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Preliminary Evaluation of the Use of Macrophage Aggregates (MA) as Fish Health Monitors

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Discrete, round to ovoid macrophage aggregations (MA) containing pigment are commonly found widely distributed in the spleen, liver, and kidney of the higher teleostii (Agius 1980). These cellular aggregations have been variously named (Grove 1968), most recently melano-macrophage centers (MMC) by Roberts (1975). The latter author, Ellis (1974), and Ferguson (1976) have suggested that MA have analogies to the germinal centers of homeotherms.

Information on the function of these aggregations has been provided by studies of the fate of injected particulate matter such as carbon or latex beads. Injected particles are ingested by phagocytic cells which then migrate to the MA suggesting their function is centralization of excess or foreign material for destruction, detoxification or reuse (Mackmull and Michaels 1932; Ferguson 1976; Ellis et al. 1976). The types and activities of the pigments in the aggregates also suggest that centralization for processing is the primary function of MA.

Histochemically the macrophages contain three pigments: hemosiderin, lipofuscin, and melanin. The origin and biochemical roles of these pigments vary. Hemosiderin is the storage form of iron; it originates in dietary sources and from the hemolysis of red blood cells. It is observed histopathologically primarily as the ferric ion. Lipofuscin, or ceroid, results from the oxidative polymerization of polyunsaturated fatty acids and protein. It has been called 'wear and tear' pigment accumulating with age and tissue destruction. The role of melanin in the macrophages is uncertain. In fish melanin may be involved in the oxidation of NADPH during the respiratory burst at low temperatures (<15°C) or it may have a protective role in aiding the conversion of superoxide anion (O_2^-) to H_2O_2 at low temperatures (10°C) than at higher temperatures (20°C).

The studies of Agius (1979a; 1979b; 1981) and Agius and Roberts (1981) have shown that the number of melano-macrophage centers (macrophage aggregations) may vary depending on the size, nutritional status, or health of the species of fish examined. These investigators observed increases in the number and size of aggregates with increasing age, starvation, and/or disease.

In 1981 Wolke et al. (1984) suggested the use of macrophage aggregates as possible monitors of the health of wild fish. It was hypothesized that increases in the number, area, and pigment content of the MA might characterize the fishes' response to chronic environmental pollution.

MATERIALS AND METHODS

A preliminary study using one species of bottom dwelling fish from clean and polluted sites in the coastal waters of the western North Atlantic was begun in 1982 and continued in 1983. One hundred seven winter flounders (Psuedopleuronectes americanus) were collected from Georges Bank (clean), the south shore of Long Island, New York from Montauk to New York City (clean and polluted), and the Arthur Kill, New Jersey (polluted). The areas sampled were designated as clean or polluted on the basis of environmental studies conducted since 1976 by the Northeast Monitoring Program of the Northeast Fisheries Center. Levels of anthropogenic pollutants in these areas have been reported by Carmody et al. in 1973, Grieg and McGrath in 1977, Waldhauer et al. in 1978, West and Hatcher in 1980, and Piotrowicz et al. in 1981. Collections were made during the months of June 1982 (Arthur Kill), November 1982 (Georges Bank) and February 1983 (Southern Long Island). The fish were examined for gross lesions, total length, gonadal maturity, and sex. Age classification was based on length. Spleens and livers were excised and fixed in 10% seawater formalin. After fixation, the tissue was mounted in paraffin, sectioned at 6 microns and stained using the Perls' Prussian Blue method (Luna 1968). This staining procedure allows differentiation of the three pigments within the macrophage aggregation. The ferric ion of the hemosiderin stains bright blue, the lipofuscin pigment is unstained and remains a waxy yellow-brown, while melanin, also unstained, appears as small (0.5-2 μm) dark brown to black irregularly-shaped granules.

The MA number (per mm^2) and area (mm^2/aggregate) were determined using the Zeiss Integrating Eyepiece Disk I after the method and reasoning of Hennig and Meyer-Arendt (1963). Pigment distribution was calculated using a Zeiss Integrating Eyepiece with 25 random points and the point counting method of Chalkey (1943) and Hennig (1958) recording 'hits' (points) for each specific pigment per 25 points.

Number, area, and pigment distribution of MA in the spleen and liver of fish from clean and polluted sites were compared with a nested ANOVA model. 'Sites' were subsamples of the 'site condition' and, therefore, nested within 'site condition'. The nested effect was then used as the error term to test the null hypothesis that the means of two groups (clean and polluted) are equal using the F statistic.

RESULTS AND DISCUSSION

A total of 96 spleens and 103 livers were examined. Table 1 presents the comparisons made between MA number, area, and pigment distribution in spleens and livers of winter flounder from clean and polluted water. The mean number ($P=0.038$) and area ($P=0.014$) of MA were greater in the spleens of fish from polluted sites. Hemosiderin was also more prevalent ($P=0.026$). In the liver, only the size of aggregates was greater ($P=0.004$) in fish from polluted sites. Gonadal maturity, sex, and the presence of gross lesions had no effect on the overall model.

This preliminary study supports the hypothesis that differences in the number, area, and pigment distribution of MA may be used to monitor fish (and environmental) health. The hypothesis is based on three assumptions. First, that MA will localize the products of excessive (pathological) tissue destruction and will exhibit an easily observable pigment. The products of tissue destruction include the lipoproteins, lipofuscin and ceroid, all of which increase during starvation or tissue necrosis, and hemosiderin which is a product of hemolysis or erythrocyte breakdown and also is elaborated secondary to starvation. The third pigment which may accumulate in the MA is melanin, presumably to further aid in the removal of substances ingested by phagocytes and concentrated in the MA (Sealy et al. 1980).

Second, that increases in numbers, area, and pigment distribution of the macrophage aggregations signify an undue stress on the physiological homeostatic mechanisms of the fish and accordingly, a change in the health status of the fish. If health is defined as a physiological balance with the environment, then undue tissue destruction, hemolysis, starvation, or a propensity to infection by microorganisms all indicate an upset in this delicate balance and, therefore, a deterioration of health, a situation certainly not conducive to the growth and reproduction of the affected fish.

The third assumption upon which the hypothesis rests is that the MA evaluation process can be quantitated with acceptable error rate. The independent variables which may affect the dependent variables are undoubtedly numerous.

In the present study the ages (as estimated by total length) of winter flounder sampled from clean and polluted areas were approximately equal. However, ambient water temperatures were not equal since the fish from polluted sites were collected in summer and fish from clean sites were collected in winter. One could hypothesize, therefore, that the observed increases in size, number, and pigment content of macrophage aggregates (MA) in winter flounder from polluted environments were more a reflection of temperature than pollution. Examination of winter flounder collected from Boston Harbor, Massachusetts (polluted) in April 1984 (water temperature 6.5°C) and Casco Bay, Maine

(clean) in January 1984 (water temperature 3.2°C) showed substantial differences in numbers of MA (Murchelano, unpublished). There are considerable differences in environmental quality between these two sites. The MA hyperplasia and frequency of other lesions observed in Boston Harbor fish is more likely pollution induced than temperature mediated since the temperature difference was only 3.3°C. The mean size of Casco Bay winter flounder was larger than that of Boston Harbor winter flounder. Higher temperatures alone do not cause MA hyperplasia, they may however, increase the magnitude of the fish's response to environmental insult.

Statistically designed experiments must be conducted to partition the effects of age, sex, ambient temperature, and maturity before considering in detail the type and degree of site pollution. Nonetheless, 'worst-best' subsets of this type of study as noted herein indicate highly significant differences between MA characteristics of fishes from clean and polluted water. Site condition and the organ studied show a promising two factor ANOVA model predicting the dependent variables; however, the error term is large, suggesting unmeasured variability. A discriminant factor to predict clean versus polluted sites may also be obtainable given a more balanced design.

Table 1. Winter flounder (Psuedopleuronectes americanus) MA parameter mean comparisons

Site Condition	Polluted Mean±SE	Clean Mean±SE	Pr. of No Difference Ho: $\bar{x}p = \bar{x}c$
Spleen	n = 38	n = 58	
Number centers/mm^2	8.00±0.90	5.71±0.62	0.038
Mean aggregate area ($mm^2 x 10^{-3}$)	3.14±0.29	1.58±0.15	0.014
Iron (points)	16.18±0.81	12.53±0.88	0.026
Lipofuscin (points)	3.24±0.54	5.55±0.63	0.092
Melanin (points)	5.58±0.50	6.05±0.49	0.459
Liver	n = 40	n = 63	
Number centers/mm^2	2.29±0.51	1.74±0.40	0.392
Mean aggregate area ($mm^2 x 10^{-3}$)	0.48±0.08	0.20±0.04	0.004
Iron (points)	6.05±0.91	6.22±0.93	0.912
Lipofuscin (points)	7.93±1.20	5.17±0.80	0.090
Melanin (points)	0.40±0.28	0.11±0.05	0.248
Fish	n = 42	n = 65	
Size (cm)	24.98±1.24	28.40±1.11	0.157

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