Stress Response of Red Snapper (*Lutjanus argentimaculatus*) Exposed to Sublethal Concentrations of Crude Oil

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Summary

Stress response indices including plasma cortisol, glucose, cholesterol, and total plasma proteins of red snapper (Lutjanus argentimaculatus) exposed to three different concentrations of water soluble fraction (WSF) of a crude oil were investigated. The experiment was conducted in a flow-through seawater system under tropical conditions and the concentrations of the toxicant were assayed based on naphthalenes contents of WSF of crude oil as 0.107, 0.052 and 0.028 ppm. Sublethal exposure to WSF of crude oil had a marked impact ($P<0.05$) on plasma cortisol, glucose, cholesterol and total plasma protein. Dose-response relationships were observed in most of the affected parameters. The results revealed that, in the experimental fish both the primary and secondary stress responses were activated following exposure to different concentrations of the toxicant. The immunosuppressive effects of WSF of crude oil on fish following activation of stress responses are discussed.

Key words: stress response, crude oil, cortisol, glucose, cholesterol, plasma proteins, red snapper, fish

Introduction

Crude oil is one of the most widespread and dangerous substances that pollute the oceans of the world (Simonov *et al* 1984, Sikkema *et al* 1995). It is an extremely complex mixture of hydrocarbon compounds (Kiceniuk & Khan 1983). In spite of the fact that various parts of tropical marine and estuarine environments are

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contaminated by crude oil (Nain *et al* 1998, Zakaria *et al* 2001), as yet there is no available information on the pathophysiology of petroleum hydrocarbons (PH) on fish in this region.

To induce physiological response in fish using PH as a stressor, Thomas *et al.* (1980) showed that exposure to WSF of No. 2 fuel oil influences interrenal activations followed by pathological responses in striped mullet (*Mugil cephalus*). They realised that plasma cortisol as the primary response rises significantly in fish exposed to 5, 10 and 20% of toxicant but not in those exposed to 1%. In another study, plasma cortisol remained elevated in mummichog (*Fundulus heteroclitus*) continuously exposed to naphthalene for 15d (DiMichele & Taylor 1978). Cortisol levels in both studies were reported to be proportional to the toxicant concentrations in the water. Similar to other stressors such as pesticides (Carballo *et al* 1995, Studnicha *et al* 2000), and handling conditions (Warning 1992), exposure to PH is known to produce chemical and biochemical (DiMichele & Taylor 1978, Thomas *et al.* 1980, Aabel & Järvi 1990) changes in fish blood picture. As the secondary responses, hyperglycaemia, increase in plasma cholesterol and osmolality were reported in striped mullet by Thomas *et al.* (1980) and Branuer *et al* (1999) in short-term exposure to WSF of crude oil.

Red snapper is an important commercial species throughout the Indo-Pacific region (Allen 1985) and has been introduced to mariculture system in some Southeast Asian countries. The purpose of present study was to determine whether sublethal concentration of a Malaysian crude oil causes any primary and secondary responses in red snapper. The dose response relationship based on exposure to naphthalenes contents of crude oil, the time course of elevated plasma cortisol level and changes in some of the blood parameters were investigated.

Materials and Methods

Exposure design and sampling of the fish. Thirty-five red snappers (211±15g) were exposed continuously to each of the sublethal concentrations of WSF of crude

oil containing 0.107, 0.053 and 0.028ppm naphthalenes. The exposure systems consisted of 3500 l concrete tanks fed with flow-through seawater, which has been described in Akbari *et al* (2002). The experiment was run in three replicates and maintained under identical conditions for each of the three concentrations. Control fish were maintained similarly but without oil. All fish were continuously exposed to WSF of crude oil for 96h, and fed with trash fish at 3% body weight per day. Unconsummated food was removed daily from the tanks by suction.

At 0, 1, 3, 6, 12, 24, 48 and 96h post exposure, five fish were gently transferred from stock tanks to a 100 l tank containing 200ppm tricaine methane sulphonate (MS222) in seawater, as the effective concentration for anaesthesia of *L.argentimaculatus*. After the fish were anaesthetised, they were bled from their caudal vein, using 5ml heparinized syringe, 23 gauge needle during 4-5min procedure. To prevent stress due to sudden changes in pH value, as the fish were introduced to MS222 solution, an alkaline reagent made by dissolving 100g of sodium citrate and 5g of sodium hydroxide in 500ml of water (Parsons *et al* 1984) was added to MS222 treated seawater. The blood (3ml) was collected into heparinized tubes and used stepwise for biochemical studies and cortisol assay. The blood was centrifuged at 10500rpm to separate plasma for 5min. The plasma from each sample was transferred into 300µl sterile plastic vials and kept in -80°C for cortisol and biochemical analysis.

Measurement of plasma glucose, plasma cholesterol, and total plasma protein. The samples were allowed to thaw at room temperature. The biochemical parameter: plasma glucose, plasma cholesterol, and total plasma protein were analysed, using diagnostic kits (Sigma). The samples for determination of each parameter were transferred to the tubes containing corresponding reagents for development of the colours at defined time for reaction in each tube. After the colour development for total plasma protein, plasma glucose and plasma cholesterol their absorbencies were read on spectrophotometer at 750, 505 and 500nm, respectively. The absorbencies of these parameters were converted to concentrations of each parameter by comparing

them with their standard concentration curves.

Measurement of cortisol level in plasma. Analysis of cortisol was done by radioimmunoassay (RIA) method described by Sufi et al (1983). Based on this method, radiolabelled (hot, also known as trace) cortisol was added to standards containing known amounts of radioinert (cold) cortisol, and to plasma samples (100µl) containing unknown amounts of cold cortisol. One hundred microlitres of polyclonal, antisera capable of binding about 50% of the hot cortisol was added and allowed to reach dynamic equilibrium at 4°C for 24h. Dextran-coated charcoal was used to absorb the unbound steroids and removed from the suspension by centrifuging at 4°C. Supernatant containing the antibody-bound hormone was counted by scintillation counter in pg/ml of plasma samples. Cortisol level of the plasma samples was determined by using the WHO immunoassay Data Processing Program, Version 5.1 with an Apple IIe computer.

The precision of the assay, inter-assay and intra-assay variations were determined by inserting triplicate quality control (QC) tubes in different positions for all assays and between five assays, respectively. The variations in plasma cortisol and biochemical parameters of blood at each sampling time and also for different exposure concentrations were calculated by analysis of variance (ANOVA). Multiple range analysis was used to identify the differences at various exposure times and also for each concentration. Significant differences between each parameter were stabilised at the 0.05 levels. The relationship between all transformed variations (square root of Y+0.5) and each variation with transformed exposure concentrations of naphthalenes were compared by using correlation analysis.

Results

When the fish were introduced into the experimental tanks, they lost schooling behaviour. They were anorexic and breathing faster than the fish in control tanks. This behaviour was observed until the end of the experiment in the tanks with the two highest concentrations of the toxicant.

Changes in primary stress response. Plasma cortisol levels were elevated in fish exposed to different concentrations of WSF of crude oil (Fig. 1A). There was similar corticosteroid response in fish exposure to crude oil containing 0.053 and 0.028ppm of naphthalenes, but the magnitude of the rise was lower. Dose-response relationships and significant differences ($P<0.05$) were observed in plasma cortisol levels at elevated points after exposure to various concentrations of crude oil.

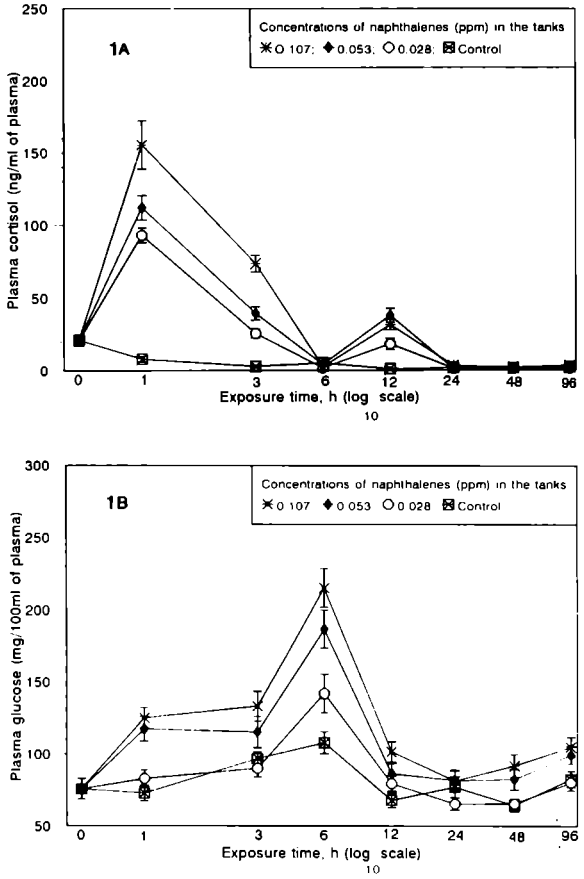
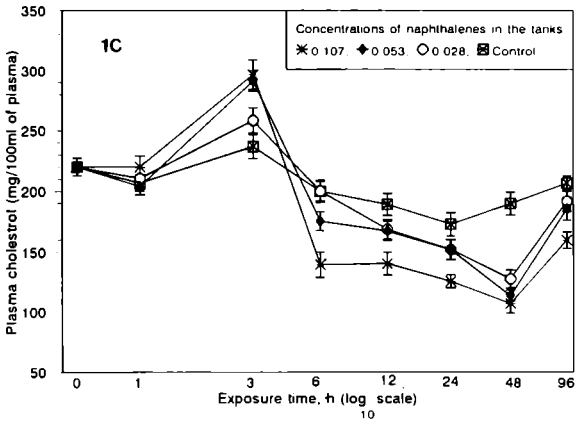
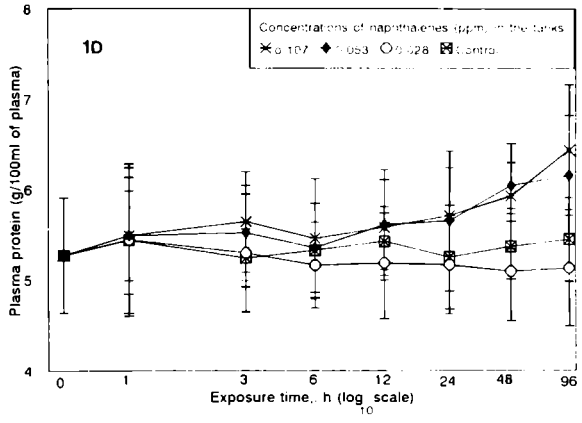


Figure 1. *Plasma cortisol and biochemical indices of blood* in L.argentimaculatus exposed to different concentrations of WSF of crude oil during 96 h sublethal toxicity test (1A) plasma cortisol, (1B) plasma glucose, (1C) plasma cholestero,l (1D) total plasma protein. Each value represents the Mean±SD of minimum nine fish*





Changes in secondary stress responses. Exposure to crude oil caused an increase in plasma glucose level of red snapper (Fig. 1B). The highest exposure dosage of toxicant caused an increase in plasma glucose up to 215±13.6mg/dl. Hyperglycaemia was noted until the end of the experiment, except a short decrease in plasma glucose level at 24h exposure. There were significant differences ($P<0.05$) and also dose-response relationship in the hyperglycaemic response to different dosages of the toxicant. Red snapper also demonstrated an increase in plasma cholesterol 3h after exposure to different concentrations of crude oil (Fig. 1C). The increase in plasma cholesterol was not long lasting. The highest exposure

concentration of crude oil caused an increase in plasma cholesterol up to 296.44±12.3mg/dl at 6h and down to 107.1±7.67mg/dl at 48h after exposure to crude oil. Changes in plasma cholesterol of fish were significantly different ($P<0.05$) when compare to the control for all concentrations of crude oil in exposed water. There was a dose-response relationship in the plasma cholesterol during the exposure.

Exposure to different concentrations of crude oil caused an increase in total plasma protein for the two highest exposure concentrations of crude oil containing 0.107 and 0.053ppm naphthalenes. There was continuous elevation in total plasma protein after 12h exposure (Fig. 1D). The total plasma protein increased markedly ($P<0.05$) at 48h after exposure, and further increased to 6.44±0.72 and 6.16±0.66g/dl at 96h for the exposure concentrations of crude oil, respectively. Statistically, dose-response relationship was not observed after exposure but the changes were significant in the fish exposed to the two higher concentrations of toxicant.

Correlation analysis of cortisol and biochemical parameters showed positive relationship between changes in the cortisol level and the other three parameters, but at different significance levels (Table 1).

Discussion

Exposure to WSF of crude oil produced changes in primary and secondary stress responses in red snapper during 96h study. The changes mimic those that occur as part of the response of fish to acute stress (Pickering 1984, Carballo *et al* 1995). The observations in this study on a tropical fish confirm most of changes with related studies, conducted in the temperate regions (DiMichele & Taylor 1978, Thomas *et at* 1980, Aabel and Järvi 1990, Brauner *et al* 1999).

Changes in primary stress response. In this study, induction of hypothalamo-pituitary-interrenal axis was observed by the production of a corticosteroid followed

by hyperglycaemic stress response. There was no correlation between the exposure time and induction of cortisol as the primary stress response. The plasma cortisol concentration started to decrease to control values after 1 h of exposure that was when, the concentration levels of naphthalenes were stable in the exposure water. The cortisol levels were at normal concentrations by 24 h, although the naphthalenes in exposure water were still stable. The hypothalamo-pituitary-interrenal axis did not become refractory to further stimuli until the end of experiment.

Table 1. *A correlation analysis of transformed (square root of Y+0.5) variations of plasma cortisol and blood parameters of L. argentimaculatus exposed to different concentrations of water soluble fractions of crude oil during 96h sublethal toxicity test*

Parameters	Cortisol	Cholestrol	Protein	Glucos
Concentration of	0.301[a]	-0.274	0.553	0.434
toxicant	(32)[b]	(32)	(32)	(32)
	0.093[c]	0.129	0.001	0.013
	-0.491	-0.336	0.316	-0.201
Exposure time	(32)	(32)	(32)	(32)
	0.004	0.060	0.079	0.270
	1.000	0.480	0.033	0.179
Cortisol	(32)	(32)	(32)	(32)
	0.000	0.005	0.859	0.327
	0.480	1.000	-0.319	0.024
Cholestrol	(32)	(32)	(32)	(32)
	0.005	0.000	0.075	0.896
	0.033	0.319	1.000	0.128
Protein	(32)	(32)	(32)	(32)
	0.859	0.075	0.000	0.486
	0.179	0.024	0.128	1.000
Glucose	(32)	(32)	(32)	(32)
	0.327	0.896	0.486	0.000

[a] Coefficient, [b] (sample size), and [c] significance level

By 12 h, when the exposure concentrations of naphthalenes were stable, a small secondary rise in cortisol concentrations occurred. Biphasic response in cortisol

secretion has been reported in sockeye salmon (*Oncorhynchus nerka*) (Donaldson and Dye 1975) exposed to low concentration of copper and in striped mullet (Thomas *et al* 1980) exposed to WSF of No.2 fuel oil.

Changes in plasma cortisol in the present study are similar to the findings of Thomas *et al* (1980), but the magnitude of response was higher. They exposed striped mullet to different concentrations of WSF of No.2 fuel oil and found that the level of plasma cortisol, at the highest exposure concentrations of oil (containing 0.366ppm naphthalenes), was calculated as 116ng/ml of plasma after 1h exposure. Red snapper also showed a different cortisol response when compared to mummichog in DiMichele and Taylor (1978) study. They exposed mummichog to different concentrations of naphthalene for 15 d. The level of cortisol in their study at the end of experiment was 229ng/ml of serum in the fish exposed to 0.2 ppm naphthalene.

In the present study, based on time-response changes in the levels of plasma cortisol, after an initial increase, adaptation to WSF of crude oil occurred in the fish, since concentration of toxicant were stable in the tanks. Adaptation in corticosteroid response has been observed in mullet exposed to PH (Thomas *et al* 1980) and in brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus m*ykiss) under crowding conditions (Pickering & Pottinger 1987). In the last report, despite the relatively rapid interrenal acclimation, the WBC counts of both species were significantly reduced during the period of crowding.

The fall in corticosteroid response, after reaching a maximum level in 1h after exposure to toxicant, may be due to inhibition of adrenocorticotrophic release in a feed back mechanism. The evidence of corticosteroid feed back on adrenocorticotrophic secretion from pituitary gland has been reported in goldfish (*Carassius auratus*) by Fryer & Peter (1977). Also, it can be suggested that induction of hepatic mixed function oxygenase (MFO) by polyaromatic hydrocarbons in fish increase cortisol metabolism and its clearance from the blood.

It has been reported that steroid compounds are endogenous substrates for certain types of MFO (Jimenez & Stegeman 1990). DiMichele & Taylor (1978) reported another case study, which showed the inhibition of corticosteroid response, in mummichog. They investigated pathological effects of naphthalene on different vital organs of the fish together with assaying serum cortisol level. They found that necrosis of interrenal cells was correlated with decreasing in serum cortisol. However, according to Mezeaud *et al* (1977) a high initial response to stress, as was observed in the present study, may result in subsequent depletion of the corticosteroid reserve which accelerates the passage of the fish into the third phase of stress response i.e., exhaustion.

Lymphoid tissue and lymphocyte's activity in fish is strongly suppressed by corticosteroids secreted from the interrenal tissue (Pickering 1984, Pickering *et al.* 1987, Carballo *et al* 1995). Immunosuppression is usually associated with increase infectious diseases (Pickering & Pottinger 1987, Khan 1990, Khan *et al* 1994, Carballo *et al* 1995, Studnicha *et al* 2000). Information on the immunosuppressive effects of crude oil on fish, are mostly involve parasitic infection. Depending on the location of the parasites in the fish, exposure to crude oil has different prognoses (Khan and Kiceniuk 1988, Khan 1987b, 1990, 1991). The prevalence and intensity of gut parasite infected winter flounder (*Pseudopleuronectes americanus*) and Atlantic cod (*Gadus morhua*) exposed chronically to WSF of crude oil were very low comparing to the control (Khan and Kiceniuk 1983). The workers concluded that direct toxic effect of drinking water, which contains WSF of crude oil and/or any modification of hydrocarbons by the gut, might have caused the low number of worms in the host. Both prevalence and intensity of monogeneids, *Gyrodactylus* sp., were higher in the Atlantic cod exposed to WSF of Venezuelan crude oil comparing to the control (Khan and Kiceniuk 1988). The same results were obtained with tricodinosis in Atlantic cod, longhorn sculpin (*Myoxocephalus octodecemspinosus*) and sculpin (*Oligocottus maculosus*) chronically exposed to crude oil (Khan 1990).

The effects of blood protozoan on cod and winter flounder in different life stages, chronically exposed to contaminated sediment were the most convincing evidence (Khan 1987a, 1987b). Mortality in juvenile and adult of winter flounder was 89% and 46% respectively for the trypanosome-infected and oil-treated fish. The mortality of control fish in this experiment was much lower and similar results were obtained for the subadult cod (Khan 1987a).

The adaptive nature of stress response of the experimented fish could mainly be related to catabolic action of corticosteroid and catecholamines. It has been revealed that, the fish increases its energy expenditure in an attempt to maintain homeostasis (Schreck 1981). It has long been recognised that different kinds of stress induce an elevation in plasma glucose level and changes in plasma cholesterol (DiMichele & Taylor 1978, Thomas *et al* 1980). The change in energy flow to the gills during stress condition also is responsible for disturbance of active Na^+ /K^+ pump between water and blood through the gills. In fish adapted to seawater there is an increase in plasma osmotic pressure accompanied by increasing in total plasma protein (Mazeaud *et al* 1977, Mazeaud & Mazeaud 1981).

Changes in secondary stress responses. In the present study, elevation of cortisol levels was accompanied by a marked rise in plasma glucose, changes in cholesterol and total plasma protein. The correlation between corticosteroid response, 1 h after exposure to various doses of WSF of crude oil, and an increase in glucose concentrations 2h later, was not an unexpected phenomenon. Hyperglycaemic stress response is mediated via increases in plasma corticosteroid and catecholamine concentrations (Mazeaud et al 1977, Mazeaud & Mazeaud 1981). High cortisol concentrations were associated with hyperglycaemia in mummichog (DiMichele & Taylor 1978) exposed to naphthalene, in mullet (Thomas et al 1980) exposed to WSF of No.2 fuel oil and in Atlantic salmon (Aabel & Järvi 1990) exposed to crude oil. In the last study plasma cortisol was not investigated. Various

duration of hyperglycaemia has been reported in above mentioned reports which suggest that the duration of hyperglycaemia depend on species under investigation.

There was a correlation between the level of plasma cortisol and the presence of cholesterol in the blood. Thomas *et al* (1980) report showed hypercholesterolemia in mullet during exposure to WSF of No.2 fuel oil. In the present study, there was an increase in plasma cholesterol 3h after exposure, which declined under control level 3h later. Hypocholesterolemia have been reported in eel (*Anguilla anguilla*) by Ferrando & Andreu-Moliner (1991) and in carp (*Cyprinus carpio*) by Guth & Hanke (1985) exposed to lindane. According to these authors, the reduction in circulating cholesterol level is believed to cause by higher utilisation of cholesterol during corticosteroidogenesis, as it is the precursor for steroid hormones, which are secreted during stressful conditions. The initial elevation of plasma cholesterol, in the present study, could be explained by the fact that the fish were feeding well before exposure to toxicant and cholesterol production could be enhanced in the liver. After exposure to WSF of crude oil, anorexia was common in experimental fish, which may have caused the decrease of plasma cholesterol. Cellular and biochemical alterations in the liver of oil-exposed fish are common (Sabo and Stegeman 1975, DiMichele and Taylor 1978). However, according to Mazeaud & Mazeaud (1981), who reviewed lipid metabolism in teleost under stress condition, reported that "the consequences of stress on lipid metabolism are far from clear".

Changes in total plasma protein in the present study showed a typical response of marine fish in stressful conditions. Elevations of total plasma protein in this study resembled the study by DiMichele & Taylor (1978) who exposed mummichog to different concentrations of naphthalene. They found a significant dose-response relationship in elevation of total plasma protein of the fish exposed to more than 0.02ppm naphthalene compared to the control, but it was not observed in fish exposed to 0.002ppm.

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