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Stress-Related Bioindicator Anomalies in Feral Male Winter Flounder (*Pleuronectes americanus*) Exposed to Effluent from Two Pulp and Paper Mills in Newfoundland

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Several studies have reported adverse biochemical, physiological and reproductive effects in freshwater fish living downstream from pulp and paper mills (Munkittrick et al. 1994). Effluent discharged by sulphite-bleaching mills at the surface of coastal areas has also affected resident marine fish including the winter flounder, *Pleuronectes americanus*, and shorthorn sculpin, *Myoxocephalus scorpius* (Khan et al. 1992, 1994; Barker et al. 1994a,b). These effects included external and tissue lesions, an enlarged liver, elevation of detoxication enzymes, a decline in circulating lymphocytes and endoparasites but an increase of ectoparasites (Khan et al. 1992; Barker et al. 1994a,b). Although the outfall reduced gonadal size and delayed sexual maturation in freshwater fish, neither of these effects was observed in male winter flounder during spring sampling (McMaster et al. 1991; Barker et al. 1994a; Munkittrick et al. 1994). The present study was designed to ascertain the effect of the effluent on a number of biological variables including gonadal development in male winter flounder in the latter part of summer when induction occurs (Scott and Scott 1988). It compared variables between samples collected near two mills and five reference sites.

MATERIALS AND METHODS

Male winter flounder were captured at depths of 3-10m (bottom temperature, 14-20°C) near two sulphite-bleaching pulp and paper mills and a number of reference sites. All of these sites in western Newfoundland connect with the Gulf of St. Lawrence. The fish were sampled over a 4-day period during August, 1996 (Fig. 1). Both mills use black spruce (*Picea mariana*) and balsam fir (*Abies balsamea*) for paper production. The mill at Corner Brook discharged untreated effluent containing suspended solids (79-93 mg/L) and resin acids (27 mg/L) whereas the other at Port Harmon, which received primary treatment prior to discharge, contained fluctuating levels of resin acids ($\leq 10 \times 10^3$ mg/L), both at the surface of marine inlets (Environment Canada, unpubl. data). Sediment at the sampling sites near both mills at Port Harman and Birchy Cove consisted of dark mud mixed with bark and fibre and emitted a strong odour of hydrogen sulphide. Both sites were devoid of macro-invertebrates. While a small quantity of wood derivatives occurred in the sediment at Meadows, the latter consisted of white sand and contained a variety of macro-

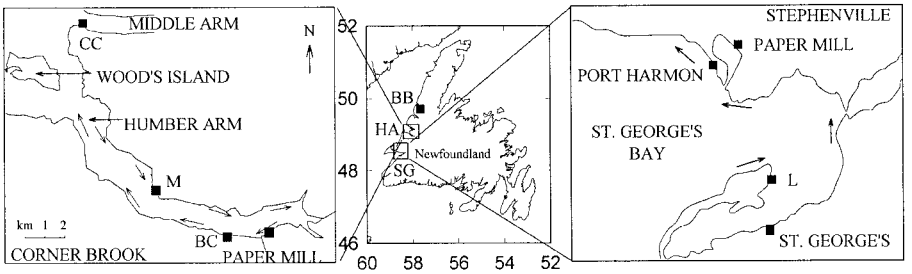

Figure 1. Location of sampling sites in St. George's Bay (SG) at Port Harmon and St. George's [near the shoreline and the lighthouse (L)], the Humber Arm (HA) at Middle Arm at Birchy Cove (BC) and Meadows (M), Cox's Cove (CC) and Gad's Cove, Bonne Bay (BB), Newfoundland. Arrows indicate current direction.

invertebrates including molluscs, echinoderms and polychaetes that were also observed at other reference sites. With the exception of Cox's Cove, Middle Arm, which received untreated domestic effluent, the reference sites at St. George's and a lighthouse in St. George's Bay and Gad's Cove, Bonne Bay were considered pristine without any anthropogenic disposal.

Fifteen adult fish were sampled at each site (total length 18-25 cm, eviscerated body weight 110-145 g) and ages, determined from otoliths, were 4-6 years. After capture, the flounder were autopsied on location. Prevalence of external lesions and black spots caused by metacercariae of the digenetic trematode, *Cryptocotyle lingua*, were recorded for each fish. Blood was removed from the caudal artery using a heparinised needle and syringe for determination of lymphocytic levels in Giemsa-stained blood smears (number per 1,000 erythrocytes). Total length and eviscerated body and liver mass were measured and used to calculate condition (K) factor (body mass/length^3 x10^{-4}) and hepatosomatic index (liver mass/body mass x10^{-3}) were noted from each fish. Samples of liver, spleen and testes were fixed in buffered formalin, processed by conventional histological methods and stained with hematoxylin and eosin. Additional samples of liver were frozen in liquid nitrogen for determination of detoxification enzymic activity, viz., ethoxyresorufin-O-de-ethylase (EROD).

Testes were classified according to the stage of development observed in stained histological sections. Developing testes exhibited seminiferous tubules, seminal lobules and primary spermatocytes whereas all of these were absent in the undeveloped organ. The mean number of macrophage aggregates/mm^2 and prevalence of clear cell foci in the liver were estimated in tissue sections. Sections of spleen were also stained with Perl's Prussian blue for hemosiderin deposits, which were estimated by digital image analysis and expressed as a percentage of the area scanned (Khan and Nag 1993). Samples of frozen liver were assayed for EROD by the method of Porter et al. (1989) within a month after collection. Briefly, the liver

was homogenized in ice-cold 50 mM Tris-HCl (pH 7.5). The reaction mixture for the reaction (final volume, 1.25 ml) contained 53 nmol Tris-sucrose buffer (50 mM, pH 7.5), 50-100 µl of the homogenate, 2.25 nmol 7-ER (150 µM) and 0.16 mg NADPH (1.25 mg/ml). The reaction was terminated after 15 min of incubation at 25°C by addition of 2.5 ml of ice-cold, spectro-analysed methanol. EROD activity was then assayed fluorimetrically. The digestive tract was examined for the digenean, *Steringophorus furciger*, and its mean abundance recorded.

Eleven variables were compared in samples originating from the seven sites (Fig. 1) by a one-way ANOVA, ANCOVA (using site as a fixed factor) and Tukey's HSD test. The non-parametric test of Kruskal-Wallis was used for comparison of parasitic abundance in fish taken from different locations. Differences were considered significant when $P \leq 0.05$. All statistical analyses were performed using the SPSS software package. Means, with standard errors or percentages were calculated for each fish group and graphically shown in Figure 2.

RESULTS AND DISCUSSION

Significant differences in selected biological variables were observed in male winter flounder sampled near the two pulp and paper mills when compared to the reference sites. Macroscopic observations revealed that the percentage of skin lesions, consisting of fin necrosis and/or epidermal ulcers, metacercariae of *C. lingua* encapsulated in the epidermis and discoloration, primarily necrosis appearing as opaque foci, in the liver were significantly greater in fish sampled near the two mills at Birchy Cove and Port Harmon than at the reference locations (Fig. 2). Condition (K) factor, hepatosomatic index and the percentage of undeveloped testes were also significantly different between flounder captured near the mills and the respective reference sites. Similarly, lymphocytic values declined while EROD activity was significantly elevated in fish living near the mills than at the reference locations. Differences in microscopic lesions were also apparent. The number of macrophage aggregates and the percentage of clear cell foci in the liver as well as the percentage of hemosiderin pigment/mm^2 in the spleen were significantly greater in flounder sampled near the mills than at the other locations. Moreover, the mean abundance of the gastrointestinal digenean, *S. furciger*, was significantly greater in fish taken at the reference sites than near the mills.

Results from the present study indicate that the health of winter flounder living near two pulp and paper mills was impaired in comparison to samples examined at reference sites. This conclusion is based on the high prevalence of macroscopic and microscopic lesions, the low body condition factor, an enlarged liver associated with elevated levels of detoxication enzymes (EROD), the decline in the number of circulating lymphocytes in the blood and delayed induction of gonadal development. Skin lesions, especially fin necrosis, have been reported previously in fish captured near mills using either chlorine- or sulphite-bleaching chemicals (Lindesjö and Thulin 1990; Khan et al. 1992, 1996). Similarly, condition factor was reduced in both freshwater and marine fish exposed to effluent from the mills (McMaster et al.

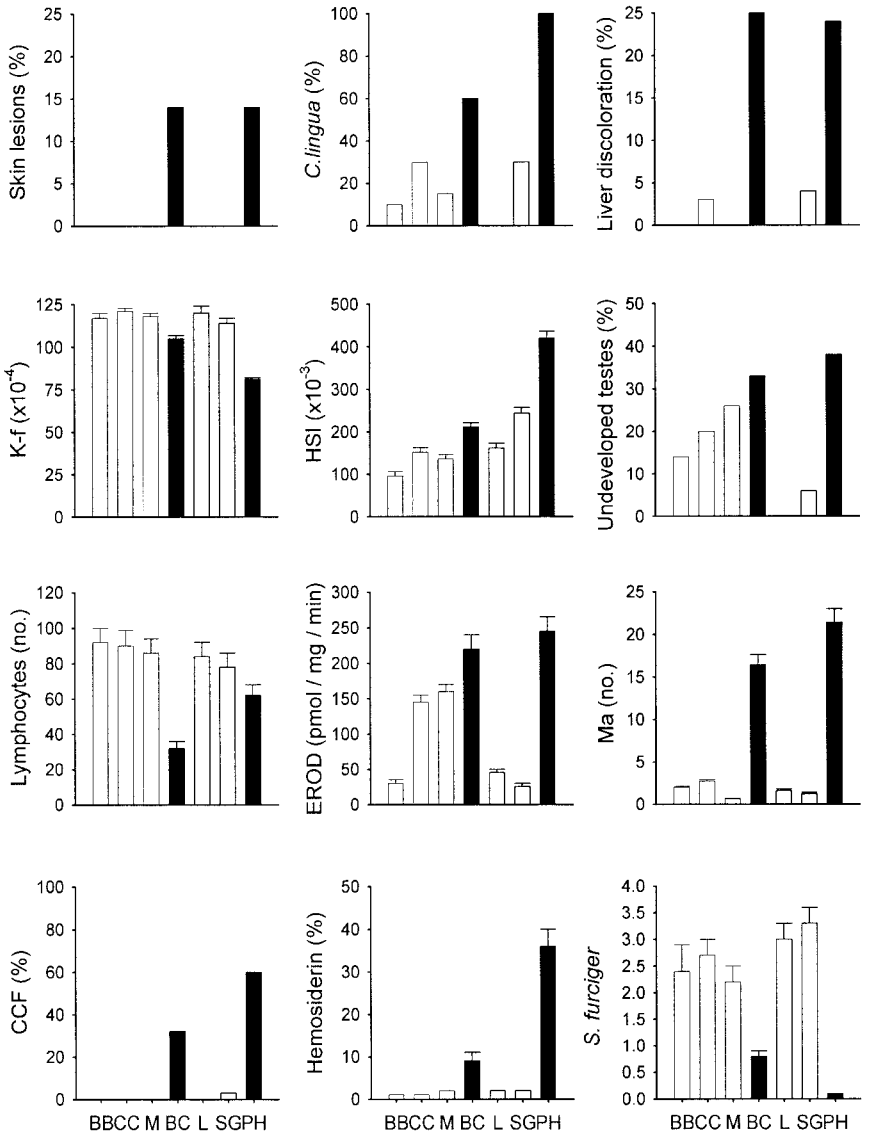

Figure 2. Comparison of prevalence (%) or mean abundance ($\bar{x} \pm$ se) of skin lesions, epidermal black spots caused by metacercariae of the digenean, *C. lingua*, liver discoloration, K-factor, hepatosomatic index (HSE), undeveloped testes, lymphocytes number (no.)/1000 /erythrocytes, EROD activity (p/mol/mg/(min)) macrophage aggregates (Ma) and clear cell foci (CCF) in the liver, hemosiderin deposits in the spleen and the digenean, *S. furciger* in the digestive tract of male winter flounder that were all significantly different between the two pulp and paper mills (solid bars) and the respective reference sites (open bars) at Bonne Bay (BB), Cox's Cove (CC), Meadows (M), Birchy Cove (BC) a lighthouse (L) offshore from St. George's (SG) and Port Harmon (PH) in August, 1996.

1991; Munkittrick et al. 1994; Barker et al. 1994a). Some of these studies also revealed that elevated hepatosomatic indices were attributed to increased activity of detoxication enzymes, EROD or cytochrome P4501A, in feral species (Munkittrick et al. 1994). Elevated levels of EROD activity in winter flounder captured at Meadows and Cox's Cove were probably associated with exposure to the effluent down-current or to untreated domestic discharges respectively (present study). McMaster et al. (1991) and Munkittrick et al. (1994) noted that disruption of gonadal development was caused by low levels of circulating sex steroids in freshwater fish sampled near bleached kraft mill effluent (BKME) or unbleached sulphite mills. It is likely that disruption of sex steroids activity was responsible for interruption of gonadal development in male winter flounder observed in the present study. Although reports of a decline in circulating lymphocytes in fish exposed to paper mill effluent are few, there is evidence of impaired macrophage activity and immunologically reactive cells, which suggest immunosuppression (Jokinen et al. 1995; Barker et al. 1994a; Jeney et al. 1996; Fournier et al. 1998; Aaltonen et al. 2000).

Pathological lesions observed in the liver and spleen of feral winter flounder sampled near the two pulp and paper mills in Newfoundland are indicative of chronic toxicity. The number of macrophage aggregates in the liver were significantly greater in fish sampled in the vicinity of the two mills than at the reference sites while clear cell foci were restricted to samples taken near the mills. The latter represent pre-neoplastic lesions and have been observed previously in flounder living in marine habitats contaminated with xenobiotics (Myers et al. 1987; Khan et al. 1994; Moore and Stegeman 1994). A high concentration of hemosiderin within macrophage aggregates occurred in the spleen of fish living near the pulp and paper mills and also in habitats contaminated with toxic compounds (Myers et al. 1994; Khan et al. 1994; Fournie et al. 2001). Its presence in tissues such as the liver, spleen and kidney represents a useful biomarker of environmental contamination in fish after long-term exposure (Myers et al. 1987; Khan et al. 1994; Fournie et al. 2001).

The level of infection with two parasites differed significantly between winter flounder sampled near the two pulp and paper mills and those collected at the reference sites. The prevalence of metacercariae of *C. lingua* in the skin was greater while the mean abundance of the endoparasitic digene, *S. furciger*, was lower in samples captured near the two mills than in the reference groups. Since lymphocytic levels, in the present study, had declined in flounder infested with *C. lingua*, it is likely that the immune response was compromised resulting in greater levels of infection. A decline of the immune response in roach (*Rutilus rutilus*), exposed to BKME, was shown previously to be associated with an increased abundance of ectoparasitic monogenes, *Gyrodactylus* spp. (Jeney et al. 1996). Low abundance of *S. furciger* in flounder was likely caused either by voiding or interruption of its life cycle at the level of the larval stage following exposure to xenobiotics (Khan and Thulin 1991). A decline of endoparasites have been reported previously in several fish species following environmental change. Both laboratory and field studies have observed a decrease in the abundance of gastrointestinal digenes in fish living in or exposed to sediment containing petroleum hydrocarbons, PCBs, pulp and paper mill effluent or acidic water bodies (Khan and Payne 1997; Marcogliese and Cone 1997; Valtonin et al. 1997). Consequently, some parasites of fish can be useful as bioindicators in aquatic environments affected by contaminants.

In conclusion, male winter flounder sampled near two sulphite-bleaching pulp and paper mills during the latter part of summer exhibited evidence of stress-related anomalies. This is based on a number of biological indicators which included the prevalence of macroscopic and microscopic lesions, condition factor, the presence of an enlarged liver attributed to elevation of detoxication enzymes (EROD) activity, delayed gonadal development, unusually low concentration of circulating lymphocytes and differences in the prevalence or abundance of two parasites when compared to reference samples. Future studies on the level of sex steroids in the blood might elucidate the impact of the effluent discharged by the pulp and paper mills on reproduction.

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