Reproductive biology and endocrinology of the greenback flounder *Rhombosolea tapirina* (Günther, 1862).

by

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

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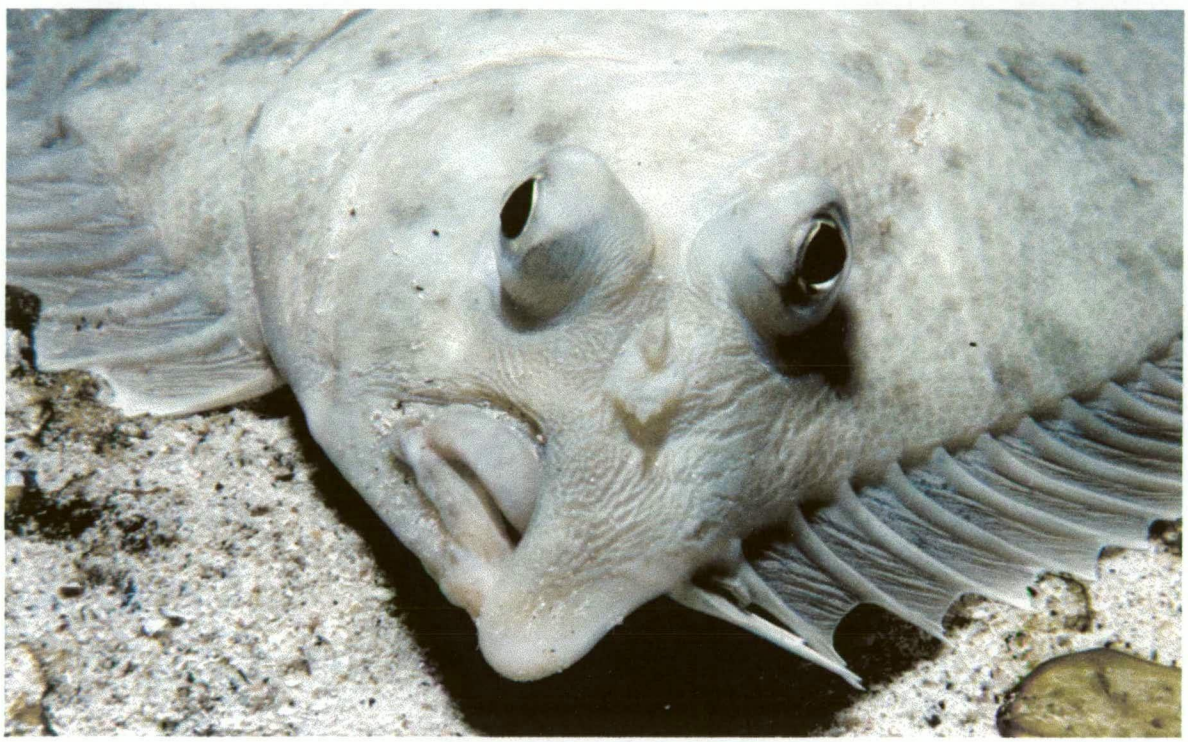
Frontispiece: Greenback flounder (*Rhombosolea tapirina*)
(Photo by Polly and Mark Hilder)



ABSTRACT

The greenback flounder *Rhombosolea tapirina* (Pleuronectidae) is distributed around Southern Australia and New Zealand. There is growing interest in developing an aquaculture industry for greenback flounder in Tasmania and pilot aquaculture schemes are already underway, however, current programs are limited by unreliable egg production. This study provided baseline information on reproductive events under normal reproductive conditions in wild greenback flounder, the stress response of greenback flounder to common husbandry and laboratory aquaculture practices, developed induced ovulation protocols, examined the mechanism of action of hormone treatment and assessed *in vitro* effectiveness of putative maturational steroids on ovarian tissue.

Oocyte size frequency distributions showed that female greenback flounder are multiple ovulators and have group synchronous oocyte development. Plasma and ovarian levels of testosterone (T) and 17β-estradiol (E_2) were elevated in association with vitellogenesis. Plasma levels of 17α20β-dihydroxy-4-pregnen-3-one (17,20βP), and 17,20βP-sulphate but not 17,20βP-glucuronide were significantly elevated in ovulated females, whereas ovarian levels of 17,20βP were elevated in association with final oocyte maturation (FOM) and ovulation.

Changes in macroscopic testis stage were characterised by few changes in proportions of gamete types between gonad stages, indicating low milt production, even in mature fish. Plasma levels of T were elevated in association with spermatogenesis, but not spermiation. No significant changes in plasma 11-ketotestosterone (11KT), 17,20βP, 17,20ßP-sulphate and 17,20βP-glucuronide were detected with change in gonad stage of males.

The latency of the plasma cortisol response to stress was approximately 10 min. Plasma levels of cortisol were significantly higher in wild fish sampled after capture, confinement and transport, and some routine husbandry practices had the capacity to stimulate elevated cortisol levels for up to 48 h. Hematocrit (Hct) did not change significantly in response to stress, suggesting that either Hct is not effected by stress in this species, or changes in Hct were not detected within the sampling protocol. After

exercise, muscle lactate did not significantly change, however, plasma lactate and muscle [H^+] significantly increased and plasma [H^+] significantly decreased, indicating that muscle and blood physiology of greenback flounder do change in response to exercise, but unlike other flatfish, there was little evidence for *in situ* glycogenesis within white muscle tissue.

Female greenback flounder were induced to repeat ovulate using a range of exogenous hormone treatments. Des Gly^{10} [$D\text{-}Ala^6$] LHRH ethylamide (LHRH-a) and human chorionic gonadotropin (hCG), significantly increased the number of ovulations above control levels. Co-administration of LHRH-a and the dopamine antagonist PIM provided no significant advantage over treatment with LHRH-a alone. Dopamine appeared to have an inconsistent effect on reproductive function in greenback flounder. Co-treatment of LHRH-a + T significantly enhanced the ovulatory effects of LHRH-a, suggesting steroid feedback enhances pituitary responsiveness to GnRH.

In vitro bioassays indicated that ovarian fragments required pre-treatment with hCG before they were receptive to steroids. In most cases, the maturational response to steroids after priming with hCG exceeded the maturational response to hCG treatment alone. Ovarian fragments were receptive to all steroids at all concentrations tested. There was considerable inconsistency in maturational responses to each steroid and each dose, however, 20α and 20β- hydroxylated steroids were most effective at inducing maturation, and 5-pregnene and 5β-pregnane steroids were least effective.

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Chapter 1

General Introduction.

1. General Introduction.

1.1. Requirement for marine fish farming in Australia

There is growing demand for fish products world wide, and there are worrying predictions that fisheries resources will not be able to supply future requirements (FAO 1996). Australia has one of the most diverse marine faunas and largest fishing zones in the world, however, due to low nutrient conditions, this is not reflected in fishing resource abundance or productivity (Kailola et al., 1993). Many Australian fisheries species are fully or heavily exploited, and the fishing industry can no longer expect to rely on increased catches. The alternatives are to increase the value of existing stocks, or develop farming of seafoods. Aquaculture is a growth industry in Australia and already contributes to 25% of the national fisheries production (ABARE, 1996), however, almost all of this aquaculture input is derived from 5 industries, pearl oysters, Atlantic salmon, rainbow trout, Pacific and Sydney rockoysters and ornamental fish (Kailola et al., 1993). With continued development of existing species, but also new species, Australia will be well placed to, compete on the international export market, supply the domestic market which is heavily subsidised by imports at present (Kailola et al., 1993) and contribute to stock enhancement programs.

1.2. Marine fish farming in Tasmania

Aquaculture has become a major growth industry in Tasmania and in 1994/95, supplied 12% of Australia's total fisheries production, 51% of Tasmania's total seafood production contributing $110 million (ABARE, 1996) and employed about 1500 people (SDAC, 1996). Most of the farmed seafood in Tasmania in the past 6 years has been Atlantic salmon and Pacific oysters, however, among the 142 marine farms operating in the state, some are for new species, albeit in the experimental stage (SDAC, 1996).

1.3. Current status of greenback flounder culture

1.3.1. General biology

Greenback flounder (*Rhombosolea tapirina*) is a right eyed flounder belonging the family Pleuronectidae and is distributed around southern Australia and New Zealand, in

estuaries, marine embayments and inshore coastal waters to depths of 100m (Kailola et al., 1993). It has a prolonged spawning season (March to October), shows serial spawning and probably spawns in deeper coastal waters (Kurth, 1957; Crawford, 1984a). Wild fish attain a length of 31-34 cm (400-500g) in 3+ years (Kurth, 1957), but cultured fish grow considerably faster in captivity (Purser, 1996). Greenback flounder forms a minor fishery of approximately 140 t per annum in Southern Australia (Kailola et al., 1993) and is largely caught by inshore trawl and gill nets (SDAC, 1996).

1.3.2. Aquaculture potential

A good aquaculture species should be biologically manageable, have a suitable growth profile, realistic promise of financial return, and have existing markets and routes of commercialisation identified. In Tasmania the infrastructure base for aquaculture research at the Department of Aquaculture, University of Tasmania and Department of Primary Industries and Fisheries, Marine Research Laboratories, Taroona, Tasmania, provide suitable environments to critically assess the aquaculture potential of a species before considerable finances are allocated to aquaculture development. Many of the characteristics essential for aquaculture development of a species have already been investigated in greenback flounder, and to date, indicate that greenback flounder is a suitable candidate for aquaculture.

- Biologically manageable: The life cycle has been closed. Larvae are highly amenable to hatchery conditions, show high survival and ween early (Crawford, 1984b, 1986; Hart, 1991, 1994). Growout has been successfully demonstrated at the Department of Aquaculture, University of Tasmania and Department of Primary Industries and Fisheries, Marine Research Laboratories, Taroona, Tasmania,

- Desirable biological characteristics: Greenback flounder have a prolonged spawning season (Kurth, 1957; Crawford, 1984a), if spawning periods can be manipulated by photoperiod control, then year round production will be possible. Market size fish can be produced within 20 months of hatching and improvements are expected with triplody and /or sterilisation (Purser, 1996). Cultured fish show a better condition factor than wild fish and recovery rates from whole fish to gutted (gill and gonad included) are around 95% (Purser 19967). Greenback flounder show extreme

tolerance of low salinity (Girling, 1997) and preliminary live transport experiments indicated that flounder can be chilled for 24 h with 100% survival (Purser, 1996).

- Taste: Sensory test results were favourable and on parr with the imported New Zealand flounder species *Rhombosolea plebia* (Purser, 1996).

- Stocking density: There was no significant increases in variability in food consumption, or growth rates when fish were held at stocking densities approximating aquaculture conditions, suggesting social interactions did not influence individual rates of food consumption (Shelverton, 1995).

- Market assessment: The Australian flatfish market is estimated at 735 t and valued at $4.4m, however, New Zealand imports (mostly trawled frozen product) supply 90% of the market. Wholesale prices for fresh fish range from $6-10.kg^{-1} however preliminary market enquires indicate prices for live fish ranging from $12-25.kg^{-1}. There is considerable potential for Australian contribution to the Asian market, as in 1994, 83,440 t of flatfish was imported to Japan, with prices for live product ranging from A$10-15 kg^{-1} (Hart, 1993; Purser, 1996).

- Technology transfer of culture techniques: There is worldwide interest in the culture of pleuronectid species, therefore, existing technology and information is potentially applicable to culture of greenback flounder.

- Supportive research: There is a considerable research history on greenback flounder relevant to the aquaculture development of this species, including studies on the general biology (Kurth, 1957; Crawford, 1984a), early lifestages of hatchery-reared greenback flounder (Crawford, 1986; Hart, 1991, 1994; Hart and Purser, 1995), feeding behaviour of larvae (Cox, 1997), nutritional and disease studies (Pattle, 1995) and osmoregulation (Girling, 1997). Other research projects on greenback flounder have been funded by the Australian Research Council (awarded to Professor Pankhurst, University of Tasmania) and the Fisheries Research and Development Corporation (awarded to Dr Purser, University of Tasmania). Collectively, these research projects provide an essential groundwork for continuing research and development of greenback flounder as an aquaculture species.

- Industry involvement: Two growers are involved in pilot scale growout assessment in Tasmania, and a small hatchery and growout facility is operating in Victoria. In addition, there are further calls of interest in Tasmania, and South Australia (Purser, 1996)

1.3.3. Further research requirements

- Market potential: No aquaculture product is currently sold , therefore all figures are based on wild caught fish (Hart, 1993; Purser, 1996).

- Product quality: A small proportion of fish in each batch show mal-pigmentation and skeletal deformities (bent spinal cord and shortened gill coverings) (Purser, 1996). This problem may be eliminated by further investigation of nutritional requirements.

- Disease: In some instances *Trichodinosis* , *Flexibacter maritimus, Vibriosis anguillarum, Aeromonas salmonicida* and microsporidium have been identified in cultured fish (Pattle, 1995; Whittington et al., 1995; Munday, 1996; Soltani et al., 1996; Handlinger et al., 1997). There is a requirement for improved understanding of transmission pathways and treatment of diseases.

- Stress response: Most fish species in captivity show some degree of stress response to aquaculture practices, including poor water quality, capture, handling, sorting, grading, transport and confinement (reviewed in Barton and Iwama, 1991; Barton, 1997; Wendelaar Bonga, 1997). An evaluation of greenback flounders stress response to husbandry and laboratory practices is required so that appropriate husbandry techniques can be implemented and potential effects of stress on experimental results can be clarified.

- Gamete supply: Cultured fish readily undergo vitellogenesis, but do not reliably ovulate without hormonal induction and current induced ovulation protocols frequently result in variable egg production and quality (Crawford, 1984b; Hart, 1991). Wild fish do not acclimate easily to captivity, but good quality eggs can be obtained from freshly caught wild fish, however, this practice may not be suitable for commercial practice due to disease risks.

- Maturation: High levels of maturation were recorded in 1 year old fish. Triploidy should overcome this problem, and high triploid rates have been achieved experimentally, although grow-out trials are yet to be conducted (Purser, 1996). Fish rarely spawn spontaneously in captivity and ovulation normally results in egg retention and even peritonitis if eggs are not regularly stripped. Improved understanding of gonadal development and the associated endocrine changes should contribute to controlled reproduction of viable eggs.

1.4. Objectives of this study

- Examine endocrine correlates of reproduction in wild flounder.
- Examine the stress response to common husbandry and experimental practices in cultured fish.
- Develop techniques for artificial induction and control of ovarian development and ovulation.
- Assess the effect of induced ovulation protocols on ovarian development and plasma levels of gonadal steroids with view to assessing the mechanism of action of hormone treatment.
- Examine the *in vitro* effectiveness of putative maturational steroids on ovarian tissue.

1.4.1. Control of reproduction

In order for reproduction to occur in captive fish, they have to undergo gonadal development and maturation, undergo gamete final maturation resulting in ovulation in females (release of eggs into the oviduct) and sperm production in males, and spawn (behavioural interaction between males and females during which eggs and sperm are released and fertilised) (Pankhurst, 1998). Bottlenecks can occur at any of these stages, possibly as a result of inappropriate environmental conditions (social and physical) and/or chronic stress imposed by the conditions of captivity (Pankhurst, 1998). Controlled approaches to managed reproduction rely on a solid understanding of the pattern of gamete development, duration and frequency of spawning events, and

associated endocrine changes. Armed with this information, potential bottlenecks can be identified and management strategies implemented.

The process of gamete growth in females is marked by germinal tissue differentiation to oogonia followed by proliferation via mitotic division to form primary or pre-vitellogenic oocytes. Ovaries arrested at this stage are referred to as immature, but regressed ovaries will also contain only pre-vitellogenic oocytes. Primary oocytes grow via the process of vitellogenesis involving accumulation of yolk proteins synthesised by the liver. There is a considerable increase in size of oocytes and ovarian follicles during this second growth phase (vitellogenesis). The transition to the tertiary growth stage is marked by the resumption of meiosis resulting in final oocyte maturation (FOM) which is characterised by nuclear migration and breakdown, lipid and yolk droplet coalescence, large increase in oocyte size due to hydration and oil droplet formation. Oocytes that have completed FOM, rupture from the maternal follicle and are expelled into the ovarian lumen, or in salmonids the body cavity (reviews given in Wallace and Selman, 1981; deVlaming, 1983; Pankhurst, 1998).

Ovarian development can be classified into 3 basic types, (1) synchronous - species that spawn once then die such as Pacific salmon, are characterised by a single clutch of oocytes that grow in unison. (2) Group synchronous and multiple group synchronous- at least two clutches of oocytes can be distinguished in the ovary at the same time, hence, these species are capable of spawning more than once. Group synchronous species spawn more than once in a life time, but typically once per season whereas multiple group synchronous species have multiple spawning episodes within a single reproductive season. (3) Asynchronous - in these species, oocytes are at a mixture of stages and no distinct clutches can be identified, although, asynchronous development is most likely an extreme case of multiple group synchrony (reviews given in Wallace and Selman, 1981; deVlaming, 1983; Pankhurst, 1998). The pattern of male gamete development broadly reflects gamete synchrony shown in females (Pankhurst, 1998).

Fish respond to changes in the environment, so that reproduction is timed to coincide with environmental conditions that are most favourable for reproduction. In many species gonad growth is commonly influenced by changes in temperature and photoperiod because collectively, they are reliable cues which foretell the advent of the appropriate time for spawning (Lam and Munro, 1987). Close to the time of gamete

final maturation appropriate physical cues (such as water flow or level, availability of suitable physical space and availability of spawning substrate) are often required for ovulation and/spawning (Stacey, 1984). Knowledge of environmental factors can be used to advance, induce, or inhibit gonad growth, spawning and recrudescence (Lam, 1982).

Internal and external factors collectively govern the process of gonadal development. External factors such as temperature and photoperiod, determine when endogenous factors will be initiated and endogenous factors, mainly circulating hormones, initiate and mediate gonadal development (Stacey, 1984). Endocrine control of reproduction is regulated via the hypothalamic-pituitary-gonad axis (HPG). The release of gonadotropins (GtHs) from the pituitary, is regulated by the stimulatory actions of gonadotropin releasing hormone (GnRH) and in most species, but not all, the inhibitory actions of dopamine (DA). Gonadotropins are released into circulation and exert their actions by binding to membrane-bound receptors in the ovary and testes, stimulating the production of steroids involved in gonadal development. Many different forms of immunoreactive GnRH have been identified in brains of teleosts (reviewed in Peter and Yu, 1997). Two GtHs have been identified (GtH-I and GtH-II), which have temporally separated actions, GtH-I being present during gametogenesis and GtH-II being predominate during maturation. The HPG axis is not simply a 1-way process that ends with steroid production. Feedback mechanisms exist whereby steroids feedback via central action to regulate their own production in a positive or negative fashion. T and E_2 for example are important feedback regulators of GtH release in goldfish and exert positive effects to increase pituitary responsiveness to LHRH-a, but basal serum GtH levels are maintained by steroid enhanced increases in DA (reviews given in Pankhurst, 1998; Peter et al., 1991; Peter and Yu, 1997).

The role of the steroids T and E_2 is standard among teleost species. T is a precursor to E_2 (Kagawa et al., 1984; Matsuyama et al., 1988) and E_2 stimulates synthesis of the yolk precursor vitellogenin which is incorporated into the growing oocyte during vitellogenesis (reviewed in Specker and Sullivan, 1993). 17α20β-dihydroxy-4-pregnen-3-one (17,20βP) is the maturation inducing steroid (MIS) in most species in which it has been investigated (Scott and Canario, 1987). In sciaenid fish however,

17α20β,21-trihydroxy-4-pregnen-3-one (20βS) has been identified as the main MIS (Thomas, 1994).

In male teleosts, plasma T levels tend to be highest during spermatogenesis (transition from spermatogonia to spermatids), and drop off prior to spermiation sperm release) (Wingfield and Grimm, 1977; Scott et al., 1984; Fostier et al., 1987; Pankhurst and Conroy, 1987; Harmin et al., 1995b; Carolsfeld et al., 1996). In many species 11-ketotestosterone (11KT) is elevated during spermatogenesis and the early stages of spermiation (release of spermatozoa into the sperm ducts), (Scott et al., 1984; Fostier et al., 1987; Dedual and Pankhurst, 1992; Methven et al., 1992; Barnett and Pankhurst, 1994; Borg, 1994; Harmin et al., 1995b; Carolsfeld et al., 1996) and is thought to be more effective than T at stimulating spermatogenesis, secondary sexual characteristics, and stimulating reproductive behaviour (Borg, 1994). 17,20βP appears to have a role in stimulating spermiation and milt production (Fostier et al., 1987; Pankhurst, 1994; Carolsfeld et al., 1996).

Steroids are often metabolised or conjugated quite rapidly after production. Conjugated steroids are formed when hydroxyl groups of steroids are conjugated with glucuronic acid (glucuronides) or sulphuric acid (sulphates). These steroid derivatives are more water soluble than the parent steroids, and are therefore readily excreted in the bile and /or urine (Scott and Vermeirssen 1993). Conjugated steroids can be measured from blood plasma or urine and may provide information regarding key biologically active steroids that are difficult to detect in blood plasma because they are released in a pulsatle fashion, or in small amounts, or are rapidly metabolised (Scott and Vermeirssen 1993).

In the absence of normal gonadal development in captivity, various exogenous hormones can be administered to induce reproductive processes (Donaldson and Hunter, 1983; Peter and Yu, 1997). Classically, fish were treated with pituitary preparations of piscine or mammalian GtH. Piscine gonadotropins are now less favoured mainly due to their expense, limited supply, lack of standardisation and species specificity (reviwed in Donaldson and Hunter, 1983; Lam, 1982; Zohar, 1988). In contrast, the mammalian gonadotropin, human chorionic gonadotropin (hCG) is relatively cheap, readily obtainable, its biological activity is readily standardised, but it

may have lower biopotency (Pankhurst, 1998). The preferred exogenous hormone treatments are synthetic analogues of fish GnRH (GnRH-a) and analogues of mammalian GnRH referred to in this volume as luteinising hormone releasing hormone analogue (LHRH-a) (Peter and Yu, 1997; Zohar, 1988), particularly in the presence of a dopamine antagonist such as pimozide, to block the inhibitory action of dopamine (Chang and Peter, 1983; DeLeeuw et al., 1985, 1987; Lin et al., 1985; Peter et al., 1988;). LHRH-a or GnRH-a in the form of an injection or slow release pellet is highly effective at inducing ovulation in fish which have completed vitellogenesis, but failed to undergo FOM (Peter and Yu, 1997) and stimulates milt hydration and spermiation in males (Pankhurst, 1994). LHRH-a or GnRH-a pellet implants sustain elevated GtH levels over long periods and have proven to be effective at inducing multiple ovulations in some species (Almendras et al., 1988; Mylonas and Zohar 1995; Berlinsky and King 1996; Mylonas et al., 1996) and ovarian development in immature fish (Crim et al., 1988; Harmin et al., 1995a; Holland et al., 1995).

Male greenback flounder will reliably undergo sperm production in captivity, although the potential to enhance sperm volume and quality has not been thoroughly assessed. Female greenback flounder undergo maturation in captivity, but do not reliably ovulate. Previous studies successfully induced ovulation with the exogenous hormone treatment hCG and the commercially available hormone mixture ovaprim (containing [D-Arg^6, Pro^9 NEt]-sGnRH and a dopamine antagonist (domperidone) (Peter et al, 1993)), although, egg quality and subsequent larval survival were variable (Crawford, 1984b; Hart, 1991; Hart and Purser,1995). In the absence of any information on the reproductive endocrinology of this species, we examined the relationship between patterns of gonadal development and endocrine changes in reproductive steroids and steroid conjugates in wild greenback flounder. The outcomes of this provided a framework for developing our understanding of the status of oocyte MIS/s in this species, and the development and interpretation of induced ovulation protocols in the absence of spontaneous ovulation in this species.

The actions of hCG and a range of C_{21} steroids on FOM were investigated by means of *in vitro* bioassay techniques. Steroids were chosen depending on their position in the steroid pathway, proven effectiveness as a MIS in other species (Scott and Canario,

1987; Thomas, 1994) and/or presence *in vitro* and *in vivo* in other species (Scott and Canario, 1987; Canario 1991; Scott and Canario, 1992).

The potential for inducing ovulation and the physiological mechanisms involved, were investigated by examining the effectiveness of a range of induced ovulation protocols. This study assessed whether greenback flounder were differentially sensitive to hypothalamic versus pituitary hormones by comparing the effectiveness of hCG and luteinising hormone releasing hormone analogue (LHRH-a), and the effectiveness of delivery mode of LHRH-a by comparing LHRH-a injections with slow release pellets. This study also assessed the potential for enhancing ovulatory success by co-treating with LHRH-a and the DA antagonist pimozide to reduce the inhibitory actions of DA on GtH secretion, and in the absence of a gonadotropin assay for this species, examined the role of positive steroid feedback actions on GnRH release, by assessing the potentiating effects of T on ovulation and steroid profiles in the absence and presence of LHRH-a.

1.4.2. Stress and aquaculture

There is by no means an established definition of stress (Barton ,1997), however, in the context of this study, stress refers to heightened metabolic or physiological function in response to environmental factors. Fish have a natural capacity to cope with environmental disturbances, although if response mechanisms are forced beyond normal limits, the response becomes detrimental (Barton and Iwama, 1991; Barton, 1997). Stress results in a series of physiological processes which are often classified as primary, secondary and tertiary, depending on when they are elicited, and the mechanisms involved. These responses can be measured to give an indication of the severity and duration of stress. The primary stress responses are largely catecholamine (adrenaline and noradrenaline) and corticosteroid (cortisol) regulated, and are generally considered adaptive responses, because they enable the animal to cope with the stressful situation imposed upon them (Barton and Iwama 1991; Sumpter 1997; Wendelaar Bonga 1997). The catecholaminergic response is difficult to measure because it is so quick (latency of seconds to minutes and resolution to normal levels within 10 min) (Mazeaud et al., 1977; Barton and Iwama 1991; Sumpter, 1997). The corticosteroid response latency is considerably more generous, generally <15 min (Laidley and Letherland, 1988; Robertson et al., 1988; Young and Cech, 1993), and

therefore provides a window during which basal levels can be established. In most situations, cortisol remains elevated as long as the stressor remains (Barton, 1997; Sumpter, 1997), consequently, the corticosteroid response provides a useful indicator of the duration of the stress. The fact that cortisol remains elevated with the stressor, means fish can be exposed to chronic elevation of cortisol if held in stressful situations. There is a considerable array of secondary stress responses that can be used as indicators of stress in fish, examples include haematology (haematocrit, leucocrit, erythrocyte and leucocyte numbers), metabolic (plasma glucose and lactic acid dissociation to $lactate^-$ and H^+), hydromineral (plasma chloride, sodium, potassium, protein and osmolality), and structural parameters (interrenal cell size, number and diameter) (reviewed in Barton, 1997). Tertiary stress responses generally occur at the whole animal and even population level, and can have profound effects on fish performance and health (Barton, 1997). Some of these responses have particular relevance to aquaculture, ie. detrimental effects on immunocompetence, energy metabolism, potentially affecting growth rates, and inhibition of various reproductive processes (reviewed in Barton, 1997; Pankhurst and Van Der Kraak, 1997; Wendelaar Bonga, 1997)

Common causes of stress in aquaculture can be capture, handling, sorting, grading, transport and confinement (Barton and Iwama, 1991; Barton, 1997; Wendelaar Bonga, 1997), which are unavoidable components of husbandry and experimental procedures. In this study, we established a baseline of stress indicators in normal unstressed fish and evaluated the impact of common husbandry and experimental practices on the stress response. An evaluation of the extent to which aquaculture practices impact on physiological processes provides some indication of the species biological manageability, requirement for improved husbandry techniques and potential for impact on other biological processes.

1.5. Thesis structure

Chapters 2 - 6 have been, or will be submitted for publication, hence there is some planned overlap of introduction and methods sections.

Chapter 2 - Barnett, C.W. and Pankhurst, N.W., 1998. The effects of common laboratory and husbandry practices on the stress response of greenback flounder *Rhombosolea tapirina* (Günther, 1862). Aquaculture. In Press.

Chapter 3 - Barnett, C.W. and Pankhurst, N.W., 1998. Reproductive biology and endocrinology of greenback flounder *Rhombosolea tapirina* (Günther, 1862). Marine and Freshwater Research. In Press.

Chapter 4 - Barnett, C.W. and Pankhurst, N.W., 1998. Effect of treatment with LHRH analogue and hCG on ovulation, plasma and ovarian levels of gonadal steroids in greenback flounder *Rhombosolea tapirina* (Günther, 1862). Journal of the World Aquaculture Society. Under review.

Chapter 5 - Barnett, C.W. and Pankhurst, N.W., 1998. Potential for steroid feedback and dopamine inhibition on GtH release in greenback flounder *Rhombosolea tapirina* (Günther, 1862): indirect assessment by measurement of gonadal steroids and ovulation. In preparation. To be submitted to Gen. and Comp. Endocrinol.

Chapter 6 - Barnett, C.W. and Pankhurst, N.W., 1998. Effects of gonadal steroids and hCG on final oocyte maturation *in vitro* in the greenback flounder *Rhombosolea tapirina* (Günther, 1862). In preparation. To be submitted to Mar. Freswater Res.

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Chapter 2

The effects of common laboratory and husbandry practices on the stress response of greenback flounder *Rhombosolea tapirina* (Günther, 1862).

2. The effects of common laboratory and husbandry practices on the stress response of greenback flounder *Rhombosolea tapirina* (Günther, 1862).

2.1. Summary

This study examined the stress response of the greenback flounder *Rhombosolea tapirina* to common laboratory and aquaculture practices. Plasma levels of cortisol in wild fish sampled within 2 min of capture were comparable with plasma cortisol values in other species captured from the wild and sampled immediately. Plasma levels of cortisol were significantly higher in wild fish sampled after capture, confinement and transport. The latency of the plasma cortisol response to stress was approximately 10 min. Cultured greenback flounder, exposed to normal husbandry conditions had low plasma cortisol levels. Three hours of crowding combined with 5 min chasing (simulated grading) resulted in significantly elevated cortisol levels for up to 48 h. Plasma cortisol was significantly higher in fish held at medium and high stocking density than at low density. The plasma cortisol stress response of greenback flounder is similar to that shown by other marine teleosts. Hematocrit (Hct) did not change significantly in response to stress, suggesting that either Hct is not affected by stress in this species, or changes in Hct were not detected within the sampling protocol. Plasma lactate levels in wild fish sampled after capture, confinement and transport, were considerably higher than levels in fish sampled within 3 min of capture, or exposed to 30 min exercise. No significant changes in muscle lactate were observed in response to exercise, however, there were significant increases in plasma lactate, and muscle $[H^+]$ and a significant decrease in plasma $[H^+]$ following exercise, indicating that muscle and blood physiology of greenback flounder do change in response to exercise. Unlike other flatfish, there was little evidence for *in situ* glycogenesis within white muscle tissue after exercise and there was some indication that greenback flounder have higher aerobic scope than other flatfish studied to date. This study showed that some routine husbandry practices have the capacity to stress greenback flounder.

2. 2. *Introduction*

It is well established that fish are stressed by capture and handling (Billard et al., 1981; Barton and Iwama, 1991; Pickering, 1992; Wendelaar Bonga, 1997) and that the stress response is characterised by disturbances in biochemistry and physiology, which may appear within seconds and can persist for hours or days (Mazeaud et al., 1977). The perturbations resulting from stress are often classified as primary, secondary or tertiary, depending on when they are elicited, and the mechanism involved. Primary responses include rapid changes in plasma levels of catecholamines and corticosteroids, and are generally considered adaptive responses because they enable the animal to cope with stressful conditions imposed upon them. However, response mechanisms may be pushed beyond their normal limits leading to a cascade of detrimental secondary and tertiary effects (Mazeaud et al., 1977). Stress has been demonstrated to affect energy metabolism, potentially affecting growth rates, suppress the immune response, inhibit various reproductive processes (reviewed in Barton and Iwama, 1991; Wendelaar Bonga, 1997; Pankhurst and Van Der Kraak, 1997), and influence flesh quality post-mortem (Wells et al., 1986; Watanabe et al., 1991; Lowe et al., 1993). Capture, handling, crowding, confinement, transport and anaesthesia, are all components of aquaculture and laboratory practice that can stress fish. Therefore, in any aquaculture species, it is important to establish the nature and time course of the stress response, so that husbandry practices can be managed to minimise the effects of stress.

In the present study, the stress response of the greenback flounder *Rhombosolea tapirina* (Pleuronectidae) was examined in both wild and cultured fish, in response to common laboratory and husbandry practices. The greenback flounder is distributed throughout the waters surrounding southern Australia and New Zealand (Ayling and Cox, 1982). There is currently considerable interest in greenback flounder as a potential aquaculture species, as it is amenable to culture conditions, has a fast growth rate, and has a developing market profile (Hart, 1993).

In order to fully assess the suitability of a species for aquaculture, a range of stress indices should be measured in response to common aquaculture practices. Plasma cortisol is the most commonly measured indicator of stress, and usually provides a good reflection of the severity and duration of the stress (Donaldson, 1981; Barton

and Iwama, 1991; Sumpter, 1997), however, the lack of a cortisol response may not always indicate the absence of stress (Wendelaar Bonga, 1997).Toxic substances which may cause death or impair fish health do not necessarily evoke cortisol increases (Grant and Mehrle, 1973; Schreck et al., 1989), and poor water quality and pollutants can suppress the corticosteroid response to subsequent stress (Pickering and Pottinger, 1987). Fish health can also influence the cortisol response; for example, experimentally induced blood parasite infection in rainbow trout *(Oncorhynchus mykiss)* did not elicit a cortisol response (Laidley et al., 1988), and diseased chinook salmon were less able to elevate plasma cortisol after three successive disturbances than healthy fish (Barton et al., 1986). Given these findings, it would therefore seem wise to measure a number of different variables when determining the stress response of an animal.

Hematological features such as red blood cell numbers, blood hemoglobin or Hct are sensitive to impaired osmoregulation and provide useful indicators of stress in fish (Morgan and Iwama, 1997). These variables can increase following stress due to increased red blood cell numbers resulting from splenic release or erythrocyte swelling through the actions of epinephrine to enhance O_2 binding affinity (Ling and Wells, 1985; Nikinmaa, 1990).

Exercise is an unavoidable component of capture and handling and during strenuous activity, fish exceed their capacity to cope aerobically and begin exercising anaerobically (Wood and Perry, 1985; Heisler, 1986). Under extreme conditions, strenuous activity can result in post-capture mortality (Graham et al., 1982; Ferguson and Tuft, 1992). Exercise generates large amounts of lactate and H ions within muscle tissue, much of which may enter into the bloodstream. Elevated lactate and low pH in both muscle and plasma have been measured in association with stress and exercise in a range of teleost species (Dando, 1969; Turner et al., 1983a, b; Schwalme and Mackay, 1985; Girard and Milligan, 1992), and are useful to measure as additional stress indicators.

Before investigating the stress response of domestic or captive fish stocks, it is essential to determine the levels of parameters to be used as stress indicators in normally active fish. Studies are potentially misleading without such information (reviewed in Pankhurst and Sharples, 1992). In this study, basal plasma cortisol and lactate levels were determined in wild greenback flounder, caught and blood sampled

within 2 min of capture. For comparison, blood samples were collected from wild fish after capture, confinement and transportation to shore. These data were then compared with plasma or muscle parameters in domesticated fish exposed to a range of normal husbandry practices, or maintained for short periods at differential stocking densities.

2.3. Materials and Methods

2.3.1. Fish collection and husbandry

Wild fish were hand speared while snorkelling or hand-netted by scuba divers from George's Bay (148° 16′ E, 41° 19′ S), and Bicheno (148° 18′ E, 41° 52′ S) on the east coast of Tasmania in September 1995, and April and June 1996 respectively. Blood samples were collected by caudal puncture within 2 min of capture. Fish caught while snorkelling were sampled on the boat, whereas fish caught by scuba divers were sampled underwater using the technique given by Pankhurst, (1990). These samples were used as an indicator of pre-stress condition. Blood samples were also collected from wild fish after capture in a gill net (6-12 h set time), transportation to shore by boat (15 min) and transportation to the sampling site by truck (15 min). Fish were transported in a 50 l container with partial water changes every 15 min. All blood samples were transported to the laboratory on ice for centrifugation and then frozen for storage at -20°C. The average body weight was 458 ± 52 g.

Cultured fish were either bred at Department of Primary Industries and Fisheries, Marine Research Laboratories, Taroona, Tasmania, or the Department of Aquaculture aquatic facility at the University of Tasmania in Launceston. Fish were subsequently maintained in Launceston at ambient temperature and photoperiod in recirculating systems incorporating a biofilter, coarse solids filter and aeration system. Fish were routinely exposed to normal recirculation system maintenance, where tank disturbances included flushing of sumps and tank cleaning. All tanks were covered with shade cloth, and disturbances in the tank room were minimised. The average body weight was 105 ± 12 g, age ranged from 1-2 years and all fish used in experiments were reproductively regressed.

2.3.2. Experiment 1 protocol: Effect of simulated grading (capture, transfer and confinement)

Fish were held in a 4000 l community tank at a stocking density of 4 $kg.m^{-3}$. Blood samples were collected from 10 fish as an indicator of 'resting condition,'. Removal and blood sampling of these fish from the community tank took 25 min. Another 70 fish were then removed from the community tank, and transferred to a 380 l tank at a stocking density of 19 $kg.m^{-3}$ and blood samples were collected from ten different fish at 0.5, 1 and 3 h after transfer. After 3 h confinement, the remaining 40 fish were placed into 380 l tanks at a stocking density of 3.9 $kg.m^{-3}$. Further blood samples were taken at 24 h, 2, 4 and 6 days after start of confinement. In this and all following experiments (except experiment 5), blood samples were taken by caudal puncture from fish anaesthetised in 0.05% 2-phenoxy ethanol and transferred to the laboratory on ice for centrifugation and then frozen for storage at -20°C.

2.3.3. Experiment 2 protocol: Effect of simulated grading (capture, transfer, crowding and chasing)

Fish were held at nominal low density (3.8 $kg.m^{-3}$) for 14 days prior to the start of the experiment. Blood samples were taken from 8 fish as an indicator of resting condition. Capture and blood sampling of these fish took 5 min. Forty fish were then captured and transferred to a 380 l tank at a stocking density of 11 $kg.m^{-3}$, chased with a net for 5 min and then left confined in crowded conditions for 3 h. Eight different fish were removed and blood sampled at 0.5, 1 and 3 h after transfer. After 3 h of crowding, the remaining fish were placed into 380 l tanks at a stocking density of 2.5 $kg.m^{-3}$. Further samples were collected at 24 h, and 2 days after the initial transfer. The exact experimental protocol was repeated with collection of blood samples 0, 0.5 h, 4, 6, 8, 10 and 12 days after transfer. Due to limited holding facilities, it was not possible to conduct both parts of the experiment simultaneously.

2.3.4. Experiment 3 protocol: Effect of repeat sampling

Blood samples were collected from 12 fish as they were removed from a 4000 l community tank (0.7 $kg.m^{-3}$ stocking density) and transfered to a 1000 l aerated tank (3 $kg.m^{-3}$ stocking density). The fish were confined for 3 h, and blood samples were collected at 0.5, 1 and 3h.

2.3.5. Experiment 4 protocol: Effect of stocking density

Fish were removed from a 4000 l community tank (4.1 $kg.m^{-3}$) and placed into 380 l tanks at 4.8 $kg.m^{-3}$, 9.6 $kg.m^{-3}$ or 14.4 $kg.m^{-3}$, which we nominated as low, medium or high relative stocking density respectively (2 replicates per stocking density). Stocking densities were chosen to span the range currently used in pilot growout systems. Fish were held at these stocking densities for 14 days, and fed at 3% body weight once/day. Blood samples were collected and body weight recorded from 8 fish per tank.

2.3.6. Experiment 5 protocol: Effect of stress and exercise

Seven fish were removed from a 500 l tank (stocking density 2.6 $kg.m^{-3}$), killed by a blow to the head and measurements and samples were collected immediately as an indicator of the pre-stress and exercise condition. The remaining fish were vigorously chased for 30 min and seven different fish were then sampled at 0.5, 1, 3, 6 and 24 h after the start of exercise. At each sample period approximately 0.5 g of muscle was removed, frozen in liquid nitrogen then transferred to -80°C for frozen storage, blood samples were collected and extracellular muscle pH was measured by cutting a narrow slit in the muscle tissue and inserting a glass spear pH probe (HANNA 9025) (Sigholt et al., 1997).

2.3.7. Analytical methods

Plasma cortisol was measured from samples collected during experiments 1-5. Cortisol was extracted from 100 µl aliquots of plasma using 1 ml ethyl acetate, and 50µl aliquots of the ethyl acetate extract were transferred to assay tubes for evaporation. Extraction efficiency was calculated as recovery of ^{3}H-labelled steroid extracted with plasma was on average 85%, and assay values were corrected accordingly. Cortisol was measured using (1,2,6,7-^{3}H) cortisol (Amersham) and an antiserum to cortisol from Bioanalysis Ltd, Cardiff. Assay protocol was as described in Pankhurst and Conroy (1987). Assay detection limit was 0.6 $ng.ml^{-1}$ plasma. Hematocrit (Hct) (% red blood cell packed volume) was measured after 3 min centrifugation in micro haematocrit tubes from blood samples collected during experiment 2. Levels of plasma lactate were measured from plasma samples collected during experiments 2 and 5. Plasma lactate was extracted from 50 µl aliquots of plasma, using 100 µl of cold 0.6

M perchloric acid (PCA), followed by 5 minutes on ice and centrifugation at 8000 g for 3 min. Muscle samples collected during experiment 5 were trimmed, weighed and approximately 0.25 g was homogenised for 45 sec in 1.5 ml of cold PCA. The homogenate was centrifuged for 10 min at 3000 rpm. Fifty seven µl aliquots of muscle lactate supernatant or plasma lactate extracts were analysed enzymatically using a Sigma 826-UV kit. Prior to measurement of plasma pH during experiment 5, plasma samples were left in uncapped vials for 3 h, to allow release of respiratory CO_2.

2.3.8. Statistical analysis

T-tests, analysis of variance (ANOVA), repeated measures ANOVA, nested ANOVA, and Tukey-HSD mean comparison tests were performed using the computer package SPSS for Windows. Data were log or square root transformed to satisfy normality and homogeneity of variance requirements. A significance level of $\alpha < 0.05$ was used for all statistical tests. Plasma and muscle pH values were converted to $[H^+]$ for numerical analysis.

2.4. Results

2.4.1. Plasma cortisol and lactate levels in wild fish

Wild fish sampled within 2 min of capture had (mean ± S.E. (n)) plasma cortisol and lactate levels of 3.9 ± 2.5 (10) $ng.ml^{-1}$ and 0.25 ± 0.09 (10) $mmol.l^{-1}$ respectively. In contrast, wild fish exposed to net capture and transport to shore had significantly elevated plasma levels of both cortisol and lactate of 61.9 ± 3.1 (20) $ng.ml^{-1}$ and 7.15 ± 0.50 (20) $mmol.l^{-1}$ respectively.

2.4.2. Experiment 1: Effect of simulated grading (capture, transfer and confinement)

Plasma cortisol levels were already high (47 $ng.ml^{-1}$) at first sampling and remained high for at least 2 days, albeit with a decreasing trend. Plasma cortisol levels in fish sampled from 2-6 days were lower than fish sampled at 0.5 h, and levels in fish sampled at 4-6 days were lower than those sampled at 0, 0.5 and 1h (Fig. 2.1).

2.4.3. Experiment 2: Effect of simulated grading (capture, transfer, crowding and chasing)

Resting plasma cortisol levels were uniformly low (1.6 $ng.ml^{-1}$) (Fig. 2.2a). Plasma cortisol levels were significantly higher than resting levels at 0.5, 1, and 24 h after the start of crowding, with maximum plasma cortisol levels of (72 $ng.ml^{-1}$). Plasma Hct did not change significantly throughout the experiment (14.29 ± 0.47, total mean ± SE). Resting plasma lactate levels were undetectable, but increased significantly 0.5 h after start of crowding and were significantly higher than resting levels at all other time periods except 8 and 12 days after start of crowding (Fig. 2.2b). Maximum plasma lactate levels (0.8 $mmol.l^{-1}$) were recorded at 1 h.

2.4.4. Experiment 3: Effect of repeat sampling

Plasma levels of cortisol were close to non detectable limits in fish sampled within 10 min of first disturbance, but increased considerably in fish sampled after 13 min (Fig. 2.3a), indicating a response latency period of 10-13 min. Plasma levels of cortisol in the same fish were significantly higher than resting levels 1 and 3 h after start of confinement (Fig. 2.3b).

2.4.5. Experiment 4: Effect of stocking density

Plasma levels of cortisol were significantly higher in fish held at medium and high stocking density than in fish held at low stocking density, but did not vary significantly between medium and high stocking density (Fig. 2.4a). The order tanks were sampled from, or the order in which fish were sampled in those tanks, did not appear to influence the results, with no evidence that fish sampled last had higher cortisol levels (Fig. 2.4b). There was no relationship between plasma cortisol and body weight (r^2 = 0.02, 0.06, 0.19, for low, medium and high stocking density respectively).

2.4.6. Experiment 5: Effect of stress and exercise

Plasma cortisol was significantly elevated above resting levels at all other sample times (Fig. 2.5a). Maximum plasma cortisol levels (22 $ng.ml^{-1}$) were recorded 3 h after the start of exercise, and there was a decrease thereafter. Plasma lactate was significantly elevated above resting levels 0.5 and 1 h after start of exercise (Fig. 2.5b). Plasma $[H^+]$ was significantly different from resting values 12 h after the start of exercise (Fig. 2.5c). Muscle lactate and $[H^+]$ were not significantly elevated above resting levels at any time; however, the muscle $[H^+]$ recorded 0.5 h after the start of exercise, was significantly higher than muscle $[H^+]$ recorded from 3-12 h, post-exercise, and muscle $[H^+]$ at 1 h was higher than levels at 3 and 12 h post exercise (Fig. 2.6).

Fig. 2.1. Plasma levels of cortisol in relation to a 3 h period of confinement (experiment 1, simulated grading). Values are mean ± S.E. (n = 10). Values that are not significantly different ($P>0.05$) share common superscripts.

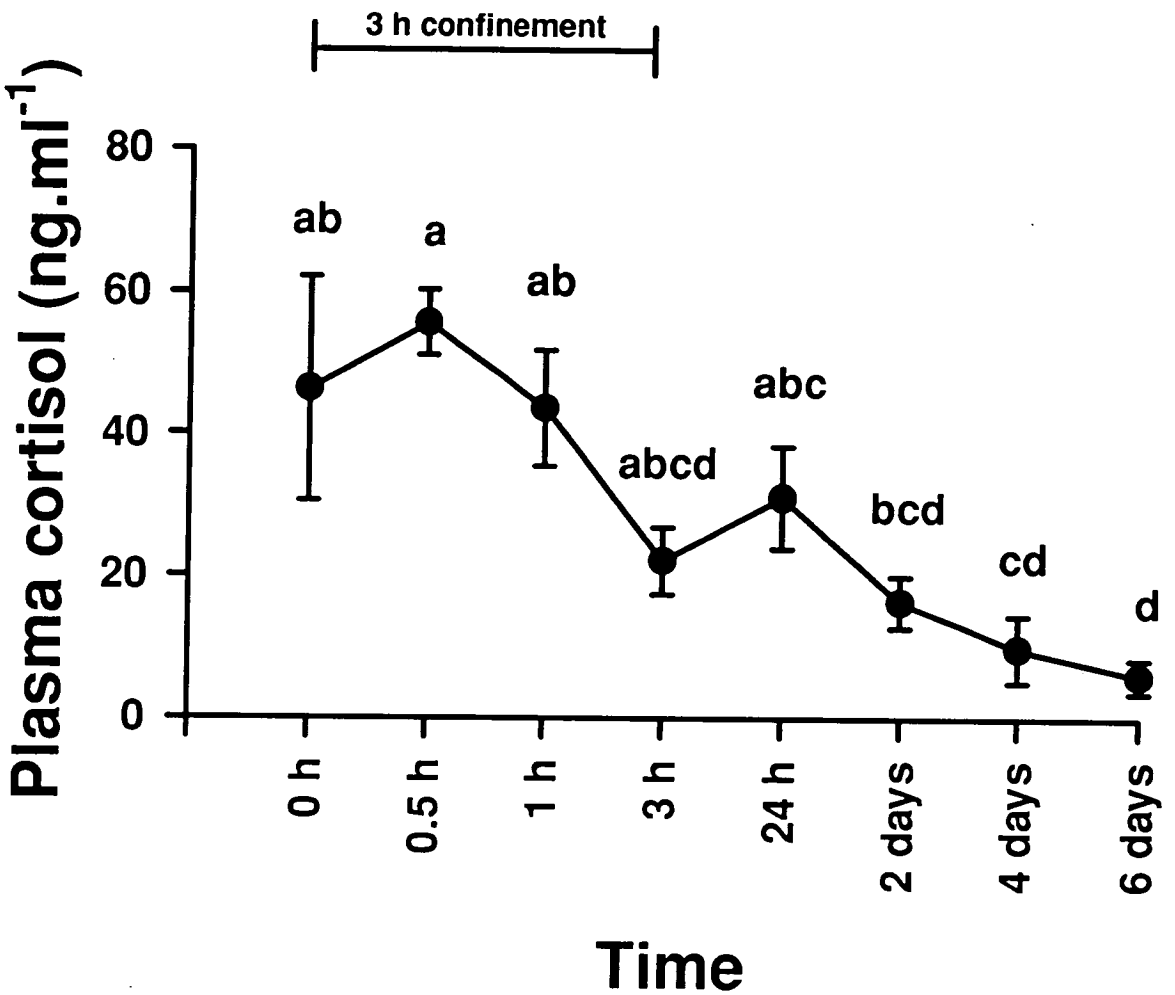

3 h confinement
Plasma cortisol (ng.ml-1)
80
60
40
20
0
ab
a
ab
abcd
abc
bcd
cd
d
0 h
0.5 h
1 h
3 h
24 h
2 days
4 days
6 days
Time

Fig. 2.2. Plasma levels of a) cortisol and b) lactate in relation to a 3 h period of crowding and 5 min chasing (experiment 2, simulated grading). First trial (•), second trial (o). Values are mean ± S.E. (n = 8). Values that are not significantly different ($P>0.05$) share common superscripts. Statistical comparisons are within each trial only.

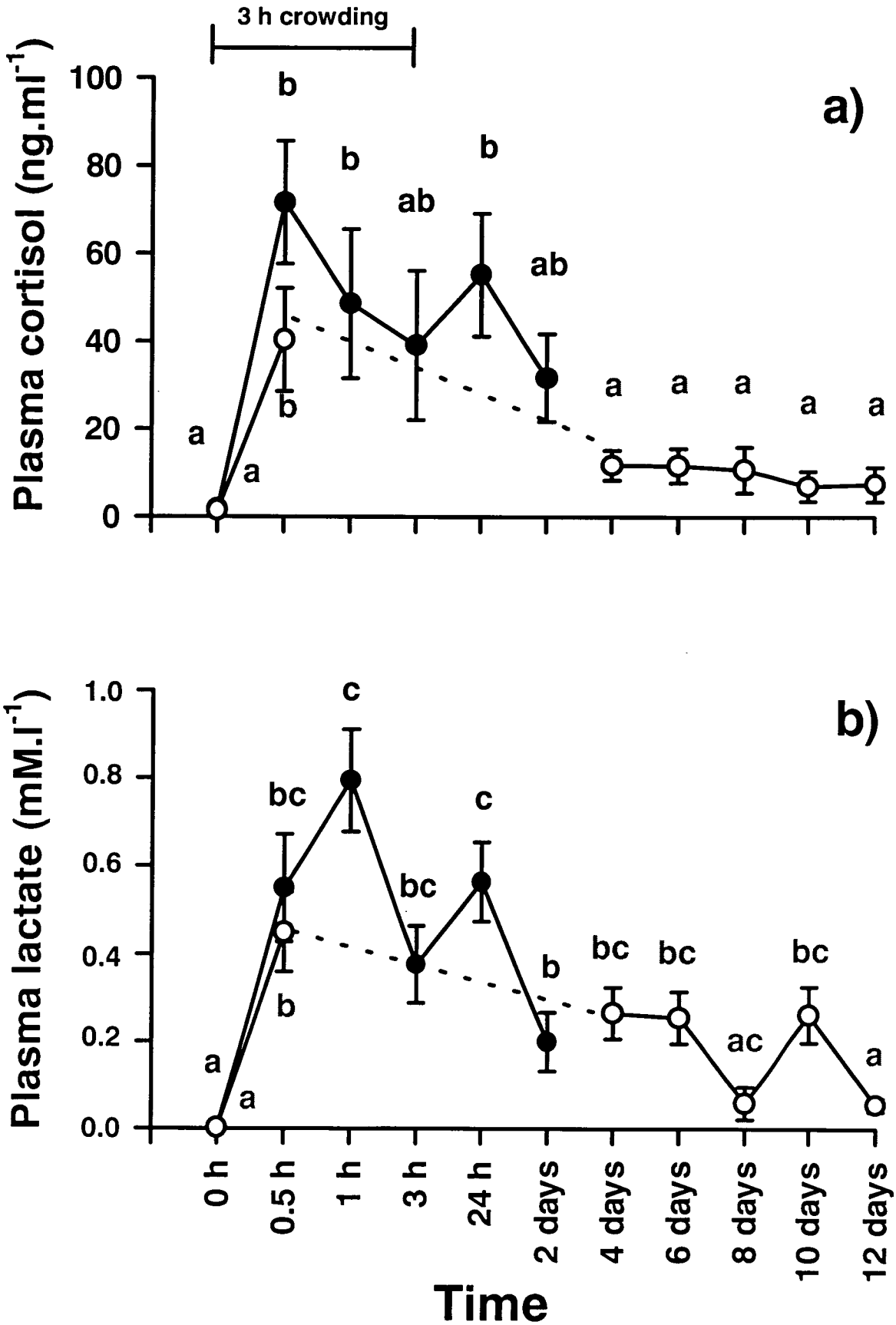

3 h crowding
Plasma cortisol (ng.ml-1)
100
80
60
40
20
0
a)
b
b
ab
b
ab
a
a
b
a
a
a
a
a
Plasma lactate (mM.l-1)
1.0
0.8
0.6
0.4
0.2
0.0
b)
c
bc
c
bc
b
bc
bc
bc
ac
a
a
b
a
0 h
0.5 h
1 h
3 h
24 h
2 days
4 days
6 days
8 days
10 days
12 days
Time

Fig. 2.3. Plasma levels of a) cortisol in fish sampled at '0 h', in relation to time from first disturbance of the tank until sampling (experiment 3, effect of serial sampling) and b) cortisol in the same fish serially sampled over 3 h (experiment 3). Values are for individual fish in a) and mean ± S.E. (n = 12) in b). (o) = mean of '0 h' samples taken from fish sampled within 10 minutes of first disturbance. Values that are not significantly different ($P>0.05$), share common superscripts.

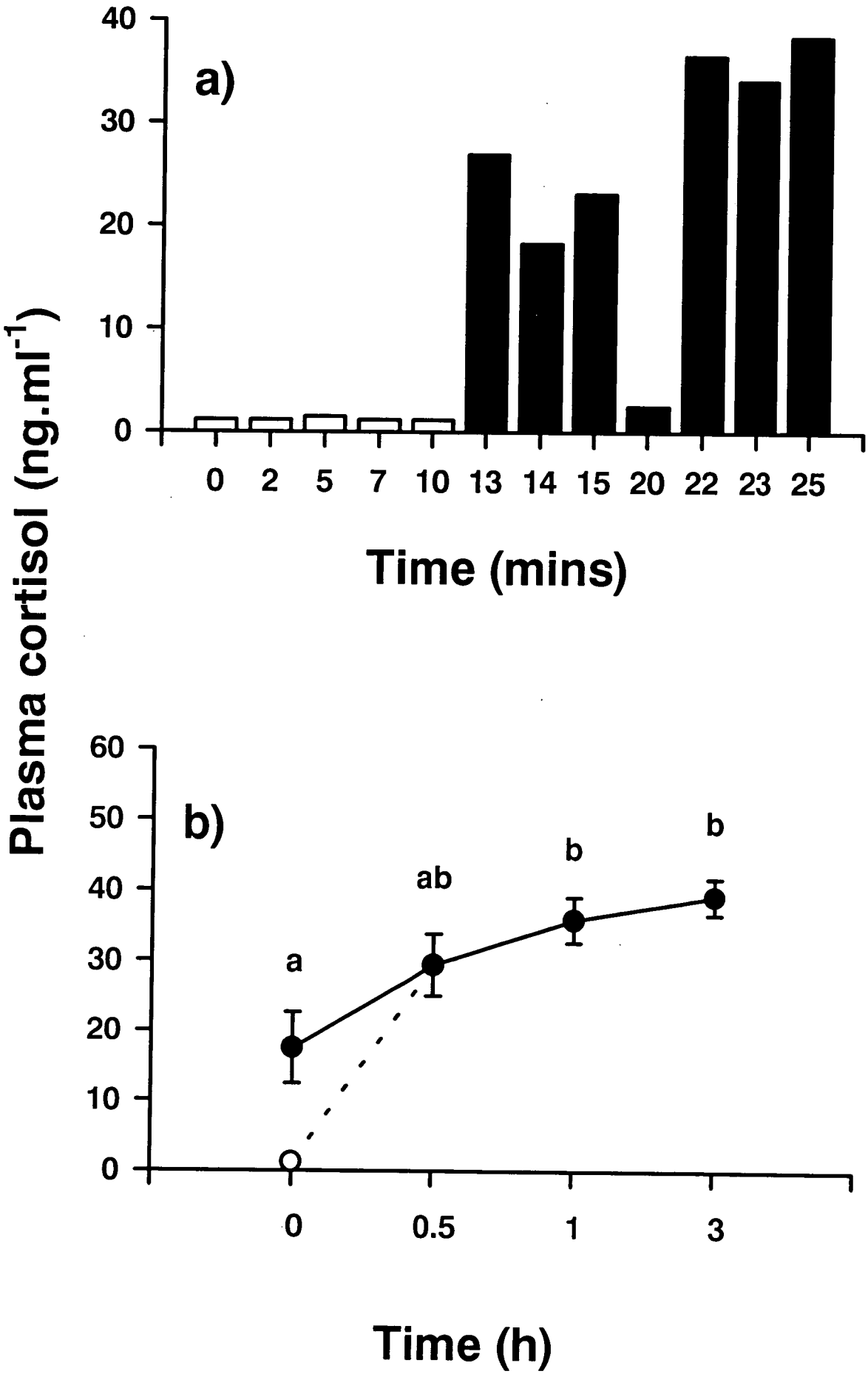

a)
40
30
20
10
0
0 2 5 7 10 13 14 15 20 22 23 25
Time (mins)
Plasma cortisol (ng.ml-1)
b)
60
50
40
30
20
10
0
a
ab
b
b
0 0.5 1 3
Time (h)

Fig. 2.4. Plasma levels of a) cortisol in fish held at low, medium or high stocking density (numbers denote order in which tanks were sampled) and b) cortisol in individual fish from these tanks in the order that fish were sampled. Values in a) are mean ± S.E. (n = 8). Values that are not significantly different ($P>0.05$) share common superscripts.

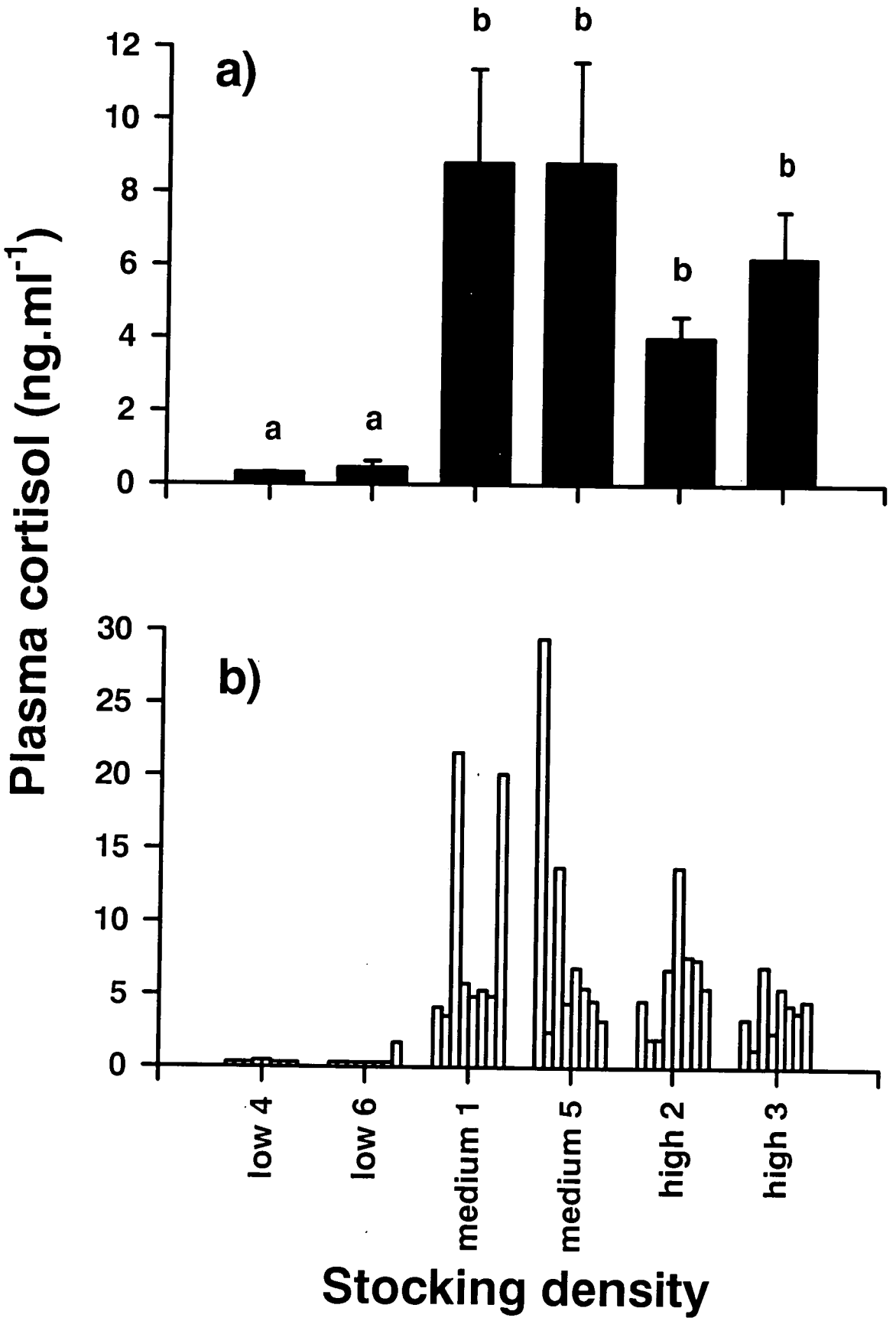

a)
b)
Plasma cortisol (ng.ml-1)
12
10
8
6
4
2
0
30
25
20
15
10
5
0
a
a
b
b
b
b
low 4
low 6
medium 1
medium 5
high 2
high 3
Stocking density

Fig. 2.5. Plasma levels of a) cortisol, b) lactate and c) $[H^+]$, in relation to 0.5 h exercise (experiment 5, effect of exercise). Values are mean ± S.E. (n = 7). Values that are not significantly different ($P>0.05$) share common superscripts.

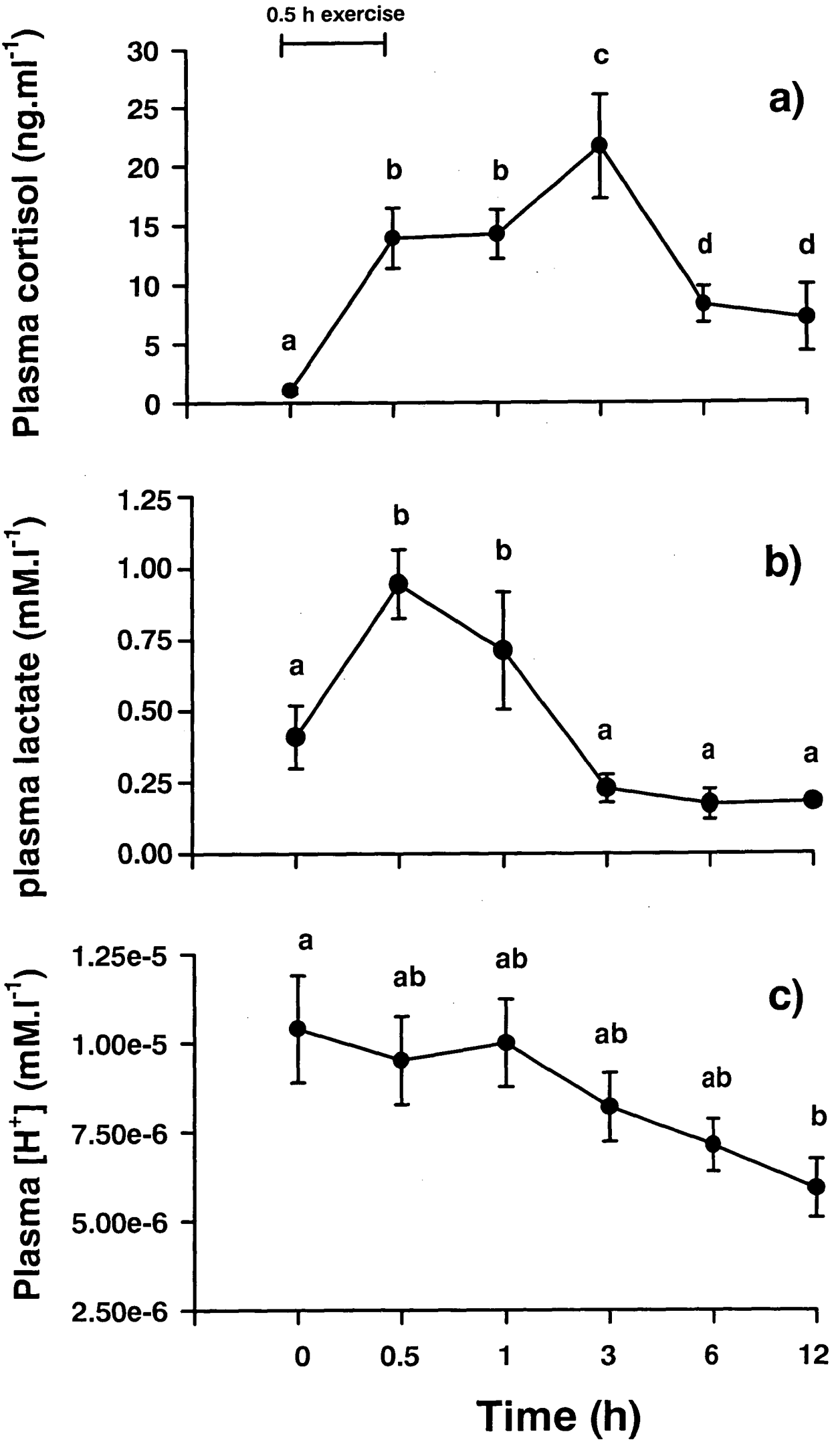
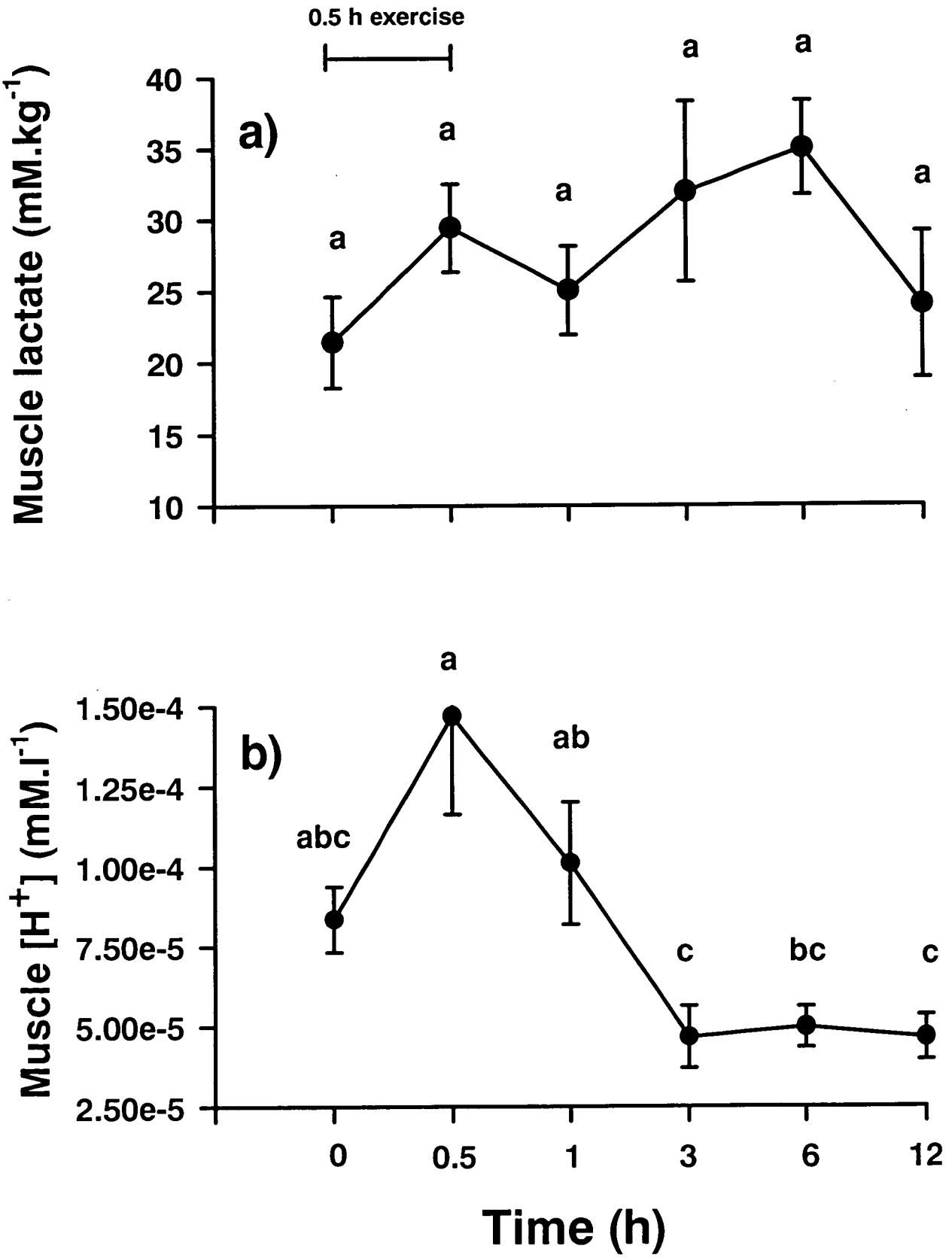

0.5 h exercise
Plasma cortisol (ng.ml-1)
30
25
20
15
10
5
0
a
b
b
c
d
d
a)
plasma lactate (mM.l-1)
1.25
1.00
0.75
0.50
0.25
0.00
a
b
b
a
a
a
b)
Plasma [H+] (mM.l-1)
1.25e-5
1.00e-5
7.50e-6
5.00e-6
2.50e-6
a
ab
ab
ab
ab
b
c)
0
0.5
1
3
6
12
Time (h)

Fig. 2.6. Muscle concentrations of a) lactate and b [H^+], in relation to 0.5 h exercise (experiment 5, effect of exercise). Values are mean ± S.E. (n = 7). Values that are not significantly different ($P>0.05$) share common superscripts.


0.5 h exercise
a)
Muscle lactate (mM.kg-1)
40
35
30
25
20
15
10
a
a
a
a
a
a
b)
Muscle [H+] (mM.l-1)
1.50e-4
1.25e-4
1.00e-4
7.50e-5
5.00e-5
2.50e-5
abc
a
ab
c
bc
c
0
0.5
1
3
6
12
Time (h)

2.5. *Discussion*

Plasma cortisol levels in wild fish sampled within 2 min of capture, were assumed to represent basal cortisol concentrations in unstressed fish. These levels are similar to plasma cortisol values in other species captured from the wild and sampled immediately (typically < 5 $ng.ml^{-1}$), and notably lower than levels in fish caught from the wild and sampled with some delay (reviewed in Pankhurst and Sharples, 1992). Plasma levels of cortisol were significantly higher in netted wild greenback flounder exposed to capture, confinement and transport, than in unstressed fish, indicating that commercial capture is highly stressful. Similarly, post stress plasma cortisol levels were high in wild plaice (*Pleuronectes platessa*) (158 $ng.ml^{-1}$) (White and Fletcher, 1989) and wild winter flounder (*Pseudopleuronectes americanus*) (83 $ng.ml^{-1}$) (Campbell. et al., 1976) caught from the wild and sampled after some delay. Reported pre-stress plasma cortisol levels in other teleosts are extremely variable, ranging from < 1 to 544 $ng.ml^{-1}$ (Barton and Iwama 1991). It is now clear that plasma cortisol levels at the higher end of the range are most likely to be a reflection of fish being stressed at the time of sampling, rather than real variation between species in basal cortisol levels (Wendelaar Bonga, 1997). Pre-confinement plasma cortisol levels obtained from greenback flounder during simulated grading with confinement (Fig. 1) were much higher than those recorded from fish in the second grading experiment (Fig. 2a), or resting levels measured in wild fish. Sampling of fish in experiment 1 took 25 min due to the difficulty of removing fish from the 4000 l community tank, and this appears to have generated the high cortisol levels recorded. This is confirmed by the subsequent pre-stress plasma cortisol levels measured during the repeat sampling experiment (Fig. 3), which indicated that serial sampling from a common tank induced a cortisol response in the remaining fish 10-13 minutes after first disturbance. This is similar to the response latency found in most other teleosts. Serial sampling from a common tank induced a stress response in <15 min in cultured red drum (*Sciaenops ocellatus*) (Robertson et al., 1988), 12-14 min in rainbow trout (Laidley and Leatherland, 1988), 15 min in young-of-the-year striped bass (*Marone saxatilis*) (Young and Cech, 1993) and 1-2 h in the sea raven (*Hemitriptrerus americanus*) (Vijayan and Moon, 1994). This indicates that appropriate measurement of resting cortisol levels in greenback flounder requires blood samples to be collected within 10 min of first disturbance. Sampling of resting fish in experiment 2, where low basal cortisol levels were recorded

took 5 min, and plasma cortisol levels were consistent with cortisol levels measured in unstressed wild greenback flounder and other species. This shows that domesticated greenback flounder exposed to maintenance conditions have low plasma cortisol levels and are presumably unstressed. Our results further demonstrate the necessity to critically evaluate sampling procedures designed to measure cortisol as a stress parameter.

Differential cortisol profiles were found in fish in the present study according to the type of stress imposed. Similarly other studies have shown that plasma cortisol response profiles can vary for a single species depending on severity and duration of the stress and experimental procedures used (Pickering and Pottinger, 1989; Barton and Iwama, 1991; Pankhurst and Sharples, 1992; Sumpter, 1997). Greenback flounder showed a slow return (48h) of cortisol to basal levels following a stress episode. This is towards the end of the range of recovery periods reported for other species. Plasma cortisol recovery profiles can vary between species and even strain, and tend to be related to the severity and type of stress (Pickering and Pottinger, 1989; Barton and Iwama, 1991; Sumpter 1997). Plasma cortisol levels returned to resting levels 1 h after 90 sec of net confinement in young-of-the -year-striped-bass (Young and Cech, 1993), ≈ 4 h after 30 sec emersion stress in brown trout (*Salmo trutta*) and rainbow trout (Pickering and Pottinger, 1989), 48 h in Atlantic salmon (*Salmo salar*), 24 h in the flounder (*Platichthys flesus*) and turbot (*Scophthalamus maximus*) after capture and 9 min net confinement (Waring et al., 1992, 1996), ≈ 8h for brown trout and ≈ 24 h for rainbow trout after handling and 1 h confinement (Pickering and Pottinger, 1989), 24 h after capture from the wild in rainbow trout (Pankhurst and Dedual, 1994), 24 h in domesticated brown trout following capture and handling (Pickering et al., 1982), 24 h in the sea raven after air exposure and chasing (Vijayan and Moon, 1994), and 48 h in snapper after capture from the wild and transport to the laboratory (Pankhurst and Sharples, 1992). In general terms, theses studies indicate that recovery from an acute stress takes place within 48 h, and even less if the stress is of short duration (minutes). In contrast, chronic stress such as long term confinement can result in significantly elevated plasma cortisol levels for periods of up to 4 weeks before acclimation occurs (Pickering and Pottinger, 1989).

The fact that crowding and confinement generated increases in plasma cortisol in greenback flounder suggests that as well as being stressed by husbandry practices, flounder could suffer stress from maintenance at inappropriate stocking densities. This was confirmed by demonstration that plasma cortisol levels were significantly lower in fish held at low stocking density, than in fish held at medium and high stocking density. Plasma cortisol also increased with increasing stocking density in chinook salmon (*Oncorhynchus tshawytscha*) (Mazur and Iwama 1993) and brown trout (Pickering and Pottinger, 1985), and plasma cortisol was significantly higher in red drum held at medium and high density, than at low density (Robertson et al., 1987). In the present study plasma cortisol levels did not vary significantly between medium and high stocking density. High plasma cortisol levels found in one or two fish in both medium density tanks. This response could not be attributed to the order that fish were sampled, but may have been related to the development of behavioural hierarchies in the tanks, at medium stocking density. A previous study on juvenile greenback flounder demonstrated the existence of feeding dominance hierarchies in fish held in small groups, even at high feeding rations, whereas feeding hierarchy strength was low at high stocking density (Shelverton, 1995). Studies on other species have demonstrated that plasma cortisol is negatively correlated with increasing social dominance in coho salmon (*Oncorhynchus kisutch*) (Ejike and Schreck, 1980) and European eels (*Anguilla anguilla*) (Hyde and Perry, 1990). Studies on rainbow trout have clearly demonstrated that plasma cortisol levels are significantly higher when fish are stocked in pairs, than in isolation or larger groups, apparently due to intense social interactions under such conditions (Laidley and Leatherland, 1988; Pottinger and Pickering, 1992). Brown trout and red drum acclimate to high population densities and this is accompanied by decreasing plasma cortisol levels (Pickering and Stewart, 1984; Robertson et al., 1987), and acclimation is faster at high stocking density than medium stocking density in red drum. Acclimation to stocking density in greenback flounder cannot be determined from our experiment because multiple samples were not taken during the 14 day confinement period, but in brown trout and red drum crowding resulted in significant elevation of cortisol levels for 25 and 48 days respectively (Pickering and Stewart, 1984; Robertson et al., 1987). Because the previous studies on greenback flounder showed that food acquisition was positively correlated with body size (Shelverton 1995), we tested whether smaller animals were characterised by having higher plasma cortisol levels. However, there was no correlation between

plasma cortisol and body weight, suggesting that if differential cortisol levels did arise from dominance interactions, these were not primarily related to fish size.

In other species, the effect of elevated cortisol on various physiological parameters is often inconsistent. Cortisol treatment decreased growth rates in channel catfish, *Ictalurus punctatus* and rainbow trout, whereas a handling protocol used to acutely elevate endogenous plasma cortisol daily, was insufficient to affect the growth rate of rainbow trout over a 10 week period (reviewed in Barton and Iwama, 1991). Both positive and negative effects of elevated cortisol levels on growth hormone (GH) have been reported (Barton and Iwama, 1991; Pankhurst and Van der Kraak, 1997; Wendalaar Bonga, 1997), hence the interaction between cortisol and GH requires further study. Many studies have reported that stress induced elevation in cortisol impaired reproductive function (reviewed in Barton and Iwama, 1991; Pankhurst and Van der Kraak, 1997; Wendalaar Bonga, 1997). However, there is conflicting evidence as to whether cortisol is responsible for the decrease in egg quality demonstrated by stressed fish, and the lack of a consistent effect of cortisol on ovarian steroidogenesis suggests that the effects of cortisol on reproductive function are indirect, possibly via cortisol-induced changes in metabolism or immunocompetence (reviewed in Pankhurst and Van der Kraak, 1997). Cortisol has generally been implicated in having inhibitory effects on the immune response. Stress induced elevation in plasma cortisol levels have been associated with reduced lymphocyte levels and antibody production and increased susceptibility to disease (reviewed in Barton and Iwama, 1991; Wendalaar Bonga, 1997), hence the negative effects of elevated cortisol on the immune response should not be ignored by the aquaculturist. The precise mechanisms of stress on growth and reproduction still requires further study. However, whether cortisol is the culprit or not, the important issue for aquaculturist, is that stressful husbandry practices do affect reproduction, growth and the immune response (reviewed in Barton and Iwama, 1991; Pankhurst and Van der Kraak, 1997; Wendalaar Bonga, 1997).

In common with the blue mao mao (*Scorpis violaceus*) (Pankhurst et al., 1992), greenback flounder showed no significant changes in Hct in response to stress. This is in contrast to a range of other species such as Parore (*Girella tricuspidata*) (Ling and Wells 1985), rainbow trout (Wells and Weber 1991), and young-of-the-year striped

bass (Young and Cech 1993), which showed significant increases in Hct in response to stress. As suggested with blue mao mao, this may indicate that adrenergic effects occur rapidly and Hct values already show some adrenergic effect which is maintained throughout the experiment (Pankhurst et al., 1992). Alternatively, stress may have limited impact on red blood cell size or splenic release in greenback flounder, possibly because enhanced O_2 carrying capacity and and/or hemoconcentration associated with high Hct, are of little consequence in less active, benthic dwelling species. This is supported by low Hct values prior to and following stress in flathead sole (*Hippoglossides elassodon*) (Turner et al., 1983) and starry flounder (*Platichthys stellatus*) (Wood et al., 1977) versus non-flatfish species (Ling and Wells 1985; Wells and Weber, 1991; Pankhurst et al., 1992;Young and Cech 1993). These results suggests that Hct is not a useful stress indicator in greenback flounder and possibly other flatfish species.

Plasma lactate in rested wild greenback flounder and unstressed or exercised cultured greenback flounder were within the range of resting levels found in other flatfish species (Dando, 1969; Wood et al., 1977; Wardle, 1978; Girard and Milligan, 1992; Waring et al., 1992, 1996). Exercise induced significant increases in plasma lactate levels 0.5 and 1 h post exercise, but the values were lower than plasma lactate increases after exercise protocols found in starry flounder (Wood et al., 1977), plaice (Wardle, 1978), and winter flounder (Girard and Milligan, 1992), but higher than post exercise plasma lactate increases found in flounder (*Platichthys flesus* L.) (Waring et al., 1992), and net confined turbot (Waring et al., 1996). Plasma levels of lactate in commercially caught greenback flounder were significantly higher than lactate levels in resting wild fish, approximately 10 fold higher than maximum plasma lactate levels measured in response to simulated grading and exercise, considerably higher than levels reported in other flatfish species after exercise protocols (Wood et al., 1977; Girard and Milligan, 1992; Waring et al., 1992) higher than plaice sampled immediately after trawling in one study (Dando, 1969), and similar to levels after trawling in another (Wardle (1978). The exact period of capture of greenback flounder in the seine net cannot be determined, but may have been up to 12 h, suggesting that extreme exercise conditions can induce considerable increases in plasma lactate. It has been reported that trout acclimated to 18°C have blood lactate levels approximately twofold higher than trout acclimated to 5°C (Kiefer et al., 1994). Wild greenback

flounder were caught from sea temperatures of 16 ± 1 °C which is higher than water temperatures in studies on other flatfish species (Wood et al., 1977; Wardle, 1978; Girard and Milligan, 1992), and may explain interspecific differences.

Greenback flounder that were chased for 30 min had plasma lactate levels of similar magnitude to fish exposed to 5 min of chasing and crowding, but post exercise recovery was more rapid. It is possible that fish became hypoxic during crowding and chasing, which in combination with anaesthesia (fish were not anaesthetised after 30 minutes of chasing) resulted in greater hypoxic stress. Fish exposed to simulated grading had considerably higher plasma cortisol levels than fish that were chased but not confined for 30 min. Cortisol is often associated with hyperglycemia (reviewed in Pankhurst and Van Der Kraak, 1997), and it is possible that the energy mobilising properties of cortisol increased the availability of blood glucose for lactate production during anaerobic respiration.

In teleost fish the major fate of lactate during recovery from exercise is via *in situ* glycogenesis within the white muscle, as opposed to the mammalian pattern of releasing lactate into the plasma for oxidative glyconeogenesis via the Cori cycle in the liver. In flatfish, the post exercise plasma lactate increase is small when compared to other more active teleost species (with the proviso that most of the data are from salmonids) (Turner et al., 1983a, b; Waring et al., 1992; Carragher and Rees, 1994; Pankhurst and Dedual, 1994), and *in situ* glycogenesis seems to be much more significant in flatfish species than in the more active teleost species (Wardle, 1978; Batty and Wardle, 1979; Turner et al., 1983b; Milligan and McDonald, 1988; Girard and Milligan, 1992). The proposed advantage of retaining lactate in the muscle rather than releasing it to the plasma, is to avoid possible loss of new glucose formed in the muscle, to other tissues, which may be important for sedentary species with a lower aerobic capacity. Retention of muscle lactate and *in situ* glycogenesis in most flatfish is associated with marked post-exercise increases in muscle lactate. This was not the case in greenback flounder where muscle lactate was not elevated above resting levels following stress and exercise. It is possible that the exercise protocol in this experiment was not sufficient to produce an anaerobic response, however, it was considerably longer than those used on plaice (Wardle, 1978) and winter flounder (Girard and Milligan, 1992). Resting levels of muscle lactate in greenback flounder appear to be

higher than resting levels reported in other flatfish species (Dando, 1969; Wardle, 1978; Girard and Milligan, 1992). It is also possible that the fish used in our experiment were exercised prior to the start of the experiment, and that muscle lactate levels were not indicative of real resting muscle lactate levels, but we consider this unlikely. Greenback flounder are most active at night time and during feeding, as indicated by increased oxygen consumption (personal communication, B. Crear, Department of Aquaculture, University of Tasmania), but it is unlikely that this activity would be sufficient to significantly elevate muscle lactate levels. Even if this were the case, we would have expected to see recovery of muscle lactate over the time course of the experiment (as in other flatfish species eg Girard and Milligan, 1992), and this did not occur.

The fact that muscle lactate did not increase post-exercise in greenback flounder, suggests that the low plasma lactate response was perhaps not due to muscle accumulation of lactate, and may mean that greenback flounder have quite high aerobic scope. This is supported by the fact that plasma lactate does increase to high levels under very extreme exercise or stress conditions as it did in netted wild fish. High aerobic scope is a promising characteristic for aquaculture of greenback flounder, as lactate accumulation due to anaerobic exercise has been linked to post-capture mortality (Graham et al., 1982; Ferguson and Tuft, 1992) and poor flesh quality in other species (Wells et al., 1986; Watabe et al., 1991; Lowe et al., 1993). Alternatively, fish size is reported to affect exercise metabolism in rainbow trout, with larger fish producing more muscle lactate post-exercise than small fish (Pearson et al., 1990). Laboratory fish used in our experiment were considerably smaller than the wild greenback flounder and wild fish used in other flatfish studies (Wood et al., 1977; Wardle, 1978; Girard and Milligan, 1992). It is also possible that cultured greenback flounder react differently to exercise conditions than wild fish. Studies on wild populations of salmonids have reported low mortality following capture, whereas exercise in laboratory stocks result in much higher mortality (reviewed in Pankhurst and Dedual, 1994).

Plasma $[H^+]$ gradually decreased following stress and exercise and was significantly lower than pre-exercise levels 12 h after exercise in greenback flounder. These results are in contrast to studies on other flatfish, in which there were significant increases in

plasma [H^+] after exercise in the flathead sole (Turner et al., 1983b) and starry flounder (Wood et al., 1977). A number of teleost species show differential release of protons and lactate from muscle (Turner et al., 1983b; Schwalme and Mackay, 1985; Waring et al., 1992). For example, in the flathead sole, metabolic plasma [H^+] was much higher than plasma lactate, indicating that protons were released to the plasma at a faster rate than lactate, suggesting that lactate was retained in the muscle for *in situ* recycling. The reverse appears to occur in greenback flounder, with muscle [H^+] peaking in concert with post-exercise increases in plasma lactate.

This study indicates that some routine husbandry procedures elicit stress responses in greenback flounder. Whether or not this has an impact on productivity has yet to be determined. Further studies need to be carried out to determine the impact of stressful husbandry practices on growth, reproduction and the immune response of greenback flounder. Until this information becomes available, stress management should be considered an important component of technology development for this emerging aquaculture species.

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Chapter 3

Reproductive biology and endocrinology of greenback flounder *Rhombosolea tapirina* (Günther, 1862).

3. Reproductive biology and endocrinology of greenback flounder *Rhombosolea tapirina* (Günther, 1862).

3.1. Summary

The relationship between patterns of gonadal development and endocrine changes during reproductive development was examined in the greenback flounder (*Rhombosolea tapirina*). Different female macroscopic gonad stages were characterised by statistically significant differences in GSI. Oocyte size frequency distributions and histological examinations showed that female greenback flounder have group synchronous oocyte development, and that there are multiple ovulations. Plasma and ovarian levels of testosterone (T) and 17ß-estradiol (E_2) were elevated in association with vitellogenesis. Plasma levels of 17α20ß-dihydroxy-4-pregnen-3-one (17,20ßP) were significantly elevated in ovulated females, whereas ovarian levels of 17,20ßP were elevated in association with final oocyte maturation (FOM) and ovulation. Plasma levels of 17,20ßP-sulphate but not 17,20ßP-glucuronide were elevated in association with FOM and ovulation. Changes in macroscopic testis stage were not accompanied by significant changes in GSI, and histological examination of testes showed very few changes in proportions of gamete types between gonad stages. Plasma levels of T were elevated in spermatogenic and partially spermiated males. No significant changes in plasma 11-ketotestosterone (11KT), 17,20ßP, 17,20ßP-sulphate and 17,20ßP-glucuronide were detected with change in gonad stage of males.

3.2. Introduction

The greenback flounder *Rhomobosolea tapirina* (Pleuronectidae) is distributed throughout the waters surrounding southern Australia and New Zealand (Ayling and Cox 1982). Approximately 140 t per annum are harvested commercially in southern Australia (Kailola et al., 1993). Although there is considerable interest in developing aquaculture of greenback flounder in Tasmania (Hart 1993) and pilot aquaculture schemes are underway, egg production and egg quality have been variable (Crawford, 1984; Hart, 1991; Hart and Purser, 1995). Successful management depends on an understanding of the physiological mechanisms initiating and mediating gonadal

development. Previous studies have indicated that the species has a prolonged reproductive season, from March to October (Kurth 1957; Crawford 1984), is a multiple spawner and has a fecundity per gram body weight ranging from 4343 - 5250 (Crawford 1984); however, nothing is known about the reproductive endocrinology of greenback flounder.

Changes in reproductive condition were examined in relation to plasma levels of the gonadal steroids testosterone (T), 17ß-estradiol (E_2), 17α20ß-dihydroxy-4-pregnen-3-one (17,20ßP), and 11-ketotestosterone (11KT). These steroids were chosen for measurement because they are markers of reproductive events in other teleost species. E_2 and T are commonly measured in females as indicators of ovarian development (Pankhurst and Carragher 1991), whereas 17,20ßP is the maturation inducing steroid in a number of teleost species (reviewed in Scott and Canario 1987). In male teleosts, elevated plasma levels of T are often associated with spermatogenesis (Fostier et al., 1987; Pankhurst and Conroy 1987, 1988; Harmin et al., 1995). 11KT is also suggested to play a role in spermatogenesis (Scott et al., 1984; Dedual and Pankhurst 1992; Borg 1994; Harmin et al., 1995) and possibly spermiation (Fostier et al., 1987; Pankhurst and Conroy 1987; Carolsfeld et al., 1996). 17,20ßP is often elevated in association with spermiation (Scott et al., 1984; Fostier et al., 1987; Carolsfeld et al., 1996), and treatment of males of some species with 17,20ßP generates an increase in milt volume (reviewed in Pankhurst 1994).

Steroids may be metabolised or conjugated soon after production with the result that free steroids may be difficult to detect (Scott and Vermeirssen 1993). Conjugated steroids are formed when hydroxyl groups of steroids are conjugated with glucuronic acid (glucuronides) or sulphuric acid (sulphates). These steroid derivatives are more water soluble than the parent steroids, and are therefore readily excreted in the bile and /or urine (Scott and Vermeirssen 1993). Conjugated steroids can be measured from blood plasma or urine and may provide information regarding key biologically active steroids that are difficult to detect in blood plasma because they are released in a pulsatle fashion, or in small amounts, or are rapidly metabolised (Scott and Vermeirssen 1993). Accordingly, we also measured plasma levels of the steroid conjugates 17,20ßP-sulphate and 17,20ßP-glucuronide, for comparison with changes in plasma 17,20ßP and reproductive condition.

Most studies that investigate reproductive steroid cycles in teleosts measure levels of steroids from the plasma (reviewed in Pankhurst and Carragher, 1991). A few studies, however, have demonstrated that plasma and ovarian steroid levels are not always correlated (Bradford and Taylor 1987; Singh and Singh 1987; Hobby and Pankhurst 1997), probably because of the potential for metabolism and conjugation of free steroid by the processes discussed above. To provide a more thorough assessment of reproductive events in greenback flounder, in this study we measured both plasma and ovarian levels of T, E_2, and 17,20ßP.

It is well established that fish are stressed by capture and handling (Billard et al., 1981; Barton and Iwama 1991; Pickering 1992) and stress has the capacity to suppress plasma levels of E_2 and T (Carragher and Pankhurst 1991; Clearwater and Pankhurst 1997). Plasma cortisol is commonly measured as an indicator of physiological stress (Donaldson 1981; Barton and Iwama 1991). In this study, to evaluate the possible effect of capture stress on plasma steroid levels, we measured plasma levels of cortisol, E_2 , T and 17,20ßP in a subsample of fish sampled within 2 mins of capture (assumed to represent resting cortisol levels) for comparison with values from the majority of fish, which experienced commercial capture.

3.3. Materials and Methods

Fish were obtained by commercial gill netting from George's Bay (148° 16' E, 41° 19' S) and the Tamar River (147° 45' E, 41° 19' S), on the east and north coast of Tasmania respectively. Additional fish were obtained by hand spear while snorkelling or hand netted by scuba divers from Georges Bay and Bicheno (148° 18' E, 41° 52' S) on the east coast of Tasmania. Fish caught while snorkelling were sampled on the boat, and fish sampled by scuba divers were sampled under water using the technique given by Pankhurst (1990), both within 2 mins of capture. Blood samples were collected by caudal puncture. Fish were collected between July 1994 and September 1996 (Table 3.1). Total body weight, gonad weight, liver weight and macroscopic gonad stage were recorded for all fish. Gonadosomatic index (GSI) was calculated as (gonad weight / body weight) x 100. Oocyte diameters, histological samples and blood samples were collected from fish deemed representative of the population.

3.3.1. Gonad staging

Criteria for staging of whole gonads are given in Table 3.2. Oocyte diameters were measured from fresh ovarian tissue randomly selected from the ovaries (oocyte diameters did not differ significantly between left and right gonads, or between anterior, middle and posterior positions in the left and right gonad, data not shown). The tissue was separated by gentle aspiration through a glass pipette and spread throughout a sorting tray. Oocytes were classified as primary, cortical alveoli, vitellogenic, germinal vesicle migration (GVM) and germinal vesicle breakdown (GVBD), hydrated or atretic. Primary and cortical alveoli oocytes were very abundant but were not sampled representatively. Instead, a standard sample of 100 primary and cortical alveoli oocytes was measured under a dissecting microscope, whereas two hundred and fifty vitellogenic and mature oocytes were measured as encountered. Classifications of oocyte staging were verified by clearing oocytes of each type in sera solution (ethanol:foramlin:acetic acid, 6:3:1 v/v), to determine the presence or position of the germinal vesicle (GV). Vitellogenic oocytes had a centrally located GV, mature oocytes had an eccentric GV or had undergone GV migration and breakdown. Atretic oocytes did not clear in sera solution. Macroscopic staging of gonads was verified by examination of ovarian and testicular sections prepared using standard paraffin wax histology (Fig. 3.1). Hisological sections were stained with haemotoxin eosin stain. Oocyte stages were assigned according to criteria derived from Wallace and Selman (1981), Janssen et al., (1985) and Clearwater and Pankhurst (1997) (Table 3.3). Spermatogenic stages were assigned according to criteria derived from Grier (1981) (Table 3.3), and frequency of male gamete types were recorded from under the intercepts of a 64 point grid (640 measurements per section).

3.3.2. Steroid measurement

Plasma levels of cortisol, T, and 17,20ßP, were measured for males and females, 11KT for males and E_2 for females by radioimmunoassay (RIA). One hundred microlitres of plasma were extracted with 1 ml of ethyl acetate and 100µl of extract were added to each assay tube for evaporation and resuspension in assay buffer, using the reagents and protocol given in Pankhurst and Conroy(1987). Extraction efficiency was determined by recovery of [^{3}H]-labelled steroid extracted with plasma, and was on average 95%, 92%, 95%, 82% and 84% for cortisol, T, 17,20ßP, 11KT and E_2 respectively. Assay values were corrected accordingly. Assay detection limits were 0.6,

0.28, 0.29, 0.25 and 0.25 ng.ml^{-1} plasma for cortisol, T, 17,20ßP, 11KT and E_2 respectively. Steroid levels in ovarian tissue were measured by homogenising 0.125 g of tissue in 1 ml of ethyl acetate, and centrifuging the homogenate at 3000 g for 10 minutes. One hundred microlitres of extract were measured in assays as before. Glucuronide and sulphate conjugates, were measured by mixing 100 µl of plasma with 1 ml of ethyl acetate in a stoppered test tube, shaking, centrifuging for ten minutes at 800 g, freezing at -80°C for 1 hr and pouring off the solvent fraction. 17,20ßP-glucuronide was determined by treating the aqueous residue with 50 µl of ß-glucuronidase (Sigma) (10,000 U.mL^{-1}) and incubating for 24 hr at 37°C. A further 50 µl of ß-glucuronidase were added to the residue and the mixture incubated for another 24 hr at 37°C. Free steroid liberated was extracted with ethyl acetate and measured as before. 17,20ßP-sulphate was determined by acid solvolysis using reagents and protocols given in Scott and Canario (1992), except HCl/ethyl acetate (1/100, v/v) was used instead of trifluroacetic acid/ ethyl acetate. The residue was redissolved in 100 µl of assay buffer and free steroid was measured as before. Interassay variability was measured using a 100 µl pooled steroid standard giving %CV's of 9% (n=4), 9% (n=5), 18.5% (n=9) and 12% (n=5) for cortisol, T, 17,20ßP and E_2 respectively. 11KT was measured in a single assay.

3.3.3. Statistical analysis

Analysis of variance, mutivariate analysis of variance (MANOVA), mean comparison tests and correlations were performed using the computer package SPSS for Windows and JMP for Macintosh. Data were log or square root transformed to satisfy normality and homogeneity of variance requirements. A significance level of $\alpha<0.05$ was used for all statistical test

Table 3. 1. Date of capture and number of fish collected of each macroscopic gonad stage.stage.

Sex	Stage	Date of capture								
		July 94	Aug 94	Sept 94	Oct 94	Aug 95	April 96	June 96	Aug 96	Sept 96
Female	1	1								
	2						2			
	3	18	12	8	5	2		6	2	2
	4			4					1	3
	5			5						7
	6	3	11	4	3			3		
Male	1				1					
	2	3	2	3	2			1		
	3			1	8			2	2	
	4		2		3	1		2	1	

Table 3. 2. Criteria for macroscopic staging of greenback flounder gonads

Modified from Crawford 1984; Scott et al., 1993; Barnett and Pankhurst 1994.

Sex	Stage	Classification	Macroscopic appearance
Female	1	Immature	Ovary clear thread
	2	Regressed	Ovary small, semi-firm, grey-orange
	3	Vitellogenic	Ovary large, yellow-orange, vitellogenic oocytes may be visible through the epithelium
	4	Final oocyte maturation	Ovary large and plump, yellow-orange. Vitellogenic and hyaline oocytes visible through the epithelium
	5	Ovulated	Oocytes can be freely expelled from the oviduct with gentle pressure.
	6	Spent	Ovary flaccid, grey, degenerating ooctyes or no oocytes visible
Male	1	Immature	Testis translucent thread
	2	Spermatogenic	Testis small firm white thread
	3	Partially spermiated	Testis firm, white and viscous milt expressible under pressure
	4	Fully spermiated	Testis plump, firm, white and milt flows freely under gentle pressure
	5	Spent	Testis bloody and flaccid, no milt expressible

Fig. 3.1. Micrographs of histological sections from greenback flounder ovaries and testes, showing (a and b) stage 3 ovary, (c) stage 4 ovary, (d) stage 3 testis. CA = cortical alveolar, CN = chromatin nucleolar, GVM = germinal vesicle migration, n = nucleus, PN = perinucleolar, SC1 = primary spermatocytes, SC2 = secondary spermatocytes, SPD = spermatids, SPZ = spermatozoa, V = vitellogenic. Scale bars (a-c) = 100 μm, (d) = 10 μm.

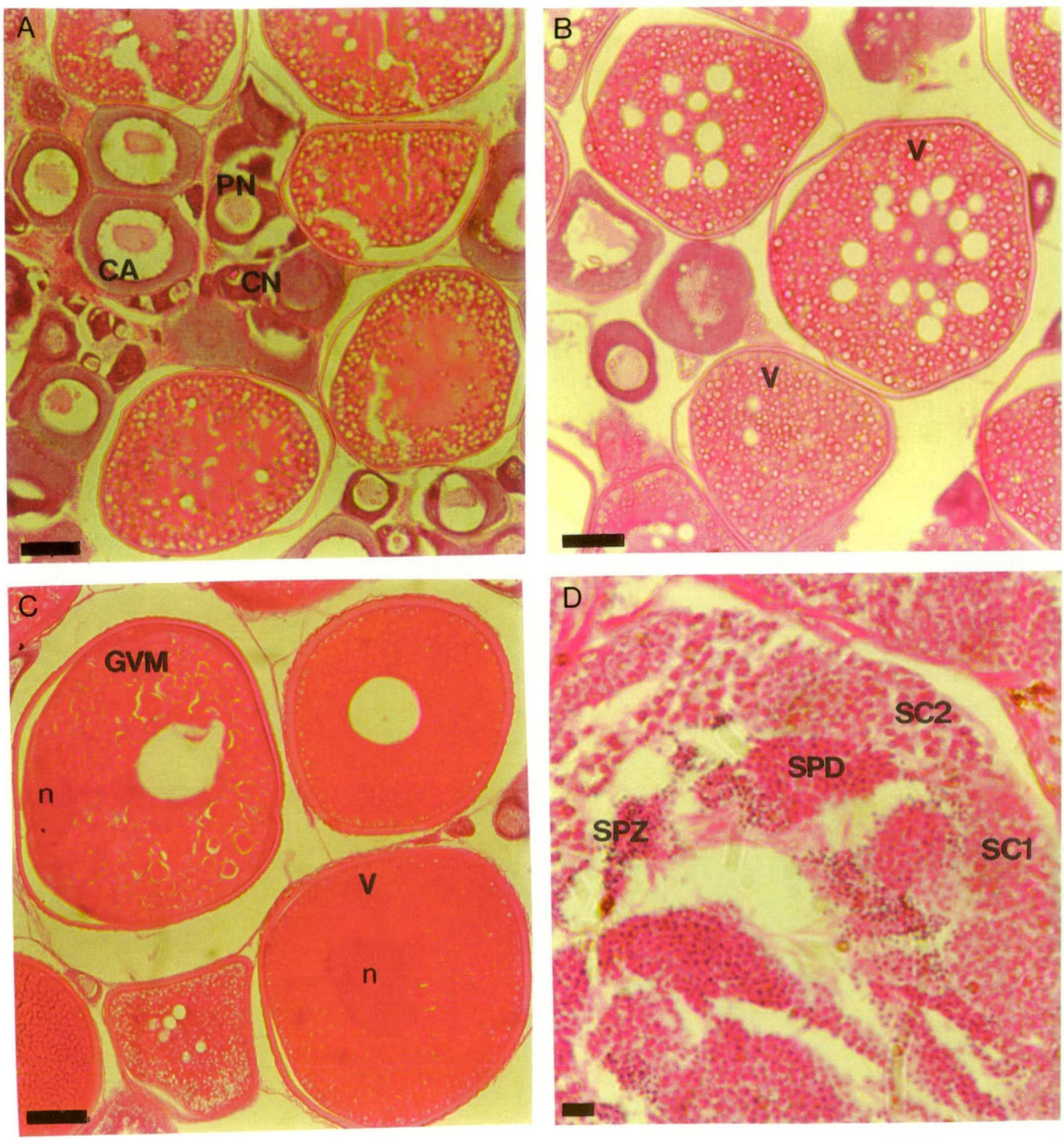
A
PN
CA
CN
B
V
V
C
GVM
n
V
n
D
SC2
SPD
SPZ
SC1

Table 3. 3. Criteria for histological staging of greenback flounder gonads modified from Grier 1981; Wallace and Selman 1981; Janssen et al., 1995; Clearwater and Pankhurst 1997.

FEMALES		
Cell classification	Diameter (μm)	Description
Chromatin nucleolar	10-60	Low cytoplasm: nucleus ratio. Single dark staining nucleolus within light staining nucleus. Cytoplasm stains dark purple.
Perinucleolar	50-120	Nucleus contains multiple dark staining nucleoli around the periphery. Cytoplasm stains light purple.
Cortical alveolar	120-240	Nucleoli are closely associated with the nuclear membrane. Yolk vesicles appear within the cytoplasm. Zona radiata becomes visible beneath the follicle layer.
Vitellogenic	200-450	Oocyte increases in size. Red yolk granules increase in size and number within the cytoplasm. Zona radiata becomes thicker and stains red.
Germinal vesicle migration	350-490	Nucleus moving towards animal pole.
Hydrated	470-600	Oocyte much enlarged, no nucleus present, but single oil droplet may be visible. Cytoplasm appears red-pink, follicle layer stretched to a thin layer.
Postovulatory follicles		A single layer of granulosa cells may appear as a thin strip or an irregularly shaped mass.
Atretic		Loss of cellular organisation and spherical shape. Granular inclusions and vacuoles in cytoplasm.
MALES		
Cell classification	Diameter (μm)	Description
Spermatogonia	5-7	Largest cell visible in the testis, light staining with visible nucleus.
Primary spermatocytes	3-4	Light staining, granular appearance.
Secondary spermatocytes	3-4	Dark staining with dense nucleus.
Spermatids	1.5	Small cell with dense staining nucleus and clear cytoplasm.
Spermatozoa	<1	Small dense staining heads, tails often visible. Found in luminal ducts in the testis.

3.4. Results

Seventy four percent of the fish collected were females. Most of the female fish collected were stage 3 (vitellogenic), and stage 6 (spent) females were next most abundant. Very few stage 4 (final oocyte maturation (FOM)) or stage 5 (hydrated) females were encountered, and only during September 1994 and August-September 1996. Only two stage 2 (resting) females and one stage 1 (immature) female were captured. Most of the male fish were stage 3 (partially spermiated). Stage 2 (spermatogenic) males were next most abundant followed by stage 4 (fully spermiated) males. Only one immature male, and no spent males were found.

The mean GSIs for stage 3, 4 and 5 females (mean ± S.E. of 13.2 ± 0.6, 18.3 ± 3.5 and 15.3 ± 2.9 respectively) were significantly higher than the mean GSI for stage 6 females (4.3 ± 0.3), and the mean GSI for stage 4 females was significantly higher than the mean GSI for stage 3 females, but there was no difference in GSI between stage 4 and 5 females. There were no significant differences in mean GSI between male gonad stages (mean ± S.E. of 1.16 ± 0.18, 1.21 ± 0.07 and 1.43 ± 0.14) for stage 2, 3 and 4 males respectively.

Size-frequency distributions of oocytes showed that oocyte development is group synchronous (Fig. 3.2). Stage 3 ovaries were characterised by the highest proportions of vitellogenic oocytes which were recruited into FOM in stage 4 and 5 ovaries. Stage 4 ovaries contained some hydrated oocytes, and were the only ovarian stage to contain oocytes undergoing GVM and GVBD. Stage 5 ovaries were characterised by high proportions of hydrated oocytes. Both stage 4 and 5 ovaries contained only small numbers of vitellogenic oocytes. Stage 6 ovaries contained a large population of degenerating vitellogenic oocytes undergoing atresia. All ovarian stages had large proportions of primary and cortical alveoli stage oocytes.

Changes in macroscopic stages of ovaries from female greenback flounder were accompanied by significant changes in the proportions of oocyte types recorded from histological sections (MANOVA - Pillais trace statistic: Prob>F = 0.0001) (Fig. 3.3). Proportions of oocyte stages from histological sections were similar to proportions of oocytes from fresh tissue and also indicated group synchronous oocyte development. Stage 3 ovaries were characterised by the highest proportions of vitellogenic oocytes,

stage 4 ovaries contained small proportions of pre-vitellogenic and vitellogenic oocytes, large proportions of hydrated oocytes and were the only stage to contain oocytes undergoing GVM. Stage 5 ovaries were characterised by hydrated oocytes and stage 6 ovaries were characterised by higher proportions of atretic oocytes. All ovarian stages contained pre-vitellogenic and vitellogenic stage oocytes.

There were no statistically significant changes in the proportions of gamete stages between male gonad stages (MANOVA - Pillais trace statistic: Prob>F = 0.4486), (Fig. 3.4).

Different macroscopic stages of gonads in female greenback flounder were characterised by significant changes in plasma and ovarian levels of T, E_2 and 17,20ßP (Fig. 3.5) and plasma levels of 17,20ßP-sulphate, but not 17,20ßP-glucuronide (Fig. 3.6a). Stage 3 females had significantly higher plasma and ovarian tissue levels of T and E_2 than stage 4, 5 or 6 females. There were no significant differences in plasma or ovarian tissue levels of T or E_2 between stage 4, 5 and 6 females. Plasma levels of 17,20ßP were significantly higher in stage 5 females, than in stage 3, 4 or 6 females, but there were no significant differences in plasma levels of 17,20ßP between stage 3, 4, and 6 females (Fig. 3.5a) . Ovarian levels of 17,20ßP were significantly higher in stage 4 and 5 females than stage 3 females, but there was no significant difference in ovarian levels of 17,20ßP between stage 4, 5 and 6 females or between stage 3 and 6 females (Fig. 3.5b). Stage 4 and 5 females had significantly higher plasma levels of 17,20ßP-sulphate, than stage 3 or 6 females, and there was no significant difference in plasma levels of 17,20ßP-sulphate between stage 3 and 6 females and stage 4 and 5 females.

Changes in male gonad stage were accompanied by significant changes in T, but not 11KT, 17,20ßP (Fig. 3.7), 17,20ßP-sulphate or 17,20ßP-glucuronide (Fig. 3.6b). Plasma levels of T were significantly higher in stage 3 males than stage 4 males, but there was no significant difference in plasma levels of T between stage 2 and 3 or stage 2 and 4 males.

Plasma levels of cortisol were lower in stage 3 and 6 females sampled within 2 mins of capture compared with commercial capture (Table 3.4). Plasma levels of T, E_2 and

17,20ßP were higher in rapidly sampled stage 3, but not stage 6 females than commercially caught stage 3 females.

Fig. 3.2. Frequency of oocyte diameters from each macroscopic ovarian stage (see materials and methods for details on sampling procedure). Sample sizes are shown in parentheses.

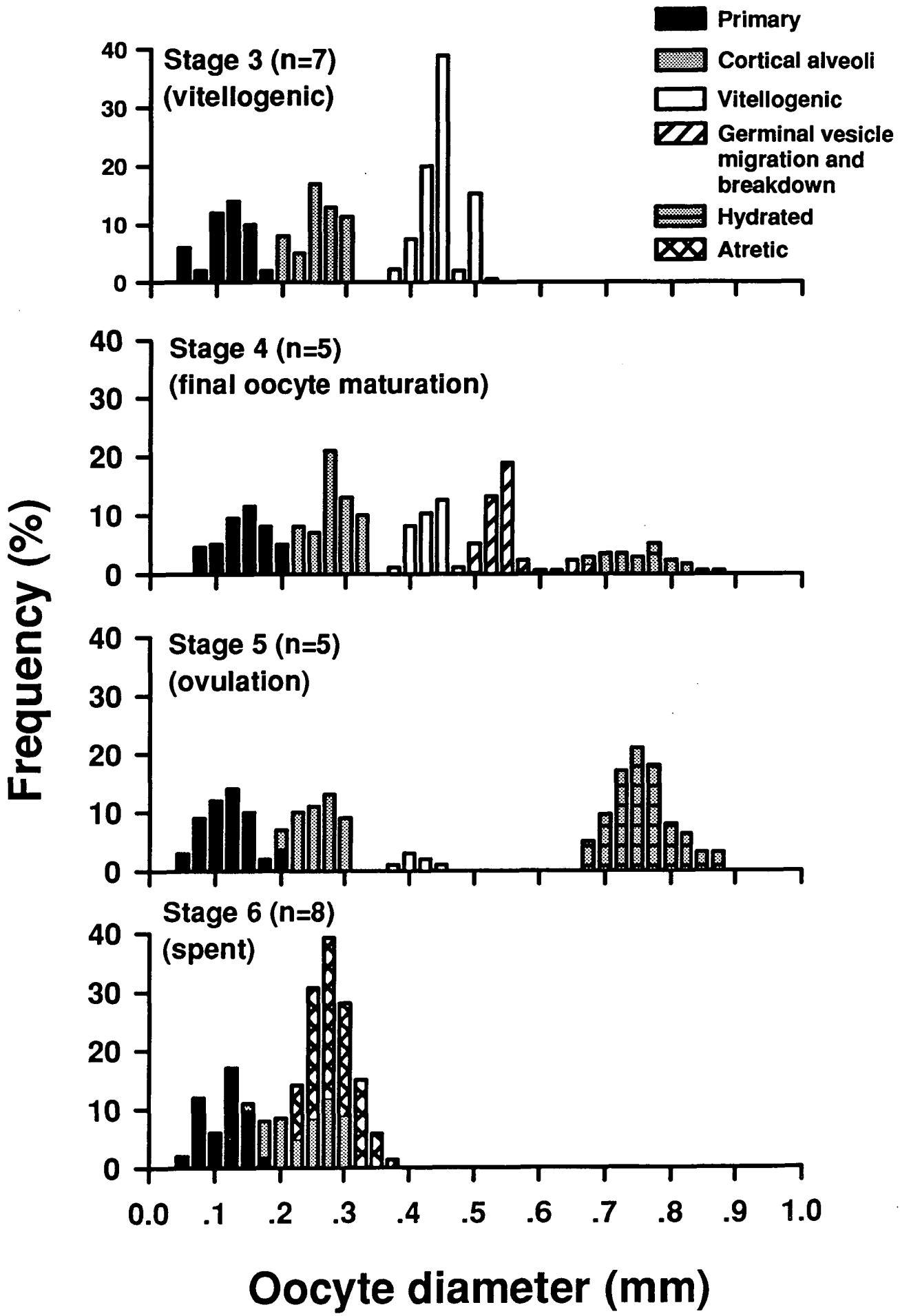
Primary
Cortical alveoli
Vitellogenic
Germinal vesicle migration and breakdown
Hydrated
Atretic
Stage 3 (n=7)
(vitellogenic)
Stage 4 (n=5)
(final oocyte maturation)
Stage 5 (n=5)
(ovulation)
Stage 6 (n=8)
(spent)
Frequency (%)
0
10
20
30
40
0.0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
Oocyte diameter (mm)

Figure 3.3. Frequency of oocyte stages in histological sections of each macroscopic ovarian stage. Values are means + S.E. Sample sizes are shown in parentheses. AT = atretic, CA = cortical alveolar, CN = chromatin nucleolar, EF = evacuated follicles, GM = germinal vesicle migration, HY = hydrated, PN = perinucleolar, V = vitellogenic. Oocyte types are described in Table 3.3.

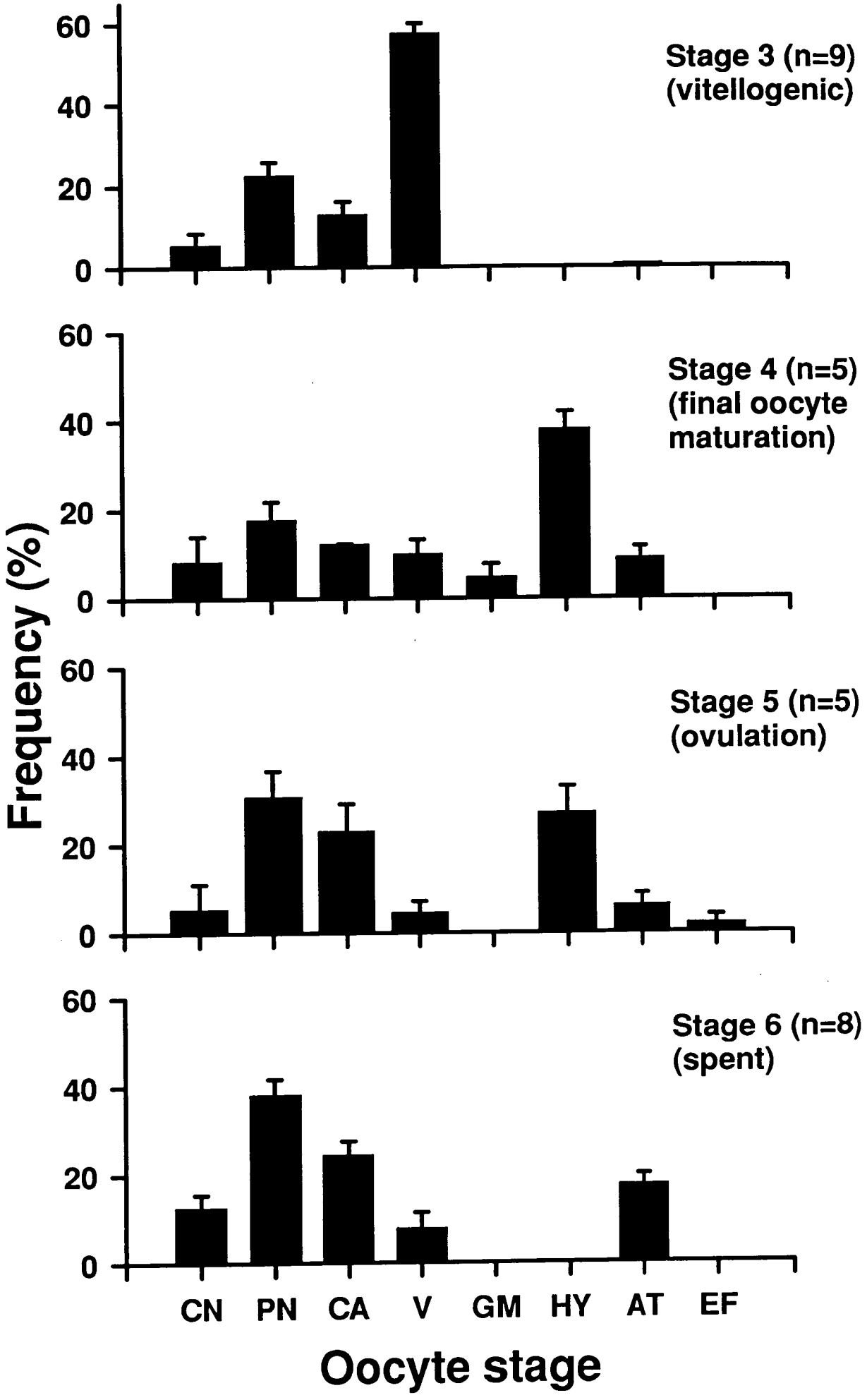
Stage 3 (n=9)
(vitellogenic)
Stage 4 (n=5)
(final oocyte
maturation)
Stage 5 (n=5)
(ovulation)
Stage 6 (n=8)
(spent)
Frequency (%)
60
40
20
0
CN
PN
CA
V
GM
HY
AT
EF
Oocyte stage

Fig. 3.4. Frequency of male gamete stages in histological sections of each macroscopic testicular stage. Values are means + S.E. SC1 = primary spermatocytes, SC2 = secondary spermatocytes, SPD = spermatids, SPZ = spermatozoa. Gonad stages are described in Table 2.

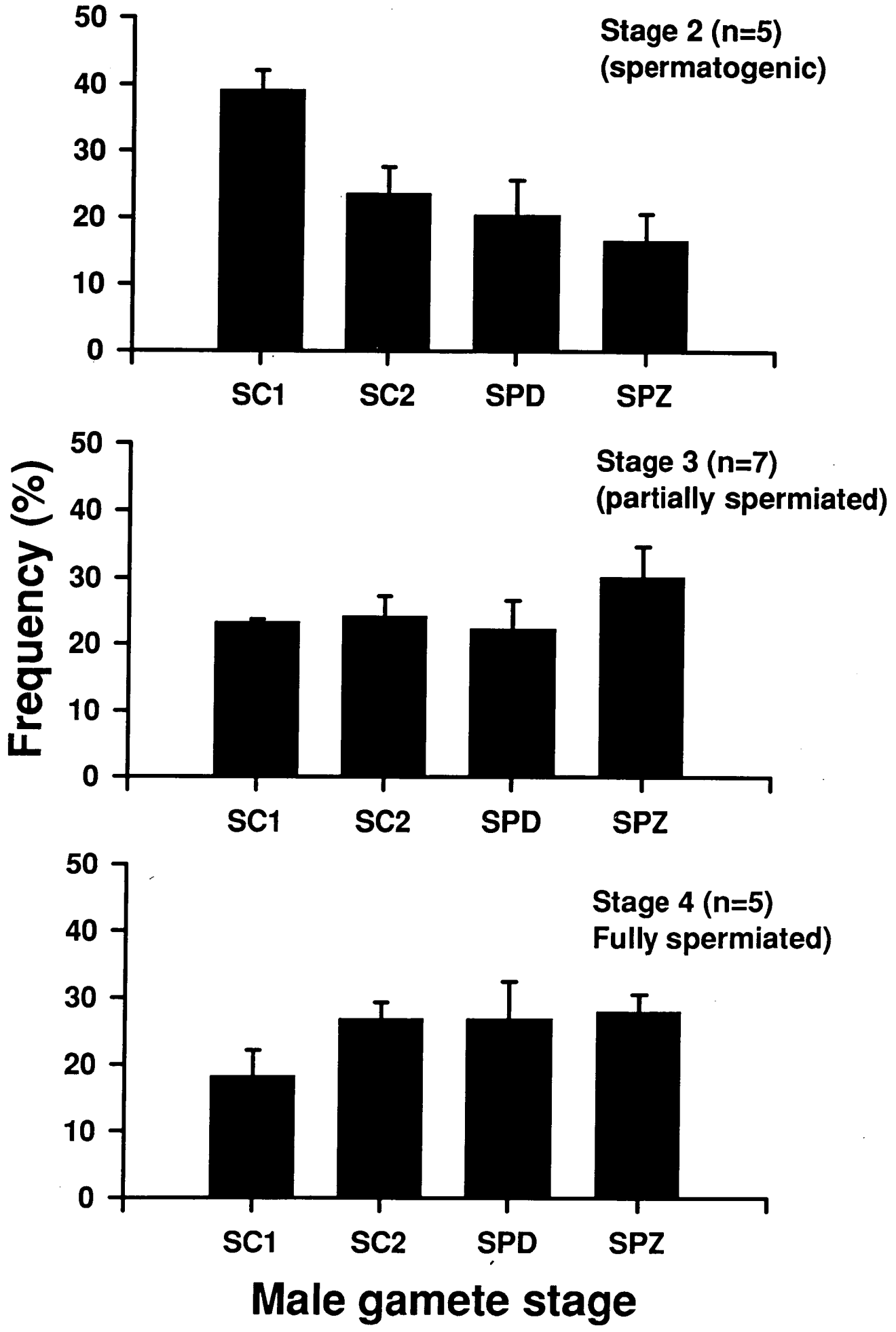
Stage 2 (n=5)
(spermatogenic)
Stage 3 (n=7)
(partially spermiated)
Stage 4 (n=5)
Fully spermiated)
Frequency (%)
50
40
30
20
10
0
SC1
SC2
SPD
SPZ
Male gamete stage

Fig. 3.5. (a) Plasma and (b) ovarian levels of reproductive steroids in relation to female gonad stage. Values are mean + S.E. F3, F4, F5 and F6 = female gonad stages three (n = 42), four (n = 7), five (n = 12) and six (n = 18) respectively. Gonad stages are described in Table 2. Values that are not significantly different ($P>0.05$) share common superscripts.

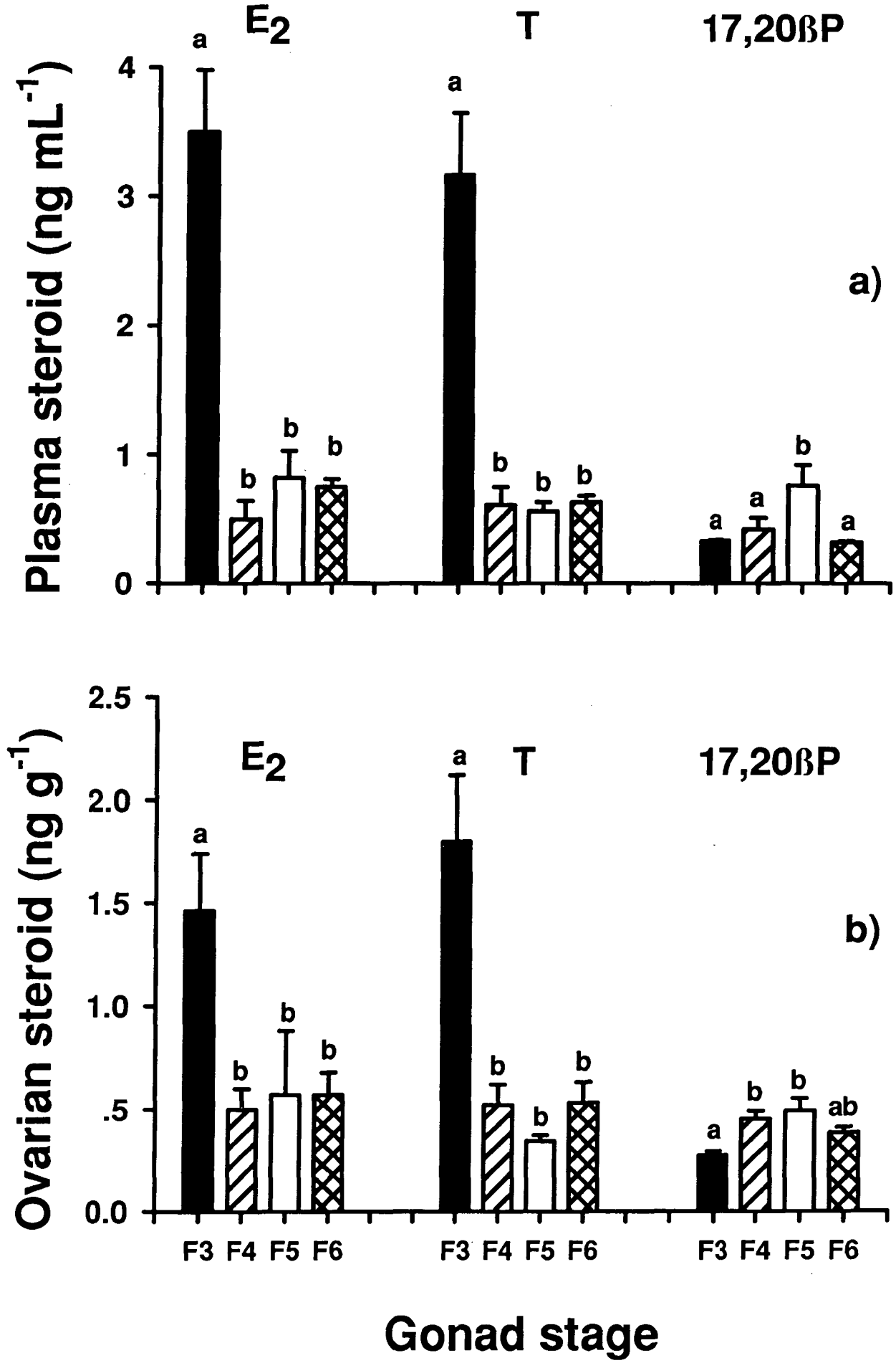
E2
T
17,20ßP
Plasma steroid (ng mL-1)
a)
Ovarian steroid (ng g-1)
b)
F3 F4 F5 F6
Gonad stage

Fig. 3.6. Plasma levels of 17,20ßP conjugates in relation to a) female and b) male gonad stage. Values are mean + S.E. F3, F4, F5 and F6 = female gonad stages three (n = 42), four (n = 7), five (n = 12) and six (n = 18) respectively. M2, M3 and M4 = male gonad stages two (n = 8), three (n = 10) and four (n = 9) respectively. Gonad stages are described in Table 2. Values that are not significantly different (P>0.05) share common superscripts.

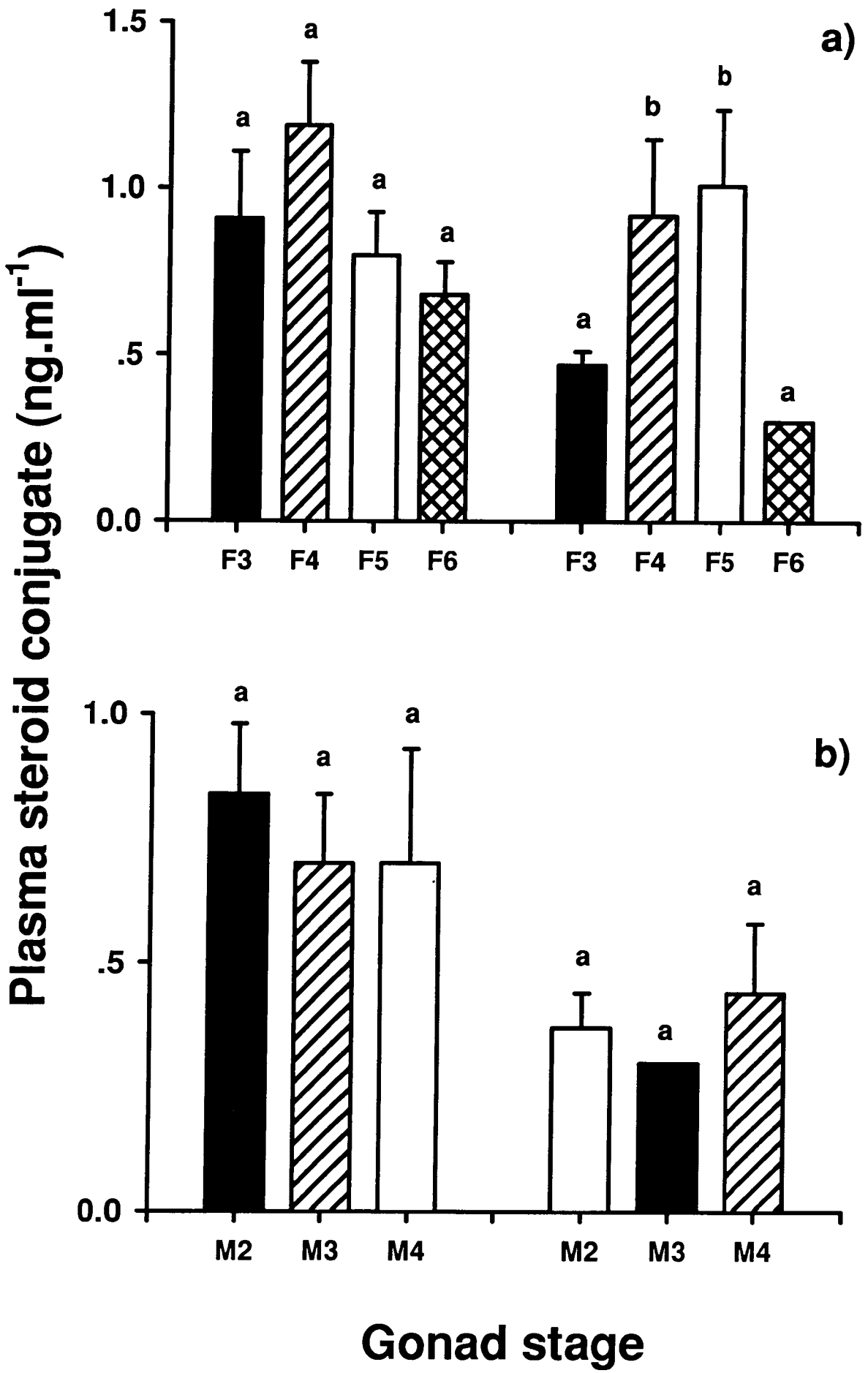
17,20ßP-glucuronide
17,20ßP-sulphate
a)
b)
1.5
1.0
.5
0.0
a
a
a
a
a
b
b
a
F3
F4
F5
F6
F3
F4
F5
F6
1.0
.5
0.0
a
a
a
a
a
a
M2
M3
M4
M2
M3
M4
Plasma steroid conjugate (ng.ml-1)
Gonad stage

Fig. 3.7. Plasma levels of reproductive steroids in relation to male gonad stage. Values are mean + S.E. M2, M3 and M4 = male gonad stages two (n = 8), three (n = 10) and four (n = 9) respectively. Gonad stages are described in Table 2. Values that are not significantly different ($P>0.05$) share common superscripts.

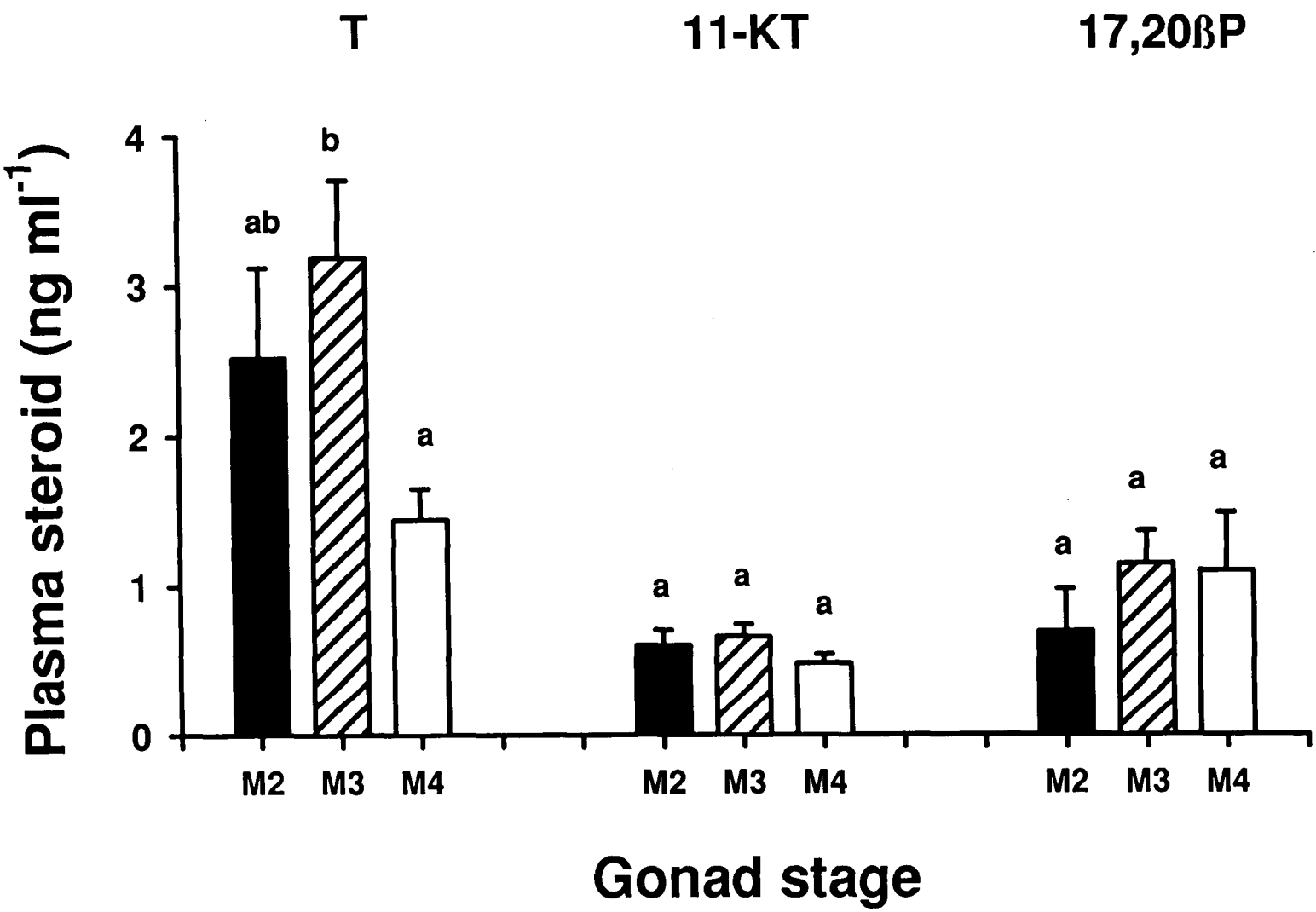
T
11-KT
17,20ßP
Plasma steroid (ng ml-1)
4
3
2
1
0
ab
b
a
a
a
a
a
a
a
M2
M3
M4
M2
M3
M4
M2
M3
M4
Gonad stage

Table 3. 4. Plasma levels of steroids in female fish sampled within 2 mins of capture, compared with commercial capture. Values show means ± S.E.

Gonad stage	Steroid	2 mins of capture	commercial capture
		(n=3)	(n=42)
3	cortisol	0.6 ± 0.17	25.9 ± 2.33
	T	7.9 ± 1.22	3.2 ± 0.48
	E_2	9.9 ± 0.94	3.5 ± 0.48
	17,20ßP	0.5 ± 0.06	0.3 ± 0.01
		(n=4)	(n=18)
6	cortisol	4.8 ± 3.35	24.3 ± 5.14
	T	0.5 ± 0.04	0.6 ± 0.05
	E_2	0.5 ± 0.03	0.75 ± 0.06
	17,20ßP	0.4 ± 0.03	0.32 ± 0.01

3.5. Discussion

Previous studies on greenback flounder showed that the reproductive season was very prolonged (March to October), and that females were more abundant in shallow water (<10 m), whereas males were more abundant in deep water (10-25 m) (Kurth 1957; Crawford 1984). It was also observed that the highest percentage of mature females sampled in any one month occurred in deeper waters, and greater abundances of larvae were found near the mouths than further up estuaries, suggesting that spawning occurs in deeper water and that females move into deeper offshore waters for spawning (Crawford 1984). In our study, fish were collected in shallow water by gill net, which may explain why females were more commonly caught than males, and why there were relatively few stage 4 males or stage 4 and 5 females. Crawford (1984) did report that there were very few running ripe females sampled at any depths, suggesting that FOM and hydration and spawning occupy relatively short periods (Crawford 1984).

GSI was shown to increase with maturity in female greenback flounder. GSI has also been found to increase in association with ovarian development in other teleost species (deVlaming 1983; Singh and Singh 1987; Johnson et al., 1991; Carragher and Pankhurst 1993; Clearwater and Pankhurst 1994) and provides a useful basic measurement of gonadal anabolism. Changes in oocyte diameters and oocyte types during gonadal development indicated that female greenback flounder have distinct groups of oocyte stages. This indicates that oocyte development is group synchronous with a capacity for multiple ovulations within a reproductive season. Hatchery fish induced to ovulate with exogenous hormones will ovulate on a daily basis after stripping (Barnett and Pankhurst, 1998a and b, chapters 4 and 5 this volume). Our unpublished observations show that naturally ovulating hatchery fish, naturally ovulated wild fish brought into captivity and wild fish brought into captivity and induced to ovulate with exogenous hormones, also ovulate daily after stripping, for a period of 3-5 days. We assume that this also occurs in the wild. In the absence of information on short term changes in ovarian cycling from individual fish, it is not clear whether oocytes are recruited from a batch of cortical alveoli, vitellogenic or mature stage oocytes. The likelihood of daily ovulations from cortical alveoli stage oocytes is not known, however, in repeat ovulating striped trumpeter (*Latris lineata*), oocytes progress from cortical alveolus stage to ovulation every 3 days (Morehead et al.,

1998). This is in contrast to species such as the New Zealand snapper (*Pagrus auratus*) which ovulates on a daily basis for most of the spawning period, and this is reflected in asynchronous ovarian development with a complete range of vitellogenic oocyte sizes present in the ovary at any one time (Scott, et al., 1993). The fact that plasma levels of T and E_2 were not elevated in stage 4 and 5 females suggests that oocytes were most likely recruited on a daily basis from a clutch of mature oocytes, however, examination of short term (< 24 hours) biopsy samples would be required to fully establish the nature of the greenback flounder ovulatory cycle.

Male greenback flounder differ from many other species in which GSI and proportions of gamete stages were reported to change during testicular development (Dedual and Pankhurst 1992; Carragher and Pankhurst 1993; Harmin et al., 1995; Carolsfeld et al., 1996). The absence of statistical differences in GSI or histological (cell type) differences between male gonad stages, indicates that seasonal changes in testis condition are small. This suggests that either testis development occurs early in the season and there are no subsequent changes as a result of spawning activity, or there is continuous recruitment of male gametes with periodic spawning of low volumes of milt. Although we have no information on the gonadal cycling of individual male greenback flounder, a previous study shows that a very high proportion of the male population is spermiated throughout the entire reproductive season (Crawford 1984).

Elevated levels of plasma and ovarian T and E_2 in vitellogenic female greenback flounder are consistent with changes in T and E_2 in other species. E_2 controls synthesis of the yolk precursor vitellogenin, which is incorporated into the growing oocyte during vitellogenesis (reviewed in Specker and Sullivan 1994). T acts as a precursor for the synthesis of E_2 (Kagawa et al., 1984; Matsuyama et al., 1988) and is also involved in positive feedback stimulation of the pituitary synthesis of gonadotrophin (GtH) (Crim et al., 1981; Trudeau et al., 1993).

Plasma levels of T and E_2 were a good reflection of concurrent production of T and E_2 in the ovary in greenback flounder. Similar relationships existed between plasma and ovarian levels of T and E_2 in the demoiselle (*Chromis dispilus*), but not for plasma and ovarian levels of T and E_2 in the New Zealand snapper (Hobby and Pankhurst 1997), for E_2 in the killifish (Bradford and Taylor 1987), and T in the catfish (*Clarias batrachus*) (Singh and Singh 1987). Suggestions provided to explain the poor

correlations between ovarian and plasma T and E_2 were high retention of steroids in the plasma by binding proteins (Bradford and Taylor 1987), and indistinct steroid peaks if the ovarian cycle is of short duration as in the daily ovulating New Zealand snapper (Hobby and Pankhurst 1997). Greenback flounder also ovulate daily, however, plasma and ovarian levels of T and E_2 are still correlated, probably because of the distinct separation of the developing oocyte clutches.

Plasma and ovarian levels of 17,20ßP in female greenback flounder were significantly elevated in association with ovulation or FOM and ovulation respectively, however, levels were always low. In many marine species, particularly pleuronectiformes and partial ovulators, plasma 17,20ßP levels often show similar lack of variation in association with FOM (Pankhurst and Conroy 1987; Scott and Canario 1987; Dedual and Pankhurst 1992; Carragher and Pankhurst 1993; Barnett and Pankhurst 1994). There are several suggestions explaining low or non-significant changes in 17,20ßP in relation to oocyte maturation; (1) changes in plasma levels of 17,20ßP are pulsatile, and are not detected in the sampling regime; (2) 17,20ßP is synthesised and utilised in the ovarian follicle but very little is released into the plasma without either conjugation or further metabolism; (3) 17,20ßP is rapidly deactivated by reduction and/or conjugation; (4) 17,20ßP is not the maturational inducing steroid in greenback flounder. There is good evidence to suggest that 17α20ßP,21-trihydroxy-4-pregnen-3-one (20ß-S) is the major maturation inducing steroid in the spotted sea trout (*Cynoscion nebulosus*) and Atlantic sea trout (*Micropogonias undulatus)* (Thomas and Trant 1989; Thomas 1994). Our unpublished observations on the effects of steroids and human chorionic gonadotrophin on *in vitro* oocyte maturation in greenback flounder indicate that greenback flounder oocytes are receptive to a broad range of C_{21} steroids including 17,20ßP and 20ß-S.

Plasma levels of 17,20ßP-sulphate were significantly elevated during FOM in the absence of significant increases in plasma 17,20ßP at this time. This suggests that plasma levels of 17,20ßP were not always representative of events in the ovary, most likely as a result of different rates of metabolism and conjugation. Similarly, plasma and ovarian 17,20ßP levels were poorly correlated in the New Zealand snapper and the demoiselle (Hobby and Pankhurst 1997).

Plasma levels of 17,20ßP-sulphate but not 17,20ßP-glucuronide were significantly elevated in females undergoing FOM and hydration, however, levels were considerably lower than plasma levels reported in plaice (*Pleuronectes platessa*). Plaice ovaries contain very active reducing and conjugating enzymes (Scott and Canario 1990), hence plasma levels of 17,20ßP in mature female plaice were < 1 ng. ml^{-1}, whereas plasma and urine levels of 17,20ßP-sulphate were 11 ng. ml^{-1} and 1500 ng. ml^{-1} respectively (Scott and Canario 1992). A recent study indicates that 17,20ßP-sulphate is not the most abundant sulphated C_{21} steroid metabolite in plasma and urine of females plaice undergoing FOM (Scott et al., 1997). It is quite possible that female greenback flounder produce high levels of some other sulphated metabolite.

In some male teleosts, plasma T levels tend to be highest throughout spermatogenesis, and drop off just before spermiation (Wingfield and Grimm 1977; Scott et al., 1984; Fostier et al., 1987; Pankhurst and Conroy 1987, 1988; Harmin et al., 1995; Carolsfeld et al., 1996). This is consistent with the results of our study, indicating that T probably plays a role in the earlier stages of testis development.

Plasma 11KT and 17,20ßP did not change with gonadal stage in male greenback flounder. In many studies, plasma 11KT levels are elevated during spermatogenesis, (Scott et al., 1984; Fostier et al., 1987; Dedual and Pankhurst 1992; Methven et al., 1992; Barnett and Pankhurst 1994; Borg 1994; Harmin et al., 1995; Carolsfeld et al., 1996), and levels often remain elevated into the early stages of spermiation (Campbell et al., 1976; Fostier et al., 1987; Methven et al., 1992; Carolsfeld et al., 1996). Elevated levels of 17,20ßP are associated with spermiation in some teleosts (Scott et al., 1984; Fostier et al., 1987; Carolsfeld et al., 1996), and treatment with exogenous 17,20ßP stimulates an increase in milt volume in snapper, and a range of other species (reviewed in Pankhurst 1994). There are several possibilities that may explain non-significant changes in plasma levels of 11KT and 17,20ßP in male greenback flounder. (1) Changes in plasma levels of 11KT and 17,20ßP are pulsatile, and are not detected in the sampling regime. (2) 11KT and 17,20ßP are synthesised and utilised in the testis but very little is released into the plasma without either conjugation or further metabolism. In future studies it would be interesting to assess testis levels of reproductive steroids. (3) 11KT and 17,20ßP are rapidly deactivated by reduction and/or conjugation. Neither plasma 17,20ßP-sulphate nor 17,20ßP-glucuronide were

significantly elevated at any stage in male greenback flounder. This is in contrast to male plaice in which high levels of 17,20ßP-sulphate and 17,20ßP-glucuronide were detected in the plasma (Scott and Canario 1992). As suggested for females, it is possible that male greenback flounder produce high levels of some other conjugated metabolite. (4) 11KT does not play a role in spermatogenesis and 17,20ßP is not the maturational inducing steroid in male greenback flounder. Studies on a range of other species showed that 11KT did not change with gonadal stage (reviewed in Pankhurst and Carragher, 1991). In addition, there is good evidence for some species that 11 KT is more strongly associated with morphological and behavioural changes than spermatogenesis and spermiation (reviewed in Pankhurst and Carragher 1991; Barnett and Pankhurst 1994; Borg 1994; Thorarensen et al., 1996). This calls into question the assertion based on correlational data that 11KT plays a role in spermatogenesis in teleosts generally. In other species, plasma 17,20ßP is often not detectable or at very low levels in spermiated fish (Pankhurst and Conroy 1987, 1988; Pankhurst and Carragher 1991; Pankhurst and Kime 1991; Dedual and Pankhurst 1992; Barnett and Pankhurst 1994). (5) 11KT and 17,20ßP may not need to be elevated much to induce spermatogenesis or spermiation. As previously described, male greenback flounder have small testes, and during gonadal development only low proportions of gamete stages advance into sperm production. If 11KT and 17,20ßP mediate these processes in greenback flounder as in some other species (reviewed in Pankhurst 1994), then low 11KT and 17,20ßP levels may be sufficient to maintain spermatogenesis and spermiation.

Plasma levels of cortisol were significantly higher in wild greenback flounder caught in gill nets, than in fish sampled within 2 minutes of capture, indicating that commercial capture is highly stressful. Stress is known to inhibit reproduction in some species probably by suppressing levels of sex steroids (Sumpter et al., 1987; Maule et al., 1989; Carragher and Pankhurst 1991; Clearwater and Pankhurst 1997). The effects of capture stress on plasma T and E_2 were evident as early as 1h after the onset of the stress in wild New Zealand snapper (Carragher and Pankhurst 1991), and at the first sample time (24 h) in wild red gurnard (*Chelidonichthys kumu*) (Clearwater and Pankhurst 1997). In greenback flounder, plasma levels of T, E_2 and 17,20ßP were lower in commercially caught than rapidly sampled stage 3 females, but appeared unaffected by commercial capture in stage 6 females. The latter is not suprising as

stage 6 females would not be expected to have the elevated plasma gonadal steroid levels. With the proviso that for logistical reasons we were only able to sample a small number of fish soon after capture, these results suggest that plasma levels of T, E_2 and 17,20ßP may have been underestimated in commercially caught females. We are assuming that the effects are not differentially expressed on stage 3, 4 or 5 fish and that relative changes in hormone levels remain unaffected. This further emphasizes the sensitivity of reproductive processes in wildfish to the stress imposed by capture.

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Chapter 4

Effect of treatment with LHRH analogue and hCG on ovulation, plasma and ovarian levels of gonadal steroids in greenback flounder *Rhombosolea tapirina* (Günther, 1862).

4. Effect of treatment with LHRH analogue and hCG on ovulation, plasma and ovarian levels of gonadal steroids in greenback flounder *Rhombosolea tapirina* (Günther, 1862).

4.1. Summary

Female greenback flounder *Rhombosolea tapirina* were induced to ovulate using either des Gly_{10} [D-Ala^6] LHRH ethylamide (LHRH-a) at 50 $\mu g.kg^{-1}$ or 100 $\mu g.kg^{-1}$ intraperitoneal injection (ipi), 100 $\mu g.kg^{-1}$ cholesterol pellet (LHRH-a pellet) implanted intraperitoneally, or human chorionic gonadotropin (hCG) 1000 $IU.kg^{-1}$ ipi. Treatment with hCG, LHRH-a (50 $\mu g.kg^{-1}$) ipi and LHRH-a pellet increased the total number of ovulations and repeat ovulations above control levels, and LHRH-a pellet induced more ovulations and repeat ovulations than LHRH-a (100 $\mu g.kg^{-1}$) ipi. Oocyte diameters increased and oocyte stages significantly advanced in response to all exogenous hormone treatments, and this was accompanied by increases in plasma and ovarian levels of 17β-estradiol (E2), in most cases plasma and ovarian levels of testosterone (T). Plasma and ovarian levels of 17α20β-dihydroxy-4-pregnen-3-one (17,20βP) were not consistently elevated in association with reproductive events, hence, the role of 17,20βP as a marker of impending ovulation was unclear.

4.2. Introduction

Greenback flounder *Rhombosolea tapirina* (Pleuronectidae) occurs around Southern Australia and New Zealand (Ayling and Cox, 1982) and is the only flatfish in Southern Australia that is large and abundant enough to be harvested commercially (Edger, 1997). There is growing interest in developing an aquaculture industry for greenback flounder in Tasmania, as it is amenable to culture conditions, it has a fast growth rate, an expanding market profile (Hart, 1993), and pilot aquaculture schemes are already underway, however, current programs are limited by unreliable and variable egg production (Crawford, 1984; Hart, 1991, 1993; Hart and Purser, 1995). Continued expansion of greenback flounder culture is dependent on the development of suitable induced ovulation protocols to control the timing and production of eggs for larval production.

The reproductive endocrine system in teleosts is regulated by the hypothalamic-pituitary-gonad axis (HPG). Pituitary release of gonadotropin (GtH) stimulates the gonad to synthesise steroids which in turn regulate gonadal development. Two forms of GtH have been identified in most species in which it has been investigated (GtH-I and GtH-II), with GtH-I present during gametogenesis while GtH-II predominates during maturation (reviewed in Pankhurst, 1998; Peter and Yu, 1997). Gonadotropin release is regulated at the hypothalamic level by the stimulatory effects of gonadotropin releasing hormone (GnRH), and in most species where it has been examined, the inhibitory actions of dopamine (DA) on gonadotrophs and GnRH release (reviewed in Peter and Yu, 1997).

Reproductive development in captive fish is often impeded because some levels in the endocrine cascade are sensitive to modification by exogenous factors associated with husbandry (reviewed in Pankhurst and Van Der Kraak, 1997). Various exogenous hormone treatments acting at different levels in the HPG axis, can be administered to override the detrimental effects of captivity on reproduction (reviewed in Donaldson and Hunter 1983; Donaldson and Devlin 1996; Peter and Yu 1997). Injection of piscine and/or mammalian gonadotropins (hypophysation) has successfully been used to induce ovulation in a wide range of species (reviewed in Lam 1982; Donaldson and Hunter 1983). Piscine GtHs have restricted usefulness because they are expensive, supply can be limiting, dose is difficult to standardise and they have relatively high species specificity (Lam, 1982; Donaldson and Hunter, 1983; Zohar, 1988). In contrast, the mammalian gonadotropin, human chorionic gonadotropin (hCG) can be readily obtained and biological activity is readily standardised, although hCG has low biopotency in some species (Pankhurst, 1998), resulting in the need for high doses and/or multiple injections (Smigielski 1975; Lam, 1982; Saidin et al., 1988). Use of GtH preparations have now been largely superseded by fish GnRH analogues (GnRH-a) or mammalian GnRH analogues refered to in this volume as luteinising hormone releasing hormone analgoues (LHRH-a). These analogues stimulate the release of the native GtH (reviewed in Zohar 1988). GnRH-a or LHRH-a are substantially more potent than their parent peptide due to structural modifications which render them more resistant to enzyme degradation (Goren et al., 1987; Zohar et al., 1990) and enhance binding affinity (Donaldson and Hunter, 1983; Zohar et al., 1990; Peter and Yu, 1997). GnRH-a or LHRH-a are particularly successful when administered in the

form of slow release systems which sustain elevated gonadotropin levels over long periods. Not only does sustained GnRH-a or LHRH-a release stimulate multiple ovulation (Almendras et al., 1988; Mylonas and Zohar, 1995; Berlinsky and King, 1996; Mylonas et al., 1996), but in some species it can also stimulate ovarian development in immature fish (Crim et al., 1988; Matsuyama et al., 1993; Harmin et al., 1995a; Holland et al., 1995).

In the absence of reliable natural ovulation in captivity, egg production is currently dependent on induced ovulation using exogenous hormones, hence it is important to characterise the response to these treatments. The aim of this study was to determine the potential for inducing ovulation in greenback flounder using the exogenous hormones, hCG and LHRH-a, and to assess whether greenback flounder are differentially responsive to hCG, LHRH-a or the dose and/or delivery mode of LHRH-a. The exogenous hormone doses were chosen based on their success at inducing ovulation in other species (Almendras et al, 1988; Peter et al, 1988; Harmin et al, 1995; Berlinsky et al, 1996; Morehead et al, 1998). The efficacy of induced ovulation protocols was assessed by examining incidence and frequency of ovulation and the associated changes in oocyte diameters and plasma and ovarian sex steroid levels.

In the absence of specific GtH-II assays for most species, gonadal steroids remain the most useful endocrine markers. E_2 and T are commonly measured as indicators of ovarian development (Pankhurst and Carragher, 1991) and 17α20β-dihydroxy-4-pregnen-3-one (17,20βP) is often measured as the potential MIS as it has been identified as the MIS in many marine and freshwater species (Scott and Canario, 1987). In wild greenback flounder, plasma and ovarian levels of testosterone (T) and 17β-estradiol (E_2) were elevated in association with vitellogenesis (Barnett and Pankhurst, 1998b, chapter 3 this volume). It has not been established whether 17,20βP is the maturational inducing steroid in greenback flounder, however, plasma levels of 17,20βP were significantly elevated in ovulated females, whereas ovarian levels of 17,20βP were elevated in association with (final oocyte maturation) FOM and ovulation. Our observations of captive greenback flounder indicate that oocyte development is arrested at the final stages of vitellogenesis, just prior to final oocyte maturation and ovulation.

4.3. Materials and methods

4.3.1. Fish and fish maintenance

Fish used in these experiments were obtained from either Camerons of Tasmania PTY LTD Dunalley, Tasmania, or the Department of Primary Industries and Fisheries, Marine Research Laboratories, Taroona, Tasmania. Fish were maintained at the Department of Aquaculture aquatic facility at the University of Tasmania in Launceston, in recirculating systems incorporating a biofilter, coarse solids filter and aeration system. Fish with ovaries at the vitellogenic stage of development were chosen for the experiments, these fish had large firm ovaries, bulging above the musculature, extending to the caudal peduncle and no oocytes were released upon gentle pressure on the abdomen. Fish used were fed at 3% body weight daily and body weight ranged from 105-370 g in body weight. Water temperature was 12°C and LD 10:14 h during both experiments.

4.3.2. Experiment 1

Fish used in this experiment were collected from Camerons of Tasmania PTY LTD and were hormone treated and first sampled on site. Time of treatment was 1030 h. Fish were divided into four treatment groups (n=7), anaesthetised in a 0.02% 2-phenoxyethanol (Sigma) water bath, and treated with either (1) saline, (2) hCG at 1000 IU. kg^{-1} body weight, (3) LHRH-a at 50 $\mu g.kg^{-1}$ body weight or (4) LHRH-a at 100 $\mu g.kg^{-1}$ body weight. All treatments were administered by intraperitoneal injection (ipi) in an injection volume of 100 $\mu L.kg^{-1}$. After recovery from anaesthesia, fish were placed in oxygenated seawater in 50 L plastic bags, transported to Launceston and transferred to 1000 L tanks in a recirculating system for the remainder of the experiment. Each fish was anaesthetised then bled 0, 24, 48, and 72 h after treatment and checked for ovulation by gentle pressure on the abdomen 0, 24, 48, 72, 96,120, 144, and 168 h after treatment. Any ovulated eggs were stripped at each sample time and visually examined for opacity, shape and the presence of single or multiple oil droplets.

4.3.3. Experiment 2

Fish used in this experiment were transported in an 800 L oxygenated portable tank from the Department of Primary Industries and Fisheries, Marine Research

Laboratories, Taroona, to Launceston. Fish were first sampled and hormone treated at 1500 h, immediately upon arrival at the Launceston facility. Fish were divided into four treatment groups (n=7) and treated with either (1) saline ipi (2) hCG ipi at 1000 IU. kg^{-1} body weight (3) LHRH-a ipi at 100 $\mu g.kg^{-1}$ body or (4) LHRH-a 100 $\mu g.kg^{-1}$ in a 95 % cholesterol + 5 % cocoa butter pellet (Lee et al., 1986), implanted intraperitoneally. Injections were administered in a volume of 100 $\mu L.kg^{-1}$. Each fish was anaesthetised, bled and ovarian biopsied at 0, 6, 24, 48, 72 and 96 h after treatment and checked for ovulation at 0, 6, 24, 48, 72, 96,120, 144, 168 and 192 h after treatment. Ovarian biopsies were collected by inserting a catheter ("Endometrial biopsy" - Laboratoire CCD 60, Paris) through the genital pore and into the gonad. Entry into the gonad was narrow and convoluted and the entrance to the genital pore was shared by the rectal opening, therefore sampling sometimes resulted in misadventure into the gut and occasionally rupture of the gut wall, hence this procedure was limited to experiment 2 only. Ovarian tissue was separated by gentle aspiration through a glass pipette and spread throughout a sorting tray in an acidified saline solution. One hundred oocytes were measured as encountered under a dissecting microscope, and classified as vitellogenic, undergoing germinal vesicle migration and breakdown, or hydrated, from representative fish in each treatment. Classification of oocyte stage was verified by clearing oocytes of each type in sera solution (ethanol:foramlin:acetic acid, 6:3:1 v/v), to determine the presence or position of the germinal vesicle. For logistical reasons, this experiment did not include a blank pellet control, and although we have statistically compared responses from unpelleted control fish with LHRH-a pellet treated fish, our results do not account for potential pellet implant effects.

4.3.4. Steroid measurement

Blood samples were collected by caudal puncture, and plasma concentrations of E_2, testosterone T and 17,20βP were measured by radioimmunoassay (RIA). One hundred microlitres of plasma were extracted with 1 mL of ethyl acetate and added to each assay tube for evaporation and resuspension in assay buffer, using the reagents and protocol given in (Pankhurst and Conroy, 1987). Extraction efficiency was determined by recovery of [3H]-labelled steroid extracted with plasma, and was on average 93%, 96%, and 93% for E_2, T and 17,20βP respectively. Assay values were corrected accordingly. Assay detection limits were 0.28, 0.29 and 0.28 $ng.mL^{-1}$ for E_2, T and

17,20βP. Ovarian concentrations of E_2, T and 17,20βP were measured by homogenising 0.125 g of ovarian tissue in 1 mL of ethyl acetate, and centrifuging the homogenate at 3000 rpm for 10 minutes. One hundred microlitres of extract were measured in assays as before. Interassay variability was measured using a pooled steroid standard giving %CVs of 10% (n=10), 12% (n=10), 14% (n=10) for E_2, T and 17,20βP respectively.

4.3.5. Statistical analysis

Repeated measures analysis of variance (ANOVA), mean comparison tests, planned contrasts and logistic analysis and curve fits were performed using the computer packages SAS, JMP for Macintosh and Curve fit for DOS. Data were log or square root transformed to satisfy normality and homogeneity of variance requirements. A significance level of (<0.05 was used for all statistical tests. In some instances, variances were still heterogeneous after transformation, however, the data was also assessed by multivariate analysis of variance and canonical discriminant analysis, and in all cases the outcomes were unchanged. We chose to present ANOVA results because of the utility of mean comparison tests and the familiarity of these tests within the literature.

4.4. Results

Spontaneous ovulation occurred during experiment 1 (as indicated by the ovulation of 3 control fish). Logistic analysis indicated that treatment with hCG and LHRH-a ipi (50 $\mu g.kg^{-1}$) significantly increased the total number of ovulations above control levels (Fig. 4.1). Treatment with LHRH-a ipi (100 $\mu g.kg^{-1}$) did not significantly increase the total number of ovulations above control levels, however, there was no significant difference in the total number of ovulations between fish treated with either hCG, LHRH-a ipi (50 $\mu g.kg^{-1}$) or (100 $\mu g.kg^{-1}$). Fish treated with exogenous hormones ovulated at each time period between 24 - 168 h after treatment. All but one fish that ovulated in response to exogenous hormones ovulated more than once, most of these fish ovulated more than twice and most ovulations occured at daily intervals. There were four mortalities during this experiment.

During experiment 2, only 1 control fish ovulated, and logistic analysis indicated that treatment with hCG, LHRH-a ipi (100 $\mu g.kg^{-1}$) and LHRH-a pellet all significantly

increased the total number of ovulations (Fig. 4.2). Treatment with LHRH-a pellet, stimulated more ovulations than treatment with LHRH-a ipi (100 $\mu g.kg^{-1}$) but there was no significant difference in the total number of ovulations between fish treated with hCG and LHRH-a (100 $\mu g.kg^{-1}$), or hCG and LHRH-a pellet. The majority of fish ovulated between 72 and 192 h after treatment. All fish that ovulated in response to exogenous hormones ovulated more than once, most of these fish ovulated more than twice and most ovulations occured at daily intervals. There were three mortalities during this experiment.

In both experiments, stripped eggs were clear in appearance, and although some eggs contained multiple oil droplets, trial fertilisations indicated that these eggs were fertile.

Oocyte diameter distributions measured from a randomly selected control fish did not significantly change from 0 - 96 h (Fig. 4.3), and logistic analysis indicated that there was no significant change in oocyte types throughout the experiment. In contrast, all exogenous hormone treatments induced significant increases in oocyte diameters and advancement of vitellogenic oocytes to germinal vesicle migration (GVM) and breakdown (GVBD) followed by oocyte hydration (Figs 4.4-4.6). Ovaries from ovulated fish were characterised by high proportions oocytes undergoing GVM , GVBD and hydration and few remaining vitellogenic oocytes.

During experiment 1, exogenous hormone treatment significantly increased plasma levels of E_2 above control levels from 24 - 72h (Fig. 4.7a), and treatment with LHRH-a ipi (50 $\mu g.kg^{-1}$) significantly increased plasma levels of T above control levels from 24 and 48h after treatment (Fig. 4.7b). Exogenous hormone treatment did not stimulate significant increases in plasma levels of 17,20ßP (Fig. 4.7c).

During experiment 2, exogenous hormone treatment significantly increased plasma and ovarian levels of E_2 from 24 - 96h (Figs 4.8a and 4.9a), plasma levels of T from 6-96 h, (except fish treated with hCG 48 h after treatment) (Fig. 4.8b) and ovarian levels of T from 6-96 h, (except fish treated with hCG and LHRH-a pellet 72 h after treatment and hCG 96 h after treatment) (Fig. 4.9b). In fish treated with exogenous hormones, plasma levels of 17,20βP were significantly elevated above control levels at 24 h (Fig. 4.8c). Plasma levels of 17,20βP were significantly elevated above pre-treatment levels in control fish and fish treated with exogenous hormones 48 h after treatment, but

levels were higher in fish treated with hCG than control fish. Ovarian levels of 17,20βP were not significantly elevated by treatment with exogenous hormones (Fig. 4.9c).

Plasma and ovarian levels of E_2 and plasma levels of T significantly decreased from pre-treatment levels in control fish throughout experiments 1 and 2 (Figs 4.7 and 4.8).

Fig. 4.1. Percentage of fish that ovulated in each treatment group at each sample time during experiment 1. Treatments that are not significantly different ($P>0.05$) share common superscript letters. Fish are labelled 1-7 within each treatment group and numbers denote which fish ovulated within each treatment at each sample time.

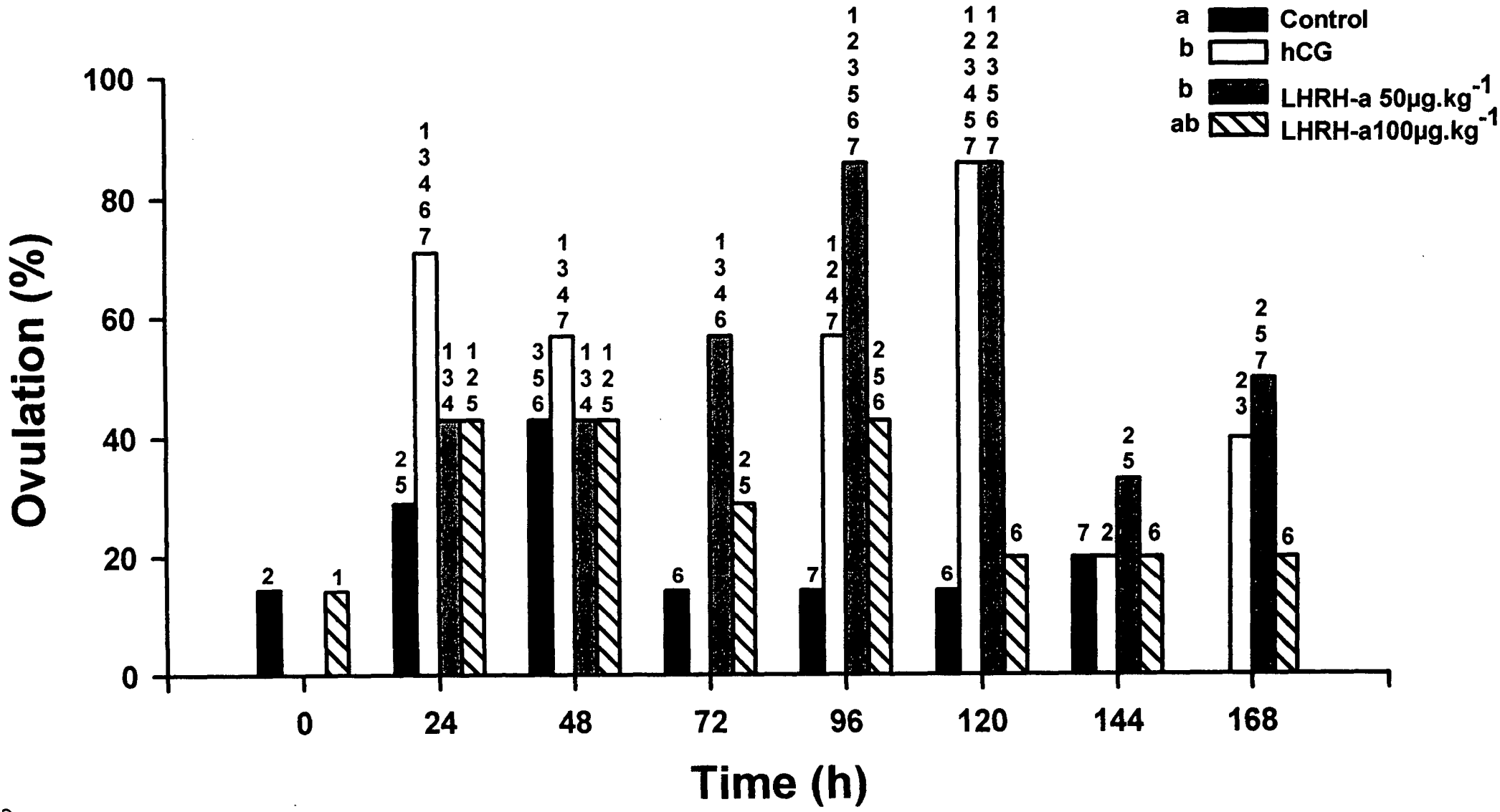

a Control
b hCG
b LHRH-a 50µg.kg-1
ab LHRH-a100µg.kg-1
Ovulation (%)
Time (h)
0
24
48
72
96
120
144
168

Fig. 4.2. Percentage of fish that ovulated in each treatment group at each sample time during experiment 2. Treatments that are not significantly different ($P>0.05$) share common superscript letters. Fish are labelled 1-7 within each treatment group and numbers denote which fish ovulated within each treatment at each sample time.

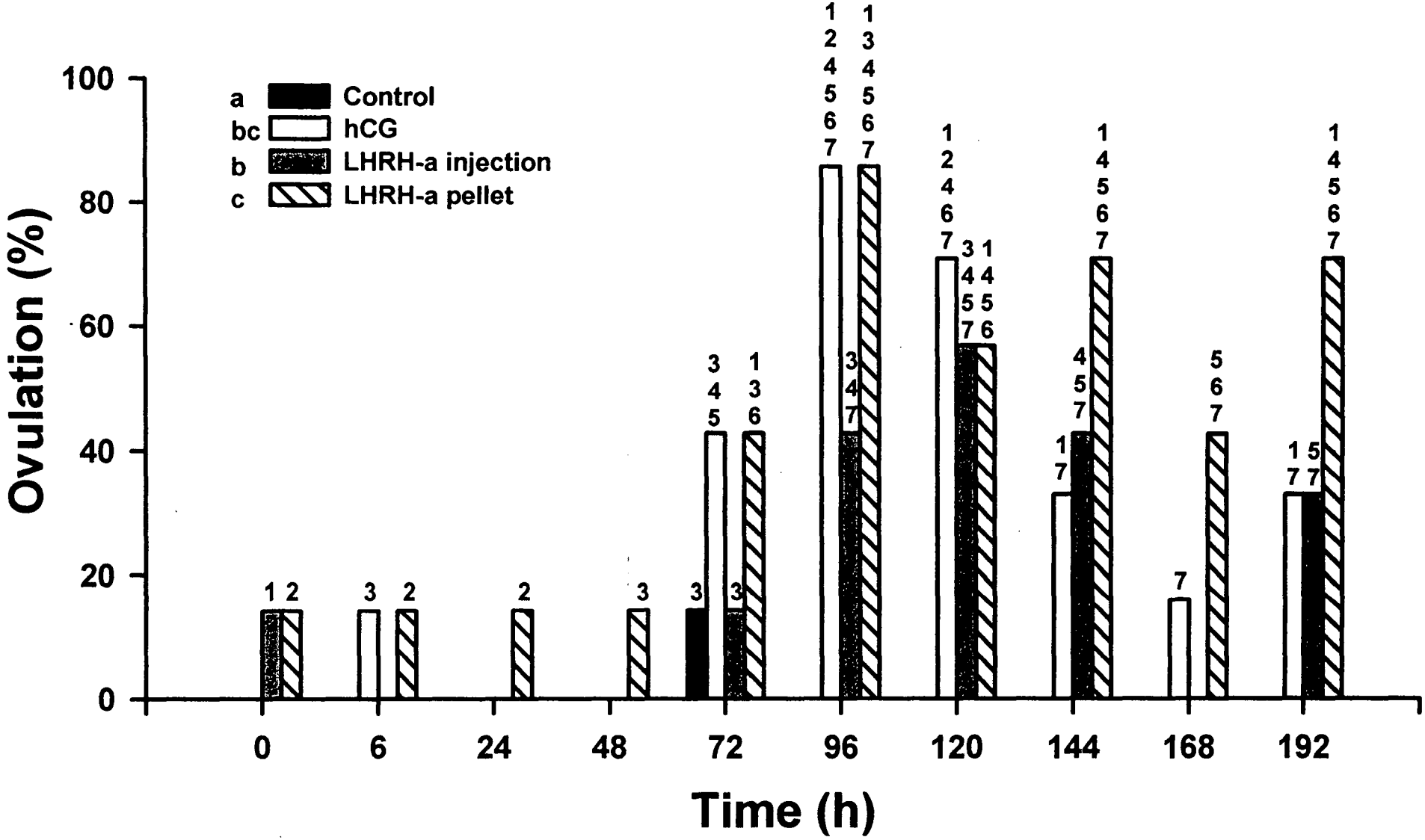

a Control
bc hCG
b LHRH-a injection
c LHRH-a pellet
Ovulation (%)
100
80
60
40
20
0
0
6
24
48
72
96
120
144
168
192
Time (h)

Fig. 4.3. Frequency of oocyte diameters at each sample time from a randomly selected fish treated with saline during experiment 2.

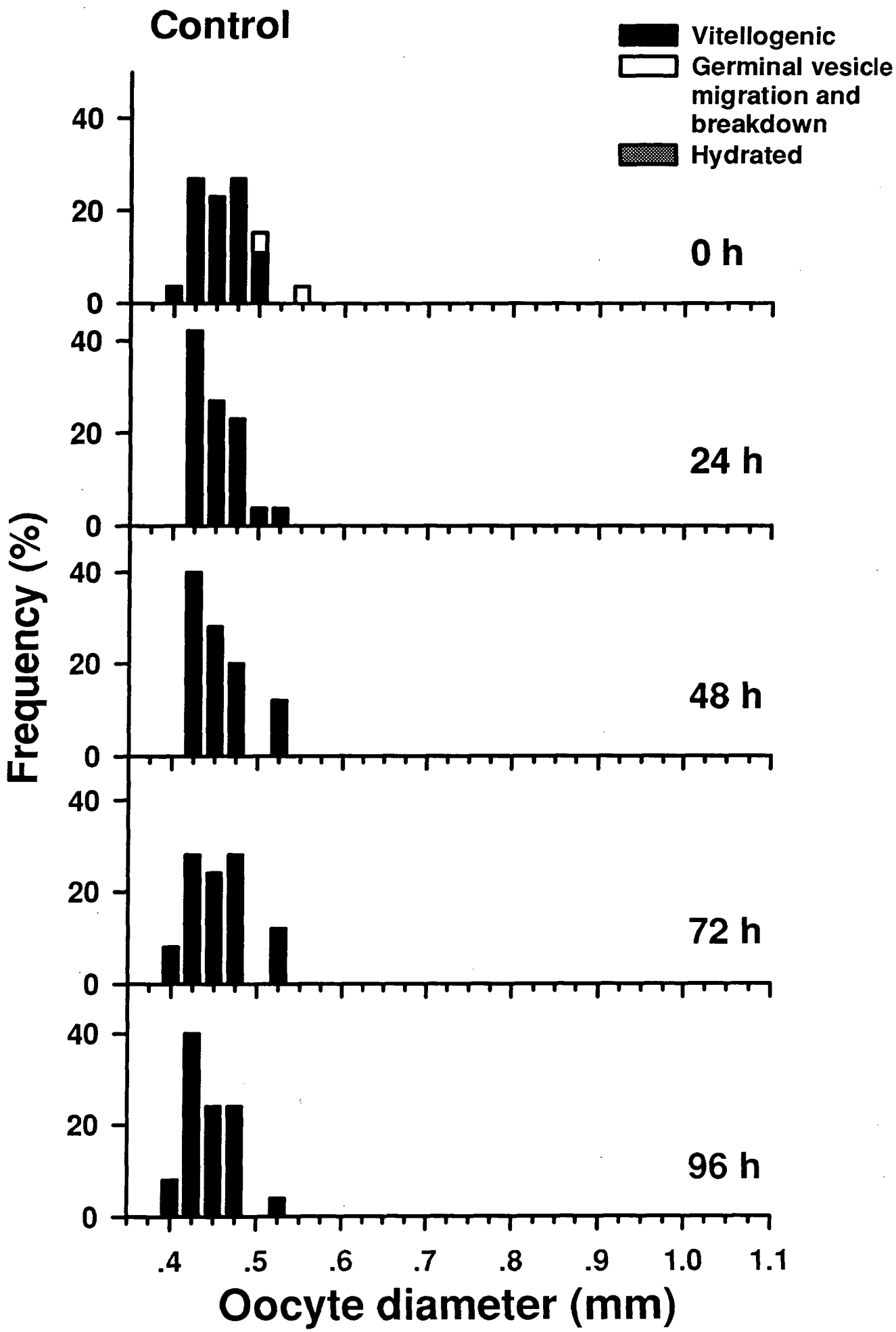

Control
Vitellogenic
Germinal vesicle migration and breakdown
Hydrated
0 h
24 h
48 h
72 h
96 h
Frequency (%)
0
20
40
.4
.5
.6
.7
.8
.9
1.0
1.1
Oocyte diameter (mm)

Fig. 4.4. Frequency of oocyte diameters at each sample time from a randomly selected fish treated with hCG during experiment 2. Asterisks indicate when the fish ovulated.

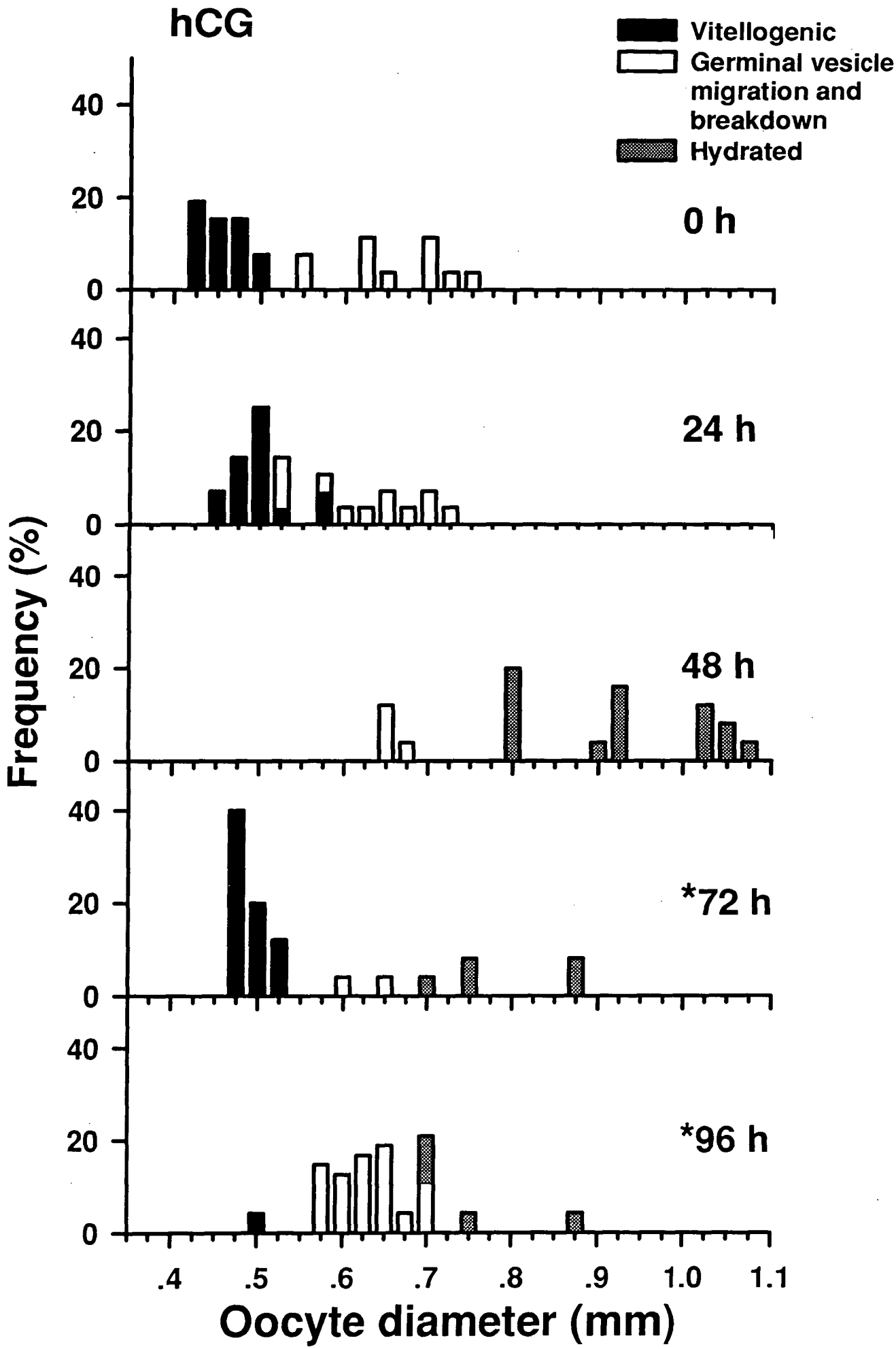

hCG
Vitellogenic
Germinal vesicle migration and breakdown
Hydrated
0 h
24 h
48 h
*72 h
*96 h
Frequency (%)
0
20
40
.4
.5
.6
.7
.8
.9
1.0
1.1
Oocyte diameter (mm)

Fig. 4.5. Frequency of oocyte diameters at each sample time from a randomly selected fish treated with LHRH-a 100 $\mu g.kg^{-1}$ ipi during experiment 2. Asterisks indicate when the fish ovulated.

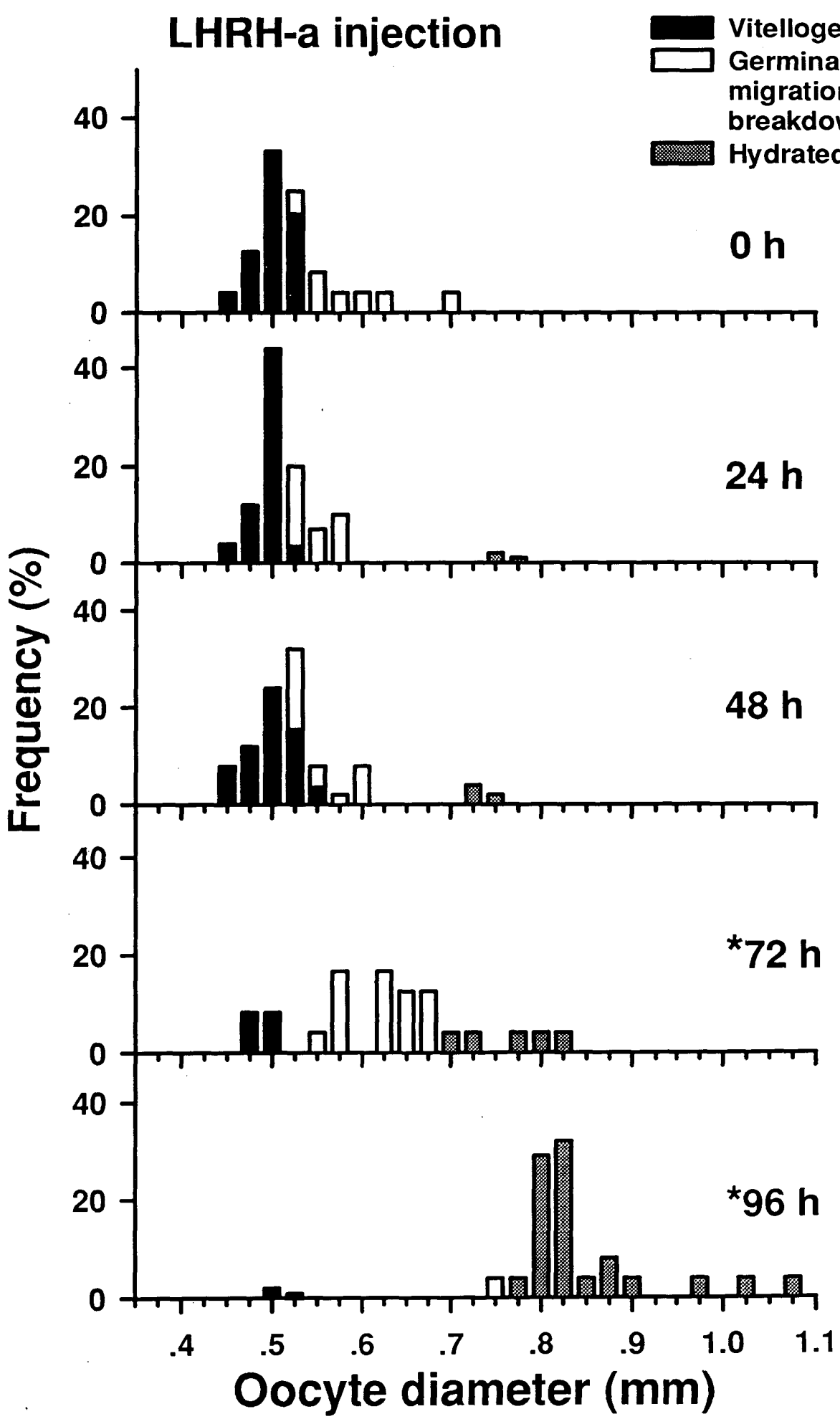

LHRH-a injection
Vitellogenic
Germinal vesicle migration and breakdown
Hydrated
0 h
24 h
48 h
*72 h
*96 h
Frequency (%)
0
20
40
.4
.5
.6
.7
.8
.9
1.0
1.1
Oocyte diameter (mm)

Fig. 4.6. Frequency of oocyte diameters at each sample time from a randomly selected fish treated with LHRH-a pellet during experiment 2. Asterisks indicate when the fish ovulated.

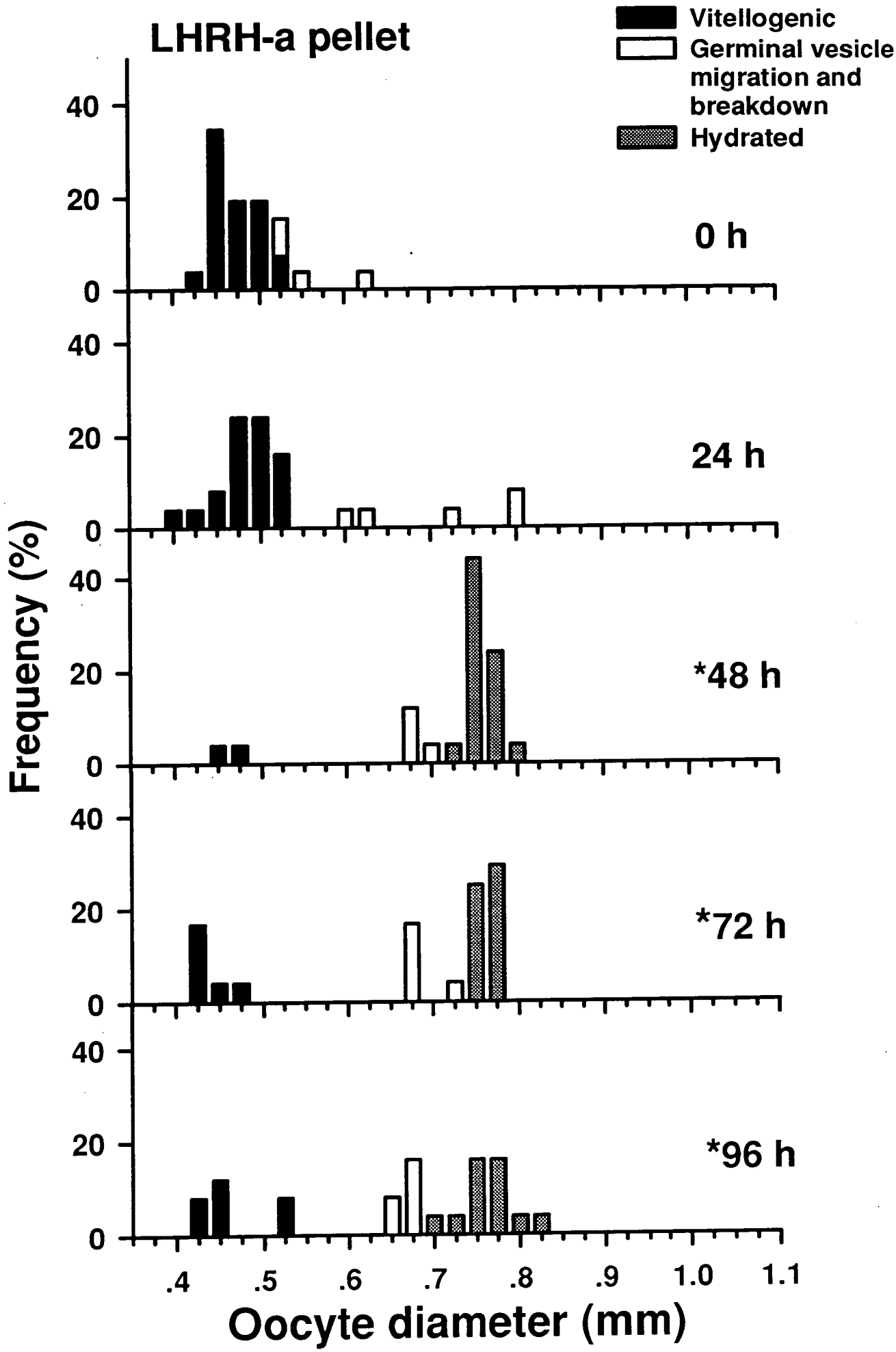

LHRH-a pellet
Vitellogenic
Germinal vesicle migration and breakdown
Hydrated
0 h
24 h
*48 h
*72 h
*96 h
Frequency (%)
0
20
40
.4
.5
.6
.7
.8
.9
1.0
1.1
Oocyte diameter (mm)

Fig. 4.7. Plasma levels of (a) E_2, (b) T and (c) 17,20ßP in each treatment group at each sample time during experiment 1. Values are mean + S.E. Values that are not significantly different (P>0.05) share common superscripts. Superscript letters show comparisons within times between groups, asterisks show controls that are significantly different from pre-treatment control levels, n=7 per treatment.

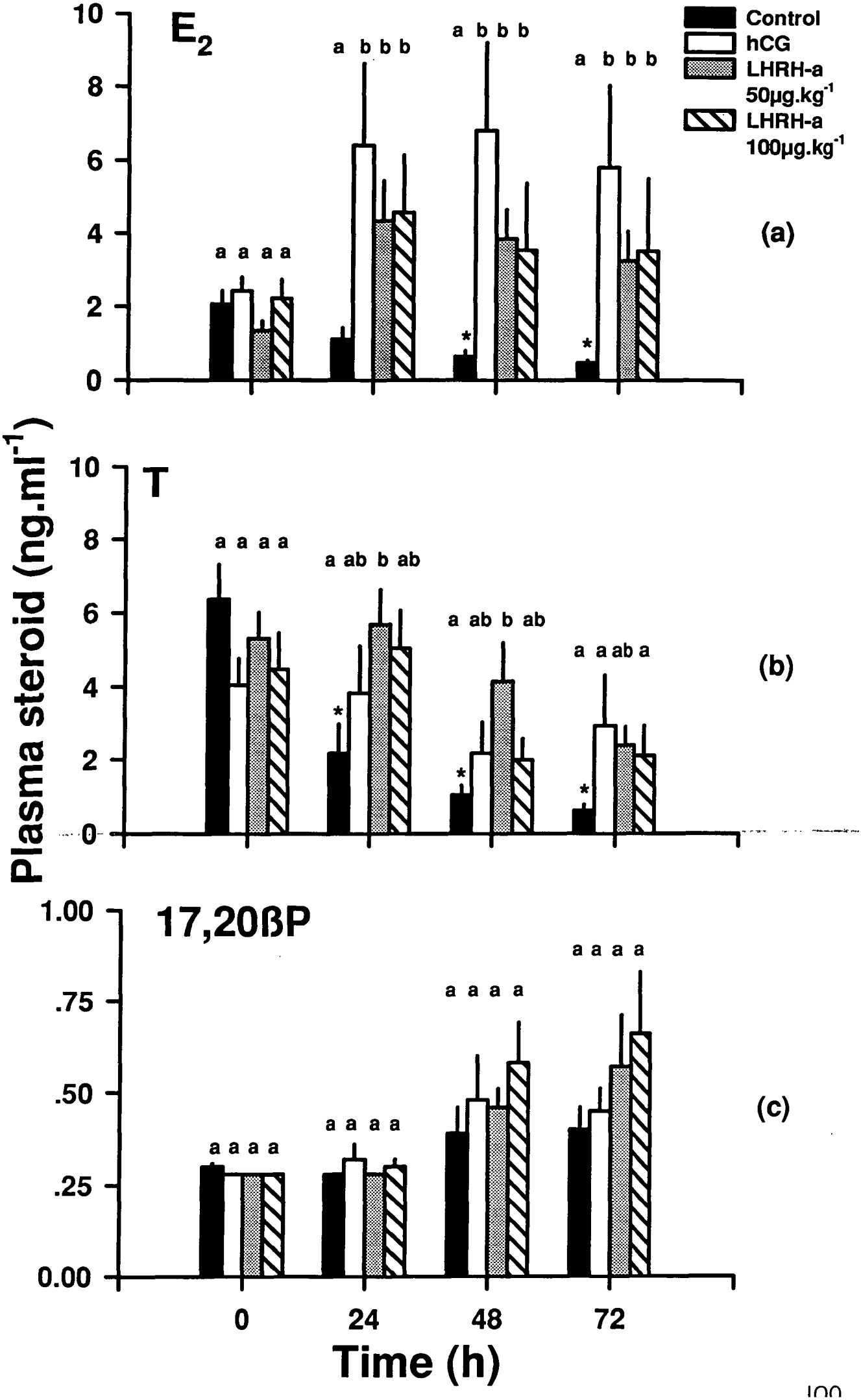

Control
hCG
LHRH-a 50µg.kg-1
LHRH-a 100µg.kg-1
E2
T
17,20ßP
(a)
(b)
(c)
Plasma steroid (ng.ml-1)
Time (h)
0
24
48
72
10
8
6
4
2
0
1.00
.75
.50
.25
0.00
a a a a
a b b b
a ab b ab
a a ab a
a a a a
*

Fig. 4.8. Plasma levels of (a) E_2, (b) T and (c) 17,20ßP in each treatment group at each sample time during experiment 2. Values are mean + S.E. Values that are not significantly different (P>0.05) share common superscripts. Superscript letters show comparisons within times between groups, asterisks show controls that are significantly different from pre-treatment control levels, n=7 per treatment.

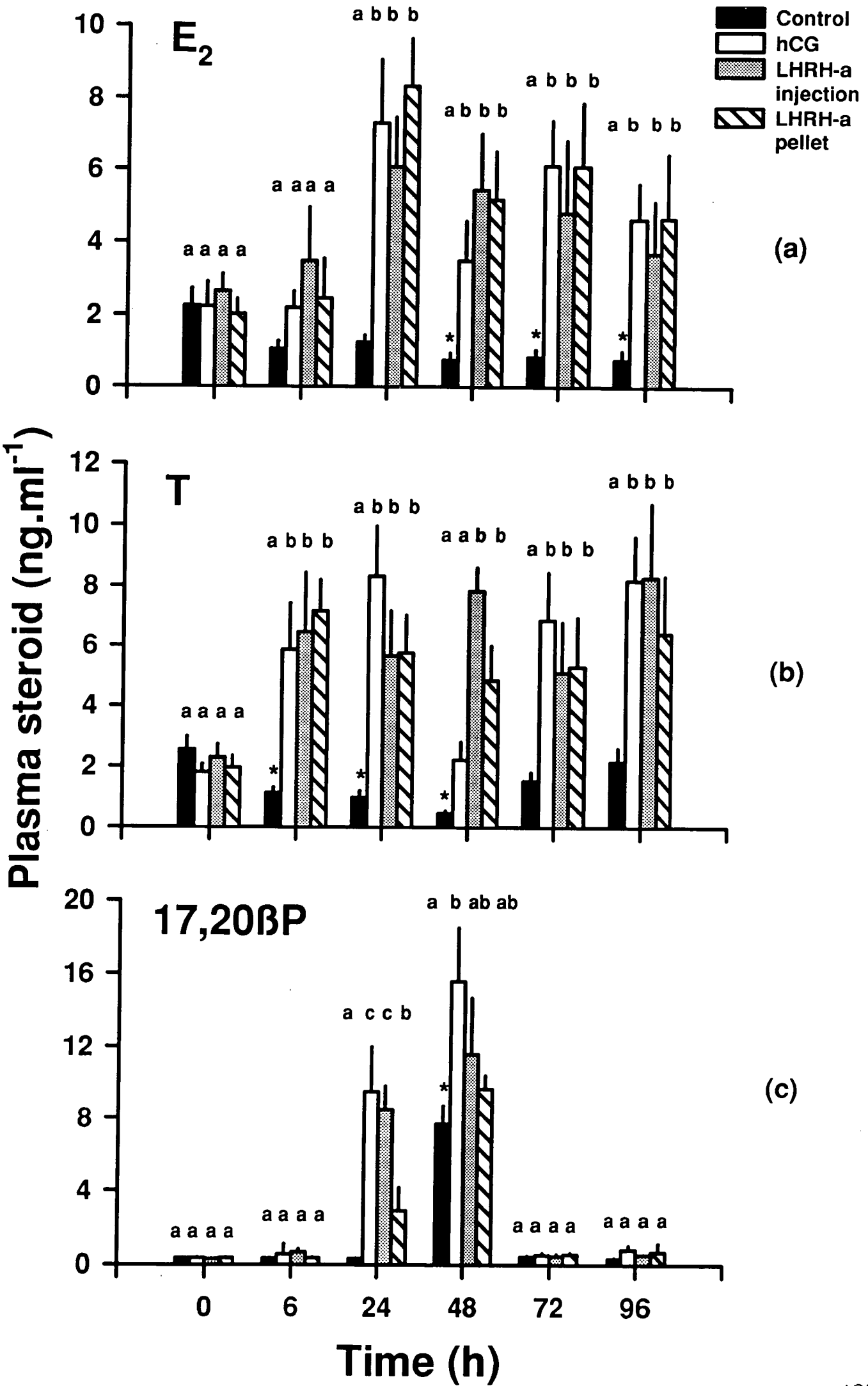
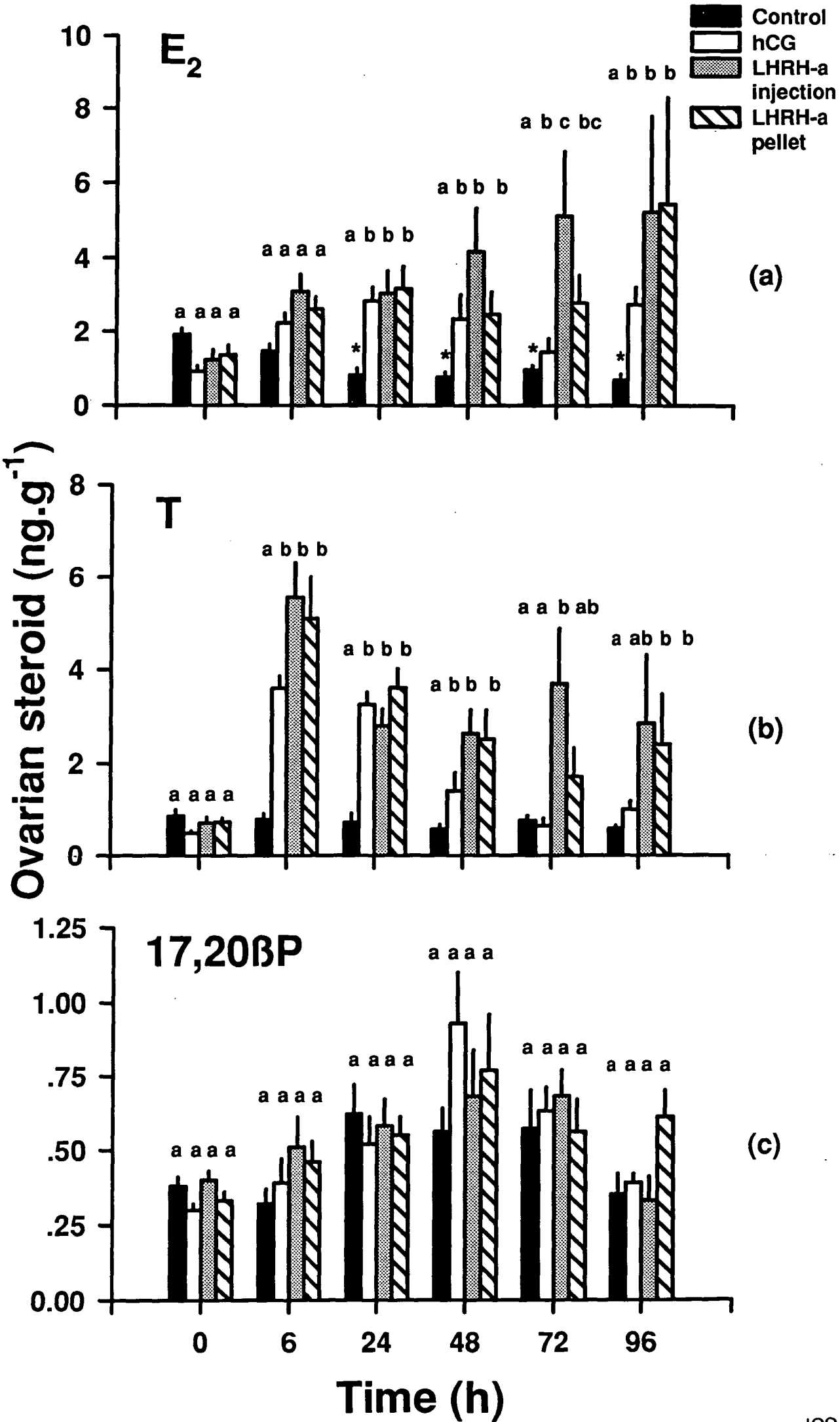

Control
hCG
LHRH-a injection
LHRH-a pellet
E2
T
17,20ßP
(a)
(b)
(c)
Plasma steroid (ng.ml-1)
Time (h)
0
6
24
48
72
96

Fig. 4.9. Ovarian levels of (a) E_2, (b) T and (c) 17,20ßP in each treatment group at each sample time during experiment 2. Values that are not significantly different (P>0.05) share common superscripts. Superscript letters show comparisons within times between groups, asterisks show controls that are significantly different from pre-treatment control levels, n=7 per treatment.


Control
hCG
LHRH-a injection
LHRH-a pellet
E2
a a a a
a a a a
a b b b
a b b b
a b c bc
a b b b
(a)
T
a a a a
a b b b
a b b b
a b b b
a a b ab
a ab b b
(b)
17,20ßP
a a a a
a a a a
a a a a
a a a a
a a a a
a a a a
(c)
Ovarian steroid (ng.g-1)
0
2
4
6
8
10
0.00
.25
.50
.75
1.00
1.25
0
6
24
48
72
96
Time (h)

4.5. Discussion

HCG and LHRH-a successfully induced repeat ovulations on a daily basis in greenback flounder which was accompanied by significant increases in oocyte diameters and the proportions of maturing oocytes. Oocyte size frequency profiles obtained from wild greenback flounder (Barnett and Pankhurst, 1998b, chapter 3, this volume), unpublished observations on naturally ovulating cultured fish and the ovulatory response and pattern of oocyte growth after exogenous hormone treatment in this study, confirmed that female greenback flounder are multiple ovulators and have group synchronous oocyte development. The summer flounder (*Paralichthys dentatus*) also demonstrated group synchronous oocyte development and ovulated daily after stripping for a period of 3-8 days, with recruitment of oocytes from a vitellogenic clutch in response to treatment with LHRH-a (Berlinsky et al., 1997). Sea bass (*Dicentrarchus labrax*) (Alvariño et al., 1992) and striped trumpeter (*Latris lineata*) (Morehead et al 1998) also demonstrated multiple spawning with group synchronous oocyte development after treatment with LHRH-a, however, the interval between consecutive spawnings in sea bass and striped trumpeter was approximately 3 days rather than daily as in greenback flounder. Sea bass recruited successive clutches from a vitellogenic population, whereas striped trumpeter demonstrated multiple clutch recruitment from cortical alveoli oocytes. In the absence of information on short term changes (<24 h) in ovarian cycling from individual fish, it is not clear whether oocytes are recruited from a batch of cortical alveoli, vitellogenic or mature stage oocytes.

In many teleost species, mammalian GtHs have orders of magnitude lower potency than teleost GtHs (reviewed in Pankhurst, 1998) and treatment with hCG may be ineffective, or large and repeat doses are required to induce ovulation (Smigielski, 1975; Lam, 1982; Donaldson and Hunter, 1983; Saidin et al., 1988; Berlinsky et al., 1997). However, in some teleost species, hCG will effectively induce ovulation (reviewed in Lam, 1982; Peter et al., 1988; Suzuki et al., 1991) and is often more successful when ovarian development is more advanced (Zohar and Gordin, 1979; Ramos, 1986; Mylonas et al., 1996; Berlinsky et al., 1997). Greenback flounder belong to the latter group, indicating that receptor recognition is at least 'good enough' to stimulate ovulation and steroid production, with the proviso that the effect may be dependent on oocytes being quite advanced at the time of treatment. Our unpublished

observations of *in vitro* oocyte maturation in greenback flounder, show that ovaries dominated by early stage vitellogenic oocytes do not respond to hCG and a range of C_{21} steroids, whereas more mature ovaries do.

LHRH-a (100 $\mu g.kg^{-1}$) ipi resulted in fewer ovulations and repeat ovulations than treatment with hCG, LHRH-a (50 $\mu g.kg^{-1}$) ipi and LHRH-a pellet. Other studies suggest that the optimal effective dose of GnRH-a or LHRH-a varies considerably between species, (although it is worth pointing out that time to ovulation and ovulatory success may in part be related to the degree of maturity at time of treatment and the GnRH-a or LHRH-a pellet matrix (Sherwood et al, 1988)). The minimum effective dose determined for grey mullet was 300-400 $\mu g.kg^{-1}$ (Lee et al., 1987) but was as low as 1-5 $\mu g.kg^{-1}$ LHRH-a in milkfish (Tamaru et al., 1988). In the sea bass, low doses of pelleted LHRH-a resulted in higher spawning and fertilisation success than high doses (Garcia, 1989), only an intermediate dose of LHRH-a significantly increased serum gonadotropin in mature male goldfish (*Carassius auratus*) (Peter, 1980) and in striped trumpeter no advantage was attained by using 200 $\mu g.kg^{-1}$dose over 100 $\mu g.kg^{-1}$ of pelleted LHRH-a (Morehead et al 1998). In goldfish, continuous administration of GnRH (Habibi, 1991) and multiple injections of LHRH and LHRH-a at high doses (Peter, 1980) resulted in desensitisation of pituitary GtH release. It is possible that a similar effect occurred in response to treatment with LHRH-a (100 $\mu g.kg^{-1}$) ipi during the present study, although the lack of difference in the steroid profiles between exogenous hormone treatments argues not.

In some species, GnRH-a or LHRH-a sustained delivery systems in the form of a cholesterol pellet or the more recently developed polyanhydride microspheres, stimulate long term gonadotropin production, and provide a desirable alternative to injected GnRH, GnRH-a, or GtH which are rapidly cleared from the blood stream (Cook and Peter, 1980; Sherwood and Harvey, 1986). In this study, LHRH-a pellet provided no significant advantage over LHRH-a (50 $\mu g.kg^{-1}$) ipi or hCG ipi. Given that the clearance rate of injected injected GnRH, GnRH-a or GtH is rapid in other species, this may indicate that a transient increase in GtH was sufficient to stimulate the processes of FOM, ovulation and self perpetuation of the maturation processes following clearance of injected hormones from the bloodstream in greenback flounder. Sustained LHRH-a delivery may be superfluous when vitellogenesis is well advanced,

but may be advantageous for inducing gonadal development in immature or regressed greenback flounder. LHRH-a copolymer pellets were used to induce gonadal maturation and spawning in completely immature red sea bream (*Pagrus major*) (Matsuyama et al., 1993).

It is well established from other studies that C_{19} and C_{18} steroids are secreted during ovarian development (Hirohiko et al., 1984; Matsuyama et al., 1988; Specker and Sullivan, 1993) and C_{21} steroids are secreted during gonadal maturation (Canario, 1991). In this study, plasma and ovarian levels of E_2 and T were significantly elevated in fish that had ovaries containing ovulated oocytes. This may have been a function of steroidogenic activity of maturing vitellogenic oocytes co-existing with unspawned ovulated oocytes, which is consistent with the suggestion that greenback flounder have group synchronous oocyte development, ovulate daily, but do not spawn spontaneously in captivity. Daily ovulations in red sea bream were accompanied by diurnal fluctuations in plasma E_2 and T with a periodicity associated with the developmental state of the ovary (Matsuyama, 1988) and similar diurnal steroid rhythms may also occur in greenback flounder. It is not known whether the periodicity of the steroid profiles was dictated by the time that the exogenous hormone treatment was administered. Plasma and ovarian levels of E_2 and T were lower in wild greenback flounder undergoing FOM and ovulation than in fish undergoing vitellogenesis (Barnett and Pankhurst, 1998b, chapter 3, this volume). However, wild fish were sampled at various times during the day, and sampling may have missed periods of steroidogenic activity. Alternatively, exogenous hormone treatments may have stimulated higher than normal plasma and ovarian steroid levels than normally occur in wild fish. Similarly, plasma levels of E_2 and T were significantly higher in winter flounder induced to ovulate using GnRH-a (Harmin et al., 1995a) than naturally ovulating winter flounder (Harmin et al., 1995b)

When either GnRH or GtH are administered by ipi, they are cleared from the blood stream quite rapidly. Plasma concentration of exogenous GnRH were halved in 12 and 12.5 min in goldfish and seabream respectively (Sherwood and Harvey, 1986; Gothilf and Zohar, 1991), the half life times of two different LHRH analogs were 18 and 22 mins (Gothilf and Zohar, 1991), and the half-disappearance time of exogenous GtH was 13 min in goldfish at 12°C (Cook and Peter, 1980). In the present study, plasma

and ovarian levels of E_2 and T remained elevated in fish treated with exogenous hormones for the duration of the experiments. Given that clearance of exogenous hormones is probably equally rapid in greenback flounder, this suggests that elevated endogenous GtH or steroids persisted after the clearance of exogenous GnRH or GtH respectively. In other species, GtH-II secretion is regulated by steroid feedback. In goldfish for example, positive steroid feedback enhances pituitary responsiveness to GnRH, but basal serum GtH-II levels are maintained by an increase in pituitary DA turnover rates (Trudeau et al., 1991b, 1993a, b). Exogenous estrogens or aromatisable androgens increase pituitary GtH levels in juvenile rainbow trout (Oncorhynchus mykiss), (Crim et al., 1981, Crim and Evans, 1983), Atlantic salmon (Salmo salar), (Crim and Peter, 1978), European eel (Anguilla anguilla) (Dufour et al., 1983, 1989) and goldfish (Trudeau et al., 1993a, b), and T treatment enhances pituitary responsiveness to exogenous GnRH-a or LHRH-a, resulting in enhanced serum GtH release in juvenile rainbow trout, goldfish, common carp and Chinese loach (Crim and Evans, 1983; Trudeau et al., 1991a, 1993a, b). Positive steroid feedback resulting from initial plasma T and E2 increases caused by exogenous hormone treatment, may function in a similar manner in greenback flounder.

Plasma T did not significantly increase in response to exogenous hormone treatment during experiment 1, although this may have been a result of rapid aromatisation of T and/or detrimental effects of stress on plasma steroid levels which was clearly demonstrated by decreasing plasma levels of E_2 and T and ovarian levels of E_2 in control fish. Stress has been reported to depress plasma levels of gonadal steroids in many other species (reviewed in Pankhurst and Van Der Kraak, 1997) and a previous study on greenback flounder indicates that experimental sampling protocols and routine husbandry practices have the capacity to stress greenback flounder (Barnett and Pankhurst, 1998c, chapter 2, this volume).

Plasma and ovarian levels of 17,20βP in wild female greenback flounder, were significantly elevated in association with ovulation or FOM and ovulation respectively, but levels were always low (Barnett and Pankhurst, 1998b, chapter 3, this volume). This seems to be common in many marine species, particularly in pleuronectiformes and partial ovulators (Scott and Canario, 1987). Plasma levels of 17,20βP changed very little throughout experiment 1, although, plasma 17,20βP increased to levels far in

excess of values we have previously found in greenback flounder (Barnett and Pankhurst, 1998b) during experiment 2. It is not clear why a similar response was not recorded during experiment 1, however, fish in the 2 experiments were sampled at different times of the day (1030 and 1500 h respectively). Diurnal changes in plasma levels of 17,20βP during the spawning season in red sea bream indicated low plasma steroid levels at 2000 h, maximal steroid levels at 0400 h and minimal steroid levels at 1600 h (Kagawa et al., 1991). If changes in plasma levels of 17,20βP in greenback flounder are short lived as in red sea bream, then sampling in experiment 1 may have missed peaks in 17,20βP. We currently have limited information on the precise timing of ovulation in this species and do not know what diel changes in plasma 17,20βP in wild or hormone treated fish might be.

In common with our previous work on induced ovulation in greenback flounder (Barnett and Pankhurst, 1998a; chapter 5 this volume), in some cases plasma levels of 17,20βP were significantly elevated above pre-treatment levels in all treatment groups including the control, but levels were not consistently elevated in association with reproductive events. 17,20βP therefore appears to be an ambiguous marker of impending ovulation. Plasma increases in 17,20βP levels may have been of interrenal origin and/or associated with a stress response; this is discussed in more detail in Barnett and Pankhurst, 1998a; chapter 5 this volume.

The average time to ovulation in this study and another study (Barnett and Pankhurst 1998a; chapter 5 this volume) was 48-72 h, and time to ovulation did not differ depending on the type of exogenous hormone treatment used. This is prolonged in comparison to the majority of species in which induced ovulation protocols have been investigated (Lam, 1982; Peter et al., 1987), however, it should be acknowledged that time to ovulation after treatment will depend on ovarian condition prior to treatment, which is variable between studies. The delay between hormone treatment and ovulation may result from indirect hormone action of the type suggested earlier. Alternatively, reproductive processes may simply be slower at lower temperatures, as a result of slower biochemical and physiological rates at lower temperatures (Withers, 1992). Studies on goldfish have indicated stimulatory effects of warm temperatures on pituitary secretion GtH rates, plasma GtH levels, and metabolic clearance rate of exogenous GtH in sexually regressed goldfish (Cook and Peter, 1980a, b, c). An examination of published literature detailing the use of exogenous hormones to induce

ovulation, indicates that time to ovulation is negatively correlated with holding temperature in a wide range of species (See Table 4.1 and Fig. 4.10) and greenback flounder are in fact similar to other species held at low temperatures. This does not preclude the possiblity that ovulation is also delayed by indirect effects of exogenous hormone treatment via steroid feedback in greenback flounder and other species.

Because different exogenous hormone treatments act at different levels of the HPG axis, and some also regulate the actions of DA inhibition, we may expect some treatments to induce ovulation more rapidly than others. In other species, there is some indication that gonadotropin releasing hormones + dopamine antagonists induce ovulation more rapidly than other treatments at holding temperatures > 10 °C (Fig. 4.9). However, there was insufficient data from studies on cold water species to make conclusions across the full temperature spectrum.

4.6. Acknowledgments

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Table 4. 1. Time to ovulation following exogenous hormone treatment vs holding temperature. Exogenous hormone treatments as follows: 1 = pituitary extracts, 2 = gonadotropin releasing hormone and analogue injections, 3 = gonadotropin releasing hormone/analogue + dopamine antagonist injections, 4= gonadotropin releasing hormone pellet implants. In the case of multiple injection protocols, time to ovulation was calculated from the first injection. Data in the form of graphical summaries, was summarised as time to 50% ovulation, data in the form of tables was summarised as average time to ovulation. (See Appendix 1 for references).

Species	Time h	Temperature °C	Treatment	Reference
African catfish *Clarias gariepinus*	11 12.5 16	25 25 25	1 3 1	(Bromage and Roberts, 1995)
sea bass *Dicentrarchus labrax*	60 60	12 12	1 2	(Bromage and Roberts, 1995)
gilt head sea bream *Sparus auratus*	60	18.5	2	(Bromage and Roberts, 1995)
trumpeter *Latris lineata*	96	13	4	(Morehead et al., 1997)
goldfish *Carassius auratus*	24	19	3	(Sokolowska et al., 1984)
sea Bass *Lates calcarifer*	42 24	27.5 27.5	2 4	(Almendras et al., 1988)
dab *Limanda limanda*	127	7.5	1	(Canario and Scott, 1990)
ayu *Plecoglossus altivelis*	48	16.5	3	(Hirose et al., 1983)
grass carp *Ctenopharyngodon idellus*	17	23.5	2	(Donaldson and Hunter, 1983)
common carp *Cyprinus carpio*	30	24	2	(Drori et al., 1994)
loach *Paramisgurnus Dabryanus*	24	18.5	3	(Lin et al., 1985)
Chinese loach *Paramisgurnus dabryanus*	12.5	23	3	(Lin et al., 1991)
winter flounder *Pseudopleuronectes americanus*	312	5	4	(Harmin and Crim, 1992)
grey mullet *Mugil cephalus*	41	27	2	(Lee et al., 1987)
catfish *Clarias macrocephalus*	15.5	28.5	1	(Mollah and Tan, 1983)
golden Pearch *Macquaria ambigua*	31	23	1	(Rowland, 1983)
ayu *Plecoglossus altivelis*	48	16	1	(Hirose et al., 1977)
red snapper *Lutjanus campechanus*	49	26	1	(Minton et al., 1983)
snapper *Pagrua auratus*	24	17	1	(Pankhurst, 1994)
greenback flounder *Rhombosolea tapirina*	60 60 60 60	12 12 12 12	1 2 3 4	(Barnett and Pankhurst, 1998) and this study

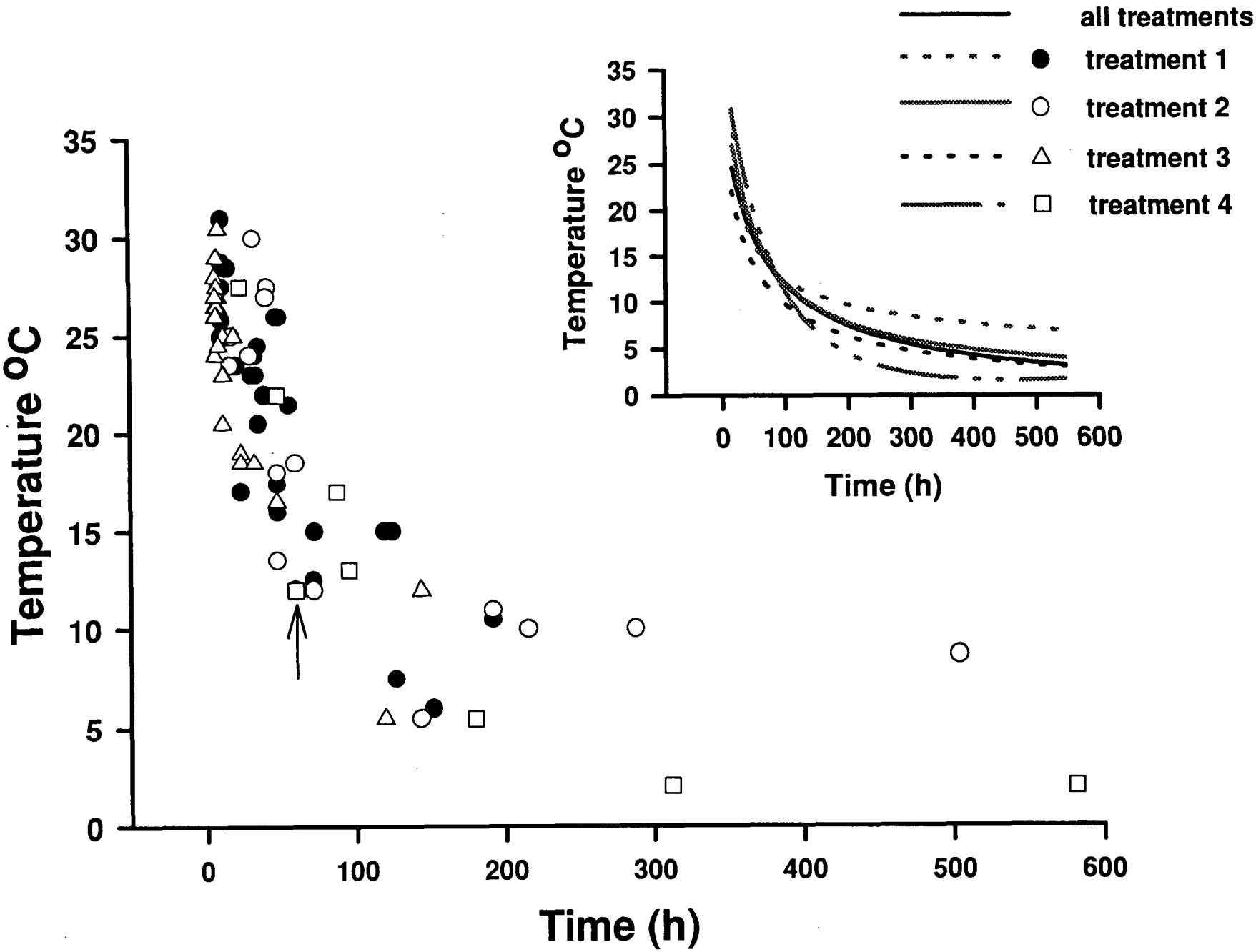
Table 4.1 continued

Indian major carps	9.5	27.5	1	(Lam, 1982)
koi *Cyprinus carpio*	11.5	25.8	1	(Lam, 1982)
Chinese carps	11.5	27.5	1	(Lam, 1982)
	11.5	31	1	
	11.5	28.8	1	
	33.5	24	1	
European catfish *Silurus glanis*	39.5	22	1	(Lam, 1982)
	34	23	1	
	21	23.5	1	
danube salmon *Hucho hucho*	152	6	1	(Lam, 1982)
summer flounder *Paralichthys dentatus*	120	15	1	(Lam, 1982)
grey mullet *Mugil cephalus*	40	22	1	(Lam, 1982)
rabbitfish *Siganus oramin*	47.5	26	1	(Lam, 1982)
rabbitfish *Siganus rivulatus*	36	24.5	1	(Lam, 1982)
sea bass *Dicentrarchus labrax*	48	13.5	2	(Alvariño et al., 1992)
Asian catfish *Clarias batrachus*	19.5	25	3	(Manickam and Joy, 1989)
Japanese flounder *Limanda yokohamae*	72	12.5	1	(Lam, 1982)
sea bass *Dicentrarchus labrax*	72.5	15	1	(Lam, 1982)
yellowfin porgy *Acanthopagrus latus*	56	21.5	1	(Leu and Chou, 1996)
white bass *Marone chrysops*	48	22	4	(Mylonas et al., 1996)
southern flounder *Paralichthys lethostigma*	88	17	4	(Berlinsky and King, 1996)
sea bass *Dicentrarchus labrax*	33	30	2	(Garcia, 1989)
grass carp *Ctenopharyngodon idellus*	18.25	25	2	(Rottmann and Shireman, 1985)
Japanese flounder *Limanda yokohomae*	72	12	1	(Hirose et al., 1979)
	72	12	2	
carp *Cyprinus carpio*	33	18.5	3	(Bieniarz et al., 1986)
common sole *Solea solea*	48	18	2	(Ramos, 1986)
goldfish *Carrassius auratus*	60	12	2	(Chang and Peter, 1983)
	60	12	3	
silver carp *Hypophthalmichthys molitrix*	12	20.5	3	(Peter et al., 1987)
	9	27	3	
	9	26.5	3	
	8.5	29	3	
mud carp *Cirhinus molitorella*	7	24	3	(Peter et al., 1987)
	7	26	3	
	7	27	3	
	7	28	3	
bream *Parabramis pekinenis*	9.5	24.5	3	(Peter et al., 1987)
	7	26.5	3	
			3	
			3	

Table 4.1 continued

grass carp *Ctenopharyngodon idellus*	12 9 8 8	20.5 26.5 27.5 26.5	3 3 3 3	(Peter et al., 1987)
bighead carp *Aristichthys nobilis*	12 8	23 26	3 3	(Peter et al., 1987)
black carp *Mylopharyngodon piceus*	7	27	3	(Peter et al., 1987)
Thailand mud carp	8 7.5	29 26	3 3	(Peter et al., 1987)
African catfish *Clarias gariepinus*	9.5	30.5	3	(Peter et al., 1987)
english sole *Parophrys vetulus*	192	10.5	1	(Sanborn and Misitano, 1991)
horse mackerel *Trachurus japonicus*	36	20.5	1	(Kurnuma and Fukusho, 1984)
yellowtail *Seriola quinqueradiata*	17.4	48	1	(Mushiake et al., 1994)
summer flounder *Paralichthys dentatus*	125	15	1	(Smigielski, 1975)
coho salmon *Oncorhynchus kisutch*	216	10	2	(Van Der Kraak et al., 1985)
rainbow trout *Salmo gairdneri*	582	2	4	(Crim et al., 1983)
rainbow trout *Salmo gairdneri*	144	12	3	(Pankhurst and Thomas, 1997)
rainbow trout *Salmo gairdneri*	192	11	2	(Billard et al., 1984)
brown trout *Salmo trutta*	144 120 180	5.5 5.5 5.5	2 3 4	(Billard et al., 1984)
coho salmon *Oncorhynchus kisutch*	504	8.7	2	(Van Der Kraak et al., 1984)
sablefish *Anoplopoma fimbria*	288	10	2	(Solar et al., 1987)

Figure 4.10. Time to ovulation following exogenous hormone treatment vs holding temperature. Exogenous hormone treatments as follows:1 = pituitary extracts and GtH preparations, 2= gonadotropin releasing hormone and analogue injections,3 = gonadotropin releasing hormone/analogue + dopamine antagonist injections, 4 = gonadotropin releasing hormone pellet implants. ↑ represents greenback flounder. Inset shows curve fits of time to ovulation vs holding temperature for each exogenous hormone treatment, curve fit equations given in appendix 2.


all treatments
treatment 1
treatment 2
treatment 3
treatment 4
Temperature °C
Time (h)
0
5
10
15
20
25
30
35
0
100
200
300
400
500
600

4.7. References

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Chapter 5

Potential for steroid feedback and dopamine inhibition on GtH release in greenback flounder *Rhombosolea tapirina* (Günther, 1862): indirect assessment by measurement of gonadal steroids and ovulation.

5. Potential for steroid feedback and dopamine inhibition on GtH release in greenback flounder *Rhombosolea tapirina* (Günther, 1862): indirect assessment by measurement of gonadal steroids and ovulation.

5.1. Summary

Greenback flounder were treated with either des Gly^{10} [D-Ala^{6}] LHRH ethylamide (LHRH-a), pimozide (PIM), testosterone (T), LHRH-a + PIM or LHRH-a + T. LHRH-a and PIM were administered by intraperitoneal injection (ipi) and T was implanted intraperitoneally as a silastic pellet. Treatment with LHRH-a, LHRH-a + PIM and LHRH-a + T significantly increased the number of ovulations above control levels. LHRH-a was more effective than LHRH-a + PIM and PIM in 1 out of 2 experiments. LHRH-a + T was more effective than LHRH-a and T in both experiments. PIM significantly increased the total number of ovulations in 1 out of 2 experiments, but treatment with T alone had no effect.

LHRH-a, PIM, LHRH-a + PIM and LHRH-a + T significantly increased the percentage of fertilised eggs that developed through to 4 cell stage above control levels, and treatment with LHRH-a resulted in significantly more eggs surviving to the 4 cell stage than fish treated with PIM and LHRH-a + PIM. Fish treated with LHRH-a and LHRH-a + PIM had higher plasma 17β-estradiol (E_2) and T levels than control fish or fish treated with PIM. Plasma E_2 levels were significantly higher in fish treated with LHRH-a and LHRH-a + T than in control fish or fish treated with T. Plasma levels of 17α20ß-dihydroxy-4-pregnen-3-one (17,20ßP) were not elevated above control levels in fish treated with exogenous hormones, but were elevated above pre-treatment levels in all treatment groups in some cases. These results suggest that dopamine has an inconsistent effect on reproductive function in greenback flounder and T potentiates the ovulatory effects of LHRH-a, possibly by enhancing pituitary responsiveness to GnRH.

5.2. Introduction

A common problem in fish culture is the failure of fish to undergo final oocyte maturation, ovulation and spawning, and consequently, a range of reproductive management techniques have been developed which act on various levels of the endocrine cascade to induce these processes (Donaldson and Hunter, 1983; Fauvel et al., 1993; Peter and Yu, 1997). Gonadotropin-II is the pituitary hormone primarily responsible for stimulating maturational steroid synthesis by the gonad (Kawauchi et al., 1989), it is secreted under stimulation by gonadotropin-releasing hormone (GnRH) and in most species examined to date, secretion is inhibited by dopamine (DA) (reviewed in Trudeau and Peter, 1995; Peter and Yu, 1997). Co-treatment of a superactive analog of fish GnRH (GnRH-a) or a mammalian GnRH analogue (referred to in this volume as luteinising hormone relasing hormone (LHRH-a)) to stimulate GtH-II release and a DA receptor antagonist such as pimozide (PIM) to reduce the inhibitory tone imposed by DA action, is very successful for inducing ovulation in most species in which it has been trialed. Examples include the Chinese loach (*Paramisgurnus dabryanus*) (Lin et al., 1985, 1986a), goldfish (*Carassius auratus*) (Chang and Peter, 1983b; Sokolowska et al., 1984), African catfish *(Clarias lazera*) (DeLeeuw et al., 1985, 1987), common carp (*Cyprinus carpio*) (Bieniarz et al., 1986) and many other freshwater species in China (Lin et al., 1986b; Peter et al., 1987, 1988). In the coho salmon (*Oncorhynchus kisutch*), gilthead seabream (*Sparus aurata*) and Atlantic croaker (*Micropogonias undulatus*) however, DA inhibition of GtH secretion is weak or non-existent (Van der Kraak et al., 1986; Zohar et al., 1987; Copeland and Thomas, 1989) and in the gilthead seabream and Atlantic croaker, co-treatment of GnRH-a and a DA receptor antagonist offers no significant advantage over GnRH-a treatment alone.

The greenback flounder *Rhombosolea tapirina* (Pleuronectidae) is a potential aquaculture species in Tasmania (Hart, 1993), however, egg production is variable and further development of the industry is reliant on the development of reliable induced ovulation techniques (Crawford, 1994; Hart, 1991, 1993; Hart and Purser, 1995). In a previous study (Barnett and Pankhurst, 1998b; chapter 4 this volume), greenback flounder with ovaries at the vitellogenic stage of development treated with human

chorionic gonadotropin (hCG) or luteinising-hormone-releasing-hormone analogue (LHRH-a) underwent ovulation, and plasma and ovarian levels of E_2 and T remained elevated in ovulating fish from 24-96 h post treatment. This suggested to us that elevated steroids persisted after the clearance of exogenous LHRH or hCG respectively and that hormone treatment had resulted in prolonged elevation of endogenous GtH. Steroid feedback via central action plays a role in regulating gonadotropin in other species. In goldfish for example, E_2 and T exert a combination of positive and negative feedback actions, whereby pituitary responsiveness to GnRH is enhanced, but basal serum GtH-II levels are maintained by an increase in pituitary DA turnover rates (Trudeau et al., 1991b, 1993a, b). Treatment with estrogens or aromatisable T increases pituitary GtH content and pituitary responsiveness to exogenous GnRH-a or LHRH-a in a number of species (Crim and Peter, 1978; Crim et al., 1981, Crim and Evans, 1983; Dufour et al., 1983, 1989; Trudeau et al., 1993a, b). Steroid feedback may also function in a similar fashion in greenback flounder, which suggests potential for exogenous steroid treatment in enhancing the ovulatory response to LHRH-a..

In this study, we tested the potential of the DA antagonist PIM to further enhance ovulatory success in the presence of LHRH-a, and also examined the potentiating effects of T on ovulation and LHRH-a induced ovulation. In the absence of a GtH assay for greenback flounder we chose to assess the efficacy of hormone treatments by examining incidence and frequency of ovulation and the associated changes in plasma sex steroid levels. As the point of induced ovulation is to provide viable eggs, the fertility of eggs produced using various treatments was also assessed.

5.3. Materials and methods

5.3.1. Fish and fish maintenance

Experiments were conducted on second generation cultured fish produced by either the Department of Primary Industries and Fisheries, Marine Research Laboratories, Taroona, Tasmania or the Department of Aquaculture aquatic facility at the University of Tasmania in Launceston. Fish were maintained at the Launceston facility, in recirculating systems incorporating a biofilter, coarse solids filter and aeration system. Fish with ovaries at the vitellogenic stage of development were chosen for the

experiments. These fish had large firm ovaries, bulging above the musculature, extending to the caudal peduncle but no oocytes were released upon gentle pressure on the abdomen. In all experiments, fish were fed at 3% body weight daily, body weights ranged from 95-285 g, water temperature was 10-12 °C, daylength was 10 h (lights on 0645 h), and all experiments commenced at 0900 h.

5.3.2. Experimental protocols

Experiments 1 and 2: Fish were divided into four treatment groups (n=7) anaesthetised and treated with either (1) saline and pimozide (PIM) vehicle (0.7% NaCl / 1 $g.l^{-1}$ sodium metabisulphite / 0.2 $ml.l^{-1}$ acetic acid (Chang and Peter, 1983a)) (2) des Gly_{10} [D-Ala6] LHRH ethylamide (LHRH-a) and PIM vehicle (3) saline and PIM, or (4) LHRH-a and PIM. LHRH-a and PIM were administered at a dose of 100 $\mu g.kg^{-1}$ and 10$mg.kg^{-1}$ respectively and each treatment was administered in an injection volume of 100$\mu l.kg^{-1}$ by intraperitoneal injection (ipi). Each fish was anaesthetised then bled 0, 24, 48, 72 and 96 h after treatment and checked for ovulation by gentle pressure on the abdomen 0, 24, 48, 72, 96, 120, 144, 168 and 192 h after treatment. Any ovulated eggs were stripped.

Experiments 3 and 4: Fish were divided into four treatment groups (n=7), anaesthetised, and treated with either (1) an injection of saline and a blank silastic pellet (2) an injection of LHRH-a and a blank silastic pellet (3) an injection of saline and T in a silastic pellet (4) an injection of LHRH-a and T in a silastic pellet. Silastic pellets were made with 1 g Silastic 382 unpolymerised medical grade elastomer (Dow Corning Corporation), + 20 µl of accelerator and crystalline T (200 $mg.g^{-1}$ elastomer) spread into 2 x 2 x 30 mm moulds to give 0.8 mg T. mm^{-1} silastic strip. The mixture was left to set and pellets were cut to the appropriate length to give a dose of 100 $mg.kg^{-1}$ body weight. Pellets were inserted into the intraperitoneal cavity using a 12G hypodermic needle. LHRH-a was administered at a dose of 100 $\mu g.kg^{-1}$ and LHRH-a and saline were administered as before. Each fish was anaesthetised, bled and stripped as for experiments 1 and 2.

5.3.3. Egg fertilisation

During experiments 2 and 3, sperm was collected from 3 - 4 spermiated males held separately from females, at each sample period in a 3 ml syringe, and stored on ice.

Ovulated females were stripped and 500 µl of eggs were mixed with 50µl sperm and 500 µl of sterilised seawater by gentle agitation for 3 min, after which the mixture was washed by additions of clean sea water, the fertilised eggs were incubated in 50 ml of clean seawater at 12°C (Hart and Purser, 1995) and examined for embryonic cleavage to 4 or more cells, 5 h after fertilisation.

5.3.4. Steroid measurement

Blood samples were collected by caudal puncture and plasma concentrations of E_2, T and 17,20ßP were measured by radioimmunoassay (RIA). One hundred microlitres of plasma were extracted with 1 ml of ethyl acetate and added to each assay tube for evaporation and resuspension in assay buffer, using the reagents and protocol given in (Pankhurst and Conroy, 1987). Extraction efficiency was determined by recovery of [^{3}H]-labelled steroid extracted with plasma, and was on average 94%, 93%, and 93% for E_2, T and 17,20ßP respectively. Assay values were corrected accordingly. Assay detection limits were 0.28 ng.ml^{-1} for E_2, T and 17,20ßP. Interassay variability was measured using a pooled steroid standard giving %CVs of 9% (n=10), 13% (n=10), 7% (n=10) for E_2, T and 17,20ßP respectively.

5.3.5. Statistical analysis

Repeated measures analysis of variance, mean comparison tests, planned contrasts and logistic analysis were performed using the computer packages SAS or SPSS for Windows. Data were log or square root transformed to satisfy normality and homogeneity of variance requirements. A significance level of $\alpha < 0.05$ was used for all statistical tests. Homogeneity of variance was not satisfied in all instances, however, the data were also assessed by multivariate analysis of variance and canonical discriminant analysis, and in all cases the outcomes were consistent. We have presented ANOVA results because of the utility of mean comparison tests and the familiarity of these tests within the literature.

5.4. Results

Logistic analysis indicated that treatment with LHRH-a and LHRH-a + PIM significantly increased the number of ovulations above control levels during experiments 1 and 2, and LHRH-a induced more ovulations than LHRH-a + PIM in experiment 2 (Figs 5.1 and 5.2). Treatment with PIM significantly increased the total

number of ovulations above control levels in experiment 2, but not experiment 1. During experiment 1, all but one fish that ovulated in response to exogenous hormones ovulated more than once, most of these fish ovulated more than twice and most ovulations occured at daily intervals. During experiment 2, all fish that ovulated in response to exogenous hormones ovulated more than once, most of these fish ovulated more than twice and most ovulations occured at daily intervals.

Treatment with LHRH-a and LHRH-a + T, significantly increased the total number of ovulations above control levels, and LHRH-a + T induced significantly more ovulations than LHRH-a alone in experiments 3 and 4 (Figs 5.3 and 5.4). Treatment with T alone did not significantly increase the number of ovulations above control levels. The majority of ovulations were recorded 48 - 144 h, 96 - 168 h, 120 - 168 h and 96 - 168 h after exogenous hormone treatment for experiments 1 - 4 respectively. During experiments 3 and 4, all but one fish that ovulated in response to exogenous hormones ovulated more than once, most of these fish ovulated more than twice and most ovulations occured at daily intervals.

Logistic analysis indicated that, treatment with LHRH-a, PIM and LHRH-a + PIM significantly increased the percentage of fertilised eggs that developed through to 4 cell stage of embryonic development and treatment with LHRH-a resulted in significantly more eggs surviving to the 4 cell stage than fish treated with PIM or LHRH-a + PIM (Fig. 5.5a). Treatment with LHRH-a and LHRH-a + T both increased the percentage of fertilised eggs that developed through to 4 cell stage above control levels and fish treated with T. There was no difference between LHRH-a and LHRH-a + T (Fig. 5.5b).

Fish treated with LHRH-a + PIM and LHRH-a had significantly higher plasma levels of E_2 than control fish and fish treated with PIM during experiments 1 and 2 (Figs 5.6a and 5.7a), and fish treated with LHRH-a + PIM had significantly higher levels of E_2 than fish treated with LHRH-a at 24 h during experiment 1. Those fish treated with PIM showed no significant increases in plasma levels of E_2 above control levels during experiments 1 and 2. Plasma levels of T were significantly higher in fish treated with LHRH-a at 96 h in experiment 1 and 24-72 h in experiment 2, than in control fish or fish treated with PIM (Figs 5.6b and 5.7b). Plasma levels of T were also significantly higher than controls or fish treated with PIM, in fish treated with LHRH-a + PIM at

48- 96 h in experiment 1 and 24-96 h in experiment 2. Fish treated with LHRH-a + PIM had significantly higher levels of T than fish treated with LHRH-a at 48 h during experiment 1. Fish treated with PIM showed no significant changes in plasma T levels during both experiments 1 and 2.

Treatment with LHRH-a and LHRH-a + T resulted in significantly higher plasma levels of E_2, 72 and 96 hours after treatment during experiments 3 and 4 than in control fish and fish treated with T (Figs 5.8a and 5.9a). Fish treated with T showed no significant changes in plasma in E_2 during experiments 3 and 4. Plasma T was significantly higher at 24 -96 h in fish treated with T and LHRH-a + T than in control fish and fish treated with LHRH-a during experiments 3 and 4 (Figs 5.8b and 5.9b). Plasma levels of T did not differ between fish treated with T and LHRH-a + T throughout experiments 3 and 4.

Plasma levels of 17,20ßP were not elevated above control levels in any fish treated with exogenous hormones throughout experiments 1 - 4 (Figs 5.6c-5.9c), although, plasma levels of 17,20βP were significantly elevated above pre-treatment levels in control fish and fish treated with exogenous hormones, 24 and 48 h after treatment during experiment 2.

Fig. 5.1. Percentage of fish that ovulated in each treatment group at each sample time during experiment 1. Treatments that are not significantly different ($P>0.05$) share common superscript letters. Fish are labelled 1-7 within each treatment group and numbers denote which fish ovulated within each treatment at each sample time.

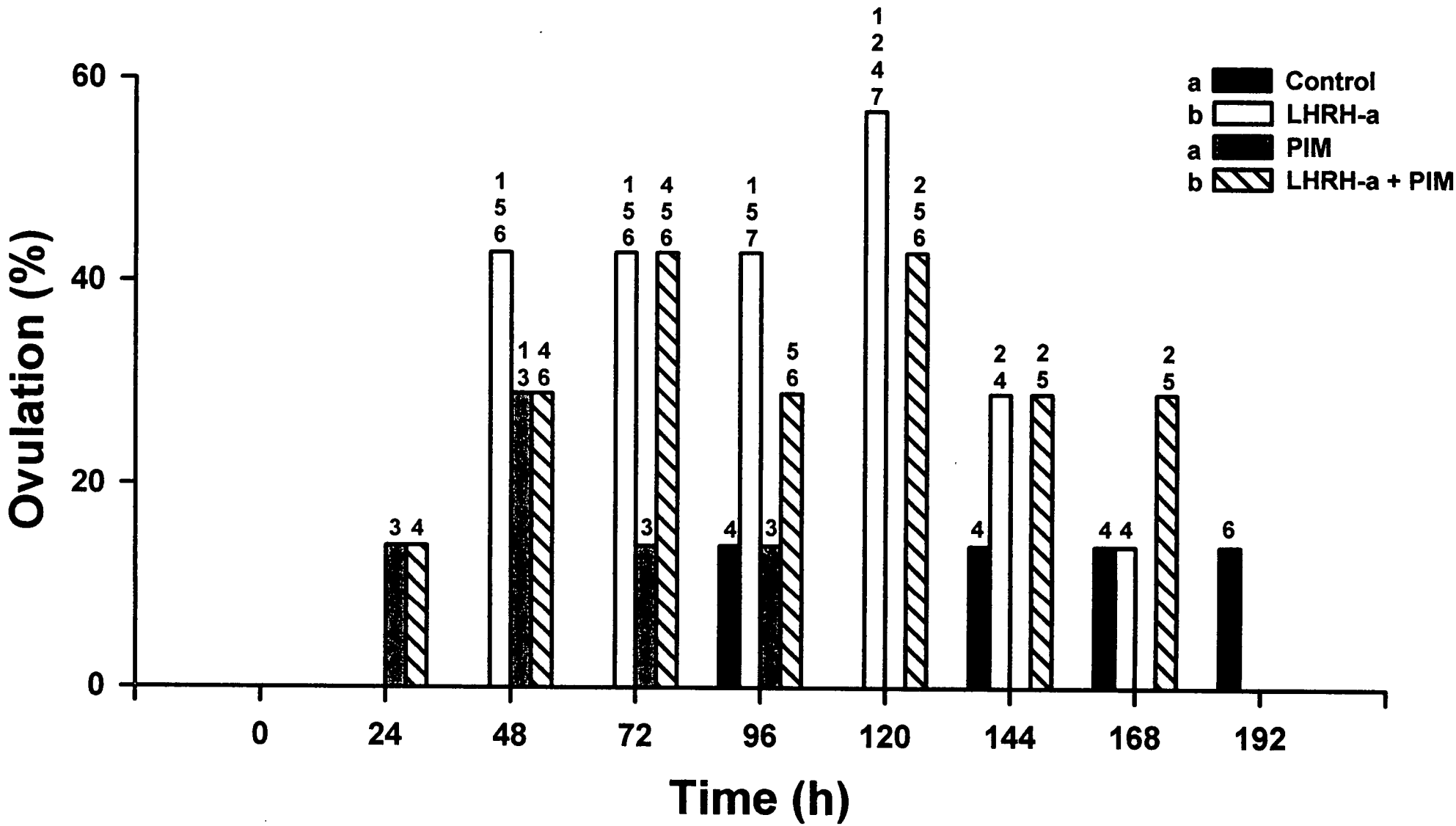

Ovulation (%)
60
40
20
0
0
24
48
72
96
120
144
168
192
Time (h)
a Control
b LHRH-a
a PIM
b LHRH-a + PIM
3 4
1 5 6
1 3 4 6
1 5 6
3
4 5 6
4
1 5 7
3
5 6
1 2 4 7
2 5 6
4
2 4
2 5
4 4
2 5
6

Fig. 5.2. Percentage of fish that ovulated in each treatment group at each sample time during experiment 2. Treatments that are not significantly different ($P>0.05$) share common superscript letters. Fish are labelled 1-7 within each treatment group and numbers denote which fish ovulated within each treatment at each sample time.

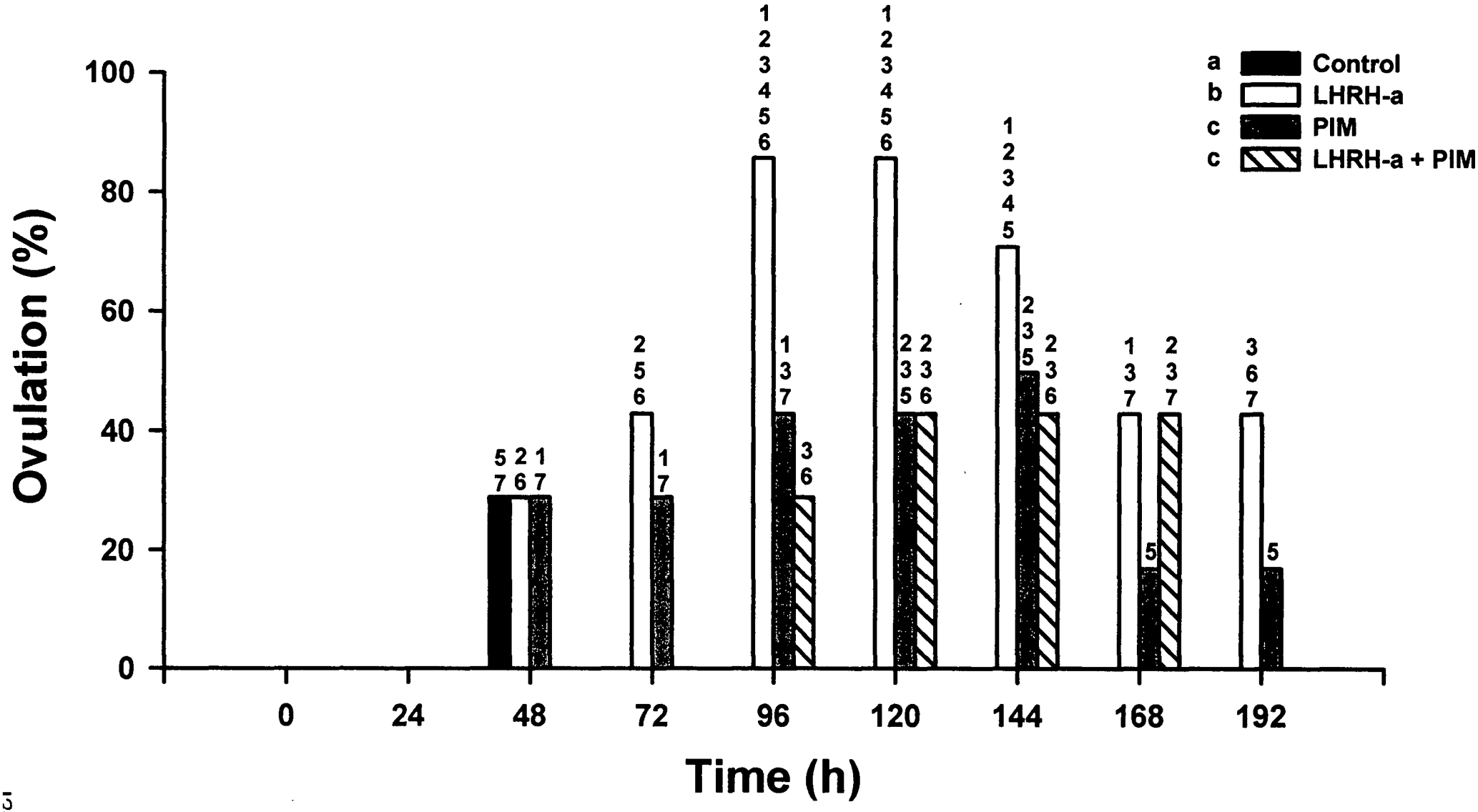

Ovulation (%)
100
80
60
40
20
0
0
24
48
72
96
120
144
168
192
Time (h)
a Control
b LHRH-a
c PIM
c LHRH-a + PIM
5 7
2 6
1 7
2 5 6
1 7
1 2 3 4 5 6
1 3 7
3 6
1 2 3 4 5 6
2 3 5
2 3 6
1 2 3 4 5
2 3 5
2 3 6
1 3 7
5
2 3 7
3 6 7
5

Fig. 5.3. Percentage of fish that ovulated in each treatment group at each sample time during experiment 3. Treatments that are not significantly different ($P>0.05$) share common superscript letters. Fish are labelled 1-7 within each treatment group and numbers denote which fish ovulated within each treatment at each sample time.

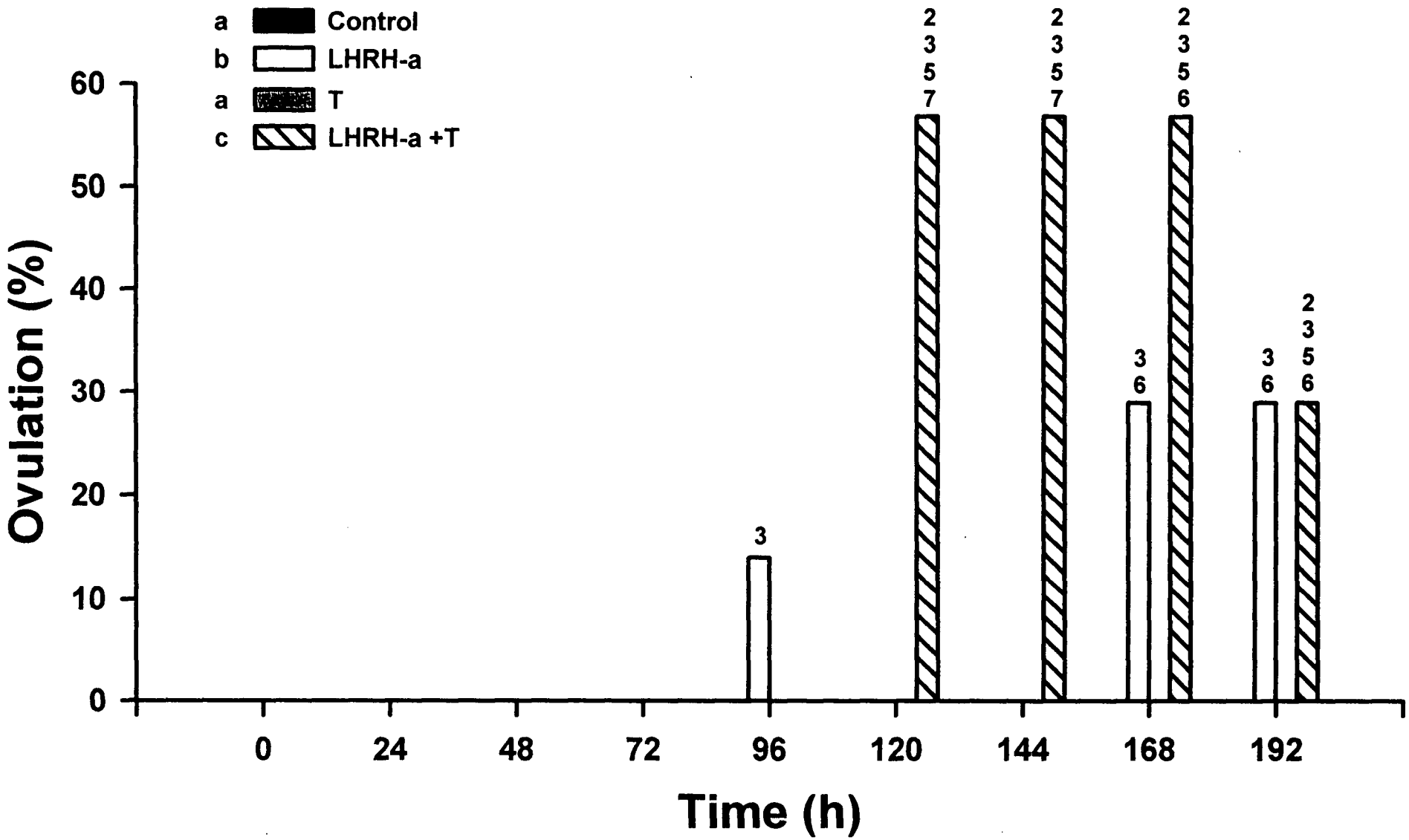

a Control
b LHRH-a
a T
c LHRH-a +T
Ovulation (%)
60
50
40
30
20
10
0
0
24
48
72
96
120
144
168
192
Time (h)
3
2 3 5 7
2 3 5 7
2 3 5 6
3 6
3 6
2 3 5 6

Fig. 5.4. Percentage of fish that ovulated in each treatment group at each sample time during experiment 4. Treatments that are not significantly different ($P>0.05$) share common superscript letters. Fish are labelled 1-7 within each treatment group and numbers denote which fish ovulated within each treatment at each sample time.

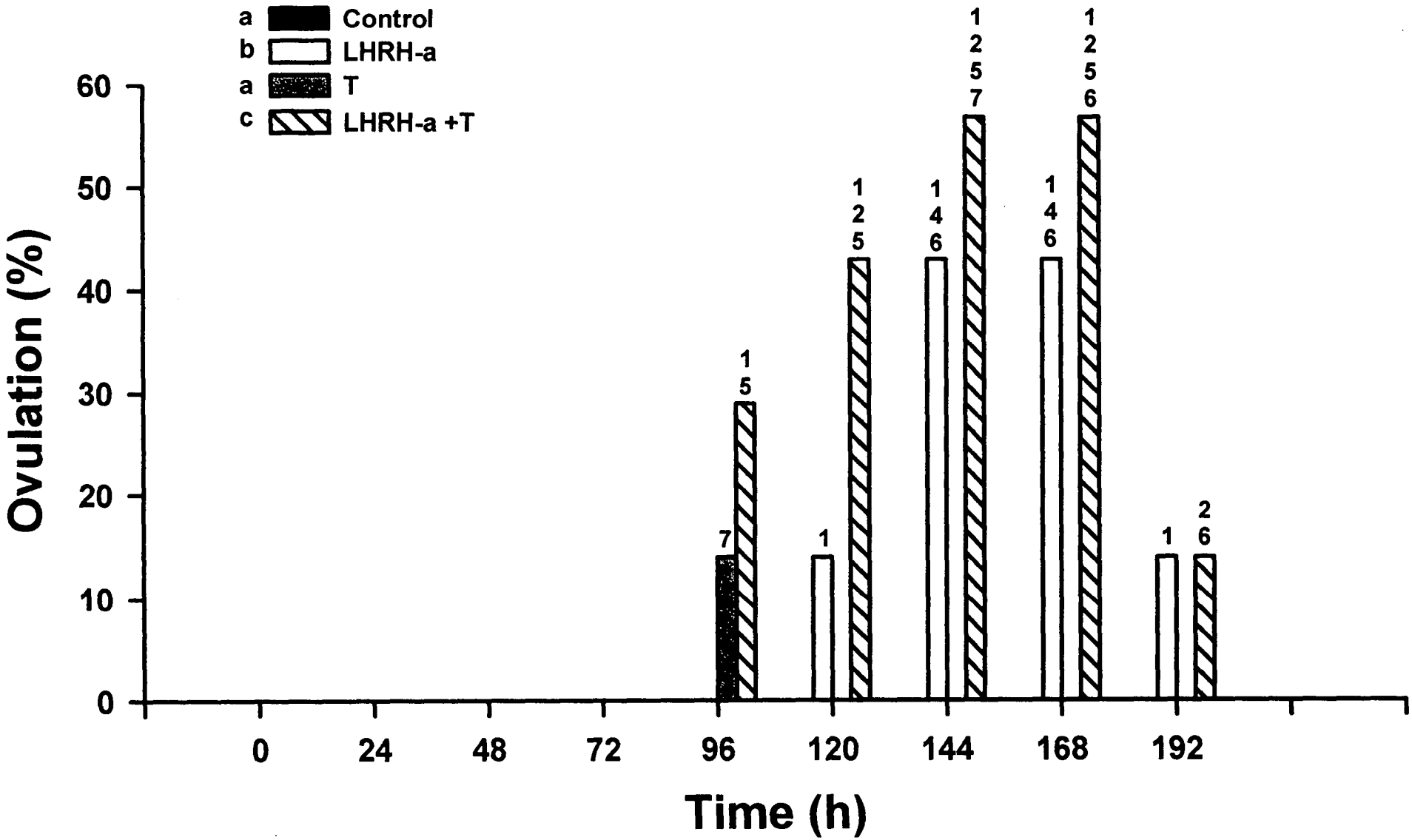

a Control
b LHRH-a
a T
c LHRH-a +T
Ovulation (%)
60
50
40
30
20
10
0
0
24
48
72
96
120
144
168
192
Time (h)
7
1 5
1
1 2 5
1 4 6
1 2 5 7
1 4 6
1 2 5 6
1
2 6

Fig.5.5. Percentage of eggs that were stripped and fertilised from fish in each treatment group, and developed through to the 4-cell stage of embryonic development (a) Experiment 2 (b) experiment 3. Treatments that are not significantly different ($P>0.05$) share common superscripts.

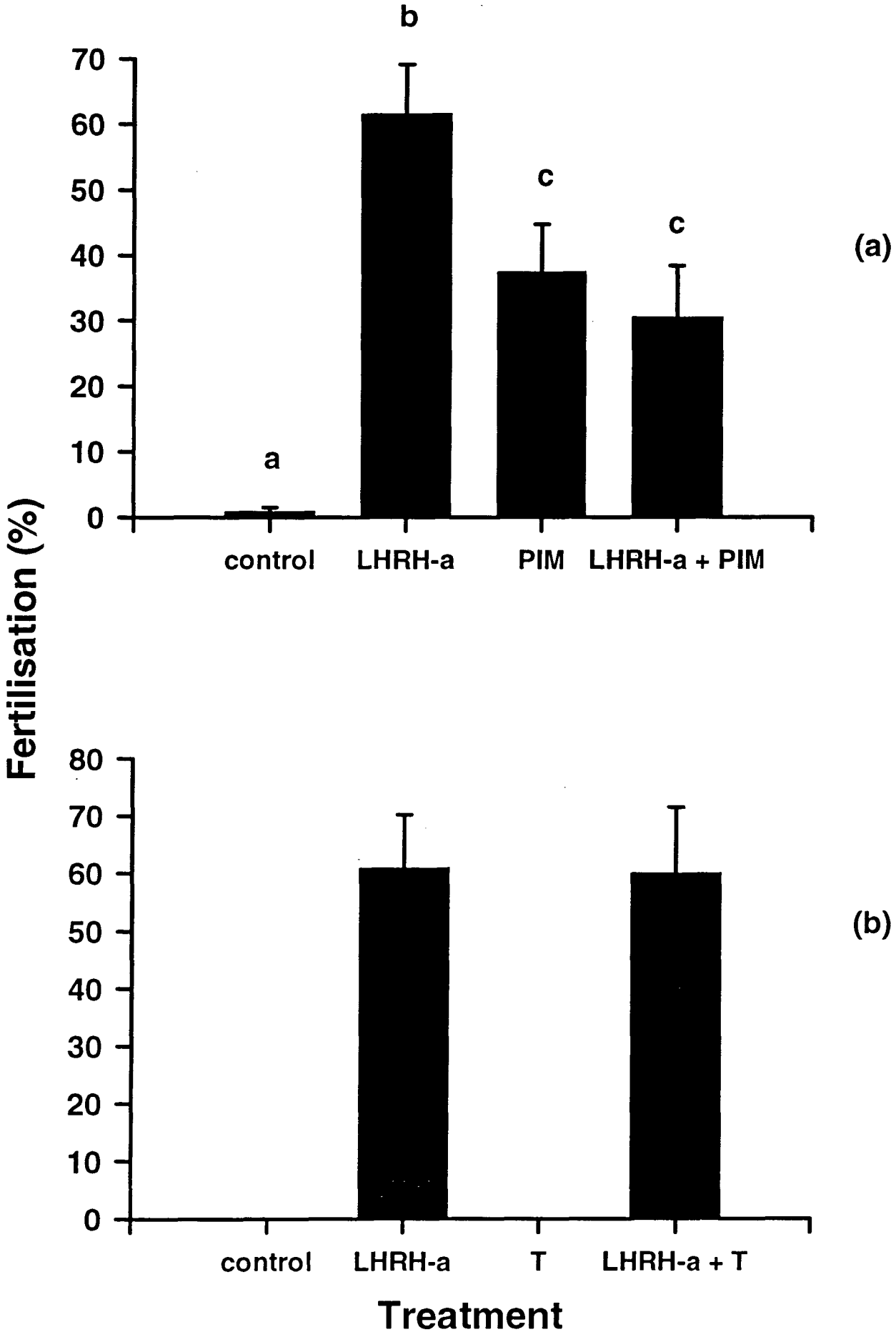

b
c
c
a
70
60
50
40
30
20
10
0
control
LHRH-a
PIM
LHRH-a + PIM
(a)
Fertilisation (%)
80
70
60
50
40
30
20
10
0
control
LHRH-a
T
LHRH-a + T
(b)
Treatment

Fig. 5.6. Plasma levels of (a) E_2, (b) T and (c) 17,20ßP in each treatment group at each sample time during experiment 1. Values are mean + S.E. Values that are not significantly different (P>0.05) share common superscripts, comparisons are within times between groups, n=7 per treatment.

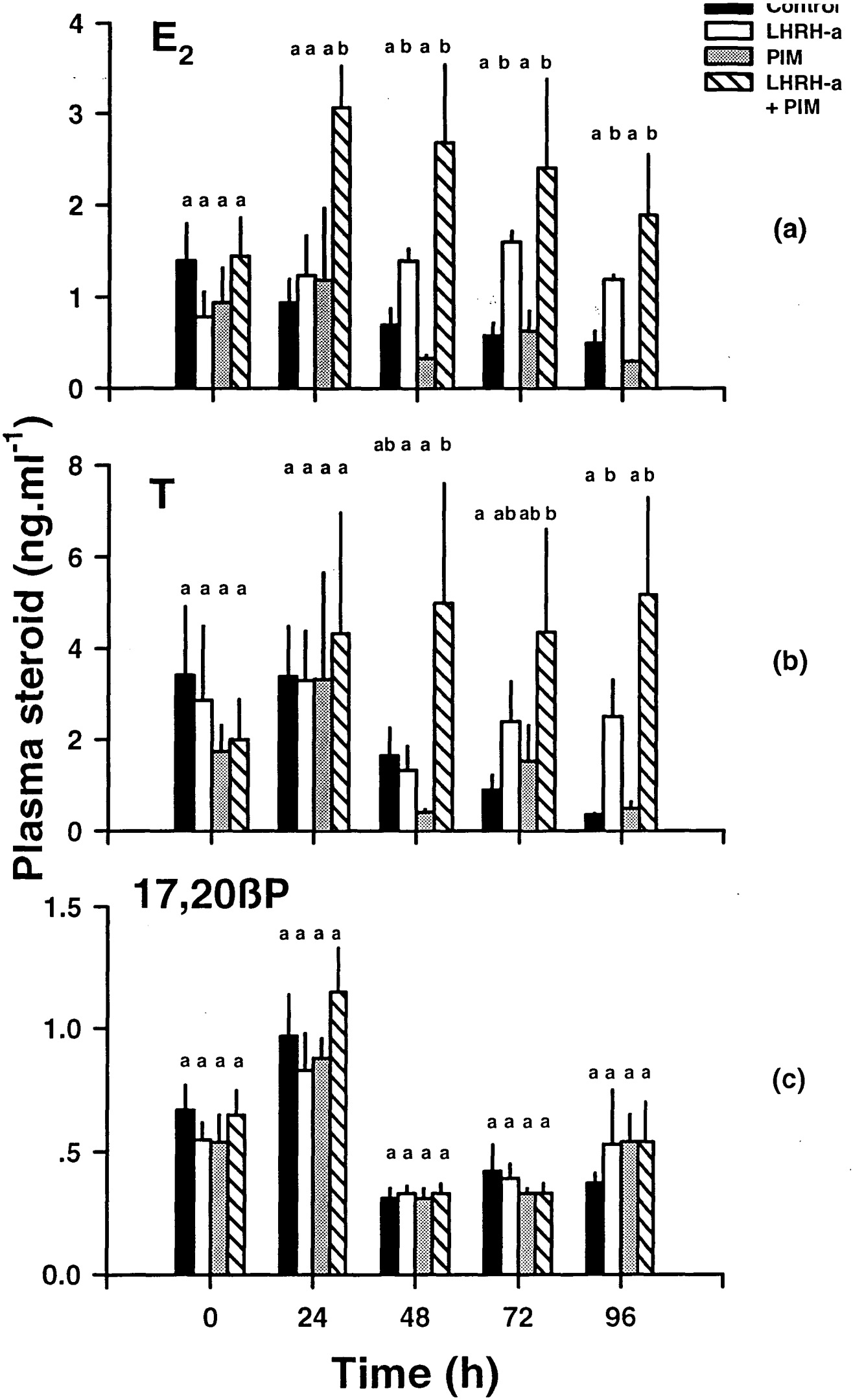

Control
LHRH-a
PIM
LHRH-a + PIM
E2
a a a b
a b a b
a b a b
a b a b
a a a a
(a)
T
a a a a
a a a a
ab a a b
a ab ab b
a b ab
(b)
Plasma steroid (ng.ml-1)
17,20ßP
a a a a
a a a a
a a a a
a a a a
a a a a
(c)
0
24
48
72
96
Time (h)

Fig. 5.7. Plasma levels of (a) E_2, (b) T and (c) 17,20ßP in each treatment group at each sample time during experiment 2. Values are mean + S.E. Values that are not significantly different (P>0.05) share common superscripts, comparisons are within times between groups, n=7 per treatment.

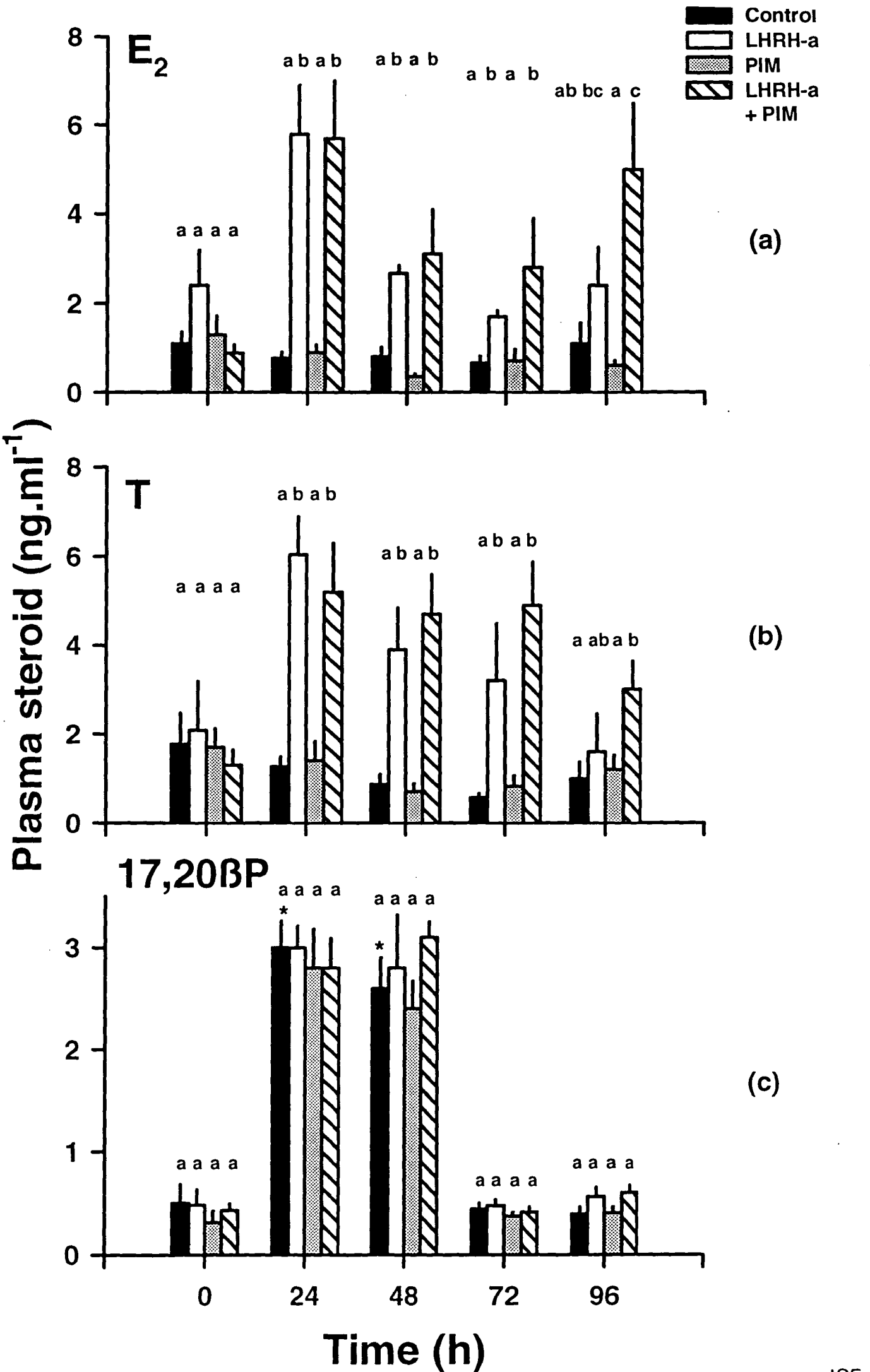

Control
LHRH-a
PIM
LHRH-a + PIM
E2
a a a a
a b a b
a b a b
a b a b
ab bc a c
(a)
T
a a a a
a b a b
a b a b
a b a b
a aba b
(b)
17,20ßP
a a a a
a a a a
a a a a
a a a a
a a a a
*
*
(c)
Plasma steroid (ng.ml-1)
0
1
2
3
4
6
8
0
24
48
72
96
Time (h)

Fig. 5.8. Plasma levels of (a) E_2, (b) T and (c) 17,20ßP in each treatment group at each sample time during experiment 3. Values are mean + S.E. Values that are not significantly different (P>0.05) share common superscripts, comparisons are within times between groups, n=7 per treatment.

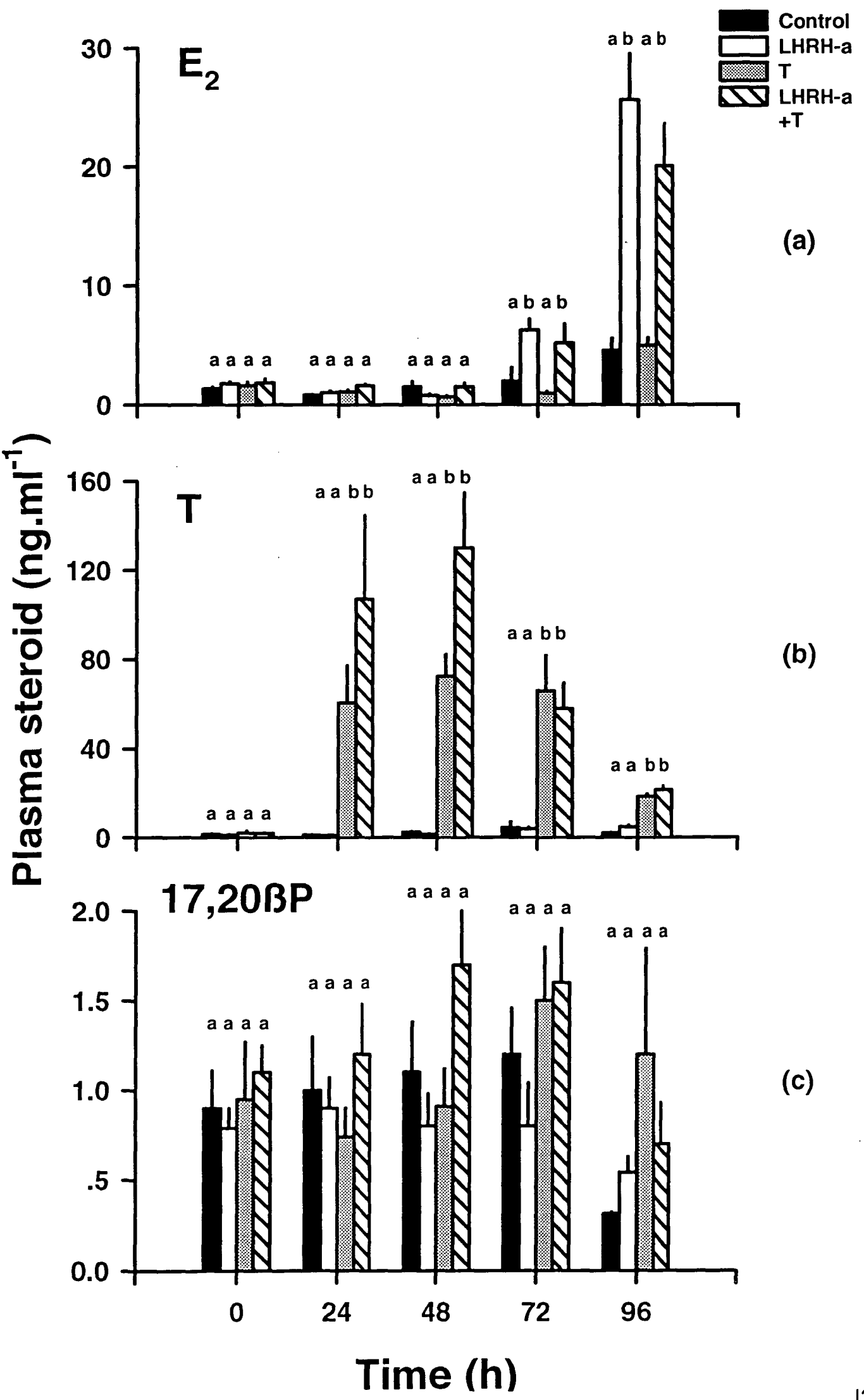
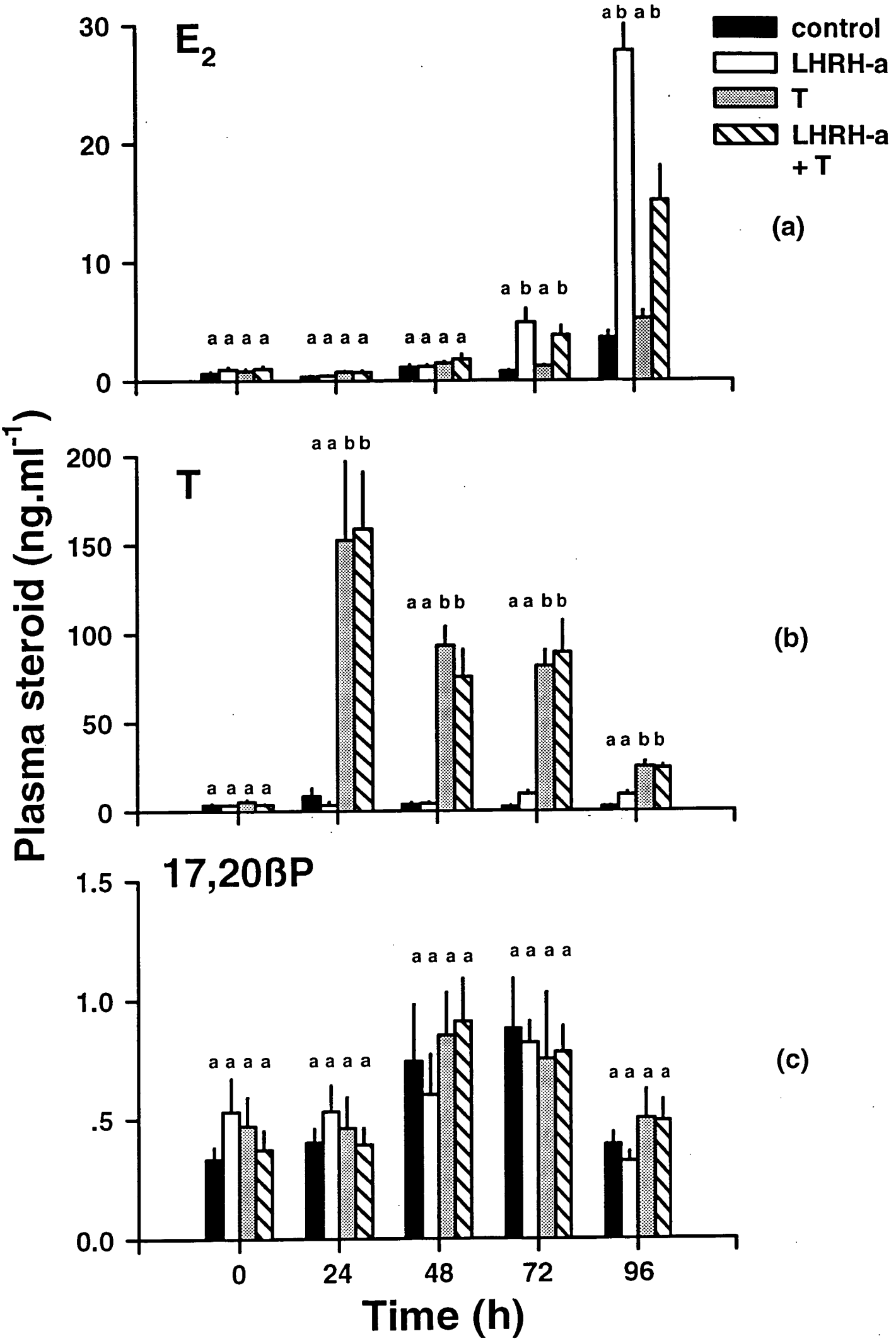

Control
LHRH-a
T
LHRH-a +T
E2
(a)
T
(b)
17,20ßP
(c)
Plasma steroid (ng.ml-1)
Time (h)
0
24
48
72
96
a b a b
a a a a
a a b b

Fig. 5.9. Plasma levels of (a) E_2, (b) T and (c) 17,20ßP in each treatment group at each sample time during experiment 4. Values are mean + S.E. Values that are not significantly different (P>0.05) share common superscripts, comparisons are within times between groups, n=7 per treatment.


E2
T
17,20ßP
control
LHRH-a
T
LHRH-a + T
(a)
(b)
(c)
Plasma steroid (ng.ml-1)
Time (h)
0
24
48
72
96
30
20
10
0
200
150
100
50
0
1.5
1.0
.5
0.0
a b a b
a a a a
a a b b

5.5. Discussion

As in many other species and consistent with our earlier work on greenback flounder (Barnett and Pankhurst 1998b, chapter 4, this volume), LHRH-a was effective at inducing ovulation in greenback flounder. Co-treatment with LHRH-a and PIM did not improve the efficacy of LHRH-a in inducing ovulation. Similarly, fish treated with LHRH-a showed persistent elevation of plasma T and E_2, but in most cases, this increase was not further augmented by co-treatment with PIM and LHRH-a. With the proviso that plasma levels of gonadal steroids only provide indirect evidence of GtH release, it appears that DA may not have strong inhibitory action on GtH release in greenback flounder. Although there is a strong body of literature demonstrating DA inhibition of GtH release (reviewed in Trudeau and Peter, 1995; Peter and Yu, 1997), a smaller number of studies indicate that DA inhibitory action on GtH release may be limited or even absent in some species. A study on coho salmon concluded that DA had a minor role in the regulation of gonadotropin in comparison to cyprinids (Van Der Kraak et al., 1986). DA antagonists did not enhance GnRH-a induced GtH release in the gilthead seabream (Zohar et al., 1987), and in Atlantic croaker, there was no evidence for the inhibitory effects of DA or DA agonists on GtH secretion, and some evidence that DA antagonists suppressed the effects of LHRH-a on GtH (Copeland and Thomas, 1989).

T treatment stimulates GtH release into the plasma of immature rainbow trout (*Oncorhynchus mykiss*), and sexually regressed female goldfish exposed to environmental conditions conducive for spawning (Crim and Evans, 1983; Kobayashi, et al., 1989). T treatment alone had no effect on ovulation in greenback flounder, however, T treatment potentiated the stimulatory effect of LHRH-a on ovulation, suggesting that T treatment enhances pituitary responsiveness to LHRH-a. Increased pituitary GtH content occurs following treatment with estrogens or aromatisable T in juvenile rainbow trout (Crim et al., 1981, Crim and Evans, 1983), Atlantic salmon *(Salmo salar)*, (Crim and Peter, 1978), European eel (*Anguilla anguilla*) (Dufour et al., 1983, 1989) and goldfish (Trudeau et al., 1993a, b). T treatment also enhanced pituitary responsiveness to exogenous GnRH-a or LHRH-a resulting in serum GtH release in juvenile rainbow trout, goldfish, common carp and Chinese loach (Crim and

Evans, 1983; Trudeau et al., 1991a, 1993a, b). It is reasonable to conclude from this study, that positive steroid feedback in greenback flounder (where DA inhibition is inconsistent) will involve stimulation of pituitary GtH but not GtH release.

Time to ovulation was negatively correlated with holding temperature in a wide range of species examined, and greenback flounder were similar to other species held at low temperatures (Reviewed in Barnett and Pankhurst, 1998b, chapter 4 this volume). In addition to the effects of temperature on delayed time to ovulation in greenback flounder, results from this study indicate that the indirect effects of exogenous hormone treatment on ovulation via steroid feedback, may, also delay ovulation.

Considerable, progress has been made in identifying other neuropeptides and neurohormones which may also have stimulatory and inhibitory actions on GtH release. Melatonin has stimulatory effects GtH release in Atlantic croaker with fully developed gonads (Khan and Thomas, 1996) and serotonin (5-hydroxytryptamine, 5-HT) stimulates GtH release in goldfish (Somoza and Peter, 1991) and the Atlantic croaker (Khan and Thomas 1991, 1994). The neurotransmitter γ-aminobutyric acid (GABA) has prominent stimulatory actions on GtH release in gonadally regressed goldfish (Kah et al., 1991; Trudeau and Peter 1995) and gonadally regressed Atlantic croaker, and inhibitory effects in mature Atlantic croaker (Khan and Thomas 1995; Trudeau and Peter 1995). It has been hypothesised that the regulation of GtH release may be under the combined effects of GnRH (stimulatory), DA (inhibitory) and GABA (modulatory) and the differential effects of GABA may in turn be modulated by gonadal steroids (Khan and Thomas 1995; Trudeau and Peter 1995). Positive and negative steroid feedback may also modulate GABA's actions on GtH-II release in greenback flounder and the role of DA inhibition may in part be superseded by the modulatory effects of GABA on GnRH.

At 72 and 96 h post treatment, plasma levels of E_2 in fish that were treated with T + LHRH-a and LHRH-a, were far in excess of values we have previously found in wild greenback flounder (Barnett and Pankhurst, 1998c, chapter 3 this volume) or greenback flounder induced to ovulate with exogenous hormones (Barnett and Pankhurst 1998b, chapter 4 this volume). The fact that similar increases in E_2 were not observed in fish treated with T alone, suggests that this was not a result of aromatisation of exogenous T to E_2. Other studies however, indicate that exogenous

steroids can pass relatively freely in and out of fish and can be detected by untreated fish (Budworth and Senger, 1993; Vermeirssen and Scott 1996). This raises the possibility that exogenous T was taken up by fish in all treatments, but was only aromatised to E_2 in the presence of GtH stimulated by treatment with LHRH-a. There were however, no significant increases in T in fish treated with LHRH-a alone and significant increases in plasma E_2 were delayed until 72 and 96 hours after treatment.

Plasma levels of 17,20βP were not significantly elevated above control levels during this study, although, in some cases plasma 17,20βP levels were significantly elevated above pre-treatment levels in all treatment groups. This response was infrequent and wasn't clearly associated with reproductive events. Similar observations have been made in another study on greenback flounder (Barnett and Pankhurst, 1998b; chapter 4 this volume), which implies that 17,20βP is an ambiguous marker of impending ovulation. This indicates a requirement to assess the production 17,20βP by maturing oocytes *in vitro*, and the role of 17,20βP in FOM *in vitro*.

In previous studies, we demonstrated that some husbandry and laboratory practices can induce a stress response in greenback flounder (Barnett and Pankhurst, 1998a; chapter 2 this volume), and reproductive processes are sensitive to stress. Plasma levels of T and E_2, but not 17,20βP significantly decreased in control fish in response to experimental procedures (Barnett and Pankhurst, 1998b, chapter 4, this volume), suggesting gonadal steroids are differentially sensitive to stress. Increases in plasma 17,20βP levels observed in this study and a previous study (Barnett and Pankhurst, 1998b, chapter 4, this volume), may have been of interrenal origin and/or associated with a stress response. A study on *in vitro* biosynthesis of 17,20βP in Atlantic salmon indicated that among other tissues, the interrenal is an excellent cellular source of 17,20βP (Sangalang and Freeman, 1988). Increases in 17,20βP have been associated with increased cortisol levels (Carragher and Pankhurst, 1991), 17, 20βP is an excellent substrate for cortisol production by the rainbow trout interrenl (Barrt et al., 1997) and cortisol potentiated GtH-stimulated production of 17,20βP in rainbow trout (Jalabert and Fostier, 1984).

In other studies on greenback flounder, intraperitoneal injections of the commercially available hormone mixture ovaprim (containing [D-Arg^6, Pro^9 NEt]-sGnRH and a

dopamine antagonist (domperidone) (Peter et al, 1993)), resulted in mean fertilisation rates of 49% (Hart, 1994) and up to 93% (Hart and Purser, 1995), although, LHRH-a treatment alone was not assessed in these studies. In this study fertilisation rates were within this range, and were consistent between experiments for the same treatment. Egg fertility was higher than levels reported for summer flounder (*Paralichthys dentatus*) treated with GnRH-a pellet implants (Berlinsky et al., 1997) and within the range of fertility reported for Southern flounder (*Paralichthys lethostigma*) and winter flounder (*Pseudopluronectes americanus*) treated with GnRH- pellet implants (Harmin and Crim, 1992; Berlinsky and King, 1996). Stress can have detrimental effects on egg quality in some species (reviewed in Pankhurst and Van Der Kraak, 1997), and the fact that some laboratory and husbandry procedures have the capacity to induce periods of acute stress in greenback flounder (Barnett and Pankhurst, 1998a; chapter 2 this volume), suggests that the experimental protocol in this study may have compromised egg quality. In the absence of experimental sampling, induced ovulation protocols may result in even higher fertilisation rates.

Treatment with LHRH-a and LHRH-a + T resulted in equivalent egg fertility. Frequency of egg fertility and % ovulations were significantly higher in fish treated with LHRH-a, than fish treated with LHRH-a + PIM or PIM, suggesting that PIM had an inhibitory effect on ovulation and egg quality during this experiment. Similarly, poor egg quality was observed in rainbow trout treated with PIM and LHRH-a + PIM, whereas egg quality was higher and less variable in fish treated with LHRH-a alone (Billard et al., 1984). Both LHRH-a alone and LHRH-a + T are the most effective treatments in terms of egg fertility, however, LHRH-a + T stimulated more ovulations than LHRH-a alone, and is therefore the most effective treatment in terms of egg production.

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Chapter 6

Effects of gonadal steroids and human chorionic gonadotropin on *in vitro* final oocyte maturation in the greenback flounder *Rhombosolea tapirina* (Günther, 1862).

6. Effects of gonadal steroids and human chorionic gonadotropin on *in vitro* final oocyte maturation in the greenback flounder *Rhombosolea tapirina* (Günther, 1862).

6.1. Summary

This study investigated the effect of reproductive steroids and human chorionic gonadotropin (hCG) on final oocyte maturation (FOM) in the greenback flounder (*Rhombosolea tapirina*). Fragments of ovarian lamellae containing vitellogenic oocytes were incubated with varying concentrations of steroids and hCG, and the maturational effect of these hormones was assessed by examining the percentage of oocytes that underwent FOM. Ovarian fragments required pre-treatment with hCG before they were receptive to steroids, and in most cases, the maturational response to steroids after priming with hCG exceeded the maturational response to hCG treatment alone. Ovarian fragments primed with hCG were receptive to all steroids at all concentrations tested, although there was considerable inconsistency in maturational responses to each steroid and each dose. 17α,20α-dihydroxy-4-pregnen-3-one (17,20αP) was most frequently ranked as the most effective MIS, but was closely followed by 20ß-hydroxy-4-pregnen-3-one (20ßP), 17α20ß-dihydroxy-4-pregnen-3-one (17,20ßP) and 17α,20ß, 21-trihydroxy-4-pregnen-3-one (20ßS). The least effective steroids were the steroid precursor 17P followed by 17-Preg and 3α,17, 20α-P-5ß. These results suggests that 20α and 20ß- hydroxylated steroids were most effective at inducing maturation in greenback flounder, and 5-pregnene and 5ß-pregnane steroids were least effective.

6.2. Introduction

Some studies have investigated the actions of C_{21} steroids on final oocyte maturation (FOM) (the resumption of meiosis after prophase arrest) in teleosts, by means of *in vitro* bioassay techniques (reviewed in Scott and Canario, 1987). This approach involves incubating oocytes in culture in the presence of known amounts of steroids and measuring the proportion of oocytes that undergo FOM. Bioassays are often not

specific enough to provide the exact identify of the true MIS, but will generally provide information regarding the structural requirements for maturational activity.

There is good evidence that 17α,20ß, 21-trihydroxy-4-pregnen-3-one (20ßS), is the major maturation inducing steroid in the spotted sea trout (*Cynoscion nebulosus*) and Atlantic croaker (*Micropogonias undulatus)* (Thomas and Trant, 1989; Thomas, 1994) and some evidence for its action in turbot (*Scophalmus maximus*) (Mugnier et al., 1995) and tbinumeri-dragonet (*Repomucenus beniteguri*) (Asahina et al., 1991). 17α20ß-dihydroxy-4-pregnen-3-one (17,20ßP) is however, the most active MIS in most other species in which it has been tested (reviewed in Scott and Canario, 1987).

Recently we have described changes in plasma and ovarian levels of 17,20ßP and plasma levels of the conjugates 17,20ßP-sulphate and 17,20ßP-glucuronide in greenback flounder *Rhombosolea tapirina*, on the basis of 17,20ßP being the main MIS in some other species (Barnett and Pankhurst, 1998b, chapter 3 this volume). Plasma levels of 17,20ßP were significantly elevated in ovulated females, whereas ovarian levels were elevated in association with final oocyte maturation (FOM) and ovulation. Plasma levels of 17,20ßP-sulphate but not 17,20ßP-glucuronide were elevated in association with FOM and ovulation. This suggests that 17,20ßP might play a role in FOM in greenback flounder. The aim of the present study was to assess whether 17,20ßP was effective at inducing FOM *in vitro. In vitro* biopotency was assessed against other C_{21} steroids at a range of doses.

The steroids chosen were based on their presence and proven effectiveness *in vitro* and *in vivo* in other species, and also their position in the steroid biosynthesis pathway. Dab (*Limanda limanda*) ovaries for example, have very active 20α-hydroxy-steroid dehydrogenase (20α-HSD) activity, plaice (*Pleuronectes platessa*) have very active 21-hydroxlylase and conjugating activity and both species have strong 5ß-reductase activity (Canario, 1991; Scott and Canario, 1992). Therefore, we also measured the effectiveness of 20α- and 20ß- hydroxylated, and 5ß-pregnane steroids.

There is some evidence suggesting that gonadotropin (GtH) stimulates maturational competence (Kobayashi et al., 1988; Canario and Scott, 1990; Thomas and Patiño, 1991; Degani and Boker, 1992; Kagawa et al., 1994; Zhu et al., 1994). We therefore tested the effectiveness of steroids on FOM in the presence or absence of human

chorionic gonadotropin (hCG). We do not have a homologous preparation of greenback flounder GtH, however, we have previously used hCG to successfully induce ovulation in greenback flounder *in vivo* (Barnett and Pankhurst, 1998a, chapter 4, this volume).

6.3. Materials and Methods

6.3.1. Fish capture and maintenance

Experiments were conducted on second generation cultured fish or wild fish. Cultured fish were produced by either the Department of Primary Industries and Fisheries, Marine Research Laboratories, Taroona, Tasmania or the Department of Aquaculture aquatic facility at the University of Tasmania in Launceston, and were maintained at the Launceston facility, in recirculating systems incorporating a biofilter, coarse solids filter and aeration system. Wild fish were either caught in hand nets by scuba divers at Bicheno (148° 18' E, 41° 52' S) on the east coast of Tasmania from June-September 1996 or by commercial gill netting from Georges Bay (148° 16' E, 41° 19' S) on the east coast of Tasmania from September-October 1996. Fish 1, 2, 3, 7 and 13 were cultured fish, all remaining fish were wild fish.

6.3.2. Tissue preparation

Fish were anaesthetised in 0.05% 2-phenoxy-ethanol, killed by spinal transection and ovaries immediately dissected and placed into ice cold Leibowitz L15 medium (Sigma) adjusted to 405 $mOs.kg^{-1}$ (an approximation of the osmolality of marine teleost plasma) and pH of 7.6. Ovaries from Launceston fish were immediately transported to the laboratory for processing, whereas, ovaries from wild fish were transported in L15 on ice to Launceston by road (1.5 and 2 h for Bicheno and Georges Bay respectively). Not all gonads were at the same stage, and ranged from vitellogenic through late vitellogenic with a scattering of hydrated oocytes. Fragments of ovarian lamellae containing 20-50 vitellogenic oocytes were dissected out and incubated in tissue culture plates (Corning) in a final volume of 1 ml of L15 medium and varying concentrations of steroids and hCG, with 4 replicates per treatment.

6.3.3. Incubation protocol

Steroids were dissolved in ethanol and added to the incubation medium in a volume of 10 µl, and 10 µl ethanol was added to the control incubates. HCG was dissolved in L15 medium and added in a volume of 100 µl. The potency of 17P was measured due to it's role as a precursor to 17,20ßP (Richter et al., 1987) and the effectiveness of 17-Preg was measured so as to assess the potency of 5-pregnen steroids versus 4-pregnen steroids. The steroids tested were :17α-hydroxy-4-pregnene-3,20-dione (17P), 17α,20ß-dihydroxy-4-pregnen-3-one (17,20ßP), 17α,20α-dihydroxy-4-pregnen-3-one (17,20αP), 17α,20ß, 21-trihydroxy-4-pregnen-3-one (20ßS), 20ß-hydroxy-4-pregnen-3-one (20ßP), 3ß,17α- hydroxy-5-pregnen-20-one (17-preg) and 3α,17α, 20α-trihydroxy-5ß-pregnane (3α,17, 20α-P-5ß). All steroids were purchased from Sigma.

Experiments were performed sequentially and the experimental protocols were modified on the basis of outcomes from preceding experiments (details are given in Table 6.1). *Experiment 1 (fish 1-6):* Ovarian fragments were incubated with either L15 alone (controls), hCG at 1, 10 and 100 IU.ml^{-1}, steroids or steroids + hCG 100 IU.ml^{-1}. *Experiment 2 (fish 7-9):* Ovarian fragments were incubated with either L15 alone, hCG at 10 and 100 IU.ml^{-1}, 17P, 17-Preg, 17P + hCG and 17-Preg + hCG at 100 IU.ml^{-1}. The remaining ovarian fragments were incubated with hCG 100 IU.ml^{-1} for 24 h, then treated with either 17P, 17-Preg, 17,20ßP or 20ßS. *Experiments 3 and 4 (fish 10-16):* Ovarian fragments were incubated with hCG 100 IU.ml^{-1} for 24 h, then treated with either 17P, 17,20ßP, 17,20αP, 20ßS, 20ßP, 17-Preg, 3α,17, 20α-P-5ß or hCG at 100 IU.ml^{-1}.

Ovarian fragments were incubated at 12°C and at each sample time the incubation medium and hormones were replaced. Before treatment, and at each sample time the developmental stage of all oocytes was identified as vitellogenic or mature. Vitellogenic oocytes had an opaque white or fine granular appearance, some maturing oocytes had a coarse granular appearance but most were clear with a single oil droplet. Oocyte staging was verified by clearing approximately 60 oocytes from each of 4 fish in sera solution (ethanol: formalin: glacial acetic acid (6 : 3 : 1 v/v)) to determine the presence or position of the germinal vesicle (GV). Vitellogenic oocytes had a centrally located or eccentric located GV, maturing oocytes with a coarse granular appearance

had a peripheral GV or had undergone GV breakdown (GVBD), all remaining mature oocytes had undergone GVBD. The maturity status of granular oocytes with a peripheral GV, is ambiguous and may not be considered equivalent in terms of endocrine status and maturity to oocytes that have undergone GVBD (Goetz, 1983; Billard and Jensen, 1996). However, very few maturing oocytes with a peripheral GV were observed (<6% of maturing oocytes), indicating oocytes underwent GV migration relatively quickly.

Table 6. 1.Details of different experiments carried out *in vitro* with greenback flounder ovarian fragments, with various steroids at different concentrations, in the presence (+) or absence (-) of hCG, with (+) or withou (-) 24 h of hCG priming.

Experiment	Steroids	Concentration ($ng.ml^{-1}$)	Presence of hCG	hCG primed	Sample frequency (h)	Incubation time (h)
1 (fish 1-6)	17P, 17,20ßP, 20ßS,	1, 10, 100	+ and —	—	24	72-120
2 (fish 7-9)	17P, 17,20ßP, 17,20αP, 20ßS, 20ßP, 17-preg	10, 100	+ and —	+	24	72-120
3 (fish 10-13)	17P, 17,20ßP, 17,20αP, 20ßS, 20ßP, 17-preg 3α,17, 20α-P-5ß	1, 10, 100, 1000	—	+	24	72
3 (fish 14-16)	17P, 17,20ßP, 17,20αP, 20ßS, 20ßP, 17-preg 3α,17, 20α-P-5ß	0.01, 0.1, 1, 10	—	+	6	42-58

6.3.4. Statistical analysis

Repeated measures analysis of variance and mean comparison tests were performed using the computer package JMP for Macintosh. Data were transformed to satisfy normality and homogeneity of variance requirements. A significance level of $\alpha < 0.05$ was used for all statistical tests. Percent maturation responses to steroids during experiments 2, 3 and 4 were ranked 9 = best response, 1 = lowest response for each fish and each dose, from the steepest part of response curve which was 72, 48, 48, 72, 48, 36, 18 and 30 h for fish 7, 8, 9, 10, 11, 12, 14 and 15 respectively.

6.4. Results

Spontaneous maturation in control fish was ≤ 5% (excluding initial counts). Oocytes from fish 3, 13 and 16, showed no response to treatment with hCG or steroids (ie. maximal % maturation was ≤ 8% excluding initial counts). Although these oocytes appeared normal in appearance, some of these oocytes failed to clear in sera solution.

Percentage maturation was significantly enhanced by treatment with 10 $IU.ml^{-1}$ hCG at 48 h, 100 $IU.ml^{-1}$ hCG at 24 and 48 h and 100 $IU.ml^{-1}$ hCG + steroids at 24 and 48 h post treatment in fish 1-6 during experiment 1 (Fig. 6.1). Treatment with steroids and hCG were no more effective than treatment with 100 $IU.ml^{-1}$ hCG alone. One $IU.ml^{-1}$ hCG and steroids alone had no effect on oocyte maturation.

During experiment 2, % maturation was significantly enhanced by treatment with 100 $IU.ml^{-1}$ hCG (Figs 6.2-6.4. and Tables 6.2-6.4.). In all cases, pre-treatment with 100 $IU.ml^{-1}$ hCG followed by 20ßS significantly enhanced % maturation. and in most cases co-treatment or pre-treatment with hCG followed by 17P, 17-Preg or 17,20ßP also significantly enhanced % maturation. Pre-treatment with hCG followed by steroids was not statistically more effective than hCG alone. Steroid treatment alone or 10 $IU.ml^{-1}$ hCG did not significantly enhance % maturation.

Ovarian fragments were receptive to all steroids, however, there was no indication of a dose response to steroids over the range of doses tested. Steroid effectiveness was not consistent between doses within fish and between fish in any experiments, hence, dose response curves were incoherent (data not shown). Comparative steroid effectiveness was assessed by ranking treatments in decreasing order of effectiveness during the

steepest part of the response curve (Table 6.5.). 20ßS and 17P were the most effective treatments at 10 ng.ml^{-1} and 20ßS and 17,20ßP were the most effective treatments at 100 ng.ml^{-1}. HCG at 10IU.ml^{-1} and steroid treatments administered without hCG were the least effective treatments.

During experiment 3, pre-treatment with 100 IU.ml^{-1} hCG followed by steroids significantly enhanced % maturation in fish 10 and 11, and in some cases in fish 12 (Figs 6.5-6.7. and Tables 6.6-6.8.). Percentage maturation was not significantly enhanced by treatment with 100 IU.ml^{-1} hCG alone, and in most cases, pre-treatment with hCG followed by steroids was statistically more effective than treatment with hCG alone.

Pre-treatment with 100 IU.ml^{-1} hCG followed by steroids during experiment 4, significantly enhanced % maturation in fish 14 and 15 (Figs 6.8-6.9. and Tables 6.9-6.10.). Percentage maturation was significantly enhanced by treatment with 100 IU.ml^{-1} hCG alone, and in many cases, pre-treatment with hCG followed by steroids was statistically more effective than treatment with hCG alone.

During experiments 3 and 4, ovarian fragments were receptive to all steroids, however, there was no indication of a dose response to steroids over the range of doses tested. Comparative steroid effectiveness was assessed by ranking treatments in decreasing order of effectiveness during the steepest part of the response curve (Tables 6.11.). Total ranks were 17,20αP, 20ßP, 17,20ßP and 20ßS equally, 17-preg and 17P equally, 3α,17, 20α-P-5ß, hCG, and control respectively, in decreasing order, however, the total score of the ranks indicates that 17,20αP, 20ßP, 17,20ßP, 20ßS were similarly effective.

Fig.6.1. Percentage maturation in oocytes from fish 4 (representative of experiment 1), for each hormone dose at each sample time. Oocytes were treated with either (a) 17P, (b) 20βS or (c) 17,20βP, in the absence or presence of hCG or (d) hCG alone. Values are mean ± s.e., n=4 per treatment dose. Asterisks shows means that are significantly different between treatments and across times (P>0.05).

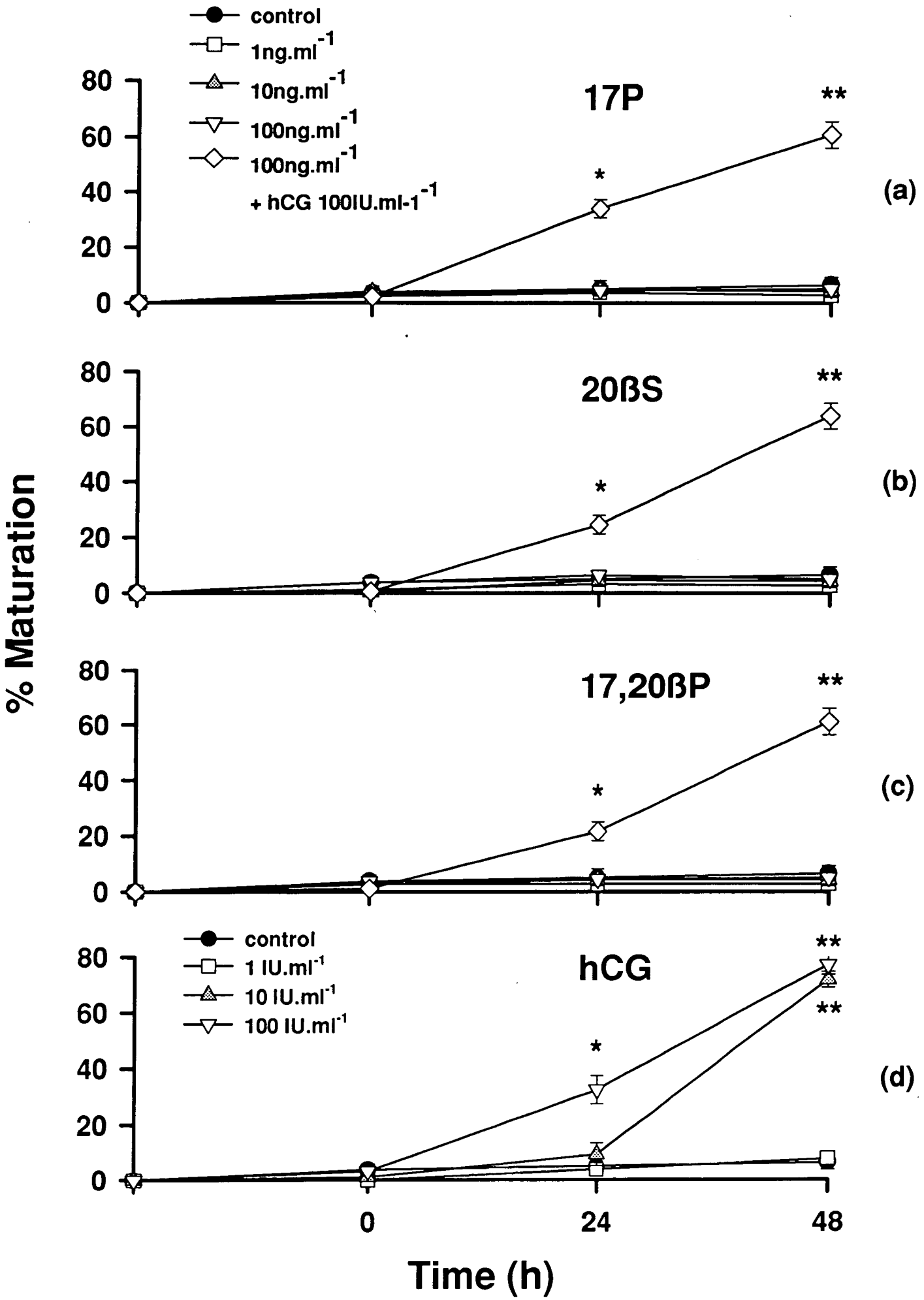
control
1ng.ml-1
10ng.ml-1
100ng.ml-1
100ng.ml-1
+ hCG 100IU.ml-1-1
17P
20ßS
17,20ßP
hCG
control
1 IU.ml-1
10 IU.ml-1
100 IU.ml-1
(a)
(b)
(c)
(d)
% Maturation
Time (h)
0
24
48
80
60
40
20
0
*
**

Fig 6.2. Percentage maturation in oocytes from fish 7, for each hormone at each sample time. Hormones were administered at (a) 10 $ng.ml^{-1}$ steroid, 10 or 100 $IU.ml^{-1}$ hCG (b) 100 $ng.ml^{-1}$ steroid, 10 or 100 $IU.ml^{-1}$ hCG or (c) 100 $ng.ml^{-1}$ steroid + hCG 100 $IU.ml^{-1}$, 10 or 100 $IU.ml^{-1}$ hCG, see Table 6.1. for details. Asterisks indicate incubations that were not primed with hCG 100 $IU.ml^{-1}$, n=4 per treatment dose.

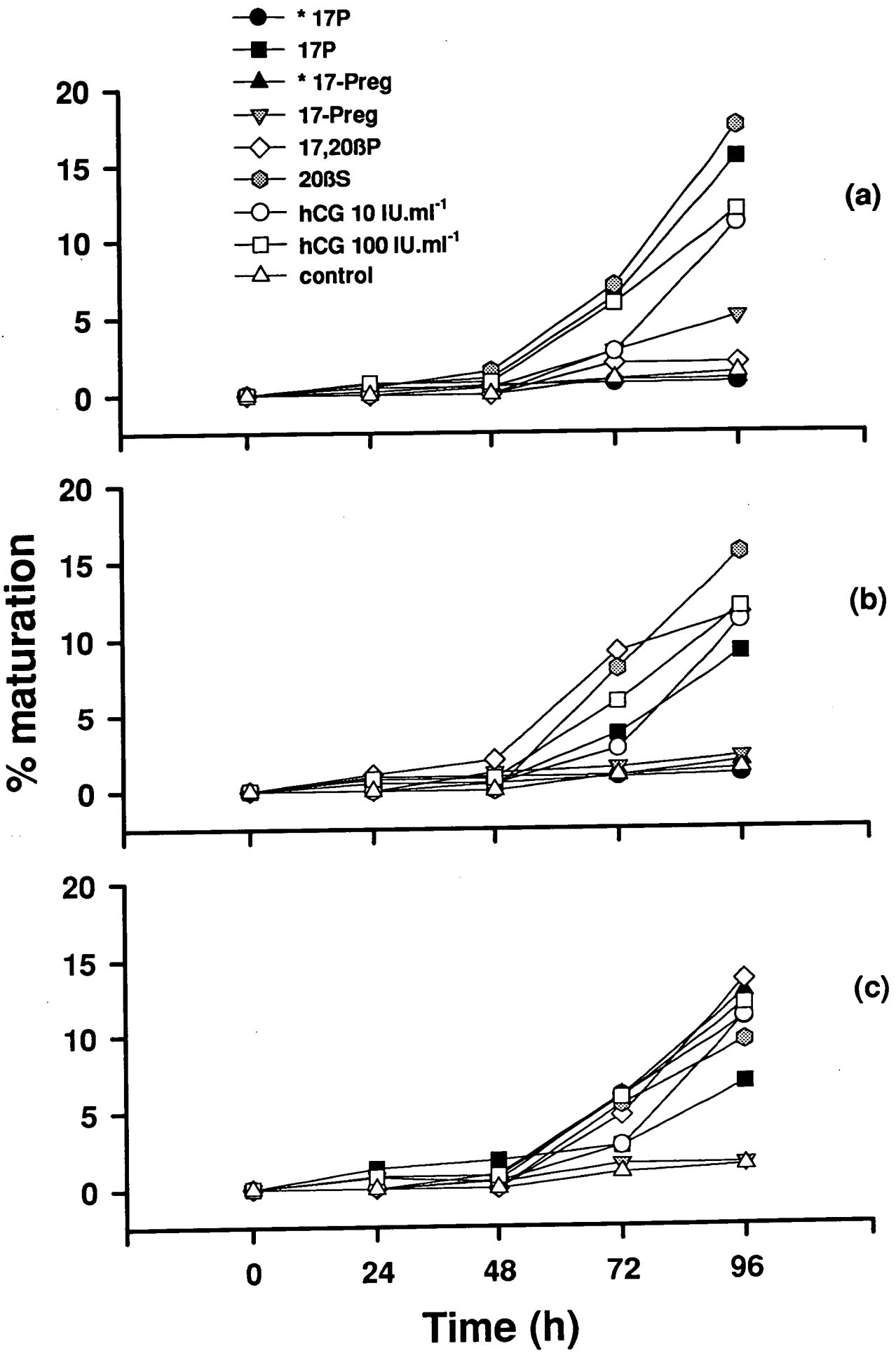
* 17P
17P
* 17-Preg
17-Preg
17,20ßP
20ßS
hCG 10 IU.ml-1
hCG 100 IU.ml-1
control
(a)
(b)
(c)
% maturation
Time (h)
0
24
48
72
96
20
15
10
5
0

Table 6.2. Statistical results for Fig. 6.2. Different superscripts show significant differences in percentage maturation in oocytes from fish 7, for each hormone at each dose and at each sample time. See Table 6.1. for details of experimental protocol. Asterisks indicate steroid treatments that were not primed with hCG 100 IU.ml^{-1}, n=4 per treatment dose.

Treatment	Dose	0 h	24 h	48 h	72 h	96 h
* 17P	10ng.ml^{-1}	a	a	a	a	a
17P	10ng.ml^{-1}	a	a	a	a	b
* 17-Preg	10ng.ml^{-1}	a	a	a	a	a
17-Preg	10ng.ml^{-1}	a	a	a	a	a
17,20ßP	10ng.ml^{-1}	a	a	a	a	a
20ßS	10ng.ml^{-1}	a	a	a	a	b
hCG	10 IU.ml^{-1}	a	a	a	a	ab
hCG	100 IU.ml^{-1}	a	a	a	a	ab
control	control	a	a	a	a	a
* 17P	100ng.ml^{-1}	a	a	a	a	a
17P	100ng.ml^{-1}	a	a	a	ab	ab
* 17-Preg	100ng.ml^{-1}	a	a	a	a	a
17-Preg	100ng.ml^{-1}	a	a	a	a	a
17,20ßP	100ng.ml^{-1}	a	a	a	b	ab
20ßS	100ng.ml^{-1}	a	a	a	ab	b
hCG	10 IU.ml^{-1}	a	a	a	ab	ab
hCG	100 IU.ml^{-1}	a	a	a	ab	ab
control	control	a	a	a	a	a
* 17P	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	a	a	bc
17P	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	a	a	ab
* 17-Preg	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	a	a	bc
17-Preg	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	a	a	a
17,20ßP	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	a	a	b
20ßS	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	a	a	bc
hCG	10 IU.ml^{-1}	a	a	a	a	bc
hCG	100 IU.ml^{-1}	a	a	a	a	bc
control	control	a	a	a	a	a

Fig 6.3. Percentage maturation in oocytes from fish 8, for each hormone at each sample time. Hormones were administered at (a) 10 ng.ml^{-1} steroid, 10 or 100 IU.ml^{-1} hCG (b) 100 ng.ml^{-1} steroid, 10 or 100 IU.ml^{-1} hCG or (c) 100 ng.ml^{-1} steroid + hCG 100 IU.ml^{-1}, 10 or 100 IU.ml^{-1} hCG, see Table 6.1. for details. Asterisks indicate incubations that were not primed with hCG 100 IU.ml^{-1}, n=4 per treatment dose.

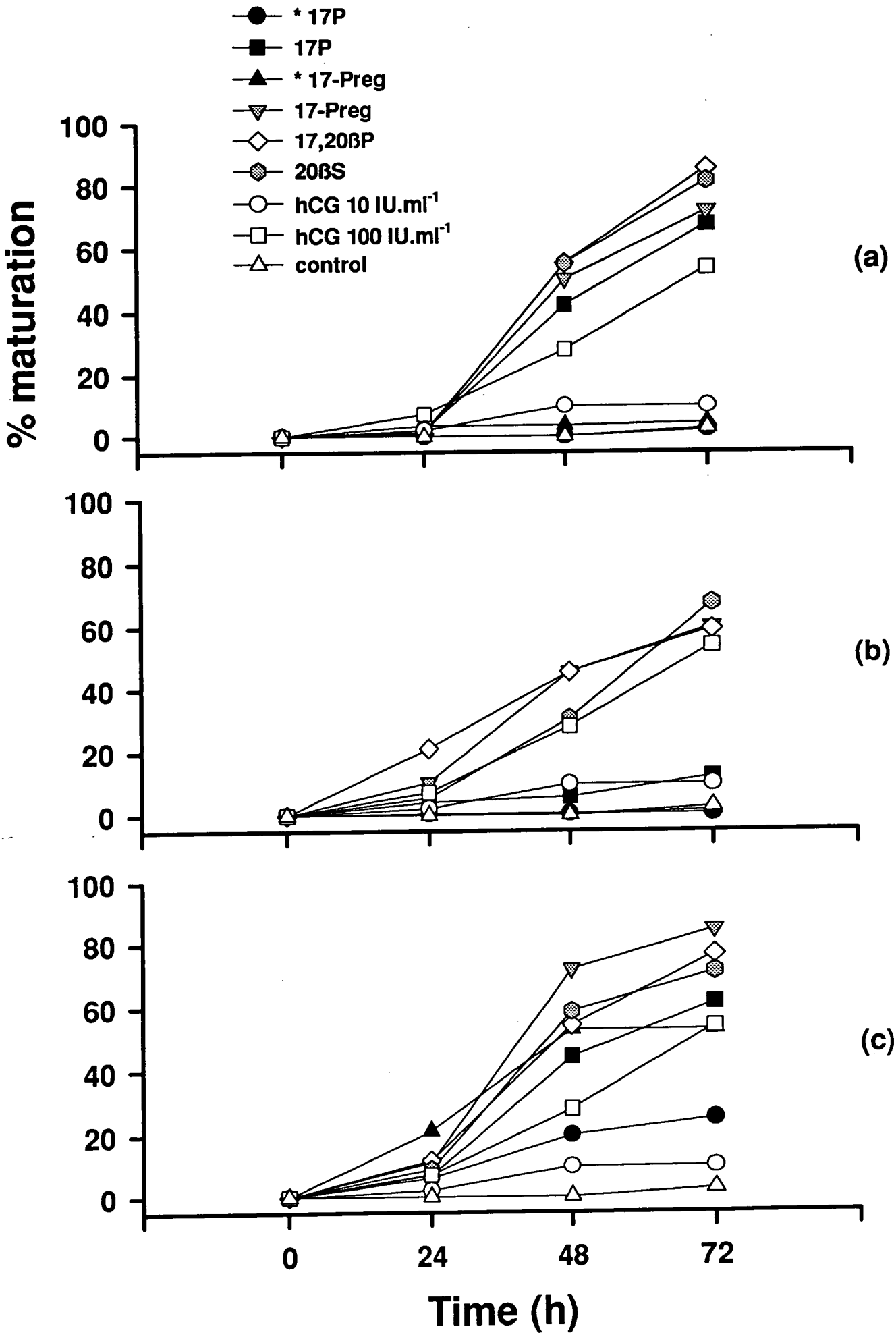
* 17P
17P
* 17-Preg
17-Preg
17,20ßP
20ßS
hCG 10 IU.ml-1
hCG 100 IU.ml-1
control
% maturation
100
80
60
40
20
0
(a)
(b)
(c)
0
24
48
72
Time (h)

Table 6.3. Statistical results for Fig. 6.3. Different superscripts show significant differences in percentage maturation in oocytes from fish 8, for each hormone at each dose and at each sample time. See Table 6.1. for details of experimental protocol. Asterisks indicate steroid treatments that were not primed with hCG 100 IU.ml^{-1}, n=4 per treatment dose.

Treatment	Dose	0 h	24 h	48 h	72 h
* 17P	10ng.ml^{-1}	a	a	a	a
17P	10ng.ml^{-1}	a	a	b	b
* 17-Preg	10ng.ml^{-1}	a	a	a	a
17-Preg	10ng.ml^{-1}	a	a	b	b
17,20ßP	10ng.ml^{-1}	a	a	b	b
20ßS	10ng.ml^{-1}	a	a	b	b
hCG	10 IU.ml^{-1}	a	a	a	a
hCG	100 IU.ml^{-1}	a	a	ab	b
control	control	a	a	a	a
* 17P	100ng.ml^{-1}	a	a	a	a
17P	100ng.ml^{-1}	a	a	a	a
* 17-Preg	100ng.ml^{-1}	a	a	a	a
17-Preg	100ng.ml^{-1}	a	a	b	b
17,20ßP	100ng.ml^{-1}	a	a	b	b
20ßS	100ng.ml^{-1}	a	a	ab	b
hCG	10 IU.ml^{-1}	a	a	a	a
hCG	100 IU.ml^{-1}	a	a	ab	b
control	control	a	a	a	a
* 17P	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	ab	ab
17P	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	bc	c
* 17-Preg	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	bc	bc
17-Preg	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	c	c
17,20ßP	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	bc	c
20ßS	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	c	c
hCG	10 IU.ml^{-1}	a	a	ab	ab
hCG	100 IU.ml^{-1}	a	a	abc	bc
control	control	a	a	a	a

Fig. 6.4. Percentage maturation in oocytes from fish 9, for each hormone at each sample time. Hormones were administered at (a) 10 $ng.ml^{-1}$ steroid, 10 or 100 $IU.ml^{-1}$ hCG (b) 100 $ng.ml^{-1}$ steroid, 10 or 100 $IU.ml^{-1}$ hCG or (c) 100 $ng.ml^{-1}$ steroid + hCG 100 $IU.ml^{-1}$, 10 or 100 $IU.ml^{-1}$ hCG, see Table 6.1. for details. Asterisks indicate incubations that were not primed with hCG 100 $IU.ml^{-1}$, n=4 per treatment dose.

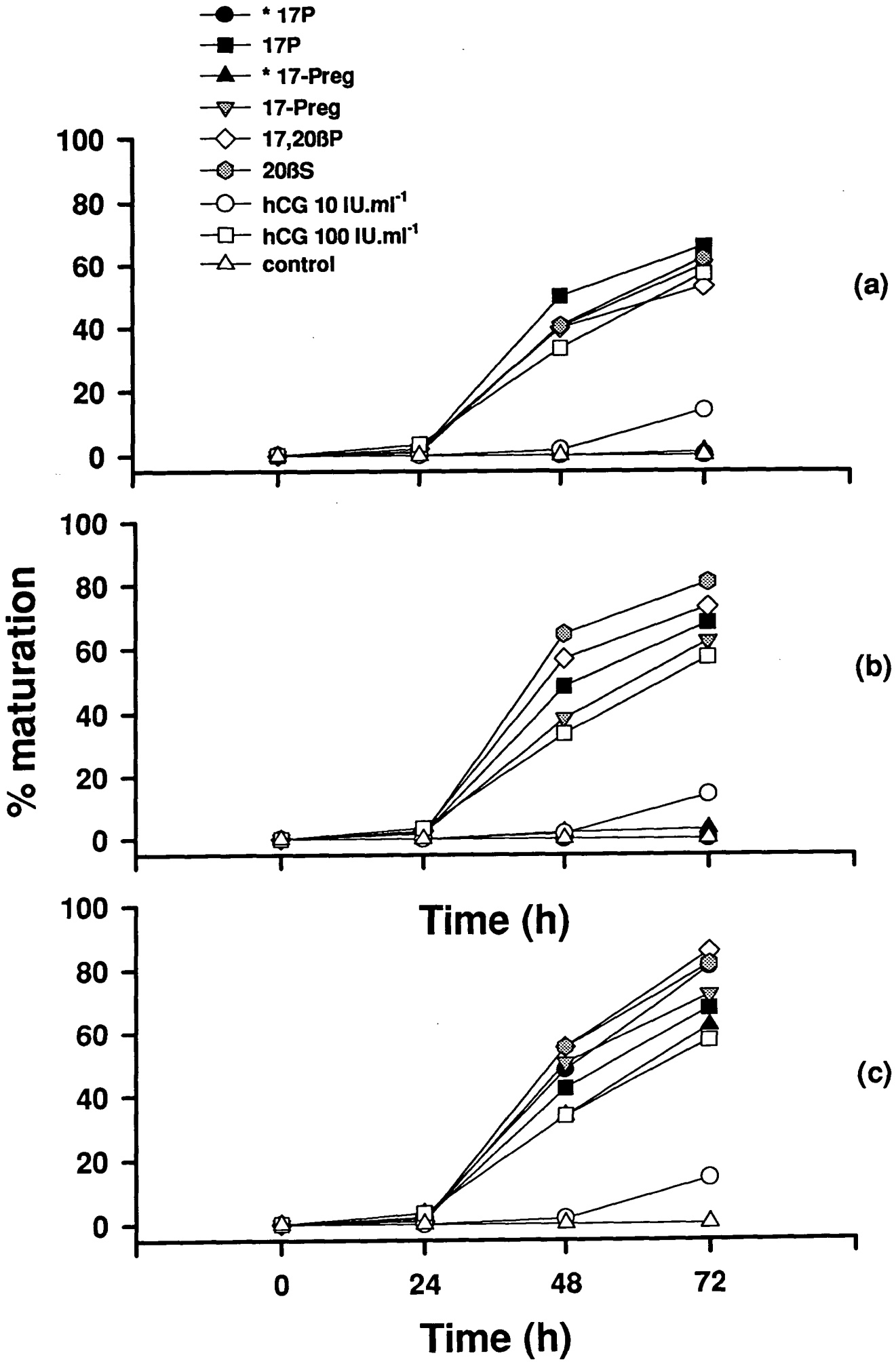
* 17P
17P
* 17-Preg
17-Preg
17,20ßP
20ßS
hCG 10 IU.ml-1
hCG 100 IU.ml-1
control
100
80
60
40
20
0
(a)
(b)
(c)
% maturation
Time (h)
0
24
48
72
Time (h)

Table 6.4. Statistical results for Fig. 6.4. Different superscripts show significant differences in percentage maturation in oocytes from fish 9, for each hormone at each dose and at each sample time. See Table 6.1. for details of experimental protocol. Asterisks indicate steroid treatments that were not primed with hCG 100 IU.ml^{-1}, n=4 per treatment dose.

Treatment	Dose	0 h	24 h	48 h	72 h
* 17P	10ng.ml^{-1}	a	a	a	a
17P	10ng.ml^{-1}	a	a	b	b
* 17-Preg	10ng.ml^{-1}	a	a	a	a
17-Preg	10ng.ml^{-1}	a	a	b	b
17,20ßP	10ng.ml^{-1}	a	a	b	b
20ßS	10ng.ml^{-1}	a	a	b	b
hCG	10 IU.ml^{-1}	a	a	a	a
hCG	100 IU.ml^{-1}	a	a	ab	b
control	control	a	a	a	a
* 17P	100ng.ml^{-1}	a	a	a	a
17P	100ng.ml^{-1}	a	a	b	b
* 17-Preg	100ng.ml^{-1}	a	a	a	a
17-Preg	100ng.ml^{-1}	a	a	b	b
17,20ßP	100ng.ml^{-1}	a	a	b	b
20ßS	100ng.ml^{-1}	a	a	b	b
hCG	10 IU.ml^{-1}	a	a	a	a
hCG	100 IU.ml^{-1}	a	a	b	b
control	control	a	a	a	a
* 17P	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	b	b
17P	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	b	b
* 17-Preg	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	b	b
17-Preg	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	b	b
17,20ßP	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	b	b
20ßS	100ng.ml^{-1} + hCG 100 IU.ml^{-1}	a	a	b	b
hCG	10 IU.ml^{-1}	a	a	a	a
hCG	100 IU.ml^{-1}	a	a	b	b
control	control	a	a	a	a

Table 6. 5. Rank of hormone effectiveness on % maturation (9 = best and 1 = worst) for each fish during experiment 2. Ranks were assigned from the steepest part of the curve.

Steroid dose ng.ml-1	10	10	10	Rank 10	100	100	100	Rank 100	100 + hCG 100 IU.ml-1	100 + hCG 100 IU.ml-1	100 + hCG 100 IU.ml-1	Rank 100 + hCG 100 IU.ml-1
Fish no.	7	8	9	7-9	7	8	9	7-9	7	8	9	7-9
* 17P	1	1.5	3	1	1	3	1.5	2	8	3	6	5
17P	8	6	9	8	6	4	7	5	3	5	5	3
*17-Preg	3	3	3	3	3	1.5	4	3	9	6	4	6
17-Preg	6	7	7	7	4	8	6	6.5	2	9	7	7
17,20BP	4	9	6	6	9	9	8	9	5	7	9	8
20ßS	9	8	8	9	8	7	9	8	6	8	8	9
hCG 10 IU	5	4	4	4	5	5	3	4	4	2	2	2
hCG 100 IU	7	5	5	5	7	6	5	6.5	7	4	3	4
control	2	1.5	3	2	2	1.5	1.5	1	1	1	1	1

Fig. 6.5. Percentage maturation in oocytes from fish 10, for each hormone at each sample time. Steroids were administered after 24 h priming with hCG 100 IU.ml^{-1}. Hormones were then administered at (a) 1 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (b) 10ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (c) 100 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} or (d) 1000 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1}, see Table 6.1. for details, n=4 per treatment dose.

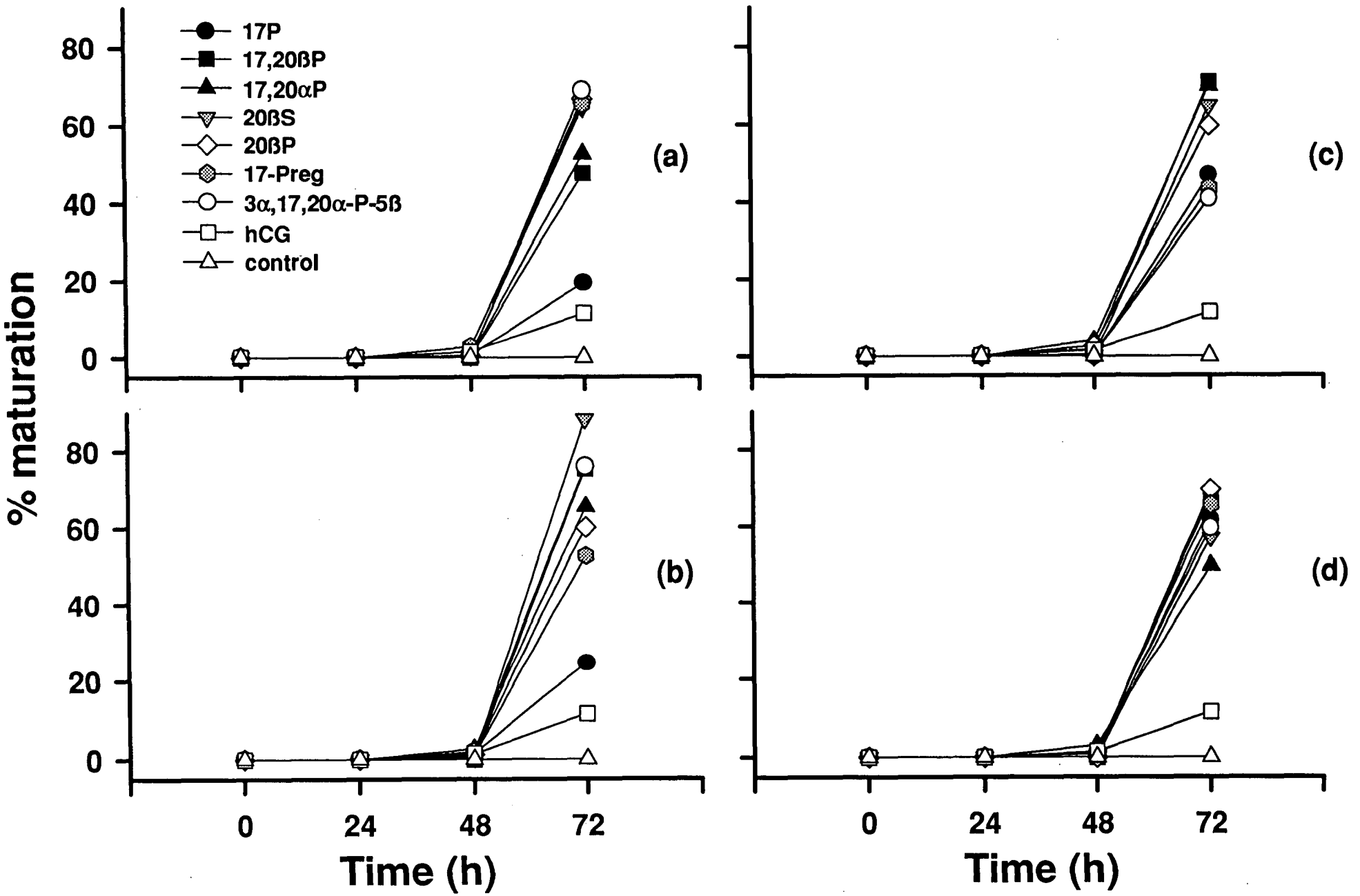
17P
17,20ßP
17,20αP
20ßS
20ßP
17-Preg
3α,17,20α-P-5ß
hCG
control
% maturation
80
60
40
20
0
(a)
(b)
(c)
(d)
0
24
48
72
Time (h)

Table 6.6. Statistical results for Fig. 6.5. Different superscripts show significant differences in percentage maturation in oocytes from fish 10, for each hormone at each dose and at each sample time. See Table 6.1. for details of experimental protocol. Asterisks indicate steroid treatments that were not primed with hCG 100 IU.ml^{-1}, n=4 per treatment dose.

Treatment	Dose	0 h	24 h	48 h	72 h
17P	1ng.ml^{-1}	a	a	a	a
17,20ßP	1ng.ml^{-1}	a	a	a	b
17,20αP	1ng.ml^{-1}	a	a	a	b
20ßS	1ng.ml^{-1}	a	a	a	b
20ßP	1ng.ml^{-1}	a	a	a	b
17-Preg	1ng.ml^{-1}	a	a	a	b
3α,17,20α-P-5ß	1ng.ml^{-1}	a	a	a	b
hCG	100 IU.ml^{-1}	a	a	a	a
control	control	a	a	a	a
17P	10ng.ml^{-1}	a	a	a	a
17,20ßP	10ng.ml^{-1}	a	a	a	b
17,20αP	10ng.ml^{-1}	a	a	a	b
20ßS	10ng.ml^{-1}	a	a	a	b
20ßP	10ng.ml^{-1}	a	a	a	b
17-Preg	10ng.ml^{-1}	a	a	a	b
3α,17,20α-P-5ß	10ng.ml^{-1}	a	a	a	b
hCG	100 IU.ml^{-1}	a	a	a	a
control	control	a	a	a	a
17P	100ng.ml^{-1}	a	a	a	b
17,20ßP	100ng.ml^{-1}	a	a	a	b
17,20αP	100ng.ml^{-1}	a	a	a	b
20ßS	100ng.ml^{-1}	a	a	a	b
20ßP	100ng.ml^{-1}	a	a	a	b
17-Preg	100ng.ml^{-1}	a	a	a	b
3α,17,20α-P-5ß	100ng.ml^{-1}	a	a	a	b
hCG	100 IU.ml^{-1}	a	a	a	a
control	control	a	a	a	a
17P	1000ng.ml^{-1}	a	a	a	b
17,20ßP	1000ng.ml^{-1}	a	a	a	b
17,20αP	1000ng.ml^{-1}	a	a	a	b
20ßS	1000ng.ml^{-1}	a	a	a	b
20ßP	1000ng.ml^{-1}	a	a	a	b
17-Preg	1000ng.ml^{-1}	a	a	a	b
3α,17,20α-P-5ß	1000ng.ml^{-1}	a	a	a	b
hCG	100 IU.ml^{-1}	a	a	a	a
control	control	a	a	a	a

Fig. 6.6. Percentage maturation in oocytes from fish 11, for each hormone at each sample time. Steroids were administered after 24 h priming with hCG 100 IU.ml^{-1}. Hormones were then administered at (a) 1 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (b) 10ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (c) 100 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} or (d) 1000 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1}, see Table 6.1. for details, n=4 per treatment dose.

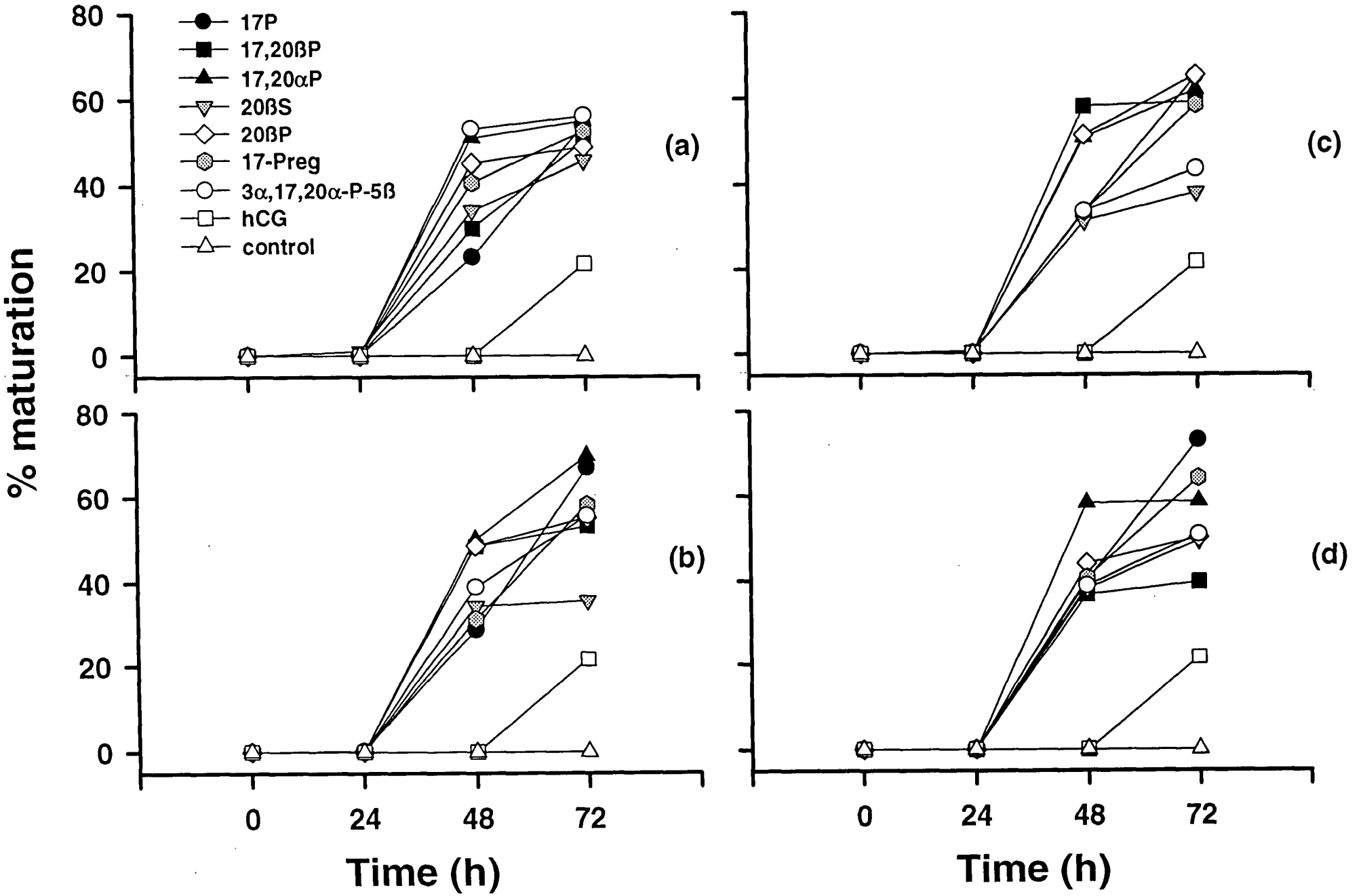
17P
17,20ßP
17,20αP
20ßS
20ßP
17-Preg
3α,17,20α-P-5ß
hCG
control
% maturation
80
60
40
20
0
(a)
(b)
(c)
(d)
0
24
48
72
Time (h)

Table 6.7. Statistical results for Fig. 6.6. Different superscripts show significant differences in percentage maturation in oocytes from fish 11, for each hormone at each dose and at each sample time. See Table 6.1. for details of experimental protocol. Asterisks indicate steroid treatments that were not primed with hCG 100 IU.ml^{-1}, n=4 per treatment dose.

Treatment	Dose	0 h	24 h	48 h	72 h
17P	1ng.ml^{-1}	a	a	ab	b
17,20ßP	1ng.ml^{-1}	a	a	bc	b
17,20αP	1ng.ml^{-1}	a	a	bc	b
20ßS	1ng.ml^{-1}	a	a	bc	b
20ßP	1ng.ml^{-1}	a	a	bc	b
17-Preg	1ng.ml^{-1}	a	a	bc	b
3α,17,20α-P-5ß	1ng.ml^{-1}	a	a	c	b
hCG	100 IU.ml^{-1}	a	a	a	a
control	control	a	a	a	a
17P	10ng.ml^{-1}	a	a	b	d
17,20ßP	10ng.ml^{-1}	a	a	b	cd
17,20αP	10ng.ml^{-1}	a	a	b	d
20ßS	10ng.ml^{-1}	a	a	b	bc
20ßP	10ng.ml^{-1}	a	a	b	cd
17-Preg	10ng.ml^{-1}	a	a	b	cd
3α,17,20α-P-5ß	10ng.ml^{-1}	a	a	b	cd
hCG	100 IU.ml^{-1}	a	a	a	ab
control	control	a	a	a	a
17P	100ng.ml^{-1}	a	a	b	c
17,20ßP	100ng.ml^{-1}	a	a	b	c
17,20αP	100ng.ml^{-1}	a	a	b	c
20ßS	100ng.ml^{-1}	a	a	b	bc
20ßP	100ng.ml^{-1}	a	a	b	c
17-Preg	100ng.ml^{-1}	a	a	b	c
3α,17,20α-P-5ß	100ng.ml^{-1}	a	a	b	c
hCG	100 IU.ml^{-1}	a	a	a	ab
control	control	a	a	a	a
17P	1000ng.ml^{-1}	a	a	b	d
17,20ßP	1000ng.ml^{-1}	a	a	b	bc
17,20αP	1000ng.ml^{-1}	a	a	b	cd
20ßS	1000ng.ml^{-1}	a	a	b	cd
20ßP	1000ng.ml^{-1}	a	a	b	cd
17-Preg	1000ng.ml^{-1}	a	a	b	cd
3α,17,20α-P-5ß	1000ng.ml^{-1}	a	a	b	cd
hCG	100 IU.ml^{-1}	a	a	a	ab
control	control	a	a	a	a

Fig. 6.7. Percentage maturation in oocytes from fish 12, for each hormone at each sample time. Steroids were administered after 24 h priming with hCG 100 IU.ml^{-1}. Hormones were then administered at (a)1 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (b) 10 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (c) 100 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} or (d) 1000 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1}, see Table 6.1. for details, n=4 per treatment dose.

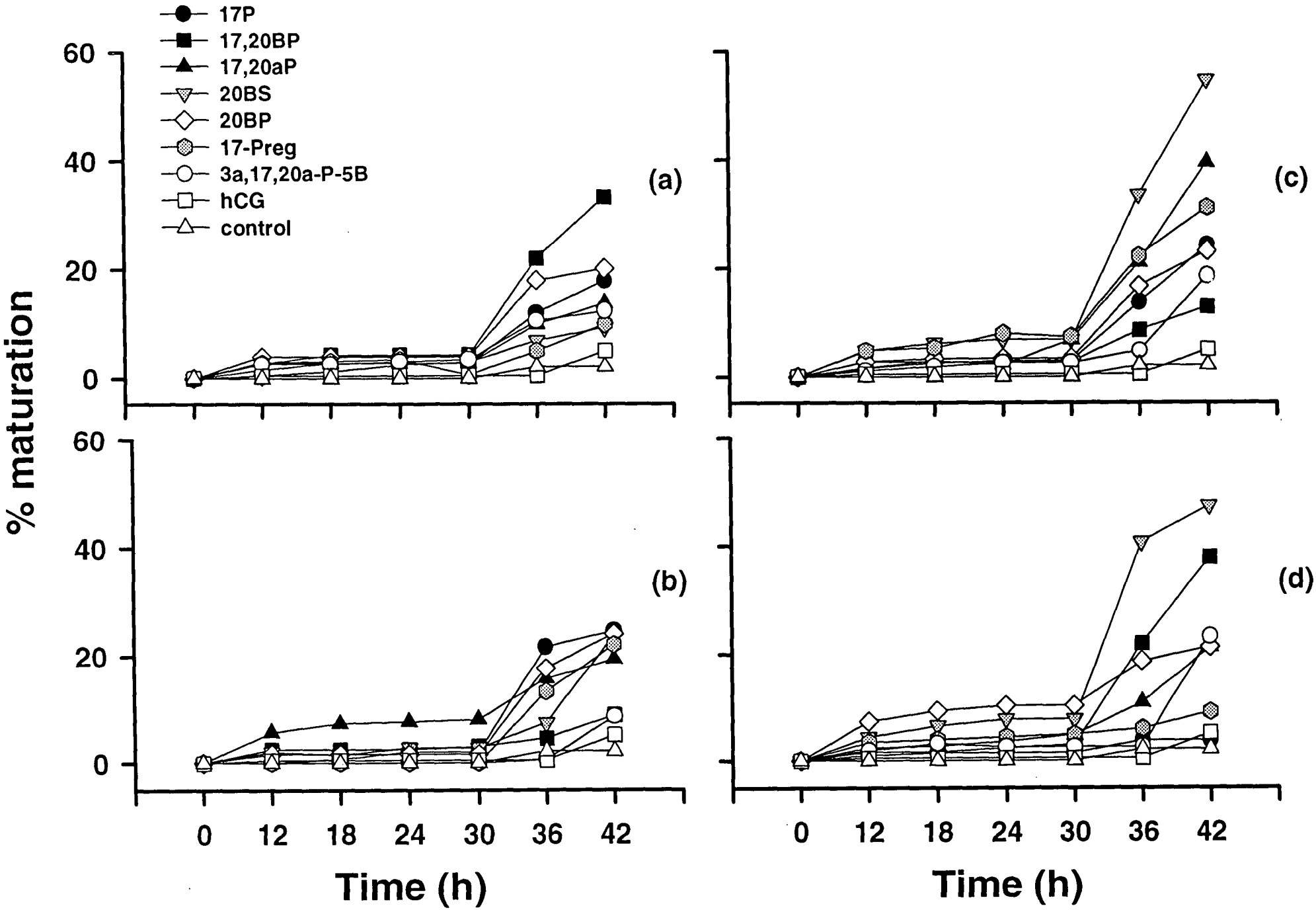
17P
17,20BP
17,20aP
20BS
20BP
17-Preg
3a,17,20a-P-5B
hCG
control
% maturation
60
40
20
0
(a)
(b)
(c)
(d)
0
12
18
24
30
36
42
Time (h)
Time (h)

Table 6.8. Statistical results for Fig. 6.7. Different superscripts show significant differences in percentage maturation in oocytes from fish 12, for each hormone at each dose and at each sample time. See Table 6.1. for details of experimental protocol. Asterisks indicate steroid treatments that were not primed with hCG 100 $IU.ml^{-1}$, n=4 per treatment dose.

Treatment	Dose	Dose	0 - 30 h	36 h	42 h
17P	$1ng.ml^{-1}$	$0.01ng.ml^{-1}$	a	ab	ab
17,20ßP	$1ng.ml^{-1}$	$0.01ng.ml^{-1}$	a	b	b
17,20αP	$1ng.ml^{-1}$	$0.01ng.ml^{-1}$	a	ab	ab
20ßS	$1ng.ml^{-1}$	$0.01ng.ml^{-1}$	a	ab	ab
20ßP	$1ng.ml^{-1}$	$0.01ng.ml^{-1}$	a	ab	ab
17-Preg	$1ng.ml^{-1}$	$0.01ng.ml^{-1}$	a	ab	ab
3α,17,20α-P-5ß	$1ng.ml^{-1}$	$0.01ng.ml^{-1}$	a	ab	ab
hCG	$100\ IU.ml^{-1}$	$100\ IU.ml^{-1}$	a	ab	a
control	control	control	a	a	a
17P	$10ng.ml^{-1}$	$0.1ng.ml^{-1}$	a	b	b
17,20ßP	$10ng.ml^{-1}$	$0.1ng.ml^{-1}$	a	ab	ab
17,20αP	$10ng.ml^{-1}$	$0.1ng.ml^{-1}$	a	ab	ab
20ßS	$10ng.ml^{-1}$	$0.1ng.ml^{-1}$	a	ab	ab
20ßP	$10ng.ml^{-1}$	$0.1ng.ml^{-1}$	a	ab	b
17-Preg	$10ng.ml^{-1}$	$0.1ng.ml^{-1}$	a	ab	ab
3α,17,20α-P-5ß	$10ng.ml^{-1}$	$0.1ng.ml^{-1}$	a	ab	ab
hCG	$100\ IU.ml^{-1}$	$100\ IU.ml^{-1}$	a	a	ab
control	control	control	a	ab	a
17P	$100ng.ml^{-1}$	$1ng.ml^{-1}$	a	ab	bc
17,20ßP	$100ng.ml^{-1}$	$1ng.ml^{-1}$	a	ab	ab
17,20αP	$100ng.ml^{-1}$	$1ng.ml^{-1}$	a	ab	bc
20ßS	$100ng.ml^{-1}$	$1ng.ml^{-1}$	a	b	d
20ßP	$100ng.ml^{-1}$	$1ng.ml^{-1}$	a	ab	bc
17-Preg	$100ng.ml^{-1}$	$1ng.ml^{-1}$	a	b	cd
3α,17,20α-P-5ß	$100ng.ml^{-1}$	$1ng.ml^{-1}$	a	ab	abc
hCG	$100\ IU.ml^{-1}$	$100\ IU.ml^{-1}$	a	a	a
control	control	control	a	a	a
17P	$1000ng.ml^{-1}$	$10ng.ml^{-1}$	a	a	a
17,20ßP	$1000ng.ml^{-1}$	$10ng.ml^{-1}$	a	ab	c
17,20αP	$1000ng.ml^{-1}$	$10ng.ml^{-1}$	a	a	ab
20ßS	$1000ng.ml^{-1}$	$10ng.ml^{-1}$	a	b	c
20ßP	$1000ng.ml^{-1}$	$10ng.ml^{-1}$	a	a	b
17-Preg	$1000ng.ml^{-1}$	$10ng.ml^{-1}$	a	a	ab
3α,17,20α-P-5ß	$1000ng.ml^{-1}$	$10ng.ml^{-1}$	a	a	b
hCG	$100\ IU.ml^{-1}$	$100\ IU.ml^{-1}$	a	a	a
control	control	control	a	a	a

Fig. 6.8. Percentage maturation in oocytes from fish 14, for each hormone at each sample time. Steroids were administered after 24 h priming with hCG 100 IU.ml^{-1}. Hormones were then administered at (a) 0.01 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (b) 0.1 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (c) 1 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} or (d) 10 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1}, see Table 6.1. for details, n=4 per treatment dose.

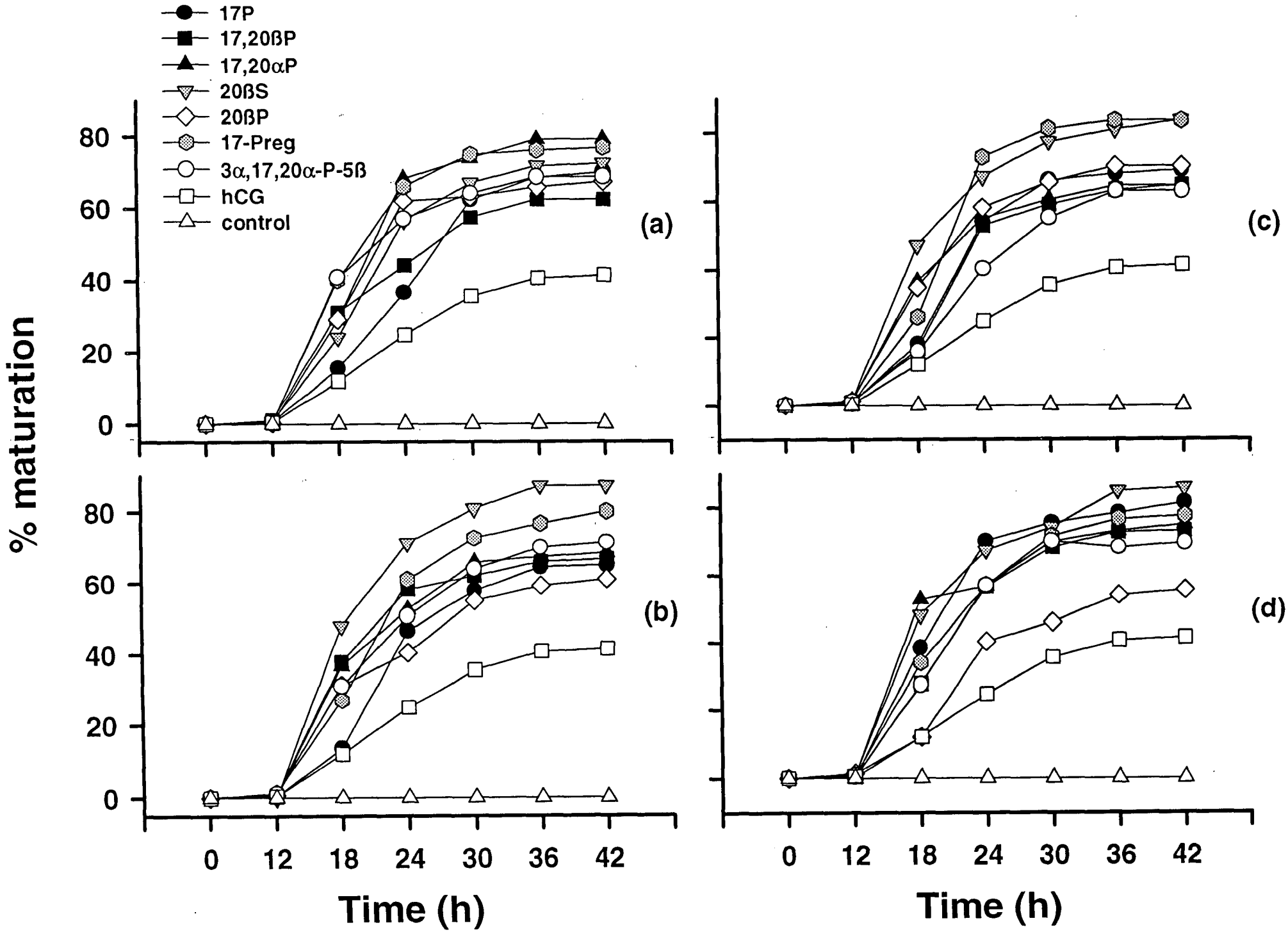
17P
17,20ßP
17,20αP
20ßS
20ßP
17-Preg
3α,17,20α-P-5ß
hCG
control
% maturation
(a)
(b)
(c)
(d)
0
20
40
60
80
0
12
18
24
30
36
42
Time (h)
Time (h)

Table 6.9. Statistical results for Fig. 6.8. Different superscripts show significant differences in percentage maturation in oocytes from fish 14, for each hormone at each dose and at each sample time. See Table 6.1. for details of experimental protocol. Asterisks indicate steroid treatments that were not primed with hCG 100 IU.ml^{-1}, n=4 per treatment dose.

Treatment	Dose	0 h	12 h	18 h	24 h	30 h	36 h	42 h
17P	0.01ng.ml^{-1}	a	a	ab	bc	bc	c	bc
17,20ßP	0.01ng.ml^{-1}	a	a	ab	bc	bc	bc	bc
17,20αP	0.01ng.ml^{-1}	a	a	ab	c	c	c	c
20ßS	0.01ng.ml^{-1}	a	a	ab	ab	c	c	bc
20ßP	0.01ng.ml^{-1}	a	a	ab	c	c	bc	bc
17-Preg	0.01ng.ml^{-1}	a	a	b	c	c	c	c
3α,17,20α-P-5ß	0.01ng.ml^{-1}	a	a	b	c	c	bc	bc
hCG	100 IU.ml^{-1}	a	a	ab	ab	b	b	b
control	control	a	a	a	a	a	a	a
17P	0.1ng.ml^{-1}	a	a	ab	bc	c	bc	bc
17,20ßP	0.1ng.ml^{-1}	a	a	ab	c	bc	bc	bc
17,20αP	0.1ng.ml^{-1}	a	a	ab	bc	c	bc	bc
20ßS	0.1ng.ml^{-1}	a	a	b	c	c	c	c
20ßP	0.1ng.ml^{-1}	a	a	ab	bc	bc	bc	bc
17-Preg	0.1ng.ml^{-1}	a	a	ab	c	c	c	c
3α,17,20α-P-5ß	0.1ng.ml^{-1}	a	a	ab	bc	bc	bc	bc
hCG	100 IU.ml^{-1}	a	a	ab	ab	b	b	b
control	control	a	a	a	a	a	a	a
17P	1ng.ml^{-1}	a	a	ab	c	c	c	c
17,20ßP	1ng.ml^{-1}	a	a	ab	c	bc	bc	bc
17,20αP	1ng.ml^{-1}	a	a	b	c	c	bc	bc
20ßS	1ng.ml^{-1}	a	a	b	c	c	c	c
20ßP	1ng.ml^{-1}	a	a	b	c	c	bc	bc
17-Preg	1ng.ml^{-1}	a	a	ab	c	c	c	c
3α,17,20α-P-5ß	1ng.ml^{-1}	a	a	ab	bc	bc	bc	bc
hCG	100 IU.ml^{-1}	a	a	ab	ab	b	b	b
control	control	a	a	a	a	a	a	a
17P	10ng.ml^{-1}	a	a	bc	c	c	bc	dc
17,20ßP	10ng.ml^{-1}	a	a	ab	bc	bc	bc	dc
17,20αP	10ng.ml^{-1}	a	a	c	bc	bc	bc	dc
20ßS	10ng.ml^{-1}	a	a	c	c	c	c	d
20ßP	10ng.ml^{-1}	a	a	ab	bc	b	b	bc
17-Preg	10ng.ml^{-1}	a	a	bc	bc	bc	bc	dc
3α,17,20α-P-5ß	10ng.ml^{-1}	a	a	ab	bc	bc	bc	dc
hCG	100 IU.ml^{-1}	a	a	ab	ab	c	b	b
control	control	a	a	a	a	a	a	a

Fig. 6.9. Percentage maturation in oocytes from fish 15, for each hormone at each sample time. Steroids were administered after 24 h priming with hCG 100 IU.ml^{-1}. Hormones were then administered at (a) 0.01 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (b) 0.1 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} (c) 1 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1} or (d) 10 ng.ml^{-1} steroid or hCG 100 IU.ml^{-1}, see Table 6.1. for details, n=4 per treatment dose.

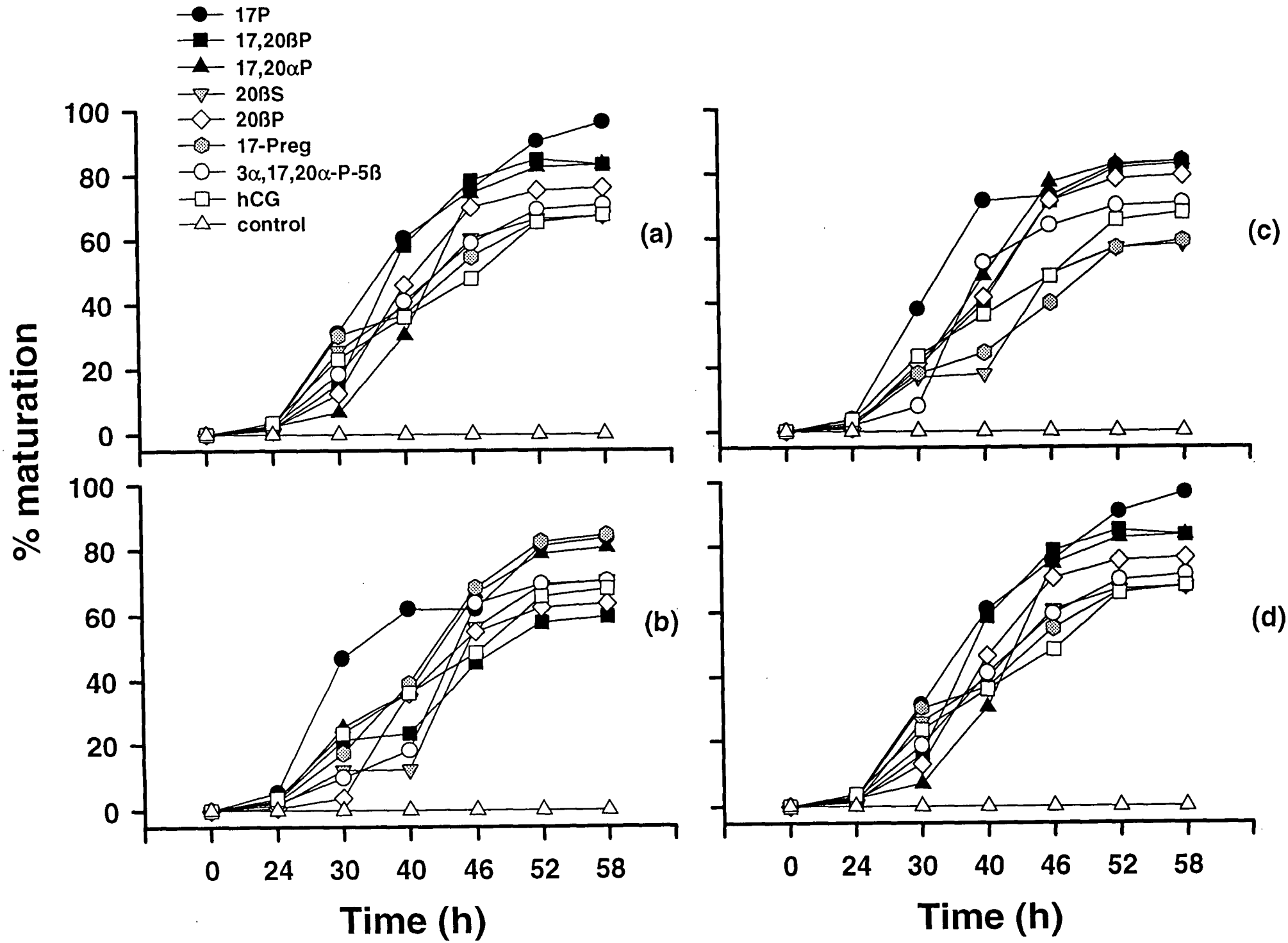
17P
17,20ßP
17,20αP
20ßS
20ßP
17-Preg
3α,17,20α-P-5ß
hCG
control
% maturation
100
80
60
40
20
0
(a)
(b)
(c)
(d)
0
24
30
40
46
52
58
Time (h)
Time (h)

Table 6.10. Statistical results for Fig. 6.9. Different superscripts show significant differences in percentage maturation in oocytes from fish 15, for each hormone at each dose and at each sample time. See Table 6.1. for details of experimental protocol. Asterisks indicate steroid treatments that were not primed with hCG 100 $IU.ml^{-1}$, n=4 per treatment dose.

Treatment	Dose	0 h	24 h	30 h	40 h	46 h	52 h	58 h
17P	$0.01ng.ml^{-1}$	a	a	a	b	b	b	b
17,20ßP	$0.01ng.ml^{-1}$	a	a	a	b	b	b	b
17,20αP	$0.01ng.ml^{-1}$	a	a	a	ab	b	b	b
20ßS	$0.01ng.ml^{-1}$	a	a	a	b	b	b	b
20ßP	$0.01ng.ml^{-1}$	a	a	a	b	b	b	b
17-Preg	$0.01ng.ml^{-1}$	a	a	a	b	b	b	b
3α,17,20α-P-5ß	$0.01ng.ml^{-1}$	a	a	a	b	b	b	b
hCG	$100\ IU.ml^{-1}$	a	a	a	b	b	b	b
control	control	a	a	a	a	a	a	a
17P	$0.1ng.ml^{-1}$	a	a	b	b	b	b	b
17,20ßP	$0.1ng.ml^{-1}$	a	a	ab	a	b	b	b
17,20αP	$0.1ng.ml^{-1}$	a	a	ab	ab	b	b	b
20ßS	$0.1ng.ml^{-1}$	a	a	ab	a	b	b	b
20ßP	$0.1ng.ml^{-1}$	a	a	a	ab	b	b	b
17-Preg	$0.1ng.ml^{-1}$	a	a	ab	ab	b	b	b
3α,17,20α-P-5ß	$0.1ng.ml^{-1}$	a	a	a	a	b	b	b
hCG	$100\ IU.ml^{-1}$	a	a	ab	ab	b	b	b
control	control	a	a	b	a	a	a	a
17P	$1ng.ml^{-1}$	a	a	b	c	d	b	b
17,20ßP	$1ng.ml^{-1}$	a	a	ab	bc	cd	b	b
17,20αP	$1ng.ml^{-1}$	a	a	ab	bc	d	b	b
20ßS	$1ng.ml^{-1}$	a	a	ab	ab	bc	b	b
20ßP	$1ng.ml^{-1}$	a	a	ab	bc	cd	b	b
17-Preg	$1ng.ml^{-1}$	a	a	ab	ab	b	b	b
3α,17,20α-P-5ß	$1ng.ml^{-1}$	a	a	ab	bc	bcd	b	b
hCG	$100\ IU.ml^{-1}$	a	a	ab	bc	bc	b	b
control	control	a	a	a	a	a	a	a
17P	$10ng.ml^{-1}$	a	a	a	b	b	b	b
17,20ßP	$10ng.ml^{-1}$	a	a	a	b	b	b	b
17,20αP	$10ng.ml^{-1}$	a	a	a	b	b	b	b
20ßS	$10ng.ml^{-1}$	a	a	a	b	b	b	b
20ßP	$10ng.ml^{-1}$	a	a	a	b	b	b	b
17-Preg	$10ng.ml^{-1}$	a	a	a	b	b	b	b
3α,17,20α-P-5ß	$10ng.ml^{-1}$	a	a	a	b	b	b	b
hCG	$100\ IU.ml^{-1}$	a	a	a	b	b	b	b
control	control	a	a	a	a	a	a	a

Table 6. 11. Rank of hormone effectiveness on % maturation (9 = best and 1 = worst) for each fish during experiments 3 and 4. Ranks were assigned from the steepest part of the curve.

Steroid dose ng.ml^{-1}	0.01	0.01	sub total rank	0.1	0.1	sub total rank	1	1	1	1	1	sub total rank	10	10	10	10	10	sub total rank	100	100	100	sub total rank	1000	1000	1000	sub total rank	Total rank	Total score	
Fish no.	14	15		14	15		10	11	12	14	15		10	11	12	14	15		10	11	12		10	11	12				steroid
17P	3	9	7	3	9	6	3	3	7	5	9	5	3	3	9	7	9	7	5	5	5	4.5	6	6	4	4	4.5	113	17P
17,20ßP	7	4	5.5	8	6	8	4	4	9	4	6	5	7	8	4	5	4	5.5	9	9	4	8.5	8	3	8	7.5	6.5	121	17,20ßP
17,20αP	6	2	3.5	7	8	9	5	8	5	8	5	8	6	9	7	9	2	8	8	7	7	8.5	3	9	6	6	9	127	17,20aP
20ßS	4	7	5.5	9	4	7	6	5	4	9	3	5	9	5	5	8	7	9	7	3	9	6.0	4	4	9	5	6.5	121	20ßS
20ßP	5	3	3.5	6	2	2.5	8	7	8	7	7	9	5	7	8	3	3	4	6	8	6	7.0	9	8	7	9	8	123	20ßP
17-Preg	8	8	9	4	5	4.5	7	6	3	6	4	3	4	4	6	6	8	5.5	4	4	7	4.5	7	7	5	7.5	4.5	113	17-Preg
3a,17,20a-P-5ß	9	5	8	5	3	2.5	9	9	6	3	2	7	8	6	2	4	5	3	3	6	5	3.0	5	5	3	3	3	103	3a,17,20a-P-5ß
hCG-100IU	2	6	2	2	7	4.5	2	2	1	2	8	2	2	2	1	2	6	2	2	2	3	2.0	2	2	1	1.5	2	56	hCG-100IU
control	1	1	1	1	1	1	1	1	2	1	1	1	1	2	3	1	1	1	1	2	1	1.0	1	2	2	1.5	1	26	control

6.5. Discussion

Ovarian fragments from greenback flounder, matured in response to steroids, but only in the presence of hCG. In most cases, the maturation response to steroids after priming with hCG, exceeded the maturation response to hCG treatment alone, indicating that the effect was not simply due to hCG stimulus of endogenous MIS production. The capacity to respond to MIS is dependent on having MIS receptors. In salmonids, the maturation inducing action of 17,20ßP in reinitiating meiosis is through binding to specific membrane receptors (Weisbart et al., 1991). Studies on spotted sea trout (Thomas and Patiño, 1991), kisu (*Sillago japonica*) (Kobayashi et al., 1988; Zhu et al., 1994), Tobinumeri-dragonet (Zhu et al., 1994), blue gourami (*Trichogaster trichopterus*) (Degani and Boker, 1992), red sea bream (*Pagrus major*) (Kagawa et al., 1994) dab and plaice (Canario and Scott, 1990), suggest that induction of MIS receptors and receptor activity is stimulated by GtH. GtH increases MIS receptor concentrations (Thomas and Patiño, 1991), induces RNA and protein synthesis related to the development of maturational competence (Patiño and Thomas, 1990; Kagawa et al., 1994) and possibly plays a role in the synthesis of steroid converting enzymes in some species (Kagawa et al., 1994; Nagahama et al., 1985; Planas et al., 1995; Yaron et al., 1995; Pankhurst, 1998).

Ovarian fragments that did not respond to hormone treatments were apparently normal in external appearance, although, some of these oocytes failed to clear in sera solution, suggesting that the oocytes were entering a state of atresia. A non response to hormones did not appear to be related to state of maturity before incubation. In another study, outwardly normal looking oocytes from some plaice and dab, also failed to respond to hCG and steroids, and did not clear in sera solution (Canario and Scott, 1990). Follicles from cultured fish in the present study tended to show a poorer response to treatment with hCG or steroids than wild fish, which suggests that culture conditions may effect oocyte quality. Broodstock nutrition is an important factor in determining egg quality in gilthead seabream (*Sparus auratus*) and European sea bass (*Dicentrarchus labrax*) (Harel et al., 1995; Navas et al., 1995). Acute and chronic stress can have subtle effects on gamete quality (reviewed in Pankhurst and Van der Kraak, 1997), and a previous study indicated that some routine husbandry practices

have the capacity to stress greenback flounder (Barnett and Pankhurst, 1998c, chapter 2 this volume).

Ovarian fragments primed with hCG were receptive to all steroids at all concentrations tested and the most effective treatments were not consistent between doses within fish or between fish. This suggests that the receptors had broad specificity and/or steroids were metabolised into a more biologically active form. If the latter were the case, we may have expected the most likely MIS to stimulate maturation more rapidly, and at lower concentration than other steroids. For example, in the New Zealand snapper (*Pagrus auratus*) there was window of time (<10 h) in which the authentic MIS 17,20ßP, was clearly more effective at lower doses (Ventling and Pankhurst, 1995) than a range of other steroids. However, if steroids are quickly metabolised into the biologically active form, such a pattern may not have been detected within this sampling protocol. There is evidence that *in situ* metabolism can be quite rapid. When ovarian homogenates from the European eel (*Anguilla anguilla*) were incubated with the radiolabeled precursor pregnenolone (25°C), 92% of the precursor was metabolised within 30 min (Lambert et al., 1991). If steroids were rapidly metabolised within the sampling intervals of this study (6-24 h), then less effective steroids could be converted to active forms and hence have a similar measured effect at the same endpoint.

There are suggestions from studies on other species that steroid converting enzyme activity may in part be GtH dependent (Nagahama et al., 1985; Kagawa et al., 1994; Planas et al., 1995; Yaron et al., 1995; Pankhurst, 1998). This suggests that hCG treatment may have enhanced the activity of steroid converting enzymes, whereby steroids were converted into the biologically active MIS before the true maturational effect of the added steroids could be detected.

The fact that greenback flounder ovarian fragments were receptive to a broad range of steroids is not unusual as virtually any steroid will induce FOM *in vitro* if administered at a high enough dose (Scott and Canario, 1987). We would however, expect some steroids to be effective only at high doses. *In vitro* incubations of New Zealand snapper oocytes were responsive to 17,20ßP over a range of doses, but were only responsive to 20ßS, 20ßP, and 17P at high doses (Ventling and Pankhurst, 1995). In this study, all steroids were effective at the range of doses tested, and this raises the possibility

that we did not test a sufficiently wide range of steroid doses, although, the doses tested were within the upper range of other studies and lower than doses tested in turbot (1.5-250 $ng.ml^{-1}$)(Mugnier, et al., 1995), Japanesese flounder (0.1-1000 $ng.ml^{-1}$) (*Limanda yokohamae*) (Hirose et al., 1987) dab and plaice (>3 and <1700 $ng.ml^{-1}$) (Canario and Scott, 1990). It is possible that flounder ovarian follicles are far more sensitive to steroids than that of most other species in which it has been investigated.

Despite the inconsistency in maturational responses to each steroid, 17,20αP was most frequently ranked as the most effective MIS *in vitro*, but was closely followed by 20ßP, 17,20ßP and 20ßS, the least effective steroids were the steroid precursor 17P followed by 17-Preg and 3α,17, 20α-P-5ß. This suggests that 20α and 20ß-hydroxylated steroids were most effective at inducing maturation in greenback flounder, and 5-pregnene and 5ß-pregnane steroids were least effective. In most teleosts there seems to be a common pattern in the biological activity of steroids depending on the conformation of the A/B rings junction, whereby 4-pregnene steroids are more biologically active, than 5-pregnen or 5ß-pregnane steroids, and addition of a hydroxyl group at the 17 and 20ß positions further augments biopotency (Nagahama et al., 1983; Scott and Canario, 1987; Canario and Scott, 1988; Canario and Scott 1990).

Establishing the potency of steroids on FOM *in vitro* is by no means the endpoint in establishing the identity of the MIS, particularly given that so many species undergo FOM in response to a range of steroids (Canario, 1991). More convincing evidence is provided when bioassays are used in combination with chromatographic, chemical or mass spectrometric techniques to identify steroids produced in the presence of neutral and/or radiolabeled precursors, or radioimmunassay to measure levels of C_{21} steroids produced *in vitro* and *in vivo*. However, even after extensive investigation, the answers may not always be straight forward. For example, in dab and plaice, 17,20ßP has been established as the MIS, and both 17,20ßP and 20ßS are the most potent steroids to induce FOM *in vitro* (Canario and Scott, 1990). However, the major steroids synthesised *in vitro* by dab ovaries are 17,20αP and 3ß,17α, 20α-trihydroxy-5ß-pregnane, and by plaice ovaries 17,21-dihydroxy-4-pregnene-3,20dione and 3α,17, 21-trihydroxy-5ß-pregnane-20-one (Canario, 1991), and blood levels of 17,20ßP are not clearly correlated with maturation. Canario and Scott, (1987 and 1989), have shown

that 17,20ßP has a high capacity for conjugation and reduction and can be found at high levels in blood and urine in the conjugated form.

It was established in a previous study that plasma and ovarian levels of 17,20ßP and plasma levels of 17,20ßP-sulphate are elevated in association with maturation events *in vivo* in wild greenback flounder (Barnett and Pankhurst, 1998b, chapter 3 this volume).Given that greenback flounder oocytes did not respond to steroids in a dose dependent manner, despite a wide range of doses being tested, suggests *in vitro* bioassay techniques may not be useful for assessing the potency of MIS/s on FOM in greenback flounder. However, this study has at least indicated that 20α and 20ß-hydroxylated steroids are more potent at inducing FOM *in vitro* than 5-pregnene and 5ß-pregnane steroids. We need to establish what other C_{21} steroids and conjugates greenback flounder are capable of producing *in vivo* and *in vitro* before we can speculate the identity of the MIS in this species.

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6.7. References

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Chapter 7

General discussion and overall summary.

7. General discussion and overall summary

7.1. Background of this study

Greenback flounder is being investigated as a potential aquaculture species in Tasmania, and looks to have many desirable aquaculture characteristics (reviewed in chapter 1, this volume), however, there are some essential requirements for propagation that have not been assessed in greenback flounder, which were addressed by this study.

7.2. The stress response to common laboratory and husbandry practices: implications for management.

Stress induced inhibition of reproduction, growth and the immune response can have severe consequences for the management of wild and domestic populations of fish. Stressors are an unavoidable component of aquaculture, however, the extent to which stress impacts on physiological function in a species, can have huge implications on the success of an aquaculture programme. Stress has been shown to inhibit plasma levels of gonadotropin (GtH) in the white sucker (*Catostomus commersoni*) and plasma levels of androgens and 17β-estradiol (E_2) in brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), snapper (*Pagrus auratus*), red gurnurd (*Chelidonichthys kumu*) and spotted sea trout (*Cynoscion nebulosis*) (reviewed in Pankhurst and Van Der Kraak, 1997). Hence, stress often inhibits reproduction at the level of gametogenesis. Chronic stress from inappropriate maintenance can be reflected in increased susceptibility to disease as demonstrated in salmonids (Pickering and Pottinger, 1987; Maule et al., 1989) and winter flounder (*Pleuronectes americanus)* (Carlson et al., 1993), and there is evidence suggesting cortisol may be in part responsible for the suppression of immunocompetence in response to stress in red drum (*Sciaenops ocellata*) and winter flounder (Thomas and Lewis, 1987; Carlson et al., 1993). Stress is also known to inhibit various parts of the growth-regulating endocrine cascade (reviewed in Barton and Iwama, 1991; Pankhurst and Van Der Kraak, 1997) and results in suppressed growth rates in brown trout and rainbow trout (Peters and Schwarzer, 1985; Pickering and Stewart, 1985).

The stress response to common husbandry and experimental practices was investigated in this study by examining a range of stress parameters (Barnett and Pankhurst, 1998e, chapter 2, this volume). Our results show that some routine husbandry practices have the capacity to stress greenback flounder, although, normal maintenance should not cause chronic stress, provided that stocking densities are appropriate. The plasma cortisol response to acute stressors such as capture, confinement and transport, in greenback flounder is consistent with the response seen in a range of other species (reviewed in Barnett and Pankhurst, 1998e, chapter 3, this volume). Unlike many other species (Ling and Wells 1985; Wells and Weber 1991;Young and Cech 1993), hematocrit (Hct) did not change significantly in response to stress, indicating that either changes in Hct were rapid and not detected by the sampling protocol ,or that Hct is not affected by stress in this species. The latter appears to be a characteristic shared by flathead sole (*Hippoglossides elassodon*) (Turner et al., 1983) and starry flounder (*Platichthys stellatus*) (Wood et al., 1977), suggesting, enhanced O_2 carrying capacity and and/or hemoconcentration associated with high Hct, are of little consequence in less active, benthic dwelling species. Muscle and blood physiology of greenback flounder change in response to exercise, but unlike other flatfish, there was little evidence for *in situ* glycogenesis within white muscle tissue after exercise and some indication that greenback flounder have higher aerobic scope than other flatfish studied to date.

Plasma levels of 17α20ß-dihydroxy-4-pregnen-3-one (17,20ßP) are generally not affected by stress, and in some cases increase during stressful periods (reviewed in Pankhurst and Van Der Kraak, 1997). Inhibition of final oocyte maturation (FOM) and spawning generally results from inappropriate environmental conditions (e.g. rising or decreasing temperature, lack of social interaction and spawning substratum, and inappropriate holding volumes) (reviewed in Pankhurst and Van Der Kraak, 1997). The fact that greenback flounder reliably undergo gametogenesis, but not FOM in captivity, suggests that environmental conditions essential to normal FOM and spawning are missing in captivity. However, this does not preclude the possibility that stress also inhibits FOM. Some experimental procedures resulted in significant decreases in plasma levels of testosterone (T) and E_2 (Barnett and Pankhurst, 1998b and d, chapters 4 and 5, this volume). Given that T positive feedback is probably an essential component of stimulation of pituitary GtH synthesis in this species (Barnett,

1998d, chapter 5, this volume), stress induced inhibition of circulating T levels could well contribute to failure of FOM in captivity.

Failure to undergo FOM in captivity is not unusual in aquaculture species, hence the development of techniques to artificially induce FOM and ovulation (reviewed in Pankhurst, 1998). However, induced ovulation protocols can be stressful for broodstock, and don't guarantee spontaneous spawning, resulting in the need for manual egg stripping. This procedure in itself, can be stressful for broodstock, particularly if the appropriate time for stripping is unknown, requiring the need to check for ovulation frequently. Given that stress is known to effect egg quality in some species (reviewed in Pankhurst and Van Der Kraak, 1997), it is desirable to limit handling procedures as much as possible, and better still, strive for natural spawning. Percentage fertilisation in greenback flounder treated with exogenous hormones was comparable to fertilisation rates in other species after exogenous hormone treatment (Harmin and Crim, 1992; Berlinsky and King, 1996; Berlinsky et al., 1997). However, in the absence of experimental sampling, induced ovulation protocols may result in even higher fertilisation rates.

This study established a baseline of stress indicators in normal unstressed fish, evaluated the impact of common husbandry and experimental practices on the stress response and concluded that some routine husbandry practices elicit stress responses in greenback flounder. This is by no means uncommon in an aquaculture species (Barton and Iwama, 1991), and should not impede the development of this emerging aquaculture species. Future studies however, need to assess the extent to which stressful husbandry practices impact on productivity (growth, reproduction and immune response). Meanwhile, stress management should not be ignored in experimental design and technology development for greenback flounder.

7.3. Controlled reproduction

Exogenous hormones such as the mammalian gonadotropin-hormone-releasing hormone analogue (LHRH-a) can sometimes be used to induced gametogenesis and ovulation in immature fish (Matsuyama et al., 1993), but are most effective at inducing ovulation and sometimes spawning in fish that have completed gametogenesis (reviewed in Peter and Yu, 1997; Pankhurst, 1998). Even when fish do spawn

spontaneously in captivity, exogenous hormone treatments allow greater control of reproductive events, resulting in more efficient and effective use of hatchery and grow-out facilities. In the absence of spontaneous FOM in captive greenback flounder, we developed techniques for artificial induction and control of ovarian development and ovulation. Successful reproductive management depends on an understanding of the physiological mechanisms initiating and mediating gonadal development, hence, we examined endocrine correlates of reproduction in wild flounder, and then went on to examine the effects of induced ovulation protocols on ovarian development and plasma levels of gonadal steroids with view to assessing the mechanism of action of hormone treatment.

In wild fish, changes in oocyte stages and diameters indicated group synchronous oocyte development and multiple ovulations (Barnett and Pankhurst, 1998c, chapter 3, this volume). Elevated plasma and ovarian levels of T and E_2 were found to be good indicators of vitellogenesis, levels of both steroids were significantly lower in fish undergoing FOM and ovulation. Plasma levels of 17,20ßP were significantly elevated during ovulation but not FOM. Ovarian levels of 17,20ßP were significantly elevated during FOM and ovulation, hence plasma levels of 17,20ßP were not always representative of events at the ovary. This was most likely a result of steroid metabolism and conjugation, as suggested by significantly elevated levels of plasma 17,20ßP-sulphate during FOM and ovulation.

In male greenback flounder, there was little variation in testis morphology and levels of the classical steroids 11 KT and 17,20ßP remained low. In many male teleosts plasma 11KT levels are elevated during spermatogenesis, (Scott et al., 1984; Fostier et al., 1987; Dedual and Pankhurst 1992; Methven et al., 1992; Barnett and Pankhurst 1994; Borg 1994; Harmin et al., 1995; Carolsfeld et al., 1996), and levels often remain elevated into the early stages of spermiation (Campbell et al., 1976; Fostier et al., 1987; Methven et al., 1992; Carolsfeld et al., 1996). Plasma 11KT did not change with gonadal stage in male greenback flounder. Similar observations have been made in some other species. 11 KT was not detected in male Gulf killifish (*Fundulus grandis*) (Greeley et al., 1988) or *R. saba* (Yeung and Chan, 1987) and in bluecod (*Parapercis colias*) and demoiselles (*Chromis dispilus*) changes in plasma 11KT levels were not related to gonadal condition (Pankhurst and Kime, 1991, Barnett and Pankhurst,

1994). There is good evidence for some species that 11 KT is more strongly associated with morphological and behavioural changes than spermatogenesis and spermiation (reviewed in Pankhurst and Carragher, 1991; Barnett and Pankhurst, 1994; Borg, 1994; Thoranensen et al., 1996). For example, in stoplight parrotfish (*Sparisoma viride*) and male blue cod plasma levels of 11KT were more strongly associated with territorial occupation (Liley et al., 1987; Pankhurst and Kime, 1991) and in male demoiselles, plasma levels of 11KT were elevated in association with spawning behaviour and territorial occupation and levels were higher in males in areas of high population density (Barnett and Pankhurst 1994, 1995). The role of 11KT could be further investigated in greenback flounder by assessing: the effect of exogenous 11KT on spermatogenesis or spermiation *in vitro* and *in vivo*, GtH stimulated 11KT production from testis tissue *in vitro*, the effect of exogenous 11KT on behavioural status, production of 11KT from testis tissue *in vivo* and the potential for conjugation and metabolism of 11KT.

Plasma levels of 17,20ßP are associated with spermiation in some teleosts (Scott et al., 1984; Fostier et al., 1987; Carolsfeld et al., 1996), and treatment with exogenous 17,20ßP stimulates an increase in milt volume in snapper, and a range of other species (reviewed in Pankhurst 1994). In contrast, plasma levels of 17,20ßP did not change in relation to gonadal condition in greenback flounder. This is not unusual, as low or undetectable plasma levels of 17,20ßP have been found in association with testis condition in many other species (Pankhurst and Conroy 1987, 1988; Pankhurst and Carragher 1991; Pankhurst and Kime 1991; Dedual and Pankhurst 1992; Barnett and Pankhurst 1994). We have already established that the fate of plasma 17,20ßP is not conjugation into the form of 17,20ßP-sulphate or 17,20ßP-glucuronide conjugates, however, this does not preclude the possibility that plasma 17,20ßP is metabolised into some other unmeasured conjugate in male greenback flounder. A further possiblilty is that 17,20ßP may not need to be elevated much to induce spermiation. We have established that male greenback flounder have small testes, and during gonadal development only low proportions of gamete stages advance into sperm production (Barnett and Pankhurst 1998c, chapter 3 this volume). If 17,20ßP mediates this process in greenback flounder as in some other species (reviewed in Pankhurst 1994), then low 17,20ßP levels may be sufficient to maintain spermiation. Future studies could assess the effect of exogenous 17,20ßP on spermiation *in vitro* and *in vivo*, GtH

stimulated 17,20ßP production from testis tissue *in vitro*, and the potential for conjugation and metabolism of 17,20ßP into other steroid conjugates.

Human chorionic gonadotropin (hCG), luteinising-hormone-releasing-hormone analogue (LHRH-a) intraperitoneal injections (ipi), LHRH-a slow release pellets and co-administration of LHRH-a + pimozide (PIM) and LHRH-a + T, successfully induced repeat ovulations in greenback flounder (Barnett and Pankhurst, 1998b and d, chapters 4 and 5, this volume). This was accompanied by significant increases in oocyte diameters and proportions of maturing oocytes, in a manner consistent with group synchronous oocyte development and multiple ovulations predicted from the examination of wild fish. In common with wild greenback flounder, these events were accompanied by significant increases in plasma and ovarian levels of E_2, and in most cases plasma and ovarian levels of T. Ovarian levels of 17,20βP did not change in response to exogenous hormone treatments, although in some cases, plasma levels of 17,20βP increased quite markedly. However, levels were not consistently elevated in association with reproductive events, hence, neither plasma or ovarian levels could be used as a marker of impending ovulation. Plasma increases in 17,20βP levels may have been of interrenal origin and/or stimulated by a stress response.

Greenback flounder were not differentially responsive to hCG, LHRH-a or the delivery mode of LHRH-a (Barnett and Pankhurst, 1998b, chapter 4, this volume). This indicates that both hypothalamic and pituitary hormones have the same effect on ovulatory success, and sustained delivery provided no significant advantage over injections, suggesting that a transient increase in exogenous GtH is sufficient to stimulate repeat ovulations. If we accept that clearance of exogenous hormones is as rapid in greenback flounder as it is in other species (< 25 min, (reviewed in Barnett and Pankhurst, 1998b, chapter 4, this volume)), then the fact that steroids and probably endogenous GtH persisted after the clearance of exogenous hormones, suggests that exogenous hormone treatments have an indirect effect on ovulation.

In some species, steroids have a positive feedback effect on pituitary responsiveness to exogenous fish gonadotropin relasing hormone analogue (GnRH-a) or LHRH-a, resulting in serum GtH release (Crim and Evans, 1983; Trudeau et al., 1991, 1993a, b). T treatment potentiated the stimulatory effect of LHRH-a on ovulation in greenback flounder (Barnett and Pankhurst, 1998d, chapter 5, this volume), suggesting that

exogenous T enhanced pituitary responsiveness to LHRH-a. Positive steroid feedback resulting from initial plasma T and E_2 increases caused by exogenous hormone treatment, may enhance pituitary GtH-II production in greenback flounder.

We did not assess the effects of HCG on fertilisation rate, but of the other exogenous hormone treatments, both LHRH-a and LHRH-a + T produced the highest % fertilisation, however, LHRH-a + T stimulated more ovulations than LHRH-a alone, and was therefore the most effective treatment in terms of egg production. Better fertilisation rates may be possible in the absence of experimental sampling or occurrence of natural ovulation and spawning. Fertilisation rates are quite acceptable as they stand, however, this study has laid the groundwork for future studies to achieve spontaneous FOM and spawning in captivity, which may result in even better fertilisation.

There is considerable interest in aquaculture development of flatfish species worldwide, and the most advanced industries are for Atlantic halibut (*Hippoglossus hippoglossus*) and turbot (*Scophthalmus maximus*). Ovulation occurs naturally in these species and gamete production is dependent on stripping. Egg viability after ovulation is extremely time dependent, however, by establishing ovulatory rhythms for individual fish, fertilisation rates of 100% can be achieved (McEvoy, 1984; Bromage and Roberts, 1995). In Chilean flounder (*Paralichthys microps*) fertilisation rates from natural spawning were 33.4% and 7.6% for groups 1 and 2 respectively (Silvia, 1994). It is hoped that improved understanding of temperature control and diet will improve egg production and fertilisation success in this species. Gamete production in other flatfish is generally dependent on induced ovulation with exogenous hormones. Fertilisation rates of 95% have been achieved in Japanese flounder (*Limanda yokohamae*) treated with hCG or salmon gonadotropin (Hirose et al., 1979). Egg fertility in this study was higher than levels reported for summer flounder (*Paralichthys dentatus*) in which fertility rates were on average 8%, 19% and 8% after treatment with GnRH-a pellets, carp pituitary extract (CPE) or hCG respectively (Berlinsky et al., 1997). In general, fertilisation success in this study is within the range of fertility reported for other flatfish species. Fertility reported for Southern flounder (*Paralichthys lethostigma*) treated with GnRH-a pellet was on average 64% (Berlinsky and King, 1996). Fertility in winter flounder treated with GnRH- pellet implants was on average 71% (Harmin

and Crim, 1992). Fertility results for English sole (*Parophrys vetulus*) injected with LHRH-a or CPE were on average 58% and 73%, respectively (Sanborn and Misitano, 1990).

The average time to ovulation in greenback flounder was prolonged in comparison to the majority of non-flatfish species in which induced ovulation protocols have been investigated, whereas the average time to ovulation was generally faster in comparison to other flatfish species (Lam, 1982; Peter et al., 1987, Barnett and Pankhurst, 1998b, chapter 4, this volume). An examination of published literature detailing the use of exogenous hormones to induce ovulation, indicates that time to ovulation is negatively correlated with holding temperature in a wide range of species (Barnett and Pankhurst, 1998b, chapter 4, this volume). Since flatfish are generally confined to cooler temperate waters, the time to ovulation is more prolonged.

Many of the founding principles of teleost endocrinology have historically been based on studies of salmonids and cyprinids. It is becoming increasingly apparent, that some aspects of endocrine regulation are not directly transferable between species. Examples include the role of 17,20βP as the maturation inducing steroid (MIS) and the role of dopamine (DA) on GtH-II regulation. There is a large body of literature mostly dominated by studies on cyprinids, which demonstrates strong DA inhibition of GtH release (reviewed in Trudeau and Peter, 1995; Peter and Yu, 1997), but a smaller number of studies indicate that DA inhibitory action on GtH release may be limited or even absent in some species (Van Der Kraak et al., 1986; Zohar et al., 1987; Copeland and Thomas, 1989). Greenback flounder appear to fall into the latter category, i.e. DA may not have strong inhibitory action on GtH release in greenback (Barnett and Pankhurst, 1998d, chapter 5, this volume), with the proviso that ovulation and not plasma GtH per se, was measured here. The significance of this finding for greenback flounder culture is that induced ovulation procedures don't require DA antagonists.

In other species considerable progress has been made in identifying other neuropeptides and neurohormones which may also have stimulatory and inhibitory actions on GtH release. Examples include melatonin which has stimulatory effects on GtH release in Atlantic croaker (*Micropogonias undulatus)* with fully developed gonads (Khan and Thomas, 1996) and serotonin (5-hydroxytryptamine, 5-HT) which stimulates GtH release in goldfish (Somoza and Peter, 1991) and the Atlantic croaker

(Khan and Thomas 1991, 1994). The neurotransmitter γ-aminobutyric acid (GABA) has prominent stimulatory actions on GtH release in gonadally regressed goldfish (Kah et al., 1991; Trudeau and Peter 1995) and gonadally regressed Atlantic croaker, and inhibitory effects in mature Atlantic croaker (Khan and Thomas 1995; Trudeau and Peter 1995). If a GtH-II assay could be developed for greenback flounder, in future studies it would be interesting to establish whether the role of DA inhibition on GtH-II, may in part be superseded by other neuropeptides and neurohormones in combination with steroid feedback in greenback flounder.

17,20ßP is the most active MIS in most species in which it has been tested (reviewed in Scott and Canario, 1987). However, there is good evidence that 17α,20ß, 21-trihydroxy-4-pregnen-3-one (20ßS), is the major maturation inducing steroid in the spotted sea trout (*Cynoscion nebulosus*) and Atlantic croaker (Thomas and Trant, 1989; Thomas, 1994) and some evidence for its action in turbot (Mugnier et al., 1995) and tbinumeri-dragonet (*Repomucenus beniteguri*) (Asahina et al., 1991). In other species, evidence for the identify of the MIS has been provided from combined studies using *in vitro* bioassays assessing the potency of steroids on FOM, chromatographic, chemical or mass spectrometric techniques to identify steroids produced in the presence of neutral and/or radiolabelled precursors, and radioimmunassay to measure levels of C_{21} steroids produced *in vitro* and *in vivo* (Canario, 1991). To date, we know that plasma levels of 17,20ßP were significantly elevated in ovulated females, and ovarian levels of 17,20ßP were elevated in association with FOM and ovulation (Barnett and Pankhurst, 1998c, chapter 3, this volume). However, plasma and ovarian levels were not consistently elevated in association with reproductive events in fish induced to ovulate using exogenous hormones (Barnett and Pankhurst, 1998b and d), chapters 4 and 5, this volume), and although 17,20ßP significantly enhanced FOM *in vitro*, 17,20ßP was not consistently more effective than any of the other 20α and 20ß-hydroxylated steroids tested (Barnett and Pankhurst, 1998a, chapter 6, this volume). Ovarian fragments may have very broad sensitivity or very low threshold, hence, *in vitro* bioassay techniques may not be useful for assessing the potency of MIS/s on FOM in greenback flounder. The identity of the MIS in greenback flounder is still not apparent, and future studies need to establish what other C_{21} steroids and conjugates

greenback flounder are capable of producing *in vivo* and *in vitro* before we can speculate on the nature of the MIS in this species.

In many marine fish species, particularly pleuronectiformes and partial ovulators, plasma 17,20ßP levels often show similar lack of variation in association with FOM (reviewed in Barnett and Pankhurst 1994, chapter 3, this volume). Although it is now accepted that 17,20ßP is the MIS in dab (*Limanda limanda*) and plaice (*Pleuronectes platessa*), the evidence is not as obvious as that provided for salmonids and cyprinids. The most convincing explanation for low levels of 17,20ßP in these species, is that 17,20ßP is rapidly deactivated by reduction and/or conjugation (Canario and Scott, 1987; 1989; 1990; Scott et al, 1998). Although plasma levels of 17,20ßP-sulphate were significantly elevated in female greenback flounder undergoing FOM and hydration, levels were considerably lower than plasma levels reported in plaice. A recent study indicates that 17,20ßP-sulphate is not the most abundant sulphated C_{21} steroid metabolite in plasma and urine of female plaice undergoing FOM (Scott et al., 1997). It is quite possible that female greenback flounder also produce high levels of some other sulphated metabolite.

The behavioural act of spawning can be the hardest part of the reproductive process to achieve under artificial conditions, yet it is the key to reliable production of high quality gametes, and the real control of managed reproduction in aquaculture. Evidence from other studies strongly suggest that for many aquaculture species, the appropriate hormonal, behavioral and social cues that occur during normal reproduction are missing. Recently there has been an increase in the number of studies relating reproductive endocrine status to reproductive behavioral and social events in teleosts (Liley & Stacey, 1983; Liley et al, 1987; Pankhurst & Barnett, 1993; Pankhurst, 1995). These studies strongly indicate that reproductive control is not a one-way system, whereby hormones regulate all reproductive processes, but a more complex system in which social, environmental and behavioral interactions also play a role in regulating reproduction (Liley et al, 1987; Pankhurst, 1995). There is also evidence that many freshwater teleost species release odors or pheromones that can affect the sexual behavior and reproductive physiology of conspecifics. Studies on goldfish have demonstrated that a primer pheromone synchronizes male - female spawning readiness, and that a releaser pheromone stimulates spawning behaviour (Stacey, 1989, 1991;

Stacey & Cardwell, 1995). Interactions between endocrine status, environment and behaviour are complex, and a thorough understanding of these relationships is dependent on a complete understanding of the animals' basic reproductive biology and endocrinology. This study has provided a good knowledge base of the reproductive biology and endocrinology of the greenback flounder, which provides the basic framework for future studies to investigate the social-behavioral-endocrine mechanisms that regulate and synchronize reproductive events and stimulate natural spawning of greenback flounder.

7.4. Summary

1. Cultured greenback flounder exposed to normal husbandry conditions had low plasma cortisol levels, however, some routine husbandry practices have the capacity to stress greenback flounder.

    - The plasma cortisol stress response of greenback flounder is similar to that shown by other marine teleosts.
    - The latency of the plasma cortisol response to stress was approximately 10 min.
    - Plasma levels of cortisol were significantly elevated in wild fish sampled after capture, and in cultured fish after simulated grading.
    - Plasma cortisol was significantly higher in fish held at medium and high stocking density than at low density.
    - Hematocrit did not change significantly in response to stress.
    - Muscle and blood physiology of greenback flounder changed in response to exercise, although there was little evidence for *in situ* glycogenesis within white muscle tissue after exercise and some indication that greenback flounder have higher aerobic scope than other flatfish studied to date.

2. Ovarian development in wild fish was characterised by significant changes in gonadal morphology and gonadal steroid levels, whereas testis development showed

very little variation in testis morphology and plasma levels of classical steroids remained low.

- Female greenback flounder demonstrated group synchronous oocyte development, with multiple ovulations.
- Plasma and ovarian levels of T and E_2 were elevated in association with vitellogenesis.
- Plasma levels of 17,20ßP were significantly elevated in ovulated females.
- Ovarian levels of 17,20ßP were elevated in association with FOM and ovulation.
- Plasma levels of 17,20ßP-sulphate but not 17,20ßP-glucuronide were elevated in association with FOM and ovulation.

3. Exogenous hormone treatments successfully induced repeat ovulations which were accompanied by changes in gonadal morphology and gonadal steroid levels similar to those found in wild fish.

- Treatment with hCG, LHRH-a (50 and 100$\mu g.kg^{-1}$) ipi and LHRH-a pellet increased the total number of ovulations and repeat ovulations above control levels, and LHRH-a pellet was more effective than LHRH-a (100$\mu g.kg^{-1}$) ipi.
- In another study, treatment with LHRH-a, LHRH-a + PIM, PIM and LHRH-a + T, significantly increased the number of ovulations above control levels. LHRH-a was more effective than LHRH-a + PIM and PIM in 1 out of 2 experiments, and LHRH-a + T was more effective than LHRH-a and T in both experiments. PIM significantly increased the total number of ovulations in 1 out of 2 experiments, but treatment with T alone had no effect.
- Co-treatment with LHRH-a and PIM did not improve the efficacy of LHRH-a, suggesting dopamine has weak inhibitory action on GtH release in greenback flounder.

- T potentiates the ovulatory effects of LHRH-a. It is reasonable to conclude that positive steroid feedback in greenback flounder stimulates pituitary GtH but not GtH release.

- Exogenous hormone treatment stimulated increases in oocyte diameters and advances in oocyte stages, and this was accompanied by increases in plasma and ovarian levels of E_2, and in most cases plasma and ovarian levels of T.

- Plasma and ovarian levels of 17,20βP were not consistently elevated in association with reproductive events. Plasma increases in 17,20βP levels may have been of interrenal origin and/or associated with a stress response

- LHRH-a, PIM, LHRH-a + PIM and LHRH-a + T significantly increased % fertilisation above control levels, and treatment with LHRH-a was more effective than PIM and LHRH-a + PIM.

- In greenback flounder, and a range of other species, time to ovulation was negatively correlated with holding temperature, and the type of exogenous hormone treatment had no influence on the time to ovulation.

4. Ovarian fragments required pre-treatment with hCG before they were receptive to steroids, however, the effect was not simply due to hCG stimulus of endogenous MIS production, as in most cases, the maturational response to steroids after priming with hCG exceeded the maturational response to hCG treatment alone.

    - Although ovarian fragments primed with hCG were receptive to all steroids at all concentrations tested, there was considerable inconsistency in maturational responses to each steroid and each dose.

    - 20α and 20ß- hydroxylated steroids were most effective at inducing maturation in greenback flounder, and 5-pregnene and 5ß-pregnane steroids were least effective.

7.5. References

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Chapter 8

Appendices

8. Appendices

8.1. Appendix 1

References for Table 4.1. showing time to ovulation following exogenous hormone treatment vs holding temperature for a range of species.

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8.2. Appendix 2

Curve fit equations for Fig. 4.9. Exogenous hormone treatments as follows: 1 = pituitary extracts, 2 = gonadotropin releasing hormone and analogue injections, 3 = gonadotropin releasing hormone/analogue + dopamine antagonist injections, 4= gonadotropin releasing hormone pellet implants.

Treatment	equation	A	B	C	r value
combined	y = A • e ^ ((lnX - B) ^ 2 / C)	27.1221	1.8410	-9.3395	0.75
1	y = A * B ^ (1/X) * X ^ C	335.8000	0.00009914	0.7004	0.71
2	y = A * B ^ (1/X) * X ^ C	48.4148	605.3296	-0.3083	0.63
3	y = A • e ^ ((lnX - B) ^ 2 / C)	30.9837	0.6069	-13.9426	0.85
4	y = A • e ^ ((X - B) ^ 2 / C)	1.5917	464.0030	67665.3	0.96