ELSEVIER

Comparative Biochemistry and Physiology Part A 124 (1999) 329–334

CBP

www.elsevier.com/locate/cbpa

Modulation of stress hormones in rainbow trout by means of anesthesia, sensory deprivation and receptor blockade

Lena Gerwick *, Nora E. Demers [1], Christopher J. Bayne

Department of Zoology, Oregon State University, Corvallis, OR 97331-2914, USA

Received 8 January 1999; received in revised form 30 August 1999; accepted 1 September 1999

Abstract

Sympathetic activation leading to increased levels of blood catecholamines, and stimulation of the hypothalamic–pituitary–inter-renal axis leading to increased cortisol, are difficult to avoid when handling animals. Yet, in research on effects of acute stress, elicitation of such responses must be minimized in the control groups. The work examines means to achieve a minimally disturbed state in rainbow trout (*Oncorhynchus mykiss*). Level of arousal was determined by adrenaline and cortisol concentrations in plasma, and by the spleen:somatic index. Fish were prepared for bleeding by rapid capture and concussion, by infusion of anesthetic into the undisturbed home tank, by confinement in black boxes, or by being fed α- and β-receptor antagonists. Even when done quickly, netting and concussion yielded fish with ca. 200-pmol adrenaline/ml plasma. Cortisol was elevated (to >10 ng/ml) within 30 s of stress initiation. Surreptitious infusion of anesthetic (2-phenoxyethanol, PE) into tanks yielded fish with lower adrenaline levels (means 19.34 and 19.58 pmols/ml in home tank and black boxes, respectively). Among fish given phentolamine and propranolol, spleen:somatic indices and plasma adrenaline were higher than in diet controls, whether undisturbed or stressed, indicative of successful receptor blockade. Since careful infusion of 2-PE yielded the lowest adrenaline levels, and requires no special apparatus, it is the method of choice for obtaining minimally stressed fish.

Keywords: Stress; Anesthesia; Antagonist; Catecholamine; Cortisol; *Oncorhynchus*; Spleen; Trout; 2-Phenoxyethanol

1. Introduction

The disruptive effects of stressors on homeostasis have been of concern to biologists for over a century [3,4]. Although a great deal is now known about both the mechanisms and the effects of stress [20,21,23], consensus has been hampered by the general lack of precision in terminology. Furthermore, immediate and acute effects have been less the focus than the longer-term consequences of stressors. Though commonly stated, the view that stress is harmful is not universally held [13]. In the aquaculture industry, fish are subjected to a variety of stressful handling protocols. While such stress levels are considered undesirable, and efforts are made to minimize them, they are also considered largely unavoidable, and their negative effects are tolerated.

The types of stress that are clearly harmful tend to be chronic and inescapable. Acute stressors that are brief but sufficient to elicit the fight-or-flight response, such as unexpected encounters with dangerous predators, do not always have negative physiological consequences. The fight-or-flight response, associated with activation of the sympathetic nervous system, involves altered states in multiple organ systems: sensory perceptiveness increases, cardio-vascular functions change, and the status of innate immune effector systems is also changed [6,11,12,14]. In vertebrate species, these changes are coordinated centrally when appropriate sensory information, channeled through the hypothalamus, elicits action potentials in adrenergic fibers of the sympathetic nervous system. The end effects of this coordination are contraction of smooth muscle in the spleen and hair follicles, and the release of adrenaline

* Corresponding author. Tel.: +1-541-737-5353; fax: +1-541-737-0501.

E-mail address: gerwickl@bcc.orst.edu (L. Gerwick)

[1] Present address: College of Arts and Sciences, Florida Gulf Coast University, Ft. Myers, FL 33965-6565, USA.

1095-6433/99/$ - see front matter
PII: S1095-6433(99)00126-9

from the adrenal medulla (chromaffin cells of the pronephros in teleosts). While catecholamines mediate the most immediate somatic responses, a surge in plasma adrenaline is usually followed by a surge in plasma corticosteroid [22].

What, if any, selective advantage is there in the release of stress hormones? Immunologists, for example, have explored the possible beneficial effects of cortisol and adrenaline, and a number of inferences have been drawn from experimental data [6,19]. In general, cortisol is immunosuppressive whereas catecholamines (adrenaline and noradrenaline) seem to have pleiotrophic effects. Feedback between these two hormonal systems, and differential effects on shared target cells complicate interpretations. In vitro, fish neutrophils react instantaneously to catecholamines, with altered metabolism of reactive oxygen species [1,2]. In vivo, sympathectomy increased lymphocyte responses to antigen [7,8]. Furthermore, blood clotting and killing of phagocytosed yeast were accelerated in trout that had experienced brief stressors [18], and several plasma proteins increased in concentration within minutes of the initiation of a stressful experience [5,6]. To facilitate further investigation of the effects of acute stressors on innate immunity, a means was needed to obtain blood and other tissue samples from fish, which were minimally stressed or alarmed. Reliance on blood vessel cannulation [9] was rejected as the surgical trauma was considered likely to induce an acute phase response that would complicate interpretation of immunological data. As corticosteroids have generally been found to respond over a time of several minutes [16] and to have delayed (often transcription-dependent) effects, whereas catecholamines respond within seconds and tend to have 'instantaneous' membrane receptor-mediated effects, it was chosen to focus on adrenergic events, while monitoring corticosteroid levels in the plasma. The work addresses concerns of any physiologist for whom the reduction or elimination of stress responses is desirable. Several protocols were used in an effort to reduce the catecholamine surge, including surreptitious anesthesia in the home tank, concussion, acclimation in dark boxes, and the addition of α- and β-adrenergic receptor blockers to the diet. Efficacy was determined by the concentrations of catecholamines in the plasma, and by spleen:somatic indices. It was found that the appropriate administration of a selected anesthetic offers an easy means to minimize sympathetic activation and its consequences.

2. Materials and methods

2.1. Animals

Shasta strain rainbow trout (*Oncorhynchus mykiss*) were bred and raised at the Food Toxicology Laboratory, Oregon State University. The trout were maintained on a 12 h light/dark cycle with constantly flowing, single-pass well water at 12°C and fed three times weekly with Oregon Moist pellet.

2.2. Handling stress treatment

Ten fish (150–300 g) were stocked in each of three 400 l tanks 1 week prior to experiments to allow for acclimatization. Food was withheld on sampling days. Samples were taken between 08:00 and 10:00 h to minimize possible temporal variations of the hormone levels. On each day of sampling, an initial fish was quickly netted, killed by cerebral concussion and bled from the caudal vein using a heparinized syringe. Four more fish, from the same tank, were then netted and held in the air for 30 s. One fish was concussed and bled immediately and the other three were placed in a bucket (30 cm in diameter) containing water that was only half as deep as the dorso-ventral axis of the fish. After 30, 60 and 90 s individual fish were recaptured from the bucket, concussed and bled. This was repeated in each tank on 2 separate days, 1 week apart. Blood samples were kept on ice for approximately 30 min until centrifuged at $500 \times g$ and 4°C. Plasma was aliquotted and stored at −80°C until assayed for catecholamines and cortisol levels.

2.3. Anesthesia in the home tank

Ten fish were stocked in each of five 400 l tanks and allowed to acclimate for 2 weeks. On each of 5 separate days of the experiment, 100 ml of 2-phenoxyethanol (2-PE) (Sigma, Saint Louis, MO), mixed by shaking with 100 ml of water, was infused over 30 seconds into the tank via the water inflow to avoid unnecessarily disturbing the fish. This was calculated to yield 0.025% of 2-PE. The authors worked quietly and did not lift the tank lid until 2 min after the introduction of 2-PE. Each fish was then bled (approx. 1 ml) from the caudal vein using heparinized syringes and placed in a separate net either in the tank with the anesthetic (resting group) or, after being revived in clean water, the fish were held in the air for 30 s, then placed in separate buckets containing shallow, fresh, clean water (stressed group). Fish were re-bled 10 min later. Samples were stored on ice until centrifuged. Plasma aliquots were frozen at −80°C until assayed for levels of catecholamines and cortisol.

2.4. Anesthesia in black box

Two black Plexiglas boxes (L 40 cm × W 30 cm × D 7 cm), with six slots each and a black lid covering the

entire box [9], were stocked with one trout (100–150 g each) per slot, 1 week prior to experiment. Each box was supplied with constantly flowing (4 l/min) 12°C well water. On the day of sampling, the 12 fish were anesthetized; each box received 7 ml of 2-PE mixed with 13 ml of water infused over 30 s. One minute later, the water flow was turned off so that the fish would remain anesthetized when blood samples were taken for catecholamine determination. After 1 more min, the fish were bled from the caudal vessel. The blood samples were stored on ice then centrifuged at $500 \times g$ at 4°C; plasma was aliquotted and frozen at −80°C for subsequent analysis of adrenaline concentrations.

2.5. Effects of α- and β-adrenergic antagonists administered in the diet

Ten fish were stocked in each of four tanks (400 l) and allowed to acclimatize for 1 week. Food was withheld for the last 2 days to improve the appetite of the fish before the treatment diet was given. On the day of the treatments, fish in two tanks (treatment resting and treatment stressed) were fed propranolol (β-receptor antagonist) (Sigma) and phentolamine (α-receptor antagonist) (Sigma) mixed into their regular food at a concentration of 10 mg of each drug/1.5 g of food. The control group was fed drug-free diet. The food (control and treatment) was supplied at a rate of 3% of the total estimated body weight of the fish in the tank. Four hours after feeding, the fish were anesthetized and bled. The fish in the *resting* groups (control and drug-treated, ten fish in each group) were anesthetized with 2-PE using the tank treatment described above. Fish in the two *stressed* groups (control and drug-treated) were netted and held in the air for 8 min, then killed by cerebral concussion. Spleens were quickly removed, carefully dissected free of any extraneous tissue, and placed in pre-weighed tubes on ice for subsequent weighing and calculation of the spleen:somatic index — see below). After the removal of the spleen, the fish were bled from the caudal vein with a heparinized syringe. The blood was placed on ice, centrifuged, and the plasma was aliquotted and stored at −80°C until assayed for catecholamines and cortisol levels. This experiment was repeated once.

2.6. Catecholamine analysis

Catecholamine levels (noradrenaline and adrenaline) in plasma were measured on a Shimadzu HPLC system with an electrochemical detector. Plasma (100 μl) was mixed with 1 ml 0.1 M of perchloric acid. Then 3,4-dihydroxybenzylamine (DHBA) was added as an internal standard (50 μl of a 4.5 nmol/ml aqueous solution). After being vortexed, samples were centrifuged ($8000 \times g$, 4°C), then 1 ml of the deproteinized supernatants were transferred to a tube containing 10 mg of acid washed alumina (Sigma), 25 μl of 5 mM sodium metabisulfite (Sigma) and 25 μl of 10% ethylene diamine tetra-acetic acid (wt:v) (Sigma). Tris buffer (600 μl, 2.0 M, pH 8.6) was added and samples were vigorously vortexed. The tubes were then placed on a rocker and the catecholamines were allowed to adsorb onto the alumina for 30 min. This was followed by a centrifugation at $2000 \times g$ and the alumina was subsequently washed three times with 1 ml of chilled Nanopure water. The catecholamines were then eluted with 100 μl 0.1 M perchloric acid and stored at −80°C until analysis of 10 μl aliquots on the HPLC. Adrenaline and noradrenaline levels were calculated by the relative areas of their peaks compared to the peak area for the internal standard (DHBA).

2.7. Cortisol analysis

Cortisol levels were determined by Radio Immuno Assay [17] in the laboratory of Carl Schreck (Department of Fisheries and Wildlife, Oregon State University).

2.8. Spleen index

Tubes containing spleens were re-weighed, and the spleen weights were determined by difference. Each fish was weighed, and its spleen:somatic index (SSI) was calculated by the formula SSI = spleen weight/body weight × 100.

3. Results

3.1. Handling stress treatment

This was designed to mimic a handling stress typically encountered by trout in hatcheries and in research laboratories. Short-term changes of catecholamine and cortisol levels were measured. The stress of netting and a 30 s air exposure elicited increases of both adrenaline from 196 ± 22.26 to 395 ± 77.68 pmols/ml and cortisol from 2.37 ± 1.69 to 16.83 ± 10.08 ng/ml (Fig. 1). When the fish were placed in a bucket of shallow water, both the adrenaline and cortisol concentrations initially decreased, to 229 ± 29.17 pmols/ml for the adrenaline and 1.80 ± 0.79 ng/ml for the cortisol. However, the levels started climbing again; after being captive 30 s more in the shallow bucket, adrenaline reached its highest level in this experiment, 582 ± 98.31 pmols/ml. After an additional 30 s in the bucket the adrenaline level was

decreased to 445 ± 75.45 pmols/ml. The cortisol levels followed a similar trend, increasing to 13.58 ± 8.6 ng/ml at 90 s after the initial netting and 22.53 ± 10.45 ng/ml after 120 s. There were a significant increase of the adrenaline levels after 30 s ($P < 0.05$) and the cortisol levels 90 s after the stressor was initiated ($P < 0.05$), using a randomized block design and ANOVA.

3.2. Anesthesia in the home tank

Adrenaline and cortisol levels were determined in plasma from control trout as well as trout exposed to a 30 s air stress followed by 10 min in a bucket with shallow water. The average resting adrenaline level was 19.39S.E. ± 2.69 pmol/ml ($n = 25$) (Fig. 2), which is similar to the adrenaline level seen in the black box treatment (see below). The values for cortisol and lysozyme levels in these fish have also been reported [6]: the resting cortisol levels ranged from 1.25 to 17.60 ng/ml (mean = 7.88 ± 3.55). The resting adrenaline and cortisol levels were analyzed from two other similar experiments.

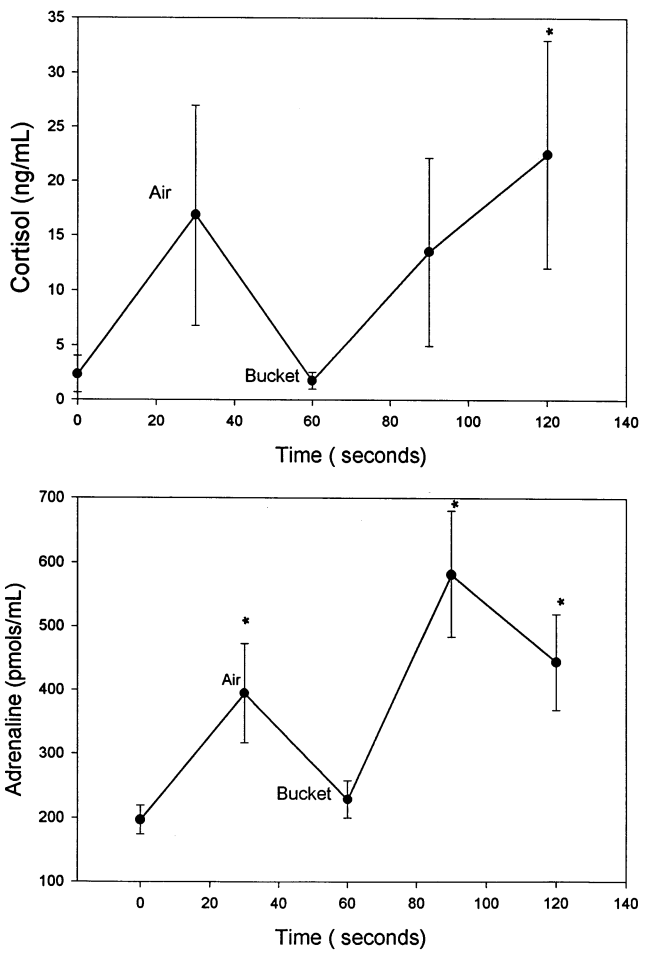

Fig. 1. The kinetics of change in cortisol and adrenaline levels in trout undergoing a 'handling stress'. The fish were netted (time = 0), held in the air for 30 s (time = 30), and bled after cerebral concussion, or placed in bucket of shallow water for 30, 60 or 90 additional s (time = 60, 90 and 120) and subsequently re-captured and bled. $N = 5$ for each data point. There were significant increases of the adrenaline levels after 30 s ($P < 0.05$) and the cortisol levels 120 s after the stressor was initiated ($P < 0.05$), using a randomized block design and analysis of variance (ANOVA).

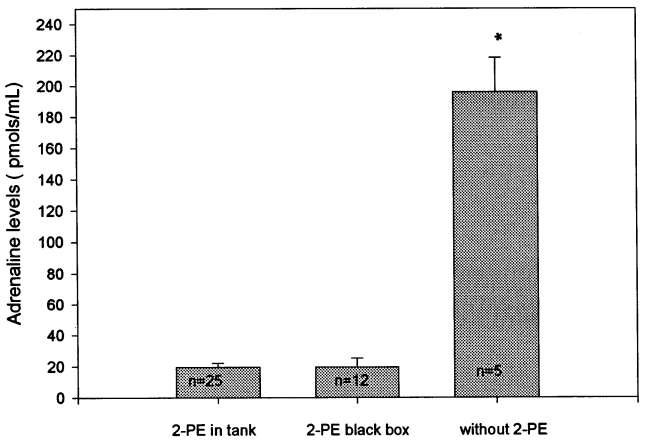

Fig. 2. Adrenaline levels in trout plasma following different treatments: anesthesia in the home tank, anesthesia in a black box, and netting and concussing without anesthesia. Left and middle bars: 2-phenoxyethanol (PE) was surreptitiously infused into the inflow water and the trout were left undisturbed for 2 min until they were anesthetized. Right bar: fish were captured in a 400 l tank without the use of anesthetic, concussed and bled quickly (generally less than 1 min).

3.3. Black box treatment

The adrenaline levels measured in the black box treatment experiment (Fig. 2) remained low in all 12 fish, with an average of 19.58 ± 5.65 pmols/ml.

3.4. Effects of α and β-antagonists administered in the diet

Among the fish that were not intentionally stressed ('resting'), those given phentolamine and propranolol had significantly higher adrenaline levels [379.9 ± 82.77 pmols/ml ($n = 12$)] than those that were drug-free [63.25 ± 9.74 pmols/ml ($n = 13$)] ($P < 0.001$, Mann–Whitney rank sum test) (Fig. 3). Cortisol levels were also higher in these resting drug-treated fish (66.32 ± 24.22 ng/ml) than in the resting drug-free controls (17.5 ± 4.84 ng/ml). However, the difference was not significant using the Mann–Whitney rank sum test.

Both adrenaline and cortisol values increased with stress. Among drug-free fish, cortisol increased with stress (to 24.5 ± 4.6 ng/ml), but in the drug-treated fish, cortisol (already high — see above) was not increased further as a result of stress (59.9 ± 15.6 ng/ml). However, among this stressed group there was high variance in the data (S.D. = 58.5 ng/ml). Adrenaline increased to 1555 ± 366 pmols/ml in the drug-free fish when

stressed, and to 1601 ± 146 pmols/ml in the drug-treated fish when stressed.

The SSI were significantly different ($P < 0.001$ using the Mann–Whitney rank sum test) between the control (0.084 ± 0.010) and drug-treated (0.124 ± 0.009) resting groups, as well and in the stressed state (0.065 ± 0.005 in controls, and 0.119 ± 0.008 in drug treated fish; $P = 0.0003$) (Fig. 4).

4. Discussion

A common means of preparing fish for experimental handling is to capture them and transfer them to a smaller container containing anesthetic, commonly MS-222 or buffered benzocaine [10]. This procedure exposes the fish to the stress of being chased, netted, passed through the air, put into a small, unfamiliar container frequently crowded with other individuals, before the anesthetic begins to take effect. In rainbow trout, this procedure, even when executed efficiently, is clearly sufficient to elicit sympathetic activation. It was satisfying, therefore, to note the markedly lower levels of plasma adrenaline that were obtained by the surreptitious infusion of 2-PE into the home tank prior to capturing the fish. While use of this drug is not permitted in fish destined for human consumption, its property of minimally eliciting sympathetic arousal [10] makes it an important drug for fish physiological research. However, lower levels of catcholamines have been measured in cannulated fish, but for our purposes cannulation was not an option because of the risk of activating the immune system. The observation of reduced levels of both cortisol and adrenaline at one minute, after increased levels at 30 s, was surprising. The speed of the initial rises certainly implies the existence of presynthesized pools of both adrenergic and steroid hormones, or their precursors. It remains unclear whether the subsequent transient decreases were due to a relief that was perceived on return to water, whether these were due to natural depletion of the putative presynthesized pools and metabolism of the released hormones, with a delay before de novo synthesis could re-supply the molecules or that the free cortisol had time to bind to the cortisol binding protein that circulates in plasma.

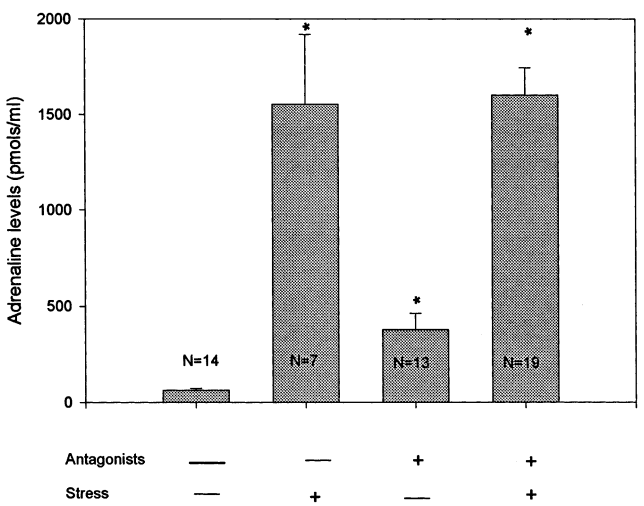

Fig. 3. Effects of α and β-adrenergic receptor antagonists, supplied in the diet, on adrenaline levels in trout plasma.

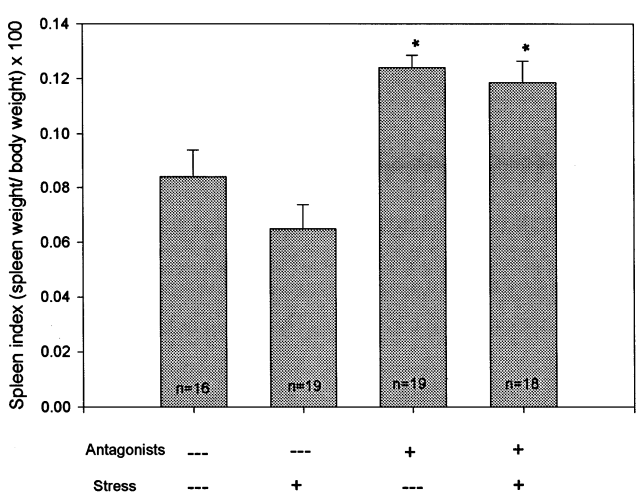

Fig. 4. The influence of dietary α and β-antagonists on spleen:somatic indices (SSI). The antagonists blocked the sympathetic response evidenced by a reduced contraction of the spleen in fish fed the treatment diet as compared to fish fed a regular diet.

Others have used black boxes, together with cannulation of the fish, as means to obtain quiescent animals [9]. The work confirmed the efficacy of black box confinement. Cannulation has been avoided, since the ultimate goal is to obtain fish without activating the immune system, and invasive surgery invariably induces an acute phase response. An advantage of confinement in black boxes may be the need for significantly reduced amounts of anesthetic: six fish could be anesthetized with 7.0 ml 2-PE in the black boxes, while 100 ml was needed to anesthetize ten fish in the home tanks.

However, since levels of stress hormones in the plasma were no lower in fish held in black boxes than in those anesthetized in their home tanks, the simpler system (home tank anesthesia) is preferred.

The elevated plasma levels of adrenaline that were observed in resting fish fed with α and β-antagonists are taken to imply homeostatic feedback pathways in these fish. By inhibiting adrenergic receptors, these drugs may have elicited an increased need for the native ligands, leading to the values we observed. Alternatively, clearance may have been slowed due to receptor occupation by the antagonists. It is clear from the SSI for these fish that the drugs were effective as used. When effective, adrenergic receptor blockers bind to adrenergic receptors on target tissues, antagonizing the normal physio-

logical responses (such as the contraction of smooth muscles in the spleen) to the relevant ligands (adrenaline and noradrenaline). Thus, the larger SSI seen in the drug-treated fish indicated that the antagonists blocked the normal physiological response. The antagonists had the anticipated effect on SSI, presumably resulting from a reduction of smooth muscle contraction. The fact that SSI values reported here are lower than those reported by others [15] suggests that room exists for further refinement of the regimen: both dose and time need to be optimized if adrenergic receptor blockers are to be effectively administered per os

This work has demonstrated an easy and practical means to prepare aquatic vertebrates for experimental manipulations in cases where the perception of stress, and the stress response, should be kept to a minimum. Both the anesthetic (2-PE) and the manner of its administration are likely important for the efficacy of the treatment.

Acknowledgements

We are grateful to members of the Carl Schreck Laboratory, Oregon State University, for determining levels of cortisol in plasma samples, to David Williams for access to the HPLC apparatus, and Gail Orner for instruction in its use, and to George Bailey and others in the Marine Freshwater Biomedical Center at Oregon State University for the provision and husbandry of the rainbow trout. We thank Ben Gilchriest and an anonymous referee for suggestions that improved the manuscript. Financial assistance was received from the NIEHS grants ES-03850 and ES-00210 supporting respectively Oregon State University's Marine-Freshwater Biomedical Research Center and Environmental Health Sciences Center.

References

[1] Bayne CJ, Levy S. Modulation of the oxidative burst in trout myeloid cells by ACTH and catecholamines: mechanisms of action. J Leukocyte Biol 1991;50:554–60.

[2] Bayne CJ, Levy S. The respiratory burst of rainbow trout phagocytes is modulated by sympathetic neurotransmitters and the 'neuro' peptide ACTH. J Fish Biol 1991;38:609–19.

[3] Bernard C. Lectures on the physiological properties and the pathological alterations of the liquids of the organism. In: Langley LL, editor. Homeostasis: Origins of the Concept'. Stroudsburg: Dowden, Hutchinson and Ross, 1973. Originally published in: Claude Bernard, Lecons sur les proprietes physiologiques et les alterations pathologiques des liquides de l'organisme, Paris: Bailliere, 1859.

[4] Cannon WB. Bodily changes in pain, hunger, fear and rage. An Account of Recent Researches into the Function of Emotional Excitement. New York: D. Appleton and Co, 1929.

[5] Demers NE, Bayne CJ. Plasma proteins of rainbow trout (*Oncorhynchus mykiss*): Immediate responses to acute stress. In: Modulators of Fish Immune Responses. Models for Environmental Toxicology/Biomarkers, immunostimulators, vol. I. Fair Haven: SOS, 1994.

[6] Demers NE, Bayne CJ. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. Dev Comp Immunol 1997;21:363–73.

[7] Flory CM. Autonomic innervation of the spleen of the coho salmon, *Oncorhynchus kisutch*: a histochemical demonstration and preliminary assessment of its immunoregulatory role. Brain Behav Immun 1989;3:331–4.

[8] Flory CM. Phylogeny of neuroimmunoregulation: effects of adrenergic and cholinergic agents on the in vitro antibody response of splenic leukocytes of the rainbow trout, *Oncorhynchus mykiss*. Dev Comp Immunol 1990;14:283–94.

[9] Gamperl AK, Vijayan MM, Boutilier RG. Epinephrine, norepinephrine, and cortisol concentrations in cannulated seawater-acclimated rainbow trout (*Oncorhynchus mykiss*) following black-box confinement and epinephrine injection. J Fish Biol 1994;45:313–24.

[10] Iwama GK, McGeer JC, Pawluk MP. The effects of five anesthetics on acid–base balance, hematocrit, blood gases, cortisol, and adrenaline in rainbow trout. Can J Zool 1988;67:2065–73.

[11] Kiecolt-Glaser JK, Cacioppo JT, Malarkey WB, Glaser R. Acute psychological stressors and short-term immune changes: what, why, for whom, and to what extent? Psychosom Med 1992;54:680–5.

[12] Matthews KA, Gaggiula AR, McAllister CG, Berga SL, Owens JF, Flory JD, Miller AL. Sympathetic reactivity to acute stress and immune response in women. Psychosom Med 1995;57:564–71.

[13] Maule AG, Tripp RA, Kaattari SL, Schreck CB. Stress alters immune function and disease resistance in chinook salmon (*Oncorhynchus tshawytscha*). J Endocrinol 1989;120:135–42.

[14] Naliboff BD, Solomon GF, Gilmore SL, Fahey JL, Benton D, Pine J. Rapid changes in cellular immunity following a confrontational role-play stressor. Brain Behav Immun 1995;9:207–19.

[15] Pearson MP, Stevens ED. Size and hematological impact of the splenic erythrocyte reservoir in rainbow trout, (*Oncorhynchus mykiss*). Fish Physiol Biochem 1991;9:39–50.

[16] Pottinger TG, Moran TA. Differences in plasma cortisol dynamics during stress in two strains of rainbow trout (*Oncorhynchus mykiss*). J Fish Biol 1993;43:121–30.

[17] Redding JM, Schreck CB, Birks EK, Ewing RD. Cortisol and its effects on plasma thyroid hormones and electrolyte concentrations during seawater acclimation in yearling coho salmon, *Oncorhynchus kisutch*. Gen Comp Endocrinol 1984;56:146–55.

[18] Ruis MAW, Bayne CJ. Effects of acute stress on blood clotting and yeast killing by phagocytes of rainbow trout. J Aquat Anim Health 1997;9:190–5.

[19] Schreck CB. Immunomodulation: endogenous factors. In: Iwama G, Nakanishi T, editors. The Fish Immune System. Fish Physiology, vol. 15. New York: Academic Press, 1996.

[20] Selye H. Stress and the general adaptative syndrome. Br Med J 1950;1:1383–92.

[21] Selye H. The evolution of the stress concept. Am Sci 1973;61:692–9.

[22] Thomas P. Molecular and biochemical responses of fish to stressors and their potential use in environmental monitoring. In: Adams SM, editor. Biological Indicators of Stress in Fish. Am Fish Soc Symp 1990;8:9–28.

[23] Wedemeyer GA, Barton BA, MacLeay DJ. Stress and acclimation. In: Schreck CB, Moyle A, editors. Methods for Fish Biology. Bethesda, MD: American Fisheries Society, 1990:451–77.