Article (refereed) - postprint

Pottinger, T.G.; Carrick, T.R.; Hughes, S.E.; Balm, P.H.M.. 1996 Testosterone, 11-ketotestosterone and estradiol-17β modify baseline and stress-induced interrenal and corticotropic activity in trout. *General and Comparative Endocrinology*, 104 (3). 284-295. 10.1006/gcen.1996.0173

Testosterone, 11-ketotestosterone and estradiol-17β modify baseline and stress-induced interrenal and corticotropic activity in trout

T. G. Pottinger, T. R. Carrick, S. E. Hughes and P. H. M. Balm[1]

Institute of Freshwater Ecology, The Windermere Laboratory, The Ferry House, Far Sawrey, Ambleside, Cumbria, LA22 0LP, United Kingdom; and [1]Department of Animal Physiology, Faculty of Science, University of Nijmegen, Toernooiveld 1, 6526 ED Nijmegen, The Netherlands

Tel.: 015394 42468 fax: 015394 46914 Email: T.Pottinger@ife.ac.uk

Keywords: Stress response, cortisol, ACTH, testosterone, estradiol, 11-ketotestosterone, rainbow trout, brown trout

Running head: Modulation of stress response in trout

ABSTRACT

Estradiol-17β (E), 11-ketotestosterone (KT), and testosterone (T) were administered to immature rainbow and brown trout by implantation of steroid-containing cocoa-butter pellets. This procedure elevated the levels of these hormones in the blood of the treated fish and had significant effects on plasma ACTH and cortisol levels in both unstressed and stressed rainbow trout and in stressed brown trout. E treatment significantly elevated resting levels of ACTH and cortisol and KT significantly suppressed resting ACTH levels in rainbow trout, although no effect of KT was noted on baseline cortisol levels. One hour of confinement stress increased ACTH levels in rainbow trout, but less so in T- and KT-implanted fish than in sham-implanted fish. A similar pattern was observed in stress-induced plasma cortisol levels where T and KT treatment of rainbow trout resulted in a more than 50% attenuation of plasma cortisol levels while E-implantation significantly increased stress-induced plasma cortisol levels. In brown trout subjected to confinement stress for 96h, within 1h of the onset of confinement the stress-induced increase in plasma ACTH and plasma cortisol was significantly lower in T- and KT-implanted fish than in sham-implanted controls. However, these differences were not sustained at subsequent sample points during the 96h period of continuous confinement. Nonetheless, overall mean ACTH levels for the entire confinement period were significantly enhanced in E-implanted brown trout and significantly reduced in KT-implanted fish. Overall mean cortisol levels were significantly lower in T- and KT-implanted fish. The enhancement of stress responsiveness observed in E-treated immature fish was not observed during confinement stress in untreated mature female trout, with naturally high plasma E levels. However, untreated mature male trout displayed a significantly reduced cortisol response to confinement. It is suggested that gonadal steroids are involved in the regulation of both baseline and stress-induced activity of the pituitary-interrenal axis in salmonid fish.

INTRODUCTION

Many factors may modify the pituitary-interrenal response to stressful stimuli in fish. These include water temperature (Sumpter *et al*. 1985), and quality (Pickering and Pottinger, 1987), strain of fish (Pottinger and Moran, 1993), individual variation (Pottinger *et al*., 1992a), and repetition of the stimulus (Barton *et al*., 1986). Early observations that the corticosteroid response to stress in salmonid fish is also affected by sexual maturity (Donaldson and Fagerlund, 1970, 1972; Fagerlund, 1970; Fagerlund and Donaldson, 1969) have been confirmed and expanded (Sumpter *et al*., 1987; Pottinger *et al*., 1995). Mature male rainbow trout display a markedly attenuated cortisol response to both acute (1h confinement) and chronic (24h confinement) stress relative to immature fish of both sexes. This reduced cortisol response to stress appears to be mediated primarily by a reduction in pituitary ACTH output during stress (Pottinger *et al*., 1995), although other factors such as increased clearance of cortisol and changes in the sensitivity of the interrenal tissue to ACTH cannot be dismissed.

Mammals also display gender-related differences in responsiveness to stress (Aloisi *et al*., 1994; Heuser *et al*., 1994; Spinedi *et al*, 1994) and reduced responsiveness of the pituitary-adrenal axis to stress in mammals has been linked to elevated androgen levels (Boissy and Bouissou, 1994; Bingaman *et al*., 1995) while enhancement of responsiveness has been linked to estrogen in the female (Burgess and Handa, 1992). Androgenic suppression of the HPA axis has evolved in mammals to reduce the impact of stress on reproductive processes (Handa *et al*., 1994) while the enhanced stress responsiveness of the female may be a mechanism whereby environmental conditions unfavourable to reproduction can inhibit reproductive processes (Viau and Meaney, 1991).

As in other vertebrates, blood levels of gonadal steroids increase during sexual maturation in salmonid fish and administration of androgens to gonadectomized sockeye salmon reduces stress responsiveness, a result which was unequivocal despite a sample size of only 3 - 4 fish (Fagerlund and Donaldson, 1969).

The present study aimed to determine whether high levels of gonadal steroids are responsible for the reduced responsiveness of sexually mature male rainbow trout to stress, and to investigate the effects of estradiol-17β on this parameter. Immature rainbow trout and brown trout were given slow-release intra-peritoneal implants of testosterone, 11-ketotestosterone, or estradiol-17β, and the pituitary-interrenal response of the fish to acute and chronic stress was assessed by the confinement stress paradigm. 11-Ketotestosterone is the dominant circulating androgen in male rainbow and brown trout; testosterone is present in large amounts throughout the reproductive cycle in the blood of both male and female fish, and estradiol-17β is found predominantly in female fish (Scott and Sumpter, 1983; Baynes and Scott, 1985; Pottinger and Pickering, 1985a). There are no data on the effect of sexual maturity in female fish on the response of the pituitary-interrenal axis to stress so the corticosteroid responses of intact immature, and mature male and female rainbow trout to the confinement stress paradigm were examined.

MATERIALS AND METHODS

Experimental fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) and brown trout (*Salmo trutta* L.) were maintained in circular 1500-liter outdoor tanks, supplied with a constant flow of lake water (25 liters min^{-1}) and fed five times weekly on commercial feed (Trouw Aquaculture) at the manufacturers recommended rate according to fish size and water temperature. Fish were stressed by net transfer from their holding tank into a lidded, rectangular polypropylene tank (50-liters) supplied with a constant flow of lake water (15 liters min^{-1}). One h after transfer (Experiments 1, 2, 4) or at intervals during a longer period of confinement (Experiment 3), fish were netted from the tanks, anaesthetized (1ml 2-phenoxyethanol: 2000 ml lake water), and blood samples were removed from the Cuverian sinus into 1.0 ml heparinized syringes. Samples from unstressed fish were obtained by netting fish directly from the 1500 -liter holding tanks into anaesthetic. Blood was placed on ice, centrifuged (1000g), and plasma frozen at -20°C until assay. For those samples in which ACTH, α-MSH or N-acetyl-β-END

were to be determined, heparin was replaced by EDTA (1.5 mg ml^{-1} blood) and aprotinin (3000 KIU ml^{-1} blood) was included.

Hormone Assays

Plasma cortisol levels were determined using a validated radioimmunoassay (Pickering *et al*., 1987). Plasma ACTH levels were determined following Balm and Pottinger (1993) and Balm *et al*. (1994). Sumpter and Donaldson (1986) encountered problems when assaying plasma from mature female salmonids, which led to unrealistically low ACTH levels in these fish. A plasma pool from the E-implanted fish diluted in parallel with the standard curve indicating that elevated vitellogenin levels or other E-related effects did not interfere with the quantification of the hormone in this study. α-Melanocyte stimulating hormone (α-MSH) and N-acetyl-β-endorphin (N-acetyl-β-END) levels were measured according to Balm *et al*. (1995). The α-MSH antibody recognizes des-, mono-, and di-acetylated α-MSH equally well. Comparing this antibody and an antibody (R6B4; a gift from B. I. Baker) related to one used by Sumpter (Rodrigues and Sumpter, 1984; R6FB, also from B. I. Baker) in our RIA system yielded similar concentrations of α-MSH immunoreactivity in rainbow trout plasma pools. The N-acetyl-β-END antibody was a gift from Prof. H. Kawauchi (Kitasato University, Japan) and has also been used previously (Sumpter *et al*., 1985). Plasma testosterone and 11-ketotestosterone levels, and plasma estradiol-17β levels were determined by methods described by Pottinger and Pickering (1985a) and Pottinger and Pickering (1990) respectively.

Experiment 1: Preliminary examination of the effect of androgen and estrogen administration on plasma levels of cortisol, ACTH, α-MSH, and N-ac-β-END during a 1 h confinement stress in rainbow trout.

The aim of this small-scale experiment was to determine whether there was justification for carrying out a full-scale study to examine the hypothesis that the administration of gonadal steroids to fish modifies the endocrine response to stress. During August 1994 (water temperature ~16^{o}C) forty-eight rainbow trout (South African strain, 3+ years old, mixed sex, mean weight ± SEM, n = 48, 328 ± 11 g) were individually tagged (opercular tags). Twelve fish were implanted with 0.5 ml cocoa butter (cb) containing 20 mg testosterone, twelve with

0.5 ml cb containing 20 mg 11-ketotestosterone, and twelve with 0.5 ml cb containing 20 mg estradiol-17β(steroids purchased from Sigma and Koch-Light). A further twelve fish received 0.5 ml cb only (sham-implanted). The fish were returned to their holding tank. All treated groups were maintained in the same tank. At 10, 14, 19 and 24 days following implantation the fish were netted from the holding tank and transferred to eight lidded, rectangular polypropylene tanks (50-liters) supplied with a constant flow of lake water (15 liters min^{-1}), six fish per tank (confinement density, 39 g l^{-1}). One h after transfer the fish were netted from the tanks, anaesthetized (1 ml 2-phenoxyethanol: 2000 ml lake water), and blood samples were removed from the Cuverian sinus into 1.0 ml heparinized syringes. The fish were returned to the 1500-liter holding tank after sampling. Blood was placed on ice, centrifuged (1000g) and plasma frozen at -20ºC until assay. On day 24, for those samples in which ACTH, α-MSH or N-acetyl-β-END were to be determined, heparin was replaced by EDTA (1.5 mg ml^{-1} blood) and aprotinin (3000 KIU ml^{-1} blood) was included.

Experiment 2: Effect of androgen and estrogen administration on plasma levels of cortisol and ACTH in rainbow trout before and following a 1 h confinement stress.

During July 1995 (water temperature ~15ºC) two hundred rainbow trout (Isle-of-Man strain, 3+ years old, mean weight of sub-sample ± SEM, n = 40, 490 ± 18 g) were divided evenly between eight 1500-liter tanks, twenty-five fish per tank. Two tanks of fish were injected with cb containing testosterone, two with cb containing 11-ketotestosterone, two with cb containing estradiol-17β (doses as for Expt. 1) and two with cb alone (sham-implanted). The replication of tanks within treatments was considered necessary to overcome the possibly confounding effects of tank-to-tank variation. Seven days after implantation, five fish were netted from each tank into anaesthetic and blood sampled as described above. After sampling, the fish were killed by a blow to the head. Immediately after this sample of undisturbed fish, a further five fish were transferred from each tank into 50-liter confinement tanks (confinement density, 49 g l^{-1}). These fish were blood-sampled 1 h after the start of confinement. A second batch of fish from the same treatment groups were sampled pre- and post-confinement at 14 days after implantation. This design provided a total of 10 fish per treatment per sample time. All samples were analysed for cortisol, ACTH, testosterone, 11-ketotestosterone, and estradiol-17β.

Experiment 3: Effect of androgen and estrogen administration on plasma levels of cortisol and ACTH during a 96 h confinement stress in brown trout.

During July 1995 (water temperature ~15^{o}C) forty brown trout (Dunsop Bridge strain, 3+ years old, mean weight ± SEM, n = 40, 365 ± 14 g) were individually tagged with intraperitoneal PIT tags (Passive Integrated Transponder, FishEagle). The fish were transferred from their 1500-liter holding tank to 50-liter confinement tanks, in groups of five. After 1 h, blood samples were taken as described above. This comprised the pre-implantation sample. The following day, fish received intraperitoneal implants of cb containing either testosterone, 11-ketotestosterone, estradiol-17β, or cb alone, ten fish per treatment (doses as for Expt. 1). After 12 days, the fish were netted from their holding tank and transferred to the 50-liter confinement tanks, five fish per tank (confinement density, 36.5 g l^{-1}). The fish were anaesthetised and blood was collected at 1, 4, 24, 48, 72, and 96 h after the onset of confinement. Blood samples were treated as described above and each sample was assayed for cortisol and ACTH. Testosterone, 11-ketotestosterone, and estradiol-17β levels were determined for the 1 h and 48 h samples only.

Experiment 4: A comparison of the cortisol response of mature male and female, and immature, rainbow trout to 1 h confinement stress.

The observations made during experiments 1 - 3 on the impact of estradiol-17β on stress responsiveness guided this experiment to compare the effect of sexual maturity on the stress response of mature female trout, with that of mature male, and sexually immature, trout. During December 1995 (water temperature ~8^{o}C), approximately 6 weeks prior to final maturation, ten immature, ten mature male, and ten mature female rainbow trout from a single 1500-liter holding tank (Stirling strain, 3+ years old) were transferred to 50-liter confinement tanks. After 1h the fish were transferred to anaesthetic and blood sampled as before. Plasma was analysed for cortisol, testosterone, 11-ketotestosterone, and estradiol-17β.

Statistical Analysis

The data were subjected to Analysis of Variance (ANOVA, Genstat) with time, treatment (sham, E, T, 11KT), and tank as factors where appropriate.In cases where the mean and

variance were not independent an appropriate transformation of the data was carried out (square root or log). The data are presented as arithmetic means ± SEM.

RESULTS

Experiment 1. Preliminary examination of the effect of androgen and estrogen administration on plasma levels of cortisol, ACTH, α-MSH, and N-ac-β-END during a 1 h confinement stress in rainbow trout.

Plasma levels of testosterone (T), estradiol-17β (E), and 11-ketotestosterone (KT) were significantly elevated in the respective groups of implanted fish and low to negligible in groups not receiving that implant (Table 1). The mean plasma cortisol levels after 1 h of confinement stress for all four sample days combined were significantly higher in E-implanted fish (169 ± 14 ng ml^{-1}, $p<0.001$, $n = 48$) and significantly lower in KT-implanted fish (80 ± 5 ng ml^{-1}, $p<0.05$, $n = 48$) than in sham-implanted fish (124 ± 11 ng ml^{-1}). Cortisol levels in T-implanted fish (116 ± 11 ng ml^{-1}) did not differ significantly from sham-implanted fish (Fig. 1a). Plasma ACTH levels on day 24 were significantly higher in E-implanted fish (155 ± 15 pg ml^{-1}, $p<0.05$, $n = 12$) than in sham-implanted fish (99 ± 11 pg ml^{-1}, Fig. 1b). Steroid implantation had no effect on the plasma levels of MSH or END on day 24.

Experiment 2. Effect of androgen and estrogen administration on plasma levels of cortisol and ACTH in rainbow trout before and following a 1 h confinement stress.

The implantation procedure successfully elevated blood levels of the selected steroids (Table 2). Cortisol and ACTH data from the two sample points were combined for analysis. ANOVA revealed there to be significant effects of treatment overall ($p<0.001$). Androgen and estrogen implantation had significant effects on both baseline and post-stress plasma levels of ACTH and cortisol. E-implanted fish had significantly higher resting levels of ACTH ($p<0.001$, 76.6 ± 5.5 pg ml^{-1}, $n = 20$) and KT-implanted fish significantly lower levels of ACTH ($p<0.001$, 50.3 ± 2.5 pg ml^{-1}) than sham-implanted fish (64.2 ± 4.0 pg ml^{-1}) (Fig. 2a). E-implanted fish also had significantly higher resting levels of plasma cortisol than

sham-implanted fish ($p<0.05$; 7.1 ± 2.0 cf. 4.3 ± 1.3 ng ml^{-1}) (Fig. 2c). Following 1 h of confinement, plasma ACTH and cortisol levels were significantly elevated in all treatment groups. However, plasma ACTH levels were significantly lower in T-implanted ($p<0.05$, 145.2 ± 11 pg ml^{-1}) and KT-implanted ($p<0.001$, 120.1 ± 14.8 pg ml^{-1}) fish than in sham-implanted fish (200.9 ± 14.8 pg ml^{-1}) (Fig. 2b). Although ACTH levels in E-implanted fish were higher than those in the sham-implanted fish this difference was not statistically significant. Post-stress plasma cortisol levels in T-implanted (25.9 ± 2.6 ng ml^{-1}) and KT-implanted (18.8 ± 3.8 ng ml^{-1}) groups were also significantly lower ($p<0.01$, $p<0.001$ respectively) than plasma cortisol levels in control fish (53.0 ± 8.1 ng ml^{-1}). In addition, E-implanted fish had significantly higher plasma cortisol levels post-stress ($p<0.05$, 71.4 ± 6.3 ng ml^{-1}) than control fish (Fig. 2d). There were no significant effects of treatment on weight or condition factor (100.weight/$length^3$).

Experiment 3. Effect of androgen and estrogen administration on plasma levels of cortisol and ACTH during a 96 h confinement stress in brown trout.

Gonadal steroid levels were measured at 1h and 48h after the start of confinement to confirm the efficiency of the implants. The mean levels detected are presented in Table 3. In each case the implant increased plasma steroid levels to within a physiologically relevant range. ANOVA revealed steroid implantation to have had significant effects on both ACTH ($p=0.002$) and cortisol ($p<0.001$) levels during confinement stress. There was no significant difference between the ACTH responses of the four groups of fish to a 1h confinement stress prior to administration of the steroid implants (Fig. 3a). However, following implantation, 1 h after the onset of confinement, plasma ACTH levels were significantly lower in T-implanted ($p<0.05$, 469 ± 63 pg ml^{-1}, n = 10) and KT-implanted ($p<0.001$, 344 ± 31 pg ml^{-1}) groups than in sham-implanted fish 586 ± 66 pg ml^{-1}). There was a significant ($p<0.001$) decline in plasma ACTH levels in all groups between 1h and 24h following the onset of confinement and no further significant differences were observed between groups at subsequent time points until the 96h sample when E-implanted fish had significantly higher ACTH levels ($p<0.05$, 190 ± 22 pg ml^{-1}) than sham-implanted fish (119 ± 13 pg ml^{-1}). ANOVA revealed there to a highly significant effect of steroid implantation on ACTH levels overall during the 96h confinement period which was resolved as significantly higher levels of ACTH in

E-implanted fish ($p<0.05$, 231 ± 23 pg ml^{-1}, n = 60) and significantly lower levels of ACTH overall in KT-implanted fish ($p<0.05$, 164 ± 15 pg ml^{-1}) than in sham-implanted fish (214 ± 26 pg ml^{-1}) (Fig. 3b). In contrast with the ACTH data, there was a significantly higher cortisol response to confinement for 1h following implantation in the sham-implanted group compared to pre-implantation values ($p<0.01$, Fig. 4). There were no significant differences in the cortisol response to stress between treatment groups prior to implantation whereas at 1h following the onset of confinement cortisol levels in T-implanted (114 ± 18 ng ml^{-1}) fish and KT-implanted fish (99 ± 22 ng ml^{-1}) were significantly ($p<0.01$, $p<0.001$ respectively) lower than those in sham-implanted fish (165 ± 22 ng ml^{-1}). There was a significant decline in plasma cortisol levels between 1h and 48h following the onset of confinement and no further significant differences were resolved at individual sample points during confinement. However, overall, taking all confinement sample points into consideration, ANOVA revealed that T-implanted fish (60 ± 7 ng ml^{-1}, n = 60) and KT-implanted fish (59 ± 7 ng ml^{-1}) had significantly lower plasma cortisol levels than sham-implanted fish (89 ± 8 ng ml^{-1}) ($p<0.01$, $p<0.001$, respectively; Fig. 4b). There were no significant effects of treatment on weight or condition factor

Experiment 4. A comparison of the cortisol response of mature male and female, and immature, rainbow trout to 1 h confinement stress.

Plasma levels of testosterone were significantly ($p<0.001$) higher in mature female (85 ± 4 ng ml^{-1}) than mature male (42 ± 10 ng ml^{-1}) rainbow trout (Fig. 5b). Estradiol levels were high only in mature females (Fig. 5c) and 11-ketotestosterone was present in substantial amounts only in mature male fish (Fig. 5d). Confinement for 1h resulted in high plasma cortisol levels in immature, mature female, and mature male rainbow trout (Fig. 5a). However, stress-induced plasma cortisol levels in mature male fish (42 ± 10 ng ml^{-1}, n = 10) were significantly lower than levels in immature ($p<0.05$; 86 ± 18 ng ml^{-1}) fish or mature female fish ($p<0.01$; 85 ± 4 ng ml^{-1}).

DISCUSSION

The results of these experiments clearly indicate that the presence of elevated levels of gonadal steroids can modify the function of the pituitary-interrenal (PI) axis in both rainbow and brown trout. As far as we are aware, this is the first report of estrogen effects on the PI axis in fish. These data therefore strongly suggest that the reduced ACTH and plasma cortisol response to acute and chronic stress observed in mature male rainbow trout (Pottinger *et al*., 1995) is a function of the elevated androgen levels present during sexual maturation. Plasma N-acetyl-β-endorphin and α-melanocyte-stimulating hormone levels were measured during the pilot experiment in view of their possible participation in the stress response (Balm *et al*., 1995) but given the apparently specific effects of the gonadal steroids on the corticotropes, the melanotropic peptides were not considered during the remainder of this study.

Administration of both KT and T affected ACTH and cortisol responses to stress in both rainbow trout and brown trout. Plasma KT levels achieved by implantation of KT-containing cocoa butter pellets (5-17 ng ml^{-1}) were not as high as peak levels observed in naturally maturing male brown trout (~75 ng ml^{-1}; Pottinger and Pickering, 1985a) or rainbow trout (~150 ng ml^{-1}; Baynes and Scott, 1985) yet pronounced effects were observed as a result of the implants. It is possible that levels were higher immediately following implantation and declined subsequently, or that a metabolite of 11KT, not detected by the assay, is responsible for the observed effects. T was administered at the same relative dose as KT but plasma levels of this steroid were considerably higher (~25-75 ng ml^{-1}) in implanted fish than was observed for KT. Differences in measurable levels of steroids administered to fish in ostensibly identical doses have been previously noted (Carragher *et al*., 1989) and such differences were assumed to reflect dissimilar modes of metabolism/clearance of the steroids. It is perhaps surprising that administration of T in Experiments 2 and 3 resulted in significant attenuation of the plasma ACTH and cortisol response during stress. No evidence for an effect of T was noted during the pilot experiment despite similar plasma levels of T being present. Although KT is the dominant androgen in sexually mature male salmonids, and T occurs in large amounts in the plasma of sexually mature female salmonids, administration of T to trout has in the past been reported to evoke male-type secondary sexual characters

(Pottinger and Pickering, 1985b) and T receptors have been identified in androgen-sensitive tissues (Pottinger, 1987). It is therefore not without precedent that T should apparently promote male-specific effects in salmonid fish. There was no evidence for conversion of T to E in implanted fish, E levels were low in T-implanted fish and *vice versa*, suggesting low aromatase activities.

The suppressive effect of androgen administration on the PI response to stress in fish observed in this study is consistent with results in studies on mammals. T-treatment of heifers results in a reduced cortisol response to stress (Boissy and Bouissou, 1994) and in male rats the ACTH response to stress is potentiated by gonadectomy, and plasma corticosterone levels are higher in gonadectomized animals than in gonadectomized males receiving dihydrotestosterone (DHT; Bingaman *et al*., 1995). Similarly, T or DHT treatment of gonadectomized male rats reduced the ACTH and corticosterone response to stress to similar levels to those observed in intact males (Handa *et al*., 1994).

In mammals, there is a distinct sexual dimorphism in the response of the hypthalamic-pituitary-adrenal axis to stress. The suppression of response observed in male rats contrasts with a hyperresponsiveness in female animals exposed to stress (Spinedi *et al*., 1994). This difference is not simply a reflection of the attenuated response of males but represents a positive enhancement of responsiveness by estradiol (E), with ovariectomy reducing responsiveness and E replacement returning stress-responsiveness to levels observed in intact females (Le_niewska *et al*., 1990). There are numerous reports of the enhancement of the stress response by E in mammals (e.g. Handa *et al*., 1994) but no studies have examined this phenomenon in fish. A marked anomaly in the present study is that although E administration had positive effects on both baseline and stress-induced activity of the PI axis in immature trout, there is no evidence that the stress response of untreated mature female rainbow trout was enhanced. Despite high levels of E in the plasma of the mature female fish, examined in Experiment 4, their cortisol response did not differ significantly from that of immature fish in the same experiment. It is possible that the simultaneous presence of high levels of T in the mature female fish opposed any E-induced enhancement of the response. If so, this appears to be a major deviation from the mammalian pattern. It is equally conceivable

that the response of the female trout to stress changes during the reproductive period as relative levels of E and T alter.

It is interesting to note that the steroid implants had significant effects on "resting" levels of cortisol and ACTH in immature rainbow trout (Experiment 2). This suggests that not only stress-induced output of the PI axis is susceptible to modulation by gonadal steroids but that baseline regulation of the system is affected. It might be argued that removal of the experimental fish from holding tanks resulted in activation of a stress response, the earliest phase of which was detected in the "unstressed" sample. This is unlikely as fish were netted and transferred to anaesthetic well within the time required to observe an increase in ACTH during time-course studies (2 mins; Sumpter *et al*., 1986). Female rats have also been reported to show higher basal, as well as higher stress-induced, corticosterone levels than male rats (Aloisi *et al*., 1994).

The effect of the gonadal steroid implants on brown trout was broadly similar to that observed in rainbow trout. After 1h of confinement stress T- and KT-implanted fish displayed attenuated responses compared with sham and E-implanted fish. Plasma ACTH levels in stressed brown trout in this study (≤600 pg ml^{-1}) were markedly higher than those of rainbow trout in this and previous studies (≤200 pg ml^{-1}). This is consistent with previous measurements of ACTH in brown trout (Pickering *et al*., 1986). Although, overall, the mean ACTH and cortisol levels for the full 96h period of confinement indicated an effect of the implants, this was difficult to resolve at individual time points. In this respect, the implantation procedure does not fully mimic the natural state. Mature male rainbow trout sustained significantly lower plasma cortisol levels than immature rainbow trout throughout a 24 h period of confinement (Pottinger *et al*., 1995). However, in a second experiment in that study there was some evidence of convergence in ACTH and cortisol levels in immature and mature fish during a confinement stress. The downward trend in plasma ACTH and cortisol with time in confined brown trout is typical of the hormone profile observed under such conditions and presumably represents a degree of acclimation/accomodation to the stress (Pottinger *et al*., 1992b). It is unclear whether the significant elevation of ACTH in E-treated fish 96h after the onset of confinement represents a delayed effect of E. Taking all time points

into account E does have a significant effect on cortisol levels but this is not resolved at any individual samples other than that at 96h. There was no accompanying elevation of plasma cortisol in the E-treated fish.

These results pose obvious questions; by what mechanism(s) do the gonadal steroids exert their influence on stress responsiveness and what is the physiological significance of these effects? Early work on sockeye salmon suggested that metabolic clearance rate of cortisol increased in mature males and was reduced by gonadectomy (Donaldson and Fagerlund, 1970), and that androgen treatment of gonadectomised males restored the original pattern (Fagerlund and Donaldson, 1969). It was also reported that the peripheral conversion of cortisol to cortisone in mature male fish was reduced by gonadectomy (Donaldson and Fagerlund, 1972). However, from the results of the present study it is clear that a primary factor affecting the cortisol response to stress in gonadal steroid-implanted fish is modification of ACTH production by the pituitary although it is possible that alterations in clearance and peripheral conversion of cortisol may well accompany this. A mechanism whereby gonadal steroids modify stress responsiveness has yet to be defined in mammals although evidence suggests that E may enhance expression of the corticotropin-releasing hormone (CRH) gene (Vamvakopoulos and Chrousos, 1993) and could inhibit corticosterone-mediated negative feedback (Burgess and Handa, 1992). The androgenic effect on stress responsiveness appears to be mediated via an androgen receptor mechanism and does not involve changes in pituitary sensitivity to CRH or changes in hypothalamic corticosteroid receptor concentrations (Handa *et al*., 1994). It is likely that similar mechanisms operate in trout and account for the results of the present study. However, the absence of a pronounced enhancement of stress responsiveness in mature female rainbow trout subjected to confinement is puzzling, as noted above, given the consistent effects of E administered via implants. It has recently been established that estrogen receptors are present in those areas of the brain of rainbow trout which are concerned with neuroendocrine control of pituitary function (Anglade *et al*., 1994) and this may be the route by which the observed effects in implanted fish were mediated. The functional significance of gonadal steroid-mediated effects on stress responsiveness is open to speculation. It has been suggested that androgenic suppression of the HPA axis has evolved in mammals to reduce the impact of

stress on reproductive processes (Handa *et al*., 1994) but this clearly could not apply to the female in which stress-responsiveness is enhanced. It has also been suggested that the enhanced stress responsiveness of the female is a means by which environmental conditions unfavourable to reproduction can inhibit reproductive processes (Viau and Meaney, 1991). Again, given the results of this study, in which E administered alone augmented the stress response in trout, but females with naturally high levels of E show no such enhancement, a different situation may pertain in trout. The effects of the gonadal steroids on behaviour must also be considered. Androgens promote dominant social behaviour both in mammals (Bouissou and Gaudioso, 1982) and fish (Munro and Pitcher, 1985) and reduce fearfulness in cattle (Boissy and Bouissou, 1994). These traits are clearly desirable during the reproductive period when intraspecific competituion among males is intense. A reduction in stress responsiveness of androgen-treated animals may therefore reflect an advantageous physiological correlate of a behavioural effect.

ACKNOWLEDGEMENTS

This work was funded by the Natural Environment Research Council (NERC), U. K. and the Netherlands Organization for Scientific Research (NWO).

REFERENCES

Aloisi, A. M., Steenbergen, H. L., van de Poll, N. E., and Farabollini, F. (1994). Sex-dependent effects of restraint on nociception and pituitary-adrenal hormones in the rat. *Physiol. Behav*. **55**, 789-793.

Anglade, I., Pakdel, F., Bailhache, T., Petit, F., Salbert, G., Jego, P., Valotaire, Y., and Kah, O. (1994). Distribution of estrogen receptor-immunoreactive cells in the brain of the rainbow trout (*Oncorhynchus mykiss*). *J. Neuroendocrinol*. **6**, 573-578.

Balm, P. H. M. and Pottinger, T. G. (1993). Acclimation of rainbow trout (*Oncorhynchus mykiss*) to low environmental pH dos not involve an activation of the

pituitary-interrenal axis, but evokes adjustments in branchial ultrastructure. *Can. J. Fish. Aquat. Sci*. **50**, 2532-2541.

Balm, P. H. M. and Pottinger, T. G. (1995). Corticotrope and melanotrope POMC-derived peptides in relation to interrenal function during stress in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol*. **98**, 279-288.

Balm, P. H. M., Pepels, P., Helfrich, S., Hovens, M. and Wedelaar Bonga, S. E. (1994). Adrenocorticotropic hormone (ACTH) in relation to interrenal function during stress in Tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol*. **96**, 447-460.

Balm, P. H. M., Hovens, M. L. M. and Wendelaar Bonga, S. E. (1995). Endorphin and MSH in concert form the corticotropic principle released by tilapia (*Oreochromis mossambicus*; Teleostei) melanotropes. *Peptides* **16**, 463-469.

Barton, B. A., Schreck, C. B. and Sigismondi, L. A. (1986). Multiple acute disturbances evoke cumulative physiological stress responses in juvenile chinook salmon. *Trans. Am. Fish. Soc*. **115**, 245-251.

Baynes, S. M. and Scott, A. P. (1985). Seasonal variations in parameters of milt production and in plasma concentrations of sex steroids of male rainbow trout (*Salmo gairdneri*). *Gen. Comp. Endocrinol*. **57**, 150-160.

Bingaman, E. W., van de Kar, L. D., Yracheta, J. M., Li, Q., and Gray, T. S. (1995). Castration attenuates prolactin response but potentiates ACTH response to conditioned stress in the rat. *Am. J. Physiol*. **269**, R856-R863.

Boissy, A. and Bouissou, M. F. (1994). Effects of androgen treatment on behavioural and physiological responses of heifers to fear-eliciting situations. *Horm. Behav*. **28**, 66-83.

Bouissou, M. F. and Gaudioso, V. (1982). Effect of early androgen treatment on subsequent social behaviour in heifers. *Horm. Behav*. **16**, 132-146.

Burgess, L. H. and Handa, R. J. (1992). Chronic estrogen-induced alterations in adrenocorticotropin and corticosterone secretion, and glucocorticoid receptor-mediated functions in female rats. *Endocrinology* **131**, 1261-1269.

Carragher, J. F., Sumpter, J. P., Pottinger, T. G., and Pickering, A. D. (1989). The deleterious effects of cortisol implantation on reproductive function in two species of trout, *Salmo trutta* L. and *Salmo gairdneri* Richardson. *Gen. Comp. Endocrinol*. **76**, 310-321.

Donaldson, E. M. and Fagerlund, U. H. M. (1970). Effect of sexual maturation and gonadectomy at sexual maturity on cortisol secretion rate in sockeye salmon (*Oncorhynchus nerka*). *J. Fish. Res. Board Can*. **27**, 2287-2296.

Donaldson, E. M. and Fagerlund, U. H. M. (1972). Corticosteroid dynamics in Pacific salmon. *Gen. Comp. Endocrinol*. **Suppl. 3**., 254-265.

Fagerlund, U. H. M. (1970). Response to mammalian ACTH of the interrenal tissue of sockeye salmon (*Oncorhynchus nerka*) at various stages of sexual maturation. *J. Fish. Res. Board Can*. **27**, 1169-1172.

Fagerlund, U. H. M. and Donaldson, E. M. (1969). The effect of androgens on the distribution and secretion of cortisol in gonadectomized male sockeye salmon (*Oncorhynchus nerka*) *Gen. Comp. Endocrinol*. **12**, 438-448.

Handa, R. J., Nunley, K. M., Lorens, S. A., Louie, J. P., McGivern, R. F., and Bollnow, M. R. (1994). Androgen regulation of adrenocorticotropin and corticosterone secretion in the male rat following novelty and footshock stressors. *Physiol. Behav*. **55**, 117-124.

Heuser, I. J., Gotthardt, U., Schweiger, U., Schmider, J., Lammers, C.-H., Dettling, M., and Holsboer, F. (1994). Age-associated changes of pituitary-adrenocortical hormone regulation in humans: importance of gender. *Neurobiol. Aging* **15**, 227-231.

Le_niewska, B., Mi_kowiak, B., Nowak, M., and Malendowicz, L. K. (1990). Sex differences in adrenocortical structure and function. XXVII. The effect of ether stress on ACTH and corticosterone in intact, gonadectomized, and testosterone- or estradiol-replaced rats. *Res. Exp. Med*. **190**, 95-103.

Munro, A. D. and Pitcher, T. J. (1985). Steroid hormones and agonistic behaviour in a cichlid teleost, *Aequidens pulcher*. *Horm. Behav*. **19**, 353-371.

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

Pickering, A. D., Pottinger, T. G., and Sumpter, J. P. (1986). Independence of the pituitary-interrenal axis and melanotroph activity in the brown trout, *Salmo trutta* L., under conditions of environmental stress. *Gen. Comp. Endocrinol*. **64**, 206-211.

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

Pottinger, T. G. (1987). Androgen binding in the skin of mature male brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol*. **66**, 224-232.

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

Pottinger, T. G. and Pickering, A. D. (1985a). Changes in skin structure associated with elevated androgen levels in maturing male brown trout, *Salmo trutta* L. *J. Fish Biol*. **26**, 745-753.

Pottinger, T. G. and Pickering, A. D. (1985b). The effects of 11-ketotestosterone and testosterone on the skin structure of brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol*. **59**, 335-342.

Pottinger, T. G. and Pickering, A. D. (1990). The effect of cortisol administration on hepatic and plasma estradiol-binding capacity in immature female rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol*. **80**, 264-273.

Pottinger, T. G., Pickering, A. D., and Hurley, M. A. (1992a). Consistency in the stress response of individuals of two strains of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **103**, 275-289.

Pottinger, T. G., Moran, T. A., and Cranwell, P. A. (1992b). The biliary accumulation of corticosteroids in rainbow trout, *Oncorhynchus mykiss*, during acute and chronic stress. *Fish Physiol. Biochem*. **10**, 55-66.

Pottinger, T. G., Balm, P. H. M., and Pickering, A. D. (1995). Sexual maturity modifies the responsiveness of the pituitary-interrenal axis to stress in male rainbow trout. *Gen. Comp. Endocrinol*. **98**, 311-320.

Rodrigues, K. T. and Sumpter, J. P. (1984). The radioimmunoassay of α-melanocyte-stimulating hormone and endorphin in trout (*Salmo gairdneri*) and the effects of blinding on the plasma levels of these peptides. *Gen. Comp. Endocrinol*. **54**, 69-76.

Scott, A. P., and Sumpter, J. P. (1983). A comparison of the female reproductive cycles of autumn-spawning and winter-spawning strains of rainbow trout (*Salmo gairdneri* Richardson). *Gen. Comp. Endocrinol*. **52**, 79-85.

Spinedi, E., Salas, M., Chisari, A., Perone, M., Carino, M., and Gaillard, R. C. (1994). Sex differences in the hypothalamo-pituitary-adrenal axis response to inflammatory and neuroendocrine stressors. *Neuroendocrinology* **60**, 609-617.

Sumpter, J. P. and Donaldson, E. M. (1986). The development and validation of a radioimmunoassay to measure plasma ACTH levels in salmonid fishes. *Gen. Comp. Endocrinol*. **62**, 367-376.

Sumpter, J. P., Pickering, A. D. and Pottinger, T. G. (1985). Stress-induced elevation of plasma α-MSH and endorphin in brown trout, *Salmo trutta* L. *Gen. Comp. Endocrinol*. **59**, 257-265.

Sumpter, J. P., Dye, H. M. , and Benfey, T. J. (1986). The effects of stress on plasma ACTH, MSH, and cortisol levels in salmonid fishes. *Gen. Comp. Endocrinol*. **62**, 377-385.

Sumpter, J. P., Carragher, J., Pottinger, T. G., and Pickering, A. D. (1987). The interaction of stress and reproduction in trout. *In* "Reproductive Physiology of Fish" (D. R. Idler, L. W. Crim and J. M. Walsh, Eds.), pp. 299-302. Memorial Univ. Press, St John's, Newfoundland, Canada.

Vamvakopoulos, N. C. and Chrousos, G. P. (1993). Evidence of direct estrogenic regulation of human corticotropin-releasing hormone gene expression. Potential implications for the sexual dimorphism of the stress response and immune/inflammatory reaction. *J. Clin. Invest*. **92**, 1896-1902.

Viau, V. and Meaney, M. J. (1991). Variations in the hypothalamic-pituitary-adrenal response to stress during the estrous cycle in the rat. *Endocrinology* **129**, 2503-2511.

TABLES

Table 1. Plasma levels of testosterone (T), estradiol-17β (E), and 11-ketotestosterone (KT) in sham- and steroid-implanted rainbow trout in Experiment 1 at various intervals following implantation. Each value is the mean ± SEM, n = 12. nd, not detected.

	Plasma levels of steroid in implanted fish (ng ml^{-1})											
Treatment	Day 10			Day 14			Day 19			Day 24		
group	T	E	KT	T	E	KT	T	E	KT	T	E	KT
Sham	3.0 ± 0.5	0.5 ± 0.2	nd	4.4 ± 0.6	0.3 ± 0.1	nd	6.0 ± 0.9	1.5 ± 0.7	nd	9.8 ± 1.2	1.9 ± 0.8	nd
T	66.4 ± 5.6	0.1 ± 0.01	nd	97.1 ± 10	0.1 ± 0.01	nd	64.6 ± 3.4	0.4 ± 0.06	nd	65.9 ± 3.3	0.5 ± 0.05	nd
E	3.4 ± 2.0	19.7 ± 2.4	nd	3.7 ± 1.5	11.0 ± 1.6	nd	8.1 ± 2.9	18.8 ± 3.0	nd	5.1 ± 0.9	12.0 ± 1.5	nd
KT	4.3 ± 0.8	0.1 ± 0.01	5.8 ± 1.1	4.3 ± 0.3	0.1 ± 0.01	4.3 ± 0.7	5.9 ± 0.4	0.1 ± 0.02	3.6 ± 0.5	8.2 ± 0.9	0.3 ± 0.04	4.3 ± 0.4

Table 2. Plasma levels of testosterone (T), estradiol-17β (E), and 11-ketotestosterone (KT) in sham- and steroid-implanted rainbow trout in Experiment 2 at seven and fourteen days following implantation. Samples were analysed from fish both prior to confinement (unstressed) and following 1h confinement (stressed). Each value is the mean ± SEM, n = 10. nd, not detected.

	Plasma levels of steroid in implanted fish (ng ml^{-1})											
	7 days						14 days					
Treatment	unstressed			stressed			unstressed			stressed		
group	T	E	KT	T	E	KT	T	E	KT	T	E	KT
Sham	0.5 ± 0.16	4.63 ± 0.7	nd	0.4 ± 0.07	4.26 ± 1.0	nd	1.48 ± 0.3	2.64 ± 0.8	nd	1.2 ± 0.4	2.1 ± 0.5	nd
T	79.9 ± 5.8	5.7 ± 0.7	1.3 ± 0.1	60.6 ± 8.7	7.0 ± 3.0	nd	60.1 ± 4.2	2.2 ± 0.7	nd	57.8 ± 7.1	2.1 ± 0.9	nd
E	0.2 ± 0.01	59.4 ± 7.2	nd	0.1 ± 0.02	70.1 ± 4.9	nd	0.33 ± 0.1	50.7 ± 7.2	nd	0.2 ± 0.04	49.3 ± 7.3	nd
KT	3.2 ± 0.8	6.91 ± 0.9	30.4 ± 4.8	2.5 ± 0.3	6.5 ± 0.7	7.6 ± 1.4	4.8 ± 1.3	1.9 ± 0.5	13.8 ± 2.5	4.5 ± 1.0	2.6 ± 0.9	17.5 ± 1.8

Table 3. Plasma levels of testosterone (T), estradiol-17β (E), and 11-ketotestosterone (KT) in sham- and steroid-implanted brown trout in Experiment 3, at 1h and 48h after the start of confinement. Each value is the mean ± SEM, n = 10. nd, not detected.

Treatment	Plasma levels of steroid in implanted fish (ng ml^{-1})					
	1h			48h		
group	T	E	KT	T	E	KT
Sham	1.42 ± 0.2	9.9 ± 2.1	nd	0.69 ± 0.1	10.43 ± 4.3	nd
T	26.4 ± 2.2	13.3 ± 4.1	nd	29.1 ± 2.1	4.7 ± 0.7	nd
E	1.35 ± 0.4	83.2 ± 13.9	nd	0.5 ± 0.1	86.3 ± 8.8	nd
KT	3.03 ± 0.9	6.0 ± 0.9	11.5 ± 2.1	1.99 ± 0.2	5.3 ± 1.3	11.62 ± 1.3

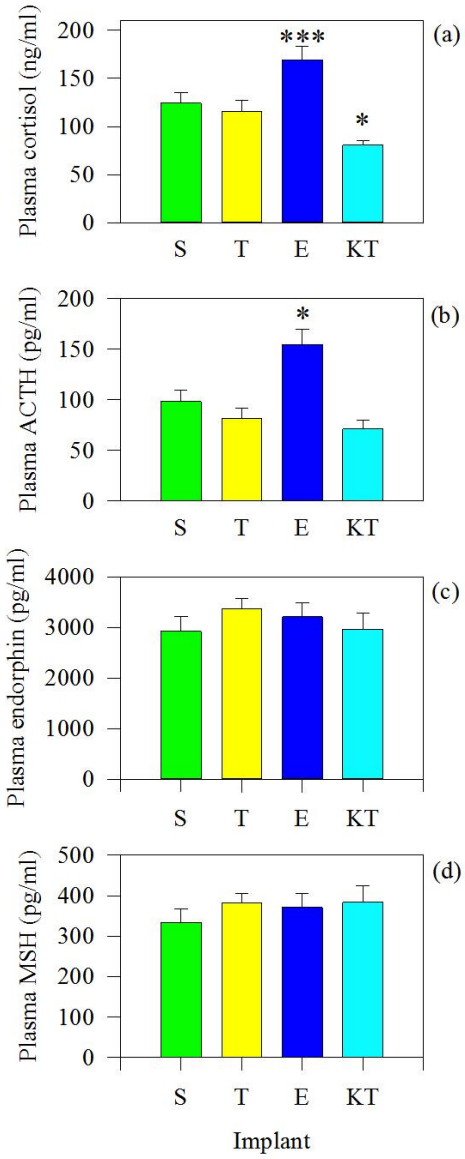

Figure 1. Experiment 1. Levels of (a) plasma cortisol, (b) plasma ACTH, (c) plasma N-acetyl-β-endorphin (END), and (d) plasma α-MSH (MSH) in rainbow trout implanted with either cocoa-butter alone (S) or containing testosterone (T), estradiol-17β (E), or 11-ketotestosterone (KT). The fish were sampled after a 1h period of confinement. Each value is the mean ± SEM, n = 12, except for cortisol where n = 48. Significant differences between treatments and the control (S) are denoted by asterisks. * $p<0.05$; *** $p<0.001$.

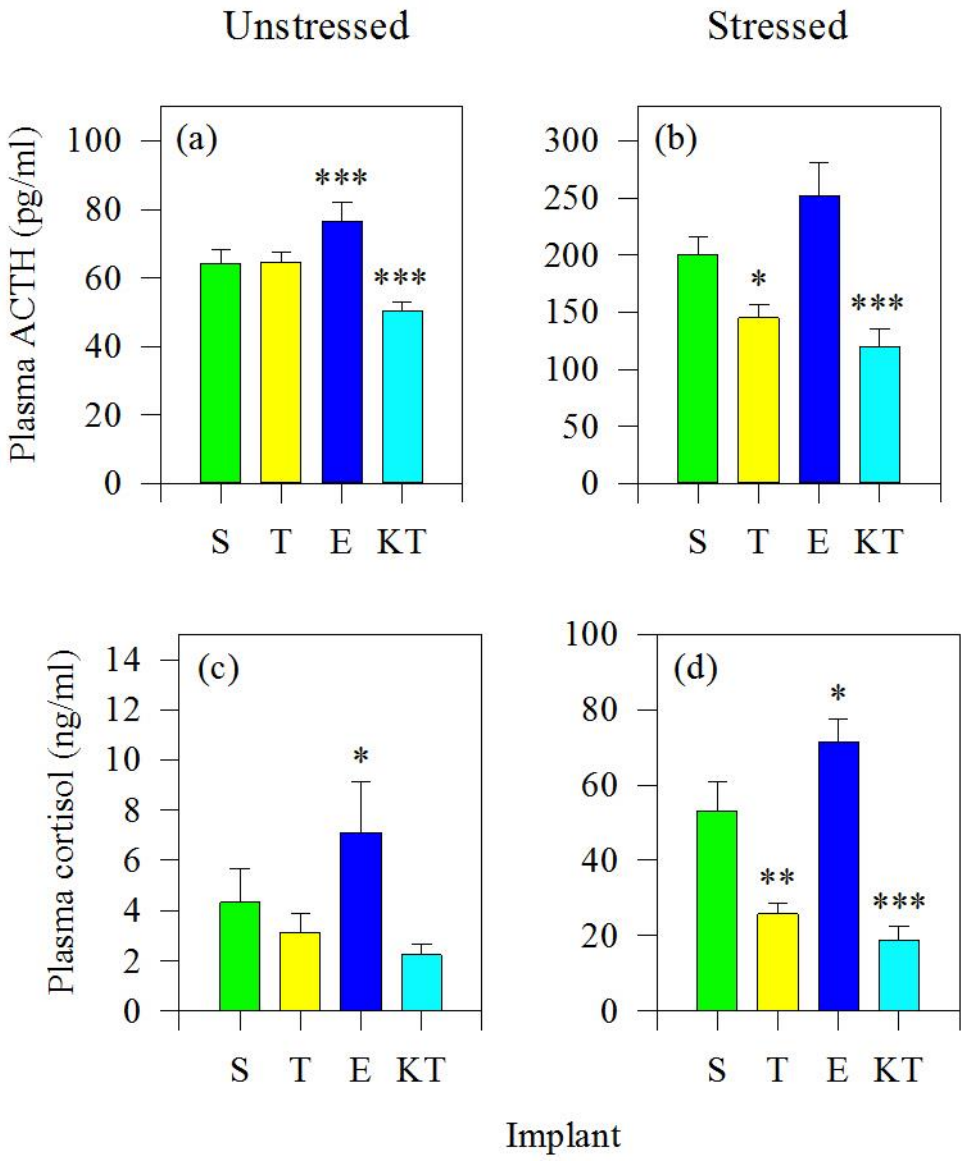

Figure 2. Experiment 2. Levels of (a, b) ACTH and (c, d) cortisol in the plasma of (a, c) unstressed or (b, d) stressed rainbow trout implanted with either cocoa-butter alone (S) or containing testosterone (T), estradiol-17β (E), or 11-ketotestosterone (KT). The fish denoted as stressed were sampled after a 1h period of confinement. The data for fish stressed 7 and 14 days after implantation have been combined. Each value is the mean ± SEM, n = 20. Significant differences between treatments and the control (S) are denoted by asterisks. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

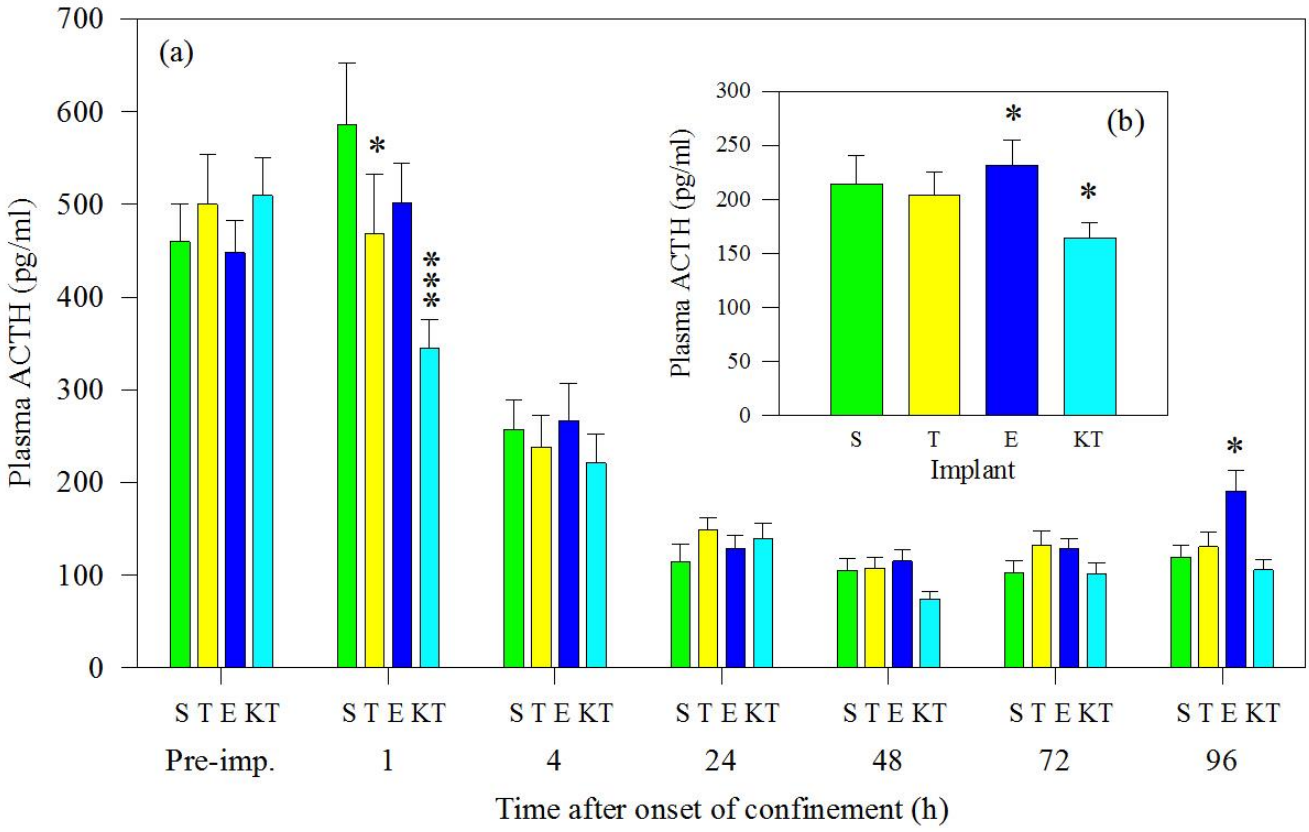

Figure 3. Experiment 3. (a) Plasma ACTH levels in brown trout implanted with either cocoa-butter alone (S) or containing testosterone (T), estradiol-17β (E), or 11-ketotestosterone (KT). The fish were sampled prior to implantation following a 1h period of confinement (Pre.-imp.) and after implantation, at intervals during a 96h period of confinement. Each value is the mean ± SEM, n = 10. (b) Mean plasma ACTH levels for all time points during the confinement period. Each value is the mean ± SEM, n = 60. Significant differences between treatments and the control (S) are denoted by asterisks. * $p<0.05$; *** $p<0.001$.

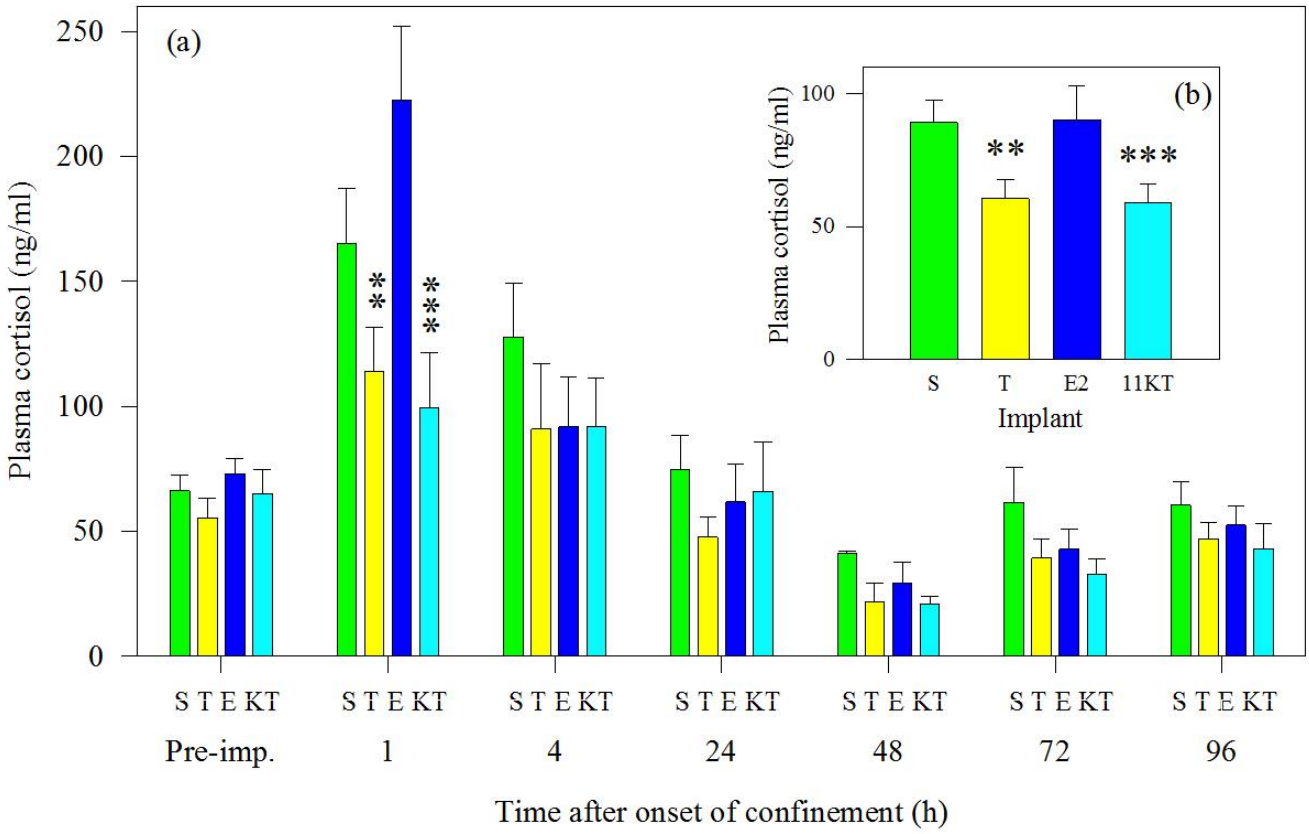

Figure 4. Experiment 3. (a) Plasma cortisol levels in brown trout implanted with either cocoa-butter alone (S) or containing testosterone (T), estradiol-17β (E), or 11-ketotestosterone (KT). The fish were sampled prior to implantation following a 1h period of confinement (Pre.-imp.) and after implantation, at intervals during a 96h period of confinement. Each value is the mean ± SEM, n = 10. (b) Mean plasma cortisol levels for all time points during the confinement period. Each value is the mean ± SEM, n = 60. Significant differences between treatments and the control (S) are denoted by asterisks. ** $p<0.01$; *** $p<0.001$.

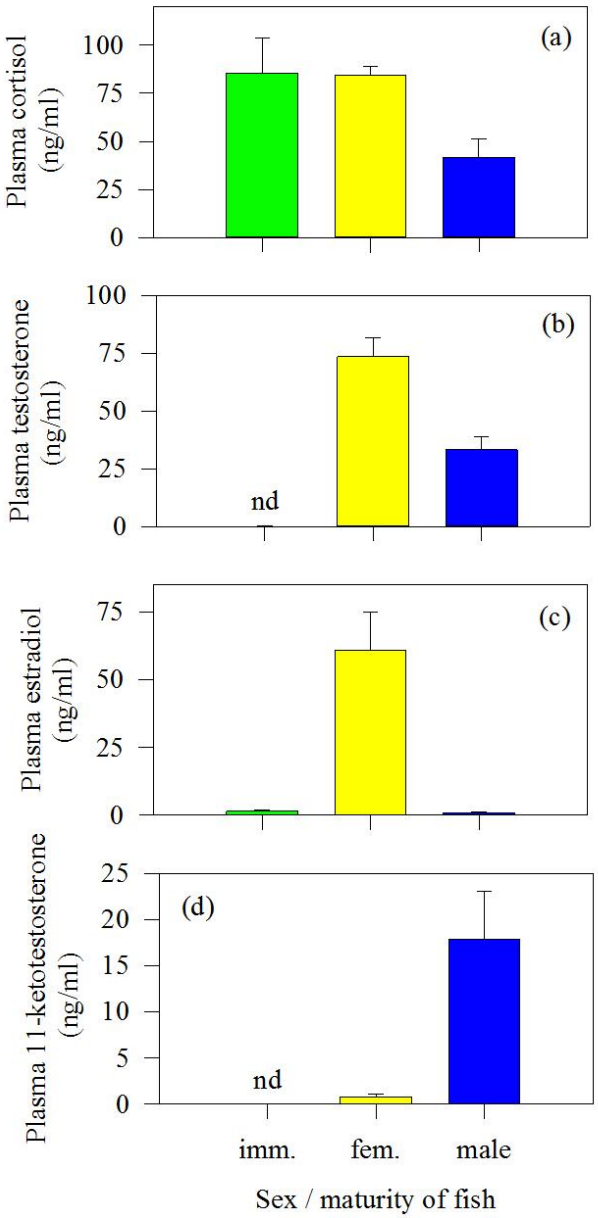

Figure 5. Experiment 4. Plasma levels of (a) cortisol, (b) testosterone, (c) estradiol-17β, and (d) 11-ketotestosterone in immature (imm.), mature female (fem.) and mature male (male) rainbow trout following a 1h period of confinement. nd, not detectable. Each value is the mean ± SEM, n = 10. Mature male plasma cortisol levels (a) are significantly lower than immature fish ($p<0.05$) and mature female fish ($p<0.01$). Mature male testosterone levels (b) are significantly lower than mature female levels ($p<0.001$).