

## **Parasitism and Phagocytic Function Among Sand Lance *Ammodytes hexapterus* Pallas Exposed to Crude Oil-Laden Sediments**

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Chemical exposure, especially to petroleum hydrocarbons, can result in altered immune response as well as decreased resistance to pathogens. For example, juvenile chinook salmon, *Oncorhynchus tshawytscha*, from urban estuaries polluted with PCBs and aromatic hydrocarbons demonstrated both altered humoral response and increased susceptibility to bacterial challenge (Arkoosh et al. 1998). Hydrocarbon exposure in demersal fishes often results in an increase in gill parasites (Khan and Thulin 1991, MacKenzie et al. 1995) as well. Changes in parasite levels have been proposed as a biomarker of demersal fish health. The link between hydrocarbon-induced immuno-suppression and increases in gill parasites, although often theorized, has never been tested.

Adult Pacific sand lance, *Ammodytes hexapterus*, inhabit the nearshore sand habitat, an area especially vulnerable to oil contamination. These fish serve as one of the most important forage fishes in both North Pacific and North Atlantic waters, forming an important link between zooplankton and larger consumers in the food chain. Their relatively small size, large abundance, and high fat content make them preferred prey for both salmon, which target larval sand lance, and groundfishes, which prey on both larvae and adults. Sand lance are also important prey for a wide range of marine mammals and birds, presumably serving as a vector for moving lipophilic pollutants to higher trophic consumers.

The monogenetic gill trematode *Gyrodactylus* sp. has been reported in greater abundance in both Atlantic cod, *Gadus morhua*, exposed to aromatic hydrocarbons in the water column (Khan and Kiceniuk 1988) and in American plaice, *Hippoglossoides platessoides*, exposed to natural sediments contaminated with mixtures of aromatic hydrocarbons and PCB's (Marcogliese et al. 1998). These investigators speculated that the increase in parasitism may have been the result of immunosuppression. To examine the possibility that susceptibility to parasites is coupled with reduced non-specific immune response, we exposed adult Pacific sand lance to oil contaminated sediments for three months. We tested whether oiled sediment exposure could be correlated with either trematode prevalence or such non-specific immune responses as superoxide anion production by activated neutrophils or percent phagocytosis.

## MATERIALS AND METHODS

Adult sand lance were collected by seine from a skiff in Lynn Canal, Alaska, on 15 August, 1998 and transported to the Auke Bay Laboratory in Juneau, Alaska, where they were held for five weeks to ensure active feeding before testing. The 700 L fiberglass holding tank had a 3 cm layer of sand sediment (250  $\mu\text{m}$  average grain size) and received a flowthrough input of saltwater (30 ‰ salinity, 8°C) of 3 L/min. Fish averaged 5.3 g (11.5 cm total length) and were fed *ad libitum* on a combination of newly hatched *Artemia* sp. and frozen mysids during the short period every morning when they emerged from the sand.

Oiled and control sediments were prepared using the methods of Moles and Norcross (1998). Alaska North Slope crude oil was artificially weathered at 70°C overnight until its initial mass was reduced by 30% (Carls et al. 1998). This removed monoaromatic fractions, simulating the composition of oil present in sediments following chronic pollution or an oil spill. The oil was mixed with sieved sand sediment at 0.05% and 0.005% by volume, corresponding approximately to the concentrations of hydrocarbons in the shallow subtidal (10-30 m) following the *Exxon Valdez* oil spill (O'Clair et al. 1996). At day 0, sediments from each tank were sampled for total hydrocarbons by ultraviolet fluorescence (Short et al. 1996). Initial concentrations were 61  $\mu\text{g/g}$  and 28  $\mu\text{g/g}$  total hydrocarbons. There is good agreement between ultraviolet fluorescence estimates of total hydrocarbons and data derived from the more expensive GC/MS measurements of total polynuclear aromatic hydrocarbons (TPAH), which often constitute only about 2% of the hydrocarbons present in crude oil (Babcock et al. 1996).

To determine the effects of oiled sediment on phagocytic function and parasite abundance (the number of parasites per infected host), we exposed 126 adult Pacific sand lance (3 concentrations x 3 replicate tanks/concentration x 14 fish/tank) to one of three concentration (0, 10, and 61  $\mu\text{g/g}$  total hydrocarbons - not TPAH) of oil in sand for 90 d beginning 21 September, 1998. Each tank received a constant flow of ambient seawater at 3L/min at 28 ‰ salinity and 8°C. Fish were fed throughout the experiment as previously described. At the end of the test, fish from each tank were sampled for superoxide production, phagocyte function, and gill parasites.

To determine the ability of activated neutrophils to produce superoxide anions, we used the nitroblue tetrazolium (NBT) slide assay developed by Anderson et al. (1992). The production of oxygen radicals reduces a soluble NBT dye to insoluble formazan. Blood from the severed caudal peduncle of anaesthetized (MS-222) fish was removed using ammonium-heparinized capillary tubes. A drop of blood from each fish was placed on each of two glass coverslips, which were incubated at 22°C for 30 min in petri dishes with a wet paper towel. This allowed the activated neutrophils to adhere to the glass. The coverslips were

rinsed with phosphate buffered saline - PBS (pH 7.4) and inverted onto a slide with a drop of 0.2% filtered NBT solution for 30 min. Four random fields on each coverslip were examined at 280x for the presence of dark blue staining neutrophils. The eight fields were averaged for each fish.

We evaluated *ex vivo* ingestion capacity of phagocytes using a modification of the technique of Collazas et al. (1995). After collecting sufficient blood for the NBT test, the remaining blood in the fish was drained into a petri dish, which was incubated at 22°C for 30 min. The adherent monolayer was rinsed with PBS (pH 7.4). Latex beads of 1.09 µm diameter (20 µm diluted to 1% in PBS) were added and incubated at 22°C for 30 min. The plates were washed with PBS, fixed with methanol, and stained with Wright-Giemsa dye. The percentage of phagocytes containing ingested particles was read in four random fields at 280x .

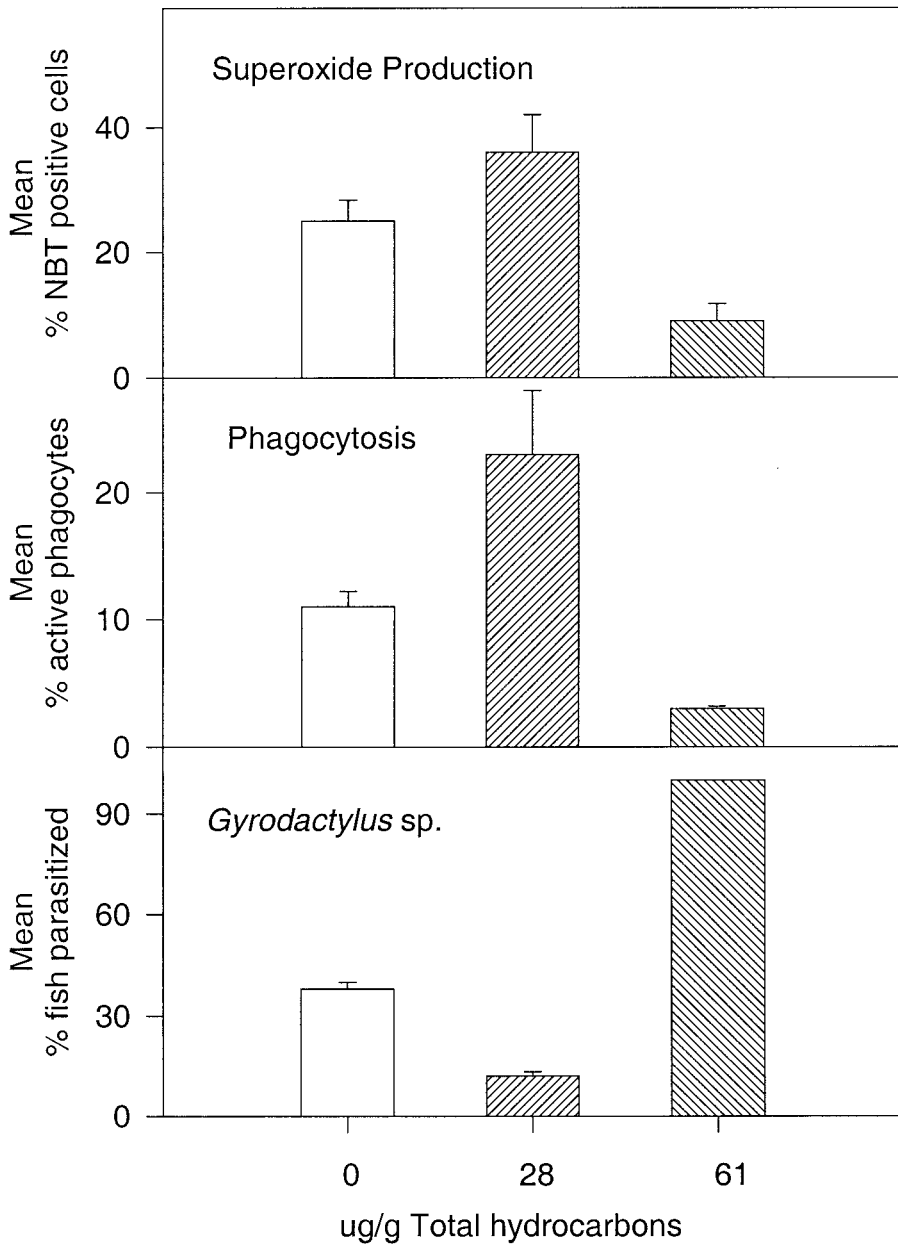
All percentage values for superoxide production and phagocytosis were arcsin transformed and analyzed using a two-way nested ANOVA for significant differences due to replicate tanks ( $p>0.87$ ). Data from replicate tanks were then combined for a total of 42 fish per treatment. The effect of oil exposure on phagocyte function was analyzed using a one-way ANOVA followed by a Dunnett's test to compare treatment means;  $p<0.05$  was taken as the minimum significance level.

After blood was sampled, the head was excised, removed, and the gills were immersed in a 0.025% solution of formalin for 40 min to release attached parasites. Additional hot formalin was added to yield a 10% solution for fixation. Prevalence (the percentage of fish with the parasite) and abundance was determined for each treatment. The prevalence of fish infected with *Gyrodactylus* sp. was compared between treatment groups using 2X2 contingency tables analysis. Parasite abundance for each fish was divided by fish length to account for the possible influence of fish size on parasite abundance. Distribution of the abundance data was analyzed by using the non-parametric Mann-Whitney U test. A probability of 0.05 or less was judged to be statistically significant.

## RESULTS AND DISCUSSION

Prevalence of *Gyrodactylus* sp. on the gills was inversely related to the non-specific immune response of the sand lance exposed to oiled sediment (Figure 1). Exposure to the low concentration of oiled sediment (10 µg/g) resulted in significantly enhanced superoxide production and phagocytosis ( $p<0.05$ ) relative to controls. Sand lance at this concentration had fewer gill trematodes than the controls. In contrast, exposure to the high concentration (61 µg/g) resulted in significantly reduced superoxide production and phagocytosis, but both the prevalence and mean abundance of *Gyrodactylus* sp. infection increased.

The NBT reduction assay measures superoxide production to determine the ability



**Figure 1.** Superoxide production, phagocytosis, and percent of fish infested with *Gyrodactylus* sp. in Pacific sand lance exposed to weathered hydrocarbon laden sediments. Error bars are SE.

of leucocytes to catabolize phagocytosed particles using a respiratory burst. Exposure to low concentrations of weathered hydrocarbons in the current experiment stimulated superoxide production in sand lance, whereas exposure to the higher concentration of hydrocarbons interfered with the activity of the leucocytes. Fish exposed to the low concentration of oiled sediment responded with the highest numbers of glass-adherent NBT-positive cells. An average of 36% of the neutrophils were NBT-positive at the low dose, as compared to 25% in the control groups. The percent of NBT-positive cells was significantly reduced at the high concentration; only 9% of the neutrophils stained positive.

The phagocytosis slide assay measures the percentage of active phagocytes. Phagocytic activity was stimulated as shown by increased burst activity and percent phagocytosis at the low concentration. Exposure to 61  $\mu\text{g/g}$  total petroleum hydrocarbons, however, suppressed phagocytotic activity after 90 d. Phagocytosis was also significantly elevated among fish exposed to 10  $\mu\text{g/g}$  TPH: 23% versus 11% among fish from control tanks. Only 3% of the samples from 61  $\mu\text{g/g}$  tanks showed phagocytic activity.

Prevalence of *Gyrodactylus* sp. among control sand lance after 90 d of exposure was 38% with a mean abundance of 3 parasites per fish (Fig. 1). The trematode was present in only 14% of the fish exposed to the low dose, with a mean abundance of 1.5 parasites per fish. All fish in the high dose had some trematodes with a mean abundance of 17.5 parasite per fish. Both prevalence and abundance differed significantly from controls ( $p < 0.05$ )

No fish died during the 90 d exposure; narcosis and distinctive lesions noted by Pearson et al. (1984) after 48 hr of exposure to 100-1000 p.p.m. in oiled sand were not present, probably due to the weathered nature and much lower concentrations of oil used in our study. Weathered oil, such as is present in subtidal sediments following an oil spill, contains virtually none of the monoaromatic hydrocarbons such as were present in oil effects studies conducted in the 1970s and 1980s.

Gill parasites proliferate among fish exposed to high concentrations of crude oil, often in conjunction with alterations in blood parameters. Marcogliese et al. (1998) observed a high abundance of *Gyrodactylus* sp. on the gills of American plaice held for five months on naturally contaminated sediments containing 40  $\mu\text{g/g}$  polynuclear aromatic hydrocarbons (TPAH) in addition to various PCB's. Khan and Kiceniuk (1988) also found an increase in *Gyrodactylus* sp. among Atlantic cod *Gadus morhua* exposed for four months to water soluble fractions of crude oil. Other gill parasites, such as the ciliated gill parasite *Trichodina* sp., are also more prevalent in demersal fishes reared on sediments containing high concentrations of crude oil (~32  $\mu\text{g/g}$  PAH) than on controlled sediments (Khan 1991; Moles and Norcross 1998). Our's is the first laboratory study to note increases in gill parasites in sediments at the low concentrations of crude oil that might be encountered in the subtidal regions following an oil spill.

It is likely that these fish were immunologically compromised due to the stress of exposure to oiled sediments. Longhorn sculpin *Myoxocephalus octodecemspinosus* exposed to oiled sediment had fewer lymphocytes and macrophage aggregates coupled with increases in *Trichodina* sp. (Khan 1991). Moles and Norcross (1998) also noted declines in macrophage aggregates and an increase in *Trichodina* sp. infestation among juvenile flatfishes of various species following exposure to oiled sediments.

Crude oil exposure can stimulate non-specific immune responses in fish. For example, Pacific herring exposed to weathered crude oil had fewer tissue leucocytes (Carls et al. 1998). Abundance of pericholangial eosinophilic glandular leucocytes also declined as the concentration of crude oil and the subsequent incidence of viral infection increased. Several studies have demonstrated that stress hormones can suppress immune function (eg., Weeks et al. 1992), suggesting that xenobiotic exposure acts as a stressor. Subsequent infestation with parasites would be as natural an outcome of a compromised immune system as would a pathogenic infection.

The pattern of an increase in immune activity with low concentrations of crude oil and reductions at higher doses is similar to the changes in macrophage aggregates following oil exposure noted by several investigators (Payne and Fancey 1989; Moles and Norcross 1998). These authors reported increases in the number of macrophage aggregates at low concentrations but declines in the number of aggregates at higher concentrations. Payne and Fancey (1989) hypothesized that low concentrations of oil may activate macrophage activity, whereas higher levels may reduce the number. Tahir et al. (1993) found increased numbers of lymphocytes and hematocrits at low concentrations of fuel oil but reductions at higher concentrations. In the present study, higher levels of exposure not only resulted in lower non-specific immune responses but also were correlated with an increase in the prevalence of the gill trematodes. Conversely, when the immune response was elevated, both prevalence and abundance of the parasite infestations was reduced. The reductions in activity at the higher concentrations may indicate that the system was overwhelmed.

Immune-based biomarkers are useful measures of environmental quality. The stress associated with exposure to PAH can both stimulate and suppress phagocytic activity, depending on concentration. The prevalence and abundance of gill parasites appears to be correlated with phagocytic activity and may provide a field estimate of animal health. If the ability of demersal fishes to tolerate gill parasites is a function of their non-specific immune system, hydrocarbon concentrations elevated enough to suppress the immune system would likewise result in increased numbers of parasites.

These reductions in health following exposure to very low concentrations of oil suggest that sand lance are very sensitive to oil exposure. In addition, sand lance are particularly susceptible to the effects of oil because of their intimate

association with a single type of sediment. Unlike other forage fishes, the eel-like sand lance lead a semi-pelagic existence by day and bury themselves at night in soft bottom sediments to avoid predators; they overwinter by burying in sediments. Sand lance have a definite preference for fine or coarse sand substrate and have rarely been found in other substrates such as silt, clay, granule, or rock. Because fine grained littoral sediments are also the major source of long-term sequestration of petroleum hydrocarbons, sand lance are a potential link between any pollutants in the sediments and the pelagic fish, birds, and mammals that feed on them.

When exposed to heavily oiled sediments for short periods, sand lance respond by choosing to remain in the water column (Pearson et al. 1984). When exposed to sand oiled with 306 ppm (measured by infrared spectroscopy) Prudhoe Bay crude oil for 3-48 hr, sand lance spent 20% less time buried in the sand. When repeating the experiment the next year with fish having a higher condition index (weight/length ratio of 0.44 instead of 0.34), decreased burial time occurred only at an oil concentration of 3384 ppm. Pearson et al. (1984) concluded that the increased energetic reserves of the fatter sand lance the second year was an important factor in the relative allocation of swimming (predatory risk) versus burial time (exposure).

Sand lance have a high lipid content, nearly 40% of dry mass during our test, and lipophilic aromatic hydrocarbons are likely to be sequestered in tissues at concentrations far in excess of less fatty fish such as salmon or flatfishes. Hydrocarbons would then be bioavailable to predators feeding on the oiled sand lance at levels far in excess of that present in the sediments. Some of these predators, such as pelagic fishes, are quite sensitive to oil. Most fishes metabolize hydrocarbons and do not accumulate concentrations that would be significant for predators. Sand lance may be the exception to this generalization due to long-term sediment exposures coupled with a high lipid content capable of sequestering hydrocarbons from metabolic pathways. If sand lance are capable of transferring these lipophilic hydrocarbons to their predators, this would introduce a significant concentration of hydrocarbons far removed from either the nearshore or benthic habitat. Changes in the prevalence of marker parasites of certain ecologically important forage fishes may provide a biomarker of ecosystem health.

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