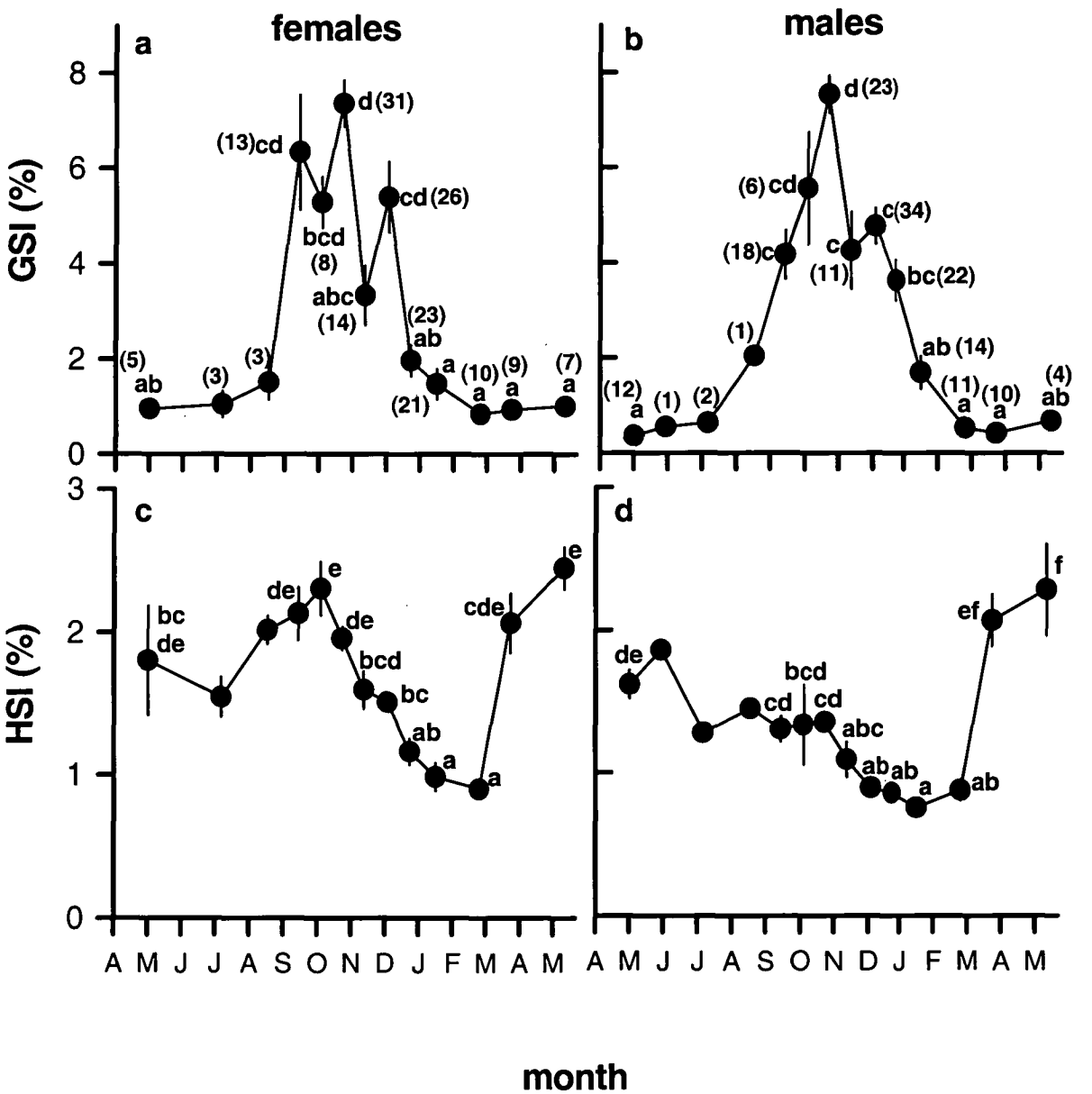


Fig. 2.1. Annual changes in gonadosomatic index (GSI) and hepatosomatic index (HSI) in wild black bream. All values are mean ± s.e. Values that are significantly different have different superscripts ($P<0.05$); values without superscripts were not included in the analysis due to low n values. (n values given in parenthesis).

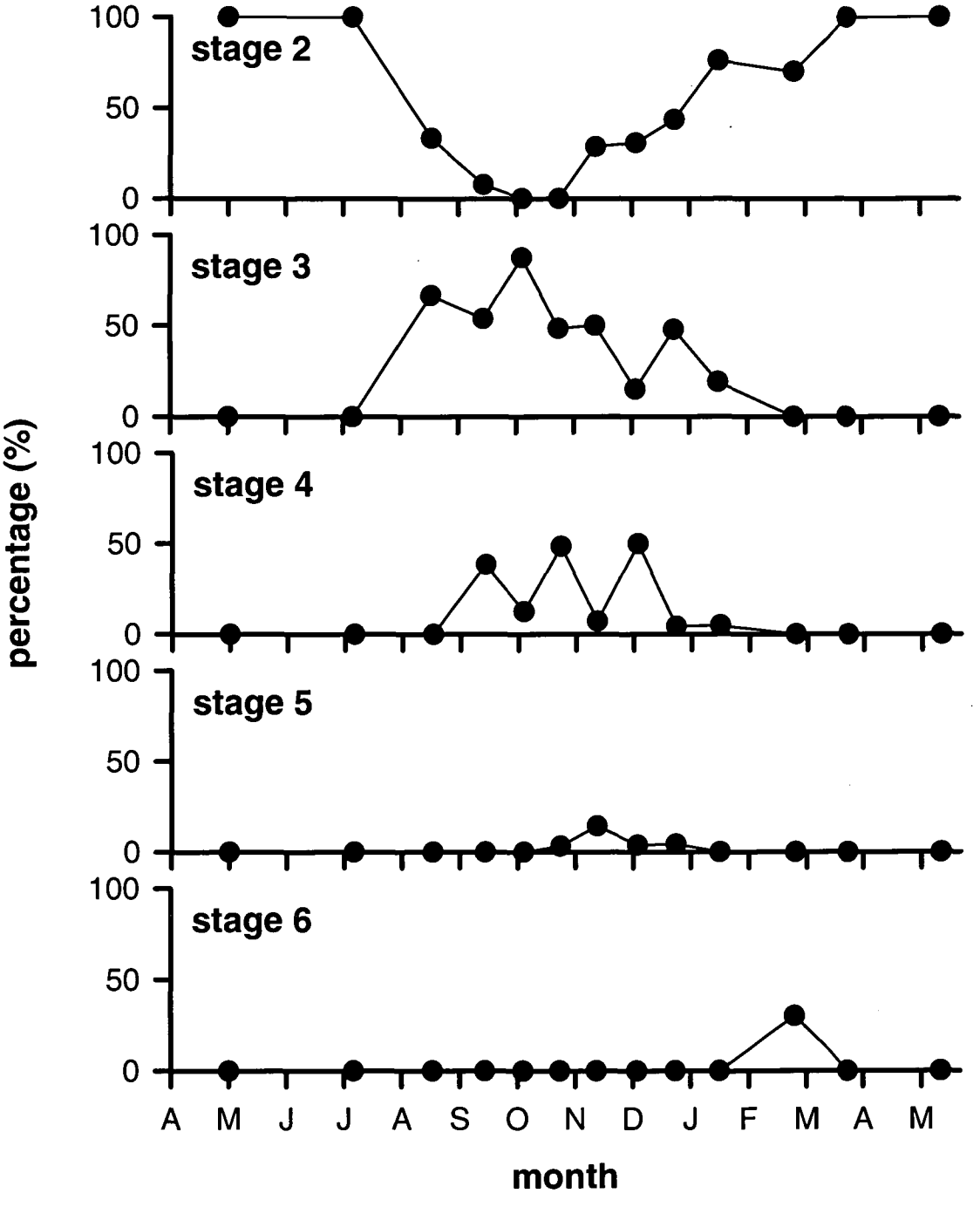


Fig. 2.2. Annual variation in proportions of female black bream with particular macroscopic gonad stages as described in Table 2.1.

The initial increase in GSI and the appearance of vitellogenic fish did not coincide between the two estuaries, with the presence of vitellogenic fish and subsequent increase in GSI being delayed by 1 month in the Swan River. With the exception of 1 captured fish during flood conditions, all fish with hydrated oocytes were captured in the upper reaches of both estuaries, however, their presence in the two estuaries did not overlap (9 Sept to 10 Nov for the Meredith compared with 28 Nov to 12 Jan for the Swan River). It is possible that spawning activity may have occurred earlier in the Swan but was not detected because of low numbers of fish captured in September.

All male fish had regressed testes (stage 2) from March to July (Fig. 2.3). Partially spermiated (stage 3) males first appeared in August and were associated with the initial increase in GSI. The proportion of partially spermiated fish gradually dropped as the GSI increased, until none were present at the beginning of November. At the end of November, partially spermiated fish reappeared and remained present until March. Fully spermiated (stage 4) fish first appeared in September and were present throughout the period of high GSI and began declining in January before disappearing by March. No spent (stage 5) males were caught.

Seasonal changes in plasma hormone concentrations

Plasma concentrations of E_2 in females were low (< 0.6 ng mL^{-1}) during autumn and winter, began to rise in September, before peaking in late October at 4.2 ng mL^{-1} then returning to low concentrations in January (Fig. 2.4). Plasma concentrations of T were lower than E_2 throughout, but followed a similar pattern with a peak in late October at 2.9 ng mL^{-1}. Concentrations of 17,20βP were low (< 0.7 ng mL^{-1}) throughout the season, but concentrations in late October were significantly higher than concentrations in March.

Plasma concentrations of T were low (< 0.3 ng mL^{-1}) in males during autumn and winter (Fig. 2.5). Concentrations rapidly increased in September and remained elevated (≈ 1.2 ng mL^{-1}) throughout the spawning season with the exception of a distinct but non-significant drop in plasma T concentrations in early October. Plasma concentrations of 17,20βP were not significantly elevated at any time; however, they followed a similar pattern to T, with highest values of 1.2 ng mL^{-1} in November.

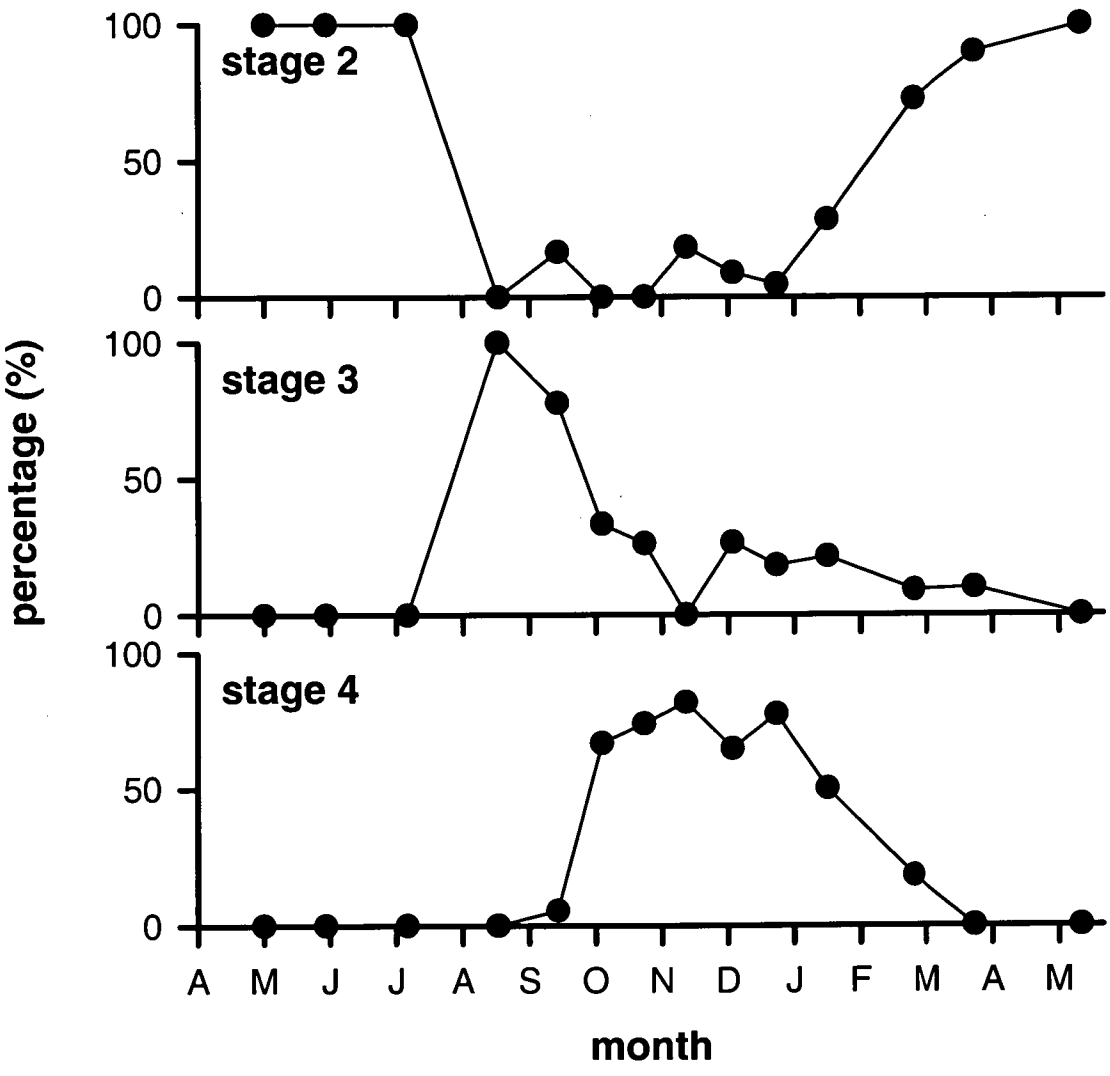


Fig. 2.3. Annual variation in proportions of male black bream with particular macroscopic gonad stages as described in Table 2.1.

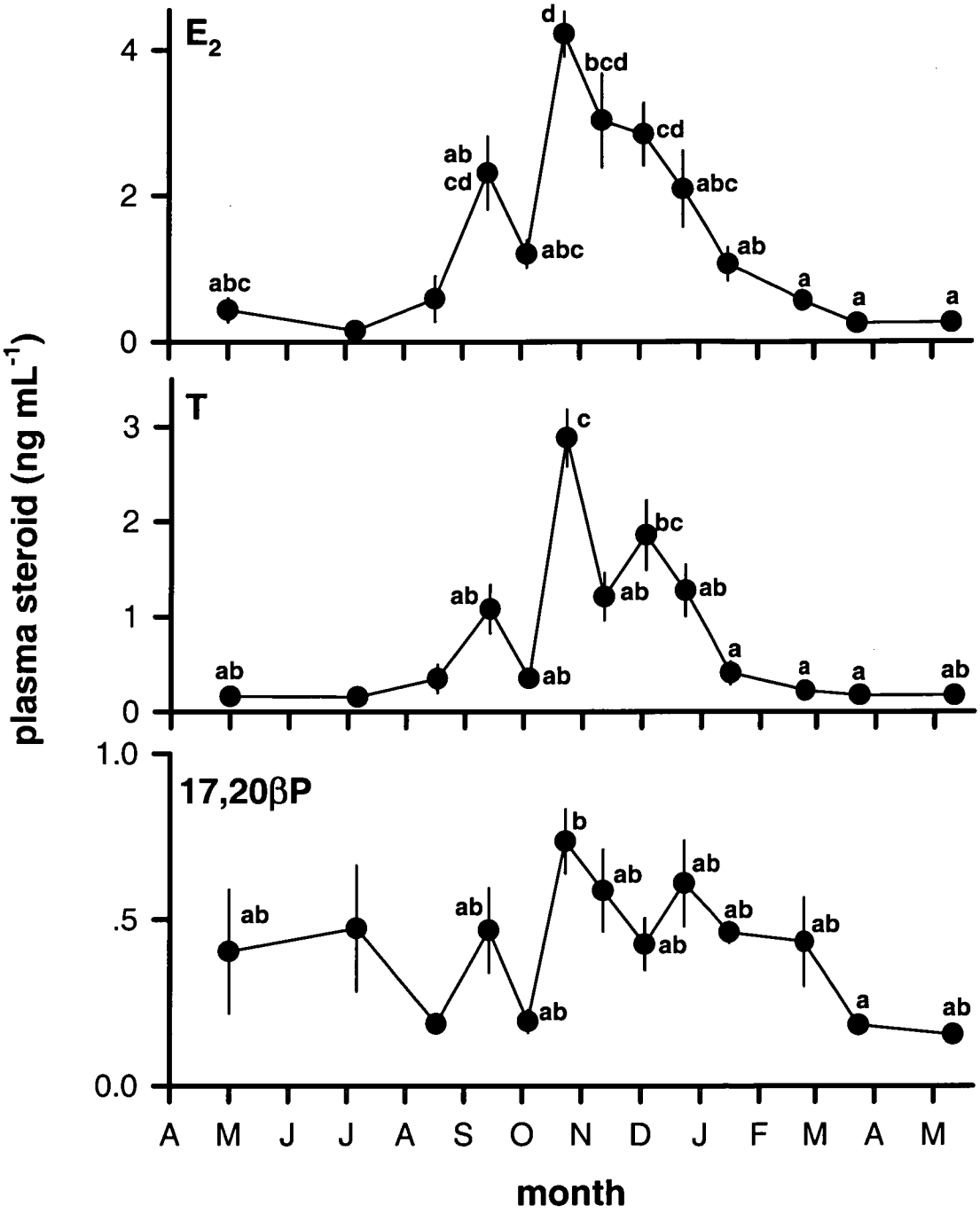


Fig. 2.4. Annual changes in plasma levels of estradiol (E_2), testosterone (T) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) in female black bream. Other details as for Fig. 2.1.

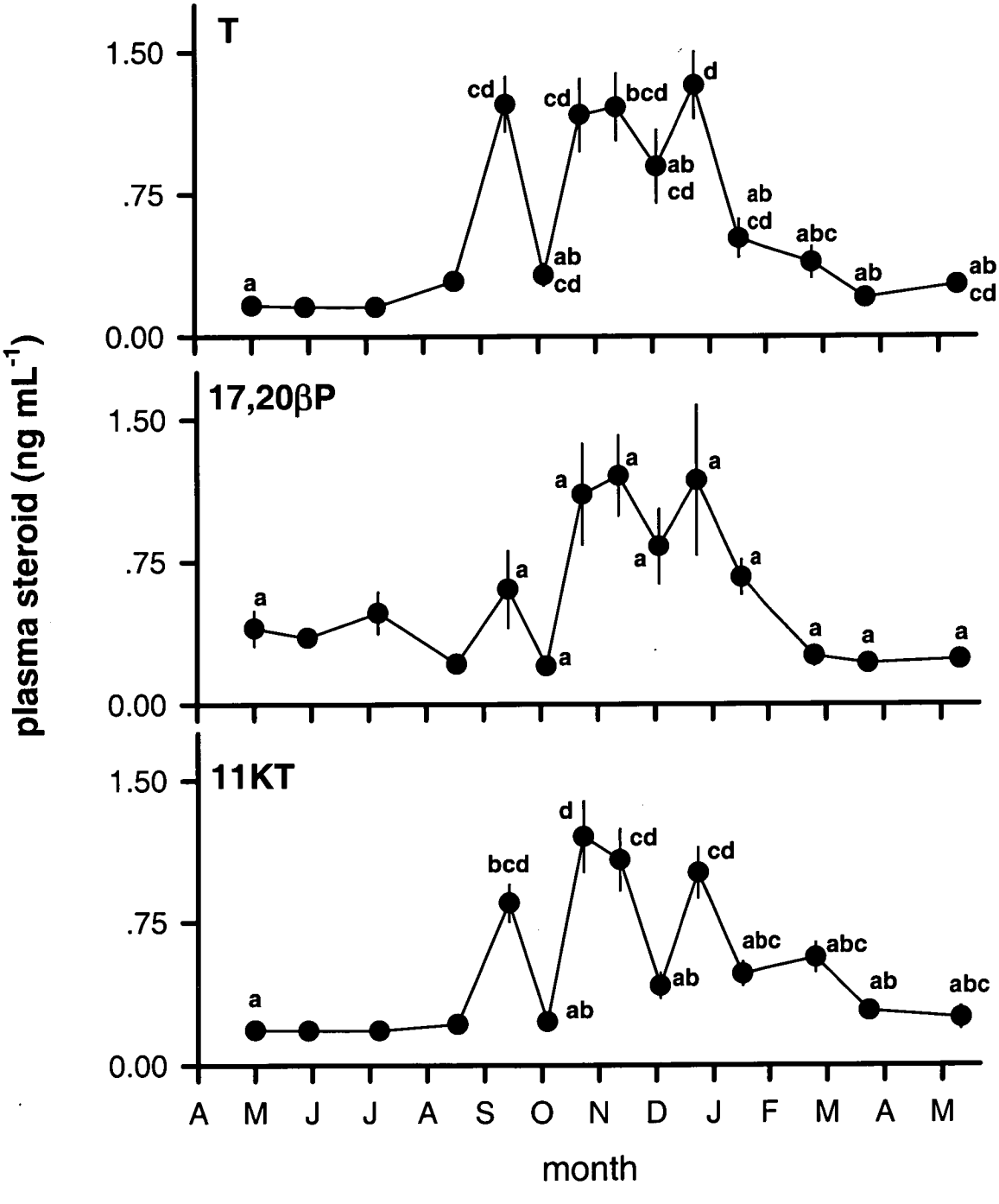


Fig. 2.5. Annual changes in plasma levels of testosterone (T), 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 11-ketotestosterone (11KT) in male black bream. Other details as for Fig. 2.1.

Plasma concentrations of 11KT were highest in late October (1.2 ng mL^{-1}) and also followed a similar pattern to T.

The majority of fish were bled within 5 minutes of hooking (92 and 95% for females and males respectively). The mean plasma cortisol concentrations of these fish were 2.8 ng mL^{-1} (n=140) for females and 1.9 ng mL^{-1} (n=140) for males.

Changes in plasma hormone concentrations with gonad stage

Plasma concentrations of E_2 and T in females were lowest in fish with ovaries that were regressed or spent (Fig. 2.6). Concentrations of E_2 increased sequentially until peaking in ovulated fish; however concentrations were not significantly different from stage 4 fish. Plasma T concentrations followed a similar pattern, but there was not a significant difference between stage 3, 4 or 5 fish. Plasma concentrations of 17,20βP were significantly elevated in fish with hydrated oocytes over fish with regressed ovaries.

Males with regressed testes had the lowest concentrations of all the sex steroids. Plasma concentrations of T and 11KT were similar in partially and fully spermiated fish, and were significantly higher than in fish with regressed testes. Plasma concentrations of 17,20βP in fully spermiated fish were significantly elevated over concentrations in fish with regressed testes.

Diel rhythm of gonad condition and plasma hormone concentrations

Proportions of females with vitellogenic ovaries were highest at midnight (2100-0259h) and progressively decreased throughout the day (Fig. 2.7). Proportions of fish with hydrated oocytes in the ovaries were highest at midday (0900-1459h) and lowest at midnight. Ovulated fish were present only in the later half of the early evening (1500-2059h). Male fish were all fully spermiated at midday. Proportions of partially spermiated fish increased from the early evening to the early morning (0300-0859h), before disappearing at midday.

Plasma concentrations of E_2 in females were not significantly different at any time of the day in sexually mature fish (Fig. 2.8). Plasma concentrations of T and 17,20βP were significantly elevated at midday relative to concentrations at midnight.

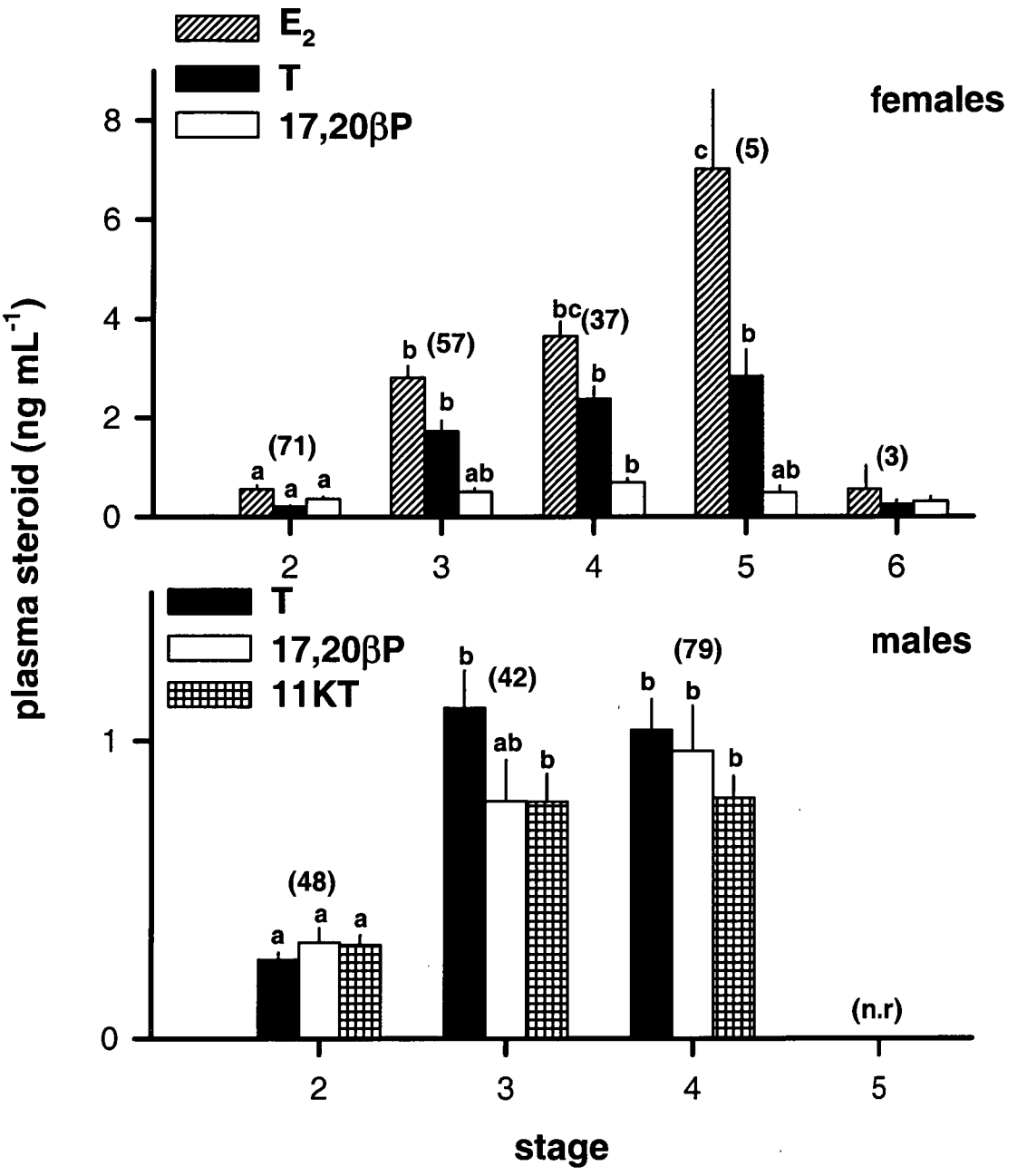


Fig. 2.6. Changes in plasma levels of estradiol (E_2, females only), testosterone (T) 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 11-ketotestosterone (11KT, males only) in black bream with particular macroscopic gonad stages as described in Table 1. Other details as for Fig 2.1.

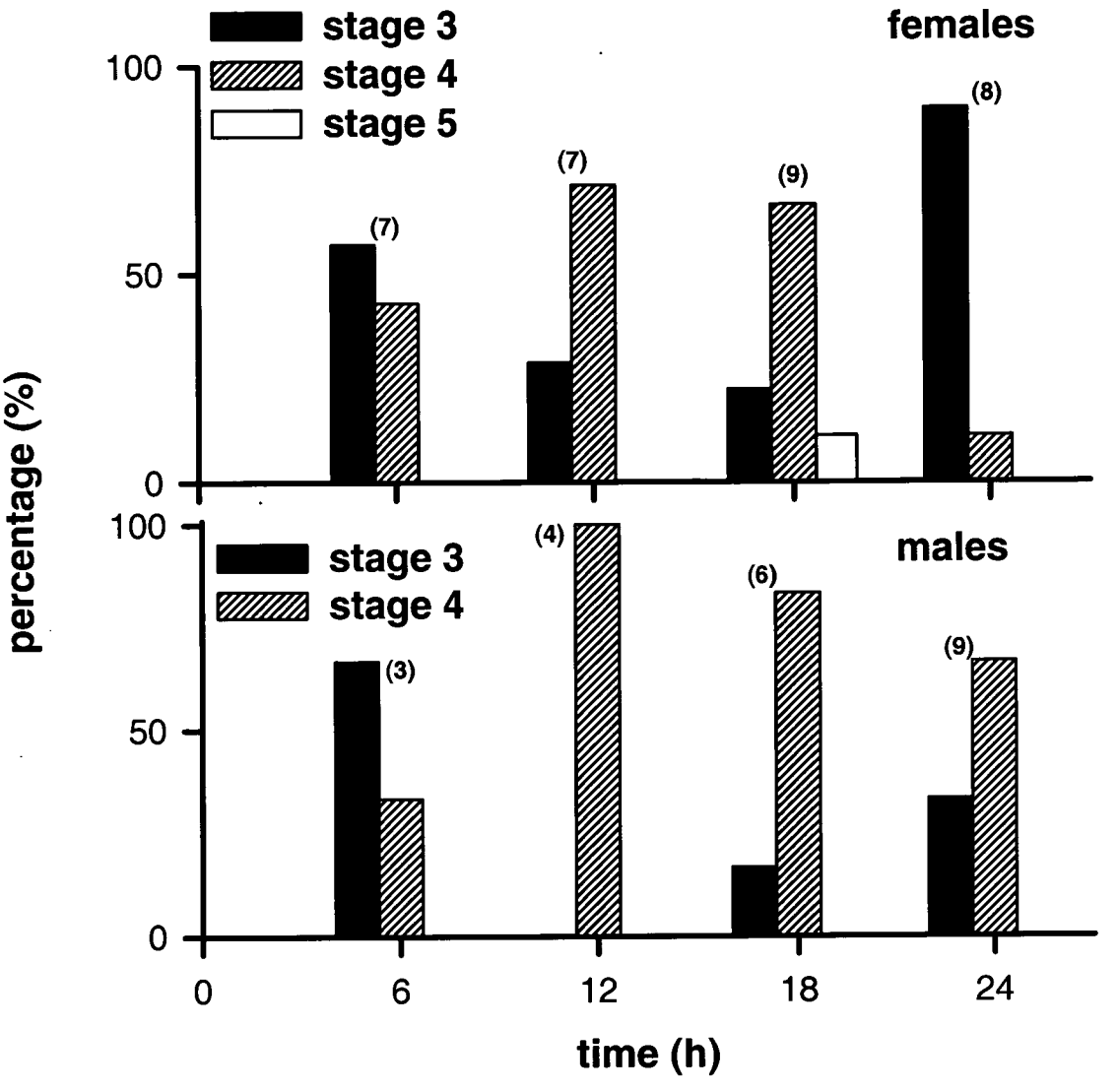


Fig. 2.7. Diel changes proportions of female and male black bream with particular macroscopic gonad stages as described in Table 2.1. (n values at each sample time in parenthesis).

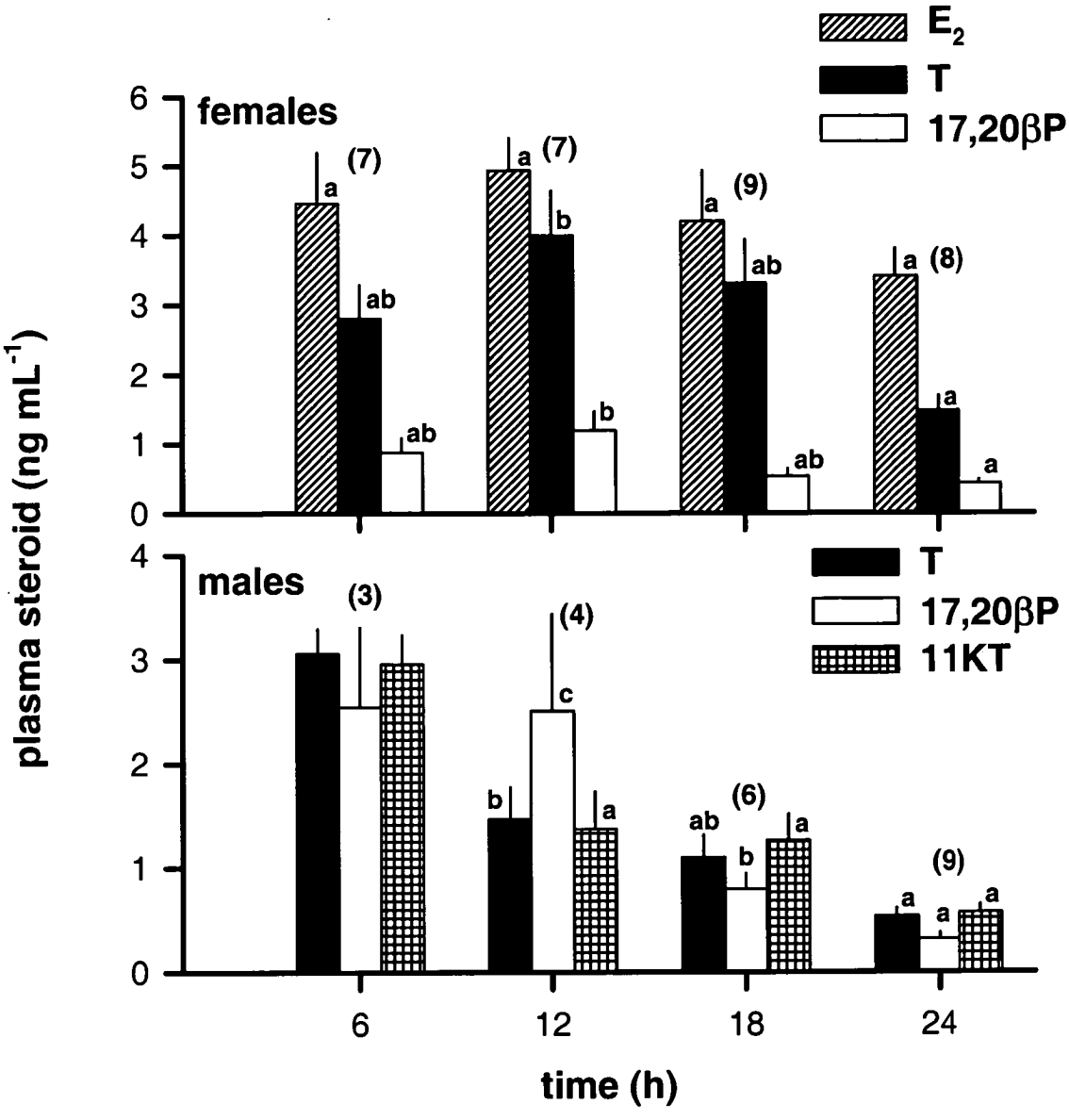


Fig. 2.8. Diel changes in plasma levels of estradiol (E_2, females only), testosterone (T) 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 11-ketotestosterone (11KT, males only) in black bream. Other details as for Fig. 2.1. (n values shown in Fig. 2.7.).

Plasma concentrations of T in males were highest in the early morning and at lowest concentrations at midnight. Plasma concentrations of 11KT were not significantly different at any time, although values from the small number of fish captured between 0300 and 0900h suggest that an early morning peak may occur. Plasma concentrations of 17,20βP were significantly elevated in fish captured between 0900 and 1500h and at lowest concentrations in fish captured between 2100 and 0300h.

Estuary descriptions and water quality ranges during spawning

The Meredith River estuary has a catchment area of 86 km^2, is small (500 m long) and shallow (maximum depth 4 m), and the mouth is seasonally closed. The upper reaches had a maximum temperature and salinity of 29.1°C and 35 g Kg^{-1} respectively. In contrast, the Swan river estuary has a catchment area of 448 km^2 is relatively long (10 Km) and deep (maximum depth 10 m), and is permanently open. The upper reaches had a maximum temperature and salinity of 27.5°C and 29 g Kg^{-1} respectively. Female fish which were ovulated or had hydrated oocytes were captured over a sub-surface (≥ 1m) salinity range of 13.9-35.0 g Kg^{-1}, temperature range of 15.5-26.2°C and DO range of 4.2-13.6 mg L^{-1}. At the same sample times, surface values of salinity, temperature and DO ranged from 0-19.2 g Kg^{-1}, 12.4-25.1°C and 6.1-8.4 mg L^{-1} respectively.

2.5 Discussion

This study confirms anecdotal reports that black bream spawn in spring and early summer in Tasmania. Seasonal changes in female GSI values, and proportions of gonad stages indicate that gametogenesis occurs rapidly, with the period from the initiation of vitellogenesis to the onset of spawning occupying less than 1 month. This pattern is also found in members of other families of temperate water fishes such as snapper (Sparidae, Scott and Pankhurst 1992), sweep (Kyphosidae, Dedual and Pankhurst 1992) and blue cod (Pinguipedidae, Pankhurst and Conroy 1987). The pattern and duration of reproductive development in black bream was consistent with other *Acanthopagrus* species (Abu-Hakima 1984; Pollock 1985; Chang and Yueh 1990) and highlights that reproductive development in sparids is essentially the same between euryhaline and stenohaline species.

Spawning began earlier in the Meredith than in the Swan River and was associated with the higher salinities and temperatures in the Meredith River. Studies of other sparids have shown that the timing of the onset of spawning is temperature sensitive (Kojima 1981; Scott and Pankhurst 1992). The role of salinity in regulating the time of reproduction has yet to be critically examined. Evidence for the acute influence of temperature and/or salinity on reproductive activity in black bream was observed in early October, when due to rainfall, a flood of cold (9°C) fresh water entered the estuaries. During this period, fish were caught only in the lower reaches of both estuaries, and there was a fall in gonadal sex steroid concentrations in both male and female fish.

The combination of the water quality data from the two estuaries has shown that black bream are reproductively active over a very wide range of environmental conditions. Sexually mature fish were caught over a temperature range of 15.5 to 26.2°C, DO concentrations of 4.2 to 13.6 mg L^{-1} and a salinity range of 13.9 to 35 g Kg^{-1}. Previous reports have suggested that black bream spawn over a salinity range of 11-18 g Kg^{-1} (Sherwood and Backhouse 1982) but clearly reproductively mature fish are present over a much wider range.

Seasonal changes in HSI occurred in both sexes with liver weights being highest early in spawning, suggesting that liver energy reserves may be used for ovarian recrudescence. Similar relationships have also been reported in other teleosts (eg Wingfield and Grimm 1977; Htun-Han 1978). In contrast, the HSI of snapper has been shown to increase in concert with the GSI and was thought to reflect the increased metabolic activity of the liver during the synthesis of vitellogenin (Scott and Pankhurst 1992). This suggests that patterns of seasonal change in HSI vary even among quite closely related species. The difference may relate to local variation in seasonal availability of food.

Protandrous sex inversion is known to occur in other species of *Acanthopagrus* (Pollock 1985; Chang and Yueh 1990); however, it is currently not known whether sex inversion occurs in black bream. In the present study, 98 % of male fish had ovarian tissue (previtellogenic oocytes only) present in the dorsal section of the testis. In contrast only 13 % of females had a residual testis present on the ventral section of the gonad. The sex ratio showed no marked differences with age (data not shown);

however, the majority (97%) of fish caught were on the basis of unvalidaded growth rings in otoliths or scales all estimated to be over three years old. In the black porgy, fish are males for the first two years of life and begin to sexually invert during their third year (Chang et al., 1994). Therefore, it is also possible that protandrous sex inversion occurs in black bream at a young age, but was not detected in the present study.

Stress generally inhibits reproduction in fish (reviewed in Pankhurst and Van Der Kraak 1997), and short episodes of stress have been shown to significantly reduce plasma concentrations of gonadal steroids in snapper (Carragher and Pankhurst 1991). Mean plasma cortisol concentrations in black bream were similar to values from snapper captured and sampled underwater (Pankhurst and Sharples 1992), and suggest that capture and sampling occurred before there was a significant elevation of plasma cortisol. Accordingly we consider the concentrations of sex steroids reported in this study are unlikely to have been influenced by capture stress.

Plasma concentrations of E_2 and T in females were found to increase in concert with gonadal recrudescence, with the highest concentrations being recorded in ovulated fish. It appears that E_2 has a universal role in stimulating vitellogenin (Vtg) synthesis in the liver of female teleosts (reviewed in Specker and Sullivan 1994) with direct evidence for E_2 stimulation of Vtg production in *Acanthopagrus* species (Chang et al., 1996). Peaks in T presumably relate to its role as a precursor for E_2 (Kagawa et al., 1982). The apparently paradoxical high E_2 and T concentrations found in ovulated fish probably relate to the fact that around ovulation, the next batch of vitellogenic oocytes is well advanced in development. Peak seasonal plasma concentrations of 17,20βP (considered to be the maturation-inducing steroid (MIS) in most teleosts; Scott and Canario 1987) coincided with those of E_2 and T, and were significantly elevated in fish undergoing FOM. However, overall changes in plasma concentrations of 17,20βP were low (<0.7 ng mL^{-1}), similar to many marine species (Pankhurst and Carragher 1991). In some species, low plasma concentrations of 17,20βP result from rapid conjugation to the glucuronated or sulphated form (Scott et al., 1990). This does not appear to be the case in sparids where measured plasma concentrations of conjugates are low (Carragher and Pankhurst 1993). An alternative explanation for low plasma concentrations of 17,20βP in black bream is the presence of a different MIS. For

example, 17,20β,21-trihydroxy-4-pregnen-3-one (17,20β21P) is the major MIS in the spotted sea trout (*Cynoscion nebulosus*) and Atlantic sea trout (*Micropogonias undulatus*) (reviewed in Thomas 1994). 17,20βP is highly effective at inducing FOM in snapper oocytes, *in vitro* (Ventling and Pankhurst 1995). 17,20β21P concentrations were not determined in the present study and the biopotency of 17,20βP relative to other C_{21} steroids in inducing FOM in black bream remains to be tested.

Plasma T and 11KT concentrations in males were consistent with the view that T and 11KT are involved in initiating and maintaining spermatogenesis (reviewed in Fostier et al., 1987). Elevated concentrations of T and 11KT during the spawning season have also been reported for the closely related black porgy (Chang et al., 1995a) and sobaity (Kime et al., 1991). Although plasma concentrations of 17,20βP were not significantly different at any stage of the season, plasma concentrations higher than 1 ng mL^{-1} were associated with the appearance of fully spermiated fish and occurred 1 month after the increases in T and 11KT. Furthermore, plasma concentrations of 17,20βP were significantly elevated in fully spermiated fish over non spermiated fish, suggesting that 17,20βP is associated with spermiation in black bream. Experimental evidence for the role of 17,20βP in spermiation in sparids is provided by a study on snapper, where milt volumes in fish treated with 17,20βP were significantly elevated over controls (Pankhurst 1994).

Diel changes in gonad stage in the present study indicate that black bream have a diurnal rhythm of oocyte maturation, with spawning occurring in the early evening. Daily spawning is common in the family Sparidae (Scott et al., 1993) and is usually accompanied by diel changes in plasma steroid concentrations (Kadmon et al., 1984; Matsuyama et al., 1988; Zohar et al., 1988; Hobby and Pankhurst 1997). Hobby and Pankhurst (1997) reported that in snapper, ovarian E_2 concentrations were high in the late afternoon/evening, but the same pattern was not expressed by plasma E_2. It was suggested that in species with short ovulatory periodicity, plasma concentrations of steroids may not adequately reflect the reproductive status of the fish during short-term cyclic ovarian changes. A similar effect could explain the failure to detect a diel variation in plasma E_2 in black bream in the present study. In contrast to E_2, T concentrations in black bream did show diel change with lower values at midnight than midday. This is consistent with the findings of Hobby and Pankhurst (1997) for

snapper. Plasma concentrations of 17,20βP were highest in black bream at midday when the majority of fish were in the final stages of maturation. Kadmon et al. (1984) reported similar results in the gilthead sea bream, where 17,20βP concentrations peaked 6 hrs before spawning (2 hrs before ovulation) and reached their lowest concentrations 6 hours after spawning. Studies on snapper have also indicated that 17,20βP concentrations are low in the evening, and high in the early morning (times vary from 0400 to 0900h) when FOM is occurring (Kagawa et al., 1991; Hobby and Pankhurst 1997).

Male black bream also appear to have a diurnal rhythm of gonadal cycling with the highest proportions of fully spermiated fish present at midday, presumably in preparation for spawning later that afternoon or evening. A similar rhythm exists in male blue cod where fully spermiated fish dominate in the late afternoon (close to the assumed time of spawning) and proportions of partially spermiated fish are highest in the early morning (Pankhurst and Kime 1991). High early-morning plasma concentrations of T and 11KT in black bream suggest that fish may be undergoing daily spermatogenesis. Diel variations of T and 11KT have also been demonstrated in male snapper with concentrations being highest between 0800 and 1200h (Carragher and Pankhurst 1993). Short-term fluctuations of plasma concentrations of T and 11KT have also been found in male blue cod, but were not related to acute changes in spermatogenic activity or the degree of spermiation (Pankhurst and Kime 1991). In contrast to T and 11KT, plasma concentrations of 17,20βP in male black bream remained high until midday, which is consistent with its role in spermiation (Fostier et al., 1987; Pankhurst 1994). Owing to the relatively small numbers of males sampled, the results need to be viewed with caution. However, it appears that gonadal cycling in males of daily spawning species, may be as dynamic as that of females.

2.6 Acknowledgments

Thanks are extended to Michael Attard, Sam Fenney, Marcus Keetch, James Hall, Rupert Hall, Paul Hannon, Zac Robinson, Brendan Spillman and Belinda Wood for their assistance in fish capture and to Prof. P.W. Alexander for the provision of the water quality monitoring equipment. This study was supported in part by Australian Research Council infrastructure and Large grants awarded to N.W.P., and University of Tasmania School of Science and Technology and Australian Postgraduate awards to J.A.H.

2.7 References

Abu-Hakima, R., 1984. Some aspects of the reproductive biology of *Acanthopagrus* spp. (Family: Sparidae). J. Fish Biol. 25, 515-526.

Bye, V.J., 1987. Environmental management of marine fish reproduction in Europe. In: Idler, D.R., Crim, L.W., Walsh, J.M., (Eds.), Reproductive Physiology of Fish 1987, Memorial University of Newfoundland, St John's. pp. 289-298.

Carragher, J.F., Pankhurst, N.W., 1991. Stress in a commercially important marine fish, *Pagrus auratus* (Sparidae). In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolfe, M.S., (Eds.), Reproductive Physiology of Fish 1991, FishSymp 91, Sheffield. pp. 253-255

Carragher, J.F., 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.

Chang, C.-F., Yueh, W.-S., 1990. Annual cycle of gonadal histology and steroid profiles in the juvenile males and adult females of the protandrous black porgy, *Acanthopagrus schlegeli.* Aquaculture 91, 179-196.

Chang, C.-F., Lee, M.-F., Chen, G.-R., 1994. Estradiol-17β associated with sex reversal in protandrous black porgy, *Acanthopagrus schlegeli.* J. Exp. Zool. 268, 53-58.

Chang, C.-F., Lau, E.-L., Lin, B.-Y., 1995a. Estradiol-17β suppresses testicular development and stimulates sex reversal in protandrous black porgy, *Acanthopagrus schlegeli.* Fish Physiol. Biochem. 14, 481-488.

Chang, C.-F., Lau, E.-L., Lin, B.-Y., 1995b. Stimulation of spermatogenesis or of sex reversal according to the dose of exogenous estradiol-17β in juvenile males of protandrous black porgy, *Acanthopagrus schlegeli*. Gen. Comp. Endocrinol. 100, 355-367.

Chang, C.-F., Lau, E.-L., Lin, B.-Y., Jeng, S.-R., 1996. Characterisation of vitellogenin induced by estradiol-17β in protandrous black porgy, *Acanthopagrus schlegeli.* Fish Physiol. Biochem. 15, 11-19.

Dedual, M., Pankhurst, N.W., 1992. Plasma steroid hormone concentrations in relation to the reproductive cycle of the sweep *Scorpis lineolatus* (Kyphosidae) caught from the wild. Aust. J. Mar. Freshwat. Res. 43, 753-763.

Foscarini, R., 1988. A review: Intensive farming procedure for red sea bream (*Pagrus major*) in Japan. Aquaculture 72, 191-246.

Fostier, A., Le Gac, F., Loir, M., 1987. Steroids in male reproduction. In:. Idler, D.R., Crim, L.W., Walsh, J.M., (Eds.), Reproductive Physiology of Fish 1987, Memorial University of Newfoundland, St John's. pp. 239-245.

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

Htun-Han, M., 1978. The reproductive biology of the dab *Limanda limanda* (L.) in the North Sea: gonadosomatic index, hepatosomatic index and condition factor. J. Fish Biol. 13, 369-378.

Kadmon, G., Yaron, Z., Gordin, H., 1984. Patterns of estradiol and 17α20β-dihydroxyprogesterone in female *Sparus aurata.* Gen. Comp. Endocrinol. 53, 453.

Kagawa, H., Tanaka, H., Okuzawa, K., Matsuyama, M., Hirose, K., 1991. Diurnal changes in plasma 17α20β-dihydroxy-4-pregnen-3-one levels during spawning season in the red sea bream *Pagrus major*. Nippon Suisan Gakkaishi 57, 769.

Kime, D.E., Lone, K.P., Al-Marzouk, A., 1991. Seasonal changes in serum steroid hormones in a protandrous teleost, the sobaity (*Sparidentex hasta* Valenciennes). J Fish Biol. 39, 745-753.

Kojima, K., 1981. Spawning of the red sea bream *Pagrus major* in the waters around Iki Island and Mishima Island in the western part of the Japan sea. Bull. Seik. Reg. Fish. Res. Lab. 56, 71-87.

Matsuyama, M., Adachi, S., Nagahama, Y., 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.

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, 185-97.

Pankhurst, N.W., 1998. Reproduction. In: Black, K.D., Pickering, A.D. (Eds.), Biology of Farmed Fish. Sheffield Academic Press, Sheffield. pp. 1-26.

Pankhurst, N.W., Conroy, A.M., 1987. Seasonal changes in reproductive condition and plasma levels of sex steroids in the blue cod, *Parapercis colias* (Bloch and Schneider) (Mugiloididae). Fish Physiol. Biochem. 4, 15-26.

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

Pankhurst, N.W., 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, 337-47.

Pankhurst, N.W., Kime, D.E., 1991. Plasma sex steroid concentrations in male blue cod, *Parapercis colias* (Bloch and Schneider) (Pinguipedidae) sampled underwater during the spawning season. Aust. J. Mar. Freshwat. Res. 42, 129-137.

Pankhurst, N.W., Sharples, D.F., 1992. Effects of capture and confinement on plasma cortisol concentrations in snapper *Pagrus auratus*. Aust. J. Mar. Freshwat. Res. 43, 345-356.

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

Pollock, B.R., 1982. Spawning period and growth of yellowfin bream, *Acanthopagrus australis* (Gunther), in Moreton Bay, Australia. J. Fish Biol. 21, 349-355.

Pollock, B.R., 1985. The reproductive cycle of yellowfin bream, *Acanthopagrus australis* (Gunther), with particular reference to protandrous sex inversion. J. Fish Biol. 26, 301-311.

Scott, A.P., Canario, A.V.M., 1987. Status of oocyte maturation-inducing steroids in teleosts. In:. Idler, D.R., Crim, L.W., Walsh, J.M., (Eds.), Reproductive Physiology of Fish 1987, Memorial University of Newfoundland, St John's. pp. 224-234.

Scott, A.P., Canario, A.V.M., Prat, F., 1990. Radioimmunoassay of ovarian steroids in plasmas of ovulating female sea bass (*Dicentrarchus labrax*). Gen. Comp. Endocrinol. 78, 299-302.

Scott, S.G., Pankhurst, N.W., 1992. Interannual variation in the reproductive cycle of the New Zealand snapper *Pagrus auratus* (Bloch & Schneider) (Sparidae). J. Fish Biol. 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). Environ. Biol. Fish. 36, 149-156.

Sherwood, J.E., Backhouse, G.N., 1982. Hydrodynamics of salt wedge estuaries - implications for successful spawning in black bream (*Acanthopagrus butcheri*). Warrnambool Institute of Advanced Education, Faculty of Applied Science and Technology. Research Report 82/3, 1-18.

Specker, J.L., Sullivan, C.V., 1994. Vitellogenesis in fishes: Status and perspectives. In: Davey, K.G., Peter, R.E., Tobe,. S.S., (Eds.), Perspectives in Comparative Endocrinology, National Science and Engineering Research Council of Canada, Ottawa. pp. 304-315.

Stewart, P.C., Greive, C., 1993. Bream, *Acanthopagrus* species. In: Kailola, P.J., Williams, M.J., Stewart, P.C., Reichelt, R.E., McNee, A., Grieve, C., (Eds.), Australian Fisheries Resources, Bureau of Resource Sciences and Fisheries Research and Development Corporation, Canberra. pp. 311-314.

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

Ventling, A.R., Pankhurst, N.W., 1995. Effects of gonadal steroids and human chorionic gonadotrophin on final oocyte maturation *in vitro* in the New Zealand snapper *Pagrus auratus* (Sparidae). Aust. J. Mar. Freshwat. Res. 46, 467-473.

Wingfield, J.C., Grimm, N.S., 1977. Seasonal changes in plasma cortisol, testosterone and oestradiol-17β in the plaice, *Pleuronectes platessa* L. Gen. Comp. Endocrinol. 31, 1-11.

Zohar, Y., Pagelson, G., Tosky, M., 1988. Daily changes in reproductive hormone levels in the female gilthead seabream *Sparus aurata* at the spawning period. In: Zohar, Y., Breton, B., (Eds.), Reproduction in Fish - Basic and Applied Aspects in Endocrinology and Genetics, INRA, Paris. 119-125.

CHAPTER 3

Stress-induced changes in concentrations of plasma sex steroids in black bream, *Acanthopagrus butcheri*.

3. Stress-induced changes in concentrations of plasma sex steroids in black bream, *Acanthopagrus butcheri.*

3.1 Summary

Black bream, *Acanthopagrus butcheri* were captured by rod and line and blood sampled at capture before confinement in a 400l tank. Fish were then removed from the tank and blood sampled from 15 min to 24 h after capture. Cortisol levels at capture, which were similar in both sexes, did not change with time of day, gonadal stage or season and were 1.9±0.2 and 2.8±0.4 ng ml^{-1} for male and female fish respectively. Confinement resulted in significantly elevated cortisol levels at all time periods; however, levels after 24 h of confinement were significantly lower than peak cortisol levels (15 min for males and 1 h for females). Confinement stress resulted in reduced levels of estradiol (E_2) and testosterone (T) within 1 h in sexually mature females. In mature males, suppression of T and 11-ketotestosterone (11KT) occurred after 30 min and 6 h of confinement respectively. The relationship between confinement stress and levels of 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) was more complex, with levels in males being elevated after 15 min and 24 h and suppressed after 6 h of confinement. In contrast, 17,20βP levels in females were elevated after 1 hour of confinement. In regressed females, plasma E_2 and T concentrations were low at capture and were not affected by confinement stress whereas plasma 17,20βP was elevated within 1 h. This study indicates that stress exerts a rapid inhibitory effect on gonadal steroidogenesis.

3.2 Introduction

The aquaculture production of any species is dependent on a regular supply of high quality eggs. For new species this is usually attained by the capture and hormonal induction of ovulation of wild-caught broodstock. However, the quality and quantity of eggs obtained from this approach is quite variable. This is particularly evident in stenohaline sparids, such as snapper *Pagrus auratus* (Bloch and Schneider), where the stress generated by capture and handling of wild fish results in depression of plasma levels of gonadal steroids, and an unreliable ovulatory response to exogenous hormones (Carragher and Pankhurst 1991; Cleary 1998). Black bream *Acanthopagrus butcheri* (Munro) is a euryhaline sparid which shows considerable potential for grow-out in inland Australia using saline groundwater (Haddy and Pankhurst 1998). However it is currently unknown whether black bream show the same susceptibility to capture stress as snapper.

It is now well established that stress has the capacity to inhibit reproductive processes in most fish (reviewed in Pankhurst and Van Der Kraak, 1997). There is a consistent association between stress, elevated plasma cortisol concentrations and decreased levels of plasma androgens and estrogens in a variety of teleost families including salmonids (Pickering et al., 1987; Sumpter et al., 1987; Pankhurst and Dedual 1994), triglids (Clearwater and Pankhurst 1997), catostomids (Jardine et al., 1996) latrids (Morehead 1998) and sparids (Carragher and Pankhurst 1991; Cleary 1998). In contrast to plasma androgens and estrogens, plasma 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) concentrations decrease, increase, or remain unchanged after stress (Carragher and Pankhurst 1991; Van Der Kraak et al., 1992; Pankhurst and Dedual 1994; Cleary 1998). Changes in plasma sex steroid concentrations have been recorded as early as 1 h after imposition of stress in several fish species (Safford and Thomas 1987; Sumpter et al., 1987; Carragher and Pankhurst 1991; Jardine et al., 1996; Cleary 1998), but, few studies have investigated the effects of shorter periods of stress. Information on the timecourse of stress-induced changes in plasma steroid concentrations is essential in interpreting results where delayed sampling may have occurred, and for providing additional information into the mechanisms of stress-induced changes of plasma sex steroids. In the present study, we examined the stress response and its effect on reproductive steroids in wild black bream by measuring

plasma steroid concentrations at capture and after various confinement times from 15 min to 24 h after the imposition of stress.

3.3 Materials And Methods

Sampling

Black bream were captured by rod and line from the Meredith (148°7'S, 42°4'E) and Swan Rivers (148°4'S, 42°4'E) at Swansea, on the east coast of Tasmania, from September 1997 to December 1997. Additional fish from a companion seasonal study (Haddy and Pankhurst 1998; September 1996 - May 1997) were included to extend baseline information on normal basal plasma cortisol values. All fish were blood sampled by caudal puncture using heparinized syringes at capture, with 95% (n=106) of fish being sampled within 5 min (mean of 2.8±0.2 min). Fish were then confined in a 400l tank for 15 or 30 min (males only; insufficient females were caught for examination of these time periods), or 1, 3, 6, 12 or 24 h (both sexes) prior to a second blood sampling before being killed for gonadal examination. Fish were confined in batches and removed from their confinement tanks and processed at intervals according to the time of capture. Blood was stored on ice, plasma obtained by centrifugation, then frozen and stored at -18°C until assay. Sex and macroscopic gonad condition (Haddy and Pankhurst 1998) were recorded for each fish. Female fish were classified as sexually mature if the ovary contained vitellogenic oocytes. Male fish were classified as sexually mature on the basis of the presence of milt in the sperm duct.

Steroid Measurement

Plasma steroid concentrations were measured by radioimmunoassay, using the reagents and protocols given in Pankhurst and Carragher (1992) for E_2, T, 17,20βP and cortisol, and Pankhurst and Kime (1991) for 11KT. Extraction efficiency was determined by recovery of [^{3}H]-labelled steroid extracted with plasma, and assay values were corrected accordingly. Assay detection limits in plasma were 0.15 ng ml^{-1}; for 11KT, E_2, T and 17,20βP and 0.3 ng ml^{-1} for cortisol. Values that were below the detection limit were treated as being equal to the detection limit. Interassay variability (%CV) measured using aliquots of a pooled standard was as follows: 11KT = 16.6%

(n=2); E_2 = 7.0% (n=2); T = 10.6% (n=4); 17,20βP = 14.8% (n=4) and cortisol = 18.3% (n=12).

Statistics

Paired t-tests, One way ANOVA and Tukey's multiple comparison of means tests were performed using the SPSS statistical package. Where necessary, data were log transformed to satisfy homogeneity of variance requirements.

3.4 Results

Plasma cortisol concentrations at capture showed no significant difference within the 5 minute sampling window, time of day (Fig. 3.1), season, gonadal stage (Fig. 3.2), or sex. Mean plasma cortisol levels at capture were 1.9±0.2 and 2.8±0.4 ng ml^{-1} for male (n=140) and female (n=140) fish respectively.

Sexually Regressed Females

Plasma cortisol concentrations after 1 h were markedly higher than first bleeds but were not significantly different (P=0.066, two tailed t-test), however, concentrations after 6 h were significantly elevated (Fig. 3.3). Plasma 17,20βP concentrations were significantly elevated after 1 h of confinement, but not different from concentrations at capture after 6 h. Plasma T and E_2 concentrations were low at capture and were not affected by capture and confinement.

Sexually Mature Females

Cortisol concentrations were not different among first bleeds, but were elevated after 1 h of confinement and remained significantly different from levels at capture for all confinement times (Fig. 3.4). Cortisol concentrations in second bleeds were higher after 1 h of confinement than at 6 or 24 h. Plasma 17,20βP concentrations were not different among first bleeds (range 0.93 to 1.58 ng ml^{-1}) but were significantly elevated after 1 h of confinement. Mean plasma E_2 and T concentrations from first bleeds were significantly different and ranged from 3.23 to 8.75 ng ml^{-1} and

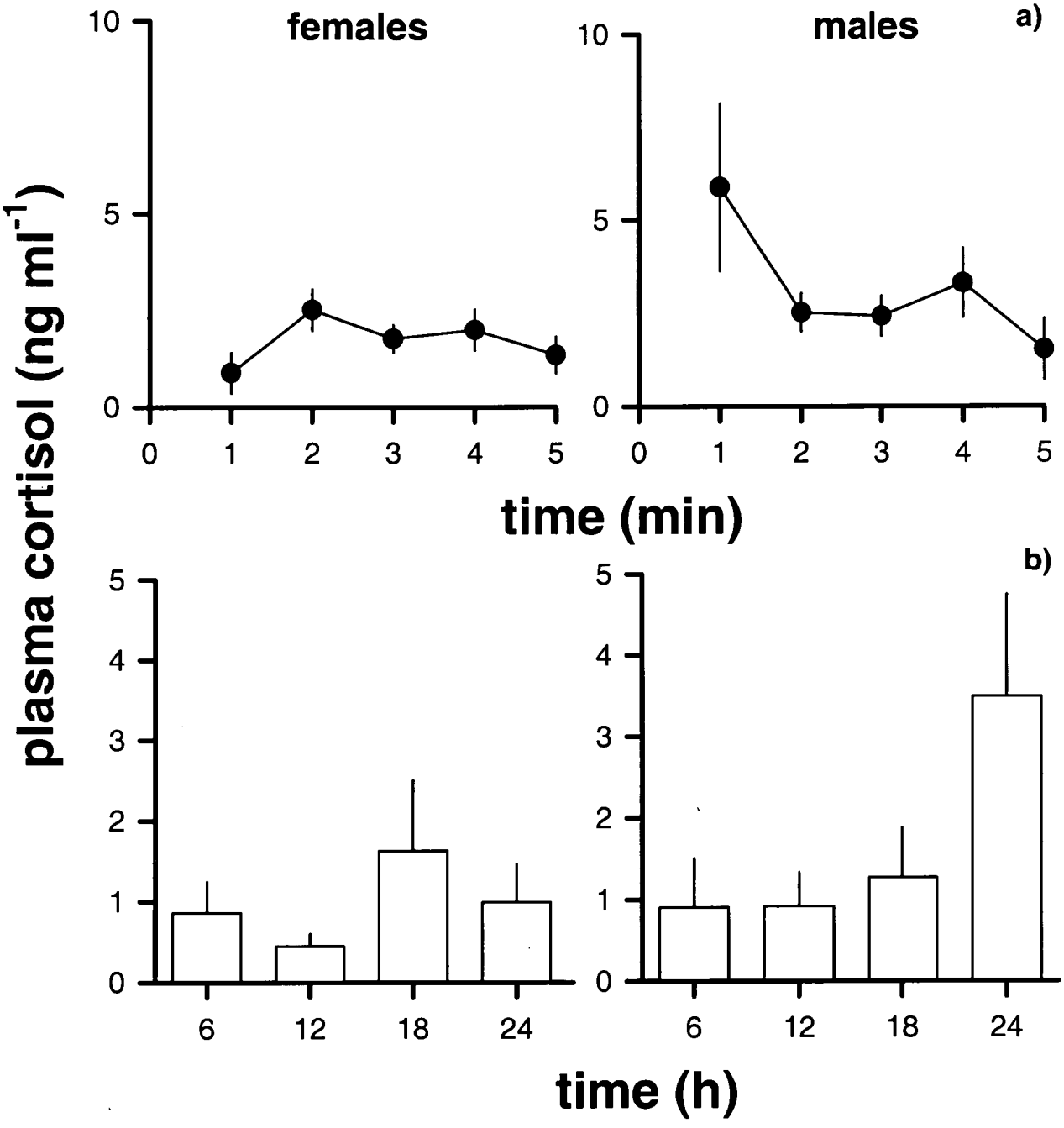


Fig. 3.1. Effects of a) interval between hooking and sampling time, and b) time of day on plasma cortisol concentrations in female and male black bream. All values are mean ± s.e. No values were different from any other (P>0.05).

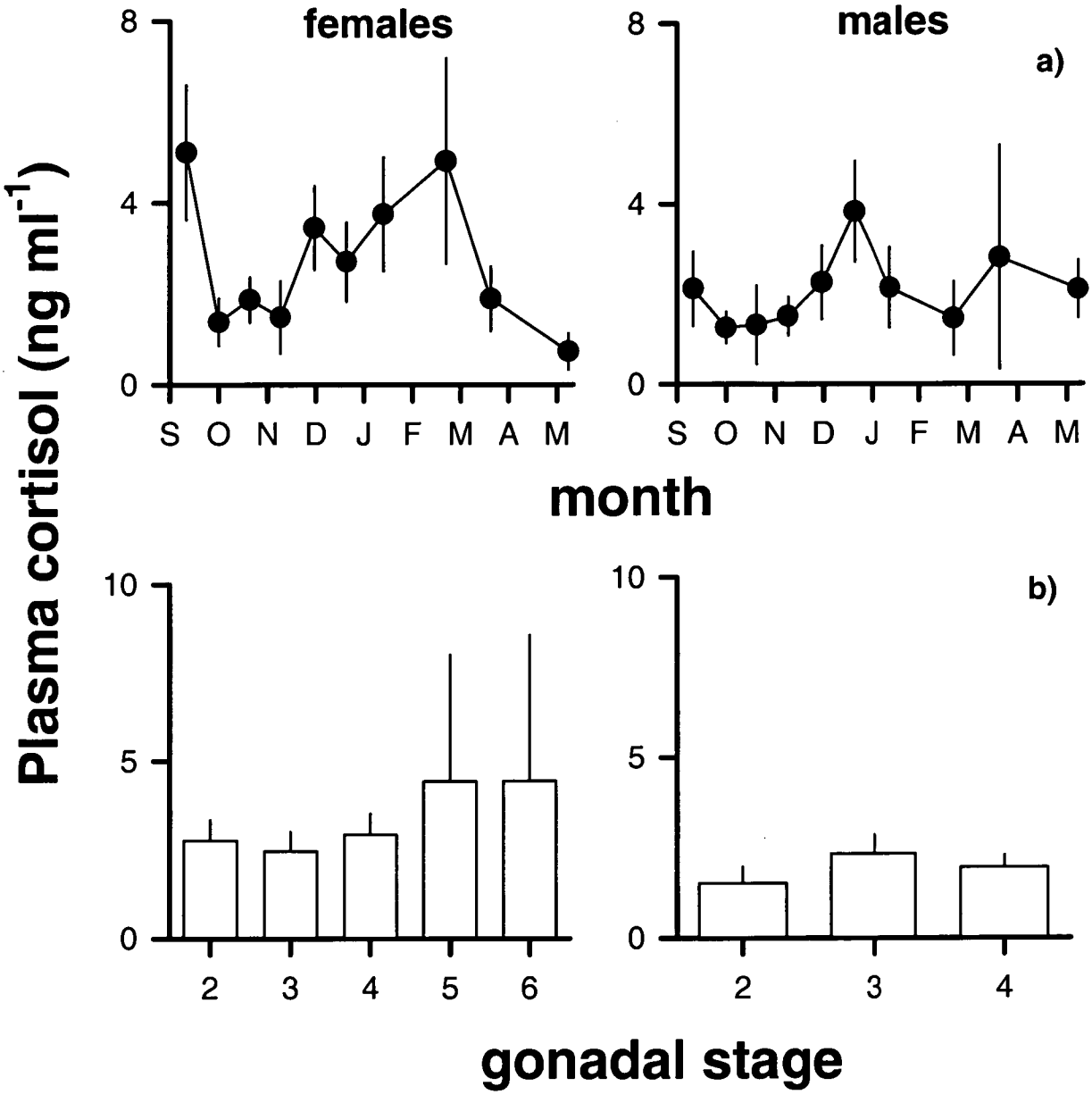


Fig. 3.2. Effects of a) season, and b) gonadal stage on plasma cortisol concentrations in female and male black bream. Gonad stages: Female; 2= previtellogenic, 3= vitellogenic, 4 = final oocyte maturation, 5= ovulated and 6= spent. Male; 2= spermatogenic, 3= partially spermiated and 4= fully spermiated. No values were different from any other ($P>0.05$). Other details as for Fig 3.1.

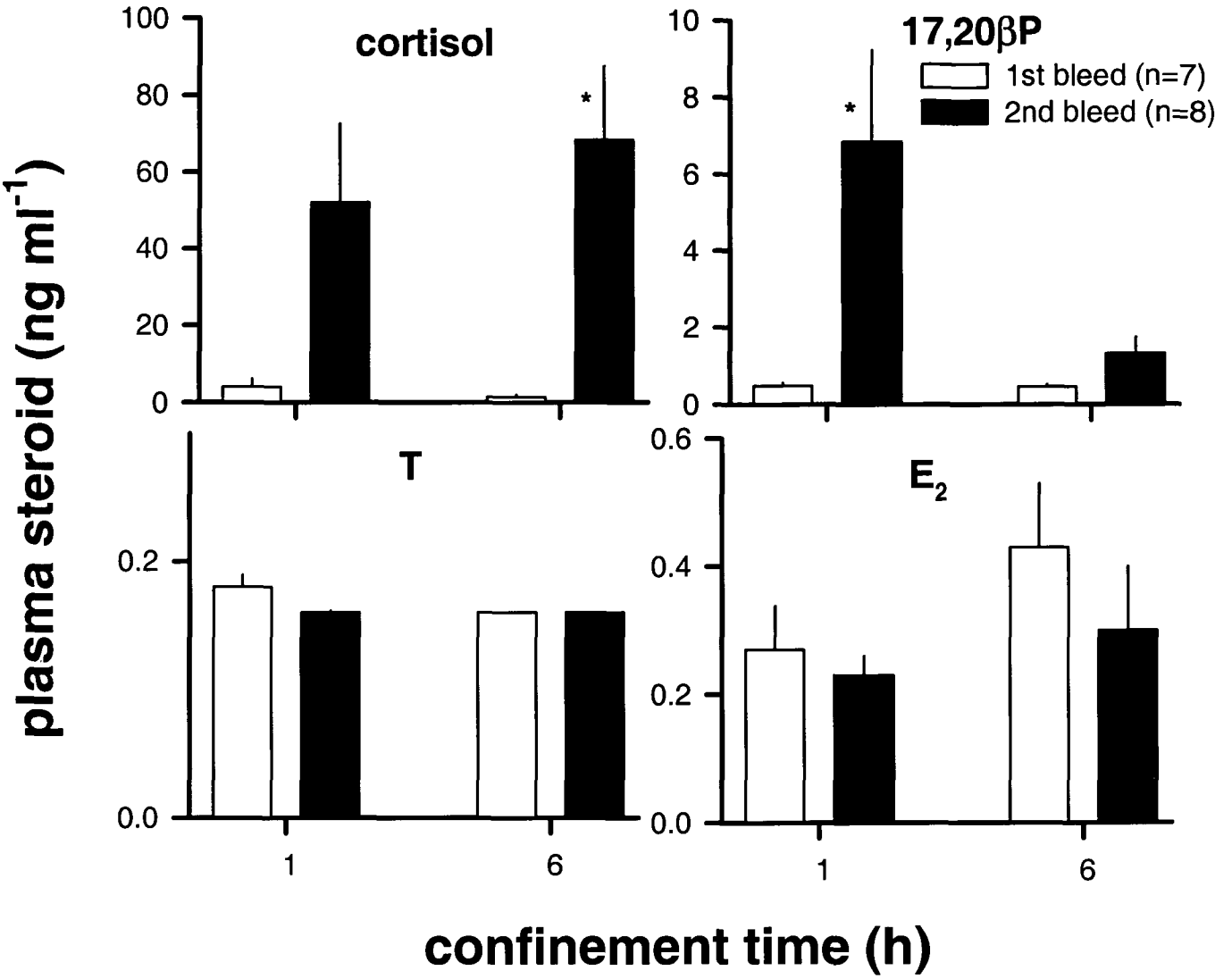


Fig. 3.3. Effects of capture and confinement on plasma cortisol, 17β-estradiol (E_2), testosterone (T) and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) concentrations in sexually regressed female black bream. * indicates 1st and 2nd bleeds are significantly different ($P<0.05$). Other details as for Fig. 3.1.

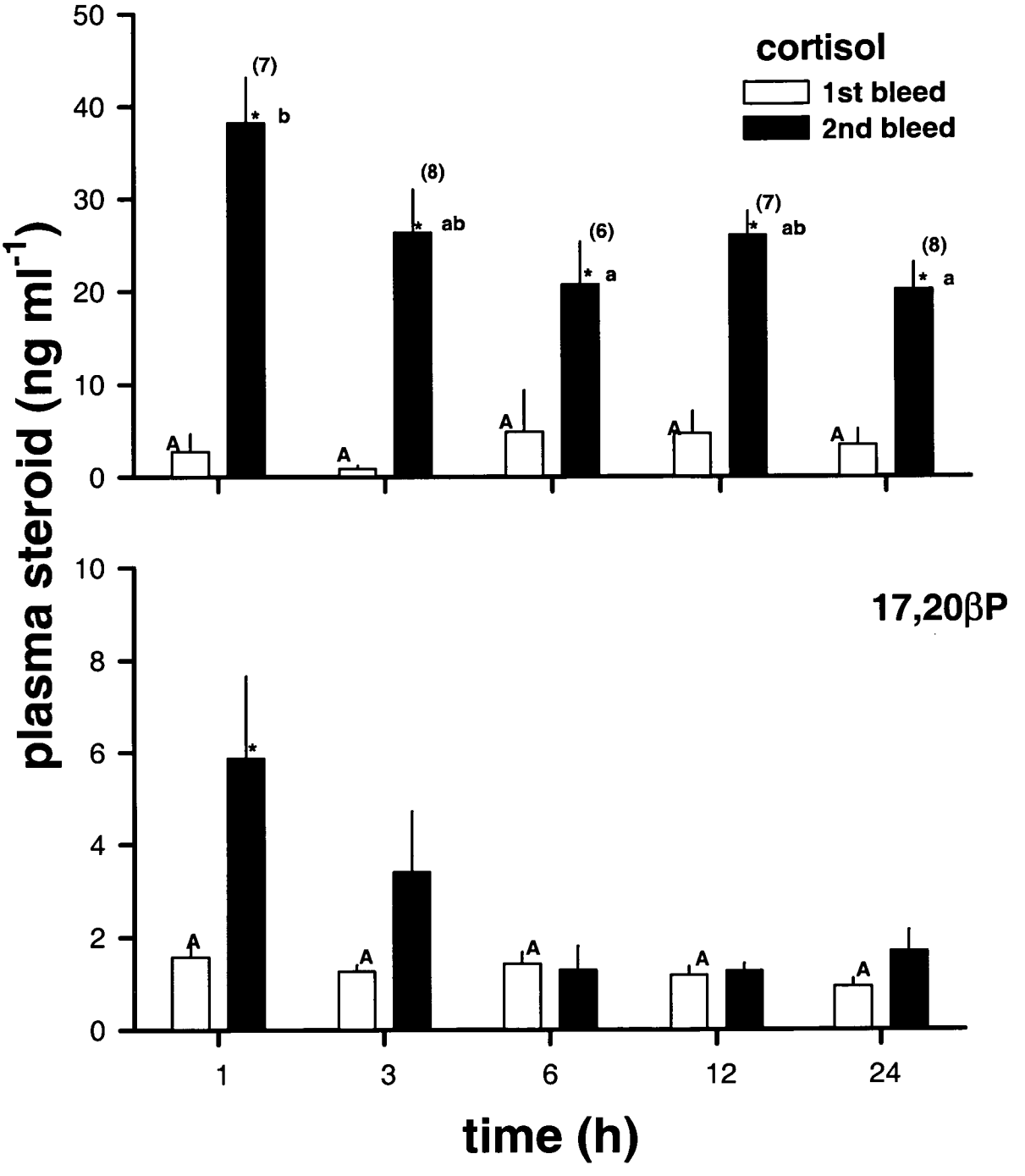


Fig. 3.4. Effects of capture and confinement on plasma cortisol and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) concentrations in sexually mature female black bream. Values that are significantly different have different superscripts ($P<0.05$). Uppercase and lowercase superscripts indicate differences between 1st and 2nd bleeds respectively. Other details as for Figs. 3.1 and 3.3.

2.62 to 8.58 ng ml^{-1} respectively (Fig. 3.5). Levels of both steroids were significantly suppressed within 1 h and remained significantly lower than first bleeds for all subsequent confinement times. The significant differences between 1st bleeds for plasma T and E_2 concentrations were associated with differences in plasma T and E_2 levels between sampling trips (data not shown).

Males

Plasma cortisol concentrations were not different between first bleeds at any time, and were elevated after 15 min of confinement and remained elevated over first bleeds for all subsequent confinement times (Fig. 3.6). Plasma cortisol concentrations were highest after 15 min of confinement and declined to significantly lower concentrations within 1 h and reached lowest post-confinement concentrations after 24 h. Mean plasma 17,20βP concentrations at capture were significantly different and ranged from 0.35 to 2.23 ng ml^{-1}. Plasma 17,20βP concentrations were significantly elevated after 15 min and 24 h, and significantly depressed after 6 h of confinement. Mean plasma T concentrations at capture were significantly different and ranged from 0.68 to 2.34 ng ml^{-1}. Plasma T concentrations were significantly suppressed within 30 min and remained significantly lower than levels at capture for all confinement times except 1 h (Fig. 3.7). Mean plasma 11KT concentrations at capture were significantly different and ranged from 0.35 to 2.03 ng ml^{-1}. Plasma 11KT concentrations were significantly suppressed for all confinement times after 6 h. At 0.5, 1 and 3 h, differences in plasma 11KT levels approached significance (P= 0.069, 0.122 and 0.168 respectively, two tailed t-tests) and at 1 and 3 h, second bleed 11KT levels were at or less than the detection limit. The significant differences between 1st bleeds for plasma T, 11KT and 17,20βP concentrations were associated with significant differences in plasma T and E_2 levels between sampling trips (data not shown).

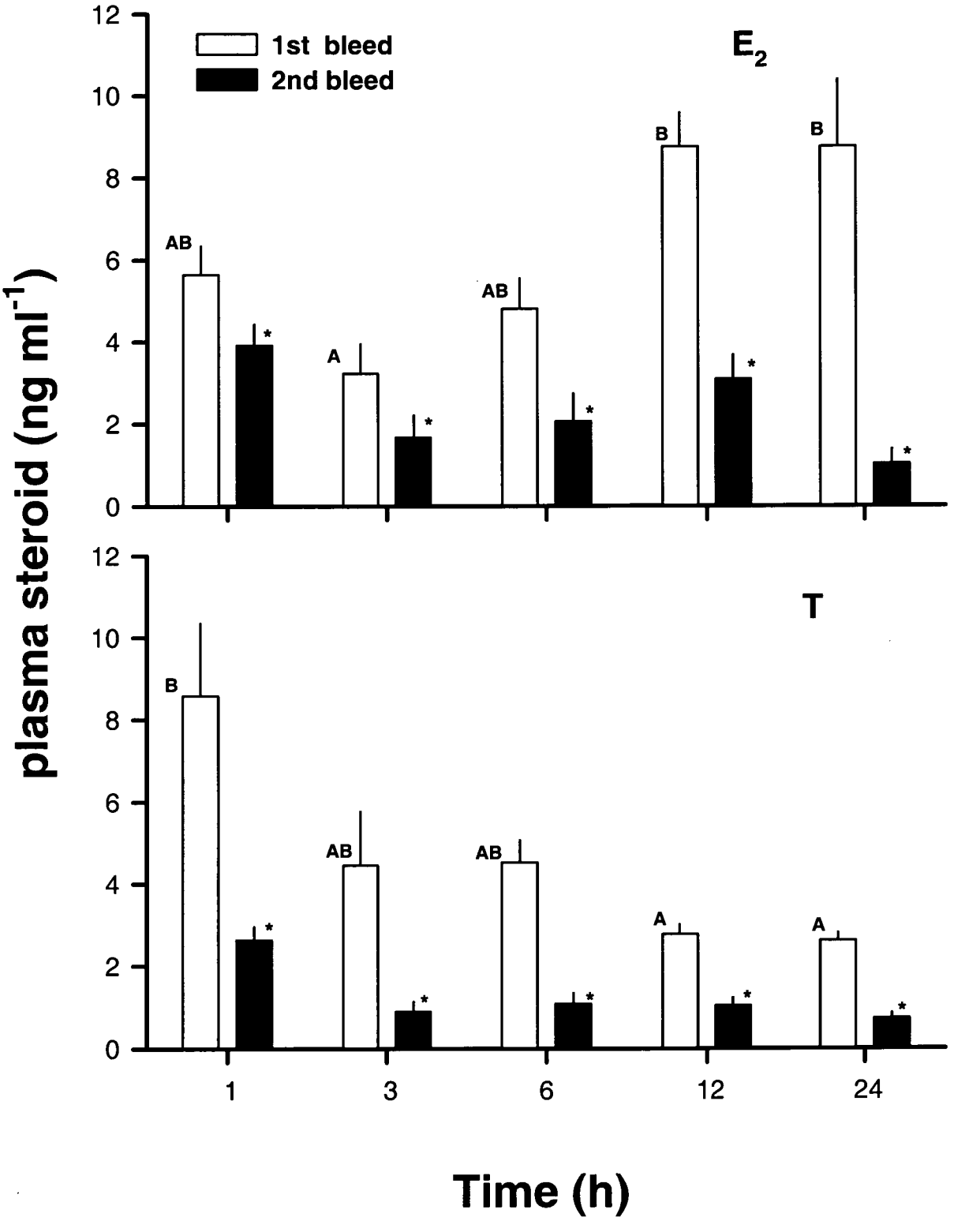


Fig. 3.5. Effects of capture and confinement on plasma 17β-estradiol (E_2), and testosterone (T) concentrations in sexually mature female black bream. Other details as for Figs. 3.1, 3.3 and 3.4.

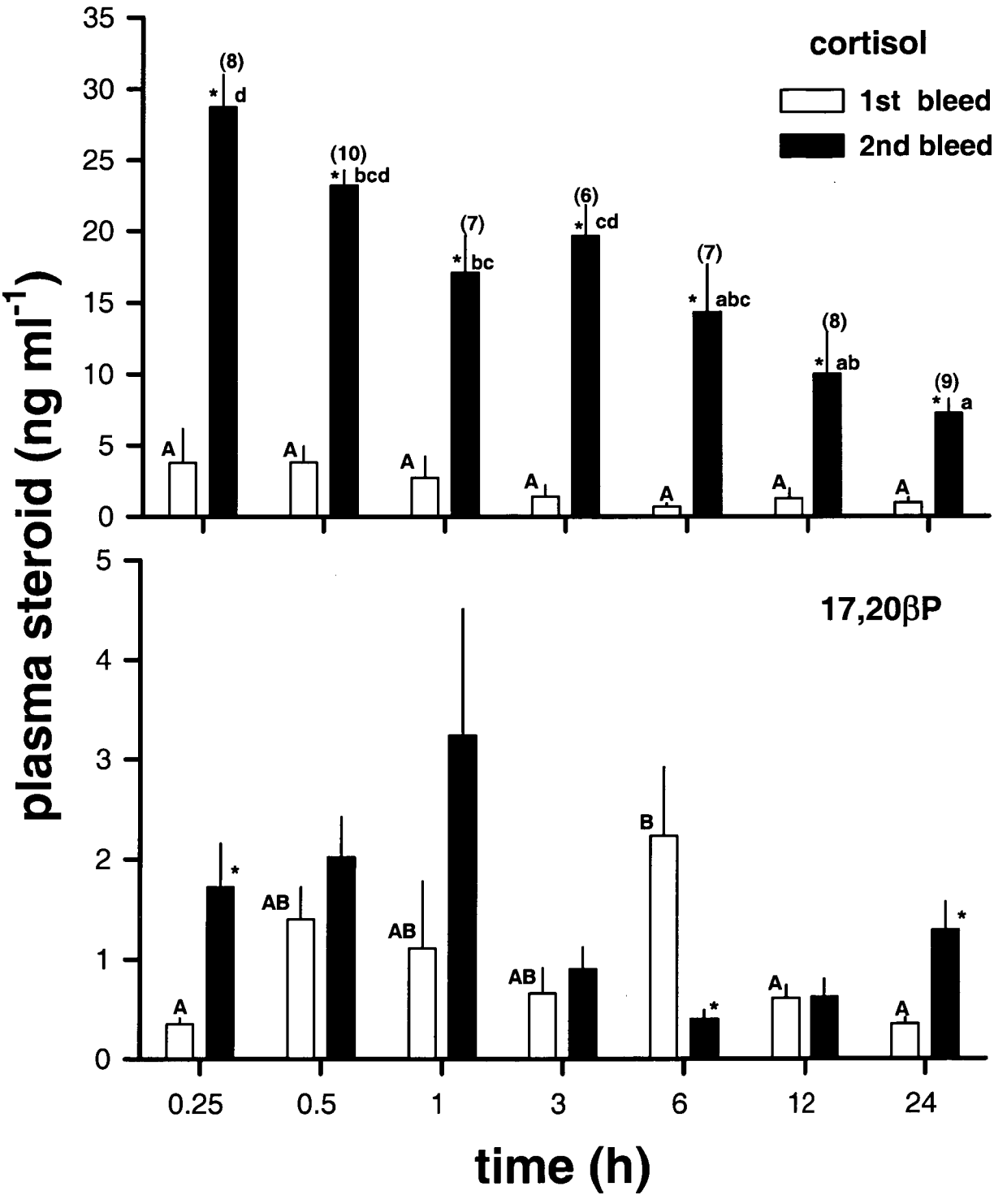


Fig. 3.6. Effects of capture and confinement on plasma cortisol and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) concentrations in sexually mature male black bream. Other details as for Figs. 3.1, 3.3 and 3.4.

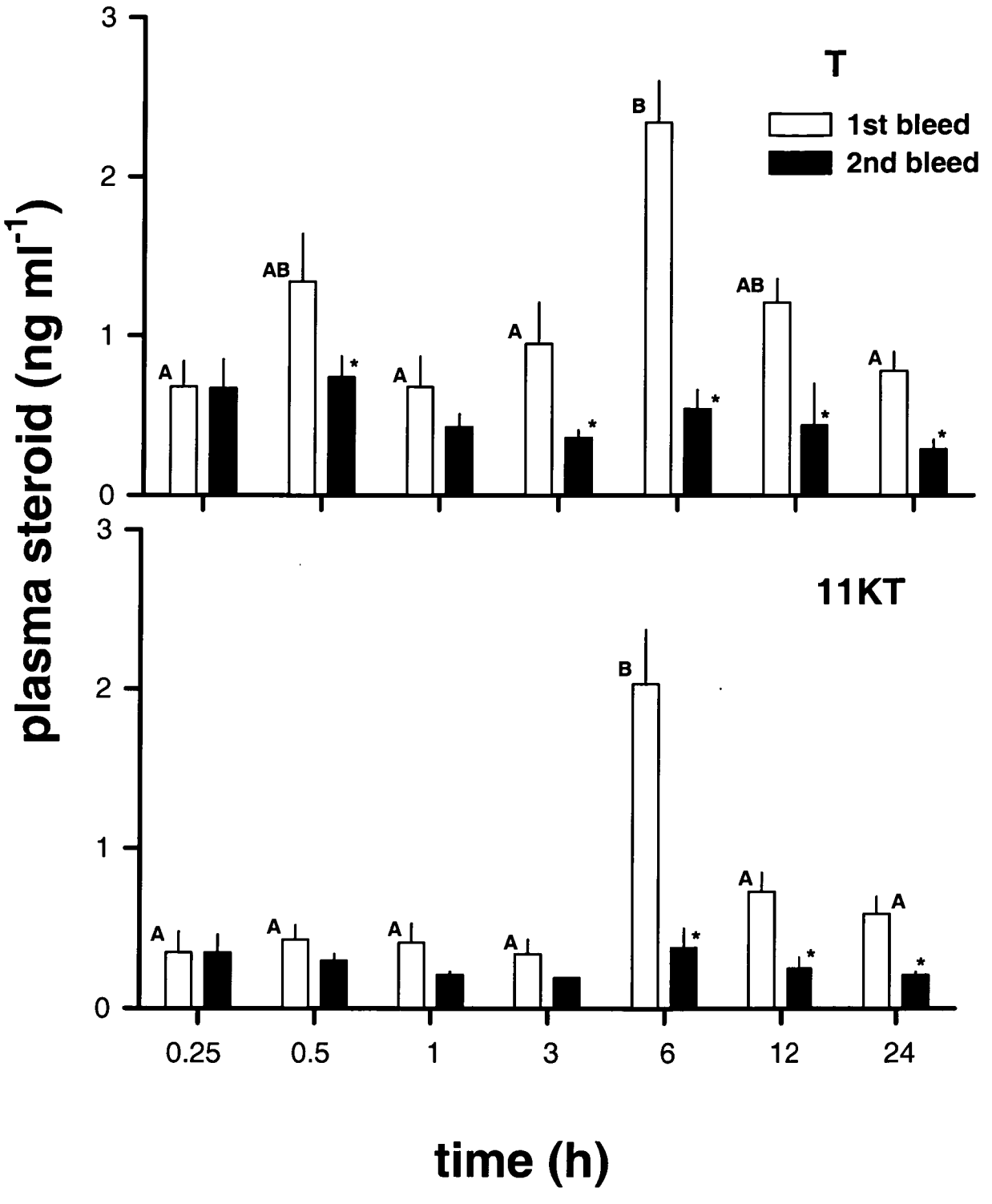


Fig. 3.7. Effects of capture and confinement on plasma testosterone (T) and 11-ketotestosterone (11KT) concentrations in sexually mature male black bream. Other details as for Figs. 3.1, 3.3 and 3.4.

3.5 Discussion

A common problem in comparing stress responses in fish is the determination of basal cortisol levels, as factors such as the method of capture, the stage of sexual maturity, season, time of day and sex can all influence plasma cortisol levels (Barton and Iwama 1991; Pankhurst and Sharples 1992; Foo and Lam 1993a Sumpter 1997). However, in black bream these factors did not appear to influence plasma cortisol concentrations. At capture, plasma cortisol ranged from undetectable (<0.3 ng ml^{-1}) to 27.5 ng ml^{-1}, with only 6% of all fish (n=338) having cortisol concentrations higher than 10 ng ml^{-1}. Mean cortisol concentrations were similar to basal values from snapper captured and sampled underwater (Pankhurst and Sharples 1992). This indicates that cortisol concentrations for black bream in the present study have been minimally influenced by sampling activity. In contrast to cortisol concentrations, initial sex steroid concentrations were variable and were associated with differences between sampling trips. Haddy and Pankhurst (1998) showed that plasma sex steroids are influenced by season, time of day and gonadal stage in both sexes, which in the present study, may have contributed to the variation observed between 1st bleeds.

In the present study, cortisol concentrations peaked at the earliest sample time (15 min and 1 h for male and female fish respectively), and dropped significantly over time, with the lowest concentrations being recorded after 24 h of confinement. This could indicate that interrenal exhaustion (Sumpter 1997) or down regulation of the hypothalamic-pituitary-interrenal axis had occurred (Bradford et al., 1992) as a result of sustained stress. Alternatively, a limited degree of recovery from stress could have occurred suggesting that tank confinement was less stressful than the initial capture and handling. Recovery to resting levels of cortisol in wild caught snapper takes at least 24-48 hours (Pankhurst and Sharples 1992; Cleary 1998). This is consistent with the persistence of elevated cortisol levels for at least 24 h in black bream in the present study.

In female black bream, capture and confinement resulted in a significant decrease in plasma concentrations of T and E_2 within 1 h and this was sustained for up to 24 h. It is possible that, as in males, a more rapid change in plasma concentrations of T and E_2 occurred, but was not detected due to the sampling regime. The rapid depression (within 1 h) of plasma T and E_2 has also been recorded in snapper (Carragher and

Pankhurst 1991; Cleary 1998) and white sucker (Jardine et al., 1996). Once steroidogenesis has been inhibited by stress, circulating steroids are likely to be rapidly cleared from the plasma (Pankhurst et al., 1986; Baroiller et al., 1987).

The mechanism by which stress affects steroid production is unknown. There is equivocal evidence as to whether the effects of stress on reproduction are mediated by cortisol. Cortisol implantation results in a reduction of plasma T and E_2 concentrations in tilapia *Oreochromis mossambicus* (Peters) (Foo and Lam 1993b) and brown trout *Salmo trutta* L. (Carragher et al., 1989). Carragher and Sumpter (1990) reported that cortisol has a direct suppressive effect on the secretion of T and E_2 by trout follicles. However, repetition of the work showed that the direct inhibition of ovarian steroidogenesis by cortisol was not the principal mechanism at work (Pankhurst et al., 1995a; Pankhurst 1998a). Furthermore, exogenous cortisol has no effect on *in vitro* steroidogenesis of ovarian follicles from goldfish *Carassius auratus* L., common carp *Cyprinus carpio* L. or snapper (Pankhurst et al., 1995b).

Capture and confinement of male black bream caused a significant decrease in plasma concentrations of T within 30 min. In contrast, concentrations of 11KT were not significantly reduced until 6 h of confinement, however, 11KT concentrations were showing trends of suppression within 30 min. Several other studies have reported rapid (within 1 h) stress-induced decreases in circulating androgens in males (Pickering et al., 1987; Safford and Thomas 1987; Sumpter et al., 1987; Jardine et al., 1996; Cleary 1998). As in females, cortisol implantation causes the depression of plasma T concentrations in male tilapia (Foo and Lam 1993a) and brown trout (Carragher et al., 1989). Carragher et al. (1989) further showed that cortisol implantation reduces the concentrations of gonadotropin (GtH) in the plasma of maturing male rainbow trout and brown trout. However, stress-induced changes in GtH are not always inhibitory. Sumpter et al. (1987) showed that a 1 hour handling and confinement stress, which resulted in the suppression of plasma T in male brown trout, significantly elevated plasma gonadotropin concentrations.

The relationship between plasma 17,20βP concentrations and stress is more complex. 17,20βP is associated with final oocyte maturation and spermiation in female and male black bream respectively. Carragher and Pankhurst (1991) reported that there was a positive correlation between cortisol and 17,20βP concentrations in

snapper with levels of both steroids being elevated after five days of confinement. In contrast, Cleary (1998) found that in the same species plasma 17,20βP concentrations levels either decreased, increased or showed no change over 48 h of confinement and were low or non-detectable after longer confinement periods.

Interestingly, sexually regressed female black bream also produced significant amounts of 17,20βP after 1 h. This suggests that 17,20βP may be of interrenal rather than gonadal origin. Sangalang and Freeman (1988) have shown that the interrenal tissue of Atlantic salmon *Salmo salar* (L.) is capable of synthesising 17,20βP *in vitro*. Furthermore, Barry et al. (1997) found that cortisol production by interrenal tissue from rainbow trout could be stimulated by providing 17,20βP as a substrate. A similar mechanism in black bream could explain the initial stimulation and subsequent drop of 17,20βP concentrations, with 17,20βP being of interrenal and/or gonadal origin and cortisol synthesis occurring from plasma and interrenal 17,20βP once other endogenous precursors become limiting.

In both male and female black bream in the present study, plasma concentrations of androgens and estrogens showed no signs of recovery within 24 hours. This finding is consistent with other studies on wild fish, in which there was no documented recovery of plasma T and E_2 during the experimental period (Carragher and Pankhurst 1991; Pankhurst and Dedual 1994; Jardine et al., 1996; Clearwater and Pankhurst 1997). Falling plasma concentrations of E_2 precede the onset of ovarian atresia in most fish studied (Matsuyama et al., 1988; Clearwater and Pankhurst 1997; Cleary 1998). Janz and Van Der Kraak (1997) found that E_2 is involved in maintaining follicular integrity via its role in suppressing apoptosis. Atretic oocytes are probably not steroidogenic with the result that once atresia has begun the recovery of plasma T and E_2 will not occur until the next batch of oocytes is recruited (Clearwater and Pankhurst 1997). Depressed T and E_2 levels caused by cortisol implantation, accompany the retardation of oocyte growth in tilapia (Foo and Lam 1993b) and reduced plasma vitellogenin concentrations and ovary weight in brown trout (Carragher et al., 1989; Pottinger et al., 1991). Campbell et al. (1994) showed that in chronically stressed maturing female rainbow trout, reduced egg size, and subsequent egg quality, was associated with depressed plasma vitellogenin levels. However, plasma E_2 was not affected by stress, suggesting that the mechanisms that disrupt vitellogenesis are more

complex than simply reduction of vitellogenin production caused by stress-induced reductions in plasma E_2 levels (Campbell et al., 1994).

In male teleosts, T and 11KT are involved in initiating and maintaining spermatogenesis, and 17,20βP stimulates spermiation (reviewed in Pankhurst 1998b). Very few studies have investigated the effects of stress on sex steroid levels and testicular development. Depressed T levels, caused by cortisol implantation, accompanied reduction in testes weight in brown trout (Carragher et al., 1989). Campbell et al. (1992) showed that repeated episodes of acute stress over 9 months reduced sperm counts in male rainbow trout, but, this difference did not affect fertility. In snapper, 1 week of confinement resulted in an increase in the proportions of primary spermatocytes and spermatozoa and decreased the proportions of secondary spermatocytes and spermatids (Cleary 1998). Cleary (1998) suggested that the rate of development of secondary spermatocytes and spermatids into spermatozoa was increased due to stress-induced increases in 17,20βP.

The results from the present study have implications for the collection and husbandry of broodstock. Hormonal induction of ovulation in wild caught fish is a common practice in aquaculture (Lam 1982; Zohar 1986). However, fish are usually caught from the wild and transported to laboratory prior to being treated with exogenous hormones. This would allow the rapid depression of plasma steroids to already be in effect prior to treatment. It is currently not known whether delayed injection of exogenous hormones will alter the endocrine response, and quantity or quality of the gametes produced. We are now investigating this possibility. This study has shown that black bream are extremely stress sensitive and if naturally spawning captive populations are to be maintained every attempt must be made to minimise stress. Furthermore in stress sensitive species a "hands off" approach during the spawning season may be essential to achieve normal reproductive development.

3.6 Acknowledgments

This study was supported by grants from University of Tasmania Faculty of Science and Engineering, the Co-operative Research Centre for Aquaculture and an Australian Postgraduate Scholarship awarded to JAH. Thanks are extended to S. Shaw for

permission to fish on private property and M. Attard, A. Hobby, A. Shepherd, and J. Welsford for assistance with fish capture and sampling.

3.7 References

Baroiller, J.F., Fostier, A., Zohar, Y., Marcuzzi, O., 1987. The metabolic clearance rate of estradiol in rainbow trout, *Salmo gairdneri* R., estimated by both single injection and constant infusion methods increase during oocyte maturation. Gen. Comp. Endocrinol. 66, 85-94.

Barry, T.P., Riebe, J.D., Parrish, J.J., Malison, J.A., 1997. Effects of 17α,20β-dihydroxy-4-pregnen-3-one on cortisol production by rainbow trout interrenal tissue *in vitro*. Gen. Comp. Endocrinol. 107, 172-181.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann. Rev. Fish Dis. 1, 3-26.

Bradford, C.S., Fitzpatrick, M.S., Schreck, C.B., 1992. Evidence for ultra-short-loop feedback in ACTH-induced interrenal steroidogenesis in coho salmon: Acute self-suppression of cortisol secretion *in vitro*. Gen. Comp. Endocrinol. 87, 292-299.

Campbell, P.M., Pottinger, T.G., Sumpter, J.P., 1992. Stress reduces the quality of gametes produced by rainbow trout. Biol. Reprod. 47, 1140-1150.

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

Carragher, J.F., Pankhurst, N.W., 1991. Stress and reproduction in a commercially important fish, *Pagrus auratus* (Sparidae). In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolfe, M.S., (Eds.), Reproductive Physiology of Fish 1991, FishSymp 91, Sheffield. pp. 253-255.

Carragher, J.F., Sumpter, J.P., 1990. The effects of cortisol on the secretion of sex steroids from cultured ovarian follicles of rainbow trout. Gen. Comp. Endocrinol. 77, 403-407.

Carragher, J.F., Sumpter, J.P., Pottinger, T.G., 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. Gen. Comp. Endocrinol. 76, 310-321.

Clearwater, S.J., Pankhurst, N.W., 1997. The response to capture and confinement stress of plasma cortisol, plasma sex steroids and vitellogenic oocytes in the marine teleost, red gurnard. J. Fish Biol. 50, 429-441.

Cleary, J.J., 1998. The effects of stress on reproduction in snapper (*Pagrus auratus*) Unpublished PhD Thesis, University of Tasmania. 162p.

Foo, J.T.W., Lam, T.J., 1993a. Serum cortisol response to handling stress and the effect of cortisol implantation on testosterone in the tilapia, *Oreochromis mossambicus.* Aquaculture 115, 145-158.

Foo, J.T.W., Lam, T.J., 1993b. Retardation of ovarian growth and depression of serum steroid levels in the tilapia, *Oreochromis mossambicus*, by cortisol implantation. Aquaculture 115, 133-143.

Haddy, J.A., Pankhurst, N.W., 1998. Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). Mar. Freshwat. Res. 49, 389-397.

Janz, D.M., Van Der Kraak, G., 1997. Suppression of apoptosis by gonadotropin, 17β-estradiol, and epidermal growth factor in rainbow trout preovulatory ovarian follicles. Gen. Comp. Endocrinol. 105, 186-193.

Jardine, J.J., Van Der Kraak, G.J., Munkittrick, K.R., 1996. Capture and confinement stress in white sucker exposed to bleached kraft pulp mill effluent. Ecotoxic. Environ. Saf. 33, 287-298.

Lam, T.J., 1982. Applications of endocrinology to fish culture. Can. J. Fish. Aquat. Sci. 39, 111-137.

Matsuyama, M., Adachi, S., Nagahama, Y., 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.

Morehead, D.T., 1998. Effect of capture, confinement and repeated sampling on plasma steroid concentrations and oocyte size in female striped trumpeter *Latris lineata* (Latrididae). Mar. Freshwat. Res. 49, 373-377.

Pankhurst, N.W., 1998a. Further evidence of the equivocal effects of cortisol on in vitro steroidogenesis by ovarian follicles of rainbow trout *Oncorhynchus mykiss*. Fish Physiol. Biochem. 19, 315-323.

Pankhurst, N.W., 1998b. Reproduction. In: Black, K.D., Pickering, A.D. (Eds.), Biology of Farmed Fish. Sheffield Academic Press, Sheffield. pp. 1-26.

Pankhurst, N.W., 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., Dedual, M., 1994. Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. J. Fish Biol. 45, 1013-1025.

Pankhurst, N.W., Kime, D.E., 1991. Plasma sex steroid concentrations in male blue cod, *Parapercis colias* (Bloch and Schneider) (Pinguipedidae) sampled underwater during the spawning season. Aust. J. Mar. Freshwat. Res. 42, 129-137.

Pankhurst, N.W., Sharples, D.F., 1992. Effects of capture and confinement on plasma cortisol concentrations in snapper *Pagrus auratus*. Aust. J. Mar. Freshwat. Res. 43, 345-356.

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

Pankhurst, N.W., Stacey, N.E., Peter, R.E., 1986. An evaluation of techniques for the administration of 17β-estradiol to teleosts. Aquaculture 52, 145-155.

Pankhurst, N.W., Van Der Kraak, G., Peter, R.E., 1995a. A reassessment of the inhibitory effects of cortisol on ovarian steroidogenesis. In: Goetz, F.W., Thomas, P., (Eds.), Reproductive Physiology of Fish 1995, Fish Symposium 95, Austin. p. 195.

Pankhurst, N.W., Van Der Kraak, G., Peter, R.E., 1995b. Evidence that inhibitory effects of stress on reproduction in teleost fish are not mediated by the action of cortisol on ovarian steroidogenesis. Gen. Comp. Endocrinol. 99, 249-257.

Pickering, A.D., Pottinger, T.G., Carragher, J.F., 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. Gen. Comp. Endocrinol. 68, 249-259.

Pottinger, T.G., Campbell, P.M., Sumpter, J.P., 1991. Stress-induced disruption of the salmonid liver-gonad axis. In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolfe, M.S., (Eds.), Reproductive Physiology of Fish 1991, FishSymp 91, Sheffield. pp. 114-116.

Safford, S.E., Thomas, P., 1987. Effects of capture and handling on circulating levels of gonadal steroids and cortisol in the spotted seatrout, *Cynoscion nebulosus*. In: Idler, D.R., Crim, L.W., Walsh, J.M., (Eds.), Reproductive Physiology of Fish 1987, Memorial University of Newfoundland, St John's. p. 312.

Sangalang, G.B., Freeman, H.C., 1988. *In vitro* biosynthesis of 17α,20β-dihydroxy-4-pregnen-3-one by the ovaries, testes, and head kidneys of the Atlantic salmon *Salmo salar*. Gen. Comp. Endocrinol. 69, 406-415.

Sumpter, J.P., 1997. The endocrinology of stress. In: Iwama, G.K., Pickering, A.D., Sumpter, J.P., Schreck, C.B., (Eds.), Fish, Stress and Health in Aquaculture, Cambridge University Press, Cambridge. pp. 95-118.

Sumpter, J.P., Carragher, J.F., Pottinger, T.G., Pickering, A.D., 1987. Interaction of stress and reproduction in trout. In: Idler, D.R., Crim, L.W., Walsh, J.M., (Eds.), Reproductive Physiology of Fish 1987, Memorial University of Newfoundland, St John's. pp. 299-302.

Van Der Kraak, G., Munkittrick, M.E., McMaster, M.E., Portt, C.B., Chang, J.P., 1992. Exposure to bleached kraft mill effluent disrupts the pituitary-gonadal axis of white sucker at multiple sites. *Toxic. Appl. Pharmacol.* 115, 224-233.

Zohar, Y., 1986. Gonadotropin releasing hormone in spawning induction in teleosts : basic and applied considerations. In: Zohar, Y., Breton, B., (Eds.), Reproduction in Fish - Basic and Applied Aspects in Endocrinology and Genetics, INRA, Paris. pp. 47-61.

CHAPTER 4

The efficacy of exogenous hormones in stimulating changes in plasma steroids and ovulation in wild black bream *Acanthopagrus butcheri* is improved by treatment at capture.

4. The efficacy of exogenous hormones in stimulating changes in plasma steroids and ovulation in wild black bream *Acanthopagrus butcheri* is improved by treatment at capture.

4.1 Summary

Sexually mature female black bream were captured by rod and line and injected with saline, human chorionic gonadotropin (hCG) or luteinizing hormone releasing hormone analogue (LHRHa) at capture, or 24 hrs post capture (saline and LHRHa treatments only). All fish were bled and checked for ovulation for 5 days post injection. Plasma levels of estradiol (E_2), testosterone (T), 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and cortisol were determined by radioimmunoassay. Saline injected fish ovulated only on day 1, whereas treatment with LHRHa or hCG resulted in fish ovulating throughout the experiment. Treatment with LHRHa at capture resulted in a better ovulatory response than treatment with hCG at capture or LHRHa 24hrs post capture. Plasma E_2 levels in saline injected fish were high at capture and had significantly dropped 1 day after capture. Injection with hCG or LHRHa at capture resulted in plasma E_2 levels remaining significantly elevated for 2 days post injection. Injection of LHRHa 24 hrs post-capture failed to significantly elevate plasma E_2 levels over controls. Plasma T levels essentially mimicked E_2 profiles. Plasma levels of 17,20βP were not significantly different between any treatments, but showed a tendency to increase after capture. Plasma cortisol levels showed no treatment effects and were initially low at capture before becoming elevated between 1-2 days post-capture. These results show that capture and handling stress reduces the responsiveness of fish to exogenous hormone treatment and that best results are obtained if hormonal treatment is administered at the time of capture.

4.2 Introduction

One of the difficulties in developing new species for aquaculture is obtaining reliable quantities of viable eggs. In the initial development stages of a new species with aquaculture potential, eggs are usually obtained by the capture and hormonal induction of ovulation of wild-caught females. This approach commonly involves the capture and transportation of fish to holding facilities, with fish being treated with exogenous hormones some time after capture. Although the effects of exogenous hormones on ovulatory and endocrine events have been examined in detail in many species (see reviews by Lam 1982; Donaldson and Devlin 1996; Peter and Yu 1997) few studies have investigated the effects of a delayed treatment on subsequent endocrine and ovulatory responses. This is likely to be a particular problem in species that are severely stress sensitive, where plasma levels of E_2 and T are rapidly depressed within 1 hour of capture (Carragher and Pankhurst 1991; Jardine et al., 1996; Cleary 1998; Haddy and Pankhurst 1999). However, it is currently unknown whether stress-induced disruption of the hypothalamic-pituitary-gonad axis affects the subsequent ability of fish to respond to exogenous hormone treatment.

Induced ovulation protocols most commonly utilise human chorionic gonadotropin (hCG) or luteinizing hormone releasing hormone analogues (LHRHa). LHRHa stimulates the release of endogenous gonadotropin (GtH), whereas, hCG mimics endogenous GtH (reviewed in Donaldson and Devlin 1996). In some species, hCG has a low biopotency and high doses or multiple injections are required, and this has led to a more widespread use of LHRHa (Donaldson and Devlin 1996).

The Australian sparid *Acanthopagrus butcheri* (black bream) is currently under investigation as an aquaculture candidate for inland saline water culture. Initial stages of culture are likely to be dependent on hormonal manipulation of sexually mature fish captured from the wild. Given that this species shows acute and profound sensitivity to stress (Haddy and Pankhurst 1999), this study investigated whether delay after capture changed the efficacy of treatment with LHRHa at inducing changes in plasma levels of gonadal steroids, and the occurrence or frequency of ovulation. The ovulatory response of fish to treatment and handling was assessed in terms of the number of ovulations, and the quantity and quality of the eggs produced. In the absence of a specific GtH assay for black bream, the endocrine response of fish to

treatment and handling was assessed by measuring plasma levels of cortisol, E_2, T and 17,20βP. The possibility that fish were differentially responsive to hCG or LHRHa was assessed in a second experiment once the appropriate treatment window was determined.

4.3 Materials and methods

Fish capture, sampling and maintenance

Sexually mature female black bream were captured by rod and line from the Meredith (148°7'S, 42°4'E) and Swan Rivers (148°4'S, 42°4'E) at Swansea, Tasmania. Fish were blood sampled by caudal puncture using heparinized syringes, fin clipped for individual identification and the time of hooking and blood sampling recorded. Blood was stored on ice, plasma obtained by centrifugation, then frozen and stored at -18°C until required for assay. Fish were placed in 400L plastic tanks with oxygenation until transportation to the laboratory (3-20 hrs experiment 1; 3-7 hrs experiment 2), where they were placed in 1000L temperature controlled (18-20°C) tanks supplied with recirculating sea water.

Experiment 1: Effect of delayed injection time

Fish were caught from October to November 1997 between 17:35 and 20:50h or 5:45 and 13:34h. Sixteen fish (mean weight = 564 ± 50 g) were blood sampled at capture without anaesthesia, placed into the holding tanks and transported to the laboratory. Twenty four hours after capture, fish were anaesthetised in 0.05% 2-phenoxyethanol, blood sampled and biopsied for macroscopic gonad condition (Haddy and Pankhurst 1998). Fish that contained mature vitellogeneic oocytes in the biopsy were then weighed, injected intraperitoneally with 50 μg kg^{-1} body weight of 50 μg ml^{-1} des-Gly^{10} (D-Ala^6)-luteinizing hormone releasing hormone ethylamide (LHRHa) (Sigma) (n=9) or 1 ml kg^{-1} of teleost saline (n=7) and checked for ovulation. Treatments were allocated on an alternating basis. LHRHa and saline treated fish were combined into tanks with 3-4 male fish. Ovulated females were manually stripped and the eggs fertilised in seawater of 35 ‰ salinity, using fresh sperm pooled from 3-4 males. Males were stripped by wiping dry the genital duct region and milt

expressed using slight abdominal pressure. The first portion of stripped milt was not collected to ensure milt was not contaminated with urine. Milt was collected while being expressed into dry 5ml syringes and placed on ice until use. Fertilised eggs were viewed under a dissecting microscope and the fertility (division to 2-8 cell stage) of the first 100 eggs encountered recorded. Thereafter, fish were bled and checked for ovulation daily for 5 days. Another 14 fish (mean weight = 428 ± 20 g) were treated as described above except fish were anaesthetised, blood sampled, biopsied, and treated with LHRHa (n=7) or saline (n=7) at the time of capture. Owing both to limited laboratory holding space, and the dependence on capture of mature wild fish the experiment was performed 6 separate groups of fish. Three groups of fish captured between 16/10/97 to 4/11/97 were allocated to treatment 24 hours after capture with the remaining groups of fish captured between 5/11/97 to 26/11/97 allocated to treatment at capture. As all fish were of a similar reproductive state it is assumed that they were equally responsive to treatment.

Experiment 2: Effect of LHRHa, HCG and teleost saline

Fish were caught from October to December 1998 between 5:45 and 12:03h and transported to the laboratory. Fish (mean weight = 615 ± 39 g) were handled as before and injected at capture with 50 μg kg^{-1} body weight LHRHa (n=7), 1000 U kg^{-1} body weight of 1000 U ml^{-1} hCG (n=8) or 1 ml kg^{-1} of teleost saline (n=7). Treatments were allocated in a cyclic fashion at the time of capture. LHRHa, hCG and saline treated fish were combined into tanks with 3-4 male fish.

Steroid measurement

Plasma steroid concentrations were measured by radioimmunoassay, using the reagents and protocols given in Pankhurst and Carragher (1992). Extraction efficiency was determined by recovery of [^{3}H]-labelled steroid extracted with plasma, and assay values were corrected accordingly. Assay detection limits in plasma were 0.15 ng ml^{-1}; for E_2, T and 17,20βP and 0.3 ng ml^{-1} for cortisol. Values that were below the detection limit were treated as being equal to the detection limit. Interassay

variability (%CV) measured using aliquots of a pooled standard was as follows: E_2 = 5.5% (n=7); T = 3.8% (n=7); 17,20βP = 10.8% (n=7) and cortisol = 9.8% (n=9).

Statistics

Repeated measures ANOVA, one way ANOVA and Tukey's multiple comparison of means tests were performed using the SPSS statistical package. Data were log transformed to satisfy homogeneity of variance requirements. In some instances, variances were still heterogeneous, however, the data were also assessed by repeated measures ANOVA for days 0-2, and in most cases the outcomes were unchanged. Where necessary, the degrees of freedom for within-subject factors and their interaction, were adjusted (Huynh-Feldt epsilon) to account for violations of the sphericity assumption. Although data were in violation of independence, we chose to present one way ANOVA results because of the utility of means comparison tests, the ability of the tests to handle a decrease in sample numbers over time and the common use of these tests elsewhere.

4.4 Results

Experiment 1.

The proportions of fish ovulating and the number of repeat ovulations were highest in fish injected with LHRHa at capture (Tables 4.1 and 4.2), with one fish serially ovulating for 4 days. Injection of LHRHa on the day following capture resulted in a smaller number of ovulations, with only 2 fish ovulating twice. Saline injected fish ovulated on day 1 only. Spontaneous ovulations from saline treated fish produced both infertile eggs (saline injection at capture) or eggs with high fertility (saline injection 24 hrs post-capture). Fertility from induced ovulations was substantially higher in fish treated at capture than at 24 hrs post-capture.

Mean plasma cortisol levels ranged from 4.3 - 25.7 ng ml^{-1} at capture, were significantly elevated on day 1 and remained elevated thereafter for all treatments except in fish injected with saline at capture, where plasma cortisol levels were not significantly elevated until day 2 (Fig. 4.1). There were no differences in cortisol

Table 4.1. Summary of the percentage of fish ovulating for the duration of experiments 1 and 2.

	Time (days)					
Treatments	**0**	**1**	**2**	**3**	**4**	**5**
Experiment 1						
At capture:						
Saline	0	57	0	0	0	0
LHRHa	0	28	57	66	25	33
24hrs post capture:						
Saline	0	43	0	0	0	0
LHRHa	0	22	11	0	33	16
Experiment 2						
Saline	0	57	0	0	0	0
LHRHa	0	86	86	100	100	0
HCG	0	62	37	75	71	33

Table 4.2. Summary of the proportions of fish ovulating, and quality of eggs collected in experiments 1 and 2.

Treatments	**proportion of ovulating fish (%)**	**proportion of fish serially ovulating (%)**	**mean number of ovulations per fish***	**mean fertilisation (%)****
Experiment 1				
At capture:				
Saline	57	0	1	0 (1)
LHRHa	85	57	2.4	60±16.5 (3)
24hrs post capture:				
Saline	43	0	1	98 (1)
LHRHa	44	22	1.5	20 (1)
Experiment 2				
saline	57	0	1	43.0±22.4 (3)
LHRHa	100	100	3.14	46.9±7.4 (16)
HCG	87.5	75	2.86	41.1±7.7 (9)

* calculated from ovulating fish only ** Fish numbers in parentheses

* NOTE:

The data presented in graph A were analysed by a single One Way ANOVA, where all values (both LHRHa and saline treatments) were compared across time. Differences between means were subsequently determined by Tukeys multiple comparisons of means test. The data presented in graph B were analysed in the same manner in a separate analysis.

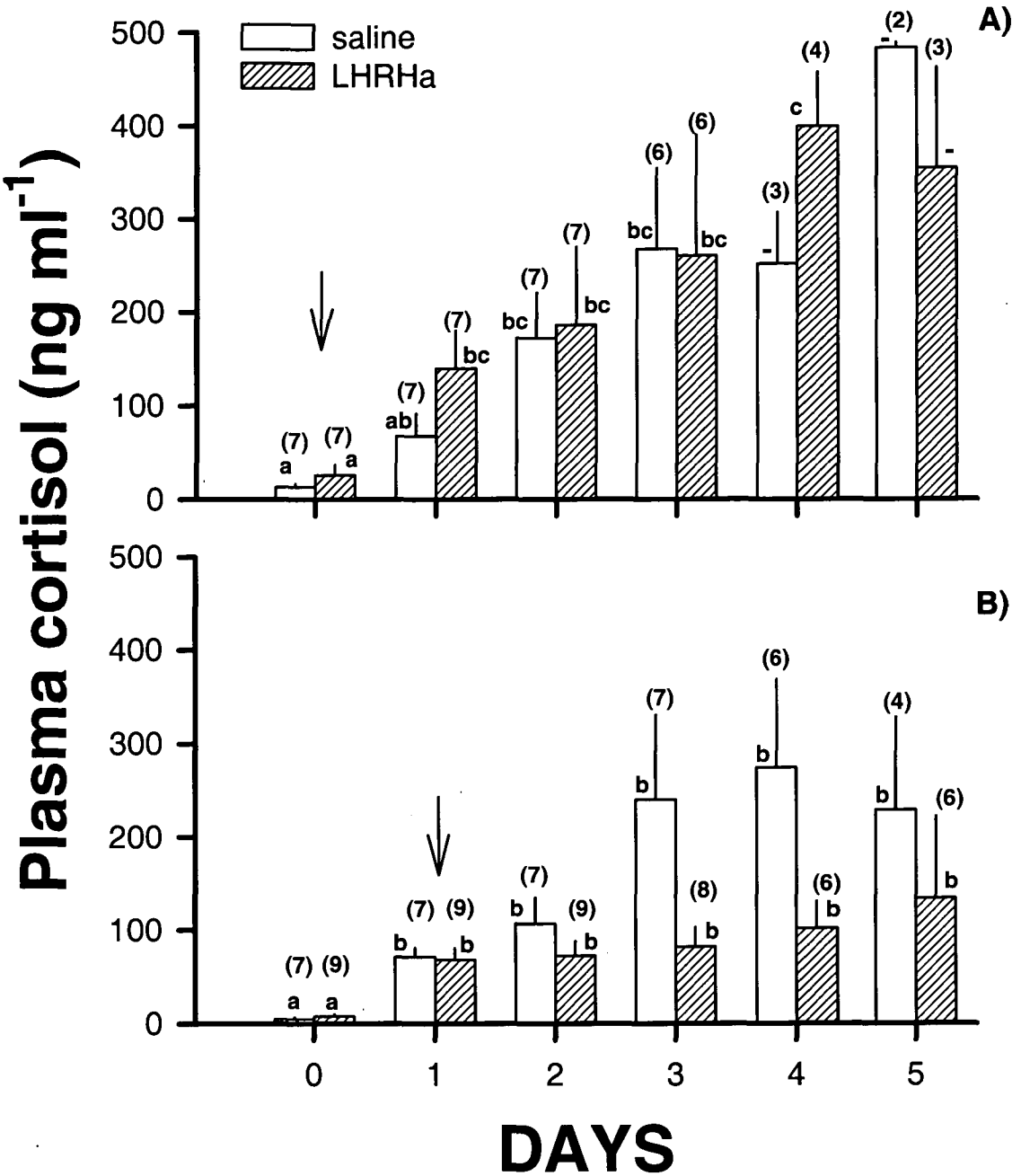


Fig. 4.1. Plasma cortisol concentrations (mean ± se) in black bream injected (indicated by arrow) with either saline or 50 μg kg^{-1} LHRHa at capture (A) or 24hrs post capture (B). Separate one way ANOVAs were conducted for graphs A and B respectively. Values that are significantly different ($P<0.05$) have different letters; values indicated by - were not included in the analysis due to low n values (given in parenthesis).

levels between fish treated with saline or LHRHa. Plasma E_2 levels were above 2 ng ml^{-1} at capture in all treatments (Fig. 4.2). In fish injected with saline either at capture, or 24 hrs post-capture, plasma E_2 levels were significantly suppressed by day 1 and remained low thereafter. Plasma E_2 levels in fish injected with LHRHa at capture were significantly elevated over controls on days 1 and 2 post-injection. Treatment of fish with LHRHa 24 hrs post-capture resulted in a variable response with no significant increase in plasma E_2 levels over saline treated fish, but plasma E_2 levels after treatment were of similar magnitude to values found at capture. Mean plasma T levels ranged from 0.8 to 1.9 ng ml^{-1} at capture and followed the same pattern as E_2 in saline injected fish (Fig. 4.3). Plasma T levels in fish injected with LHRHa at capture were significantly elevated over controls on day 1 but there was no significant effect of LHRHa in fish treated 24 hrs after capture. However, as for E_2 levels, the response was highly variable with mean plasma T levels in LHRHa-injected fish approaching pre-treatment values. Plasma 17,20βP levels were not different among fish injected at capture at any time (Fig. 4.4). Treatment with LHRHa 24 hours after capture resulted in 17,20βP levels that were elevated over those at capture on days 1, 3 and 4 , but values were not different from same day controls.

Experiment 2

All fish injected with LHRHa ovulated more than once, with 2 fish serially ovulating for 4 days (Tables 4.1 and 4.2). In fish injected with hCG, 6 out of 8 fish serially ovulated, with 2 fish ovulating for 4 days. Saline injected fish ovulated on day 1 only. Ovulations from all groups produced similar proportions of fertile eggs (ranging from 41-47%).

Mean plasma cortisol levels ranged from 4.7 - 28.6 ng ml^{-1} at capture, were significantly elevated on day 1 and remained elevated thereafter (Fig. 4.5). Hormone treatment had no effect on plasma cortisol levels. Mean plasma E_2 levels for all treatments were above 2 ng ml^{-1} at capture (Fig. 4.6). Plasma E_2 levels were significantly suppressed by day 1 in fish injected with saline, and remained low thereafter. Plasma E_2 levels in fish injected with LHRHa or hCG were significantly elevated over levels in controls on days 1 and 2, but there were no differences

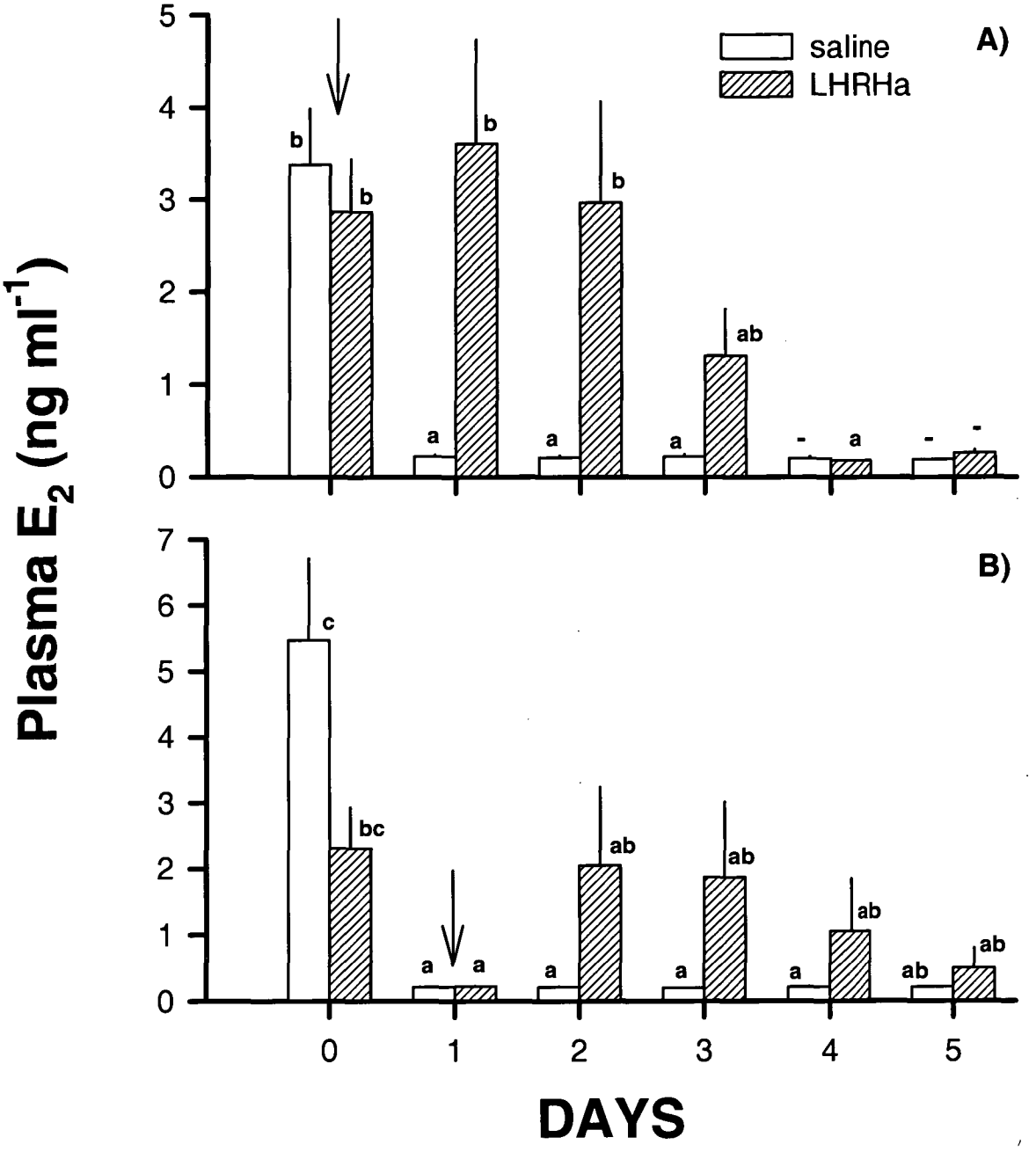


Fig. 4.2. Plasma E_2 concentrations in black bream injected (indicated by arrow) with either saline or 50 μg kg^{-1} LHRHa at capture (A) or 24hrs post capture (B). Other details as for Fig. 4.1.

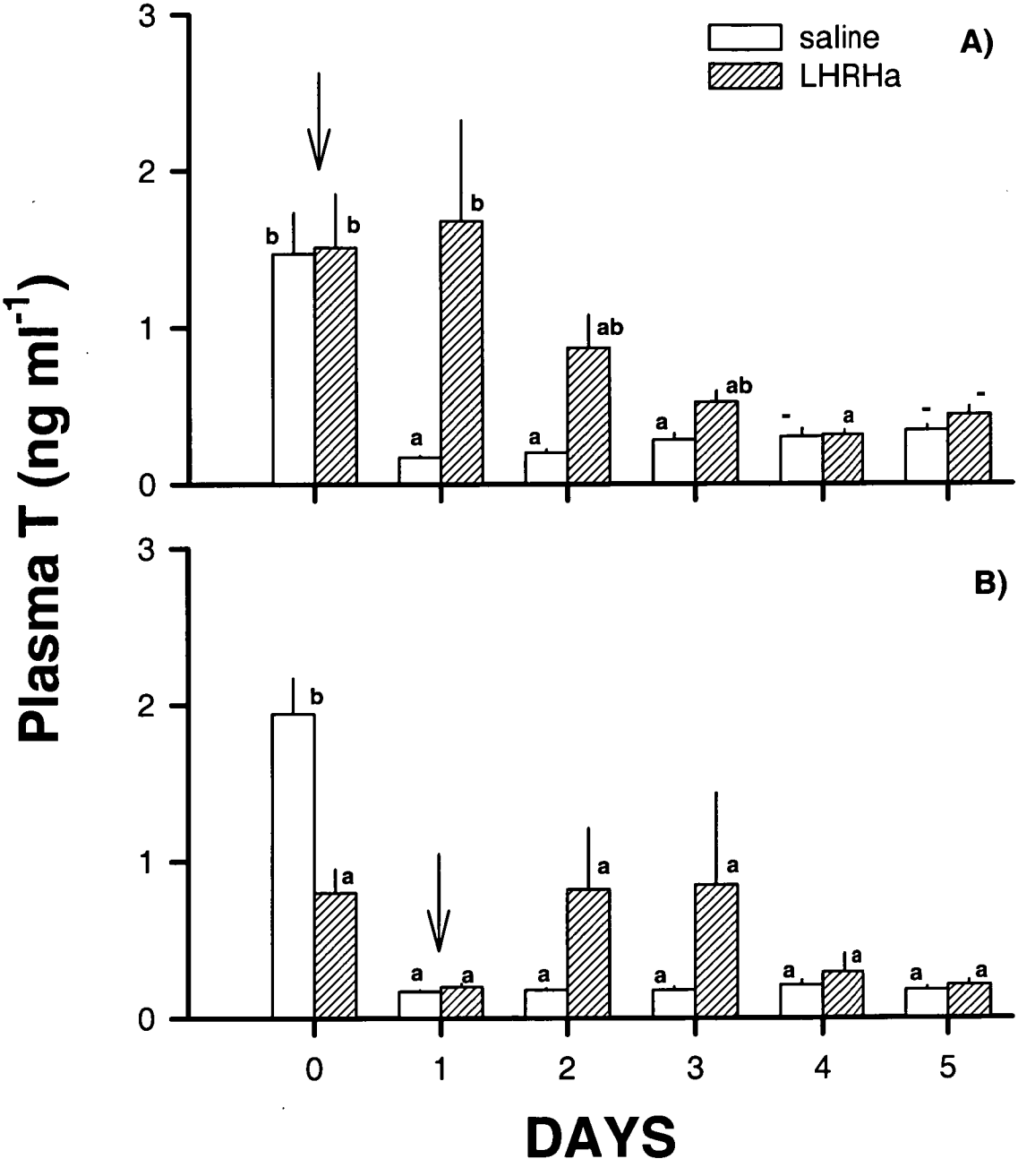


Fig. 4.3. Plasma T concentrations in black bream injected (indicated by arrow) with either saline or 50 μg kg^{-1} LHRHa at capture (A) or 24hrs post capture (B). Other details as for Fig. 4.1.

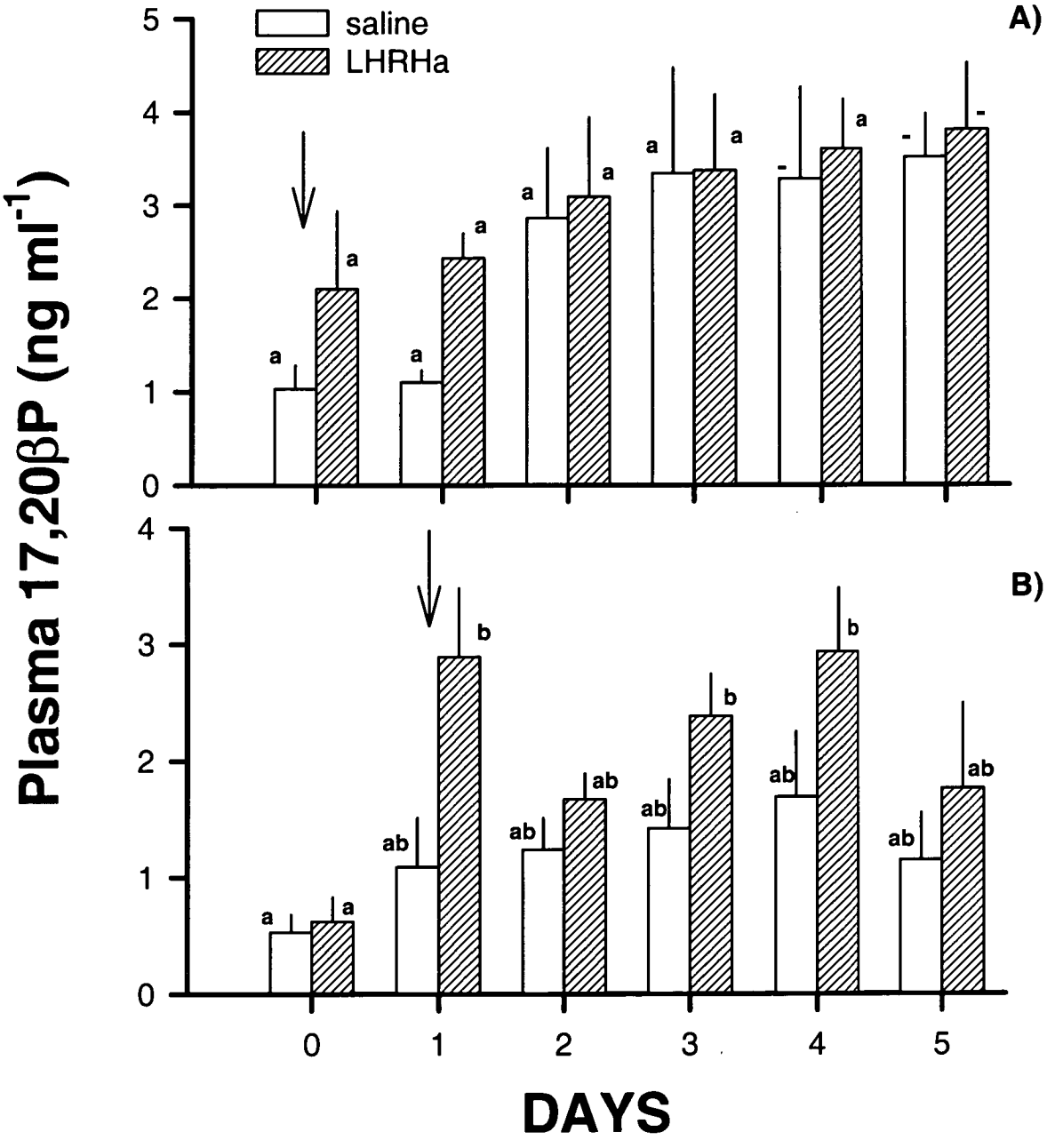


Fig. 4.4. Plasma 17,20βP concentrations in black bream injected (indicated by arrow) with either saline or 50 μg kg^{-1} LHRHa at capture (A) or 24hrs post capture (B). Other details as for Fig. 4.1.

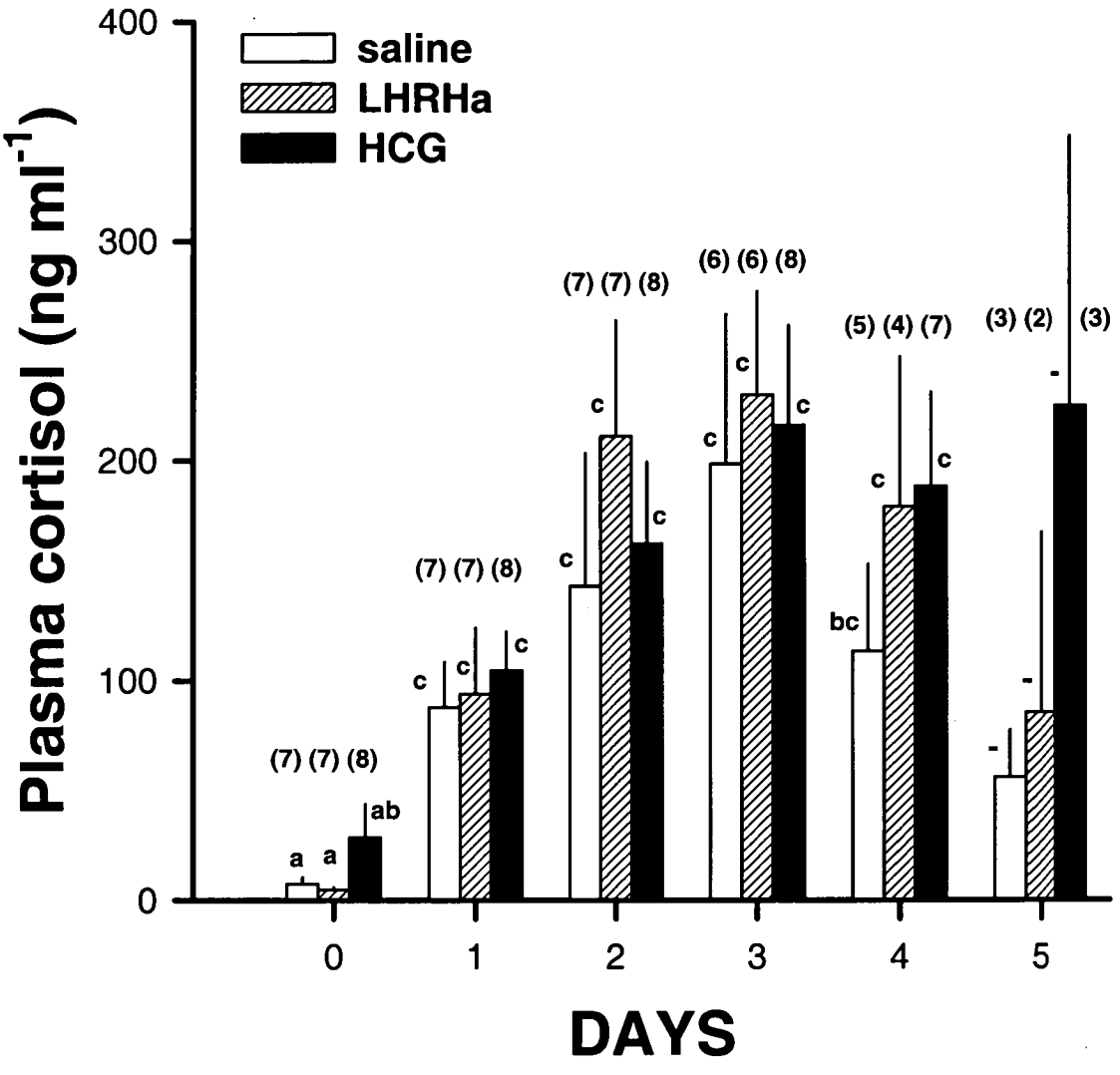


Fig. 4.5. Plasma cortisol concentrations in black bream injected with either saline, 50 μg kg^{-1} LHRHa or 1000 U kg^{-1} hCG at capture. Other details as for Fig. 4.1.

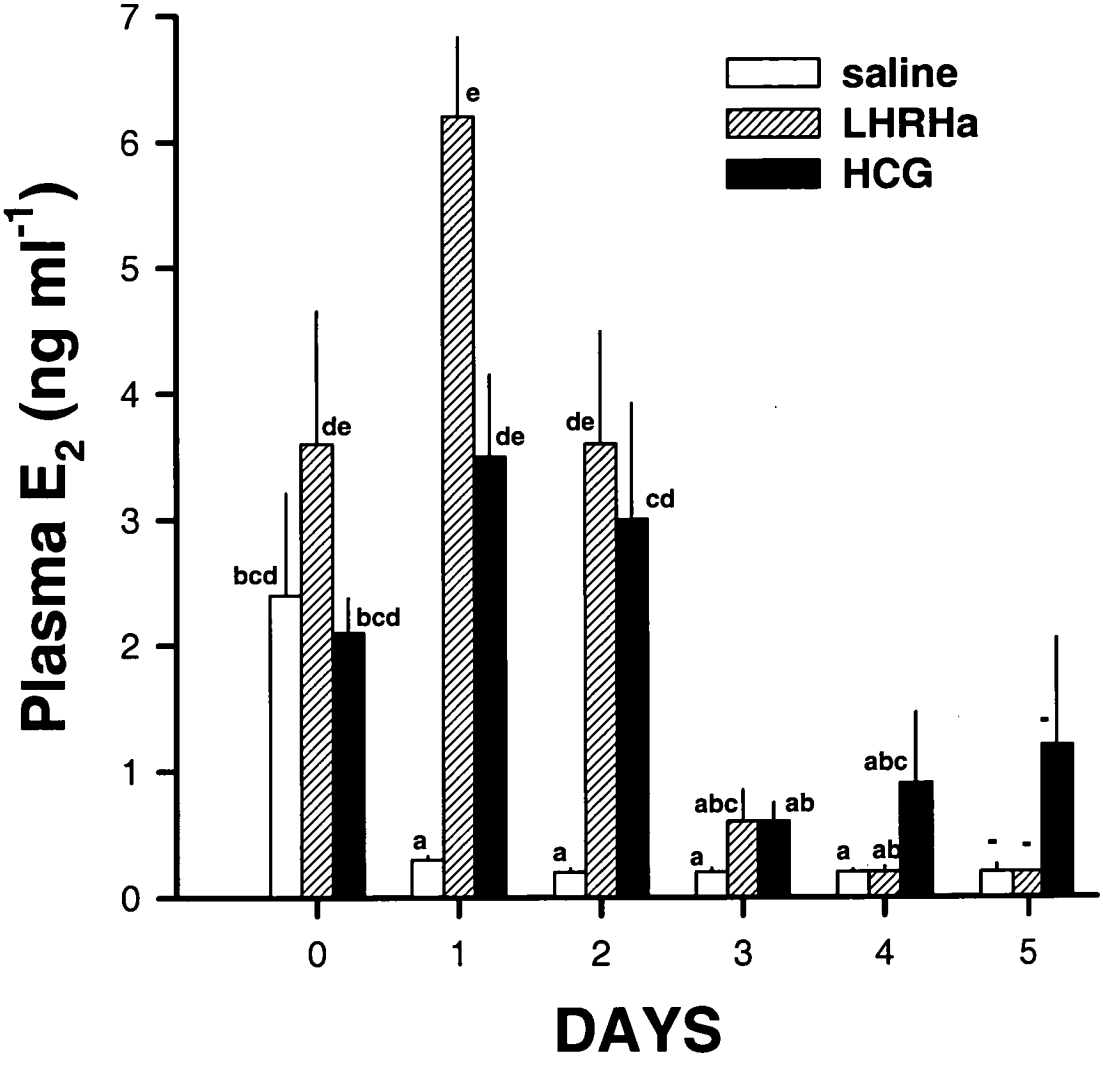


Fig. 4.6. Plasma E_2 concentrations in black bream injected with either saline, 50 μg kg^{-1} LHRHa or 1000 U kg^{-1} hCG at capture. Other details as for Fig. 4.1.

between the two hormone treatments at any time. Mean plasma T levels at capture ranged from 1.3 to 2.2 ng ml^{-1} and followed a similar pattern to E_2 in saline injected fish, with a fall from pre-injection values to 'near detection' levels (Fig. 4.7). Plasma T levels in fish injected with LHRHa and hCG were significantly elevated over plasma T levels in saline injected fish on days 1 and 2, and day 1 respectively. Plasma T levels were not different between LHRHa or hCG-injected fish at any time. Plasma 17,20βP levels were not different among treatments at any time, however, there was a general tendency for 17,20βP levels to increase over the course of the experiment (Fig. 4.8).

4.5 Discussion

Results from the present study show that broodstock collection and daily handling stress in black bream causes the sustained elevation of plasma cortisol in all fish, and suppression of plasma T and E_2 in saline-injected fish within 1 day of treatment. Studies on stress sensitive species caught from the wild, including black bream, have shown that stress-induced suppression of reproductive steroids occurs within 1 hour of capture (Carragher and Pankhurst 1991; Jardine et al., 1996; Cleary 1998; Haddy and Pankhurst 1999) Furthermore, in wild fish subjected to daily handling, plasma T and E_2 levels remain suppressed and show no signs of recovery during the experimental period (Carragher and Pankhurst 1991; Clearwater and Pankhurst 1997; Morehead 1998). Low plasma E_2 concentrations are associated with the onset of ovarian atresia, and once atretic, follicles are unlikely to be steroidogenic (Clearwater and Pankhurst 1997; Janz and Van Der Kraak 1997). The failure of plasma E_2 and T levels to recover in saline-injected fish in the present study is consistent with the previously demonstrated effects of stress on reproductive endocrine function in fish.

Black bream are serial spawners with a daily spawning pattern (Haddy and Pankhurst 1998). In the present study, 43-57% of saline treated fish ovulated on the day following capture but failed to continue to ovulate. Cleary (1998) showed similar effects in snapper *Pagrus auratus*. Carragher and Pankhurst (1991) found that untreated New Zealand snapper serially ovulated for up to 4 days after capture but as in the present study there was a major fall within 2 days of capture. Shut down of the daily spawning pattern after capture is most likely to be due to stress-induced

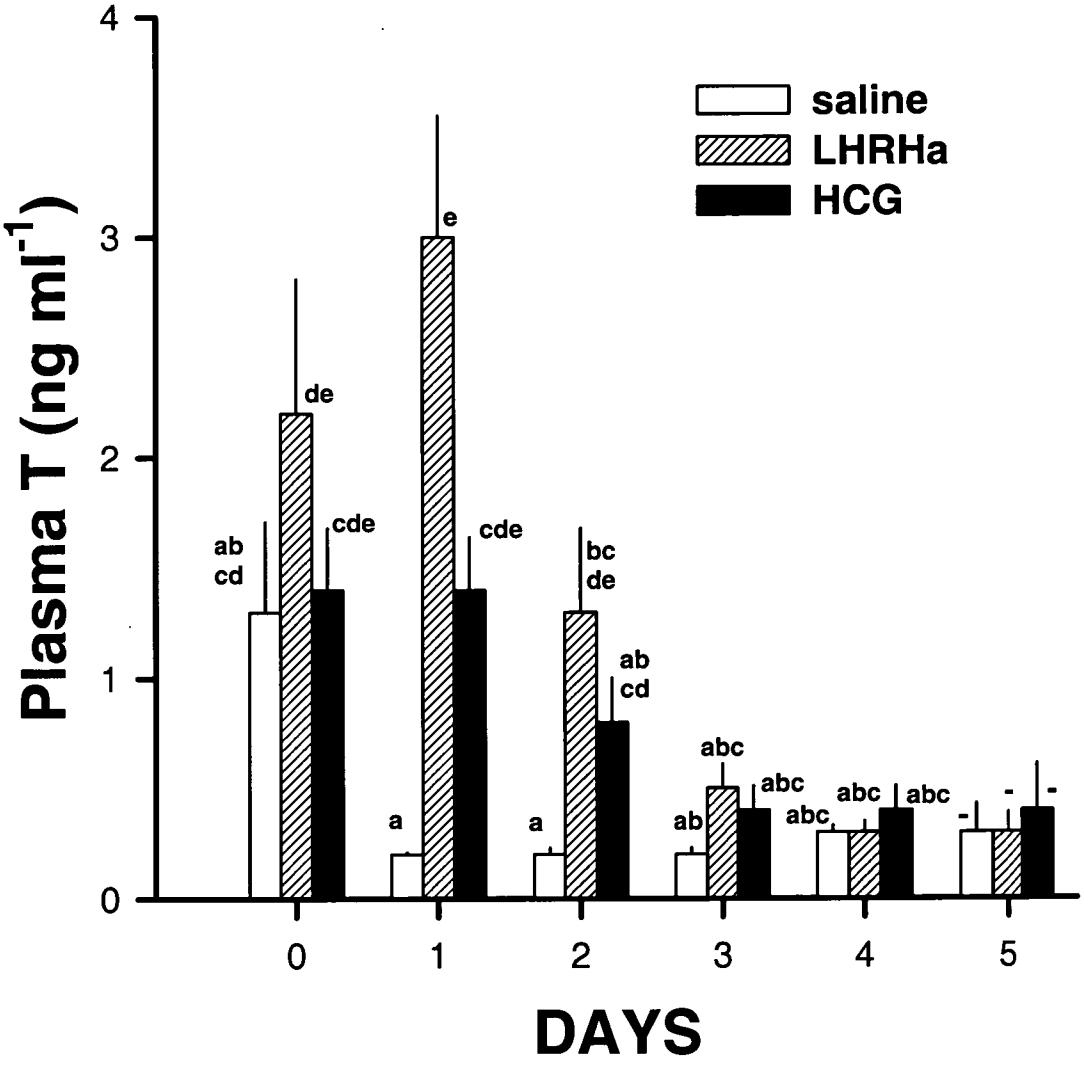


Fig. 4.7. Plasma T concentrations in black bream injected with either saline, 50 μg kg^{-1} LHRHa or 1000 U kg^{-1} hCG at capture. Other details as for Fig. 4.1.

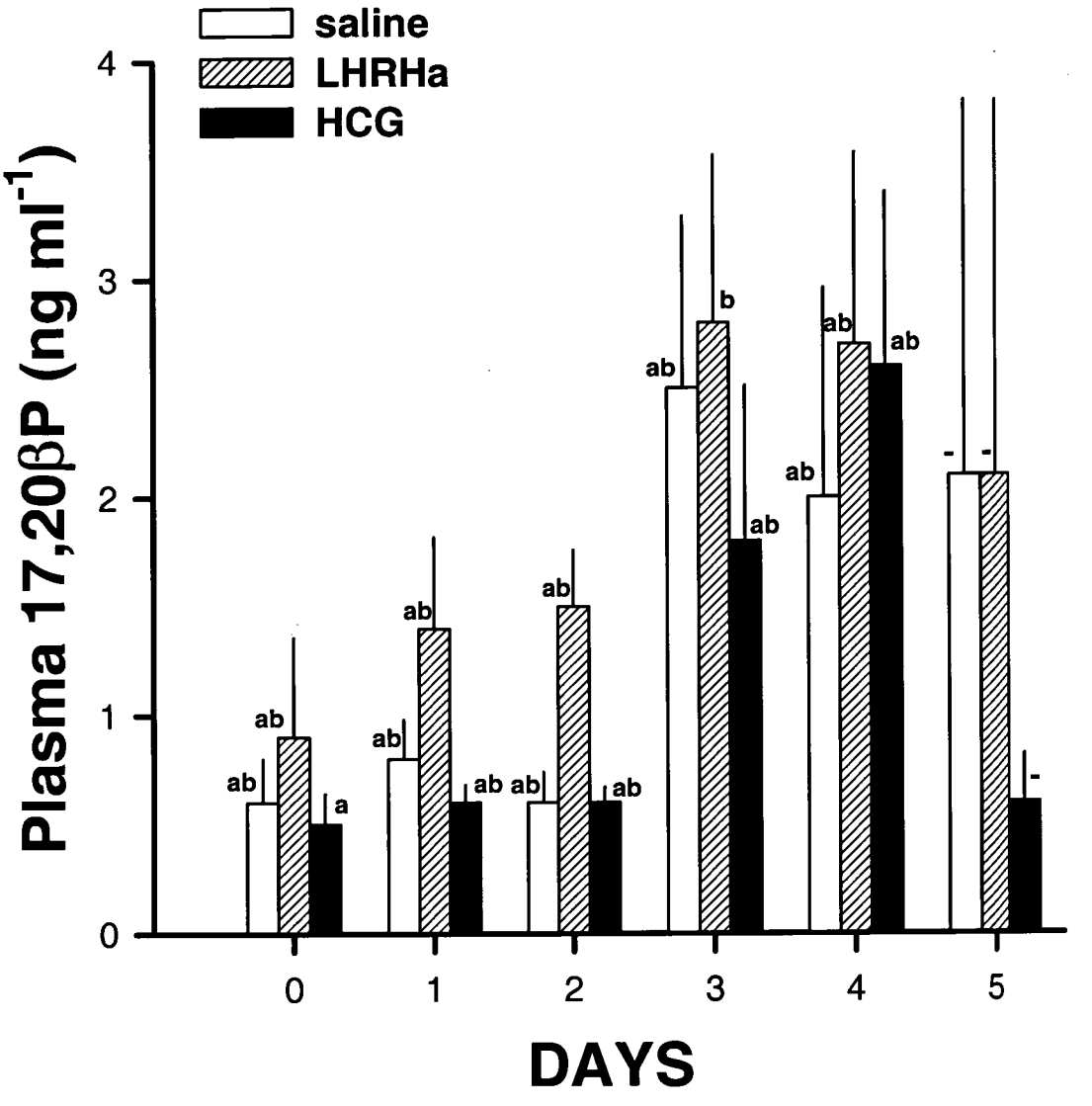


Fig. 4.8. Plasma 17,20βP concentrations in black bream injected with either saline, 50 μg kg^{-1} LHRHa or 1000 U kg^{-1} hCG at capture. Other details as for Fig. 4.1.

suppression of reproductive steroids discussed above, with initial ovulations on day 1 reflecting the outcomes of maturational events already in progress at the time of capture. It is currently unknown why saline treated fish in experiment 1 showed a higher proportion of ovulation than LHRHa treated fish on day 1, as fish were allocated to treatments in an alternating fashion at capture.

Injection of LHRHa at capture resulted in more ovulations and greater fertilisation than in fish treated with LHRHa on the day following capture. This difference was associated with the impaired endocrine response in fish injected with LHRHa on the day following capture. Cleary (1998) showed that in snapper, a 24 hour delay in hormone treatment with hCG or LHRHa did not markedly affect the numbers of ovulating fish, but did reduce the volume and quality of eggs produced. De Montalembert et al. (1978) found that the ratio of ovulated oocyte weight to initial ovary weight in northern pike *Esox lucius* declined from 96% to 40% in fish treated with exogenous hormones at capture or 3 days after capture respectively. However, egg fertility was not affected by delayed treatment in northern pike.

In the present study, the fertility of stripped eggs was highly variable and ranged from 0 to 98%. In many repeat spawning species, including sparids, post-ovulatory egg viability decreases with time, with peak fertility (above 50%) extending for only 6 hours after ovulation (Scott et al., 1993; Hobby and Pankhurst 1997). In the present study, fish were checked at 24 hour intervals in an attempt to minimise handling stress, with the result that it is not known exactly when fish ovulated. Undetected variation in the time between ovulation and stripping may account for the variability in fertility seen here.

HCG and LHRHa both successfully induced repeat ovulations in black bream. In some teleosts, mammalian GtHs have a lower biopotency than piscine GtHs (Lam 1982; Pankhurst 1997) and treatment with hCG may be ineffective, or large and repeat doses may be required to induce ovulation (Lam 1982; Berlinsky et al., 1997). HCG has been successfully used to induce ovulation in several sparids including snapper (Pankhurst and Carragher 1992; Battaglene and Talbot 1995; Cleary 1998), gilthead seabream *Sparus aurata* (Zohar and Gordin 1979), yellowfin porgy *Acanthopagrus latus* (Leu and Chou 1996) and black bream (present study). In the gilthead seabream, hCG doses as low as 100 U kg^{-1} have been successfully used to induce serial

ovulations (Zohar and Gordin 1979). In contrast, injection of 500 U kg^{-1} of hCG in the yellow fin porgy failed to induce natural spawning whereas injection with 1000 U kg^{-1} hCG resulted in partial spawning. Results from the present study indicate that in black bream hCG has biopotency at 1000 U kg^{-1} *in vivo* and that receptor recognition is at least good enough to stimulate ovarian steroidogenesis and subsequent ovulation. However, more work is required to establish the minimum effective dose. LHRHa was effective in black bream in the present study at 50 μg kg^{-1}. In the gilthead seabream injection of LHRHa at doses as low as 7.5 μg kg^{-1} have been successfully used to induce ovulation (Zohar 1986). In yellowfin bream *Acanthopagrus australis*, injection of 15-20 μg kg^{-1} of LHRHa is the minimum effective dose to reliably induce spawning (Cowden 1995). As with hCG, the minimum effective dose of LHRHa in black bream has yet to be established.

LHRH analogues are becoming the hormone of choice for reproductive manipulation in fish culture (Donaldson and Devlin 1996; Pankhurst 1998). In some species LHRHa treatment is ineffective without the addition of a dopamine (DA) antagonist (Trudeau and Peter 1995; Peter and Yu 1997). DA inhibition of GtH secretion appears to be weak or non-existent in the gilthead seabream with co-treatment of LHRHa and a DA antagonist offering no significant advantages over LHRHa treatment alone (Zohar et al., 1987a). This is consistent with the present study where injection of LHRHa alone successfully induced multiple ovulations. In the closely related yellowfin bream, injection of Ovaprim (a mixture of LHRHa and the DA antagonist domperidone, Syndel) was less effective than LHRHa alone (Cowden 1995). Co-treatment with LHRHa and a DA antagonist remains to be explored in black bream, but results from the present study provide indirect evidence that DA does not play a pivotal role in GtH release in this species.
Administration of LHRHa in sparids results in a rapid surge of plasma GtH, with GtH levels peaking within 30 minutes to 1.5 hours post injection then gradually decreasing thereafter (Zohar et al., 1987b; Zohar et al., 1990; Tanaka et al., 1993). In the gilthead seabream, plasma GtH levels remain significantly elevated following injection with LHRHa for 48 hours. Although plasma GtH levels were not measured in the present study, plasma E_2 and T profiles suggest that LHRHa-induced increases in GtH are of a similar duration in black bream, with E_2 and T remaining elevated for 2 days post

injection. In contrast, plasma T levels in response to hCG returned to control values 1 day sooner than LHRHa treated fish. This is most likely due to a quicker clearance of injected hCG than LHRHa which resulted in a prolonged secretion of endogenous GtH. The use of slow release LHRHa implants sustain elevated gonadotropin levels over longer periods than acute administration (Breton et al., 1990) and in sequential spawners result in inducing several successive ovulations (Mylonas et al., 1995; Cowden 1995). However, the use of LHRHa implants in black bream remains to be investigated.

The short term conservation of plasma T and E_2 levels following hCG or LHRHa treatment of black bream indicates maintenance of the steroidogenic activity of vitellogenic follicles (Haddy and Pankhurst 1998). Morehead et al. (1998) showed that multiple ovulations in striped trumpeter *Latris lineata* treated with LHRHa were dependent on recruitment of previtellogenic oocytes into vitellogenesis, and were associated with elevated plasma T and E_2 levels. This suggests that multiple ovulations in black bream may also have been supported by serial recruitment of follicles from various stages of vitellogenesis.

Plasma 17,20βP levels in black bream in the present study were unaffected by hormonal treatment, and typically showed a tendency to increase over time due to capture and handling. Capture and confinement of black bream results in a rapid increase in plasma 17,20βP levels, with stress-induced increases in 17,20βP levels thought to be of interrenal origin (Haddy and Pankhurst 1999). In the gilthead seabream, 17,20β21-trihydroxy-4-pregnen-3-one is the most likely candidate as the maturation inducing hormone (MIH) (Canario et al., 1995), whereas in snapper 17,20βP appears to be the MIH (Adachi et al., 1988; Kagawa et al., 1991; Ventling and Pankhurst 1995). 17,20βP has not yet been conclusively identified as the MIH of black bream, but has been associated with final oocyte maturation and shows seasonal peaks during the spawning season (Haddy and Pankhurst 1998). The results of the present study emphasise that 17,20βP levels in black bream appear to be an ambiguous marker of impending ovulation in stressed fish.

In summary, fish capture caused the shut down of reproductive activity but injection of LHRHa or hCG at capture resulted in the maintenance of plasma T and E_2 levels, and was accompanied by the induction of multiple ovulations. Delayed

injection of LHRHa resulted in a poorer ovulatory and a dampened endocrine response. LHRHa and hCG treatments resulted in a similar steroidogenic response, however LHRHa produced a more consistent ovulatory response. These results confirm that in stress sensitive species such as black bream, wild fish should be treated with LHRHa or hCG as soon as possible after capture for optimal responses.

4.6 Acknowledgments

This study was supported by grants from University of Tasmania Faculty of Science and Engineering, the Co-operative Research Centre for Aquaculture, an Australian Research Council Infrastructure Grant held by NWP and an Australian Postgraduate Scholarship awarded to JAH. Thanks are extended to S. Shaw for permission to fish on private property and to M. Attard, A. Hobby, P. Polhner, R. Morrison, K. Engin, M. Ferhangi, A. Shepherd and B. Wood, for assistance with fish capture and sampling, and to N. Moltschaniwskyj for statistical advice.

4.7 References

Adachi, S., Ouchi, K., Hirose, K., Nagahama, Y., 1988. Induction of oocyte maturation *in vitro* by steroid hormones in the red sea bream *Pagrus major*. Nippon Suisan Gakkaishi 54, 1665.

Battaglene, S.C., Talbot, R.B., 1992. Induced spawning and larval rearing of snapper, *Pagrus auratus* (Pisces:Sparidae), from Australian waters. NZ J. Mar. Freshwat. Res. 26, 179-183.

Berlinsky, D.L., King, W., Hodson, R.G., Sullivan, C.V., 1997. Hormone induced spawning of summer flounder *Paralichthys dentatus*. J. World Aquaculture Soc. 28, 79-86.

Breton, B., Weil, C., Sambroni, E., Zohar, Y., 1990. Effects of acute versus sustained administration of GnRHa on GtH release and ovulation in the rainbow trout, *Oncorhynchus mykiss*. Aquaculture 91, 373-383.

Canario, A.V.M., Couto, E., Vilia, P., Kime, D.E., Hassin, S., Zohar, Y., 1995. Sex steroids during the ovulatory cycle of the gilthead seabream (*Sparus aurata*). In: Goetz, F.W., Thomas, P. (Eds.), Reproductive Physiology of Fish 1995. Fish Symposium 95, Austin. pp. 290-292.

Carragher, J.F., Pankhurst, N.W., 1991. Stress and reproduction in a commercially important fish, *Pagrus auratus* (Sparidae). In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolfe M.S. (Eds) Reproductive Physiology of Fish 1991. FishSymp 91, Sheffield. pp. 253-255.

Clearwater, S.J., Pankhurst, N.W., 1997. The response to capture and confinement stress of plasma cortisol, plasma sex steroids and vitellogenic oocytes in the marine teleost, red gurnard. J. Fish Biol. 50, 429-441.

Cleary, J.J., 1998. The effects of stress on reproduction in snapper (*Pagrus auratus*) Unpublished PhD Thesis, University of Tasmania, Launceston, Australia. 162p.

Cowden, K.L., 1995. Induced spawning and culture of yellowfin bream, *Acanthopagrus australis* (Günther, 1859) and Mangrove Jack, *Lutjanus argentimaculatus* (Forsskal, 1775). Unpublished PhD Thesis, James Cook University, Townsville, Australia. 270p.

De Montalembert, G., Jalabert, B., 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., Devlin, R.H., 1996. Uses of biotechnology to enhance production. In: Pennell, W., Barton, B.A. (Eds.), Developments in Aquaculture and Fisheries Science, Volume 29; Principles of Salmonid Culture. Elsevier Science B.V. Amsterdam. pp. 969-1020.

Haddy, J.A., Pankhurst, N.W., 1998. Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). Mar. Freshwat. Res. 49, 389-397.

Haddy, J.A., Pankhurst, N.W., 1999. Stress-induced changes in concentrations of plasma sex steroids in black bream. J. Fish Biol. 55, 1304-1316.

Hobby, A.C., Pankhurst, N.W., 1997. Post-ovulatory egg viability in the snapper *Pagrus auratus* (Sparidae). Mar. Freshwat. Res. 48, 385-389.

Janz, D.M., Van Der Kraak, G., 1997. Suppression and apoptosis by gonadotropin, 17β-estradiol, and epidermal growth factor in rainbow trout preovulatory ovarian follicles. Gen. Comp. Endocrinol. 105, 186-193.

Jardine, J.J., Van Der Kraak, G.J., Munkittrick, K.R., 1996. Capture and confinement stress in white sucker exposed to bleached kraft pulp mill effluent. Ecotoxicol. Environ. Saf. 33, 287-298.

Kagawa, H., Tanaka, H., Okuzawa, K., Matsuyama, M., Hirose, K., 1991. Diurnal changes in plasma 17α20β-dihydroxy-4-pregnen-3-one levels during spawning season in the red sea bream *Pagrus major*. Nippon Suisan Gakkaishi 57, 769.

Lam, T.J., 1982. Applications of endocrinology to fish culture. Can. J. Fish. Aquat. Sci. 39, 111-137.

Leu, M-Y., Chou, Y-H., 1996. Induced spawning and larval rearing of captive yellowfin porgy, *Acanthopagrus latus* (Houttuyn). Aquaculture 143, 155-166.

Morehead, D.T., 1998. Effect of capture, confinement and repeated sampling on plasma steroid concentrations and oocyte size in female striped trumpeter *Latris lineata* (Latrididae). Mar. Freshwat. Res. 49, 373-377.

Morehead, D.T., Pankhurst, N.W., Ritar, A.J., 1998. Effect of treatment with LHRH analogue on oocyte maturation, plasma sex steroid levels and egg production in female striped trumpeter *Latris lineata* (Latrididae). Aquaculture 169, 315-331

Mylonas, C.C., Zohar, Y., Richarsson, B.M., Minkkinen, S.P., 1995. Induced spawning of wild American shad *Alosa sapidissima* using sustained administration of gonadotropin-releasing hormone analog (GnRHa). J. World Aquaculture Soc. 26, 240-251.

Pankhurst, N.W., 1997. *In vitro* steroid production by isolated ovarian follicles of the striped trumpeter. J. Fish Biol. 51, 669-685.

Pankhurst, N.W., 1998. Reproduction. In: Black, K.D., Pickering, A.D. (Eds.), Biology of Farmed Fish. Sheffield Academic Press, Sheffield. pp. 1-25.

Pankhurst, N.W., 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.

Peter, R.E., Yu, K.L., 1997. Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. Rev. Fish Biol. Fish. 7, 173-197.

Scott, S.G., Zeldis J.R., Pankhurst, N.W., 1993. Evidence of daily spawning in natural populations of the New Zealand snapper *Pagrus auratus* (Sparidae). Environ. Biol. Fish. 36, 149-56.

Tanaka, H., Kagawa, H., Okuzawa, K., Hirose, K., 1993. Purification of gonadotropins (PmGTH I and II) from red seabream (*Pagrus major*) and development of a homologous radioimmunoassay for PmGTH II. Fish. Physiol. Biochem. 10, 409-418.

Trudeau, V.L., Peter R.E., 1995. Functional interactions between neuroendocrine systems regulating GtH-II release. In: Goetz, F.W., Thomas, P. (Eds.), Reproductive Physiology of Fish 1995. Fish Symposium 95, Austin. pp. 44-48.

Ventling, A.R., Pankhurst, N.W., 1995. Effects of gonadal steroids and human chorionic gonadotrophin on final oocyte maturation *in vitro* in the New Zealand snapper *Pagrus auratus* (Sparidae). Aust. J. Mar. Freshwat. Res. 46, 467-73.

Zohar, Y., 1986. Gonadotropin releasing hormone in spawning induction in teleosts : basic and applied considerations. In: Zohar, Y., Breton, B. (Eds.), Reproduction in Fish - Basic and Applied Aspects in Endocrinology and Genetics. INRA, Paris. pp. 47-61.

Zohar, Y., Gordin, H., 1979. Spawning kinetics in the gilthead sea-bream, *Sparus aurata* L. after low doses of human chorionic gonadotropin. J. Fish Biol. 15, 665-670.

Zohar, Y., Pagelson, G., Tosky, M., Finkelman, Y., 1987a. GnRHa control of gonadotropin secretion, ovulation and spawning in the gilthead seabream *Sparus aurata.* In: Idler, D.R., Crim, L.W., Walsh, J.M. (Eds.), Reproductive Physiology of Fish 1987. Memorial University of Newfoundland, St John's. p. 106.

Zohar, Y., Schreibman, M.P., Margolis-Nunno, H., Tosky, M., Pagelson, G., Cepriano, L., 1987b. Gonadotropin biodynamics following GnRH administration in the gilthead seabream *Sparus aurata:* A combined radioimmunoassay (RIA) and immunocytochemical (ICC) study. In: Idler, D.R., Crim, L.W., Walsh, J.M. (Eds.), Reproductive Physiology of Fish 1987. Memorial University of Newfoundland, St John's. p. 46.

Zohar, Y., Breton, B., Sambroni, E., Fostier, A., Toskey, M., Pagelson, G., Leibovitz, D., 1990. Development of a homologous radioimmunoassay for gonadotropin of the gilthead seabream, *Sparus aurata.* Aquaculture 88, 189-204.

CHAPTER 5

The effects of salinity on reproductive development, plasma steroid levels, fertilisation and egg survival in black bream *Acanthopagrus butcheri*.

5. The effects of salinity on reproductive development, plasma steroid levels, fertilisation and egg survival in black bream *Acanthopagrus butcheri.*

5.1 Summary

The effects of salinity of holding water of 5, 20 or 35‰ on seasonal reproductive development, plasma steroid levels, the efficacy of luteinizing hormone releasing hormone ethylamide (LHRHa) to stimulate ovulation, sperm motility, and egg fertility and development to hatching were investigated. Fish were captured from the wild from December to February, placed into salinity regimes in May and held until the normal times of spawning the following November. Blood samples were taken in August, September and November. Female fish were injected with saline or LHRHa (50μg kg^{-1}) in November and bled and checked for ovulation for 5 days. Gonadal maturation was unaffected by salinity in both sexes. In females, seasonal plasma steroid levels were unaffected by salinity, whereas in males, plasma levels of 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 11-ketotestosterone were higher in fish held at 35‰ than in fish held at 5‰ in September, and in fish held at 5 and 20‰ in November respectively. Plasma estradiol (E_2) and testosterone (T) levels in saline-injected fish, either remained low or were significantly suppressed. LHRHa treatment resulted in the short term elevation of plasma E_2 and T levels at all salinities, whereas plasma 17,20βP levels were elevated over controls on days 1 and 2 post-injection in fish held in 20‰, but remained unchanged in fish held in 35 or 5‰. All but 1 fish ovulated in response to LHRHa, however, the number of ovulations and egg volumes were lowest in fish held at 5‰. Both fertilisation and sperm motility were significantly reduced at 5‰. Naturally fertilised eggs (35‰; 2-cell stage) were incubated to hatching at salinities of 0, 5, 10, 15, 20, 25, 30 or 35‰. Eggs hatched in all salinities except 0‰, but with lowered survival at 5 and 10‰. Larvae showed high levels of deformity at salinities below 15‰.

5.2 Introduction

Black bream, *Acanthopagrus butcheri* is an important recreational and commercial species endemic to southern Australia, and is currently being investigated as an aquaculture candidate for inland saline water culture. As black bream is an euryhaline species that spawns in the mid to upper reaches of estuaries at the interface between freshwater and the underlying salt wedge, it has been suggested that salinity may play a role in regulating reproductive activity (Sherwood and Backhouse 1982; Haddy and Pankhurst 1998). Current information indicates that the spawning of black bream occurs over a salinity range of 11-35 ‰ (Sherwood and Backhouse 1982; Haddy and Pankhurst 1998). Environmental factors such as salinity are known to directly effect fertilisation, survival and normal development of fish eggs in other species (reviewed in Holliday 1969; Alderdice 1988), however , to date there is no information on the effects of salinity on the early life stages of black bream.

Additional interest in black bream has been created by the problem of salination of agricultural land. Approximately 20% of Australia's inland aquifers are classified as being either brackish or saline with salinities ranging from 1.5-20.5‰ (Ingram et al., 1996). Rising saline groundwater is causing major loss of agricultural land, and current practice to reduce the height of the water table involves the pumping of saline groundwater into evaporation basins (Clayton 1998; Blackwell 1999). These saline ponds are currently not exploited and may be suitable for inland mariculture of a variety of species, including black bream (Ingram et al., 1996; Fielder et al., 1999). Techniques for the hormonal induction of ovulation have recently been developed for black bream (Haddy and Pankhurst under review), however, information on the maturation of black bream in captivity and the effectiveness of hormonal treatment at differing salinities are yet to be investigated. Such information is critical to the selection of inland sites for the artificial propagation of black bream, and in determining suitable inland lakes for the development of new fisheries where natural recruitment can occur.

The aims of this study were to investigate the effect of holding water salinity on, reproductive development of wild-caught broodstock and subsequent fertilisation, egg development, and hatching. The effects of salinity on reproductive activity in adults were assessed by macroscopic gonadal condition, seasonal changes in plasma levels of

cortisol, estradiol (E_2), testosterone (T), 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and 11-ketotestosterone (11KT), and the efficacy of LHRHa treatment at inducing changes in plasma levels of gonadal steroids, and subsequent ovulation and egg production. The effects of salinity on gamete quality were assessed in terms of fertilisation, sperm activity, and survival and development of eggs to hatching.

5.3 Materials and methods

Fish capture and maintenance

Black bream were captured by rod and line from the Meredith (148°7'S, 42°4'E) and Swan Rivers (148°4'S, 42°4'E) at Swansea, Tasmania at the end of the spawning season from December 97 to February 98. Fish were transported to the laboratory and placed in 1000L temperature (20°C) controlled tanks supplied with recirculating sea water, under natural photoperiod. Fish were sexed by the presence of milt, and males and "presumed females" kept in separate tanks. At the beginning of May (when the GSI is low and the gonads are regressed in wild fish, Haddy and Pankhurst 1998), fish were allocated to experimental tanks with recirculating sea water (37‰) and the salinity adjusted to 35 ‰, 20 ‰ (over 2 weeks) or 5 ‰ (over 4 weeks) by weekly water exchanges with fresh water. Salinities were held constant thereafter, and were checked weekly with a refractometer. Water exchanges and tank cleaning were conducted as required. Fish were fed to satiation on an in house marine fish pellet, and disturbances kept to a minimum. Temperatures were dropped from 20 to 16°C at the end of April and thereafter dropped by 1°C every 15 days until August (minimum of 11°C) when temperatures were raised by 1°C every 15 days to simulate the natural temperature cycle in eastern Tasmania (Haddy and Pankhurst 1998).

Seasonal sampling

On the 1st August, fish were removed from tanks, placed into a 400 L holding tank and the time of first disturbance recorded for each tank. Fish were anaesthetised in 0.05% 2-phenoxyethanol, blood sampled by caudal puncture using heparinized syringes and 22G needles, dart tagged for individual identification and males checked for spermiation. The presumed sexes were then equally distributed into 2 tanks at each salinity. Fish were blood sampled again on the 4th September and 15th November.

Blood was stored on ice, plasma obtained by centrifugation, then frozen and stored at -18°C until required for assay.

Induced ovulation experiment

On the 15th of November, female fish were blood sampled as described above, biopsied for macroscopic gonad condition (Haddy and Pankhurst 1998), weighed, and injected intraperitoneally with either 50 μg kg^{-1} body weight of 50 μg ml^{-1} des-Gly^{10} (D-Ala^{6})-luteinizing hormone releasing hormone ethylamide (LHRHa; pGlu-His-Trp-Ser-Try-$_{D}$-Ala-Leu-Arg-Pro-NHEt) or 1 ml kg^{-1} of teleost saline. Owing to logistical constraints there was only a single tank available for each treatment. This means that the possibility of tank effects cannot be discounted, however, this is viewed as unlikely due to the identical nature of the tank systems and conditions. Fish were bled daily and checked for ovulation twice daily for a period of 5 days. Eggs released into the tanks were collected in egg collectors and the volumes recorded. Ovulated females were manually stripped of eggs, egg volumes recorded and the eggs from fish at each salinity (volumes over 10 mls) fertilised at 35, 20 and 5 ‰ salinity using fresh sperm pooled from 3-4 males held at 35 ‰ salinity. Fertilised eggs were viewed under a dissecting microscope and the viability (division to 2-8 cell stage) of the first 300 eggs encountered recorded. Males were stripped by wiping dry the genital duct region and milt expressed using slight abdominal pressure. The first portion of stripped milt was not collected to ensure milt was not contaminated with urine. Milt was collected while being expressed into dry 5ml syringes and placed on ice until use. Sperm motility was assessed by mixing a drop of freshly stripped milt with a drop of water at 5, 20 or 35‰ in a cavity slide. A coverslip was quickly placed over the cavity and sperm motility observed under a microscope. Motility was assessed within 10 seconds of activation and ratings assigned as: High; very active all sperm visibly progressing rapidly across the field of view; Medium; less energetic movement, most with forward motion; Low; slow movement, some spermatozoa progressing slowly or swimming in a spiral motion; or Not activated; no swimming activity. Sperm was collected from 3 males held at 35 ‰ salinity, and sperm motility ranked at 1 minute intervals over 5 minutes. The salinity at which sperm motility was first initiated was also determined for the 3 males by testing sperm motility at 1‰ intervals between 5 and 10‰.

Egg incubations

Fertilised eggs were collected from a group of naturally spawning bream held at 35 ‰ salinity. Between 5-11 eggs were pipetted into each well of a 24 well plastic tissue culture plate (Corning) using a modified pipette tip. Egg were viewed under a dissecting microscope, and the numbers of fertile eggs (division to 2-8 cell stage) in each well recorded. Eggs were then incubated in 1 ml water of salinity 0 (distilled water), 5, 10, 15, 20, 25, 30 or 35 ‰ (6 replicates per salinity) at 20°C without light. The numbers of developing eggs, and hatched larvae were recorded on days 1 and 2 respectively, and the proportions of abnormal larvae noted. Incubations were run from four separate spawnings. As we did not have unhandled naturally spawning fish held at 20 or 5 ‰ we could not conduct egg incubation experiments at these salinities.

Steroid measurement

Plasma steroid concentrations were measured by radioimmunoassay, using the reagents and protocols given in Pankhurst and Carragher (1992) for E_2, T, 17,20βP and cortisol, and Pankhurst and Kime (1991) for 11KT. Extraction efficiency was determined by recovery of [^{3}H]-labelled steroid extracted with plasma, and assay values were corrected accordingly. Assay detection limits in plasma were 0.15 ng ml^{-1}; for E_2, T and 17,20βP and 0.3 ng ml^{-1} for cortisol. Values that were below the detection limit were treated as being equal to the detection limit. Interassay variability (%CV) measured using aliquots of a pooled standard was as follows: E_2 = 9.8% (n=5); T = 6.8% (n=6); 17,20βP = 7.1% (n=6), 11KT = 9.9 % (n=3), and cortisol = 20.7% (n=6).

Statistics

Kruskal-Wallis, One way ANOVA and Tukey's multiple comparison of means tests were performed using the SPSS statistical package. Percentage data were arcsin transformed, and steroid data log transformed to satisfy homogeneity of variance requirements. As some data were in violation of the assumption of independence, and in some instances homogeneity of variances, the significance level for one way

ANOVAs was set at 0.01 and the data also assessed by Kruskal-Wallis one way ANOVA. The statistical outcomes were the same regardless of whether parametric or non-parametric tests were applied. We chose to present one way ANOVA results because of the utility of means comparison tests ($P<0.05$), the ability of the tests to handle a decrease in sample numbers over time and the common use of these tests elsewhere.

5.4 Results

The effect of salinity on seasonal gonadal development and plasma steroid levels

Salinity did not effect the proportions of spermiated males with, a high percentage of males already spermiating by August and all males spermiating by November (Table 5.1). Similarly, ovarian recrudescence was not affected by salinity with only 1 fish failing to undergo vitellogenesis.

Table 5.1. Percentage of mature male (spermiated) and female (vitellogenic) fish maintained in water of 5, 20 or 35 ‰ salinity.

Treatment	Aug	Sep	Nov
Males			
5 ‰	83	83	100
20 ‰	75	100	100
35 ‰	90	90	100
Females			
5 ‰	nm*	nm	87.5
20 ‰	nm	nm	100
35 ‰	nm	nm	100

* nm= not measured

Concentrations of plasma cortisol were lowest in August and high in September and November, however, in fish held at 35‰, cortisol levels in November were not significantly elevated over cortisol levels in August (Fig. 5.1). Mean plasma E_2 levels were high in August and November but low in September, however, plasma E_2 levels in fish held at 5 and 35 ‰ in November were not significantly elevated over plasma E_2 levels in September. Plasma T levels remained unchanged except in fish held at 20‰ where plasma T levels in November were significantly higher than plasma T levels in

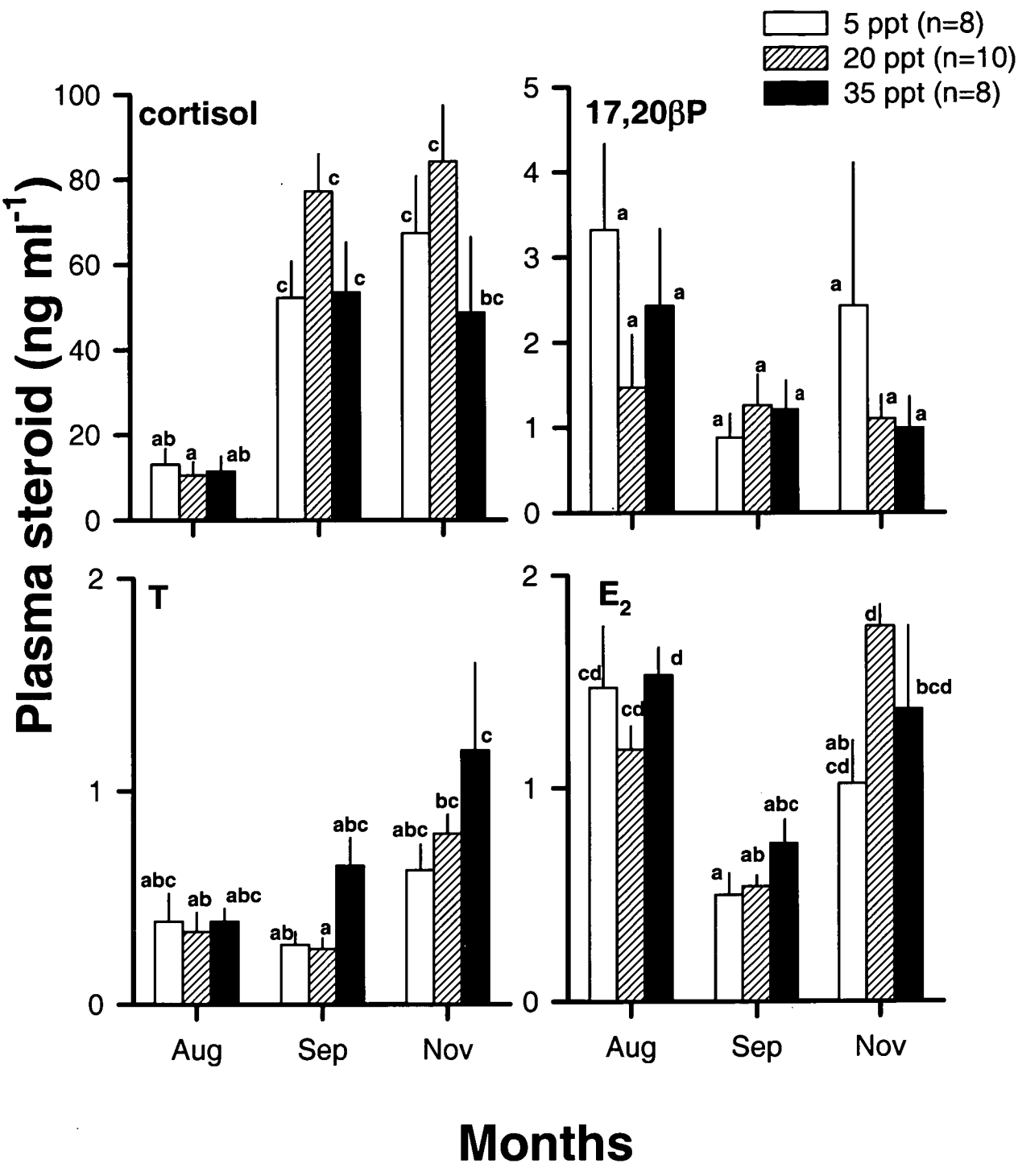


Fig. 5.1. The effect of salinity on plasma steroid levels (mean + se) in maturing captive female black bream. Values that are significantly different have different superscripts ($P<0.05$).

September. Plasma 17,20βP levels remained unchanged throughout.

Concentrations of plasma cortisol in males were low in August and high in September and November, however in fish held at 5‰, cortisol levels in November were not significantly elevated over cortisol levels in August (Fig. 5.2). Plasma T levels were not significantly different from August to November in fish held at 5 or 20‰, however in fish held at 35‰, plasma T levels in November were significantly elevated over plasma T levels in September. Plasma 11KT levels were low in August and September and peaked in November, however, in fish held at 20‰ this increase was not significant. Plasma 11KT levels in November were higher in fish held at 35‰ than in fish held at 5 or 20‰. Plasma 17,20βP levels were not effected by salinity in August or November, but were higher in September in fish held at 35‰ than in fish held at 5‰.

The effect of salinity on induced ovulation, plasma steroid levels, fertilisation and sperm motility

Ovulations first occurred 2 days post injection in all three salinities (Table 5.2). No saline injected fish ovulated. One fish, held at 5 ‰, failed to ovulate in response to LHRHa injection. All fish injected with LHRHa and held in 35 and 20‰ serially ovulated with a mean of 3.2 and 3.5 ovulations per fish respectively, whereas LHRHa treatment of fish in 5‰ resulted in 3 out of 5 fish serially ovulating with a mean of 2 ovulations per fish. In fish injected with LHRHa, total egg production was low in fish held at 5‰ and high in fish held in 20 or 35 ‰ (statistical comparison not made due to egg production being a mixture of spontaneously released eggs and manually stripped eggs). Egg fertility in fish held at 35 ‰ was significantly reduced when eggs were fertilised at 5‰ (Table 5.3). A similar trend was also evident in eggs from fish held at 5‰ (statistical comparison not made due to small sample size). In eggs from fish held at 20‰, fertility was higher at 20 than 5‰ but there was no difference between 35 and 20‰ or 35 and 5‰. Initial sperm motility was high when sperm was activated by water of 20 or 35‰, with sperm activity decreasing within 1 to 2 minutes (Table 5.4). Sperm were not activated at a salinity of 5‰, with sperm motility being initiated at 6, 7 and 10‰ for the three males tested (data not shown).

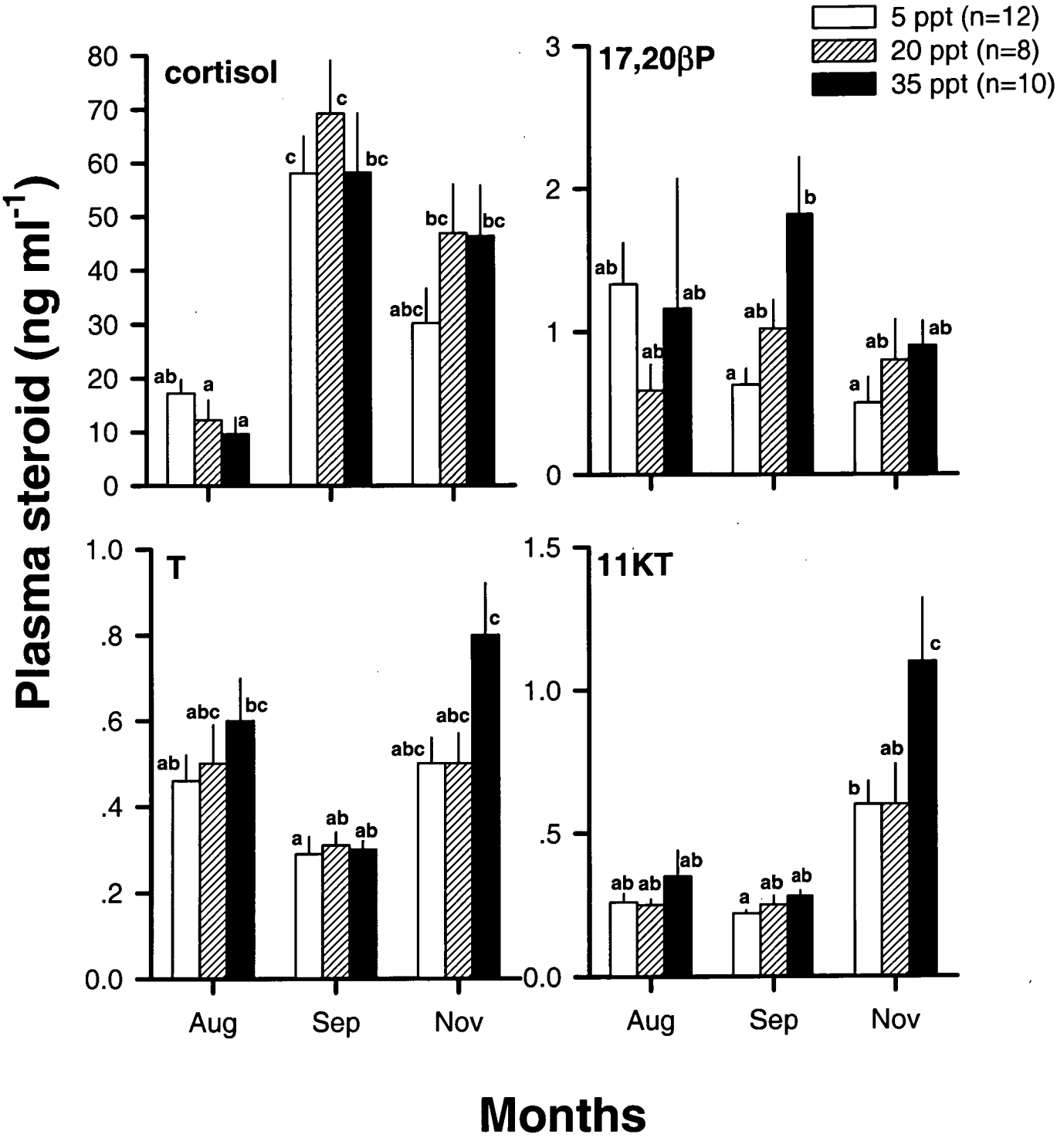


Fig. 5.2. The effect of salinity on plasma steroid levels in maturing captive male black bream. Other details as for Fig. 5.1.

Table 5.2. Summary of the proportions of fish ovulating and quantity of eggs produced from fish maintained in water of 5, 20 or 35 ‰ salinity and injected with LHRHa.

	DAYS (PI)						
Treatments*	**0**	**1**	**2**	**3**	**4**	**5**	**Totals**
			ovulating fish (%)				
5 ‰ LHRHa	0	0	60	80	20	40	80
20 ‰ LHRHa	0	0	80	100	100	75	100
35‰ LHRHa	0	0	80	100	100	40	100
			total egg volumes (ml kg^{-1})				
5 ‰ LHRHa	0	0	23.2	72.1	35.0	5.9	136.2
20 ‰ LHRHa	0	0	36.9	373.4	237.8	44.9	693.1
35 ‰ LHRHa	0	0	113.2	283.3	274.9	88.3	759.7

* no saline treated fish ovulated.

Table 5.3. The effect of salinity on egg fertilisation from fish maintained in water of 5, 20 or 35 ‰ salinity.

Holding salinity	**Fertilising salinity**		
	35 ‰	**20 ‰**	**5 ‰**
5 ‰ (n=2)	14.5±8.2	14.8±12.8	0±0
20 ‰ (n=9)	38.9±10.7 ab	41.3±10.8 b	7.4±5.2 a
35 ‰ (n=13)	65.8±4.9 b	57.9±7.8 b	19.7±5.8 a

Table 5.4. Summary of sperm activity at 5, 20 and 35 ‰.

	Time (mins)					
Salinity	**0**	**1**	**2**	**3**	**4**	**5**
5 ‰	N	N	N	N	N	N
20 %	H	H	M	M	M-L	L
35 ‰	H	H-M	M	L	L	L

H=high, M=medium, L=low and N=not activated

Plasma cortisol concentrations were high (ranging from 29.1-96.8 ng ml^{-1}) at the time of injection and showed no change over time in all treatments (data not shown). At all three salinities, treatment with LHRHa significantly increased plasma estradiol levels over controls on days 1-3 post injection (Fig. 5.3). In fish held at 20‰ and injected with saline, plasma E_2 levels were significantly suppressed by day 1 and remained low thereafter, however, in saline-injected fish held at 5 or 35‰, plasma E_2 levels were low at the time of injection and remained unchanged. Plasma T levels in LHRHa injected fish peaked on day 1 at all salinities and were significantly elevated over controls on days 1 and 2, days 1-3, and day 1 in fish held at 5, 20 and 35‰ respectively (Fig. 5.4). In saline injected fish, plasma T levels followed a similar pattern to E_2 except in fish held at 20‰ where plasma T levels were not significantly suppressed until days 4 and 5. Plasma 17,20βP levels were variable and not affected by LHRHa treatment in fish held at 35 or 5‰, however, in fish held at 20‰, plasma 17,20βP levels were elevated over controls on days 1 and 2 (Fig. 5.5).

The effect of salinity of egg development and hatching

The incubation salinity significantly affected both egg survival (day 1) and survival to hatch (day 2) (Fig. 5.6). Egg survival on day 1 was high in eggs incubated in water of 10-35‰, whereas eggs incubated in 5‰ had a significantly lower survival, than at salinities above 10‰ No eggs survived when incubated in distilled water. Eggs hatched on day 2 in salinities from 5-35‰. Survival to hatch was high in eggs incubated from 15-35‰, whereas, survival to hatch at lower salinities (10 and 5‰) was lower. Abnormalities of larvae at hatching were observed at all salinities where eggs hatched, and were characterised by curvature of the spine and tail flexure. All larvae that hatched at 5‰ were abnormal (Fig. 5.7). Normal larvae first appeared at 10‰ with the proportions of normal larvae being highest at salinities from 20-35‰. Eggs incubated in 15‰ resulted in highly variable proportions of normal larvae with larval deformities ranging from 5 to 93%.

* NOTE:

The data presented at 35 ppt were analysed by a single One Way ANOVA, where all values (both LHRHa and saline treatments) were compared across time. Differences between means were subsequently determined by Tukeys multiple comparisons of means test. The data presented at 20 and 5 ppt were analysed in the same manner in separate analysis.

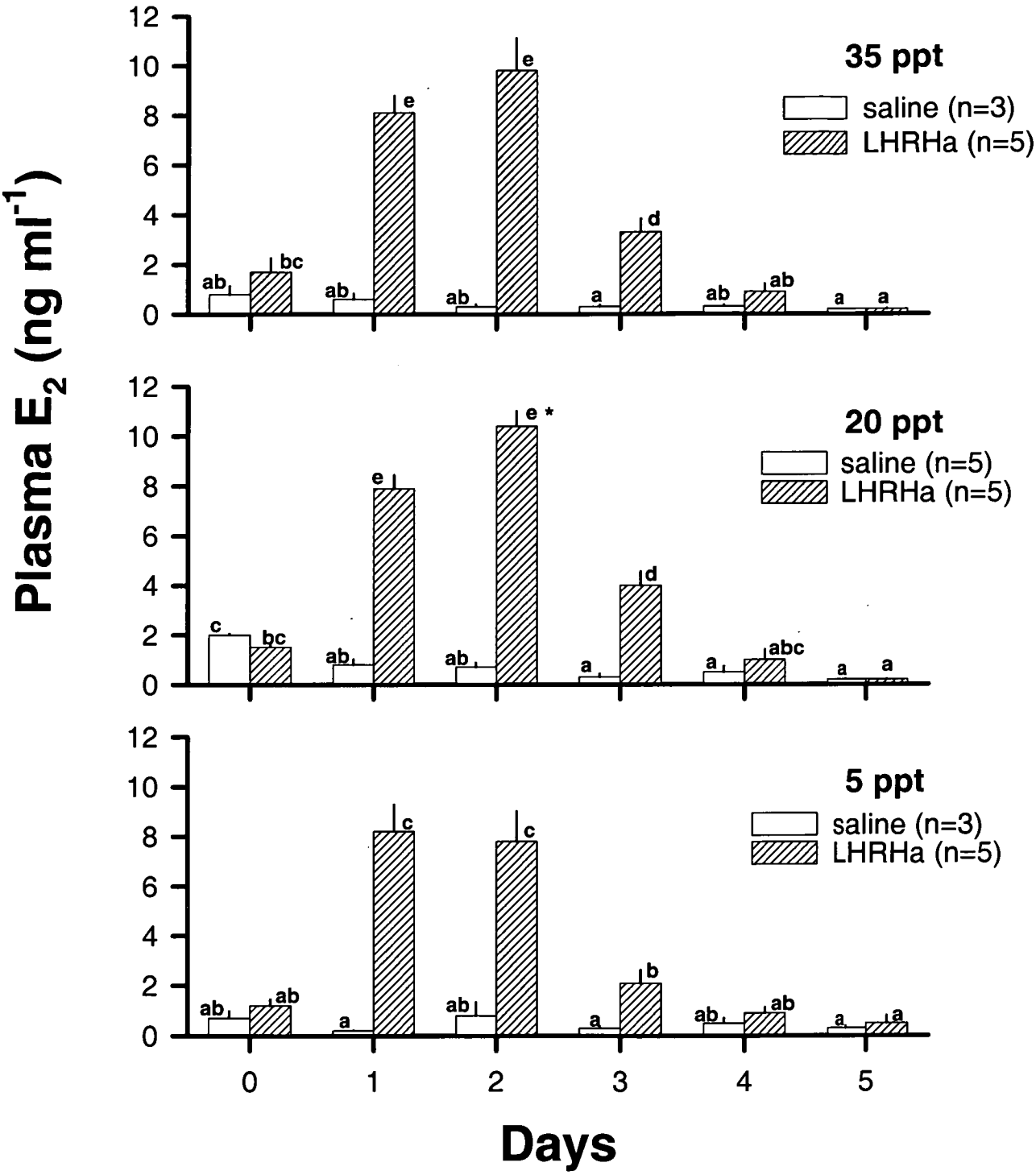


Fig. 5.3. Plasma E_2 concentrations in female black bream maintained at 5, 20 or 35 ‰ salinity and injected with either saline or 50 μg kg^{-1} LHRHa. * indicates mortality, n=4 thereafter. Separate one way ANOVAs were conducted on each test salinity. Other details as for Fig. 5.1.

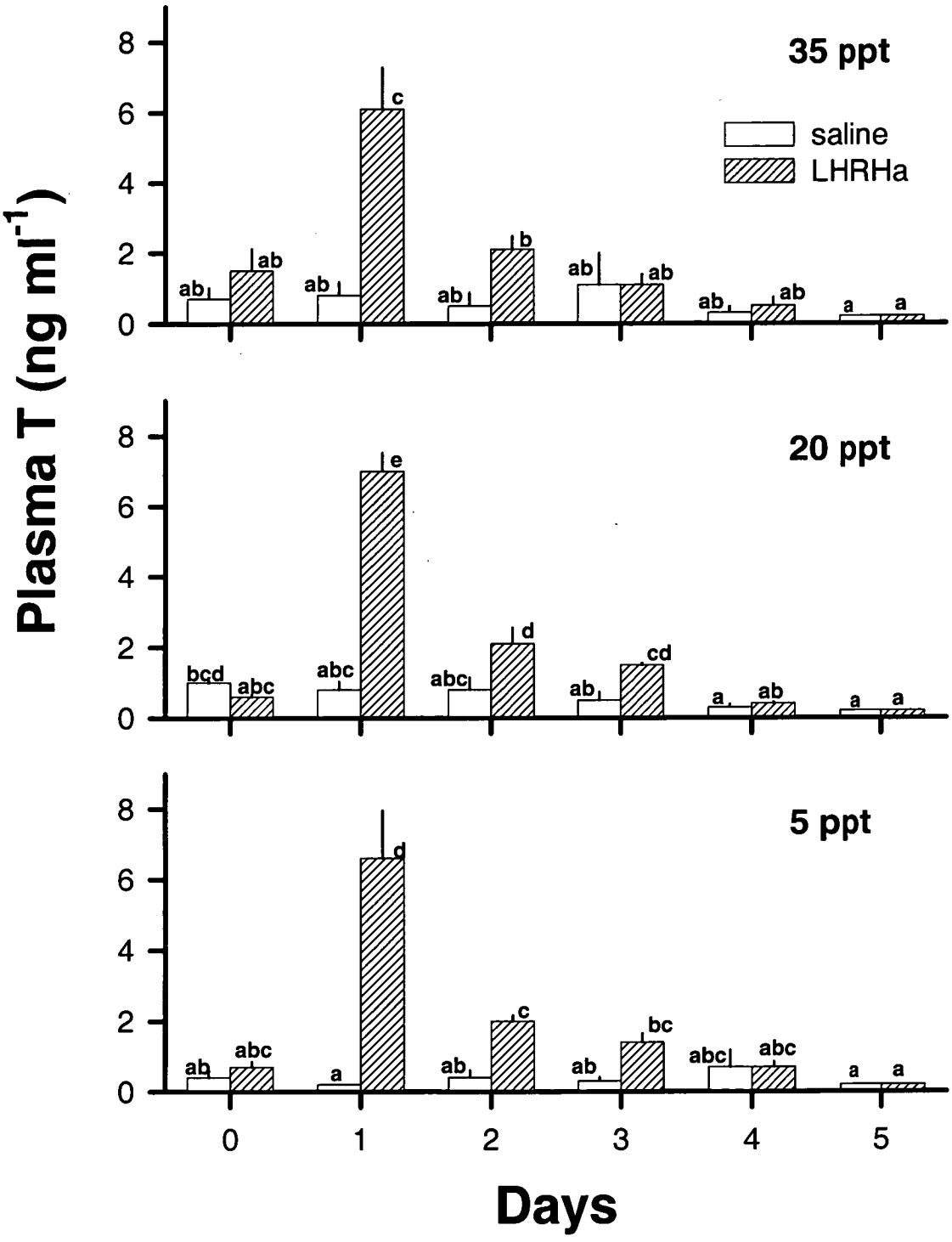


Fig. 5.4. Plasma T concentrations in female black bream maintained at 5, 20 or 35 ‰ salinity and injected with either saline or 50 μg kg^{-1} LHRHa. Other details as for Figs. 5.1 and 5.3.

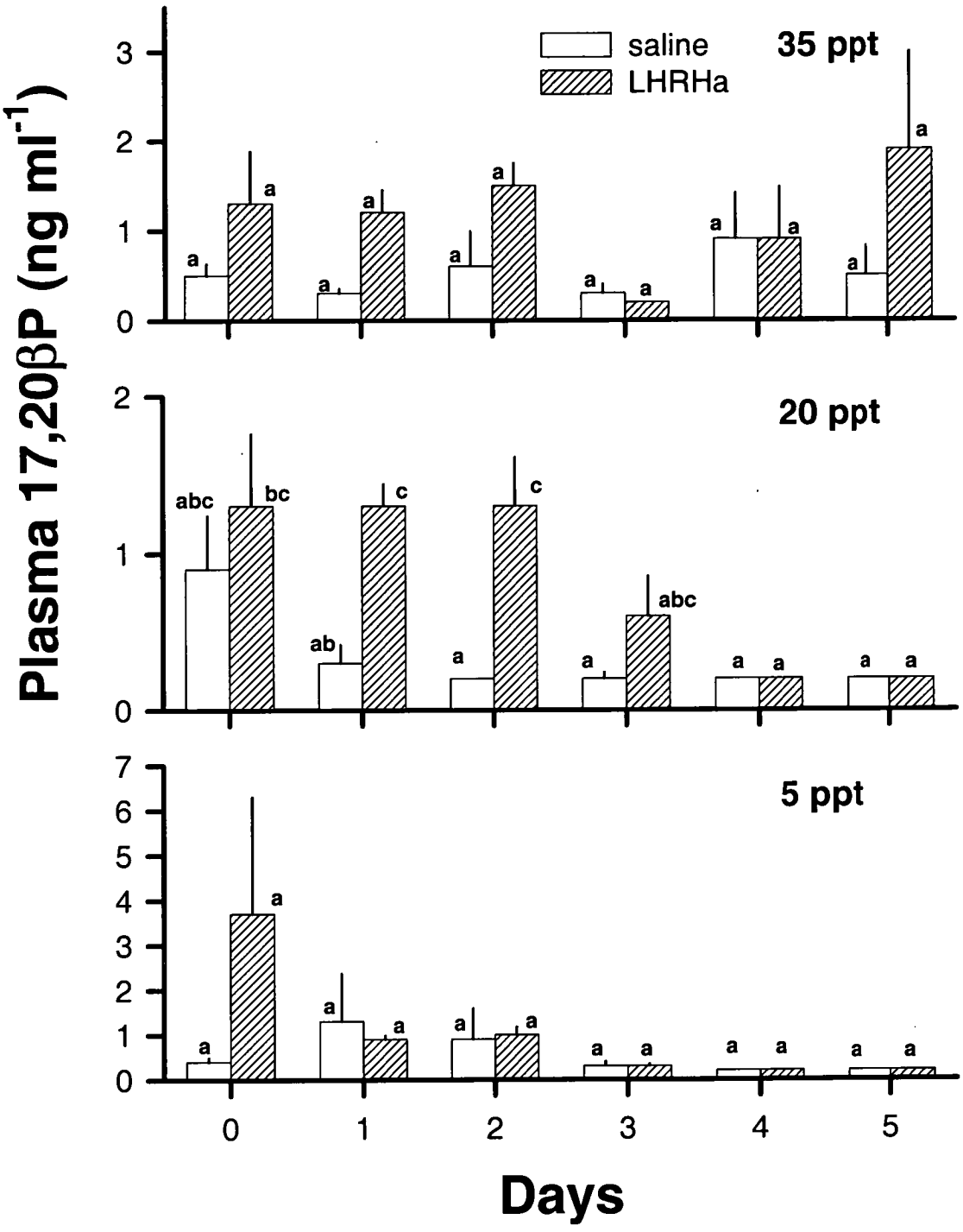


Fig. 5.5. Plasma 17,20βP concentrations in female black bream maintained at 5, 20 or 35 ‰ salinity and injected with either saline or 50 μg kg^{-1} LHRHa. Other details as for Figs. 5.1 and 5.3.

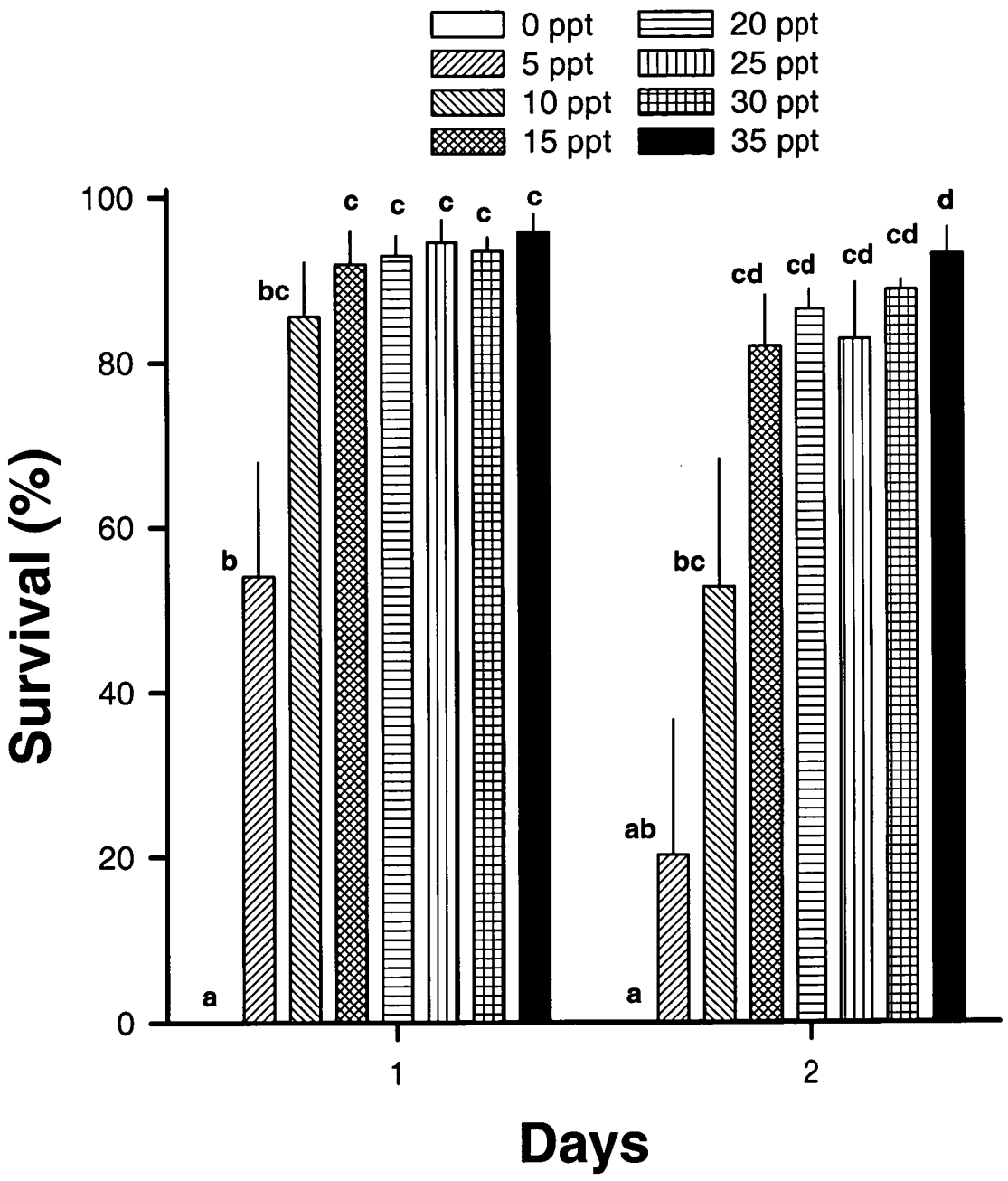


Fig. 5.6. The effect of salinity on black bream egg survival (Day 1) and hatching (Day 2). Values are mean + se (n=4). Separate one way ANOVAs were conducted on days 1 and 2 respectively.

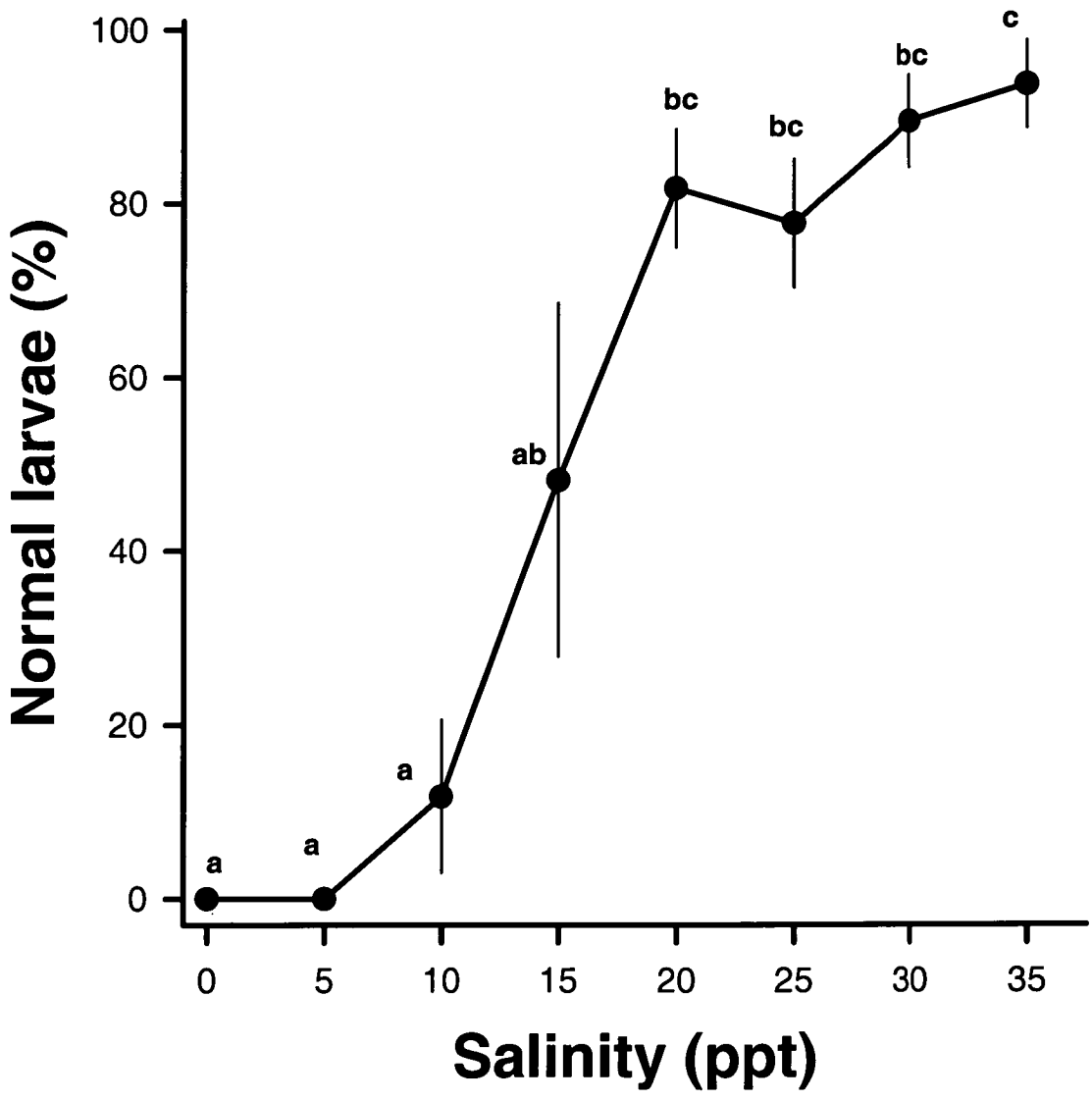


Fig. 5.7. Proportions of normal black bream larvae hatched from eggs incubated at salinities of 0-35‰. Other details as for Fig. 5.6.

5.5 Discussion

Capture stress is known to rapidly inhibit reproductive activity and initiate ovarian atresia in many sparids including black bream (Carragher and Pankhurst 1991; Cleary 1998; Haddy and Pankhurst under review). The success of using wild caught fish as prospective broodstock is, therefore, dependent on the recovery from the stress of capture and captivity before the next cycle of gametogenesis (reviewed in Pankhurst 1998). Cleary (1998) reported that wild snapper *Pagrus auratus* held for 5 years showed little evidence of acclamation to captivity. In contrast, results from the present study show that wild black bream will acclimatise to captivity, and undergo gonadal development within their first year of capture. Cortisol profiles in the present study indicate that captive black bream are still highly sensitive to stress, with handling resulting in the elevation of plasma cortisol and temporary suppression of gonadal steroids in both sexes. However when fish were maintained with minimal disturbance, (no handling from September to mid November) plasma sex steroid levels either recovered or began to increase. This highlights that stress management is a key factor in the success of broodstock maintenance in stress sensitive species such as black bream.

There is relatively little information on the effects of salinity on ovarian recrudescence and reproductive physiology in fish. Vitellogenin synthesis can be induced by estradiol administration in both freshwater and saltwater adapted eels *Anguilla anguilla* (Petersen and Korsgaard 1989). Tamaru et al. (1994) showed that female striped mullet *Mugil cephalus* initiated vitellogenesis at salinities ranging from 0-35‰, but females maturing in freshwater exhibited a slower rate of oocyte growth, with a significantly lower number of females completing vitellogenesis. In contrast, Zanuy and Carrillo (1984) reported that changes in oocyte diameter and the time of gonadal recrudescence were similar in sea bass *Dicentrarchus labrax* reared at 3.5 or 37.8‰. In the present study, salinity did not apparently affect the plasma steroid levels or ovarian development in female black bream. Therefore the initiation and maintenance of vitellogenesis in black bream appears to more dependent on the classical cues of photoperiod and temperature rather than salinity.

Results from the present study demonstrate that black bream are responsive to LHRHa treatment at salinities ranging from 5-35‰. LHRHa treatment in black bream

caused the short term elevation of plasma E_2 and T and resulted in ovulations at all three salinities. In repeat spawning species, hormonal treatment aids the induction of multiple ovulations by the maintenance of development of successive clutches of vitellogenic oocytes (Morehead et al., 1998; Haddy and Pankhurst under review). Therefore, our results are consistent with the established roles of plasma E_2 and T in ovarian recrudescence (Pankhurst 1998), and demonstrate that reproductive endocrine processes remain intact in black bream over a wide range of salinity. In contrast to LHRHa treated fish, saline-injected fish did not ovulate, and E_2 and T levels either remained low or became significantly suppressed. The effects of daily handling on plasma cortisol levels and plasma E_2 and T levels in saline injected fish are consistent with the effects of stress on reproduction in this and other species (Carragher and Pankhurst 1991; Clearwater and Pankhurst 1997; Cleary 1998; Haddy and Pankhurst 1999).

Although 17,20βP levels in black bream show seasonal peaks during the spawning season and have been associated with final oocyte maturation (Haddy and Pankhurst 1998), 17,20βP has not yet been conclusively identified as the maturational inducing steroid. In the present study, plasma levels of 17,20βP were unaffected by LHRHa treatment in fish held in 5 and 35‰, whereas 17,20βP levels in fish held in 20‰ were significantly elevated over controls on days 1 and 2. The physiological relevance of this finding is currently unclear, as the differences in 17,20βP levels were not associated with a marked difference in the proportions of fish ovulating or the volume of eggs produced. In stressed fish, 17,20βP levels are highly variable and are linked to the stress response, with stress-induced increases in 17,20βP levels thought to be of interrenal origin (Haddy and Pankhurst 1999). Therefore, in common with our previous work on induced ovulation in black bream (Haddy and Pankhurst under review), 17,20βP levels in black bream appear to be an ambiguous marker of impending ovulation in hormone-treated fish.

Although there was no marked differences in the endocrine response to treatment with LHRHa treatment at differing salinity in female black bream, the proportions of ovulating fish, the numbers of serial ovulations and the volume of eggs produced were all lower in fish held at 5‰ than in fish held at higher salinities. Zanuy and Carrillo (1984) showed that although sea bass matured in low salinities, spawning did not take

place unless the fish were transferred to sea water. Striped mullet can be induced to spawn over a salinity range of 0-35‰, however, no fertilised eggs were obtained in freshwater (Lee et al., 1992). Results from the present study show that the induction of final oocyte maturation and ovulation can be achieved over a wide salinity range in black bream, however, the efficacy of LHRHa treatment is reduced at low salinities (5‰). The cause of this effect is unknown.

In male black bream, T and 11KT are associated with spermatogenesis, whereas 17,20βP increases with spermiation (Haddy and Pankhurst 1998). In the present study, plasma T levels were unaffected by salinity whereas, plasma levels of 17,20βP and 11KT varied with salinity in September and November respectively. However, these differences had little apparent influence on the state of maturity of fish held at different salinities. This suggests that, male black bream have the capacity to synthesise sufficient steroids for testicular development and milt production over a salinity range of 5-35‰.

The percentage of fertilised black bream eggs was significantly reduced at 5‰, suggesting that the viability of either the egg and/or the sperm is impaired at low salinity. Several studies have demonstrated a reduction in percentage of fertilised eggs at low salinities in euryhaline and stenohaline fish (Holliday 1969; Lee et al., 1992; Hart and Purser 1995). Results from the present study clearly demonstrate that sperm activity is impaired at low salinities, with sperm motility being lost between 6-10‰. Similar findings have also been reported for other teleosts including other *Acanthopagrus* species (Harris 1986; Lee et al., 1992; Thorogood and Blackshaw 1992; Palmer et al., 1994; Litvak and Trippel 1998). Palmer et al. (1994) showed that in pikey bream *Acanthopagrus berda,* sperm motility was most intense at salinities of 25-35‰ and the duration of activity longest at salinities above 15‰. Results from the present study indicate that intense sperm motility is short lived, and drops within 5 minutes. This is consistent with studies on black porgy *Acanthopagrus schlegeli,* where sperm activated with artificial sea water exhausted their energy supply within 5 minutes (Gwo 1995).

In the present study naturally spawned eggs were removed from the spawning salinity of 35‰, and placed into the incubation salinity at the 2-8 cell stage. At this stage of development, the osmoregulatory capacity of the fertilised egg is one of

resistive maintenance, achieved through a tight plasma membrane and limited transmembrane water and ion fluxes (Alderdice 1988). Lee and Menu (1981) demonstrated that in striped mullet, naturally spawned fertilised eggs transferred at the gastrula stage were more tolerant to salinity change than were those transferred at the 2 blastomere stage. In the present study no eggs incubated at 0‰ survived and only 54% of eggs survived to day 1 (neurula stage) when incubated at 5‰, and egg survival was highly variable. By day 2 the percentage of hatched larvae was high (>80%) and unaffected by salinity from 15-35‰. Similarly, fertilised eggs of Australian bass *Macquaria novemaculeata,* cease developing within 2-3 hours post transfer to fresh water and at 5 and 10‰ only a small percentage of larvae hatch, but hatching success increases to above 75% when eggs are incubated at salinities of 15-35‰ (Van Der Wal 1985). It has been suggested that failure to successfully hatch at low salinities results from poorly developed tail musculature and/or larvae finding it difficult to free themselves from the chorion (Holliday 1969; Young and Dueñas 1993). Results in the present study support this suggestion, as the hatching success of larvae dropped at low salinities, and in some cases larvae died in a partly emerged state.

In summary, this study has shown that black bream adapt well to captivity, but remain highly sensitive to stress and must be maintained with minimal disturbances. Under these conditions reproductive development proceeds normally and is unchanged over a salinity range of 5-35‰. However, the ovulatory response to LHRHa is impaired at low salinity, with fish ovulating less frequently and producing smaller volumes of eggs in response to treatment. Fertilisation of eggs was significantly reduced at low salinity, most likely due to a reduction in sperm activity at 5‰. Finally, fertilised black bream eggs developed over a wide range of salinities with viable larvae being produced over a salinity range of 10-35‰, however the proportion of viable larvae was highest at salinities above 20‰. These result suggest that some natural recruitment could occur in saline lakes with salinities above 10‰, and highlight that black bream is a potential candidate for the establishment of an inland mariculture industry.

5.6 Acknowledgments

This study was supported by grants from University of Tasmania Faculty of Science and Engineering, the Co-operative Research Centre for Aquaculture an Australian Research Council Infrastructure grant awarded to NWP, and an Australian Postgraduate Scholarship awarded to JAH. Thanks are extended to S. Shaw for permission to fish on private property, to M. Attard, P. Cassidy, A. Hobby, A. Shepherd and J. Welsford for assistance with fish capture and sampling and to P. Hilder, and J. Sadler for assistance with induction experiments and finally to C. Carter for the formulation and production of pelleted feed.

5.7 References

Alderdice, D.F., 1988. Osmotic and ionic regulation in teleost eggs and larvae. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology vol. 11A. Academic Press, London. pp. 163-251.

Blackwell, J., 1999. Using serial biological concentration to combine irrigation and saline aquaculture in Australia. In: Smith, B., Barlow, C. (Eds.), Inland Saline Aquaculture. Australian Centre for International Agricultural Research (ACIAR) Proceedings No. 83, Canberra. pp. 26-29.

Carragher, J.F., Pankhurst, N.W., 1991. Stress in a commercially important marine fish, *Pagrus auratus* (Sparidae). In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolfe, M.S. (Eds.), Reproductive Physiology of Fish 1991. FishSymp 91, Sheffield, pp. 253-255.

Clayton, B., 1998. Beating the salt problem at Shepparton. Civil Engineers Australia, July, pp. 50-53.

Cleary, J.J., 1998. The effects of stress on reproduction in snapper (*Pagrus auratus*) Unpublished PhD Thesis, University of Tasmania, Launceston, Tasmania. 162p.

Fielder, D.S., Bardsley, W.J., Allan, G.L., 1999. Culture of snapper *Pagrus auratus* in saline groundwater from western NSW, Australia. In: The Annual International Conference and Exposition of the World Aquaculture Society, Book of Abstracts, World Aquaculture 99. p. 78.

Gwo, J.-C., 1995. Ultrastructural study of osmolality effect on sperm of three marine teleosts. In: Goetz, F.W., Thomas, P. (Eds.), Reproductive Physiology of Fish 1995. Fish Symposium 95, Austin. p. 119.

Haddy, J.A., Pankhurst, N.W., 1998. Annual change in reproductive condition and plasma concentrations of sex steroids in black bream, *Acanthopagrus butcheri* (Munro) (Sparidae). Mar. Freshwat. Res. 49, 389-397.

Haddy, J.A., Pankhurst, N.W., 1999. Stress-induced changes in concentrations of plasma sex steroids in black bream. J. Fish Biol. 55, 1304-1316.

Haddy, J.A., Pankhurst, N.W., Under review. The efficacy of exogenous hormones in stimulating changes in plasma steroids and ovulation in wild black bream *Acanthopagrus butcheri* is improved by treatment at capture. Aquaculture. Accepted subject to suitable revision. Currently under re-review.

Harris, J.H., 1986. Reproduction of the Australian bass *Macquaria novemaculeata* (Perciformes:Percichthyidae) in the Sydney basin. Aust. J. Mar. Freshwat. Res. 37, 209-235.

Hart, P.R., Purser, G.J., 1995. Effects of salinity and temperature on eggs and yolk sac larvae of the greenback flounder (*Rhombosolea tapirina* Günther, 1862). Aquaculture 136, 221-230.

Holliday, F.G.T., 1969. The effects of salinity on the eggs and larvae of teleosts. In: Hoar, W.S., Randall, D.J., (Eds.), Fish Physiology, vol. 1. Academic Press, London. pp. 293-311.

Ingram, B., Gooley, G., McKinnon, L., 1996. Potential for inland mariculture in Victorian saline groundwater evaporation basins. Austasia Aquaculture 10, 61-63.

Lee, C.-S., Menu, B., 1981. Effects of salinity on egg development and hatching in grey mullet *Mugil cephalus* L. J. Fish Biol. 19, 179-188.

Lee, C.-S., Tamaru, C.S., Kelley, C.D., Moriwake, A., Miyamoto, G.T., 1992. The effect of salinity on the induction of spawning and fertilisation in the striped mullet, *Mugil cephalus*. Aquaculture 102, 289-296.

Litvak, M.K., Trippel, E.A., 1998. Sperm motility patterns of Atlantic cod (*Gadus morhua*) in relation to salinity: effects of ovarian fluid and egg presence. Can. J. Fish. Aquat. Sci. 55, 1871-1877.

Morehead, D.T., Pankhurst, N.W., Ritar, A.J., 1998. Effect of treatment with LHRH analogue on oocyte maturation, plasma sex steroid levels and egg production in female striped trumpeter *Latris lineata* (Latrididae). Aquaculture 169, 315-331.

Palmer, P.J., Hogan, A.E., Barlow, C.G., 1994. Chilled storage of pikey bream (*Acanthopagrus berda*) sperm and activation in different salinities. Asian Fish. Sci. 7, 35-40.

Pankhurst, N.W., 1998. Reproduction. In: Black, K.D., Pickering, A.D. (Eds.), Biology of Farmed Fish. Sheffield Academic Press, Sheffield. pp. 1-26.

Pankhurst, N.W., 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., Kime, D.E., 1991. Plasma sex steroid concentrations in male blue cod, *Parapercis colias* (Bloch and Schneider) (Pinguipedidae) sampled underwater during the spawning season. Aust. J. Mar. Freshwat. Res. 42, 129-137.

Petersen, I., Korsgaard, B., 1989. Experimental induction of vitellogenin synthesis in eel (*Anguilla anguilla*) adapted to sea-water or freshwater. Comp. Biochem. Physiol. 93B, 57-60.

Sherwood, J.E., Backhouse, G.N., 1982. Hydrodynamics of salt wedge estuaries - implications for successful spawning in black bream (*Acanthopagrus butcheri*). Warrnambool Institute of Advanced Education, Faculty of Applied Science and Technology. Research Report 82/3, 1-18.

Tamaru, C.S., Lee, C.-S., Kelley, C.D., Miyamoto, G., Moriwake, A., 1994. Oocyte growth in the striped mullet *Mugil cephalus* L. maturing at different salinities. J. World Aquaculture Soc. 25, 109-115

Thorogood, J., Blackshaw, A., 1992. Factors affecting the activation, motility and cryopreservation of the spermatozoa of the yellowfin bream, *Acanthopagrus australis* (Günther). Aquacult. Fish. Man. 23, 337-344.

Van Der Wal, E.J., 1985. Effects of temperature and salinity on the hatch rate and survival of Australian bass (*Macquaria novemaculeata*) eggs and yolk-sac larvae. Aquaculture 47, 239-244.

Young, P.S., Dueñas, C.E., 1993. Salinity tolerance of fertilised eggs and yolk-sac larvae of the rabbitfish *Siganus guttatus* (Bloch). Aquaculture 112, 363-377.

Zanuy, S., Carrillo, M., 1984. Delayed spawning of sea bass after rearing at low salinity. In: Barnabe, G., Billard, R. (Eds.), The Aquaculture of Sea Bass and Sparids. INRA, Paris. pp. 73-80.

CHAPTER 6

General discussion.

6. General discussion

6.1 Background

At the onset of this study (1996), considerable interest was being generated by the aquaculture potential of black bream for stocking inland saline waters (reviewed in chapter 1, this volume). However very little information was available on the reproductive biology of black bream, or the effect of salinity and common aquacultural practices on their reproductive activity. This study addressed this by investigating various aspects of the reproductive endocrinology of black bream.

6.2 Reproduction in wild black bream

Black bream have an annual reproductive cycle with a 3 month spawning season in spring / early summer with spawning occurring in the mid to upper reaches of the estuary. The period from the onset of vitellogenesis to the initiation of spawning activity was short (< 1 month), and occurred when both photoperiod and water temperature were increasing. Gonad staging data and oocyte distributions indicated that gonadal development followed a multiple group synchronous model. Spawning occurred in the evening and was associated with daily cycles of gonadal maturation. This pattern is consistent with the reproductive development of many temperate marine fish, including other sparids (Pankhurst and Conroy 1987; Chang and Yueh 1990; Dedual and Pankhurst 1992; Scott and Pankhurst 1992).

Gonadal development in black bream was associated with both seasonal and daily variations in plasma steroid levels. Elevated levels of plasma estradiol-17β (E_2; females only), testosterone (T) and 11-ketotestosterone (11KT; males only) were associated with the initiation and maintenance of vitellogenesis and spermatogenesis. Elevated plasma 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) levels were associated with final oocyte maturation (FOM) and spermiation in female and male fish respectively. These findings are consistent with the established roles of sex steroids in reproductive function in teleosts (reviewed by Pankhurst and Carragher 1991; Pankhurst 1998a)

6.3 Stress

The extent to which stress impacts on physiological function in teleosts varies with both the severity and duration of the stressor, and between species (reviewed by Barton and Iwama 1991). The effects of stress can have huge implications to the success of an aquaculture program, and it is now universally accepted that stress exerts an inhibitory effect on reproductive processes in fish (reviewed by Pankhurst and Van Der Kraak 1997). Reproductive shut down is a common problem when attempting to collect eggs from wild fish, and in acclimatising fish to captivity for use as potential broodstock (Cleary 1998; Pankhurst and Van Der Kraak 1997). The suppressive effects of stress are typically reflected in depressed plasma levels of circulating androgens and estrogens, and in females, the onset of ovarian atresia (Carragher and Pankhurst 1991, Clearwater and Pankhurst 1997, Cleary 1998; Morehead 1998). However, the mechanisms by which stress inhibits reproductive function in fish is currently unclear.

Black bream were shown to be highly stress sensitive, with capture and confinement resulting in the rapid increase in plasma cortisol levels, and depression of circulating levels of plasma E_2, T and 11KT. The speed in which plasma steroids were depressed suggests that stress directly affects gonadal steroidogenesis via impairment of the activity of one or more of the enzymes in the steroid cleavage pathway. Cleary (1998) showed that in snapper *Pagrus auratus*, 17α-hydroxylase and/or 17,20-lyase activity (measured indirectly by *in vitro* conversion of 17α-hydroxyprogesterone to T by ovarian follicles) is impaired by capture and confinement stress. In addition, recent *in vivo* studies on rainbow trout *Oncorhynchus mykiss,* suggest that the inhibitory effects of stress are mediated by cortisol and that the effect does not involve the inhibition of GtH secretion (Pankhurst and Van Der Kraak 2000). However, the failure of cortisol to exert a direct effect on ovarian steroidogenesis *in vitro,* suggests that cortisol acts in concert with another hitherto factor that is not present (or consistently present) in the *in vitro* system (Pankhurst et al., 1995; Pankhurst 1998b; Pankhurst and Van Der Kraak 2000).

In contrast to the affects of stress on plasma levels of E_2, T and 11KT, plasma levels of 17,20βP increased during short confinement times. The possibility that these increases were of extragonadal origin was further supported by the fact that sexually

regressed females also showed significant peaks in plasma levels of 17,20βP after 1 hour of confinement. Studies on salmonids suggest that the interennal tissue may be responsible for stress induced changes in 17,20βP levels (Sangalang and Freeman 1988; Barry et al., 1997). This possibility remains to be explored in black bream.

Further understanding of stress and how it affects reproduction is essential in designing management protocols that minimise reproductive impairment due to husbandry practices. The rapid onset of the inhibitory effects of stress on plasma steroid levels highlights that at least part of the effect is generated by something other than a classical steroid effect on gene activation. The implications for broodstock management are that normal maintenance should be timed to avoid chronic stress, and that the duration of husbandry procedures be kept short and to a minimum when keeping stress sensitive species. The collection of wild fish is always going to be stressful irrespective of good stress management practices. Therefore, industry reliance on wild fish as a source of gametes is unlikely to be a viable option for the longer term sustainable development of aquaculture.

6.4 The use of exogenous hormones

Failure of fish to undergo FOM, either after capture or in captivity, has initiated the development of techniques to artificially induce FOM and ovulation (reviewed by Lam 1982; Zohar 1988; Donaldson and Devlin 1996; Peter and Yu 1997; Pankhurst 1998). The most common endocrine tool used to induce FOM is treatment with exogenous hormones such as gonadotropin preparations (GtH) and gonadotropin releasing hormones (GnRH). The use of exogenous hormones provides a degree of control over reproductive events which allows more efficient use of hatchery and grow out facilities. Human chorionic gonadotropin (hCG) and luteinizing hormone releasing hormone analogue (LHRHa), both induced serial ovulations in black bream which were associated with short term elevations or conservation of plasma E_2 and T levels. This suggests that multiple ovulations in black bream are supported by serial recruitment of follicles from various stages of vitellogenesis. Although 17,20βP is the most likely MIS in black bream, stress induced increases due to daily handling highlight that 17,20βP levels are an ambiguous marker of impending ovulation in stressed fish.

In some freshwater species, GtH release is regulated by a gonadotropin release inhibiting factor, such as dopamine (DA), and treatment with GnRH analogues is unsuccessful unless a DA antagonist is included in the treatment (reviewed by Trudeau and Peter 1995; Peter and Yu 1997). The fact that LHRHa treatment successfully induced elevations in plasma levels of E_2 and T, which was subsequently followed by multiple ovulations, suggest that DA inhibition of GtH release is less important in black bream. Induced ovulation procedures for black bream appear not to require the use of DA antagonists. A similar lack of DA inhibition of GtH release has been demonstrated in several marine fish, which suggests that in general marine fish do not require DA antagonists in addition to GnRHa to induce secretion of GtH (Copeland and Thomas 1989; Zohar 1989; Zohar et al., 1995).

Administration of exogenous hormones by injection results in a transitory rise and fall in plasma sex steroid levels, which in black bream, was subsequently followed by a drop in the ovulatory response 4-5 days post injection. Once the supply of exogenous hormones is cleared from the circulation, the inhibitory effects of stress appear to come back into effect. The use of sustained release delivery methods for GnRHa, maintain elevated GtH levels over longer periods than acute administration protocols (Breton et al., 1990). The implication here is that in serial spawning species, the ovulatory response could be maintained for longer periods without increased handling. The use of sustained release implants of GnRHa in black bream remains to be investigated.

Poor ovulatory responses of wild fish to hormone treatments is a common problem in aquaculture (Foscarni 1988; Battaglene and Talbot 1992; Carrillo et al., 1995; Zohar et al., 1995). The use of wild fish as prospective broodstock usually involves the capture and transportation of fish prior to being treated with exogenous hormones. This allows the inhibitory action of stress on endocrine function to already be in effect before treatment. Pre-treatment stress reduces ovulation volumes in response to exogenous hormones in snapper and Northern Pike *Esox lucius* (De Montalembert et al., 1978; Cleary 1998). Similarly, delayed treatment with LHRHa in black bream results in a poorer ovulatory and dampened endocrine response. Therefore, in stress sensitive species, wild fish should be treated with exogenous hormones as soon as possible after capture for optimal responses. The mechanisms by

which stress reduces the efficacy of LHRHa treatment is currently unknown. Differential abilities of stressed and unstressed fish to respond to hormone treatments, and the speed in which stress can exert an inhibitory effect on reproductive processes, highlights that broodstock capture methods must also be rapid. Capture methods such as long lining, fish traps and netting can cause considerable damage to fish and / or result in fish already being stressed at the time of landing (Pankhurst and Sharples 1992; Battaglene 1995). Rapid capture techniques such as rod and line or handlines appear to be the most suitable methods for broodstock capture.

Induced ovulation protocols can be stressful to broodstock, and do not guarantee spontaneous spawning. Under these conditions broodstock must be manually stripped of ovulated eggs, which results in additional handling stress. Post ovulatory egg viability decreases with time in all species studied (Hobby and Pankhurst 1998), and if the appropriate time of stripping is unknown, fish must be checked for ovulation on a regular basis. The variable and generally poor egg fertility achieved in the induced ovulation experiments in the present study is most likely due to inappropriate stripping times. The route to achieve high quality fertilised eggs from black bream, appears to be through the establishment of natural spawning in captive fish. This has been achieved in a number of fish species through the domestication of broodstock, and manipulation of environmental parameters such as photoperiod, temperature and tank size (Foscarni 1988; Carrillio et al., 1995; Thomas et al., 1995; Zohar et al., 1995).

6.5 Black bream in captivity

Failure to initiate and complete vitellogenesis in captive fish is a common bottleneck in egg production, and is most likely due to culture-induced stress and / or inappropriate environmental cues (Pankhurst 1998a). Cleary (1998) found that wild snapper failed to complete vitellogenesis despite, 5 years of captivity in which to acclimatise. In contrast, black bream are highly adaptable to captivity, and when maintained with minimal disturbances vitellogenesis proceeds normally. The fact that black bream adapted to captivity and naturally spawned within 1 year, makes this species an ideal brood fish, and highlights that stress management is a key component of fish husbandry in stress sensitive species. Jenkins et al. (1999) also supports this

method, and showed that when black bream are provided with good quality water and minimal disturbances, natural spawning can be achieved within the first or second season of captivity.

The natural spawning observed in the present study occurred after a brief disturbance when a few fish were netted from the tank. It is assumed that this response was due to the mature fish failing to undergo FOM due to inappropriate holding conditions, and the subsequent handling disturbance resulting in stress induced increases in 17,20βP levels, which initiated FOM, ovulation and behavioural spawning. In-tank (1000L) spawning then occurred in the evenings for a period of 7 days. After 7 days of non-spawning activity the fish were again disturbed, which induced a subsequent week of behavioural spawning. The fact that spawning activity in the present study was short, suggests that the environmental conditions in the tanks were not adequate for maintenance of natural spawning. The development of culture conditions which allow successful spawning to occur is one of the most demanding aspects of broodstock management. In pelagic spawners, spawning behaviour seems to require a critical water volume or depth. This appears to be the case in sparids where spawning involves chasing and a rush to the surface where gamete release occurs (Smith 1986; Jenkins et al., 1999; personal observation). Holding volumes are critical for natural spawning of snapper, gilthead sea bream, *Sparus auratus* and sea bass *Dicentrarchus labrax* (Carrillo et al., 1995; Zohar et al., 1995; Cleary 1998). Minimum tank volumes which, enable natural spawning will vary between species because of differences in courtship and spawning behaviour. Jenkins et al. (1999) indicated that black bream broodstock should be held in a tank with a capacity of 10000 L or greater, and when held in these tanks with minimal disturbances fish spawn naturally over a 3-4 month period without hormonal intervention.

6.6 Reproduction and salinity in black bream

Completion of vitellogenesis depends on the normal functioning of the vitellogenic machinery, including the synthesis of GnRH, GtH, GnRH and GtH receptors, ovarian steroidogenesis, hepatic synthesis of vitellogenin and the sequestration of vitellogenin into developing oocytes (reviewed by Mommsen and Walsh 1988; Tyler 1991; Specker and Sullivan 1994). In the present study, the

endocrine processes involved in vitellogenesis and the hormonal induction of FOM and ovulation of black bream remained intact over a salinity range of 5-35‰. In the wild, black bream have been found in spawning condition in salinities ranging from as low as 3.5-8‰ in the Moore River estuary to as high as 41-45‰ in the Wellstead estuary (Sarre and Potter 1999). Therefore, the reproductive processes appear to be very robust in the face of considerable variation in salinity. This very wide range in salinity in which reproductive activity proceeds normally is advantageous in attempts to establish self-supporting populations of black bream in the inland saline waters of Australia.

While it is evident that reproductive activity of adult black bream is maintained over a wide range of salinities, the effects of salinity on larval survival is less clear. Salinity is known to affect sperm activity, fertilisation, egg development and hatching success in a variety of species (Holliday 1969; Van Der Wal 1985; Harris 1986; Lee et al., 1992; Thorogood and Blackshaw 1992; Palmer et al., 1994). In black bream, fertilisation is impaired at low salinity due to sperm motility being lost over a salinity range of 6-10 ‰. Egg development and hatching is also impaired at low salinity in black bream, with the percentage of normal larvae and hatching success decreasing at salinities at or below 15‰. The implication of these findings is that recruitment is likely to be poor at salinities below 15 ‰ in inland saline waters. For the hatchery rearing of black bream water salinities above 20 ‰ may be suitable, however, it would be desirable to use water with a salinity greater than 30 ‰ as this would allow the separation of good quality eggs from poor quality eggs (that usually sink) and wastes from the bottom of the tank. The upper salinity limit for black bream egg development and hatching remains to be determined, and further work is required to determine the salinity tolerances of black bream larvae. The effects of an impaired reproductive capacity of adult fish, and low or no larval survival during periods of low salinity, could explain the fact that in some estuaries black bream populations display an episodic pattern of recruitment (Morison et al., 1998).

6.7 Future outlook for black bream

The first commercial production of black bream occurred in western Australia in 1997 (Jenkins et al., 1999). Development of hatchery techniques for black bream

culture has been extremely rapid due to the pre-existing technology for closely related species (Foscarni 1988; Battaglene 1995), and the fact that black bream readily adapt to captivity with natural spawning being achieved within 1-2 years after capture. The main concern with the viability of commercial culture is growth rate. In the present study 3 year old fish had mean fork lengths of 23.7 and 20.5 cm and mean total weights of 308 and 195 g for the Meredith and Swan river estuaries respectively (unpublished data). The growth rate in wild fish is dependent on factors such as temperature and feed availability, and less-than-optimum conditions are likely to be experienced for at least part of the year in the natural environment. However, research into the growth of black bream in saline dams is indicating that growth rates can exceed those found in wild populations when fish are provided with additional feed (Gavin Sarre pers com 2000). The development of low cost diets which replace fish meals with plant proteins while maintaining good growth should be a research priority, especially as black bream are known to consume large amounts of algae in the wild (Sarre et al., 2000). Grow-out trials are currently being conducted in aquaria and saline farm dams in Western Australia. Preliminary data indicate that growth is unaffected at salinities between 12-48 ‰ (Jenkins et al., 1999). The remarkable euryhaline nature of black bream and ease of production make it an ideal species for inland saline aquaculture.

6.8 Overall Summary

1. Reproductive development and activity in wild black bream was characterised by seasonal and daily changes in gonadal condition and plasma sex steroid levels.

- Black bream demonstrated multiple group synchronous gonad development, with spawning occurring in the evening on a daily basis in spring / early summer.

- Elevated plasma levels of E_2 and T were associated with vitellogenesis.

- Elevated plasma levels of T and 11KT were associated with spermatogenesis.

- Elevated plasma levels of 17,20βP levels were associated with FOM and spermiation in male and female fish respectively.

- Fish that were undergoing FOM or were ovulated were caught over a sub surface (≥ 1m) salinity range of 13.9-35.0 ‰, a temperature range of 15.5-26.2°C, and DO range of 4.2-13.6 mg L^{-1}.

2. Capture and confinement of wild fish significantly reduces concentrations of plasma sex steroids.

- Capture and confinement elevated plasma cortisol levels.

- Capture and confinement reduced plasma levels of E_2 and T within 1 h in females, and suppressed plasma levels of T and 11KT after 30 min and 6 h respectively in males.

- Plasma levels of 17,20βP increased, decreased or remained unchanged in response to capture and confinement.

3. Exogenous hormone treatment successfully induced serial ovulation which was accompanied by changes in plasma sex steroid levels, however, treatment delay reduced the responsiveness of fish to exogenous hormones.

- Treatment with LHRHa or hCG resulted in multiple ovulations.

- Capture and handling stress reduced the responsiveness of fish to exogenous hormone treatment and better results were obtained if hormonal treatment is administered at the time of capture.

- Injection with hCG or LHRHa at capture resulted in the short term elevation of plasma E_2 and T, whereas, injection of LHRHa 24 hrs post-capture did not elevate plasma E_2 or T levels over controls.

- Plasma levels of cortisol and 17,20βP were unaffected by hormone treatment.

4. Black bream have the capacity to successfully reproduce over a wide range of salinity, however, egg production, sperm motility and egg development, and hatching success were reduced at low salinity.

- Gonadal maturation and seasonal plasma steroid levels were essentially unaffected by salinity (5-35 ‰) in both sexes.

- Hormone therapy resulted in the typical endocrine and ovulatory response in 5, 20 and 35 ‰ salinity, however, egg production was reduced in fish held at 5 ‰.

- Both fertilisation and sperm motility were significantly reduced at 5‰.

- Egg development and hatching success were best at salinities above 20 ‰.

6.9 References

Barry, T.P., Riebe, J.D., Parrish, J.J., Malison, J.A., 1997. Effects of 17α,20β-dihydroxy-4-pregnen-3-one on cortisol production by rainbow trout interrenal tissue *in vitro*. Gen. Comp. Endocrinol. 107, 172-181.

Barton, B.A., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann. Rev. Fish Dis. 1, 3-26.

Battaglene, S.C., Talbot, R.B., 1992. Induced spawning and larval rearing of snapper, *Pagrus auratus* (Pisces: Sparidae), from Australian waters. NZ J. Mar. Freshwat. Res. 26, 179-183.

Breton, B., Weil, C., Sambroni, E., Zohar, Y., 1990. Effects of acute versus sustained administration of GnRHa on GtH release and ovulation in the rainbow trout, *Oncorhynchus mykiss*. Aquaculture 91, 373-383.

Carragher, J.F., Pankhurst, N.W., 1991. Stress and reproduction in a commercially important fish, *Pagrus auratus* (Sparidae). In: Scott, A.P., Sumpter, J.P., Kime,

D.E., Rolfe, M.S., (Eds.), Reproductive Physiology of Fish 1991, FishSymp 91, Sheffield. pp. 253-255.

Carrillo, M., Zanuy, S., Prat, F., Cerda, J., Ramos, J., Mananos, E., Bromage, N., 1995. Sea bass (*Dicentrarchus labrax*). In: Bromage, N., Roberts, R.J., (Eds.), Broodstock management and egg and larval quality, Blackwell Science, Oxford. pp. 138-168.

Chang, C.-F., Yueh, W.-S., 1990. Annual cycle of gonadal histology and steroid profiles in the juvenile males and adult females of the protandrous black porgy, *Acanthopagrus schlegeli.* Aquaculture 91, 179-196.

Clearwater, S.J., Pankhurst, N.W., 1997. The response to capture and confinement stress of plasma cortisol, plasma sex steroids and vitellogenic oocytes in the marine teleost, red gurnard. J. Fish Biol. 50, 429-441.

Cleary, J.J., 1998. The effects of stress on reproduction in snapper (*Pagrus auratus*) Unpublished PhD Thesis, University of Tasmania. 162p.

Copeland, P.A., Thomas, T., 1989. Control of gonadotropin release in the Atlantic croaker (*Micropogonias undulatus*): Evidence for a lack of dopaminergic inhibition, Gen. Comp. Endocrinol. 74, 474-483.

Dedual, M., Pankhurst, N.W., 1992. Plasma steroid hormone concentrations in relation to the reproductive cycle of the sweep *Scorpis lineolatus* (Kyphosidae) caught from the wild. Aust. J. Mar. Freshwat. Res. 43, 753-763.

De Montalembert, G., Jalabert, B., 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., Devlin, R.H., 1996. Uses of biotechnology to enhance production. In: Pennell, W., Barton, B.A. (Eds.), Developments in Aquaculture and Fisheries Science, Volume 29; Principles of Salmonid Culture. Elsevier Science B.V. Amsterdam. pp. 969-1020.

Foscarini, R., 1988. A review: Intensive farming procedure for red sea bream (*Pagrus major*) in Japan. Aquaculture 72, 191-246.

Harris, J.H., 1986. Reproduction of the Australian bass *Macquaria novemaculeata* (Perciformes: Percichthyidae) in the Sydney basin. Aust. J. Mar. Freshwat. Res. 37, 209-235.

Hobby, A.C., Pankhurst, N.W., 1997. Post-ovulatory egg viability in the snapper *Pagrus auratus* (Sparidae). Mar. Freshwat. Res. 48, 385-389.

Holliday, F.G.T., 1969. The effects of salinity on the eggs and larvae of teleosts. In: Hoar, W.S., Randall, D.J., (Eds.), Fish Physiology, vol. 1. Academic Press, London. pp. 293-311.

Jenkins, G.I., Frankish, K.R., Partridge, G.J., 1999. Manual for the hatchery production of black bream (*Acanthopagrus butcheri*). Aquaculture Development Unit, Fremantle Maritime Centre South Metropolitan College of TAFE, Fremantle, Western Australia. 125p.

Lam, T.J., 1982. Applications of endocrinology to fish culture. Can. J. Fish. Aquat. Sci. 39, 111-137.

Lee, C.-S., Tamaru, C.S., Kelley, C.D., Moriwake, A., Miyamoto, G.T., 1992. The effect of salinity on the induction of spawning and fertilisation in the striped mullet, *Mugil cephalus*. Aquaculture 102, 289-296.

Morehead, D.T., 1998. Effect of capture, confinement and repeated sampling on plasma steroid concentrations and oocyte size in female striped trumpeter *Latris lineata* (Latrididae). Mar. Freshwat. Res. 49, 373-377.

Mommsen, T.P., Walsh, P.J., 1988. Vitellogenesis and oocyte assembly. In: Hoar, W.S., Randall, (Eds.), Fish Physiology, Vol. 11, Part A. Academic Press, London. pp. 347-406.

Morison, A.K., Coutin, P.C., Robertson, S.G., 1998. Age determination of black bream, *Acanthopagrus butcheri* (Sparidae), from the Gippsland lakes of south-eastern Australia indicates slow growth and episodic recruitment. Mar. Freshwat. Res. 49, 491-498.

Palmer, P.J., Hogan, A.E., Barlow, C.G., 1994. Chilled storage of pikey bream (*Acanthopagrus berda*) sperm and activation in different salinities. Asian Fish. Sci. 7, 35-40.

Pankhurst, N.W., 1998a . Reproduction. In: Black, K.D., Pickering, A.D. (Eds.), Biology of Farmed Fish. Sheffield Academic Press, Sheffield. pp. 1-26.

Pankhurst, N.W., 1998b. Further evidence of the equivocal effects of cortisol on in vitro steroidogenesis by ovarian follicles of rainbow trout *Oncorhynchus mykiss*. Fish Physiol. Biochem. 19, 315-323.

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

Pankhurst, N.W., Conroy, A.M., 1987. Seasonal changes in reproductive condition and plasma levels of sex steroids in the blue cod, *Parapercis colias* (Bloch and Schneider) (Mugiloididae). Fish Physiol. Biochem. 4, 15-26.

Pankhurst, N.W., Sharples, D.F., 1992. Effects of capture and confinement on plasma cortisol concentrations in snapper *Pagrus auratus*. Aust. J. Mar. Freshwat. Res. 43, 345-356.

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

Pankhurst, N.W., Van Der Kraak, G., 2000. Evidence that acute stress inhibits ovarian steroidogenesis in rainbow trout in vivo, through the action of cortisol. Gen. Comp. Endocrinol. 117, 225-237.

Pankhurst, N.W., Van Der Kraak, G., Peter, R.E., 1995. Evidence that inhibitory effects of stress on reproduction in teleost fish are not mediated by the action of cortisol on ovarian steroidogenesis. Gen. Comp. Endocrinol. 99, 249-257.

Peter, R.E., Yu, K.L., 1997. Neuroendocrine regulation of ovulation in fishes: basic and applied aspects. Rev. Fish Biol. Fish. 7, 173-197.

Sangalang, G.B., Freeman, H.C., 1988. *In vitro* biosynthesis of 17α,20β-dihydroxy-4-pregnen-3-one by the ovaries, testes, and head kidneys of the Atlantic salmon *Salmo salar*. Gen. Comp. Endocrinol. 69, 406-415.

Sarre, G.A., Potter, I.C., 1999. Comparisons between the reproductive biology of black bream *Acanthopagrus butcheri* (Teleostei: Sparidae) in four estuaries with widely differing characteristics. Int. J. Salt Lake Res. 8, 179-210.

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

Smith, P.J., 1986. Spawning behaviour of snapper, *Chrysophrys auratus*, in captivity (Note). NZ. J. Mar. Feshwat. Res. 20, 513-515.

Specker, J.L., Sullivan, C.V., 1994. Vitellogenesis in fishes: Status and perspectives. In: Davey, K.G., Peter, R.E., Tobe,. S.S., (Eds.), Perspectives in Comparative Endocrinology, National Science and Engineering Research Council of Canada, Ottawa. pp. 304-315.

Thomas, P., Arnold, C.R., Holt, G.J., 1995. Red drum and other sciaenids. In: Bromage, N., Roberts, R.J., (Eds.), Broodstock management and egg and larval quality, Blackwell Science, Oxford. pp. 118-137.

Thorogood, J., Blackshaw, A., 1992. Factors affecting the activation, motility and cryopreservation of the spermatozoa of the yellowfin bream, *Acanthopagrus australis* (Günther). Aquacult. Fish. Man. 23, 337-344.

Trudeau, V.L., Peter R.E., 1995. Functional interactions between neuroendocrine systems regulating GtH-II release. In: Goetz, F.W., Thomas, P. (Eds.), Reproductive Physiology of Fish 1995. Fish Symposium 95, Austin. pp. 44-48.

Tyler, C.R., 1991. Vitellogenesis in salmonids In: Scott, A.P., Sumpter, J.P., Kime, D.E., Rolf, M.S., (Eds.), Reproductive Physiology of Fish 1991. FishSymp 91, Sheffield. pp. 295-299.

Van Der Wal, E.J., 1985. Effects of temperature and salinity on the hatch rate and survival of Australian bass (*Macquaria novemaculeata*) eggs and yolk-sac larvae. Aquaculture 47, 239-244.

Zohar, Y., 1988. Gonadotropin releasing hormone in spawning induction in teleosts : basic and applied considerations. In: Zohar, Y., Breton, B. (Eds.), Reproduction in Fish - Basic and Applied Aspects in Endocrinology and Genetics. INRA, Paris. pp. 47-61.

Zohar, Y., 1989. Endocrinology and fish farming : Aspects in reproduction, growth, and smoltification. Fish Physiol. Biochem. 7, 395-405.

Zohar, Y., Harel, M., Hassin, S., Tandler, A., 1995. Gilt-head sea bream (*Sparus aurata*). In: Bromage, N., Roberts, R.J., (Eds.), Broodstock management and egg and larval quality, Blackwell Science, Oxford. pp. 94-117.