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T.G. Pottinger, Patrick Prunet, A.D. Pickering. The effects of confinement stress on circulating prolactin levels in rainbow trout (*Oncorhynchus mykiss*) in fresh water. *General and Comparative Endocrinology*, 1992, 88, pp.454-461. 10.1016/0016-6480(92)90240-K . hal-02716090

HAL Id: hal-02716090

<https://hal.inrae.fr/hal-02716090>

Submitted on 1 Jun 2020

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The Effects of Confinement Stress on Circulating Prolactin Levels in Rainbow Trout (*Oncorhynchus mykiss*) in Fresh Water

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Accepted May 15, 1992

Rainbow trout were confined for 48 hr, during which time water quality either was allowed to deteriorate (resulting in elevated NH_3 , elevated free CO_2 , and reduced dissolved O_2) or was maintained at preconfinement levels. Fish were removed and blood samples taken at 0, 2, 4, 8, 24, and 48 hr after the onset of confinement from both stressed (confined) and unstressed (unconfined) fish. Plasma cortisol and plasma prolactin (PRL) levels were determined using specific RIAs. Chronic confinement of rainbow trout, accompanied by a decline in water quality, resulted in significant elevation of plasma cortisol, maintained for the period of confinement. Plasma PRL levels were significantly lower in stressed fish, by up to 60% relative to control fish, during the first 24 hr of confinement. The stress of confinement alone, in the absence of deterioration in water quality, produced similar results, with the change in prolactin levels being less rapid but more prolonged under these conditions. © 1992 Academic Press, Inc.

Environmental stress has profoundly deleterious effects on many physiological functions in teleost fish, some of which have an endocrine basis. Activation of the hypothalamic-pituitary-interrenal (HPI) axis is pivotal in stress-induced loss of immunocompetence (Maule *et al.*, 1987; Wiik *et al.*, 1989) and in reproductive dysfunction (Carragher *et al.*, 1989; Pottinger and Pickering, 1990) and contributes to stress-related growth suppression (Pickering, 1990). However, not all stress-induced physiological changes are ascribable directly to changes in activity of the HPI axis. For example, suppression of growth hormone levels during stress may contribute to the reduction in growth observed under such conditions (Pickering *et al.*, 1991).

Structurally related to growth hormone, the pituitary hormone prolactin (PRL) is implicated in a range of physiological functions (Clarke and Bern, 1980; Hirano, 1986; Prunet *et al.*, 1990a) including, in teleost fish, osmoregulation. Prolactin has marked

sodium-retaining activity in many species (Grau *et al.*, 1984; Hasegawa *et al.*, 1986) and influences branchial diffusional and osmotic permeability, as well as intestinal and renal osmoregulatory processes (Oduleye, 1975; Hirano, 1986). There is also evidence that PRL influences reproduction in teleost fish (Singh *et al.*, 1988; Prunet, Haux, and Bjornsson, unpublished data). In view of the involvement of PRL in these physiological processes, an understanding of the influence of stress on PRL levels is important. Although widely studied in mammals (see, for example, Delitala *et al.*, 1987) the effect of stress on PRL levels in fish has been examined in only two previous studies, with contradictory results. Spieler and Meier (1976) reported a stress-induced reduction in plasma PRL levels while, more recently, Avella *et al.* (1991) observed an elevation of plasma PRL following stress. The present study was designed to clarify the effects of stress on PRL levels in fish by examining whether stress, in the form of

confinement, with and without an accompanying deterioration in water quality, affects the circulating levels of PRL in freshwater-adapted rainbow trout, *Oncorhynchus mykiss*.

MATERIALS AND METHODS

Fish. Two-year-old rainbow trout (Annandale strain, reared from egg at the IFE, mean weight = 447 ± 9 g, $n = 30$) were maintained at a density of 150 fish per tank (~ 45 g liter $^{-1}$) in 1500-liter outdoor circular fiberglass tanks, each supplied with a constant flow of lake water (35 liters min $^{-1}$). Water temperature during the experimental period (October 1989) was within the range 12–13°. Fish were fed once daily, except during the experimental period, with commercial feed (BP Mainstream) at a rate in accordance with the manufacturer's instructions. Within the population as a whole, 21% of the fish were maturing. These were distributed evenly within the experimental groups.

Experiment 1. Determination of the effects of the combined stress of confinement and water quality deterioration on cortisol and prolactin levels. At time 0, 40 fish were netted from a single rearing tank and distributed randomly among 10 50-liter, lidded, polypropylene tanks, 4 fish per tank (~ 36 g liter $^{-1}$). Each tank was supplied with a constant flow of lake water, flow rate adjusted prior to the start of the experiment to 0.6 liters min $^{-1}$. At this flow rate and number and size of fish, dissolved O $_2$ levels within these tanks were reduced to approximately 4.0 mg liter $^{-1}$. Simultaneously, a second operator removed 8 fish from the rearing tank into anesthetic (2-phenoxyethanol, 1:2000). After 1 min, blood samples were removed from the sinus venosus of these fish into heparinized syringes. Fish were weighed, and any external evidence of sexual maturity was noted. The fish were then returned to a recovery tank. Once sampled, fish took no further part in the experiment. Plasma was stored frozen at -70° prior to assay. Two hours after this initial sample 4 more fish were netted from each of the two previously undisturbed rearing tanks, and 4 fish were removed from each of two confinement tanks. Both groups were anesthetized and blood samples were removed as above. This procedure was repeated to provide samples at 0, 2, 4, 8, 24, and 48 hr after the onset of confinement. Dissolved O $_2$ levels within each confinement tank were determined immediately prior to each sample using a Jenway portable oxygen meter which was calibrated before each set of measurements. The whole experimental procedure was repeated to increase the number of replicates within each sample to 16, using a second group of undisturbed fish.

Experiment 2. Determination of the effect of preventing water quality deterioration on the plasma cor-

tisol and prolactin response to prolonged confinement of rainbow trout. This experiment was carried out to distinguish the effects of water quality deterioration from the effects of handling and confinement. The same experimental procedure was employed as described for Experiment 1 except that flow rates in the confinement tanks were adjusted to 15 liters min $^{-1}$, and aeration was supplied to each confinement tank via a portable compressor. This experiment was carried out once only, giving 8 replicates within each sample.

Chemical analyses. Between 24 and 48 hr during both experiments, water samples were removed from four tanks of each experimental group for chemical analysis. Free CO $_2$ was calculated from pH, alkalinity, and temperature data (Mackereth *et al.*, 1978), and total ammonia levels were measured using the indophenol blue method (Chaney and Marbach, 1962).

Hormone analyses. Cortisol was measured in plasma samples by RIA (Pickering *et al.*, 1987). The limit of detection of the assay was 0.2 ng ml $^{-1}$. The mean intraassay coefficient of variation (CV) was 12.1%. The mean interassay CV was 14.5%. Prolactin was determined using a homologous RIA (Prunet *et al.*, 1985). Sensitivity of the assay was 20 pg PRL per assay tube. The mean intraassay CV was 8.8%. The interassay CV was 16%.

Statistical analyses. Data were analyzed using multifactorial analysis of covariance (ANCOVA, Genstat) with treatment (stressed, unstressed), time, and replicate experiment number (Experiment 1 only) as factors. Maturity (immature, mature) was included in the analyses as a covariate. Data were log transformed, prior to analysis, to improve homogeneity of variance.

RESULTS

Experiment 1. Confinement of fish under conditions of reduced water flow rate significantly reduced dissolved O $_2$ levels relative to control tanks (3.6 cf. 8.9 mg liter $^{-1}$, $P < 0.001$, Table 1), a significant increase in free CO $_2$ (136.3 cf. 60.0 μ E liter $^{-1}$, $P < 0.05$, Table 1) and a significant increase in total ammonia (NH $_3$ + NH $_4^+$, 306 cf. 51.5 μ g liter $^{-1}$, $P < 0.01$, Table 1).

Analysis of covariance revealed a highly significant effect of stress on both plasma cortisol and plasma PRL levels ($P < 0.001$ in each case). Plasma cortisol levels in stressed fish increased rapidly from 4.3 ± 0.8 ng ml $^{-1}$ to 90.3 ± 13.8 ng ml $^{-1}$ ($x \pm$ SEM, $n = 16$) within 2 hr of the onset of confinement (Fig. 1). Cortisol levels in

TABLE 1
WATER QUALITY DURING CONFINEMENT STRESS EXPERIMENTS

	Total ammonia ($\mu\text{g/liter}$)	Free CO_2 ($\mu\text{E/liter}$)	Dissolved O_2 (mg/liter)
Stock tanks (control)	51.5 ± 5.0 ($n = 4$)	60.0 ± 5.5 ($n = 4$)	8.9 ± 0.1 ($n = 22$)
Confinement tanks (low flow, no aeration)	$306 \pm 45.0^{**}$ ($n = 7$)	$136.3 \pm 15.0^*$ ($n = 4$)	$3.6 \pm 0.2^{***}$ ($n = 20$)
Confinement tanks (high flow, aeration)	40.0 ± 2.7 ($n = 4$)	48.0 ± 2.1 ($n = 4$)	$9.6 \pm 0.1^{***}$ ($n = 10$)

Note. Values are $\bar{x} \pm \text{SEM}$. Asterisks denote values significantly different from controls: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

these fish remained significantly higher ($P < 0.001$) than those in control unstressed fish throughout the experiment. There was, however, a significant ($P < 0.001$) fall in cortisol levels in the stressed fish from $81.7 \pm 2.3 \text{ ng ml}^{-1}$ at 4 hr to $31.2 \pm 6.2 \text{ ng ml}^{-1}$ at 8 hr before significant elevation by 24 hr ($P < 0.001$) to reach $125.2 \pm 18.5 \text{ ng ml}^{-1}$.

There was a significant increase in PRL levels in control fish, from $2.3 \pm 0.4 \text{ ng ml}^{-1}$ at 0 hr to $4.0 \pm 0.7 \text{ ng ml}^{-1}$ at 8 hr ($P < 0.05$, Fig. 2), which was sustained at 24 hr. The control value at 48 hr was not significantly different from that at 0 hr, however. Within 2 hr of the onset of confinement PRL levels in stressed fish were significantly ($P < 0.01$) lower than levels in control fish (2.0 ± 0.6 cf. $3.2 \pm 0.4 \text{ ng ml}^{-1}$,

Fig. 2) and remained significantly ($P < 0.001$) lower until 24 hr after the onset of stress, by which time levels in stressed fish were similar to levels in control fish.

Experiment 2. Confinement of fish under conditions of high flow, together with aeration, resulted in ammonia, free CO_2 , and dissolved O_2 levels similar to those in control tanks (Table 1).

Again, ANCOVA showed a highly significant effect of stress on both cortisol and PRL levels ($P < 0.001$ in each case). Significant increases in cortisol levels were observed in confined fish, rising to $109.6 \pm 19.1 \text{ ng ml}^{-1}$ within 2 hr of confinement, compared to $7.5 \pm 2.4 \text{ ng ml}^{-1}$ in control fish ($P < 0.001$, Fig. 3). At all subsequent times, cortisol levels in stressed fish were

FIG. 1. Changes in plasma cortisol levels in rainbow trout confined and exposed to deteriorating water quality (broken line) compared to unconfined control fish (solid line). Each point is the mean $\pm$ SEM, $n = 16$. Values significantly different from corresponding controls are denoted by asterisks: $^{***}P < 0.001$.

FIG. 2. Changes in plasma PRL levels in rainbow trout confined and exposed to deteriorating water quality (broken line) compared to unconfined control fish (solid line). Each point is the mean $\pm$ SEM, $n = 16$. Values significantly different from corresponding controls are denoted by asterisks: $^{**}P < 0.01$, $^{***}P < 0.001$.

FIG. 3. Changes in plasma cortisol levels in rainbow trout confined (broken line) with water quality maintained as that of the control fish (solid line). Each point is the mean $\pm$ SEM, $n = 8$. Values significantly different from corresponding controls are denoted by asterisks: *** $P < 0.001$.

significantly higher ($P < 0.001$) than those in control fish. The cortisol profile in stressed fish showed a moderate decline at 8 hr, similar to that observed in Experiment 1, but in this case it was not significant.

There was no significant change with time in PRL levels in the control fish in Experiment 2. Prolactin levels in stressed fish were significantly lower than those in control fish at 8 hr (2.2 ± 0.4 cf. 5.1 ± 1.2 ng ml $^{-1}$, $P < 0.01$) and 24 hr (2.8 ± 0.6 cf. 5.2 ± 1.3 ng ml $^{-1}$, $P < 0.05$), but had recovered by 48 hr to a level statistically indistinguishable from that of the controls.

Prolactin levels were not found to be related to the state of sexual maturity of fish in either experiment.

DISCUSSION

Confinement, with or without an accompanying deterioration in water quality, evoked a marked and prolonged increase in plasma cortisol levels (Figs. 1 and 3). Cortisol levels in unstressed control fish remained below 10.0 ng ml $^{-1}$ throughout the experiments. Elevation of plasma cortisol is widely employed as an index of stress and these results agree with previous investigations in which confinement was shown to activate the hypothalamic-pituitary-

interrenal axis (Sumpter *et al.*, 1985; Pickering *et al.*, 1986, 1987; Pickering and Pottinger, 1987). The present experiments revealed cortisol levels in the stressed fish to show a biphasic pattern; although the decline at 8 hr was significant only in Experiment 1 (Fig. 1) a similar trend is apparent in Experiment 2 (Fig. 3). Similar responses have previously been noted (Pickering and Pottinger, 1985; Tomasso *et al.*, 1981) but have yet to be accounted for. The confinement procedure employed, although not resulting in major alterations in density, restricts spatial freedom of the fish, reducing their ability to avoid social confrontation, and constitutes a reliable means of inducing chronic stress (Pottinger and Pickering, 1992).

Mean plasma PRL levels in unstressed fish during the present investigation lie within the range 2–5 ng ml $^{-1}$, similar to levels reported to occur in unstressed coho salmon, *Oncorhynchus kisutch* (Avella *et al.*, 1991; ~ 1 –7 ng ml $^{-1}$), sockeye, *O. nerka*, and amago, *O. rhodurus*, salmon (Yada *et al.*, 1991; < 1 –4 ng ml $^{-1}$), but lower than those previously reported in rainbow trout (Prunet *et al.*, 1985; ~ 10 –30 ng ml $^{-1}$). Plasma PRL levels in fish stressed by confinement and exposure to deteriorating water quality were significantly lower than those in control unstressed fish (Fig. 2) for up to 24 hr after the onset of confinement. Fish stressed by confinement alone also exhibited significantly lower PRL levels relative to unstressed controls (Fig. 4), although in this case the difference in PRL levels was not apparent until 8 hr after the onset of stress, compared to 2 hr in the first experiment. The differences in PRL levels between stressed and control groups would therefore appear not to be solely a function of deteriorating water quality but are stimulated, at least in part, by the stress of handling and confinement. The delay in PRL response in the second experiment may indicate that the combination of stressors present during the

FIG. 4. Changes in plasma PRL levels in rainbow trout confined (broken line) with water quality maintained as that of the control fish (solid line). Each point is the mean $\pm$ SEM, $n = 8$. Values significantly different from the corresponding controls are denoted by asterisks: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

first experiment was a more potent stimulus to the factors controlling PRL levels than handling and confinement alone. In both experiments, PRL levels in confined fish were statistically indistinguishable from those in control fish within 48 hr although at this point cortisol levels were still significantly elevated. The significant elevation of plasma PRL in control fish during the first 8 hr of Experiment 1, although not observed in Experiment 2, may be evidence of diel variation in PRL levels. Complex diel variation of plasma PRL is known to occur in mammals (Willoughby, 1980). Little is known of the variability of baseline PRL levels in fish, although previous studies on PRL dynamics in salmonid fish reveal there to be considerable variation with time in control fish (Prunet *et al.*, 1985; Avella *et al.*, 1991; Yada *et al.*, 1991). Indeed, it might be argued that at least in Experiment 1, rather than reducing plasma PRL levels, the effect of stress is to oppose an elevation occurring in unstressed fish.

Stress is known to influence PRL levels in mammalian systems—many studies reveal a consistent elevation of plasma PRL levels in response to a wide range of stressors (Delitala *et al.*, 1987; Tache *et al.*, 1978; Kant *et al.*, 1983; Klemcke *et al.*, 1987). It has recently been proposed that stress-induced PRL release has a protective function in moderating harmful consequences of

stress (Drago *et al.*, 1989). Prolactin is believed to act by suppressing corticosterone secretion, inducing analgesia, and opposing stress-induced immunosuppression. Until recently, direct examination of the effect of stress on PRL levels in fish has not been possible because of the lack of a specific, homologous RIA. Spieler and Meier (1976) reported a significant reduction in circulating PRL levels in goldfish, *Carassius auratus*, subjected to capture and restraint, using a heterologous RIA (anti-pollack PRL, labeled ovine PRL) and, although partially validated for use with goldfish, the results must be interpreted with caution (Nicoll, 1975). Avella *et al.* (1991), employing the same specific RIA as the present investigation, demonstrated that chronic stress (confinement for up to 5 days) elevated plasma PRL levels in juvenile coho salmon. Although it should be noted that in Avella's study fish at the presmolt and smolt stages were employed, these results are diametrically at variance with the observations made in the present study in which circulating levels of PRL were lower in stressed fish than in control fish. Interestingly, Kelley *et al.* (1990) reported that cortisol inhibits the synthesis and release of PRL by coho salmon pituitaries *in vitro*. It might be argued that if the effect of cortisol is on transcriptional processes, the response we have observed is too rapid to be accounted for by such a mechanism. However, if release of PRL were impaired then a more rapid decline in plasma levels might be envisaged. In both the present experiments, PRL levels in stressed fish returned to control values although plasma cortisol remained elevated, suggesting that any interaction between the two hormones is likely to be complex.

In conclusion, subjecting rainbow trout to a 48-hr period of confinement, with or without an accompanying deterioration in water quality, evokes a classical stress-induced elevation of plasma cortisol and results in significantly lower levels of circu-

lating PRL in stressed fish than in control fish. Plasma PRL levels remain significantly lower in stressed fish for up to 24–48 hr after the onset of stress but recover to control values despite the presence of the stressful stimuli and continued elevation of cortisol levels. In view of the involvement of PRL in a range of physiological processes, further work is required to clarify the effects of nonspecific environmental perturbations on this important hormone.

ACKNOWLEDGMENTS

The authors thank Mr. S. Killeen for maintaining the experimental fish. This work was funded by the Natural Environment Research Council.

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