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Plasma cortisol and 17β-estradiol levels in roach exposed to acute and chronic stress

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Running head: Stress response of roach

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ABSTRACT

Plasma cortisol levels were measured as an indicator of physiological stress in roach subjected to brief handling, or to a 14 day period of confinement, and in undisturbed control fish, during winter (water temperature 5^{o}C) and summer (16^{o}C), at which time plasma 17β-estradiol levels were also determined. Cortisol levels in undisturbed roach were low (mean 8.1 ng ml^{-1} at 5^{o}C; 1.4 ng ml^{-1} at 16^{o}C) and both handling and handling + confinement significantly elevated blood cortisol levels to 400 and 140 ng ml^{-1} respectively (at 5^{o}C) and 700 and 600 ng ml^{-1} respectively (at 16^{o}C). Blood cortisol levels had almost returned to baseline within 4 hours following handling alone but in fish subjected to handling and prolonged confinement cortisol levels remained elevated for up to 168 hours. Differences in baseline and post-stress levels of cortisol, and in the rate of recovery from acute stress, were observed at the two different temperatures and the possible factors underlying these differences are discussed. Circulating levels of 17β-estradiol were significantly reduced within 24 hours of exposure to either acute handling or chronic confinement indicating that the reproductive endocrine system in roach is sensitive to disruption by stressors.

Key words: Roach, *Rutilus rutilus*, stress, cortisol, 17β-estradiol

INTRODUCTION

The considerable body of work which addresses the impact of environmental stress on fish is comprised predominantly of studies on salmonid fish, in particular the rainbow trout, *Oncorhynchus mykiss* (Walbaum), and Pacific salmon of the genus *Oncorhynchus* (Walbaum). Of the non-salmonids present in the freshwaters of the British Isles only the response of the carp, *Cyprinus carpio* L., to environmental stress has been studied (Yin *et al*., 1995; Van Raaij *et al*., 1996; Pottinger, 1998). There is increasing interest in the measurement of physiological indices in non-salmonid fish as indicators of environmental degradation (Aaltonen *et al*., 1997; Brodeur *et al*., 1997). If physiological data are to be collected from populations of fish in the natural environment it is essential that the range of a given physiological parameter in a target species is established in individuals under controlled conditions, both undisturbed and subject to disturbance. The interpretation of data collected in the field will be difficult otherwise and may be confounded by a lack of appreciation of capture-related changes in the parameters of interest (Jardine *et al*., 1996). While it is therefore possible to predict in qualitative terms, based on existing data for other teleost species, the response of roach, *Rutilus rutilus* (L.), to acute and chronic stressors, the quantitative characteristics of the response in this particular species remain to be determined. As a preliminary step to examining the physiological status of roach in the natural environment we have characterised partially the response of the pituitary-interrenal axis of roach to an acute (handling) and a chronic (handling + confinement) stressor.

The study was carried out in both winter and summer in order to evaluate the effect of seasonal temperature changes on the response of roach to environmental stress. Plasma cortisol levels were measured because of the pivotal role of cortisol in the stress response of

vertebrates and its wide acceptance as an index of stress in fish (Barton, 1997), and in addition plasma 17β-estradiol levels were measured in stressed and unstressed pre-spawning female roach to assess the sensitivity of the pituitary-gonad axis to disruption by external stressors in roach.

MATERIALS AND METHODS

Fish maintenance

In January 1997 100 2-year old roach were transferred to each of fifteen 1000 litre circular glassfibre tanks, each supplied with a constant flow of lake water at ambient temperature (range during the acclimation period 4 - 7^{o}C). The fish were maintained under these conditions for two months with food (Trouw Fry 03) being offered three times weekly. During the experimental studies, food was withheld from all treatment groups to avoid potentially confounding effects of stress on appetite and food intake.

Time-course: Control (undisturbed)

Holding tanks 1 to 6 were employed for this study. On day 0 (March, water temperature 5^{o}C) eight fish [weight 24.9 ± 0.3g, _ ± standard error of the mean (SEM), n = 192] were netted from tank 1 and placed into anaesthetic (2-phenoxyethanol, 1:2000 in lake water). When fully anaesthetized, a blood sample was collected as described below. Further batches of eight fish were sampled without disturbance at intervals of 1, 4, 24, 48, 72 (tanks 2-6), 168 and 336 h (re-sampled tanks 1 and 2). The samples at 168 and 336 h were taken from tanks 1 and 2 because of constraints on tank space. It was assumed that sufficient time had elapsed for these fish to have recovered fully from the effects of the earlier sampling episode as has been demonstrated for brown trout, *Salmo trutta* L. (Pickering *et al*., 1982). These fish,

undisturbed other than by the sampling procedure, comprised the control group.

Time-course: Acute stressor (handling only)

On day 0 eight fish were removed from tank 7 to provide the 0h unstressed sample before the remaining fish from tanks 7 - 12 were transferred into six 50 l fibreglass tanks containing lake water and confined for 5 minutes before being returned to their holding tanks. These fish comprised the acutely stressed group. Subsequently, blood samples were taken from batches of eight fish from each of tanks 7-12 at the same time intervals as for the control group (7 and 8 resampled at 168 and 336 h as per control tanks 1 and 2).

Time-course: Chronic stressor (handling + prolonged confinement)

On day 0 eight fish were removed from tank 13 to provide an unstressed time 0 sample. The remaining fish in tanks 13-15 were allocated in groups of approximately 50 to each of six 50 l confinement tanks, containing 20 l of water with a constant flow-through (2 l min^{-1}). These fish comprised the chronically stressed group. Batches of eight fish were sampled from single confinement tanks at the same intervals as for the control and acutely stressed groups.

The same experimental procedure was carried out 5 months later (August, water temperature 16^{o}C) when the mean weight of the fish was 22.7 ± 0.3g (_ ± SEM, n = 192).

Collection of blood and sample processing

Blood samples were collected into a heparinized glass micropipette from the caudal vessels of each fish following severance of the caudal peduncle. The fish were killed by a blow to the

head. The blood samples were placed on ice prior to being transferred to capped 0.5 ml polypropylene tubes and centrifuged at 1000 rpm for 10 minutes at 4ºC to separate cells from plasma. The plasma was transferred to clean tubes and stored frozen (-20ºC) until required for assay. Approximately 5 minutes elapsed between the onset of complete immobility and removal of blood from the last fish in each batch.

Analyses

Plasma cortisol (C) and 17β-estradiol (E2) levels were determined using previously characterised radioimmunoassays (RIA) but employing a new antibody (IgG-F-2; IgG Corporation) for the cortisol RIA (Pickering *et al*., 1987a; Pottinger & Pickering, 1990). Cross-reactivity of the anti-cortisol antibody with cortisone, the major potential competing steroid in the plasma, was 2.6%. The data were evaluated statistically using ANOVA (Genstat), with time and treatment as factors, and significant differences were determined by comparing a limited number of means using the estimated standard error of the difference between means. Gonadosomatic index (GSI; gonad weight/body weight.100) was calculated for the female fish in the final 336 h sample.

RESULTS

Plasma cortisol levels in undisturbed roach remained low throughout each experiment with mean levels in August (1.4 ± 0.5 ng ml^{-1}, _ ± SEM, n = 63) being significantly (P<0.001) lower than those in March (8.1 ± 1.1 ng ml^{-1}, n = 63). Both handling and confinement initiated a highly significant elevation of cortisol in roach (P<0.001; Fig. 1). The magnitude of this response was considerably greater at 15ºC (~600 - 700 ng ml^{-1}; Fig. 1b) than at 5ºC (~150 - 400 ng ml^{-1}; Fig. 1a). Although levels of cortisol in the acutely stressed group

returned toward control values within 4 h (Fig. 1b; 15^{o}C) to 48 h (Fig. 1a; 5^{o}C) they remained marginally but significantly (P<0.01-P<0.001) higher than control levels throughout the remainder of both studies, other than at 336 h at 5^{o}C where levels in the acutely stressed fish were indistinguishable from those of the control fish. Plasma levels of cortisol were elevated for markedly longer in the confined fish than the acutely stressed fish at both temperatures, declining towards, but not reaching, control values by the end of each study. Levels of cortisol in the confined fish were higher than those in acutely stressed fish at, and subsequent to, 24 h at 5^{o}C (Fig. 1a) and 4 h at 15^{o}C (Fig. 1b).

Plasma E2 levels in female roach were low (~2 - 3 ng ml^{-1} ; Fig. 2), and indistinguishable between the treatment groups until the 24 h sample point at which time E2 levels in both confined and acutely stressed roach declined significantly below those in control fish. E2 levels in the chronically confined fish remained lower than control values for the remainder of the study. In the acutely stressed group E2 levels were statistically indistinguishable from control values at 72 h and 336 h but not at 168 h. There was no significant difference in GSI between the groups at 336 h after the imposition of the stressors but the mean values were suggestive nonetheless of a treatment-related effect (control: 13.3 ± 1.0; acute stress: 12.2 ± 0.8; chronic stress: 11.8 ± 0.8; n = 8). Insufficient numbers of maturing males were present in the population to allow androgen levels to be compared between groups.

DISCUSSION

These data represent the first report of an endocrine response to an acute and chronic stressor in the roach. Qualitatively, the response of roach to stress resembles that of other teleost fish, with an acute stimulus (netting and brief confinement) eliciting a rapid elevation of blood

cortisol levels followed by recovery, and a chronic stimulus (continuous confinement) resulting in prolonged elevation of cortisol levels with gradual acclimation. The species most closely related to roach (Cypriniformes, Ostariophysi) for which comparable data are available is the carp. Baseline and stress-stimulated levels of blood cortisol reported previously for carp (unstressed: ~10 - 50 ng ml^{-1}; stressed ~300 ng ml^{-1}; Dabrowska *et al*., 1991; Roelants *et al*., 1993; Yin *et al*., 1995; Pottinger, 1998) are within the same range as the roach examined in the present study, and in at least two studies stress-induced levels of cortisol in carp have been reported to approach or exceed the maximum levels of ~700 ng ml^{-1} observed in roach during the present study (Davis & Parker, 1986; Van Raaij *et al*., 1996). Plasma cortisol levels in salmonids have rarely been reported to exceed 200 - 300 ng ml^{-1} even following exposure to severe stressors (Barton & Iwama, 1991). At a water temperature of 15ºC, cortisol levels in rainbow trout exposed to stressors of a severity comparable to those employed in the present study do not exceed 300 ng ml^{-1} (Pottinger *et al*., 1996) although severe anoxia at a similar temperature has been reported to elicit levels of cortisol as high as 700 ng ml^{-1} in trout (Van Raaij *et al*., 1996). There is inadequate information available with which to assess whether exposure to stressors of a similar severity elicits a greater cortisol response in cyprinids than salmonids, although the indications are that this may be the case.

The magnitude of the cortisol response to stress was markedly different at the two sample times, March and August. There was an effect on the absolute level of cortisol attained following exposure to the stressor, on the rate of recovery from acute stress (more rapid at 16ºC), and on baseline levels in undisturbed fish (lower at 16ºC). A number of factors may have contributed to this seasonality. It is possible that the stress response in roach is

temperature dependent, as for other species of fish (Sumpter *et al*., 1985; Pickering & Pottinger, 1987; Barton, 1997). Physiological processes in poikilothermic animals are generally temperature dependent. For example, whole-body lactate concentrations of juvenile roach subjected to exhaustive exercise are almost twice as great, and the rate of recovery more than four times as rapid, at 20^{o}C compared to 4^{o}C (Dalla Via *et al*., 1989). It is also possible that diffusional loss of steroids, including cortisol, across the gill surfaces (Vermeirssen & Scott, 1996) is greater in warmer water as a consequence of higher ventilatory activity (Butler & Metcalfe, 1983). Other potential modulators of blood steroid levels, including biosynthesis and clearance, may have contributed to the observed differences, but their relative importance is unknown.

A surprising aspect of the present study was the failure of plasma cortisol levels in acutely stressed roach to return fully to levels comparable with the controls after the initial elevation. Although levels in recovering acutely stressed fish were very close to those in unstressed fish after 48 h (at 5^{o}C) and 4 h (at 16^{o}C) they remained significantly higher. Given that successive samples were removed from different tanks for both control and acutely stressed fish, and that the phenomemon occurred during both studies, the consistency of this observation is convincing. We are not aware of reports of similar effects in other fish species. The failure to return to a true baseline may represent an effect of the acute stressor on the set point of baseline activity of the hypothalamic-pituitary-interrenal axis, such that the homeostatic feedback mechanism which maintains cortisol levels in the blood is redefined or disturbed by an incidence of stress. We are unaware of any similar data from other vertebrate studies, whether this hypothesis is valid and has any implication for the sensitivity of the fish to subsequent stressors requires further investigation.

Plasma E2 levels in sexually mature female roach in this study were within the range reported previously for wild-caught roach during spring (1 - 5 ng ml^{-1}; Rinchard *et al*., 1997). The slight increase observed in control fish between 0 and 336 hours probably represents the normal seasonal cycling of E2 levels (Rinchard *et al*., 1997). Both handling and confinement caused a significant decline in E2 levels in roach relative to undisturbed controls. The inter-individual variability in plasma E2 levels was substantial and, combined with the small sample size, may have contributed to the apparently anomalous absence of significant differences between control and acutely-stressed fish at 168 h. Stress disturbs the reproductive endocrine system in salmonid (Pickering *et al*., 1987b; Pankhurst & Dedual, 1994) and non-salmonid fish (Safford & Thomas, 1987; Carragher & Pankhurst, 1991) via activation of the hypothalamic-pituitary-interrenal axis. The precise mechanism by which gonadal steroid levels are suppressed by stress remains to be elucidated (Bonga, 1997). We are unaware of any previous studies which demonstrate this effect in cyprinid fish.

In conclusion, roach respond to acute and chronic stressors with an elevation of blood cortisol. The increase is rapid and the rate of recovery, and absolute levels achieved, are temperature dependent. The maximum stress-induced levels of cortisol observed in roach in this study are considerably higher, in proportion to baseline levels, than for most other species of teleost so far studied. Both acute and chronic stressors significantly reduce plasma levels of 17β-estradiol in roach. This observation suggests that stress-induced reproductive dysfunction is likely to occur in roach which experience prolonged exposure to sublethal stressors, as is the case for other species of fish (Campbell *et al*., 1992, 1994).

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References

Aaltonen, T. M., Valtonen, E. T. & Jokinen, E. I. (1997). Immunoreactivity of roach, *Rutilus rutilus*, following laboratory exposure to bleached pulp and paper mill effluents. *Ecotoxicology and Environmental Safety* **38**, 266-271.

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

Barton, B. A. & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases* **1**, 3-26.

Bonga, S. E. W. (1997). The stress response in fish. *Physiological Reviews* **77**, 591-625.

Brodeur, J. C., Sherwood, G., Rasmussen, J. B. & Hontela, A. (1997). Impaired cortisol secretion in yellow perch (*Perca flavescens*) from lakes contaminated by heavy metals: *in vivo* and *in vitro* assessment. *Canadian Journal of Fisheries and Aquatic Sciences* **54**, 2752-2758.

Butler, P. J. & Metcalfe, J. F. (1983). Respiration and circulation. In *Control Processes in Fish Physiology* (Rankin, J. C., Pitcher, T. J. & Duggan, R. eds.) pp. 41-65. London: Croom Helm.

Campbell, P. M., Pottinger, T. G. & Sumpter, J. P. (1992). Stress reduces the quality of gametes produced by rainbow trout. *Biology of Reproduction* 47, 1140-1150.

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

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

Dabrowska, H., Dabrowska, K., Meyer-Burgdorff, K., Hanke, W. & Gunthers, K.-D. (1991). The effect of large doses of vitamin C and magnesium on stress responses in common carp, *Cyprinus carpio*. *Comparative Biochemistry and Physiology* **99A**, 681-685.

Dalla Via, J., Huber, M., Wieser, W. & Lackner, R. (1989). Temperature-related responses of intermediary metabolism to forced exercise and recovery in juvenile *Rutilus rutilus* (L.) (Cyprinidae: Teleostei). *Physiological Zoology* **62**, 964-976.

Davis, K. B. & Parker, N. C. (1986). Plasma corticosteroid stress response of fourteen species of warmwater fish to transportation. *Transactions of the American Fisheries Society* **115**, 495-499.

Jardine, J. J., Van Der Kraak, G. J. & Munkittrick, K. R. (1996). Capture and confinement stress in white sucker exposed to bleached kraft pulp mill effluent. *Ecotoxicology and Environmental Safety* **33**, 287-298.

Pankhurst, N. W. & Dedual, M. (1994). Effects of capture and recovery on plasma levels of cortisol, lactate and gonadal steroids in a natural population of rainbow trout. *Journal of Fish Biology* **45**, 1013-1025.

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

Pickering, A. D., Pottinger, T. G. & Christie, P. (1982). Recovery of the brown trout, *Salmo trutta* L., from acute handling stress: a time-course study. *Journal of Fish Biology* **20**, 229-244.

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

Pickering, A. D., Pottinger, T. G. & Sumpter, J. P. (1987b). On the use of dexamethasone to block the pituitary-interrenal axis in the brown trout, *Salmo trutta* L. *General and Comparative Endocrinology* **65**, 346-353.

Pottinger, T. G. (1998). Physiological changes in carp, *Cyprinus carpio* L., retained in anglers keepnets. *Journal of Fish Biology* (submitted for publication)

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

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**, 284-295.

Rinchard, J., Kestemont, P. & Heine, R. (1997). Comparative study of reproductive biology in single and multiple-spawner cyprinid fish. II. Sex steroid and plasma protein phosphorous concentrations. *Journal of Fish Biology* **50**, 169-180.

Roelants, I., Epler, P., Miklajczyk, T., Sokolowska-Mikolajczyk, M., Breton, B. & Ollevier, F. (1993). The effect of handling stress on the circadian release pattern of cortisol and gonadotropin (GTH_2) in female carp (*Cyprinus carpio* L.). *Polskie Archiwum Hydrobiologii* 40, 347-353.

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

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

Van Raaij, M. T., Van Den Thillart, G. E. E. J. M., Vianen, G. J., Pit, D. S., Balm, P. H. M. & Steffens, A. B. (1996). Substrate mobilisation and hormonal changes in rainbow trout (*Oncorhynchus mykiss*, L.) and common carp (*Cyprinus carpio*, L.) during deep hypoxia and subsequent recovery. *Journal of Comparative Physiology B* **166**, 443-452.

Vermeirssen, E. L. M. & Scott, A. P. (1996). Excretion of free and conjugated steroids in rainbow trout (*Oncorhynchus mykiss*): evidence for branchial excretion of the maturation-inducing steroid, 17,20β-dihydroxy-4-pregnen-3-one. *General and Comparative Endocrinology* **101**, 180-194.

Yin, Z., Lam, T. J. & Sin, Y. M. (1995). The effects of crowding stress on the non-specific immune response in fancy carp (*Cyprinus carpio* L.). *Fish & Shellfish Immunology* **5**, 519-529.

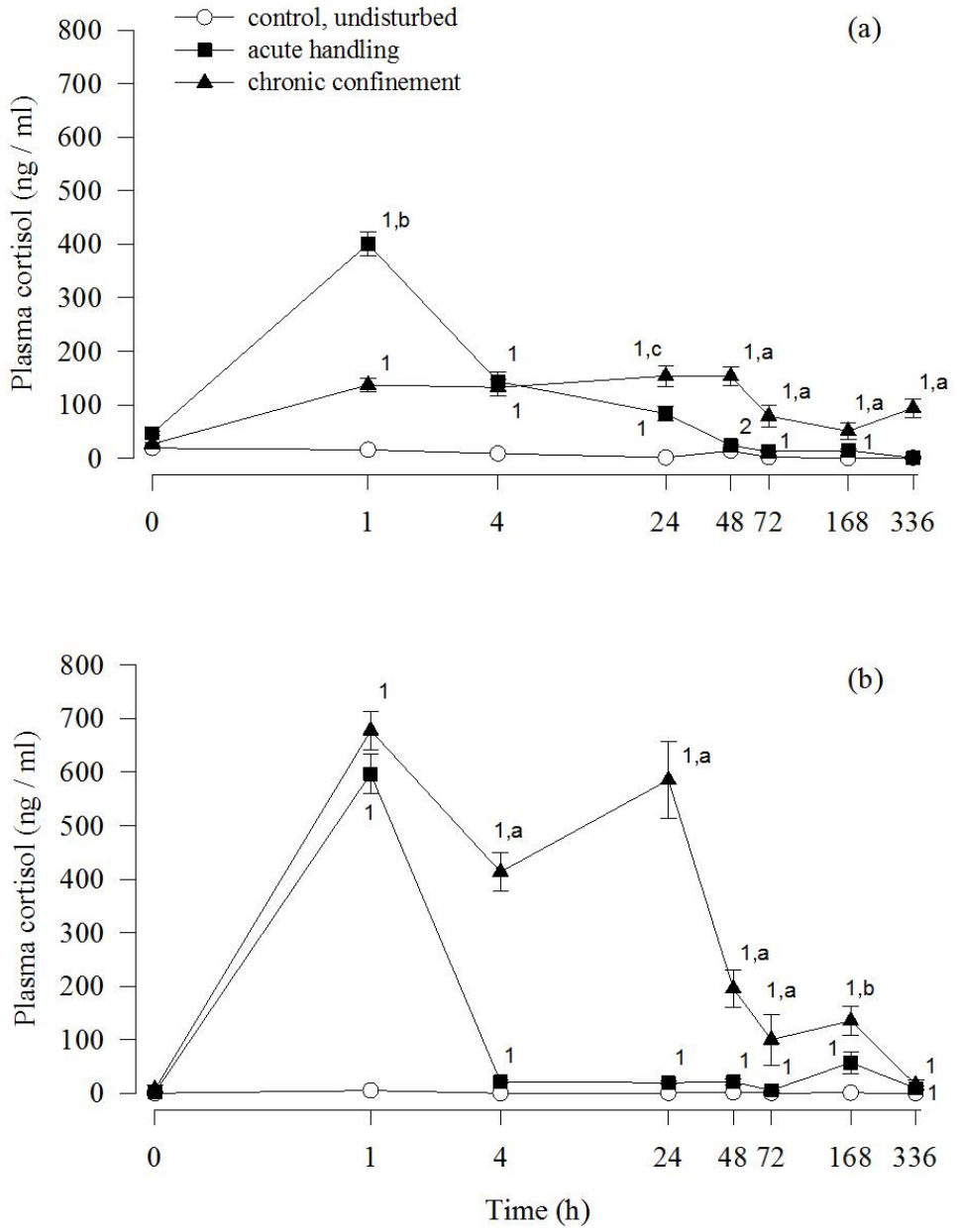

Figure 1. Plasma cortisol levels in undisturbed roach (❍), roach subject to a brief handling stress (■) and roach subject to chronic confinement (Δ) determined at intervals prior to and up to 336 hours after the initial disturbance at either (a) 5°C or (b) 16°C. Each value represents the mean of eight fish ± SEM. Significant differences between stressed fish and undisturbed controls are denoted by 1: $P<0.001$; 2: $P<0.01$; and between chronically confined fish and acutely handled fish by a: $P<0.001$; b: $P<0.01$; c: $P<0.05$. At some points the error bars are obscured by the symbol.

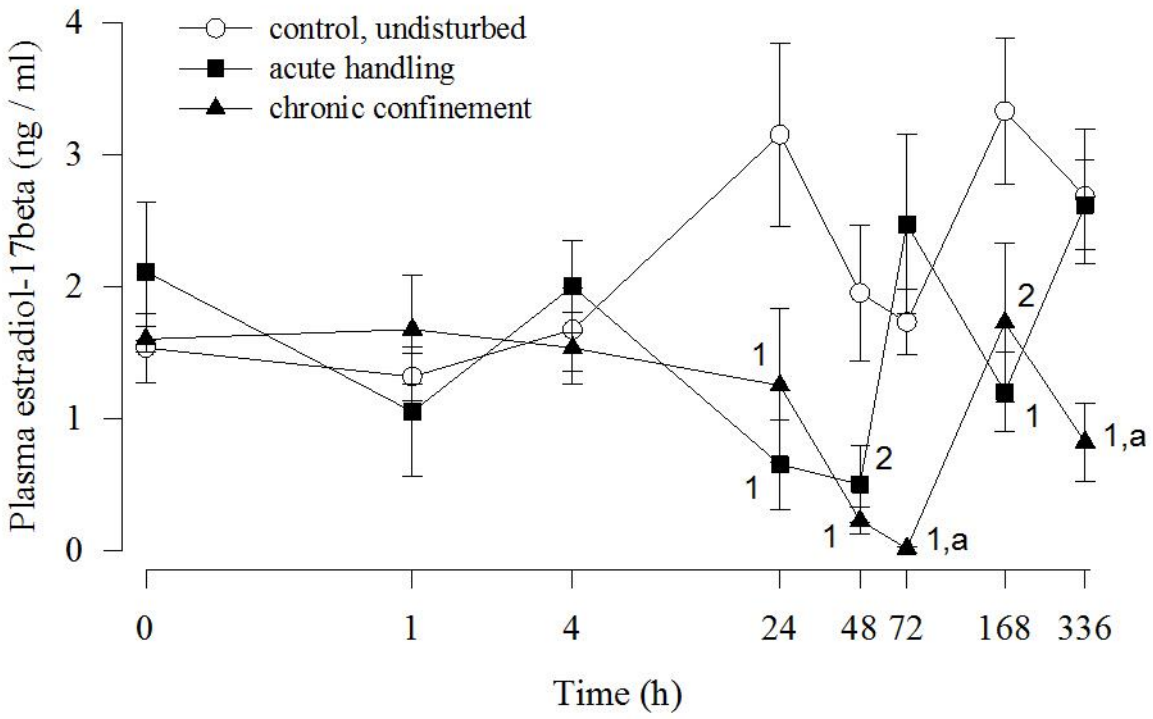

Figure 2. Plasma 17β-estradiol levels in undisturbed roach (❍), roach subject to a brief handling stress (■) and roach subject to chronic confinement (Δ) determined at intervals prior to and up to 336 hours after the initial disturbance at 5°C. Each value represents the mean of 3-8 fish ± SEM. Significant differences between stressed fish and undisturbed control fish are denoted by 1: $P<0.001$; 2: $P<0.01$; and between chronically confined fish and acutely handled fish by a: $P<0.001$.