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Jarmo Lappivaara

Modifications of Acute Physiological Stress  
Response in Whitefish, *Coregonus Lavaretus*,  
after Prolonged Exposures to Water of  
Anthropogenically Impaired Quality



UNIVERSITY OF JYVÄSKYLÄ

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## ABSTRACT

Lappivaara, Jarmo

Modifications of acute physiological stress response in whitefish after prolonged exposures to water of anthropogenically impaired quality

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Yhteenveto: Ihmistoiminnan aiheuttaman veden laadun heikentymisen vaikutukset planktonsiian fysiologisessa stressivasteessa  
Diss.

Hatchery-reared, 1+- and 2+-year-old juvenile European whitefish, *Coregonus lavaretus*, were exposed to two kinds of anthropogenic waste waters, bleached kraft mill effluents (BKME) and iron supplemented peat water in altogether five laboratory and field experiments. Besides the traditional "expose and execute"- method that addresses merely momentary effects of impaired water qualities on aquatic animals, the main target of the study was set to reveal possible functional alterations in the acute physiological stress response of exposed fish. Therefore, some of the fish were after each exposure period (21 - 42 days) subjected to a short stressful handling disturbance and, after precisely defined recovery periods (5 min - 5 h), sampled similarly to the fish sacrificed before the additional handling procedure. A complementary experiment was carried out to merge some of the effects of previous waterborne exposures with those originating from the oral administrations of iron overdose and dehydroabietic acid, obviously the most hazardous agents involved in the previous studies. Moreover, to ascertain the reliability of the field results and to compare the impacts of a natural factor to those derived from the anthropogenic water quality impairment, the effects of four different acclimation temperatures were studied within two experiments. In accordance with the integrated nature of the general stress response, a wide variety of end points, both at the primary and the secondary levels of physiological responses were applied. The results indicate the following points: 1) Due to the time-dependency and sensitivity of the physiological stress response, the evaluation of recovery periods is overwhelming for the reliability of the method. 2) Both the primary and the secondary level of the integrated stress response may be modified by the applied impaired water quality regimes. 3) Deviations in the characteristics of the secondary stress response between different experiments are generally smaller in unexposed than in exposed fish. Thus, the nature of responses, and the reliability of the results therein, may be more precisely predicted in fish maintained in uncontaminated water quality. 4) In whitefish maintained in dechlorinated tap water, the physiological stress response is influenced by acclimation temperature, whereas after a subchronic exposure to impaired water quality, the effects of ambient conditions in whitefish disappear to a great extent. 5) The interpretation of data may be easily confused due to a capture of freely swimming fish. To obtain reliable and comparable results in field studies, the sampling method should be either fast or time-dependently standardised. 6) The paucity of the reports considering methodological aspects of this kind together with the widely accepted employment of stressful catching methods involving a delayed sampling of wild fish, suggests ignorance associated with ecotoxicological studies.

Key words: Acclimation temperature; BKME; catecholamines; cortisol; handling; iron overdose; peat water; stress response; whitefish.

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## LIST OF ORIGINAL PAPERS

The thesis is based on the following papers which are referred to in the text by their Roman numbers:

- I Lappivaara, J. and Oikari, A. 1999. Altered challenge response in whitefish subchronically exposed in areas polluted by bleached kraft mill effluents. - *Ecotoxicol. Environ. Saf.* 43: 212-222.
- II Lappivaara, J., Kiviniemi, A. and Oikari, A. 1999. Bioaccumulation and subchronic physiological effects of waterborne iron overload on whitefish exposed in humic and nonhumic water. - *Arch. Environ. Contam. Toxicol.* 37: 196-204.
- III Lappivaara, J., Mikkonen, J., Soimasuo, M., Oikari, A., Karels, A and Mikkonen, A. 2001. Attenuated carbohydrate and gill Na<sup>+</sup>-K<sup>+</sup>-ATPase stress responses in whitefish caged near bleached kraft mill discharges.- *Ecotoxicol. Environ. Saf.* (in press)
- IV Lappivaara, J. 2001. Effects of acute handling stress on whitefish *Coregonus lavaretus* after prolonged exposure to biologically treated and untreated bleached kraft mill effluent. - *Arch. Environ. Contam. Toxicol.* 41: 55-64.
- V Lappivaara, J. and Marttinen, S. 2001. Acute physiological stress response of whitefish to a short air challenge is impaired by winter conditions, cold water alone, and intermittent exposure to waterborne iron overload. - Submitted to *Can. J. Aquat. Sci.*

## Responsibility of Jarmo Lappivaara in the papers of the thesis

Paper I. The study was designed, set up and performed by the two authors together with Markus Soimasuo, Harri Leppänen, Aarno Karels, and Jarno Mikkonen. I conducted the physiological measurements, handled the data and wrote the draft of the article.

Paper II. The study was planned by myself together with Aimo Oikari. Ari Kiviniemi and I were responsible in setting up and running the experiment. Ari Kiviniemi conducted the Fe analyses together with the staff of the Institute for Environmental Research, Jyväskylä. I conducted the physiological measurements, handled the data and wrote the draft of the article.

Paper III. The study was designed, set up and performed by the authors together with Harri Leppänen and Pia Vehviläinen. I conducted the physiological measurements, handled the data and wrote the draft of the article.

Paper IV. I was responsible in designing, setting up and running the experiment. Jatta Karonen conducted the chromatographic analyses with support from Harri Leppänen. Ahti Haaparanta and Pia Vehviläinen took part in the sampling procedure. I conducted the physiological measurements, handled the data and wrote the draft of the article.

Paper V. I was responsible in designing, setting up and running the experiments. Sanna Marttinen conducted the chromatographic analyses. Ahti Haaparanta and Jarno Mikkonen took part in the sampling procedures. Armi Puttonen and I conducted the Fe analyses together with the staff of the Institute for Environmental Research, Jyväskylä. I conducted the physiological measurements, handled the data and wrote the manuscript.

Kuopio, November 30.,2000



Jarmo Lappivaara

# 1 INTRODUCTION

## 1.1 Stress concepts: stress response and stress

The term stress is applied to various meanings in different biological and psychological contexts. This confusing appearance originates from the obscurity and multiplicity of the definition itself. Consequently, terms like toxicity, stimulus, fear or pain may be understood as equal to, or replaced by, the term stress even in international scientific journals involved in the categories of environmental or medical sciences.

Within physiological disciplines (Selye 1950, Donaldson 1981, Pickering 1981, Wedemeyer et al. 1990, Barton & Iwama 1991, Wendelaar Bonga 1997, Mommsen et al. 1999), physiological stress should not be understood as an external stimulus (stressor instead) or as a specific toxic influence. The "general stress response" involves three different stages. The acute (or alarm) phase is an integrated internal avalanche of reactions responding to innumerable kinds of external stimuli, starting from the level of afferent fibres of central nervous system and ending, through an extremely complicated network of hierarchically regulated humoral and cellular messengers, to finely controlled alterations in the physiological functions of the individual. Normally, a passed stimulus is followed by a total recovery or physiological adaptation, i.e. an attainment of a new internal equilibrium without notable impediments. This transient response is one of the most indispensable abilities of all healthy vertebrates. After a prolonged, combined, or otherwise extreme stimulus, however, the acute stress response may result in a slight or a more severe loss of homeostatic control instead of the recovery. This resistance phase may, after a highly variable time period, end up either as adaptation to the new situation or to physiological exhaustion (phase) eventually leading to death.

The physiological compensatory functions involved in the stress response may be equally activated and stimulated during the different phases. These physiological alterations, whether adaptive or maladaptive, are vital to

the homeostatic maintenance. For example, elevated blood pressure in gill lamellae and an increased blood glucose level are typical changes found in the physiological stress response. They are also necessary for adaptation to threatening situations during which the increased physiological capacity ameliorates possibilities to survive ("fight-or-flight" -reaction). Some of the consequences of these alterations, like increased electrolyte losses and groundless energy expenditure, are, however, harmful in the long run. Thus, the (chronic) stress between the original homeostasis, including the acute stress response, and death, relates to maladjustment of necessary functions. It is extremely difficult to know, whether the level of a single function, or a value of a measured variable reflecting the function concerned, is maladaptive or within the beneficial range in terms of the entire homeostasis. The problem diminishes with the increasing number of variables measured (or functions known).

## **1.2 Functions of the general stress response: primary and secondary levels**

The uniqueness and impulsiveness of the general stress response in all vertebrate groups to a wide variety of internal and external stimuli, justifies the ultimate necessity of the reaction for an individual to get along through threatening challenges. At the onset of the acute phase, adequate stimulation of the central nervous system awakes two practically quiescent routes, neuroendocrinological and endocrinological, for physiological and behavioural acclimation of an organism. Sympathetic cholinergic fibers stimulate chromaffin cells in the head kidneys and in the post cardinal veins, whereas the release of the hypothalamic corticotrophine releasing factor (CRF) awakes an immediate release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary resulting in rapid stimulation of interrenal cells of the head kidney (HPI-axis) (Mazeaud & Mazeaud 1981, Wendelaar Bonga 1997). After the signals, impulsive secretion of catecholamines (mainly adrenaline and noradrenaline) and corticosteroids (mainly cortisol in fish) from these tissues, respectively, to blood circulation mediates the primary (hormonal) response to the entire organism via cellular hormone receptors of peripheral tissues (onset of the secondary response). Generally, the catecholamines bring about a tremendously increased ability to energy expenditure resulting mainly from the elevated ventilation and circulatory effectiveness, the increased oxygen carrying capacity of red blood cells and stimulated glucose production from glycogen stores (Thorpe & Ince 1974, Morata et al. 1982b, Ottolenghi et al. 1985, Janssens & Lowrey 1987, Wright et al. 1989, Wendelaar Bonga 1997). The effects of cortisol are even more integrated, aiming at the long-term homeostatic maintenance of an organism (Pickering 1993, Mommsen et al. 1999). For example, the stimulation of liver gluconeogenesis i.e. the production of glycogen for glucose utilisation from glycerol (Vijayan et al. 1991), amino acids (Milligan 1997,

Vijayan et al. 1997c) and lactate (Mommmsen et al. 1999), the modification of  $\beta$ -adrenoceptor mediated cellular functions in several important tissues, like e.g. in liver (Reid et al. 1992, Vijayan et al. 1994a) and an interference with other hormones (Vijayan et al. 1994b, Mommmsen et al. 1999) all target at the homeostatic maintenance of an organism. The most typical, and for a serious researcher, the most problematic characteristics of the physiological stress response is the never-ending web of feedback loops (Roillant et al. 2000).

### 1.3 Modification of the general stress response

Similarly to all necessary physiological phenomena, the extent and duration of the general stress response are modified by both intrinsic and extrinsic factors of which acclimation temperature (Mazeaud & Mazeaud 1981, Barton & Schreck 1987, Davis & Parker 1990) and nutritional status (Barton et al. 1988, Hemre et al. 1992) are obviously the most effective. Accordingly, the "natural" range of variation on a single function of a certain species is relatively large and tied with other responses arisen at molecular, biochemical and physiological levels. Anthropogenic activities give rise to "unnatural" stress response modification in fish in innumerable ways. Inorganic and organic toxicants affect directly the function of the HPI-axis (Ilan & Yaron 1983, Quabius et al. 1997), the centre of the general response, and the basic functions of peripheral tissues responding to the signals of the HPI-axis. In addition, poor water quality (Pickering & Pottinger 1987) or otherwise altered ambient conditions like fluctuated water temperature or food availability, exert their effect with or without those of toxic factors resulting in an extremely complicated impact of combined factors derived from the anthropogenically impaired water quality. Therefore, it is not feasible to try to find a clear dose-response relationship between a single toxicant and "unnatural" modification of the general stress response. Instead, a dose-response relationship may be predicted to occur between the level of chronic stress i.e. the dysfunction of the internal equilibrium, and the "unnatural" stress response modification. It goes without saying that the "natural" modification of stress response is as reasonable for the well-being of an individual as is the modification of all other adaptive functions, whereas "unnatural" modification is not evolutionary controlled.

In previous field studies, exposures to BKMEs (Hontela et al. 1997, Hontela 1998), cadmium (Schreck & Lorz 1978, Gill et al. 1993), mixtures of organic contaminants and heavy metals (Hontela et al. 1992, 1995, Brodeur et al. 1997, Girard et al. 1998) as well as cadmium and zinc contaminated stream water (Norris et al. 1997, 1999) have been shown to suppress the cortisol response to several kinds of handling disturbances or to an ACTH stimulus in a variety of fish species. Besides impaired interrenal function, as identified also by morphological changes (Norris et al. 1997), adrenocortical and hypothalamic changes attributable to altered ACTH synthesis have been detected (Norris et al. 1997, 1999). Despite the consistency of these studies, unchanged cortisol

responses together with unaffected interrenal morphology have also been detected in fish captured and challenged within the same exposure conditions at a different time of the year (McMaster et al. 1994, Girard et al. 1998) or after a less severe handling disturbance (Norris et al. 1999). In addition, an erratic cortisol response together with impaired metabolic response to handling disturbance has been observed in rainbow trout after a toxic challenge (Vijayan et al. 1997b). In all of the previous experiments, the applied exposure conditions have been considered both toxic and stressful. Consequently, the suppressed cortisol response has been regarded as an indicator of prolonged stress, chronically stimulated HPI-axis function and failure to cope with threatening challenges (Norris et al. 1997, 1999). It is noteworthy that both experiments (Barton et al. 1985; Pickering & Pottinger 1987) describing enhancement of plasma cortisol response to an acute handling disturbance have been conducted in water impaired by reduced pH.

Beyond the dysfunction of HPI-axis, the possible impacts of impaired water quality on other variables affected by the acute physiological stress response have been poorly studied. In the existing studies, inconsistent effects of exposure to BKME (McMaster et al. 1994, Jardine et al. 1996) or combined toxicants (Girard et al. 1998) have been noted on plasma reproductive steroids and carbohydrate metabolism. Among other methodological factors, like season, time of the day and applied handling regime, the inconsistency of the results between these studies may be due to the high levels ( $> 100$  ng / ml) of plasma cortisol before the applied stressful handling regimes or species-specificity in the regulation of HPI-axis function.

#### **1.4 Ecological relevance of stress response modification**

A severe impairment of the acute cortisol response is, by physiological means, fatal to vertebrates, including human, as exemplified by the intensive international discussion in medical journals in the beginning of the 1980's (e.g. Fellows et al. 1983, Ledingham & Watt 1983, Ledingham et al. 1983, Wagner et al. 1984, Preziosi & Vacca 1988). Beyond physiology, the ecological relevance of impaired stress response is based on integrated and homeostatic alterations in a stressed individual. In fish, the basic functions taking care of the proper maintenance of the internal equilibrium, namely, energetic metabolism, ionic and acid-base regulation, oxygen transport and consumption as well as the immune system, are all affected by stress hormones to an extent exceeding or being equal to the significance of other regulatory hormones (Wendelaar Bonga 1997, Mommsen et al. 1999). In addition, stress related alterations in hormonal status exert their influences on (social) behaviour (Sloman et al. 2001, mental condition, reproductive physiology (Pickering et al. 1987, Pankhurst & Dedual 1994) and growth (Barton & Iwama 1991). Generally, immunity, reproduction and growth are more often negatively than positively affected (Barton & Iwama 1991, Wendelaar Bonga 1997, Gregory & Wood 1999). Therefore, each of these

functions may be transiently, lasting from hours to a few days, modified along with the modification of a single stress response. This notion is supported by the well-known fact that fluctuations on the strength of the physiological stress response, say, for example, in the level of plasma cortisol increase (Pankhurst & Van Der Kraak 2000) or/and the level of corticotropin releasing hormone (CRH) expression in the hypothalamic paraventricular nucleus (PVN) (Aguilera 1998), may have intricate and far-reaching physiological and ecological consequences in terms of either short-term impairment in e.g. reproductive physiology (Pickering et al. 1987, Pankhurst & Dedual 1994, Haddy & Pankhurst 1999, Pankhurst & Van Der Kraak 2000) or even lifelong changes in reproductive success (Campbell et al. 1992). Because wild fish in their natural environment frequently encounter stressful challenges, e.g. chasing by predators, competitions for spawning or territory as well as abiotic fluctuations, that are directly associated with their ability to survive or to breed successfully, even the consequences of stress response modification alone may appear fatal within a relatively short time. In addition, if the recovery time after a single stress stimulus is extended (Mesa et al. 2000) or the time period between individual threats is shortened due to chronic stress caused by anthropogenic factors affecting water quality, the cumulative effects of acute stress leading to physiological exhaustion of fish increase (Barton & Iwama 1991). Therefore, the state of chronic stress may be both physiologically and ecologically attributable to the modification of the stress response by hazardous exposure. Thus, all the functions mentioned above may be chronically affected in fish exerting a modified physiological stress response to acute challenge.

Despite the obvious connection between the stress response modification and the capacity of an individual to survive and breed, scientific reports considering this aspect are rare. It has, however, been reliably demonstrated that the vulnerability of prey fish to predator fish (or the effectiveness of predator as well) (Olla & Davis 1989, Olla et al. 1992) as well as the function of the immune system (Maule & Schreck 1991) and the susceptibility of fish to diseases (Pickering & Duston 1983, Barton & Iwama 1991, Narnaware et al. 1994) are remarkably modified by the acute stress response or cortisol administration. There is no reason to doubt that the modulation of the general stress response by anthropogenic water quality would not affect these and other phenomena of ecological relevance as well. Overall, natural conditions that cause chronic stress in fish are rare, whereas such anthropogenic conditions are by far more common (Barton & Iwama 1991). Natural challenges like e.g. spawning and predation as well as pathogens and parasites are more easily coped by the animals living in water conditions without additional anthropogenic stress stimuli.

## 1.5 Objectives of the study

The main purpose of this study was to reveal whether the physiological stress response of juvenile whitefish is modified by water conditions closely resembling anthropogenic water impairment in Finland. As appears from the above, this study is not a single or the first one considering the modulation of stress response. Rather, the earlier paucity of the reports has turned into a sharp increase during the last few years. Though there is a tremendous amount of older and recent literature concerning the basics and physiological consequences of the general stress response, a lot of questions are still unanswered. Therefore, the whole subject is still in its infancy, regarding both the amount of reports and the general awareness. The ultimate importance of physiological stress response to an individual and its definitive role in a variety of intra- and interspecific interactions within the entire field of vertebrate ecology, ensure a growing interest in anthropogenic modifications of physiological stress response in the near future. Consequently, a side-objective of this thesis was to ascertain the importance of subsequent studies targeting also the effects of stress response modulation on ecologically relevant phenomena in water ecosystems affected by anthropogenic activities.

Until today, only a couple of articles (e.g. McMaster et al. 1994, Jardine et al. 1996, Quabius et al. 1997, Vijayan et al. 1997a, 1997b, Girard et al. 1998; Jørgensen et al. 2001) have concerned water quality or toxicity based modification of acute stress response in fish in terms of other physiological variables besides those directly involved in the function of HPI-axis. Therefore, in addition to answering the question presented in the beginning, the objective was extended to improve and broaden the understanding of general stress response by paying particular attention to the integrated nature of the stress response through revealing a wide variety of secondary responses at both general and cellular levels of physiology.

The methodological target for data interpretation was based on the fact that a wild fish can be very rarely, if ever, captured without causing an acute stress response. Despite this, seine, purse-seine, net, electricity, spinning, as well as hook and line with changeable caging periods, have all been widely accepted and used catching methods within the ecotoxicological field studies for a long time. The possible effects of stress response have usually been, however, either entirely ignored or insufficiently clarified. Thus, the final target of the study was to disclose and lay stress on the evident defect hidden under the interpretation of physiological data involving stressful capture of fish.



## 2 MATERIALS AND METHODS

### 2.1 European whitefish *Coregonus lavaretus*

The characteristics of the general stress response as well as the sensitivity of response to various external stimuli differ to a certain extent between different fish species (Sloman et al. 2001) due to the internal factors including biological rhythms, strain, sex and reproductive status. Therefore, to enable a decent comparison of the results of all the experiments, they were carried out with a juvenile stage (1+- and 2+-year classes) of one species, European whitefish *Coregonus lavaretus*. European whitefish, a member of the widely distributed subgenus *Coregonus*, was primarily chosen because of its excellent applicability to both laboratory and field caging experiments as a generalist feeder (Næsje et al. 1991), and due to its ecological significance and commonness. Whitefish is also extensively hatchery-reared for release into natural water ecosystems, and of a relatively high economic value. In addition, there is a distinct deficiency in the scientific reports concerned with the physiology of coregonines although their ecology has been more intensively studied (Lindsey & Woods 1970, Morin et al. 1982, Christie & Regier 1988, Næsje et al. 1991, Griffiths et al. 1992, Morin et al. 1992).

### 2.2 States of rest and stress in short-term studies

In the majority of researches concerned with the physiological stress in fish, plasma cortisol concentration is used to characterize the states of rest and stress. This is reasonable for short-term studies focusing on stress related changes lasting from a few minutes to a couple of days, since plasma cortisol response is extremely abrupt and time-dependently predictable in healthy fish, and, easily measured due to the uniqueness of the molecule in most vertebrate groups.

Other widely used, sensitive and reliable indicators of transient stress include plasma ACTH and catecholamine concentrations, which may, however, be even too sensitive and variable for some otherwise useful sampling regimes. In addition, the scientific background knowledge on these variables is by far more scarce, and the methods of measurements a bit more complicated and expensive than those for plasma cortisol.

For most fish species, the measurable concentration of plasma cortisol in healthy and unstressed fish should be, depending on internal and external rhythms and factors, at the level of 20 - 50 ng/ml at most (Woodward & Strange 1987, Vijayan & Moon 1992, Gamperl et al. 1994, Kennedy et al. 1995, Barton & Dwyer 1997, Roillant & Tort 1997). In the study conducted first for this thesis (V), as well as in all the others thereafter, this was the case also in whitefish *Coregonus lavaretus*. The whitefish turned out to be a "normal" fish species also in regard to the awakening of acute physiological stress response, as plasma cortisol responded to the handling disturbance by a moderate increase (~ 100 ng/ml) already after five minutes from the onset of handling (IV). Thus, the classification of the whitefish into unstressed and stressed groups was in each experiment based on the duration of time period, 0-4 min and 5 - 300 min respectively, between the onset of handling and the stunning of each individual, and on the mean plasma cortisol concentrations measured. It should be emphasized, however, that plasma cortisol level is not necessarily equal to the stress of the fish i.e. chronically stressed fish may possess normal or even lower basal levels of plasma cortisol although there may be some other changes or disorders in the function of HPI-axis (Wendelaar Bonga 1997, Blom et al. 2000, Jørgensen et al. 2001). In addition, a brief cortisol response, lasting a few hours at maximum, may often result in much more prolonged changes in secondary functions (Mømmesen et al. 1999, Blom et al. 2000).

### 2.3 Handling regimes

Three kinds of acute handling procedures were applied in the experiments. A short air exposure (I, II, IV, V), consisting of air exposures repeated three (10 s in air - 10 s in water - 20 s or 30 s in air - 10 s in water - 10 s in air) or two (10 s + 10 s) times, was applied as a standardized high-level stressor in order to elicit a distinct "fight- or-flight" -reaction to a life threatening challenge in all the fish handled. The other two handling procedures, underwater transportation (ca 10 min) (I, III) and low (ca 10 cm) water depth (5 min) (IV) were low-level stressors revealing the impacts of moderate disturbances, which are obviously more often met by feral fish. Both high- and low-level stressors resembling these disturbances are unavoidable incidents in all field studies concerning freely swimming fish.

The duration of the recovery periods (10 min - 5 h) was originally chosen for the first experiments (I, III, V) on the basis of earlier studies describing the time-dependent progression of plasma cortisol and blood carbohydrate responses in

several fish species, and modified thereafter to reveal more rapid (within 5 min) responses (IV, V). During the recovery, the experimental whitefish were either in a cage or in their original tank, but always in the same water milieu as before the handling.

## 2.4 Exposure regimes: environmental relevance

Exposure waters were chosen to represent the characteristics of anthropogenic water loading in Finland. Environmental relevance was achieved by using dilutions of biologically treated elemental chlorine free bleached kraft mill effluents (I, III, IV) and iron-rich run-off waters from peat production areas (II, V). Both BKMEs and iron-rich humic waters are known to cause molecular, biochemical, physiological and whole animal level effects in several fish species (Smith et al. 1973, Andersson & Nyberg 1984, Andersson et al. 1988, Santos et al. 1990, Hodson et al. 1992, Wepener et al. 1992, Goksøyr et al. 1994, Peuranen et al. 1994, Adams et al. 1996, Jeney et al. 1996, Baker et al. 1997, Hontela et al. 1997, Soimasuo 1997, Payne et al. 1998, Vuorinen et al. 1999, Pacheco & Santos 1999). In order to compare the physiological influences of these water milieus to those caused by similar, but more severely impaired waters, untreated BKMEs (IV) and iron (2 - 8 mg Fe/l) supplemented waters (II, V) were involved. In addition, some of the effects of waterborne exposures were compared to those connected with oral administrations of dehydroabietic acid (DHAA) and Fe (V), the two main components of the effluents known to possess hazardous impacts on biochemical and physiological functions of fish (refs. above). No effort was made, however, to reveal possible specific and direct effects of the harmful chemicals involved, since the main target of the work was set on the integrated nature of the general stress response. The present scientific knowledge clearly shows that interactions and combined consequences of stress and toxicity are by far too complicated to be specified and distinguished as both affect each other in innumerable ways. The water temperatures applied to fish acclimation were 4, 8.5, 13 - 13.5 and 18.5°C in laboratory, and ca. 10 - 17.5°C in field experiments. The optimal temperature for the critical swimming speed, the metabolic scope for activity, the maximum oxygen consumption (active metabolism) (Bernatchez & Dodson 1985), the locomotor activity (Scherer & Harrison 1988) and the growth (Christie & Regier 1988) of the lake whitefish *Coregonus clupeaformis* is 12 - 13°C. The duration of subchronic and sublethal exposures was 21 - 42 days.

## 2.5 Sampling procedure and analyses

The fish were carefully hand-netted either one by one or in pairs, stunned with a blow on the neck, after which blood samples were taken from their caudal veins, and other tissue samples cut off. All the samples were immediately stored in liquid nitrogen till different assays. Plasma adrenaline and noradrenaline were extracted with the alumina absorption method (Anton and Sayre 1962, Brown and Whitehead 1995) and measured with High Pressure Liquid Chromatography (Perkin Elmer 200) supplied with electrochemical detector (ESA Coulochem II) (V). Plasma cortisol (I-V), estradiol (II, IV, V) and testosterone (IV) concentrations were measured with commercial enzyme assays (Fenzia EIA kit, Orion Diagnostica) based on competitive binding of known (labelled) and unknown concentrations of steroid molecules on solid-phase secondary antibodies. The activities of glycogen phosphorylase (*a + b*) in liver (II, IV, V) tissue samples, and Na<sup>+</sup>-K<sup>+</sup>-ATPase in gill (III, IV, V) tissue samples were determined with two buffer systems based on the methods described in Morata et al. (1982a) and Schrock et al. (1994), respectively. The concentrations of liver glycogen (Lim & Ip 1989), blood glucose, lactate (Boehringer Mannheim, 124036 & 256773) and hemoglobin levels as well as the total concentrations of tissue proteins (Bio-Rad Prot. Assay Kit) along with P<sub>i</sub> liberated within GPase and Na<sup>+</sup>-K<sup>+</sup>-ATPase assays, were measured spectrophotometrically with iEMS platereader (Labsystems). Plasma and red blood cell Na<sup>+</sup> and K<sup>+</sup> concentrations were analysed with Corning 435 Flame Photometer and liver EROD activity (Burke et al. 1985) fluorometrically with Ascent platereader (Labsystems).

### 3 RESULTS

Under the subheadings, the results are generally expressed in the following order: 1) General effects of the applied exposures on the basal levels of variables. Basal levels denote the data derived from whitefish sampled before additional handling disturbance. 2) The effects of additional handling on the reference whitefish. 3) The effects of exposures on the stress response caused by additional handling. 4) The effects of modified ambient conditions, i.e. primarily acclimation temperatures below (4°C & 8.5°C) or above (18.5°C) the one applied in all experiments (13.0 ± 1.0°C). In the field studies the range of water temperature was, at the moment of sampling, 11.5 - 17.5°C. In addition, remarks considering the combined effects of the three applied factors (i.e. handling, exposure, ambient conditions) are included.

#### 3.1 Stress hormones

Plasma adrenaline and noradrenaline concentrations were decreased in whitefish exposed for 30 days to periodic iron overload (0.5 - 3 mg/L) in diluted (5 %) peat water (V). Though the short air exposure appeared to increase the levels of both catecholamines in each group, the changes were not statistically significant due to the relatively small amount of individuals, and extensive deviations between individuals. The influence of waterborne iron exposure was, however, expressed as lowered plasma catecholamine levels after handling in both simulated winter and spring conditions. In winter group, however, the differences of plasma catecholamine levels were smaller between the exposed and reference fish, because cold water (+4°C) together with a short day length (8 h : 16 h; L : D) and restricted food availability (0.5 % / w day) was also connected with lowered plasma catecholamine levels. The decrease was thereby more prominent in spring than in winter conditioned whitefish, both before and after the handling disturbance. The applied exposure regime totally faded the effects of ambient conditions on plasma catecholamine levels noted in

the reference whitefish at all three sampling times (before, 15 min and 2 h after the air exposure) (Fig. 1) (V).

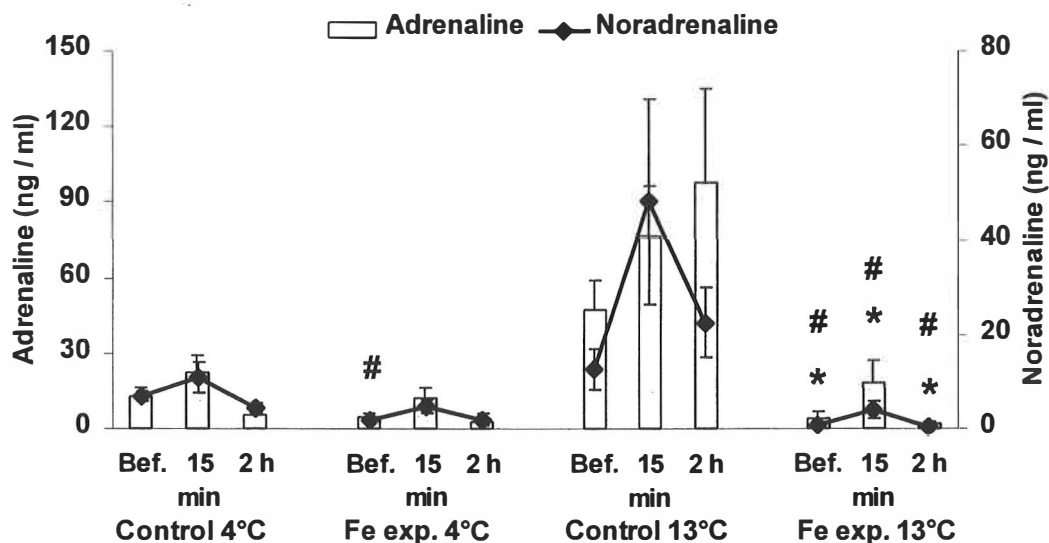


FIGURE 1 Plasma adrenaline and noradrenaline concentrations (means  $\pm$  SE) in whitefish exposed for 30 days to episodic waterborne iron overload in iron-rich humic water (5 %) supplemented with inorganic iron (5 mg Fe/l). After exposure, part of the fish were subjected to a handling challenge by a short air exposure and left to recover for 15 min or 2 h. "Bef." denotes the fish sampled before additional handling. "Control 4°C" denotes water temperature of 4°C and photoperiod of 8L:16D. "Control 13°C" denotes water temperature of 13°C and photoperiod of 16L:8D. \* indicate significant ( $p < 0.05$ ) differences in adrenaline and # in noradrenaline between exposed and control whitefish groups within each sampling point at the same ambient conditions. N = 5 - 8 for each column.

Similarly to the decreased plasma catecholamine levels noted in the whitefish exposed to iron supplemented peat water, both the basal level of plasma cortisol and the magnitude of the increase after different handling procedures were generally suppressed in the exposed fish (II, III, IV, V;  $n = 301$ ; Fig. 2). Due to a relatively great individual variability and the apparent sensitivity of the cortisol response to minute external disturbances, however, significantly decreased plasma cortisol levels were observed only in two individual experiments (III, IV) in BKME exposed whitefish. Exposure to 8 mg of additional iron, to the highest waterborne iron concentration applied in the experiments, was connected, however, to increased plasma cortisol levels of the fish in both peat and tap water. Also in this experiment, the highest cortisol level after the handling, a factor of about 50, was noted in the reference fish, whereas it was only 8 - 22 in six other whitefish groups exposed to iron-rich

waters. The suppressed cortisol level reached in the exposed whitefish appeared to be only marginally affected by the different handling procedures and recovery periods applied in the different experiments, whereas in the reference whitefish, there was more individual variation.

The acclimation of whitefish to low water temperatures, to +8.5°C and +4°C, weakened the cortisol response to handling disturbance by making it either more transient than in the fish maintained at around +13°C, or by decreasing the level reached (V) after a 120 min recovery. As opposed to this, warm (+18.5°C) water was ineffective in cortisol response modulation.

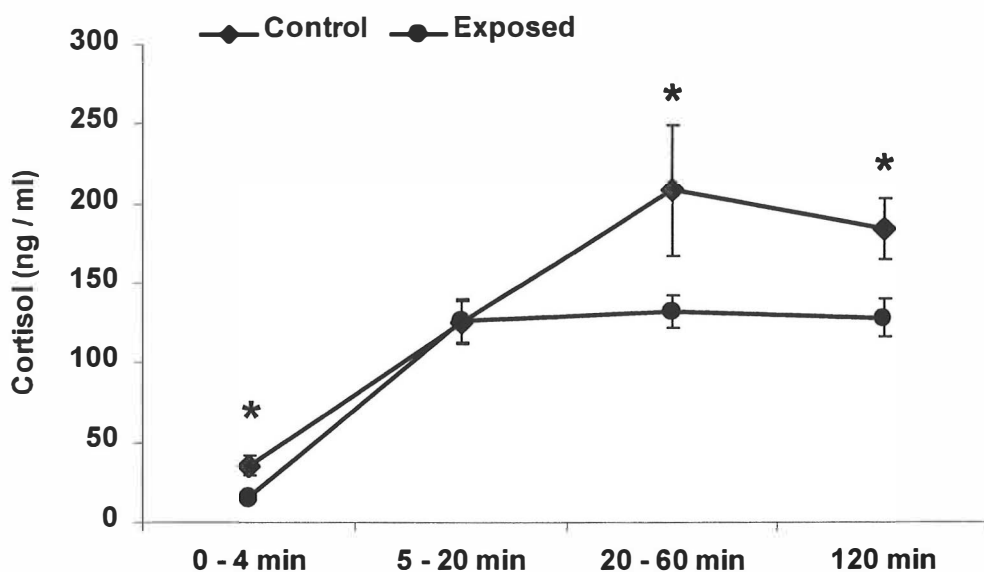


FIGURE 2 Plasma cortisol levels (mean  $\pm$  SE) in 301 juvenile whitefish. The exposed fish were maintained for 30 or 42 days under the influence of unbleached (IV) or bleached kraft mill effluents (III, IV), or iron supplemented peat water (II, V), and subjected to an acute handling disturbance by a repetitive air exposure, a low water depth or an underwater transportation. The number of the reference and exposed fish measured for the presented mean values was 32 and 75 (0 - 4 min), 27 and 59 (5 - 20 min), 19 and 57 (20 -60 min), and 13 and 19 (120 min), respectively. Asterisks denote significant ( $p < 0.05$ ) differences between the exposed and the reference fish at the same point.

### 3.2 Carbohydrate metabolism

Although each of the applied exposure regimes had some individual effects on the basal levels of liver glycogen phosphorylase activities, liver glycogen

concentration, blood glucose or lactate levels, there was no general trend in the changes except an increase in phosphorylase activity by both the waterborne (IV) and oral administrations (V) of high doses of DHAA. Similarly, an increasing trend in glucose and lactate levels and a decreasing trend in glycogen level were the general changes derived from the different handling disturbances both in the reference and the exposed whitefish (I - V). In the field studies considering caged whitefish (I, III) these metabolite responses were in most cases remarkably attenuated in the fish maintained for 30 days in the vicinities of three different BKME discharges. This phenomenon was less obvious, yet observable, in the liver glycogen responses of the whitefish exposed in laboratory conditions with controlled water temperature and food availability (IV, V). Accordingly, a handling disturbance, applied by the air exposure regime, increased liver GPase activities in reference whitefish, but not in exposed fish (IV, V) (Fig. 3).

Liver glycogen concentration and blood glucose levels were, in both experiments considering different ambient conditions for the acclimation of whitefish, decreased in the highest water temperatures. Changed acclimation temperatures, when compared to that of 13 - 13.5°C, were connected also with a changed liver GPase response pattern in a way resulting in a more transient or missing response in the reference fish acclimated to colder water (V), but not in the fish exposed to waterborne iron overload.



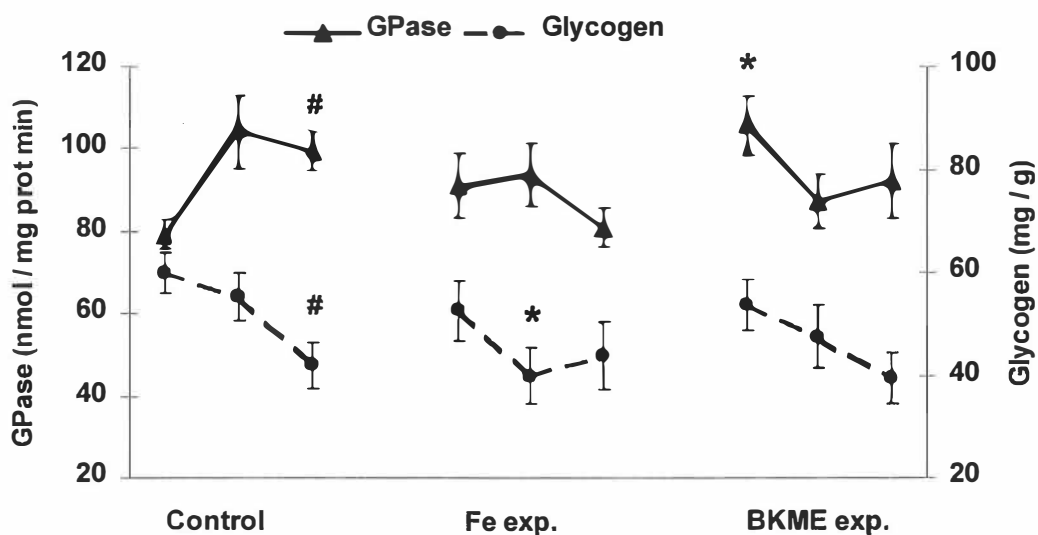


FIGURE 3 Liver glycogen phosphorylase activity and glycogen concentration (mean  $\pm$  SE) in 152 juvenile whitefish sampled before (the first point in each line) or either 5 - 20 min (the middle point) or 120 - 150 min (the third point) after the onset of the additional handling disturbance. The handling consisted of an air exposure repeated three times (10 s, 30 s, 10 s), and of a subsequent recovery in a submerged cage (50-l). Exposed fish were, within two waterborne exposures, maintained for 42 or 30 days under the influence of BKMEs (IV) or iron supplemented peat water (V), respectively, or fed for 21 days with DHAA (150  $\mu$ g/g) or iron (3 mg/g) supplemented food pellets (V). DHAA fed fish are included in the points reflecting BKME exposed whitefish. The number of the reference fish and exposed (Fe, BKME) fish measured for the presented mean values was 22, 14 and 22 (before), 18, 10 and 21 (5 - 20 min), and 17, 10 and 19 (120 - 150 min), respectively. # denote significant ( $p < 0.05$ ) differences between the first and either of the two subsequent sampling points. Asterisks denote significant ( $p < 0.05$ ) differences between the exposed and reference fish at the same point.

### 3.3 Osmoregulation

In the whitefish sampled without additional handling, increased gill  $\text{Na}^+\text{-K}^+$ -ATPase activities were noted after exposures to unbleached kraft mill effluent (4 - 8 %) (IV) and to iron supplemented (5 mg Fe/l) peat (5 %) water in simulated winter conditions (V), and decreased plasma  $\text{Na}^+$  and  $\text{K}^+$  levels after the exposure to a nominal 8 mg Fe/l in dechlorinated tap water (II).

When compared to the measured hormonal and metabolic variables, which generally exhibited one-way trends in the reference whitefish after handling procedures, the effects of handling disturbances on gill  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activities were more variable. An apparent increase (IV, V), a decrease (III) or a prolonged decrease (V) were observed in reference whitefish maintained under different experimental and ambient conditions, and after variable recovery periods. In contrast, branchial  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity was not affected by the air exposure or underwater transportation in BKME exposed fish (III, IV). Accordingly, plasma cationic responses to handling procedures were variable (I, IV, V) and unpredictably modified by the exposures. There was, however, an apparent decrease (20 min) followed by a rapid recovery in plasma potassium concentrations in two-year-old reference whitefish, while the iron exposed fish exhibited, in both of the ambient conditions applied, a coherent trend of increase (V).

In the reference whitefish acclimated in the simulated winter conditions, the gill  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity was lower, both before and after the air exposure, than in the other two reference groups reared in the spring conditions. In the iron exposed fish, however, these effects of applied ambient conditions, once again, vanished totally (V).

### 3.4 Red blood cells (RBC)

In the whitefish sampled without additional handling, red blood cell  $\text{Na}^+$  concentrations were increased both after the exposure to iron supplemented peat water (V, spring) and after caging in three areas affected by different BKMEs (III), but not affected after an exposure to one of these BKME waters in laboratory conditions (IV). Despite only few minute changes were observed in RBC ATPase activity, potassium concentration and water percent of RBCs after different exposures (III, IV, V), blood hemoglobin and hematocrite levels were regularly affected by both types of impaired water quality (I - V). BKME exposure had an increasing effect on basal hemoglobin levels (I, IV).

As in the case of gill  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity, the effects of handling and recovery periods on RBC cationic concentrations in the reference whitefish were dissimilar between the experiments (III, IV, V). In the laboratory experiments, a transient  $\text{Na}^+$  increase was followed by a decrease, whereas  $\text{K}^+$  response was exactly the inverse (IV, V). In the field experiment, however, responses appeared to be totally opposite (III). In a few cases, RBC responses to handling were more prolonged in exposed whitefish. After the exposure to iron supplemented peat water, RBC sodium concentrations seemed to be decreased and water percent increased in both ambient water conditions after a 120 min recovery from the onset of acute handling, while the RBCs of the reference whitefish appeared to be recovered at this point (V). A similar trend was seen after the field exposure in one mill area (III). Instead, the handling caused an increase in blood hemoglobin level, a typical response of the reference whitefish

to all the handling procedures applied (I - V), which was noted to be attenuated after iron (II) and BKME exposures (III; IV).

RBC potassium concentration and blood hematocrite level were decreased, and the RBC cationic responses to the handling disturbance attenuated in the reference and exposed whitefish acclimated to low water temperature. The response of RBC water percent to additional handling was, however, modified by ambient conditions in the reference whitefish, but not in the iron exposed whitefish (V).

### 3.5 Reproductive steroids and EROD activity

The exposure of one-year-old whitefish to a high amount of additional iron (a nominal, 8 mg Fe/l) in both dechlorinated tap water and in slightly diluted peat water (80 %) resulted in elevated plasma estradiol levels, whereas the simultaneous exposure to a lower amount of additional iron (a nominal, 2 mg Fe/l) was ineffective both in tap water and in the diluted (20 %) peat water (II). In the two-year-old whitefish, the effect of iron supplemented (5 mg Fe/l) diluted (5 %) peat water exposure on plasma estradiol concentrations was opposite to that noted in one-year-old fish, since plasma estradiol levels were decreased after the iron exposure in both ambient conditions applied (V). In the one-year-old fish, in their turn, 42 days of laboratory exposure to unbleached or bleached kraft mill effluents affected neither estradiol nor testosterone levels (IV). 7-ethoxyresorufin O-deethylase activity (EROD), indicating enzymatic capacity of separated liver microsomes to react with compounds resembling planar polycyclic and chlorinated hydrocarbons, was decreased in the whitefish exposed to the highest additional iron concentration (8 mg Fe/l) in tap water, but not in those kept in peat water (II). 21 and 42 days of exposure to diluted (4 - 8 %) kraft mill effluents increased EROD activity (IV).

Although a clear increase in plasma estradiol concentrations was noted in both reference and iron exposed one-year-old whitefish after a two days fasting and a subsequent handling stress introduced by air exposure (II), a bare air exposure with a 120 min recovery period in a 50-l cage did not have effects on the levels of plasma reproductive steroids (IV, V; Fig 4a). Simultaneously with the increased plasma estradiol levels discussed above, a decrease of liver EROD activity took place both in the iron exposed fish and reference fish (II). An air exposure decreased liver EROD activities also in the BKME exposed whitefish, but not in the reference fish, resulting in the multiplicity of data interpretation (IV; Fig 4b).

Plasma estradiol concentrations in the one-year-old whitefish maintained in dechlorinated tap water were affected by the applied ambient conditions, whereas the effect of simulated winter and spring conditions was absent also when regarding this variable in the whitefish exposed to waterborne iron overload (Fig 4a).

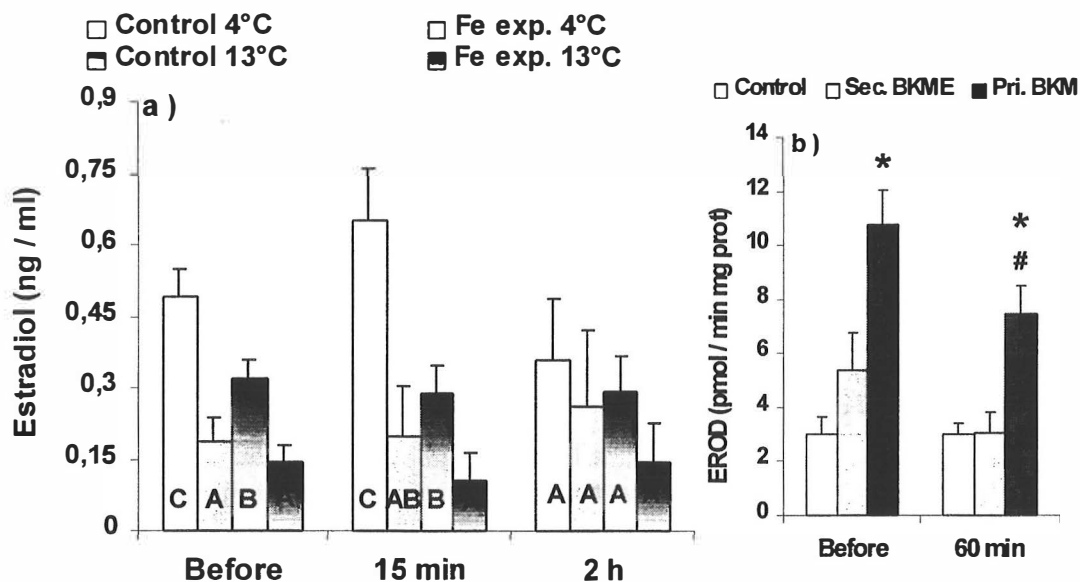


FIGURE 4 a) Plasma estradiol levels (mean  $\pm$  SE) in the control and in the iron exposed (30 days) whitefish sampled either before or 15 min or 2 h after a short air exposure. Different capitals indicate significant differences between the groups in each sampling point. N is 8 (Before) or 5 (15 min and 2 h) for each column (V). b) Liver microsomal EROD activities (mean  $\pm$  SE) in the control and in the kraft mill effluent exposed (21 or 42 days) whitefish sampled either before or 60 min after a 5 min exposure to a low water depth (10 cm). # denote significant ( $p < 0.05$ ) difference between the different sampling points at the same fish group. Asterisks denote significant ( $p < 0.05$ ) differences between the exposed and control fish at the same point. Number of fish is 16 for each column (IV).

## 4 DISCUSSION

### 4.1 Effects of exposures on whitefish sampled without additional handling

Prolonged exposure of whitefish to the applied water conditions reflecting actual and potential influences of anthropogenic emissions on Finish water ecosystems changed the physiological status of whitefish in terms of the used variables. The variance in the changes between the different experiments was most probably connected with dissimilarities in the applied exposure regimes, in water chemistry and in ambient conditions as well as caused by specific impacts of hazardous substances. Non-specificity, i.e. dependency on several factors, and stress sensitivity of some of the variables were probably also involved. In general, these results that consider only momentary reflections of anthropogenic impacts on the exposed animals are in accordance with the current trend in biological research. Mechanistic, functional and behavioural aspects should be preferred instead of producing scattered information. Physiological and biochemical data gained from one-point sampling after *in situ* exposure rarely indicates the true meaning of an observed alteration because of the effectiveness and multiplicity of feedback mechanisms and the relatively great natural variability of physiological functions especially in cold blooded animals. In fact, it is almost impossible to distinguish direct toxic impacts and compensatory responses from each other without molecular level investigations and, thereby, figure out whether the outcomes reflect maladaptive or adaptive processes (Wendelaar Bonga 1997).

Despite the general inconsistency in the directions of changes within a certain variable measured, a decreasing trend in plasma stress hormone levels (III, IV, V) and liver glycogen concentration (I, III) as well as an increased red blood cell sodium concentration (III, V) and gill  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity (IV, V) were observed both in the BKME and iron exposed whitefish. Though a lowered level of plasma cortisol in the fish sampled without an additional

handling may have been a consequence of decreased ACTH responsiveness of interrenal cells due to a prolonged stress (Quabius et al. 1997), it is obvious that an attenuated or delayed (Norris et al. 1999) response to minor stressors of sampling regimes (III, V) was also involved. Decreased levels of cortisol in blood circulation, in their turn, are one possible reason behind the decreased liver glycogen concentrations as cortisol acts as an important gluconeogenic factor (Vijayan et al. 1994b, Vijayan et al. 1997c, Milligan 1997). On the other hand, however, slow-release cortisol implants resulting in prolonged increase in plasma cortisol levels have been noted to cause depletion of liver glycogen stores (Andersen et al. 1991, Vijayan & Leatherland 1989, Vijayan et al. 1991), which suggests that lowered cortisol levels would not have the same effect. Thus, increased hepatic GPase activity (Fig. 3, IV, V), alterations in the functions of peripheral cortisol receptors (Pottinger 1990, Vijayan et al. 1997a), other cellular effects of resin acids (Råbergh et al. 1999) or histopathological changes like cellular necrosis (Adams et al. 1996) may have taken place in the BKME exposed whitefish. McLeay & Brown (1979) noted the same effect in coho salmon (*Oncorhynchus kisutch*) exposed for 30 days to environmentally realistic concentration of BKME. The notions concerning increased gill  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity suggest that the possible inhibitory effects of toxic substances *i.e.* resin acids and iron on branchial chloride cells have been overcome by compensatory factors such as increased number, functional area or turnover rate of these cells (Wendelaar Bonga 1997). Overall, disturbances in water and ion homeostasis and branchial fluxes are typical characteristics of any kind of stress in fish.

Some of the apparently contradictory results, like the ones of plasma estradiol alterations, may also be explained to be derived from non-specific stress related functions: plasma estradiol concentration was either clearly increased (II) or distinctly decreased in the whitefish (V) exposed to the iron-rich humic peat water supplemented with additional inorganic iron, whereas both treated and untreated BKMEs were totally ineffective (IV). At first glance, this result seems peculiar as most BKMEs are known to include estrogenic compounds like  $\beta$ -sitosterol (Mellanen et al. 1996, Tremblay & van der Kraak 1998), while iron-rich humic waters are not. In both exposures carried out with the one-year-old whitefish (II, IV), however, plasma estradiol exhibited an increasing trend in handling-stressed fish groups along with an increased level of plasma cortisol, while this phenomenon was totally absent in the two-year-old whitefish (V). Thus, a change in the primary source of plasma estradiol, from the interrenals to the gonads, may be suggested to occur during the second year of whitefish life resulting in a disconnection of these steroid responses. The reduction of plasma estradiol concentrations in two-year-old iron-exposed whitefish (V) is clearly consistent with earlier studies indicating depreciatory effect of both prolonged handling stress (Coward et al. 1998, Morgan et al. 1999) as well as cellular toxicity of iron overload (Lucesoli et al. 1999) and lifelong BKME exposure (Janz et al. 1997) on reproductive physiology. Overall, however, inconsistent and multiple effects of stress and toxicity on reproductive functions are closely familiar to endocrinologists (Barton & Iwama 1991).

## 4.2 Modification of the general stress response by exposure to impaired water quality

### 4.2.1 Impairment of the primary stress responses

Although impaired water conditions, like those derived from low pH and waterborne aluminium (Witters et al. 1991, Brown & Whitehead 1995), have shown to be capable of inducing catecholamine response in fish, the studies evaluating possible catecholamine response modifications are extremely scarce. Both a prolonged physical stress (Reid et al. 1994) and an exposure to organic water contaminants (Nilsson & Block 1991) disturb, however, the responsiveness of chromaffin tissue to physiological stimuli elicited by an acute disturbance. These considerations are coherent with the notion of distinctly lowered plasma adrenaline and noradrenaline levels in handling disturbed whitefish exposed periodically to iron-rich humic peat water supplemented with an environmentally realistic amount of inorganic iron (V). This may be a secondary consequence due to alterations in acid/base balance (Tang & Boutilier 1988) and in oxygen uptake (Wright et al. 1989) caused by disturbed gill morphology and ochre suffocation (Peuranen et al. 1994), or, either an indirect (humoral, neuronal or paracrine) or a direct (humoral, neuronal or paracrine) implication of prolonged stress, or, indirect (e.g. alteration in intracellular  $Ca^{2+}$  homeostasis) or direct (e.g. oxygen radical formation) toxic effect of iron overload on chromaffin cells. For example, a prolonged physical stress alone has been noted to affect adrenal chromaffin cells of rainbow trout *Oncorhynchus mykiss* in a way resulting in an order of magnitude desensitization of catecholamine responsiveness to cholinoreceptor stimulation (Reid et al. 1994), which is considered to be one of the primary mechanisms (Nilsson 1984) controlling exocytotic functions in this tissue type (Burgoyne 1991). On the other hand, Kajiwara et al. (1999), demonstrated that the extent of low density lipoprotein (LDL) oxidation, which proceeds in the presence of transition metals (like iron) *in vitro* (Lamb et al. 1995), is in correlation with the magnitude of the inhibition of catecholamine secretion in bovine adrenal medullary cells. In overall, however, the several possible mechanisms behind the modulation of plasma catecholamine response to acute disturbance are extremely complex and not sufficiently investigated in fish (Gfell et al. 1997, Reid et al. 1998, Perry et al. 1999).

The recent reports describing an attenuated plasma cortisol response in feral fish after a life-long exposure to impaired water qualities (Hontela et al. 1992, 1995, Brodeur et al. 1997, Girard et al. 1998, Hontela 1998, Norris et al. 1999), like due to BKMEs (Hontela et al. 1997), are consisted with *in vitro* studies (Ilan & Yaron 1980, Brodeur et al. 1997, Quabius et al. 1997, Girard et al. 1998, Hontela 1998), revealing decreased responsiveness of interrenal cells to ACTH challenge. It has been suggested that direct toxic mechanism is involved, either dependently (Quabius et al. 1997) or independently (Ilan & Yaron 1983) on cellular cAMP stores. On the other hand, this exhaustion of the HPI axis may

occur at the pituitary/hypothalamic level by involving an impaired ACTH release from the adenohypophysis. This impairment might be due to the prolonged overstimulation of hypothalamus by a reduced cortisol feedback (Norris et al. 1999, Roillant et al. 2000). In contrast to these contributions, and particularly the one presented by Ilan & Yaron (1983) considering o,p'DDD exposed *Sarotherodon aureus*, Jørgensen et al. (2001) have recently demonstrated that exposure of arctic charr (*Salvelinus alpinus*) to o'p-DDD does not alter plasma cortisol and ACTH responses to acute handling stress. In concert with the complexity of these results, the extent of cortisol response impairment noted within the present work was not connected with the amount of toxicants in exposure waters (IV). Furthermore, the individual deviations in plasma cortisol level reached were small between the different experiments and were not at all affected by the duration of recovery period after the attainment of the stress level (Fig. 2). This result, which together with the decreased prestress level of plasma cortisol in the exposed fish obviously is the most important finding of this study due to the overall significance of cortisol and HPI-axis response, suggests two wider consequences. First, only one (IV) out the five experiments concerned with the cortisol response in the exposed whitefish, gave, to a statistically significant extent, the same result as the overall picture. Thus, either the amount of experimental whitefish was too small in relation to the extent of individual variability derived from e.g. sampling procedures and genetic factors or, the handling and sampling procedures themselves were too variable for this sensitive indicator of stress. Obviously, similar problems may be hidden under the reports performed by other researchers as well. Secondly, this result does not support the idea that a certain specific toxic mechanism would exist under the impairment of plasma cortisol response in fish. Rather, a non-specific and toxicant independent mechanism resulting in either interrenal or pituitary exhaustion (exhaustion of the HPI axis) (Norris et al. 1999, Mesa et al. 2000), or stimulated cortisol degradation (Vijayan et al. 1997a, Mommsen et al. 1999, Blom et al. 2000), or both, are most probably involved. Though impaired stress response is, at least in the respect of attenuated cortisol response, considered to express physiological dysfunction, it may also serve as an energetic advantage to exposed and stressed animals having a disturbed energy balance. Thus, impaired stress response appears to be in line with reduced growth and fecundity, phenomena connected with the reduced energy stores of a chronically stressed individual. Consistently, ovulating female whitefish caught from spawning grounds exert extremely high plasma cortisol levels whereas those from other sites do not (Fuller et al. 1976). If the cortisol excretion would be seriously impaired, like for example due to the water of impaired quality, there certainly would appear some extra troubles during the spawning as energetic resources of fish would not be prepared for stressful situation.



#### 4.2.2 Modification of secondary responses

Modifications of secondary stress responses are primarily derived from two different mechanisms as well as their combined effects *i.e.* indirectly through hormonal alterations and directly by specific toxic actions. Accordingly, the observed modifications of primary responses (see above) were most probably accompanied by hazardous impacts of both BKME derived toxic compounds (e.g. Bogdanova & Nikinmaa 1998, Nikinmaa et al. 1999, Råbergh et al. 1999) and iron overdose (e.g. Brock et al. 1994, Kim et al. 1995, Payne et al. 1998) on cellular functions. Together with the variability of the applied exposure regimes and handling procedures, this suggests improbability of coherent outcomes in secondary stress response modification. Also other reports (Leatherland 1985, Barton & Iwama 1991) have argued against purely causal relationships between primary and secondary stress responses. Indeed, the most outstanding result in respect to the secondary response modification was higher variability of handling responses in the exposed whitefish than in the reference whitefish. This phenomenon was particularly well established in the field experiments (I, III), and showed also as vanished effects of ambient acclimation conditions on the physiological responses of whitefish exposed to waterborne iron overdose (V).

The suppression of primary responses during the acute phase of general stress response occurred simultaneously with alterations in several individual responses at the secondary level, modified liver glycogen mobilisation being most evident of these. This result appears feasible, as both cortisol (the major glucocorticosteroid) and catecholamines exert powerful regulatory roles in carbohydrate metabolism during the acute stress response in fish. Generally, attenuated liver glycogen phosphorylase responses were noted more frequently (IV, V) than attenuated liver glycogen (I, III, IV), plasma glucose (I, III, IV, V) and lactate (I, III) responses in whitefish groups exposed either to BKMEs or iron supplemented iron rich humic peat water. This result is firmly consistent with the notion of attenuated catecholamine response in the iron exposed whitefish groups and suggests, together with less affected plasma cortisol response, that adrenergically mediated hepatic glycogenolysis (Wright et al. 1989, Wendelaar Bonga 1997) was more strongly disturbed by the exposures than cortisol mediated gluconeogenesis (Wendelaar Bonga 1997, Mommsen et al. 1999). This suggestion is not exactly in the same line, but not in contrast either, with the one presented by Vijayan et al. (1997b), who observed also a modified cortisol response and an impaired carbohydrate metabolism response in rainbow trout. A field exposure was a more probable cause for response modification than a laboratory exposure. Differences in the nutritional status of fish (Barton et al. 1988; Vijayan & Moon, 1992) and the extended duration of handling in the field experiment are probable causes for this outcome.

Similarly, Hontela et al. (1995) found higher liver glycogen concentrations and lower plasma glucose and tyrosine levels in acutely stressed immature yellow perch, *Perca flavescens*, caught from polluted (PAHs, PCBs, Hg, Cd, As, Zn) areas and suffering from an impaired cortisol response to a

more prolonged handling stress. This finding is supported by the report of Vijayan and Leatherland (1989) in which a prolonged plasma cortisol increase was shown to affect carbohydrate metabolism of coho salmon by increasing clearance rate for  $T_3$ , and stimulating gluconeogenesis and mobilization of glycogen stores. Several other studies have supported the opinion that cortisol acts as an important glyconeogenetic factor by facilitating the replenishment and maintenance of liver glycogen stores during acute and chronic stress (Leach & Taylor 1980, Vijayan et al. 1991, Milligan 1997, Vijayan et al. 1997c, Mommsen et al. 1999) though contradictory results have also been presented (Andersen et al. 1991).

The study by McMaster et al. (1994), on the other hand, revealed a lowered plasma lactate and unchanged plasma glucose responses to a 5 min air exposure in BKME exposed male white sucker, *Catostomus commersoni*, exhibiting also a reduced plasma cortisol level before handling stress. A result resembling, but not equal to those of the present study focused on whitefish caged for 30 days in areas affected by BKMEs (I, III). Impaired water qualities of very different characteristics are, therefore, capable of attenuating both stress hormone and carbohydrate responses of fish to acute handling disturbances by unknown and probably highly complicated mechanism as stated already by Andersson et al. (1987, 1988). One of the functions taking part in this overall phenomenon appears to be reduced responsiveness of liver glycogen phosphorylase activity to handling disturbance (IV, V), which may be at least partly caused by a decreased number of  $\beta$ -adrenoceptors in the surfaces of hepatocytes due to decreased circulating cortisol concentrations (Reid et al. 1992). Glycogen phosphorylase enzymes ( $a + b$ ) are the initiators of the glycogen breakdown cascade in the liver and stimulated by catecholamines (Janssens & Lowrey 1987, Wright et al. 1989) and handling (Morata et al. 1982a). Thus, an attenuated catecholamine response due to the 30 days exposure to iron-rich humic water with additional (5 mg Fe/l) inorganic iron (V), as also a prolonged stress alone (Reid et al. 1994), may directly retard the production of glucose via glycogenolysis. Notions concerning direct actions of toxicants on hepatocytes (Nikinmaa et al. 1999) and those describing alterations in responsiveness of these cells to catecholamines (Reid et al. 1992) by, e.g. a neuroendocrinological dysfunction (Hontela et al. 1997), however, make overall conclusions impossible.

Contradictory to the rather parallel modifications of hormonal and carbohydrate responses, there were no clear trends in osmoregulatory and in red blood cell response modifications in the exposed whitefish. One reason for this is the inconsistent nature of these responses in the control whitefish, a phenomenon arisen obviously from the high efficiency of handling disturbance and circulating catecholamines to stimulate water and ion movements through branchial epithelium and erythrocyte plasma membrane as also to elicit volumetric changes (Eddy 1981, McDonald et al. 1991, Okimoto et al. 1994). Increases in plasma sodium concentration and blood hemoglobin and hematocrite levels after handling were either transient (I, II, III, V), sustained (II; V) or absent (IV), whereas the response of other variables was not at all

predictable. In the case of red blood cell cationic concentrations, this appeared to be derived from tight time-dependency, e.g. short-term deviations in the intracellular  $K^+ / Na^+$  value were followed by similar, but opposite changes (III, IV, V). The slight and relative rapid increases in branchial  $Na^+-K^+$ -ATPase activity within 20 min after handling (IV, V) are not explainable by other studies concerned with this variable, while the decrease (III, V) may be due to adrenergic inhibition (Benzis & Bornanzin 1984).

Overall, in the control groups of whitefish subjected either to air exposure (IV, V) or to underwater transport (I, III), statistically significant changes in plasma cationic concentrations were noted in 6 out of 10 cases, while in red blood cells in 6 out of 8 cases. Instead, in the exposed whitefish groups the numbers were 4 out of 14 and 8 out of 14, respectively. In a very broad aspect, also this result may be considered to indicate attenuated response of the exposed whitefish to a handling disturbance. Also Mesa et al. (2000) considered this kind of overall attenuation in stress responses and suggested it to be possibly connected with the exhaustion of the HPI axis (discussed above). It is, however, evident that also prolonged responses of red blood cells (III, IV) to acute disturbances may take place in exposed fish, possibly due to e.g. increased  $\beta$ -adrenergic responsiveness (Reid & Perry 1991) or plasma membrane permeability (Toivola & Isomaa 1991).

### 4.3 Interpretation of the data

According to the present results, a momentary handling disturbance may lead to multiplicity in data interpretation in three different ways. 1) Even in the cases in which the effects of a short handling on measurable variable is negligible, the original effects of exposure may be faded through increased inter-individual deviations (V; Fig 4a). 2) Dissimilar physiological impacts of handling disturbance on reference and exposed fish may entirely wipe out the actual impact of the exposure (IV, Fig. 3 GPase; Fig. 4b). 3) The most common and meaningful problem for the reliability of data interpretation in field experiments is the one arisen as a consequence of variable time periods and dissimilar stress responses. Namely, if fish are sampled after different (recovery) periods in natural reference areas and, for example, in BKME exposed lake areas, the results may be easily lost or even totally inverted although the effects of handling would not be significantly different between the two areas involved (I, III). These notions are supported by the discrepancy of the results of earlier field studies as well as by the outcomes of the experiments describing multiple effects of cortisol, ACTH or acute stress also on detoxification enzyme systems (Lee et al. 1993, Mommsen et al. 1999, Blom et al. 2000), reproductive hormones (Pickering et al. 1987) and  $Na^+-K^+$ -ATPase activity (Langdon et al 1984, Quabius et al. 1997) of fish, and, particularly, by a couple of studies concerned with the data interpretation of field experiments (McMaster et al. 1994, Jardine et al. 1996).

## 5 CONCLUSIONS

The presented experiment indicates the following points:

- 1) Due to the time-dependency and sensitivity of the physiological stress response, the evaluation of recovery periods is overwhelming for the reliability of the method.
  - 2) Both primary and secondary levels of the integrated stress response may be modified by a prolonged exposure of fish to anthropogenically impaired water quality
  - 3) Deviations in the characteristics of the secondary stress response between different experiments are generally smaller in unexposed than in exposed fish. Thus, the nature of responses, and the reliability of results therein, may be more precisely predicted in fish maintained in uncontaminated water quality.
  - 4) In whitefish maintained in dechlorinated tap water, the physiological stress response is modified by acclimation temperature, whereas the effects of ambient conditions are, to a great extent, disappeared in whitefish after a subchronic exposure to impaired water quality.
  - 5) The interpretation of data may be easily confused due to a capture of freely swimming fish. Thus, to obtain reliable and comparable results in field studies, the sampling method should either be very fast (< 5 min) or at least time-dependently standardised.
  - 6) The paucity of the reports considering these kind of methodological aspects, together with the widely accepted employment of stressful catching methods involving a delayed sampling of wild fish (or other animals), suggests hazardous ignorance associated with ecotoxicological studies.
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## YHTEENVETO

### **Ihmistoiminnan aiheuttaman veden laadun heikentymisen vaikutukset planktonsiian, *Coregonus lavaretus*, fysiologisessa stressivasteessa**

Nykyinen teknologinen kulttuuri muokkaa maa- ja vesiympäristöjä vauhdilla, joka on luonnonvaraisten eliöiden evolutiiviseen sopeutumisprosessiin verrattuna nopea. Täten syntyvät ympäristön muutokset luovat eliöille uusia ja yllättäviä ulkoisia vaatimuksia ja haasteita. Kehittyvä ilmastonmuutos äärevine ilmasto-oloineen, maaperän happamoituminen ja eroosio, ravinnepestöt vesistöjä rehevöittävine vaikutuksineen sekä teollisuusprosessien tuottamat luke mattomat haitta-aineet ovat eräitä niistä ihmisperäisesti aiheutuneista tekijöistä, jotka muokkaavat vesiympäristöjä tavalla, johon evolutiivisesti kehittyneet eliöt eivät ole ehtineet sopeutua. Ihmistoiminnan muuttamiin oloihin joutuneilla kaloilla on yksilön ja populaation säilymisen ylläpitämisen kannalta katsoen vaihtoehtoina joko fysiologinen sopeutuminen, elintapojen muuttaminen tai sopivamman elinympäristön etsiminen. Myös jälkimmäiset vaihtoehdot edellyttävät yleensä fysiologista sopeutumista. Koska sopeutumiskyky on yksilöllinen ominaisuus ja koska fysiologisen sopeutumisen ja stressitilan ero on monessa suhteessa liukuva, on edullinen sopeutuminen (ns. adaptaatio) käytännön tasolla usein erittäin vaikeasti erotettavissa epäedullisesta elimistön sisäisen tasapainotilan häiriintymisestä (ns. krooninen stressi).

Altistuminen erilaisille muutoksille ja uhkatekijöille on kuitenkin eräs luonnonvaraisten eläinten elämän keskeisimpiä piirteitä. Kyky selviytyä näistä useimmiten ulkoiselta taholta eläimeen kohdistuvista vaaroista on, ravinnonhankinnan ja lisääntymiskyvyn ohella, eräs tärkeimmistä perusedellytyksistä yksilön ja lajin geneettisen perimän säilymisen kannalta. Yleinen fysiologinen stressivaste (GAS = general adaptation syndrome) kuuluu siis välttämättömänä osana kaikkien terveiden selkärankaisten eläinyksilöiden elintoimintoihin. Stressivaste on epäspesifinen reaktio hyvin erilaisille häiriötekijöille ja tapahtuu samalla tavalla kaikilla selkärankaisilla eläinryhmillä. Psykologinen uhka, fyysinen käsittely, lämpötilan äkillinen muutos, altistuminen erilaisille kemika-

leille, haitta-aineille tai saalistukselle sekä lajinsisäiset parittelu- tai reviiirikiistat ovat esimerkkejä tilanteista, jotka vaativat yleisen fysiologisen stressivasteen olemassaoloa. Stressivasteen tarkoituksena on sopeuttaa eläinyksilö haastavaan olosuhteeseen säilyttämällä sisäinen fysiologinen tasapainotila (homeostaasi) uusia ympäristöolosuhteita ts. muuttunutta suorituskykyä vastaavalla tasolla. Ulkoinen tai sisäinen tekijä, joka muuttaa yksilön kykyä sopeutua esim. em. tilanteisiin ts. kykyä fysiologiseen stressivasteeseen, muuttaa yhtälailla yksilön menestymistodennäköisyyttä alati muuttuvissa ja uusia haasteita tarjoavissa ympäristöoloissa. Muuttunut stressivaste onkin eräs kroonisen stressitilan tunnusmerkkejä.

Tämän väitöskirjatyön tarkoituksena oli selvittää lyhytaikaisen stressivasteen käyttöä pitkittyneen stressitilan ilmentäjänä, ts. vastata kysymykseen muuttaako kalan altistuminen ihmisperäisesti heikentyneelle veden laadulle fysiologista stressivastetta (mahdollisesti epäedulliseen suuntaan). Tutkimuksissa selvitettiin tyypillisten suomalaisia sisävesiä muuttaneiden häiriöiden, sellutehtaiden päästövesien sekä turvetuotantoalueiden valumavesien, pitkäaikaista vaikutusta planktonsiian fysiologiseen stressivasteeseen kahden kenttäkokeen sekä kolmen luonnonoloja jäljittelevän laboratoriokokeen puitteissa. Lisäksi yhdessä kokeessa altistettiin siikoja em. häiriötekijöiden tyypillisimmille haitta-ainelle (dehydroabietiinihappo ja rauta) ravinnon kautta annosteltuina puhdasaine-altistuksina. Myös luonnollisten ympäristöolosuhteiden vaihteluiden, kuten veden lämpötilan (4, 8.5. 13-13.5 ja 18.5°C), aiheuttamaa stressivasteemuuntelua selvitettiin kahdessa osakokeessa. Stressivasteen herkkyyden ja tulosten vertailukelpoisuuden vuoksi kaikki kokeet tehtiin saman kalalajin nuorilla (1+ ja 2+ vuotiaat) yksilöillä. Äkillisenä lisähäiriönä käytettiin 3 - 6 viikon altistusjaksojen jälkeen lyhytaikaista käsittelyhäiriötä joko nostamalla kalat toistuvasti (10 s + 20 - 30 s + 10 s) ylös vedestä, altistamalla matalalle vedelle (10 cm, 5 min) tai kenttäkokeeseen välttämättömänä osana liittyvälle vedenalaiselle kuljetukselle (10 min). Näiden lyhytaikaisten lisähäiriöiden jälkeen kalojen annettiin toipua tarkasti määritelty ajanjakso (5 min - 5 h) ennen kalojen tainnutusta ja varsinaista näytteenottoa.

Fysiologinen stressivaste on koko elimistöä koskettava kokonaisvaltainen tapahtumasarja, jossa ulkoista ärsykettä seuraa plasman stressihormonien, kortikosteroidien ja katekoliamiinien, pitoisuuksien jyrkkä nousu sekä edelleen näistä johtuvat moninaiset muutokset mm. hapenotossa ja -kuljetuksessa, energiametaboliassa sekä ionitasapainon säätelyssä. Tästä syystä tutkimuksissa määritettyjen muuttujien määrä on suuri ja kuvastaa kalan elintoimintoja sekä fysiologisella että biokemiallisella tasolla. Jokaisessa osakokeessa lyhytaikainen käsittelyhäiriö aiheutti planktonsiioissa äkillisen stressivasteen, jota ilmensivät plasman kortisoli- ja katekoliamiinipitoisuuksien, veren glukoosi- ja laktaattipitoisuuksien sekä hemoglobiini- ja hematokriittiarvojen nouseminen kuten myös maksan glykogeenifosforylaasiaktiivisuuden nousu ja glykogeenipitoisuuden lasku puhtaassa vedessä pidetyissä kaloissa. Plasman estradiolipitoisuuden, kidusten Na<sup>+</sup>-K<sup>+</sup>-ATPaasi aktiivisuuden sekä plasman ja punasolujen ionipitoisuuksien suhteen muutokset olivat vaihtelevampia johtuen ilmeisesti erilaisista koeolosuhteista ja -järjestelyistä sekä käytettyjen palautumisjaksojen

eroista. Etelä-Saimaalla suoritetuissa kenttäkokeissa ja vastaavalla sellujätevedellä suoritettussa laboratoriokokeessa havaittiin altistettujen siikojen stressivasteen mm. plasman kortisolivasteen sekä hiilihydraattivasteen muuttuminen puhtaalla alueella sumputettuihin sekä puhtaassa, hiilisuodatetussa laboratoriovedessä pidettyihin kontrollisiikoihin verrattuna. Turvetuotantoalueen valuma-altaan rautapitoisen humusveden ei sellaisenaan havaittu aiheuttavan suuria fysiologisia muutoksia, mutta epäorgaaninen lisärauta aiheutti sitä vastoin muutoksia sekä humuspitoiseen turvetuotantoalueen valumaveteen että puhtaaseen laboratorioveteen lisättyinä kuin myös ravinnon kautta annosteltuna. Tässä suhteessa merkittävimpänä tuloksena oli adrenergisen vasteen eli plasman katekoliamiinipitoisuuden ja maksan glykogeenifosforylaasi-aktiivisuuden nousujen vaimeneminen lisärautaa (5 mg Fe/l) sisältäneelle turvetuotantovedelle (5 %) altistetuilla kaloilla.

Tässä työssä esitetyt kokeet toivat ilmi seuraavat seikat: 1) Fysiologisen stressivasteen aikasidonnaisuudesta, häiriöherkkyydestä ja kokonaisvaltaisuudesta johtuen palautumisajanjaksojen tarkka seuranta on vastaavan tyyppisten kokeiden tulosten luotettavuuden kannalta ensiarvoisen tärkeää. 2) Planktonsiian pitkäaikainen altistuminen kokeissa käytettyjen kaltaisille ihmistoiminnan muuttamille ympäristöoloille voi aiheuttaa muuntelua sekä primaarisessa että sekundaarisessa stressivasteessa. Heikentyneeseen hormonaaliseen vasteeseen liittyy hiilihydraatti-aineenvaihduntaa sekä vähäisemmässä määrin ionisäätely- ja hapenkuljetuskykyä kuvaavien muuttujien vastemuutokset. 3) Altistamattomilla kaloilla fysiologisen stressivasteen yksilöllinen vaihtelu on pienempi kuin altistetuilla kaloilla. Täten stressivasteen luonne ja tulosten luotettavuus on tarkemmin ennustettavissa altistamattomilla kaloilla. 4) Hiilisuodatetussa vesijohtovedessä pidettyjen planktonsiikojen fysiologinen stressivasteen voimakkuus ja aikasidonnaisuus riippuu kalojen sopeuttamislämpötilasta. Sitä vastoin pitkäaikaisesti altistetuilta kaloilta fysikaalisten ympäristöolojen fysiologiset vaikutukset ovat heikentyneet tai kokonaan hävinneet. 5) Altistuksen seurauksena muuttunut stressivaste haittaa oleellisesti sellaisten tavallisten vastetutkimusten tulosten luotettavuutta, joissa stressivasteen alaisia elimistön toimintojen muutoksia ei havainnoida ajallisella seurannalla. Tämä siksi, että varsinkin luonnon olosuhteissa vapaasti uivilla kaloilla tehtyihin kokeisiin liittyy aina kalojen stressaantuminen pyynnin ja näytteenoton yhteydessä. Muuttunut stressivaste muuttaa siis myös saatavia tuloksia ennustamattomaan suuntaan ja saattaa johtaa joidenkin muuttujien osalta jopa täysin päinvastaisiin tuloksiin.



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**Original papers**

**I**

**Altered challenge response in whitefish subchronically exposed  
in areas polluted by bleached kraft mill effluents**

by

Jarmo Lappivaara and Aimo Oikari, 1999

Ecotoxicology and Environmental Safety 43, 212-222

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II

**Bioaccumulation and subchronic physiological effects of  
waterborne iron overload on whitefish exposed in humic  
and nonhumic water**

by

Jarmo Lappivaara, Ari Kiviniemi and Aimo Oikari, 1999

Archives of Environmental Contamination and Toxicology 37,  
196-204

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<https://doi.org/10.1007/s002449900506>

III

**Attenuated carbohydrate and gill Na<sup>+</sup>-K<sup>+</sup>-ATPase stress  
responses in whitefish caged near bleached kraft mill  
discharges**

by

Jarmo Lappivaara, Jarmo Mikkonen, Markus Soimasuo, Aimo  
Oikari, Aarno Karels and Anni Mikkonen, 2001

Ecotoxicology and Environmental Safety, in press

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<https://doi.org/10.1006/eesa.2000.1998>

IV

**Effects of acute handling stress on whitefish *Coregonus lavaretus*  
after prolonged exposure to biologically treated and  
untreated bleached kraft mill effluent**

by

Jarmo Lappivaara, 2001

Archives of Environmental Contamination and Toxicology 41,  
55-64

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<https://doi.org/10.1007/s002440010220>

V

**Acute physiological stress response of whitefish to a short air challenge is impaired by winter conditions, cold water alone, and intermittent exposure to waterborne iron overload**

by

Jarmo Lappivaara and Sanna Marttinen

Manuscript

Submitted to Canadian Journal of Fisheries & Aquatic Sciences

<https://doi.org/10.1016/j.ecoenv.2004.01.003>