WATER QUALITY AND STRESS INDICATORS IN MARINE AND FRESHWATER ECOSYSTEMS: LINKING LEVELS OF ORGANISATION (INDIVIDUALS, POPULATIONS, COMMUNITIES)

Edited by

DAVID W. SUTCLIFFE

Published by the Freshwater Biological Association

Invited papers from a joint Associations specialised conference

held at

Napier University, Edinburgh on 6-7 September 1993

by

The Freshwater Biological Association,

The Ferry House, Far Sawrey, Ambleside, Cumbria LA22 0LP, England

and

The Marine Biological Association of the United Kingdom,

The Laboratory, Citadel Hill, Plymouth PL1 2PB, England

and

The Scottish Association for Marine Science,

PO Box 3, Oban, Argyll PA34 4AD, Scotland

ISBN 0-900386-54-1

Effects of stress on the immune system of fish

A. L. PULSFORD[1], S. LEMAIRE-GONY[1,2] AND S. FARLEY[1]

[1]*Plymouth Marine Laboratories, Citadel Hill, Plymouth PL1 2PB, UK*
[2]*Laboratoire d'Ecologie Fondamentale et d'Ecotoxicolgie Université de Bordeaux 1, Faculté des Sciences, Avenue des Facultés 33405, Talence, France*

The effects of stress on the immune system of various fish species including dab *Limanda limanda*, flounder *Platichthys flesus*, sea bass *Dicentrarchus labrax* and gobies *Zosterisessor ophiocephalus*, were investigated from laboratory and field experiments, using various assays to measure immunocompetence, correlated with histological and ultrastructural observations. Modulation of the immune system was demonstrated at tissue, cellular and biochemical levels following exposure to various stressors. The spleen somatic index was depressed in dab stressed in the laboratory and gobies collected from polluted sites in the Venice Lagoon. Differential blood cell counts consistently showed an increase in phagocytes and decrease in thrombocytes in fish exposed to various stressors. Phagocytic activity from spleen and kidney adherent cells was stimulated in dab stressed by transportation but depressed in fish exposed to chemical pollutants. Respiratory burst activity in phagocytic cells was also stimulated in stressed dab but depressed in sea bass exposed to cadmium. The results are discussed in relation to current concepts on stress in fish and the regulation of the immune system.

Introduction

The aquatic environment is continually subjected to natural and anthropogenic stressors, including temperature changes and pollutants. Fish exposed to such stressful environments respond, in an attempt to regain homeostasis, by a physiological response known as "stress". Selye (1950) defined stress as the sum of all physiological responses by which an animal tries to maintain or re-establish a normal metabolism in the face of a physical or chemical force. The physiological response is similar whether the stressor is psychological, physical or environmental, and appears to be similar throughout all the vertebrate groups that have been studied (Khansari *et al.* 1990).

Environmental stimuli induce neuroendocrine responses that often include the activation of the sympathetic nervous system and the hypothalamic–pituitary–adrenal axis. The stressor is perceived by the hypothalamus, which releases CRF (corticotropin releasing factor) which stimulates the corticotrophs of the pituitary to secrete and release ACTH (adrenocorticotrophic hormone) and probably other peptides derived from the precursor propiomelanocortin (POMC). In turn ACTH stimulates the synthesis and secretion of cortisol from the interrenal gland in the kidney, a hormone which can have a wide range of effects, often referred to as the secondary effects of stress.

The overall result is an increased stimulation of adrenocortical secretion with an increase in serum glucocorticoids and activation of the sympathetic nervous system, followed by a release of catecholamines (Donaldson 1981). The adrenergic function of fish is very sensitive to stress and results in an increase in the concentration of plasma catecholamines (Donaldson 1981; Mazeaud & Mazeaud 1981). Catecholamines may be elevated as a primary effect of stress and remain elevated for many hours after the cessation of stress. The effects of catecholamines on

the immune response are complex and further complicated by the fact that adrenoreceptors are up-and-down modulated during the activation of the immune cells (Besedovsky *et al.* 1985; Bayne & Levy 1991). It is the effect of these hormones on the cells of the immune system that is thought to mediate the stress-induced immunomodulation.

A wide range of environmental stressors, including pollutants such as heavy metals (Zelikoff 1993), pesticides (Dunier *et al.* 1991), chlorinated dioxins, polycyclic aromatic hydrocarbons (PAHs) and various sources of radiation, drugs and sewage (Secombes *et al.* 1991, 1992; Tahir *et al.* 1993), have all been shown to have an effect on the immune system of fish. However, not all pollution-induced immunomodulatory effects are inhibitory, and immunomodulatory substances can enhance responses in certain circumstances, or can go from a stimulatory to inhibitory effect with increasing concentration (Dunier *et al.* 1991).

Fish living on sediments contaminated with heavy metals, PCBs and PAHs, resulting from the accumulation of industrial discharges, may be subjected to chronic stress which may modulate the responses of the cells of the immune system (Secombes *et al.* 1991, 1992; Tahir *et al.* 1993). This may result in an increased susceptibility to infectious agents which may not be pathogenic in unstressed environments (Anderson 1990). In studies of environmental contamination on fish in the wild and the laboratory, effects on macrophage phagocytosis (Weeks & Warriner 1984), chemotaxis (Weeks *et al.* 1986) pinocytosis (Weeks *et al.* 1987) and respiratory burst activity (Secombes *et al.* 1991) have all been demonstrated.

One of the primary although difficult goals of environmental immunological research is to establish causative links between environmental stressors and immune modulation in wild fish to determine whether they can be used as biomarkers of exposure (Weeks *et al.* 1990a,b). The important questions in studies on contaminant exposure are whether the recorded effects at the whole organism, tissue, cellular and biochemical levels are a result of direct exposure to pollutants, or the indirect effect of the physiological response to stress as a consequence of reduced water quality. Linking the different levels of measurement is one of the main challenges of research in environmental stress on the immune system.

Immunological assays have been standardised and used in medical and veterinary diagnostic medicine for many years (Weeks & Kavas 1979; Pick & Mizel 1981). These assays have also been increasingly applied to measure the effects of pollutant stress on fish (Weeks & Warinner 1984; Seeley *et al.* 1990). Measurements may be correlated with immune modulation and the severity of the stressor, such as the concentration of the pollutant, which can act as a "biomarker" of contamination (Weeks *et al.* 1987). Assays range in complexity from simple microscopic counting methods to spectrophotometric microplate assays. Although the effects of stress on the immune system can be assessed by physiological assays, results should also be compared with data at other levels of physiological response and take seasonal effects into account.

Public attention has been drawn to the possible effects of pollution-induced immunosuppression by the increased incidence of visible cancers and lesions on fish collected from contaminated sites (Milne 1987; Sindermann 1993), and this has stimulated research into stress-induced immunosuppression.

Fish living in intimate contact with sediments which are sites of pollutant accumulation are more susceptible and have been used for studies on the effects of contaminant stress. The dab *Limanda limanda* is a flatfish which has been used extensively in monitoring biological effects (Bucke *et al.* 1989) as it is an important food fish in Europe, abundant in the North Sea and was, until recently, thought to maintain well defined feeding grounds and thus provide a link between pollution and immunomodulation (McVicar *et al.* 1988). The flounder *Platichthys flesus*, another flatfish, is an estuarine species which tends to maintain defined home ranges

within estuaries (Dando 1980; Vethaak 1993), returning to these after spawning at sea, and therefore may be a good pollution indicator (Pulsford *et al.* 1992a).

The goby *Zosterisessor ophiocephalus* burrows into the sediments and has been used to investigate the biological effects of pollutants, in a study of the Venice Lagoon (Pulsford *et al.* 1994a). For laboratory exposure experiments other commercially important species, such as sea bass *Dicentrarchus labrax*, have been used.

Experimental observations

Various experiments have been carried out at the Plymouth Marine Laboratory and in collaboration with MAFF laboratories, Weymouth, to assess the effects of environmental stress on the immune systems of fish. Assays to measure immunocompetance have been correlated with histological and ultrastructural studies.

Laboratory experiments

(a) Acute stress effects on the dab immune system. In a laboratory experiment exposing dab to acute stress from simulated transportation (Pulsford *et al.* 1994b) various haematological and immune changes were recorded. The spleen somatic index was depressed and an increase in groups of thrombocytes forming clumps together with a general increase in the number of damaged cells in the peripheral blood was recorded. Stress also stimulated overall phagocytic activity and the phagocytic indices of both spleen and kidney macrophages. The amount of hydrogen peroxide released during the respiratory burst from stimulated kidney macrophages was also significantly increased in stressed fish (Fig. 1).

(b) Effect of cortisol on phagocytosis. The effects of physiologically relevant levels of cortisol (i.e. a concentration of 320 ng ml^{-1}) on phagocytosis and respiratory burst activity were investigated *in vitro* and showed a suppression of phagocytosis in both spleen and kidney macrophages (Fig. 2). Cortisol stimulated the basal respiratory burst activity of spleen and kidney cells but depressed the stimulated activity (Fig. 3).

(c) Effect of exposure of sea bass to cadmium and benzo(a)pyrene. Sea bass exposed in laboratory aquaria to cadmium (at 40 $\mu g\ l^{-1}$) and benzo(a)pyrene have shown decreased phagocytic activity on exposure to cadmium alone, but cadmium and benzo(a)pyrene together depressed the phagocytic activity and the phagocytic index. Exposure to cadmium also depressed the numbers of thrombocytes in the peripheral blood and depressed the total serum proteins. Ultrastructural changes included a fibrillar deposit on the gill epithelial cells and an increase in the endoplasmic reticulum in the hepatocytes of cadmium-exposed fish (Fig. 4).

(d) Exposure of flounder to fluoranthene and contaminated sediments. Exposure of juvenile flounder to 200 $\mu g\ ml^{-1}$ fluoranthene had a slight stimulatory effect on phagocytosis (Pulsford *et al.* 1994c) while adult flounder collected from sediments contaminated with particulate metals have shown by X-ray microanlaysis that these may be taken up and sequestered in the melanomacrophage centres of the spleen and kidney (Pulsford *et al.* 1992a) (Fig. 5).

(e) Laboratory exposure of fish to contaminated sediments. Dab were exposed in laboratory aquaria at the MAFF laboratories, Weymouth, to two sediments obtained from Mersey Docks (Liverpool Bay and Marsden Bay) of similar particle size but different contaminant levels. Sediments were analysed at the MAFF laboratory, Weymouth for heavy metals, PCBs and PAHs. The contaminated sediment generally had higher levels of PCBs and PAHs (Livingstone *et al.* 1993). Blood samples were taken at 144 days post-exposure, from the exposed (contaminated group) and control (reference group) fish, and blood smears and serum

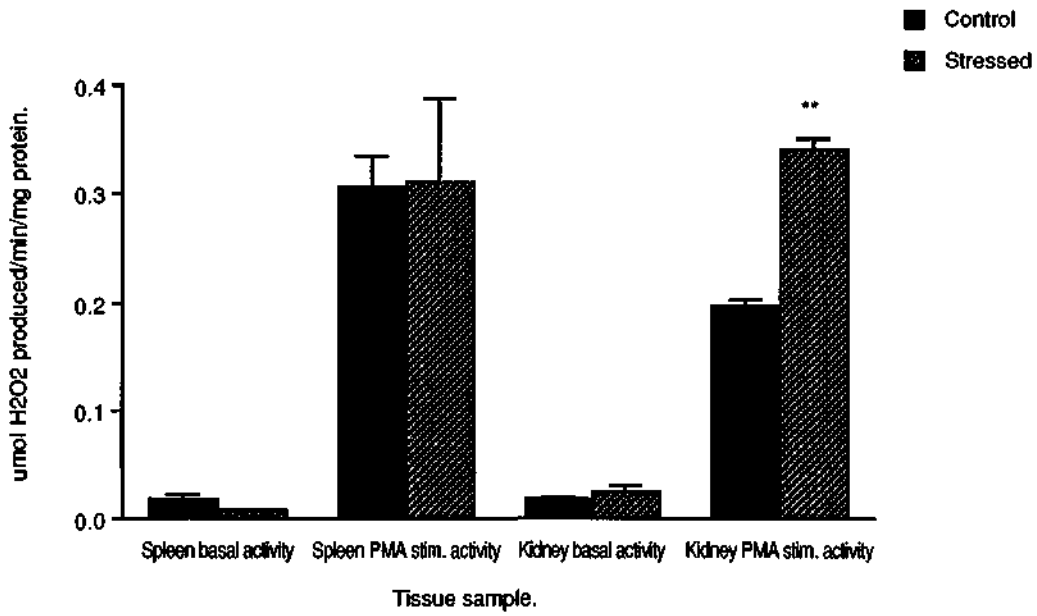

Figure 1. Respiratory burst activity measured by release of hydrogen peroxide (μmol produced per minute per mg protein) from spleen and kidney macrophage taken from stressed and unstressed dab (*Limanda limanda*). Spleen macrophages from stressed fish showed a significant increase in PMA-stimulated activity. PME = phorbol–12–myristate–13–acetate. The T bars indicate 1 standard error (SE) of the mean; ** = $p<0.01$ for significant differences between the control and stressed fish.

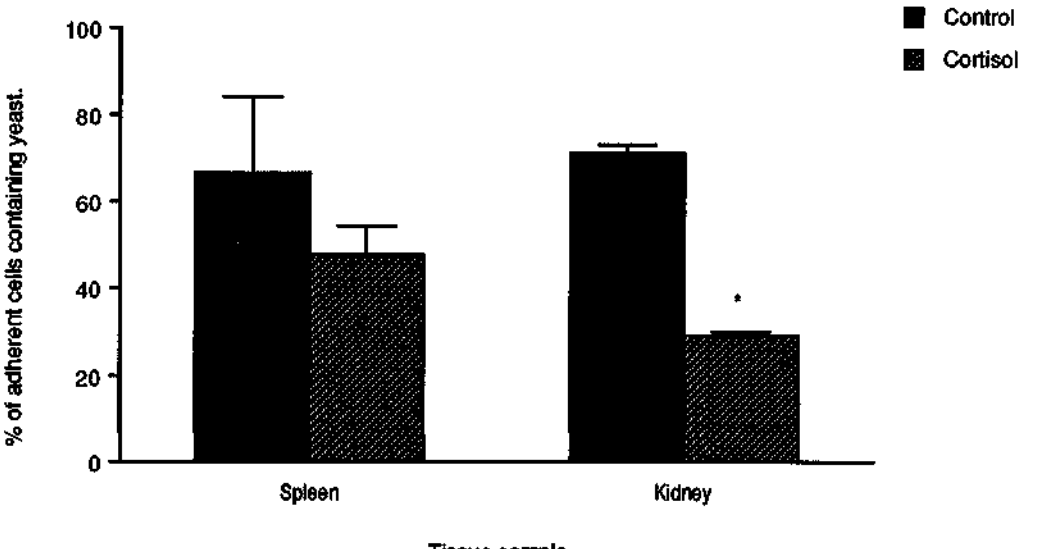

Figure 2. Phagocytic activity of spleen and kidney macrophages (estimated by the percentage of adherent cells containing yeast) from dab (*Limanda limanda*) and the effect of *in vitro* addition of cortisol (320 ng ml^{-1}) to the culture medium. Both spleen and kidney activity was depressed. T bars = 1 SE; * = $p<0.05$ for significant differences between the control and cortisol-exposed cells.

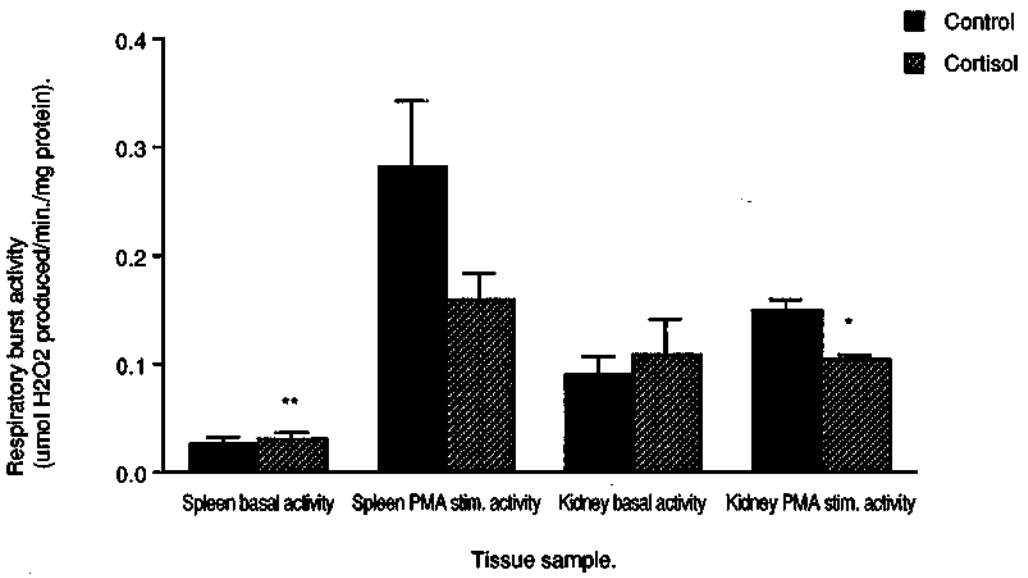

Figure 3. Respiratory burst activity measured by release of hydrogen peroxide (µmol produced per minute per mg protein) from stimulated and unstimulated spleen and kidney cells from dab (*Limanda limanda*) and the effects of adding cortisol at a concentration of 320 ng ml^{-1}. Basal activity of spleen cells was stimulated, and stimulated activity of kidney cells was suppressed. T bars = 1 SE; * = $p < 0.05$ and ** = $p < 0.01$ for significant differences between the control and cortisol-exposed cells.

samples were collected. Haematological and serological parameters were compared with a sample of dabs caught off Plymouth (known as the "wild" population). The phagocyte proportion was higher in the experimental fish than in the wild population (8.9 ± 1.0%). The difference was significant in the contaminated group (16.5 ± 1.4%) but not the reference group (13.3 ± 1.5%). The thrombocyte proportion was significantly lower in fish from the contaminated (10.1 ± 1.2%) and reference sediments (19.8 ± 1.0%), which was similar to the wild population (Fig. 6).

Total serum proteins from reference and contaminated groups were within the normal range for teleosts, and both were slightly lower than the wild population (Table 1). The difference was significant when comparing the contaminated and wild populations.

Table 1. *Concentrations of total serum proteins from dab* (Limanda limanda) *experimentally exposed to contaminated sediments (see the text), compared with a "wild" population and a control (reference) group.*

Values are means (mg total serum proteins per ml blood) ± S.E.

Wild	Reference	Contaminated
36.20 ± 1.13	33.61 ± 2.50	28.55 ± 2.50

Field experiments

North Sea Task Force

During July 1991 the North Sea Task Force sampled five dabs from the estuaries of the Firth of Forth, Humber, Tees, Thames and Weser in Northern Europe (Pulsford *et al.* 1992b). Yeast

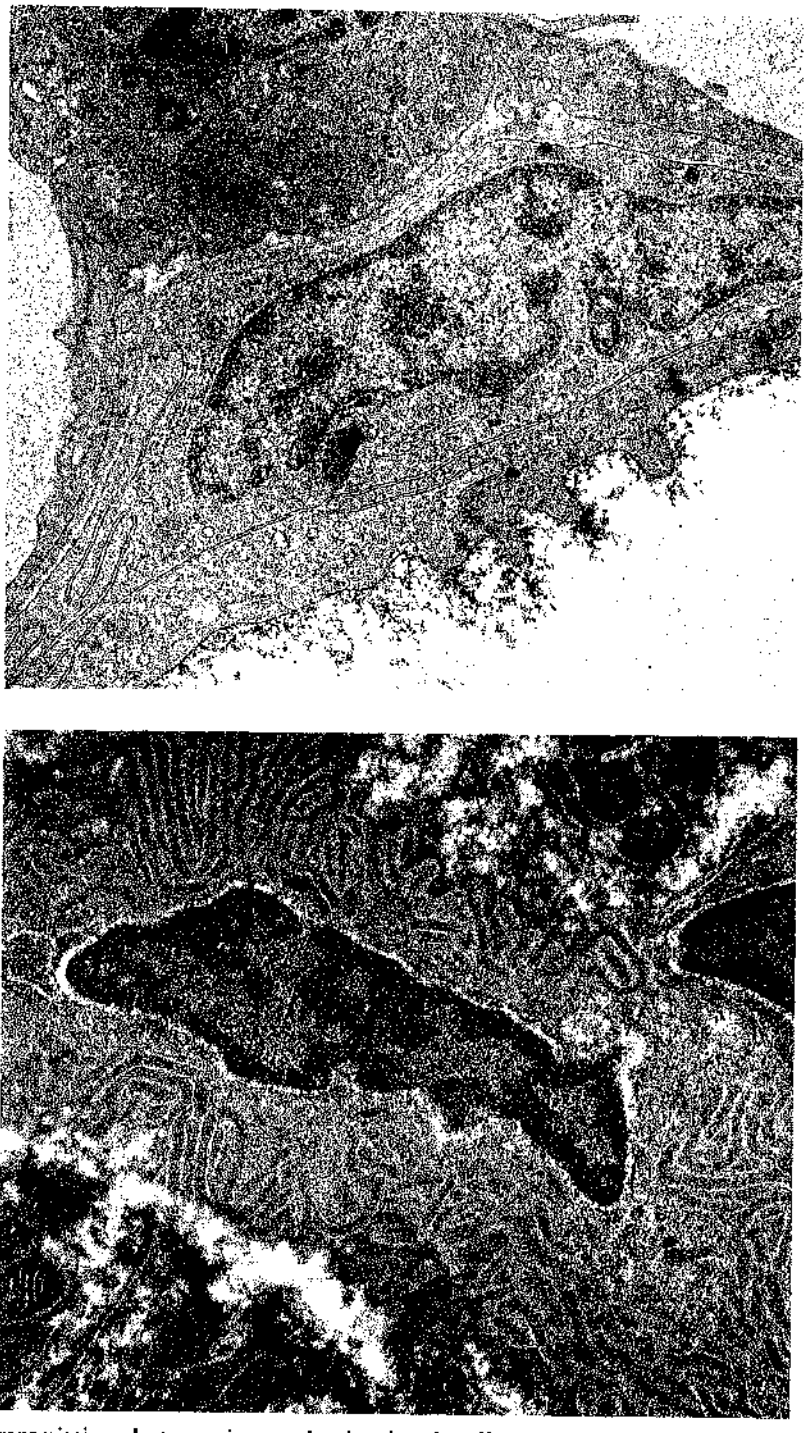

Figure 4. Transmission electron micrographs showing the effects of experimental exposure of sea bass (*Dicentrarchus labrax*) to cadmium. *Above*: Gill epithelial cells (magnification x 6,800) with fibrillar deposits on the cells at bottom right of the picture. *Below*: Hepatocyte from the liver (magnification x 8,200). Structural modifications, after exposure to cadmium, included dilation of the nuclear membrane and the endoplasmic reticulum.

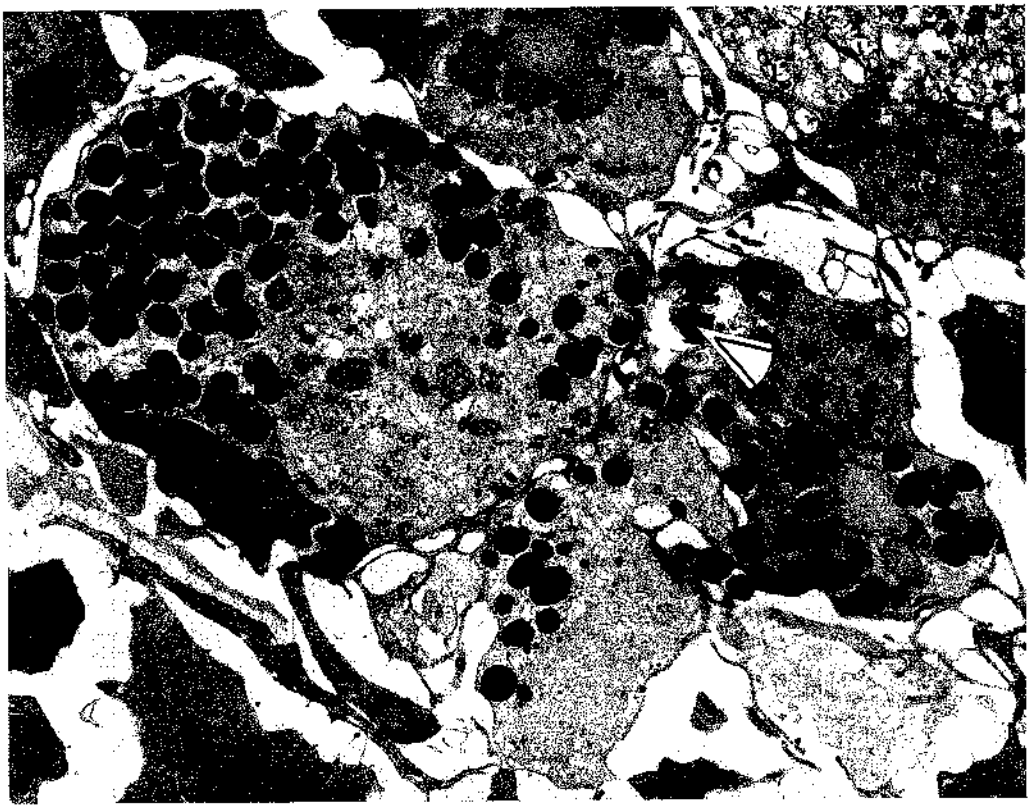

Figure 5. Transmission electron micrograph (magnification x 10,000) of melanomacrophage centres from the kidney of a flounder (*Platichthys flesus*) naturally exposed to sediments contaminated with particulate metals. The arrow centre right points to a metal inclusion; the other numerous dark particles, more rounded in appearance, are pigment granules (melanin).

suspensions were prepared in advance of the cruise and the phagocytosis assay was carried out on board ship, on adherent cells isolated from freshly caught dab spleen and kidney. Phagocytosis by kidney macrophages was always significantly higher than spleen macrophages. Dabs from the Tees and Humber had significantly higher rates of phagocytosis in spleen and kidney cells than dabs from the Thames, Weser and Firth of Forth (Fig. 7).

Venice Lagoon study

The goby *Zosterisessor ophiocephalus*, a bottom-dwelling fish, was used in this study and sampled from various sites in the Venice Lagoon during April, June and October 1992. The fish collected from the polluted site at Porto Marghera, a major oil port, showed depressed spleen somatic indices, depressed spleen and kidney phagocytic activity, and an increased phagocyte proportion but depressed thrombocyte proportion in the peripheral blood (Pulsford *et al.* 1994a)

Discussion

Using a range of *in vitro* and *in vivo* laboratory experiments and field studies it has been demonstrated that environmental stressors can affect the tissues and cells of the immune

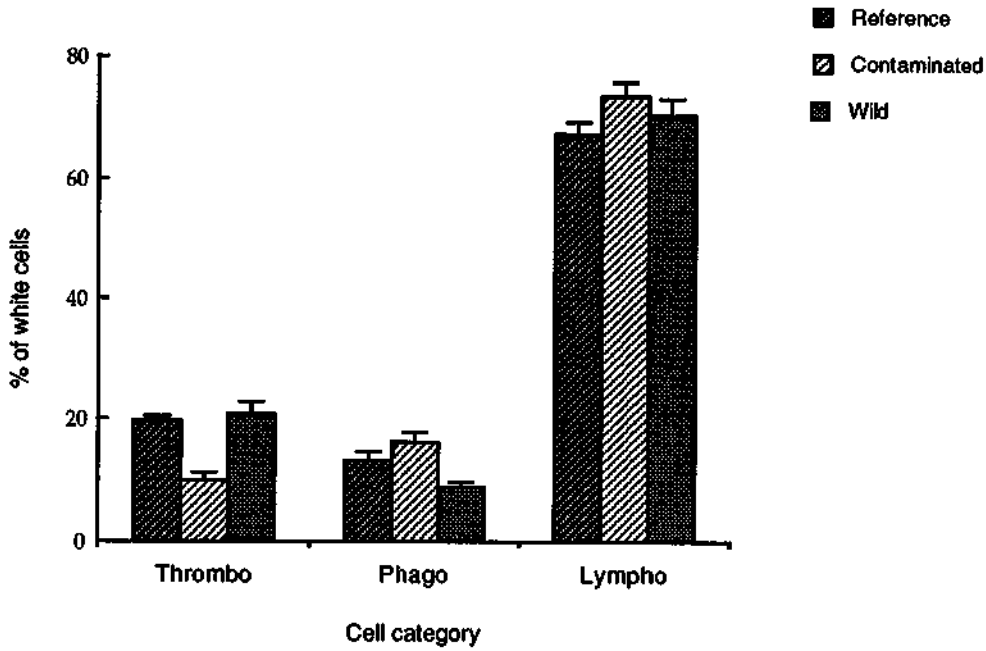

Figure 6. Peripheral blood cells from dab (*Limanda limanda*) experimentally exposed to contaminated sediments (high levels of PCBs, PAHs and heavy metals) and "reference" sediments (lower levels of pollutants), taken from Mersey Docks, compared with a "wild" (uncontaminated) population of fish caught at Plymouth. There is a significant reduction in the thrombocyte proportion and increase in the phagocyte proportion in fish from the contaminated sediments, relative to the wild population.

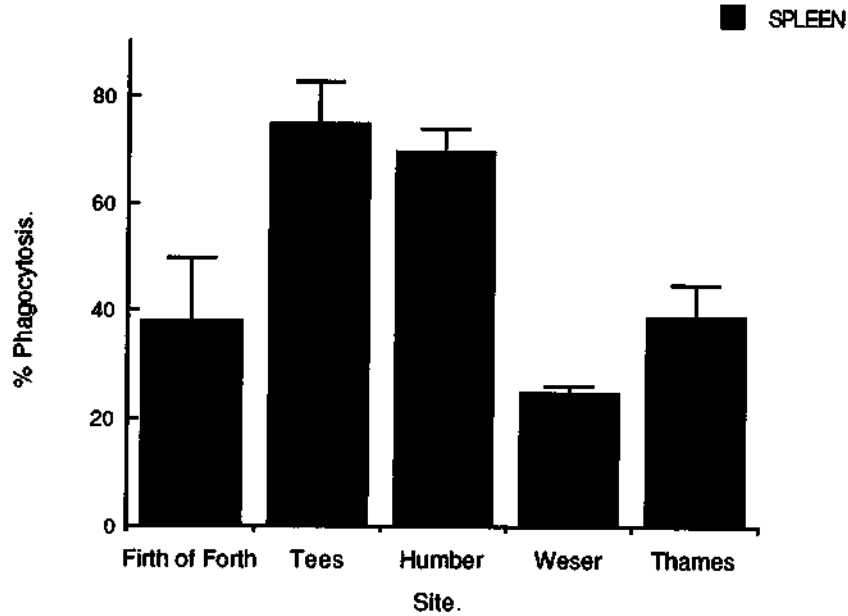

Figure 7. Phagocytosis of spleen macrophages from dab (*Limanda limanda*) collected from various estuaries in the North Sea.

system of fish at tissue, cellular and biochemical levels (Schreck & Lorz 1978; Donaldson 1981; Ellis 1981; Mazeaud & Mazeaud 1981; Pickering 1981; Pickering & Stewart 1984; Pickering & Pottinger 1987; Pickering *et al.* 1989; Faisal *et al.* 1989; Khansari *et al.* 1990; Pankhurst *et al.* 1992; Pulsford *et al.* 1992b, 1994a,b; Weeks *et al.* 1990a,b).

All vertebrates, including fish, when subjected to stressful conditions display neuroendocrine responses. These include a rise in serum adrenocorticotrophic hormone (ACTH), cortisol and catecholamines (Mazeaud & Mazeaud 1981; Peters *et al.* 1991). A discharge of corticosteroids and catecholamines occurs within minutes of stimulation of the interrenal tissue and chromaffin cells during the stress response in fish (Ellis 1981). Corticosteroids are thought to be the most immunosuppressive hormones released during the stress response, although other hormones may be involved.

The initial period of exposure to a moderate stressor often elicits the greatest endocrine response in fish, resulting in an acute transient increase in plasma cortisol (Barton & Peter 1982). Secondary stress responses, such as changes in blood and liver sugar levels, immunosuppression and blood cell profiles, are thought to be a response to the elevation in corticoid hormones and catecholamines. Cortisol is the main corticosteroid in most teleost groups that have been studied (Fagerlund 1970; Pickering 1981) and the elevation of plasma cortisol in response to stress is well documented (Pickering 1981; Pickering *et al.* 1989; Thomas & Robertson 1991). Plasma ACTH is elevated within two minutes of acute stress (Tripp *et al.* 1987; Thomas & Robertson 1991) and the resultant cortisol increase may persist for several hours (Pickering 1981). MacArthur *et al.* (1984) suggested that one of the most important effects of cortisol in teleosts is its ability to inhibit leucocyte recruitment at sites of tissue injury.

Chronic stress also elevates blood cortisol but the elevation may persist for several days or weeks (Pankhurst *et al.* 1992). However, cortisol determinations may not be a good indicator of the general health of fish because normal titres have also been demonstrated in fish which are severely stressed (Schreck & Lorz 1978).

Exposure to conditions of reduced water quality may induce a stress response in fish, resulting in changes in the immune system. Similar responses have also been recorded from socially stressed, hierarchical or aggressive fish (Peters & Schwartzer 1985; Cooper *et al.* 1989; Peters *et al.* 1991). Subordinate (stressed) trout had elevated levels of circulating catecholamines, cortisol, lactate and glucose, and were more readily infected experimentally (Faisal *et al.* 1989). Fish eventually show an adaptation to stress, similar to that demonstrated in mammals. Plasma cortisol levels become acclimated and relatively reduced in situations of chronic stress over a period of time (Pickering & Stewart 1984).

In the present studies the spleen somatic index was significantly depressed in dabs subjected to acute stress, and also in gobies collected from the contaminated site in the Venice Lagoon. This may be because the spleen, which is innervated by catecholamine-containing nerves (Randall & Perry 1992), contracts during stress and forces erythrocytes out into the circulation. However, an alternative explanation for the low spleen weight in gobies from the Venice Lagoon may be that anaemia reduces the number of erythrocytes stored in the spleen (Pulsford *et al.* 1994a).

Haematological changes were detected in fish exposed to pollutants in laboratory studies. A significant decrease in the thrombocyte proportion was recorded in dabs exposed to the contaminated sediments and in sea bass following cadmium exposure. Secombes *et al.* (1991, 1992) also found the thrombocyte fraction significantly reduced in dabs exposed to sewage sludge. Ellsaesser & Clem (1986) found a significant decrease in peripheral blood thrombocytes following handling and transport stress, and Pickering & Pottinger (1987) also found that chronic stress caused a reduction in the proportion of circulating thrombocytes.

In contrast, a significant increase in the proportion of circulating phagocytes was recorded in experimental dabs maintained on both the reference and contaminated sediments, compared with the wild population, and in gobies collected from the contaminated site in the Venice Lagoon (Pulsford *et al.* 1994a). This is in agreement with Secombes *et al.* (1991, 1992) and Tahir *et al.* (1993) who found an increase in the number of phagocytes in fish exposed to contaminated sediments and sewage sludge. The phagocyte classification adopted in the present studies included both neutrophils and monocytes, and increases in peripheral blood neutrophils have been shown to be indicative of stress in fish (Ellsaesser & Clem 1986). Bly *et al.* (1990) suggested that two populations of neutrophils may exist in fish and a marginated pool may be released into the circulation following stress.

The process of phagocytosis is an important and effective part of the non-specific defence system of fish (MacArthur & Fletcher 1985). It is also affected by stress, resulting in a depression or stimulation of the phagocytic cells (MacArthur *et al.* 1984). Phagocytic activity in spleen and kidney adherent cells was affected in the present studies, and both suppression and enhancement of activity were recorded. Acute stress stimulated phagocytic activity of spleen and kidney cells in dabs, but *in vitro* application of cortisol depressed activity. Exposure to cadmium or benzo(a)pyrene in sea bass slightly stimulated activity of kidney macrophages but both contaminants together depressed activity. This is in agreement with Dunier *et al.* (1991) who found effects on the immune system were often dose related.

Gobies collected from the polluted site in the Venice Lagoon also showed depressed phagocytic activity in both spleen and kidney phagocytes at all times of the year. Dabs collected from estuaries during the North Sea Task Force also showed significant differences in phagocytic activity, but without detailed chemical analyses from the collection sites it was not possible to correlate the findings with contaminant exposure.

Weeks & Warriner (1984) have also shown that phagocytic activity of pronephric macrophages was depressed in two species of fish from a river polluted with polycyclic aromatic hydrocarbons, compared with fish from an unpolluted river. Percentage phagocytosis by pronephric macrophages was decreased by 50% and 65% respectively in fish which had suffered pollution stress for a prolonged period. However, uptake into the cells by pinocytosis was stimulated (Weeks *et al.* 1987), which underlines the requirement to carry out a range of assays to measure immunocompetence.

Recent studies suggest that in fish, events occuring within phagocytic cells during the respiratory burst are similar to those in mammals (Chung & Secombes 1988) and a range of reactive oxygen species are produced which are used to kill microorganisms. The wide variety of reactive oxygen species generated during the respiratory burst is considered to be one of the most important microbicidal components in the armoury of fish phagocytes. These oxygen radicals may be detected by a range of *in vitro* biochemical assays (Pick & Mizel 1981) adapted from mammalian studies and have been shown to be sensitive indicators of stress (Secombes *et al.* 1991, 1992).

In the present studies, acute stress stimulated the amount of hydrogen peroxide produced by the stressed dab macrophages during the respiratory burst, but the cortisol-treated, stimulated cells showed depressed activity. In sea bass exposed to cadmium and benzo(a)pyrene, the respiratory burst measured by production of superoxide anion was increased. Secombes *et al.* (1991, 1992) found that in dab exposed to sewage sludge, oxygen free-radical production (superoxide anion) was suppressed in phagocytes from the kidney.

Exposure to sewage sludge also resulted in a decrease in total serum proteins (Secombes *et al.* 1992) which agrees with our findings on the dab exposed to contaminated sediments. Although immunoglobulin levels were lower following sewage sludge exposure there was no effect on

serum lysozyme, which indicates that it was not simply a non-specific effect on all serum proteins. Differences recorded in our experiment following laboratory exposure to contaminated sediments could result from stress effects on protein synthesis due to laboratory confinement, a poor diet related to the wild fish, or undetected infections in the confined fish. The present consensus is that stress, including that induced by poor water quality, can impair immune function in fish and lead to decreased resistance to disease (Peters & Schwartzer 1985; Anderson 1990; Sindermann 1993; Vethaak 1993). Acute and delayed mortality of fish after exposure to aquaculture stressors such as handling, transportation and poor water quality is a major problem in fish culture and can result in considerable economic losses (Anderson 1990).

However, not all responses to stress are immunosuppressive; some stressors can be stimulatory (Dantzer & Kelley 1989) and the effects may be dependent on the concentration of the stressor (Dunier *et al.* 1991). As some aspects of the immune system may be stimulated while others are suppressed, it is necessary to apply a range of assays in order to obtain a clear evaluation of changes in the immune system. A range of assays have now been developed, with emphasis on the microplate methods for speed of data collection and reproducibility of results, and should be applied to measurements of stress on the immune system of fish.

This work was funded in part by the Department of the Environment contract 7/7/386. We appreciate the help of Barry Evans, Nicola Collingwood, Maggie Thomas and Jackie Coles in collecting the data for the environmental monitoring. The contaminated sediments experiment was carried out at MAFF laboratories, Weymouth under the direction of David Bucke.

References

Anderson, D. P. (1990). Immunological indicators: effects of environmental stress on immune protection and disease outbreaks. *American Fisheries Society Syposium*, **8**, 38-50.

Barton, B. A. & Peter, R. E. (1982). Plasma cortisol stress response in fingerling rainbow trout, *Salmo gairdneri* Richardson to various transport conditions, anaesthesia and cold shock. *Journal of Fish Biology*, **20**, 39-51.

Bayne, C. J. & Levy, S. (1991). The respiratory burst of rainbow trout *Oncorhynchus mykiss* (Walbaum) phagocytes is modulated by sympathetic neurotransmitters and the 'neuro' peptide ACTH. *Journal of Fish Biology*, **38**, 609-619.

Besedovsky, H. O., del Rey, A. E. & Sorkin, E. (1985). Immune-Neuroendocrine interactions. *Journal of Immunology*, **135**, 750-754.

Bly, J. E., Miller, N. W. & Clem, W. L. (1990). A monoclonal antibody specific for neutrophils in normal and stressed channel catfish. *Developmental and Comparative Immunology*, **14**, 211-221.

Bucke, D., Dixon P. F. & Feist, S. W. (1989). The measurement of disease susceptibility in dab *Limanda limanda* L. following long term exposure to contaminated sediments: preliminary studies. *Marine Environmental Research*, **28**, 363-367.

Chung, S. & Secombes, C. J. (1988). Analysis of events occurring within teleost macrophages during the respiratory burst. *Comparative Biochemistry and Physiology*, **89B**, 539-544.

Cooper, E. L., Peters, G., Ahmed, I .I., Faisal, M. & Ghoneum, A. (1989). Aggression in *Tilapia* affects immunocompetent leukocytes. *Aggressive Behaviour*, **15**, 13-22.

Dantzer, R. & Kelley, K. W. (1989). Stress and immunity: an integrated view of relationships between the brain and the immune system. *Life Sciences*, **44**, 1995-2008

Dando, P. (1980). Local fish stocks. Flounders. (In the Report of the Council). *Journal of the Marine Biological Association of the United Kingdom*, **60**, 1104-1105.

Donaldson, E. M. (1981). The pituitary-interrenal axis as an indicator of stress in fish. In *Stress and Fish* (ed. A. D. Pickering), pp. 11-47. Academic Press, London.

Dunier, M., Siwicki, A. K. & Demael, A. (1991). Effects of organophosphorus insecticides: effects of trichlorfon and dichlorvos on the immune response of carp (*Cyprinus carpio*). III *In vitro* effects on lymphocyte proliferation and phagocytosis and *in vivo* effects on humoral response. *Ecotoxicology and Environmental Safety*, **22**, 79-87.

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

Ellsaesser, C. F. & Clem, L. W. (1986). Haematological and immunological changes in channel catfish by handling and transport. *Journal of Fish Biology*, **28**, 511-521.

Fagerlund, U. H. M. (1970). Determining cortisol and cortisone simultaneously in salmonid plasma by competitive protein binding. *Journal of the Fisheries Research Board of Canada*, **27**, 596-600.

Faisal, M., Chiappelli, F., Ahmed, I. I., Cooper, E. L. & Weiner, H. (1989). Social confrontation "stress" in aggressive fish is associated with an endogenous opiod-mediated suppression of proliferative response to mitogens and nonspecific cytotoxicity. *Brain Behaviour and Immunity*, **3**, 223-233.

Khansari, D. N., Murgo, A. J. and Faith, R. E. (1990). Effects of stress on the immune system. *Immunology Today*, **11**, 170-175.

Livingstone, D., Lemaire, P., Matthews, A., Peters, L., Bucke, D. & Law, R. (1993). Pro-oxidant, antioxidant and 7.ethoxyresorufin O-deethylase (EROD) activity responses in liver of dab (*Limanda limanda*) exposed to sediment contaminated with hydrocarbons and other chemicals. *Marine Pollution Bulletin*, **26**, 602-606.

MacArthur, J. I. & Fletcher, T. C. (1985). Phagocytosis in Fish. In *Fish Immunology* (eds M. J. Manning & M. F. Tatner), pp. 29-46. Academic Press, London.

MacArthur, J. I., Thomson, A. W. & Fletcher, T. C. (1985). Aspects of leucocyte migration in the plaice, *Pleuronectes platessa* L. *Journal of Fish Biology*, **27**, 667-676.

Mazeaud, M. M. & Mazeaud, F. (1981). Adrenergic responses to stress in fish. In *Stress and Fish* (ed. A. D. Pickering), pp. 49-75. Academic Press, London.

McVicar, A. H., Bruno, D. W. & Fraser, C. O. (1988). Fish diseases in the North Sea in relation to sewage sludge dumping. *Marine Pollution Bulletin*, **19**, 169-173.

Milne, R. (1987). Pollution and politics in the North Sea. *New Scientist*, **19 November** 1987, 53-58.

Pankhurst, N. W., Wells, R. M. G. & Carragher, J. F. (1992). Effects of stress on plasma cortisol levels and blood viscosity in blue Mao mao, *Scorpius violaceus* (Hutton), a marine teleost. *Comparative Biochemistry and Physiology*, **101A**, 335-339.

Peters, G., Nubgen, A., Raabe, A. & Mock, A. (1991). Social stress induces structural and functional alterations of phagocytes in rainbow trout *Oncorhynchus mykiss*. *Fish and Shellfish Immunology*, **1**, 17-21.

Peters, G. & Schwartzer, R. (1985). Changes in hemopoietic tissue of rainbow trout under influence of stress. *Diseases of Aquatic Organisms*, **1**, 1-10.

Pick, E. & Mizel, D. (1981). Rapid microassays for the measurement of superoxide and hydrogen peroxide by macrophages in culture using an automatic enzyme immunoassay reader. *Journal of Immunological Methods*, **46**, 211-226.

Pickering, A. D. (1981). Introduction: the concept of biological stress. In *Stress and Fish* (ed. A. D. Pickering), pp. 1-9. Academic Press, London.

Pickering, A. D. & Pottinger, T.G. (1987). Poor water quality suppresses the cortisol response of salmonid fish to handling and confinement. *Journal of Fish Biology*, 30, 363-374.

Pickering, A. D. & Stewart, A. (1984). Acclimation of the interrenal tissue of the brown trout, *Salmo trutta* L., to chronic stress. *Journal of Fish Biology*, **24**, 731-740.

Pickering, A. D., Pottinger, T. G. & Carragher, J. F. (1989). Differences in sensitivity of brown trout, *Salmo trutta* L., and rainbow trout, *Salmo gairdneri* Richardson, to physiological doses of cortisol. *Journal of Fish Biology*, **34**, 757-768.

Pulsford, A. L., Lemaire-Gony, S. & Farley, S. R. (1992b). *Effects of Environmental Stress on Dab* Limanda limanda *Immune System*. I.C.E.S.C.M. 1992/E14 Marine Environmental Quality Committee.

Pulsford, A. L., Ryan, K. P. & Nott, J. (1992a). Metals and melanomacrophages in the flounder *Platichthys flesus* spleen and kidney. *Journal of the Marine Biological Association of the United Kingdom*, **72**, 483-498.

Pulsford, A. L., Thomas, M. E., Lemaire-Gony, S., Coles, J., Fossato, V. U. & Pipe, R. K. (1994a). Studies on the immune system of the goby, *Zosterisessor ophiocephalus*, from the Venice Lagoon. *Marine Pollution Bulletin* (in press).

Pulsford, A., Lemaire-Gony, S., Farley, S. R., Tomlinson, M., Collingwood, N. & Glynn, P. J. (1994b).

Effects of acute stress on the immune system of the dab *Limanda limanda*. *Comparative Biochemistry and Physiology* (in press).

Pulsford, A. L., Tomlinson, M., Lemaire-Gony, S. & Glynn, P. J. (1994c). Development and immunocompetence of the juvenile flounder, *Platichthys flesus*. *Fish and Shellfish Immunology* (in press).

Randall, D. J. & Perry, S. F. (1992). Catecholamines. In *Fish Physiology, Volume XII (Part B), The Cardiovascular System* (eds W. S. Hoar, D. J. Randall & A. P. Farrell), pp. 255-300. Academic Press, London.

Schreck, C. B. & Lorz, H. W. (1978). Stress response of coho salmon (*Oncorhynchus kisutch*) elicited by cadmium and copper and potential use of cortisol as an indicator of stress. *Journal of the Fisheries Research Board of Canada*, **35**, 1124-1129.

Secombes, C. J., Fletcher, T. C., O'Flynn, J. A., Costello, M. J., Stagg, R. & Houlihan, D. F. (1991). Immunocompetence as a measure of the biological effects of sewage sludge pollution in fish. *Comparative Biochemistry and Physiolology*, **100C**, 133-136.

Secombes, C. J., Fletcher, T. C., White M. J., Costello M. J., Stagg R. & Houlihan, D. F. (1992). Effect of sewage sludge on immune responses in the dab *Limanda limanda* L. *Aquatic Toxicology*, **23**, 217-230.

Seeley, K. R., Gillespie, P. D. & Weeks, B. A. (1990). A simple technique for the rapid spectrophotometric determination of phagocytosis by fish macrophages. *Marine Environmental Research*, **30**, 37-41.

Selye, H. (1950). Stress and the general adaptation syndrome. *British Medical Journal*, **1**, 1383-1392.

Sindermann, C. J. (1993). Interactions of pollutants and disease in marine fish and shellfish. In *Pathobiology of Marine and Estuarine Organisms* (eds J. A. Couch & J. W. Fournie), pp. 451-482. CRC Press, London.

Tahir, A., Fletcher, T. C., Houlihan, F. & Secombes, C. J. (1993). Effects of short-term exposure to oil-contaminated sediments on the immune response of dab, *Limanda limanda*. *Aquatic Toxicology*, **27**, 71-82.

Thomas, P. & Robertson, L. (1991). Plasma cortisol and glucose stress responses of red drum (*Sciaenops ocellatus)* to handling and shallow water stressors and anesthesis with MS-222, quinaldine suphate and metomidate. *Aquaculture*, **96**, 69-86.

Tripp, R. A., Maule, A. G., Schreck, C. B. & Kaattari, S. L. (1987). Cortisol mediated suppression of salmonid lymphocyte responses *in vitro*. *Developmental and Comparative Immunology*, **11**, 565-576.

Vethaak, A. D. (1993). *Fish diseases and marine pollution*. Thesis, University of Amsterdam.

Weeks, B. A. & Kavas, A. F. (1979). Macrophage chemotaxis and phagocytosis in guinea pigs: influence of age and nutrition. *Journal of the Reticuloendothelial Society*, **26**, 502-506.

Weeks, B. A. & Warinner, J. E. (1984). Effects of toxic chemicals on macrophage phagocytosis in two estuarine fishes. *Marine Environmental Research*, **14**, 327-335.

Weeks, B. A., Huggett, R. J. & Hargis, W. J. (1990a). Integrated chemical, pathological and immunological studies to assess environmental contamination. In *In situ Evaluation of Biological Hazards of Environmental Pollutants* (eds S. S. Sandhu, W. R. Lower, F. J. De Serres, W. A. Suk & R. R. Tice), pp. 233-240. Plenum Press.

Weeks, B. A., Huggett, J. E., Warinner, J. E. & Matthews, E. S. (1990b). Macrophage responses of estuarine fish as bioindicators of toxic contamination. In *Biomarkers of Environmental Contamination* (eds J. F. McCarthy & L. R. Shugart), pp. 193-201. L. R. Lewis Publishers, CRC Press, Florida.

Weeks, B. A., Keisler, A. S., Warinner, J. E. & Matthews, E. S. (1987). Preliminary evaluation of macrophage pinocytosis as a technique to monitor fish health. *Marine Evironmental Research*, **22**, 205-213.

Weeks, B. A., Warinner, J. E., Mason, P. L. & McGinnis, D. S. (1986). Influence of toxic chemicals on the chemotactic response of fish macrophages. *Journal of Fish Biology*, **28**, 653-658.

Zelikoff, J. T. (1993). Metal pollution-induced immunomodulation in fish. *Annual Review of Fish Diseases*, **3**, 305-325.