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

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Abstract

Sexually mature female black bream were captured by rod and line and injected with saline, human chorionic gonadotropin (hCG) or luteinizing hormone releasing hormone analogue (LHRHa) at capture, or 24 h post capture (saline and LHRHa treatments only). All fish were bled and checked for ovulation for 5 days post injection. Plasma levels of oestradiol (E_2), testosterone (T), 17,20β-dihydroxy-4-pregnen-3-one (17,20βP) and cortisol were determined by radioimmunoassay. Saline-injected fish ovulated only on day 1, whereas treatment with LHRHa or hCG resulted in fish ovulating throughout the experiment. Treatment with LHRHa at capture resulted in a better ovulatory response than treatment with hCG at capture or LHRHa 24 h post capture. Plasma E_2 levels in saline-injected fish were high at capture and had significantly dropped 1 day after capture. Injection with hCG or LHRHa at capture resulted in plasma E_2 levels remaining significantly elevated for 2 days post injection. Injection of LHRHa 24 h post capture failed to significantly elevate plasma E_2 levels over controls. Plasma T levels were similar to E_2 profiles. Plasma levels of 17,20βP were not significantly different between any treatments, but showed a

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tendency to increase after capture. Plasma cortisol levels showed no treatment effects and were initially low at capture before becoming elevated between days 1 and 2 post capture. These results show that capture and handling stress reduce the responsiveness of fish to exogenous hormone treatment and that best results are obtained if hormonal treatment is administered at the time of capture.

Keywords: Induced ovulation; Reproduction; *Acanthopagrus butcheri*; Stress; Cortisol; Oestradiol; Testosterone

1. Introduction

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

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

The Australian sparid *Acanthopagrus butcheri* (black bream) is currently under investigation as an aquaculture candidate for inland saline water culture. Initial stages of culture are likely to be dependent on hormonal manipulation of sexually mature fish captured from the wild. Given that this species shows acute and profound sensitivity to stress (Haddy and Pankhurst, 1999), this study investigated whether delay after capture changes the efficacy of treatment with LHRHa at inducing changes in plasma levels of gonadal steroids, and the occurrence or frequency of ovulation. The ovulatory response of fish to treatment and handling was assessed in terms of the number of ovulations, and the quantity and quality of the eggs produced. In the absence of a specific GtH assay for black bream, the endocrine response of fish to treatment and handling was assessed by measuring plasma levels of cortisol, E_2, T and 17,20β-dihydroxy-4-pregnen-3-one (17,20βP). The possibility that fish were differentially responsive to hCG or LHRHa

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

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

was assessed in a second experiment once the appropriate treatment window was determined.

2. Materials and methods

Sexually mature female black bream were captured by rod and line from the Meredith (148°7′S, 42°4′E) and Swan River estuaries (148°4′S, 42°4′E) at Swansea, Tasmania. Fish were blood-sampled by caudal puncture using heparinized syringes, fin clipped for individual identification and the time of hooking and blood-sampling recorded. Blood was stored on ice, plasma obtained by centrifugation, then frozen and stored at −18°C

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

Treatments	Proportion of ovulating fish (%)	Proportion of fish serially ovulating (%)	Mean number of ovulations per fish[a]	Mean fertilisation (%)[b]
Experiment 1				
At capture				
Saline	57	0	1	0 (1)
LHRHa	85	57	2.4	60 ± 16.5 (3)
24-h post capture				
Saline	43	0	1	98 (1)
LHRHa	44	22	1.5	20 (1)
Experiment 2				
Saline	57	0	1	43.0 ± 22.4 (3)
LHRHa	100	100	3.14	46.9 ± 7.4 (16)
HCG	87.5	75	2.86	41.1 ± 7.7 (9)

[a] Calculated from ovulating fish only.
[b] Fish numbers in parentheses.

until required for assay. Fish were placed in 400-l plastic tanks with oxygenation until transportation to the laboratory (3–20 h experiment 1; 3–7 h experiment 2), where they were placed in 1000-l, temperature-controlled (18–20°C) tanks supplied with recirculating seawater.

2.1. Experiment 1: effect of delayed injection time

Fish were caught from October to November 1997 between 17:35 and 20:50 h or 5:45 and 13:34 h. Sixteen fish (mean weight = 564 ± 50 g) were blood-sampled at capture without anaesthesia, placed into the holding tanks and transported to the laboratory. Twenty four hours after capture, fish were anaesthetised in 0.05% 2-pheno-

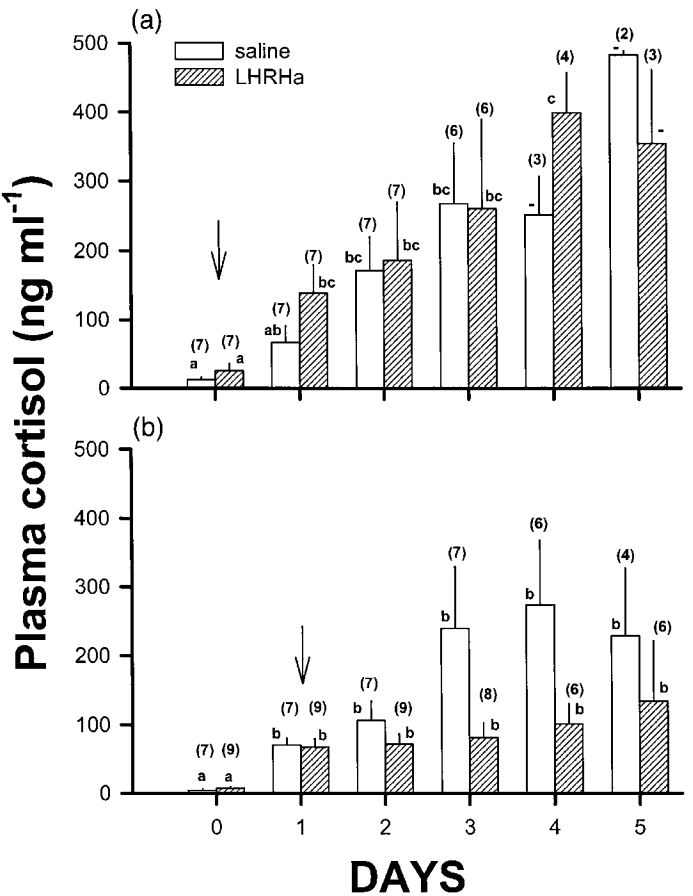

Fig. 1. Plasma cortisol concentrations (mean + SE) in black bream injected (indicated by arrow) with either saline or 50 μg kg^{-1} LHRHa, at capture (a), or 24-h post capture (b). Values that are significantly different ($P < 0.05$) have different letters; values indicated by (-) were not included in the analysis due to low n values (given in parenthesis).

xyethanol, blood-sampled and biopsied for macroscopic gonad condition (Haddy and Pankhurst, 1998). Fish that contained mature vitellogenic oocytes in the biopsy were then weighed, injected intraperitoneally with 50 μg kg^{-1} body weight of 50 μg ml^{-1} des-Gly^{10} (D-Ala^6)-luteinizing hormone releasing hormone ethylamide (LHRHa) (Sigma) ($n = 9$) or 1 ml kg^{-1} of teleost saline ($n = 7$) and checked for ovulation. Treatments were allocated on an alternating basis. LHRHa- and saline-treated fish were combined into tanks with 3–4 male fish. Ovulated females were manually stripped and the eggs fertilised in seawater of 35‰ salinity, using fresh sperm pooled from 3–4 males. Males were stripped by wiping dry the genital papilla region and milt expressed using slight abdominal pressure. The first portion of stripped milt was not collected to ensure milt was not contaminated with urine. Milt was collected while being expressed into dry 5-ml syringes and placed on ice until use. Fertilised eggs were viewed under a dissecting microscope and the fertility (division to 2–8-cell stage) of the first 100 eggs encountered recorded. Thereafter, fish were bled and checked for ovulation daily for 5 days. Another 14 fish (mean weight = 428 ± 20 g) were treated as described above except fish were

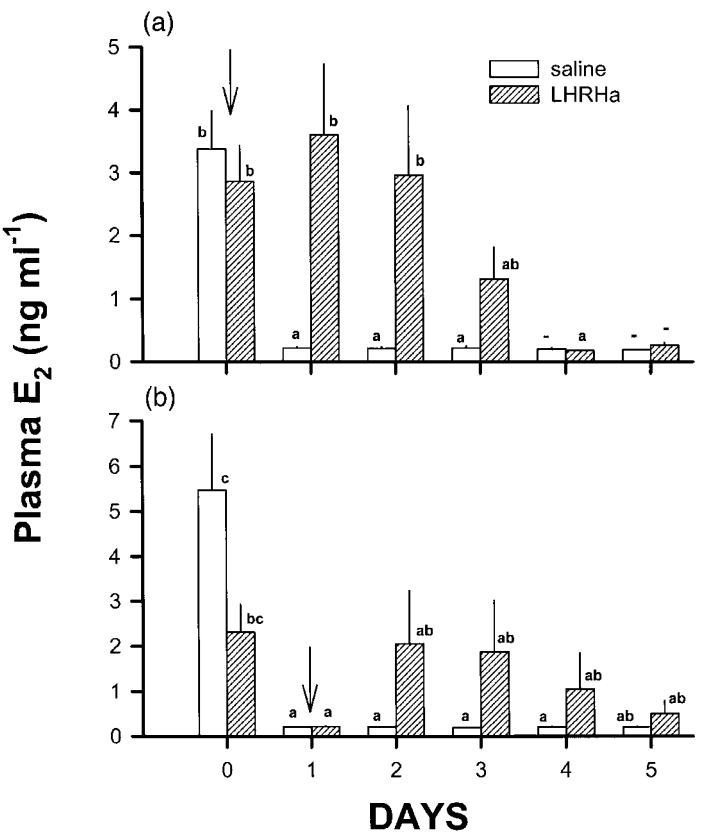

Fig. 2. Plasma E_2 concentrations (mean + SE) in black bream injected (indicated by arrow) with either saline or 50 μg kg^{-1} LHRHa, at capture (a), or 24-h post capture (b). Other details as for Fig. 1.

anaesthetised, blood-sampled, biopsied, and treated with LHRHa ($n = 7$) or saline ($n = 7$) at the time of capture. Owing to limited laboratory-holding space, and the dependence on capture of mature wild fish, the experiment was performed on six separate groups of fish. Three groups of fish captured between 16/10/97 and 4/11/97 were allocated to treatment 24 h after capture, with the remaining groups of fish captured between 5/11/97 and 26/11/97 allocated to treatment at capture. As all fish were of a similar reproductive state, it is assumed that they were equally responsive to treatment.

2.2. Experiment 2: effect of LHRHa, HCG and teleost saline

Fish were caught from October to December 1998 between 5:45 and 12:03 h and transported to the laboratory. Fish (mean weight = 615 ± 39 g) were handled as before and injected at capture with 50 μg kg^{-1} body weight LHRHa ($n = 7$), 1000 U kg^{-1} body weight of 1000 U ml^{-1} hCG ($n = 8$) or 1 ml kg^{-1} of teleost saline ($n = 7$).

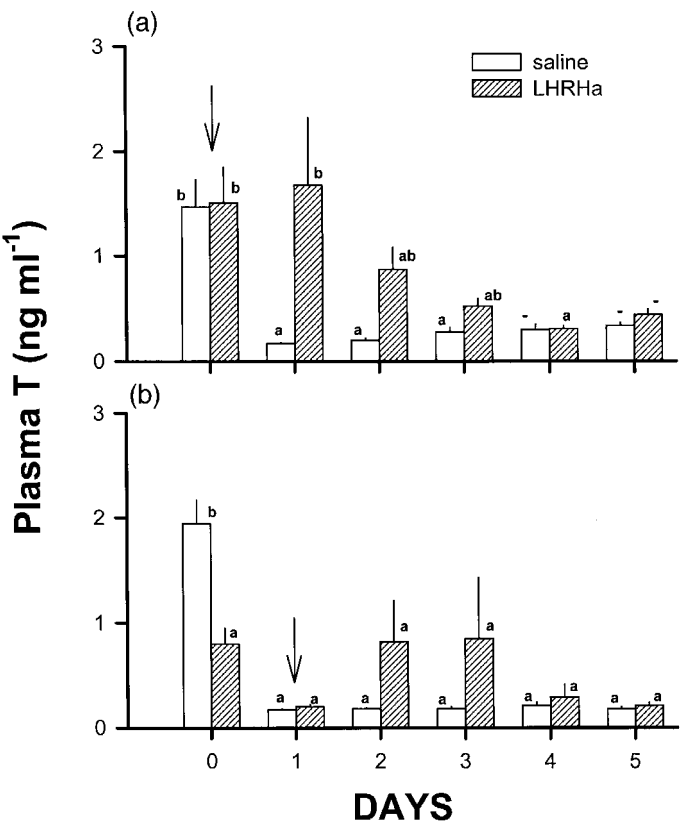

Fig. 3. Plasma T concentrations (mean + SE) in black bream injected (indicated by arrow) with either saline or 50 μg kg^{-1} LHRHa, at capture (a), or 24-h post capture (b). Other details as for Fig. 1.

Treatments were allocated in a cyclic fashion at the time of capture. LHRHa-, hCG- and saline-treated fish were combined into tanks with 3–4 male fish.

2.3. Steroid measurement

Plasma steroid concentrations were measured by radioimmunoassay, using the reagents and protocols given in Pankhurst and Carragher (1992). Extraction efficiency was determined by recovery of [^{3}H]-labeled steroid extracted with plasma, and assay values were corrected accordingly. Assay detection limits in plasma were 0.15 ng ml^{-1} for E_2, T and 17,20βP, and 0.3 ng ml^{-1} for cortisol. Values below the detection limit were treated as being equal to the detection limit. Interassay variability (%CV) measured using aliquots of a pooled standard was as follows: E_2 = 5.5% (n = 7); T = 3.8% (n = 7); 17,20βP = 10.8% (n = 7); cortisol = 9.8% (n = 9).

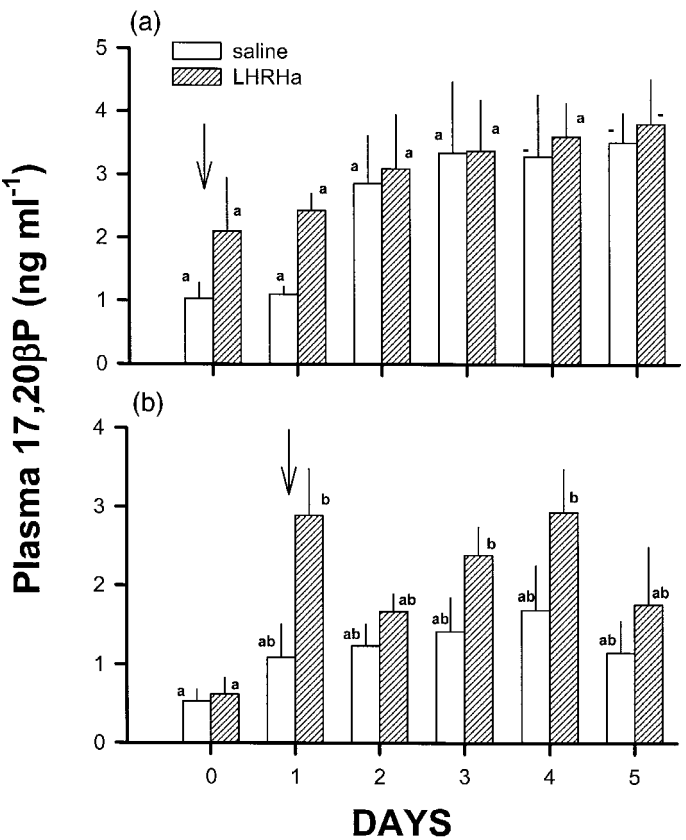

Fig. 4. Plasma 17,20βP concentrations (mean + SE) in black bream injected (indicated by arrow) with either saline or 50 μg kg^{-1} LHRHa, at capture (a), or 24-h post capture (b). Other details as for Fig. 1.

2.4. Statistics

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

3. Results

3.1. Experiment 1

The proportions of fish ovulating and the number of repeat ovulations were highest in fish injected with LHRHa at capture (Tables 1 and 2), with one fish serially ovulating for 4 days. Injection of LHRHa on the day following capture resulted in a smaller

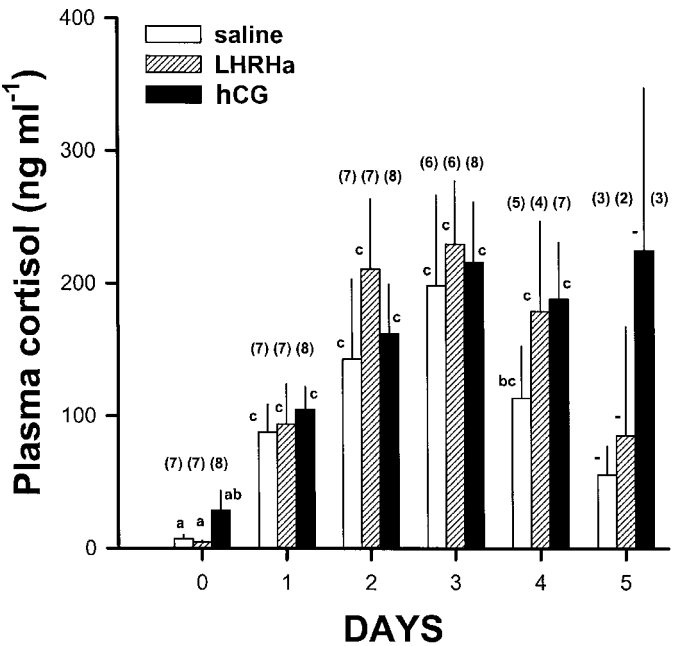

Fig. 5. Plasma cortisol concentrations (mean + SE) in black bream injected with either saline, 50 μg kg^{-1} LHRHa or 1000 U kg^{-1} hCG, at capture. Other details as for Fig. 1.

number of ovulations, with only two fish ovulating twice. Saline-injected fish ovulated on day 1 only. Spontaneous ovulations from saline-treated fish produced infertile eggs (saline injection at capture; $n = 1$) or eggs with high fertility (saline injection 24 h post capture; $n = 1$). Fertility from induced ovulations was substantially higher in fish treated at capture than at 24 h post capture.

Mean plasma cortisol levels ranging from 4.3 to 25.7 ng ml^{-1} at capture were significantly elevated on day 1 and remained elevated thereafter for all treatments except in fish injected with saline at capture, where plasma cortisol levels were not significantly elevated until day 2 (Fig. 1). There were no differences in cortisol levels between fish treated with saline or LHRHa. Plasma E_2 levels were above 2 ng ml^{-1} in all groups prior to treatment (Fig. 2). In fish injected with saline either at capture, or 24 h post capture, plasma E_2 levels were significantly suppressed by day 1 and remained low thereafter. Plasma E_2 levels in fish injected with LHRHa at capture were significantly elevated over controls on days 1 and 2 post injection. Treatment of fish with LHRHa 24 h post capture resulted in a variable response with no significant increase in plasma E_2 levels over saline-treated fish, but plasma E_2 levels after treatment were of similar magnitude to values found at capture. Mean plasma T levels ranged from 0.8 to 1.9 ng ml^{-1} at capture and followed the same pattern as that of E_2 in saline-injected fish (Fig. 3). Plasma T levels in fish injected with LHRHa at capture were significantly elevated over controls on day 1 but there was no significant effect of LHRHa in fish treated 24 h after capture. However, as for E_2 levels the response was highly variable, with mean

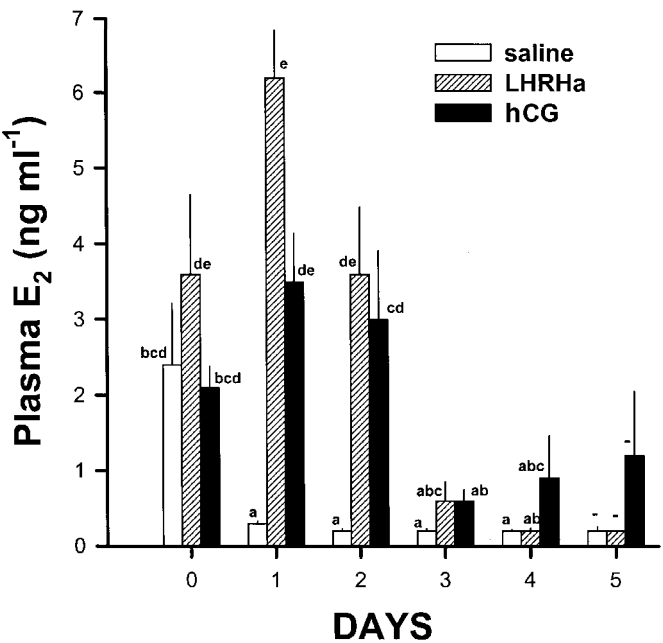

Fig. 6. Plasma E_2 concentrations (mean + SE) in black bream injected with either saline, 50 μg kg^{-1} LHRHa or 1000 U kg^{-1} hCG, at capture. Other details as for Fig. 1.

plasma T levels in LHRHa-injected fish approaching pre-treatment values. Plasma 17,20βP levels were not different among fish injected at capture at any time (Fig. 4). Similarly, treatment with LHRHa 24 h after capture did not affect 17,20βP levels, however, 17,20βP levels in LHRHa-treated fish were elevated over initial capture values on days 1, 3 and 4.

3.2. Experiment 2

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

Mean plasma cortisol levels ranging from 4.7 to 28.6 ng ml^{-1} at capture, were significantly elevated on day 1 and remained elevated thereafter (Fig. 5). Hormone treatment had no effect on plasma cortisol levels. Mean plasma E_2 levels for all treatments were above 2 ng ml^{-1} at capture (Fig. 6). Plasma E_2 levels were significantly suppressed by day 1 in fish injected with saline, and remained low thereafter. Plasma E_2 levels in fish injected with LHRHa or hCG were significantly elevated over levels in controls on days 1 and 2, but there were no differences between the two hormone treatments at any time. Mean plasma T levels at capture ranged from 1.3 to 2.2

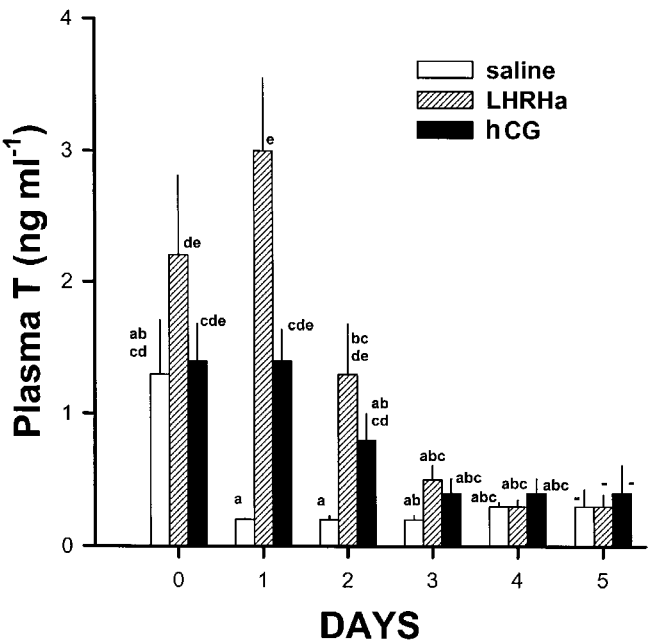

Fig. 7. Plasma T concentrations (mean + SE) in black bream injected with either saline, 50 μg kg^{-1} LHRHa or 1000 U kg^{-1} hCG, at capture. Other details as for Fig. 1.

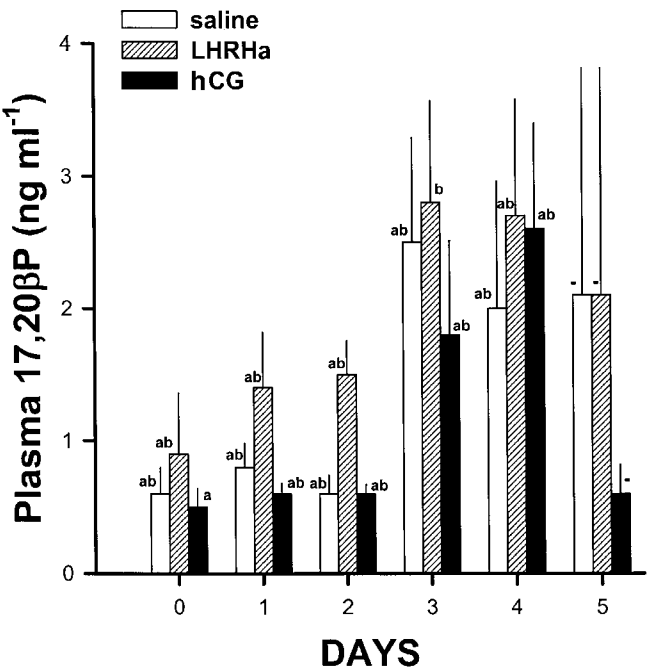

Fig. 8. Plasma 17,20βP concentrations (mean + SE) in black bream injected with either saline, 50 μg kg^{-1} LHRHa or 1000 U kg^{-1} hCG, at capture. Other details as for Fig. 1.

ng ml^{-1} and followed a pattern similar to that of E_2 in saline-injected fish, with a fall from pre-injection values to "near detection" levels (Fig. 7). Plasma T levels in fish injected with LHRHa and hCG, were significantly elevated over plasma T levels in saline-injected fish on days 1 and 2, and day 1, respectively. Plasma T levels were not different between LHRHa- or hCG-injected fish at any time. Plasma 17,20βP levels were not different among treatments at any time, however, there was a general tendency for 17,20βP levels to increase over the course of the experiment (Fig. 8).

4. Discussion

Results from the present study show that broodstock collection and daily handling stress in black bream causes the sustained elevation of plasma cortisol in all fish, and suppression of plasma T and E_2 in saline-injected fish within 1 day of treatment. Studies on stress-sensitive species caught from the wild, including black bream, have shown that stress-induced suppression of reproductive steroids occurs within 1 h of capture (Carragher and Pankhurst, 1991; Jardine et al., 1996; Cleary, 1998; Haddy and Pankhurst, 1999) Furthermore, in wild fish subjected to daily handling, plasma T and E_2 levels remain suppressed and show no signs of recovery during the experimental period (Carragher and Pankhurst, 1991; Clearwater and Pankhurst, 1997; Morehead, 1998).

Low plasma E_2 concentrations are associated with the onset of ovarian atresia, and once atretic, follicles are unlikely to be steroidogenic (Clearwater and Pankhurst, 1997; Janz and Van Der Kraak, 1997). The failure of plasma E_2 and T levels to recover in saline-injected fish in the present study is consistent with the previously demonstrated effects of stress on reproductive endocrine function in fish.

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

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

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

HCG and LHRHa both successfully induced repeat ovulations in black bream. In some teleosts, mammalian GtHs have a lower biopotency than piscine GtHs (Lam, 1982; Pankhurst, 1997) and treatment with hCG may be ineffective, or large and repeat doses may be required to induce ovulation (Lam, 1982; Berlinsky et al., 1997). However, hCG has been successfully used to induce ovulation in several sparids including snapper (Pankhurst and Carragher, 1992; Battaglene and Talbot, 1992; Cleary, 1998), gilthead seabream *Sparus aurata* (Zohar and Gordin, 1979), yellowfin porgy *Acanthopagrus latus* (Leu and Chou, 1996) and black bream (present study). In the gilthead seabream, hCG doses as low as 100 U kg^{-1} have been successfully used to induce serial ovulations (Zohar and Gordin, 1979). In contrast, injection of 500 U kg^{-1} of hCG in the yellow fin porgy failed to induce natural spawning, whereas injection with 1000 U kg^{-1} hCG resulted in partial spawning. Results from the present study indicate that in black bream hCG has biopotency at 1000 U kg^{-1} in vivo. However, more work is required to

establish the minimum effective dose. LHRHa was effective in black bream in the present study at 50 μg kg^{-1}. In the gilthead seabream, injection of LHRHa at doses as low as 7.5 μg kg^{-1} have been successfully used to induce ovulation (Zohar, 1986). However, in yellowfin bream *Acanthopagrus australis*, injection of 15–20 μg kg^{-1} of LHRHa is the minimal effective dose to reliably induce spawning (Cowden, 1995). As with hCG, the minimal effective dose of LHRHa in black bream has yet to be established.

LHRH analogues are becoming the hormone of choice for reproductive manipulation in fish culture (Donaldson and Devlin, 1996; Pankhurst, 1998), however, in some species LHRHa treatment is ineffective without the addition of a dopamine (DA) antagonist (Trudeau and Peter, 1995; Peter and Yu, 1997). DA-inhibition of GtH-secretion appears to be weak or non-existent in the gilthead seabream with co-treatment of LHRHa, and a DA antagonist offering no significant advantages over LHRHa treatment alone (Zohar et al., 1987a). This is consistent with the present study where injection of LHRHa alone successfully induced multiple ovulations. In the closely related yellowfin bream, injection of ovaprim (a mixture of LHRHa and the DA antagonist domperidone, Syndel) was less effective than LHRHa alone (Cowden, 1995). Co-treatment with LHRHa and a DA antagonist remains to be explored in black bream, but results from the present study provide indirect evidence that DA does not play a pivotal role in GtH-release in this species.

Administration of LHRHa in sparids results in a rapid surge of plasma GtH, with GtH levels peaking within 30 min to 1.5 h post injection, then gradually decreasing thereafter (Zohar et al., 1987b; Zohar et al., 1990; Tanaka et al., 1993). In the gilthead seabream, plasma GtH levels remain significantly elevated following injection with LHRHa for 48 h. Although plasma GtH levels were not measured in the present study, plasma E_2 and T profiles suggest that LHRHa-induced increases in GtH are of a similar duration in black bream, with E_2 and T remaining elevated for 2 days post injection. In contrast, plasma T levels in response to hCG returned to control values 1 day sooner than LHRHa-treated fish. This is most likely due to a quicker clearance of injected hCG than LHRHa which resulted in a prolonged secretion of endogenous GtH. The use of slow-release LHRHa implants sustains elevated gonadotropin levels over longer periods than acute administration (Breton et al., 1990) and in sequential spawners, results in inducing several successive ovulations (Mylonas et al., 1995; Cowden, 1995). However, the use of LHRHa implants in black bream remains to be investigated.

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

Plasma 17,20βP levels in black bream in the present study were unaffected by hormonal treatment, and typically showed a tendency to increase over time due to capture and handling. Capture and confinement of black bream result in a rapid increase

in plasma 17,20βP levels, with stress-induced increases in 17,20βP levels thought to be of interrenal origin (Haddy and Pankhurst, 1999). In the gilthead seabream, 17,20β21-trihydroxy-4-pregnen-3-one is the most likely candidate as the maturation inducing hormone (MIH) (Canario et al., 1995), whereas in snapper 17,20βP appears to be the MIH (Adachi et al., 1988; Kagawa et al., 1991; Ventling and Pankhurst, 1995). 17,20βP has not yet been conclusively identified as the MIH of black bream, but has been associated with final oocyte maturation and shows seasonal peaks during the spawning season (Haddy and Pankhurst, 1998).

In summary, fish capture caused the shut down of reproductive activity but injection of LHRHa or hCG at capture resulted in the maintenance of plasma T and E_2 levels, and was accompanied by the induction of multiple ovulations. Delayed injection of LHRHa resulted in a poorer ovulatory and a dampened endocrine response. LHRHa and hCG treatments resulted in a similar steroidogenic response, however, LHRHa produced a more consistent ovulatory response. These results confirm that in stress-sensitive species such as black bream, wild fish should be treated with LHRHa or hCG as soon as possible after capture for optimal responses.

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