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NCMR
NATIONAL CENTRE FOR MARINE RESEARCH

Aghios Kosmas, 166 04 Hellenikon, Athens, Greece, Tel.: +30 1 9821354, Fax: +30 1 9811713

INSTITUTE OF MARINE BIOLOGICAL RESOURCES

SHORT REPORT

(4.1.1999 - 3.5.2002)

PROJECT TITLE:

" FISCI: A new bio-index for the assessment of stress condition in cultured marine fish "

CONTRACT N°: FAIR-PL98-4217

PROJECT STARTING DATE: 4.1.1999 - DURATION: 40 MONTHS

Partners

National Centre for Marine Research, (NCMR)

The Hebrew University of Jerusalem (HUJ)

Katholic University of Nijmegen (KUN)

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INTRODUCTION

Objectives

The **overall objective** of the project is to develop a non-invasive method for detecting general and specific stressor-related stress effects in fish reared in aquaculture. Within the general research framework the project aims at **two interrelated objectives**:

1. To detect, analyse and quantify stress effects, under both laboratory and fish farm conditions, in two Mediterranean marine fish species, gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*), which are of major economic importance for European aquaculture, using as parameters changes in the skin and gill epithelia, and the mucus layer covering the skin: (a) changes in tissue morphology (b) enzymatic activity in skin and skin mucus (c) concentrations of the stress hormone cortisol in blood serum and skin mucus and (d) non-enzymatic biochemical parameters in skin mucus samples. The stressors applied (low dissolved oxygen levels, high ammonia levels, and handling stress) are relevant to aquaculture.

2. To select subsequently the analytical methods and stress parameters that will prove to be reliable and easy to perform, using a sampling procedure from which the sampled fish can easily recover. The selected methods will be combined as bio-indicators through the establishment of a new stress-index, the «Fish Stress Condition Index » (FISCI), that will enable the characterisation of stress condition in cultured fish and will reflect both fish health and water quality This will provide fish farmers with a protocol method to check the stress condition of their fish.

Work carried out

During the project the work carried out concerned:

Preliminary experimentation on cortisol transfer to the skin

Exposure of fish to different stressors under laboratory conditions. The stressors applied were:

- Increased NH_3 levels for sea bass and sea bream at high and low temperatures
- Reduced O_2 levels for sea bass and sea bream at high temperatures
- Combination of increased NH_3 and reduced O_2 levels for sea bass and sea bream at high temperatures.
- Handling and confinement stress for sea bass and sea bream at high and low temperatures

Exposure of fish to different stressors under aquaculture conditions. The stressors applied were:

- Vaccination procedure at low temperatures in the sea bass
- Sorting procedure at low temperatures in the sea bream,
- Low oxygen conditions at high temperatures in the sea bream

Analyses of samples obtained from the above experiments

- Serum composition i.e. cortisol, ion, osmolarity, hematocrit, leucocrit, haemoglobin, lactate, glucose and free fatty acids.
- Mucous cortisol
- Skin LM analysis i.e.epidermis thickness, number and/or area, size and shape of mucous cells, melanosome dispersion and morphometric analysis.
- Gill LM analysis, i.e. lamellar fusion, epithelial lifting, mucous cells, chloride cells, leucocytes infiltration and morphometric analysis
- Skin EM analysis, i.e. ultrastructural changes of dermis chromatophores, basement membrane, pavement cells, epidermis filament cells

- Gill EM analysis i.e. apoptosis, necrosis.
- Immunohistochemistry

Monitoring of water quality of fish farms

- Arrangements were made with the farms participating in the project for monitoring the O_2 and NH_3 content in certain cages together with values of T and pH during summer 2000 and summer 2001.

RESULTS AND DISCUSSION

EFFECTS OF STRESS ON PLASMA COMPOSITION

NH_3 exposure

NH_3 addition seems to be stressful to sea bass at high levels as it was indicated by the high plasma cortisol compared to the controls for several sampling points during the stressor exposure. This does not appear however to have an effect on the osmotic balance of fish. No systematic change in plasma glucose and lactate could be seen. In sea bream subjected to similar levels of NH_3 at high T, plasma glucose did not appear, as well, to follow a systematic trend and no significant differences were apparent for lactate at all sampling points during treatment. Effects at low temperatures are of similar or of smaller magnitude; glucose changes in sea bass are not consistent and a small increase is observed for some hrs after exposing fish to NH_3 for sea bream, while lactate indicates decreased values compared to the controls some time after exposure, with no significant differences for most of the cases. Osmolarity does not appear also to be affected. The same holds for free fatty acid levels for both low and high temperature experiments. Also the changes of other plasma parameters appear to be small.
The mean 96-h LC50 value for ammonia has been reported to be 23.7 mg/l TAN for fish of 0.4-3.2g in weight (Wajsbrot et al., 1991). For larger sea bream of 135g a threshold of 30.8 mg/g (2.2 mmol/l) has been reported (Person le Ruyet et al., 1998) and similar values of 2-2.7mmol/l have been also reported for 32 gr sea bream and 29-32gr sea bass. (Person le Ruyet et al., 1995). The values used in this work were lower than the threshold values for fish of similar size. The moderate responses observed for the plasma ion, glucose and lactate levels, which are considered classical stress parameters, are in line with the conclusions that the stress levels induced were moderate. These experiments indicate that plasma cortisol level is a more sensitive stress marker than plasma osmolarity or plasma ion levels and glucose or lactate concentrations.

Low oxygen exposure

Low oxygen exposure resulted also in increased plasma cortisol values for sea bass, which returned to normal quite fast. The effect on plasma osmolarity was very small with a significant increase in the value measured only for the low O_2 group 24h after the stress application. Results on osmolarity indicate a small effect of the reduction of oxygen levels on this parameter and only for the low O_2 group. It has to be noted that despite the fact that a decrease of O_2 level to 1ppm, occurred in the low O_2 group about 24 h after the initiation of the experiment, the osmolarity values at this point were indistinguishable than those of the corresponding control. Plasma glucose levels and lactate were however affected for both species but only for the low O_2 group, since significant increases for both parameters were observed for certain sampling points during the exposure to the stressor. FFA levels seemed also to be affected in sea bream since a number of values of the low O_2 groups decreased relative to the controls during the exposure period.

In most fish species hypoxia or anoxia results in a mobilization of hepatic and muscular glycogen resulting in hyperglycemia. Activation of anaerobic metabolism also occurs resulting in increased levels of lactic acid (Vanden Thillart and Van Raaij, 1995). The

increases in the values of both glucose and lactate observed in Sea bream and Sea bass indicate that these fish conform to the general pattern of response to reduced O_2 levels. A level of O_2 of 4ppm does not appear to be adequate for a response of this type since increased values are observed at the lower level of 2 ppm.

There is evidence that catecholamines released during the stress response of fish are involved in the mobilization of FFAs which are important energy substrates. Reports on the effect of stress on FFA are contradictory since both increases and decreases have been found (Wendelaar Bonga, 1997). The results of the present work indicate reduction of FFA with hypoxia in sea bream. . Also from these experiments it appeared that plasma cortisol levels are more sensitive to the stressor than the other parameters, while, on the other hand, plasma glucose and lactate levels are more sensitive than osmolarity and ion levels.

Exposure to high NH_3 and low O_2

The application of combined levels of high NH_3 and low O_2 was performed at milder conditions than those used in the previous studies. These conditions are possible to be found in aquaculture units and furthermore the effect of both stressor applied at the same time do not appear to be simply additive since the toxic effect of ammonia has been found to decrease at low O_2 levels (Wajsbrot et al., 1991). Plasma values of metabolites (glucose – lactate) as well as osmolarity and plasma ion levels do not appear to be affected by the treatment. In sea bass plasma cortisol levels confirming that cortisol levels are the most sensitive plasma parameter were higher in all experimental groups, with a tendency for recovery after the exposure period, in the high nitrogen groups. This was also reflected in the high oxygen high nitrogen group for the mucus cortisol levels.

Exposure to handling stress

Plasma cortisol levels were generally increased in both sea bass and sea bream stressed by handling at high temperatures. However due to a fluctuation of the values statistically significant differences among stressed group and respective controls were obvious only in few cases. Osmolarity values indicated significant increases of a short period after the application of the stressor (up to 4h), for both sea bass and sea bream at high temperatures mainly for the air exposed groups (the same being apparent for the Na^+ values measured for both species at high temperatures).

Osmolarity increases in these 4-7 hrs after the stress application values were also apparent at low temperatures but statistically significant were only for sea bream. Significant increases in both glucose and lactate levels were also apparent, for both species and temperatures, for some period after the application of the stress mainly for the air exposed group. No clear effect on the FFA levels was obvious. So in general no consistent effects were observed for FFA.

EFFECTS OF STRESS ON MUCUS COMPOSITION

Mucus cortisol levels appear to be a valuable index of the stress condition some time after the stress application. The values measured indicate a clear response at 0.5 hr after the application of the stress in ammonia exposure of sea bass and at 10 hrs in low O_2 exposure of the same fish at high temperature. This response is observed at the highest stressor level. In handling experiments at high temperature the high mucus cortisol values of the air exposed groups persist for a longer period. This is about 7 hrs for both air exposed sea bass and sea bream, while the weighing procedure gives a statistically significant increase in the values only for sea bass up to 1.5 hr after the stress application. This indicates that mucus cortisol can be used as a stress indicator for more acute stress, and for a slightly longer time than plasma cortisol levels, after the stress application. A great advantage of mucus cortisol levels above plasma cortisol levels is the fact that the former are much less affected by the capture

and sampling prodecures than the latter, and thus give a better reflection of the effect of the stressor.

Other compounds that have been tried to be included as stress indices were peroxidase and haemoglobin. The first one did not give reliable results and the second one could not be applied due to the very low quantities of Hb excreted in the mucus. As appeared from the experiments in the first year, the biochemical mucus peroxidase assay does not give very reproducible results since the enzyme is less resistant to storage and extraction from sticky mucus matrix is more difficult than cortisol. Haemoglobin is present in too small quantities to be detected reliably. The last results further show that an increase in peroxidase and haemoglobin concentrations require at least moderate to high stress levels, in contrast to mucus cortisol levels. So the sensitivity of the peroxidase and haemoglobin assays is also less than that of cortisol in the mucus. A further complication of the peroxidase assay was the "over the hill effect", as appeared during the validation of this method in the laboratory: the concentration of peroxidase in the mucus increased with the intensity of stress, but only during mild and moderate stress levels. At high stress levels the concentration decreased. This was attributed to the damage caused, during high stress, to the upper layers of the skin epithelium: these cells, which produce the peroxidase, show apoptiosis and necrosis, and sometimes disappear completely.

MICROSCOPICAL EM STUDIES

EM studies in skin and gills indicated a number of changes that were obvious at both moderate and low levels of stress. These changes span all layers of skin and gills and seem to persist for all the time of stress application as well as for a significant time during the recovery period. Thus, these parameters seem to be sensitive stress parameters and stressor non-specific, which is a prerequisite for a useful stress indicator.

Skin EM

Stress effects in skin tissue are easily distinguishable using EM. The basement membrane between dermis and epidermis is one of the "stress sites", where structural changes indicate that the skin is reacting to adverse conditions. The two other "stress sites" in the skin where adverse conditions are swiftly signalized by cytological alterations are at the chromatophore level of the dermis and the pavement cell level of the epidermis. The cytological changes in the filament cells of the epidermis appear afterwards. Changes which occur and can be used for determining stress effects are:

- *Dispersion of melanosomes* into the dendritic processes of melanocytes surrounding iridophores. Linear formation of iridocytes together with melanosomes and creation of a light barrier along the inner part of the dermis (more pronounced in sea bream). This results to a darkening of fish skin. This change is very fast and might occur as a response to handling stress during sampling of fish. However since change in skin color is the first step in the visible stress reaction, darkening of skin could be one of the FISCI indices to enable aquaculturist to recognize that stress has occurred in the aquaculture unit. This procedure was much more intense in sea bream than sea bass.
- *Aggregates between groups of xanthophores with melanophores*. these occur at the distal region of the dermis, near the epidermis.
- *Changes in the basement membrane*. The appearance of the basement membrane can give an indication of the severity of stress application. At the beginning the pinocytotic vesicles at the dermis-epidermis boundary disappear, while the boundary is still a straight line. Then the continuous straight structure at the dermis-epidermis boundary becomes wavy as stress continues the size and amount of waves increases and in heavily stressed fish the continuity of the membrane is interrupted, with destruction of basal lamina. The recovery of the integrity begins during the post stress recovery period.

- *Penetration chromatophores into the epidermis*. The appearance of these cells into the epidermis is occasional in non stressed fish. Application of stress increases their number. Frequent finding of these cells in the epidermis is useful as a stress indication.
- *Changes in the structure of epidermis*. Significant changes occur in the structure of the epidermis as a response of stress including, opening of intercellular connecting structures, vacuolization, increased disconnection of filament cells from each other and loss of perpendicular orientation to the basement membrane, increased apoptosis of epidermal cells
- *Changes in pavement cell structure*. These include the disappearance of ridges, the electron lucidity of cytoplasm and the dissociation of the cells from the epidermis. These changes are easily distinguisable and their intensity depends on the intensity of stressor

Gills EM

At the electron microscopical level stress can be easily identified in the gill tissue. On the basis of a comparison of the different stressors applied in the present project we conclude that this method is very sensitive and reliable, although time consuming. The best parameters, which are found after all types of stressors when at an adequate strength:

- *Increased chloride cell numbers.* The increase is due to the development of young chloride cells, which becomes noticeable already after 24 h (probably by differentiation of immature cells and not cell proliferation) and were pronounced after 96 h (probably as a result of cell proliferation).
- *Increased apoptosis of chloride cells.* Whereas the numbers of chloride cells can also be determined reliably (and faster) at the light microscope level, the percentage of apoptotic cells appeared to require cell counts at the electron microscope level. Standard apoptotic techniques for the light microscope did not give reliable results, at least for the chloride cells of the species examined. The increase of apoptotic cells, which are very scarce in healthy control fish, is easily detected at the EM-level, and is a very sensitive parameter for stress. It most probably reflects increased cell death by aging as a result of enhanced chloride cell activity (ion-transport between water and fish).
- *Increased necrosis of chloride cells*. Cell death by necrosis can be easily distinguished from cell death by apoptosis, but only at the electron microscope level. At the light microscope level it appeared almost impossible to identify necrotic chloride cells, since this requires double staining (NaK ATPase staining for the identification of chloride cells; uptake of specific markers for necrosis) and both staining methods are not easily combined. However, necrosis did not appear to be a sensitive parameter for stress: the numbers of necrotic cells increase only slightly when compared to apoptotic cells and for obtaining statistically significant results a large number of cells have to be examined.
- *Increased intercellular spaces infiltrated by leucocytes.* The occurrence of enlargement of intercellular spaces in the lamellar epithelium of the gills is a sensitive and reliable marker of stress, irrespective of the stressor. The same conclusion applies to the infiltration of these spaces by leucocytes (in particular lymphocytes and macrophages). Apparently these cells migrate from the blood into the gill tissue immediately after the start of a stress response. These cells may be attracted by antigens released by the tissue of entering the fish from the water. At a later stage the macrophages are involved in removing necrotic and apoptotic cells. These cells can be easily detected at the electron microscope level. At the light microscope level specific antibodies against these cells are required, but so far these are not available. Their availability would greatly facilitate the detection of these cells.

LM STUDIES

Skin

From the histological results at the LM level, it is evident that elevated levels of ammonia have the most marked effects on the quality of the skin tissue in both the sea bream

and the sea bass. These effects are visible in the dispersion of the melanosomes and in the number of the mucous cells. The first stress indicator, namely an *increase in the dispersion of the melanosomes* appears to be species non specific, characterized by an increase in the dispersed melanosomes in both the sea bream and the sea bass. The effect however on the mucous cells appears to be species specific, characterized by an *increase in the (neutral) mucous cells in the bream* and a *decrease in the (acid) mucous cells of the bass*. The other parameters examined, i.e. epidermis thickness (in both species) and size and shape of the mucous cells (in the sea bream) showed no clear trend, with the possible indication of mucous cells hypertrophy in the high ammonia experiments in the sea bream.

Reduced oxygen, in contrast had no visible effects on the quality of the skin at the LM level. Finally, no clear trend could be detected in the case of the combined high ammonia-low oxygen experiments, possibly due to the lower ammonia concentrations used in these experiments. The same trend i.e. increase in mucous cells is detected in the air exposure - weight procedure handling experiments and the low oxygen in field conditions experiment. In the sorting and vaccination handling experiments however no clear trend was evident.

Gills

*Increased chloride cell numbers.*This is a reliable parameter that reliably and sensitively reflects chronic stress and also more pronounced types of acute stress. At the light microscope level these cells can be easily and reliably revealed by NaK ATPase histochemistry.

Epithelial lifting is a parameter that is associated with chronic mild and strong stressors and with acute marked stressors. It probably reflects elevated blood pressure. It can be easily measured in routine histological sections.

Lamellar fusion is a rather fluctuating phenomenon that does not seem to be related to the stressors used in this project. In the present study it was only consistently observed at high ammonia levels. Apparently it is not a non-specific stress parameter. On the basis of literature we conclude that it is mainly linked to water pollution with toxic substances, and this is in line with our findings.

FISCI INDEX

From the results obtained and evaluated it appears that skin and gill structure is responding to stress application and a number of the changes observed can be used as an index of stress. The effects of stress can be seen both at ultrastructural (EM) and structural level (LM). The last holds promise as an easier and cheaper way of stress detection. These changes usually last as long as the stress is applied and require a period of recovery for returning to normal values. Furthermore these appear to be stressor non specific but in some cases (mucous cells in the skin) species specific. The mucus cortisol is also a useful index of stress for a short time after the stress application since it is not affected by stress imposed by handling during the sampling of the fish.

A thorough evaluation of the parameters selected could provide a solid base for the FISCI Index, including the following parameters as stress indices of fish:

Mucus

Cortisol levels in mucus for all types of stressed groups for some time after the stress application

Skin (LM)

Mucous cells hyperplasia (in the sea bream)
Neutral mucous cells hyperplasia and acid mucous cells depletion (in the sea bass)
Increased dispersion of melanosomes in both species

Skin (EM)
Dispersion of melanosomes in the dendrites of melanocytes.
Linear formations of iridocytes-melanosomes
Aggregates of xanthophores-melanophores
Disappearance of pinocytic vesicles
Deformation of dermis-epidermis boundary
Destruction of basal lamina area
Penetration of chromatophores from dermis in epidermis.
Destruction of pavement cell layer (outer epidermal layer)
Vacuolation and disconnection of epidermal cells
Loss of perpendicular orientation of the epidermal cells at the dermis epidermis boundary.

Gills (LM)
Increased chloride cell numbers
Increased epithelial lifting

Gills (EM)
Increased chloride cell numbers
Increased apoptosis of chloride cells
Increased intercellular spaces infiltrated by leucocytes.

Table 1 summarizes the stress indices incorporated in the FISCI index.

Table 6. 1. Stress parameters incorporated in the FISCI INDEX.

Parameters	low O_2		high NH_3		high NH_3 / low O_2		Handling (Astakos)		Air-exposure (Astakos)	
PLASMA	bream	bass	bream	bass	bream	bass	bream	bass	bream	bass
glucose	↑	-	-	-			↑	↑	±↑	↑
lactate	↑	±↑	-	-			-	↑↑	↑	↑↑
FFA	±↓		-	-			-	-	-	-
osmolarity	±↑	-	-	±↓			↑	↑↑	↑↑	↑↑
Na	-	-	-	-	-	-	-	↑↑	↑	↑↑
Cl	-	-	±↓	±↓	~	~	↑	↑	±↑	±↑
K	-	-	-	-	-	-	-	-		-
cortisol	-	±↑	±↑	↑	~	↑	±↑	↑	±↑	↑
MUCUS										
cortisol	±↑	±↑	↑	↑↑	-	-	-	↑	↑	↑↑
SKIN-LM										
neutral mucous cells	-	-	↑↑	↑↑	-	-	±↑		±↑	
acid mucous cells		-		↓↓		-		±↑		±↑
melanosome dispersion	-	-	↑	↑	-	-	±↑	±↑	±↑	±↑
SKIN-EM										
dispersion of melanosomes	+	+	+	+	+	+	+	+	+	+
linear formations of iridocytes-melanosomes	+	+	+	+	+	+	+	+	+	+
aggregates of xanthophores-melanophores	+	+	+	+	+	+	+	+	+	+
disappearance of pinocytic vesicles	+	+	+	+	+	+	+	+	+	+
deformation of dermis-epidermis boundary	+	+	+	+	+	+	+	+	+	+
destruction of basal lamina	+	+	+	+	+	+	+	+	+	+
penetration of chromatophores in epidermis	+	+	+	+	+	+	+	+	+	+

Table 6. 1. Stress parameters incorporated in the FISCI INDEX. (continued)

Parameters	low O_2		high NH_3		high NH_3 / low O_2		Handling (Astakos)		Air-exposure (Astakos)	
	bream	bass	bream	bass	bream	bass	bream	bass	bream	bass
SKIN-EM (continued)										
destruction of pavement cell layer	+	+	+	+	+	+	+	+	+	+
vacuolation, disconnection of epidermal cells	+	+	+	+	+	+	+	+	+	+
loss of perpendicular orientation of epidermal cells	+	+	~	~	~	~	+	+	+	+
GILLS-LM										
chloride cells	~	-	↑	↑	±↑	±↑	±↑	↑	±↑	~
lamellar fusion (%)	-	-	±↑	-	-		-	-	-	-
lamellar lifting (%)	-	-	↑	±↑	↑	±/~↑	-	-	±↑	-
capillaries	-	-	-	-	~	~	-	-	↑	↑
PCNA/mitosis	-	-	-	-	~	~	±↑	±↑	±↑	±↑
GILLS-EM										
cc-cells-apoptosis	~	↑	±↑	↑	↑	↑	±↑	↑	↑	↑↑
cc-cells-necrosis	~	↑	±↑	↑	~	~	-	↑	↑	↑
cc-cells-juvenile	±↑	±↑	↑	↑	-	-	-	↑	↑	↑
pavement cell-apoptosis	±↑	↑	~	±↑	~	~	±↑	↑	↑	↑
pavement cell-necrosis	~	~	~	±↑	~	~	-	-	↑	↑
intercellular spaces	~	↑	↑	↑	↑	~	↑	↑	↑↑	↑↑
leucocyte infiltration	±↑	↑	↑	↑	↑	↑	↑	↑↑	↑	↑↑

↑ increase, moderate
↓ decrease, moderate
~ no clear effect
– no effect
+ effect

↑↑ increase, strong
↓↓ decrease, strong

±↑ tendency to increase
±↓ tendency to decrease

CONCLUSIONS

The results of this program represent for the first time a comprehensive multifaceted analysis of the effects of stressors on marine fish. This brings the knowledge of the species concerned, with respect to stress at the same level as, or even further than, that of the most extensive studied freshwater fish species. Our program included:

- two marine species of great economical importance, seabream and seabass
- a great number of stress parameters were examined, for plasma, skin and gills
- several tressors: low oxygen, high ammonia (separated or in combination), weighing, sorting, vaccination, air exposure
- all parameters in all experiments were analysed in a standardized way

In these respects the present program was unique. The results give not only an extensive data set for marine fish, but also form the basis for analysis of the most suitable parameters for routine establishment of the stress condition of fish in aquaculture. On the basis of the results adequate management procedures can be taken to reduce the negative impact of stress in fish (reduced growth, reduced disease resistence, and, where appropriate, reduced reproduction). We expect that the resulting FISCI-INDEX is not only useful for establishing stress intensity in aquaculture in a marine environment but also in freshwater aquaculture, since other studies of the participants have already shown that the stress responses forming the basis of the FISCI-INDEX can also be examined in stressed freshwater fish. Thus, the deliverables of the present programme are:

- The presentation of a profound and fundamental analysis of the effects of different stressors on blood, skin and gills of two marine species,
- An extensive data set providing the basis for the FISCI-INDEX for marine fish in aquaculture,
- The FISCI-INDEX for application in marine aquaculture for the evaluation of stress,
- An index that may also be useful for application for freshwater aquaculture.

The following comments have to be kept in mind:

1.The FISCI-INDEX contains a listing of stress parameters of skin and gills that are useful over a range of stress intensities, are sensitive, reliable and reproducible, and do not seem to be species-specific. At the moment these parameters still have to be read out with classical biochemical, light microscopical end electron microscopical techniques. They are all standard techniques, but require some specific expertise and equipment that is present in most modern laboratories for for veterinary practices, but not available in aquaculture farms. Therefore, although our FISCI-INDEX is now available for experienced hands, it should be adapted for making it more widely available. To this end further reaserch is necessary to simplify the methodology.

2. If we compare the stress parameters included in the FISCI-INDEX with the list of criteria that should be fulfilled (as presented in the Introduction to this report, we conclude that not all FISCI-parameters fulfil all criteria. However, the idea behind the FISCI-INDEX is that stress can not be evaluated by one parameter only, because it is such a complex phenomenon, that can only be adequately addressed with a multifatorial approach. It is therefore not necessary (and can not be expected) that each stress parameter will fulfil all five criteria. However, each parameter should fulfil at leastmost of the criteria, and all together they should cover the whole sprectrum. Confrontation of the FISCI-parameters with the criteria leads to the following conclusion:

- Criterium 1: The parameters should allow differentiation between acute and chronic stress.Most criteria can be used for the evaluation of acute (minutes to hours), semi-chronic (a few hours to a week) and chronic (one week and longer) stress. We have not done chronic experiments in our program, but the first result of additional experiments on freshwater fish show that the FISCI-parameters are also applicable to chronic stress. The

mucus cortisol levels decrease however after the first hours, similar as in the blood (this program) and they remain lower in the period afterwards, although well above control levels. The cell proliferation (PCNA-test) in the gills is an example of a parameter that is not useful for acute stress since it does not show an increase in the first few days of stress. On the other hand, these parameters contribute to the differentiation between acute and chronic stress.

- Criterium 2: The parameter should be sensitive, reproducible and not complicated to determine. All parameters fulfil this criterium, although the necessary expertise and equipment are limited to laboratories (see under 1).
- Criterium 3: The samples necessary for the measurement of a parameter should be obtained easily and in a reproducible way. All parameters fulfil this criterium, although some care (and thus some expertise) is needed, as was demonstrated during our experiments.
- Criterium 4: Samples should be obtained without the necessity to kill the fish. This is possible with all the FISCI-parameters. During the program the fish were killed because we needed blood samples for comparison with our results on gills and skin. For the FISCI-INDEX the use of blood is no longer necessary: one of our major results is the replacement of the plasma cortisol values by the cortisol concentration of the mucus that can be blotted from the skin of the fish. Biopsies of the gills and skin can be obtained without killing the fish (although preferably after anesthetization), as we have already demonstrated. The wounds of small biopsies are healing rapidly.
- Criterium 5: It should be possible to store and transport the samples. All parameters of gills and skin can be measured in fixed tissues or in mucus. The latter can be stabilized by fixation (in e.g. ethanol) as well, and thus no cooling or other treatments that make transport difficult (such as use of dry ice) are necessary. Our results showed that the enzyme parameters (peroxidase and alkaline phosphatase), that were investigated as potential FISCI-parameters did not work because the “over the hill” effect, and thus special and expensive care to maintain enzyme activity in the samples is not necessary. In fact all samples can be stored at room temperature.

Thus, in conclusion, although not each of the FISCI- parameters fulfils all criteria, in general all the criteria are amply covered.

3. It remains necessary to validate the stress parameters included in the FISCI-INDEX further:

- for more stressors; in particular for more toxic water pollutants than ammonia;
- for more species, from freshwater as well seawater; although we have indications that they can be used for many species, for each species baseline levels should be established.

4. Dissemination of the results only started recently and should be intensified using classical (reports, scientific papers, and papers in professional journals on aquaculture) as well as electronic means (websites).