

**INTERACTION OF STRESS, PATHOGENS  
AND DEVELOPMENT  
ON THE BEHAVIOR OF TELEOSTS**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Fishes are frequently exposed to multiple, sequential or concurrent stressors. It appears that the physiological response to such events is adaptive in terms of resisting the stressors but could be maladaptive in terms of allostatic load (Sterling and Eyer, 1988; McEwen, 1998). For fish this consists of tradeoffs between the immediate benefits of short term survival and diminished longer-term fitness as evidenced in reduced growth, reproductive capacity, and disease resistance (Schreck, 2000).

Juvenile chinook salmon, *Oncorhynchus tshawytscha*, in the Columbia River system are exposed to multiple, sequential stressors as they migrate into lower

river systems and make the transition to seawater. As judged from eight years' research on stress and health physiology based on (1) the primary stress factor cortisol, (2) energetic factors such as glucose and lactate, (3) specific and non-specific immune system responses including number of antibody producing cells and respiratory burst activity, (4) activity levels of enzymes in the circulation, (5) osmoregulatory capacity assessed by saltwater tolerance and saltwater preference tests, and (6) resistance to bacterial pathogens such as *Renibacterium salmoninarum* we predicted that stressed or pathogen-infected salmonids would have retarded or reversed smoltification and hence have impaired ability to successfully enter the ocean.

Under laboratory conditions, mildly stressed chinook salmon "smolts" in a horizontal fresh-saltwater gradient had reduced preference for salt water compared to unstressed controls that essentially all selected saltwater. More severe stress exacerbated the saltwater avoidance, as did infection with *R. salmoninarum*. Simulation of stress associated with passage through the Snake and Columbia River hydropower system (eight dams) also reduced saltwater preference relative to unstressed controls. While such a shift in behavior would be ecologically risky, the physiology of the fish suggested that they were "hardened" by the exposure to the multiple stressors since they recovered faster from the eighth than from the first stressful experience. Threat of avian predation (a simulated hazard from above) resulted in transient saltwater selection by stressed smolts that avoided salt water otherwise. However, avoidance behavior of a simulated threat from below by saltwater-preferring smolts consisted of fish swimming up to but not through the halocline.

Salmon entering the Columbia River estuary have been either stressed by passage through as many as eight dams or by a management program in which fish are transported and released upstream of the estuary in barges. Our experiments in the laboratory and assessment of fish in the field suggest that stress and pathogens affect success at seawater entry and responsiveness to threat of predation. Radiotelemetry of juvenile salmon in the Columbia River showed that that nearly 100% of the fish successfully migrate the last 200 km to the upper estuary, but that approximately 15% are taken by avian predators while traveling the last 25 km to the ocean.

We expanded a life history-based population simulation model designed to assess risk in salmon (Oosterhout, 1999) to include effects of stress, improper development (smoltification), pathogens, and predation as hazards. The model was run for 1,000 iterations per year for 100 years using present-day chinook

salmon abundance as a starting point to generate population trends. Employing data and inferences from stress physiology from either our field or laboratory studies as variables, the model revealed that stress encountered upstream on mortality of salmon at ocean entry is significant in terms of recovery of stocks at risk of extinction. However, stress in juvenile salmon appears to be an important population regulating factor only when habitat conditions are poor.

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**BEHAVIORAL AND NEUROENDOCRINE CORRELATES  
OF SOCIAL STRESS IN FISH**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Salmonid fish often develop strong dominance hierarchies, especially at juvenile life stages when they are highly aggressive and territorial in nature. Socially subordinate fish are characterized by a general behavioral inhibition, including suppressed aggressive behavior, an inhibition of appetite and feed intake, and lowered spontaneous locomotor activity (Øverli *et al.*, 1998). In addition, subordinate fish usually display chronically elevated plasma levels of cortisol (Winberg and Lepage, 1998; Øverli *et al.* 1999).

Behavioral and neuroendocrine stress responses are to a large degree controlled and integrated by control mechanisms within the brain, and the central monoaminergic systems appear to play important roles in these control mechanisms. We have observed that escalated fights for social dominance in pairs of juvenile rainbow trout (*Oncorhynchus mykiss*) results in a rapid

activation of the norepinephric, dopaminergic and serotonergic systems in the brain, of both winners and losers, e.g. future dominant and subordinate pair members (Øverli *et al.*, 1999). However, in the dominant fish brain monoaminergic activity as well as plasma cortisol levels rapidly returns to baseline levels following the settlement of the fight for social dominance, whereas in subordinates brain serotonergic activity and plasma cortisol levels remain elevated. Especially the stress-induced activation of the brain serotonergic systems shows very weak signs of habituation, and brain serotonergic activity remains elevated in subordinate fish even after long-term social interaction in established dominance hierarchies (Winberg and Nilsson, 1993).

Social subordination also results in a rapid and chronic elevation of pituitary pro-opiomelanocortin (POMC) mRNA levels in rainbow trout (Winberg and Lepage, 1998). In recent experiments we have shown that socially subordinate Arctic charr (*Salvelinus alpinus*) display an elevation in plasma levels of melanocytostimulating hormone (MSH) (Höglund *et al.*, in press). In Arctic charr, social subordination also results in skin darkening, an effect that appears to be mediated by a MSH, and that may serve as a social signal decreasing aggressive behavior in established dominance hierarchies (Höglund *et al.*, in press).

Serotonin (5-hydroxytryptamine, 5-HT) has been suggested to inhibit aggressive behavior, locomotor activity and feed intake, and also to stimulate the hypothalamic-pituitary-adrenocortical axis in mammals. We have obtained results demonstrating that 5-HT has similar effects in teleost fish, and that brain 5-HT may be involved in mediating both the behavioral and endocrine effects of social subordination in salmonids. In subordinate rainbow trout the ratio of 5-hydroxyindoleacetic acid (5-HIAA, the major 5-HT metabolite) to 5-HT in the hypothalamus, an index 5-HT activity, correlates with plasma levels of cortisol (Winberg and Lepage, 1998). Further, 8-OH-DPAT, a specific 5-HT<sub>1A</sub> agonist, elevates plasma levels of cortisol in cannulated rainbow trout in a dose dependent manner (Winberg *et al.*, 1997). Using receptorautoradiography with <sup>3</sup>H-8-OH-DPAT as the radioligand we have been able to show that 5-HT<sub>1A</sub> receptors are present in the rainbow trout brain, including the hypothalamus. The stress-induced elevation of plasma MSH, and thus skin darkening, may also, at least in part, be mediated by 5-HT. Plasma levels of MSH correlates with brain 5-HIAA/5-HT ratios (Höglund *et al.*, in press), and 8-OH-DPAT (i.p.) induces skin darkening in Arctic charr.

Pharmacological stimulation of the brain 5-HT system inhibits spontaneous locomotor activity and general behavioral responsiveness in Arctic charr, whereas pharmacological inhibition of the brain 5-HT system has the opposite effects (Winberg and Nilsson, 1993). Serotonin is synthesised from the amino acid L-tryptophan (TRP), and the rate of 5-HT synthesis *in vivo* appears to be restricted by TRP availability in both fish and mammals. In a recent experiment we observed that dietary supplementation of TRP elevates brain 5-HT activity and suppresses aggressive behavior in juvenile rainbow trout.

In conclusion, our studies have shown that social subordination results in chronic stress, a stress that initially is related to losing aggressive interactions but that later on when the hierarchy is established seems to be more related to lack of control and the constant threat imposed by dominant fish. Socially subordinate fish are characterized by a chronic activation of the brain 5-HT system. This monoaminergic neurotransmitter system appears to play a key role in controlling and integrating behavioral and neuroendocrine stress responses. A stress-induced activation of the brain 5-HT system may mediate several other behavioural and physiological effects of social subordination in salmonid fish, e.g. activation of the hypothalamic-pituitary-interrenal axis, elevation of plasma MSH levels causing skin darkening, inhibition of aggressive behavior, and a general suppression of behavioral responsiveness to environmental stimuli.

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**WHY DID THE SALMON CROSS THE ROAD?**

**THE NEUROCRINE CONTROL**

**OF LOCOMOTOR BEHAVIOUR**

**IN JUVENILE SALMONIDS**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Activation of the stress response generally alters locomotory behaviours in ways that may function to increase survivability. A large body of evidence exists showing that among mammals a number of neuroactive chemicals are involved in controlling stress induced locomotor activity. However, the neurocrine/endocrine factors controlling locomotory movements in fish are less understood. We have investigated the role of the peptide corticotropin releasing hormone (CRH) and the neurotransmitters serotonin, dopamine, and (-amino-n-butyric acid (GABA) on locomotor activity in juvenile chinook salmon (*Oncorhynchus tshawytscha*). These four systems were manipulated by injecting both agonists and antagonists into the third ventricle to allow for diffusion to various brain sites. Locomotor activity following recovery was monitored by remotely activated video cameras placed above the tanks. Activity was later quantified by superimposing a grid onto the image and counting the number of line crossings within a specific period.

CRH and serotonin acted synergistically to increase spontaneous locomotor activity during the test. The combined action of CRH and serotonin was inhibited by GABA. In contrast, GABA alone stimulated activity, an effect that appears to be mediated through the dopaminergic system. The results of these experiments suggest that the interactions amongst the CRH, serotonergic, dopaminergic and GABAergic systems are important for determining the final behavioural output. CRH is believed to be the primary activator of the hypothalamic-pituitary-interrenal axis in fish, and as such is likely to play a central role during the stress response. The secretion of serotonin, dopamine, and GABA is also significantly altered during the stress response. Therefore, we hypothesise that CRH may co-ordinate both the behavioural and physiological changes observed during the stress response in fish by interaction with a number of neurocrine systems.

To investigate whether the activating effects of CRH noted above might have adaptive value during the stress response we tested experimentally the preference of fish for light or dark and their ability to find cover following ICV injections of CRH or saline. The behaviour of fish during the light/dark preference experiments was complex although fish injected with CRH did spend less time in the dark. However, we think that this may be due primarily to increased activity alone. Recently, we found that the ability of fish to find cover following a stressor was significantly reduced in fish that had received an injection of the CRH antagonist,  $\nabla$ -helical CRH. This suggests that during the stress response endogenous CRH may be important for reducing the likelihood of predation by facilitating cover seeking.

**PHYSIOLOGICAL EFFECTS OF BEHAVIOURAL INTERACTIONS  
IN ARTIFICIAL AND NATURAL ENVIRONMENTS**

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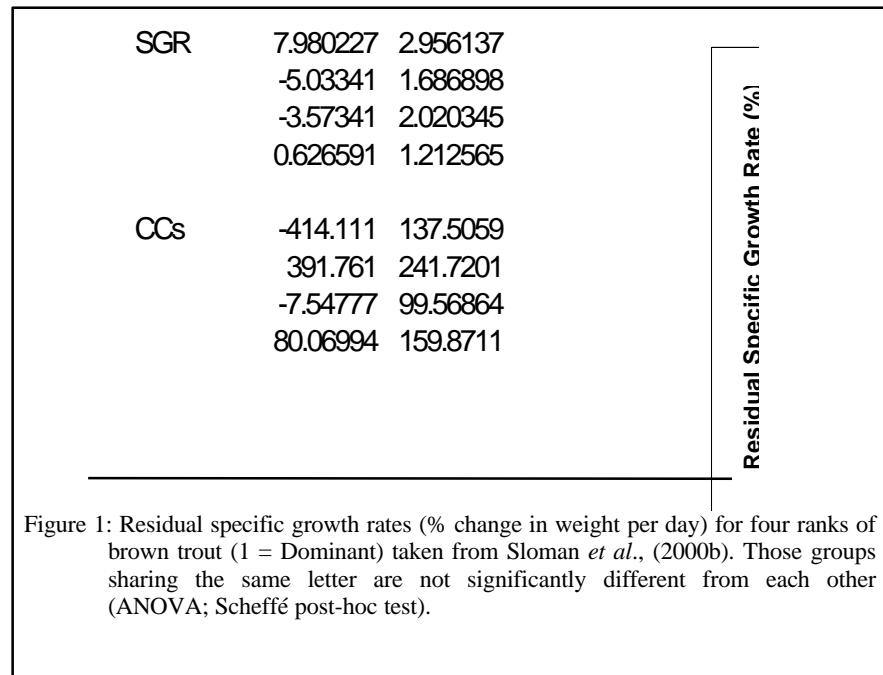
**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Social interactions and aggression among salmonid fish are known to induce stress responses, particularly in subordinate fish, and the physiological effects of these stress responses have implications for both aquaculture and fish in natural environments. The physiological responses exhibited by subordinate fish vary depending on the nature and extent of the social interaction and can also be influenced by the environment of the fish. Artificial environments generally elicit a larger stress response in the subordinate fish due to fish being held in close confinement. A time course study investigating the effects of social stress in rainbow trout, *Oncorhynchus mykiss*, and brown trout, *Salmo trutta*, confined in pairs demonstrated that after 48 h of confinement subordinate fish exhibited elevation of the stress hormone cortisol. In the more aggressive rainbow trout cortisol elevation was apparent after 4 h of confinement (Sloman *et al.*, unpublished a), demonstrating that the amount of social interaction and aggression has an effect on the cortisol response and can vary between salmonid species.

An increase in stress may also cause an elevation of metabolism. When brown trout were confined in pairs, the subordinate fish showed a significant increase in standard metabolic rate (Sloman *et al.*, unpublished b). The potential of the increases in blood plasma cortisol concentrations associated with subordination, to elicit chloride cell proliferation in the gill epithelia was also investigated, since artificial elevation of cortisol concentrations is known to induce

proliferation of chloride cells. However, when trout were confined in pairs no chloride cell proliferation was observed, although cortisol elevation occurred in subordinates (Sloman *et al.*, 2000a).

Dominance hierarchies do not always lead to stress responses in subordinates. In a study carried out under semi-natural conditions (in stream tanks), the formation of dominance hierarchies amongst groups of four brown trout did not elicit plasma cortisol elevation in subordinate fish. However, dominant fish still appeared to have a physiological advantage over subordinate fish in terms of growth; the growth of subordinates was significantly higher than in the other three ranks of fish (Sloman *et al.*, 2000b; Fig. 1).



In this particular study, sub-dominant (second-ranking) fish had the lowest growth rate and it is suggested that these fish adopted a high cost/high return strategy, competing with the dominant fish and therefore expended more energy than the other two ranks of subordinate fish which adopted a low cost/low return

strategy. Interestingly, sub-dominant fish demonstrated significantly higher numbers of chloride cells (Fig. 2), but whether the proliferation of chloride cells is related to changes in plasma cortisol concentrations remains unclear.

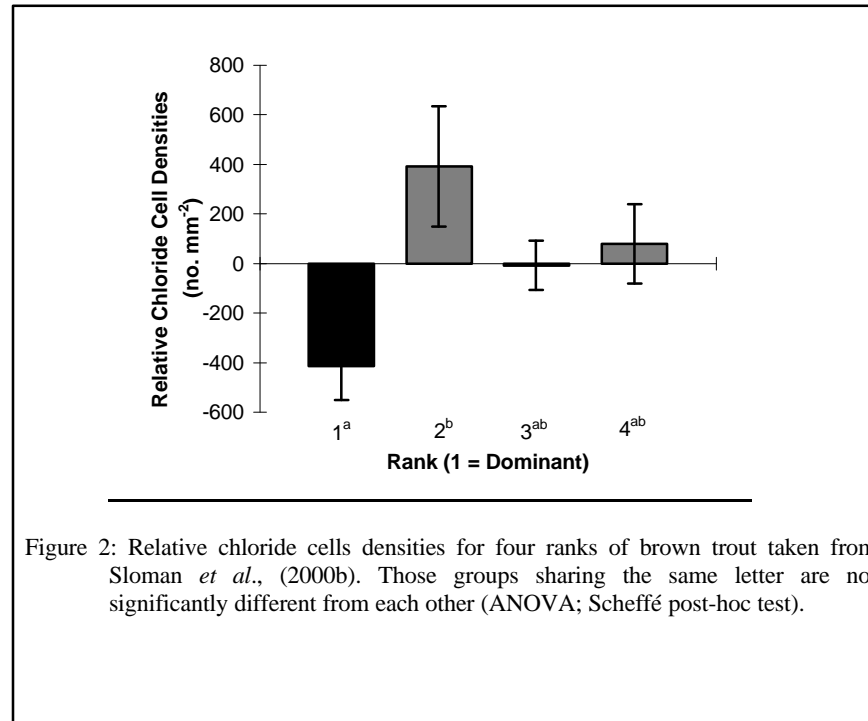


Figure 2: Relative chloride cells densities for four ranks of brown trout taken from Sloman *et al.*, (2000b). Those groups sharing the same letter are not significantly different from each other (ANOVA; Scheffé post-hoc test).

Further studies using semi-natural conditions investigated the effects of environmental perturbations on previously established dominance hierarchies (Sloman *et al.*, unpublished c). The environmental perturbation of lowered water levels, simulating drought, was found to disrupt the dominance hierarchy and the social structure of the groups of four brown trout. The normal growth advantages gained by dominant fish under constant semi-natural conditions were lost, and all fish exhibited similar growth rates. No effects of the environmental perturbation on plasma cortisol concentrations were seen suggesting that (a) environmental perturbations may disrupt social hierarchies and (b) these

perturbations are not necessarily stressful in themselves so the absence of a higher growth rate of dominant fish in the experimental groups was dependent on changes in social structure.

In conclusion, the physiological responses to social interaction and the formation of dominance hierarchies in salmonid fish are affected by the environment of the fish e.g. whether the environment is artificial or natural, and stable or subject to fluctuations. Known effects of social stress include decreases in growth rate and condition, and increases in plasma cortisol and standard metabolic rate. The potential of social stress to affect chloride cell proliferation is currently being investigated further.

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**IMPAIRED ADRENAL STEROIDOGENESIS  
IN FISH CHRONICALLY EXPOSED  
TO ENVIRONMENTAL XENOBIOTICS.**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

The increase in plasma cortisol levels in response to acute exposures to abiotic and biotic stressors has been documented in numerous laboratory studies with teleost fish. However, physiological responses to chronic exposures are less well understood, particularly the responses to chronic environmental exposures to pollutants. A reduced capacity to elevate plasma cortisol in response to a confinement stress has been reported in yellow perch, *Perca flavescens* and northern pike, *Esox lucius*, sampled at sites contaminated by mixtures of organic contaminants and metals (Hontela, 1998). The mechanisms through which chronic environmental exposures disrupt cortisol secretion have not been elucidated thus far. Therefore, the objective of the present study was to determine if a dose-response relationship can be established between the environmental exposure to pollutants, the tissue burden of pollutants and effects on the physiological and endocrine status of the fish.

**Materials and methods**

Fish were captured in six lakes situated along a metal contamination gradient in a mining region in northwestern Québec. Following capture, fish were placed for 24 hrs into floating enclosures in the lake to facilitate recovery from capture and reduce variability due to handling of the fish. Fish were sampled the next day between 10:00 and 11:00hr. One group was blood sampled following a 1 hr confinement stress and anesthesia (« stress » group). A second group was blood

sampled without the confinement stress and the head kidneys were dissected for the *in vitro* tests. To test the functional integrity of the interrenal tissue *in vitro*, individual head kidneys were cut into small fragments and divided among three microplate wells. Following a preincubation period to reach basal levels of cortisol secretion, the head kidney fragments were stimulated with ACTH (2 I.U.), dbcAMP (4 mM) or medium (MEM) only. Cortisol was assayed in the supernatants collected from the *in vitro* tests; cortisol, chloride and glucose were assayed in plasma from fish sampled with or without confinement. Metals (Cd, Zn and Cu) and metallothionein levels were measured in the liver and head kidneys of all the fish.

## Results

Levels of Zn, Cu and Cd, as well as metallothionein increased along a gradient, ranking the lakes in the following order of contamination: L. Dufault > L. Osisko > L. Vaudray > L. Bousquet > L. Opasatica > L. Dasserat. Concentrations of metals and metallothioneins were higher in the liver than in the head kidney (Table 1). The capacity to increase plasma cortisol levels in response to a standardized confinement stress decreased in relation to tissue burdens of metals in the liver and head kidney of the yellow perch. Stressed fish from the reference and intermediate lakes were able to increase their plasma glucose while this response was significantly impaired in fish from the most contaminated lake. No effects of contamination on plasma chloride levels were detected but confinement stress decreased significantly plasma chloride in all the fish (Table 2). *In vitro* response to ACTH and dbcAMP was lower in head kidneys from L. Osisko (contaminated lake) compared to L. Dasserat (reference lake). No differences in cortisol production were detected for the unstimulated head kidneys from the two lakes tested (Table 2).

**Table 1.** Concentrations of Zn, Cu, and Cd (mean±SE, µg.g dry wt), and correlations between concentrations of metallothionein (MT) [nmol metal binding sites (g dry wt)<sup>-1</sup>] and metal (M), in pooled samples of liver or interrenal tissue of adult yellow perch collected in six lakes from a mining area.

Lakes	Liver			Head kidney		
	[Zn]	[Cu]	[Cd]	[Zn]	[Cu]	[Cd]
OP	92.4±3.6 a	10.4±1.8 a	2.9±0.4 a	104.6±2.3 a	2.3±0.2 a	0.9±0.1 a
DS	98.6±4.2 ab	10.8±0.9 a	5.3±0.6 b	118.8±2.6 b	2.9±0.3 a	1.7±0.2 b
BO	106.5±5.4 ab	20.4±4.5 b	20.3±2.9 c	96.08±1.2 a	2.2±0.1 a	3.8±0.2 c
VA	108.9±1.6 b	12.9±0.7 ab	25.1±1.7 c	127.6±4.7 b	2.7±0.2 a	5.7±0.7 cd
OS	177.2±9.0 c	246.5±29.8 c	45.7±3.2 d	153.6±6.6 c	6.5±1.0 b	8.0±0.5 de
DT	151.1±3.7 c	148.5±11.1 c	61.3±5.3 d	227±10.3 d	6.4±1.5 b	12.6±0.4 e
Correlation	0.93 *	0.95 *	0.91 *	0.82 *	0.77 *	0.95 *

Means followed by the same letter are not significantly different, comparison between lakes only ( $p < 0.01$ , Tukey-Kramer HSD test). Number of replicates for each measure 7-8.\* -  $P < 0.001$  (Pearson's test).

Table 2. Cortisol *in vivo* post-stress and *in vitro* post-ACTH or dbcAMP, chloride and glucose (change post-stress (means  $\pm$  SE) in yellow perch collected in lakes from a mining area.

Lakes	Plasma parameters			Cortisol secretion by head kidney <i>in vitro</i> ng/ml/mg		
	Cortisol (ng/ml plasma)	Glucose ( $\Delta$ mg.ml plasma)	Chloride ( $\Delta$ mg.ml plasma)	ACTH	dbcAMP	none
OP	260 $\pm$ 11 ab	+0.12 $\pm$ 0.02* *	-15.0 $\pm$ 4*	2.5 $\pm$ 0.4 a	2.6 $\pm$ 0.2 a	1.5 $\pm$ 0.3 a
DS	340 $\pm$ 16 a					
BO	240 $\pm$ 19 abc					
VA	270 $\pm$ 20 ab	+0.11 $\pm$ 0.7* ab	- 17 $\pm$ 8 *			
OS	177 $\pm$ 14 c	0.00 $\pm$ 0.01	-33.0 $\pm$ 4 *	2.0 $\pm$ 0.3 b	1.8 $\pm$ 0.3 b	1.3 $\pm$ 0.3 b
DT	110 $\pm$ 10 d					

\*Significantly different from the no stress group ( $p < 0.05$ , t-test).

## Discussion

The capacity to secrete cortisol was significantly lower in perch from contaminated lakes, compared to reference lakes. The cortisol impairment was detected *in vivo* in fish subjected to a confinement stress, and also *in vitro* following a challenge with ACTH and dbcAMP. The secretory impairment could not be reversed by stimulation with dbcAMP, suggesting that intracellular steps following the cAMP generation may be impaired in corticosteroidogenic cells of fish from the most contaminated lakes. Head kidney and liver burdens in metals, as well as tissue levels of metallothionein followed a gradient similar to the secretory impairment, in a dose-related pattern. Our data provide evidence that accumulation of metals in the head kidney may be responsible for the functional impairment of the steroidogenic cells. Although *in vivo* exposures of rainbow trout to environmental levels of Cd up to 30 days in the laboratory elevates plasma cortisol and increases the responsiveness of the head kidney to ACTH *in vitro* (Brodeur et al., 1998), recent laboratory studies demonstrated a dose-dependant secretory impairment in rainbow trout head kidney cells acutely exposed *in vitro* to Cd, Zn, Cu or o,p'-DDD (Leblond and Hontela, 1999; Benguira and Hontela, 2000). These results suggest that the mechanism of the cortisol impairment in fish chronically subjected to environmental pollutants may be a disruption of adrenal steroidogenesis occurring when critical burdens of the xenobiotics, resulting from the chronic environmental exposures, are reached in the head kidney tissue.

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**BRAIN MONOAMINE CONCENTRATIONS  
AS PREDICTORS OF GROWTH INHIBITION  
IN CHANNEL CATFISH EXPOSED TO AMMONIA**

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A means of predicting the impact of environmental ammonia on channel catfish (*Ictalurus punctatus*) growth in ponds would be of value to farmers. Unfortunately, a simple correlation between ammonia concentration and growth would not be applicable in pond production systems because of the fluctuating nature of ammonia concentrations due to daily variation of pH (which affects the ratio of ammonia to the relatively-nontoxic ammonium) and seasonal variations in temperature and feeding rates (which affect the ammonia:ammonium ratio and nitrogen input rates, respectively). The use of physiological indicators may be an alternative. In this study, we determined the dynamics of selected monoamine neurotransmitters (i.e., indoleamine and catecholamine groups of neurotransmitters) in the brain of channel catfish chronically exposed to ammonia.

Growth (as indicated by weight gain and increase in total length) and condition factor decreased significantly with increasing exposure to ammonia during the 9-week study. The number of fish completing the study ( $80 \pm 14.1\%$ ) was not related to ammonia exposure and ranged from six to ten fish per recirculating system.

Concentrations of brain 5-hydroxytryptamine (serotonin, 5-HT, Figure 1A) and dopamine (DA, Figure 1B) decreased significantly with increasing exposure. The concentration of brain 5-hydroxyindoleacetic acid (5-HIAA) did not

increase due to ammonia exposure (Figure 1C); however, the 5-HIAA:5-HT ratio increased significantly (Figure 1D). Concentrations of 3,4-dihydroxyphenylacetic acid, epinephrine, 5-hydroxytryptophan (5-HTP), and norepinephrine (NE) did not change due to ammonia exposure.

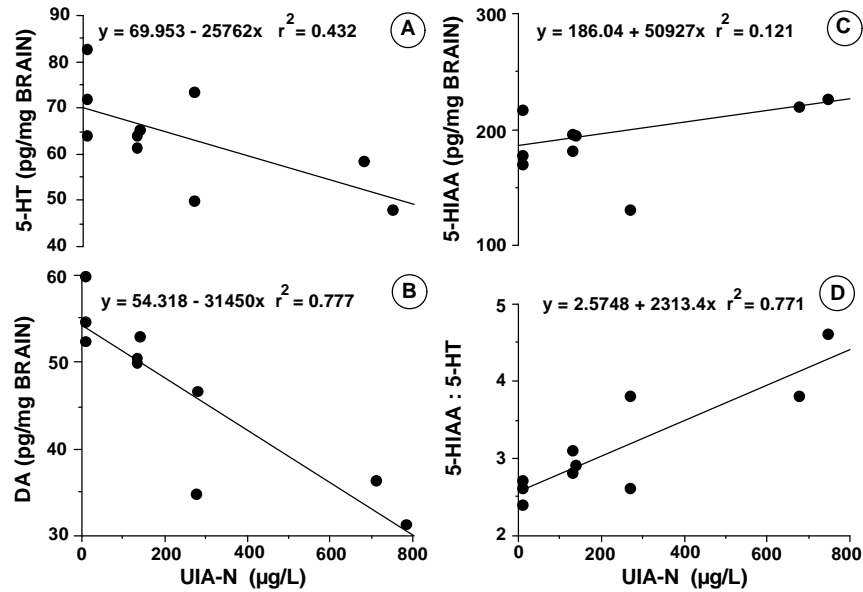


Figure 1. Brain serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA) and 5-HIAA/5-HT ratios in channel catfish after exposure for 9 weeks to un-ionized ammonia (UIA-N). The slopes of the 5-HT, DA and 5-HIAA:5-HT lines were significant ( $P \leq 0.05$ ).

The stepwise regression indicated that 88% of the ammonia-induced inhibition of growth could be predicted with two inputs: DA (partial  $r^2=0.7352$ ;  $P=0.0015$ ) and 5-HIAA:5-HT (partial  $r^2=0.1401$ ;  $P=0.0263$ ) with the model:

$$\text{Percent growth} = 101.21 - 52.23 (5\text{-HIAA:}5\text{-HT ratio}) + 4.02 (\text{DA as pg/mg}).$$

Inputs of the other monoamines measured did not significantly improve the predictive value of the model.

Stress-induced changes in serotonergic activity have been correlated with subordinate behavior and appetite loss in fish, resulting in reduced food intake, growth and production (Winberg et al. 1993, Alanära et al. 1998, De Pedro et al. 1998). These neuroendocrine changes have been suggested as a physiological mechanism underlying altered behavior leading to appetite loss and, thus, poor growth of captive fishes which may partly explain the correlation between the monoamine changes and growth inhibition observed in this study.

This study demonstrates the potential to predict ammonia-induced inhibition of growth in channel catfish with physiological changes. However, before such an approach can be applied to production situations, the dynamics of the physiological changes need to be better characterized with respect to short-term changes in ammonia concentrations. For example, if brain monoamine concentrations change in response to the daily rhythms of environmental ammonia in ponds, then sampling times must be fixed at the same time of day. Also, other stressors present in the pond that may effect brain monoamine concentrations must be identified to ensure that observed changes are interpreted correctly.

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**STREAM TEMPERATURE AFFECTS GROWTH AND  
STRESS INDICES IN JUVENILE CHINOOK SALMON**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Forest harvesting has the potential to impact freshwater aquatic ecosystems by causing greater variation in discharge, increased suspended sediment concentration and turbidity levels, increased mean temperature, and greater daily and seasonal fluctuations in temperature (Gregory et al. 1991). There is increasing evidence that retention of riparian vegetation adjacent to streams may mitigate some of the impacts of forest harvesting on physical changes to the aquatic ecosystem (Barton et al. 1985), but there have been limited studies to examine the effects on fish residing within these streams.

Chinook salmon (*Onchorynchus tshawytscha*) numbers are high in small tributaries of the Torpy River in central British Columbia, suggesting these streams are important juvenile rearing habitat (Shrimpton, Bourgeois, Quigley and Blouw, *unpublished data*). Our objective was to determine the effect of forest harvesting on fish populations residing in these small tributary streams. We conducted a sampling program to measure indices of growth and stress in juvenile chinook salmon. Fish were captured by electrofishing in streams flowing through harvested and non-harvested areas, and the mainstem river. In early summer, there was no significant difference in any of the parameters measured among the sampling sites. In late summer, however, fish sampled from streams flowing through non-harvested areas showed greater growth, as assessed by muscle RNA/DNA ratios and liver ornithine decarboxylase activity,

than fish from streams flowing through cut-blocks. Fish from the mainstem had the lowest indices of growth, lower condition factor and were smaller (Table 1).

**Table 1.** Mean water temperatures for August, 1998. Ornithine decarboxylase activity and condition factor were measured in fish sampled on September 3, 1998. Values are means  $\pm$  SD.

	Temperature $^{\circ}\text{C}$	ODC Activity $\text{pmol CO}_2 \text{ hr}^{-1} \text{ mg protein}^{-1}$	Condition Factor $100 \text{ g cm}^{-3}$
Non-harvested	$11.26 \pm 1.05$	$2.65 \pm 0.48$	$1.06 \pm 0.02$
Harvested	$12.45 \pm 2.12$	$1.83 \pm 0.26$	$1.04 \pm 0.02$
Mainstem	$16.75 \pm 1.89$	$1.71 \pm 0.18$	$1.02 \pm 0.01$

Plasma cortisol levels were not correlated with rearing environment, however gill cortisol receptors concentrations (Bmax) were higher in tributary streams than the mainstem. A significant inverse correlation was found between cortisol receptor Bmax and water temperature (Figure 1). Lower concentrations of gill cortisol receptor have been linked to a downregulation of receptors due to stress (Shrimpton and Randall, 1994), and may reflect chronic stress in fish sampled from streams with higher mean and greater daily variation in water temperature.

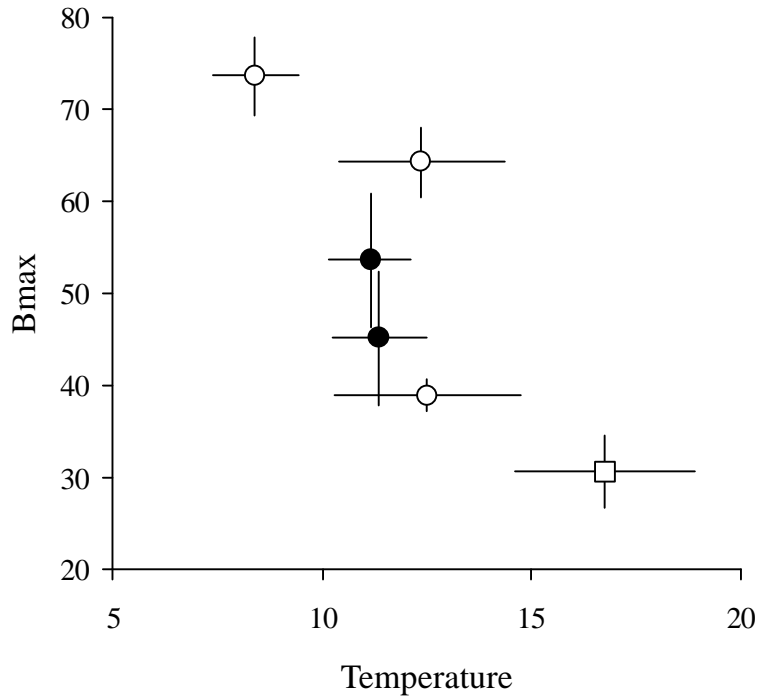


Figure 1. Corticosteroid receptor concentration ( $B_{max}$ ,  $\text{fmol mg protein}^{-1}$ ) versus temperature for juvenile chinook salmon sampled in early September. ●-fish caught in control (non-harvested) tributaries, ○-fish caught in streams adjacent to harvested areas, and □-fish caught in the mainstem river. Values are means  $\pm$  1 SD.

The importance of riparian reserves is reflected in harvest prescriptions mandated by the British Columbia Forest Practices Code (FPC). The FPC defines the dimensions and management guidelines for streamside buffer strips according to physical and biological stream attributes. For example, the FPC prescribes a 20 m riparian management zone (RMZ) for streams less than 1.5 m wide. This RMZ allows for a wide range of practices within the riparian zone of the stream, including removal of all timber. Following the experimental removal of riparian vegetation, we found water that flows through the

unbuffered creeks heats more than the water in the buffered creeks. As a consequence the diurnal change in temperature is greater in the unbuffered creeks. The diurnal changes in temperature and mean temperature, however, are greatest in the mainstem river.

Water temperature is an important parameter influencing stream ecology and fish productivity as it influences all biological processes including metabolic rates, growth, behaviour, and survival of fish populations. In addition, daily temperature fluctuations are stressful for fish and impact on energy reserves (Thomas et al. 1986). As the variation in temperature was greater in harvested streams and the mainstem river, these fish would be expected to experience the greatest stress. Additionally the higher temperature of the mainstem would affect the stress response of the fish as Barton and Schreck (1987) showed fish held in warmer water exhibit a more rapid and sustained response to stress than fish in cooler water. Turbidity of the mainstem was also significantly greater than the tributary streams. In juvenile salmonids, increases in suspended sediments have been shown to be stressful, leading to increases in circulating cortisol levels (Redding et al. 1987). As juvenile chinook show extensive use of tributary streams for rearing throughout their early life history, forestry practices that alter temperature regimes in these streams may be detrimental to growth and survival. This work emphasizes the importance of the riparian zones for the aquatic environment and particularly for fish residing in these streams.

### **Acknowledgments**

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**ARE BEHAVIOUR AND BODY COLOURATION  
EFFECTIVE DIAGNOSTIC TOOLS FOR ASSESSING  
ENVIRONMENTAL STRESS IN FISH?**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Physiological determinants of environmental stressors generally require either invasive or terminal sampling techniques, themselves imposing further stress and constraints on the animal subjects. Non-invasive methodologies utilising stress induced behavioural changes have resulted in successful studies of sub-lethal effects of stress. Video monitoring of behavioural interactions and general activity have been used within our laboratory in an attempt to integrate these observations within the known physiological and metabolic consequences of environmental stress.

For some time, there has been anecdotal evidence for colouration ('darkness') being a useful indicator of general stress in fish, but there are few published examples of quantification of this method in relation to environmental factors. A recently published study has quantified the effects of social stress and hierarchy on colour changes in salmon (O'Conner et al. 1999). In our presentation, we would like to focus upon this technique by examining the effects of environmental factors on these colour changes.

Using aluminium as the model stressor, we exposed brown trout (*Salmo trutta*) to a sub-lethal concentration of Al ( $13 \mu\text{g.l}^{-1}$  ramping from zero over a period of six hours) following acclimation to soft water and pH 5.2. Observations of swimming behaviour and body colour were made using CCTV and video

recording over 40 days exposure to these and control conditions. Correlations were made with physiological condition in parallel groups and by terminal sampling.

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**COMPARISON OF SHORT-TERM CHANGES IN CONDITION  
OF HATCHERY-RAISED CHINOOK SALMON  
BETWEEN POLLUTED AND REFERENCE ESTUARIES**

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**Abstract**

To assess the bioavailability and effects of polycyclic aromatic hydrocarbons (PAHs) to organisms residing near an aluminium smelter, hatchery raised, outmigrating juvenile chinook salmon were analysed histopathologically, biochemically and chemically. Salmon stocks were first sampled from the Kitimat hatchery prior to release into the Kitimat River or the Dala and Kildala Rivers. Two weeks after release, fish were collected in the inner harbour of Kitimat estuary, near an aluminum smelter and the Kildala estuary, which is remote from heavy industry. The chinook salmon showed differences in short term physiological stress responses related to sampling site. The Kitimat estuary is heavily contaminated with PAHs and uptake of these compounds was indicated by pyrenol-conjugate concentrations measured by synchronous scan fluorescence spectrometry. PAHs were about 100-fold greater in bile of fish from the smelter harbour than from the Kildala estuary or the hatchery. Observed physiological differences between the hatchery and field fish are attributed to crowded hatchery conditions and changes related to a fresh to salt water transition. The superior immune status of the Kildala fish, measured by phagocytic respiratory burst activity, and decreased interrenal cell activity,

determined by nuclear diameter measurements, suggest that these fish have adapted to their environment better than the Kitimat estuary fish.

## **Introduction**

Marine environments near industrial and urban centers are exposed to a wide range of chemicals that may be transformed, either biologically or chemically, into new potentially toxic compounds (Malins and Hodgins 1981). Research has shown a positive link (by association) between certain xenobiotic chemicals present in sediments, seawater or food organisms, with histological and biochemical changes in demersal fish species. For instance, differences in overall health (condition factor measurements and various hematological variables), immune status (respiratory burst activity of phagocytes (Lemaire-Gony et al., 1995) and plasma lysozyme), stress responses involving the endocrine system (increase in interrenal cell nuclear diameters (MacDonald et al., 1988)) plus incidences of liver (Myers et al., 1987) and/or gill lesions (Haensly et al., 1982), have all been correlated with exposure to xenobiotics.

The Kitimat hatchery produces between one and three million chinook salmon, *Oncorhynchus tshawytscha*, annually for release into the Kitimat River, its tributary Hirsch Creek, and the Kildala and Dala Rivers. After release from the hatcheries the juvenile salmon reside in and are dependent on the neighboring estuaries for their food and shelter. During the time of this study, total polycyclic aromatic hydrocarbon (PAH) concentrations in Kitimat Estuary and Arm sediments varied. Mean PAH values exceeded 200 mg/kg near the aluminum smelter to less than 3 mg/kg at a distance of 3 km across or down the Arm (Payne et al., 1996). In order to assess the bioavailability of PAHs to organisms residing in the Kitimat estuary, a study was conducted using hatchery-raised juvenile chinook salmon following the lead of researchers at the Northwest Fisheries Science Center in Seattle, Washington (Stein et al., 1995). These researchers measured various biomarkers of exposure to PAH of hatchery-raised juvenile chinook salmon that were caught 2-3 weeks after release from hatcheries in various marine sites in Puget sound. In this study, we examined whether outmigrating juvenile chinook salmon assimilate contaminants that produce histopathological, biochemical and chemical changes that compromise their ability to survive.

## **Materials and Methods**

### *Field Collection*

Junvenile chinook salmon, *Oncorhynchus tshawytscha*, were obtained from the Kitimat Hatchery at peak emergence (May 2, 1994) and from the Kildala estuary (May 24, 1994) and Kitimat Harbour (May 30, 1994) two weeks after release. Fish were collected by enticing them to the surface with fish feed followed by capturing them with a dip net. Fish were housed in coolers with aerated seawater. Meristic data were recorded and dissections done in a temporary field laboratory or at the hatchery.

### *Histology*

Gill, liver and interrenal tissues were examined from a total of 25 fish from each site. The salmon were euthanized by spinal severance and fixed whole in Deitrich's formalin (Gray 1954). Samples were decalcified in Cal-EX (Fisher Scientific) overnight at room temperature, washed in running water, dehydrated in graded ethanol, cleared in toluene, and embedded in paraffin (Humason 1979). Serial sections were cut at 6  $\mu\text{m}$  and stained with Gill's hematoxylin and eosin for general histology. Findings were classified, respectively, using nomenclature consistent with Myers et al. (1987) for liver lesions or Mallatt (1985) for gill changes. Alterations in interrenal cell nuclear diameters as defined by Donaldson (1984), were measured using a computer program for video microscopy (Cohu Solid State Video Camera) and measurements (BioScan Optimas version 3.14. Edmonds, WA. 1992). Twenty five cells were counted in one section from each of the 25 fish per group.

Statistical analyses were performed using the PC-based program, SPSS standard version for Windows (version 10.0.5, 1999). Coding schemes for gill lesions were used, where gills were ranked from 1 to 4 depending on the number and types of lesions. To investigate if gill lesions are dependent on collection site the nonparametric Kruskal Wallis test was used followed with the Wilcoxin paired T test. To test if there was a difference in the interrenal nuclear diameters with site of capture, two way ANOVAs with repeated measures were used. Post-hoc comparison of means using the Tukey HSD test was performed to clarify significant main effects.

### *Immune Status*

For general health and immune status, additional chinook salmon (*Oncorhynchus tshawytscha*) were sampled from the Kitimat hatchery (n=125), Kildala estuary (n=52), and the Kitimat estuary (n=53). The general health of each fish was examined from weight, fork length and condition factor measurements. Blood was collected from each fish to assess various hematological variables; hematocrit, erythrocyte cell counts and mean erythrocyte volume. The activity of the natural immune system was also examined in each fish. Head kidney material was isolated and the respiratory burst activity of glass adhered phagocytes determined using the nitro-blue tetrazolium (NBT) assay (Anderson 1992). In addition, the activity of plasma lysozyme was determined using the lysoplate method (Osserman & Lawlor 1966 with modifications by Yousif et al., 1994).

Differences between the sample sites for each of the above variables were determined using Kruskal-Wallis tests, and the comparisons between the sites were made using Dunn's pairwise comparison tests. Differences were noted where  $p < 0.05$ . The computer software program Sigmastat (Jandel Scientific, San Rafael, CA) was used for all the analyses.

### *PAH Metabolite Analysis*

Bile samples for PAH metabolite analysis were taken from a separate set of fish. 60 fish were combined in one composite sample and 4 composite samples were taken for each site. Gall bladders were removed from euthanized salmon, placed into precleaned amber vials, frozen over dry ice, stored at  $-20^{\circ}\text{C}$  and then at  $-80^{\circ}\text{C}$  until analysed. PAH metabolite conjugates in bile were measured by synchronous-scan fluorescence spectrometry (SFS) at Simon Fraser University following the method of Ariese et al. (1993). The samples of bile were diluted 1/500 with a 1:1 HPLC grade ethanol and HPLC water solution (Fisher Scientific Ltd.). The fluorescence response was measured on a Perkin Elmer Luminescence spectrophotometer LS-50 with FL data manager operating on an IBM-PC compatible computer. Both excitation and emission monochromators were scanned synchronously with a fixed wavelength difference of 37 nm. The

area of the fluorescence emission response was measured from 335-356 nm on the emission monochromator. A six point calibration curve of 1-hydroxypyrene was used to calibrate the fluorescence spectrophotometer. The fluorescent emission of the 1-hydroxypyrene standards was measured from 340-361 nm. A conversion factor of 2.2 was applied to account for the difference in fluorescence yield between 1-hydroxypyrene and its conjugate, 1-pyrenyl glucuronide, which is the major conjugated metabolite of pyrene in the bile. Protein analyses of fish bile were done by the method of Lowry et al. (1951) using a Bausch and Lomb Spectronic 20 spectrometer.

Principal components analysis (PCA) was performed using the multivariate statistical software package *Pirouette* v. 2.60. The data were autoscaled prior to performing the analysis.

## Results and Discussion

### *Histology*

Histologically the liver tissues examined from all the juvenile chinook salmon, *Oncorhynchus tshawytscha*, were normal. A two week exposure time to contaminated sediments is too short to reveal any idiopathic liver lesions. For instance, it takes approximately three years for English sole, *Pleuronectes vetulus*, inhabiting the contaminated sediments of Puget Sound to develop preneoplastic liver lesions (pers. comm. M.S. Myers, NOAA, Seattle, WA).

Analysis of the interrenal nuclear diameters (Figure 1 & Table 1) revealed a statistically significant increase ( $p > 0.001$ ) in fish collected from the hatchery and from Kitimat Harbor compared to those from the Kildala estuary. The mean diameters were as follows: Kildala estuary =  $5.2 \pm 0.27\mu\text{m}$ , Kitimat Harbour =  $5.9 \pm 0.31 \mu\text{m}$  and Kitimat hatchery =  $5.9 \pm 0.32 \mu\text{m}$ . Interrenal cells in elasmobranchs and bony fishes represent the equivalent of the mammalian adrenal cortex. These cells produce corticosteroids, with cortisol being the most quantitatively important in teleosts. This hormone has potent effects on intermediary metabolism and is important for seawater adaptation. In addition, cortisol levels can be elevated after exposure of teleost fishes to some stressors, suggesting that it is a major factor in the piscine stress response (Ferguson 1989). Interrenal cell activity in salmonids has been shown to exhibit transient increases due to stress from: exposure of butoxyethanol ester of 2,4-dichlorophenoxyacetic acid; with density-related social interaction; and a

transitory stress response when smolts are released from a hatchery directly into sea water (McBride et al., 1981; Donaldson et al., 1984; McDonald et al., 1988). Plasma cortisol changes and histological examination of interrenal tissues, has been utilized as an indicator of primary effects of stress. For instance, Brown et al. (1986) reported rainbow trout exposed to high acid levels revealed plasma cortisol and interrenal cell nuclear diameters to be higher than for control levels.

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- Fig. 1 Chinook salmon head kidney showing interrenal cells (IR), kidney tubule (KT) and hemopoietic tissue (H), H & E. Bar = 50  $\mu$ m.
- Fig. 2 Gill. Primary and secondary lamella ( $1^{\circ}$   $2^{\circ}$ ), chondrocyte (c), pillar cells (p), mucus cell (m), and erythrocytes (e). H & E. Bar = 50  $\mu$ m.
- Fig. 3 Slight epithelial lifting (small arrow) of the secondary lamella and hyperplasia (large arrow) of the epithelial cells. H & E. Bar = 50  $\mu$ m.
- Fig. 4 Severe epithelial lifting and hyperplasia leading to fusion of the secondary lamella. H & E. Bar = 50  $\mu$ m.

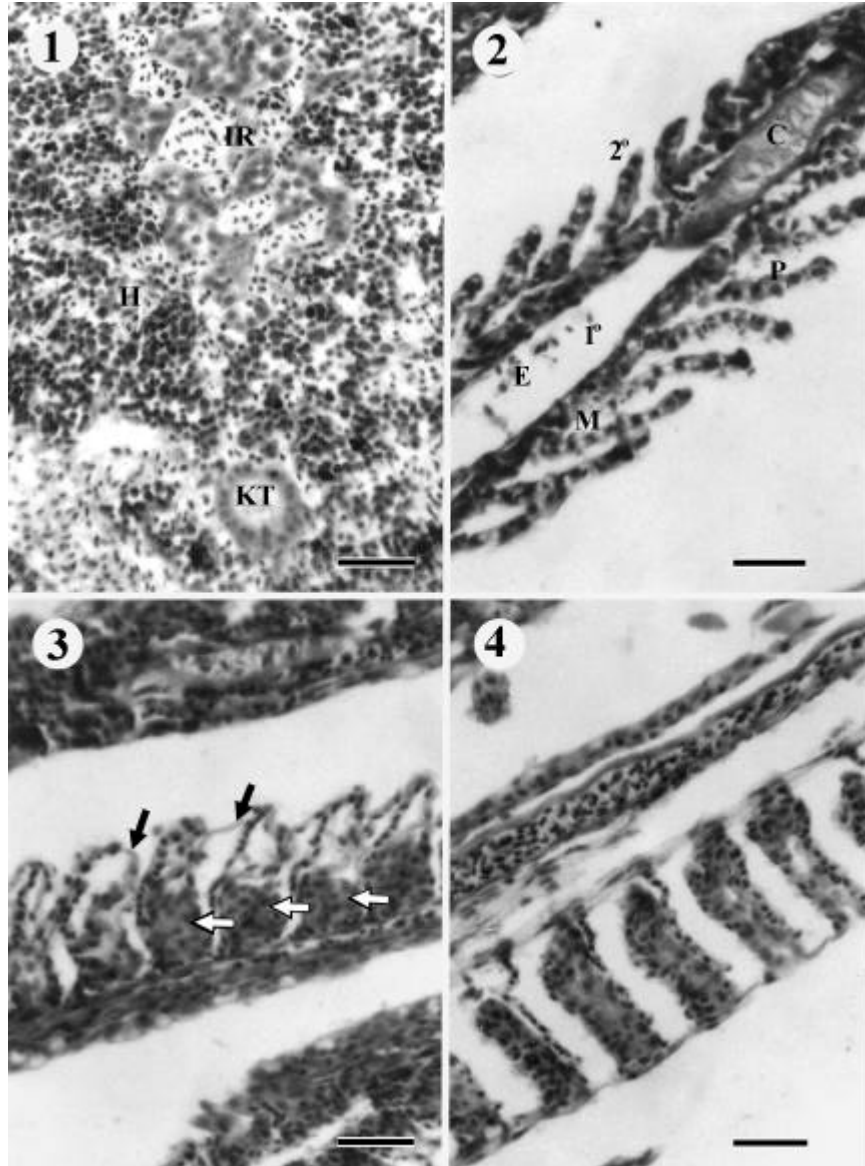


Table 1. Summary of general condition, hematological data and immune status for juvenile chinook salmon sampled from the hatchery and from the Kildala and Kitimat estuaries two weeks after release.

	Kitimat Hatchery	Kildala Estuary	Kitimat Estuary
Weight (g)	8.6 ± 0.11 (125) <sup>b</sup>	8.9 ± 0.22 (52) <sup>a</sup>	8.3 ± 0.12 (53) <sup>b</sup>
Fork Length (cm)	9.1 ± 0.04 (125) <sup>a</sup>	9.3 ± 0.05 (52) <sup>b</sup>	9.4 ± 0.04 (53) <sup>b</sup>
Condition Factor (x10 <sup>4</sup> g/(cm <sup>3</sup> ))	112 ± 0.86 (125) <sup>a</sup>	109 ± 2.33 (52) <sup>a</sup>	101 ± 0.66 (53) <sup>b</sup>
Hematocrit (%)	52.0 ± 0.5 (125) <sup>a</sup>	46.9 ± 0.8 (52) <sup>b</sup>	48.2 ± 0.6 (51) <sup>b</sup>
Erythrocytes (x10 <sup>6</sup> /mm <sup>3</sup> )	0.57 ± 0.00 (125) <sup>a</sup>	0.63 ± 0.015 (52) <sup>b</sup>	0.68 ± 0.017 (53) <sup>c</sup>
MEV (µm <sup>3</sup> )	941 ± 18.2 (125) <sup>a</sup>	760 ± 18.0 (52) <sup>b</sup>	736 ± 21.5 (51) <sup>b</sup>
Plasma Lysozyme Activity (U/mL)	144 ± 11.8 (109) <sup>a</sup>	69.4 ± 8.0 (46) <sup>b</sup>	75.8 ± 3.9 (49) <sup>b</sup>
Phag. Resp. Burst Activity (%NBT pos.)	2.45 ± 0.112 (125) <sup>a</sup>	5.65 ± 1.05 (51) <sup>b</sup>	1.86 ± 0.13 (53) <sup>a</sup>
Gill lesion index*	3.16 ± 0.18 (25) <sup>b</sup>	2.56 ± 0.23 (25) <sup>a</sup>	2.60 ± 0.18 (25) <sup>a</sup>
Interrenal nuclear diameter (µm)	5.9 ± 0.01 (750) <sup>a</sup>	5.2 ± 0.01 (750) <sup>b</sup>	5.9 ± 0.01 (750) <sup>a</sup>

<sup>a</sup> X±SE (n), different from b at the 95% confidence level, <sup>b</sup> X±SE (n), different from a at the 95% confidence level, <sup>c</sup> X±SE (n), different from a and b at the 95% confidence level. \*Gill lesion index is at the 93% confidence level

In this study, an increase in interrenal cell nuclear diameters was observed with respect to site of capture. In other words, fish collected from Kitimat hatchery and harbour have nuclear diameters that are 13.5% larger than in fish collected from the Kildala estuary. The differences observed in the interrenal cell nuclear diameters suggests that the hatchery salmon are exhibiting physiological stress due to crowded hatchery conditions. Recovery, after release appears to be occurring for the Kildala estuary fish, but is not evident in the Kitimat estuary stock.

The manner in which gill changes occur is often an accurate indicator of the causative agent, *e.g.*, bacteria, diet, or chemical. However, various agents may produce gill lesions simultaneously. Extensive damage from a specific agent may overshadow or mask gill injury produced by a second or third. The changes in gill lamellar structure however, can affect gas exchange, osmoregulation, as well as altering susceptibility to a variety of disease-causing organisms (Sinderman 1979). Normal gill tissue (figure 2) as well as gill lamellae with epithelial lifting (figure 3) or partial fusion and hyperplasia (figure 4) occurred at varying degrees of severity in all fish collected from all sites. Gill lesions were ranked from 1 to 4 depending on the number of and lesion types, present. Statistical analysis revealed that the degree of severity in gill lesions of the hatchery fish was significantly greater compared to the fish from Kitimat Harbour ( $p > 0.05$ ) and the Kildala estuary ( $p > 0.02$ ). This difference may be related to factors associated with overcrowding, such as changes in oxygen, urine, and pH levels.

#### *Immune Status*

The general condition of the fish was found to vary with the sample site (Table 1). The weight and fork length measurements were used to calculate the condition factor. The condition factor is an indicator of the nutritional state or “well being” of a fish (Busacker et al., 1990). Our sampling revealed that the condition factor in the fish from the Kitimat estuary was significantly lower than the other sites. Perhaps the growth rate of these fish was compromised due to lack of available food.

The hematological data (Table 1) reveals differences between the fish sampled from the 3 sites. Hematocrit values were significantly higher in the hatchery group, while the erythrocyte counts were significantly lower than the other sites. The mean erythrocyte volume (MEV), which is calculated from the hematocrit

and erythrocyte count data, was significantly higher in the hatchery group. These hematological results are likely due to the osmoregulatory status of the fish. At the Kildala and Kitimat estuary sites, the fish were in seawater and were perhaps dehydrated due to the hypertonic environment. The high erythrocyte counts observed in the Kildala and especially the Kitimat estuary fish, may also reflect reduced O<sub>2</sub> carrying capacity in the seawater or alternatively hemoconcentration due to gill damage

The status of the natural immune system of the fish from the 3 sites was found to be significantly different (Table 1). The plasma lysozyme activity was significantly higher in the hatchery fish. Lysozyme has been shown to decrease during smoltification, when cortisol (membrane stabilizer) levels increase and neutrophil (lysozyme producing leucocytes) numbers decrease (Muona and Soivio 1992). The respiratory burst activity of head kidney phagocytes was significantly higher in the fish sampled from the Kildala site. The reduced respiratory burst activity in the hatchery and Kitimat estuary fish may be associated with stress and/or contaminant exposure. Pentachlorophenol (PCP) has been shown to suppress the respiratory burst activity of phagocytes (Anderson & Brubacher 1993), and macrophages (Roszell & Anderson 1994). Benzo(a)pyrene has also been shown to inhibit respiratory burst activity (Lemaire-Gony et al., 1995). Various environmental contaminants have been shown to suppress the immune system of fish (Dunier & Siwicki 1993).

#### *PAH Metabolites*

The PAH metabolite conjugate concentrations in composited bile of 60 fish are reported as 1-pyrenyl glucuronide equivalents (Table 2). Although the methods show a high degree of specificity, it may overestimate the actual concentration of 1-pyrenyl glucuronide. Pyrene sulphates, other pyrene glucuronides, alkylpyrene glucuronides sulphates, and other conjugates of PAH having the pyrenoxy chromophore may be present within the bile and contribute to the signal (Ariese et al., 1993). These interferences would be minor, but nonetheless, the SFS method employed here is considered more for screening than for absolute quantitation.

The 1-pyrenyl glucuronide equivalent concentrations in bile composites from the hatchery and those caught after release at the head of Kildala estuary were all below the detection limit of 0.26 µg/ml with the exception of one composite of Kitimat River stock fish from the hatchery. The five composites from the Kitimat estuary ranged from 25.7 to 47.1 µg/ml.

Table 2: Synchronous-scan fluorescence spectrometric analysis of juvenile chinook salmon bile for PAH metabolite conjugate concentrations reported as 1-pyrenyl glucuronide equivalents (Pyr.gluc). Values expressed as mean  $\pm$  standard deviation of four composites with 60 fish each.

Sample ID*	Pyr.gluc. / Protein ( $\mu\text{g}/\text{mg}$ )	Detection limits ( $\mu\text{g}/\text{mg}$ )	Pyr.gluc. / Biliverdin ( $\mu\text{g}/\text{mg}$ )	Detection limits ( $\mu\text{g}/\text{mg}$ )
KID	0.067 $\pm$ 0.089	0.067 $\pm$ 0.089	0.783 $\pm$ 0.707	0.783 $\pm$ 0.707
KIT	0.029 $\pm$ 0.009	0.028 $\pm$ 0.010	1.010 $\pm$ 0.730	0.815 $\pm$ 0.799
KD	0.026 $\pm$ 0.003	0.026 $\pm$ 0.003	0.958 $\pm$ 0.104	0.958 $\pm$ 0.104
KT	3.174 $\pm$ 0.613	0.024 $\pm$ 0.002	180.6 $\pm$ 76.69	0.446 $\pm$ 0.153

\* KID, hatchery salmon-kildala stock; KIT, hatchery salmon-kitimat stock; KD, Kildala estuary salmon; KT, Kitimat estuary salmon

The PAH metabolite concentration depends on feeding status of the fish, where levels increase when the fish are not feeding and water in the bile is resorbed (Collier and Varanasi, 1991). Thus, for comparison purposes PAH concentrations in the bile are generally expressed relative to the concentrations of protein or biliverdin whose concentrations in bile also increase and decrease with feeding status.

The concentrations of proteins in the bile composites of juvenile chinook salmon from the Kitimat inner harbour varied slightly, indicating similar feeding status (not unexpectedly since composites of 60 fish were used). The PAH metabolite conjugate concentrations in the Kitimat harbour samples ranged from 2.40 - 3.88  $\mu\text{g}/\text{mg}$  bile protein which was 100 fold greater when compared with the samples from the Kildala estuary and hatchery.

#### ***Data Summarised using Principal Components Analysis***

Principal Components Analysis modelling illuminates the differences in the fish from the two field sites and the hatchery (Figure 5). Thus, the first principal

component distinguishes the two field sites from the hatchery, and the second, the field sites from each other. The phagocytic respiratory burst activity, weight and condition factor make a strong positive contribution and the interrenal nuclear diameter makes a strong negative contribution to PC 2, resulting in a clear distinction between the Kildala estuary and the other two sites. The condition factor also loads positively to the PC 1, helping distinguish the Kildala estuary and hatchery from the Kitimat estuary. The mean erythrocyte volume, gill lesion index, plasma lysozyme activity, and % hematocrit load strongly positive on PC 1, while the fork length and erythrocyte concentration load strongly negative. These variables mainly contribute to the distinction between the hatchery and the other two sites along PC1. The plasma lysozyme activity and gill lesion index load almost the same on both PCs, indicating a particularly strong correlation between these two variables.

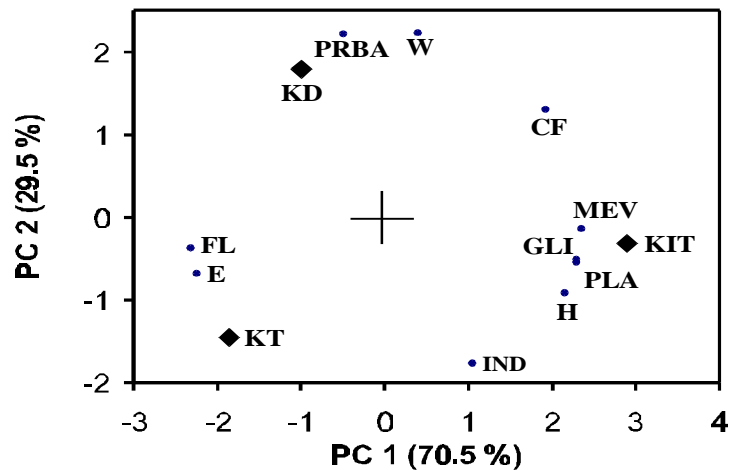


Figure 5. Biplot of sample site scores (♦) and scaled variable loadings (•) for PCA model for juvenile chinook salmon from three sites (KIT, KD and KT) in the Kitimat study area. Data for the model were autoscaled before processing. The scale factors for the variables in the biplot were 5.93 and 3.69 for PC1 and PC2, respectively. (W = weight, FL = fork length, CF = condition factor, H = hematocrit, E = erythrocyte, MEV = mean erythrocyte volume, PLA = plasma lysozyme activity, PRBA = phag. resp. burst activity, GLI = gill lesion index, IND = interrenal nuclear diameter)

## Conclusion

In general, the histopathological and biochemical differences could be explained by the differing rate of recovery from crowding stress in the hatchery and saltwater stress in the estuaries. Chemical exposure is known to cause impaired function in fish and our results suggest that the presence of chemicals related to aluminium smelting activity in the Kitimat estuary may hinder physiological processes involved in smoltification. Further work is required to determine whether the observed differences affect the juvenile chinook salmon's survival to adulthood.

## Acknowledgements

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**MOLECULAR MECHANISMS FOR CONTENDING  
WITH OSMOTIC STRESS**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Atlantic salmon (*Salmo salar*) must contend with hyperosmotic stress as juveniles on a seaward migration from their natal freshwater stream. This osmotic stress is more acute in routine aquaculture practice, in which juveniles are directly transferred from freshwater hatcheries to netpens in full salinity seawater ( $\cong$  1100 mOsm). Plasma chloride can rise from 110 to  $\geq$  200 mMol/L in 12 hours (Handeland et al., 1996). Individuals incapable of regaining osmotic/ionic homeostasis die, or grow at a reduced rate (Björnsson et al., 1988; Duston, 1994; Koch and Evans, 1959). In this report, we present investigations of the molecular mechanisms by which salmon adapt to hyperosmotic stress. At least 6 genes have been found to be upregulated in tissues of salmon exposed to osmotic stress *in vitro* or *in vivo*. Accumulation of mRNA for heat shock protein (hsp) 70, the major stress protein of fish, increased as much as 500 % in branchial lamellae, hepatic tissues and erythrocytes. Incubation of branchial lamellae in medium containing the membrane permeable solute, glycerol, also caused a prominent increase in the concentration of hsp70. A 54 kDa protein, Osp54, also was found to be induced in branchial lamellae and erythrocytes by osmotic stress caused by NaCl or by the membrane impermeable solute, mannitol. Similar to hsp70, mRNA coding for Hsp90 accumulated in the branchial lamellae in response to osmotic stress *in vitro* and *in vivo*. Although, hsp90 was upregulated in both branchial lamellae and kidney by thermal stress, osmotic stress did not stimulate expression in the kidney. Two novel genes,

expressed in branchial lamellae and kidney in response to osmotic stress, were isolated using differential expression analysis and cloned. Nucleotide sequence analysis indicates that one of the cDNAs codes for a protein 160 amino acids in length and rich in glycine. Hydropathy analysis revealed two domains with the second (amino acids 83 to 160) containing G<sub>2</sub>-Y-G<sub>2</sub> repeats. Of significant relevance to osmotic stress, the protein exhibited a high degree of similarity (58% at the deduced amino acid level) to a glycine-rich RNA binding protein upregulated by drought stress in plants. Partial sequence analysis revealed that the other cDNA contains a RING box protein motif.

The results of these investigations suggest that a complex array of mechanisms, similar to those observed in the renal tubules of terrestrial mammals, are involved in adaptation of salmon to seawater. Research is underway in our laboratory to identify additional genes and the mechanisms involved in adaptation to osmotic stress.

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**STRESS-ASSOCIATED MODULATION IN THE EXPRESSION OF  
CYTOKINE AND RELATED GENES IN RAINBOW TROUT,  
*ONCORHYNCHUS MYKISS.***

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

In mammals, the immunomodulatory effects of physiological/psychological stress and the reciprocal effects of proinflammatory cytokines on the stress (HPA) axis are well established (Turnbull and Rivier, 1995; Buckingham et al., 1996). Similarly, elements of both specific and innate immune responses, such as phagocytosis, B cell proliferation/differentiation and apoptosis are also known to be modulated by stress in fish, although the involvement of fish cytokines and related molecules in immune and stress homeostatic processes is presently unknown (Wendelaar Bonga, 1997; Weyts et al., 1999).

As a result of our recent developments in obtaining the full gene sequence and recombinant protein of the multifunctional prototypic cytokine, IL-1b it is now

possible to examine the impact of stress on the expression of IL-1b and its effect on the activity of the stress (HPI) axis in rainbow trout (*Oncorhynchus mykiss*). Furthermore, success in cloning one of the enzyme executioners of apoptosis, caspase-6, may provide an additional tool in furthering our understanding of immune and neuroendocrine interactions occurring in response to stress in fish.

## **Methods**

### *The effect of confinement stress on IL-1b and caspase-6 gene expression.*

Two approaches were used in order to address the impact of stress on caspase-6 and IL-1b gene expression. Initially, fish were subjected to confinement stress for 1-168hr, followed by the extraction of total RNA and subsequent RT-PCR analysis from gill, head kidney, spleen, brain and pituitary tissues samples. In the second study, fish were confined for 1-24hr and isolated head kidney leucocytes stimulated with LPS (5µg/ml) for 4hr, followed by the extraction of total RNA. Subsequent IL-1b expression was quantified by Northern blot and densitometric analysis. In each study, the resulting stress response was monitored by measuring both plasma glucose and cortisol levels.

### *In vitro studies.*

*In vitro* experiments were also conducted in order to examine the impact of LPS (5µg/ml) and/or cortisol (320 and 1000ng/ml) on the expression of caspase-6 in head kidney leucocytes. Isolated cells were incubated with or without LPS and cortisol for 4hr followed by RNA extraction and RT-PCR analysis.

### *Effect of exogenous IL-1b*

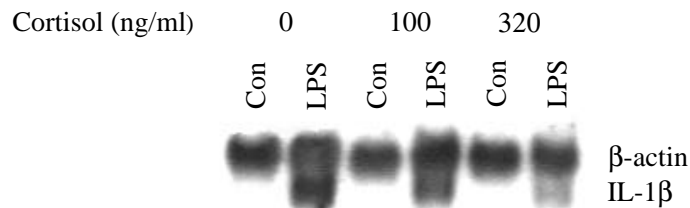
To examine the effect of IL-1b on the activity of the HPI axis, fish were injected, intraperitoneally, with trout recombinant IL-1b (µg) and plasma cortisol levels monitored from 1-168hr post injection.

### *Statistical analysis*

Cortisol and glucose data were subjected to analysis of variance (ANOVA) where significance was set at  $P \leq 0.05$ .

## Results and discussion

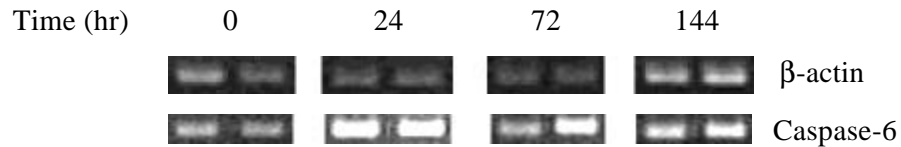
Preliminary results indicate that after 3 days of confinement, significant expression of IL-1b was detected by RT-PCR in head kidney and spleen, with minimal or no detectable expression observed in gill, brain and pituitary samples. Furthermore, with the exception of spleen, no clear expression was observed at earlier time points. *Ex-vivo* LPS-induced IL-1b expression in head kidney leucocytes was significantly suppressed following 1-8hr confinement stress, with mRNA levels recovering by 24hr post confinement. These findings are consistent with our previous *in vitro* studies describing the suppression of IL-1b in head kidney leucocytes by exogenous cortisol (Fig. 1; Zou et al., in press).



**Figure 1.** Northern blot analysis demonstrating the effect of exogenous cortisol (100 and 320ng/ml) on the LPS-induced expression of IL-1b in trout head kidney leucocytes.

Intraperitoneal injection of recombinant IL-1b stimulated an increase in the levels of plasma cortisol 24hr post injection, thus providing evidence for the involvement of IL-1b in the activation of the HPI axis.

Finally, with respect to apoptosis, the expression of caspase-6 was found to be strongly induced in head kidney leucocytes following 24hr of confinement, suggesting, in line with mammalian apoptotic mechanisms, the induction of a similar caspase enzyme cascade during stress responses (Fig. 2). *In vitro* studies revealed an apparent synergy between LPS and cortisol in stimulating increases in caspase-6 expression. Although, when treatments were administered individually, no impact on gene expression was observed.



**Figure 2.** RT-PCR analysis from 2 representative fish demonstrating the effect of confinement stress (24-144hr) on the expression of caspase-6 in trout head kidney leucocytes.

The implications of these findings with respect to bilateral communication between the immune system and the neuroendocrine system in fish, along with the potential use of IL-1b and caspase-6 gene expression as biomarkers for fish stress are discussed.

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**CHARACTERIZATION OF THE IMPACT OF REARING DENSITY  
ON THE RESPONSES OF YELLOW PERCH, *PERCA FLAVESCENS*,  
TO SUBSEQUENT INFLAMMATORY CHALLENGES**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

Yellow perch (*Perca flavescens*) aquaculture in the United States is characterized by wide variation in intensity, ranging from extensive pond culture to intensive rearing systems. Increasingly, high-density intensive aquaculture operations are being used to rear yellow perch but little information is available on the impact of chronic rearing density stressors on their responses to subsequent challenges. The objective of these studies was to determine the impact of rearing density on the responses of yellow perch to an acute inflammatory challenge.

In our first experiment we injected yellow perch with bacterial lipopolysaccharide (LPS; 3 mg/kg) to induce an acute inflammatory response and sampled plasma from these fish at 1.5, 3, 6, and 22 h after injection. Plasma was assayed for cortisol to examine the glucocorticoid response associated with the inflammatory challenge over time. The responses of LPS-injected fish were compared with those of saline-injected fish and fish that were handled but not injected. At 6 h following injection LPS-injected fish had significantly larger amounts of cortisol in the plasma than both groups of control fish (Figure 1); the

6-h interval was then used for subsequent tests as representative of a phenomena unique to LPS injections rather than handling.

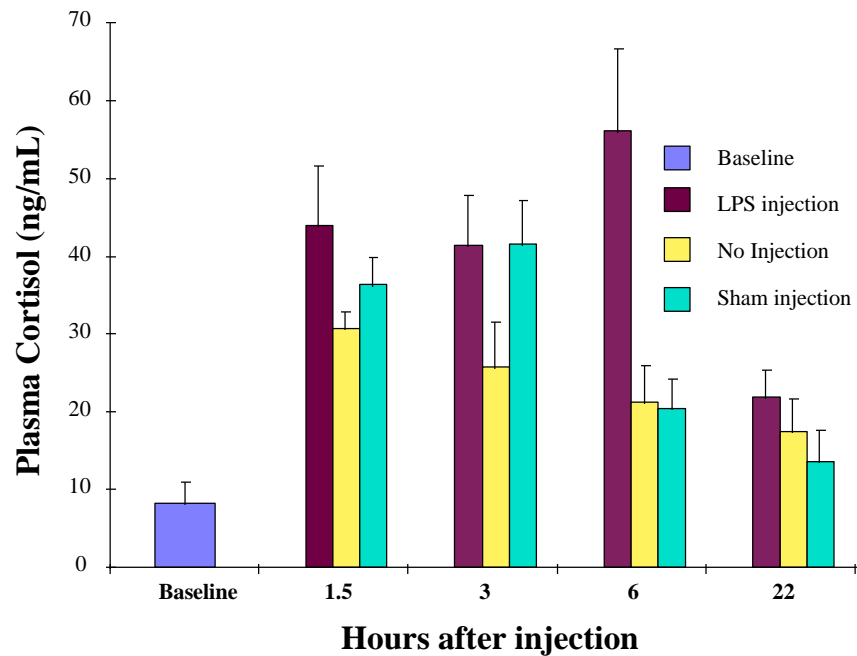


Figure 1. Concentrations of cortisol in the plasma from yellow perch at intervals after injection with 3 mg LPS/kg fish weight, a sham injection with saline, and by handling alone.

In our second experiment we held groups of yellow perch at two rearing densities (8 kg/m<sup>3</sup> or 16 kg/m<sup>3</sup>) for 3, 7, or 14 d. Two groups of high density fish were maintained; fish held at high density for the assigned duration and immediately injected with LPS (HD) and fish held at high density for the assigned duration but allowed to recover at reduced density for 24 h before injection (HDR). At each duration we sampled fish and collected plasma before injection and 6 h after an intraperitoneal injection of LPS (3 mg/kg fish weight).

Rearing density treatments altered the responses of these fish to LPS injection. After 3 and 7 d of rearing at the different densities, fish from the HDR group

appeared the most responsive to LPS injections (Figure 2). After 3 d of rearing the mean concentration of cortisol in the plasma from fish in the low density group (LD) was 94.7 ng/mL which was significantly smaller than that observed in the HDR fish, 166 ng/mL (Figure 2). After 7 d of rearing, HD fish had significantly smaller concentrations of cortisol in the plasma than HDR fish, 125 ng/mL versus 165 ng/mL (Figure 2). After 14 d of rearing, fish reared at the lowest density appeared most sensitive to the LPS injection and had significantly larger amounts of cortisol in the plasma than high density fish, 77.5 versus 127 ng/mL (Figure 2).

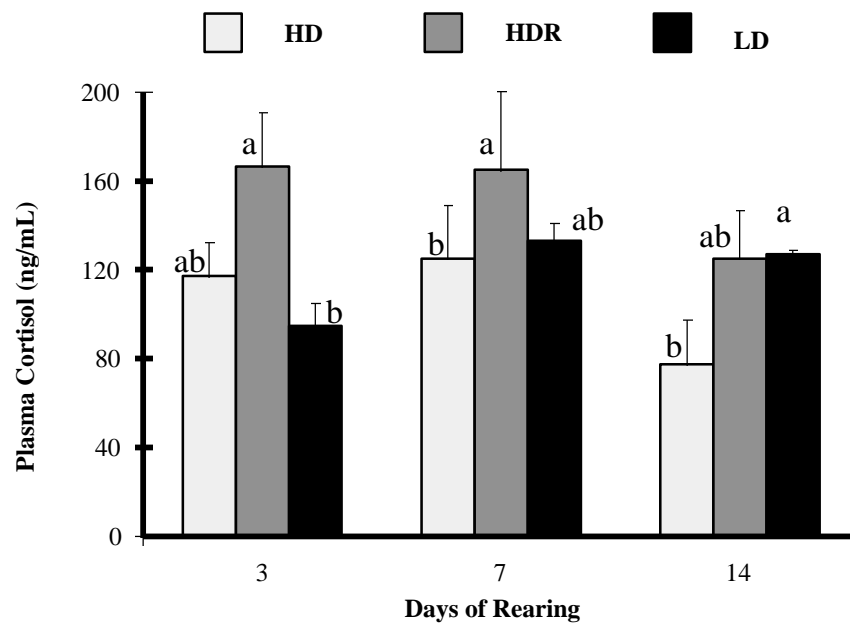


Figure 2. Concentrations of cortisol 6 h after injection with 3 mg LPS/kg fish weight for groups of yellow perch reared at 8 and 16 kg/m<sup>3</sup>: low density (LD), high density (HD), and high density followed by 24 h recovery at reduced density before injection of inflammatory challenge (HDR). Within each of the intervals, bars shown with similar letters did not differ significantly ( $P < 0.05$ )

These data demonstrate that fish reared at different densities differ in their response to an acute inflammatory challenge. In other evaluations conducted in our laboratory, walleye (*Stizostedion vitreum*), showed a similar pattern by responding with a reduced response to an inflammatory challenge when reared at high density for 14 d. Presently, we are assaying neurotransmitters in the brains of the yellow perch described above to determine if there is a correlation between monoaminergic activity and changes observed in the glucocorticoid response following an inflammatory challenge.

### **Acknowledgments**

We thank students Justin Sipiorski, Dan James, and Catherine Sykes for their technical assistance throughout the course of these experiments.

**INVOLVEMENT OF CORTICOTROPIN-RELEASING FACTOR  
AND UROTENSIN I IN MEDIATING  
THE APPETITE-SUPPRESSING EFFECTS OF STRESS IN FISH**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

**Introduction**

A decrease in appetite frequently accompanies the response of fish to environmental stress. The related neuropeptides corticotropin-releasing factor (CRF) and urotensin I (UI), the key hypothalamic regulators of the hormonal stress response in fish, have also been implicated in the regulation of food intake in mammals (Negri et al., 1985; Spina et al., 1996). While there is some evidence that central administration of CRF suppresses appetite in goldfish (De Pedro et al., 1993), it is not known whether UI plays a role in the control of food intake in fish. We therefore examined the effects of exogenous and endogenous UI and CRF upon food intake in goldfish.

**Methods**

To determine the acute effects of UI on appetite relative to those of CRF, fish were given intracerebroventricular (icv) injections of goldfish UI and rat/human CRF (0.2-200 ng/g) and food intake was assessed for a 2 hour period starting 30 min post-injection. The potential involvement of central CRF receptors in mediating the effects of CRF and UI on appetite was also determined by measuring food intake in fish given icv injection of either UI or CRF (10 ng/g) alone, or in combination with the CRF receptor antagonist  $\beta$ -hCRF<sub>(9-41)</sub> (100 ng/g). To assess whether endogenous UI and CRF modulate fish appetite, goldfish were given intraperitoneal implants of the glucocorticoid receptor

antagonist, RU-486 (100  $\mu\text{g/g}$ ), or the cortisol synthesis inhibitor, metyrapone (200  $\mu\text{g/g}$ ), and food intake was monitored daily over the following 72 hours. In a parallel experiment, the effects of blocking the negative feedback action of cortisol on CRF and UI gene expression with RU-486 and metyrapone implants were assessed by measuring brain mRNA levels of UI and CRF at 0, 24, 48, and 72 hours post-implantation by slot blot. Finally, the effects of RU-486 and metyrapone on food intake were determined in fish pre-treated with either a sham or  $\beta\text{-hCRF}_{(9-41)}$  icv implant.

### Results and Discussion

Injections of UI and CRF (0.2-200 ng/g) both suppressed food intake in a dose-related manner and UI [ $\text{ED}_{50}$  (median effective dose) = 3.8 ng/g] was significantly more potent than CRF ( $\text{ED}_{50}$  = 43.1 ng/g). Pre-treatment with  $\beta\text{-hCRF}_{(9-41)}$  reversed the reduction in food intake induced by icv UI and CRF. Fish treated with implants of either RU-486 (Figure 1) or metyrapone were characterized by an increase in hypothalamic UI mRNA levels, telencephalon-preoptic CRF mRNA levels, and a chronic reduction in food intake. Pre-treatment with icv implants of  $\beta\text{-hCRF}_{(9-41)}$  partially reversed the appetite-suppressing effects of RU-486 and metyrapone.

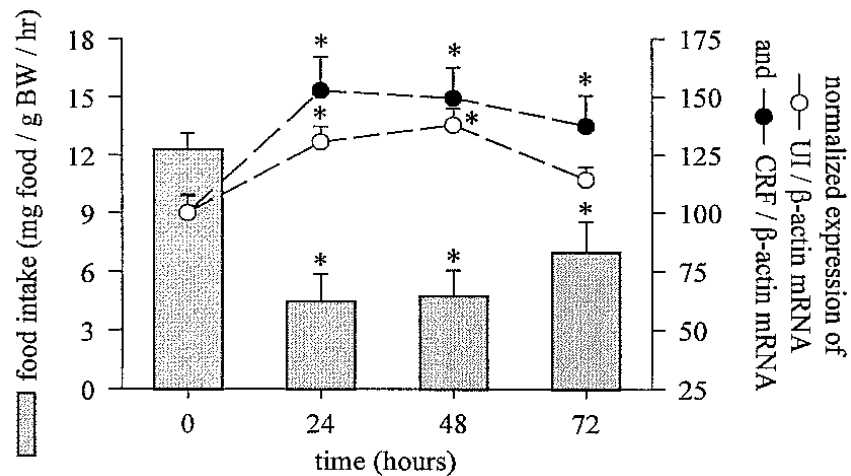


Figure 1. Time course of changes in food intake (gray bars), telencephalon-preoptic corticotropin-releasing factor (CRF; solid circle) and

hypothalamic uotensin I (UI; open circle) mRNA levels of goldfish given an implant of the glucocorticoid receptor antagonist, RU-486 (100 µg/g; N = 12). An asterisk indicates a significant difference from the 0-min control value for a given parameter ( $P < 0.05$ ). Values are means + S.E.M.

Together, these results suggest that UI is a potent anorectic peptide in goldfish and that endogenous CRF-related peptides can modulate fish appetite. As in mammals (Negri et al., 1985; Spina et al., 1996), icv UI displays a higher potency than CRF in reducing feeding in goldfish and the appetite-suppressing effects of UI and CRF appear to be mediated through CRF receptors. In addition, our results provide further support for a cortisol-mediated negative feedback loop on CRF gene expression (Bernier et al., 1999) and suggest that cortisol is involved in the regulation of UI synthesis in the hypothalamus. Given the key role of CRF-related peptides in orchestrating the response to stress among vertebrates, our results suggest that both UI and CRF may be endogenous factors in the brain responsible for the effects of stress on fish appetite.

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### **Acknowledgements**

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**IN-VITRO REGULATION OF CORTISSOL AND ACTH OUTPUT  
FROM INTERRENAL AND PITUITARY TISSUE  
OF GILTHEAD SEA BREAM (*Sparus aurata*)**

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**Introduction**

The involvement of hypothalamus and pituitary in the control of cortisol production has been established for teleost fish, and adrenocorticotrophic hormone (ACTH) has been considered the major factor controlling synthesis and release of cortisol from interrenal cells in the head kidney. The endocrine control of cortisol secretion in teleost is complex, whereas cortisol has been shown to effect self suppression by negative feed-back of its secretion directly at different levels. ACTH has been considered the major factor controlling the synthesis and release which in turn is regulated by hypothalamic factors such as corticotrophic releasing hormone (CRH). However, little is known, specially in marine fish, about the contribution and dynamics of hypothalamic-pituitary hormones on the stress response. We therefore investigated the differences regarding hypothalamus-pituitary-interrenal communication in *Sparus aurata* compared to other fish by testing the *in vitro* responsiveness of interrenal tissue to ACTH and the responsiveness of ACTH producing cells to CRH and by characterising the response using a CRH antagonist.

## Material and methods

### *Animals*

Sexually immature gilthead sea bream weighing 75 to 160g were kept under constant conditions of photoperiod (12L:12D), salinity (38‰), temperature ( $17\pm 1^\circ\text{C}$ ) and density ( $7\text{Kg.m}^{-3}$ ). To investigate the responsiveness of ACTH producing cells to CRH, a total number of sixty fish were used, ten groups of six fish were captured and the pituitary glands were excised and superfused. To investigate the responsiveness of interrenal tissue to ACTH, forty five fish were used, nine groups of 5 fish were captured and the head kidneys were excised and superfused. To study the in-vivo and in-vitro effect of cortisol on the sensitivity of cortisol producing tissue to ACTH, fish were pre-treated 36h and 6h with cortisol before sampling. Thus a total of 24 fish (8 controls, 8 cortisol *in-vitro* and 8 cortisol *in-vivo*) were sampled.

### *Pituitaries and Head Kidneys*

Pituitaries and head kidneys were excised. Single pituitaries and head kidneys were superfused with a Hepes (15mM; pH 7.4) buffer solution containing 0.25% (w/v) glucose and 0.03% (w/v) bovine serum albumin. For pituitary glands, medium, either supplemented with CRH competitive antagonist ( $\alpha$ -helical CRH (9-41) or not supplemented. The flow was 40  $\mu\text{l}/\text{min}$ . After 220 min of superfusion, medium supplemented with different concentrations of CRH was given for 20 min. For head kidney superfusion, tissue was stimulated with ACTH at different concentrations (Fig.1) and at a concentration of 5nM (Table.1) (hACTH<sub>1-39</sub>, Sigma) during 20 minutes. To study the “in-vitro” effect of exogenous cortisol, medium was supplemented with different concentrations of hemisuccinate-cortisol. The maximum cortisol release due to ACTH stimulation was compared with the basal release in order to obtain the stimulation factor of ACTH, (maximum release – basal release) / (basal release).

### *Biochemical and Statistical analysis*

Cortisol and ACTH were measured by well-established and validated radioimmunoassay (RIA) for gilthead sea bream. Results are given as mean  $\pm$  SEM for each group. Differences among groups were assessed by means of One-way Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test. The level for accepted statistical significance was (\*  $p < 0.05$ ;

\*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). The concentration-effect curves were fitted using a non-linear data analysis program describing a sigmoid curve (Jandel Sigma plot, Scientific graphic software, Version 3.0).

## Results

In vitro responsiveness of ACTH producing cells to CRH and by characterising the response using a CRH antagonist.

The results show that CRH stimulated the ACTH in a concentration-dependent manner in pituitaries of non-disturbed sea bream. The maximum level of stimulation were about 1000% of basal release for ACTH and the EC50 values defined as the required CRH dose for half-maximal response, were  $1.5 \times 10^{-9}$  M (Fig.1). In the presence of 400nM  $\alpha$ -helical CRH (9-41), the ACTH-releasing activity of CRH was greatly reduced. Thus the antagonist blocked the secretion of ACTH that was stimulated by a dose of 1 nM CRH by a 75% and 10nM of CRH by 50%.

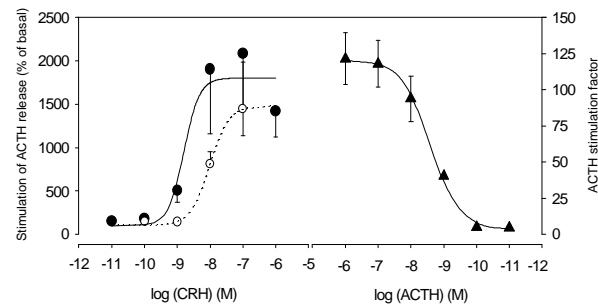


Figure 1. Effect of increasing doses of CRH on the ACTH release from sea bream pituitaries superfused in the presence (dotted line; calculated from (○) or absence (solid line: calculated from (●) of 400nM  $\alpha$ -helical CRH (9-41). Data represent mean $\pm$ SEM (n=6). Concentration effect curve showing maximal stimulation of cortisol release during superfusion of control head kidneys (▲). Values are mean $\pm$ SEM; (n=5 for all points).

*In vitro* responsiveness of interrenal tissue to ACTH and effect of cortisol administration

ACTH stimulated cortisol release in a concentration-dependent manner in interrenal tissue of control fish. The EC50 value was  $2.6 \times 10^{-9}$  M (Fig.1).

Table 1. Sensitivities of interrenal tissues of control to ACTH (expressed as ACTH stimulation factor) under cortisol administration.

	Plasma cortisol (ng.mL <sup>-1</sup> )	Medium cortisol (ng.mL <sup>-1</sup> )	ACTH stimulation factor
<i>Control</i>	4.2±2	-	79±10
Cortisol "In-vitro"	3.4±1	20 200	62±14 73±11
Cortisol fed	21.2±2*	-	81±12

Table 1 shows the steroidogenic response (expressed as ACTH stimulation factor) of head kidneys from sea bream *in vitro* superfused with a medium either supplemented with cortisol (20 and 200 ng.ml<sup>-1</sup>) or not supplemented and both stimulated with 5 nM of hACTH<sub>1-39</sub> for 20 minutes. The ACTH sensitivity of interrenal tissue of non-cortisol-fed sea bream (expressed as ACTH stimulation factor) was 79±10 in the control and was not altered by cortisol administration *in vitro* at the two different concentration tested. In cortisol fed fish, again the ACTH stimulation factor was not different from non-cortisol-fed sea bream.

### Discussion

CRH stimulates the pituitary ACTH release in a concentration-dependent manner. Immunocytochemistry studies using antisera directly against

mammalian CRH allowed the identification of a CRH-like system in a number of teleosts. In all except one species examined, CRH-like immunoreactivity is concentrated in the parvocellular and magnocellular areas of the nucleus preopticus (NPO). However, it has been reported (Mancera et al., 1995) that in gilthead sea bream, most of the CRH-like immunoreactivity was found in the nucleus lateralis tuberis (NLT) but not in the NPO. Among the different explanations discussed by the authors it was suggested that perhaps CRH was not a releasing factor for ACTH in this species. Although our *in vitro* approach does not necessarily mimic the *in vivo* situation, the superfusion results do not support that explanation and otherwise they are in accordance with most of the previous works (Balm et al., 1994) showing that CRH is able to stimulate the release of ACTH from sea bream pituitary gland, as in all other fish species studied to date. Hence, when applying the same antagonist ( $\alpha$ -helical-CRF(9-41) previously used to antagonise the ACTH-releasing activity of CRH in fish (Weld et al., 1987), a blocking of the ACTH in a dependent CRH concentration is observed. Thus, the superfusion results are consistent with CRH being a physiological regulator of ACTH secretion from pituitary glands of gilthead sea bream, a similar situation than in other teleosts. The ACTH stimulates the interrenal cortisol release in a concentration-dependent manner. Cortisol administration either *in vivo* and *in vitro* had no effect on the sensitivity of the interrenal cells to ACTH (expressed as ACTH stimulation factor. Table 1). Thus, the data suggest that sea bream interrenal cells are relatively insensitive to direct cortisol feedback, which may seem surprising in view of previous “*in vitro*” results on salmon (Bradford et al. 1992). However, this discrepancy could be attributed to the use of the different *in vitro* superfusion systems.

In summary, the present work shows that the results of *in vitro* investigation demonstrate that CRH stimulates the release of ACTH from sea bream pituitaries. Moreover, they demonstrate that  $\alpha$ -helical CRH (9-41) antagonises the ACTH-releasing activity of CRH from the sea bream pituitary as it has been shown for another teleost (Weld et al., 1987) and for the mammalian pituitary (Rivier et al., 1984), suggesting that CRH plays important roles in control of the stress response in sea bream. Furthermore, The interrenal ACTH sensitivity in sea bream is probably not regulated by cortisol. Thus, in sea bream the ACTH interrenal sensitivity would be regulated at the hypothalamus-pituitary levels and communicated via circulating ACTH levels, supporting similar mechanisms identified in mammals.

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**THE REGULATORY ROLES OF CORTISOL AND  
CATECHOLAMINES IN TROUT WHITE MUSCLE GLYCOGEN  
METABOLISM STUDIED *IN VITRO*.**

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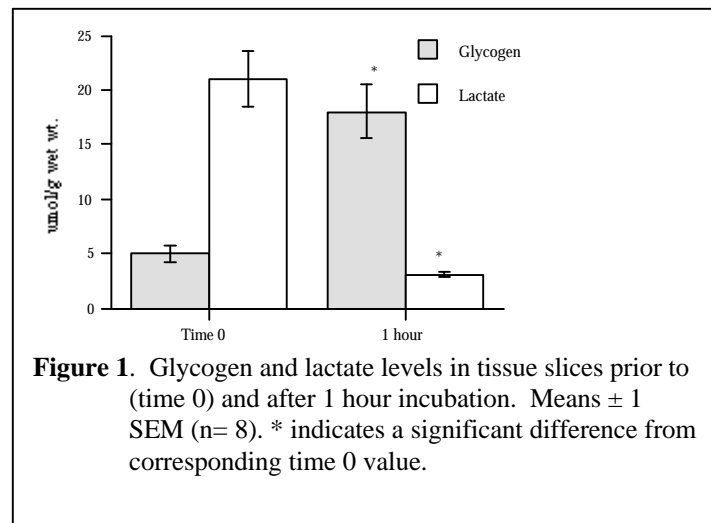
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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

In fish, high intensity exercise results in a near total depletion of white muscle glycogen stores. Glycogen replenishment and hence, restoration of sprint performance, was thought to be a slow process, requiring in excess of 6 hr. The time period required for metabolic recovery from a bout of exhaustive exercise may, in fact, be considerably shorter (Pagnotta *et al.*, 1994; Eros & Milligan, 1996; Milligan *et al.*, 2000), if the post-exercise elevation in plasma cortisol is prevented. Under these conditions, muscle glycogen and lactate are restored to pre-exercise levels within 2 h after exercise. The story emerging from these *in vivo* studies is that cortisol is involved in the regulation of muscle glycogen metabolism, though its mechanism of action is not understood.

To test the hypothesis that cortisol is having a direct regulatory role in muscle glycogen metabolism and determine what that role might be, we developed an *in vitro* white muscle slice preparation from rainbow trout. Fish, either at rest or after 5 min of exhaustive exercise, were killed by anaesthetic overdose and a block (~1.5 cm<sup>3</sup>) of the dorsal epaxial muscle was excised and placed in ice-cold Cortland's saline. Tissue slices (~1.1-1.3 mm thick) were obtained with a Stadie-Riggs microtome. One slice was immediately freeze-clamped under liquid nitrogen and served as a "time 0" point of reference for each 1 hour

incubation. The other slices were individually incubated in 3.5 ml Cortland's saline containing 10 mM pyruvate, and 5 mM lactate and continuously aerated with humidified 99.5% O<sub>2</sub>: 0.5% CO<sub>2</sub> for 1 hour at 15 °C in a shaking water bath. At the end of 1 hour, slices were removed, blotted dry and freeze-clamped under liquid nitrogen.



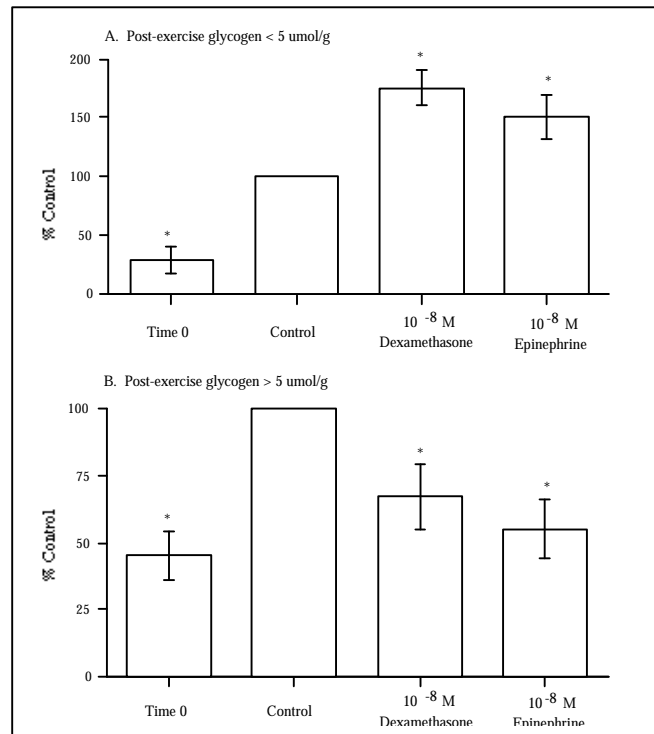
**Figure 1.** Glycogen and lactate levels in tissue slices prior to (time 0) and after 1 hour incubation. Means  $\pm$  1 SEM (n= 8). \* indicates a significant difference from corresponding time 0 value.

Muscle slices obtained from exhaustively exercised trout were capable of significant glycogen synthesis and lactate clearance (Figure 1). After 1 hour incubation in Cortland's saline containing 5 mM lactate, approximately 13 umol of glycogen was synthesized and 18 mol of lactate was cleared. This is unequivocal evidence that trout white muscle is capable of *in situ* glycogenesis.

The regulatory roles of cortisol and catecholamines are complex and dependent upon the endogenous levels of glycogen (figure 2). In muscle slices where glycogen levels were > 5 umol/g, both dexamethasone (a cortisol analogue) and epinephrine stimulated glycogenolysis and the relative activity of glycogen phosphorylase. However, when glycogen levels were <5 umol/g, both glycogenesis and the relative activity of glycogen synthase were stimulated.

In conclusion, these data indicate that the *in vitro* white muscle slice preparation is a good model system for studying the regulation of glycogen metabolism.

Furthermore, cortisol and epinephrine have direct, though complex effects on white muscle glycogen metabolism.



**Figure 2.** The effects of exogenous dexamethasone-21-phosphate and epinephrine on muscle glycogen levels *in vitro*. Data are presented as % of control, the control representing a tissue slice incubated in the absence of any exogenous hormones. The time 0 data are the glycogen levels immediately after the slice is obtained from the fish. Means  $\pm$  1 S.E.M., n=8. \* Indicates a significant difference from control value.

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**LOCOMOTORY DYNAMICS AS INDICATORS OF STRESS IN FISH –  
REMOTE MEASURES UTILIZING ACTIVITY TRANSMITTERS**

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**Abstract**

Stress related physiological disturbances can result in either locomotory impairments or hyperactivity. These behavior-altering disturbances, however, are difficult to quantify. Undoubtedly, for free-swimming fish in non-laboratory settings, it is these sub-lethal disturbances that result in changes in activity that can increase the susceptibility to predation, elevate energy expenditure, and generally disrupt the ecology of the species. Devices capable of remotely monitoring and quantifying the locomotory activity of free-swimming fish have been developed and have been applied widely within fisheries science, particularly for the determination of activity patterns and the calibration of bioenergetics models. The application of these devices to the measurement of environmental or anthropogenic stressors provides the opportunity to link behavior and physiology in an effort to understand the influences of these stressors from both an energetic and ecological framework. We present examples of data generated from several novel uses of activity transmitters in the areas of aquaculture production research, thermal effluent monitoring, and handling and angling related disturbances. The approach of coupling behavior

and physiology as outlined in this paper, is particularly robust, providing what we believe to be an important component of our comprehension of how stress manifests itself at the organismal level.

## **Introduction**

Aquatic organisms, and in particular, fish, pose several challenges to scientists interested in assessing the behavioral and physiological responses to stress under both natural and artificial conditions. Ideal approaches to data collection would be generally non-invasive and would permit evaluation of *in situ* conditions without repeatedly handling or terminally sampling individuals. Approaches used to assess the locomotory activity of free-ranging fish have included videography and locational telemetry. These approaches are undoubtedly useful, but both have limitations in many situations that either make it difficult to obtain continuous time series data or to collect data at appropriate temporal and spatial scales for detailed assessments of locomotory activity.

Activity transmitters capable of remotely measuring the activity of free-swimming fish have been developed over the past 20 years and can operate on either radio or ultrasonic frequencies. There is large variation in the transmitter configurations and how the data are actually collected (see Cooke 1999 for review). For example, some devices rely on electromyographic signals that are detected by electrodes implanted in musculature, relaying either raw or integrated muscular activity correlates. Other configurations rely on pressure transducers capable of detecting changes in the frequency and amplitude of caudal fin beats. Some devices do not directly measure any biological information from the fish, but instead infer swimming activity from changes in detected water velocities. Many of these devices have been described in detail in the literature, including both the technical details concerning their design and operation, as well as laboratory and field applications of the technologies. Some of these devices have come into commercial production. Although commercially produced devices minimize the creative energies required to develop new technologies, thus restricting further advances, it is perhaps time that researchers attempt to use a series of standardized devices to facilitate interstudy and interspecific comparisons. Initially, if a researcher was interested in obtaining remote activity measures, it required the assistance of electrical engineers among others, as well as time and financial resources. Now a researcher can order devices from several companies specializing in physiological telemetry.

### *Activity as a surrogate measure of stress*

Generally, activity transmitters are an accepted method for the assessment of the activity of free ranging fish. One area of research where activity transmitters could make a valuable contribution is in the measurement of stress. Changes in the activity levels of fish have recently been observed to be sensitive indicators of stress (Schreck 1990; Scherer 1992; Schreck et al. 1997). Swimming requires the integration of numerous physiological processes that if quantified, could provide information on the general health and stress of the fish (Schreck 1990). As a result, efforts to understand the behavioral and physiological impacts of various stressors could include a focus on activity levels. Stressors that have the potential to disrupt activity patterns or either elevate or impair swimming activity are perhaps the applications best suited for activity transmitters. Behavioral changes in free swimming fish however, have been difficult to obtain *in situ* (Beamish 1978) until the recent development of activity transmitters.

### *Case Studies – Diverse Examples*

As noted above, activity transmitters have the potential to contribute to our understanding of whole organism responses to stress. Below we provide several examples of the types of applications for which activity transmitters may be particularly suited and some data generated using this approach. The applications that we describe are diverse and include aquaculture production research, environmental monitoring and handling and angling disturbances. The data that we provide were all generated using “electromyogram” activity transmitters that emit an integrated signal (Lotek Engineering Inc, Newmarket, ON). We are not endorsing this product specifically, however, alternative technologies such as pressure transducer based activity transmitters (Vemco Inc, Shad Bay, NS) have become commercially available only recently.

### *Activity Transmitters in Aquaculture Production Research:*

Activity transmitters provide opportunities to develop quantitative animal welfare correlates for fish exposed to different husbandry practices. For example, Cooke et al. (2000) exposed adult rainbow trout (*Oncorhynchus mykiss*) to a series of increasing density treatments (15-low, 30-medium, 45-high kg/m<sup>3</sup>) and associated disturbances. Fish were first exercised in respirometers to

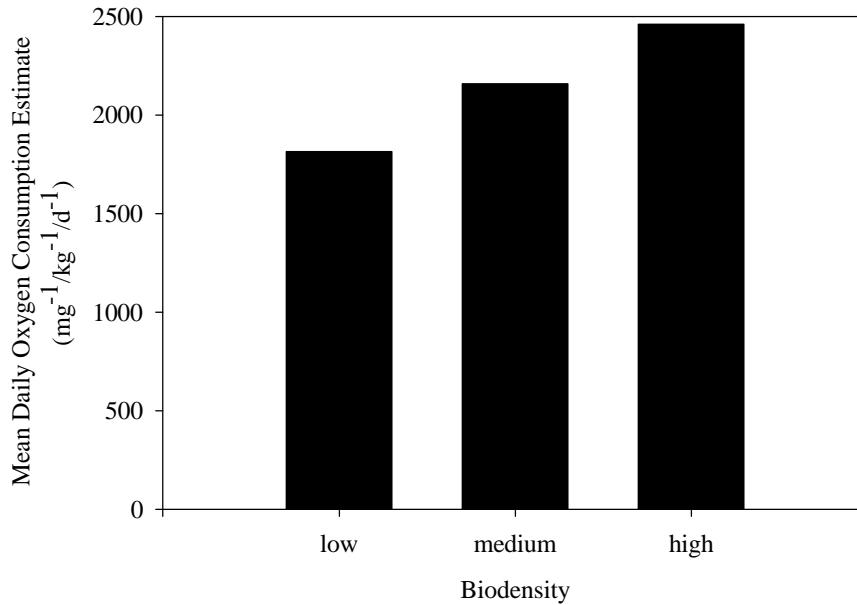


Figure 1. Effects of increasing density treatments on daily oxygen consumption rates of adult rainbow trout.

establish relationships between swimming speed, telemetered activity signals, and oxygen consumption. Signals recorded during the increasing density treatments could then be used to estimate the *in situ* energetic expenditure of fish. Overall, there was an overwhelming trend of increased activity and energetic expenditure that corresponded to the increasing densities (Figure 1).

A second example involves the assessment of fish behavior during transport while fish were being held in varying concentrations of clove oil anesthetic. It has been postulated that at low concentrations, clove oil may serve as a means of minimizing fish activity during transport (Keene et al. 1998), thus reducing physiological disturbance and stress. We transported adult rainbow trout for a period of three hours in separate 60 l containers. Concentrations of clove oil (0,

2.5, 5, 10 PPM) were maintained during this period. Interestingly, the lowest concentration of clove oil (2.5 PPM) resulted in the lowest activity levels during

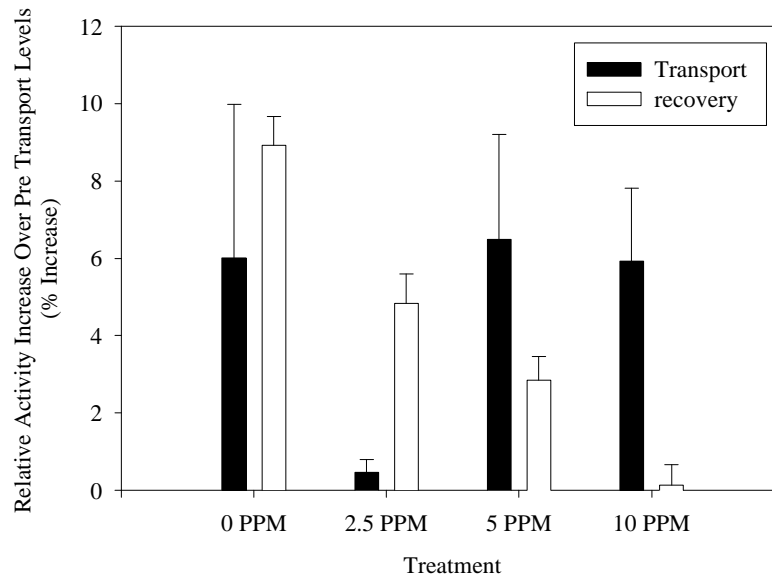


Figure 2. Effects of clove oil on activity of adult rainbow trout during transport.

transport. At higher concentrations fish lost equilibrium and spent the majority of the time struggling while upside down (Figure 2). Upon release, fish from the two highest concentrations were the least active. Control fish were the most active, with the lowest concentration showing intermediate activity levels. Activity of all fish following release was higher than prior to transport for at least 24 hours. Activity transmitters provided behavioral insights, that can be used to explain differences obtained using conventional physiological measures.

**Activity Transmitters and Environmental Monitoring**

Environmental variations and site-specific conditions experienced by fish in the wild are sometimes difficult to replicate in laboratory settings. Knowledge of the *in situ* response of fish to environmental fluctuations can provide managers with opportunities to minimize anthropogenic disturbances. We have used activity transmitters to monitor the locomotory activity of fish residing in a

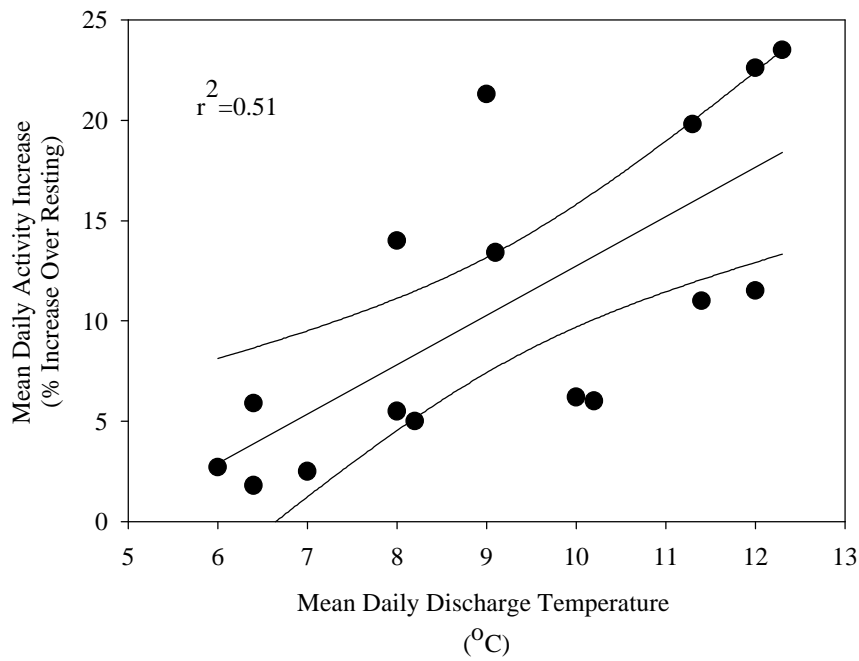


Figure 3. Relationship between mean daily water temperature and locomotory activity of adult channel catfish in a thermal discharge canal.

thermal discharge canal on Lake Erie. Here, we show data (Figure 3) for an adult channel catfish (*Ictalurus punctatus*) residing in a thermal effluent during March. In this case, we see a moderate correlation between mean daily water temperature and mean daily swimming activity. Data from activity transmitters can also be examined on a finer time scale (seconds) to detect more immediate responses to temperature fluctuations or other environmental variables (i.e., Rand and Hinch 1998).

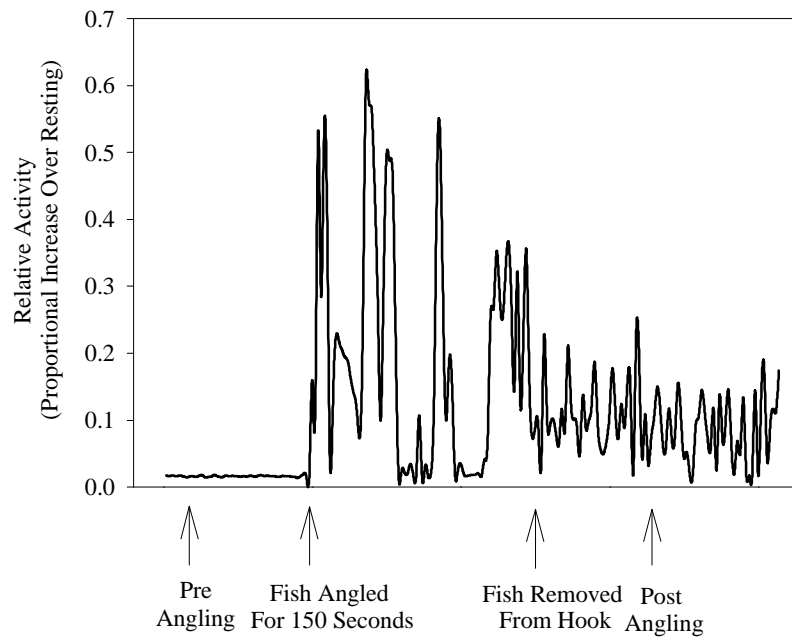


Figure 4. Pattern of adult smallmouth bass locomotory activity before, during, and after exhaustive angling.

### *Activity Transmitters and Angling Disturbances*

Measurements of the activity associated with catch-and-release angling, as well as behavior and activity of fish during the recovery period, would be useful in further understanding how this practice may affect free swimming fish. To this

end, we have conducted several studies examining locomotory activity before, during, and after angling. Here we present an example of an adult smallmouth bass (*Micropterus dolomieu*) angled at 14°C for a period of 150 seconds (Figure 4). The fish was observed to be inactive and under cover prior to angling. Activity increased dramatically upon hooking the fish. During the angling period, there was a decrease in activity as the fish became exhausted. The fish exhibited somewhat heightened activity relative to pre angling levels after release.

### **Conclusion**

We are strong proponents of continued research and development on the remote collection of indices of locomotory activity (and other relevant measures). We also urge more researchers to begin adopting some of these techniques. Because numerous studies utilizing activity transmitters now exist illustrating a variety of different techniques for surgical implantation (see Beddow and McKinley 1999; Bunt 1999), data collection, and data analysis, it is intuitive to encourage the standardization of approaches to facilitate comparisons across studies. We also encourage the adoption of approaches that incorporate other indicators of an organisms physiology and behavior. In this paper we have provided several novel examples of how commercially available activity transmitters can be used to generate useful data. When this technique is combined with other more conventional methodologies, scientists will have a robust set of tools to integrate both behavior and physiology in understanding how fish respond to stress. Behavioral studies only provide information on what animals are doing. An understanding of the proximate basis for a given behavior, i.e. how they are physiologically able to perform the activity, is essential to understand ultimate questions of why they behave as they do.

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**AN EXPERIMENTAL TEST OF SUBSTRATE-INDUCED STRESS  
IN TWO BENTHIC FISH SPECIES, BURBOT (*LOTA LOTA*)  
AND STONE LOACH (*BARBATULA BARBATULA*)**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Behavioural and respiratory responses of burbot (*Lota lota*) and stone loach (*Barbatula barbatula*) to different types of substrate were tested. Both species showed distinct diel cycles in activity and respiration rate with highest values immediately after dusk and lowest values during the day. In burbot, a 30% increase in mean respiration rate was observed on pebble substrates compared to cobbles in a 24-h cycle. During daytime, these effects increased to 86%, indicating a high level of substrate induced stress in juvenile burbot when no adequate shelter is available. In contrast, no significant differences in respiration rate between substrates were found during the night. The results show that the lack of adequate shelter may substantially affect metabolism and somatic growth rates in benthic fish species. These effects occur when no predator is actually present and shelter is not essential for survival. The results provide evidence that current theories on the effects of substrate and predation pressure should be carefully applied to the benthic community. Because most of these theories are derived from epi-benthic or pelagic 'model' - species, where the actual presence of a threat is needed to modify behaviour and finally specimen's metabolism, their validity for the benthic community seems to be limited. Especially for fish with high substrate affinity, the availability of adequate shelter may be of more importance than other environmental resources, even when no predator is actually present.



## **METABOLIC IMPACT OF HANDLING**

**ON *Pseudoplatystoma coruscans*,**

**A WIDESPREAD TELEOST FISH**

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### **Introduction**

The aquatic environment is physically more restrictive than the terrestrial. Water bodies characteristics fluctuate and are dependent on many surrounding factors. Variables such as temperature, pH, and oxygen concentration, among others, are important factors for fish culture. In addition to water quality factors, fish culture procedures such as handling and transportation can be important sources of stress in intensive aquaculture. Handling, followed by transportation of the largest number of fish into the smallest possible volumes of water necessitates correctly designed systems to prevent adverse water quality changes. Basic physiological needs require continuous circulation of freshly aerated water to all parts of hauling tanks. Another adverse situation that must be minimized is the accumulation of ammonia during fish transport operations. This is usually the main shock imposed on the fish during crowding and transportation.

Different techniques of handling are more or less stressing, sometimes simulating disturbances resulted from chasing (Schwalme and Mackay, 1990; Waring et al.,1995). In view of this, several metabolic responses may proceed from such stressful conditions and different adjustments are expected. Metabolic costs associated with stress resulting from physical disturbances have been described (Saunders, 1963; Barton and Schreck, 1987; Fletcher, 1992). If the fish has a metabolic demand to supply the stress cost, this means less available energy for other performance components (Barton and Iwama, 1991). Cortisol

seems to have an important role in metabolic responses from stress (Barton and Schreck, 1987).

Activity of certain metabolic enzymes should be affected by cortisol (Davis et al., 1985; Vijaian et al., 1991). As well, an increase in metabolic intermediates, like glucose, is ascribed to cortisol (Leach and Taylor, 1982; van der Boon et al., 1991) but other investigations are needed to clarify this effect.

The kinds of responses to stress are different among fishes and are displayed at different levels (Davis and Parker, 1983; 1986; Sumpter et al., 1986; Pickering and Pottinger, 1989). However, differences among species seem to be, in part, attributed to strains (Barton et al., 1986; Pottinger and Moran 1993; Noga et al., 1994), discrete stocks (Iwama et al., 1992) or between wild and hatchery fish, suggesting a “domestication” effect. Moreover, the stress response seems to have a genetic component (Heath et al., 1993) and some fish may be genetically predisposed to exhibit different responses to cortisol (Pottinger et al., 1992) albeit heritability estimates to date are low (Fevolden et al., 1993). In light of this fact, selection of the best strains of fish for aquaculture systems is one point to be considered.

This work reports preliminary data concerning metabolic and hematological responses of a single population of the tropical catfish (pintado) *Pseudoplatystoma coruscans* after stress caused by handling, a common practice in tropical aquaculture systems.

## **Material and Methods**

### *Fish*

Young specimens of *P. coruscans* weighting  $8.0 \pm 2.0$  g were purchased from Projeto Pacu – Campo Grade – MS, Brazil and transported in hauling boxes to the laboratory. The animals were stocked in 2,000L plastic tanks at  $25 \pm 3^\circ\text{C}$  under natural photoperiod with filtered, well aerated water and nourished with carnivorous fish ration pellets. After 90 days under these new environmental conditions, the animals reached  $38.48 \pm 7.30$  g and were submitted to the following experimental design.

### *Experimental design*

Forty animals were carefully divided into five groups of eight fish in the same environment. After 15 days, four groups were captured and transferred to plastic 50L boxes, staying there for three minutes. After that, three groups were placed back into the tanks to recovery and one group, after withdrawing blood samples, was immediately killed to collect liver, white muscle and kidney. The three groups under recovery were sampled at different time intervals (12, 24, and 48 hours). The fifth group remained in the tank and was used as a control and the animals were sampled right after the 48 hours group.

### *Analytical procedures*

Blood was withdrawn from the caudal vein in heparinized syringes and used for hematological and plasma analyses. Tissues were excised, promptly frozen in nitrogen and kept at  $-20^{\circ}$  C for subsequent analysis. The whole blood was used for haematocrit, red cell count and hemoglobin determination (Drabkin, 1948). The whole plasma was used for triglyceride (Doles' Colorimetric Kit) and total protein (Deutscher, 1990) colorimetric determination. Free protein trichloroacetic acid extracts from plasma and tissues were used to estimate glucose (Dubois et al., 1956), lactate (Harrower and Brown, 1972) and pyruvate (Lu, 1939) by colorimetric methods. Glycogen was estimated by acid hydrolytic method (Dubois et al., 1956) in liver, kidney and white muscle after alkaline digestion and alcohol precipitation (Bidinotto et al., 1997).

### *Statistical analysis*

The means  $\pm$  standard deviation were compared with the control using an ANOVA test and the Kolmogorov-Smirnov test was fitted to a normal distribution. If any difference was detected among samples, the Dunnett test was used to group the means. Samples of non-homogeneous variances were applied by non-parametric ANOVA (Kruskal-Wallis) to the row data and the same Dunnett test was used to group the means. The confidence interval used was  $p < 0.05$ .

## Results and Discussion

The effect of handling was reflected in the blood by the increase in hemoglobin/red blood cell content of the (Table 1). This effect may be caused by a decrease in blood cell volume, noticeable after only 24 hours. Immediate increase in haematocrit after handling, followed by similar increases of hemoglobin and red blood cells, suggests the release of erythrocytes into the circulatory system by hematopoietic structures. This hematological response may be to compensate for the metabolic demand resulting from physical disturbance.

The concentration of plasma triglycerides, lactate and glucose were significantly decreased after handling (Figures 1, 2 and 3). The continuous decrease in plasma triglycerides over 48 hours suggests a persistent use of this energy-rich molecule to sustain the metabolic demand resulting from the stress. Plasma glucose concentration decreased during the first 12 hours after stress but, afterwards, it tended to fluctuate around normal values. The energetic cost from this stress was very high and particularly aerobic. The amount of lipids metabolized over 48 hours after stress was very large (70 $\mu$ mol of triglycerides) as compared to glucose (3.3  $\mu$ mol). This is characteristic of aerobic preference. The liver glycogen decreased throughout the experimental recovery (Fig. 4). However, considering that glucose and lactate remained constant and plasma glucose decreased, it is plausible to suggest the use of the glycogen within the liver. In view of the large amount of glycogen hydrolyzed (183  $\mu$ mol of glucosyl-glucose per gram of wet tissue) it is improbable that its use was as an energy source. Therefore, transformation of glucose into other kinds of metabolites is suggested. It is interesting to observe that white muscle lactate decreased 12 hours after the stress, returning slightly to normal values. White muscle glycogen were also slightly decreased (1  $\mu$ mol per gram of wet tissue) during 12 hours from the stress, returning back to normal values after 48 hours. Glucose was consumed at higher rates (4  $\mu$ mol per gram of tissue) than glycogen, but not statistically significantly. However, considering the usual anaerobic preference of white muscles, this metabolic behavior suggests an inconspicuous use of anaerobic metabolism to supply the energetic demand in such tissue after handling. The kidney consumed about 7.00 $\mu$ mol of glucose/mg of wet tissue after 24 hours of handling. However, the bulk of glycogen remained almost constant (13.00 $\mu$ mol of glucosyl-glucose/mg of wet tissue). The glucose decrease was very smooth and after 24 hours, the recovery had begun. The kidney glycogen stock was 36.00 $\mu$ mol of glucosyl-glucose/mg

of wet tissue, and its consumption was not significant. However, the use of glucose was very expressive, decreasing from the stress until 24 hours later (27  $\mu\text{mol}$ s per gram of tissue). Considering that after handling lactate remained constant, it is possible to assume an oxidative metabolism in this tissue.

The hematological response observed to handling, as well as the organismal metabolic changes, are both very suggestive that *P. coruscans* presents an oxidative preference to supply the metabolic cost from physical disturbance. The large plasma triglyceride decrease compared to the slight glucose consume is a strong indication that lipids are the preferential fuel supply for handling demands from stress.

Table 1. Blood values after 3 minutes of handling, in *Pseudoplastystoma coruscans*.

	Control	0 h	12 h	24 h	38 h
Hemoglobin (g/100 mL)	5.92 $\pm$ 0.76	9.22 $\pm$ 1.28 (*)	7.49 $\pm$ 0.98	7.98 $\pm$ 1.22 (*)	6.51 $\pm$ 0.96
RBC (10 <sup>6</sup> cels/mL)	1.26 $\pm$ 0.83	1.67 $\pm$ 0.33 (*)	1.59 $\pm$ 0.28 (*)	1.25 $\pm$ 0.14	1.12 $\pm$ 0.16
Hematocrit (%)	17.58 $\pm$ 2.67	24.20 $\pm$ 1.68 (*)	19.58 $\pm$ 2.15	14.43 $\pm$ 1.93	16.00 $\pm$ 2.98
CMV (nm <sup>3</sup> )	14.04 $\pm$ 2.15	15.13 $\pm$ 3.69	12.63 $\pm$ 2.31	11.69 $\pm$ 1.48 (*)	14.52 $\pm$ 3.12
MCH ( $\mu\text{g}$ /cell)	4.73 $\pm$ 0.63	5.77 $\pm$ 1.55 (*)	4.83 $\pm$ 0.94	6.46 $\pm$ 1.11 (*)	5.91 $\pm$ 1.14 (*)

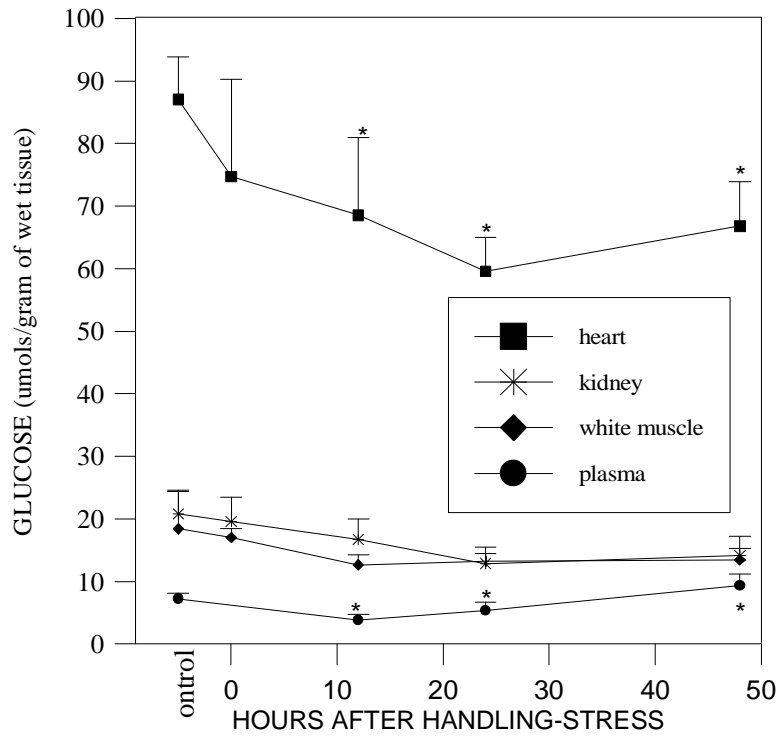


Figure 1. Tissue glucose concentration after 3 minutes of handling in *Pseudoplatystoma coruscans*.

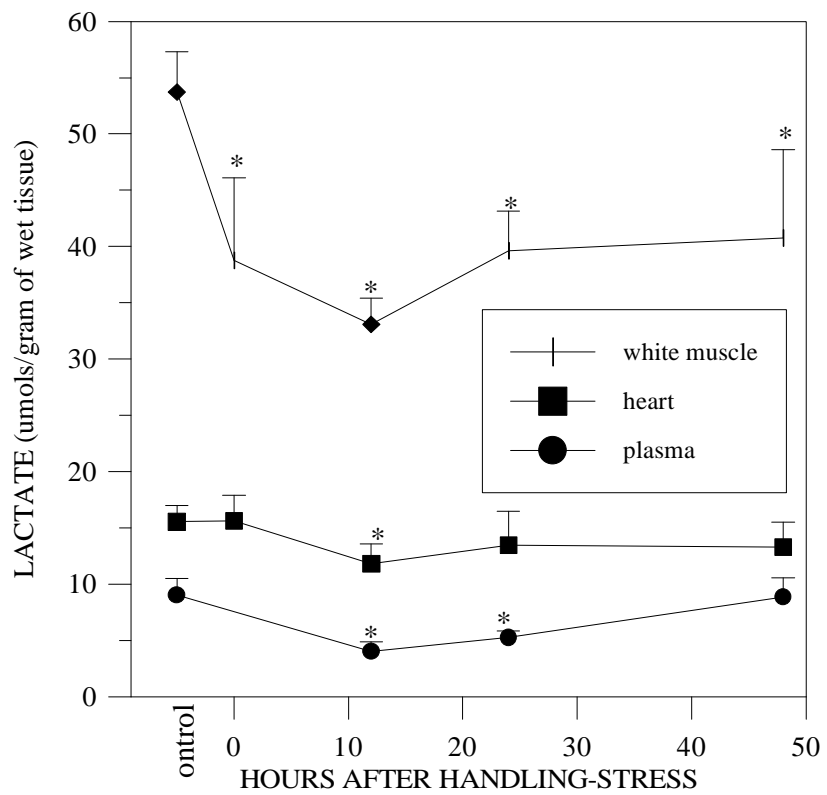


Figure 2. Tissue lactate concentration after 3 minutes of handling in *Pseudoplatystoma coruscans*.

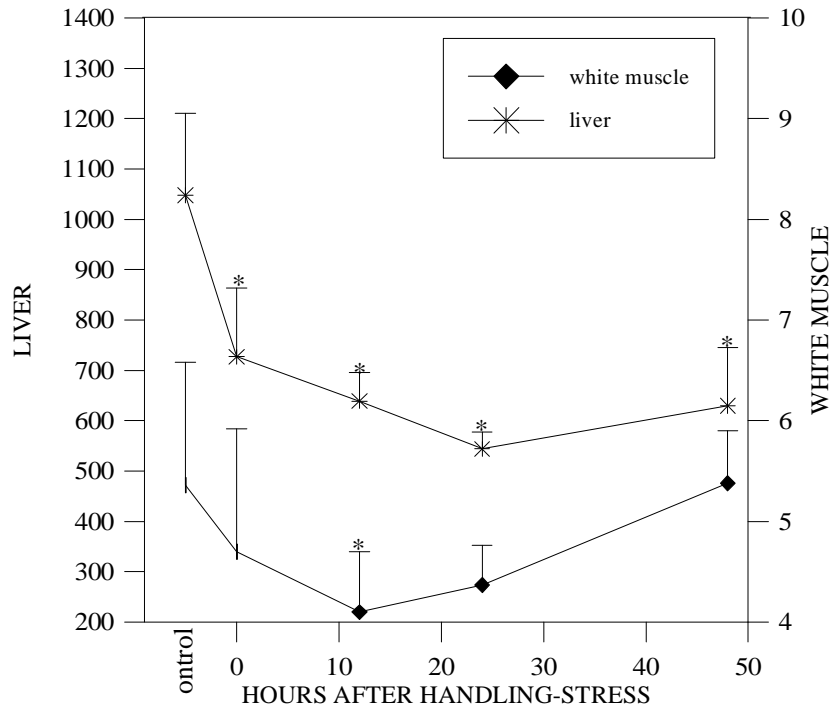


Figure 3. Tissue glycogen concentration ( $\mu\text{mols}$  glucosyl-glucose/gram of wet tissue) after 3 minutes of handling in *Pseudoplastystoma coruscans*.

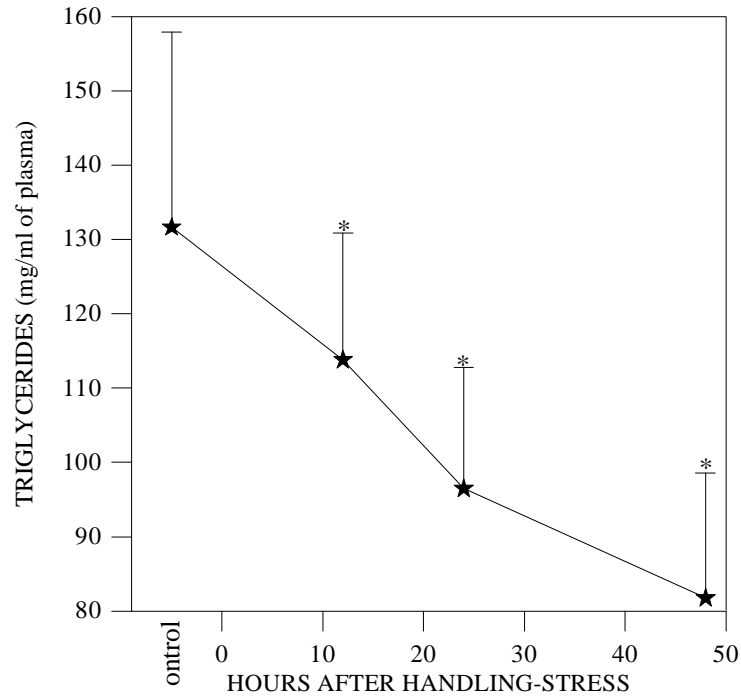


Figure 4. Plasma triglyceride concentration after 3 minutes of handling in *Pseudoplatystoma coruscans*.

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**VARIATION IN THE STRESS RESPONSE  
BETWEEN GENETICALLY DIFFERENT STRAINS  
OF COMMON CARP, *CYPRINUS CARPIO* L.**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

**Introduction**

Inter and intra-species differences in the response to stress have been well reported in the literature (Salonius & Iwama, 1991; Ruane et al., 1999). While these differences with respect to species are clear, the genetic and environmental influences causing intra-species variations in the stress response are difficult to separate. Therefore in this study isogenic carp strains with a known genetic background were used. Fish for each experiment were produced on the same day and reared under identical conditions to reduce environmental influences.

**Materials and Methods**

*Fish*

Different isogenic strains of common carp were produced by normal fertilization techniques (Komen et al., 1988) or by androgenesis (Bongers et al., 1998) in the University hatchery (De Haar Vissen, Wageningen University). Fish larvae were fed freshly hatched *Artemia* nauplii for the first 21 days and with pelleted food thereafter at a rate of 20 g/kg<sup>0.8</sup>. This level was reduced to 10 g/kg<sup>0.8</sup> at 100 days post hatch (dph) and all fish were sampled at 145 dph. Fish were weighed

every two weeks and the new feeding level was calculated based on the average weight of all strains. Two experiments were carried out each investigating the response of four strains of carp to a 3-h net confinement: one strain (E4E5 x R3R8) was used in both experiments to assess the magnitude of inter-experimental variation.

Table 1. Overview of the different carp strains used in both experiments.

Exp 1. Strain	Sex	Wt. 145 dph (g)	Exp 2. Strain	Sex	Wt. (g)
E4E5 x R3R8	XY	84.7 ± 1.8	E4E5 x R3R8	XY	84.6 ± 2.0
E4	XX	80.7 ± 5.9	41DF x R3R8	XY	88.0 ± 1.8
E5	XX	96.2 ± 6.7	0DD0 x R3R8	XY	91.1 ± 2.5
R3R8	YY	75.5 ± 6.8	EB9E x R3R8	XY	85.8 ± 1.2

Parents R3R8 : homozygous male (YY), inbred strain, Polish/Hungarian origin  
 E4 : homozygous female (XX), inbred strain, Dutch origin  
 E5 : homozygous male sex-reversed (XX), inbred strain, Dutch origin  
 E4E5 : isogenic progeny of E4 x E5  
 41DF, 0DD0, EB9E : homozygous inbred strains selected for high, high, low cortisol response to cold shocks

### Experimental Procedures and Analyses

Each carp strain was reared separately in duplicate tanks containing 20 – 30 fish each. The net confinement procedure used was described previously (Ruane et al., 1999). Plasma cortisol levels were measured by radioimmunoassay (Tanck et al., accepted) and statistically analysed using the parametric general linear model procedure (SAS Institute Inc.) with a Tukey test as post test determining differences with the control 0-h values and between the strains at each sample point.

### Results and Discussion

#### *Environmental effects*

The standard strain (E4E5 x R3R8) included in both experiments as a control showed a similar growth rate (Table 1) and also had a comparable cortisol response to the confinement stressor indicating that the environmental differences between each experiment were minimal. These data support the

suggestion by Bongers et al. (1998) that genetically uniform fish perform in a predictable manner under similar environmental conditions.

### Genetic effects

Net confinement induced a significant increase in plasma cortisol levels in all carp strains tested (Figs 1A & B). Significant differences were found in the cortisol response between the strains in experiment 1, but not in experiment 2.

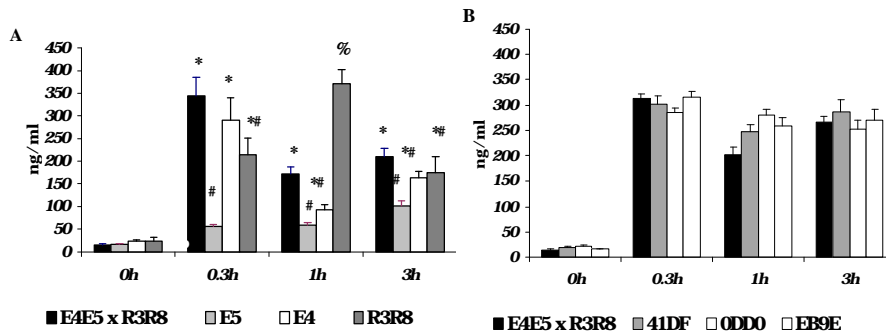


Fig 1. The effect of a 3-h net-confinement on plasma cortisol in different strains of common carp. Values are means  $\pm$  SEM,  $n = 5-10$ . Different symbols indicate significant differences at that sample point. All confined cortisol levels (0.3, 1 and 3 h) are significantly higher than the respective 0 h controls.

The female fish used in experiment 2 had previously been selected based upon a high or low cortisol response to a temperature shock (Table 1, M. Tanck & J. Komen, unpubl. data). Crossing with the R3R8 male which appeared to have a higher response (Fig. 1A) eliminated this effect. However it may also be possible that selection for a response to one type of stressor (e.g. cold shocks) may not necessarily be similar for another type (e.g. net-confinement). Further work will be carried out to determine the underlying mechanisms for the different cortisol responses in experiment 1 and whether this conveys significant benefits/costs in physiological performance.

### **Acknowledgements**

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**EFFECTS OF HATCHERY STRESS**  
**ON HEAT SHOCK PROTEIN INDUCTION**  
**IN JUVENILE ATLANTIC SALMON, *SALMO SALAR***

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

The development of sensitive and rapid methods to identify stressful conditions in animal husbandry has been the goal of numerous studies. The expression of heat shock proteins (hsps) has been considered as a possible candidate as a stress indicator. To test this hypothesis, juvenile Atlantic salmon (*Salmo salar*) were exposed to common forms of hatchery stress and the response of hsps 90, 70 and 30 determined. Treatments included exposure to:

- 1) two types of anesthesia (tricaine methanesulfonate, 75 ppm and 2-phenoxyethanol 250 ppm; fish fully anesthetized for 10 minutes followed by a one hour recovery period)
- 2) formalin (250 ppm for two hours followed by a one hour recovery period)

- 3) hypoxia (fish maintained in a static tank without aeration until gasping at surface, followed by a one-hour recovery period)
- 4) hyperoxia (99% O<sub>2</sub> bubbled into static tank for 30 min (approximately 22 ppm dissolved oxygen) followed by a one hour recovery period)
- 5) capture stress (fish netted and allowed to struggle in net out of water for 20 seconds, returned to the water for 20 seconds, repeated three times followed by a one-hour recovery period)
- 6) crowding, (15 fish (mean weight 43 g) in 20 L of water for 3 hours followed by a one hour recovery period)
- 7) starvation (fish fasted for one week)
- 8) cold shock (two hours at 4°C, followed by a one hour recovery period at ambient temperature 19°C )
- 9) Positive control - 15-minute heat shock at 26°C ( $\Delta T=10^{\circ}\text{C}$ ), followed by a one hour recovery period
- 10) Negative control – unhandled fish.

Total RNA was isolated from gill tissue following treatment, and subjected to Northern analysis with cDNA probes generated to hsps 90, 70 and 30 by polymerase chain reaction (PCR) or cloning. Assay of mRNA for actin, a prominent constitutive protein, was used to normalize hsp mRNA. Hsp mRNA was not upregulated in response to the different hatchery stresses, whereas heat shock resulted in upregulation of hsps 30 (Fig. 1) and 70. Although hsp90 mRNA levels were unaffected by the heat shock treatment used in this experiment, a subsequent investigation revealed that a 30 minute exposure to 26°C water stimulated upregulation. These data suggest that hsps 90, 70 and 30 are not sensitive indicators of hatchery stress in Atlantic salmon.

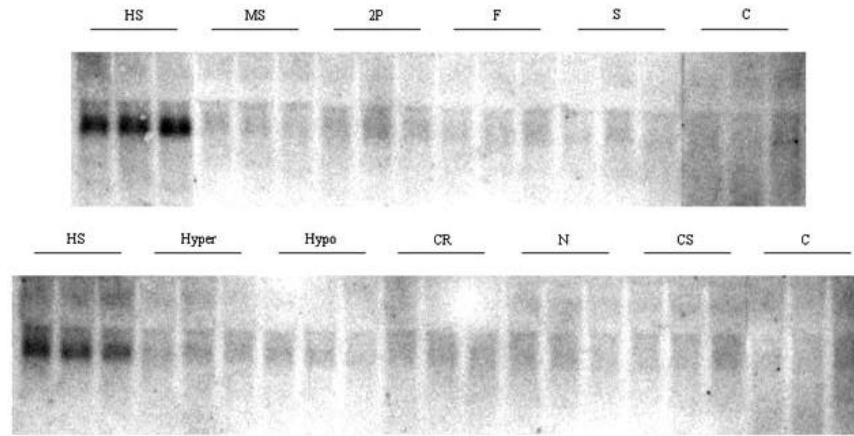


Figure 1. Northern blot of gill tissues from juvenile Atlantic salmon exposed to control and stress conditions as described in text and probed with  $\alpha$ -<sup>32</sup>P dATP labeled hsp30 cDNA. HS = heat shock, MS = tricaine methanesulfonate, 2P = 2 -phenoxyethanol, F = formalin, S = starvation, C = negative control, Hyper = hyperoxia, Hypo = hypoxia, CR = crowding, N = capture stress, CS = cold shock.

### Acknowledgements

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**NEUROTRANSMITTER ACTIVITY IN THE FORE- AND HIND-BRAIN  
OF THE PALLID STURGEON, *SCAPHIRHYNCHUS ALBUS*,  
FOLLOWING ACUTE AND CHRONIC STRESS**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

In the United States, the pallid sturgeon (*Scaphirhynchus albus*) is a federally endangered fish of the Missouri River. Because of the loss of natural spawning habitat, fisheries managers are depending on hatchery-reared fish to re-establish populations. Hatchery managers require information regarding how pallid sturgeon are affected by stress in order to maximize their health, growth, survivorship, and reproductive capacity while in captivity. Previously these sturgeon were shown to have a reduced corticosteroid response to stress relative to teleosts. Thus, the objective of this study was to characterize fore- and hind-brain neurotransmitter responses to acute and chronic stress to determine if the low stress responsiveness of this species was also apparent at the central nervous system level.

Six neurotransmitters—epinephrine (Epi), 5-hydroxytryptophan (5-HTP), 3,4 dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindoleacetic acid (5-HIAA), and 5-hydroxytryptamine (5-HT, serotonin)—were measured in the fore-brain and the hind-brain using high performance liquid chromatography. The ratio of the concentrations of DOPAC to DA was calculated as an index of dopaminergic activity. The ratios of the concentrations

of 5-HTP to 5-HT and 5-HIAA to 5-HT were calculated as indices of serotonergic activity. DOPAC, DA, 5-HTP, 5-HIAA, and 5-HT were detected in the forebrain and Epi, DA, 5-HT and 5-HIAA were detected in the hind-brain.

In our first experiment, pallid sturgeon were subjected to a 30-s (acute) handling stressor. Fore- and hind-brain tissues were sampled from these fish immediately (0 h), 1, 3, 6, and 25 h after the application of the stressor. The acute stressor did not affect any of the neurotransmitters or their activity in either of the brain regions. Additionally, concentrations of DA, 5-HIAA, 5-HT and the ratio of 5-HIAA to 5-HT were significantly higher in the fore-brain than the hind brain throughout the experiment.

In the second experiment pallid sturgeon were subjected to a chronic density stressor. Groups of fish were held at high density (4.0 kg/m<sup>2</sup>) or at low density (1.0 to 0.33 kg/m<sup>2</sup>). Fore- and hind-brain tissues were sampled at 3, 7, 13, and 21 d during the confinement. A subset of sturgeon from both density treatments at 7, 13, and 21 d was subjected to a 30 s handling stressor before tissue sampling.

There were no significant differences in the concentrations any of the six neurotransmitters between sturgeon held at high and low density over the 21 d experiment. Again, throughout the experiment, the fore-brain exhibited significantly higher concentrations of DA, 5-HIAA and 5-HT as well as higher 5-HIAA to 5-HT ratios than the hind-brain. However, in chronically confined sturgeon subjected to an additional acute stressor, there were significant differences attributed to density in DA, 5-HIAA, and 5-HT concentrations. Fore-brain DA concentrations significantly increased during the periods from 7 to 13 d and from 13 to 21 d in fish held at high density whereas fore-brain DA concentrations remained constant in low density held fish (Figure 1). A similar significant trend was apparent in fore-brain 5-HIAA concentrations (Figure 2). There was a significant increase in fore- and hind-brain 5-HT concentrations from 7 to 13 d and from 13 to 21 d in high density held fish but fore- and hind-brain levels remained constant in those held at low density (Figure 3). These results suggest that pallid sturgeon chronically confined at high density exhibit different neurotransmitter responses to acute stress than do sturgeon chronically confined at low density.

Figure 1: Mean DA levels (pg/mg) in the fore- and hind-brain of pallid sturgeon held at two different densities for 21 d and then subjected to an additional 30-s net stress at 7, 14, and 21 d. Error bars indicate standard error of the mean. Shaded bars are fore-brain DA levels and open bars are hind-brain levels. Fore-brain DA concentrations significantly increased from 7 to 13 d and from 13 to 21 d in fish held at high density whereas fore-brain DA concentrations were constant in low density-held fish.

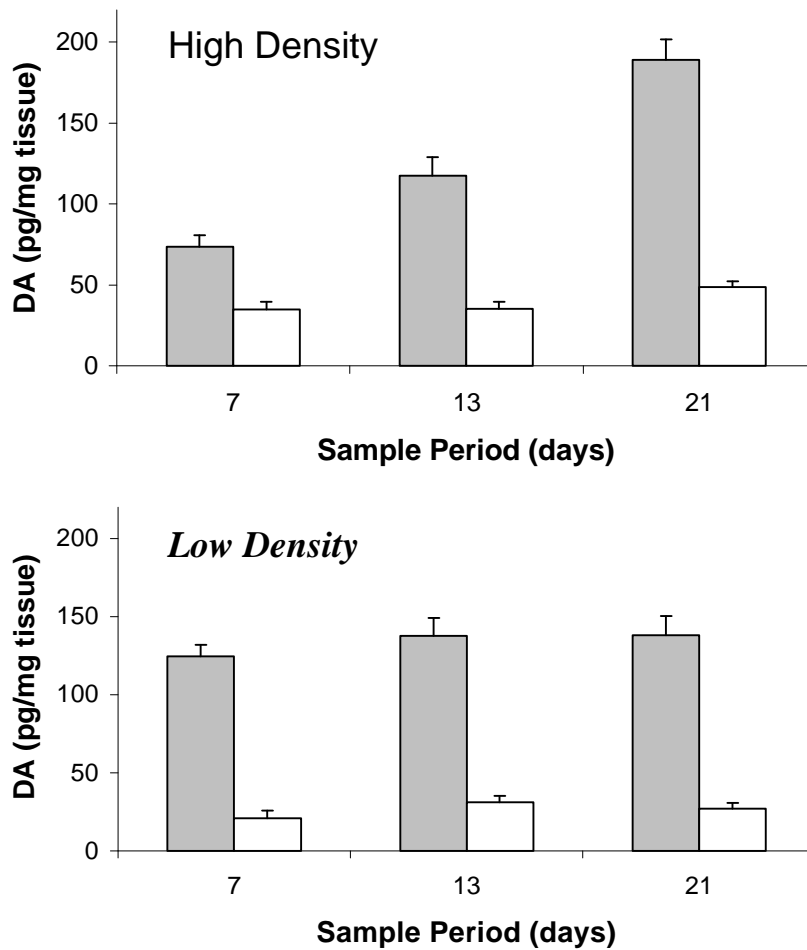
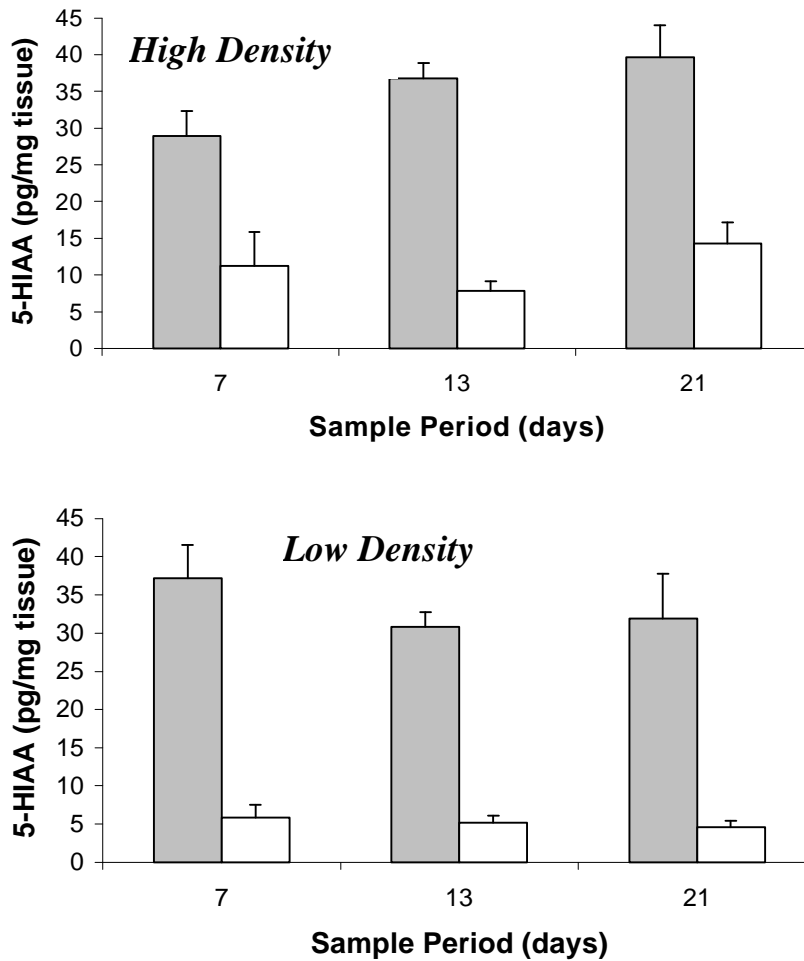
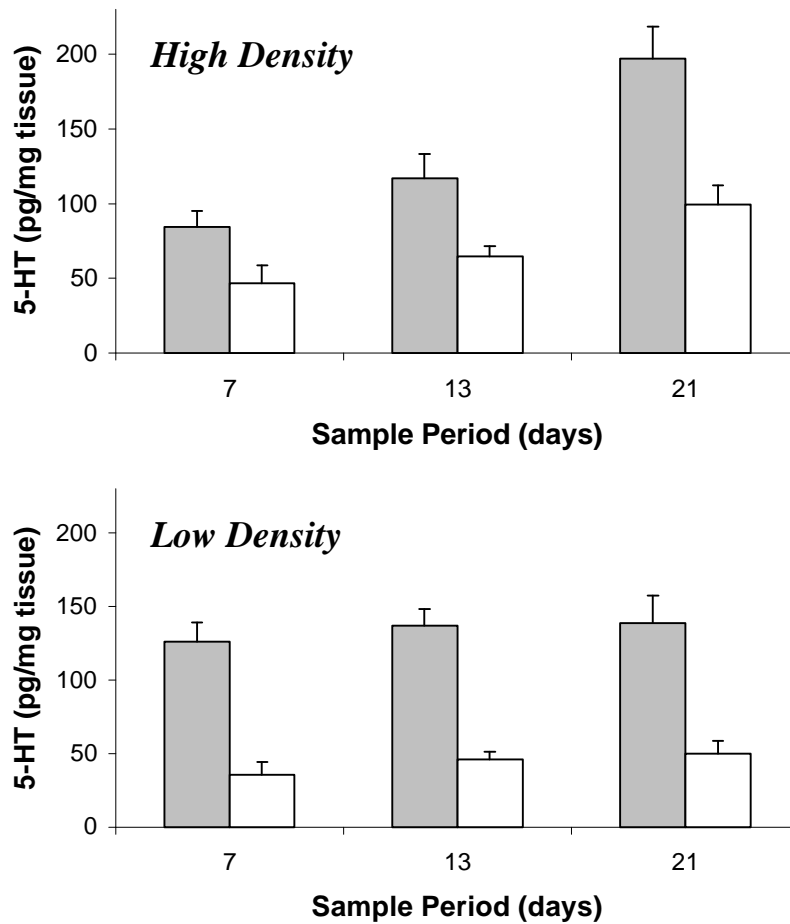


Figure 2: Mean 5-HIAA levels (pg/mg) in the fore- and hind-brain of pallid sturgeon held at two different densities for 21 d and then subjected to an additional 30-s net stress at 7, 14, and 21 d. Error bars indicate standard error of the mean. Shaded bars are fore-brain 5-HIAA levels and open bars are hind-brain levels. Fore-brain 5-HIAA concentrations significantly increased from 7 to 13 d and from 13 to 21 d in fish held at high density whereas fore-brain DA concentrations were constant in low density-held fish.



**Figure 3:** Mean 5-HT levels (pg/mg) in the fore- and hind-brain of pallid sturgeon held at two different densities for 21 d and then subjected to an additional 30-s net stress at 7, 14, and 21 d. Error bars indicate standard error of the mean. Shaded bars are fore-brain 5-HT levels and open bars are hind-brain levels. There was a significant increase in fore- and hind-brain 5-HT concentrations from 7 to 13 d and 13 to 21 d in high density fish but fore- and hind-brain levels remained constant in the low density-held group.



**Acknowledgements**

We thank Herb Bollig, U.S. Fish and Wildlife Service, for providing the fish and students Dan James, Catherine Sykes, Chris Jansen, and Rachel West for assistance in tissue and plasma collection.

**HANDLING STRESS AFFECTS AVOIDANCE BEHAVIOURAL  
RESPONSES OF JUVENILE WALLEYE, *Stizostedion vitreum***

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Walleye, *Stizostedion vitreum* (Percidae), are subjected to a variety of handling-related stressors as part of normal transport and stocking operations to support recreational fishery programs. The objective of this study was to determine the effects of handling and transport stress on the avoidance response behaviour of juvenile walleye in the context of their ability to avoid predation after stocking.

The behavioural test chamber consisted of a VHS-video camera mounted vertically on a bracket above a 1.1-m-diameter circular  $\times$  30-cm-deep fiberglass tank. The entire apparatus was surrounded by a wooden frame enclosed with heavy black plastic to block external visual stimuli. A spotlight assembly consisting of four 150-W incandescent bulbs located above and directed into the tank provided an instantaneous noxious stimulus. The video camera and spotlights were controlled by a computer programmed to command the on-off timing of the video camera and spotlight operations.

We evaluated the response of the fish visible in the field of the camera by recording each trial frame-by-frame to document the movements of individual fish. In 1997, 50% of the surface area of the tank was exposed to the light stimulus (Fig. 1, areas  $N_1$  and  $N_2$ ). The behavioural avoidance response was characterized as the amount of time elapsed for individual fish to swim out of the field of view and seek cover ( $n = 15$ ). The behavioural response was

measured in 1998 as the amount of time required for individual fish ( $n = 30$ ) to swim out of or among the individual squares within a grid marked on the floor of the pool (Fig. 1, areas  $N_1$ ,  $N_2$ ,  $N_3$  and  $N_4$ ).

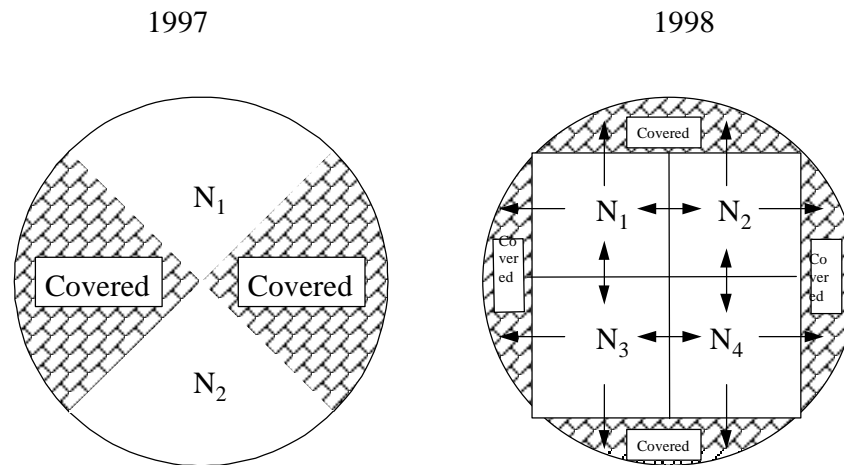


Figure 1. Layout of observation fields and cover patterns in the behaviour observation chamber for avoidance response experiments conducted with walleye fingerlings during 1997 and 1998.

In 1997, groups of fish were allowed to recover for intervals of 1, 12, 24 and 72 h following the stressor treatment (30-s handling – 1-h recovery – 30-s handling); 7-d acclimated unstressed fish served as controls. During 1998, the recovery intervals used were 1 h, 12 h after evening capture, 12 h after morning capture, and 24 h. Unstressed control groups were allowed 1 d to recover after transfer. Observations from replicate groups for each recovery period and control groups were recorded in both years. Fish were removed at the end of each observation period to collect blood samples.

The median avoidance response of fish allowed different recovery times after handling differed significantly in 1997 trials (Fig. 2). In 1997, the median time to seek cover for fish that had recovered for only 1 h was

11.4 s, which was significantly longer than for fish allowed longer periods of recovery and also for non-stressed control fish. The median times for fish to seek cover after recovering for 12, 24 and 72 h following handling, and control fish, were 3.0, 2.8 and 4.3 s, and 3.2 s, respectively, and did not differ from one another.

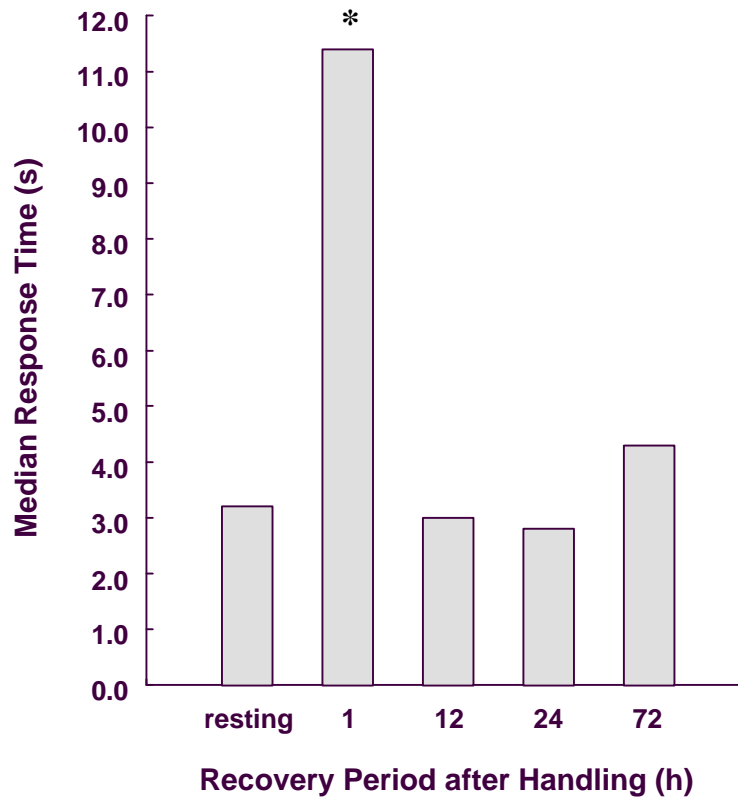


Figure 2. Median avoidance response times, pooled from two replicate trial series conducted in 1997, of juvenile walleye subjected a noxious light stimulus after varying durations of recovery following a handling stressor (\* different from resting,  $P < 0.05$ ).

Fish that had only recovered for 1 h in 1998 trials responded more slowly to the noxious light stimulus than fish given 24 h to recover and also control fish (Fig. 3). The slowest avoidance response, however, occurred in fish that were tested 12 h after a handling stressor applied in the morning (AM); the median response time for this group was 9.6 s, which was significantly longer than that in either the 1-h recovery group or in fish tested 12 h after applying the stressor in the

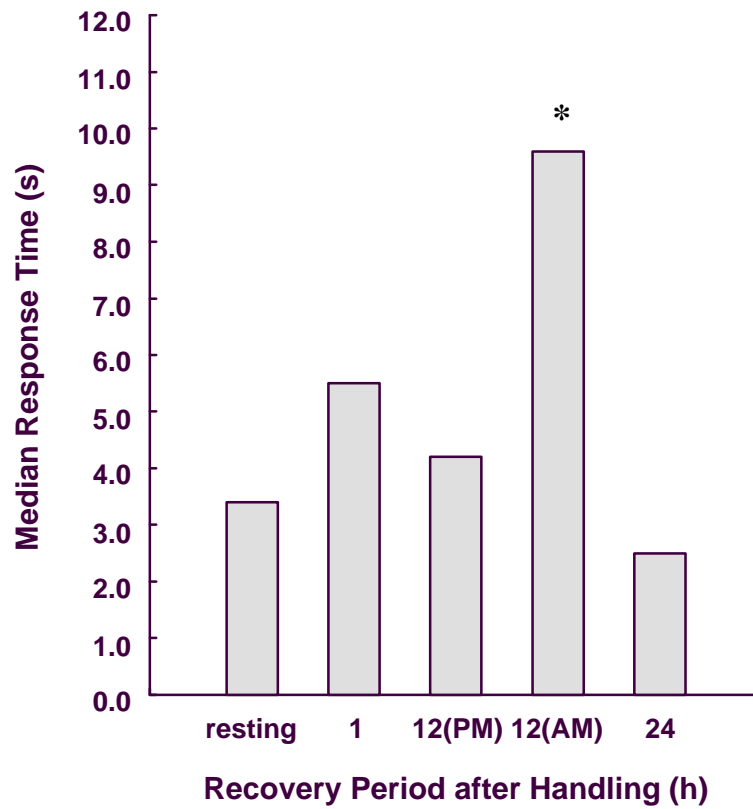


Figure 3. Median avoidance response times, pooled from two replicate trial series conducted in 1998, of juvenile walleye subjected a noxious light stimulus after varying durations of recovery

following a handling stressor (\* different from resting,  $P < 0.05$ ).

evening (PM) and sampled the next morning. In both years there were instances where individual fish did not move from the field of view for the entire 27-min observation period, but this only occurred in fish with only 1 h to recover from handling.

The group mean plasma cortisol concentrations in fish sampled after 1 h and 1 d of recovery (32 and 33 ng/mL) were higher than plasma cortisol in fish collected after observation following 12 h or 3 d of recovery (8.4 and 18 ng/mL). The lowest plasma cortisol levels were in undisturbed (resting) fish collected from the source tanks (3.1 ng/mL) and in the control fish minimally disturbed and sampled 7 d after transfer into the observation tank (6.7 ng/mL). Plasma cortisol concentrations again were lowest in resting fish (4.9 ng/mL) sampled in 1998 trials and highest at 1 h post-handling (29 ng/mL), but the mean value determined for resting fish did not differ from that in fish following 12 h recovery after handling in the morning (8.7 ng/mL). Similar levels of plasma cortisol were measured in fish sampled 1 d after handling (18 ng/mL), the minimally stressed control group (19 ng/mL), and fish that had recovered 12 h overnight after being handled in the evening (18 ng/mL).

Plasma concentrations of chloride ion in 1997 tests ranged from 86.1 meq/L in fish sampled after 1 h of recovery, to 96.1 meq/L in undisturbed fish; mean values were not different. Concentrations of plasma chloride ion in 1998 were lowest among fish that recovered from the handling stressor for 1 h, but the mean value of 98.4 meq/L for this group differed significantly only from those in fish sampled from undisturbed conditions (114 meq/L) and at 12 h after handling them in the morning (104 meq/L).

The results of these tests demonstrate that differences in behaviour of juvenile walleye can occur that depend on the amount of time fish are allowed to recover from a handling stressor. In both years the extreme example of fish not moving at all in response to the light stimulus was observed in groups of fish that only had 1 h to recover. The delayed response to the noxious stimulus suggests that juvenile walleye might be vulnerable to predation for at least an hour after being transported and stocked. The reduced ability to rapidly seek cover was a transitory phenomenon and when the fish were given more time to recover, the

median avoidance response time was reduced. The rate of recovery differed slightly between years but when fish had recovered for 24 h after handling, the median avoidance response was reduced by at least 50% in both years. No direct correlation existed between plasma indices of stress and behaviour.

### **Acknowledgements**

We thank students Robert Pugh, Justin Sipiorski, Craig Donelan, and Chris Jansen for their technical assistance.

**PHYSIOLOGICAL STRESS RESPONSES  
OF STREAM DWELLING RAINBOW TROUT  
TO CLEAR-CUT LOGGING**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

**Introduction**

Stress in fish affects a variety of functions, including reproduction, growth and resistance to disease (Wedemeyer et al. 1984). Because clear-cut logging remains the most common logging practice in British Columbia, Canada, the potential exists for impacts associated with the removal of riparian vegetation to be stressful to stream dwelling fish. We examined the effects of clear-cut logging on the health of rainbow trout (*Oncorhynchus mykiss*) in the central interior of BC, a region characterized by a temperate climate. Indicators of fish health comprised body condition and physiological stress responses (both acute and chronic).

**Methods**

Fish were sampled in September/October 1996 from 15 small (bankfull width 2.4-4.7m) streams divided into 3 categories: unlogged (control), recently logged (clear-cut to both stream banks within the last 5-10 years), and older logged (clear cut more than 25 years ago). We did not measure potential stressors in our streams, and instead assumed that our categorization of logging integrated all

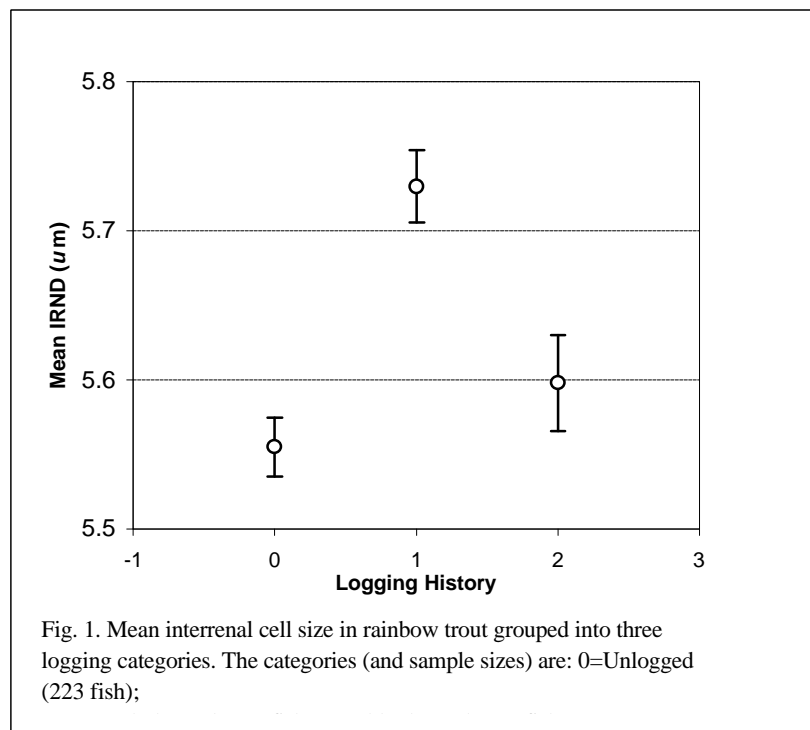
associated impacts, whatever they may be. However, high temperatures (resulting from reductions in riparian canopy cover) and increased suspended sediment levels (resulting from recent road building activities) were likely the primary stressors acting on fish in streams with recently harvested riparian zones. Likewise, habitat alterations (resulting from long-term reductions in large organic debris recruitment) were likely the primary stressors operating in older clear-cut streams. We chose plasma cortisol, glucose and chloride concentrations as indicators of acute stress (Wedemeyer et al. 1984), and interrenal nuclear diameters (IRND; Donaldson et al. 1984), impairment of the plasma cortisol response (Hontela et al. 1992), and fish condition as indicators of chronic stress. If clear-cut logging is stressful to stream dwelling fish, then we predict plasma cortisol and glucose levels, as well as interrenal nuclear diameters, would be greater in fish from logged streams compared to fish from control streams. In addition, we predict that fish from the two logged categories would show lower plasma chloride concentrations and body condition, as well as showing evidence of impairment of the cortisol response system. Mean values for IRND and for each haematological indicator were grouped according to treatment and were compared using analysis of variance (ANOVA). Fish condition was assessed by comparing length-weight relationships across our three treatments using analysis of covariance (ANCOVA).

## **Results and Discussion**

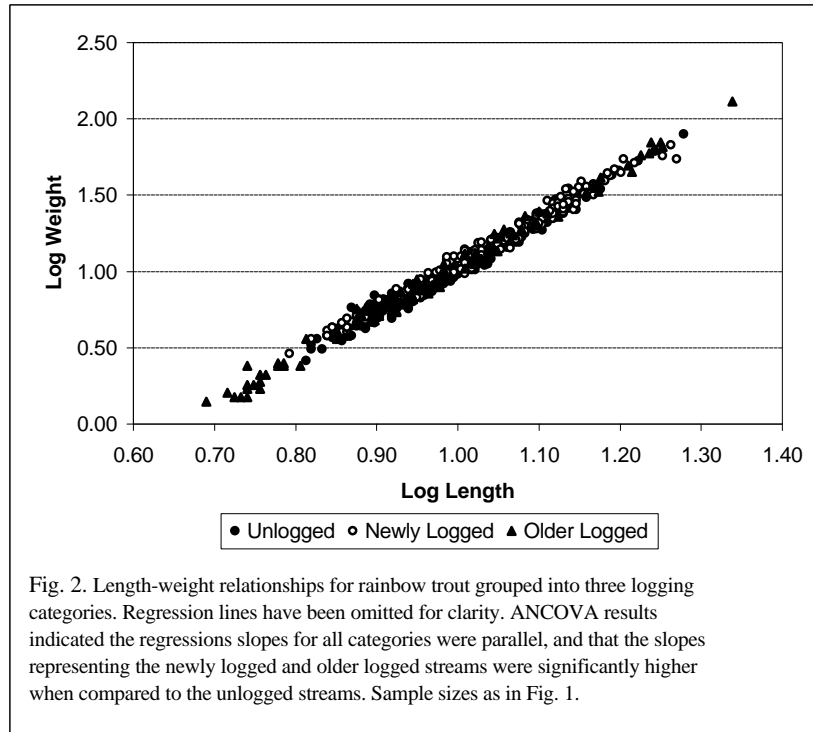
Plasma glucose concentration was the only acute stress indicator that showed a response to clear-cut logging, with fish from older logged streams exhibiting significantly higher ( $p < 0.001$ ) levels than fish from either the recently logged or control streams. However, blood glucose levels are influenced by diet and satiation levels (Barton et al. 1988), and these are factors we could not control for in our study. We therefore urge caution in interpreting the glucose results, and because cortisol concentrations do not appear to be influenced by diet and satiation level (Barton et al. 1988), we instead place greater emphasis on our cortisol results (which showed no acute responses to clear-cut logging).

With respect to our chronic stress indicators, no impairment of the cortisol response was evident in the treatment streams. On the other hand, fish from recently logged streams had significantly larger ( $p < 0.001$ ) mean IRND values than fish from either the control or older logged streams (Fig. 1), suggesting the presence of a chronic stressor(s) within that treatment category. However, the average increase in IRND was approximately 3% relative to the control streams,

compared to increases of 9-25% reported in the literature for chronically stressed fish. In addition, Donaldson et al. (1984) and Brown et al. (18984) found increases in plasma cortisol concentrations that were concomitant with increases in IRND, a finding not supported by our results. (Because interrenal cells are the cells that produce cortisol, an increase in IRND is expected to be accompanied by an increase in cortisol levels). Therefore, while our IRND results are statistically significant, we question their biological validity.



With respect to fish condition, the ANCOVA results showed that the slopes of all three length-weight regressions were homogeneous, and that the regression intercepts (treatment effects) were significantly higher ( $p < 0.001$ ) for newly logged and older logged streams when compared to the control streams (Fig. 2). This suggests that fish from streams that were clear-cut (either recently or over 25 years ago) were significantly heavier across all lengths compared to fish from unlogged streams.



## Conclusions

Our results suggest that clear-cut logging (even without riparian buffer strips) may not be detrimental to fish health (as measured by our acute and chronic stress indicators) in central interior streams of British Columbia. Furthermore, streamside harvesting may confer some benefit to fish growth (as measured using fish condition) over the time interval covered by our study. However, in the absence of further analyses (incorporating fish abundance and size distributions, as well as stream physical attributes), we must emphasize the preliminary nature of our conclusions.

### **Acknowledgements**

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**METABOLIC REARRANGEMENTS OF *Synbranchus marmoratus***

**SUBMITTED TO ENVIRONMENTAL DEHYDRATION**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Seasonal changes in the environment, like those occurring in the tropical regions, might lead species to rearrange their metabolic profile to reach proper adjustments, which help to avoid a sort of injuries. Amongst different classes of response to environmental changes, aestivation, which occurs during the dry season, probably causes the major number of transitory modifications in the metabolism.

For some fish, dry season means little water availability or even its total absence. For instance, lungfish like *Lepidosiren paradoxa* may experience confinement in a moist mud burrow and survive several months during the dry season (Dunn, 1983).

There are four problems that emerge when fish is exposed to the air: 1) Their gas exchange apparatus is not able to perform O<sub>2</sub> uptake and CO<sub>2</sub> releasing, unless they have (like Lepidosirenidae and Synbranchidae) air breathing organs. Such an inability causes anoxia and blood pH decrease. 2) Ammonia, the main

nitrogen metabolism waste, cannot be released to the environment via gills during air exposure and, as it's well known, this molecule is toxic to the nervous system even in relatively low levels. 3) Air exposition causes strong dehydration, whose consequences are changes in plasma electrolytic composition and acid-basic equilibrium and 4) Air exposed fish are not able to get food, so they suffer starvation during such exposition (Johansen, 1970; DeLaney, *et al.*, 1974).

To face the problems above, fish adopt physiological, biochemical and behavioural strategies which include decrease in metabolic rate and increase in anaerobic metabolism; changes in nitrogen metabolism, and burrowing in the mud (Long, 1995).

*Synbranchus marmoratus*, called swamp eel, can be found in many different habitats. It has a single ventral opercular opening and its branchial chambers have a well-developed vascular epithelium able to perform gas exchange (Liem, 1987). All individuals are hermaphrodites, which explains their ability to thrive in environments recently emerged. During severe dry season they aestivate and, in laboratory facilities, they were kept for 9-month aestivating (Bicudo and Johansen, 1979).

Eighteen specimens of *S. marmoratus* were kept in well-aerated 250 litre containers at 25°C for one week. Thereafter, six individuals (the control group) were killed by punching their spinal cord and then blood, liver, kidney, white muscle, heart and brain were taken. The other twelve individuals were divided into two groups of six, and each group was placed within containers with water and mud. Afterwards, water was gradually removed from the containers creating a muddy environment that induced fish to aestivate. Both groups were kept aestivating, one for 15 and the other for 45 days. After each period, the fish were killed and their tissues and blood were taken as described for control group.

Analysis of variance (ANOVA) and Tukey-Kramer Multiple Comparisons test was applied for all data, with  $p > 0,05$ .

Table1. Measures made in each tissue.

	<i>Liver</i>	<i>Kidney</i>	<i>Heart</i>	<i>Brain</i>
Glucose	§	§	§	§
Glycogen	§	§		
Lactate	§	§	§	§
Pyruvate	§	§		
f.a.a. <sup>1</sup>	§	§		
Arginase	§	§		
GS <sup>2</sup>	§	§		
GDH <sup>3</sup>	§		§	§
LDH <sup>4</sup>	§		§	§
MDH <sup>5</sup>	§		§	§

1= free amino acids; 2= glutamine synthetase; 3= glutamate dehydrogenase; 4= lactate dehydrogenase; 5= malate dehydrogenase.

The results showed that metabolic adjustments occurred in *S. marmoratus* in the way to attend its energetic demands. The increase of free amino acids in the kidney suggests the occurrence of proteolysis followed by gluconeogenesis and the exportation of glucose to the plasma. The enzyme activity of MDH, LDH and GDH suggest the preference for oxidative metabolism in brain and heart during aestivation.

The kidney seems to assume an important role in ammonia detoxification at the beginning of aestivation due to its glutamine synthetase activity. The activity of hepatic arginase suggests the role of the liver in the protein catabolism under the aestivation of swamp eel, which might provide free amino acids to gluconeogenesis pathway.

Table 2. Results. Values are mean  $\pm$  SD

		Liver	Kidney	Heart	Brain
Glucose $\mu\text{mols/g}$	Control	275,9 $\pm$ 51,3	25,1 $\pm$ 1,9	88,4 $\pm$ 15,1	19,3 $\pm$ 3,8
	15 days	280,4 $\pm$ 31,9	21,2 $\pm$ 3,4	67,7 $\pm$ 7,3	19,9 $\pm$ 1,7
	45 days	190,0 $\pm$ 56,0	24,4 $\pm$ 4,0	95,6 $\pm$ 20,9	25,2 $\pm$ 1,7
Glycogen $\mu\text{mols/g}$	Control	308,3 $\pm$ 63,2	62,7 $\pm$ 3,1	-	-
	15 days	365,6 $\pm$ 35,8	32,0 $\pm$ 5,7	-	-
	45 days	135,3 $\pm$ 40,3	33,9 $\pm$ 4,1	-	-
Lactate $\mu\text{mols/g}$	Control	20,4 $\pm$ 10,1	18,7 $\pm$ 3,5	28,4 $\pm$ 8,6	30,4 $\pm$ 5,6
	15 days	22,2 $\pm$ 6,8	11,0 $\pm$ 3,8	22,85 $\pm$ 2,5	28,5 $\pm$ 2,6
	45 days	15,7 $\pm$ 5,6	8,1 $\pm$ 1,5	18,0 $\pm$ 2,7	15,1 $\pm$ 2,7
Pyruvate $\mu\text{mols/g}$	Control	0,63 $\pm$ ,033	0,27 $\pm$ 0,16	-	-
	15 days	0,83 $\pm$ 0,32	0,47 $\pm$ 0,33	-	-
	45 days	0,97 $\pm$ 0,12	1,18 $\pm$ 0,16	-	-
f.a.a. $\mu\text{mols/g}$	Control	1,52 $\pm$ 0,21	2,0 $\pm$ 0,46	-	-
	15 days	1,61 $\pm$ 0,9	1,35 $\pm$ 0,29	-	-
	45 days	19,7 $\pm$ 0,04	16,2 $\pm$ 3,5	-	-
Arginase nmols/mg min	Control	13,1 $\pm$ 3,0	10,9 $\pm$ 3,0	-	-
	15 days	17,1 $\pm$ 1,8	5,1 $\pm$ 0,4	-	-
	45 days	21,5 $\pm$ 2,6	17,9 $\pm$ 1,6	-	-
GS nmols/mg min	Control	0,09 $\pm$ 0,02	0,04 $\pm$ 0,001	-	-
	15 days	0,0	0,1 $\pm$ 0,013	-	-
	45 days	0,0	0,0	-	-
GDH $\mu\text{mols/g/}$ min	Control	12,4 $\pm$ 3,4	-	5,0 $\pm$ 1,8	4,18 $\pm$ 0,63
	15 days	13,8 $\pm$ 3,3	-	9,9 $\pm$ 1,2	5,8 $\pm$ 1,3
	45 days	22,3 $\pm$ 4,9	-	8,5 $\pm$ 1,6	4,9 $\pm$ 0,6
LDH $\mu\text{mols/g/}$ min	Control	3,4 $\pm$ 0,5	-	115,5 $\pm$ 40,0	81,1 $\pm$ 21,4
	15 days	3,3 $\pm$ 0,4	-	232,0 $\pm$ 74,0	118,5 $\pm$ 21,4
	45 days	,2 $\pm$ 0,9	-	267,0 $\pm$ 18,4	104,2 $\pm$ 9,5
MDH $\mu\text{mols/g/}$ min	Control	18,8 $\pm$ 4,1	-	23,4 $\pm$ 4,0	6,1 $\pm$ 0,9
	15 days	18,4 $\pm$ 4,1	-	47,3 $\pm$ 7,6	-
	45 days	17,9 $\pm$ 1,2	-	43,3 $\pm$ 2,0	8,3 $\pm$ 1,5

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**EFFECTS OF DIETARY SUPPLEMENTAL TRYPTOPHAN  
ON INTRASPECIFIC AGGRESSIVE BEHAVIOUR  
IN RAINBOW TROUT:  
IMPLICATION FOR THE REARING OF FISH IN AQUACULTURE**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

In a recent series of experiments we have found that the addition of the amino acid L-tryptophan (TRP) to fish feed leads to a significant reduction of intraspecific aggressive behaviour in rainbow trout (*Oncorhynchus mykiss*). The amino acid L-tryptophan (TRP) is the precursor of the monoaminergic neurotransmitter, serotonin (5-HT). In fact the conversion of TRP to 5-hydroxytryptamin (5-HTP) by tryptophan hydroxylase is the rate limiting step in the 5-HT synthesis. Behavioural effects of 5-HT are well documented in both teleost fish and mammals, and the consensus is that 5-HT is believed to be involved in the regulation of agnostic behaviour and mediates a general

behavioural inhibition, including the reduction of aggressive behaviour. Intraspecific aggressive behaviour, and the formation of social dominance hierarchies, may constitute a substantial problem for the rearing of fish at high densities in aquaculture systems, and raise a number of problems concerning both production and animal welfare. The effect of hierarchy formation include injuries, unequal distribution of food and appetite reduction in subordinates, causing growth depensation in subordinates and stress induced, elevation of cortisol in subordinates, which negatively influence the immune system possibly related in immune suppression.

### **Methods**

Experiments were performed on fish isolated in individual 62,5 litres compartments in 250 litres glass aquaria. During the first week after transfer to social isolation fish were hand fed commercial feed (EWOS ST40) to satiation daily and individual feed intake was quantified. Following one week of acclimation the fish were tested for aggressive behaviour, using a resident-intruder test. A small rainbow trout (body wt ca 50 % of the resident fish) was introduced. The formation resident-intruder pairs pair of fish always led to unilateral aggression from the resident against the smaller intruder. The frequency of attacks during 30 min following the first aggressive act was quantified from video recording. After one hour the intruder was removed, and during the following week fish were fed an experimental wet feed prepared from herring and shrimps. For one group of fish (n=12), the experimental feed was supplemented with 1,5 % TRP (by weight), an other group (n=12) received 0,15 % TRP, while a similar number of controls received the same feed without TRP supplementation. The fish were fed to satiation daily, and the individual feed intake was recorded. Aggressive behaviour was quantified again after 3 and 7 days of TRP feeding, using the resident-intruder test. Following the last test of aggressive behaviour the fish were sacrificed, weighed, and sampled for blood and brain tissue (telencephalon, hypothalamus, brain stem). Some untested fish (n=8 in each group) were kept in isolation during the all experiment period to serve as no-stress controls for the effect of the intruder on blood and brain parameters. Blood plasma was analysed for TRP and cortisol concentrations, brain tissue for levels of TRP, serotonin, and 5-hydroxyindoleacetic acid (5-HIAA, the major 5-HT metabolite).

## Results and Discussion

The results show that dietary supplementation of TRP suppress aggressive behaviour in rainbow trout. Fish fed with 1,5 % and 0,15 % TRP supplemented feed for 7 days displayed a significant reduction in the number of aggressive acts performed against the intruder (Figure 1). However, there was no difference in aggressive behaviour between control and TRP supplemented fish after 3 days.

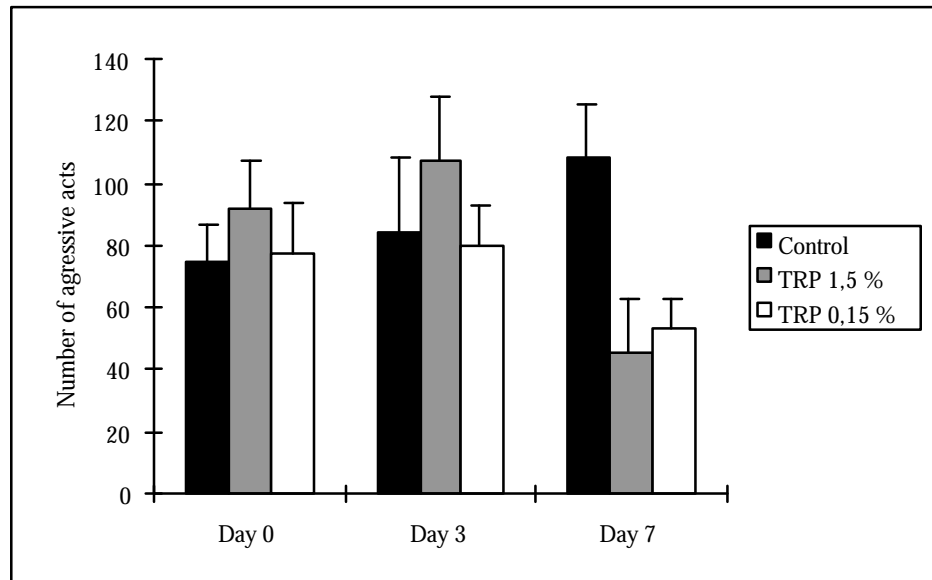


Figure 1. Number of aggressive acts performed by resident fish against an intruder during 30 minutes following the first aggressive acts, before TRP feeding (day 0), after three days (day 3), and seven days (day 7) of TRP feeding.

Plasma and brain levels of TRP were increased in fish given TRP supplemented feed as compare to controls. Brain levels of 5-HIAA, as well as 5-HIAA/5-HT ratio (an index of 5-HT activity), were increased in fish fed TRP supplemented feed. Fish subjected to resident-intruder test showed slightly higher plasma cortisol levels than undisturbed controls. There was no difference in plasma

cortisol concentration between TRP supplemented fish and controls, neither in fish subjected to resident-intruder tests, or in fish that had been kept isolated.

The observed inhibition of aggression appear to be related to a TRP-induced increase in brain serotonergic activity. The result suggest that supplementation with dietary TRP increases brain 5-HT activity, and thereby decreases aggressive behaviour in juvenile rainbow trout. The effect of TRP enhanced feed on aggressive behaviour suggest a number of experiments to precede the possible utilisation of dietary supplementation of TRP in large-scale aquaculture operations. Experiments include effects of supplemental TRP on physiological stress responses, on the immune system, and effects on social behaviour of fish during periodic feeding restrictions.

## WHY ARE SEA BASS SO SUSCEPTIBLE TO STRESS?

### INTERRENAL DYNAMICS PROVIDE SOME EXPLANATIONS

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#### EXTENDED ABSTRACT ONLY – DO NOT CITE

One of the problems regarding the culture of sea bass *Dicentrarchus labrax* is the stress susceptibility of this species. This condition does not allow the producers to often handle the fish for practices such as grading in order to get homogeneous sizes or characteristics and commercialization value decreases in such non-homogeneous batches. The present work was undertaken to study the stress response of the sea bass subjected to acute stress by assessing the interrenal function of sea bass by measuring the levels of cortisol in both plasma and interrenal tissue and the time course recovery, the in vitro sensitivity to ACTH and specific histological features of the interrenal. Previous work on stressed sea bass showed levels of plasma cortisol, but the dynamics between interrenal production and release were not yet studied.

The results show a significant increase of plasma cortisol after 30 minutes and the levels are still higher after 1 hour of stress. Samples after 4h and 24h did not show significant changes respect to initial values. Regarding cortisol values in the interrenal tissue of head kidney, the results did not show an emptying process as in other fish, but a similar dynamics than in plasma with a significant increase at 30 minutes after stress and no changes compared to initial levels after 1h, 4h, or 24 hours. ACTH stimulated in vitro the production of cortisol but both basal and maximal levels were higher than in other sparid fish. The analysis of

the diameter of interrenal cell nucleus showed an increase concomitant with the cortisol release pattern

These results are discussed in terms of the short-term interrenal response of sea bass to an acute stressor. Compared to other fish such as tilapia, trout or other sparids such as sea bream, the sea bass shows a different dynamics. In these species, there is a clear inverse relationship between plasma cortisol and interrenal cortisol during stress. The results observed in sea bass could be explained by a different dynamics of the relationship between pituitary ACTH production, interrenal stimulation rates and interrenal sensitivity. It is suggested that the sea bass interrenal reacts to stressors by mainly increasing the synthesis of cortisol rather than releasing maximal amounts. These studies can help to understand the higher stress susceptibility of the sea bass compared with other species.

**METABOLIC ACTIONS OF CORTISOL  
DURING STRESS IN FISH**

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Plasma concentration of glucose is usually elevated after an acute stress in fish. Glucose is an important fuel for energy metabolism and certain tissues rely primarily on glucose for their energy needs. As physiological adjustments to stress demands energy, the increase in plasma glucose concentration is thought to allow fish to metabolically cope with stress. The hormonal control of glucose production will be discussed in the context of adaptation to stress in fish. Epinephrine and/or cortisol play a key role in the stressor-induced production of glucose. However, my talk will primarily focus on the cortisol-mediated glucose regulation in fish. Specifically, the talk will include the genomic action of cortisol on energy substrate mobilization and liver metabolism, including glucose and glycogen metabolism. The interaction of cortisol with other glucoregulatory hormones on hepatocyte metabolism during stress will be discussed. Based on our results, a working hypothesis will be presented showing the metabolic pathways that are under cortisol stimulation, and important in allowing animals to cope with stress.