Skin Responses of Fish to Stressors

Declan Thomas Nolan

Skin Responses of Fish to Stressors

Een wetenschappelijke proeve op het gebied van de
Natuurwetenschappen, Wiskunde en Informatica

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Katholieke Universiteit Nijmegen,
volgens besluit van het College van Decanen
in het openbaar te verdedigen
op donderdag 29 juni 2000
des namiddags om 3.30 uur precies

door

Declan Thomas Nolan

Geboren op 31 mei 1965 te Portlaoise, Ierland

Promotor: Prof. Dr. S.E. Wendelaar Bonga

Manuscriptcommissie: Prof. J.C. Fenwick (University of Ottawa, Canada)
Dr. C. Hogstrand (King's College London, U.K.)
Dr. S.C. Johnson (National Research Council, Canada)
Dr. R.A.C. Lock

This thesis has been financially supported by:
KEMA Environmental Services & KEMA Power Generation, The Netherlands
M&T Graduate School of Environmental Chemistry and Toxicology, The Netherlands
Marine Institute, Ireland
National Centre for Animal Experiment Alternatives (Platform Alternatieven voor Dierproeven), The Netherlands

Printed by: PrintPartners Ipskamp, Enschede, The Netherlands.

Theme and concept: A.S. Maia
Cover design: Grafische Vormgeving

ISBN: 90-9013752

The most beautiful thing we can experience is
the mysterious. It is the source of all true art
and science. He to whom this emotion is a
stranger, who can no longer pause to wonder
and stand rapt in awe, is as good as dead:
his eyes are closed.
Albert Einstein

To my parents, for
supporting my studies
in science.

Contents

Chapter 1
General Introduction

The stress response of fish

The stress response of fish is complex and involves a series of behavioural and physiological responses considered to be compensatory and/or adaptive, which enable the animal to overcome the effects of the stressor. These responses involve all levels of animal organisation and are collectively called the integrated stress response (see review by Wendelaar Bonga, 1997). The stress parameters generally measured and used to evaluate stress levels in fish are blood cortisol and glucose levels. The primary stress response of fishes involves the activation of the higher brain centres, resulting in the massive release of corticosteroids and catecholamines, and the activation of numerous secondary stress responses at all levels throughout the animal. Increases in plasma glucose are effects of the catecholamines mobilising energy resources to fuel the responses. The release of catecholamines results from the stimulation of the chromaffin cells, which are located close to the posterior cardinal veins in the head kidney, by preganglionic cholinergic fibres of sympathetic nerves. The main effects of catecholamines are on the respiratory and cardiovascular systems, directed at increasing oxygen uptake and delivery throughout the body by increasing ventilation rate, branchial blood flow and oxygen diffusion capacity, as well as oxygen transport capacity of the blood, mostly through β-adrenergic mechanisms (Randall and Perry, 1992). The effects of catecholamines on glycogenolysis and glucose release from the liver mediate the immediate stress-related hyperglycaemia reported in many teleosts, but catecholamines also have indirect effects on hydromineral balance. The most disadvantageous side effect of catecholamines in relation to hydromineral balance is the increase in the permeability of the gills, probably via effects on the tight junctions, which increases water and ion diffusion rates through the paracellular pathways of the branchial epithelium.

Cortisol and the stress response

Increased circulating blood cortisol is mediated by the hypothalamic-pituitary-interrenal axis, where corticotrophin-releasing hormone and adrenocorticotropic hormone are the main secretagogues stimulating the synthesis and release of cortisol from the inter-renal tissue located close to the posterior cardinal veins. Although cortisol has a broad activity spectrum in fishes, its major functions are mineralocorticoid (it stimulates the differentiation of branchial chloride cells, increasing the specific activity of ion transporting enzymes, in particular Na^+/K^+-ATPase) and glucocorticoid (it stimulates gluconeogenesis in the liver, e.g. from non-protein substrates, such as the amino acid alanine and lactate; Vijayan et al., 1994a, b). However, high peak levels of circulating cortisol, or moderately elevated levels for prolonged periods may also have effects on the hydromineral balance by inducing apoptosis of the chloride cells in the gills (Bury et al., 1998) and thus impairing osmoregulation. Cortisol also mediates other stress-related changes in the skin of the

rainbow trout *Oncorhynchus mykiss*, including stimulation of mucous discharge and synthesis of vesicles in the cells of the upper epidermis (Iger et al., 1995). Cortisol is further implicated in mediating the inhibitory effects of stressors on the immune response, thus decreasing disease resistance (Ellis, 1981; Pickering and Pottinger, 1989; Anderson, 1990; Houghton and Matthews, 1990). High cortisol levels in the blood are associated with reduced circulating lymphocyte levels, decreased antibody production, lower mitogen-induced proliferation of lymphocytes and the inhibition of phagocytotic activity (Stave and Roberson, 1985; Kaattari and Tripp, 1987; Maule et al., 1987; Espelid et al., 1996). Studies with Atlantic salmon selected for high and low cortisol stress response have demonstrated that a high cortisol response was correlated with reduced non-specific immunity and increased susceptibility to pathogens (Fevolden and Roed, 1993; Fevolden et al., 1994).

Stress and the skin of fish

Nevertheless, the stress response is designed to be primarily adaptive and, within certain limits, of benefit to the fish. In this thesis stress-related responses are examined in the skin of salmonid and cyprinid fishes. The outer layer of fish skin is a complex epithelium comprised of several layers of living cells that are continuous over the body surface (Harris and Hunt, 1975; Whitear, 1986; Iger and Wendelaar Bonga, 1994; Iger et al., 1994b, c, d; Burkhardt-Holm et al., 1997, 1998). It forms the first barrier between the external and the internal environment and is protected with a chemically and functionally complex mucous coat, which is discharged by specialized mucous cells in the epidermis (Shephard, 1994). The teleostean epidermis is influenced by a number of endocrine factors (Pottinger and Pickering, 1985; Iger et al., 1995) and shows characteristic changes in fish exposed to stressors. Most research has been carried out on the gills, as they are the principal site for ionoregulation, gas exchange, and nitrogenous waste excretion (Evans et al., 1982; Perry and Laurent, 1993), and disruption at the gill has deleterious consequences for the fish (Wendelaar Bonga and Lock, 1992; Lock et al., 1994). The accepted estimate is that the gills account for 70-90% of the surface area of the fish. Therefore the role of the skin in physiological or toxicological studies was largely overlooked or assumed insignificant. The diffusion characteristics of organic chemicals for rainbow trout *O. mykiss* and channel catfish *Ictalurus punctatus* were calculated using a mathematical model and indicated that the skin could account for up to 3.5 and 8.3% of total organic toxicant uptake per species respectively (Nichols et al., 1996) and in laboratory experiments the importance of the skin in organic toxicant uptake diminished with increasing body size (McKim et al., 1996). The role of the skin with respect to inorganic toxicant uptake is not known, although the gill and the gut are considered the main uptake routes. However, several studies indicate that fish skin is very sensitive and responds strongly to a variety of heavy metals (Iger et al.,

1994a, c, d; Stouthart et al., 1995; Berntssen et al., 1997). Many of these responses can be observed in the tissue for up to 30 days. The active uptake and transport of particulate antigens by the skin is significant in *O. mykiss* and particles can still be detected in the skin at 24 days after immersion immunisation (Moore et al., 1998). These studies show that the skin, like the gill, is a dynamic organ, yet one where the functional implications of many of the responses to stressors have not been studied. Studying the responses of skin to stressors might yield suitable parameters for use as early warning signals for water pollutants and for other types of stressors. Therefore, the main objectives of this thesis were to investigate the epidermal responses associated with stressors (including pollutants), to study the function of cortisol in the regulation and induction of these responses, and to assess the usefulness of skin as an indicator of stress.

Outline and scope of this thesis

The overall emphasis is on the responses of the skin epidermis to toxic (water from the lower river Rhine) and non-toxic stressors (fish lice), and the role of cortisol in these responses. To provide a broad picture of the consequences of the effects on the whole animal, both skin and gill epithelia, as well as several other parameters including hydromineral balance and occasionally, immune capacity, have been studied in the *in vivo* experiments.

The first part of this thesis (Chapters 2-4) concerns a series of whole animal studies on the effects of environmentally relevant stressors (water from the lower river Rhine, acute temperature shock, ectoparasitic fish lice including the insidious caligid salmon louse *Lepeophtheirus salmonis*). To investigate the role of the primary stress hormone cortisol in mediating these stress-related effects, the second part of the thesis (Chapters 5 and 6) utilised low-level stress free *in vivo* cortisol administration via the food to analyse the function of this hormone in mediation of the stress-related effects. Infection with the fish louse *Argulus japonicus* was used as a stress model, as the host-parasite relationship takes place at the upper epidermal cells in the skin. Following from this, the third part (Chapters 7 and 8) utilised *in vitro* methodology to study the role of cortisol in mediating stress-related cellular responses in the skin. In this way, the influences of *in vivo* endogenous factors that may complicate the situation in the intact animal were removed. In addition, a primary skin explant culture system for future long-term *in vitro* research was developed and characterised.

- In Chapter 2, the additive effects of exposure to Rhine water and short-term acute temperature increase (+ 7°C for 3 h) were studied on the skin, gill and osmoregulation of the native sea trout smolt, *Salmo trutta*. This long-term study included histological examination of the skin and gills and determination of osmoregulatory parameters to evaluate the full impact of the stressors on the fish.

- In Chapter 3, an experiment was designed and carried out to measure the stress parameters reflecting the activity of the hypothalamo-pituitary-interrenal and brain-sympathetic axes (plasma cortisol and glucose) of rainbow trout in response to continuous exposure to Rhine water. The effects associated with chronic stress on growth and immune function are reported.
- In Chapter 4, experimental infection with low numbers of the salmon louse *L. salmonis* was used to examine whether this non-toxic stressor would induce similar responses to those reported with toxic stressors, in order to identify which stress-related responses might be endogenously mediated. Both skin and gill epithelia were studied, together with the osmoregulatory consequences of the infection.
- In Chapter 5, stress free administration of low levels of cortisol via the food was used to see whether beneficial responses could be induced in *O. mykiss* when subsequently the fish were infected with low numbers of the fish louse *A. japonicus*. In this study, the glucocorticoid receptor was demonstrated and localized in the skin, providing evidence of where cortisol may be mediating its effects.
- In Chapter 6, we examined the influence of cortisol, using similar methodology, on the skin of very small (< 1 gram) carp *Cyprinus carpio* in relation to infection with juvenile *A. japonicus.* Apoptotic and proliferating cells in the epidermis were quantified and the cortisol receptor localized to indicate which cells might be responsive to the hormone.
- In Chapter 7, a 24 hour *in vitro* incubation method with 5 concentrations of cortisol was used to investigate whether mucous, mitotic and apoptotic cells in the skin epidermis were stimulated by cortisol and to see whether responses were different between high and low doses. Furthermore, using the glucocorticoid receptor blocker mifepristone, we tried to identify which processes are mediated via the glucocorticoid receptor.
- In Chapter 8, a primary explant culture system for skin of *O. mykiss* was characterised in terms of growth, proliferation and apoptosis, in order to provide a culture system to study effects on actively growing skin epidermal cells in the longer term (to 8 days).

Chapter 2

Acute temperature elevation in tap and Rhine water affects skin and gill epithelia, hydromineral balance and gill Na^+/K^+-ATPase activity of sea trout smolts (*Salmo trutta* L.)

D.T Nolan, R.H. Hadderingh[1], F.A.T. Spaning, H.A. Jenner[2] and S.E. Wendelaar Bonga

[1]) KEMA Environmental Services, KEMA, Utrechtsweg 310, 6812 AR Arnhem, The Netherlands.

[2]) KEMA Power Generation, P.O. Box 9035, NL-6800 ET Arnhem, The Netherlands.

Canadian Journal of Fisheries & Aquatic Sciences (2000). 57:708-718

Abstract

The effects of a 3 h temperature elevation of 7°C were studied for 29 d on the sea trout *Salmo trutta* smolt in tap water and in water from the lower Rhine. The effects in the skin were apparent at 3 h and included depletion of electron dense vesicles and increased numbers of heavily stained desmosomes in the filament cells of the upper epidermis. Increased levels of apoptosis and necrosis occurred and were associated with leukocyte infiltration of the epidermis. Similar stress-related effects in the gill epithelium were mainly confined to the chloride cells. Highest levels of necrosis in skin and gill epithelia occurred in fish that were temperature-shocked in Rhine water. Effects of exposure to Rhine water alone were intermediate between those of temperature shock in tap water and in Rhine water. At 29 d, recovery was good in tap water, partial in Rhine water and poor for the fish temperature shocked in Rhine water. Although disruption of hydromineral balance was not indicated in plasma electrolytes, specific Na^+/K^+-ATPase activities in the gills were higher for all treatments at 24 h and for the groups temperature shocked in Rhine water at 8 d. Overall, temperature shock in Rhine water gives additive stress effects and poor recovery at 29 d.

Introduction

Most fishes are ectotherms (i.e. having a primarily external source of body heat), and as such, the environmental temperature dictates their body temperature. Because the heat capacity of water is a factor of 3000 higher than that of air, thermal conductance of water is also higher, and therefore fish are greatly influenced by the effects of increased temperature. Temperature affects all levels of biological organization and considerable literature exists reporting the effects of temperature on fishes (reviewed by Hazel, 1993 and Goldspink, 1995). The impact of short-term acute increases in temperature is a serious concern for fish populations and migrating salmonids are especially sensitive to acute temperature increases (Coutant, 1973; Cherry et al., 1975; Gray, 1990). Although the role of acute temperature shock was studied in a number of laboratory experiments, the effects of thermal discharges on free-moving juvenile salmonids were not determined (Gray, 1990).

Thermal discharges from anthropogenic water use (e.g. cooling waters for industry) threatens fish both directly, through temperature shock (Coutant, 1973; Cherry et al., 1975; Gray, 1990) and impingement at intake sites (Hadderingh et al., 1983), as well as indirectly through interactive effects with pollutants (Reid et al., 1997) and alterations to thermal microhabitats (Spigarelli and Thommes, 1979; Haynes et al., 1989). Thus, temperature shock alone may be considered a physical stressor, which in combination with chemical stressors, such as pollutants, may increase the toxicity. This can occur either by temperature directly affecting the physicochemical properties of the pollutants or by the additive effects of temperature stress and pollution stress.

The Rhine, the largest and most important European river, has been affected by a variety of anthropogenic factors, including both thermal and xenobiotic pollution (for overviews, see Friedrich and Muller, 1984; Van Dijk et al., 1995). The Rhine had self-sustaining salmon populations historically but these have dramatically declined (Cazemier, 1994; Van Dijk et al., 1995). Because salmonids have high water quality requirements and the anadromous lifestyle results in the utilization of the whole river system, the Atlantic salmon (*Salmo salar*) was chosen as the indicator species whose successful re-establishment would confirm the improved water quality and health of both the Rhine and its catchment.

Recent international negotiations have targeted improving the quality of the water by agreements on reducing levels of contaminated discharges into the river (Schulte-Wülwer-Leidig, 1994). However, thermal pollution is a stress factor that is often ignored and there is a thermal gradient from the upper to the lower Rhine, resulting from the utilization of river water for cooling water by industry. Migrating salmonids encounter both a thermal gradient and a number of thermal plumes in the river. In the Dutch part of the lower Rhine, cooling waters are discharged by industry at 7°C above intake temperature. A single acute 3 h temperature shock of + 7°C induces prolonged stress-related effects in the skin of freshwater rainbow trout, *O. mykiss* (Iger et al., 1994d), but it is not yet known to

what extent this may disrupt other functions, such as hydromineral balance or gill function in fresh water.

While Atlantic salmon were absent from the Rhine for many years, the sea trout (anadromous *Salmo trutta*) has always been present in some numbers (Cazemier, 1994). Trout skin (*O. mykiss* and *S. trutta*) responds to Rhine water with a number of ultrastructural changes very similar to those shown by fish stressed under laboratory conditions (Iger et al., 1994d, 1995), suggesting that salmonid populations in the river system may be stressed (Iger et al., 1994c; Nolan et al., 1998). Many of the changes reported in the skin of the fish are mediated by cortisol (Iger et al., 1995), which is the main corticosteroid in teleosts and is known to be hypersecreted during stress (Wendelaar Bonga, 1997). Together, these data indicate that exposure to present day Rhine water evokes a stress response, at least at the level of the skin epithelium. Any interactions between temperature and the pollutants in Rhine water have not been reported to date.

In the present study, we exposed native sea trout smolts (*S. trutta*) to a single acute 3 h temperature shock of +7°C in both tap water and in water from the lower Rhine. Fish were sampled at 3 h and 1, 8, 18, 22 and 29 d in order to evaluate the effect of a short-term acute temperature shock, as would be naturally experienced during their migration through a single thermal plume. The sample points were chosen based on the results from previous studies with *O. mykiss* and *S. trutta* (Iger et al., 1994c, d, 1995; Nolan et al., 1998). The fish were continuously exposed to water from the lower Rhine during the final stages of smoltification, which is the time that they are exposed to this river section in nature. In this way, we studied the effects of a temperature shock (a physical stressor) and Rhine water (a chemical stressor) on the sea trout smolt. We looked for interactive effects of temperature with Rhine water by combining the two types of stressor. Because skin and gills are affected by toxicant exposure, we combined ultrastructural analysis of skin and gills, as well as by examination of plasma ions and gill Na^+/K^+-ATPase activity. These parameters were validated as stress parameters connected with toxicity in earlier papers by our (Wendelaar Bonga and van der Meij, 1989; Iger et al., 1994c, d, 1995; Nolan et al., 1998) and other groups (Jagoe et al., 1996a, b; Burkhardt-Holm et al., 1997; Kakuta and Murachi, 1997; Lionetto et al., 1998).

Materials and Methods

Experimental set-up

Pre-smolt sea trout (weight 39.37 ± 6.16 g, fork length 15.55 ± 1.02 cm, n = 10) bred from a sea trout population naturally migrating from the North Sea into streams in Schleswig Holstein, Germany were obtained in spring. The fish were placed in groups of 30 in 400 L black plastic tanks in running non-chlorinated tap water with a flow-through rate of 600 $L \cdot h^{-1}$. Each tank was strongly aerated by air compressor via an airstone, and mechanically filtered by an Eheim 2213 external power filter, increasing circulation by a further 440 $L \cdot h^{-1}$. The fish were fed a standard pelleted trout diet at the rate of 1% of body weight daily. Water temperature and oxygen saturation levels were measured in all tanks daily. Lighting was adjusted periodically during the experiment to match the natural photoperiod (February - April) and windows in the laboratory allowed the fish to perceive the natural changes in photoperiod.

After three weeks acclimation, the water supply to 4 groups was changed from tap water to Rhine water (referred to as RW). This was achieved with minimum disturbance to the groups by changing the inlet water supply pipe to these tanks from tap water to Rhine water at source. The Rhine water was pumped up at KEMA (near Arnhem, the Netherlands) and filtered by a lamellar filter system in a sediment chamber to remove particles larger than 0.2 mm (Iger et al. 1994b). The composition for some of the major contaminants in the Rhine water during the exposure is given in Table 1 from data measured by RIZA near Lobith, The Netherlands during the experimental period. Two groups of fish remained in tapwater as controls (TW). The water temperature in two tap water and two RW groups was then increased to 7°C above ambient for 3 h (referred to as TW&T and RW&T, respectively). This temperature shock was delivered by shutting off the flow-through system and raising the water temperature over a 30-50 min period using heating coils, until 7°C elevation was achieved (Iger et al., 1994d). The groups, which were not temperature-shocked, were sham treated by shutting off the flow-through systems for the same duration as the other groups. During these periods, the oxygen saturation levels of the tanks were continuously monitored and no decrease in oxygen level were recorded, as the tanks were continuously aerated and recirculated throughout. As the TW and RW water temperatures were different (9 and 12°C respectively), the temperature shock increased water temperatures to 16 and 19°C respectively. The temperatures in the sham treated tanks were also monitored and remained stable over the period, as the laboratory was not heated. These temperatures were monitored and maintained for a 3 h period, after which the flow through system was re-opened, and the temperature returned to initial values within 60 minutes. The fourth treatment was comprised of two groups maintained continuously in Rhine water (RW).

The temperature profile for both the RW and the TW was measured daily. RW temperature was higher than TW in the beginning and both increased over time (TW was 8°C at the beginning of the experiment and 11°C at the end, while RW was 12 and 17°C respectively). Dissolved oxygen content of the water was checked daily, and varied between 8.7-11.5 mg· L^{-1} in tap water and 8.7-10.7 mg· L^{-1} in Rhine water during the experimental period.

Sampling

Fish were sampled at 3 h and at 1, 8, 18, 22 and 29 d after the beginning of the treatments. All sampling took place first thing in the morning before feeding. Four fish per treatment were sampled at each time point. Replicate tanks were sampled alternately on consecutive sample days, such that no tank was disturbed twice within the same week, and data are from replicate treatment tanks.

At sampling, skin for transmission electron microscopy (TEM) and light microscopy (LM) was sampled first. Blood was withdrawn by needle into Na^+-heparinized syringes from the caudal blood vessels. The fish were killed by spinal transection at the base of the skull, and length and weight data were collected to calculate condition factors for each fish.

Sample processing

Heparinized blood was spun for 3 min in a bench centrifuge at 13,000 r.p.m., plasma was pipetted off, aliquoted and stored at -20°C. For osmolarity and ion analysis a Roebling cryoscopic micro-osmometer and a flame photometer (Technikon model IV auto-analyzer) were used. For determination of Na^+/K^+-ATPase activity, gill filaments were trimmed from the gill arches, rinsed and placed in ice-cold buffer (0.3 M sucrose, 20 mM Na_2EDTA, 0.1 mM imidazole, pH 7.1 with HCl), frozen immediately in liquid nitrogen and stored at -80°C until analysis. The specific Na^+/K^+-ATPase activity was determined in a H_0 homogenate fraction by the method routinely used in our laboratory (Flik et al., 1984).

Table 1. Chemical parameters measured in Rhine water at Lobith during the experimental exposure of the sea trout smolts (*Salmo trutta*).

Parameter	Units	Rhine water range during experiment	Tap water values
General parameters			
PH		7.62-7.91	8.14
O_2	mg· L^{-1}	9.8-11.1	4.9
NO_2^-	µg· L^{-1}	50.0-70.0	< 20.0
NH_4^+	µg· L^{-1}	100-180	< 50
PO_4^-	µg· L^{-1}	48-67.0	< 30.0
Heavy metals			
Al	µg· L^{-1}	211.0	< 5.0
Ba	µg· L^{-1}	88.0	20.5
Fe	µg· L^{-1}	1270-1980	30
Cd	µg· L^{-1}	0.05-0.11	< 0.02
Cr	µg· L^{-1}	4.2-7.5	< 1.0
Cu	µg· L^{-1}	6.0-8.2	< 5.0
Hg	µg· L^{-1}	0.02-0.03	< 0.05
Mn	µg· L^{-1}	97.0	< 10.0
Ni	µg· L^{-1}	4.1-4.7	< 5.0
Pb	µg· L^{-1}	3.8-9.6	< 5.0
Zn	µg· L^{-1}	30.0-105.0	< 5.0
Other ions			
Ca^{2+}	mg· L^{-1}	67-77.0	34.0
Cl^-	mg· L^{-1}	78-114.0	24.0
K^+	mg· L^{-1}	4.4-5.1	2.4
Na^+	mg· L^{-1}	46-67.0	15.6

For TEM, samples were taken at 3 h, 1 and 29 d only, to study immediate effects and long-term effects. From each fish, a piece (2x3 mm) of skin from the scaleless dorsal part of the head, along with a small piece of the first gill arch, were fixed in 3% glutaraldehyde buffered in sodium cacodylate (0.09 M, pH 7.3) on ice for between 15-30 min and post-fixed for 1 h on ice with 1% osmium tetroxide and 2.5% potassium bichromate in the same buffer (Wendelaar Bonga and van der Meij, 1989). Ethanol-dehydrated tissues were embedded in Spurr's resin, after which ultra-thin sections of skin and gill were cut by diamond knife and collected on a 150 mesh copper grid. Four grids per sample were prepared; these were stained with uranyl acetate and lead citrate before viewing in a Philips EM 300 transmission electron microscope at 40 kV. Overall condition of the epithelia was examined and 10 representative views per specimen were photographed without knowledge of the treatment groups involved.

In the skin, five areas from the upper epidermis of each fish were scanned and photographed at low magnification; the negatives were enlarged 4 times and printed. From these micrographs, numbers of electron dense vesicles (EDV) and electron dense desmosomes were quantified in 7-10 filament cells per fish (each filament cell representing a cytoplasmic area of circa 250 μm^2), and averaged to give mean values per fish. Care was taken to include data only from mature filament cells with a visible nucleus, which were neither necrotic nor apoptotic. Overall ultrastructure of the epidermis was examined, and particular attention was paid to cellular necrosis (characterized by nuclei with aggregations of chromatin, swelling of the cytoplasmic compartment and loss of apical microridges) and apoptosis (characterized by progressive densification of the nucleus, organelles and cytoplasm, leading to shrinkage and loss of contacts with surrounding cells), leukocyte infiltration and epithelial integrity (evaluated by intercellular swelling and cell-cell contacts). For gill specimens, attention was focused on the interlamellar areas of the filaments where most of the chloride cells (CC) are located. Necrosis, apoptosis (characterized for chloride cells by progressive densification of the mitochondria, nucleus and cytoplasm, as well as dilation of the tubular system) and leukocyte infiltration were also evaluated. From the micrographs, the tissues were evaluated and scored semi-quantitatively against TW controls using a graded evaluation system (-, +, ++, +++, ++++) to represent incidence of each parameter.

For LM, skin samples (5 x 5 mm) were fixed in Bouin's fixative and processed to 5 µm sections by conventional methods. Then replicate series of sections were stained by either Periodic Acid Schiff's or Alcian Blue (pH 2.5), to identify total and acidophilic mucous cells respectively. The numbers of mucous cells were quantified using a calibrated brightfield microscope as described in Chapter 4.

Data handling and statistics

For statistical analysis, raw data (except condition factor and Na:Cl ratio) were log transformed and effects of treatments were analyzed by one-way ANOVA at each sample point. Tukey-Kramer Multiple Comparisons Test was used to assess the differences between treatments. For analysis of control values of gill Na^+/K^+-ATPase activity over time, one-way ANOVA of log transformed data was applied and trend analysis of raw data to 18 d was used to investigate the presence of a linear trend. For condition factor and Na:Cl ratio, differences between controls and treatments, as well as controls over time were assessed with the Mann-Whitney *U* test. In each case, data are presented as means ± SEM for a sample size of $n = 4$. Statistical significance was accepted at $P < 0.05$.

Results

Fish health and smolt status

Specific gill Na^+/K^+-ATPase activity and condition factor of control fish were used to indicate smolt status. Gill Na^+/K^+-ATPase activity of TW fish was higher after 18 d, indicated by trend analysis (slope 2.471; $r^2 = 0.4732$; $F = 7.876$; $P = 0.0038$). The condition factor decreased from this point (Figure 1). Condition factors of the different treatments did not differ from TW fish at any point. All fish fed well throughout the period, and remained in good condition and disease free. Overall mortality was less than 1.5%, evenly distributed over all treatments.

Plasma ions

Neither plasma osmolality, sodium nor chloride were significantly different between treatment groups at any sample point and were in the range considered normal for freshwater salmonids (data not shown).

Gill Na^+/K^+-ATPase activity

Specific gill Na^+/K^+-ATPase activity (Table 2) was unaffected by the treatments at 3 h but was higher than controls in TW&T, RW and RW&T groups at 24 h. Specific enzyme activity of the RW&T treatment was also significantly higher than controls at 8 d and significantly lower at 18 d. Na^+/K^+-ATPase activity was similar in all treatment groups at 22 and 29 d.

Light microscopy of skin

Total numbers of mucous cells and acidophilic mucous cells were not significantly different between treatment groups at any sample point (data not shown).

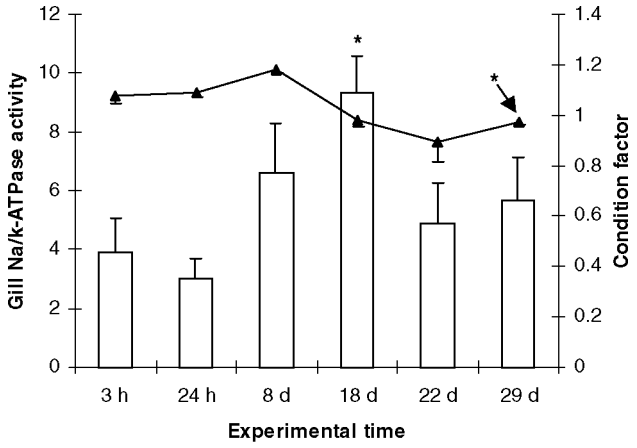


Figure 1. Gill Na^+/K^+-ATPase activity and condition factor profiles for control sea trout (*Salmo trutta*) smolts maintained in tap water during the experimental period. The bars are the enzyme activity (given as µmol P_i· mg $protein^{-1}$· h^{-1}) and refer to the left axis; the line is condition factor and refers to the right axis. * = $P < 0.05$ compared with 3 and 24 h values.

Electron microscopy of skin

The appearance of the skin of TW control smolts agreed with that described previously for trout (Iger et al., 1994c; Burkhardt-Holm et al., 1997; Nolan et al., 1998). The uppermost layer of cells was differentiated into specialized pavement cells that have apical microridges (Figure 2A). Many microfilament-containing cells below the pavement cell layer were highly active, synthesizing electron dense vesicles (EDV) (Figure 2A). These cells were interconnected by desmosomes, with little intercellular spacing (Table 3; Figure 2A). Occasional sloughing of pavement cells was observed, and necrotic and apoptotic cells were seldom seen in the inner cell layers (Table 3). At 29d, the epidermal structure of TW control fish was similar, but for the reduction in the numbers of EDVs to minimal values (Figures 3A & 4).

TW&T resulted in disruption of the pavement and filament cells at 3 h. Sloughing necrotic pavement cells, characterized by nuclei with aggregations of chromatin, swelling of the cytoplasmic compartment and loss of apical microridges, were frequently observed (Figure 2B). Populations of EDVs in the filament cells were reduced at 3 and 24 h post temperature shock (Figure 3A). The number of desmosomes per filament increased at 3 and 24 h (Figure 3B). Leukocytes and macrophages were more frequently encountered than in TW controls (Table 3). At 29 d, the epidermal condition of the TW&T fish had improved, although intercellular spaces still occurred (Figure 2C; Table 3). Numbers of filament cell EDVs were similar to TW controls (Figure 4), while numbers of desmosomes remained elevated (Figure 4). At 29 d, lymphocytes and macrophage presence was comparable with TW controls (Table 3).

Exposure to Rhine water increased apoptosis and necrosis in the filament cell and pavement cell layers within 3 h. Necrosis of the pavement cells, characterized by swollen organelles and cytoplasm, and subsequent membrane rupture, was a prominent feature (Figure 2D). Numbers of EDVs in the inner filament cells were reduced at 3 and 24 h (Figure 3A). Intercellular spaces opened up at 3 h (Figure 2D) and numbers of filament cell desmosomes were higher than in TW fish at 3 and 24 h (Figure 3B). Macrophages and lymphocytes were commonly found throughout the epidermis from 24 h onwards. At

29 d, the epidermis of RW fish showed slight improvement (Table 3). The EDVs in the filament cells were virtually absent, and large areas of epithelial disruption were evident. Sloughing pavement cells and apoptosis in the inner cell layers were commonly seen (Table 3). Numbers of electron dense desmosomes per filament cell were similar to TW controls at 29 d (Figure 4). Macrophages and lymphocytes were less frequently observed than in the earlier stages, but were more abundant than in control fish (Table 3).

RW&T increased the incidence of apoptosis and necrosis of pavement and filament cells. Both types of cell death were seen at 3 h and intercellular spaces were present (Figure 2E). Populations of EDVs in the filament cells were reduced compared with TW controls at 3 and 24 h (Figure 3a). Numbers of filament cell desmosomes were higher at 3 and 24 h (Figure 3B). Lymphocytes and macrophages were frequently seen. At 29 d, the epidermis of RW&T smolts was not recovered (Table 3). Necrotic pavement cells were common and intercellular spaces still occurred (Figure 2F). At 29 d, filament cell EDV numbers were similar to TW controls, while numbers of desmosomes were higher (Figure 4). Macrophages and lymphocytes were commonly seen throughout the epidermis (Table 3).

Electron microscopy of gill

The gill of the FW *S. trutta* smolt is composed of a series of filaments with pairs of lamellae branching alternately off on both sides. The branchial epithelium is comprised primarily of squamous epithelial cells, similar to the pavement cells of the epidermis, as well as mucous cells and CCs. Under control conditions, populations of CCs are located mainly in the interlamellar epithelium of the filament (Figure 5A). Lymphocytes and macrophages are especially common in the gill filaments of sea trout (Table 3).

TW&T fish gills were not dramatically affected by the treatment. Some disruption of the epithelia was evident from intercellular spaces and infiltration by macrophages and lymphocytes (Figure 5B). Although some swollen, necrotic chloride cells were observed, apoptotic CCs were uncommon. At 29 d, the gills were recovered and the structure was comparable to TW control fish (Table 3).

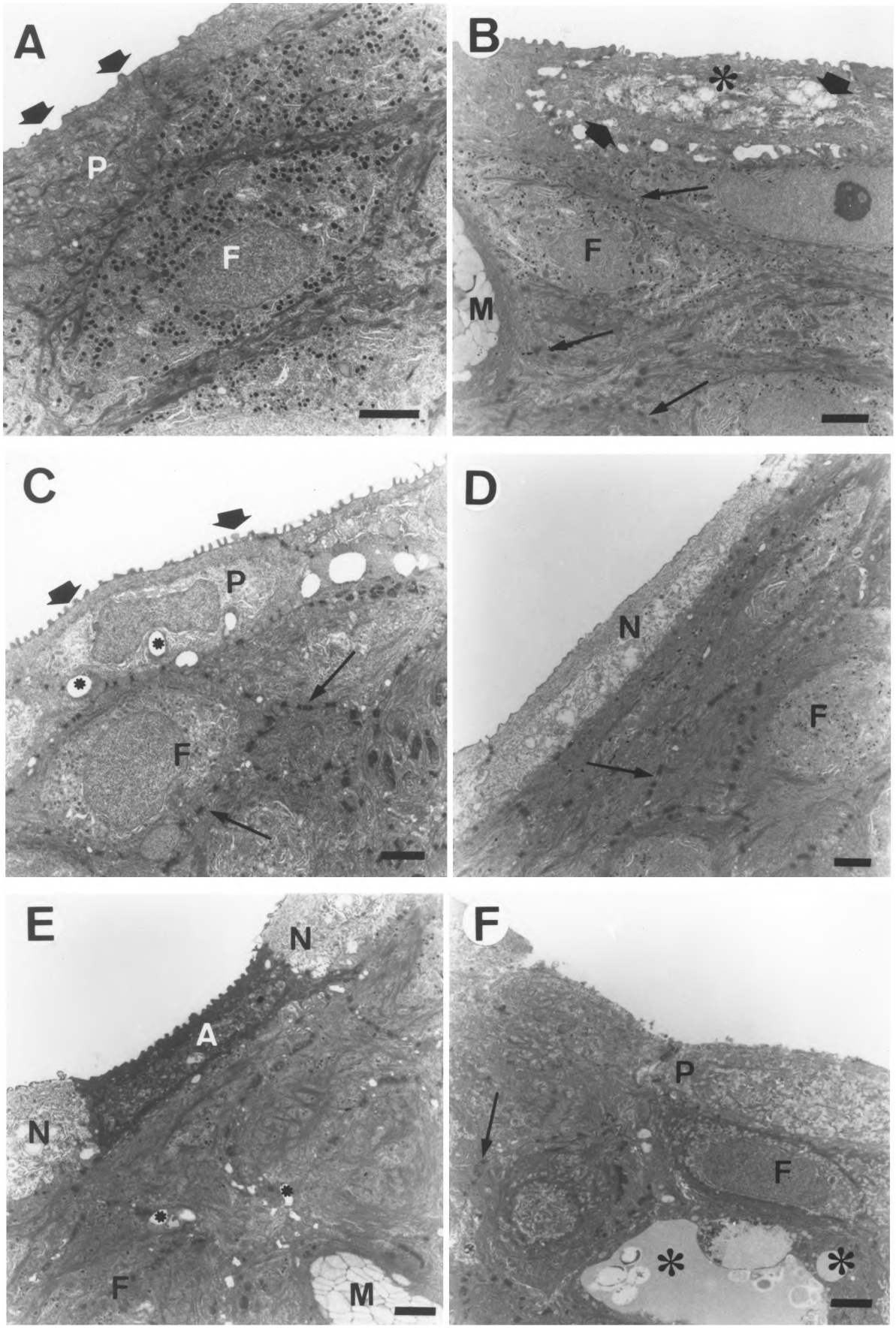


Figure 2. Electron microscopy of head skin from *Salmo trutta* smolts. Comparisons of treatments are made with TW control fish. (A) Control fish at 3 h. Pavement cells (**P**) have microridges at apical side (arrowheads) and the epithelium has good structural integrity. Filament cells (**F**) contain many electron dense vesicles. Scale bar = 5 µm (B) 3 h +7 °C temperature shock in TW. Detaching pavement cells (*) with swollen, electron lucent organelles (arrowheads) and shrinkage are characteristic. The overall structural integrity is good, although numbers of filament cells (**F**) vesicles are

lower then in unshocked fish. Heavily stained desmosomes are characteristic (arrows). **m**; mucous cell. Scale bar = 5 µm (C) 29 d post-3 h +7 °C temperature shock in tap water. Pavement cells (**P**) with basal intercellular vacuolation (*) and good apical microridge structures (arrowheads) are characteristic and the epithelial structural integrity is good. Few electron dense vesicles in the filament cells (**F**) are present and heavily stained desmosomes are clearly seen (arrows). Scale bar = 5 µm (D) 3 h in Rhine water. The pavement cell layer is necrotic (**N**) and swollen, with electron lucent organelles and without apical microridges. The epithelial integrity is good but intercellular spaces are opening up and filament cell (**F**) electron dense vesicles are depleted. Heavily stained desmosomes are very obvious (arrow). Scale bar = 5 µm (E) 3 h +7 °C temperature shock in Rhine water. The pavement cells are necrotic (**N**) and apoptotic (**A**) and the epithelial structural integrity is compromised, as intercellular spaces open up (*). Filament cell (**F**) electron dense vesicles are greatly depleted and heavily stained desmosomes are clearly seen. **M**; mucous cell. Scale bar = 5 µm (F) 29 d after 3 h +7 °C temperature shock in Rhine water. The pavement cells (**P**) are swollen contain many electron lucent vesicles. Epithelial structural integrity is compromised with large intercellular spaces present (*). Filament cell (**F**) electron dense vesicles are barely discernible and heavily stained desmosomes are present (arrow). Scale bar = 5 µm.

Exposure to RW resulted in alterations in the gill epithelia within 3 h (Figure 5C). Intercellular spaces had opened up and were invaded by many macrophages and lymphocytes (Figure 5C, Table 3). Necrosis, or sloughing of the superficial epithelial cells, was not observed. Necrotic chloride cells, characterized by swollen organelles and cytoplasm, as well as aggregated chromatin, were commonly seen (Figure 5C). Apoptotic CCs, identified by progressive densification of the cytoplasm and organelles, and dilation of the tubular system, were frequently observed. Many leukocytes contained apoptotic fragments and lysosomes containing cell debris (Figure 5C). Some CCs appeared normal at the apical pole, but atrophied at the basal pole, they appeared to have lost connections with surrounding cells, and were frequently associated with macrophages at this time (Figure 5C). At 29 d in RW, the condition of the gills in these fish was improved (Table 3). There was a reduction in intercellular spaces, although atrophying CCs were still common (Figure 5D).

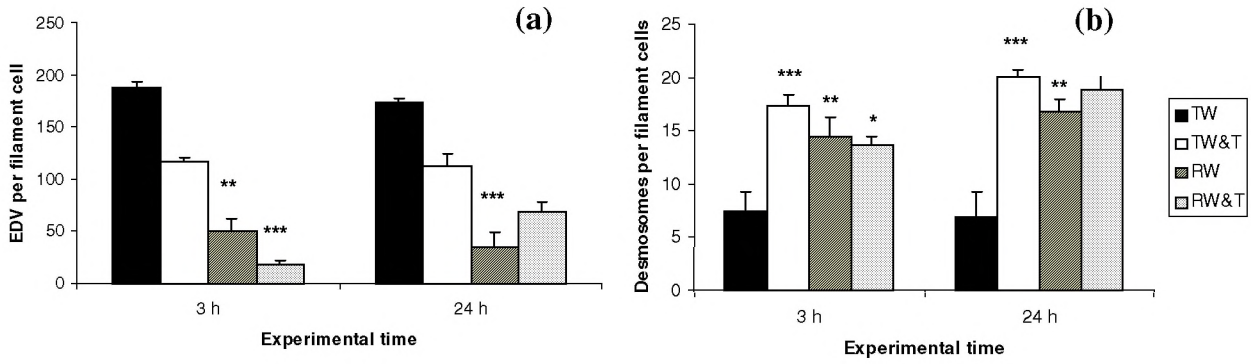


Figure 3. (A) Numbers of electron dense vesicles per filament cell from the head skin of sea trout (*Salmo trutta*) smolts at 3 and 24 h post-treatments. (B) Numbers of electron dense desmosomes per filament cell from the head skin of sea trout (*S. trutta*) smolts at 3 and 24 h post-treatments. The solid bars are the tap water control values at 3 h, the fine hatched after a 3 h +7°C temperature shock in tap water, the coarse hatched after Rhine water, the stippled after a 3 h +7°C temperature shock in Rhine water. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ compared with TW control values.

Table 2. Gill Na^+/K^+-ATPase activity (µmol $P_i \cdot$ mg $protein^{-1} \cdot h^{-1}$) of sea trout (*Salmo trutta*) smolts in tap water (TW), and following exposure to Rhine water (RW), a single 3h +7°C temperature shock in tap water (TW&T), or the combination of the two (RW&T). Data are presented as means ± S.E.M. for a sample size $n = 4$. * = significantly different from TW control at $P < 0.05$, ** = significantly different from TW control at $P < 0.01$.

Sampling point	TW	TW&T	RW	RW&T
3 h	3.9±1.1	4.9±1.2	5.8±0.9	8.4±1.5
24 h	3.0±0.7	7.9±0.7**	8.1±1.6**	8.5±1.3**
8 d	6.6±1.7	6.7±1.0	8.3±1.4	14.5±2.7*
18 d	9.3±1.3	10.5±2.3	7.8±0.9	2.3±1.3**
22 d	4.9±1.4	10.1±2.5	6.7±1.0	5.6±0.7
29 d	5.7±1.4	6.2±2.9	4.2±1.4	3.4±0.3

RW&T treatment considerably disrupted the epithelial integrity. At 3 h, substantial intercellular spaces had opened up (Figure 5E) and were infiltrated by lymphocytes and macrophages (Table 3). Necrotic chloride cells, characterized by swollen organelles and cytoplasm, as well as aggregated chromatin, were commonly seen at 3 h, at 24 h and 29 d. The swollen CCs were clearly necrotic and showed ruptured membranes and cytoplasmic leakage at late stage (Figure 5F). Apoptotic and atrophied CCs were common and persisted through to 29 d. Lymphocytes and macrophages remained common at 29 d. The condition of the gill of the RW&T fish at this time was poor (Table 3).

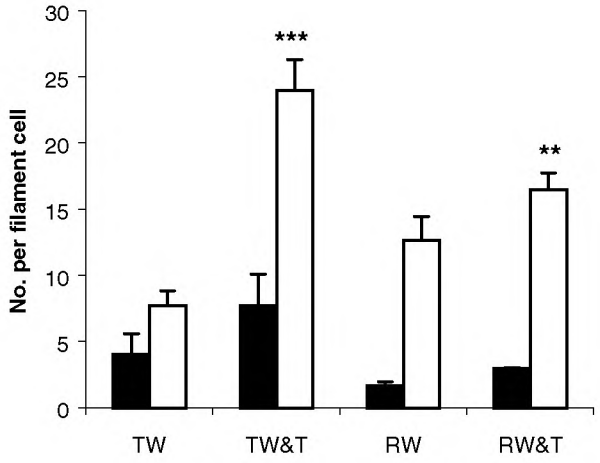


Figure 4. Numbers of electron dense vesicles and desmosomes per filament cell from the head skin of sea trout (*Salmo trutta*) smolts at 29 d post-treatments. The solid bars are electron dense vesicles while the white bars are desmosomes. ** = $P < 0.01$, *** = $P < 0.001$ compared with TW control values.

Discussion

The results of this study show that short-term, sub-lethal and acute temperature shocks, approximating the actual situation encountered by migrating salmonids in nature, induced effects in the sea trout smolt which are considered stressful and which may have deleterious effects on the health status of the fish in nature. The behavioral effects of acute heat shock are severe and were reported in terms of increased susceptibility to predation in both stenothermic salmonids (Coutant, 1973) and eurythermal cyprinids (Webb and Zhang, 1994), indicating that temperature shock effects are severe for both thermosensitive and thermotolerant species alike. In the present study, we demonstrated the effects of acute temperature shock on the skin and gill epithelia, and gill Na^+/K^+-ATPase activity at 24 h after and to 29 d post-treatment. For migrating smolts in nature, we speculate that these effects could lead to increased disease susceptibility or difficulties during seawater adaptation.

The effects of temperature elevation on the gill in TW were less profound than effects on the skin. Both tissues recovered reasonably well within the 29 d study period. Previously, prolonged effects over 14 d were reported for a comparable temperature treatment in the skin of the rainbow trout (Iger et al., 1994d). The present study showed a similar response in the skin and gill epithelia of the sea trout smolt and further indicated biochemical gill responses in terms of increased gill Na^+/K^+-ATPase activity, while hydromineral balance was maintained. There are few studies where epithelial integrity, ion-transporting ATPases and plasma electrolytes were studied together, but the results corroborate our other studies where we report changes in Na^+/K^+-ATPase without electrolyte disturbance, e.g. after ectoparasitic infection of Atlantic salmon, *S. salar*, in sea water (Chapter 4), as well as confinement stress in the tilapia *Oreochromis mossambicus* in both fresh water and sea water (Nolan et al., 1999) and in lice-exposed *O. mykiss* in sea water (Nolan et al., 2000). Many freshwater stressors induce ionoregulatory disturbances that, eventually, may result in a decrease in plasma electrolyte levels (Wendelaar Bonga, 1997). The results of the current study point to the successful adaptation of the ionoregulatory mechanisms of the gills in response to an ionoregulatory challenge associated with the temperature shock, even when ultrastructure indicates poor condition. The transient changes in the gill ATPase seen here may reflect adjustments in ionoregulatory function, but may also reflect the energetic costs of extensive apoptosis of the chloride cells (an energy dependent process indicating increased ageing of the cell population) and their replacement by newly differentiated cells. Similar effects were reported in seawater adapted post-smolt *S. salar* experimentally infected with the sea louse *Lepeophtheirus salmonis* (Chapter 4). Here, measurements of plasma Na^+ and Cl^- indicated little effect on ionoregulation, while disrupted skin and gill epithelia suggested otherwise. This was

Table 3. Ultrastructural effects in the skin and gill epithelia of sea trout (*Salmo trutta*) smolts in tap water (TW), following exposure to Rhine water (RW), a single 3h +7°C temperature shock in tap water (TW&T), or the combination of the two (RW&T). The semi-quantitative evaluation is expressed as - = unaffected to ++++ = most strongly affected.

Skin parameter	TW	TW&T	RW	RW&T
Overall apoptosis	+	++	+++	+++
Overall necrosis	+	++	+++	+++
Intercellular spaces	-	+	++	+++
Electron dense vesicles	+++	++	+	+
Filament cell desmosomes	+	+++	+++	+++
Macrophage/leukocyte infiltration	+	++	+++	+++
Epidermal recovery	-	good	partial	poor
Gill parameter				
Overall apoptosis	+	+	+	+
Overall necrosis	+	+	+	+
Intercellular spaces	-	+	++	+++
Chloride cell apoptosis	+	+	++	++
Chloride cell necrosis	-	+	++	+++
Chloride cell atrophy	-	-	+	++
Macrophage/leukocyte infiltration	+	++	+++	+++
Epithelial recovery	-	good	partial	poor

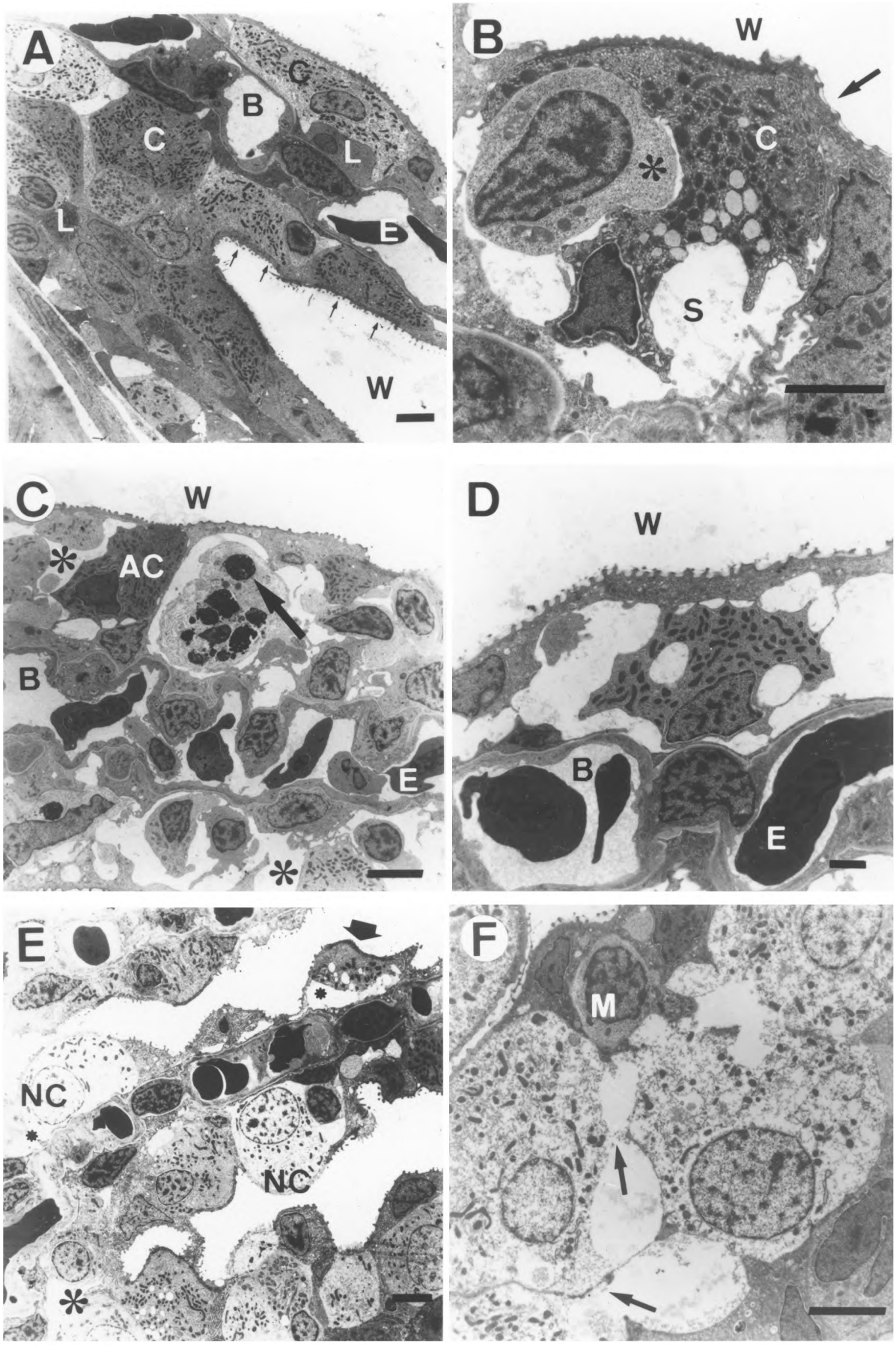


Figure 5. Electron microscopy of gills from *Salmo trutta* smolts. Comparisons of treatments are made with TW control fish. (A) Interlamellar region from TW control at 3 h sample point. Many mitochondria-rich chloride cells (**C**) are present here and the have apical microridge structures (small arrows). Epithelial integrity is good and some leukocytes (**L**) can be seen. **E**; erythrocytes; **B**, blood space; **W**, water. Scale bar = 10 μm (B) The interlamellar region at 24 h post-3 h +7

°C temperature shock in TW. A macrophage (*) engulfs an atrophying chloride cell (**C**), which is still in apical contact with the water (arrow), resulting in the development of intercellular spaces (**S**). **W**, water. Scale bar = 2 µm (C) The basal lamella after 3 h in Rhine water. The structural integrity is disrupted and intercellular, lymphatic spaces (*) occur. Atrophying chloride cells are characteristic of early apoptosis (**AC**) and macrophages contain phagocytosed material in lysosomes (arrow). **E**; erythrocytes; **B**, blood space; **W**, water. Scale bar = 10 µm (D) The lamella after 29 d in Rhine water. Atrophying chloride cells are characteristic and many of these cells have lost apical contact with the water. **e**; erythrocytes; **b**, blood space; **w**, water. Scale bar = 5 µm (E) Interlamellar 3 h +7°C temperature shock in Rhine water. The structural integrity is very disrupted and many intercellular, lymphatic spaces have opened up (*), contributing to lamellar swelling. Apoptotic (arrowhead) and necrotic (**NC**) chloride cells are characteristic. Scale bar = 10 µm (F) Necrotic chloride cells in the *S. trutta* gill 24 h after a +7°C temperature shock in Rhine water. Swollen cell and organelles, ruptured membranes, and electron lucent cytoplasm indicate chloride cells necrosis. In the late stages, cytoplasmic leakage occurs (arrows). Macrophages (**M**) are often associated with chloride cells. Scale bar = 5 µm

explained by higher gill Na^+/K^+-ATPase activity, which was associated with increased chloride cell turnover (apoptosis and necrosis), and thus hydromineral balance was maintained.

In the present experiment, there was an absolute difference in temperature of 3-5 °C between the TW and RW at the beginning and over the experimental period. This resulted from the fact that the TW originates from groundwater, while the RW is surface water. RW temperature is thus influenced by both ambient climatic factors and the many thermal discharges along its route. As these temperature differences could not be controlled during the experiment, we cannot say whether these temperature differences affected any of the parameters in the present study. There is no literature reporting the influence of such temperature differences on salmonid smolts. Gray (1990) reviewed the influence of water quality (including thermal discharges) on fish movements, migration and avoidance behavior. Natural upstream migration of sonic-tagged adult *O. tschawytscha* and *O. mykiss* in the Colombia river was not affected by thermal discharges (surface water + 0-17 °C), while juvenile *O. tschawytscha* migrating downstream avoided thermal discharges when plume temperature exceeded 9-11°C above ambient in laboratory experiments (Gray, 1990). In relation to the cellular response to temperature change, a heat shock response in fish is induced by very modest temperature increases (Iwama et al., 1998). A +4°C temperature increase resulted in the induction of stress proteins in a chinook salmon embryonic cell line (Heikkila et al., 1982). As there is a thermal gradient in the Rhine resulting from surface heating of the waters along the route and thermal discharges into the lower part of the river especially, the temperature increases experienced by the fish in RW in the present experiment are not that different to what can occur when migrating smolts pass through heavily industrialized areas.

In general, effects on hydromineral balance are a response to a variety of stressors (both real and perceived) and are brought about by the induction of an integrated stress response (Wendelaar Bonga, 1997). They are, therefore, not a stressor-specific indicator or bioindicator of toxicity *per se*. Transient increases in gill Na^+/K^+-ATPase activity in TW&T, RW&T and RW without any apparent disturbance of hydromineral balance reflects disturbance of whole animal unidirectional ionic flow rates. In a study which examined ionic flows as potential biomarkers of pollutant effects in the brook char (*Salvelinus fontinalis*), the general response to a series of metal/pH exposure combinations was increased net

sodium loss and the elimination of the net calcium inflow which occurs under normal conditions (Grippo and Dunson, 1996). In the FW-adapted rainbow trout, confinement stress for 4 or 8 h increased Na^+ and Cl^- outflow eightfold (Postlethwaite and McDonald, 1995). These observations support the view that measurement of plasma electrolytes alone are not sufficient to assess osmoregulatory disturbance and may lead to the incorrect conclusion of no effect. For such a situation, measurement of ionic flows is necessary. Increases in ion transport ATPase activities may, however, also reflect ionoregulatory disturbance (Nolan et al., 1999, 2000 and Chapter 4). From our experience, we conclude that the best method to assess effects on hydromineral balance in the absence of ion flow data is by combined plasma electrolyte and ion transporting ATPase measurements.

The effects of the treatments on the skin and gill epithelia of the sea trout smolts provided some indication of the sublethal effects that may affect migrating sea trout in the Rhine system. Our results show that temperature shocks in the form of thermal plumes in clean water (i.e. TW) compromise the integrity of the skin and gill epithelia for a considerable period and that these tissues appear to recover by 29 d post-temperature shock. However, continuous exposure to Rhine water alone resulted in incomplete recovery in both epithelia at 29 d. These results are in accordance with those reported for the skin of rainbow trout exposed for 24 d to Rhine water (Iger et al., 1994c) and brown trout (*S. trutta*) exposed to waste-water management plant effluents (Burkhardt-Holm et al., 1997). The compromised epithelia may render the fish susceptible to ionoregulatory disturbance and to secondary pathogenic infection during this time. Furthermore, increased occurrence and infiltration of the epithelia by leukocytes indicates effects on the immune system, caused by either permeation of antigens across skin and gill epithelia or as an immune response to tissue damage. Exposure to chronic stress increased disease susceptibility in fish (Pickering and Pottinger, 1989; Fevolden et al., 1994). Reduced disease resistance to challenge with *Aeromonas salmonicida* related to exposure to pollutants was reported in goldfish (*Carassius auratus*) after 30 d exposure to 5% treated sewage (Kakuta, 1997).

Significant findings in the present study are the changes in the vesicle content of the filament cells of the sea trout smolt. These secretory vesicles contained endogenous peroxidase activity (Iger et al., 1994b, d) and their synthesis is induced in rainbow trout *O. mykiss* by administration of the stress hormone cortisol (Iger et al., 1995). Our data show that the numbers of these vesicles per filament cell are more than 10 fold higher in the sea trout smolts than in the rainbow trout in the study by Iger et al. (1995). The higher density is likely to be a species difference and may be related to the smoltification process, as the amount of vesicles illustrated in the study of Burkhardt-Holm et al. (1997) in the non-smolting *S. trutta* epidermis are much lower than in our sea trout and similar to the non-smolting rainbow trout. Peroxidase is considered to be an antimicrobial component of the non-specific defense system of fish and was demonstrated in the mucus and glycocalyx on the surface of the skin (Iger et al., 1994d; Brokken et al., 1998). This secreted skin peroxidase is a biochemically distinct isoform from the

peroxidase of the blood. The significance of the enhanced secretion of peroxidase during stress is unknown at present (Brokken et al., 1998).

For the RW&T fish, the present data indicate that the combination of 3 h temperature shock and RW had the strongest effects on all parameters assessed. The significantly increased gill Na^+/K^+-ATPase activities at 24 h and 8 d post-treatment, followed by reduced gill Na^+/K^+-ATPase activity at 18 d, reflect events in the CC cell population. In studies with the tilapia *O. mossambicus* during seawater adaptation, it was shown that the functional, mature CCs degenerate during adaptation and are replaced by newly differentiated CCs (Wendelaar Bonga and van der Meij 1989). Reduced gill Na^+/K^+-ATPase activity was correlated with reduced numbers of CCs and increased levels of apoptotic CCs in the gill of SW-adapted *O. mossambicus* after confinement stress (Nolan et al., 1999).

It is not possible to say which Rhine water factors specifically potentiated the effects of the acute temperature shock and brought about prolonged degeneration of the epithelia and limited recovery. It may have been the effects of a combination of different pollutants, even at levels that individually would have no effect. Herbicide mixtures were more toxic than individual exposures to the catfish *Ictalurus punctatus* and the sunfish *Lepomis microchirus* (Abdelghani et al., 1997), while low pH increased heavy metal toxicity to the char *S. fontinalis* (Grippo and Dunson, 1996). Increased toxic effects of cadmium to the goldfish *Carassius auratus* occurred when ammonia was present (Gargiulo et al., 1996), while greater toxicity of inorganic contaminant mixtures was demonstrated in 3 endangered fish species (Buhl and Hamilton, 1996). Effects of pollutants and treatments can be additive to fish, and the present study showed that the effects of temperature shock and water from the lower river Rhine were also additive.

In conclusion, a single acute, sub-lethal temperature shock of +7°C, as legally allowed at present by European legislation, induced effects within 3 h in the native sea trout *S. trutta* smolt when delivered in tap water and in present day water from the river Rhine. These effects included disrupted skin and gill epithelia, which endured to 29 d post-treatment. While 3 h temperature elevation in de-chlorinated tap water resulted in good recovery by 29 d, fish temperature-shocked in Rhine water showed the least recovery. For migrating smolts in nature, which may encounter up to 30 such plumes, we speculate that these effects could lead to increased disease susceptibility and reduced hypoosmoregulatory ability in the marine environment.

Acknowledgments

The authors are grateful to G.H.F.M. van Aerssen and J.J. Gerritsen (KEMA) for technical support and animal care during these experiments and for the financial contribution of the Dutch Electricity Companies. A.J.H.X. Stouthart and A.E. Lamers were invaluable for assisting with sampling. Data on the Rhine water composition was kindly provided by RIWA, Lobith, The Netherlands.

Chapter 3

Exposure to water from the lower Rhine induces a stress response in the rainbow trout *Oncorhynchus mykiss*

D.T. Nolan, F.A.T. Spanings, N.M. Ruane, R.H. Hadderingh[1], H.A. Jenner[2] and S.E. Wendelaar Bonga

[1]) KEMA Environmental Services, KEMA, Utrechtsweg 310, 6812 AR Arnhem, The Netherlands.

[2]) KEMA Power Generation, P.O. Box 9035, NL-6800 ET Arnhem, The Netherlands.

Submitted for publication

Abstract

The water quality of the river Rhine has improved in recent years and populations of salmonids are increasing. At present, the water quality of the lower Rhine is a complex mixture containing low levels of many pollutants and it is not known whether exposure to such water is stressful to salmonid fish. We continuously exposed the trout *Oncorhynchus mykiss* for 31 days to water from the lower Rhine in the Netherlands and measured a variety of physiological, biochemical and histological parameters, including the stress parameters cortisol and glucose. Exposure to Rhine water significantly increased cortisol and glucose after 3 h. At 21 and 31 d, cortisol was lower in exposed fish, indicating inhibition or exhaustion of the hypothalamic-pituitary-interrenal (HPI) axis. Electron microscopical analysis of the skin and gill epithelia revealed stressor-related effects that reflected disruption of the skin epithelium, the interface between the fish and the environment. This had little influence on hydromineral balance, as although intestine and kidney specific Na^+/K^+-ATPase activities were affected, neither gill Na^+/K^+-ATPase activity nor plasma Na^+ and Cl^- were altered. Analysis of heavy metal concentrations in the liver, kidney and intestine indicated no bioaccumulation. Immunostimulation was reflected by increased respiratory burst activity of the head kidney leucocytes. From 7 d onwards, body weight of the Rhine water fish was significantly lower than control fish. Overall, the data show that acute exposure to present day water from the lower Rhine induced a stress response in the fish which, during chronic exposure, was followed by impairment of the HPI axis, reduced growth and prolonged immunostimulation.

Introduction

The stress response of an organism involves physiological and behavioural responses directed at protecting homeostasis, or restoring a disturbance of the dynamic equilibrium of the organism. The intrinsic or extrinsic stimuli threatening or disturbing this homeostasis are defined as stressors (Selye, 1973). The stress response can involve all levels of an animal's organisation and is observed in reaction to many different types of stressors. Under conditions of intense and prolonged stress, this response may lose its adaptive value, becoming dysfunctional and maladaptive, and is associated with, for instance, inhibition of growth, reproductive failure, and reduced disease resistance through immunosuppression (Anderson, 1990; Pickering, 1990; Wendelaar Bonga, 1997).

The current model of the integrated stress response is based on the dominant role of the catecholamines and glucocorticoids. These hormones are the primary messengers of the two major routes through which the brain co-ordinates the stress response: the hypothalamic-autonomic nervous system and the hypothalamic-pituitary-interrenal axis (Wendelaar Bonga, 1997). While the main neuroendocrine control mechanisms of the integrated stress response in teleosts show many similarities to that of the terrestrial vertebrates, conspicuous differences exist between the two, primarily related to the aquatic environment of fishes. Fish are directly exposed (via the water) to aquatic stressors such as pollutants, and this is compounded by the intimate relationship over a large area between the gill and skin epithelia and the ambient water environment. These epithelia are a complex assembly of many types of living cells (Whitear, 1986) and maintenance of epithelial integrity is essential for hydromineral balance, and protection against waterborne pathogens, and thus fish health.

Cortisol is a primary stress hormone in teleost fish. The synthesis and release of this corticosteroid is controlled via the hypothalamic-pituitary-interrenal axis, and mediated primarily by ACTH and αMSH, and possibly also β-endorphin (see review by Wendelaar Bonga, 1997). Cortisol is responsible for several changes in the skin of rainbow trout *in vivo* (Iger et al., 1995) and has long-term effects on hydromineral regulation, growth and immune system. Reduced cortisol secretion and lowered circulating cortisol levels result from chronic exposure to a variety of pollutants (Hontela et al., 1995) and indicate an impaired stress response through toxic actions on the hypothalamic-pituitary-interrenal axis (Hontela et al., 1997; Quabius et al., 1997).

The Rhine is an example of an ecologically important river containing a complex mixture of low-level contaminants and other anthropogenic factors (Hendriks and van de Guchte, 1997; Nienhuis et al., 1998), which have markedly influenced fish populations (Poels et al., 1980; Friedrich and Muller, 1984). Many migratory species, in particular the salmonids, have been seriously affected (Arnold and Braunbeck, 1994; Cazemier, 1994; Roche, 1994). Exposure to Rhine water induced changes in the skin epithelium of salmonids, *Salmo trutta* smolts (Nolan et al., 1998

and Chapter 2) and rainbow trout, *Oncorhynchus mykiss* (Iger et al., 1994c), including increased levels of apoptosis, necrosis and mucous cell discharge, and resulted in epithelial disruption over prolonged periods (up to 30 d). Infiltration of the epithelia by leukocytes indicated stimulation of the immune system. These are typical responses to stressors in teleost fish. Reduced growth has been reported in rainbow trout after 1 month in Rhine water during the 1970's (Poels et al., 1980). More recent studies that include growth are not available in the literature. Reduced growth of rainbow trout in Rhine may result from an integrated stress response diverting metabolic energy away from anabolic processes such as growth, into catabolic pathways to provide the energetic requirements to cope with the stressor. Increased energetic demands are required to support an activated immune system to cope with antigens penetrating across the disrupted epithelia, and increased gill Na^+/K^+-ATPase activity to defend hydromineral balance, which also result from disrupted epithelia.

In studying the stress-related effects of pollutants, only a few studies distinguished between direct and indirect effects to understand how individuals and populations may be affected. In a study of a non-toxic stressor, we examined the effects of ectoparasitic lice in post-smolt Atlantic salmon, *Salmo salar* (Chapter 4). The direct effects of the parasite were the damage caused by parasite attachment and feeding on the body surface. The indirect stress effects included the effects on the overall integrity of both the skin and gill epithelia and these included increased apoptosis and necrosis of the superficial epithelial cells and decreased numbers of mucous cells in the skin. In the gill, where no lice were found, uplifting of the epithelium, intercellular swelling and infiltration by leukocytes occurred in filaments and lamellae. High cell turnover of chloride cells was associated with significantly elevated gill Na^+/K^+-ATPase activities. These indirect stress effects are mainly hormone mediated as a consequence of the parasite being perceived by the host, causing a stress response in the fish, and likely resulting in increased levels of blood cortisol and catecholamines.

So far, studies on the stress effects of Rhine water on salmonids have centred on the effects of long-term exposure on the skin of the fish (Iger et al., 1994c; Nolan et al., 1998). However, during Rhine water exposure, not only the skin, but also the gill epithelia might be affected by external toxic (i.e. pollutants present in water from the lower Rhine; Chapter 2) and internal non-toxic factors (i.e. very high catecholamine levels; Wendelaar Bonga, 1997). The influence of disrupted surface epithelia on infiltration and bioaccumulation of waterborne Rhine water components, such as heavy metals, has not been reported for fish. Yet heavy metals have pronounced deleterious effects on fish skin (Iger et al., 1994e, f) and gill epithelia (Jagoe et al., 1996a, b; Bury et al., 1998; Dang et al., 1999), as well as the teleost immune system (Rougier et al., 1994; Kakuta, 1997).

No study to date has examined the stress parameters cortisol and glucose in the blood, bioaccumulation of waterborne toxic components, or effects on the immune system of any fish

species during long-term exposure to Rhine water. To evaluate the effects of present day water from the lower Rhine on salmonids, we exposed rainbow trout (*Oncorhynchus mykiss*, Walbaum) to Rhine water. We used blood parameters (cortisol, glucose, Na, and Cl) and Na^+/K^+-ATPase activities (of the gill, intestine and kidney) to demonstrate a stress response and effects on hydromineral balance, respectively. At time zero and after 31 d continuous exposure, heavy metal content (Cr, Cd, Pb, Fe and Cu) of kidney, intestine and liver were measured to investigate bioaccumulation of waterborne metals from Rhine water. Using electron microscopy, effects on skin and gill epithelial ultrastructure were studied to record the effects and to see whether recovery occurred within 31 d. In addition, we used respiratory burst measurements of leukocytes from the head kidney to examine effects on the immune system. Growth was assessed from body weight and condition factor.

Materials and Methods

Exposure protocol

One hundred and sixty immature 150-200 g rainbow trout (*Oncorhynchus mykiss*) (187 ± 8.9; mean ± S.E.M., n = 8) were held in 4 groups in 400-l black plastic tanks for 31 days of acclimation in non-chlorinated Arnhem tap water at a flow through rate of 600 l· $hour^{-1}$. Each tank was strongly aerated by air compressor via an airstone, and mechanically filtered by an Eheim 2213 external power filter, increasing circulation by a further 440 L· h^{-1}. The fish were fed a commercial trout feed at 1% of body weight daily. Water temperature and oxygen saturation levels were measured in all tanks daily. After acclimation, the water supply to two of the tanks was changed over 2 hours from tap water to Rhine water. The Rhine water was pumped from the river near Arnhem, The Netherlands, and filtered by a lamellar filter system (designed by KEMA, The Netherlands) into a sedimentation chamber, to remove particles larger than 0.2 mm. Data on the water quality of the Rhine during the exposure period was provided by RIWA (Lobith, The Netherlands) from their continuous monitoring programme. The water of all groups was continuously refreshed (flow through of 8-9 l min^{-1}) and well aerated.

Sampling procedure, sample processing and analysis

Fish were sampled from replicate groups at 3 and 24 h, 7, 21, and 31 d in Rhine water (RW). Two groups remained in tap water as controls (TW). All samplings took place first thing in the morning and were completed prior to late morning feeding times. Four fish from each tank were sampled each time, and pooled to provide a sample size of 8 fish per group at each time point, representing replicate treatment tanks.

Body parameters

Fish were weighed and fork length was measured to obtain data to calculate condition factors and growth. For condition factor, the calculation was made from the formula

$CF = 100 \cdot W/(FL^3)$

where CF is the condition factor, W is the body weight in g and FL is the fork length in cm.

Blood parameters

Blood was withdrawn by needle from the caudal blood vessels of irreversibly anaesthetised (2-phenoxyethanol, Sigma; 1:1000) fish into Na^+-heparinsed syringes, the plasma was separated by centrifugation and immediately frozen in liquid nitrogen for analysis of plasma cortisol, glucose, Na^+ and Cl^-. Na^+ concentrations were determined in 200x diluted plasma with a flamephotometric auto analyser (Technicon model IV) coupled to a spectrophotometer for determining Cl^- by the formation of ferrothiocynate. Plasma glucose was assayed with the Boehringer UV-test kit according to the manufacturers protocol (Boehringer, Mannheim, Germany). Plasma cortisol was assayed by RIA validated for measuring cortisol in fish plasma (Balm et al., 1994, 1995).

Na^+/K^+-ATPase activity

A 1 cm piece from the posterior part of the kidney, 1 cm of intestine from the area past the pyloric cecae (emptied of food content and rinsed in storage buffer) and the gill filaments from the second gill arch (trimmed from the arch) were dissected and placed in 1 ml of ice-cold SEI buffer (0.3 M sucrose, 20 mmol Na_2EDTA, 0.1 M imidazole, pH 7.1; Zaugg, 1982) and stored at -20°C until analysis. The assay of specific Na^+/K^+-ATPase activity was carried out on first homogenates according to the microplate method described by (Quabius et al., 1997), with the exception that absorbance was read at 690 nm, the optimal wavelength for the colorimetric method described (Nolan et al., 2000).

Heavy metal concentrations in tissues

Tissue samples from kidney (circa 1g, immediately posterior to the sample for Na^+/K^+-ATPase analysis), intestine (circa 0.1g sampled just posterior to the pyloric cecae) and liver (circa 1g from the overlying proximal lobe) were dissected for heavy metal analysis and placed in pre-weighed eppendorf tubes. The samples were weighed, frozen on dry ice and dried to constant weight under vacuum. Freeze-dried kidney, liver and intestine samples were digested for 48 h in 500 µl HNO_3 (65% nitric acid ultrapure, Merck) at 60°C. Chromium, cadmium, lead, iron and copper concentrations were analysed with Inductive Coupled Plasma Atomic Emission Spectrometry (Plasma IL200, Thermo

Electron, USA) and read against analytical standards acidified in the same way as the samples with the same nitric acid stock. Concentrations were expressed per unit wet weight.

Respiratory burst

Leukocytes isolated from head-kidney tissue from each fish were prepared as described by (Ruane et al., 1999a) and respiratory burst was determined using the reduction of nitroblue tetrazolium (NBT; Sigma), following Chung and Secombes (1988). Results were expressed as stimulation indices, where the unstimulated wells from each fish were controls and were assigned an arbitrary value of 100. The PMA stimulated wells were expressed relative to these controls.

Electron microscopy

For electron microscopy, samples of skin (5 x 3 mm) from the head and small pieces containing 4-6 filaments from the second gill arches were fixed in 3% glutaraldehyde in sodium cacodylate buffer (0.09 M, pH 7.35) on ice, post fixed in 1% osmium tetroxide in the same buffer for 60 minutes and washed several times in dH_2O, as described by (Iger et al., 1994b, c, d). For transmission electron microscopy (TEM), one replicate set of fixed skin and gill samples from 4 fish was stained for 2 h in 2% uranyl acetate, dehydrated through an ethanol series and embedded in Spurr's resin. Ultra-thin sections were cut by diamond knife, collected on 150 mesh copper grids and contrasted with lead citrate before viewing in a Philips EM 300 transmission electron microscope at 40 kV. A series of representative micrographs were made without knowledge of the treatments and effects on the epithelia were evaluated by comparing 20 micrographs representing 4 fish from RW against TW controls at each sample point.

For skin, the overall ultrastructure was observed and attention was focused on cellular necrosis (characterised by nuclei with aggregations of chromatin, swelling of the cytoplasmic compartment and loss of apical microridges) and apoptosis (characterised by progressive densification of the nucleus, organelles and cytoplasm, leading to shrinkage and loss of contacts with surrounding cells), leukocyte infiltration and epithelial integrity (evaluated by intercellular swelling and presence of intact cell-cell contacts). For gills, the overall ultrastructure and the appearance of the respiratory lamellae were observed. In addition, the interlamellar chloride cell populations were studied, and necrosis, apoptosis (characterised for chloride cells by progressive densification of the mitochondria, nucleus and cytoplasm, as well as dilation of the tubular system) and leukocyte infiltration were evaluated. A second replicate set of skin and gill samples were examined by scanning electron microscopy (SEM). These samples were dehydrated through an ethanol series, critical point dried under liquid carbon dioxide, gold sputtered in a Balzers coating

unit (CPD 020, Balzers, Switzerland) and viewed in a Jeol-JSM T 300 scanning electron microscope.

Statistical analysis

As there were no significant differences between replicate tanks, data are pooled and expressed as means ± S.E.M. for n = 8 fish per treatment at each time point. Differences between groups at each sample point were assessed with Student's t test, except for plasma Na:Cl ratio and condition factor which were tested using the Mann-Whitney *U* non-parametric test. Differences within a treatment over time were assessed by ANOVA followed by Tukey-Kramer post tests (non-parametric ANOVA for Na:Cl ratio and condition factor). Statistical significance was accepted at $P < 0.05$.

Results

Rhine water, growth and condition factor

The water quality of the Rhine was constant throughout the experimental period and none of the contaminants monitored at RIWA (Lobith, The Netherlands) fluctuated significantly during the exposure. Values for all parameters remained within permitted levels and the fish remained disease free and outwardly healthy. There were no significant differences in oxygen saturation levels or temperature profiles between tanks or between Rhine water and tap water during the experiment. The condition factors were not significantly different between groups sampled from TW and RW at any time point (Table 1). Body weights were significantly lower for fish sampled from RW at 7, 21 and 31 d; fork length was lower only at 7 and 21 d (Table 1).

Blood parameters

Plasma cortisol of TW control fish ranged from 10 to 30 ng· ml^{-1} during the experimental period (Figure 1a). The mean plasma cortisol levels of RW fish were significantly higher (65 ng· ml^{-1}) compared with the TW controls at 3 h, but were similar to TW fish at 24 h and 7 d. At 21 and 31 d in RW, plasma cortisol values were significantly lower than in TW (Figure 1a). A significant increase in plasma glucose levels in RW fish was observed at 3 h and at 31 d, compared with TW controls (Figure 1b). Plasma Na^+ concentrations of control and experimental groups remained similar throughout the experiment and were within the normal range of values reported for freshwater (FW) salmonids. Plasma Cl^- was significantly higher in RW fish at 21 d, but not at 24 h, 7d or 31d (Table 2). At 24 h, the Na:Cl ratio was significantly lower in RW fish compared with TW controls (Table 2).

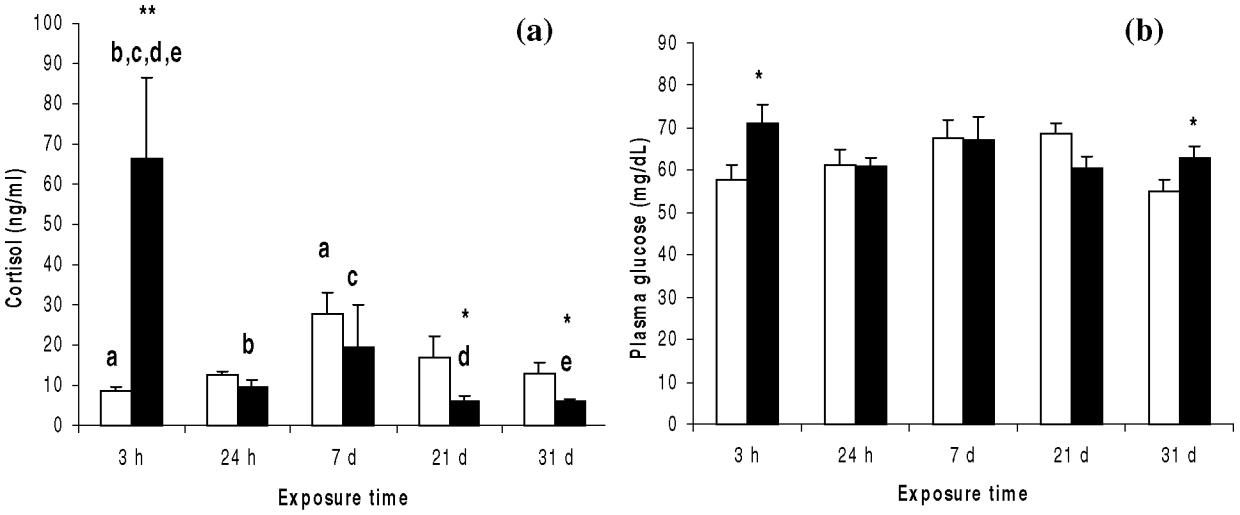


Figure 1. (a) Plasma cortisol measured in the trout *Oncorhynchus mykiss* in tap water and Rhine water. Data are expressed as ng cortisol/ml for a sample size of n = 8. Bars sharing the same letter are significantly different where a = $P < 0.05$, b,c = $P < 0.01$ and d,e = $P < 0.001$. Differences between tap water controls and Rhine water are indicated by * ($P < 0.05$) and ** ($P < 0.01$). (b) Plasma cortisol in *O. mykiss* in tap water and Rhine water. Data are expressed as mg glucose/dL for a sample size of n = 8. Differences between tap water controls and Rhine water are indicated by * ($P < 0.05$).

Na^+/K^+-ATPase activity

The Na^+/K^+-ATPase activity in gill tissues of the TW controls and the RW exposed fish remained similar throughout the experiment (Table 2). Intestinal Na^+/K^+-ATPase activity was significantly decreased in RW fish at 21 d compared with TW controls at this sample point (Table 2), but remained similar to TW fish at all other sample points. The Na^+/K^+-ATPase activity in kidney tissues of RW fish was significantly increased at 7, 21 and 31 d compared with TW fish (Table 2).

Heavy metal concentrations of tissues

All tissues from control and RW fish at each time point had similar metal concentrations (data not shown).

Respiratory burst activity of the head kidney leukocytes

The respiratory burst activity of the leukocytes of the RW fish increased progressively during the experimental period and at 31 d was significantly higher than that of TW controls (Table 3).

Table 1. Fork length, body weight and condition factor of rainbow trout (*Oncorhynchus mykiss*) in tapwater (TW) or water from the lower river Rhine (RW). Data are given as mean ± S.E.M. for n = 8. Condition factor formula is given in the text. * = $P < 0.05$; ** = $P < 0.01$ significance from TW controls; shared superscript letters indicate significance where a,b,e,f = $P < 0.05$; c,d = $P < 0.01$.

Treatment and sample point	Fork length (cm)	Body weight (g)	Condition factor
TW 3 h	25.1 ± 0.4	187.1 ± 8.9^{c}	1.18 ± 0.27
RW 3 h	24.0 ± 0.6^{b}	175.8 ± 9.9	1.28 ± 0.05
TW 24 h	25.0 ± 0.1^{a}	190.0 ± 8.0^{d}	1.21 ± 0.19
RW 24 h	24.8 ± 0.5	188.3 ± 10.3	1.22 ± 0.04
TW 7 d	26.0 ± 0.5	223.6 ± 12.4	1.27 ± 0.04
RW 7 d	24.0 ± 0.4**	169.0 ± 11.9^{**,e}	1.21 ± 0.05
TW 21 d	26.2 ± 0.4	227.6 ± 11.1	1.26 ± 0.02
RW 21 d	25.2 ± 0.3*	196.2 ± 8.9*	1.23 ± 0.03
TW 31 d	26.8 ± 0.5^{a}	249.6 ± 12.9^{c,d,e}	1.25 ± 0.02
RW 31 d	25.8 ± 0.3^{b}	210.9 ± 9.2*	1.25 ± 0.03

Skin ultrastructure

The ultrastructure of the head skin of TW rainbow trout as revealed by SEM showed the surface to be a continuous sheet of pavement cells with concentric apical microridges and good epithelial integrity through tight connections between pavement cells. In RW, there was increased incidence of mucous cells discharging at 3 h, and increased cell death in the pavement cell layer was indicated by the loss of the microridge structure in many cells (Figure 2a). Examination by TEM confirmed the occurrence of apoptosis, necrosis and loss of microridges in the upper pavement cell layers. The appearance of the epidermis of RW fish in SEM at 24 h indicated further deterioration, with extensive swelling and shedding of the pavement cells (Figure 2b). At 7, 21, and 31 d in RW, the appearance of the skin pavement cells in SEM was very comparable to that of TW controls.

Table 2. Parameters investigated in rainbow trout (*Oncorhynchus mykiss*) in tapwater (TW) or water from the lower river Rhine (RW). Data are given as mean ± S.E.M. for n = 8. Na and Cl values and mm/l and specific Na^+/K^+-ATPase activity is µmol P_i· mg $protein^{-1}$· h^{-1}. * = P < 0.05; ** = P < 0.01; *** = P < 0.001 indicate significantly different from TW controls; shared superscript letters indicate significance where d,h = P < 0.05; a,g = P < 0.01; b,c,e,f = P < 0.001.

Treatment and sample point	Plasma Na	Plasma Cl	Plasma Na:Cl ratio	Gill Na^+/K^+-ATPase	Kidney Na^+/K^+-ATPase	Intestine Na^+/K^+-ATPase
TW 3 h	148 ± 4	124 ± 3	1.19 ± 0.01^{a,b,c}	2.57 ± 0.25	3.42 ± 0.29	3.52 ± 1.34^{g}
RW 3 h	153 ± 3	120 ± 9	1.20 ± 0.04	3.07 ± 0.32	3.66 ± 0.33	6.64 ± 1.38
TW 24 h	150 ± 3	127 ± 2	1.19 ± 0.01^{d,e,f}	2.82 ± 0.29	4.27 ± 0.30	3.61 ± 0.83h
RW 24 h	138 ± 10	130 ± 4	1.06 ± 0.07*	3.83 ± 1.02	4.01 ± 0.38	4.45 ± 0.96
TW 7 d	142 ± 4	124 ± 4	1.15 ± 0.01^{a,d}	2.27 ± 0.27	3.43 ± 0.24	7.72 ± 1.04
RW 7 d	147 ± 1	129 ± 2	1.14 ± 0.01	2.74 ± 0.27	5.03 ± 1.00**	7.21 ± 1.80
TW 21 d	139 ± 4	123 ± 3	1.13 ± 0.01^{b,e}	2.75 ± 0.31	3.47 ± 0.39	12.48 ± 0.83^{g,h}
RW 21 d	150 ± 5	136 ± 4*	1.01 ± 0.01	2.65 ± 0.48	5.22 ± 0.20***	7.73 ± 0.91***
TW 31 d	142 ± 5	126 ± 4	1.13 ± 0.01^{c,f}	1.89 ± 0.17	3.39 ± 0.27	7.88 ± 2.24
RW 31 d	143 ± 4	128 ± 4	1.12 ± 0.01	2.16 ± 0.81	5.41 ± 0.35***	6.18 ± 0.72

Gill ultrastructure

The gill structure of TW control trout in SEM was composed of numbers of filaments with delicate leaflike lamellae arranged alternately on both sides (Figure 3a). The upper respiratory cells were similar to the pavement cells of the skin, with apical microridge structures. At 3 h, the lamellae of RW fish were wrinkled and the individual respiratory cells stood out in relief (Figure 3b). These respiratory cells had disrupted apical microridges in RW. TEM examination of RW interlamellar areas showed that already at 24 h, many chloride cells were swollen and necrotic and numbers of macrophages infiltrated the epithelia. The surface of the respiratory cells showed characteristic apical pit structures in the centre of many respiratory cells and this persisted through to 31 d (Figure 4a). These apical pits occurred along the filament, as well as on lamellae. At 7 d in RW, necrotic chloride cells were commonly observed and intercellular spaces were common throughout the respiratory epithelia (Figure 4b). At 21 d in RW, improved structural integrity of the gill epithelium was apparent in some individuals with new chloride cells differentiating (Fig. 5a), while extensively disruption and chloride cell necrosis was evident in others (Figure 5b). At 31 d, overall improved gill epithelial integrity was observed in SEM, and this was reflected in the TEM observations also.

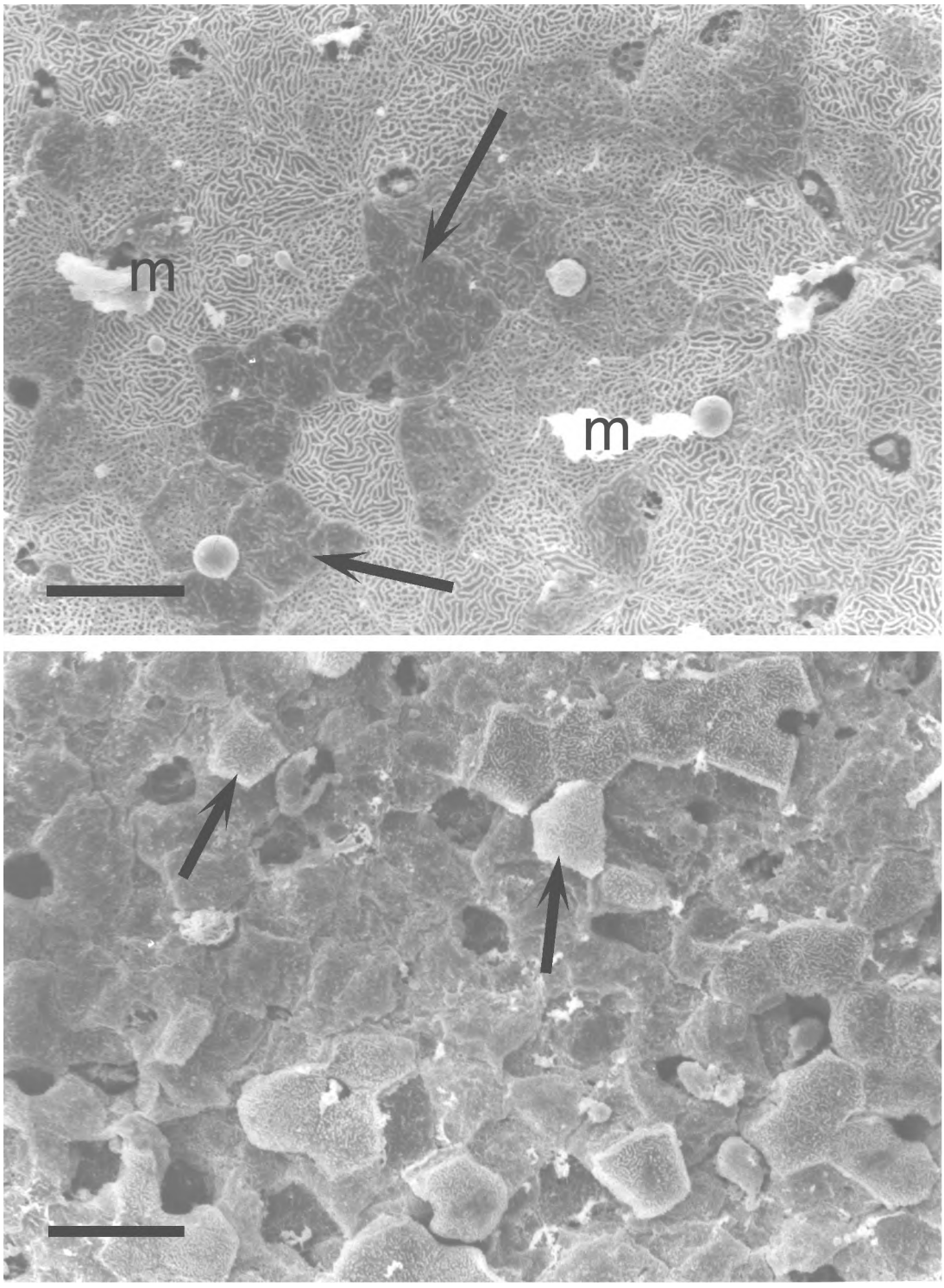


Figure 2. Scanning electron micrographs of head skin from the trout *Oncorhynchus mykiss*. (a) Rhine water, 3 h. Many pavements cells are darkened (arrows) and have lost their microridges. Mucus (**m**) discharges from mucous cell pores. Scale bar = 10 µm. (b) Rhine water, 24 h. There is extensive disruption as a result of much cell death in the pavement cells and many cells are sloughing off (arrows). Scale bar = 15 µm.

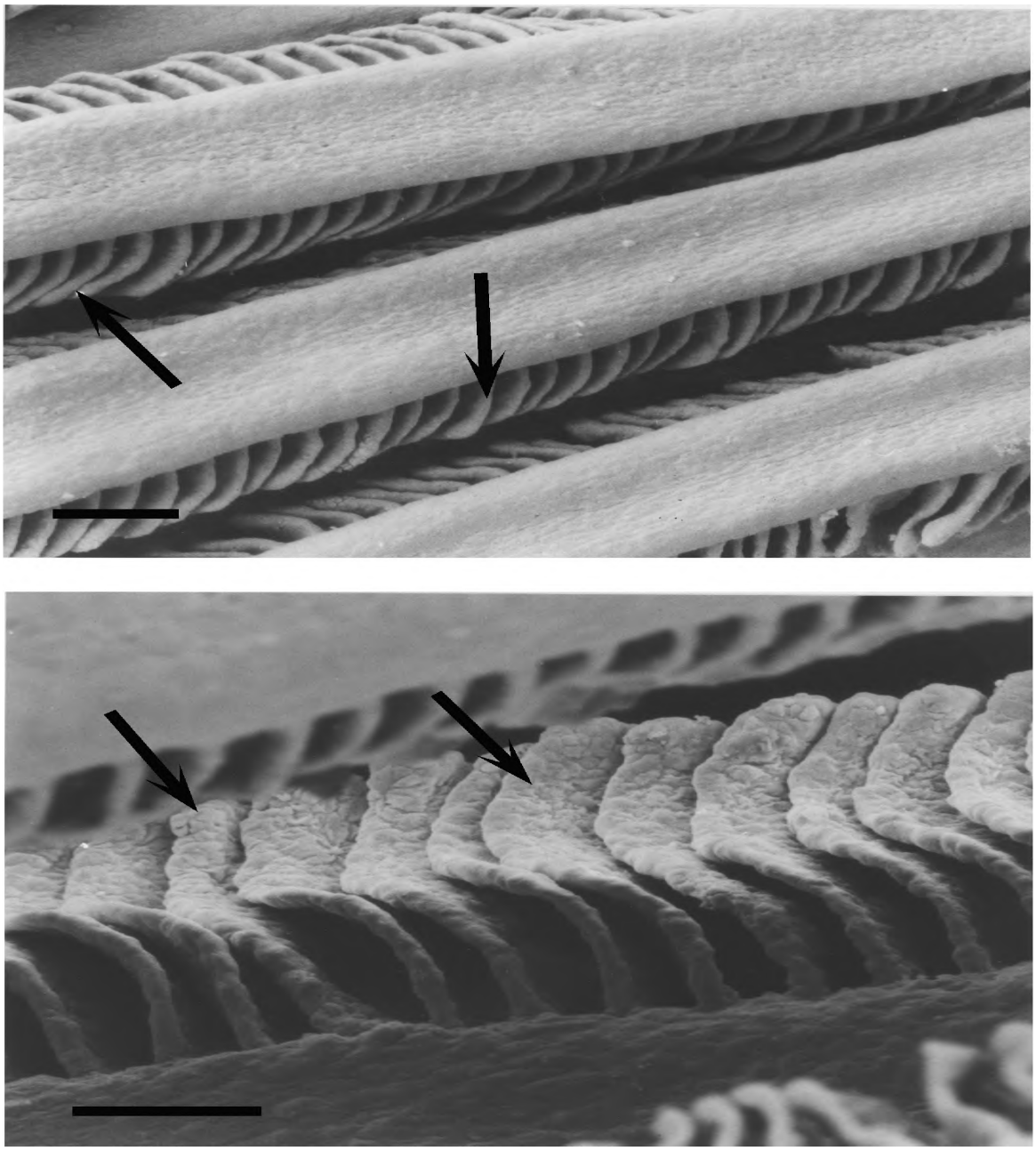


Figure 3. Scanning electron micrographs of gills from the trout *Oncorhynchus mykiss*. Upper is tap water control, 3 h. The gills are composed of filaments with leaflike lamellae branching off (arrow). Scale bar = 100 µm. Lower is Rhine water, 3 h. The lamellae are wrinkled and the respiratory cells stand out in relief (arrows). Scale bar = 50 µm.

Table 3. Respiratory burst activity of head kidney leukocytes from rainbow trout (*Oncorhynchus mykiss*) in tapwater (TW) or water from the lower river Rhine (RW). Data are given as means ± S.E.M. for n = 8. Data are given as stimulation index values, calculated from the increased oxygen radical production between PMA stimulated and unstimulated macrophages for each fish (see materials and methods for more detail). ** = $P < 0.01$ significance from TW controls; shared superscript letters indicate significance where [a] = $P < 0.01$.

Sample point	Tap water	Rhine water
3 h	117.4 ± 8.7	129.7 ± 5.3[a]
24 h	137.5 ± 8.5	144.5 ± 13.0
7 d	133.7 ± 8.7	144.8 ± 10.7
21 d	136.0 ± 12.1	165.4 ± 16.8
31 d	134.0 ± 10.1	195.6 ± 16.5[**,a]

Discussion

This is the most comprehensive study to date on the stress response and effects of RW on a single fish species. Exposure of rainbow trout to RW induced an integrated stress response, as evidenced by increased circulation in the blood of the stress indicators cortisol and glucose in the short-term. The longer-term effects of continuous exposure on anabolic processes and the immune system were reduced growth and stimulation of macrophage respiratory burst activity.

Plasma levels of cortisol of control fish in the present study ranged from 10 to 30 ng· ml^{-1} plasma during the experimental period. Marked elevation of plasma cortisol is the most widely used indicator of stress used in fish and occurs in response to a wide variety of pollutants (see review by Brown, 1993). On exposure to an acute stressor, cortisol levels rise, in general returning to control levels after a few hours, or can remain elevated in the case of chronic stress. Basal levels of cortisol for unstressed salmonid fish are normally below 10 ng· ml^{-1} (Gamperl et al., 1994; Wendelaar Bonga, 1997). The significant increase in cortisol (group mean 65 ng· ml^{-1} plasma) at 3 h in RW indicated a primary stress response in the fish on exposure to Rhine water. These levels are similar to those found in rainbow trout subjected to confinement stress (Pottinger and Moran, 1993; Ruane et al., 1999a, b). The return of cortisol plasma concentrations to basal levels at 24 h and 7 d RW might indicate that RW was an acute stressor. However, the significant decreases in plasma cortisol at 21 and 31d in RW indicated reduced cortisol levels in response to chronic exposure to pollutant stress, as has now been shown in a number of field studies of fish from chemically contaminated environments e.g. PAHs, PCBs and heavy metals in the St. Lawrence river (Hontela et al., 1992, 1995). In laboratory experiments, reduced plasma cortisol values were reported in juvenile (60 g) rainbow trout exposed to 5 µg· l^{-1} cadmium (but not when exposed to 1µg· l^{-1}) for 30 d (Ricard et al.,

1998), indicating that effects on blood cortisol levels are affected by both toxicant concentration and duration of exposure.

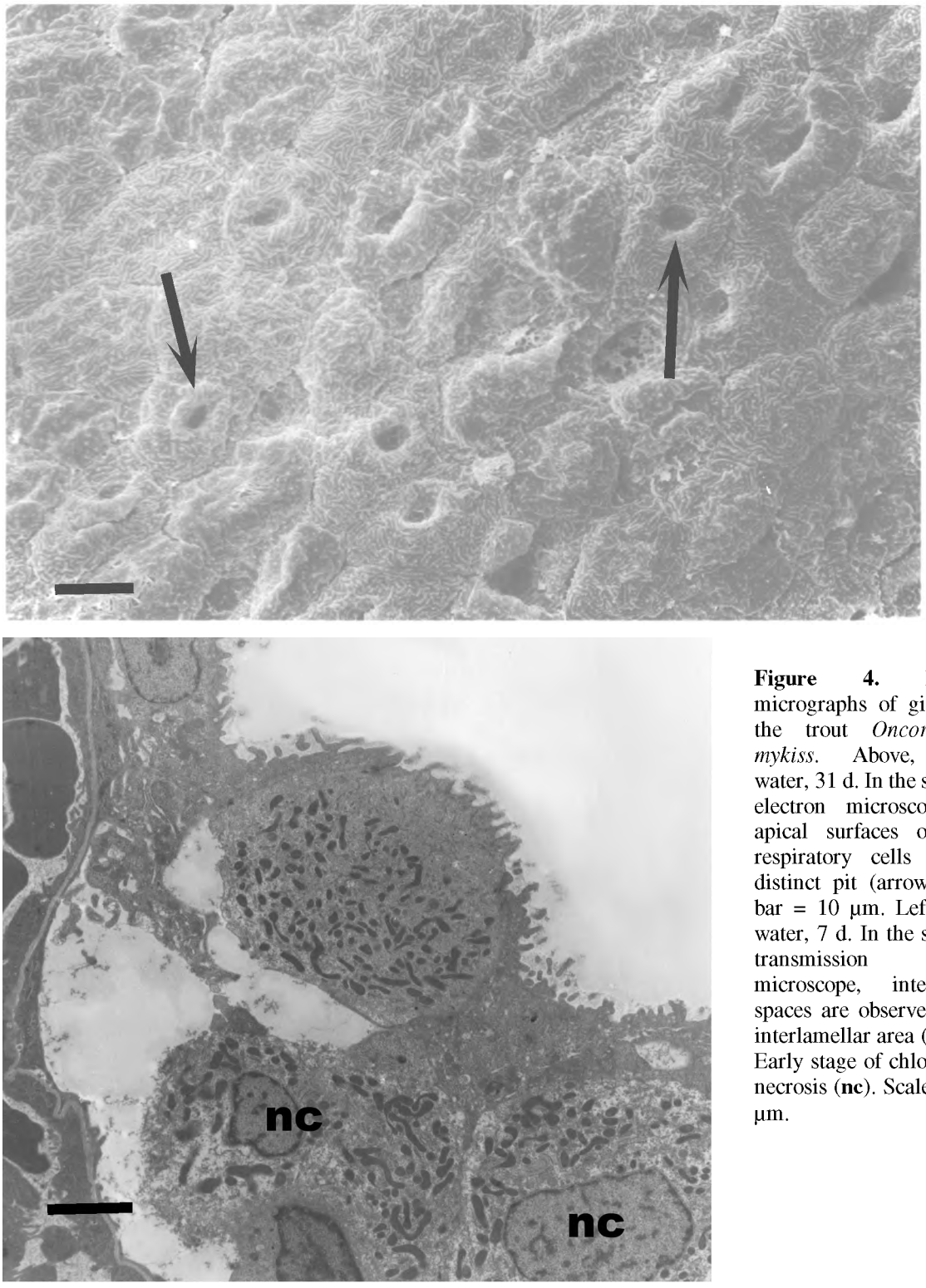


Figure 4. Electron micrographs of gills from the trout *Oncorhynchus mykiss*. Above, Rhine water, 31 d. In the scanning electron microscope, the apical surfaces of many respiratory cells have a distinct pit (arrow). Scale bar = 10 µm. Left, Rhine water, 7 d. In the scanning transmission electron microscope, intercellular spaces are observed in the interlamellar area (arrows). Early stage of chloride cell necrosis (**nc**). Scale bar = 5 µm.

Two major cortisol actions in fish are regulation of hydromineral balance and energy metabolism. The elevated plasma glucose levels at 3 h in RW probably represent stress-related hyperglycaemia, but the elevated plasma glucose at 31 d is more difficult to explain. As the RW fish were kept in a continuous flow-through system, it is possible that a pulse event in the river system may have induced this response in the fish. Yet no change in pollutant loading was recorded at this time during the routine monitoring at RIWA station upstream at Lobith (RIWA, Lobith, unpublished data). If this were induced by a pulse event of unnoticed pollutant(s), a concomitant increase in plasma cortisol levels might be expected. However, as mentioned above, reduced interrenal responsiveness resulting from chronic exposure to polluted environments is now well documented in stress responses from polluted environments (Hontela et al., 1992, 1995; Brodeur et al., 1997) and might have prevented elevation of cortisol levels in our experiment.

In general, exposure to waterborne pollutants disrupts gill function and this results in hydromineral disturbance (Wendelaar Bonga, 1997). This can occur as a direct toxic effect of the pollutant on gill structure and function (Sola et al., 1994; Mallatt et al., 1995) or as a result of the effects of elevated blood catecholamines and glucocorticoids released during the integrated stress response on gill permeability and specific gill cell types, respectively (Wendelaar Bonga, 1997 and references therein). Fresh water stressors usually result in reduced plasma osmolarity and the loss of plasma electrolytes, typically reported as reductions in Na^+ and Cl^- (Pilgaard et al., 1994; Grippo and Dunson, 1996) or whole body ionic concentrations (Marr et al., 1995). The RW-exposed trout in the present study maintained Na^+ and Cl^- homeostasis during most of the exposure period. The transient reduction in plasma Na:Cl ratio at 24 h in RW fish might reflect a change in acid/base balance in the blood after transfer to RW, as a result of adjustments to ionic, osmotic and metabolic regulation during stress (McDonald and Milligan, 1997). The Na:Cl ratio proved to be a sensitive parameter in our earlier studies on other stressors in FW and SW fish (Nolan et al., 1998, 1999, 2000; Chapter 4). During stress in FW, Na^+ and Cl^- are usually lost through leakage at the gill (McDonald and Milligan, 1997). Increased intestinal Na^+/K^+ ATPase activity after 21 d in RW may be directed at increasing Na^+ uptake to compensate for continued losses, particularly branchial. In FW, the ion-transporting capacities of the teleost intestine involve actively taking up essential ions primarily from the food, including Ca^{2+} and Na^+. The drinking rate of FW fish is extremely low and the contribution to ionoregulation is considered negligible, especially compared with the gill (Flik and Verbost, 1995). The increased kidney Na^+/K^+-ATPase activity observed at 7, 21 and 31d RW might significantly contribute to reducing urinary ion losses. This would be advantageous for maintaining ionic homeostasis during periods of increased elimination of excess water, which enters the fish across

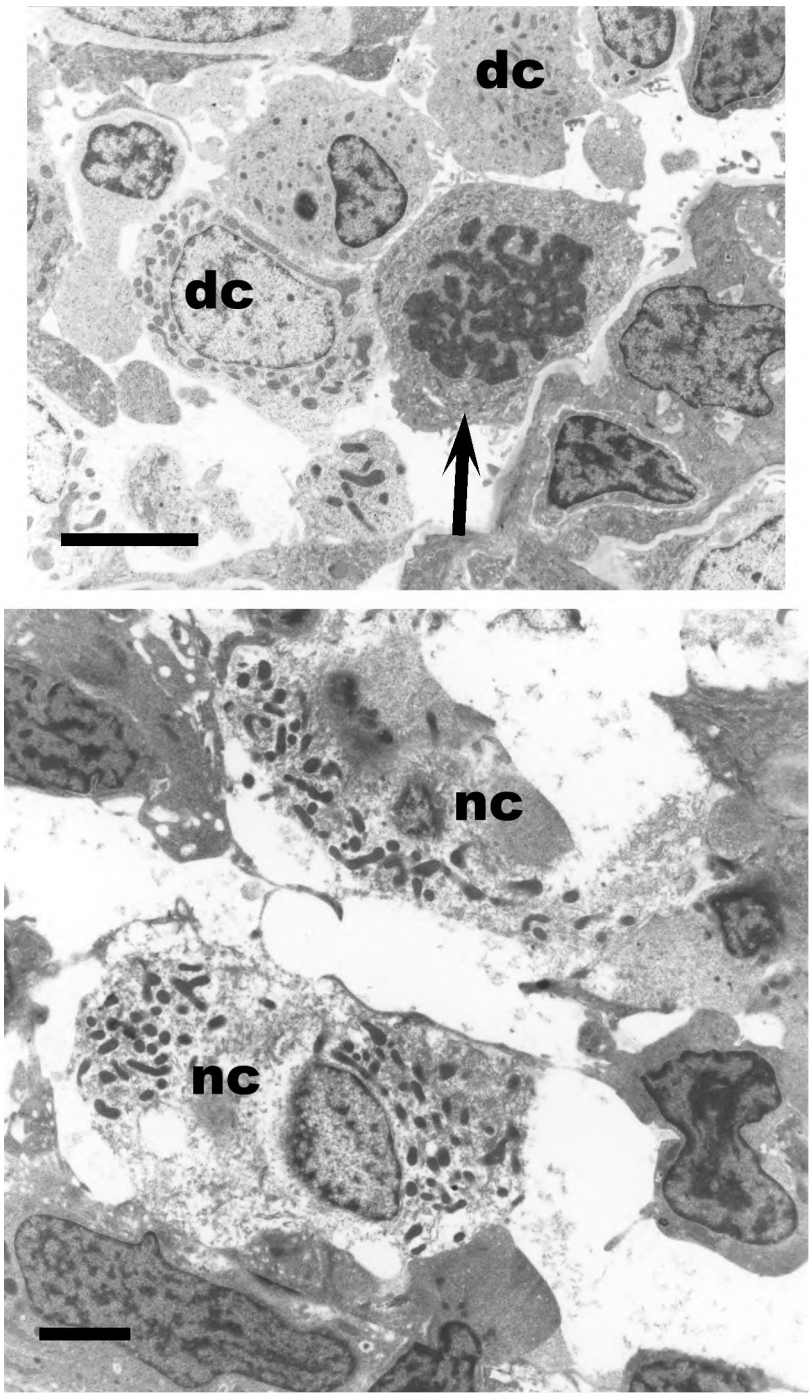


Figure 5. Transmission electron micrographs of gills from the trout *Oncorhynchus mykiss*. Above, Rhine water, 21 d. In the filament, differentiating chloride cells (**dc**) are seen alongside a mitotic cell (arrow) in the germinal layer under the interlamellar area. Scale bar = 10 µm. Below, Rhine water, 21 d. Chloride cell necrosis (**nc**) in the interlamellar area was characteristic of some fish. Scale bar = 5 µm.

permeabilised epithelia by osmotic drag, as higher renal flow requires more renal Na^+ and Cl^- reabsorption. Gill Na^+/K^+-ATPase activity was unaffected by exposure to RW in the present experiment. In other experiments with *S. trutta* smolts, plasma Na^+ and Cl^- levels were unaffected in RW fish, and this ionic homeostasis was associated with increased gill Na^+/K^+-ATPase activity at 24 h in RW (Chapter 2). The latter result may relate to the physiological status of the sea trout smolt, which is very different from that of non-smoltifying salmonids. In the Atlantic salmon smolt, high kidney Na^+/K^+ ATPase activity is associated with the FW parr stage and reduced Na^+/K^+ ATPase in the kidney occurs in preparation for seawater adaptation (McCartney, 1976).

The increased apoptosis, necrosis and mucus discharge in the skin confirmed observations from other long-term RW studies on both *O. mykiss* (Iger et al., 1994c) and *S. trutta* (Nolan et al., 1998; Chapter 2). Increased chloride cell necrosis, apical pit formation in the respiratory cells, together with stable gill specific Na^+/K^+-ATPase activity, indicated that epithelial disruption did not affect homeostatic plasma Na^+ or Cl^- values and that the ionoregulatory capacity of the animals was sufficient to prevent disturbance. Similar effects on hydromineral balance were reported from studies using both toxic, e.g. RW in *S. trutta* (Nolan et al., 1998; Chapter 2) and non-toxic stressors, e.g net confinement of *O. mossambicus* (Nolan et al., 1999), sea lice on *S. salar* (Chapter 4). These effects, together with increased glucose and cortisol in the blood, indicated that Rhine water evoked an acute stress response. Thus, exposure to present day RW is a stressor for rainbow trout, but not severe enough to disturb ionoregulation beyond the regulating capacity of the fish.

Previously, growth retardation in rainbow trout in RW was recorded after 1-2 months (Poels et al., 1980). We report reductions in growth already at 7 d in RW fish. This difference may result from the fact that the first sampling point in the previous study was at 1 month (Poels et al., 1980), thus earlier differences might have been missed. Ricard et al. (1998) report reduced growth in rainbow trout after 30 d exposure to 10 and 25 $\mu g \cdot l^{-1}$ Cd, however the Cd concentrations in RW are only 0.05-0.11 $\mu g \cdot l^{-1}$ which are considerably lower. As RW is a complex mixture of low-level pollutants, it is unlikely that reduced growth can be attributed to one specific component alone. Moreover, in the present study we measured no significant bioaccumulation of Cr, Cd, Pb, Fe and Cu in intestine, kidney or liver of the fish, indicating that bioaccumulation from the Rhine water does not occur during relatively short-term exposure to these waterborne pollutants. This does not exclude the possibility that accumulation of organic pollutants via the food chain occurs in nature. Indeed, the latter were reported to accumulate in the adipose tissues of RW-exposed fish (Poels et al., 1980). Their effects may not become apparent until they are released by lipid mobilisation during overwintering or during vitellogenesis, as occurs in the breeding stage.

Rhine water exposure induced severe effects in both the skin and gill epithelia of the rainbow trout where the effects included the stimulation of mucous discharge, the induction of apoptosis and

necrosis, epithelial infiltration by leukocytes, and increases in the intercellular spaces. These effects induced by exposure to Rhine water are similar to those reported for a variety of other waterborne toxicants (Iger et al., 1994b; Mallatt et al., 1995; Burkhardt-Holm et al., 1997). Moreover, in a study examining direct and indirect effects of sea lice on skin and gill epithelia, it was shown that attachment of ectoparasitic lice to Atlantic salmon (*S. salar*) could induce similar effects in the skin at sites distant from and unaffected by the parasites and in the gill where adult parasites do not attach (Chapter 4). In that study, necrosis was observed in the pavement cells of the skin, but not in the chloride cells of the gill. Necrosis occurred in both tissues in the present RW study. In a study of the effects of another non-toxic stressor, net confinement, on the tilapia *O. mossambicus*, chloride cell necrosis was not characteristic (Nolan et al., 1999). Chloride cell necrosis has been regularly reported for a variety of toxicants (Wendelaar Bonga et al., 1990; Mallatt et al., 1995; Verbost et al., 1995; Teh et al., 1997; Li et al., 1998), suggesting that induction of chloride cell necrosis results directly from effects of the toxicant on the cell and not as an indirect effect associated with the stress response, such as high cortisol levels in the blood. Furthermore, cortisol can protect the chloride cells against toxicant-induced necrosis (Bury et al., 1998).

By contrast, both necrosis and apoptosis were characteristic in the pavement cells of the skin in both non-toxic (Chapters 2, 4, 5 and 7) and toxic situations (Iger and Wendelaar Bonga, 1994; Iger et al., 1994b, c; Burkhardt-Holm et al., 1997; Nolan et al., 1998). These observations indicate that in the pavement cells of the skin epidermis, necrosis may be induced also by an internal, stress-related factor. Immunohistochemical localisation has shown that the distribution of the glucocorticoid receptor in fish skin is primarily in the pavement cell and underlying upper epidermal cells in the common carp *Cyprinus carpio*, (Chapter 5) and in the trout *O. mykiss* (Chapter 6). Thus, whereas toxicants may directly induce necrosis in the chloride cells in the gill (Bury et al., 1998), in the skin, pavement cell necrosis is associated with non-toxic stressors and may be cortisol dependent.

We have demonstrated that exposure to current day RW did not result in immunosuppression, although it is generally reported that chronic exposure to stressors results in suppression of the humoral immunoreactivity (Pickering and Pottinger, 1989; Anderson, 1990; Wedemeyer, 1997). The reduced respiratory burst activity reported in fish from environments contaminated with organic compounds may indicate that effects may require mobilisation (Tahir et al., 1993; Lemaire-Gony et al., 1995; Fournier et al., 1998). The fish in the present study were actively growing, and perhaps if we had initiated a starvation protocol or fed sub-optimal rations, we might have observed toxicity associated immunosuppression. The data we present showing respiratory burst stimulation in head kidney leukocytes from RW fish indicate that the cellular immune reactivity was not compromised. In the current study, as well as in others investigating the

effects of RW on salmonids, the condition factor of the fish was high and mortality low in RW, without any disease outbreak (Iger et al., 1994c; Nolan et al., 1998; Chapter 2).

In conclusion, we have demonstrated that exposure of rainbow trout to the current water quality of the river Rhine evoked an integrated stress response, resulting in elevated blood cortisol and glucose levels in the short-term and reduced growth in the long-term. Examination of the skin and gill epithelia showed that changes, similar to those reported for a variety of other stressor types, were induced and there were adjustments to the Na^+/K^+-ATPase activities in some of the ionoregulatory organs, probably directed at maintaining hydromineral balance. Moderate stressors, such as cortisol feeding alone or combined with infection with the fish louse *Argulus foliaceus* (Ruane et al., 1999a), exposure to infective life stages of the sea louse *Lepeophtheirus salmonis* (Nolan et al., 2000; Ruane et al., 2000), and dietary administration of PCB126 (Quabius et al., 1997) modulated the response to a subsequent stressor. Salmonids living in RW may also have an impaired stress response, which could result in increased susceptibility to other environmental stressors. Together, these data show that current day Rhine water is considerably below optimal for salmonids.

Acknowledgements

The authors are grateful to G.H.F.M. van Aerssen, J.J. Gerritsen and M. Van Donk (KEMA) for technical support and animal care during these experiments and for the financial contribution of the Dutch Electricity Companies. L. Parker assisted with sampling, TEM processing and early manuscript drafting. Data on the Rhine water composition was kindly provided by RIWA, Lobith, The Netherlands.

Chapter 4

Infection with low numbers of the sea louse *Lepeophtheirus salmonis* induces stress-related effects in post-smolt Atlantic salmon (*Salmo salar*)

D.T. Nolan, P. Reilly[1] and S.E. Wendelaar Bonga.

[1]) Aquaculture Development Center, University College Cork, Lee Maltings, Cork, Ireland.

Canadian Journal of Fisheries and Aquatic Sciences (1999). 56:947-959

Abstract

Infection of post-smolt Atlantic salmon with 3, 6 or 10 pre-adult and adult sea lice per fish resulted in changes to epithelial structure at sites in the skin and gill, distant from lice attachment and feeding. In the skin, increased apoptosis and necrosis occurred in the superficial epithelial cells and the number of mucous cells decreased. In the gill, where no lice were found, uplifting of the epithelium, intercellular swelling and infiltration by leukocytes occurred in filaments and lamellae. High cell turnover of chloride cells was associated with significantly elevated gill Na^+/K^+-ATPase activities. Serum chloride levels were elevated in the 3 and 6 lice/fish groups, and serum Na/Cl ratio was lower in all parasitized groups at 5 d. The results indicate that infection with low numbers of the pre-adult and adult parasite induced changes characteristic of a stress response. In the low- and medium-infested groups, homeostatic recovery had occurred by 10 d, but recovery was incomplete in the highly infected group. Thus, 10 lice/fish, which is a low infestation level in nature, is stressful and creates a long period during which the overall condition of the skin and gill epithelia may render the fish susceptible to secondary infections.

Introduction

The sea louse *Lepeophtheirus salmonis* is a caligid copepod ectoparasite of salmonids in sea water. This species is an economically important parasite of sea-farmed salmonids which frequently causes serious disease throughout the northern hemisphere (Wootten et al., 1982; Pike, 1989). In wild stocks it is normally present in low numbers (i.e. from 10 parasites/fish upwards; Berland, 1993; Holst et al., 1993), however under certain conditions, epizootics occur (Wootten et al., 1982; Tully et al., 1993; Johnson et al., 1996).

Lepeophtheirus salmonis has a direct life cycle (i.e. no intermediate host) consisting of 10 stages. These include two planktonic naupliar stages, one infectious copepodid stage, and seven on-host stages (four chalimus, two preadult and one adult; Wootten et al., 1982; Johnson and Albright, 1991). Numerous studies investigated aspects of the life cycle, population dynamics and ecology of *L. salmonis* in relation to environmental factors (Ritchie et al., 1993; Heuch et al., 1995; Johnson et al., 1996; Pike and Wadsworth, 1999). However, remarkably fewer studies examined the nature of the host-parasite relationship. A few studies have reported tissue responses at the site of attachment and feeding, as well as copepodid distribution and load on the body of the host fish (Jones et al., 1990; Johnson and Albright, 1992a; Jonsdottir et al., 1992). Physiological effects following experimental infection of post-smolt Atlantic salmon with large numbers of *L. salmonis* were only recently reported (Grimnes and Jakobsen, 1996), and these effects included disturbance of hydromineral balance and mortality attributed to the development of subadult and adult stges of the lice.

One key factor which affects the health status of fish and increases susceptibility to disease is stress (Pickering and Pottinger, 1989; Wedemeyer, 1997). While the topic of stress in fish is complex and many of the mechanisms involved are not fully understood, cortisol and the catecholamines are crucial in mediating the integrated stress response (see review by Wendelaar Bonga, 1997). Salmonids intensively raised in aquaculture have high stress levels and these are compounded by aquaculture practices such as transport, grading and routine maintenance (Barton and Iwama, 1991), increasing susceptibility to disease, including ectoparasites. Johnson and Albright (1992b) have shown that intra-peritoneal administration of cortisol influenced the salmonid host-parasite interaction and increased susceptibility of coho salmon to experimental infection with *L. salmonis* by suppressing inflammatory response and epithelial hyperplasia in skin and gills.

The skin and gills of a fish are covered by complex epithelia comprised of several layers of living cells that are continuous over the body surface (Whitear, 1986; Hinton, 1993; Iger et al., 1995; Nolan et al., 1998). They form the first barrier between the external and the internal environment and are protected by a chemically and functionally complex mucous coat that is discharged by specialized mucous cells in the epidermis (Shephard, 1994). The teleostean epidermis is apparently

influenced by a number of endocrine factors (Pickering and Pottinger, 1989; Iger et al., 1995) and shows characteristic changes in fish exposed to stressors. It is proposed that salmonids probably die from osmoregulatory problems caused by adult lice mechanically disrupting the skin (Wootten et al., 1982). Recently, osmoregulatory failure and mortality by developing adult stages have been demonstrated in Atlantic salmon (Grimnes and Jakobsen, 1996), yet possible indirect effects of the parasite on the epithelia have not been examined. Such effects will become expressed at locations distant from the feeding and attachment areas and may also adversely affect osmoregulation.

The skin epithelium of fish responds strongly to stressors (Iger et al., 1995; Nolan et al., 1998) and offers good possibilities for evaluating indirect stress effects of ectoparasites on the host fish. Several responses, such as increased apoptosis of branchial chloride cells and the pavement cells of the skin, have been shown to be under control of the stress hormone cortisol, and will therefore also occur in areas not affected directly by the parasites, including the gills. These stress parameters also allow any relationship between the effects of sea lice and susceptibility to secondary infection to be established, through examination of the skin and gill epithelia. The direct tissue damage caused by parasite grazing, and indirect epithelial effects and immunosuppression, offer opportunities for invasion by secondary pathogens if the overall integrity of the epithelia is compromised during, or remains compromised beyond, this period. These effects may disrupt hydromineral balance and can be assessed by measuring blood ion levels and the activity of key ionoregulatory Na^+/K^+-ATPases, such as in the gill.

The objective of the present study was to determine whether infection with low numbers of adult and preadult *L. salmonis* induced a stress in the Atlantic salmon. We studied parameters in the skin and gill (ultrastructure through light and electron microscopy, as well as gill Na^+/K^+-ATPase) coupled with blood parameters (serum sodium, chloride, calcium, protein and urea), to reveal the presence of a stress response.

Materials and Methods

Experimental setup

Four groups of 40 seawater-adapted Atlantic salmon (200-250 g body weight) were placed in 400-L circular green plastic holding tanks at the Bantry research facility of the National Aquaculture Development Centre, Cork, Ireland. The tanks were provided with a constant supply of temperature-controlled sea water at 15°C under a 12:12 (L:D) photoperiod. After 3 weeks acclimation, 3 groups were infected with either 120, 240 or 400 sub-adult and adult *L. salmonis* of both sexes, to achieve initial infection levels of 3, 6 and 10 lice/fish. Infection was carried out in the evening by pouring sea water containing the parasites into each tank. Seawater only was poured into the control tank. Seven fish from each group were sampled at 24 hours, 5 and 10 days post infection (1, 5 and 10 DPI).

Sampling procedure

All fish were killed by concussion at the dorsal part of the head. Blood was collected by needle from the caudal blood vessels, allowed to clot for 24 h at 4 °C and centrifuged to obtain serum. Samples of skin from the rostral part of the head above the nostrils, and small pieces from the second gill arches, were fixed in Bouin's fixative for light microscopy and 3% glutaraldehyde buffered in Na-cacodylate (0.09 M, pH 7.3) with post-fixation in 1% osmium tetroxide in the same buffer for electron microscopy (Iger et al. 1995). The samples were taken from areas where no parasites were attached or where no indications of previous feeding activity were observed. Another series of head skin samples were taken at locations where parasites were present and fixed for electron microscopy in the same way. The remaining gills were dissected, the filaments trimmed from the gill arch, placed in 1 ml of ice-cold buffer (0.3 M sucrose, 20 mM Na_2EDTA, 0.1 mM imidazole, pH 7.1 with HCl) and immediately frozen for measurement of Na^+/K^+-ATPase activity.

Sample processing

For quantification of mucous cells, Bouin-fixed skin samples from each fish were processed into paraffin blocks and 5µm sections were cut perpendicular to the plane of the epidermis. Replicate series of sections were stained with periodic acid-Schiff's stain to identify total mucous cells and the Alcian blue (pH 2.5) method to identify acidophilic mucous cells (Blackstock and Pickering, 1982). Total numbers of mucous cells were counted in ten independent 300 µm views from each sample using a calibrated micrometer in the ocular lens of a light microscope, averaged for each fish and the mean number of cells per millimeter of transverse epidermal section was calculated. Gill samples from each fish were processed by sectioning perpendicular to the plane of

the lamellae, stained with haematoxylin and eosin, and examined for general morphology by light microscopy.

For transmission electron microscopy (TEM), one replicate set of fixed skin and gill samples from 4 fish was stained for 2 h in 2% uranyl acetate, dehydrated through an ethanol series and embedded in Spurr's resin. Ultra-thin sections were cut by diamond knife, collected on 150 mesh copper grids and contrasted with lead citrate before viewing in a Philips EM 300 transmission electron microscope at 40 kV. For skin, the overall ultrastructure was observed and attention focused on cellular necrosis (characterized by nuclei with aggregations of chromatin, swelling of the cytoplasmic compartment and loss of apical microridges) and apoptosis (characterized by progressive densification of the nucleus, organelles and cytoplasm, leading to shrinkage and loss of contacts with surrounding cells), leukocyte infiltration and epithelial integrity (evaluated by intercellular swelling and cell-cell contacts). For gills, the overall ultrastructure and the appearance of the respiratory lamellae were observed. In addition, the interlamellar chloride cell populations were studied, and necrosis, apoptosis (characterized for chloride cells by progressive densification of the mitochondria, nucleus and cytoplasm, as well as dilation of the tubular system) and leukocyte infiltration were evaluated. From micrographs, the epithelia were evaluated and scored semi-quantitatively against control using a graded evaluation system (-, +, ++, +++, ++++), representing parameter unaffected (-) to strongly affected (++++).

A second replicate set of skin and gill samples were examined by scanning electron microscopy (SEM). These samples were dehydrated through an ethanol series, critical point dried under liquid carbon dioxide, gold sputtered in a Balzers coating unit (CPD 020, Balzers, Switzerland) and viewed in a Jeol-JSM T 300 scanning electron microscope.

The sera were analyzed for sodium and chloride indirectly using ion-specific electrodes, calcium using o-cresolphthalein complexone, and urea using urease and glutamate dehydrogenase. Total protein was measured with the biuret reagent. All determinations were carried out on a Hitachi 747 automatic analyzer. The assay of Na^+/K^+-ATPase activity in the gill tissue was carried out on first homogenates according to the method of Flik et al. (1984).

Statistical analysis

Data are expressed as means ± S.E.M. of 7 fish for each treatment at each time point. Differences between control groups over experimental time were assessed by ANOVA of log transformed data (except for Na:Cl ratio). Data sets for each timepoint (except Na:Cl ratio) were log transformed, analyzed by ANOVA and significance between treatments was assessed using the Bonferroni Multiple Comparisons Test. For Na:Cl ratio, data were tested using the Mann-Whitney *U* non-parametric test. Statistical significance was accepted at $P<0.05$.

Results

Skin

Ultrastructure of head skin from control salmon by SEM showed the surface was a continuous sheet of pavement cells (Figure 1A). Examination by TEM at 1, 5 and 10 DPI revealed several distinct cell populations (Figure 2A). The most numerous cell type was the filament cell, which was characterized by numbers of perinuclear microfilaments. The uppermost layer of cells was differentiated into specialized pavement cells, which had apical microridges (Figure 2A). Tight junctions and desmosomes maintained cell-cell contacts between pavement cells and filament cells. Little intercellular spacing occured in the epithelium (Figure 2A). Mucous cells differentiated in the middle layers of the epidermis and migrated through to the upper layer where they discharged their contents onto the body surface. Low numbers of leukocytes were also present (Table 1).

The skin ultrastructure from the head of parasitized fish by SEM in areas distant from the feeding sites of the parasites showed marked epithelial changes (Figure 1C), compared to control fish (Figure 1A), including swelling and shedding of the pavement cells (Figure 1C). At sites of copepod feeding, gross damage was visible as lesions of disrupted and swollen pavement cells (Figure 1B). In all fish examined, lines of ducts were observed in the scaleless region of the head (Figure 1D) and, when examined by LM, these ducts were richly-lined with mucous cells (Figure 1E).

There was no statistical difference between the counts of cells stained with the PAS or the Alcian blue (pH 2.5) method at any sample point, therefore the total numbers of cells counted from PAS staining are given. There were significantly fewer mucous cells in the non-feeding areas of the epidermis of infected fish when compared to controls at 1, 5 and 10 DPI (Table 3).

There were strong effects in the epidermis of fish infected with 3 parasites/fish at non-feeding sites at 1 DPI. (Figure 2B). Necrotic pavement cells were commonly observed. Increased intercellular spaces and increased incidence of heavily stained desmosomes were typical (Table 1, Figure 2B). Leukocytes were seen within the intercellular spaces throughout the epidermis. At 5 DPI, heavily stained desmosomes were very abundant (Figure 2C). Apoptotic cells were occasionally encountered, but necrosis in the pavement cell layer was reduced. At 10 DPI, the condition of the skin of these fish was slightly recovered compared with 5 DPI (Table 1).

Table 1. Semi-quantitative evaluation of the epidermal responses of head skin of post-smolt Atlantic salmon (*Salmo salar*) to 3 infection levels of pre-adult and adult sea louse *Lepeophtheirus salmonis*. The samples are taken away from areas of parasite attachment and feeding. (- = unaffected to ++++ = strongly affected).

Skin parameter	Control	3 lice/fish	6 lice/fish	10 lice/fish
Day 1 and day 5				
Pavement cell necrosis	-	++	+++	++++
Heavily stained desmosomes	-	+++	+++	++++
Intercellular space	-	+++	+++	++++
Mucous cell discharge	-	+++	+++	+++
Apoptotic cells	+	++	+++	+++
Leukocyte infiltration	+	++	++	++
Day 10				
Pavement cell necrosis	-	-	++	+++/+
Heavily stained desmosomes	-	++	++	+++
Intercellular spaces	-	+	++++	+++/+
Mucous cell discharge	-	++	++	++
Apoptotic cells	+	+	++	++
Leukocyte infiltration	+	++	++	+++/+

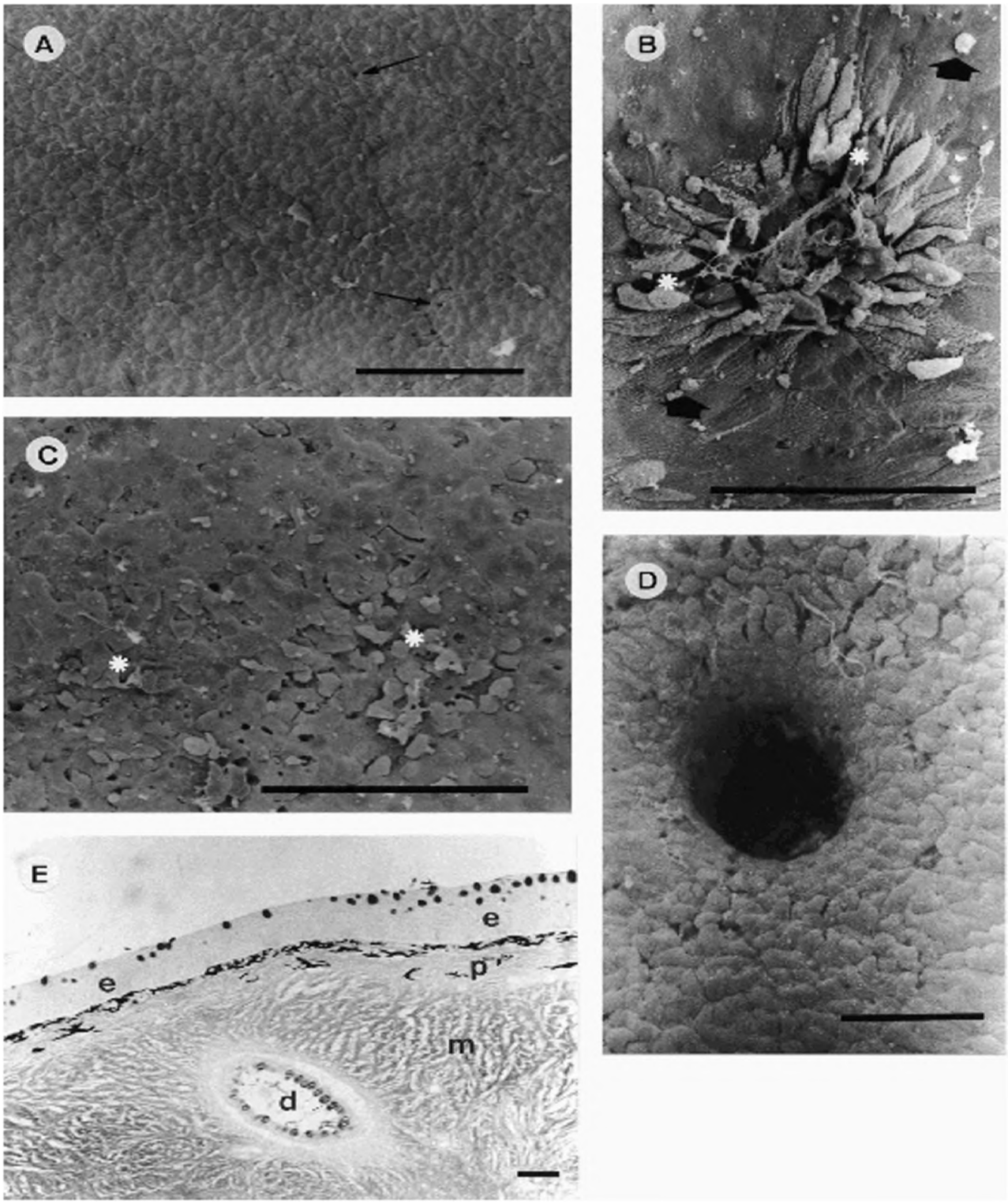


Figure 1. (A) The head skin of control *Salmo salar* seen in the scanning electron microscope. The individual pavement cells form a continuous sheet and occasional pores are discharging mucous cells (arrow). Scale bar = 100 µm. (B) Scanning electron micrograph of head skin of *S. salar* where *L. salmonis* was present. The pavement cells are mechanically damaged by the activities of the parasite and much residual mucus is visible around the lesion (*). Many pores from discharging mucous cells can be seen (arrows). Scale bar = 100 µm. (C) Scanning electron micrograph of head skin of *S. salar* at 1 DPI with *L. salmonis* (6 parasites/fish). Sample was taken away from place of attachment of parasites. Swollen pavement cells can be seen sloughing off (*), many mucous pores are visible and the integrity of the pavement cell layer is disrupted. Scale bar = 50 µm. (D) Scanning electron micrograph of a mucous cell-lined duct from the head of control Atlantic salmon *S. salar*. Scale bar = 50 µm. (E) Light microscopy view through the head skin of control *S. salar*. The stain shows mucous cells in the epithelium (**e**) and a mucous cell lined duct (**d**). **p**, pigment cells; **m**, muscular tissue. Scale bar = 100 µm.

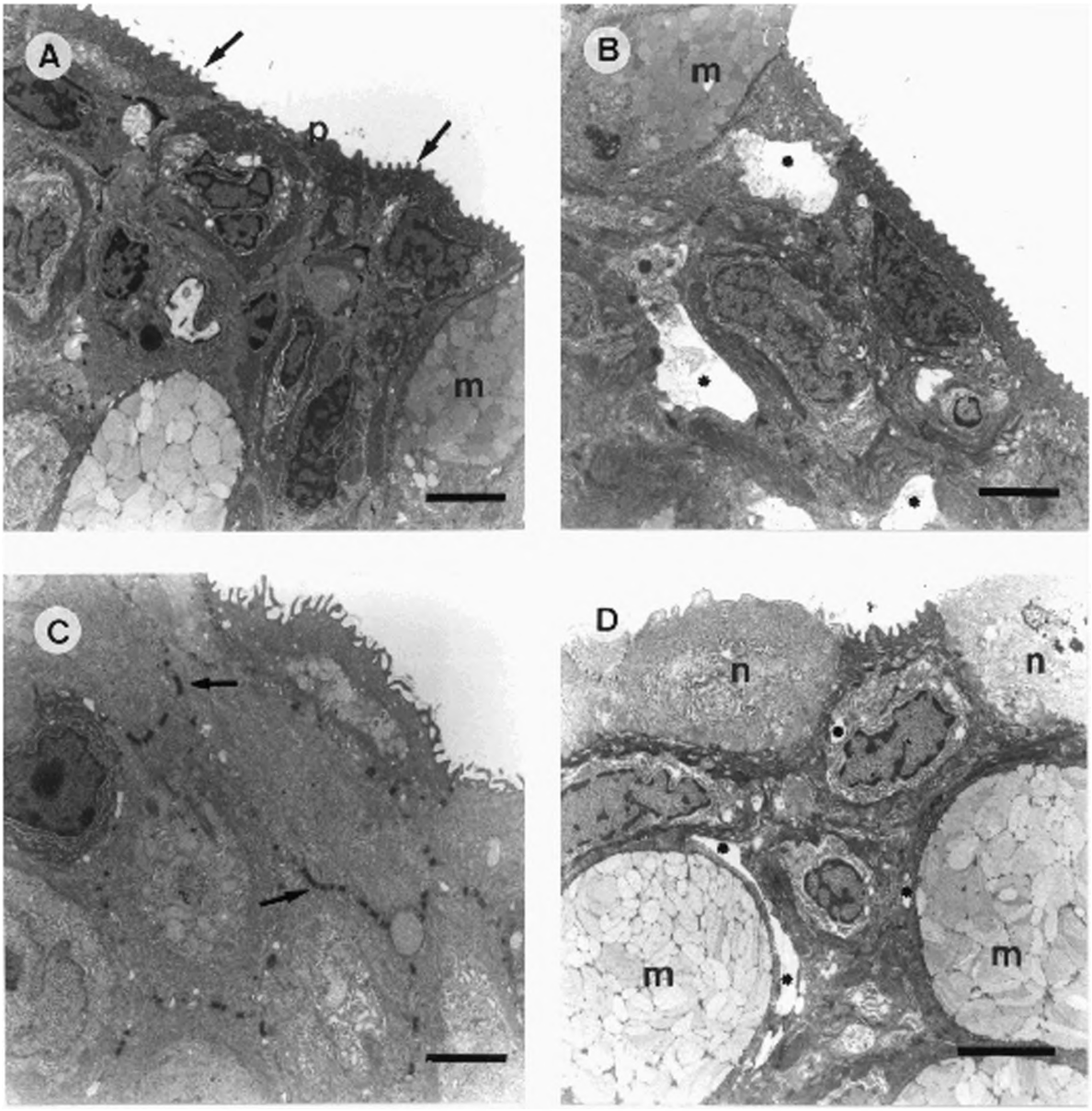


Figure 2. Transmission electron micrographs of skin epidermis *S. salar*. (A) Upper epidermis of control fish. The pavement cells (**p**) have apical microridges (arrows) and little intercellular spacing occurs. **m**, mucous cell. Scale bar = 5 µm. (B) One DPI with *L. salmonis* (3 parasites/fish). Intercellular spaces are visible (*) and epithelial integrity is disrupted. A discharging mucous cell can be seen (**m**). Scale bar = 5 µm. (C) Five DPI with 3 parasites/fish. Intercellular spaces are absent and epithelial integrity is good. Considerable numbers of electron-dense desmosomes (arrows) are seen at the borders of the filament cells. Scale bar = 5 µm. (D) One DPI with 6 parasites/fish. Many necrotic pavement cells (**n**) and intercellular spaces are present (*). Pre-discharge mature mucous cells can be seen close to the surface (**m**). Scale bar = 5 µm.

At 1 DPI the epidermal condition of fish infected with 6 parasites/fish was similar to the group infected with 3 parasites/fish (Table 1, Figure 2D). However, necrosis in the pavement cell layer was more severe, disruption of the underlying cell layers was greater and many pre-discharge mucous cells were located in the upper epidermis (Figure 2D). At 5 DPI, there was an increase in the staining intensity of the desmosomes and the intercellular spacing was reduced (Table 1, Figure 3A). Various stages of filament cell apoptosis were observed (Figure 3A). At 10 DPI, the overall situation was comparable to 5 DPI, with the exception that intercellular spaces were more extensive and necrosis of the pavement cell layer was reduced (Table 1).

The 10 parasites/fish group showed the strongest epidermal effects at all sampling points (Table 1). At 1 DPI, there was extensive necrosis of the pavement cell layer, heavily stained desmosomes and intercellular spaces were present (Figure 3B). At 5 DPI, immature mucous cells could be seen discharging at the surface, and apoptosis and necrosis of the pavement cell were common (Figure 3C). At 10 DPI, there was some variation in the condition of the epidermis of individual fish. Some individuals still had extensive pavement cell necrosis and intercellular swelling, while others showed good recovery with a well differentiated and active pavement cell layer and good epithelial integrity (Figure 3D).

Gill

The external gill structure of uninfected salmon inspected by SEM and TEM were typical for salmonids we have sampled from aquaculture conditions (personal observation). The filaments had leaflike lamellae arranged alternately on both sides (Figure 4A). The respiratory epithelium was one cell thick and leukocytes were commonly in the intercellular spaces (Figure 4B). In the interlamellar areas, populations of mitochondria-rich chloride cells and mucous cells were located (Figure 4E). Apoptotic chloride cells were seen occasionally, whereas apoptosis and necrosis were uncommon in other cell types (Table 2). The epithelium of the filaments, with the exception of the interlamellar areas, resembled that of the skin, being comprised of filament cells, mucous cells, leukocytes and an apical pavement cell layer with microridges.

In lice-infected groups, SEM observations showed the lamellae were swollen and wrinkled (Table 2, Figure 4C). In TEM observations, the lamellar respiratory epithelium was uplifted and detached from the endothelium of the central blood sinus in many places (Figure 4D). Both mucous and chloride cells were found on the filaments, as well as on the lamellae. In lice-infected fish, the chloride cells showed an extensive dilated tubular system (Figure 4F), not observed in control fish (Figure 4E, Table 2).

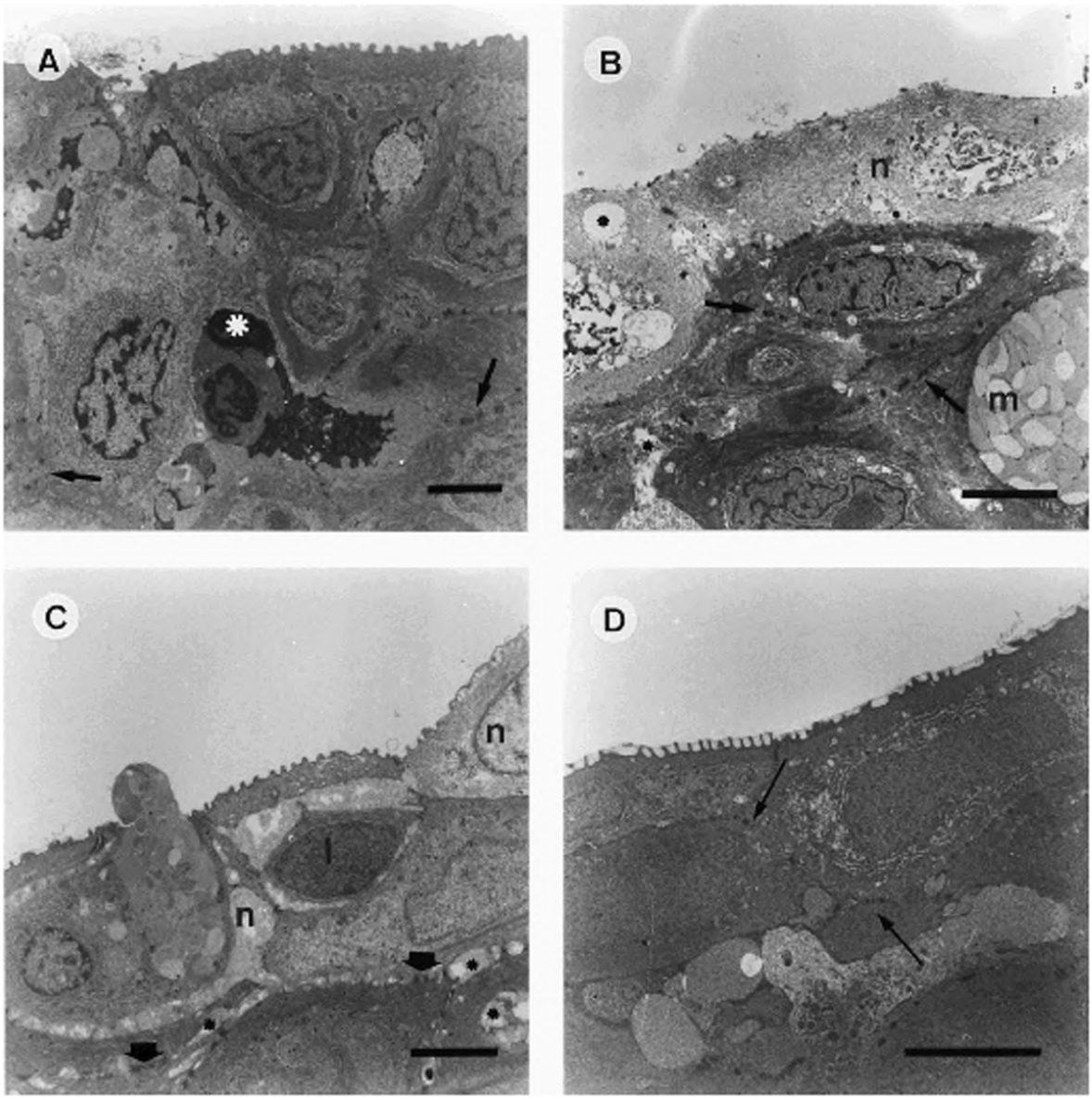


Figure 3. TEM of skin epidermis *S. salar*. (A) Upper epidermis 5 DPI with 6 parasites/fish. A macrophage (*) engulfs an apoptotic filament cell, intercellular spaces are absent, and numbers of electron-dense desmosomes (arrows) occur at the filament cell borders. Scale bar = 5 µm. (B) One DPI with 10 parasites/fish. Necrotic pavement cells (**n**) can be seen and intercellular spaces are present (*). Electron-dense desmosomes (arrows) are visible at the cell borders and a mature pre-discharge mucous cell can be seen (**m**). Scale bar = 5 µm. (C) Five DPI with 10 parasites/fish. Necrotic pavement cells (**n**) and intercellular spaces (*) still occur. A lymphocyte (**l**) infiltrates the pavement cell layer. Electron-dense desmosomes (arrows) are visible and a discharging immature mucous cell can be seen (**m**). Scale bar = 5 µm. (D) Ten DPI with 10 parasites/fish, taken from an area where there was no parasite attachment. Epithelial integrity is restored. Few electron-dense desmosomes (arrows) are visible. Scale bar = 5 µm.

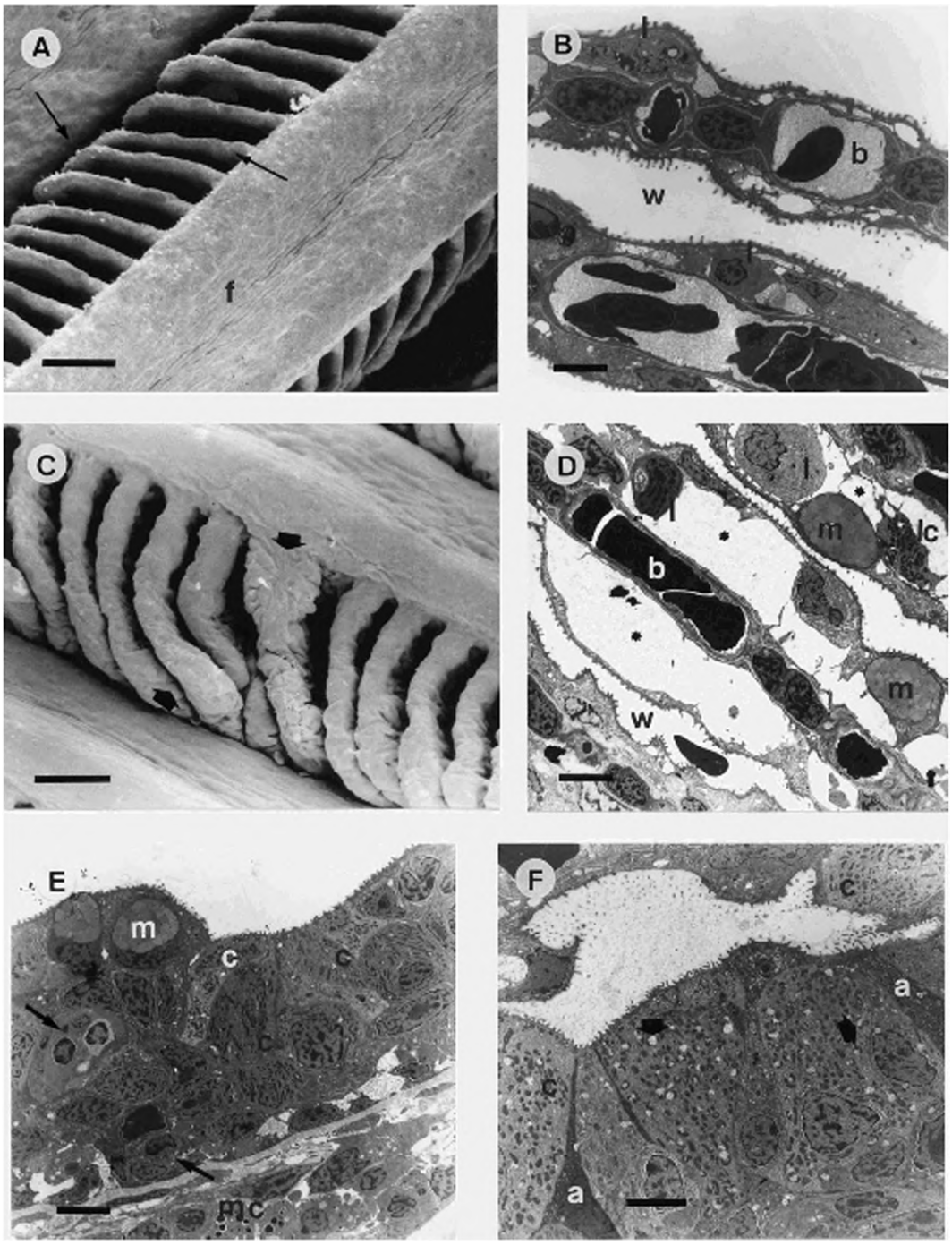


Figure 4. Micrographs of the gills from *S. salar*. (A) Gill of control fish seen in the scanning electron microscope. The filament (**f**) and lamellae (arrows) are clearly seen. Scale bar = 100 µm. (B) The ultrastructure of control gill lamellae. The blood space (**b**) and the water (**w**) are separated by a thin respiratory epithelium that is 1 cell layer thick. Leukocytes (**l**) infiltrate the epithelium. Scale bar = 5 µm. (C) One DPI with 10 lice/fish. The lamellae are wrinkled and swollen in the scanning electron microscope (arrows). Scale bar = 50 µm. (D) One DPI with 6 lice/fish. The distance between the blood space (**b**) and the water (**w**) is increased by epithelial uplifting. Numbers of leukocytes (**l**) can be seen in the epithelial intercellular spaces (*). Mucous cells (**m**) and chloride cells (**lc**) are present on the lamella. Scale bar = 5 µm. (E) Control fish. Many chloride cells (**c**) and mucous cells (**m**) are present. Epithelial integrity is good and some leukocytes (arrows) and mast cells (**mc**) can be seen. Scale bar = 10 µm. (F) One DPI with 10 lice/fish. The tubular systems of the chloride cells (**c**) are dilated (arrows) and apoptotic chloride cells are present (a). Scale bar = 5 µm.

Table 2. Semi-quantitative evaluation of the responses of the gill of post-smolt Atlantic salmon (*Salmo salar*) to 3 infection levels of pre-adult and adult sea louse *Lepeophtheirus salmonis*. (- = unaffected to ++++ = strongly affected).

Skin parameter	Control	3 lice/fish	6 lice/fish	10 lice/fish
Day 1 and day 5				
Pavement cell necrosis	-	++	+++	++++
Heavily stained desmosomes	-	+++	+++	++++
Intercellular space	-	+++	+++	++++
Mucous cell discharge	-	+++	+++	+++
Apoptotic cells	+	++	+++	+++
Leukocyte infiltration	+	++	++	++
Day 10				
Pavement cell necrosis	-	-	++	+++/+
Heavily stained desmosomes	-	++	++	+++
Intercellular spaces	-	+	++++	+++/+
Mucous cell discharge	-	++	++	++
Apoptotic cells	+	+	++	++
Leukocyte infiltration	+	++	++	+++/+

At 1 DPI, the lamellae of fish infected with 3 parasites/fish were swollen (Table 2). In the filament, mucus discharge was stimulated and in the interlamellar area apoptotic chloride cells were frequently seen (Table 2). Apoptotic chloride cells in the interlamellar area lost apical contact with the water and were apparently replaced by neighboring new chloride cells. At 5 DPI, apoptotic bodies, the last stage of apoptotic chloride cells, could be observed (Figure 5A), in addition to other apoptotic stages (Figure 5B).

The lamellae sampled from fish infected with 6 parasites/fish at 1 DPI were comparable with those of the 3 lice/fish group (Table 2). Mucus discharge was stimulated in the filament epithelium and intercellular spaces occurred with heavy leukocyte infiltration (Figure 5C). The chloride cell population and pavement cells in the interlamellar area were also strongly affected as desmosomes were reduced and apoptotic cells atrophied (Figure 5D). At 10 DPI, the gill structure in this group was comparable with the 3 lice/fish group, with the exception of lamellar swelling which still occurred (Table 2).

The group infected with 10 parasites/fish showed the strongest lamellar effects (Table 2). Cellular shrinkage and swelling of the lamellar respiratory epithelium was severe (Figure 5E). At 1 DPI, newly differentiated cells could be seen at the base of the lamella (Figure 5F). At 5 DPI, many macrophages containing cellular debris (comprised mainly of apoptotic bodies), were observed. The high turnover of chloride cells was continuous and observed at 5 and 10 DPI in most fish examined, although there was some variation between individuals at 10 DPI (Table 2).

Blood parameters

Compared with control values, serum sodium was significantly lower in the 10 lice/fish group sampled at 1 DPI and serum chloride was significantly higher at 5 DPI in both the 3 and 6 lice/fish groups (Table 3). Although the serum sodium and chloride levels did not differ significantly in any other group at any other time, the serum Na:Cl ratio in all lice infected groups was significantly reduced at 5 DPI (Table 3). Serum total calcium levels, protein, and urea were significantly elevated in the 10 lice/fish group sampled at 1 DPI (Table 4) but not at any other time point or in any of the other groups.

Gill Na^+/K^+-ATPase

The gill Na^+/K^+-ATPase activity was significantly higher in all infected fish at 5 DPI, and remained elevated in the 10 lice/fish group sampled at 10 DPI (Table 3).

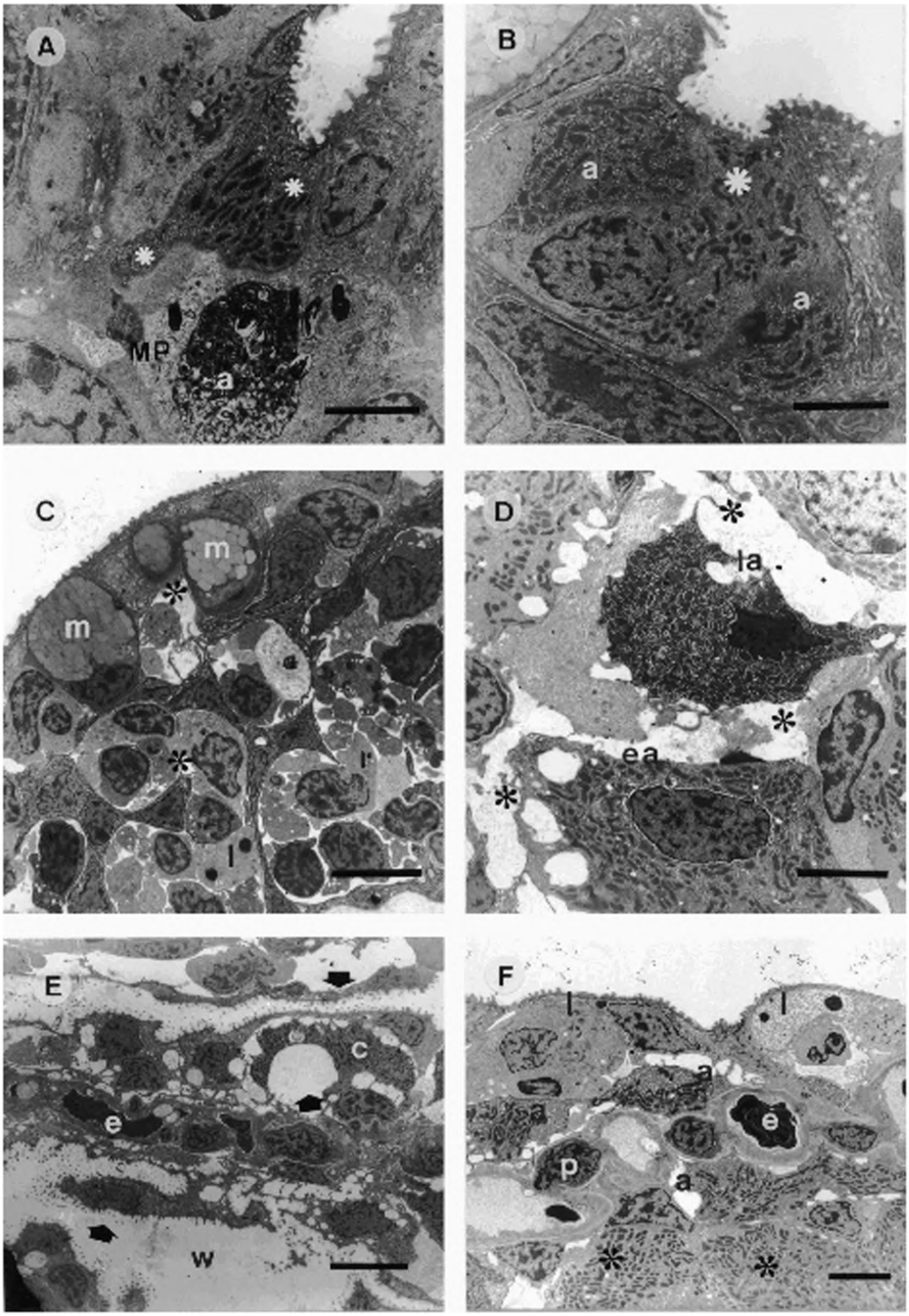


Figure 5. TEM of the gills from *S. salar*. (A) The interlamellar region at 5 DPI with 3 lice/fish. Late stage apoptotic chloride cells are present as condensed apoptotic bodies (**a**) located in a macrophage (**mp**) and electron-dense earlier stage apoptotic chloride cells with dilated tubular systems can be seen (*). Scale bar = 5 µm. (B) Five DPI with 3 lice/fish. Apoptotic chloride cells (**a**) will be replaced by newly differentiated replacement chloride cells (*). Scale bar = 5 µm. (C) The gill filament at 5 DPI with 6 lice/fish. The epithelial structure is disrupted and the intercellular spaces (*) are massively infiltrated by leukocytes (**l**); m, mucous cell. Scale bar = 10 µm. (D) Five DPI with 6 lice/fish. Late (**la**) and earlier stage apoptotic chloride cells (**ea**) can be seen; *, intercellular spaces. Scale bar = 5 µm. (E) One DPI with 10 lice/fish. The respiratory epithelium is wrinkled, individual cells are shrunken, and intercellular spaces occur (arrows). A shrunken chloride cell (**c**) is out on the lamella; **e**, erythrocyte; **w**, water. Scale bar =5 µm. (F) Lamellar chloride cells at 1 DPI with 10 lice/fish. Apoptotic chloride cells (**a**) occur in the inner layers of the epithelium while newly differentiating ones are observed below (*). Leukocytes (**l**) are in the upper layer. **p**, pillar cell; **e**, erythrocyte. Scale bar = 5 µm.

Table 3. Parameters investigated in Atlantic salmon (*Salmo salar*) infected with numbers of pre-adult and adult sea louse *Lepeophtheirus salmonis*. Data for serum sodium and chloride values are (mmol·l^{-1}), gill Na^+/K^+-ATPase activity is µmol ATP·mg protein^{-1}·h^{-1} and total numbers of mucous cells are per mm skin epidermis. Data are expressed as means ± S.E.M. for n=7. *=P<0.05, **=P<0.01, ***=P<0.001 compared with control values.

Serum parameter	Control	3 lice/fish	6 lice/fish	10 lice/fish
24 hours				
Sodium	170±2.5	164±3.1	167±2.6	159±2.2*
Chloride	141±4.5	136±2.6	142 ±4.2	134±2.2
Na/Cl Ratio	1.20±.02	1.20±.02	1.18±.02	1.19±.01
Gill Na^+/K^+-ATPase	8.2±0.97	8.3±0.41	8.7±.0.38	9.7±0.41
Epidermal mucous cells	91.9±6.2	63.5±16.9**	66.3±6.1**	65.1±8.1**
5 days				
Sodium	166±1.1	169±3.0	168±1.4	162±2.4
Chloride	132±1.5	143±3.7*	142±1.9*	137±2.4
Na/Cl Ratio	1.26±.01	1.19±.01***	1.18±.01***	1.20±.02**
Gill Na^+/K^+-ATPase	8.9±0.82	14.43±1.04**	15.88±1.30**	15.0±0.67***
Epidermal mucous cells	91.3±4.6	62.3±3.7**	65.4±7.4**	64.4±4.5**
Day 10				
Sodium	169±1.1	171±2.4	170±1.7	171±1.3
Chloride	137±1.6	136±1.9	142±1.8	135±1.0
Na/Cl Ratio	1.24±.02	1.27±.01	1.20±.01	1.26±.01
Gill Na^+/K^+-ATPase	7.0±0.27	7.9±1.42	7.8±1.04	13.3±0.70**
Epidermal mucous cells	90.8±6.2	54.2±8.2**	57.6±11.6**	50.0±6.5***

Discussion

Infection with low numbers of pre-adult and adult sea louse *L. salmonis* had a marked effect on the integrity of the skin and gill epithelia of the Atlantic salmon. This was an indirect effect of the parasite, as it was reported from areas of epithelia where there was no obvious evidence of prior parasite attachment. The results of this study lead to the conclusion that many of the epithelial changes reported are similar to those described for stressors in general (Wendelaar Bonga, 1997), including toxic agents (chemical stressors), handling, and a submissive position in hierarchy. These effects are likely hormone-mediated, as a consequence of the infection causing an integrated stress response in the fish, resulting in increased levels of circulating cortisol and catecholamines. These results demonstrate that the effects of infection with the sea louse *L. salmonis* can be divided into two distinct categories. The first are the direct effects of parasite attachment and feeding on the body surface, as reported by others (Jones et al., 1990; Johnson and Albright, 1992a; Jonsdottir et al., 1992; MacKinnon, 1993) and confirmed in this study, i.e. direct damage caused by parasite feeding etc. The second are the indirect effects of the integrated stress response on the integrity of the skin and gill epithelia, including the osmoregulatory consequences in terms of gill Na^+/K^+-ATPase, as reported here for the first time.

Table 4. Serum protein, urea and total calcium of Atlantic salmon (*Salmo salar*) at 24 hours post infection with numbers of pre-adult and adult sea louse *Lepeophtheirus salmonis*. Data are expressed as means ± S.E.M. for n=7. *=P<0.05, **=P<0.01 compared with control values.

Serum parameter	Control	3 lice/fish	6 lice/fish	10 lice/fish
Protein (mg/dl)	28.7±6	42.3±5	42.0±5	50.7±2*
Urea (mM)	2.3±0.2	2.2±0.1	2.3±0.2	3.0±0.1*
Total calcium ($mmol \cdot l^{-1}$)	2.7±0.2	3.1±0.2	3.3±0.2	3.6±0.1**

At non-feeding sites, infestation with *L. salmonis* caused necrosis in the pavement cells of the skin, increased apoptosis in the inner cell layers of the epidermis, and stimulated mucus discharge. Necrosis is a form of cellular death directly caused by toxic factors and other damaging agents. Pavement cell necrosis has been reported for a number of toxic chemicals in freshwater (Iger et al., 1994c; Burkhardt-Holm et al., 1997; Nolan et al., 1998; Chapters 2 and 3) and also during seawater adaptation (Wendelaar Bonga and van der Meij, 1989; Uchida et al., 1996) and water acidification (Wendelaar Bonga et al., 1990; Iger and Wendelaar Bonga, 1994). The necrotic cells were restricted to the pavement cell layer of parasitized salmon, suggesting that a change in the protective role of the mucus layer may have been a contributing factor, possibly by reduced protection as a result of a reduction in either total mucus coat or a change in mucus composition. The function of the mucous layer on the body surface of the fish, and its components and composition have been reviewed, although there is still uncertainty about the involvement of mucus in ionoregulation in fishes (Shephard, 1994). Increased mucus secretion in the present study was indicated by the numbers of discharging and pre-discharge mucous cells seen in TEM and reduced total numbers of epidermal mucous cells in infested fish. Ectoparasites have been shown to reduce the numbers of mucous cells in brown trout epidermis (Pottinger et al., 1984) and stimulation of mucus discharge is a cortisol-mediated effect in rainbow trout *in vivo* (Iger et al., 1995).

The deletion of cells from a tissue is achieved by apoptosis, a physiologically controlled process, which activates a genetically-programmed mechanism that induces death and elimination of a cell. The stimulation of apoptosis in the skin and gill of parasitized fish is typical of the general epithelial response reported in many fishes (Wendelaar Bonga and van der Meij, 1989; Nolan et al., 1998) and indicates increased aging, and therefore increased cell turnover, within these epithelia (Wendelaar Bonga, 1997). The mechanisms controlling this process are not fully elucidated, but recently, it has been shown *in vivo* (Iger et al., 1995) and *in vitro* (Bury et al., 1998; Chapter 7) that cortisol promotes apoptosis. The increased apoptosis in skin and gill epithelia of the sea lice infested fish in the present study may result from elevated circulating cortisol levels.

The overall structural integrity of the epithelia was disrupted, as indicated by the presence of widened intercellular spaces. These spaces are caused by hydrostatic pressure and osmotic forces and are prominent during periods of epithelial cell elimination through necrosis and apoptosis and increased mucus discharge. As numbers of cells are eliminated, the contacts between the remaining cells are reduced and the spaces become infiltrated with leukocytes. The stimulation of leukocyte infiltration and increased epithelial permeability are probably catecholamine-mediated effects (see review by Wendelaar Bonga, 1997). The increase in electron-density of the desmosomes connecting the epithelial cells of the skin was correlated with parasite infestation. These desmosomes may fortify the epidermis and reduce intercellular swelling. Heavily stained desmosomes in the present

study were observed predominantly in the epidermis of fish that had relatively high levels of damage. In a study of sea lice effects on skin of Atlantic salmon, electron dense desmosomes between cells have also been shown in the skin of control fish, but a different tissue fixation method for TEM was used (MacKinnon, 1993).

In the gill, catecholamine action also increases branchial blood flow, resulting in increased gill perfusion and blood pressure (Satchell, 1991; Perry and Laurent, 1993; Lock et al., 1994; Wendelaar Bonga, 1997). The lamellar swelling reported in the present study in parasitized fish may reflect the action of high levels of catecholamines released as a result of parasite-induced stress. We are confident that the effects we report in the gills do not result from any direct action of the parasite, as we infected our fish with pre-adult and adult parasites, while only the copepodid and chalimus stages have been reported to attach to the gills (Johnson and Albright, 1992a, b). Inspection of the gills from our infected fish by light microscopy and SEM revealed no parasites or parasite damage to the gills. In addition, at the water temperature in our experiment (15 ^{o}C), the generation time from egg to infective stage is estimated at 7.4 days (Johnson and Albright, 1991), so if successful reproduction had occurred, the infective stages would have been active shortly before the 10 DPI sample point. This was when the 3 and 6 lice/fish (but not 10 lice/fish) showed recovery.

Infestation with sea lice increased gill Na^{+}/K^{+}-ATPase activity and induced chloride cell apoptosis without greatly disrupting hydromineral balance. Elevated cortisol levels in fish stimulate chloride cell differentiation *in vitro* (McCormick, 1995) and improve hypoosmoregulation *in vivo* (Madsen, 1990; Cornell et al., 1994), as well as inducing chloride cell apoptosis *in vitro* (Bury et al., 1998). Cortisol is a pluripotent hormone whose effects can be advantageous at moderately elevated levels and deleterious at highly elevated levels (Wendelaar Bonga, 1997). In parasitized fish in the present study, the infection increased gill Na^{+}/K^{+}-ATPase activity at 5 DPI in all groups, increased aging of the mature chloride cells and stimulated differentiation of a new chloride cell population and these responses may have contributed to the maintenance or restoration of hydromineral balance. At 10 DPI, gill Na^{+}/K^{+}-ATPase activity and chloride cell apoptosis had returned to control levels in the 3 and 6 lice/fish groups. However, Na^{+}/K^{+}-ATPase activity and chloride cell apoptosis in the 10 lice/fish group remained above control levels indicating that recovery was incomplete and that the stress was greater at this infestation level.

The effects on the chloride cells of the 10 lice/fish group were the strongest. These were correlated functionally at 24 hours post-infection with decreased serum sodium and increased urea, total calcium and protein in this group. Teleosts are ammoniotelic and excrete most nitrogenous waste through the gill as ammonium, NH_4^+. This occurs through the chloride cells and elevated levels of urea in the blood are indicative of a rise in the production of nitrogenous waste products, as is typical for stressed fish (Wendelaar Bonga, 1997), and may reflect impaired excretion of these products as a result of disrupted gill function. The decreased sodium and increased calcium levels further support this. The former reflects the activity of increased gill Na^+/K^+-ATPase activity to eliminate excess sodium influx and/or effects of reduced drinking rate, while the latter cannot be explained. Another study has reported increased plasma chloride and decreased serum total protein in Atlantic salmon infested with adult *L. salmonis* (Grimnes and Jakobsen, 1996). This contrasts with the present study where we report an increase in serum protein, no increase in serum sodium and a small increase in serum chloride at 5 DPI in the 3 and 6 lice/fish groups. However, the study of Grimnes and Jakobsen (1996) involved much higher infestation levels on considerably smaller fish (median infestation intensity 64 lice/fish at 11 days and 42 lice/fish at 31 days following infection with 86-176 infective copepodids/40 g fish) and these numbers of lice caused osmoregulatory failure.

The results of this study are based on single groups of fish for each experimental treatment. Although several replicate tanks per treatment are preferable, this was not possible. However, we are confident that the effects we report here are representative, as we have carried out later experiments infecting groups of salmonids with the crustacean ectoparasite *Argulus foliaceus*. Here, using a similar methodology and replicate treatment tanks, no differences were noted between replicate tanks for any treatment group at any time (Ruane et al., 1999a).

In the present study, we report the presence of mucous cell-lined ducts in the head region of Atlantic salmon. These ducts have not been reported previously, to the best of our knowledge, and we have confirmed the presence of these ducts on other salmonids (*Salmo trutta* and *Oncorhynchus mykiss*). We propose that, under normal circumstances, these ducts provide a rich supply of mucus to flow back over the body surface. Sea lice infestation of the scaleless head region can be severe (Pike, 1989; Boxshall and Defaye, 1993). The parasites may find this area attractive because the mucus provides a rich food source, and preferentially gather there during heavy infestation, when competition between parasites for feeding resources may be high.

In conclusion, infection of post-smolt Atlantic salmon with low numbers of the sea louse *L. salmonis* had marked effects on the epithelia of the skin and gills, both locally in the skin as a result of parasite attachment and feeding (a direct effect), and overall as a result of the stress response of the fish to infection (an indirect effect). Hydromineral balance in the blood was not greatly affected,

but increased gill Na^+/K^+-ATPase activity indicated that while the epithelial integrity was compromised, a high rate of sodium turnover occurred. The fish could apparently adapt to an initial infection level of 3-6 lice/fish within 10 days, but not to 10 lice/fish. Thus, infestation was stressful and at higher infection levels, created a prolonged period during which the overall compromised integrity of the skin and gill epithelia, as well as stress-related immunosuppression, may render the fish susceptible to secondary infections.

Acknowledgments

The excellent technical assistance of J.C.A. van der Meij (transmission electron microscopy) and H.P.M. Geurts (scanning electron microscopy) is gratefully acknowledged. This work was supported by a grant from the Graduate School M&T, The Netherlands to D.T.N.

Chapter 5

The host-parasite interaction between the rainbow trout (*Oncorhynchus mykiss*) and the crustacean ectoparasite *Argulus japonicus*: short-term host response and influence of cortisol.

D.T. Nolan, N.M. Ruane, J. Rotllant[1], P.H.M. Balm and S.E. Wendelaar Bonga.

[1]) Unit of Animal Physiology, Department of Cell Biology and Physiology, Universitat Autonoma de Barcelona, 08193-Cerdanyola, Spain.

Abstract

The effects of cortisol on the host-parasite interaction between the rainbow trout *Oncorhynchus mykiss* and the fish louse *Argulus japonicus* were investigated by the administration of low levels of dietary cortisol prior to infecting the fish with low numbers of the parasite. The dietary cortisol elevated blood cortisol and glucose levels and stimulated the synthesis of vesicles in the upper epidermal cells of the skin 24 h post treatment. Infection with 6 lice per fish resulted in the infiltration of the skin epidermis by lymphocytes in areas that were not infested. The numbers of lymphocytes in the blood at 48 h post infection with the parasite were reduced. Other changes in the skin, characteristic of exposure to many types of stressors and that are stimulated by cortisol administration, were also found in the epidermis of parasitized fish, in spite of the fact that neither plasma cortisol nor glucose level was affected. Glucocorticoid receptors, localised immunohistochemically, were found in the pavement and filament cells, and in the migrating leukocytes of the upper epidermis. Cortisol feeding reduced the numbers of parasites, indicating that cortisol may mediate effects on the host skin that protect against the parasite. This study shows that a mild stress response may be adaptive when dealing with a parasitic louse. Further, we suggest that the adaptive response operates via the glucocorticoid receptor in the skin epidermis, which are located at the site of parasite attachment and feeding.

Introduction

Host-parasite relationships are highly complex and to understand this relationship, it is necessary to have knowledge about the host response to the infestation and factors such as stress level, general health, and maturation state that might interfere with this response. The branchiuran fish lice belonging to the genus *Argulus* are responsible for disease outbreaks in a wide variety of fish species throughout the world (Rushton-Mellor and Boxshall, 1994; Rahman, 1995). With some exceptions, these lice occur in fresh water and are a major problem for fish farmers in developing countries (Singhal et al., 1990; Jafri and Ahmed, 1991; Rahman, 1996), but are also a problem in European fish culture (Buchmann et al., 1995; Grignard et al., 1996). For example, a rainbow trout (*Oncorhynchus mykiss*) culture in Portugal (Menezes et al., 1990) and a Scottish rainbow trout and brown trout (*Salmo trutta*) stillwater fishery (Northcott et al., 1997) were decimated by *A. foliaceus*.

However, despite the real and potential economic losses from crustacean ectoparasites in fisheries in general and in aquaculture, little is known about the nature of the relationship between the lice and their hosts. Since the early 1970's, it has been recognised that the host fish can affect several aspects of lice biology, but the mechanisms behind these effects are poorly understood. Host rejection of the cyclopoid copepods *Lernaea cyprinacea* and *Lernaea polymorpha* was reported in both naïve and previously infected fish (Shields and Goode, 1978; Woo and Shariff, 1990) and it was thought that the rejection was in part due to cellular responses of the host. With respect to the salmon louse *Lepeophtheirus salmonis*, naïve hosts of different species of salmon showed variability in their susceptibility to infection (coho < chinook < Atlantic) (Johnson and Albright, 1992a). The coho salmon were the most resistant to parasite establishment and mounted strong tissue responses leading to parasite loss. Naïve Atlantic salmon, however, were highly susceptible to infection and failed to mount any significant tissue responses to any of the developmental stages. Cortisol administered in cocoa butter implants strongly elevated cortisol levels in the blood, suppressed the inflammatory response and the development of epithelial hyperplasia in the normally resistant coho salmon and resulted in the inability of the fish to shed *L. salmonis* (Johnson and Albright, 1992b).

The relative importance of innate and acquired immune responses in the relationship between fish lice and their hosts is not well understood, but does differ among species. In naïve hosts of some species however, it appeared that innate immune responses such as inflammation and epithelial hyperplasia, were important in the response to parasite infection (e.g. *L. salmonis* on coho salmon; Johnson and Albright, 1992a, b). Additionally, cellular responses caused changes in the distribution of *Lernaea piscinae* from the body to the cornea of the big head carp *Aristichthys nobilis* (Shariff and Roberts, 1989), and rejection of the copepod *Ergasilus labracis* together with a reduction in egg sac production on striped bass *Morone saxatilis* were attributed to a well-developed epithelial tissue response (Paperna and Zwerner, 1982). In other species including both naïve and

naturally infected Atlantic salmon, only minor host tissue responses occurred at the point of attachment of the caligid copepods *L. salmonis* and *C. elongatus* (Jones et al., 1990; Johnson and Albright, 1992a, b; Jonsdottir et al., 1992; MacKinnon, 1993).

The stress response of fish involves a series of compensatory and/or adaptive behavioural and physiological responses, which are collectively referred to as the integrated stress response (see review by Wendelaar Bonga, 1997) and in the case of ectoparasite infestations, this may protect the host by reducing parasite settlement and eliminating attached parasites, by stimulating both specific and non-specific immune responses, epithelial hyperplasia, mucous cell discharge, and affect the composition of the mucus.

The primary stress response of fishes involves the activation of the higher brain centres, which trigger a massive release of catecholamines and corticosteroids. The secondary stress responses are the manifold immediate actions and effects of these hormones and occur at all levels of organisation. Increased circulating blood cortisol is mediated by the hypothalamic-pituitary-interrenal axis, where corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) are the main tropic hormones, which ultimately stimulate the synthesis and release of cortisol from the cortisol-producing interrenal cells. Cortisol has a broad activity spectrum in fishes and has both mineralocorticoid and glucocorticoid activities. It stimulates the differentiation of branchial chloride cells and increases the specific activity of ion transporting enzymes, in particular Na^+/K^+-ATPase, actions which are mineralocorticoid (Madsen et al., 1995; McCormick, 1996; Seidelin et al., 1999), and stimulates gluconeogenesis in the liver which is a glucocorticoid action (Vijayan et al., 1994a, b). High circulating levels of cortisol of short duration or chronic but moderately elevated cortisol levels also impair hydromineral balance by inducing apoptosis in branchial cell types, including the chloride cells (Bury et al., 1998). Cortisol also mediates many of the stress-related changes in the skin of rainbow trout, where it stimulates mucus discharge, mitosis, apoptosis of epidermal cells, synthesis of vesicles in the cells of the upper epidermis, and the infiltration of the epidermis by leukocytes (Iger et al., 1995; Chapter 7).

Infection of salmonids with low numbers of the salmon louse *L. salmonis* generally has not resulted in significant increases in plasma cortisol levels (Johnson and Albright, 1992b; Bjorn and Finstad, 1997; Ross et al., 2000). However, heavy infections typically elevate plasma cortisol levels beyond those which cause immunosuppression (Mustafa et al., 2000). Johnson and Albright (1992b) reported similar plasma cortisol values in *S. salar* experimentally infected with copepodids of *L. salmonis*. Exposure of *O. mykiss* to juvenile stages of *L. salmonis* increased blood cortisol levels after 4 h net confinement to levels that were significantly higher than those in confined, but unparasitized, fish (Ruane et al., 2000). Similar results were obtained with fish confined after 21 days of infestation with adult *A. foliaceus* (Ruane et al., 1999a). Here, the effect of infestation with

low numbers of lice was studied alone, and combined with prior moderate elevation of blood cortisol level in the long term (i.e. 21 d). The objectives of the present experiment were to measure the combined effect of a low-level cortisol administration and infection with *A. japonicus* in *O. mykiss* in order to study aspects of the host-parasite interaction. Replicate groups of fish were fed cortisol in their diet to prevent any stress due to administration and were subsequently infected with 6 *Argulus* per fish. Fish were sampled after 14 days, 24 h after the last cortisol feeding, and 48 h after infection with the parasites. A number of parameters related to the stress response, hydromineral balance, immune function, and infestation levels were measured.

Materials and Methods

Experimental fish and conditions

Six groups of 30 rainbow trout (approx. 73 g) were set up in 65 l black plastic circular tanks. The water was filtered using an Eheim power filter, aerated using an airstone and continuously refreshed with dechlorinated Nijmegen tap water using a partial flow-through system. The photoperiod was 12:12 light:dark, and the water temperature was 16°C. Fish were fed with Pro-Aqua 20/4 (Trouw™) at 1% of body weight once daily. The experiment began after a 14 d acclimation period to these conditions.

Experimental design

This consisted of 3 experimental treatments, each performed in duplicate tanks. The experiment was designed to infect fish with A. japonicus, and to do so in cortisol-treated and untreated fish. The control groups consisted of fish that were sham treated for cortisol administration and parasite infection (see below). At 4 d and 2 d before infection, the normal food was substituted with the same ration of food treated with reagent grade ethanol (4 groups) or ethanol containing cortisol (2 groups). This food was prepared by mist spraying the pellets with absolute ethanol (Merck), or the same volume of ethanol containing cortisol (hydrocortisone, Sigma) to give a cortisol content of 20 mg· kg^{-1} food. The ethanol was allowed to evaporate for 5 d prior to feeding the treated pellets.

Two days after the second cortisol containing meal, two groups fed cortisol-containing food and two groups fed ethanol-sprayed food were each infected with 120 adult and sub-adult *A. japonicus*. This was accomplished by pouring beakers containing the required number of *Argulus* into each tank. Control fish were sham infected by pouring beakers of water only into their tanks. The treatment groups are referred to as CONTROL (ethanol-sprayed food and sham infected), ARGULUS (ethanol-sprayed food and *Argulus* infected) and CORT&ARG (cortisol containing food and *Argulus* infected).

Sample points and methods

Four fish from each tank were sampled at time zero (T_{ZERO}) i.e. after 14 d acclimation. Five fish per tank were sampled 24 h after the second cortisol administration (T_{CORT}) and another five fish were sampled 48 h post *Argulus* infection (T_{ARG}). The five fish were netted into a solution of 2-phenoxyethanol (1:1000; Sigma) in which they were rapidly and irreversibly anaesthetised. Blood was taken by needle from the caudal blood vessels and immediately discharged into pre-cooled eppendorf tubes containing Na_2EDTA/aprotinin (1.5 mg/3,000 KIU per ml blood; Sigma) for lymphocyte counts and plasma analysis. The fish were weighed, measured, and the number of attached parasites was counted.

For histological analysis, skin biopsies (10x10 mm) from 4 fish from each tank were taken from the anterior part of the head and fixed in Bouin's fixative for light microscopy (LM). For electron microscopy (EM), smaller pieces of skin (5x5 mm) from the same 4 fish were fixed in Na-cacodylate buffered glutaraldehyde for 20 min on ice, and post-fixed in 1% osmium tetroxide in the same buffer (Iger et al., 1995; Chapter 3). Tissues were fixed at the sample points post cortisol feeding and post *Argulus* infection.

Infestation density and condition factor

Infestation level was recorded as the total number of parasites on each fish. Condition factor was calculated as 100(body weight/fork length^3), where body weight is in g and fork length is in cm.

Sample processing

Plasma cortisol, α-MSH and ACTH were measured with radioimmunoassays developed and validated to measure these hormones in the blood of fishes (Balm and Pottinger, 1995; Balm et al., 1995). Na^+ concentrations were determined with a flame-photometric auto analyzer (Technicon model IV) coupled to a spectrophotometer for determining Cl^- by the formation of ferrothiocynate in plasma diluted 200x. Plasma glucose was assayed using the Boehringer UV-test kit following the manufacturers protocol (Boehringer, Mannheim, Germany). Lymphocyte numbers were quantified by diluting the blood 1:20 with Türck liquid, then counting them in a Bürker haemocytometer in a light microscope (Ruane et al., 1999a).

For LM, skin samples were fixed for 24 h, processed through paraffin wax and sectioned at 5 µm. Mucous cells were stained with the Alcian blue (pH 2.5) method, as described in Chapters 4 and 7. The presence and localization of cortisol receptors in the epidermis were demonstrated with an antibody against O. mykiss glucocorticoid receptors raised in rabbits (Tujague et al., 1998), as described in detail in Chapter 6. The primary antibody was used at a working dilution of 1:32,000. For the controls, primary and secondary antibodies were omitted. For transmission electron

microscopy (TEM), 4 samples per group at each time point were processed, examined and photographed without knowledge of the treatments, and analysed as described in Chapters 2, 3 and 4. Five low magnification areas from the upper epidermis of each fish were enlarged 4 times and printed. From these micrographs, the numbers of vesicles were counted in 7-10 filament cells per fish (each filament cell representing a cytoplasmic area of circa 200 μm^2). The counts were averaged to give a mean value for each fish (Chapter 2). Only data from mature filament cells with a visible nucleus, which were neither necrotic nor apoptotic, were used. The general ultrastructure of the epidermis was examined with particular attention being paid to cellular necrosis (characterized by nuclei with aggregations of chromatin, swelling of the cytoplasmic compartment and loss of apical microridges) and apoptosis (characterized by progressive densification of the nucleus, organelles and cytoplasm, leading to cell shrinkage and loss of contacts with surrounding cells), leukocyte infiltration and epithelial integrity (evaluated by intercellular swelling and cell-cell contacts). The observations were compiled and expressed semi-quantitatively as described elsewhere (Iger et al., 1994d, 1995; Chapter 2 and 4).

Data handling and statistics

As there were no statistically significant differences in the data between replicate tanks, the data were pooled giving n = 8 at T_{ZERO} and n = 10 at T_{CORT} and T_{ARG}. For LM parameters n = 8 and for EM n = 4. For all parameters except condition factor and Na:Cl ratio, effects of treatments were tested by ANOVA at each sample point and differences between treatment groups were identified with Bonferroni's multiple range test. Non-parametric data were identified with Bartlett's test and were log transformed before analysis to achieve homogeneity of variance. For condition factor and Na:Cl ratio, differences were tested by non-parametric ANOVA. Differences between ARGULUS and CORT&ARG lice numbers at T_{ARG} were tested with the Mann Whitney *U* test. Spearman Rank Correlation analysis was used to examine the relationship between the numbers of attached parasites and the circulating blood hormone levels at 24 h post infection. All data are presented as mean values ± S.E.M. Statistical significance was $P < 0.05$.

Results

Fish condition factor and parasite infestation density

No fish died during the experimental period; the fish fed well and remained disease free. The mean condition factor of the CONTROL fish was 1.055 ± 0.024 and was not significantly different from the ARG or CORT&ARG groups at any sample point (data not shown). At T_{ARG}, the number of parasites attached to ARG fish was significantly higher than on CORT&ARG fish (Table 1).

Blood parameters

Plasma Na^+ and Cl^- concentrations of all fish groups sampled, as well as Na:Cl ratios, remained similar throughout the experiment and were within the range of values reported for freshwater salmonids (data not shown). At T_{ZERO}, cortisol values were significantly higher in the CONTROL fish than in the ARG and CORT&ARG groups. Cortisol levels were all well within levels considered basal for unstressed fish (Table 1). At T_{CORT}, plasma cortisol was significantly higher in the CORT&ARG group. At T_{ARG}, the mean plasma cortisol values of ARG and CORT&ARG fish were not different than the CONTROL values (Table 1). There was a significant positive correlation between the numbers of parasites per fish and the levels of cortisol measured in the plasma of ARG fish, but there was no such correlation for the CORT&ARG fish (Table 1). Table 2 shows that the plasma glucose values were significantly higher in the CORT&ARG group at T_{CORT}, that plasma ACTH and α-MSH were not significantly different between treatments at any time point, and that the numbers of circulating lymphocytes in the blood were significantly lower in ARG and CORT&ARG groups at T_{ARG} (Table 2).

Light microscopy

There were no significant changes in the numbers of mucous cells over time or between treatments (data not shown). Cortisol receptor staining occurred mostly in the upper layer of the epidermis, primarily in the top few cell layers (pavement cells and underlying filament cells, illustrated in Figure 1 from a CONTROL fish at T_{ZERO}). Staining was also observed in the lower layer, mainly in irregularly shaped leukocyte-like cells, identified as macrophages, and small regularly shaped lymphocytes. A weaker, more discrete staining could be seen in the cytoplasm of mucous cells.

	T_{ZERO}	T_{CORT}	T_{ARG}
Plasma cortisol ($ng \cdot ml^{-1}$)			
CONTROL	5.17±1.34	2.31±0.74	6.11±1.48
ARG	1.16±0.19**	6.47±2.36	12.23±3.24
CORT&ARG	1.89±0.47*	27.62±7.61**	9.53±2.85
No. lice·fish^{-1}			
CONTROL	-	-	-
ARG	-	-	3.5±0.70[a]
CORT&ARG	-	-	1.1±0.31[a]
Spearman Rank Correlation			
CONTROL	-	-	-
ARG	-	-	R=0.69, P=0.03
CORT&ARG	-	-	R=0.44, P=0.20

Table 1. Plasma cortisol levels and numbers of attached parasites at sampling for rainbow trout (*O. mykiss*) at the beginning of the experiment (T_{ZERO}), 24 h after receiving the second of 2 cortisol-containing feeds (T_{CORT}), and 48 h post infection with 6 *A. japonicus* per fish (T_{ARG}). Data given as mean ± S.E.M. for n = 8-10. For full details, see text. * = $P < 0.05$; ** = $P < 0.01$ compared with control. Where 2 groups share the same superscript letter, significance between them is $P < 0.01$.

Table 2. Plasma glucose, ACTH and αMSH, and circulating lymphocytes in rainbow trout (*O. mykiss*) at the beginning of the experiment (T_{ZERO}), 24 h after receiving the second of 2 cortisol-containing feeds (T_{CORT}), and 48 h post infection with 6 *A. japonicus* per fish (T_{ARG}). Data are given as mean ± S.E.M. for n = 8-10. For full details, see text. ** = $P < 0.01$; *** = $P < 0.001$ compared with control.

	T_{ZERO}	T_{CORT}	T_{ARG}
Plasma glucose ($mg \cdot dl^{-1}$)			
CONTROL	60±3	71±3	83±4
ARG	54±5	73±2	80±5
CORT&ARG	54±2	94±2**	81±3
Plasma ACTH ($pg \cdot ml^{-1}$)			
CONTROL	25±2	31±5	33±7
ARG	19±6	38±6	30±5
CORT&ARG	24±3	25±3	28±4
Plasma αMSH ($pg \cdot ml^{-1}$)			
CONTROL	415±90	475±35	345±60
ARG	345±50	460±35	335±40
CORT&ARG	485±40	475±75	370±50
Lymphocytes ($x10^7 \cdot ml^{-1}$)			
CONTROL	4.0±0.5	3.9±0.4	4.3±0.5
ARG	3.6±0.3	3.8±0.4	3.0±0.2***
CORT&ARG	3.7±0.4	3.5±2.8	3.0±0.3***

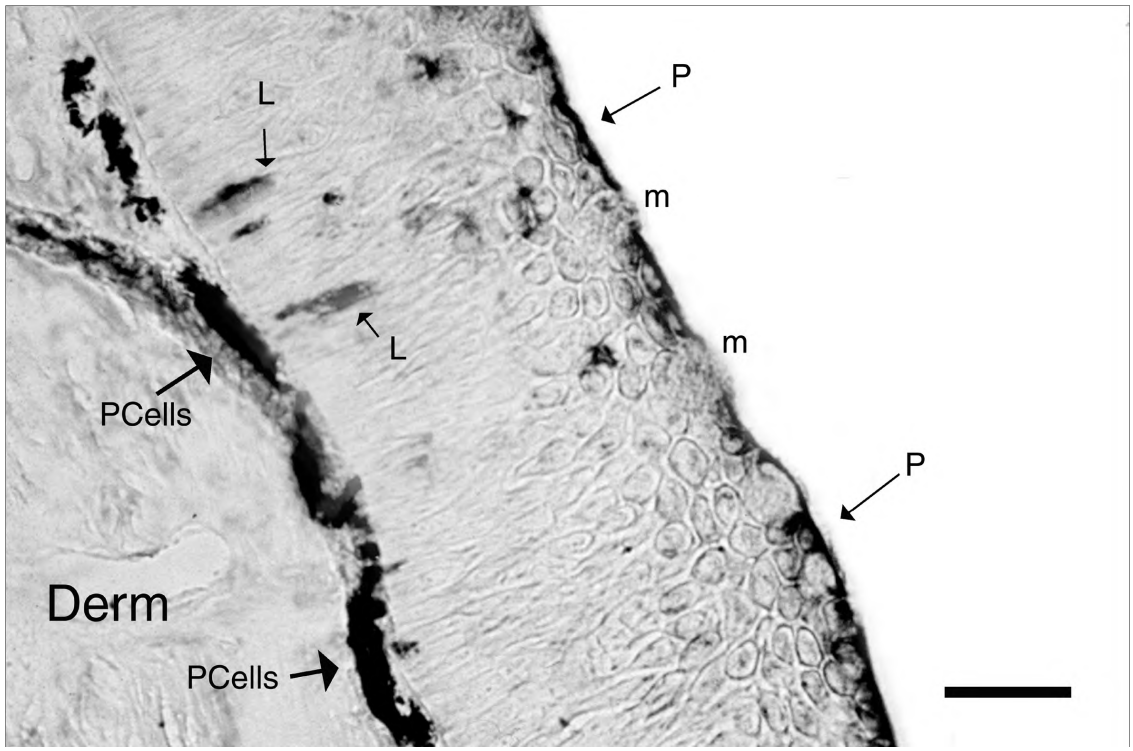


Figure 1. Immunolocalisation of the glucocorticoid receptor in the epidermis of the head skin of control *Oncorhynchus mykiss* at T_{ZERO}. Staining (arrows) is concentrated in the upper layer of the epidermis, primarily in the top few cell layers (pavement cells and underlying filament cells and in the lower layer, mainly in irregularly shaped leukocyte-like cells. **L**, leukocyte; **m**, discharging mucous cell; **p**, pavement cell; **PCells**, melanin-containing pigment cells in subepidermal layer; **Derm**, dermis. Scale bar = 30 μm.

Electron microscopy

The EM appearance of the skin epidermis of the CONTROL fish was normal, and consistent with previous reports for this species (Iger et al., 1994b, d; Chapter 3). The multilayered epithelium was composed of several cell types, with the uppermost layer of cells, those in contact with the water, was differentiated into pavement cells with elaborated apical microridges. Below this were large populations of filament and mucous cells, within which low numbers of leukocytes, mainly macrophages, granulocytes and lymphocytes, were observed. Both the pavement and filament cells contained considerable numbers of small vesicles (Figure 2a). This epidermal structure was altered in the CORT&ARG group at T_{CORT} (Figure 2b) when more discharging mucous cells were seen, vesicle numbers in the pavement cells increased, and the vesicles were located closer to the apical surface (Figure 2d) than in control fish (Figure 2c). Additionally, some limited microridge disruption was observed and the incidence of apoptotic and necrotic cells in the upper cell layers increased slightly. Intercellular spaces in the epidermis became evident (Figure 2b) and they were often infiltrated by leukocytes. The epidermal condition of the ARG group at T_{CORT} was comparable to that seen in CONTROL. A semi-quantitative overview of the condition of the epidermis is presented in Table 3.

Table 3. Semi-quantitative evaluation of the ultrastructural parameters observed in the head skin epidermis of rainbow trout (*Oncorhynchus mykiss*) after receiving the second of 2 cortisol-containing feeds, and 48 h post infection with 6 *Argulus japonicus* per fish. Data given as mean ± S.E.M.for n = 8-10. For full details, see text. For a given parameter, 0 = unaffected, - = negatively stimulated and + = positively stimulated.

Parameter	CONTROL	ARG	CORT&ARG
Post cortisol feed (T_{CORT})	Ethanol	Ethanol	Cortisol
Pavement cell microridges	0	0	0/-
Pavement cell vesicles	0	0	++
Pavement cell apoptosis	0	0	0/+
Pavement cell necrosis	0	0	0
Mucous cell discharge	0	0	0/+
Intracellular spaces	0	0	+
Leukocyte infiltration	0	0	+
Post Argulus infection (T_{ARG})	sham	+ Argulus	+ Argulus
Pavement cell microridges	0	0	0
Pavement cell vesicles	0	+	+
Pavement cell apoptosis	0	+	+
Pavement cell necrosis	0	++	+
Mucous cell discharge	0	++	+
Intracellular spaces	0	++	++
Leukocyte infiltration	0	+++	++

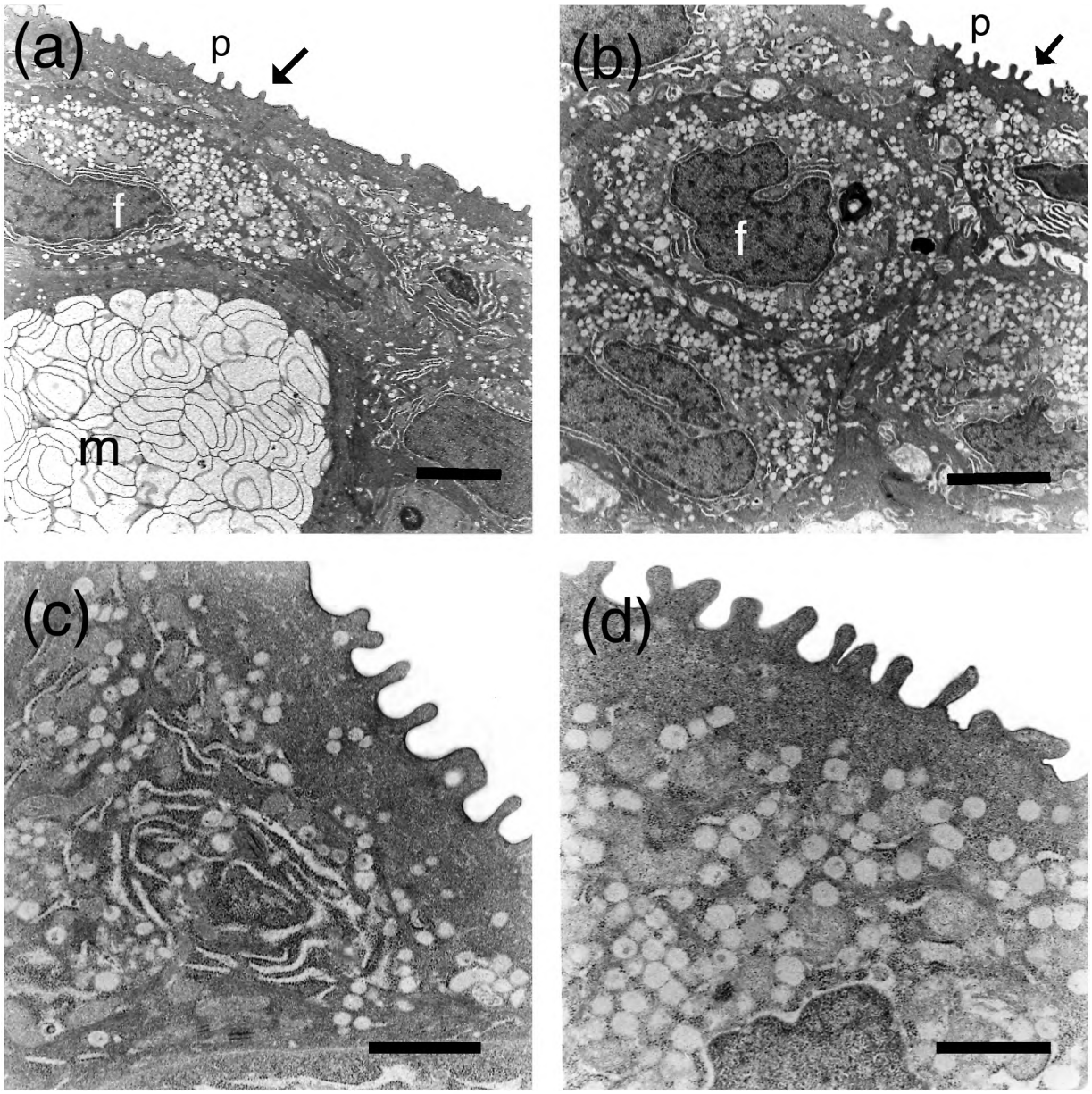


Figure 2. Transmission electron micrographs of the upper epidermis of head skin from *Oncorhynchus mykiss*. (a) The upper pavement cells (**p**) have apical microridges (arrow) and, together with the underlying filament cells (**f**), contain considerable populations of vesicles. **m** = mucous cell. (b) 24 h after receiving the second of 2 cortisol-containing meals (T_{CORT}), the vesicle populations are extensive in pavement (**p**) and filament cells (**f**). (c) Upper pavement cell of control trout at T_{CORT}, showing signs of active vesicle synthesis and normal vesicle density. (d) Upper pavement cell 24 h after receiving the second of 2 cortisol-containing meals (T_{CORT}). Vesicle density is greater and the vesicle populations are located closer to the apical side of the cells. Scale bars: a, b = 5 µm; c,d = 2 µm.

In general, the *Argulus* infestation had a greater impact on the epidermis than did cortisol feeding. In the ARG group at T_{ARG}, discharging mucous cells were more evident, vesicle numbers in some apical cells were depleted, microridges were more seriously disrupted, intercellular spaces were more extensive and contained more leukocytes, and the numbers of necrotic and apoptotic cells in the upper epidermis were increased. The responses seen in some parameters in the CORT&ARG group were less than in the ARG group. The main differences were reduced effects on pavement cell necrosis, vesicle numbers, and the extent of leukocyte infiltration. The numbers of vesicles in the upper epidermal cells of the cortisol fed fish were significantly higher at T_{CORT} (Figure 3). There were no significant differences in the numbers of vesicles between treatments at T_{ARG}. A summary semi-quantitative evaluation of these results is given in Table 3.

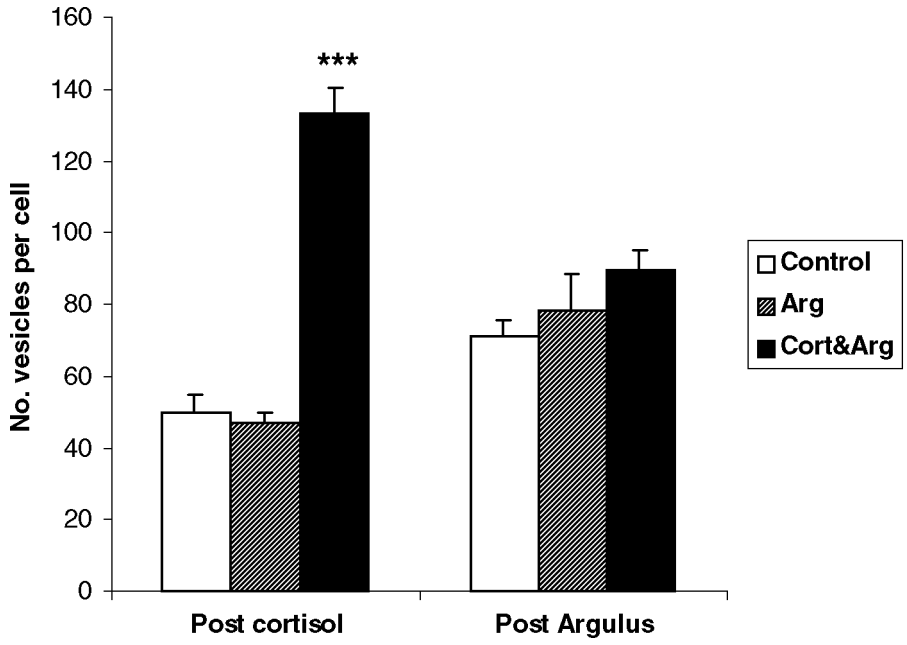


Figure 3. Numbers of vesicles quantified in the head skin epidermis of *Oncorhynchus mykiss* after receiving the second of 2 cortisol-containing meals (post cortisol), and 48 h post infection with 6 *Argulus japonicus* per fish (Post Argulus). The data represent counts per cytoplasmic area of upper epidermal cells. Data given as mean ± S.E.M.for n = 4. For full details, see text. *** = $P < 0.001$.

Discussion

This study is the first to examine the role of the stress hormone cortisol on the relationship between the fish louse *A. japonicus* and the trout, *O. mykiss*. The results show that the administration of low levels of cortisol induced adaptive and beneficial effects, which reduced the intensity of infestation. While we do not understand the exact mechanism of this adaptive response, it is possible that the cortisol-induced elevation of plasma glucose provided more energy for dealing with the energetic costs of countering the parasite. To obtain as comprehensive a picture as possible, we measured a variety of parameters in the host. In the short term, infestation with low parasite numbers resulted in the migration of lymphocytes out of the blood and into the epidermis, where the epithelial disruption was associated with an increased incidence of discharging mucous cells and moderately elevated levels of apoptosis. Infection with low numbers of the parasite did not affect hydromineral balance, nor did it cause an increase in plasma cortisol or glucose levels, which are the primary stress parameters used to assess stress in fish. From the positive correlation between numbers of lice and blood cortisol levels, we predict that higher numbers of parasites would significantly increase the levels of circulating cortisol. Our observations that low levels of infestation by this fish louse do not disrupt the hydromineral balance are consistent with other studies (Grimnes and Jakobsen, 1996; O'Flaherty et al., 1999; Nolan et al., 2000; Chapter 4).

The formation of intercellular spaces in the teleost skin epidermis and infiltration of these by leucocytes was reported in Atlantic salmon with an infestation of the sea louse *L. salmonis* (Chapter 4), as well as for a variety of other stressors (e.g. Iger and Wendelaar Bonga, 1994; Iger et al., 1994d; Nolan et al., 1998; Chapter 2). Extravasation of leukocytes from the blood vessels and their subsequent appearance in the peripheral tissues is normally observed in stressed fish (Wendelaar Bonga, 1997), where it is thought to be in part mediated by cortisol (Iger et al., 1995). This is supported by the mammalian literature where, while acute stress enhanced cell-mediated immunity in rats in vivo by directing leukocytes to the skin, chronic stress suppressed such movements. And the reaction to acute stress, and the beneficial aspects of both the stress response and immune function (principally resistance to viruses, bacteria and fungi), was positively related with circulating glucocorticoid levels in the blood of the rat (Dhabhar and McEwen, 1997). In the present study, low-level cortisol administration did not significantly reduce the number of circulating lymphocytes in the blood, but did increase their presence in the epidermis. This observation points to rapid replacement from the haematopoietic tissues. Moderately elevated cortisol levels during exposure to a stressor are thought to be adaptive whereas high cortisol levels can have deleterious effects (Wendelaar Bonga, 1997; Bury et al., 1998). Based on plasma cortisol data from previous experiments in which rainbow trout were fed cortisol in their diet, we predict that in the present experiments, the cortisol peaks would have been about 100-150 ng· mL^{-1}, which would have

occurred within 2-4 h, and would have returned to basal levels within 24-36 h (Barton et al., 1987; Balm and Pottinger, 1995). The peak values expected then would have been considerably lower than the levels reported by Iger et al. (1995).

In the present study, stress free cortisol administration via the diet increased the numbers of vesicles in the upper cell layers of the skin epidermis of trout. These secretory vesicles contain endogenous peroxidase activity (Iger et al., 1994b, d) and in rainbow trout their synthesis is stimulated by cortisol (Iger et al., 1995). Indeed many stressors induce changes in the numbers of vesicles in the upper cells of the epidermis of fish skin. The contents of these vesicles are secreted out into the glycocalyx and mucus of the fish. Peroxidase is considered to be an anti-microbial component of the non-specific defence system of fish and has been demonstrated in the mucus and glycocalyx on the surface of the skin (Iger et al., 1994b; Brokken et al., 1998). The secreted skin peroxidase is a biochemically distinct isoform from the peroxidase of the blood (Brokken et al., 1998). While the significance of the enhanced secretion of peroxidase during stress is unknown at present, the stimulation of vesicle synthesis by cortisol administration in the present study was associated with reduced establishment by *A. japonicus* on the host fish. Further, in another study, the vesicle populations in the cells of the upper epidermis of sea trout smolt (*S. trutta*) were depleted within 3 h of exposure to either Rhine water or acute temperature shock (Chapter 2). These observations are consistent with the hypothesis that these vesicles have a role in the integrated stress response to both toxic and non-toxic stressors.

Experimental infection of the Atlantic salmon with low numbers of the sea louse *L. salmonis* induced stress-related changes in the skin and gill epidermis and these occurred in a dose-dependent manner relative to the number of parasites introduced (Chapter 4). In the present experiment, fish infected with *Argulus* showed a statistically significant correlation between parasite number and plasma cortisol level, and this supports the view that increasing numbers of parasites are increasingly stressful for the fish. This relationship, however, was lost in the cortisol fed group, possibly because these fish had lower parasite numbers. This indicates that this cortisol performs a functional role in the adaptive responses of the host to the parasite, at least in the short term. Stimulation of leukocyte extravasation and their migration into the peripheral tissues is clearly one such adaptive process; stimulation of vesicle synthesis in the upper epidermis and their discharge is likely another. The positive correlation between plasma cortisol levels and parasite numbers indicates that increasing numbers of *A. japonicus* induce stronger stress responses in the fish. Using a hyperbolic model, Poole et al. (2000) demonstrated that baseline cortisol levels in wild sea trout *S. trutta* naturally infested with *L. salmonis* were significantly higher than uninfested fish. Furthermore, there was a positive correlation between infestation intensity and extrapolated baseline cortisol level. The cortisol response of trout to *A. japonicus* infestation and the effects of chronically

elevated levels of cortisol in the blood of the fish remain to be demonstrated. Ruane et al. (1999a) found no differences in unstressed plasma cortisol in *O. mykiss* 21 days post infection with 6 *Argulus* per fish, indicating that adaptation to this number of parasites had occurred.

This is the first study to demonstrate cortisol receptors in the epidermis of the rainbow trout *O. mykiss*. Effects of cortisol administration on epithelial cells of *O. mykiss* included increased synthesis and secretion rate of vesicles and increased infiltration by leukocytes (this study and Iger et al., 1995). The data support the view that these effects may be mediated via the cortisol receptor, as the cortisol receptor was found by immunohistochemistry to be located in the upper epidermis (consisting primarily of pavement cells and cells just before the start of differentiation) and in leukocytes, sites where cortisol appeared to exert effects. Low levels of cortisol can enhance carp neutrophil function and proliferation *in vitro*, while higher levels induced apoptosis in B-lymphocytes (Weyts et al., 1998). The latter observations, together with those from the present study, support the hypothesis that cortisol levels slightly above unstressed basal levels are maladaptive in teleost fish (Wendelaar Bonga, 1997; Bury et al., 1998).

In conclusion, this study shows that low levels of cortisol induce cellular responses in the skin of rainbow trout that are typical responses to a variety of stressors. Certainly, the increased synthesis and discharge of vesicles in the cells of the upper epidermis appears to be adaptive as it is associated with a lower infestation rate by the fish louse *A. japonicus*. The localization of the glucocorticoid receptor in the pavement and filament cells, as well as the presence of leukocytes in the epidermis, suggests that the effects are cortisol receptor-mediated. *Argulus* infestation increased the numbers of lymphocytes infiltrating the skin epidermis and indicated immunostimulation. The maintenance of hydromineral balance in spite of moderate epithelial disruption, together with the absence of an infestation-related increase of cortisol or glucose in the plasma, indicate that the numbers of parasites used were not excessively or chronically stressful to the fish. However the significant correlation between parasite number and plasma cortisol suggests that higher infestation levels would cause greater effects. A dose-response study to the parasite is required to examine this prediction.

Acknowledgements

The authors thank F.A.T. Spanings for design and provision of experimental set-up, fish care and sampling assistance, and J.C.A.van der Meij for analysing electron microscopy samples. The gift of the glucocorticoid receptor antibody from Dr. B. Ducouret (INRA, Rennes, France) is gratefully acknowledged.

Chapter 6

Effects of infection with the ectoparasite *Argulus japonicus* and administration of cortisol on cellular proliferation and apoptosis in the epidermis of common carp *Cyprinus carpio* skin

A.L. van der Salm, D.T. Nolan, F.A.T. Spanings and S.E. Wendelaar Bonga.

Journal of Fish Diseases (in press)

Abstract

The host-parasite interaction between the juvenile carp *Cyprinus carpio* and the ectoparasitic louse *Argulus japonicus*, together with the role of cortisol in this interaction, was examined at the level of the host skin epidermis. Epidermal mucous cell numbers, and proliferation and apoptosis of the epithelial cells were studied over 32 d. Apoptotic cell numbers in the uppermost epidermis were reduced at 26 d post-infection with the lice, while the other parameters were unaffected. Administration of cortisol-containing food resulted in reduced apoptosis in the cells in the upper skin epidermis at 24 h and at 28 d post-feeding. Cortisol feeding combined with *A. japonicus* infection reduced numbers of apoptotic cells in the upper epidermis more than either individual treatment. Further, combining the treatments also significantly increased apoptosis in the lower epidermis in cells morphologically identified as leukocytes, apparently migrating macrophages and lymphocytes. Using immunohistochemistry, the cortisol receptor was demonstrated and localised in the epidermis. Receptors only occurred in pavement cells in the upper epidermis and in leukocytes in the lower parts of the epidermis. The ectoparasites, or administered cortisol, induced effects which may be functionally adaptive in the upper pavement cells, while combining the 2 treatments also induced changes indicative of immunosuppression.

Introduction

Epizootics of ectoparasites are a very serious threat to fish populations and the Branchiuran ectoparasitic fish lice of the genus *Argulus* are serious pathogens of both farmed and wild fish populations (Menezes et al., 1990; Singhal et al., 1990; Jafri and Ahmed, 1994; Northcott et al., 1997). The skin of fish is a food source for many crustacean ectoparasites (Heckmann, 1993; Schmidt and Roberts, 1996), many of which feed on the mucous layer, the flesh and even the blood. *Argulus japonicus* is a common freshwater parasite of a variety of freshwater fish species (Basten and Cochran, 1991; Jafri and Ahmed, 1991, 1994; Lamarre and Cochran, 1992; Poly, 1998). *Argulus* species feed on the skin of its host, causing lesions, possibly disturbing hydromineral balance and are associated with increased susceptibility to disease (Singhal et al., 1990). The effects of *Argulus foliaceus* on the host include direct epithelial damage caused by attachment and feeding of the parasite, as well as localised disruption of the epithelial structure (Rahman, 1995).

The skin and gills of a fish are complex epithelia comprised of living cells which form the first barrier between the external and the internal environment and are protected with a chemically and functionally complex mucous coat which is discharged by specialised mucous cells in the epidermis (Shephard, 1994). As the skin epithelium of fish responds strongly to stressors (Iger et al., 1995; Nolan et al., 1998; Chapters 2, 3 and 4), it offers good possibilities for evaluating indirect stress effects of ectoparasites on the host fish and studying the host-parasite relationship at its first level of interaction.

Apoptosis is physiologically programmed cell death that balances the increases in cell numbers that result from proliferation and immigration. Increased necrosis (accidental cell death) and apoptosis, increased cell migration (mucous cells and leukocytes), and increased proliferation are characteristic epidermal responses to both toxic and non-toxic stressors. Stress responses in the skin, including increased apoptosis of certain cell types, are controlled by the stress hormone cortisol (Iger et al., 1995). As cortisol is present in the blood, it can have systemic activity, leading to changes in epithelia in areas not directly affected by parasite infestation.

The stressor-induced disruption of the skin and gill epithelia of the fish caused by parasites (Chapters 4 and 5), but also by a variety of stressors (e.g. Iger et al., 1994b, d; Burkhardt-Holm et al., 1997; Nolan et al., 1998; Chapters 2 and 3), are significant in relation to disease resistance, as the prolonged period of disrupted epithelia leaves the fish open to invasion by opportunistic pathogens. Effects of infection with the sea louse *Lepeophtheirus salmonis* can therefore be divided into the direct effect of parasite attachment and feeding on the body surface and the indirect effect of the integrated stress response on the integrity of the skin and gill epithelia, including the osmoregulatory consequences in terms of gill Na^+/K^+-ATPase (Chapter 4). If there is also immunosuppression resulting from elevated cortisol levels (Pickering and Pottinger, 1989; Nagae et

al., 1994), or from environmental pollutants (e.g Kakuta and Murachi, 1997), the deleterious effects of parasites are compounded and mortality can occur. Ectoparasitic infestation, as well as cortisol administration, influences the host-parasite interaction and fish skin (Johnson and Albright, 1992a, b; Eufemia et al., 1997; Chapter 5). Not all epidermal cells respond to cortisol (Iger et al., 1995; Chapter 7) and the presence and cellular localization of cortisol receptors in carp skin has not been reported.

Stressors induce changes in the numbers of vesicles in the cells of the upper epidermis of many fishes (Iger et al., 1994b, 1995; Nolan et al., 1998, 1999b; Chapter 4). These are secretory vesicles which contain endogenous peroxidase activity (Iger et al., 1994b, d) and their synthesis and release are induced in rainbow trout *O. mykiss* by oral cortisol administration (Iger et al., 1995; Chapter 5). Peroxidase, an antipathogenic component of the non-specific defence system of fish, has been demonstrated in the mucus and glycocalyx of the skin (Iger et al., 1994b; Brokken et al., 1998). Clearly, as the epidermis and mucus contain many antipathogenic substances (e.g. lysozyme, c-reactive protein, complement, lectins, proteases, immunoglobulins; see review by Shephard, 1994) and are attachment sites, habitat and food source for fish lice, studying these should help our understanding of the host-parasite interaction.

While little is known about the host-parasite interaction of fishes generally, particularly little attention has been paid to non-salmonid species. Therefore we studied the host-parasite interaction between *A. japonicus* and its host the common carp *Cyprinus carpio* at the level of the skin epidermis. We took advantage of having a group of 200 juvenile (< 1.0 g) similar sized *C. carpio* of mixed parentage available to us at a time when our laboratory population of *A. japonicus* was producing many egg strings. We administered cortisol and infected with numbers of the parasite separately to see whether there were common responses we could identify as indirect effects of the parasite and which might be mediated by cortisol. Further, we administered both treatments together to see whether the effects were additive for any of the parameters, or if there was any evidence of deleterious effects of the combination. The numbers of proliferating and apoptotic cells in the skin, as well as the numbers of mucous cells, were quantified using light microscopy techniques. The cortisol receptor was demonstrated in the epidermis and using immunohistochemistry to identify and localise cells that were expressing it. Quantitative data was qualitatively in relation to the effects at the epidermis surface was confirmed using scanning electron microscopy, as it has been used previously to show parasite-induced epithelial disruption and cell death (Chapters 4 and 5).

Materials and Methods

Experimental set-up and design

Eight groups of 25 common carp *C. carpio* aged 3 months old and weighing circa 0.8 g, bred from the Nijmegen laboratory broodstock, were placed in separate 45 l black circular tanks containing aerated fresh water, with a flow-through rate of approximately 5 l· h^{-1}. Each group was fed 1 g of finely ground commercial pelleted fish diet twice daily and maintained at a constant water temperature of 16-17 °C. The experimental design consisted of 4 treatments, each in duplicate: sham treatment (CTR), infestation with *A. japonicus* (ARG), cortisol feeding (CORT) and cortisol feeding followed by infection with *A. japonicus* (CORT+ARG).

Cortisol feeding

After an acclimatisation period of 3 weeks, 2 fish from each group were sampled as time zero controls. The following day the CORT groups and the CORT+ARG groups received 1 g of food containing cortisol (100 µg· g^{-1} food), followed 48 h later by a second 1 g of the same cortisol feed. This cortisol dose was chosen as it moderately elevates blood cortisol levels in rainbow trout (Barton et al., 1987; Balm and Pottinger, 1995) and was used in previous *Argulus* infection studies (Ruane et al., 1999a; Chapter 5). Cortisol food was prepared by dissolving 2 mg of cortisol in 1.5 ml ethanol and spraying the solution evenly over 20 g of food. The ethanol was left to evaporate for 10 d. Food for the other groups was sprayed with ethanol only. At 24 h after the second cortisol feed, 5 fish from each group were sampled and 2 treatments (ARG and CORT+ARG) were infected with *Argulus* as outlined below.

Fish lice (Argulus japonicus)

Lice were collected from Nijmegen laboratory broodstock carp 20 d and 24 h before infestation. The lice collected 20 d before infestation were placed in 9 cm Petri dishes containing aquarium water that was refreshed with non-chlorinated tap water. They were maintained at room temperature (20°C), and the water was replaced twice a week. The females began laying egg-strings within 12 h and all adult lice were dead within 8 d. Most of the eggs had hatched by 18 d. Together with a group of adult lice which were collected 24 h before infection, the infestation level was set at 10 adult and 75 metanauplius lice per group of 18 fish. Infection was carried out by introducing a Petri dish containing the lice into the tank. Non-infected groups were sham treated by the addition of a Petri dish containing water only. At 48 h post-*Argulus* infection, 5 fish from each group were sampled. The remaining fish were left for 26 d after which 5 fish from each group were sampled as described below. The remaining 8 fish were killed in the same manner and weight and length

measurements were taken to examine any effects of the treatments on these parameters. Condition factors (CF) were calculated according to the formula

$CF = (100*BW)/FL^3$

where BW is body weight in g and FL is fork length in cm.

Sampling method and sampling processing

At sampling, 5 fish per tank were rapidly netted and immediately and irreversibly anaesthetised in a solution of 2-phenoxy ethanol (Sigma) in a 1:1000 dilution. At all time points, 2 fins from each group (giving n = 4 per treatment) were fixed for electron microscopy. Fins were fixed in 3% glutaraldehyde in cacodylate buffer for 20 min, followed by post fixation in 1% osmium in the same buffer (Iger et al., 1995; Chapter 3). Immediately after removal of a fin for EM, the whole fish was fixed in Bouin fixative for light microscopic analysis. After 72 h of fixation, the samples were washed in demineralised water to remove excess picric acid, dehydrated via an ethanol series, infiltrated with paraffin and cut longitudinally in sections of 5 µm. The sections were then stained and analysed as outlined below.

Histological parameters

Mucous cells

Sections were stained with Alcian blue (pH 2.5) to identify acidophilic mucous cells and quantified by counting total cell numbers in 10 randomly selected 300 µm transverse sections of the epidermis from the dorsal part of the head of each fish as described in Chapter 4. The mean number of mucous cells per mm length of epidermis per fish was calculated. For all subsequent histological and immunohistochemical procedures, the same quantification location and method was used.

Proliferation

A monoclonal antibody, PC10 (Oncogene), against proliferating cell nuclear antigen (PCNA) was used. The sections were cleared and placed in a solution of 1% H_2O_2 in methanol for 5 min to remove endogenous peroxidase activity. The antigen was retrieved by microwaving cleared sections in 1% $ZnSO_4$ to 45 °C and maintaining them at this temperature for 5 min at 150 W. The sections were then transferred to 1% $ZnSO_4$ on ice for 2 min, microwaved again to 45 °C, maintained for 5 min and finally back to ice for 2 min. After overnight incubation with PC10 (1:10,000) at room temperature, the sections were incubated with a second antibody (goat anti-mouse, 1:200) for one hour, followed by avidin biotin complex (Vectastain, 1:100) for one hour. Sections were washed 3x between each step. The antibody complex was visualised by staining in filtered DAB-Ni (0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) with 2.5 mg/ml $(NH_4)_2SO_4.NiSO_4.6H_2O$ in Tris buffer) with

the addition of 0.0125% H_2O_2 immediately prior to use. Negative controls were included by omission of either the first or the second antibody and otherwise being incubated and stained as above. Total numbers of PCNA-positive cells were counted and expressed per mm of epidermis as described above.

Apoptosis

To determine the number of apoptotic cells, the TUNEL method was used (In Situ Cell Death Detection Kit, Fluorescein; Boehringer Mannheim). To visualise the fluorescein in the nuclei of apoptotic cells, the peroxidase-conjugated converter enzyme POD (Boehringer Mannheim) was utilised to bind to the fluorescein, and this was stained with DAB-Ni. The antigen was retrieved in 0.1% Triton-Na-citrate buffer in 10% phosphate buffered saline using the same microwaving protocol as for the PCNA above. Negative controls were included by omission of either the TUNEL enzyme or the POD converter enzyme. When quantifying apoptosis in the epidermis, a distinction was made between the upper 40 µm of the epidermis, and the lower 60µm area (Figure 1).

Cortisol receptors

The presence and localization of cortisol receptors in the epidermis was demonstrated using an antibody against glucocorticoid receptors raised in rabbits (Ducouret et al., 1995; Tujague et al., 1998). The protocol followed was the same as for the PCNA staining, including controls. The primary antibody was used at a working dilution of 1:32,000.

Scanning electron microscopy

For scanning electron microscopy, the fin samples were dehydrated via an ethanol series into warmed tertiary butanol (Inoue and Osatake, 1988). The butanol was frozen in the refrigerator, after which the samples were dried using a Virtis vacuum pump, then mounted on aluminium blocks and gold sputtered under an Argon atmosphere using a Balzers coating unit (CPD 020, Balzers, Switzerland), and viewed in a JSM T 300 SEM. Photographs were taken for a qualitative analysis of the surface ultrastructure of the epidermis.

Statistics

Data were analysed at each time point with analysis of variance (ANOVA) followed by Tukey-Kramer post-tests. All data are presented as means ± standard error. The sample size was n = 4 at the time zero and n = 10 for the other time points. These sample sizes were accomplished by pooling duplicate treatment tanks, as no significant differences between replicate treatment tanks at any sample point were found.

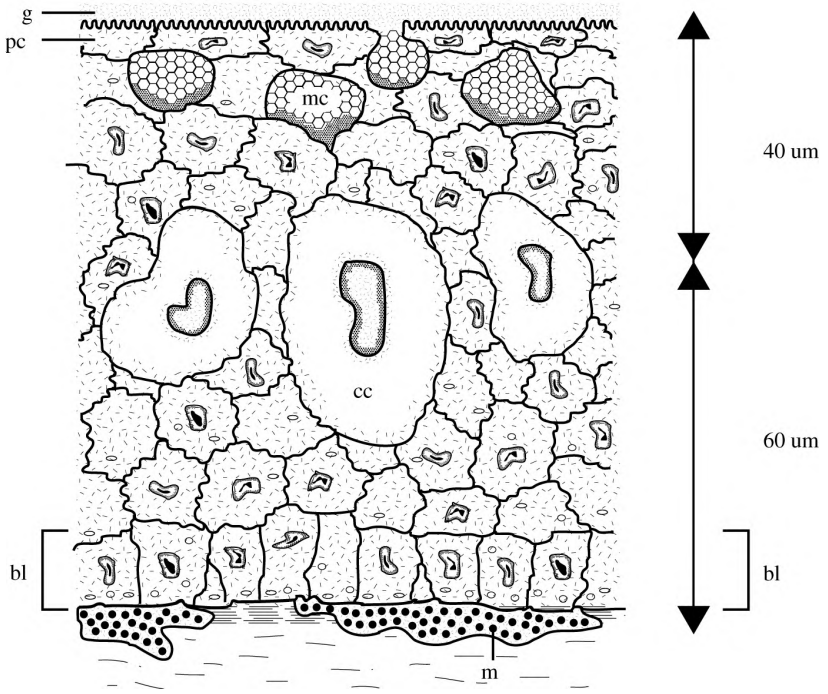


Figure 1. Schematic diagram of cross-sections of the skin of common carp *Cyprinus carpio* (modified after Iger & Wendelaar Bonga, 1994).
g glycocalyx; **pc**, pavement cell with microridges; **mc**, mucous cell; **cc** club cell; **bl**, basal cell layer; **m**, melanocyte.

Results

Fish condition and mortality

Throughout the experimental period, all fish fed well and remained in a good condition and apparently disease free. There was no mortality in any of the groups. At the end of the experiment, the body weight and condition factor of the CORT group were significantly lower than controls, while the fork length was unaffected (Table 1).

Mucous cells

The numbers of mucous cells were not significantly affected by the treatments at any sample point (Table 2).

Proliferation

The numbers of proliferating cells in the epidermis did not vary significantly between treatments at any sample point. PCNA-positive cell counts decreased with time in all 4 groups (Table 2). Negative controls showed no aspecific staining.

Table 1. Body parameters of juvenile common carp, *C. carpio* at the final sampling point. Fish were fed normal food ration (CTR), food containing 100 µg cortisol on 2 alternate days (CORT), infected with *A. japonicus* only (ARG), or cortisol feeding with *Argulus* infection 24 h after the 2nd cortisol feed (CORT+ARG) as described in the text. Values are mean ± SEM for n = 16. * = P < 0.05 compared with control (CTR) values.

	Body weight (g)	Fork length (cm)	Condition factor
CTR	1.74 ± 0.14	4.24 ± 0.11	2.23 ± 0.03
CORT	1.37 ± 0.09*	4.00 ± 0.09	2.12 ± 0.04*
ARG	1.49 ± 0.11	4.06 ± 0.12	2.18 ± 0.05
CORT+ARG	1.66 ± 0.10	4.22 ± 0.08	2.16 ± 0.03

Table 2. Number of proliferating cells and mucous cells in the epidermis of head skin from juvenile common carp, *C. carpio*. Fish were fed normal food ration (CTR), food containing 100 µg cortisol on 2 alternate days (CORT), infected with *A. japonicus* 24 h after the 2nd feed (ARG), or both treatments (CORT+ARG) as described in the text. Values are mean ± SEM for n = 8, except at time zero where n = 4. Superscript lettering indicates significant differences compared with Time zero values in the same row. [a] = p < 0.05, [b] = p < 0.01, [c] = p < 0.001.

Parameter and treatment group		Time zero	24 h post-cortisol (4 d)	48 h post-Argulus (6 d)	Final sample (32 d)
Number mucous cells·mm^{-1}	CTR	166.6 ± 12.8	138.6 ± 12.1	144.9 ± 20.7	159.8 ± 10.2
	CORT	126.8 ± 8.5	157.8 ± 10.3	166.4 ± 10.1	155.1 ± 10.0
	ARG	153.9 ± 9.4	103.1 ± 15.9	174.4 ± 10.4	184.3 ± 16.9
	CORT+ARG	118.7 ± 10.5	119.6 ± 11.0	148.0 ± 8.5	151.5 ± 10.3
Number PCNA-positive cells·mm^{-1}	CTR	313.4 ± 27.9	219.0 ± 23.1	178.6 ± 27.0 [b]	93.2 ± 7.7 [c]
	CORT	320.7 ± 11.5	176.8 ± 12.2 [c]	188.5 ± 19.8 [c]	94.4 ± 6.4 [c]
	ARG	328.5 ± 22.9	258.6 ± 17.5	177.5 ± 15.2 [c]	75.9 ± 11.9 [c]
	CORT+ARG	340.7 ± 26.3	243.0 ± 19.4	212.7 ± 32.3 [a]	89.8 ± 9.1 [c]

Apoptosis

The incidence of apoptosis in the upper epidermis was similar in all groups at t = 0. At 24 h post cortisol feeding, there was a significant reduction in the number of apoptotic cells in the upper epidermal layer of both groups (CORT and CORT+ARG) that received cortisol in the diet (Figure 2). At 48 h after infestation with *A. japonicus* there was no significant difference between treatments. At the final sampling (32 d), each treatment group had significantly lower numbers of apoptotic cells in the upper epidermis compared with control groups. This decrease in numbers of apoptotic cells occurred in a manner, such that the effects of the treatments were additive (Figure 2). There were only about 50% as many apoptotic cells in the lower epidermis as there were in the upper epidermis (Figure 3). There were no significant differences between treatments at any time point, other then the CORT+ARG group 48 h after infection with *Argulus* when the number of apoptotic cells in the lower layer of the epidermis was significantly higher than that of the control group (Figure 3). Excluding the TUNEL enzyme as a negative control resulted in staining of the cytoplasm only of club cells. Excluding the POD converter enzyme resulted in no staining at all, indicating a cross-reactivity of the POD antibody with a club cell specific cytoplasmic antigen. As the method stains apoptotic nuclei specifically, this cross-reactivity did not interfere with analysis of apoptosis.

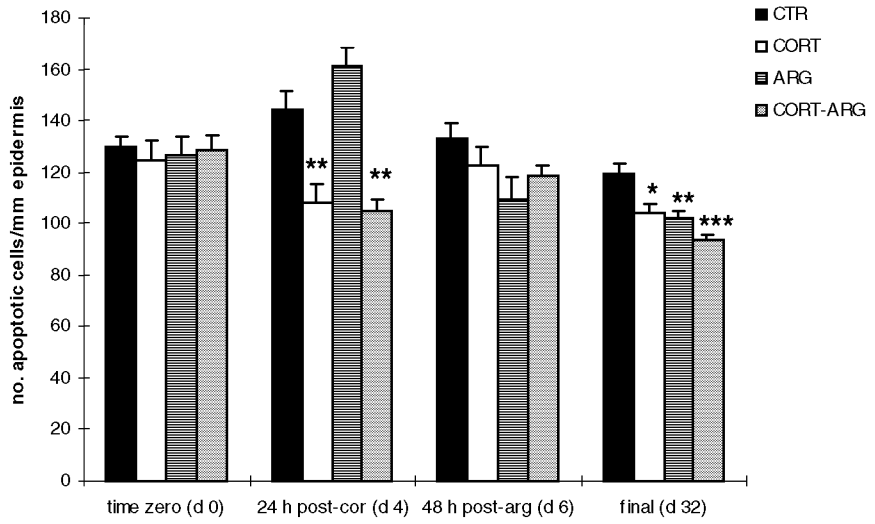


Figure 2. Numbers of apoptotic cells in the upper (± 40 µm) layer of the epidermis of carp (*C. cyprinus*) head skin of control (CTR), cortisol fed (CORT), *Argulus*-infested (ARG) and combined cortisol feeding followed by *Argulus*-infestation (CORT+ARG). Data are presented as mean (± SEM) numbers of TUNEL-positive cells per mm length of epidermis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ indicate statistically significant differences from the control group at the same time point where n = 4 at the time zero and n = 10 for the other time points.

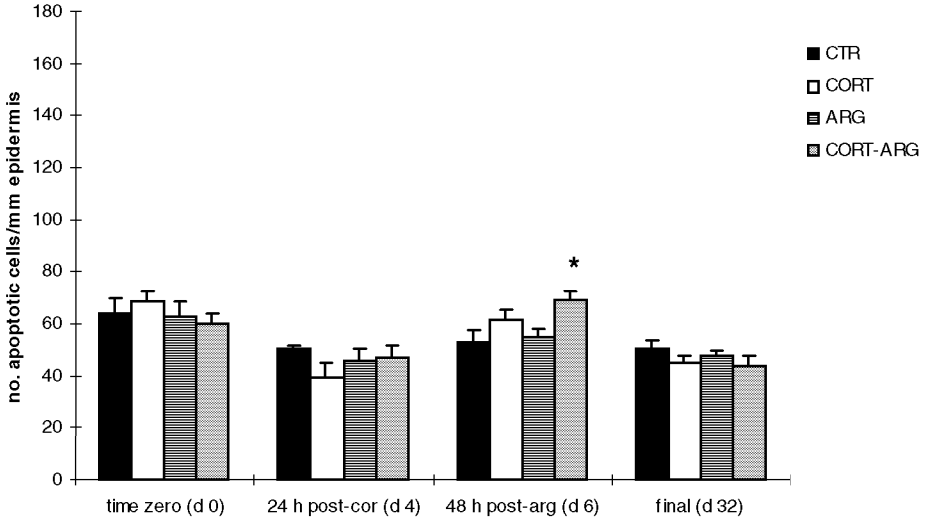


Figure 3. Numbers of apoptotic cells in the lower (± 60 µm) layer of the epidermis of carp (*C. cyprinus*) head skin of control (CTR), cortisol fed (CORT), *Argulus*-infested (ARG) and combined cortisol feeding followed by *Argulus*-infestation (CORT+ARG). Data are presented as mean (± SEM) numbers of TUNEL-positive cells per mm length of epidermis. * $P < 0.05$ indicates statistically significant difference from the control group at the same time point where n = 4 at the time zero and n = 10 for the other time points.

Cortisol-receptors

No non-specific staining was observed in staining controls (Figure 4a). Cortisol-receptor staining occurred most intensely in the upper layer of the epidermis, primarily in the top few cell layers (pavement cells and underlying filament cells, Figure 4B). Some staining was also observed in the lower layer, mainly in irregularly shaped migrating leukocyte-like cells (macrophages and lymphocytes, Figure 4C).

Scanning electron microscopy

The surface of the epidermis of the fins of CTR carp in the scanning electron microscope was comprised of polygonal cells of 7-10 µm in diameter with concentric microridges coupled tightly together by tight cell-cell borders, and many pores, mainly located at the junction of 3 cells. These are the sites of mucus discharge (Figure 5A). Apoptotic or sloughing cells were not observed. Cortisol feeding had no effect on the skin surface ultrastructure. Exposure to the parasite resulted in slight concentric structure disruption of the microridges of the pavement cells, cell-cell borders were raised above the cell surface, and enlarged pores were observed between the cells at 48 h post-infection (Figure 5B). However, apoptotic cells or sloughing cells were not observed. At 48 h post-infection, the microridges of the pavement cells of CORT+ARG fish skin were less concentric than those of controls (Figure 5C). In general, more small pores discharging mucus were observed at the surface. At 32 d the SEM appearances of all treatments were similar to controls.

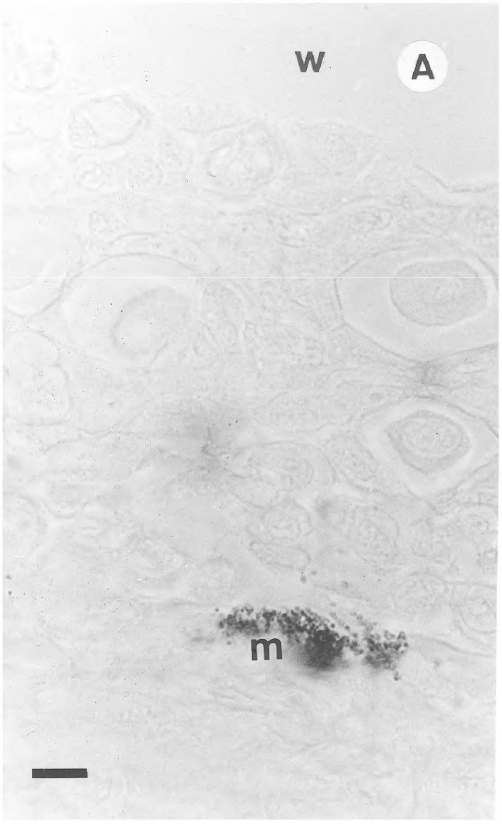
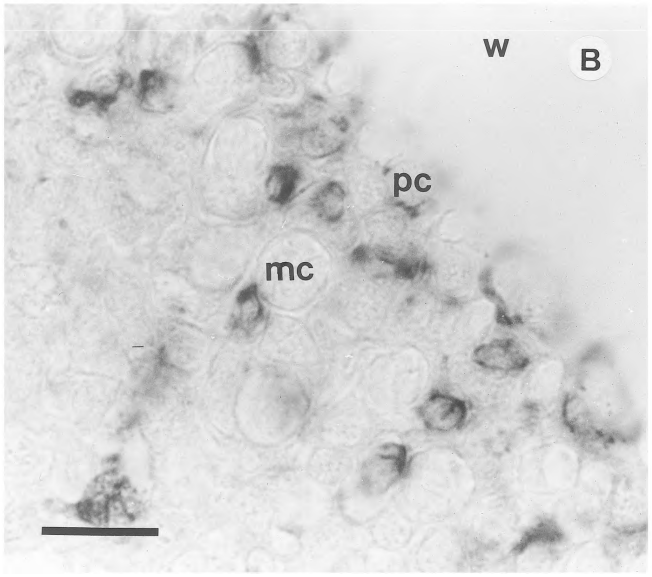
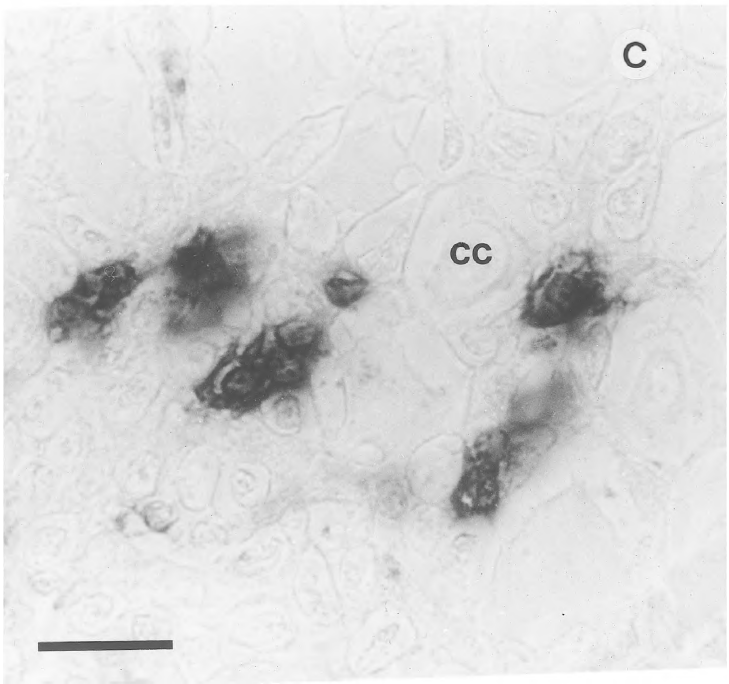


Figure 4. (A) Light micrograph of the epidermis of carp (*Cyprinus carpio*) skin. Negative control to which no cortisol receptor antibody has been applied. There is no staining visible. **w** = water side, **m** = melanocytes. Scale bar, 10 µm. (B) The upper epidermis of carp skin, stained with cortisol receptor antibody. The immunopositive cells are primarily located in the upper cell layers. **w** = water side, **mc** = mucous cell, **pc** = pavement cell. Scale bar, 15 µm. (C) The lower epidermis of carp skin, stained with cortisol receptor antibody. Morphologically, the stained cells are likely to be leukocytes. **cc** = club cell. Scale bar, 15 µm.





Discussion

This study showed that stress free administration of cortisol via the food reduced the numbers of apoptotic cells in the upper epidermis of juvenile common carp *C. carpio* within 24 h. High circulating levels of this stress hormone induced apoptosis in many teleost cell types e.g. lymphocytes (Weyts et al., 1997), activated b-cells, but not other lymphoid cells (Weyts et al., 1998), chloride cells (Bury et al., 1998), and pavement cells (Iger et al., 1995). In general, many deleterious effects attributed to cortisol were reported in the literature, and few other data on the beneficial or the additive effects are available. The stress response of fish is designed to be adaptive, enabling the organism to defend homeostatic mechanisms threatened or disturbed by a stressor (Wendelaar Bonga, 1997). Therefore, it is not particularly surprising that if levels of cortisol were moderately elevated by cortisol administration via the food in a stress-free environment and manner, responses designed to be adaptive might be recorded. Previously, similar methodology was used to study the host-parasite interaction between *A. foliaceus* and the rainbow trout *Oncorhynchus mykiss* (Ruane et al., 1999a; Chapter 5). These studies revealed that moderately elevated cortisol levels did not stimulate mucous cell discharge *in vitro* or *in vivo* and that *in vivo*, experimental cortisol elevation was associated with the stimulation of the synthesis and release of granules from the pavement cells coincident with reduced establishment of *A. foliaceus* (Chapter 5). Furthermore, incubating trout epidermis with cortisol *in vitro* for 24 h demonstrated that under these conditions, only cortisol concentrations above 500 ng· ml^{-1} increased levels of apoptosis (Nolan et al., 1999b; Chapter 7). Using *in vitro* short-term organ culture, Bury et al. (1998) showed that cortisol protects tilapia (*Oreochromis mossambicus*) chloride cells from copper toxicity, indicating a protective function of the hormone against toxic stressors.

Argulus japonicus infection resulted in reduced numbers of apoptotic cells in the upper epidermis of the carp, but only after 30 days. This is not easily explained, but is a consistent observation in all three of the treatment groups, and demonstrates how the epidermis of fish is influenced in the long-term by previous stress and stress-related experiences. This corroborates the observations from several other studies where stress-related effects were reported still present in fish skin epidermis for up to 30 days (Iger et al., 1994c, d; Burkhardt-Holm et al., 1997; Nolan et al., 1998; Chapter 2) and supports the view that some of these prolonged effects can be attributed to the effects of cortisol alone. Prolonged effects on the endocrine and immune system were demonstrated in *O. mykiss* after similar cortisol feeding (Ruane et al., 1999a).

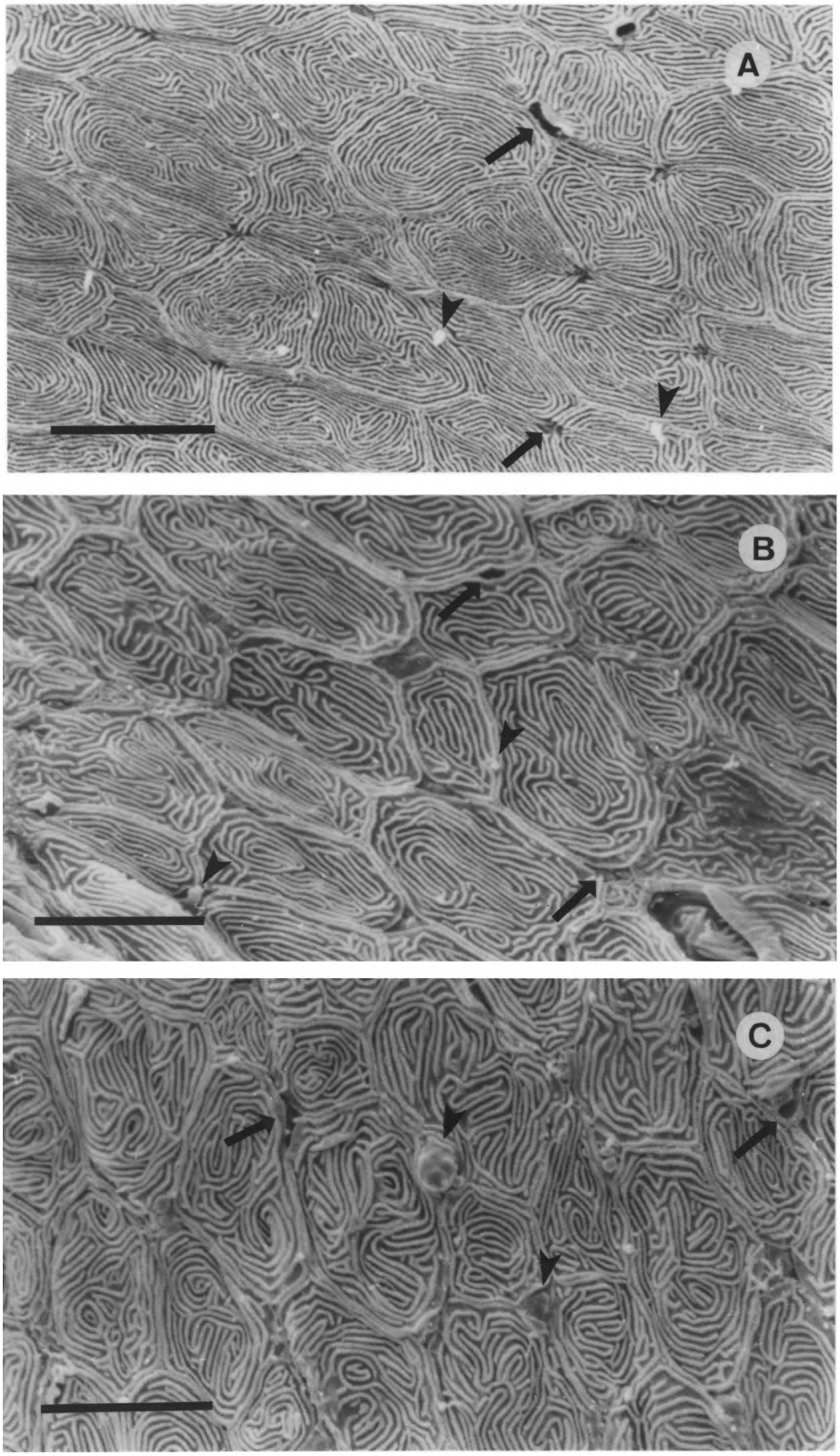


Figure 5. Scanning electron microscopy of the epidermal surface of the fin of a control carp (*Cyprinus carpio*). (A) Control at time zero. Microridges are visible as concentric lines on the surface of the pavement cells, which are tightly connected to each other. Discharged mucus (arrowheads) and pores (arrows) are visible in between. (B) *A. japonicus* infested at 48 h post-infection. The concentric structure of the microridges is disrupted. Cell-cell borders are raised above the plane of the cells. The mucous pores between the cells are enlarged (arrows). (C) Cortisol-fed and *A. japonicus* infested at 48 h post-infection. The microridge structure is altered and the overall ultrastructure is less concentric than in controls. Many pores (arrows) discharging mucus (arrowheads) are visible. Cell-cell borders are not raised and are in the same plane as those of controls. Scale bars are 10 μm.

Other studies generally report increased apoptosis associated with stress and cortisol administration, but these studies imposed strong (chronic or acute) stressors, or high cortisol doses. The results and conclusions were based on semi-quantitative evaluation of extensive transmission electron microscopy (TEM) observations (Iger et al., 1994c, d, 1995; Burkhardt-Holm et al., 1997; Nolan et al., 1998; Chapter 2), while we used quantitative analysis after TUNEL staining. The TUNEL-method is based on specific labelling of nuclear DNA fragmentation in situ through end-labelling of double stranded DNA breaks with terminal transferase (Negoescu et al., 1997). Furthermore, we are confident about the quantification of apoptotic cells we report, as the location of apoptotic cells agree well with many TEM studies on fish skin (Iger et al., 1994c, d, 1995; Burkhardt-Holm et al., 1997; Nolan et al., 1998; Chapter 2), where apoptosis was reported as being primarily concentrated in the upper epidermal cells. In addition, our SEM observations confirmed that, although there were minor effects of *Argulus* on the appearance of the pavement cell microridges, increased cell death in the upper cell layers was not characteristic in this experiment. In other studies, SEM examination of the epidermis surface has clearly demonstrated increased cell death (Chapters 2 and 4).

We also demonstrated increased levels of apoptosis in certain cell types in the lower epidermis of the CORT+ARG groups, confirming TEM observations on stressed fish (Iger et al., 1994c; Burkhardt-Holm et al., 1997; Nolan et al., 1998) and providing some evidence for potentially immunosuppressive effects. These observations reflect the combined effects of the 2 experiences, where the treatments individually induced a mild stress response and processes, which are adaptive, and when combined, induced a stronger stress response, with the negative effects considered to be maladaptive. The maladaptive aspects of the stress response of fishes, including immunosuppressive effects, are discussed by Wendelaar Bonga (1997). Additive effects of treatments have been reported for cortisol administration and infestation of *O. mykiss* with adult *Argulus* (Ruane et al. 1999), short-term moderate temperature elevation and exposure to Rhine water in the sea trout *Salmo trutta* (Chapter 2) and exposure of *Carassius auratus* to treated sewage and challenge with the pathogen *Aeromonas salmonicida* (Kakuta, 1997).

It was reported that stressors induce the synthesis and discharge of vesicles in the upper epidermal cells. As these vesicles contain peroxidase (Iger and Wendelaar Bonga, 1994; Iger et al., 1994d), the contents of the vesicles may be protective to the fish as a non-specific, probably antipathogenic, defence mechanism. The prevention of apoptosis by cortisol may represent a prolongation of the life of these vesicle-producing cells. The dose of cortisol we administered in our study was low, and expected to generate mildly elevated blood cortisol levels in the fish, following other studies (Barton et al., 1987; Balm and Pottinger, 1995; Ruane et al., 1999a). Although we have not been able to report the actual blood cortisol levels for the fish in our study due to the small size

of the fish (~0.8 g body weight), we can state that the dose provided no evidence of increased mortality or observable pathology and did not reduce growth in the long term. These observations suggest that the dose used must not have elevated blood levels excessively. Blood cortisol measurements, while useful, do not provide information about the clearance rates of the administered cortisol in the fish. In the present study, we have localised the effects of the treatments separately and in combination, as well as demonstrating and localising the presence of the cortisol receptor in the target tissue, providing strong circumstantial evidence for the role of cortisol in mediating the observed effects.

The teleost skin is a dynamic tissue and is influenced by external factors, including stressors (Iger et al., 1994c, d; Burkhardt-Holm et al., 1997; Nolan et al., 1998; Chapters 2 and 4). This not only holds for epidermal processes such as apoptosis and mitosis, but also for the production and discharge rate, as well as composition, of mucus (Pickering and Richards, 1980; Shephard, 1994; Burkhardt-Holm et al., 1997). The numbers of mucous cells in the carp epidermis were unaffected by the treatments. Reduced numbers of epidermal mucous cells have been reported for *S. salar* exposed to salmon lice *L. salmonis* (Chapter 4). Exposure to Rhine water did not affect the numbers of mucous cells in either *O. mykiss* or *S. trutta*, although increased mucous discharge and other stress-related effects such as increased apoptosis and leukocyte infiltration were reported. Other studies report stressor-induced decreases in mucous cell numbers accompanied by increased leukocyte infiltration (Martinez et al., 1994; Burkhardt-Holm et al., 1997). Together, these studies indicate that there is a complex relationship between the kind of stressor and mucous cell numbers. As the numbers of epithelial mucous cells of our carp were similar between treatments and over time, it is clear that neither cortisol feeding, infestation with *A. japonicus* alone, nor the combination of both treatments disrupted the balance between proliferation and differentiation of these cells, nor their discharge.

We used PCNA immunohistochemistry to stain and quantify proliferating cells. The proliferating cell nuclear antigen is a 36 kDa antigen called cyclin that, as an auxiliary protein for DNA-polymerase delta, is required for DNA synthesis and repair (Kurki et al., 1987; Alison, 1995). PCNA-levels in the cell begin to rise during the G1-phase and are maximal during the S-phase; the phase of DNA-replication. During the G2 and M-phase the levels drop again. Therefore, the method allows quantification of all cells in the proliferative cycle, which may not be morphologically distinguished as mitotic in TEM. This technique also allowed us to evaluate much larger areas of the epidermis than evaluated in the study of Iger et al. (1995). The method does not allow us to distinguish between different cell types undergoing mitosis, and therefore it is possible that one cell type (or subpopulation) may increase proliferative activity, while another shows a reduction, with total numbers unaffected overall, as we report here.

Johnson & Albright (1992b) reported that cortisol implants in coho salmon *O. kisutch* reduced the development of epithelial hyperplasia when the fish were subsequently infested with sea lice. However, these authors dosed their fish with high levels of cortisol, raising serum/plasma levels up to 819 ng/ml. Increased incidence of mitotic figures in fish skin in response to a variety of stressors, including cortisol administration, has been reported from electron microscopy studies (Iger et al., 1994b, c, 1995). However Iger et al. (1995) also administered a high cortisol dose, elevating plasma cortisol levels to circa 400 ng cortisol/ml. The cortisol dose we administered to the carp in the present experiment was much lower and therefore may not have been sufficient to stimulate mitosis.

This is the first study to demonstrate cortisol receptors in the skin of common carp. Effects of cortisol administration on epithelial cells of *O. mykiss* included increased synthesis and secretion of vesicles, increased cellular apoptosis and proliferation rates (thus inferring increased cell turnover rate), together with an increase in the discharge of mucus and infiltration by leukocytes (Iger et al., 1995). The data from the present study support the view that many of these effects may be directly mediated via the cortisol receptor, as most cortisol receptor immunoreactivity was found in the upper epidermis (consisting primarily of pavement cells and cells capable of differentiating into them) and in leukocytes in the basal cell layers.

Low levels of cortisol can enhance carp neutrophil function and proliferation *in vitro*, while higher levels induced apoptosis in B-lymphocytes (Weyts et al., 1998). These observations along with those from the present study support the hypothesis that low levels of cortisol induce adaptive effects, whereas higher levels may be maladaptive in teleost fish (Wendelaar Bonga, 1997). We show, for the first time, that effects of cortisol are limited to the cells in the epidermis, which express the cortisol receptor. Overall, the treatments administered to the fish in the present study and the data generated indicate that cortisol does not always induce deleterious effects and at low levels can induce effects that may be adaptive. In addition, some aspects of the host-parasite interaction are clearly indirect effects of the parasite and are mediated in the host fish by cortisol. The effects of the combined treatments indicate that there is a threshold beyond which the negative effects of stress become manifest, i.e. immunosuppression.

Acknowledgements

The antibody against the cortisol receptor was a generous gift from Dr. B. Ducouret, INRA, Rennes, France. The authors are grateful to Professor G.A. Boxshall (British Museum of Natural History, London) for identification of the *Argulus* species, and to L.J.S. Brokken who developed the PCNA immunohistochemistry and shared the method.

Chapter 7

In vitro evidence that cortisol directly modulates stress-related responses in the skin epidermis of the rainbow trout (*Oncorhynchus mykiss*)

D.T. Nolan, A.L. van der Salm and S.E. Wendelaar Bonga

Abstract

Exposure of fish to stressors leads to multiple changes in the skin epithelium, including increases in mitosis, necrosis, apoptosis, leukocyte infiltration, and increased mucous cell and pavement cell secretion. We investigated the role of the stress hormone cortisol in the control of these changes by exposing pieces of skin from the rainbow trout *Oncorhynchus mykiss* to cortisol using a 24 h *in vitro* tissue culture incubation procedure. The effects of short-term exposure of skin pieces to 4 cortisol concentrations (0, 50, 500 and 1000 ng/ml) were investigated after 24 h *in vitro* tissue culture. Numbers of mucous, mitotic and apoptotic cells were quantitatively assessed using immunohistochemical techniques, in situ nick end labelling (TUNEL), as well as conventional light and scanning electron microscopy (SEM). The glucocorticoid receptor blocker mifepristone was used to investigate whether the effects could be attributed to the direct action of the hormone. Overall, cortisol had no effect on the mucous cell population. Incubation with the receptor blocker reduced the number of mucous cells. Cell proliferation was stimulated by the addition of 50 and 500 ng cortisol/ml, but not at 1000 ng/ml. Incubation with the receptor blocker increased proliferation in the 0 ng cortisol/ml group only. Apoptosis increased at 500 and 1000 ng/ml cortisol. This increase was blocked by incubation with the receptor blocker, which resulted in lower numbers of apoptotic cells in all except the 0 ng cortisol/ml group. SEM observations corroborated the quantitative data. The study showed that the effects of stressors mentioned above on the fish epidermis are dependent on cortisol, but are not all mediated in the same manner via the glucocorticoid receptor.

Introduction

The skin epithelium of teleost fish is a complex multilayered assembly of living cells, which are mostly not, or only lightly keratinised. The upper layer of living cells is in direct contact with the ambient water. The epidermal structure of fishes changes in response to a wide variety of stressors (McBride and van Overbeeke, 1971; Roubal and Bullock, 1988; Shephard, 1994). Levels of the primary stress hormone cortisol are elevated in the blood of stressed fish and can lead to decreased disease resistance through immunosuppression, reduced growth and reduced reproduction (Anderson, 1990; Wendelaar Bonga, 1997).

Increased mucus discharge, general cellular apoptosis and necrosis, as well as proliferation and differentiation have been reported by many authors as general effects of stressors on the teleost epidermis (Iger et al., 1994b; Burkhardt-Holm et al., 1997; Chapter 6). And several of these, including increased mucous cell discharge, proliferation and apoptosis were shown to be influenced in the rainbow trout by cortisol administration *in vivo* (Iger et al., 1995). Some authors have concluded that the skin is capable of autonomic regulation of many of these processes, as data on the effects of environmental acidification on rainbow trout skin indicated that neither cortisol, α-MSH nor prolactin levels were affected, while a variety of stress-related effects were reported (Balm et al., 1995).

Although *in vivo* administration of cortisol to *Oncorhynchus mykiss* (Laurent and Perry, 1990; Ruane et al., 1999a), *O. kisutch* (Maule and Schreck, 1991; Johnson and Albright, 1992b; Shrimpton and Randall, 1994), *O. masou* (Nagae et al., 1994), *Salmo trutta* (Seidelin et al., 1999), *S. salar* (Ross et al., 2000) and *Cyprinus carpio* (Weyts et al., 1998b; Chapter 6) has produced effects which were attributed to cortisol, clear evidence about this relationship can not be obtained *in vivo* because of the presence of many endogenous factors that can interfere with, or be influenced by cortisol administration. Other hormones may also mediate similar effects on the skin epidermis (Pickering and Duston, 1983; Pottinger and Pickering, 1985; Balm et al., 1995). This was also indicated by the results of our immunohistochemical study on the localisation of the glucocorticoid receptor in the rainbow trout epidermis (Chapter 5). The receptor was only expressed in the upper cell layers of the epidermis (pavement cells and upper filament cells), and in leucocytes migrating through the tissue, although the effects of cortisol were reported for a much broader number of cell types (Iger et al., 1995). For cortisol, this suggests the involvement of another corticosteroid receptor, possibly equivalent to the mineralocorticoid receptor of mammals (Jenq et al., 1996; Kolla et al., 1999).

In vitro methods offer an attractive manner to investigate whether or not cortisol can mediate directly the effects reported in fish skin *in vivo* (Iger et al., 1994b, 1995; Balm et al., 1995; Chapters 5 and 6). Using *in vitro* methods, cortisol was shown to induce differentiation (McCormick, 1990)

and apoptosis (Bury et al., 1998) of chloride cells in the gills of the tilapia *Oreochromis mossambicus*. The latter authors demonstrated a number of effects attributed to the addition of the hormone cortisol including increased apoptosis and decreased sensitivity to copper toxicity. They also showed that the apoptosis of chloride cells was mediated by glucocorticoid receptors by using the synthetic glucocorticoid receptor blocker RU 486 (also known as mifepristone). This receptor blocker was used as a tool in several studies with rainbow trout (Van Oostrom and Bols, 1991; Dasmahapatra and Lee, 1993; Vijayan et al., 1994b; Reddy et al., 1995; Bury et al., 1998; Weyts et al., 1998a). It binds with high affinity to the cytosolic glucocorticoid receptor without exhibiting agonistic activity (discussed in Reddy et al., 1995).

In the present study, we used a short-term *in vitro* culture method to study mucous cells, mitosis and apoptosis in the trout skin epidermis in response to different cortisol levels and to identify which of the stress effects reported for fish skin in the literature can be attributed to the direct action of the hormone. We incubated pieces of skin with 0, 50, 500 and 1000 ng/ml cortisol in the absence and presence of mifepristone and measured mucous cells, proliferating cells and apoptotic cells.

Materials and methods

Culture method and sampling

Replicate biopsies of head skin measuring approximately 20 x 20 mm were sampled from 6 irreversibly anaesthetized rainbow trout of about 150 g from laboratory stock kept in Nijmegen tap water at 15 °C on a 12 h light:12 h dark photoperiod were used. The fish were fed commercial trout pellets (2% of fish body weight fed once daily). The biopsies were washed 3 times in sterile PBS for 15 min each wash. One piece from each fish was then placed in 5 ml of a modified complete fish skin culture medium, the same as that described by Mothersill et al. (1995), but without the addition of cortisol. One skin piece from each fish was placed into a series of culture media containing cortisol (Hydrocortisone; Sigma) at a concentration of 0, 50, 500 and 1000 ng/ml cortisol. Similarly, in the same way, a replicate set of skin pieces was placed in a replicate series of media containing the same cortisol concentrations plus 100 ng/ml of the glucocorticoid receptor blocker mifepristone.

After 24 h incubation at room temperature, the skin samples were divided using a sharp scalpel blade. The larger parts were fixed in Bouin's solution for 24 h and routinely processed for light microscopy, sectioned at 5 μm and attached to polylysine coated slides. For electron microscopy, smaller pieces of skin (5x5 mm) were fixed in Na-cacodylate buffered glutaraldehyde for 20 min on ice, followed by post-fixation in 1% osmium tetroxide in the same buffer (Iger et al., 1994c, 1995).

Sample processing

For scanning electron microscopy (SEM), the fixed skin samples were dehydrated through an ethanol series and freeze dried following the method developed by (Inoue and Osatake, 1988), as described in Nolan et al. (2000) and Chapter 6. The specimens were mounted on aluminium stubs, gold sputtered in a Balzers coating unit (CPD 020, Balzers, Switzerland) and viewed in a Jeol-JSM T 300 scanning electron microscope at an accelerated voltage of 15 kV. The specimens were scanned and photographed onto Agfapan 100 ASA black and white print film. Exposed film was developed and printed by conventional means. Semi-quantitative analysis was carried out by examination of the micrographs and evaluation of the treatments against the control (0 ng cortisol/ml) group in terms of the appearance of the apical surfaces of the pavements cells, occurrence of discharging mucous cells, as well as the presence of swollen or sloughing cells.

To remove endogenous peroxidase activity, the light microscope sections were incubated in 5% H_2O_2 in methanol for 15 mins, rehydrated through an alcohol series and then microwaved at 450 watt to 50 ºC for 5 min in an antigen retrieval solution (see below), plunged into the same solution on ice for 2 minutes and the heating-cooling process repeated a second time. The solutions were 1% ZnSO4 in demineralised water (immunohistochemistry) or 0.1% Triton X100/Na^+-citrate in 10% PBS (TUNEL apoptosis staining) to open the matrix. The PCNA staining was carried out using standard methods and the antibody was applied overnight (PC 10, Oncogene at 1:10000) at room temperature. The immunohistochemistry was completed by the application of appropriate secondary antibodies and the antigens visualized with an appropriate peroxidase systems to react against DAB-nickel (0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) with 2.5 mg/ml $(NH_4)_2SO_4.NiSO_4.6H_2O$ in Tris buffer with the addition of 0.0125% H_2O_2 immediately prior to use), as described in Chapter 6.

For TUNEL identification of apoptosis, the In Situ Cell Death Detection Kit (Fluorescence, Boehringer Mannheim) was used according to the manufacturers instructions and the fluorescein visualized using a peroxidase conjugated antibody (POD, Boehringer Mannheim), visualized with DAB-nickel. Negative controls were included each time by excluding either primary or secondary antibody (immunohistochemistry) and the TUNEL enzyme or POD converter antibody system (apoptosis staining). Details of the above analytical methods can be found in Chapter 6. Mucous cells were stained by the Alcian blue (pH 2.5) method. Numbers of positive cells were quantified in 6 independent 300 µm views, as described in Chapter 4.

Data handling and statistics

Data are expressed as means ± SE for n = 6 and effects of treatments between groups were investigated using ANOVA and Tukey post-hoc testing. Student's t-test (2 sided) was used to identify differences between comparable groups with and without the glucocorticoid receptor

blocker. For each parameter, trend analysis was applied to test for a relationship between cortisol dose and parameter response. Statistical significance was accepted at $P < 0.05$.

Results

The number of PCNA positive cells, indicative of cell proliferation, in the skin biopsies incubated at 50 and 500 ng cortisol/ml was significantly higher than in the control (0 ng/ml) biopsies, but not in those incubated with cortisol at 1000 ng/ml (Figure 1). Incubation with mifepristone resulted in a higher number of PCNA positive cells in the 0 ng/ml groups, but there were no other effects (Figure 1). There was no significant relationship between cortisol concentration and numbers of proliferating cells (Table 1). The number of apoptotic cells in the epidermis generally increased in response to increased cortisol concentration in all cortisol only treated groups (Figure 2) and there was a significant relationship between cortisol concentration and apoptosis (Table 1).

Table 1. Trend analysis of parameters measured from *Oncorhynchus mykiss* skin biopsies after 24 h *in vitro* incubation with cortisol alone or together with the glucocorticoid receptor blocker, mifepristone. The statistics are calculated based on n = 24.

	Slope	r^2	F	P
0, 50, 500, 1000 ng cortisol/ml				
Mucous cells	-1.970	0.0441	1.0709	0.3131
Proliferating cells	2.601	0.0362	1.2950	0.2686
Apoptotic cells	10.167	0.7811	92.1336	<0.0001
0, 50, 500, 1000 ng cortisol/ml with mifepristone (100 ng/ml)				
Mucous cells	-1.4640	0.0072	0.1888	0.6686
Proliferating cells	-0.6470	0.0043	0.0919	0.7649
Apoptotic cells	-1.272	0.0497	1.7720	0.1981

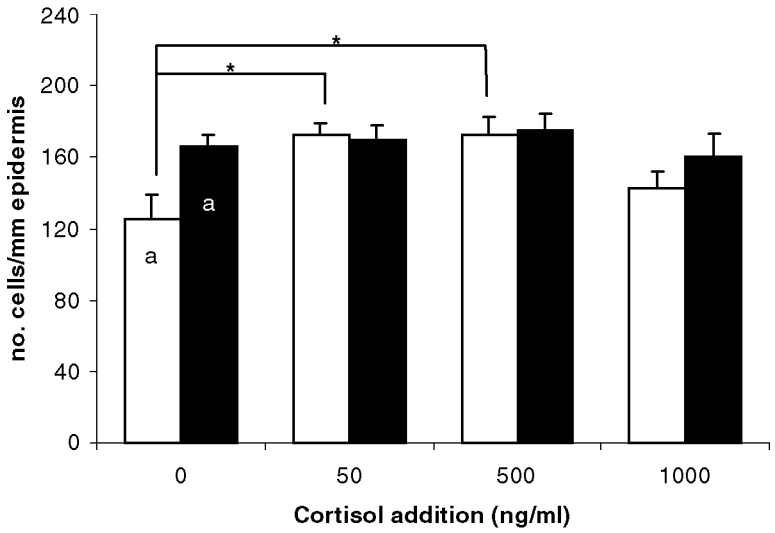


Figure 1. The numbers of proliferating cell nuclear antigen positive cells per mm epidermis identified by immunohistochemistry in biopsies of head skin from the trout *Oncorhynchus mykiss* incubated with different cortisol concentrations (clear bars) and with different cortisol concentrations with the glucocorticoid receptor blocker mifepristone (black bars). The data are expressed as mean ± SE for n = 6. * indicates significantly different from 0 ng cortisol/ml at $P < 0.05$, while bars sharing the same letter are significantly different from each other at $P < 0.05$.

Incubation in the presence of mifepristone effectively blocked this and there was no significant difference between any of the mifepristone treated groups, regardless of cortisol concentration (Figure 2). The numbers of apoptotic cells in the mifepristone incubated groups were significantly lower at than those of groups incubated without the blocker.

Mucous cell numbers were not affected by incubation with cortisol, with or without mifepristone (Table 2). However, in general there were lower numbers of mucous cells in the groups incubated with the receptor blocker (except at 500 ng/ml, where there were 3 high and 3 low values), and these reductions were significant at 50 and 1000 ng cortisol/ml (Table 2). There was no significant relationship between cortisol concentration and mucous cell numbers overall (Table 1).

SEM analysis after 24 h in culture indicated that the skin of control was in good condition generally, with intact microridge structures and fragments of mucus from discharging mucous cells at the surface. More pavement cells with characteristics of apoptosis were observed *in vitro* than is usual *in vivo* (Figure 3a). With the addition of 50, 500 and 1000 ng cortisol/ml, there was a marked increase in mucous cells discharging and this created a dramatically different appearance in many areas (Figure 3b). The upper epidermal cells of the groups at 500 and 1000 ng cortisol/ml showed many signs of dying pavement cells and increased cell turnover (Figure 4a). Pavement cells lost the microridge structure and sloughed off in a characteristic manner. Incubation with mifepristone had little effect on the 0 ng/ml treatment and the SEM appearance of the skin was generally comparable with that of skin incubated without the blocker, except that the incidence of apoptotic pavement cells appeared lower (Figure 4b). The other treatments gave inconsistent results, as some skins and areas were similar to those incubated without the receptor blocker with much cell death, while others were in good condition, more closely resembling that of the control 0 ng/ml group. A semi-quantitative overview of the effects of the treatments observed is given in Table 2.

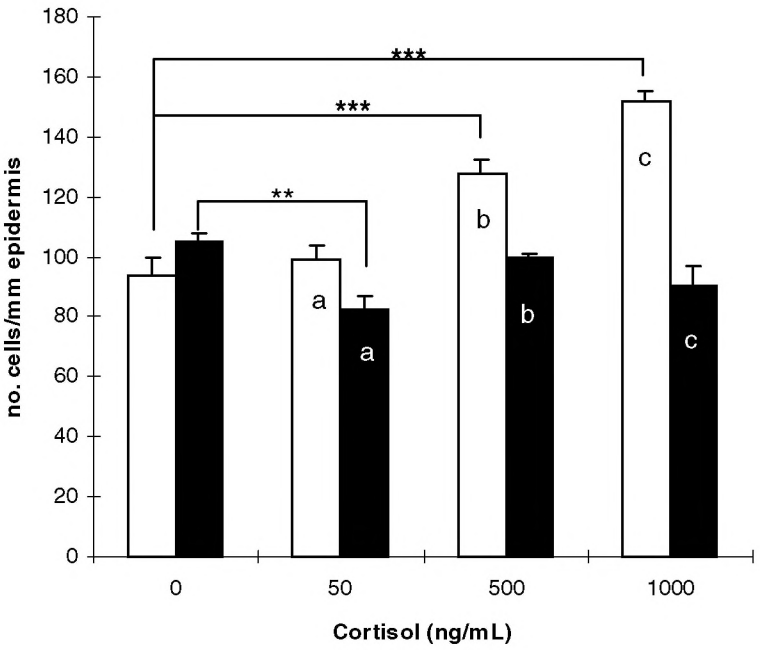


Figure 2. The numbers of apoptotic TUNEL positive cells per mm epidermis identified in biopsies of head skin from the trout *Oncorhynchus mykiss* incubated with different cortisol concentrations (clear bars) and with different cortisol concentrations with the glucocorticoid receptor blocker mifepristone (black bars). The data are expressed as mean ± SE for n = 6. * indicates significantly different from 0 ng cortisol/ml at $P < 0.05$, *** indicates significance at $P < 0.001$. Bars sharing the same letter are significantly different from each where a = $P < 0.05$; b = $P < 0.001$; c = $P < 0.0001$.

Table 2. Parameters analysed from *Oncorhynchus mykiss* skin biopsies after 24 h *in vitro* incubation with different cortisol concentrations alone, or together with the glucocorticoid receptor blocker mifepristone (100 ng/ml). The numbers of mucous cells were quantified from light microscopy, while the other parameters were semi-quantitatively evaluated from scanning electron microscopy. Data are given as mean ± SE where n = 6. Values sharing the same superscript letter are significantly different from each other (^{a} = $P < 0.01$, ^{b} = $P < 0.05$).

Treatment group	No. mucous cells/mm epidermis	Mucous cell discharge	Pavement cell swelling and sloughing
0 ng/ml cortisol only	74 ± 7	0	+
50 ng/ml cortisol only	90 ± 5 ^{a}	+	+
500 ng/ml cortisol only	73 ± 12	+	++
1000 ng/ml cortisol only	67 ± 8 ^{b}	+	++
0 ng/ml cortisol + mifepristone	54 ± 11	0	0/+
50 ng/ml cortisol + mifepristone	66 ± 5 ^{a}	+	+
500 ng/ml cortisol + mifepristone	88 ± 26	+	+/++
1000 ng/ml cortisol + mifepristone	37 ± 8 ^{b}	+	+/++

Discussion

This study shows that cell proliferation and apoptosis of trout skin epidermal cells are directly stimulated by cortisol *in vitro*. While cell proliferation was stimulated at modest levels (50 and 500 ng cortisol/ml) and could not be blocked by the glucocorticoid receptor blocker, high levels of cortisol (500 and 1000 ng/ml) induced apoptosis in skin *in vitro* and this was effectively blocked by the glucocorticoid receptor blocker mifepristone.

The cortisol treatments had no effect on the numbers of epidermal mucous cells, yet increased mucus discharge was commonly reported in response to a wide variety of stressors (Pickering and Macey, 1977; Pottinger et al., 1984; Iger et al., 1994b; Burkhardt-Holm et al., 1997; Chapter 4) and after cortisol administration (Iger et al., 1995). The data on the numerical density of the mucous cells in the skin epidermis in the present experiment were comparable with published data from this head region of salmonids *in vivo* (Iger et al., 1995; Burkhardt-Holm et al., 1998; Nolan et al., 1998, 1999b, 2000). This indicates that cortisol is not involved in the differentiation of mucous cells, but does not exclude that the hormone may have a role in inhibition of mucous cell discharge. Indeed, this view is supported by the fact that when the tissues were incubated with the glucocorticoid receptor blocker, numbers of mucous cells were lower compared with cortisol only treatments. This suggests that blocking the receptor results in accelerated discharge. The glucocorticoid receptor has been demonstrated in the skin epidermis of rainbow trout and is expressed by mucous cells (Chapter 4).

Cell proliferation is a fundamental process in health and disease, and increased proliferation can compensate for rates of apoptosis and necrosis, both of which result in a loss of cells. When the increased proliferation is in response to a stressor, then the replaced cells may be physiologically better equipped for the new situation (Wendelaar Bonga and van der Meij, 1989; Dang et al., 1999). The measurement of proliferation and advantages of the PCNA method for assessing this have been discussed Chapters 6 and 8.

Increased incidence of mitotic cells was reported generally in skin epidermis of stressed fish (Iger et al., 1994b, c; Burkhardt-Holm et al., 1997) and in fish with experimentally increased levels of circulating cortisol (Laurent and Perry, 1990; Iger et al., 1995). Therefore, as in stressed fish, it is not surprising that we report increased numbers with 50 and 500 ng/ml cortisol addition to the culture medium. However, these increases were apparently not directly mediated via the mifepristone sensitive receptors, as they were not blocked by mifepristone. Further, when added to the control group, the blocker resulted in higher numbers of proliferating cells, comparable with the

Figure 3. Scanning electron micrograph of the surface characteristics of skin from the trout *Oncorhynchus mykiss* incubated for 24 h *in vitro* (a) 0 ng cortisol/ml and 100 ng mifepristone/ml. The pavement cells form a continuous sheet of confluent cells, with mucus still attached. Electron dense areas may indicate the beginning of apoptosis in some cells (arrows). Scale bar = 10 µm. (b) 50 ng cortisol/ml. Increased incidence of discharging mucous cells and less clearly distinct borders between cells were characteristic. Scale bar = 10 µm.

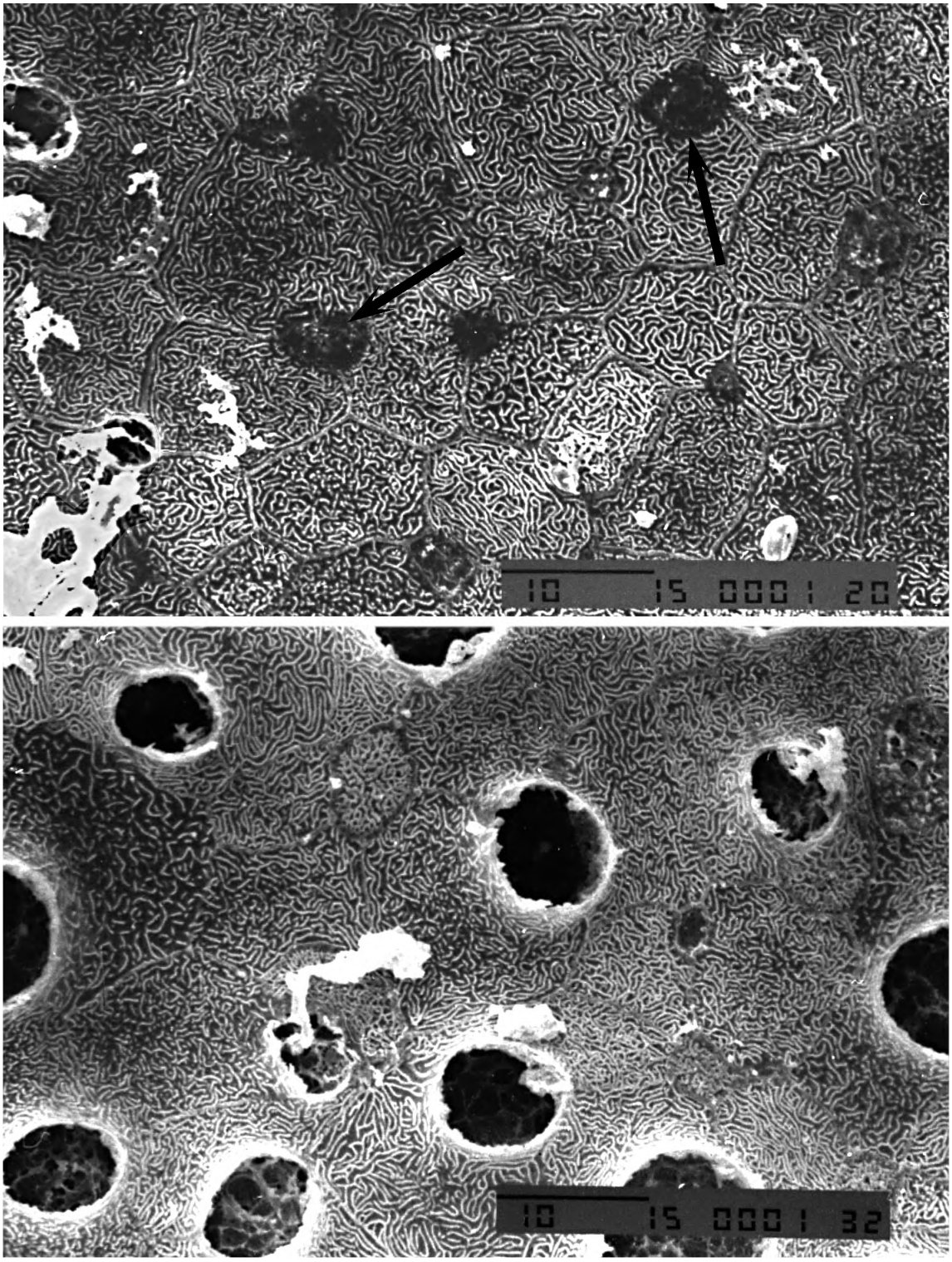
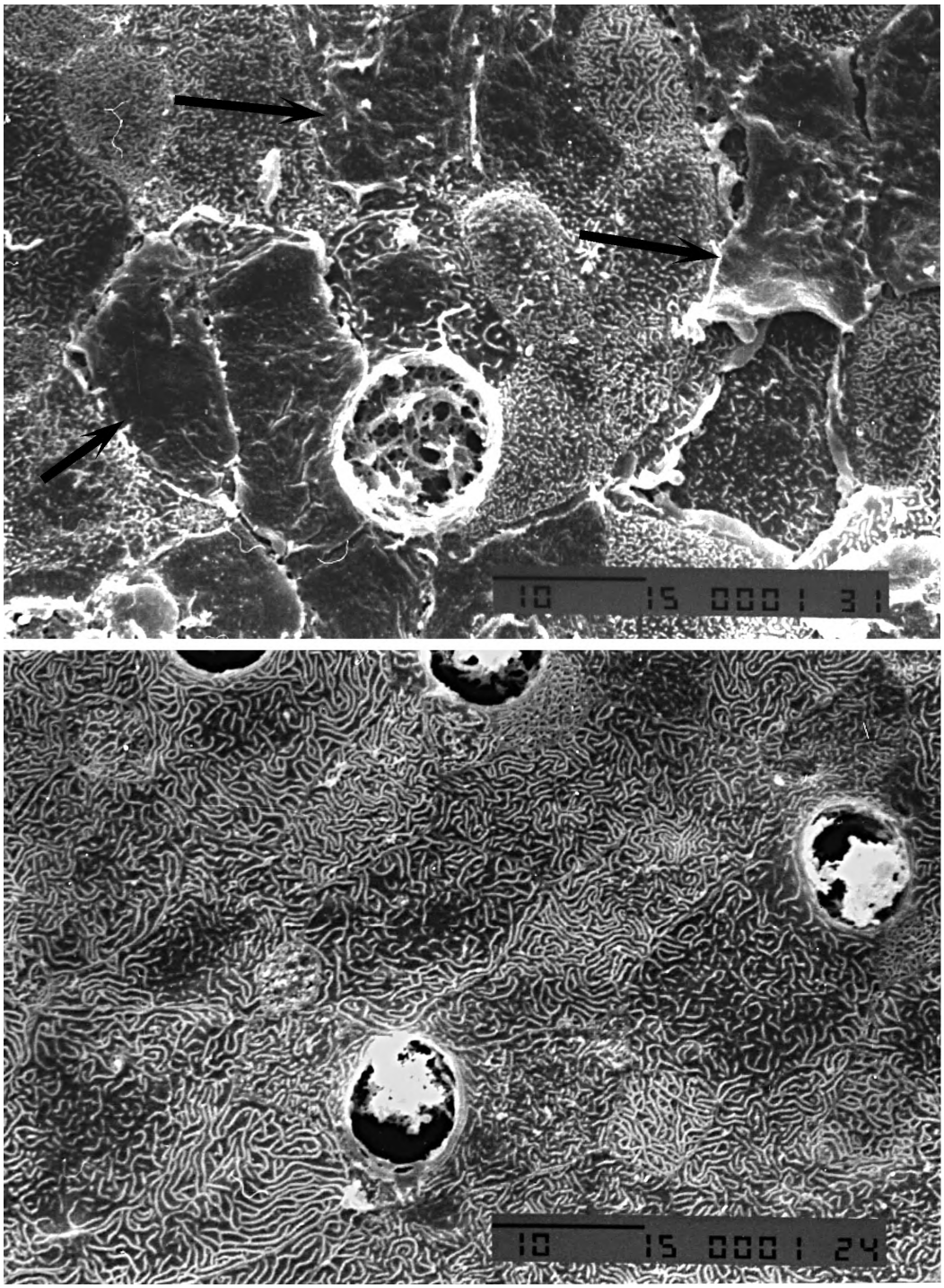


Figure 4. Scanning electron micrograph of the surface characteristics of skin from the trout *Oncorhynchus mykiss* incubated for 24 h *in vitro* (a) 500 ng cortisol/ml. Increased apoptosis of the pavement cells was characteristic. Apoptotic pavement cells lose their apical microridges and their contacts with neighbouring cells before sloughing off (arrow). (b) 1000 ng cortisol/ml and 100 ng mifepristone/ml. The appearance of the pavement cells is comparable to the group incubated with mifepristone only. Increased mucous cell discharge was characteristic and the cell-cell borders were difficult to distinguish, compared with those incubated in the absence of both cortisol and mifepristone. Scale bar = 10 µm.



tissues of all other mifepristone-treated groups, indicating that blocking the glucocorticoid receptor had a similar effect as increasing cortisol concentration. This suggests that the chemical also has some cortisol agonistic action, perhaps through stimulation of a non-glucocorticoid receptor. This could be a mineralocorticoid receptor, as suggested by Carruth et al. (2000). Mifepristone inhibited the dexamethasone-induced proliferation in a rat smooth muscle cell line after short-term pulse exposure (2 min to 6 h), while continuous exposure to both chemicals for 24 h decreased proliferation (Kawai et al., 1998). As the metabolic rate of teleost cells is lower than mammalian cells, perhaps short-term pulse methodology might be a good additional approach to explore further the role of cortisol in stimulation of proliferation in teleost cells.

Increased levels of epidermal apoptosis were consistently reported in trout skin in response to a variety of stressors (Iger et al., 1994b, c, d; Burkhardt-Holm et al., 1998); Chapters 2 and 5). In the present *in vitro* experiment, there was a statistically significant concentration-dependent response to the cortisol additions used, with higher cortisol concentrations correlated with increased numbers of apoptotic cells. Furthermore, the glucocorticoid receptor blocker mifepristone eliminated this response to cortisol. Mifepristone binds with high affinity to the cytosolic glucocorticoid receptor without exhibiting agonistic activity (discussed in Reddy et al., 1995) and has been used as a tool in several studies with rainbow trout (Van Oostrom and Bols, 1991; Dasmahapatra and Lee, 1993; Vijayan et al., 1994b; Reddy et al., 1995; Bury et al., 1998; Weyts et al., 1998a).

Apoptosis is a physiological process of genetically programmed cell death in a tissue that balances increases in cell numbers that result from proliferation and immigration. The basal level of apoptosis we report for the skin epidermis is equivalent to about 15% of the total cell numbers in the epidermis. There are only few studies that report the basal level of apoptosis in fish tissues, and in the gills, levels of apoptosis of chloride cells are <5% (Wendelaar Bonga et al., 1990; Verbost et al., 1995; Bury et al., 1998; Li et al., 1998). This is because of technical difficulties with measuring apoptosis accurately and many researchers have depended on morphological examination using electron microscopy. Morphological detection of apoptotic cells is dependent on correctly identifying structural changes associated with the later stages in the apoptotic process (i.e. chromatin condensation and the progressive densification of the nucleus, organelles and loss of contacts with surrounding cells, and membrane blebbing). Analysis is subjective, and if the researcher is inexperienced, the potential for misidentification is high. The TUNEL method using nick end labelling has several advantages. Nuclear DNA fragmentation (characteristic of both early and later stages of apoptosis) is labelled *in situ* through nick end labelling with terminal transferase (Negoescu et al., 1996). There have been some issues raised about the specificity of TUNEL staining in mammalian research (Charriaut-Marlangue and Ben-Ari, 1995; Grasl-Kraupp et al., 1995; Negoescu et al., 1997, 1998), particularly with respect to false positives caused by the method

staining fragmented DNA in necrotic cells (Grasl-Kraupp et al., 1995). However, improved methods have been developed to enhance specificity (Shi et al., 1991; Sträter et al., 1995; Negoescu et al., 1997; Rao et al., 1998; Whiteside et al., 1998). We confirmed TUNEL observations with electron microscopy and found that both showed increased apoptosis with increased cortisol concentrations administered. The TUNEL method has the advantage that it is less labour intensive than electron microscopy and larger areas can be examined, although in less detail. TUNEL is now routinely applied to fish cells (Soutschek and Zupanc, 1995; Burkhardt-Holm et al., 1998; Bury et al., 1998; Weyts et al., 1998a).

In contrast to terrestrial vertebrates where cortisol (or corticosterone) is the principle glucocorticoid and aldosterone the mineralocorticoid hormone, in fish cortisol acts as the major glucocorticoid as well as mineralocorticoid hormone (Wendelaar Bonga, 1997). The glucocorticoid receptor in mammals specifically binds glucocorticoids, while the mineralocorticoid receptor can bind both glucocorticoids and mineralocorticoids (discussed in Tujague et al., 1998). As cortisol combines these two functions in teleosts, glucorticoid and mineralocorticoid bioactivities may be mediated via separate receptors that are differentially expressed between tissues. The rainbow trout glucocorticoid receptor has been cloned and shows high affinity and specificity for cortisol and none for aldosterone (Ducouret et al., 1995). The glucocorticoid receptor has been demonstrated in a variety of salmonid tissues e.g. leucocytes and gills of *O. kisutch* (Maule and Schreck, 1991; Maule et al., 1993), gills of *O. mykiss* (Sandor et al., 1984) and brains of *O. mykiss, O. nerka kennerlyi* and *O. tshawytscha* (Knoebl et al., 1996; Allison and Omeljaniuk, 1998; Tujague et al., 1998; Carruth et al., 2000). In mammals, the glucocorticoid and mineralocorticoid receptors are differentially expressed in different cells and tissues (Miller et al., 1998), while in fish, the mineralocorticoid receptor has not been demonstrated to date.

Cortisol administration to teleost tissues *in vitro* resulted in increased proliferation of chloride cells in the gills of the tilapia *O. mossambicus* (McCormick, 1990), of fibroblasts from *O. mykiss* (Lee and Bols, 1989), and for skin *in vivo*, stimulated mitosis in *O. mykiss* epidermal filament cells (Iger et al., 1995), inhibited fibroblast activity in wound healing in *S. salar* (Roubal and Bullock, 1988) and had no effect on proliferation in juvenile *Cyprinus carpio* epidermal filament cells (Chapter 6). Immunolocalisation of the glucocorticoid receptor in the skin of *O. mykiss* did not show it to be expressed in the zone of proliferation, apart from in leukocytes which were present in that part of the tissue (Chapter 5). Thus, the evidence from localization and blocking the cortisol receptor in this study does not support the view that cortisol directly stimulated proliferation in the epidermis, although the involvement of this hormone, i.e. via a mineralocorticoid receptor, cannot be excluded.

Twenty-four hours *in vitro* culture was adequately long to expect to see many of the cortisol-related effects occurring in the skin of stressed fish. Iger et al. (1995) administered cortisol to rainbow trout and induced several effects, including increased apoptosis, after 24 h and increased filament cell mitosis at 4 and 7 d post-cortisol, phenomena that are also observed in stressed fish. In these fish, the blood cortisol levels were significantly elevated and peaked at 400-500 ng/ml during the 24 h post-feeding. In general, with the exception of mitosis (Iger et al., 1994c, d; Nolan et al., 1998), stress related effects became apparent within a few hours *in vivo* (Iger et al., 1994c, d, 1995; Chapters 2-5) and *in vitro* (Bury et al., 1998; Weyts et al., 1998a).

In conclusion, this study has shown that within 24 h *in vitro*, cortisol can stimulate mitosis and apoptosis in the skin epidermis of the trout *O. mykiss*, phenomena regularly observed and reported when fish are stressed. The 24 h *in vitro* incubation technique provides an attractive method to verify and further analyse *in vivo* observations and eliminates many of the complicating factors which must be considered when studying hormone actions *in vivo*. Although mucous cell numbers were unaffected by cortisol, reductions in these numbers when the receptor was blocked indicated that it cannot be excluded that this hormone might have some effect on the rate of secretion. The effects of mifepristone on filament cell mitosis indicated that cortisol does not act via the glucocorticoid receptor, and may act via another yet undetected receptor, or may act synergistically with other factors in stimulating proliferation of the epidermal cells. The data indicate that not all of the cells of the trout epidermis are regulated similarly. The role of cortisol in the stimulation of apoptosis is clear and shows for the first time that the increased apoptosis in the epidermis reported after exposure of fish to a wide variety of stressors is not a direct effect of the stressor, but an indirect effect and a response of the tissue to increased levels of cortisol in the intercellular fluids.

Acknowledgements

This study was funded by the Dutch Foundation for the Development of Animal Experiment Alternatives (P.A.D. grant number 96-29). The mifepristone was a generous gift from Roussel Uclaf (Romainville, France). The authors are grateful to L.J.S. Brokken who developed the PCNA immunohistochemistry and shared the technique.

Chapter 8

Characterisation of primary culture of rainbow trout (*Oncorhynchus mykiss*) skin explants: growth, cell composition, proliferation, and apoptosis

D.T. Nolan, I. Nabben, J. Li and S.E. Wendelaar Bonga

Submitted for publication

Abstract

A trout (*Oncorhynchus mykiss*) skin primary explant system was evaluated over 8 days and three distinct regions of the explant outgrowth were identified on the basis of cell composition. The area immediately adjacent to the founder tissue contained mainly small migrating cells and mucous cells. Of the former, *circa* 20% were mitotic and 6% apoptotic. The middle area was characterised by differentiated pavement cells and mucous cells, with fewer small migrating cells. Proliferation was *circa* 30% and apoptosis 5%. Over time, total cell numbers halved as pavement cells differentiated. The growing front contained many mucous and small migrating cells initially, with few pavement cells and about 50% of the cells were in proliferative phase and 5% were apoptotic. Later, there were few migrating and mucous cells, with higher numbers of pavement cells, halving total cell numbers. The number of apoptotic cells was then *circa* 9%. *Circa* 70% of the cells were proliferating. As *in vivo*, pavement cells had apical microridges, although they were vacuolated and contained phagocytosed apoptotic bodies. As this culture system is reproducible and closely approximates the epidermis of trout, it is a powerful tool to study the effects of pollutants, parasites and endocrine factors on fish, eliminating whole animal factors and reducing experimental animal numbers.

Introduction

Fish primary cell cultures and cell lines are increasingly utilised as tools in fundamental and applied research to analyse the effects of heavy metals (Lyons-Alcantara et al., 1996; Magwood and George, 1996) and xenobiotics (Celander et al., 1997; Huuskonen et al., 1998). They are also used to study pathogenic bacteria and viruses (Austin and Cross, 1998), and to examine cellular defence mechanisms such as metallothioneins or stress proteins (Burgess et al., 1993; Gagné and Blaise, 1997; Iwama et al., 1998). Cell culture systems are attractive as they reduce both the numbers of experimental animals required and the variation in data, due to inherent differences between individual animals, and they offer possibilities for analysis of cellular mechanisms without the influence of hormones and other systemic factors. However, their usefulness differs because types of tissue perform differently *in vitro* and *in vivo*. Further, whereas cultured cells may structurally and morphologically resemble the cells *in vivo*, they may not function in the same fashion *in vitro* and they may not have similar responses. For example, in the fathead minnow *Pimephales promelas*, exposure to copper induced a heat shock protein response in whole larvae *in vivo* at concentrations which were an order of magnitude lower than required for induction of this response in a cell line from the same species (Sanders et al., 1995). But in other studies, good agreement was found between *in vitro* and *in vivo* responses (Magwood and George, 1996; Austin and Cross, 1998; Leugen et al., 1998). Cell cultures and their performance are also influenced by the age and maturity of the founder animals (Burgess et al., 1993; Mothersill et al., 1995). Defining a cell culture system and validating it for a given research purpose is important and essential for a meaningful outcome. Unfortunately, there are few studies which report characterisation of fish cell cultures (Ku and Chen, 1992; Ostrander et al., 1995; Avella and Ehrenfeld, 1997; Calduch-Giner et al., 1997; Tong et al., 1997; Flaño et al., 1998) and fewer studies with fish skin (Denizeau and Marion, 1984; Bols et al., 1994; Mothersill et al., 1995; Ghioni et al., 1998).

As obligate aquatic vertebrates, fish are directly exposed (via the water) to many aquatic factors (including stressors such as thermal plumes and xenobiotics) because of the intimate relationship between the external epithelia and the ambient water environment. The effects of such factors quickly lead to epithelial disruption and osmoregulatory disturbance. The skin and gills of a fish are covered by complex epithelia comprised of several layers of living cells that are continuous over the body surface and are affected by a variety of external and internal factors (Wendelaar Bonga, 1997). Although skin epithelium has been less intensively studied than that of the gill, *in vivo* the former is extremely sensitive to pollutants and xenobiotics (Noga et al., 1991; Iger and Wendelaar Bonga, 1994; Nolan et al., 1998; Schmidt et al., 1999) and often has a longer recovery period than the branchial epithelium (Chapters 2 and 4).

Fish skin is protected by a chemically and functionally complex mucous coat which is discharged by specialised mucous cells in the epidermis, as well as an underlying glycocalyx secreted by the pavement cells as granules which contain a variety of biologically active compounds, including peroxidase (Iger and Wendelaar Bonga, 1994; Iger et al., 1994d; Brokken et al., 1998), lysozyme (Rainger and Rowley, 1993), immunoglobulins, complement, and c-reactive protein (Shephard, 1994). Thus, the presence of mucous cells and mucus is essential for normal tissue responses, *in vivo* as well as *in vitro*. A primary culture system using skin explants has been reported, which gives a differentiated epithelium comprised of several different cell types (including mucous cells), which can be grown for 3 weeks and maintained for up to 60 days (Mothersill et al., 1995).

Total numbers of cells in the epidermis and in a skin culture system are the result of several processes, namely proliferation, migration, apoptosis and necrosis. The processes of proliferation and migration have been reported for fish skin *in vivo* (Bullock et al., 1978; Iger and Wendelaar Bonga, 1994) and *in vitro* in cell cultures from gill explants (Fernandes et al., 1995). The latter study on rainbow trout particularly illustrates an important point in relation to the explant system and its uses. Removal of the cell monolayer outgrowth was carried out 3 times, twice the monolayer grew back, but the third time, no more new cells grew from the explant, and the culture ceased to grow thereafter (Fernandes et al., 1995). Apparently, the culture outgrowth resulted from proliferation, together with (e)migration from the founding tissue explant and was negatively influenced by the age of the explant. For a reasonable approximation of the *in vivo* skin response, *in vitro* experimentation should take place with a differentiated culture at a stage where the explant is actively growing through both proliferation and emigration processes. Proliferation occurs in cells located in the middle layers of the fish skin, well below the differentiated pavement cells (Iger and Wendelaar Bonga, 1994; Chapter 6). In explant culture, differentiated cells forming monolayers do not proliferate, and so outgrowth must occur as a result of migration of cells, or proliferation of undifferentiated cells close to the growing front of the explant, as was reported in trout gill explants (Leugen et al., 1998). To the best of our knowledge, this aspect of the explant in culture has not been quantitatively studied in skin to date, although methods are available for measuring proliferation in fish tissues (Negishi and Shinagawa, 1993; Moore. et al., 1994; Ortego et al., 1994; Chiu et al., 1995; Ostrander et al., 1995; Chapters 6 and 7).

Cell proliferation is a fundamental process in both health and disease. For reliable estimation of proliferation rates, the choice of analytical method is crucial (Alison, 1995). Proliferating cell nuclear antigen is a 36 kDa non-histone nuclear protein, cyclin, which is an auxiliary protein for DNA-polymerase (Tan et al., 1987; Kurki et al., 1988). Using PCNA immunocytochemistry, proliferating cells can be labelled and quantified in fish tissues (see discussion in Chapter 7). PCNA

immunocytochemistry has the advantage that it is a non-radioactive method, it does not require prior incubation with labelling precursors, as required by the bromodeoxyuridine method (Moore. et al., 1994; Alison, 1995), and it also enables localisation of the proliferating cells.

Apoptosis is a normal physiological process in a tissue and balances cell numbers which result from proliferation and immigration. The incidence of apoptosis increases in fish skin and gills in response to a variety of external (Iger and Wendelaar Bonga, 1994; Burkhardt-Holm et al., 1997; Nolan et al., 1998, 1999a; Chapters 3 and 4) and internal (Iger et al., 1995; Bury et al., 1998; Nolan et al., 1999b; Chapters 5 and 7) factors. Morphological detection of apoptotic cells is dependent on correctly identifying structural changes associated with the later stages in the apoptotic process (i.e. chromatin condensation and the progressive densification of the nucleus, organelles and loss of contacts with surrounding cells, and membrane blebbing). Analysis is subjective, only later stages can be recognised and the potential for misidentification is high, depending on the researcher's experience. To measure apoptosis, the TUNEL method using nick end labelling has several advantages. Nuclear DNA fragmentation (characteristic of both early and later stages of apoptosis), is labelled *in situ* through nick end labelling with terminal transferase (Negoescu et al., 1997). The TUNEL method allows large areas of the tissue to be examined and has successfully been applied to fish cells (Soutschek and Zupanc, 1995; Burkhardt-Holm et al., 1998; Bury et al., 1998; Weyts et al., 1998a). Unlike mammalian cells that form many apoptotic bodies in the late stages of apoptosis, apoptotic fish cells typically remain as a single body and are usually phagocytosed as a single unit (Wendelaar Bonga and van der Meij, 1989). Combining brightfield microscopy with TUNEL fluorescence staining and electron microscopy facilitates reliable quantification of apoptosis in cultured explants.

To facilitate further in depth studies on the effects of internal and external factors on fish skin, and as part of a project to reduce the numbers of experimental animals used in research, we have studied and characterised a skin primary culture system from the rainbow trout *Oncorhynchus mykiss* over 8 d. Three distinct regions of the explant outgrowth were identified and characterised in terms of cell composition using brightfield, scanning and transmission electron microscopy. Quantitative data on proliferation and apoptosis for each area were also gathered and used to evaluate the culture in relation to fish skin *in vivo*.

Materials and methods

Experimental animals and design

Immature rainbow trout of approximately 100 g body weight from laboratory stock kept in Nijmegen tap water at 15 °C on a 12 h light:12 h dark photoperiod were used. The fish were fed commercial trout pellets (2% of fish body weight fed once daily). The fish were killed by spinal

transection. Strips of skin were immediately removed from the flank of each fish, below the lateral line, and cleaned of muscle and fibrous tissue before being held in a first wash medium (RPMI culture medium with antibiotics and supplements, but without serum, see Table 1). The skin strips were washed 3 times 5 min in a series of petri dishes, bringing them into aseptic conditions in a laminar flow cabinet. All further handling took place in the laminar flow cabinet to reduce the risk of contamination.

Tissues were diced into 25 mm^2 pieces and individually placed with the scale side up in 25 cm^2 culture flasks (Costar) containing 2 ml of complete culture medium (Table 1), with the scale side placed up. Enzymatic treatments were not used. The cultures were maintained at 22 °C in a humidified incubator under normoxic atmosphere. After 24 h the medium was refreshed, and thereafter the cultures were maintained with a twice-weekly medium change.

Sampling and quantification method

Initially, a number of pilot experiments were carried out to optimise sampling technique, and tissue and culture handling. Cultures from these pilot experiments were used to optimise the fixation and staining techniques described below. To characterise and establish the performance of the explant system, 2 replicate experiments were carried out within a 4 week period. For each experiment 7 fish were used from which 8 cell cultures per fish were initiated. At each sample point, 1 cell culture per individual fish was used, resulting in 7 replicates per time point. Sampling was performed by stopping the growth of the explant by fixing the cells with 4% buffered histological formalin in phosphate buffered saline (PBS) for 24 h on a shaking table. Cultures were fixed at 24, 36, 48, 60, 135 and 200 h *in vitro*. The fixed cultures were stored in 70% ethanol at room temperature.

To access the fixed explants, the bottoms of the flasks were cut out with a laser without damaging, detaching or drying the fixed tissue. The area of the flask covered by epithelial cells was measured 3 times for each specimen, using the MOB system (Kontron) and the average of the 3 measurements per sample was expressed in cm^2.

Histochemistry of mucous cells

To identify mucous cells, Alcian blue staining at pH 2.5 was used to identify acidophilic glycoproteins, as this facilitates quantification (Chapter 4).

Immunohistochemistry of proliferating cells

Proliferating cell nuclear antigen (PCNA) immunhistochemistry was based on (Ortego et al., 1994) and was performed on tissues sampled at 48, 60 and 200 h sample points. Several

modifications, made in preliminary trials to optimise the staining, resulted in the following protocol: after rinsing the samples 3 times 10 min in PBS, endogenous peroxidase activity was removed in 1% H_2O_2 in methanol for 15 min, followed by antigen retrieval in 1% $ZnSO_4$ (heating by microwave up to 65ºC at 450 W, maintaining for 2 min at 65ºC, cooling on ice for 5 min, again heating for 2 min and cooling for 15 min). Samples were then rinsed in dH_2O 3 times 5 min, then in buffer TBST (50 mM Tris and 15 mM NaCl, adjusted to pH 7.4 with HCl + 3 ml/l Triton X) 3 times 5 min and received a protein blocker consisting of 0.5% powdered milk and 0.5% bovine serum albumin (BSA, Sigma) in TBST for 20 min. After the protein blocking step, the primary antibody was applied: antiPCNA PC 10 (Oncogene) diluted 1:1000 in 0.5% powdered milk + 0.5% BSA in TBST. Samples were incubated overnight at room temperature, after which they were rinsed in TBST containing 0.1% normal goat serum (NGS) 3 times 5 min, then treated with the secondary antibody (biotinylated goat anti-mouse; bGAM, Amersham) diluted 1:200 in TBST containing 1% NGS for 1 h. After rinsing in TBST containing 0.1% NGS 3 times 5 min, the third antibody system (Avidin Biotin Complex: ABC, Vectastain) was applied for 1 h, diluted 1:100 in TBST containing 1% NGS, followed by rinsing in TBST 3 times 5 min, then rinsing in Tris buffer (TB: 50 mM Tris, adjusted to pH 7.4 with HCl) 3 times 5 min. Negative controls were extra cultures treated as described, but without the first antibody: antiPCNA, or without the ABC visualisation system.

Staining was performed in filtered 0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma) in TB with 2.5 mg/ml $(NH_4)_2SO_4.NiSO_4.6H_2O$ with the addition of 0.0125% H_2O_2 immediately prior to use. After staining, samples were rinsed in tap water and stored in 70% ethanol.

TUNEL staining of apoptotic cells

Apoptosis in the explant outgrowth was quantified using the TUNEL method (Boehringer Mannheim), based on (Negoescu et al., 1997) and performed at 24, 36, 48 and 135 h sample points. Modifications to the protocol were as follows: the fixed samples were washed thoroughly in PBS 3 times 5 min and microwaved for 5 min at 65ºC in a solution of 0.025% Triton + 0.025% sodium citrate in PBS, cooled in demi-water for 10 min and rinsed in PBS 3 times 5 min. 50 µl freshly prepared TUNEL reaction mixture was added per outgrowth, covered with parafilm squares, and incubated for 1 h at 37ºC in the dark. After rinsing in PBS 3 times 5 min, the explant outgrowth was mounted under a coverslip in 10% glycerol in PBS. Negative controls were cultures with all treatments except the TUNEL enzyme solution was excluded. The samples were kept in darkness at 4 °C to avoid fading of the fluorescence until photographed the same or the following day.

Quantification of cell numbers

Cell numbers were calculated using brightfield microscopy, together with the percentage of PCNA and TUNEL positive cells. Cells with PCNA positive nuclei were counted using an inverted microscope (Zeiss) with the addition of a phase contrast filter, 0.32 mm^2 in view with a 10 X objective. Cells with TUNEL positive nuclei were counted from pictures taken with a camera adjusted to a fluorescent microscope (Leica), with a magnification of 6.3 to the camera and a 40x objective, resulting in 0.14 mm^2 in view. Phase contrast images and fluorescent staining were overlaid and only fluorescing nuclei were identified and counted. Five random grids were counted in each area of the explant outgrowth and averaged to give the mean value for each parameter per sample (and thus, per fish).

Electron microscopy

Four 48 h cell cultures were fixed *in situ* in 3% glutaraldehyde buffered in Na-cacodylate (0.09 M, pH 7.3, on ice) with post-fixation in 1% osmium tetroxide in the same ice cold buffer. For transmission electron microscopy, the cultures were stained for 2 h in 2% uranyl acetate, ethanol-dehydrated and embedded in Spurr's resin *in situ*. When the resin had hardened, the culture was cut into strips with a fine saw and blocks were cut from the strips with a pincer. Ultra-thin sections were cut by diamond knife and collected on a 150 mesh copper grid. Four grids per sample were prepared and stained with lead citrate before viewing in a Philips EM 300 transmission electron microscope at 40 kV. For scanning electron microscopy, fixed cultures were cut out from their culture flasks, dehydrated through an ethanol series and freeze dried following the method described by (Inoue and Osatake, 1988). In short, specimens were dehydrated stepwise for 10 min each step in absolute ethanol (50, 70, 80, 90 and twice in 100%). The ethanol was eliminated by replacement with 1 ml of tert-butyl alcohol (Merck) 3 x, 10 min. At the 3rd change of tert-butyl alcohol, the specimens were frozen into the alcohol by placing in the fridge at 4°C for 5 min, followed by evaporation under vacuum (thermocouple vacuum pump, Virtis) until the alcohol was eliminated. The specimens were mounted on aluminium stubs, gold sputtered in a Balzers coating unit (CPD 020, Balzers, Switzerland) and viewed in a Jeol-JSM T 300 scanning electron microscope at an accelerated voltage of 15 kV.

Statistics

The sample size was 7 at each sample point, representing the data from explants originating from 7 different fish. Values for percentage of cells were calculated from the mean of number of cells per $mm^{2.}$ All values are given as mean ± S.E.M. Statistical significance of differences between the areas and over time was assessed using one-way Analysis of Variance (ANOVA) on the raw

data at each time point, followed by a Bonferroni Multiple Comparisons Test and trend analysis. Statistical significance was accepted at $P < 0.05$.

Results

Attachment and growth of explants

The founding explants had attached and confluent cell layers grew out within 24 h. These layers were seen as a whitish outgrowth coming from the founding piece of tissue and were mainly composed primarily of small migrating cells, mucous cells and flattened epithelial cells. The whitish colour resulted from mucus discharged out on top of the culture by mucous cells. All cells that grew from the explant strongly adhered to the plastic flask and generally grew as a monolayer. The explants continued growing actively up to 200 h (Fig. 1), and trend analysis indicated that the increase in size was statistically significant (slope = 0.6706; $r^2 = 0.5583$; $F = 62.173$; $P < 0.0001$).

Appearance of explants

Over time, the morphology of the outgrowth changed. A schematic diagram of a typical explant outgrowth is presented in Fig 2. Initially, cells close to the explant were small and there was a gradient from multilayered to monolayer from the founding explant outwards. This part of the outgrowth is referred to as the explant side (see Fig. 1) and is shown in Figure 3. After 48 h, the first cells had differentiated, which resulted in large, angular shaped, pavement-like cells with relatively small nuclei. This differentiation resulted in a distinct recognisable region of the explant, referred to as the middle area (Fig. 4). Other differentiated cells were identified as mucous cells by staining with Alcian blue, and had a large size, circular shape and eccentric horseshoe-shaped nucleus. Small cells migrated from the explant outward through the monolayer of differentiated cells towards the leading edge, which was characterised initially by few differentiated pavement cells and many mucous cells. Later this area (referred to as the leading edge) had many pavement cells and fewer mucous cells (Fig. 5). An overview of the main characteristics of the different areas of the cultures between 0 and 48 h and between 48 and 200 h is presented in Table 2.

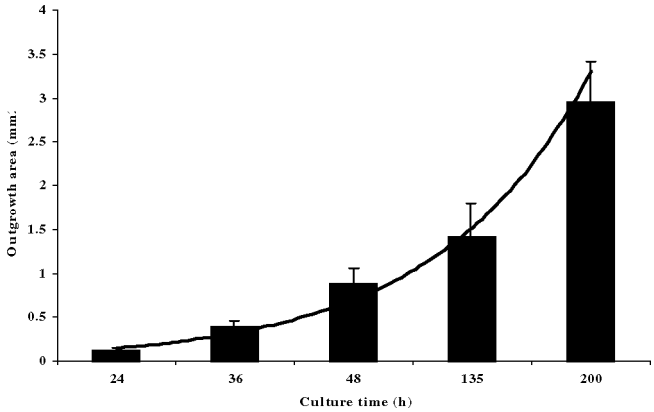


Figure 1. Net explant outgrowth area of rainbow trout *Oncorhynchus mykiss* skin explant cultures over the 200 h experimental period. The figure represents data from cultures initiated from 7 different individual fish at each sample point (mean ± SEM).

PCNA staining of proliferating cells

PCNA positive cells had a specific black staining in the nucleus. Negative controls showed no such staining. In general, proliferating cells were smaller than differentiated cells, with minimal cytoplasm and nuclei almost as large as the whole cell. At 48 h, proliferation at the leading edge of the outgrowth was significantly higher than at the explant side and at the middle, whereas proliferation at the middle of the explant outgrowth was significantly higher than at the explant side. At 60 h, the numbers of PCNA positive cells in the middle and explant side of the outgrowth were similar and lower than the explant side. At 200 h the situation was comparable, except that the percentage of proliferating cells at the leading edge was higher than previously. Overall, the highest percentage of proliferating cells occurred at the leading edge (Fig. 5) and there was a significant trend for increasing proliferation at the leading edge over time (slope = 8.901; r^2 = 0.2149; F = 12.5673; P = 0.0025).

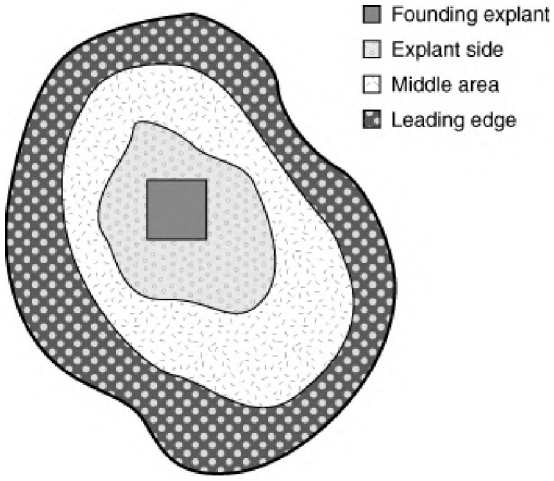


Figure 2. Schematic overview of the rainbow trout *Oncorhynchus mykiss* skin explant culture, indicating the different areas described in the text. The original founder explant is central, with the three distinguishable areas indicated.

TUNEL staining of apoptotic cells

Cells stained positive by the TUNEL method had fluorescing nuclei. In negative controls where the TUNEL enzyme was omitted, this fluorescence was not present. In addition, vesicles and inclusions in the cytoplasm of many cells fluoresced intensely when incubated with the TUNEL enzyme. Detached apoptotic cells lying above the culture monolayer, which were apparently held in the mucus, also fluoresced strongly (Fig. 6). The percentage TUNEL positive cells in different areas of the explant outgrowth are given in Fig. 7, where trend analysis indicated a significant trend for increased apoptosis at the leading edge (slope = 0.9153; r^2 = 0.1714; F = 4.9832; P = 0.0385).

Table 1. Medium composition used to culture skin explants from the rainbow trout *Oncorhynchus mykiss* The medium required for each experiment was prepared, pH checked and sterilised by filtration through 0.2 µm Millipore® filters. The final pH was 7.5.

Cell culture reagent	Quantity and units
RPMI 1640 with 25mM HEPES-buffer, without L-Glutamine (Gibco)	500 ml
Foetal calf serum (Gibco)	60 ml
Horse serum (Gibco)	40 ml
L-glutamine (Gibco)	20 mM
Penicillin (Gibco)	50 IU·ml^{-1}
Streptomycin (Gibco)	50 µg·ml^{-1}
Fungizone (Gibco)	1 µg·ml^{-1}
Human recombinant insulin (Sigma)	0.05 IU·ml^{-1}
Hydrocortisone (Sigma)	1 µg·ml^{-1}
Insulin (Novo Nordisk)	10 IU·ml^{-1}

Electron microscopy

In the scanning electron microscope, the explant appeared as a relatively flat monolayer composed of distinctive flattened cells, some of which had well formed microridges (Fig 8). Although most of the mucus is lost in the fixation and preparation, fragments of mucus were visible on the surface, together with discharging mucous cells. In the transmission electron microscope, the outgrowth was typically comprised of a monolayer of flattened cells, similar to pavement cells, with tight junctions at the cell-cell contact points (Fig. 8 inset). Some of these had well differentiated apical microridges (Fig. 9a), while the most flattened cells did not (Fig. 9b). At 48 h, leukocytes were occasionally observed in the areas close to the founder explant, but seldom far out in the outgrowth and frequently contained phagosomes. Necrotic cells were seldom seen in transmission or scanning electron microscope analysis of the cultures. Many of the pavement-like cells contained phagosomes (Fig 9b), similar to those observed in the macrophage. A distinct glycocalyx could be seen in many views (Fig 9b). Mucous cells had stacked mucosomes and were observed frequently throughout the explant outgrowth.

Figure 3. Cultured 150 h skin explant from the rainbow trout *Oncorhynchus mykiss* at the outgrowth area referred to as the explant side (see text and Fig. 2) close to the founder explant. The culture is composed of many small undifferentiated and mucous cells. The latter have characteristic horseshoe shaped nuclei (arrowheads). Scale bar = 15 µm.

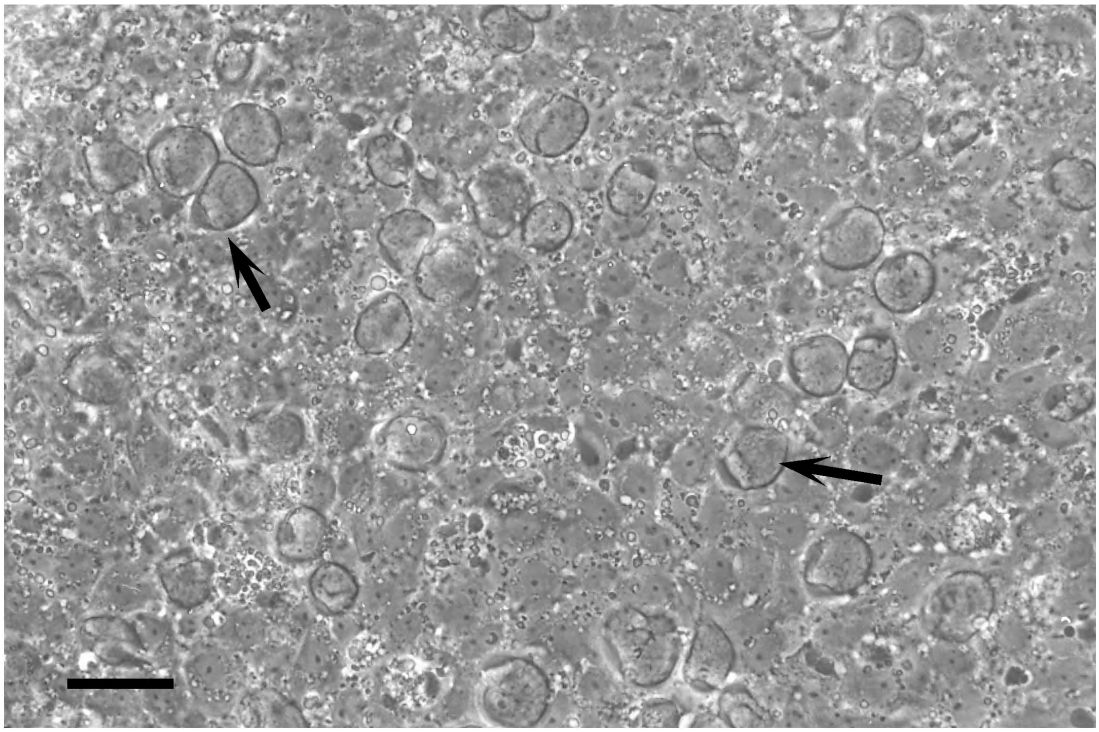


Figure 4. Cultured skin explant from the rainbow trout *Oncorhynchus mykiss* at the outgrowth area referred to as the leading edge (see text and Fig. 2) furthest away from the founder explant. The culture is composed of many large flattened differentiated pavement cells (**p**), with small, undifferentiated cells at the very front (arrows). (150 h culture). Scale bar = 20 µm.

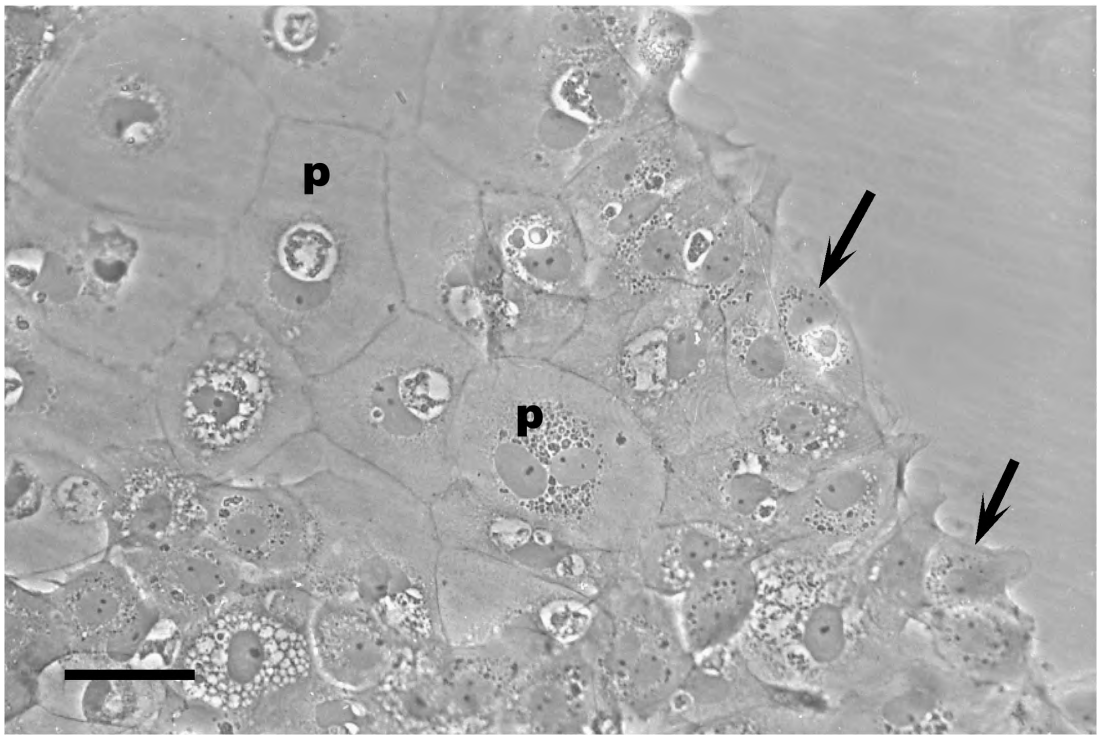


Table 2. Combined quantitative and semi-quantitative overview of the rainbow trout *Oncorhynchus mykiss* skin explant culture system. To facilitate comparison, the three areas of the explant outgrowth are described separately at the initial and final stages of the experiment. For details of the defined areas, see text and Fig. 2. Note: -, +, ++, +++, ++++ indicate increasing gradations for a given parameter, from absent to the maximum observed in the culture.

Location and parameter	**To 48 h**	**60 to 200 h**
Explant side		
no. cells per mm^2	1132 ± 72	1123 ± 58
mucous cells	+++	+++
pavement cells	-	-
small migrating cells	++++	++++
% proliferating/PCNA positive cells	21	20
% apoptotic/TUNEL positive cells	3.7	6.1
Middle		
no. cells per mm^2	928 ± 50	483 ± 54
mucous cells	+++	++
pavement cells	-	++
small migrating cells	+++	++
% proliferating/PCNA positive cells	26	31
% apoptotic/TUNEL positive cells	2.1	4.6
Leading edge		
no. cells per mm^2	589 ± 22	302 ± 15
mucous cells	+++	+
pavement cells	+	+++
small migrating cells	+++	+
% proliferating/PCNA positive cells	55	73
% apoptotic/TUNEL positive cells	4.8	9.1

Discussion

In this study, we described an *in vitro* fish skin explant culture system that closely resembles that of fish skin *in vivo*. We divided the culture into regions by analysis of cell composition, together with immunohistochemical quantification of both proliferation and apoptosis, and using electron microscopy, necrosis. We present data indicating the extent and location of these processes in the outgrowth. The explant culture system is reproducible and provides a differentiated epithelium containing several cell types which are reported in trout skin *in vivo* (Iger et al., 1995; Burkhardt-Holm et al., 1997; Nolan et al., 1998). We evaluated and quantified the levels of proliferation and physiological cell death to determine the usefulness of the system as a model for the skin of fish *in vivo*. The results show that the explant culture system performs *in vitro* similar to the skin of trout *in vivo*.

There are substantial differences between various areas of the outgrowth, and therefore the present study emphasised the importance of examining different areas of the explant and analysing them separately. In general, proliferation, as indicated by the percentage PCNA positive cells, was circa 20% at the explant side and in the middle and rose to 50-70% at the leading edge. Thus the explant grew more actively at the front edge. Mothersill et al (1995) reported 90% proliferating cells in trout skin explants in culture up to 8 d, and this percentage reduced to 19% by 15 d. These authors used the proliferation marker Ki67 and reported a higher incidence of positively stained cells at the growing edge (Mothersill et al., 1995). The reduced percentage of proliferating cells reported at 15 d in the latter study may have resulted from the differentiation of pavement cells, as we generally report similar amounts, except at the leading edge. The initial percentages reported by this group, as high as 90% are seen as excessive as other studies using monocultures of fish cell lines reported maxima of 50% of cells proliferating at any one time (Lee, K.J. and Hahn, 1988; Lee, L.E. and Bols, 1989). Further, 5-bromo-2'-deoxy-uridine incorporation methodology in primary gill explants of rainbow trout gave 5-15% proliferation (Leugen et al., 1998).

In the present study, we report levels of apoptosis in the skin explant cultures of trout of circa 5%. There are few studies that report levels of apoptosis in fish tissues, but in the gill cells, levels of apoptosis vary from <5% to 20% (Wendelaar Bonga et al., 1990a; Verbost et al., 1995; Bury et al., 1998; Li et al., 1998). As our measurements were made in the actively growing skin explant outgrowth, 5% apoptosis seems reasonable and is comparable with the basal levels reported from several mammalian studies (Burton and Kehril Jr., 1996; Louahed et al., 1996; Watson et al., 1996). Although we quantified apoptosis, we did not quantify cell death caused by necrosis (Sen, 1992; Wendelaar Bonga, 1997; Chapters 2 and 4). This is characterised by nuclei with aggregations of

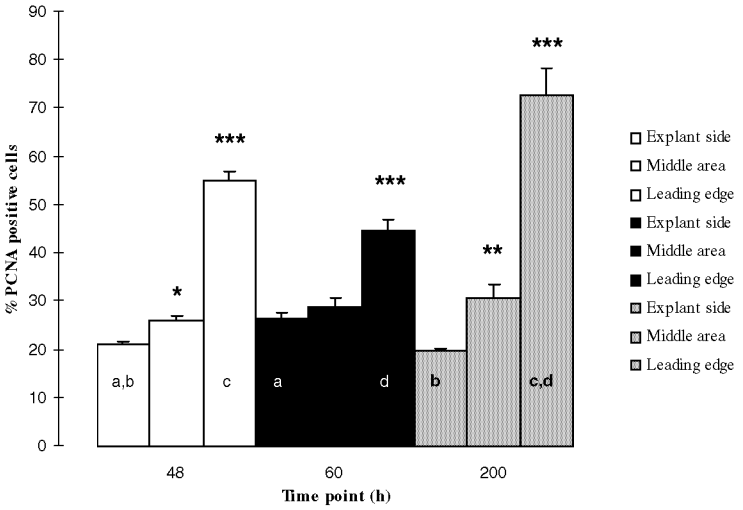


Figure 5. Percentage of proliferating cells (PCNA positive) in the different areas of the cultured skin explant (see text and Fig. 2) from rainbow trout *Oncorhynchus mykiss* at 48, 60 and 200 h in culture. The data represent 7 individual fish at each time point and are presented as mean ± SEM.

chromatin, membrane rupture, swelling of the cytoplasmic compartment and in the pavement cells of fish skin, loss of apical microridges (Wendelaar Bonga and van der Meij, 1989; Teh et al., 1997) and leakage of intracellular products into the extracellular space (Abu Damir et al., 1993; Folmar et al., 1995). Differences in culture growth rates as a result of experimental treatments may result from altered rates of proliferation or apoptosis and necrosis. In normal fish tissues, the numbers of necrotic cells are very low compared with those of apoptotic cells (Wendelaar Bonga et al., 1990a; Wendelaar Bonga, 1997) and our electron microscopy observations indicate a similar situation for the skin explant culture system. In the present study, necrotic cells were seldom encountered in TEM analysis of the cultures. As rates of *in vivo* necrosis increase during disease (Rodger et al., 1991; Turnbull et al., 1996) or exposure to toxicants (Hinton and Lauren, 1990; Bury et al., 1998; Sweet et al., 1998), higher incidence of this form of cell death may be expected by exposure of the cultures to toxic substances or pathogens.

A major advantage of the present study is that we have consistently obtained cultures with mucous cells occurring throughout the outgrowth. Mothersill et al. (1995), using a comparable method also observed mucous cells, although the distribution was restricted to explant side only. These mucous cells appear to be functional, as mucus was seen on the explant outgrowth surface and mucous cells were seen discharging in the normal manner in transmission electron microscopy. In the present study mucous cells may have appeared throughout the outgrowth because the skin explants were not treated enzymatically before culture, because larger founder explant pieces were used, and because the cultures were kept in the same medium composition throughout. As mucus is an essential feature of the normal fish skin and contains peroxidase (Iger and Wendelaar Bonga, 1994; Brokken et al., 1998), lysozyme (Rainger and Rowley, 1993), immunoglobulins, complement, c-reactive protein etc. (Shephard, 1994), the presence of mucous cells in the culture is likely to be important for the outcome of experiments with disease agents (including parasites; Nolan and

Figure 6. Identical views of cultured skin explant from the rainbow trout *Oncorhynchus mykiss* at the outgrowth area referred to as the leading edge (see text and Fig. 2) photographed for fluorescence and brightfield after TUNEL staining at 48 h in culture. A TUNEL positive nucleus (arrowhead) can be distinguished from the strongly fluorescing cytoplasmic inclusions of phagosomes (small arrows). Scale bar = 20 µm.

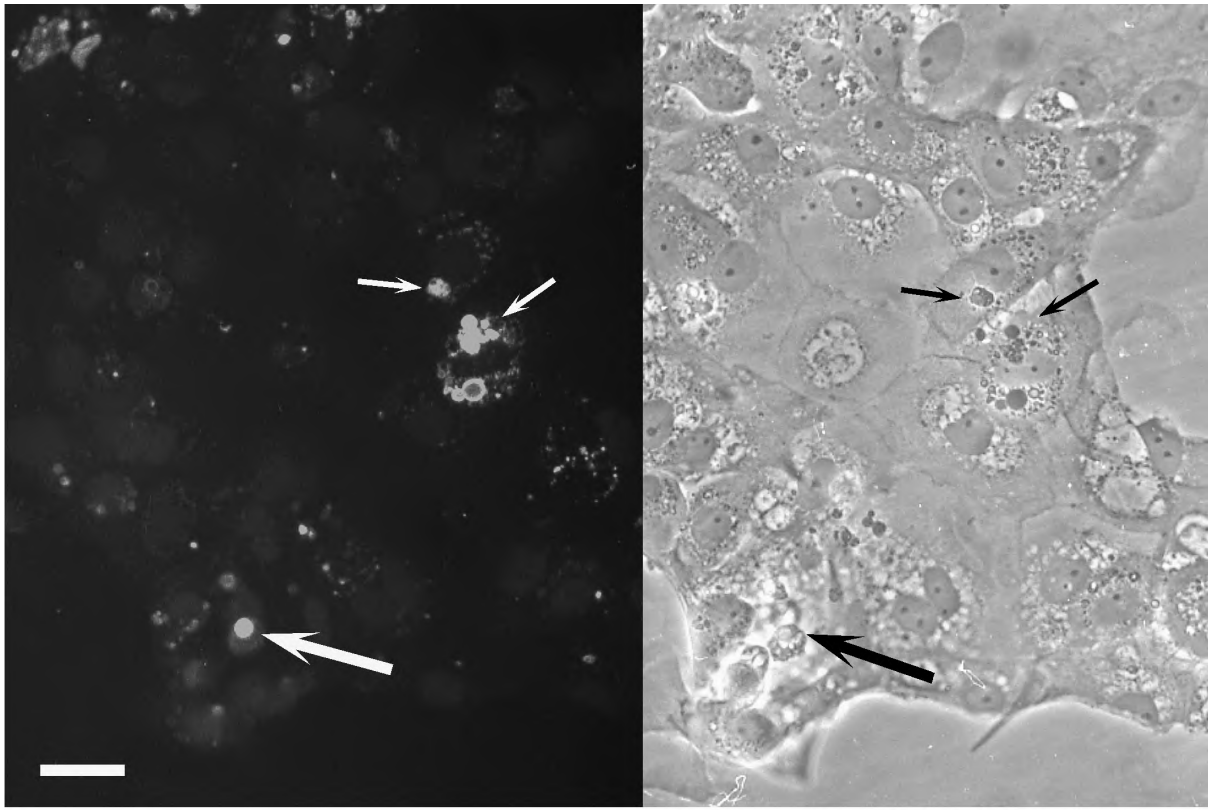
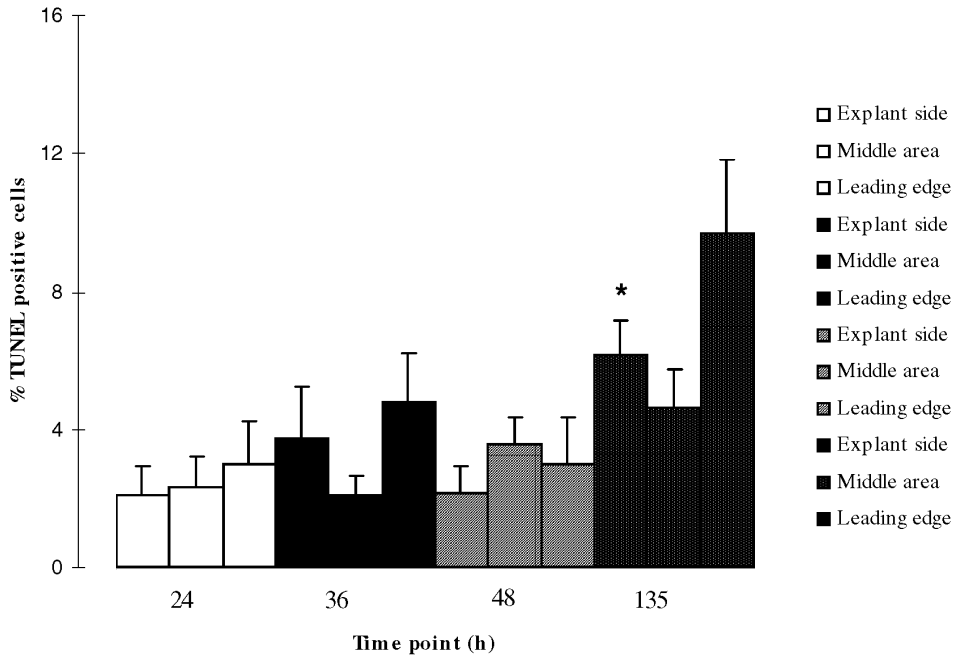


Figure 7. Percentage of apoptotic cells (TUNEL positive) in the different areas of the cultured skin explant (see text and Fig. 2) from rainbow trout *Oncorhynchus mykiss* at 24, 36, 48 and 135 h in culture. The data represent 5-7 individual fish at each time point and are presented as mean ± SEM.



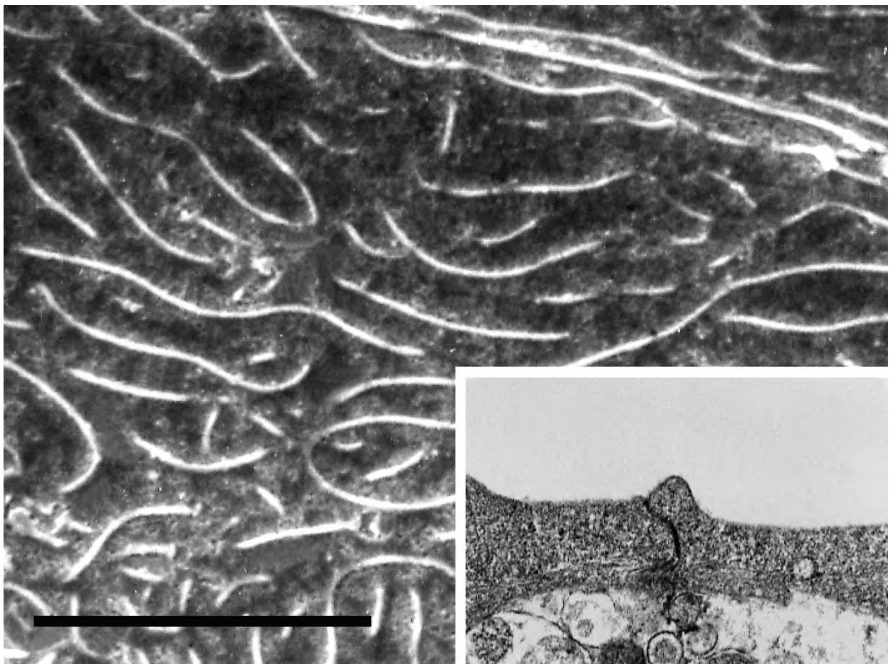
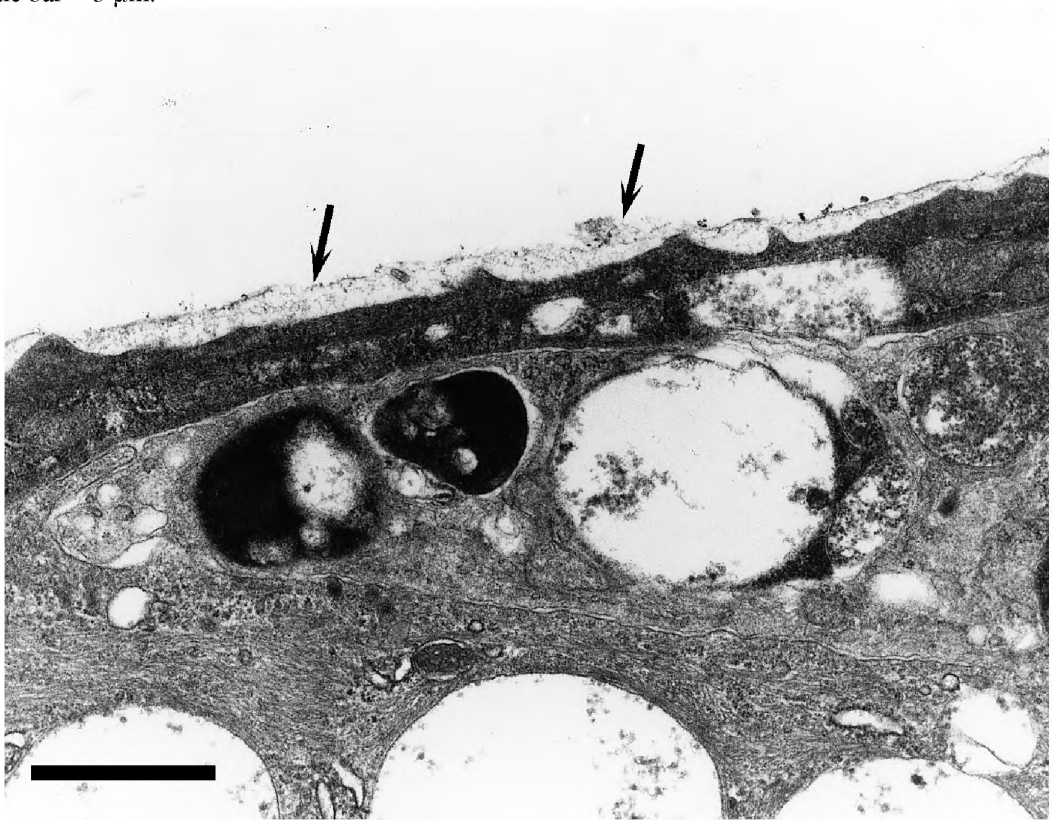


Figure 8. Scanning electron micrograph of cultured cells in the cell outgrowth from the middle area of a skin explant of the rainbow trout *Oncorhynchus mykiss* at 135 hours in culture showing the apical microridges on a differentiated pavement cell. Scale bar = 10 µm. Inset: Transmission electron micrograph of cultured cells in the outgrowth. Note the cell-cell contacts and tight junctions, similar to the *in vivo* situation in the trout epidermis.

Figure 9. Transmission electron microscopy of cultured cell outgrowth from the middle area of a skin explant of the rainbow trout *Oncorhynchus mykiss* at 48 hours in culture. At the explant side, the outgrowth can be multilayered. Here, the glycocalyx (small arrows) on the overlying pavement cells and the phagosomes in the underlying cells are clearly visible. Scale bar = 3 µm.



Johnson, 2000) and also with toxicants (Sanders et al., 1995; Lyons-Alcantara et al., 1996).

A limitation of this culture system is that it contains few macrophages or other leukocyte types. We have noted some at the explant side in the early stages, but these apparently do not occur in the middle, leading edge, or at later stages. The teleost fish have an advanced immune system which is intimately involved in disease resistance and maintaining healthy tissues (Secombes, 1994; Van Muiswinkel, 1995). *In vivo*, the epidermis of the fish skin contains numbers of leukocytes, patrolling agents of the specific immune system (e.g. lymphocytes), and non-specific defence agents (macrophages and granulocytes) (Sin et al., 1996; Dalmo et al., 1997). Their absence in culture results in the elimination of cellular debris, normally carried out by the professional phagocytes, apparently being taken over by the differentiated pavement cells. This was indicated by the presence of vacuoles containing apoptotic bodies of cells seen in many cells. The vacuolated appearance of the cells containing phagocytosed apoptotic bodies in brightfield microscopy was reported in gill explant primary cultures, and reported as indicative of senescence of the culture (Fernandes et al., 1995). In long-term cell cultures from the sea lamprey *Petromyzon marinus*, viable cells have been grown for up to 6 months, and gill cells were also reported to possess large numbers of secondary lysosomes (Ma and Collodi, 1996).

In conclusion, this primary culture system provides a differentiated epithelium closely approximating that of the teleost epidermis *in vivo*. Major cell types present include pavement cells, mucous cells and migrating undifferentiated cells, as well as low numbers of leukocytes. In the culture period described, the explant outgrowth was actively growing and the levels of proliferation and apoptosis were similar to those reported for fish epithelia *in vivo*. Furthermore, this culture has mucous cells throughout, which are active and discharge mucus onto the apical side of the outgrowth. This indicates that this system is a good model to study the effects of toxic compounds, as well as disease related aspects, such as interaction with pathogens, and may serve as an efficacious model of mucous cell function.

Acknowledgements

This study was funded by the Dutch Foundation for the Development of Animal Experiment Alternatives (P.A.D. grant number 96-29)

Chapter 9
General Discussion

In this thesis, some of the responses of fish skin epidermis to toxic and non-toxic stressors have been studied. The main findings are:

- Exposure to toxic pollutants (Rhine water) induces an integrated stress response, which at the level of the skin, manifests as increased cell death (apoptosis and necrosis), the synthesis and discharge of large numbers of vesicles in the filament and pavement cells of the upper epidermis, and invasion of the epidermis by leukocytes. Long-term exposure results in partial recovery of the epidermis, and is associated with reduced growth of the fish.
- When exposure to Rhine water is combined with a short-term acute temperature elevation, the effects of both stressors are additive.
- Fish lice, used as a non-toxic stressor also produced an integrated stress response. The effects on the skin were studied at locations where the parasites had not been present, and the changes observed indicated that some responses (e.g. increased apoptosis and mucous discharge) could be attributed to endogenous factors, such as the stress hormone cortisol.
- Cortisol administration to trout, followed by infection with parasites, was used to examine the effect of this hormone on the skin. This showed that cortisol administration stimulated the synthesis of large numbers of vesicles in the upper epidermis and this was associated with reduced establishment of the parasite on the host fish.
- With an *in vitro* approach, the role of cortisol in inducing apoptosis via the glucocorticoid receptor in the epidermis was demonstrated, together with evidence for a role of the hormone in stimulating proliferation and regulating mucous discharge, possibly via a putative mineralocorticoid receptor.
- A primary skin explant system was characterised which may facilitate the study of the effects of cortisol *in vitro* on actively growing epidermal cells that might have similar performance *in vitro* as *in vivo*.

The general discussion below will concentrate on the skin in relation to (i) the responses of fish to a toxic stressor using Rhine river water, (ii) the responses of the skin to a non-toxic stressor, such as fish lice and (iii) the mediating role of cortisol in the skin epidermis. This is followed with some perspectives.

Responses to Rhine water

The river Rhine is the largest and most important European river and has been heavily polluted by a variety of anthropogenic factors including xenobiotic pollution (Friedrich and Muller, 1984; Van Dijk and Marteijn, 1993; Van Dijk et al., 1995). The salmon populations of the Rhine have

declined dramatically in the last century (Cazemier, 1994; Van Dijk et al., 1995), as salmonids have high water quality requirements and the water quality deteriorated severely (Muskens and Hensgens, 1977; Hendriks, 1994; Schulte-Wülwer-Leidig, 1994). Recent international negotiations have targeted improving the quality of the water by agreements on reducing levels of contaminated discharges into the river (Van Dijk and Marteijn, 1993; Schulte-Wülwer-Leidig, 1994). In the 1970s, one study reported that rainbow trout exposed to Rhine river water showed reduced growth within 1 month (Poels et al., 1980). Recent studies have shown that exposure to present day river Rhine water still induces stress-related effects in the skin epithelium of sea trout *Salmo trutta* smolts (Nolan et al., 1998; Chapter 2), and in rainbow trout, *Oncorhynchus mykiss* (Iger et al., 1994c; Chapter 3), and these effects include increased levels of apoptosis, necrosis and mucous cell discharge, and result in prolonged epithelial disruption. Infiltration of the epithelia by leukocytes indicated effects on the immune system. These are typical responses in teleost to stressors in general. Together, these studies indicate that the water quality of the river Rhine is still far from optimal for salmonids, and that exposure of non-adapted fish to Rhine water is a stressful experience.

Temperature shock is a physical stressor which migrating salmonid smolts encounter as a result of thermal plumes that are discharged by industries along the course of the lower Rhine. Thus, salmonid smolts in nature encounter both a toxic (Rhine water) and non-toxic stressor (temperature shock) as they migrate through the Rhine system. Temperature stress is a strong inducer of stress responses in fish (Dietz, 1994; Iger et al., 1994d; Burkhardt-Holm et al., 1998) and induces heat shock proteins in isolated cells of fish, a stress response at the cellular level (Dyer et al., 1991; Oda et al., 1991; Iwama et al., 1998). The research described in this thesis demonstrates the individual and additive effects of Rhine water and temperature elevation, and provided some indication of which epithelial responses might be endogenously mediated by factors, such as cortisol. While many stress-related responses were induced over the experimental period, indications of recovery were observed when the treatments were given separately, but not when they were combined.

Discharge of electron dense vesicles in the upper epidermal layers of the skin occurred with all treatments and was least affected by the temperature shock. Exposure to Rhine water alone or combined with temperature shock induced a stronger response, decreasing the vesicle populations even further. These vesicles in the epidermis of *O. mykiss* contain peroxidase (Iger et al., 1994b, d), and this is discharged into the glycocalyx and mucous layer. Temperature shock alone also altered mucus biochemistry by inducing peroxidase positive mucosomes in the mucous cells of the rainbow trout (Iger et al., 1994d). The biochemistry and enzymic properties of this peroxidase are altered by stress (Brokken et al., 1998) and peroxidase is one of several enzymes that comprise part of the non-specific defense system, which occurs in the mucus of fish (Shephard, 1994). The cells of the sea trout contain many more vesicles than those of the rainbow trout (Iger et al., 1995) or the non-

smoltifying brown trout *S. trutta* (Burkhardt-Holm et al., 1997), and this may be a smolt-related phenomenon.

The transient increase in gill Na^+/K^+-ATPase activity immediately following exposure to the stressor appears to be a compensatory response, as hydromineral balance (indicated by sodium and chloride homeostasis), was maintained. Transient adjustments to the osmoregulatory Na^+/K^+-ATPase activities were associated with the maintenance of hydromineral balance, despite the disrupted skin and gill epithelia. As the smolt is in a physiologically complex life stage during which the fish preadapt for entry into seawater, we also designed an experiment to see whether exposure to Rhine water induced a stress response in the non-smoltifying rainbow trout. Increased blood cortisol and glucose levels indicated the presence of a stress response. As bioaccumulation of heavy metals did not occur, direct uptake of these from the water could not be demonstrated, indicating the toxic effects of these metals were mainly concentrated on the body surfaces.

This is the first time that exposure of rainbow trout to Rhine water was shown to induce an integrated stress response, as evidenced by increased blood levels cortisol and glucose in the short-term and by the longer-term effects of continuous exposure on anabolic processes and the immune system, specifically reduced growth and stimulation of macrophage respiratory burst activity. In general, exposure to toxicants is associated with immunotoxicity and immunosuppression (Wong et al., 1992; Kelly-Reay and Weeks-Perkins, 1994; Fournier et al., 1998). However, we found that invasion of the epithelia by leukocytes is characteristic for fish exposed to Rhine water (Chapter 2) and this confirmed an earlier study on Rhine water from this laboratory (Iger et al., 1994c). Leucocyte invasion of the skin has commonly been reported for fish exposed to water pollutants (Hinton, 1993; Iger et al., 1994e, f; Burkhardt-Holm et al., 1997), and this process is difficult to relate to immune suppression. Our finding of macrophage respiratory burst stimulation in Rhinewater-exposed fish (Chapter 3) provides data supporting the view that cellular immunity is stimulated in salmonids in the Rhine. Overall, there have not been disease-related problems reported in the salmonid populations of the Rhine, and the data we present in this thesis supports this by finding no evidence of suppression of the humoral immunity.

Together, the data indicate that present day Rhine water is unsuitable for salmonids, especially when coupled with acute temperature elevation, and that studying the skin offers good parameters to study stress-related effects in these fish. The difficulties experienced in restoring Atlantic salmon populations in the Rhine (Cazemier, 1994; Roche, 1994; Van Dijk et al., 1995), together with the low sea trout populations, indicate that the sea trout population may be sustained from resident stock in the tributaries of the Rhine, while migrating smolts may be experiencing difficulties resulting in low returns of spawning adults from the marine environment and thus low recruitment of migrating forms into the population.

Responses of fish to lice

Effects of lice on the host

We studied the stress-related responses of the Atlantic salmon *S. salar* to the salmonid-specific caligid sea louse *Lepeophtheirus salmonis* as a non-toxic stressor. The caligid fish lice are marine ectoparasitic copepods and within the family Caligidae, numerous species are economically important parasites of commercially valuable fish species, especially those reared in aquaculture. Infection with low numbers of pre-adult and adult *L. salmonis* induced strong stress-related responses in the skin and gill epithelia of the host Atlantic salmon (Chapter 4). Natural fish populations are exploited by a wide variety of parasites (Alvarez-Pellitero et al., 1993; Due and Curtis, 1995; Bakke and Harris, 1998; Byrne et al., 1999), and in wild fish stocks these parasites occur naturally and have caused disease outbreaks (Tully et al., 1993a; Birkeland, 1996; Johnson et al., 1996; Birkeland and Jakobsen, 1997).

Infection of salmonids with low numbers of the salmon louse *L. salmonis* has generally not resulted in significant increases in plasma cortisol levels (Johnson and Albright, 1992b; Bjorn and Finstad, 1997; Ross et al., 2000). However, with heavy infestations, plasma cortisol levels often increased well beyond levels known to cause immunosuppression (Mustafa et al., 2000). Johnson and Albright (1992b) reported no change in plasma cortisol values for *Salmo salar* in the laboratory after experimental infection with infective copepodids of *L. salmonis*. Bjorn and Finstad (1997) experimentally infected the sea trout *S. trutta* with *L. salmonis* in the laboratory and could not report differences in plasma cortisol of control and infected fish. Similar results were reported by Ross et al. (2000). On the other hand, Dawson (1998) found significant differences between serum glucose of lice-infested wild seawater *S. trutta* sampled from different locations in the west of Ireland, but it cannot be excluded that the large variation in the values reported for this normally tightly regulated parameter resulted from the impact of the gill netting required to catch these fish before blood sampling or from the different infestation levels of the fish. As the cortisol and glucose responses in the blood of fishes begin rapidly after disturbance, experimental design and sampling protocols should take this into account (Quabius et al., 1997; Nolan et al., 1999a). In the field, sampling true baseline cortisol values is problematic because of unavoidable catching stress, but mathematical extrapolation modelling methods may offer a solution of this problem (Poole et al., 2000).

Our work with *L. salmonis* infection was carried out on postsmolt *S. salar* adapted to seawater (Chapter 4). Infection with low numbers of pre-adult and adult *L. salmonis* induced strong responses in the skin and gill epithelia of the host Atlantic salmon. These were indirect effects of the parasite as they occurred in areas of the epithelia where there was no obvious evidence of prior parasite attachment. The results lead to the conclusion that many of the epithelial changes reported here are similar to those described for stressors in general (increased apoptosis, necrosis, mucous

discharge, invasion by leukocytes), including toxic stressors. Many of these effects are likely hormone-mediated, as a consequence of the infection causing an integrated stress response in the fish, resulting in increased levels of circulating cortisol and catecholamines. These results demonstrate that the response to infection with the sea louse *L. salmonis* can be divided into two distinct categories. The first is a direct effect of parasite attachment and feeding on the body surface, as reported by others (Jones et al., 1990; Johnson and Albright, 1992a, b; Jonsdottir et al., 1992; MacKinnon, 1993) and confirmed in Chapter 4, i.e. direct damage caused by parasite attaching to and feeding on the body surface. The second is the indirect effect of the stressor, probably mediated via the integrated stress response, on the integrity of the skin and gill epithelia, including the osmoregulatory consequences in terms of gill Na^+/K^+-ATPase. The beneficial effects of increased gill Na^+/K^+-ATPase activity could be seen in the maintenance of hydromineral balance.

Effects of the host on the lice

The responses in *L. salmonis*-infested fish did not include effects on vesicle populations in the upper epidermal cell layers. This may be related to the postsmolt status of the fish, which may differ between smoltifying and non-smoltifying species. Many of the stress-related changes reported in Chapter 4 were attributed to cortisol, which when administered via the food, promoted the synthesis of these vesicles in rainbow trout in fresh water (Iger et al., 1995). The endocrine control of the synthesis of these vesicles in the host-parasite interaction was examined by administering cortisol via the food to rainbow trout and infecting them with low numbers of the fresh water fish louse *Argulus japonicus* to see whether the host-parasite relationship could be influenced by the hormone (Chapter 5). Since the early 1970's, it has been recognised that the host affects aspects of the biology of fish lice. Host rejection of the cyclopoid copepods *Lernaea cyprinacea* and *Lernaea polymorpha* has been reported in both naïve and previously infected fish (Shields and Goode, 1978; Woo and Shariff, 1990). The mechanisms behind this rejection are poorly understood, but the contents of these vesicles might be one of these mechanisms. With respect to the salmon louse, *L. salmonis* it was demonstrated that naïve hosts of different species of salmon differ in their susceptibility to infection (coho < chinook < Atlantic; Johnson and Albright, 1992a). Coho salmon, which were found to be the most resistant to parasite establishment, mount strong tissue responses (including hyperplasia and leukocyte invasion), leading to parasite loss. The effects of cortisol have also been demonstrated in experiments with coho salmon. Implantation of cortisol suppressed the inflammatory response and the development of epithelial hyperplasia, which resulted in the inability of coho salmon to shed *L. salmonis* (Johnson and Albright, 1992b). Differences in the growth rate and the number of eggs carried by *L. salmonis* have been reported on different host species. *L. salmonis* grew faster and carried larger numbers of eggs on naturally infected Atlantic salmon when compared to naturally infected chinook salmon (Johnson, 1993). Host effects on egg production and

viability have also been reported for *Ergasilus labracis* and *Lernaea cyprinacea* (Paperna and Zwerner, 1982; Woo and Shariff, 1990). These results indicate that the host response to parasitic infection can have strong effects on the parasite, which might be beneficial to the fish.

Feeding rainbow trout twice with a cortisol-containing meal elevated blood cortisol and glucose levels and stimulated the synthesis of vesicles in the upper epidermal cells. Numbers of *A. japonicus* on cortisol fed fish were lower than on untreated fish, indicating that cortisol-mediated effects on the host skin and increased numbers of vesicles were protective against the parasite. The glucocorticoid receptor was localised and demonstrated in the pavement and filament cells, and in the migrating leukocytes of the upper epidermis. The glucocorticoid receptor has been demonstrated in teleost leukocytes before (Maule and Schreck, 1990, 1991; Maule et al., 1993; Weyts et al., 1997, 1998b), and in chloride cells in the gill (Sandor et al., 1984; Maule and Schreck, 1990, 1991; Maule et al., 1993; Weyts et al., 1997, 1998b; Dang et al., 1999), but not in the skin cells.

In Chapter 5, data showing a significant correlation between parasite number and plasma cortisol at sampling is presented. Cortisol feeding and infection with *A. japonicus* indicates that cortisol may have a protective action against the parasites, and this points to a protective function of the stress response of fish when dealing with the parasites. This effect of cortisol is likely mediated through the glucocorticoid receptor in the skin epidermis, and resulted in stimulation of the synthesis of vesicle populations in the upper epidermal cells and the release of their contents at the skin surface. Thus, we have found a new role for cortisol and demonstrated that it is a central hormone in the host-parasite relationship. Furthermore, we show this hormone induces a protective effect, and this represents an adaptive function of the integrated stress response.

Apoptosis, cortisol and lice

Another important effect of lice infestation on the skin, apart from the effects on the pavement cell vesicles, is the increased apoptosis reported in Chapters 4 and 5. In Chapter 6, epidermal mucous cell numbers and proliferation and apoptosis of the epithelial cells, were studied over 32 d in the common carp *Cyprinus carpio* to see whether these effects are cortisol mediated. These parameters have been shown to be influenced in salmonids by both parasite infestation (Chapters 4 and 5) and by cortisol administration (Iger et al., 1995 and Chapter 5). Apoptotic cell numbers in the uppermost epidermis were reduced at 26 d post-infection with the lice, while the other parameters were unaffected. Administration of cortisol-containing food resulted in reduced apoptosis in the upper epidermal cells at 24 h and at 28 d post-feeding. Cortisol feeding combined with *A. japonicus* infection reduced numbers of apoptotic cells in the upper epidermis more than did either treatment alone, but increased apoptosis in the lower epidermis in cells morphologically identified as migrating macrophages and lymphocytes. This provides evidence for

immunosuppressive effects. If levels of cortisol are moderately elevated by stress-free cortisol administration via the food in a carefully controlled environment, responses designed to be adaptive might be recorded. Infection with *A. japonicus* infection reduced numbers of apoptotic cells in the upper epidermis of the carp, but only after 30 days, demonstrating how the epidermis of fish is influenced in the long-term by previous stress experiences, similar to other studies where responses were reported in fish skin epidermis for up to 30 days (Iger et al., 1994c; Burkhardt-Holm et al., 1997; Nolan et al., 1998; Chapter 2) and supports the view that some of these prolonged effects can be attributed to the effects of cortisol alone. Prolonged effects on the endocrine and immune system have been demonstrated in *O. mykiss* after similar cortisol feeding (Ruane et al., 1999a). The glucocorticoid receptor was demonstrated to be expressed in the cells where these effects were observed, providing direct evidence for the role of cortisol in mediating the responses (Chapters 5 and 6).

In conclusion, the studies with fish lice demonstrated that fish skin responds strongly to these non-toxic stressors similar to how it responds to toxic stressors. This response occurs throughout the epithelium, which suggests these responses are mediated endogenously. *In vivo* cortisol administration showed how vesicle synthesis could be stimulated and subsequently associated with protective effects when fish were infected with lice.

In vitro studies on skin epidermis

In Chapter 7, the effect of cortisol on mucous cells, as well as on cell proliferation and apoptosis in the skin epidermis of rainbow trout was studied *in vitro*. With the objective to study the question whether cortisol effects in the skin *in vivo* were direct or indirect, the glucocorticoid receptor blocker mifepristone was included in the study. The results indicated that the regulation of the 3 processes investigated was complex. Mucous cell numbers were unaffected by cortisol. Reductions in these numbers when the receptor was blocked indicated that it could not be excluded that this hormone might have some effect on the overall rate of discharge. Within 24 hours, cortisol clearly stimulated mitosis and apoptosis in the skin epidermis. The effects of mifepristone on filament cell mitosis indicate that cortisol does not act via the glucocorticoid receptor, and may act via another yet undetected (mineralocorticoid?) receptor, or may act synergistically with other factors in stimulating proliferation of the epidermal cells. The data indicate that not all of the cells of the trout epidermis are regulated similarly. The role of cortisol in the stimulation of apoptosis is clear and shows for the first time that the increased apoptosis in the epidermis reported after exposure of fish to a wide variety of stressors is not a direct effect of the stressor, but is rather an indirect effect of elevated cortisol levels.

The characterisation of a trout (*O. mykiss*) skin primary explant system sets the scene to study the effects of cortisol on an actively growing epidermis for periods longer than 24 hours. Three distinct regions of the explant outgrowth were identified on the basis of cell composition. The proximal growth to the founding explant contained small migrating cells and mucous cells; the middle area was characterised by differentiated pavement cells and fewer mucous and small migrating cell; the distal growing front contained many mucous and small migrating cells, with few pavement cells initially. The data on proliferating and apoptotic cells indicated that the culture system provided an *in vitro* system closely approximating trout epidermis *in vivo*. In particular, the numbers of mucous cells in the culture and the presence of discharged mucus on the surface indicated that, functionally, the system might have similar performance *in vitro* as *in vivo*. This culture system may be well suited to the study of the effects of factors such as hormones and organic pollutants, and possibly toxic metals, as well as for studying the host-parasite relationship by *in vitro*.

Final comments and perspectives

The studies presented in this thesis have provided data on the effects of toxic and non-toxic stressors on fishes, and focussed on the responses in the skin. The whole animal physiology approach is time consuming, but allows the effects of the treatments applied to be evaluated and reported with more certainty about their overall impact on the fish. In this way, we have been able to demonstrate responses of skin and gill epithelia to toxic and non-toxic stressors and to show the effects on hydromineral balance. The levels of stress applied by the treatments were not excessive and treatment-related mortality was not caused. The responses reported may generally be considered adaptive and in some cases, such as the synthesis of vesicles in the upper epidermal cells, directly beneficial for reducing the impact of the stressor, as shown for ectoparasitic lice establishment. The skin epithelium is very sensitive, as it responds rapidly to many stressor types and remains affected in the long term, and the changes reported might be used as an early warning system of environmental pollutants and of other factors.

References Cited

Abdelghani, A.A., P.B. Tchounwou, A.C. Anderson, H. Sujono, and A. Monkiedje (1997). Toxicity evaluation of single and chemical mixtures of Roundup, Garlon-3A, 2,4-D, and Syndets surfactant to channel catfish (*Ictalurus punctatus*), bluegill sunfish (*Lepeomis microchirus*), and crawfish (*Procambarus* spp.). Environ. Toxicol. Water Qual. *12*:237-243.

Abu Damir, H., N.I. Eldirdiri, S.E.I. Adam, J.A. Howarth, Y.M. Salih, and O.F. Idris (1993). Experimental copper poisoning in the camel (*Camelus dromedarius*). J. Comp. Pathol. *108*:191-208.

Alison, M.R. (1995). Assessing cellular proliferation: what's worth measuring? Human Exp. Toxicol. *14*:935-944.

Allison, C.M., and R.J. Omeljaniuk (1998). Specific binding sites for [^{3}H]dexamethasone in the hypothalamus of juvenile rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. *110*:2-10.

Alvarez-Pellitero, P., A. Sitja-Bobadilla, and A. Franco-Sierra (1993). Protozoan parasites of wild and cultured sea bass, *Dicentrarchus labrax* (L.), from the Mediterranean area. Aqua. Fish. Man. *24*:101-108.

Anderson, D.P. (1990). Immunological indicators: effects of environmental stress on immune protection and disease outbreaks. Am. Fish. Soc. Symp. *8*:38-50.

Arnold, H., and T. Braunbeck (1994) Disulphoton as a major toxicant in the Rhine chemical spill at Basle in 1986: acute and chronic studies with eel and rainbow trout. In: Sublethal and Chronic Effects of Pollutants On Freshwater Fish. R. Muller, and R. Lloyd, eds. FAO, Oxford, pp. 75-87.

Austin, B., and N. Cross (1998). Infection of pronephros cell cultures derived from rainbow trout (*Oncorhynchus mykiss*, Walbaum) with bacterial fish pathogens: A comparison with whole fish infectivity studies. Methods Cell Sci. *19*:317-324.

Avella, M., and J. Ehrenfeld (1997). Fish gill respiratory cells in culture: a new model for Cl^--secreting epithelia. J. Membr. Biol. *156*:87-97.

Bakke, T.A., and P.D. Harris (1998). Diseases and parasites in wild Atlantic salmon (*Salmo salar*) populations. Can. J. Fish. Aquat. Sci. *55(Suppl 1)*:247-266.

Balm, P.H.M., and T.G. Pottinger (1995). Corticotrope and melanotrope POMC-derived peptides in relation to interrenal function during stress in rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. *98*:279-288.

Balm, P.H.M., P. Pepels, S. Helfrich, M.L.M. Hovens, and S.E. Wendelaar Bonga (1994). Adrenocorticotropic hormone in relation to interrenal function during stress in tilapia (*Oreochromis mossambicus*). Gen. Comp. Endocrinol. *96*:347-360.

Balm, P.H.M., Y. Iger, P. Prunet, T.G. Pottinger, and S.E. Wendelaar Bonga (1995). Skin ultrastructure in relation to prolactin and MSH function in rainbow trout (*Oncorhynchus mykiss*) exposed to environmental acidification. Cell Tissue Res. *279*:351-358.

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

Barton, B.A., C.B. Schreck, and L.D. Barton (1987). Effects of chronic cortisol administration and daily acute stress on growth, physiological conditions, and stress responses in juvenile rainbow trout. Dis. Aquat. Org. *2*:173-185.

Basten, D.J., and P.A. Cochran (1991). The effect of a fish medication on *Argulus japonicus*, a crustacean parasite of fish. J. Freshw. Ecol. *6*:457-458.

Berland, B. (1993) Salmon lice on wild salmon (*Salmo salar* L.) in western Norway. In: Pathogens of Wild and Farmed Fish: Sea Lice. G.A. Boxshall, and D. Defaye, eds. Ellis Horwood Limited, London, pp. 179-187.

Berntssen, M.H.G., F. Kroglund, B.O. Rosseland, and S.E. Wendelaar Bonga (1997). Responses of skin mucous cells to aluminium exposure at low pH in Atlantic salmon (*Salmo salar*) smolts. Can. J. Fish. Aquat. Sci. *54*:1039-1045.

Birkeland, K. (1996). Consequences of premature return by sea trout (*Salmo trutta*) infested with the salmon louse (*Lepeophtheirus salmonis* Krøyer): migration, growth, and mortality. Can. J. Fish. Aquat. Sci. *53*:2808-2813.

Birkeland, K., and P.J. Jakobsen (1997). Salmon lice, *Lepeophtheirus salmonis*, infestation as a causal agent of premature return to rivers and estuaries by sea trout, *Salmo trutta*, juveniles. Environ. Biol. Fish. *49*:129-137.

Bjorn, P.A., and B. Finstad (1997). The physiological effects of salmon lice infection on sea trout post smolts. Nord. J. Freshwater Res. *73*:60-72.

Blackstock, N., and A.D. Pickering (1982). Acidophilic granular cells in the epidermis of the brown trout, *Salmo trutta* L. Cell Tissue Res. *210*:259-269.

Bols, N.C., R.C. Ganassin, D.J. Tom, and L.E.J. Lee (1994). Growth of fish cell lines in glutamine-free media. Cytotechnol. *16*:159-166.

Boxshall, G.A., and D. Defaye, eds. (1993) Pathogens of Wild and Farmed Fish: Sea Lice. Ellis Horwood Limited, London.

Brodeur, J.C., G. Sherwood, J.B. Rasmussen, and A. Hontela (1997). Impaired cortisol secretion in yellow perch (*Perca flavescens*) from lakes contaminated by heavy metal: *in vivo* and *in vitro* assessment. Can. J. Fish. Aquat. Sci. *54*:2752-2758.

Brokken, L.J.S., P.M. Verbost, W. Atsma, and S.E. Wendelaar Bonga (1998). Isolation, partial characterization and localization of integumental peroxidase, a stress-related enzyme in the skin of a teleostean fish (*Cyprinus carpio* L.). Fish Physiol. Biochem. *18*:331-342.

Brown, J.A. (1993) 11. Endocrine responses to environmental pollutants. In: Fish Ecophysiology. J.C. Rankin, and F.B. Jensen, eds. Chapman & Hall, London, pp. 276-296.

Buchmann, K., A. Uldal, and H.C.K. Lyholt (1995). Parasite infections in Danish trout farms. Acta Vet.Scandinav. *36*:283-298.

Buhl, K.J., and S.J. Hamilton (1996). Toxicity of inorganic contaminants, individually and in environmental mixtures, to three endangered fishes (Colorado squawfish, bonytail, and razorback sucker). Arch. Environ. Contam. Toxicol. *30*:84-92.

Bullock, A.M., R. Marks, and R.J. Roberts (1978). The cell kinetics of teleost fish epidermis: mitotic activity of the normal epidermis at varying temperatures in plaice (*Pleuronectes platessa*). J. Zool., Lond. *184*:423-428.

Burgess, D., N. Frerichs, and S. George (1993). Control of metallothionein expression by hormones and stressors in cultured fish cell lines. Mar. Environ. Res. *35*:25-28.

Burkhardt-Holm, P., M. Escher, and W. Meier (1997). Waste-water management plant effluents cause cellular alterations in the skin of brown trout. J. Fish Biol. *50*:744-758.

Burkhardt-Holm, P., H. Schmidt, and W. Meier (1998). Heat shock protein (hsp70)in brown trout epidermis after sudden temperature rise. Comp. Biochem. Physiol. *120A*:35-41.

Burton, J.L., and M.E. Kehril Jr. (1996). Effects of dexamethasone on bovine circulating T lymphocyte populations. J. Leuko. Biol. *59*:90-99.

Bury, N.R., J. Li, R.A.C. Lock, and S.E. Wendelaar Bonga (1998). Cortisol protects against copper induced necrosis and promotes apoptosis in fish gill chloride cells *in vitro*. Aquat. Toxicol. *40*:193-202.

Byrne, C.J., C. Holland, and O. Tully (1999). Metazoan parasite community structure of sea trout on the west coast of Ireland. J. Fish Biol. *55*:127-134.

Calduch-Giner, J.A., A. Sitjà-Bobadilla, P. Alvarez-Pellitero, and Pérez-Sánchez (1997). Growth hormone as an in vitro phagocyte-activating factor in the gilthead sea bream (*Sparus aurata*). Cell Tissue Res. *287*:535-540.

Carruth, L.L., R.E. Jones, and D.O. Norris (2000). Cell density and intracellular translocation of glucocorticoid receptor-immunoreactive neurons in the kokanee salmon (*Oncorhynchus nerka kennerlyi*) brain, with an emphasis on the olfactory system. Gen. Comp. Endocrinol. *117*:66-76.

Cazemier, W.G. (1994). Present status of the salmonids Atlantic salmon and sea-trout in the Dutch part of the river Rhine. Water Sci. Technol. *29*:37-41.

Celander, M., J. Bremer, M.E. Hahn, and J.J. Stegeman (1997). Glucocorticoid-xenobiotic interactions: dexamethasone-mediated potentiation of cytochrome P4501A induction by β-naphthoflavone in a fish hepatome cell line (PLHC-1). Environ. Toxicol. Chem. *16*:900-907.

Charriaut-Marlangue, C., and Y. Ben-Ari (1995). A cautionary note on the use of the TUNEL stain to determine apoptosis. Neurorep. *7*:61-64.

Cherry, D.S., K.L. Dickson, and J. Cairns Jr (1975). Temperatures selected and avoided by fish at various acclimation temperatures. J. Fish. Res. Board Can. *32*:485-491.

Chiu, J.F., A.F. Mack, and R.D. Fernald (1995). Daily rhythm of cell proliferation in the teleost retina. Brain. Res. *673*:119-125.

Chung, S., and C.J. Secombes (1988). Analysis of events occurring within teleost macrophages during the respiratory burst. Comp. Biochem. Physiol. *89*(*B*):539-544.

Cornell, S.C., D.M. Portesi, P.A. Veillette, K. Sundell, and J.L. Speckler (1994). Cortisol stimulates intestinal fluid uptake in Atlantic salmon (*Salmo salar*) in the post-smolt stage. Fish Physiol. Biochem. *13*:183-190.

Coutant, C.C. (1973). Effect of thermal shock on vulnerability of juvenile salmonids to predation. J. Fish. Res. Board Can. *30*:965-973.

Dalmo, R.A., K. Ingebrigtsen, and J. Bogwald (1997). Non-specific defence mechanisms in fish, with particular reference to reticuloendothelial system (RES). J. Fish Dis. *20*:241-273.

Dang, Z., A.C. Lock, G. Flik, and S.E. Wendelaar Bonga (1999). Metallothionein response in gills of *Oreochromis mossambicus* exposed to copper in fresh water. Am. J. Physiol. *277*:R320-R331.

Dasmahapatra, A.K., and P.C. Lee (1993). Down regulation of CYP 1A1 by glucocorticoids in trout hepatocytes *in vitro*. In Vitro Cell. Dev. Biol. *29A*:643-648.

Dawson, L.H.J. (1998). The physiological effects of salmon lice (*Lepeophtheirus salmonis*) infections on returning post-smolt sea trout (*Salmo trutta* L.) in western Ireland. ICES J. Mar. Sci. *55*:193-200.

Denizeau, F., and M. Marion (1984). Cultured rainbow trout and human cells exposed to PCBs in the evaluation of cytotoxicity of aquatic pollutants. Water Res. *18*:247-251.

Dhabhar, F.S., and B.S. McEwen (1997). Acute stress enhances while chronic stress suppresses cell-mediated immunity *in vivo*: a potential role for leukocyte trafficking. Brain Behav. Immun. *11*:286-306.

Dietz, T.J. (1994). Acclimation of the threshold induction temperatures for 70-kDa and 90-kDa heat shock proteins in the fish *Gillichthys mirabilis*. J. Exp. Biol. *188*:333-338.

Ducouret, B., M. Tujague, J. Ashraf, N. Mauchel, N. Servel, Y. Valotaire, and E.B. Thompson (1995). Cloning of a teleost fish glucocorticoid receptor chows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. Endocrinology *136*:3774-3783.

Due, T.T., and M.A. Curtis (1995). Parasites of freshwater resident and anadromous Arctic charr (*Salvelinus alpinus*) in Greenland. J. Fish Biol. *46*:578-592.

Dyer, S.D., K.L. Dickson, E.G. Zimmerman, and B.M. Sanders (1991). Tissue-specific patterns of synthesis of heat-shock proteins and thermal tolerance of the fathead minnow (*Pimephales promelas*). Can. J. Zool. *69*:2021-2027.

Ellis, A.E. (1981) Stress and the modulation of defence mechanisms in fish. In: Stress and Fish. A.D. Pickering, ed. Academic Press, London, pp. 147-169.

Espelid, S., G.B. Lokken, K. Steiro, and J. Bogwald (1996). Effects of cortisol and stress on the immune system in Atlantic salmon (*Salmo salar* L.). Fish & Shell. Immunol. *6*:95-110.

Eufemia, N.A., T.K. Collier, J.E. Stein, D.E. Watson, and R.T. Di Giulio (1997). Biochemical responses to sediment-associated contaminants in brown bullhead (*Ameriurus nebulosus*) from the Niagara River ecosystem. Ecotoxicol. *6*:13-34.

Evans, D.H., J.B. Claiborne, L. Farmer, C. Mallery, and E.J. Krasny Jr. (1982). Fish gill ionic transport: methods and models. Biol. Bull. *163*:108-130.

Fernandes, M.N., F.B. Eddy, and W.S. Penrice (1995). Primary cell culture from gill explants of rainbow trout. J. Fish Biol. *47*:641-651.

Fevolden, S.E., and K.H. Roed (1993). Cortisol and immune characteristics in rainbow trout (*Oncorhynchus mykiss*) selected from high or low tolerance to stress. J. Fish Biol. *43*:919-930.

Fevolden, S.E., K.H. Roed, and B. Gjerde (1994). Genetic components of post-stress cortisol and lysozyme activity in Atlantic salmon; correlations to disease resistance. Fish & Shell. Immunol. *4*:507-519.

Flaño, E., P. López-Fierro, F. Alvarez, B. Razquin, and A. Villena (1998). Splenic cultures from rainbow trout, *Oncorhynchus mykiss*: establishment and characterisation. Fish & Shell. Immunol. *8*:589-606.

Flik, G., and P.M. Verbost (1995) Cellular mechanisms in calcium transport and homeostasis in fish. In: Biochemistry and Molecular Biology of Fishes. Z.Z. Hochachka, and Z.Z. Mommensen, eds. Elsevier Science B.V., Amsterdam, Vol. 5, pp. 251-263.

Flik, G., S.E. Wendelaar Bonga, and J.C. Fenwick (1984). Ca^{2+}-dependent phosphatase and Ca^{2+}-dependent ATPase activities in plasma membranes of eel gill epithelium-III. Stimulation of branchial high-affinity Ca^{2+}-ATPase activity during prolactin-induced hypercalcemia in American eels. Comp. Biochem. Physiol. *79*(*B*):521-524.

Folmar, L.C., J. Harshbarger, P.C. Baumann, G. Gardner, and S. Bonomelli (1995). Pathological and serum chemistry profiles of brown bullheads (*Ameiurus nebulosus*) from Black river and Old Woman creek, Ohio. Bull. Environ. Contam. Toxicol. *54*:50-59.

Fournier, M., A. Lacroix, I. Voccia, and P. Brousseau (1998). Phagocytic and metabolic activities of macrophages from mummichog naturally exposed to pulp mill effluents in the Miramichi river. Ecotoxicol.Environ.Safety *40*:177-183.

Friedrich, G., and D. Muller (1984) Rhine. In: Ecology of European Rivers. B.A. Whitton, ed. Blackwell Scientific Publications, Oxford, pp. 265-315.

Gagné, F., and C. Blaise (1997). Evaluation of cell viability, mixed function oxidase activity, metallothionein induction, and genotoxicity in rainbow trout hepatocytes exposed to industrial effluents: II. Validation of the rainbow trout hepatocyte model for ecotoxicity testing of industrial wastewater. Environ. Toxicol. Water Qual. *12*:305-314.

Gamperl, A.K., M.M. Vijayan, and R.G. Boutilier (1994). Experimental control of stress hormone levels in fishes: techniques and applications. Rev. Fish Biol. Fish. *4*:215-255.

Gargiulo, G., P. de Girolamo, L. Ferrara, O. Soppelsa, G. Andreozzi, R. Antonucci, and P. Battaglini (1996). Action of cadmium on the gills of *Carassius auratus* L. in the presence of catabolic NH_3. Arch. Environ. Contam. Toxicol. *30*:235-240.

Ghioni, C., D.R. Tocher, and J.R. Sargent (1998). Effects of dichlorvos and formalin on fatty acid metabolism of rainbow trout (*Oncorhynchus mykiss*) skin cells in primary culture. Fish Physiol. Biochem. *18*:241-252.

Goldspink, G. (1995). Adaptation of fish to different environmental temperature by qualitative and quantitative changes in gene expression. J. Therm. Biol. *20*:167-174.

Grasl-Kraupp, B., B. Ruttkay-Nedecky, H. Koudelka, K. Bukowska, W. Bursch, and R. Schulte-Hermann (1995). In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatol. *21*:1465-1468.

Gray, R.H. (1990). Fish behaviour and environmental assessment. Environ. Toxicol. Chem. *9*:53-67.

Grignard, J.-C., C. Mélard, and P. Kestemont (1996). A preliminary study of parasites and diseases of perch in an intensive culture system. J. Appl. Ichthyol. *12*:195-199.

Grimnes, A., and P.J. Jakobsen (1996). The physiological effects of salmon lice infection on post-smolt of Atlantic salmon. J. Fish Biol. *48*:1179-1194.

Grippo, R.S., and W.A. Dunson (1996). The body ion loss biomarker. 1. Interactions between trace metals and low pH in reconstituted coal mine-polluted water. Environ. Toxicol. Chem. *15*:1955-1963.

Hadderingh, R.H., G.H.F.M. van Aerssen, L. Groeneveld, H. Jenner, and J.W. van der Stoep (1983). Fish impingement at power stations along the rivers Rhine and Meuse in the Netherlands. Hydrobiol. Bull. *17*:129-141.

Harris, J.E., and S. Hunt (1975). The fine structure of the epidermis of two species of salmonid fish, the Atlantic salmon (*Salmo salar* L.) and the brown trout (*Salmo trutta* L.). I. General organistaion and filament-containing cells. Cell Tissue Res. *157*:553-565.

Haynes, J.M., G.P. Gerber, and J.K. Buttner (1989). Response of sport fishes to thermal discharges into the great lakes: is Somerset station, lake Ontario, different? J. Great Lakes Res. *15*:709-718.

Hazel, J.R. (1993) Thermal Biology. In: The Physiology of Fishes. D.H. Evans, ed. CRC Press, Florida, pp. 427-467.

Heckmann, R.A. (1993) Parasites of salmonid fishes. In: Fish Medicine. M.K. Stoskopf, ed. W.B. Saunders, Philadelphia, pp. 408-428.

Heikkila, J.J., G.A. Schultz, K. Iatrou, and L. Gedamu (1982). Expression of a set of fish genes following heat or metal ion exposure. J. Biol. Chem. *257*:12000-12005.

Hendriks, A.J. (1994). Monitoring and estimating concentrations and effects of microcontaminants in the Rhine-delta: chemical analysis, biological laboratory assays and field observations. Water Sci. Technol. *29*:223-232.

Hendriks, A.J., and K. van de Guchte (1997). Optimal modelling and monitoring in ecotoxological assessments: choosing instruments for applied research and management with examples from the Rhine-Meuse delta. Environ. Toxicol. Water Qual. *12*:321-333.

Heuch, P.A., A. Parsons, and K. Boxaspen (1995). Diel vertical migration: a possible host-finding mechanism in salmon louse (*Lepeophtheirus salmonis*) copepodids? Can. J. Fish. Aquat. Sci. *52*:681-689.

Hinton, D.E. (1993) Toxicologic histopathology of fishes: a systemic approach and overview. In: Pathology of Marine and Estaurine Organisms. J.A. Couch, and J.W. Fournie, eds. CRC Press, Boca Raton, Florida, pp. 177-215.

Hinton, D.E., and D.J. Lauren (1990) Integrative histopathological approaches to detecting effects of environmental stressors on fishes. In: Biological Indicators of Stress in Fish. S.M. Adams, ed. American Fisheries Society Symposium, Maryland, Vol. 8, pp. 51-66.

Holst, J.C., F. Nilsen, K. Hodneland, and A. Nylund (1993). Observations of the biology and parasites of postsmolt Atlantic salmon, *Salmo salar*, from the Norwegian sea. J. Fish Biol. *42*:962-966.

Hontela, A., J.B. Rasmussen, C. Audet, and G. Chevalier (1992). Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs, and mercury. Arch. Environ. Contam. Toxicol. *22*:278-283.

Hontela, A., P. Dumont, D. Duclos, and R. Fortin (1995). Endocrine and metabolic dysfunction in yellow perch, *Perca flavescens*, exposed to organic contaminants and heavy metals in the St. Lawrence river. Environ. Toxicol. Chem. *14*:725-731.

Hontela, A., C. Daniel, and J.B. Rasmussen (1997). Structural and functional impairment of the hypothalamo-pituitary-interrenal axis in fish exposed to bleached kraft mill effluent in the St Maurice River, Quebec. Ecotoxicol. *6*:1-12.

Houghton, G., and R.A. Matthews (1990). Immunosuppression in juvenile carp, *Cyprinus carpio* L.: the effects of the corticosteroids triamcinolone acetonide and hydrocortisone 21-hemisuccinate (cortisol) on acquired immunity and the humoral antibody response to *Ichthyophthirius multifiliis*. J. Fish Dis. *13*:269-280.

Huuskonen, S.E., M.E. Hahn, and P. Lindström-Seppä (1998). A fish hepatoma cell line (PLHC-1) as a tool to study cytotoxicity and CYP1A induction properties of cellulose and wood chip extracts. Chemosphere *36*:2921-2932.

Iger, Y., and S.E. Wendelaar Bonga (1994). Cellular responses of the skin of carp (*Cyprinus carpio*) exposed to acidified water. Cell Tissue Res. *275*:481-492.

Iger, Y., M. Abraham, and S.E. Wendelaar Bonga (1994a). Response of club cells in the skin of carp *Cyprinus carpio* to exogenous stressors. Cell Tissue Res. *277*:485-491.

Iger, Y., P.H.M. Balm, and S.E. Wendelaar Bonga (1994b). Cellular responses of the skin and changes in plasma cortisol levels of trout (*Oncorhynchus mykiss*) exposed to acidified waters. Cell Tissue Res. *278*:535-542.

Iger, Y., H.A. Jenner, and S.E. Wendelaar Bonga (1994c). Cellular responses in the skin of the rainbow trout (*Oncorhynchus mykiss*) exposed to Rhine water. J. Fish Biol. *45*:1119-1132.

Iger, Y., H.A. Jenner, and S.E. Wendelaar Bonga (1994d). Cellular responses in the skin of the trout (*Oncorhynchus mykiss*) exposed to temperature elevation. J. Fish Biol. *44*:921-935.

Iger, Y., R.A.C. Lock, H.A. Jenner, and S.E. Wendelaar Bonga (1994e). Cellular responses in the skin of carp (*Cyprinus carpio*) exposed to copper. Aquat. Toxicol. *29*:49-64.

Iger, Y., R.A.C. Lock, J.C.A. van der Meij, and S.E. Wendelaar Bonga (1994f). Effects of water-borne cadmium on the skin of the common carp (*Cyprinus carpio*). Arch. Environ. Contam. Toxicol. *26*:342-350.

Iger, Y., P.H.M. Balm, H.A. Jenner, and S.E. Wendelaar Bonga (1995). Cortisol induces stress-related changes in the skin of rainbow trout (*Oncorhynchus mykiss*). Gen. Comp. Endocrinol. *97*:188-198.

Inoue, T., and H. Osatake (1988). A new drying method of biological specimens for scanning electron microscopy: the t-butyl alcohol freeze-drying method. Arch. Histol. Cytol. *51*:53-59.

Iwama, G.K., P.T. Thomas, R.B. Forsyth, and M.M. Vijayan (1998). Heat shock protein expression in fish. Rev. Fish Biol. Fish. *8*:35-56.

Jafri, S.I.H., and S.S. Ahmed (1991). A new record of ectoparasitic crustaceans (Branchiura: Argulidae) from major carps in Sindh, Pakistan. Pakistan J. Zool. *23*:12-14.

Jafri, S.I.H., and S.S. Ahmed (1994). Some observations on mortality in major carps due to fish lice and their chemical control. Pakistan J. Zool. *26*:274-276.

Jagoe, C.H., A. Faivre, and M.C. Newman (1996a). Morphological and morphometric changes in the gills of the mosquitofish (*Gambusia holbrooki*) after exposure to mercury (II). Aquat. Toxicol. *34*:163-183.

Jagoe, C.H., P.L. Shaw-Allen, and S. Brundage (1996b). Gill Na^+,K^+-ATPase activity in largemouth bass (*Micropterus salmoides*) from three reservoirs with different levels of mercury contamination. Aquat. Toxicol. *36*:161-176.

Jenq, W., H. Rabb, M. Wahe, and G. Ramirez (1996). Hypoxic effects on the expression of the mineralocorticoid and glucocorticoid receptors in human renal cortex epithelial cells. Biochem. Biophys. Res. Commun. *218*:444-448.

Johnson, S.C. (1993) A comparison of development and growth rates of *Lepeophtheirus salmonis* (Copepoda: Caligidae) on naive Atlantic salmon (*Salmo salar*) and chinook (*Oncorhynchus tshawytscha*) salmon. In: Pathogens of Wild and Farmed Fish: Sea Lice. G.A. Boxshall, and D. Defaye, eds. Ellis Horwood Limited, London, pp. 68-80.

Johnson, S.C., and L.J. Albright (1991). Development, growth, and survival of *Lepeophtheirus salmonis* (Copepoda: Caligidae) under laboratory conditions. J. Mar. Biol. Ass. U.K. *71*:425-436.

Johnson, S.C., and L.J. Albright (1992a). Comparative susceptibility and histopathology of the response of naive Atlantic, chinook and coho salmon to experimental infection with *Lepeophtheirus salmonis* (Copepoda: Caligidae). Dis. Aquat. Org. *14*:179-193.

Johnson, S.C., and L.J. Albright (1992b). Effects of cortisol implants on the susceptibility and the histopathology of the responses of naive coho salmon *Oncorhynchus kisutch* to experimental infection with *Lepeophtheirus salmonis* (Copepoda: Caligidae). Dis. Aquat. Org. *14*:195-205.

Johnson, S.C., R.B. Blaylock, J. Elphick, and K.D. Hyatt (1996). Disease induced by the sea louse (*Lepeophtheirus salmonis*) (Copepoda: Caligidae) in wild sockeye salmon (*Oncorhynchus nerka*) stocks of Alberni Inlet, British Colombia. Can. J. Fish. Aquat. Sci. *53*:2888-2897.

Jones, M.W., C. Sommerville, and J. Bron (1990). The histopathology associated with the juvenile stages of *Lepeophtheirus salmonis* on the Atlantic salmon, *Salmo salar* L. J. Fish Dis. *13*:303-310.

Jonsdottir, H., J.E. Bron, R. Wootten, and J.F. Turnbull (1992). The histopathology associated with the pre-adult and adult stages of *Lepeophtheirus salmonis* on the Atlantic salmon, *Salmo salar* L. J. Fish Dis. *15*:521-527.

Kaattari, S.L., and R.A. Tripp (1987). Cellular mechanisms of glucocorticoid immunosuppression in salmon. J. Fish Biol. *31 (Supplement A)*:129-132.

Kakuta, I. (1997). Effect of sewage on blood parameters and the resistance against bacterial infection of goldfish, *Carassius auratus*. Environ. Toxicol. Water Qual. *12*:43-51.

Kakuta, I., and S. Murachi (1997). Physiological response of carp, *Cyprinus carpio*, exposed to raw sewage containing fish processing water. Environ. Toxicol. Water Qual. *12*:1-9.

Kawai, Y., T. Hayashi, K. Eguchi, K.-y Asazuma, K. Masamura, A. Iwamuro, Y. Takano, H. Tada, S. Matsukawa, and I. Miyamori (1998). Effects of brief glucocorticoid exposure on growth of vascular smooth muscle cells in culture. Biochem. Biophys. Res. Commun. *245*:493-496.

Kelly-Reay, K., and B.A. Weeks-Perkins (1994). Determination of the macrophage chemiluminescent response in *Fundulus heteroclitus* as a function of pollution stress. Fish & Shell. Immunol. *4*:95-105.

Knoebl, I., M.S. Fitzpatrick, and C.B. Schreck (1996). Characterization of a glucocorticoid receptor in the brains of chinook salmon, *Oncorhynchus tshawytscha*. Gen. Comp. Endocrinol. *101*:193-204.

Kolla, V., N.M. Robertson, and G. Laitwack (1999). Identification of a mineralocorticoid/glucocorticoid response element in the human Na/K ATPase α1 gene promoter. Biochem. Biophys. Res. Commun. *266*:5-14.

Ku, C.C., and S.N. Chen (1992). Characterization of three cell lines derived from colour carp *Cyprinus carpio*. J. Tiss. Cult. Meth. *14*:63-72.

Kurki, P., K. Ogata, and E.M. Tan (1987). Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J. Immunol. Methods *109*:49-59.

Kurki, P., K. Ogata, and E.M. Tan (1988). Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J. Immunol. Methods *109*:49-59.

Lamarre, E., and P.A. Cochran (1992). Lack of host species selection by the exotic parasitic crustacean, *Argulus japonicus*. J. Freshw. Ecol. *7*:77-80.

Laurent, P., and S.F. Perry (1990). Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. Cell Tissue Res. *259*:429-442.

Lee, K.J., and G.M. Hahn (1988). Abnormal proteins as the trigger for the induction of stress responses: heat, diamide, and sodium arsenite. J. Cell. Physiol. *136*:411-420.

Lee, L.E., and N.C. Bols (1989). Action of cortisol on the proliferation of rainbow trout fibroblasts. Cell Tissue Kinet. *22*:291-301.

Lemaire-Gony, S., P. Lemaire, and A.L. Pulsford (1995). Effects of cadmium and benzo(a)pyrene on the immune system, gill ATPase and EROD activity of European sea bass *Dicentrarchus labrax*. Aquat. Toxicol. *31*:297-313.

Leugen, I., M. Pisam, M. Bidet, M. Tauc, and P. Poujeol (1998). pHi regulation and ultrastructural analysis in cultured cells from freshwater and seawater-adapted trout. Fish Physiol. Biochem. *18*:297-309.

Li, J., E.S. Quabius, S.E. Wendelaar Bonga, G. Flik, and R.A.C. Lock (1998). Effects of water-borne copper on branchial chloride cells and Na^+/K^+-ATPase activities in Mozambique tilapia (*Oreochromis mossambicus*). Aquat. Toxicol. *43*:1-11.

Lionetto, M.G., M. Maffia, M.S. Cappello, M.E. Giordano, C. Storeelli, and T. Schettino (1998). Effect of cadmium on carbonic anhydrase and Na^+-K^+-ATPase in eel, *Anguilla anguilla*, intestine and gills. Comp. Biochem. Physiol. *120A*:89-91.

Lock, R.A.C., P.H.M. Balm, and S.E. Wendelaar Bonga (1994) Adaptation of freshwater fish to toxicants: stress mechanisms induced by branchial malfunctioning. In: Sublethal and Chronic Effects of Pollutants On Freshwater Fish. R. Muller, and R. Lloyd, eds. FAO, Oxford, pp. 124-134.

Louahed, J., J.-C. Renauld, J.-B. Demoulin, G. Baughman, S. Bourgeois, K. Sugamura, and J. van Snick (1996). Differential activity of dexamethasone on IL-2, IL-4-, or IL-9-induced proliferation of murine factor-dependent T cell lines. J. Immunol. *156*:3704-3710.

Lyons-Alcantara, M., J.V. Tarazona, and C. Mothersill (1996). The differential effects of cadmium exposure on the growth and survival of primary and established cells from fish and mammals. Cell Biol. Toxicol. *12*:29-38.

Ma, C., and P. Collodi (1996). Culture of cells from tissues of adult and larval sea lamprey. Cytotechnol. *21*:195-203.

MacKinnon, B.M. (1993). Host response of Atlantic salmon (*Salmo salar*) to infection by sea lice (*Caligus elongatus*). Can. J. Fish. Aquat. Sci. *50*:789-792.

Madsen, S. (1990). The role of cortisol and growth hormone in seawater adaptation and development of hypoosmoregulatory mechanisms in sea trout parr (*Salmo trutta trutta*). Gen. Comp. Endocrinol. *79*:1-11.

Madsen, S.S., M.K. Jensen, J. Nøhr, and K. Kristiansen (1995). Expression of Na^+-K^+-ATPase in the brown trout, *Salmo trutta*: *in vivo* modulation by hormones and seawater. Am. J. Physiol. *269*:R1339-R1345.

Magwood, S., and S. George (1996). *In vitro* alternatives to whole animal testing. Comparative cytotoxicity studies of divalent metals in established cell lines derived from tropical and temperate water fish species in a neutral red assay. Mar. Environ. Res. *42*:37-40.

Mallatt, J., J.F. Bailey, S.J. Lampa, M.A. Evans, and S. Brumbaugh (1995). A fish gill system for quantifying the ultrastructural effects of environmental stressors: methylmercury, Kepone, and heat shock. Can. J. Fish. Aquat. Sci. *52*:1165-1182.

Marr, J.C.A., H.L. Bergman, M. Parker, J. Lipton, D. Cacela, W. Erikson, and G.R. Phillips (1995). Relative sensitivity of brown and rainbow trout to pulsed exposures of an acutely lethal mixture of metals typical of the Clark Fork River, Montana. Can. J. Fish. Aquat. Sci. *52*:2005-2015.

Martinez, J.L., M.V. Lopezdoriga, G.G. Baschwitz, and C.F.B. Dequiros (1994). Ultrastructural observations on pigmented macrophages in the epidermis of the brown trout (*Salmo trutta* L.). J. Submicroscop. Cytol. Pathol. *26*:481-487.

Maule, A.G., and C.B. Schreck (1990). Glucocorticoid receptors in leukocytes and gill of juvenile coho salmon (*Oncorhynchus kisutch*). Gen. Comp. Endocrinol. *77*:48-455.

Maule, A.G., and C.B. Schreck (1991). Stress and cortisol treatment changed affinity and number of glucocorticoid receptors in leukocytes and gill of coho salmon. Gen. Comp. Endocrinol. *84*:83-93.

Maule, A.G., C.B. Schreck, and S.L. Kaattari (1987). Changes in the immune system of coho salmon (*Oncorhynchus kisutch*) during parr-to-smolt transformation and after implantation of cortisol. Can. J. Fish. Aquat. Sci. *44*:161-166.

Maule, A.G., C.B. Schreck, and C. Sharpe (1993). Seasonal changes in cortisol sensitivity and glucocorticoid receptor affinity and number in leukocytes of coho salmon. Fish Physiol. Biochem. *10*:497-506.

McBride, J.R., and A.P. van Overbeeke (1971). Effects of androgens, estrogens, and cortisol on the skin, stomach, liver, pancreas, and kidney in gonadectomized adult sockeye salmon, *Oncorhynchus nerka*. J. Fish. Res. Board Can. *28*:485-490.

McCartney, T.H. (1976). Sodium-potassium dependent adenosine triphosphatase activity in the gills and kidneys of Atlantic salmon (*Salmo salar*). Comp. Biochem. Physiol. *53*(*A*):351-353.

McCormick, S.D. (1990). Cortisol directly stimulates differentiation of chloride cells in tilapia opercular membrane. Am. J. Physiol. *259*:857-863.

McCormick, S.D. (1995) Hormonal control of gill Na^+,K^+-ATPase and chloride cell function. In: Cellular and Molecular Approaches to Fish Ionic Regulation. C.M. Wood, and T.J. Shuttleworth, eds. Academic Press Inc., California, pp. 285-315.

McCormick, S.D. (1996). Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na^+,K^+-ATPase in Atlantic salmon (*Salmo salar*): interaction with cortisol. Gen. Comp. Endocrinol. *101*:3-11.

McDonald, G., and L. Milligan (1997) Ionic, osmotic and acid-base regulation in stress. In: Fish Stress and Health in Aquaculture. G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck, eds. Cambridge University Press, Cambridge, pp. 119-144.

McKim, J.M., J.W. Nichols, G.J. Lien, A.D. Hoffman, C.A. Gallinat, and G.N. Stokes (1996). Dermal absorption of three waterborne chloroethanes in rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*). Fundam. Appl. Toxicol. *31*:218-228.

Menezes, J., M.A. Ramos, T.G. Pereira, and A.M. da Silva (1990). Rainbow trout culture failure in a small lake as a result of massive parasitosis related to careless fish introductions. Aquaculture *89*:123-126.

Miller, A.H., R.L. Spencer, B.D. Bradley, T.L. Pisell, Y. Azrieli, P. Tanapat, H. Moday, R. Rhee, and B.S. McEwen (1998). Glucocorticoid receptors are differentially expressed in the cells and tissues of the immune system. Cell. Immunol. *186*:45-54.

Moore, J.D., M. Ototake, and T. Nakanishi (1998). Particulate antigen uptake during immersion immunisation of fish: the effectiveness of prolonged exposure and roles of skin and gill. Fish & Shell. Immunol. *8*:393-407.

Moore, M.J., D.F. Leavitt, A.M. Shumate, P. Alatalo, and J.J. Stegeman (1994). A cell proliferation assay for small fish and aquatic invertebrates using bath exposure to bromodeoxyuridine. Aquat. Toxicol. *30*:183-188.

Mothersill, C., F. Lyng, M. Lyons, and D. Cottell (1995). Growth and differentiation of epidermal cells from the rainbow trout established as explants and maintained in various media. J. Fish Biol. *46*:1011-1025.

Muskens, P.J.W.M., and W.G.J. Hensgens (1977). Time series analysis on ammonia concentration and load values of the river Rhine. Water Res. *11*:509-515.

Mustafa, A., C. MacWilliams, N. Fernandez, K. Matchett, G.A. Conboy, and J.F. Burka (2000). Effects of sea lice (*Lepeophtheirus salmonis* Krøyer, 1837) infestation on macrophage functions in Atlantic salmon (*Salmo salar* L.). Fish & Shell. Immunol. *10*:47-59.

Nagae, M., H. Fuda, K. Ura, H. Kawamura, S. Adachi, A. Hara, and K. Yamauchi (1994). The effect of cortisol administration on blood plasma immunoglobulin M (IgM) concentrations in masu salmon (*Oncorhynchus masou*). Fish Physiol. Biochem. *13*:41-48.

Negishi, K., and S. Shinagawa (1993). Fibroblast growth factor induces proliferating cell nuclear antigen-immunoreactive cells in goldfish retina. Neurosci. Res. *18*:143-156.

Negoescu, A., P. Lorimer, F. Labat-Moleur, C. Drouet, C. Robert, C. Guillermet, C. Brambilla, and E. Brambilla (1996). In situ apoptotic cell labelling by the TUNEL method: improvement and evaluation on cell preparations. J. Histochem. Cytochem. *44*:959-968.

Negoescu, A., P. Lorimier, F. Labat-Moleur, L. Azoti, C. Robert, C. Guillermet, C. Brambilla, and E. Brambilla (1997). TUNEL: improvement and evaluation of the method for *in situ* apoptotic cell identification. Biochemica *2*:12-17.

Negoescu, A., C. Guillermet, P. Lorimier, E. Brambilla, and F. Labat-Moleur (1998). Importance of DNA fragmentation in apoptosis with regard to TUNEL specificity. Biomed Pharmacother *52*:252-258.

Nichols, J.W., J.M. McKim, G.J. Lein, A.D. Hoffman, S.L. Bertelsen, and C.M. Elonen (1996). A physiologically based toxicokinetics model for dermal absorption of organic chemicals by fish. Fundam. Appl. Toxicol. *31*:229-242.

Nienhuis, P.H., R.S.E.W. Leuven, and A.M.J. Ragas, eds. (1998) New Concepts for Sustainable Management of River Basins. Backhuys Publishers, Leiden.

Noga, E.J., J.F. Wright, J.F. Levine, M.J. Dykstra, and J.H. Hawkins (1991). Dermatological diseases affecting fishes of the Tar-Pamlico estuary, North Carolina (USA). Dis. Aquat. Org. *10*:87-92.

Nolan, D.T., and S.C. Johnson (2000) Interaction between crustacean ectoparasites and their hosts. In: Aquatic Invertebrate Cell Culture. B. Austin, and C. Mothersill, eds. Springer-Praxis, (accepted).

Nolan, D.T., R.H. Hadderingh, H.A. Jenner, and S.E. Wendelaar Bonga (1998) The effects of exposure to Rhine water on the sea trout smolt (*Salmo trutta trutta* L.): and ultrastructural and physiological study. In: New Concepts for the Sustainable Management of River Basins. P.H. Nienhuis, R.S.E.W. Leuven, and A.M.J. Ragas, eds. Backhuys Publishers, Amsterdam, pp. 261-271.

Nolan, D.T., R.L.J.M. Op 't Veld, P.H.M. Balm, and S.E. Wendelaar Bonga (1999a). Ambient salinity modulates the stress response of the tilapia, *Oreochromis mossambicus*, (Peters) to net confinement. Aquaculture *177*:297-309.

Nolan, D.T., A.L. van der Salm, and S.E. Wendelaar Bonga (1999b) *In vitro* effects of short-term cortisol exposure on proliferation and apoptosis in the skin epidermis of rainbow trout (*Oncorhynchus mykiss* Walbaum). In: Recent Developments in Comparative Endocrinology and Neurobiology. E. Roubos, S.E. Wendelaar Bonga, H. Vaudry, and A. De Loof, eds. Shaker Publishers, Maastricht, pp. 161-162.

Nolan, D.T., N.M. Ruane, Y. van der Heijden, E.S. Quabius, J. Costelloe, and S.E. Wendelaar Bonga (2000). Infection with juvenile life stages of *Lepeophtheirus salmonis* affects the skin and gill epithelia of rainbow trout *Oncorhynchus mykiss* (Walbaum) and the response to a handling procedure. Aqua. Res. (accepted).

Northcott, S.J., A.R. Lyndon, and A.D. Campbell (1997). An outbreak of freshwater fish lice, *Argulus foliaceus* L., seriously affecting a Scottish stillwater fishery. Fish. Man. Ecol. *4*:73-75.

Oda, S., H. Mitani, K. Naruse, and A. Shima (1991). Synthesis of heat shock proteins in the isolated fin of the medaka, *Oryzias latipes*, acclimatized to various temperatures. Comp. Biochem. Physiol. *98*:587-591.

O'Flaherty, G., R. Ryan, U. MacEvilly, W.R. Poole, O. Tully, and D.T. Nolan. (1999) Biochemical responses in relation to sea lice (*Lepeophtheirus salmonis* Krøyer) infestation in wild sea trout (*Salmo trutta*, L.) post smolts. 4th International Conference on Sea Lice, Trinity College Dublin. 28-30 June 1999.

Ortego, L.S., W.E. Hawkins, W.W. Walker, R.M. Krol, and W.H. Benson (1994). Detection of proliferating cell nuclear antigen in tissues of three small fish species. Biotech. Histochem. *69*:317-323.

Ostrander, G.K., J.B. Blair, B.A. Stark, G.M. Marley, W.D. Bales, R.W. Veltri, D.E. Hinton, M. Okihiro, L.S. Ortega, and W.E. Hawkins (1995). Long-term primary culture of epithelial cells from rainbow trout (*Oncorhynchus mykiss*) liver. In Vitro Cell. Dev. Biol. *31*:367-378.

Paperna, I., and D.E. Zwerner (1982). Host-parasite relationship of *Ergasilus labracis* Krøyer (Cyclopidea, Ergasilidae) and the striped bass, *Morone saxatilis* (Walbaum) from the lower Chesapeake Bay. Ann. Parasitol. (Paris) *57*:393-405.

Perry, S.F., and P. Laurent (1993) Environmental effects on fish gill structure and function. In: Fish Ecophysiology. J.C. Rankin, and F.B. Jensen, eds. Chapman & Hall, London, pp. 231-264.

Pickering, A.D. (1990). Stress and the suppression of somatic growth in teleost fish. Prog. Clin. Biol. Res. *342*:473-479.

Pickering, A.D., and J. Duston (1983). Administration of cortisol to brown trout, *Salmo trutta* L., and its effects on the susceptibility to *Saprolegnia* infection and furunculosis. J. Fish Biol. *23*:163.

Pickering, A.D., and D.J. Macey (1977). Structure, histochemistry and the effect of handling on the mucous cells of the epidermis of the char *Salvelinus alpinus* (L.). J. Fish Biol. *10*:505-512.

Pickering, A.D., and T.G. Pottinger (1989). Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. Fish Physiol. Biochem. *7*:253-258.

Pickering, A.D., and R.H. Richards (1980). Factors influencing the structure, function and biota of the salmonid epidermis. Proc. R. Soc. Edinburgh *79B*:93-104.

Pike, A.W. (1989). Sea lice- major pathogens of farmed Atlantic salmon. Parasitol. Today *5*:291-297.

Pike, A.W., and S.L. Wadsworth (1999). Sea lice on salmonids: their biology and control. Adv. Parasitol. *44*:233-337.

Pilgaard, L., H. Malte, and F.B. Jensen (1994). Physiological effects and tissue accumulation of copper in freshwater rainbow trout (*Oncorhynchus mykiss*) under normoxic and hypoxic conditions. Aquat. Toxicol. *29*:197-212.

Poels, C.L.M., M.A. van der Graag, and J.F.J. van der Kerkhoff (1980). An investigation into the long-term effects of Rhine water on rainbow trout. Water Res. *14*:1029-1035.

Poly, W.J. (1998). New state, host, and distribution records of the fish ectoparasite, *Argulus* (Branchiura), from Illinois (USA). Crustaceana *71*:1-8.

Poole, W.R., D.T. Nolan, and O. Tully (2000). Extrapolating blood baseline cortisol levels in trout: modelling capture effects in wild sea trout *Salmo trutta* (L.) infested with *Lepeophtheirus salmonis* (Krøyer). Aqua. Res. (accepted).

Postlethwaite, E.K., and D.G. McDonald (1995). Mechanisms of Na^+ and Cl^- regulation in freshwater-adapted rainbow trout (*Oncorhynchus mykiss*) during exercise and stress. J. Exp. Biol. *198*:295-304.

Pottinger, T.G., and T.A. Moran (1993). Differences in plasma cortisol and cortisone dynamics during stress in two strains of rainbow trout (*Oncorhynchus mykiss*). J. Fish Biol. *43*:121-130.

Pottinger, T.G., and A.D. Pickering (1985). The effects of 11-ketotestosterone and testosterone on the skin structure of brown trout, *Salmo trutta* L. Gen. Comp. Endocrinol. *59*:335-342.

Pottinger, T.G., A.D. Pickering, and N. Blackstock (1984). Ectoparasite induced changes in epidermal mucification of the brown trout, *Salmo trutta* L. J. Fish Biol. *25*:123-128.

Quabius, E.S., P.H.M. Balm, and S.E. Wendelaar Bonga (1997). Interrenal stress responsiveness of tilapia (*Oreochromis mossambicus*) is impaired by dietary exposure to PCB 126. Gen. Comp. Endocrinol. *108*:472-482.

Rahman, M.M. (1995). Some aspects of the biology of a freshwater fish parasite, *Argulus foliaceus* (L.) (Argulidae, Branchiura, Crustacea). Bangladesh Journal of Zoology *23*:77-86.

Rahman, M.M. (1996). Effects of a freshwater fish parasite, *Argulus foliaceus* Linn. infection on common carp, *Cyprinus carpio*. Bangladesh Journal of Zoology *24*:57-63.

Rainger, G.E., and A.F. Rowley (1993). Antibacterial activity in the serum and mucus of rainbow trout, *Oncorhynchus mykiss*, following immunisation with *Aeromonas salmonicida*. Fish Shellf. Immunol. *3*:475-482.

Randall, D.J., and S.F. Perry (1992) Catecholamines. In: Fish Physiology. W.S. Hoar, D.J. Randall, and A.P. Farrell, eds. Academic Press, San Diego, CA, pp. 255-300.

Rao, J.K.S., P. Letada, D.M. Haverstick, M.M. Herman, and J. Savory (1998). Modifications to the *in situ* TUNEL method for detection of apoptosis in paraffin-embedded tissue sections. Ann. Clin. Lab. Sci. *28*:131-137.

Reddy, P.K., M.M. Vijayan, J.F. Leatherland, and T.W. Moon (1995). Does RU486 modify hormonal responses to handling stressor and cortisol treatment in fed and fasted rainbow trout? J. Fish Biol. *46*:341-359.

Reid, S.D., D.G. McDonald, and C.M. Wood (1997) Interactive effects of temperature and pollutant stress. In: Global Warming: Implications for Freshwater and Marine Fish. C.M. Wood, and D.G. McDonald, eds. Society of Experimental Biology Seminar Series 61, Cambridge, pp. 325-349.

Ricard, A.C., C. Daniel, P. Anderson, and A. Hontela (1998). Effects of subchronic exposure to cadmium chloride on endocrine and metabolic functions in rainbow trout *Oncorhynchus mykiss*. Arch. Environ. Contam. Toxicol. *34*:377-381.

Ritchie, G., A.J. Mordue, A.W. Pike, and G.H. Rae (1993) The reproductive output of *Lepeophtheirus salmonis* adult females in relation to temperature and photoperiod. In: Pathogens of Wild and Farmed Fish: Sea Lice. G.A. Boxshall, and D. Defaye, eds. Ellis Horwood Limited, London, pp. 153-165.

Roche, P. (1994). Habitat availability and carrying in the French part of the Rhine for Atlantic salmon (*Salmo salar* L.). Water Sci. Technol. *29*:257-265.

Rodger, H.D., T.M. Murphy, E.M. Drinan, and D.A. Rice (1991). Acute skeletal myopathy in farmed Atlantic salmon Salmo salar. Dis. Aquat. Org. *12*:17-23.

Ross, N.W., K.J. Firth, A. Wang, J.F. Burka, and S.C. Johnson (2000). Changes in hydrolytic enzyme activities of naïve Atlantic salmon (*Salmo salar*) skin mucus and serum due to infection with *Lepeophtheirus salmonis* and host immunosuppression. Dis. Aquat. Org. (in press).

Roubal, F.R., and A.M. Bullock (1988). The mechanism of wound repair in the skin of juvenile Atlantic salmon, *Salmo salar* L., following hydrocortisone implantation. J. Fish Biol. *32*:545-555.

Rougier, F., D. Troutaud, A. Ndoye, and P. Deschaux (1994). Non-specific immune response of zebrafish, *Brachydanio rerio* (Hamilton-Buchanan) following copper and zinc exposure. Fish Shellf. Immunol. *4*:115-127.

Ruane, N.M., D.T. Nolan, J. Rotllant, L. Tort, P.H.M. Balm, and S.E. Wendelaar Bonga (1999a). Modulation of the response of rainbow trout (*Oncorhynchus mykiss* Walbaum) to confinement, by an ectoparasitic (*Argulus foliaceus* L.) infestation and cortisol feeding. Fish Physiol. Biochem. *20*:43-51.

Ruane, N.M., S.E. Wendelaar Bonga, and P.H.M. Balm (1999b). Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. Gen. Comp. Endocrinol. *115*:210-219.

Ruane, N.M., D.T. Nolan, J. Rotllant, J. Costelloe, and S.E. Wendelaar Bonga (2000). Experimental exposure of rainbow trout *Oncorhynchus mykiss* (Walbaum) to the infective stages of the sea louse *Lepeophtheirus salmonis* (Krøyer) influences the physiological response to an acute stressor. Fish Shellf. Immunol. (Accepted).

Rushton-Mellor, S.K., and G.A. Boxshall (1994). The developmental sequence of *Argulus foliaceus* (Crustacea: Branchiura). Journal of Natural History *28*:763-785.

Sanders, B.M., J. Nguyen, L.S. Martin, S.R. Howe, and S. Coventry (1995). Induction and subcellular localization of two major stress proteins in response to copper in the fathead minnow *Pimephales promelas*. Comp. Biochem. Physiol. *112C*:335-343.

Sandor, T., J.A. DiBattista, and A.Z. Mehdi (1984). Glucocorticoid receptors in the gill tissue of fish. Gen. Comp. Endocrinol. *53*:353-364.

Satchell, G.H. (1991) Physiology and Form of Fish Circulation, 1st ed. Cambridge University Press, Cambridge.

Schmidt, G.D., and L.S. Roberts (1996) Parasitic crustaceans. In: Gerald D. Schmidt & Larry S. Roberts' Foundations of Parasitology, 5th ed. L.S. Roberts, and J. Janovy Jr., eds. Wm. C. Brown Publishers, Dubuque, IA, pp. 513-534.

Schmidt, H., D. Bernet, T. Wahli, W. Meier, and P. Burkhardt-Holm (1999). Active biomonitoring with brown trout and rainbow trout in diluted sewage plant effluents. J. Fish Biol. *54*:585-596.

Schulte-Wülwer-Leidig, A. (1994). Outline of the ecological master plan for the Rhine. Water Sci. Technol. *29*:273-280.

Secombes, C.J. (1994). Enhancement of fish phagocyte activity. Fish Shellf. Immunol. *4*:421-436.

Seidelin, M., S.S. Madsen, A. Byrialsen, and K. Kristiansen (1999). Effects of insulin-like growth factor-I and cortisol on Na^+,K^+-ATPase expression in osmoregulatory tissues of brown trout (*Salmo trutta*). Gen. Comp. Endocrinol. *113*:331-342.

Selye, H. (1973). The evolution of the stress concept. Amer. Sci. *61*:692-699.

Sen, S. (1992). Programmed cell death: concept, mechanism and control. Biol. Rev. *67*:287-319.

Shariff, M., and R. Roberts (1989). The experimental histopathology of *Lernaea polymorpha* Wu, 1938 infection in naive *Aristichthys nobilis* (Richardson) and a comparison with the lesion on naturally infected clinically resistant fish. J. Fish Dis. *12*:405-414.

Shephard, K.L. (1994). Functions for fish mucus. Rev. Fish Biol. Fish. *4*:401-429.

Shi, S.-R., M.E. Key, and K.L. Kalra (1991). Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemistry staining based on microwave oven heating of tissue sections. J. Histochem. Cytochem. *39*:741-748.

Shields, R.J., and R.P. Goode (1978). Host rejection of *Lernaea cyprinacea* L. (Copepoda). Crustaceana *35*:301-307.

Shrimpton, J.M., and D.J. Randall (1994). Downregulation of corticosteroid receptors in gills of coho salmon due to stress and cortisol treatment. Am. J. Physiol. *267*:R432-R438.

Sin, Y.M., K.H. Ling, and T.J. Lam (1996). Cell-mediated immune response of goldfish, *Carassius auratus* (L.), to *Ichthyophthirius multifiliis*. J. Fish Dis. *19*:1-7.

Singhal, R.N., S. Jeet, and R.W. Davies (1990). The effects of argulosis-saprolegniasis on the growth and production of *Cyprinus carpio*. Hydrobiol. *202*:27-32.

Sola, F., A. Masoni, and J. Isaia (1994). Effects of lead loads on branchial osmoregulatory mechanisms in the rainbow trout *Oncorhynchus mykiss*. J. Appl. Toxicol. *14*:343-349.

Soutschek, J., and G.K.H. Zupanc (1995). Apoptosis as a regulator of cell proliferation in the central posterior/prepacemaker nucleus of adult gymontioform fish, *Apteronotus leptorhynchus*. Neurosci. Lett. *202*:133-136.

Spigarelli, S.A., and M.M. Thommes (1979). Temperature selection and estimated thermal acclimation by rainbow trout (*Salmo gairdneri*) in a thermal plume. J. Fish. Res. Board Can. *36*:366-376.

Stave, J.W., and B.S. Roberson (1985). Hydrocortisone suppresses the chemiluminescent response of striped bass phagocytes. Dev. Comp. Immunol. *9*:77-84.

Stouthart, A.J.H.X., F.A.T. Spanings, R.A.C. Lock, and S.E. Wendelaar Bonga (1995). Effects of water pH on chromium toxicity to early life stages of the common carp (*Cyprinus carpio*). Aquat. Toxicol. *32*:31-42.

Sträter, J., A.R. Günthert, S. Brüderlein, and P. Möller (1995). Microwave irradiation of paraffin-embedded tissue sensitizes the TUNEL method for *in situ* detection of apoptotic cells. Histochem. *103*:157-160.

Sweet, L.I., D.R. Passino-Reader, P.G. Meier, and G.M. Omann (1998). Fish thymocyte viability, apoptosis and necrosis: *in vitro* effects of organochlorine contaminants. Fish Shellf. Immunol. *8*:77-90.

Tahir, A., T.C. Fletcher, D.F. Houlihan, and C.J. Secombes (1993). Effect of short-term exposure to oil-contaminated sediments on the immune response of dab, *Limanda limanda* (L.). Aquat. Toxicol. *27*:71-82.

Tan, C.-K., K. Sullivan, X. Li, E.M. Tan, K.M. Downey, and A.G. So (1987). Autoantibody to the proliferating cell nuclear antigen neutralizes the activity of the auxiliary protein for DNA polymerase delta. Nucleic Acids Res. *15*:9299-9308.

Teh, S.J., S.M. Adams, and D.E. Hinton (1997). Histopathologic biomarkers in feral freshwater fish populations exposed to different types of contaminant stress. Aquat. Toxicol. *37*:51-70.

Tong, S.-L., H. Li, and H.-Z. Miao (1997). The establishment and partial characterization of a continuous fish cell line FG-9307 from the gill of flounder *Paralichthys olivaceus*. Aquaculture *156*:327-333.

Tujague, M., D. Saligaut, C. Teitsma, O. Kah, Y. Valotaire, and B. Ducouret (1998). Rainbow trout glucocorticoid receptor overexpression in *Escherichia coli*: production of antibodies for Western blotting and immunohistochemistry. Gen. Comp. Endocrinol. *110*:201-211.

Tully, O., W.R. Poole, and K.F. Whelan (1993a). Infestation parameters for *Lepeophtheirus salmonis* (Krøyer) (Copepoda: Caligidae) parasitic on sea trout, *Salmo trutta* L., off the west coast of Ireland during 1990 and 1991. Aqua. Fish. Man. *24*:545-555.

Tully, O., W.R. Poole, K.F. Whelan, and S. Merigoux (1993b) Parameters and possible causes of epizootics of *Lepeophtheirus salmonis* (Krøyer) infesting sea trout (*Salmo trutta* L.) off the west coast of Ireland. In: Pathogens of Wild and Farmed Fish: Sea Lice. G.A. Boxshall, and D. Defaye, eds. Ellis Horwood Limited, London, pp. 202-213.

Turnbull, J.F., R.H. Richards, and D.A. Robertson (1996). Gross, histological and scanning electron microscopic appearance of dorsal fin rot in farmed Atlantic salmon, *Salmo salar* L., parr. J. Fish Dis. *19*:415-427.

Uchida, K., T. Kaneko, K. Yamauchi, and T. Hirano (1996). Morphometrical analysis of chloride cell activity in the gill filaments and lamellae and changes in Na^+,K^+-ATPase activity during seawater adaptation in chum salmon fry. J. Exp. Zool. *276*:193-200.

Van Dijk, G.M., and E.C.L. Marteijn, eds. (1993) Ecological Rehabilitation of the River Rhine, the Netherlands Research Summary Report (1988-1992). Report of the Project "Ecological Rehabilitation of the Rivers Rhine and Meuse", Report No. 50. RIZA/RIVM/RIVO-DLO/IBN-DLO/SC-DLO, The Netherlands.

Van Dijk, G.M., E.C.L. Marteijn, and A. Schulte-Wülwer-Leidig (1995). Ecological rehabilitation of the river Rhine: plans, progress and perspectives. Regul. Riv. Res. Man. *11*:377-388.

Van Muiswinkel, W.B. (1995) The piscine immune system: innate and acquired immunity. In: Fish Diseases and Disorders. P.T.K. Woo, ed. CAB International, Oxon, U.K., Vol. 1: Protozoan and Metazoan Infections, pp. 729-749.

Van Oostrom, J.A., and N.C. Bols (1991). Influence of temperature on the proliferative response of rainbow trout gonadal fibroblasts to cortisol and RU 486. Fish Physiol. Biochem. *9*:261-269.

Verbost, P.M., M.H.G. Berntssen, F. Kroglund, E. Lydersen, H.E. Witters, B.O. Rosseland, B. Salbu, and S.E. Wendelaar Bonga (1995). The toxic mixing zone of neutral and acidic river water: acute aluminium toxicity in the brown trout (*Salmo trutta* L.). Water Air Soil Pollut. *85*:341-346.

Vijayan, M.M., C. Pereira, and T.W. Moon (1994a). Hormonal stimulation of hepatocyte metabolism in rainbow trout following an acute handling stress. Comp. Biochem. Physiol. *108*:321-329.

Vijayan, M.M., P.K. Reddy, J.F. Leatherland, and T.W. Moon (1994b). The effects of cortisol on hepatocyte metabolism in rainbow trout: a study using the steroid analogue RU486. Gen. Comp. Endocrinol. *96*:75-84.

Watson, R.W.G., H.P. Redmond, J.H. Wang, C. Condron, and D. Bouchier-Hayes (1996). Neutrophils undergo apoptosis following ingestion of *Escherichia coli*. J. Immunol. *156*:3986-3992.

Webb, P.W., and H. Zhang (1994). The relationship between responsiveness and elusiveness of heat-shocked goldfish (*Carassius auratus*) to attacks by rainbow trout (*Oncorhynchus mykiss*). Can. J. Zool. *72*:423-426.

Wedemeyer, G.A. (1997) Effects of rearing conditions on the health and physiological quality of fish in intensive culture. In: Fish Stress and Health in Aquaculture. G.K. Iwama, A.D. Pickering, J.P. Sumpter, and C.B. Schreck, eds. Cambridge University Press, Cambridge, pp. 35-71.

Wendelaar Bonga, S.E. (1997). The stress response in fish. Physiol. Rev. *77*:591-625.

Wendelaar Bonga, S.E., and R.A.C. Lock (1992). Toxicants and osmoregulation in fish. Nether. J. Zool. *42*:478-493.

Wendelaar Bonga, S.E., and J.C.A. van der Meij (1989). Degeneration and death, by apoptosis and necrosis, of the pavement and chloride cells in the gills of the teleost *Oreochromis mossambicus*. Cell Tissue Res. *255*:235-243.

Wendelaar Bonga, S.E., G. Flik, P.H.M. Balm, and J.C.A. van der Meij (1990a). The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water. Cell Tissue Res. *259*:575-585.

Wendelaar Bonga, S.E., G. Flik, P.H.M. Balm, and J.C.A. van der Meij (1990b). The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water. Cell Tissue Res. *259*:575-585.

Weyts, F.A.A., B.M.L. Verburg-van Kemenade, G. Flik, J.G.D. Lambert, and S.E. Wendelaar Bonga (1997). Conservation of apoptosis as an immune regulatory mechanism: effects of cortisol and cortisone on carp lymphocytes. Brain Behav. Immun. *11*:95-105.

Weyts, F.A.A., G. Flik, J.H.W.M. Rombout, and B.M.L. Verburg-van Kemenade (1998a). Cortisol induces apoptosis in activated B cells, not in other lymphoid cells of the common carp, *Cyprinus carpio* L. Dev. Comp. Immunol. *22*:551-562.

Weyts, F.A.A., B.M.L. Verburg-van Kemenade, and G. Flik (1998b). Characterisation of glucocorticoid receptors in peripheral blood leucocytes of carp, *Cyprinus carpio* L. Gen. Comp. Endocrinol. *111*:1-8.

Whitear, M. (1986) The skin of fishes including cyclostomes - Epidermis. In: Biology of the Integument, Vol 2. J. Bereiter-Hahn, A.G. Matoltsy, and K.S. Richards, eds. Springer-Verlag, Berlin, Heidelberg, New York, Tokyo, pp. 8-38.

Whiteside, G., N. Cougon, S.P. Hunt, and R. Munglani (1998). An improved method for detection of apoptosis in tissue sections and cell culture, using the TUNEL technique combined with Hoechst stain. Brain Res. Protocols *2*:160-164.

Wong, S., M. Fournier, D. Coderre, W. Banska, and K. Krzystyniak (1992) 8. Environmental immunotoxicology. In: Animal Markers As Pollution Indicators. D. Peakall, ed. Chapman & Hall, London, pp. 167-189.

Woo, P.T.K., and M. Shariff (1990). *Lernaea cyprinacea* L. (Copepoda: Caligidea) in *Helostoma temminicki* Cuvier & Valenciennes: the dynamics of resistance in recovered and naive fish. J. Fish Dis. *13*:485-493.

Wootten, R., J.W. Smith, and E.A. Needham (1982). Aspects of the biology of the parasitic copepods *Lepeophtheirus salmonis* and *Caligus elongatus* on farmed salmonids, and their treatment. Proc. R. Soc. Edinburgh *81B*:185-197.

Samenvatting (Summary in Dutch)

In dit proefschrift worden de veranderingen bestudeerd in de huid van vissen als gevolg van blootstelling aan stressoren. Dit zijn prikkels uit de omgeving die het fysiologisch evenwicht van dieren verstoren of dreigen te verstoren en waarop deze reageren met een stress-respons: het geheel van reacties bedoeld om de nadelige gevolgen van stressoren te verminderen en zo mogelijk te herstellen of ervoor te compenseren. In dit proefschrift zijn de resultaten vermeld van een onderzoek naar een giftige stressor (blootstelling van vissen aan Rijnwater) en niet-giftige stressoren (blootstelling van vissen aan een plotselinge temperatuurverhoging van het Rijnwater en aan parasieten). De belangrijkste vindingen zijn:

I. Blootstelling van vissen aan water uit de benedenloop van de Rijn veroorzaakt een stressrespons die zich, wat de huid betreft, manifesteert door een toenemende sterfte van cellen door apoptose (de zogenaamd fysiologisch gereguleerde vorm van celdood) en door necrosis (celdood door directe en dodelijke beschadiging van cellen). Ook worden er in de bovenste cellagen van de opperhuid een groot aantal secretieblaasjes gevormd die waarschijnlijk enzymen en andere stoffen bevatten die aan de slijmlaag van de huid worden afgegeven. Deze stoffen hebben waarschijnlijk een beschermende werking tegen micro-organismen die de aangetaste huid kunnen binnendringen. Ook het verschijnen van witte bloedcellen in het huidepitheel wijst op het binnendringen van dergelijke organismen en van lichaamsvreemde stoffen. Langdurige blootstelling van vissen aan Rijnwater resulteert in gedeeltelijk herstel van de huid, al gaat dit gepaard met een verminderde groei van de dieren. Het water van de Rijn is al meer dan een eeuw door toedoen van de mens vervuild met allerlei giftige en niet-giftige stoffen. Daardoor zijn de populaties van veel vissoorten sterk teruggelopen dan wel geheel uitgeroeid. Dit geldt met name voor zalmachtige vissen omdat deze dieren veelal hoge eisen stellen aan de kwaliteit van het rivierwater. Hoewel de kwaliteit van het Rijnwater in de afgelopen jaren flink is verbeterd blijkt uit de in dit proefschrift gepresenteerde resultaten dat de kwaliteit nog onvoldoende is vooruitgegaan om een terugkeer van zalmachtigen te mogen verwachten. Dit geldt in elk geval voor de benedenloop van de Rijn, waar de experimenten zijn gedaan en die de trekkende zalmachtigen zoals zalm en forel moeten passeren op weg naar hun broedgebieden dan wel op de terugweg naar zee.

II. De kwaliteit van het Rijnwater wordt niet alleen aangetast door de aanwezigheid van allerlei stoffen maar ook door verhoging van de temperatuur als gevolg van de lozing van opgewarmd koelwater afkomstig van bijvoorbeeld elektriciteitscentrales. Uit dit onderzoek blijkt dat de gevolgen van blootstelling aan Rijnwater nog aanzienlijk ernstiger zijn wanneer de temperatuur

van het water plotseling wordt verhoogd. Deze proefopzet is te vergelijken met het passeren van een vis van een koelwateruitlaat in de Rijn. Terwijl de vissen zich binnen enkele weken herstellen van de gevolgen van blootstelling aan Rijnwater, is dit niet het geval bij de gecombineerde blootstelling aan Rijnwater en een verhoogde watertemperatuur, ook al duurde de temperatuur-verhoging maar kort. De resultaten van dit onderzoek laten niet alleen de kwalijke gevolgen zien van de huidige waterkwaliteit op vissen, maar laten ook zien dat de effecten van toxische stressoren (Rijnwater) en niet-toxische stressoren (temperatuurverhoging) in veel opzichten vergelijkbaar zijn en bovendien dat deze effecten optelbaar zijn.

III. Vervolgens is nagegaan of ook infectie van vissen met parasieten een stressrespons kan veroorzaken. In dit deel van het onderzoek zijn de effecten van een infectie met zalmluizen bestudeerd bij de Atlantische zalm. De zalmluis hecht zich op een bepaalde plaats van de huid en eet dan de bovenlaag van de huid. De effecten van de zalmluisinfectie zijn bestudeerd in die gebieden van de huid waar deze parasieten juist niet aanwezig waren, om na te gaan of de zalmluizen soortgelijke veranderingen in de huid veroorzaken als blootstelling aan Rijnwater en verhoging van de watertemperatuur. Dit blijkt het geval te zijn, wat een verdere aanwijzing is dat de waargenomen effecten geen specifieke reacties zijn op Rijnwater of op temperatuurverhoging, maar algemene reacties op een stressor. De resultaten van dit onderzoek geven aan dat het heel goed mogelijk is dat de ernstige teruggang van de zalm- en forelpopulaties in bijvoorbeeld Ierland en Schotland, waar de zalmluis zich de laatste tien jaren sterk heeft uitgebreid als gevolg van de toename van de zalmteelt in de kustgebieden, (mede) wordt veroorzaakt door deze parasiet.

IV. In volgende experimenten is nagegaan welke rol het hormoon cortisol, dat een belangrijk stresshormoon is bij vissen, speelt bij het totstandkomen van de veranderingen in de huid van vissen als reactie op stressoren. Daartoe is cortisol toegediend aan forellen. Een deel van deze met cortisol behandelde dieren zijn daarna geïnfecteerd met zalmluizen. Toediening van cortisol blijkt vooral de synthese te stimuleren van de secretieblaasjes in de bovenste huidlagen van vissen en bij geïnfecteerde dieren gaat dit samen met een afname van het aantal parasieten op de dieren. Het lijkt er dus op dat cortisol, dat als reactie op infectie met luizen in verhoogde mate wordt afgegeven aan het bloed, de vissen enige bescherming kan geven tegen deze parasieten.

V. Vervolgens zijn stukjes huid in kweek gebracht en met deze methode is aangetoond dat cortisol een rol speelt zowel bij het induceren van apoptose (via binding van het hormoon aan de glucocorticoid receptor) als bij het stimuleren en reguleren van de slijmafscheiding door de huid (in dit laatste geval mogelijk via binding aan een mineralocorticoidreceptor).

VI. Tot slot is een primaire huidkweek beschreven die goede vooruitzichten lijkt te bieden voor het testen van de effecten van hormonen en andere stoffen, zoals bijvoorbeeld toxische metalen.

Acknowledgements (dankwoord)

There are very many people whose contributions are reflected in this thesis. Firstly, I thank my promotor, Sjoerd Wendelaar Bonga for inviting me to Nijmegen **for a couple of weeks** at the end of 1993. The contents of this thesis result from his enthusiasm and continual encouragement (and creativity!!) over this period. He gave me my first opportunity here and I am grateful for this, but equally grateful to him for not stopping me from grasping every other opportunity that presented itself during this period. This has enabled me to gain considerable experience with arranging transnational student placements, international meetings and training courses, grant and report writing, as well as building links with, traveling to and working with collaborators abroad during this period. These skills are essential to continue to work in the scientific community.

Secondly, my thanks also go to all of my many colleagues with whom I have shared the trials and tribulations of doing a PhD (or alternatively, who have had to share the trials and tribulations of my PhD!). This includes my so-called "**intra**departmental" colleagues in the Organismal Physiology workgroup, my "**inter**departmental" colleagues from the other 2 workgroups on the 3rd and 4th floors, and my many "**extra**departmental colleagues" throughout the university (and especially those in the Gemeenschappelijk Instrumentarium, incl. Electron Microscopie). It would be unfair to even attempt to list names, as I am afraid to leave somebody out! Suffice to say that I really appreciated all of the support, help and advice that I received from everyone.

I have had 14 STAGE/ERASMUS students working with me, 5 practicum students, and several other categories of co-workers during this time. While the majority of these were Dutch students, there were also visitors from Ireland and Spain. I would like to thank them for their work and hope that they benefited from their contact with the department. It gives me tremendous pleasure to see them complete their placements and take on new challenges, in research and beyond. I wish each of them success and happiness in their future.

My thanks to the members of the manuscript committee who evaluated this thesis and gave their comments and criticisms. These helped me greatly at a time where I was becoming blind to the contents. I am also pleased to say thank you to the people in the universities and institutes where I have collaborated and worked. These include: Aquaculture Development Centre, Cork, Ireland (Prof. M. Mulchay & Dr. R. Fitzgerald); Salmon Research Agency, Newport, Ireland (Drs. K. Whelan & R. Poole); Trinity College Dublin, Ireland (Dr. O. Tully); KEMA, Arnhem, The Netherlands (Drs. H. Jenner & R. Hadderingh); Dublin Institute of Technology, Ireland (Dr. C. Mothersill); Federal University of São Carlos, São Paulo, Brazil (Professor M. Fernandes and her group). In addition, although I have not worked directly with Dr. Stewart Johnson (Institute for Marine Biosciences, National Research Council, Canada), I would like to extend my special thanks to him for his time, helpful discussions and perspectives on the relationship between fish lice and their hosts. This exchange resulted is a substantial book chapter on the subject which is now with the publisher.

En ten slotte Maia, mijn meest belangrijke ontdekking op mijn werk. Há bichos do ar, bichos da terra e bichos do mar. Há até mesmo bichos tão pequenos que não se vêem. Amo tudos esses bichos mais seja o que for, amarei você mais do que tudo. Eu odiaria imaginar minha vida sem você. Obrigado meu amor.

Curriculum Vitae

Declan Thomas Nolan werd geboren te Portlaoise, Ierland op 31 mei 1965. Hij behaalde zijn middelbare school diploma in 1982 aan het Saint Benildus College te Kilmacud, Dublin. Daarna werkte hij als chef in een groothandel en studeerde marketing en 'retail management' voor de Dublin Chamber of Commerce aan het College of Marketing and Design te Dublin. In 1989 begon hij aan de studie Biologie aan het University College Dublin. In 1993 studeerde hij af (hoofdvak zoölogie/experimentele dierkunde) en begon in september van dat jaar in Nijmegen op de afdeling Organismale Dierfysiologie, K.U.N. met een beurs als onderzoekassistent. Na 2 jaar trad hij in dienst als assistant in opleiding. Het onderzoek beschreven in dit proefschrift werd verricht onder leiding van Prof. Dr. S.E. Wendelaar Bonga.

Declan Thomas Nolan was born in Portlaoise, Ireland on 31st May 1965. He completed his secondary school education with his leaving certificate examinations in 1982. Thereafter, he worked in retailing and studied Marketing and Retail Distribution Management for the Dublin Chamber of Commerce at the College of Marketing and Design. In 1989, he was accepted to study biology at University College Dublin and graduated with a 1st class honours Bachelor of Science degree in 1993. In September of that year, he moved to Nijmegen to the Organismal Physiology group of the Department of Animal Physiology, K.U.N. for 2 years as a research assistant, after which he entered into a PhD programme. The work presented in this thesis was supervised and guided by Prof. S.E. Wendelaar Bonga during this period.

Publications

Wendelaar Bonga, S.E., N. Bury, **D. Nolan**, and L. Jie (1997) Increased cell death by apoptosis during stress in gills and peripheral lymphocytes in fish is probably mediated by cortisol. In: Proceedings of the XIII International Congress of Comparative Endocrinology, Yokohama, Japan, pp. 1789-1792.

Nolan, D.T., R.H. Hadderingh, H.A. Jenner, and S.E. Wendelaar Bonga (1998) The effects of exposure to Rhine water on the sea trout smolt (*Salmo trutta trutta* L.): and ultrastructural and physiological study. In: New Concepts for the Sustainable Management of River Basins. P.H. Nienhuis, R.S.E.W. Leuven, and A.M.J. Ragas, eds. Backhuys Publishers, Amsterdam, pp. 261-271.

Quabius, E.S., **D.T. Nolan**, P.H.M. Balm, and S.E. Wendelaar Bonga (1998). The influence of polychlorinated biphenyl 126 on tilapia (*Oreochromis mossambicus*) liver. Comp. Biochem. Physiol. *120A*:57-63.

Nolan, D.T., R.L.J.M. Op 't Veld, P.H.M. Balm, and S.E. Wendelaar Bonga (1999). Ambient salinity modulates the stress response of the tilapia, *Oreochromis mossambicus*, (Peters) to net confinement. Aquaculture *177*:297-309.

Nolan, D.T., P. Reilly, and S.E. Wendelaar Bonga (1999). Infection with low numbers of the sea louse *Lepeophtheirus salmonis* (Krøyer) induces stress-related effects in post-smolt Atlantic salmon (*Salmo salar* L.). Can. J. Fish. Aquat. Sci. *56*:947-959.

Ruane, N.M., **D.T. Nolan**, J. Rotllant, L. Tort, P.H.M. Balm, and S.E. Wendelaar Bonga (1999). Modulation of the response of rainbow trout (*Oncorhynchus mykiss* Walbaum) to confinement, by an ectoparasitic (*Argulus foliaceus* L.) infestation and cortisol feeding. Fish Physiol. Biochem. *20*:43-51.

Nolan, D.T., A.L. van der Salm, and S.E. Wendelaar Bonga (1999) *In vitro* effects of short-term cortisol exposure on proliferation and apoptosis in the skin epidermis of rainbow trout (*Oncorhynchus mykiss* Walbaum). In: Recent Developments in Comparative Endocrinology and Neurobiology. E. Roubos, S.E. Wendelaar Bonga, H. Vaudry, and A. De Loof, eds. Shaker Publishers, Maastricht, pp. 161-162.

Ruane, N.M., **D.T. Nolan**, J. Rotllant, J. Costelloe, and S.E. Wendelaar Bonga (2000). Experimental exposure of rainbow trout *Oncorhynchus mykiss* (Walbaum) to the infective stages of the sea louse Lepeophtheirus salmonis (Krøyer) influences the physiological response to an acute stressor. Fish Shellf. Immunol. (a*ccepted).*

Nolan, D.T., I. Nabben, L. Jie, and S.E. Wendelaar Bonga (2000). Primary cell culture of rainbow trout skin explants: growth, cell composition, proliferation and apoptosis. (*submitted).*

Quabius, E.S., **D.T. Nolan**, H. Segner, and S.E. Wendelaar Bonga (2000). Confinement stress and starvation modulate the induction of EROD activity after dietary exposure to PCB 126 in the Mozambique tilapia (*Oreochromis mossambicus*). (*submitted*).

Nolan, D.T., N.M. Ruane, Y. van der Heijden, E.S. Quabius, J. Costelloe, and S.E. Wendelaar Bonga (2000). Infection with juvenile life stages of Lepeophtheirus salmonis affects the skin and gill epithelia of rainbow trout *Oncorhynchus mykiss* (Walbaum) and the response to a handling procedure. Aqua. Res. *(accepted).*

Nolan, D.T., F.A.T. Spanings, N.M. Ruane, R.H. Hadderingh, H.A. Jenner, and S.E. Wendelaar Bonga (2000). Exposure to water from the lower Rhine induces a stress response in the rainbow trout (*Oncorhynchus mykiss* Walbaum). (*submitted*).

Nolan, D.T., A.L. van der Salm, and S.E. Wendelaar Bonga (1999). The host-parasite relationship between the rainbow trout (*Oncorhynchus mykiss* Walbaum) and the ectoparasite *Argulus foliaceus* L. (Crustacea: Branchiura): epithelial mucous cell response, cortisol and factors which may influence parasite establishment. Contrib. Zool. (*in press*).

Van der Salm, A., **D.T. Nolan**, F.A.T. Spanings, and S.E. Wendelaar Bonga (2000). Effects of infection with the ectoparasite *Argulus japonicus* (Thiele) and administration of cortisol on cellular proliferation and apoptosis in the epidermis of common carp *Cyprinus carpio* (L.) skin. J. Fish Dis. (a*ccepted*).

Poole, W.R., **D.T. Nolan**, and O. Tully (2000). Extrapolating blood baseline cortisol levels in trout: modelling capture effects in wild sea trout *Salmo trutta* (L.) infested with *Lepeophtheirus salmonis* (Krøyer). Aqua. Res. (*in press*).

Nolan, D.T., and S.C. Johnson (2000) Interaction between crustacean ectoparasites and their hosts. In: Aquatic Invertebrate Cell Culture. B. Austin, and C. Mothersill, eds. Springer-Praxis (*accepted*).

Tully, O., W.R. Poole, M. Dillane, K.F. Whelan, and **D.T. Nolan** (2000). Sea lice infestation and osmoregulation of sea trout smolts along an estuarine salinity gradient. (*submitted*).

Quabius, E.S., **D.T. Nolan**, C.J. Allin, and S.E. Wendelaar Bonga (2000). The influence of dietary exposure to PCB 126 and of nutritional state on the stress response of tilapia (*Oreochromis mossambicus*) and rainbow trout (*Oncorhynchus mykiss*). (*submitted*).

Nolan, D.T., R.H. Hadderingh, F.A.T. Spanings, H.A. Jenner, and S.E. Wendelaar Bonga (2000). Effects of short-term acute temperature elevation on sea trout smolts (*Salmo trutta* L.) in tap water and in Rhine water: effects on skin and gill epithelia, hydromineral balance and gill specific Na^+/K^+-ATPase activity. Can. J. Fish. Aquat. Sci. (*in press*).