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Sexual maturity modifies the responsiveness of the pituitary-interrenal axis to stress in male rainbow trout

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ABSTRACT

A significant reduction in stress-induced plasma cortisol levels is apparent in mature male rainbow trout compared to immature fish of both sexes and of the same age and strain. Mean plasma cortisol levels in groups of immature fish subjected to a standard 1h confinement stress were consistently higher (range 93.9 ± 4.9 - 114.8 ± 4.1 ng ml^{-1}) than mean levels in mature males exposed to the same procedure (range 47.0 ± 4.3 - 71.7 ± 5.7 ng ml^{-1}), throughout the 4 month period around spawning ($p<0.001$). Body weight was not found to be a significant determinant of post-stress cortisol level. The dissimilarity in plasma cortisol levels between mature and immature fish following confinement does not stem from differences in the dynamics of the response; during a 24h period of confinement the rate of elevation of plasma cortisol levels was similar for both mature male and immature fish but mature male fish attained a significantly lower peak cortisol level (51.6 ± 5.2 ng ml^{-1}) than immature fish (89.5 ± 7.7 ng ml^{-1}), a disparity which was maintained throughout the period of stress ($p<0.05$ - $p<0.001$). The reduced responsiveness of the interrenal tissue of mature male fish during stress appears to be modulated by the hypothalamus/pituitary. Plasma ACTH levels in mature male trout (44 ± 9 pg ml^{-1}) are significantly lower than those of immature fish (71 ± 9 pg ml^{-1}, $p<0.01$) within 30 min of the onset of confinement and remain so during a 3h period of confinement. These data suggest that the cortisol/ACTH feedback equilibrium has been modified in mature fish, to a lower "set point". Furthermore, although stress caused a significant decline of plasma α-MSH levels in both immature fish and mature males, N-acetyl-β-endorphin levels were reduced only in mature male fish during confinement stress.

INTRODUCTION

The physiological response of teleost fish to environmental stress is becoming increasingly well characterised (Pickering and Pottinger, 1994; Sumpter *et al*., 1994) and the mechanisms underlying the impact of environmental stress on growth, reproduction and survival are being defined. It has been established that within a species the magnitude of the corticosteroidogenic response to a given stressor can display wide variation, associated with temperature regime (Sumpter *et al*., 1985), strain of fish (Pickering and Pottinger, 1989; Pottinger and Moran, 1993; Pottinger *et al*., 1994) and as a consequence of differences in responsiveness between individuals (Pottinger *et al*., 1992; Balm *et al*., 1994). Furthermore, it appears that the salmonid stress-response, for which most information is available, may not be an appropriate model for all fish species. Both tilapia (*Oreochromis mossambicus*; Balm *et al*., 1994) and the sea raven (*Hemitripterus americanus*; Vijayan and Moon, 1994) display patterns of response to stress which differ from those of salmonid fish. The bases for, and physiological significance of, these qualitative and quantitative differences in stress responsiveness within and between species are unclear but the factors modulating the stress response in fish may be more complex than once assumed (Donaldson, 1981).

Salmonid fish exhibit a pronounced circannual reproductive cycle, and gonadal growth and recrudescence are associated with profound changes in the endocrine environment of the fish (Hoar *et al*, 1983). It is well established that stress disrupts the normal functioning of the endocrine reproductive system (Pickering *et al*., 1987) with significant effects on reproductive success (Campbell *et al*., 1992). Furthermore, we have previously reported that the onset of sexual maturation in both rainbow (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) is associated with a marked reduction in apparent stress responsiveness in male fish (Sumpter *et al*., 1987). Earlier data on the effects of sexual maturation on corticosteroid dynamics in salmonids (Donaldson and Fagerlund, 1970, 1972; Fagerlund, 1970; Fagerlund and Donaldson, 1969) also suggest an attenuation of the corticosteroid stress response in mature male fish. However, these studies focused on

Pacific salmon, which are characterised by almost complete post-spawning mortality. The aim of this study was to investigate further these observations and establish whether sexual maturity is an additional factor that modifies the response of the brain-pituitary-interrenal axis to stress in rainbow trout. To this end, plasma cortisol levels were determined in mature male and immature rainbow trout under conditions of stress, and plasma levels of ACTH, α-MSH and N-acetyl-β-endorphin (as components of the pituitary stress response) were also measured to establish whether such modulation is exerted at the brain/pituitary or interrenal level.

MATERIALS AND METHODS

Experimental fish

Rainbow trout (*Oncorhynchus mykiss* Walbaum) 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 (Mainstream, BP Nutrition Ltd.,Northwich) at the manufacturers recommended rate. Fish were stressed by netting from their holding tank into a lidded, rectangular polypropylene tank (50-liters) supplied with a constant flow of lake water (15 liters min^{-1}). After 1 hr (Experiment 1) or at intervals during a longer period of confinement (Experiments 2 and 3), fish were removed from the troughs, anaesthetized (2-phenoxyethanol, 1:2000), and blood samples were removed from the cuverian sinus into 1.0 ml syringes, lightly dusted with heparin. Blood was placed on ice, centrifuged, and plasma stored at -20^{o}C until assay. For those samples in which ACTH was to be determined, heparin was replaced by EDTA (1.5 mg ml^{-1} blood) and aprotinin (3000 KIU ml^{-1} blood).

Assay procedures

Plasma cortisol levels were determined using a previously validated radioimmunoassay (Pickering *et al*., 1987). Plasma ACTH levels were determined as described in Balm and Pottinger (1993) and 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 the one used by Rodrigues and Sumpter (1984; R6FB; also from B. I. Baker) in the present system yielded similar concentrations of α-MSH immunoreactivity in rainbow trout plasma pools. The END antibody was a gift from Prof. H. Kawauchi (Kitasato University, Japan) and has also been used previously (Sumpter *et al*., 1985).

Experiment 1. *A comparison of the plasma cortisol response to a 1h period of confinement in mature male and immature rainbow trout of the same age and strain.*

During late summer, 48 three-year old male rainbow trout showing clear signs of the onset of sexual maturity (darkening and thickening of the skin, kype formation) were separated from non-maturing fish of the same strain and age and transferred to two holding tanks, 24 fish per tank. Forty eight of the non-maturing fish were similarly distributed in a further two tanks. Mean weight ± SE of the population was 505 ± 11 g (n = 96, range = 267 - 760 g), stocking density = 8 g l^{-1}. At monthly intervals for the following four months the fish in each of the four experimental tanks were subjected to a 1 hr period of confinement (12 fish per 50 liter tank), blood-sampled under anaesthesia, weighed and measured, and returned to the holding tanks. Cortisol levels were determined for each sample.

Experiment 2. A comparison of the time-course of the plasma cortisol response to a 24h period of confinement in mature male and immature rainbow trout of the same age and strain.

During November, twelve mature male, and twelve immature two-year old rainbow trout were netted from a population previously segregated into mature males and immature fish, anaesthetized, blood-sampled and killed by a blow to the head. Immediately after this operation, 96 fish from each group were transferred to confinement tanks, 12 fish per tank. At intervals of 0.5, 1, 2, 3, 5, 7.5, 12 and 24h after the initial disturbance, 12 mature male and 12 immature fish were sampled. Cortisol levels were determined for each sample.

Experiment 3. Determination of plasma cortisol, ACTH, α-MSH and N-acetyl-β-endorphin in mature male and immature rainbow trout during a 3h period of confinement.

During December, eight mature male and eight immature three-year old rainbow trout

were netted from a previously segregated population of fish (mean weight ± SE; 600 ± 14 g, n = 79; range = 273 - 951 g), anaesthetized, blood-sampled and killed by a blow to the head. Thirty two fish from each group were transferred to confinement tanks, 8 fish per tank (stocking density = 96 g l^{-1}). Samples of eight fish from each group were taken 0.5, 1, 2 and 3h after the initial disturbance. Fish were weighed and measured, and blood samples were assayed for cortisol, ACTH, α-MSH and β-endorphin.

Statistical analysis

Hormone and somatic data from each experiment were subjected to analysis of variance (ANOVA, Genstat) with time and state of maturity as factors. In addition, for Experiments 1 and 3, cortisol data were subjected to analysis of covariance (ANCOVA, Genstat) with weight as covariate.

RESULTS

Experiment 1. *A comparison of the plasma cortisol response to a 1h period of confinement in mature male and immature rainbow trout of the same age and strain.*

Pre-stress plasma cortisol levels were low (<8.0 ng ml^{-1}) throughout the experiment. A significant effect of maturity on post-stress plasma cortisol level was revealed by the ANOVA. This was resolved as significantly lower mean plasma cortisol levels in the mature fish following 1h of confinement than in immature fish at all four monthly sample points ($p<0.001$, Fig. 1a). In November, the mean post-stress value in immature fish was 60 % greater than that in mature males (114.8 ± 4.1 ng ml^{-1} cf. 71.7 ± 5.7 ng ml^{-1}, n = 48, mean ± SE). The corresponding differences for the following three months were 128%, 56% and 105%. During November, mature male fish were significantly heavier than immature fish (557 ± 13 cf. 452 ± 15, n=48, $p<0.001$, Fig. 1b). This disparity in size was maintained through December but in the January and February samples no significant weight differences were apparent between mature and immature fish. Analysis of covariance of cortisol and body weight revealed that body weight was not a significant determinant of cortisol level following stress. A similar effect was noted for fork length. Mature male fish were

significantly longer ($p<0.01$) than immature fish in November (33.3 ± 0.3 cf. 32.1 ± 0.3) and December but were of a similar length by January and February (35.0 ± 0.3 cf. 35.6 ± 0.4) and ANCOVA revealed no significant effect of length on cortisol levels. Comparison of post-stress cortisol levels in a sub-set of immature fish revealed no significant difference between levels in immature males (135.2 ± 12.6 ng ml^{-1}, n = 23) and immature females (144.3 ± 15.9 ng ml^{-1}, n = 23).

Experiment 2. A comparison of the time-course of the plasma cortisol response to a 24h period of confinement in mature male and immature rainbow trout of the same age and strain.

Overall, during the 24h confinement period plasma cortisol levels in mature fish were significantly lower than in immature fish (ANOVA, $p<0.001$). Plasma cortisol levels in both mature and immature fish rose rapidly from resting levels (< 9.0 ng m^{-1}), following transfer to confinement tanks, to 89.5 ± 7.7 ng ml^{-1} (n = 12) in immature fish and 51.6 ± 5.2 ng ml^{-1} in mature fish. With some variation, plasma cortisol remained at or about these levels in each group for the remainder of the experimental period. Cortisol levels were significantly lower in mature male fish ($p<0.05$ - $P<0.001$) than in immature fish at all time points during the confinement period with the exception of 5h and 8h.

*Experiment 3. Determination of plasma cortisol, ACTH, α-MSH and N-acetyl-β-endorphin in mature male and immature rainbow trout during a 3h period of confinement.*A similar pattern of change in plasma cortisol levels to that noted in Experiments 1 and 2 was observed during the three hour confinement. Plasma cortisol levels rose rapidly from a low baseline (< 2.5 ng ml^{-1}, Fig. 3a) in both mature and immature fish but attained a significantly higher peak at 1h after the onset of confinement in immature (108.7 ± 11 ng ml^{-1}, n=8) than in mature fish (49.3 ± 9.9 ng ml^{-1}, $p<0.01$). A significant difference between male and immature fish was sustained at 2h ($p<0.05$) but was not apparent at 3h. Mature male fish were significantly heavier than immature fish in the group employed for this experiment (mature: 649 ± 15 g; immature: 550 ± 21 g; $p<0.001$, n = 40). However, ANCOVA indicated that weight and cortisol were not significantly related at an individual level. A similar

difference in response to 3h confinement stress was observed for plasma ACTH - hormone levels were significantly higher overall in immature than mature fish ($p<0.001$).At time zero, plasma ACTH levels in immature fish were significantly ($p<0.05$) higher than those in mature males (18.6 ± 3.4 cf. 11.3 ± 1.5 pg ml^{-1}). Confinement significantly ($p<0.001$, Fig. 3b) elevated plasma ACTH levels in both groups of fish, and within 30 min of the onset of confinement ACTH levels were higher in immature fish (71± 9 pg ml^{-1}) than in mature male fish (44 ± 9 pg ml^{-1}; $p<0.01$). Plasma ACTH levels in immature fish remained higher than those in mature fish for the remainder of the confinement period, significantly so at 2h ($p<0.05$). Plasma MSH levels declined rapidly and significantly following the onset of confinement in both mature and immature fish (418 ± 35 to 276 ± 33, 485 ± 81 to 211 ± 17 pg ml^{-1} respectively, $p<0.001$, Fig. 3c). There was no significant difference in the response of either group and MSH levels remained significantly lower in both groups than those at 0h after 3h confinement (260 ± 23, 276 ± 31 pg ml^{-1} , $p<0.001$). The dynamics of the response of plasma N-acetyl-β-END levels to stress were quite different to those of cortisol and ACTH (Fig. 3d). Although overall, maturity was a highly significant factor in accounting for variation in N-acetyl-β-END levels ($p<0.001$) confinement stress had no significant effect on END levels in immature fish throughout the experiment (2439 ± 282 pg ml^{-1} at time 0, 2272 ± 292 pg ml^{-1} at 3h); in contrast there was a significant decline of END levels in mature fish, from 2241 ± 234 pg ml^{-1} at time 0 to 1535 ± 120 pg ml^{-1} after 2h confinement ($p<0.01$). N-acetyl-β-END levels in mature fish were significantly lower than those in immature fish at 1h ($p<0.05$) and 2h ($p<0.01$) following the onset of confinement. Levels of N-acetyl-β-END and α-MSH in individual fish were significantly correlated ($p<0.001$; $r = 0.62$ immature; $r = 0.67$ mature) but the maturity-related nature of the response of the two peptides to stress was highlighted by significant changes in the END/MSH ratio which at 0h was not significantly different for mature and immature fish (immature 5.5 ± 0.6; mature 5.4 ± 0.4) but which was so at 0.5h (immature 10.6 ± 0.8; mature 6.7 ± 0.5; $p<0.01$).

DISCUSSION

The present data demonstrate that the extent to which plasma cortisol levels increase after brief periods of confinement stress is reduced in mature male rainbow trout compared to immature rainbow trout of the same age and strain. Although the disparity in responsiveness observed following a single sample after 1 hour of confinement stress (Experiment 1) might arguably arise from an alteration in the dynamics of the cortisol response, the repeated time-point sampling of Experiments 2 and 3 demonstrate that there is a real difference in the maximum stress-induced plasma cortisol level attained between immature and mature fish. Size of the fish is not a determinant of this phenomenon; although immature fish may on average be smaller than mature fish (see months 1 and 2 of Experiment 1, and Experiment 3) analysis of covariance indicates that body weight does not contribute substantially to the variation observed in plasma cortisol levels. Furthermore, during the course of Experiment 1 the difference in weight between immature and mature fish present during November and December was absent during January and February whereas the substantial reduction in post-stress cortisol levels of the mature male fish was maintained. These data broadly support early observations on maturity-related differences in cortisol dynamics in Pacific salmon. An increase in metabolic clearance rate for cortisol has been reported to occur during sexual maturity in sockeye salmon (*O. nerka*; Donaldson and Fagerlund, 1972) and progressive decline in the total plasma 17-hydroxycorticosteroid response to stress was observed in male chinook salmon (*O. tshawytscha*) during final sexual maturation and spawning migration (Hane *et al*., 1966). However, the almost complete mortality associated with spawning and massively elevated blood corticosteroid levels in mature Pacific salmon, contrast with the higher post-spawning survival rate and relatively low blood cortisol levels of the multiple spawning rainbow trout employed in the present study. Although of the same genus, direct comparisons may not be valid.

Several questions arise; what is/are the underlying mechanism(s) accounting for the differences in responsiveness between mature and immature fish and what is the physiological significance of the attenuation of the cortisol response to stress in mature

male fish? While increased metabolic clearance of cortisol from the blood may contribute to a reduction of stress-induced levels in mature male fish, the data obtained in Experiment 3 suggest that a change in pituitary responsiveness to stress is largely responsible for the difference. In rainbow trout, experimental evidence indicates that interrenal cortisol production during confinement stress is predominantly controlled by ACTH (Sumpter *et al*., 1986; Balm and Pottinger, submitted) and the results of Experiment 3 demonstrate that plasma ACTH levels in mature male trout during stress are significantly lower than those in immature fish. This interpretation of the results is supported by earlier work which indicated that sexual maturation did not cause a significant change in interrenal responsiveness to exogenous ACTH in sockeye salmon (Fagerlund, 1970) although it contrasts with the reduced responsiveness to ACTH observed in mature chinook salmon (Hane *et al*., 1966). The release of ACTH from the pituitary in fish, as in mammals, is stimulated by corticotropin-releasing factor (CRF/CRH; Weld *et al*., 1987; Okawara *et al*., 1992). Therefore, reduced ACTH release during stress in mature fish implies either a reduction in sensitivity of the corticotropes to CRH, or reduced CRH release relative to immature fish. Negative feedback of circulating cortisol, via interaction at either the pituitary or hypothalamic level, also affects ACTH release (Fryer and Peter, 1977; Balm and Pottinger, 1995). The lower stress-induced cortisol levels in mature males, combined with lower ACTH levels than observed in the immature fish, suggests that the feedback equilibrium has been modified in mature fish, to a lower "set point". This might also account for the lower "baseline" plasma ACTH levels prior to the onset of confinement in mature than immature fish.

Although there have been few reports of maturity-related modulation of the stress response in fish during the last twenty years, the existence of gender-related differences in responsiveness to stress in mammals is well established (Vamvakopoulos and Chrousos, 1993). In mammals a reduced responsiveness of the pituitary-adrenal axis to stress is clearly linked to elevated androgen levels. For example, testosterone treatment reduces post-stress levels of ACTH and corticosterone in rats (Handa *et al*., 1994) and the increase in cortisol levels in androgen-treated heifers following exposure to a variety of stressors was

always lower than that in untreated controls (Boissy and Bouissou, 1994). Gonadal influences extend at least to the hypothalamus, where immunoreactive CRF content is reduced by gonadectomy (Haas and George, 1988).It is likely that a similar mechanism operates in fish - an early study reported that the administration of androgens to gonadectomized sockeye salmon reduced stress-induced cortisol levels in treated fish compared with controls (Fagerlund and Donaldson, 1969) and preliminary data from this laboratory indicate that administration of 11-ketotestosterone to immature rainbow trout significantly reduces the corticosteroid response to stress (T. G. Pottinger and S. E. Hughes, unpublished). The influence of androgens on adrenal/interrenal activity in unstressed animals has also been explored. Kime *et al*. (1980) summarize earlier work which provides evidence for direct effects of gonadal steroids on adrenal enzymes and indirect effects on adrenal function via the hypothalamus/pituitary. Although the interrelationships are complex, an overall picture emerges of androgens exerting a generally suppressive influence on pituitary-adrenal activity.

A surprising correlate of the maturity-related differences in ACTH and cortisol responses to stress is that N-acetyl-β-END levels also display maturity-related alterations during stress, declining significantly in mature males while remaining stable and unchanged in immature fish exposed to the same stress. A previous study examining the effects of stress on N-acetyl-β-END levels in salmonids gave different results, however. In immature brown trout confinement stress alone had no effect on circulating END levels (determined using the same antibody as that employed in the present study), but levels were significantly elevated by exposure to severe thermal shock (Sumpter *et al*., 1985).The reasons for these surprising inconsistencies are unclear and there is insufficient information available within the literature to resolve the question. The apparently different responses may reflect species and/or procedural differences; it has been reported that different forms of stress may exert differing effects on β-endorphin immunoreactivity in the neurointermediate lobe of mammals (Forman and Estilow, 1988).More recently, a significant decline in plasma N-acetyl-β-END levels has been noted in immature rainbow trout after 48h and 96h confinement but not within 3h of the onset of confinement (Balm and Pottinger, 1995). In

the present study, no change in N-acetyl-β-END levels was observed in immature fish subjected to 3h confinement whereas levels declined significantly during confinement in mature fish. In rats, androgens modulate levels of neurointermediate lobe β-endorphin immunoreactivity during stress (Forman and Estilow, 1988) and this mechanism may account for the more rapid than expected decline in plasma levels of melanotrope N-acetyl-β-END levels observed in mature fish during the course of the confinement stress.

In contrast to the other three hormones examined, there appears to be no maturity effect on the response of α-MSH to stress, a significant decline in plasma levels occurring in both immature and mature male fish. This stress-induced decline in α-MSH levels is consistent with recent studies on immature rainbow trout (Balm and Pottinger, 1995) and tilapia (Balm *et al*., 1995) but at odds with previous work on brown (Sumpter *et al*., 1985) and rainbow trout (Sumpter *et al*., 1986) in which no α-MSH response to a moderate stress was observed; rather, a significant increase in plasma α-MSH levels was observed after exposure to severe stress. Again, as discussed with regard to the N-acetyl-β-END response, there is a minimal amount of information available on these peptides in fish; until further data are available we can only speculate that species and procedural differences may account for the inconsistencies. A significant correlation between MSH and END is observed throughout, which excludes the assays as a possible source of variation. Instead, these data highlight the possibility that the melanotrope response to stress may be less fundamental than that of the corticotropes, but that under certain circumstances it is of adaptive value to increase melanotrope activity (Sumpter *et al*., 1985; 1986), to sustain melanotrope activity at a constant level (Sumpter *et al*., 1985; 1986) or to reduce the output of the melanotropes (this study; Balm and Pottinger, 1995). The functional significance of these responses is unclear but melanotropes may exert a potent corticotropic signal (Tran *et al*., 1989; Balm *et al*., 1995) and melanotrope POMC-derived products other than MSH and END may play a role in the regulation of interrenal cortisol production (Takahashi *et al*., 1985). In mammals, also, certain forms of stress increase melanotrope activity (Akil *et al*., 1985), α-MSH stimulates corticosteroid production (Vinson *et al*., 1983), and recent data indicate corticosteroid feedback on the melanotropes in rabbits (Schimchowitsch *et al*., 1994) as in

fish (Balm *et al*., 1993). The maturity-related divergence in response to stress of two peptides which are both derived from the melanotropes is surprising - during stress, α-MSH levels decline significantly in mature and immature fish while N-acetyl-β-END levels change only in mature males. A similar phenomenon, unrelated to sex or maturity, has been reported for tilapia (Balm *et al*., 1995) and may arise from the differential processing of POMC during stress.

With regard to the physiological significance of the reduction in responsiveness of mature male fish to stress, it is possible that an attenuated stress response protects the fish to some degree from the debilitating effects of corticosteroid elevation on metabolic and reproductive processes. However it may be the behavioural/nociceptive correlates of the stress responsiveness of fish which are the important factors. Further work is needed to resolve this question.

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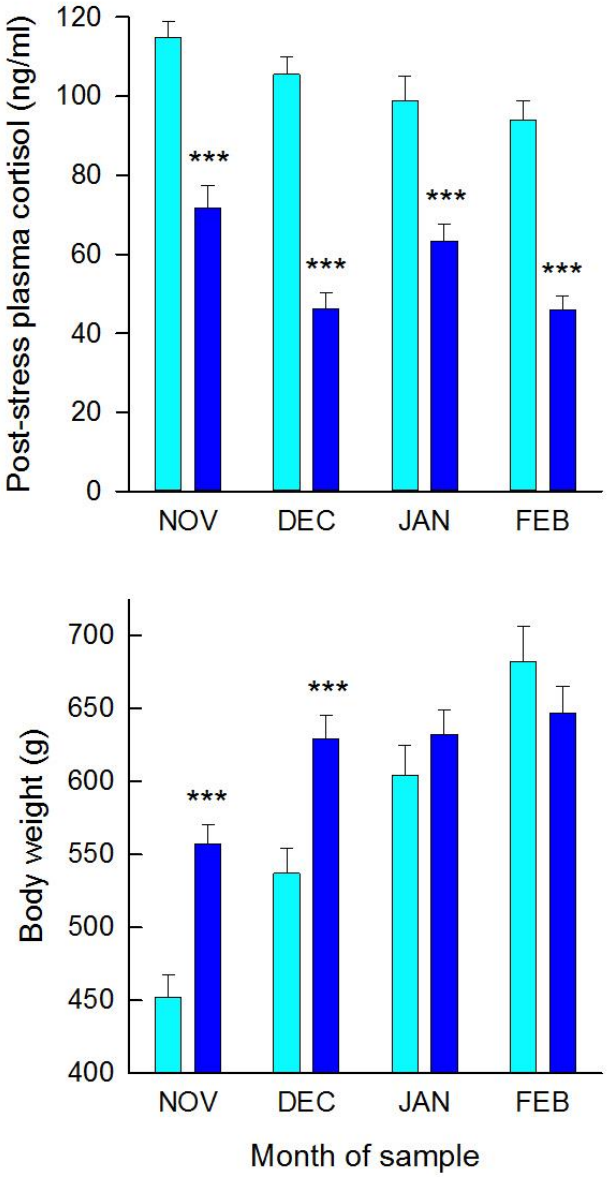

Figure 1. Plasma cortisol levels and body weight in immature (shaded bar) and mature male (solid bar) rainbow trout following a 1h period of confinement stress during the late stages of the reproductive cycle. Each point represents the mean ± SE (n = 48). Significant differences between mature and immature fish within each monthly sample are denoted by asterisks; *** $p<0.001$.

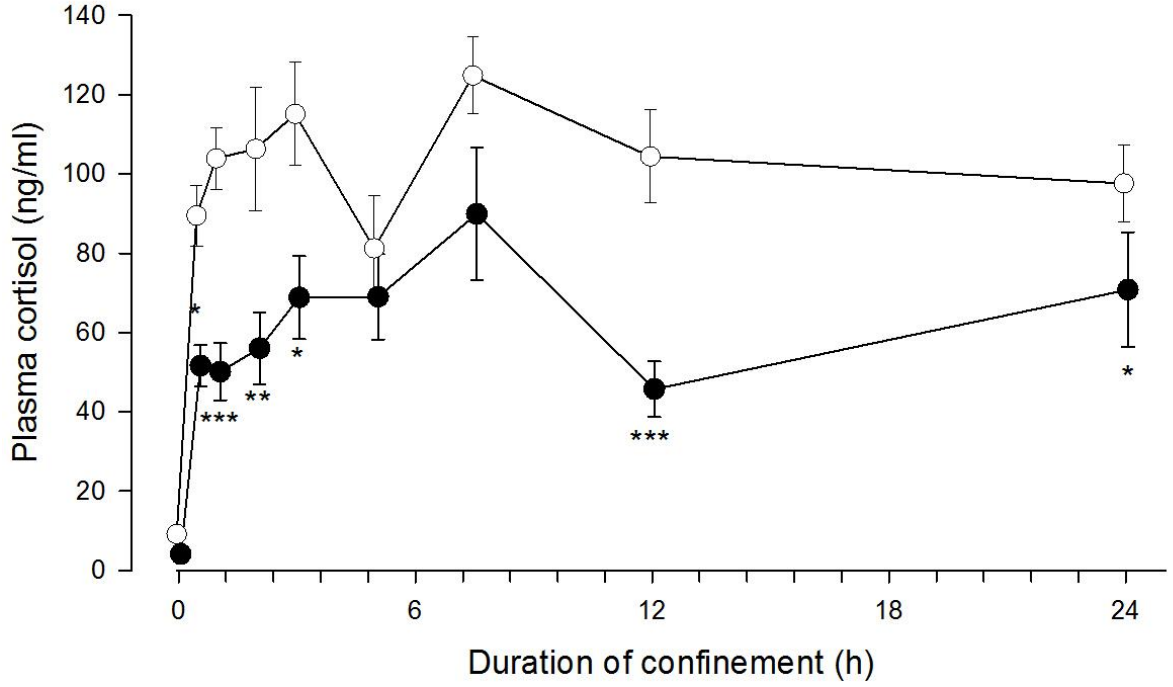

Figure 2. Changes in plasma cortisol level with time in a group of immature (°) and mature male (•) rainbow trout immediately prior to and during a 24h period of confinement. Each point represents the mean ± SE (n = 12). Significant differences between mature male and immature fish at each time point are indicated by asterisks; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

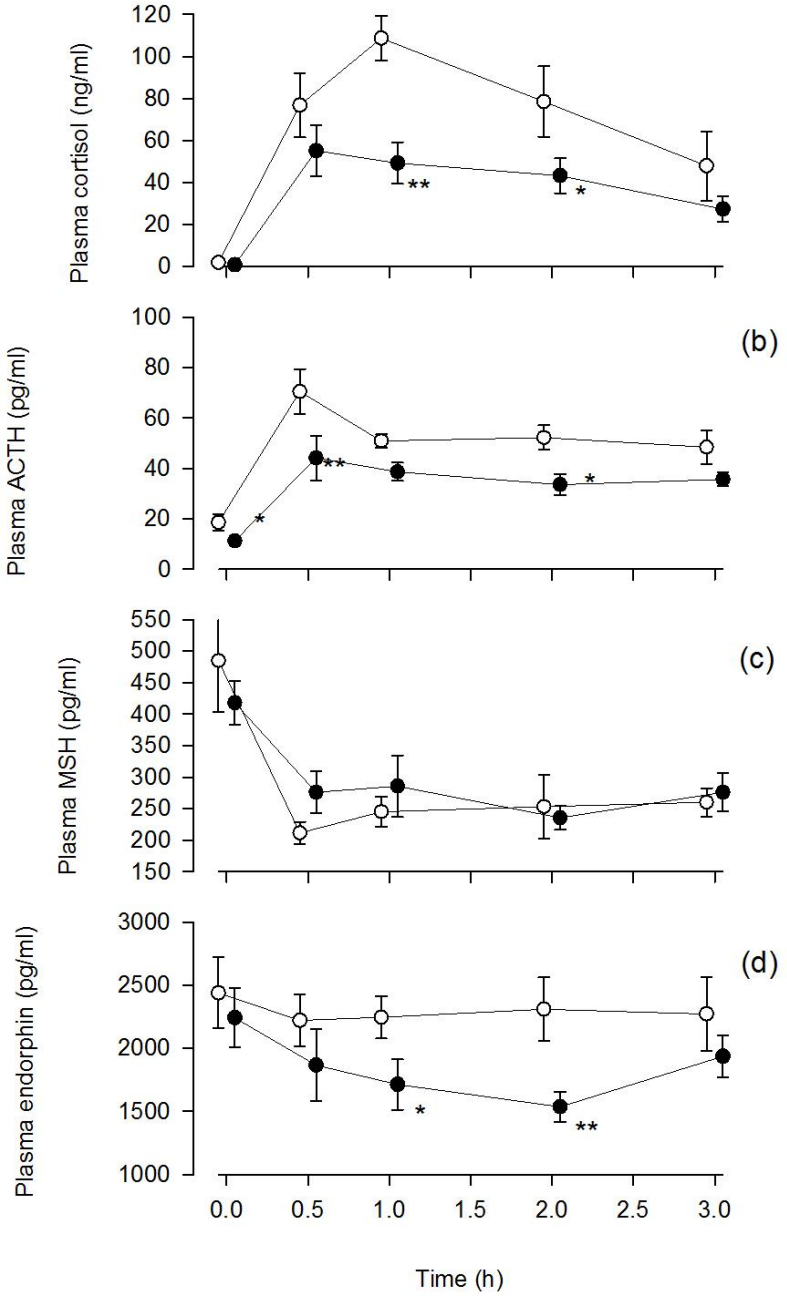

Figure 3. Changes in (a) plasma cortisol, (b) plasma ACTH, (c) plasma α-MSH and (d) plasma endorphin levels in a group of immature (°) and mature male (•) rainbow trout immediately prior to and during a 3h period of confinement. Each point represents the mean ± SE (n = 8). Significant differences between mature male and immature fish at each time point are indicated by asterisks; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.