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HEMOCYTE AND TISSUE CHANGES BY CRUDE OIL IN THE BLUE MUSSEL *MYTILUS EDULIS*

A

THESIS

Presented to the Faculty of the University of Alaska

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

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Fairbanks, Alaska

September 1983

HEMOCYTE AND TISSUE CHANGES BY CRUDE OIL IN THE

BLUE MUSSEL *MYTILUS EDULIS*

RECOMMENDED:

Chairman, Advisory Committee

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Aug. 31, 1983
Date

This thesis is dedicated to the memory

of Dr. Frederick Bang

ABSTRACT

This study examines the effects of Prudhoe Bay crude oil on the number and types of circulating hemocytes, on the phagocytic response, on spawning progression, and on internal structural changes. The number of hemocytes was reduced with 4-5 week exposure to 1000 nL/L of oil; a significant number of individuals showed a higher than average cell count with longer exposure. An increase in agranulocytes in the 8-9 week control population does not occur in populations exposed to 1000 nL/L and 500 nL/L of oil for 8-9 weeks, but, the phagocytic response was significantly depressed. The Chi-square test showed that oil interferes significantly with progression of spawning. Analysis of internal tissue structure indicates that oil can affect adipo-granular storage cells, vesicular tissue, and digestive tubule cells. The changes occurring in circulating hemocytes are not necessarily consistent with changes in internal morphology.

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I. INTRODUCTION

When studying the effects of oil pollution, consideration should be paid to the potential impact on the whole animal, the whole population, and to environmental and physiological factors (Jeffries, 1972). Individual maladaptive response to change can result in pathology, recognized as a deviation from normal morphology and metabolic function. Pathological alterations may be associated with symptoms of disease and may result from microbial infection, genetic alteration, immunological or cellular dysfunction, or from environmental stressors. Environmental stressors include habitat change, reduction in food supply, parasites, or pollutants. Any of these can decrease the fitness of an animal and impose physiological adjustments with energetic costs. Through homeostatic mechanisms, internal adjustments establish a new balance, accommodating needs to environmental demands (Bayne, *et al.*, 1976).

Attempts to associate oil pollution and pathology of marine organisms are often based upon circumstantial but sometimes unconvincing evidence of a cause and effect relationship (Malins, 1982). This is due to the complexity of the route and fate of oil through the marine system and to the varied adaptive responses of individuals and populations. This problem is exacerbated by incomplete knowledge of pathological mechanisms and by an insufficient understanding of threshold tolerances toward environmental and physiological stresses.

OIL AS AN ENVIRONMENTAL STRESSOR

The direct effects of oil may be difficult to identify but the strains they may cause the animal may be evident in the physiological reactions to maintain homeostasis. Oil may be considered a stressor and its effect may cause a strain on the functional physiology of the animal, identified generally as stress. Levy (1983) defines stress as the result of all the physical, chemical, and other factors that cause physiological or psychological tension in the individual or population. Bayne, *et al.* (1976) defines stress as "a measurable alteration of a physiological (behavioral, biochemical, or cytological) steady-state which is induced by an environmental change and which renders the individual (the population, or the community) more vulnerable to further environmental change."

Stebbing (1981) considers stress as any external force (including oil) whether natural or man induced that elicits a generalized adaptive response in organisms. Stress results in a load, according to Stebbing, on the homeostatic processes of organisms, requiring energy to counteract the disturbance. As load renders the homeostatic process more susceptible to further load through summation, the capacity of the system ultimately can be exceeded. At threshold level, any additional increase in load may have a significant effect on an organism inspite of the fact that prior to reaching the threshold level the stress showed no effect. Sindermann (1981) considers petroleum contamination of marine waters an additional source of stress to marine organisms, often reflected in pathological change.

He concludes that petroleum in sufficient concentration can be toxic and such toxicity may be expressed in morphological change, i.e., pathology. Levy (1983) believes that the potential impact of oil must be considered in terms of its additional effect to the overall level of stress endured by the individual or population. Thus, Levy, Stebbing, and Sindermann all support the need to consider the impact of pollution, i.e. of oil, as an added stress to be examined within the context of the biology of the animal and the population, in relation to the environmental and physiological stresses normally encountered.

DETERMINING THE SUBLETHAL EFFECTS OF OIL

In determining the effects of oil, the examination of individual physiological responses in isolation of biological phenomena may be enigmatic. Subtle changes may be identified but the consequence may not be understood or may prove insignificant due to variability in biological response and the inherent mechanisms of animals to maintain homeostatic control. Subtle changes add up to sublethal physiological stress to make the animal unfit to meet environmental stresses or to disrupt the timing of particular life history patterns, which fall out of synchrony with environmental timing.

The biology of *Mytilus edulis* is well known (Bayne, 1976). *Mytilus edulis* is a hardy intertidal invertebrate considered by some as resistant to the effects of petroleum and pollution in general (Roberts, 1976). Its hardiness and availability, however, make it a good experimental animal, and much study has been done in terms of its

ability to concentrate potential human pathogens, heavy metals, and pollutants. In this regard this species has been the animal selected by the International Mussel Watch Program to monitor the health of the coastal waters of the world (IMWW, 1980). Research performed on *Mytilus edulis* and bivalvia in general provides an extensive scientific background upon which the understanding of subtle changes by known pollutants can be measured and compared.

Hemocytes are important mediators in physiological requirements and may be critical in reacting to physiological strain as well as to the direct effects of oil. The importance of examining their changes lies in their functions in homeostasis, including immunological defense (Cheng 1981). They are transported in the hemolymph throughout the bivalve body, including its ctendia and open cavity, the hemocoel. They are capable of non-self recognition and can transport and dispose of foreign substances including food, waste products, necrotic and degenerate tissues, toxic substances, and invading microorganisms. Hemocytes appear to function in resorption of gonadal degenerate material following spawning (Moore and Lowe, 1977) and shell and wound repair (Cheng, 1981). Unlike the specialized blood cells of vertebrate species with humoral immunoglobulins and cell mediated responses in immunological defense, bivalve mollusks maintain such defense through cellular recognition and are mostly unaided by opsonic factors. Non-self recognition is manifested in their phagocytic response, i.e., the ability to recognize and engulf foreign particles or particles of degenerate tissue. The sequence occurring

during phagocytosis and killing of microorganisms, first described by Metchnikoff (1887), is their attraction to the microbe whereupon they send out pseudopodia to engulf and subsequently digest the phagosome (the microbe) in vacuoles by intracellular release of hydrolytic enzymes concentrated in lysosomes of cells.

Lysosomes are an important feature of the digestive process of bivalve mollusks (Owen, 1972). In bivalves, lysosomes are well developed in the digestive tubules (Owen, 1972) and in phagocytic blood cells (Sminia, 1981). Petroleum hydrocarbons have been reported to destabilize lysosome membranes causing cytolysis (Moore *et al.*, 1978) as a result of certain forms of sublethal injury (Goldstein, 1974). Lysosomal hydrolytic enzymes may be released from cells into the extra cellular environment or released from lysosome organelles into the cytoplasm (Goldstein, 1974), causing cytolysis. The effect may accelerate autolysis of cellular material with subsequent deterioration of the health of the individual. Release of hydrolytic enzymes may deplete hemocytes or, because of homeostatic mechanisms, increase the number of circulating hemocytes. However, normal variability in bivalve hemocyte counts makes the determination of higher and lower than normal counts difficult. Nevertheless a norm may be determined upon which to measure some change, as suggested by IMWW (1980).

Fluctuations in hemocyte numbers within and among individuals are great (Huffman and Tripp, 1982). Localized increases in the number of hemocytes are known to be induced by parasitism, injury, and tissue

resorption as determined by histological examination, (IMWW, 1980). An increase in the number of circulating cells results from increased heart rate which is influenced by temperature increase and mechanical stimuli (Feng, 1965; Pauli, 1971) as well as feeding. Nevertheless, as IMWW (1980) states, "Hemocyte numbers generally remain within acceptable limits, bearing in mind natural variability... Once the hemocyte numbers exceed acceptable limits and the cells are evident in vary large numbers throughout the whole connective tissue network, this is taken to be a manifestation of stress and is classified as being indicative of loss of condition."

Petroleum may interfere with hemocyte functioning. The lipophilic nature of aromatic hydrocarbons, the toxic constituent of petroleum, may disrupt membrane structure (Roubal, 1974) if the rate of metabolism is less than the rate of diffusion. Phagocytic success is dependent upon their ability to recognize, phagocytize, and digest or dispose of non-self substances (Feng, *et al.*, 1977). Bivalve phagocytes are not known to have enhancing opsonic factors (Fries and Tripp, 1980). Bivalve phagocytic recognition appears to be totally dependent upon intact membranes for general chemotactic surface recognition but recognition mechanisms involved in phagocytosis in general are still poorly understood (Wilkinson, 1982; Vasta, *et al.*, 1982). In filter-feeding bivalves, hemocytes circulate throughout the gills to be bathed continuously with environmental pollutants. The toxic lipophilic components of crude oil can readily be intercalated into membrane surfaces to alter their function (Roubal, 1974), and

possibly to interfere with surface recognition in phagocytic response. Hemocytes may be responding to high hydrocarbon body burdens and the changes may make them less fit for winter survival or gametogenesis. Certain hemocytic functions may be disrupted and certain kinds of hemocytes may be produced at the cost of other important functions. Hemocytic functional changes during critical post-spawning recovery may affect winter survival and gametogenesis. Initiation of gametogenesis in the summer requires both a suitable temperature range and adequate food supply but once started, gametogenesis occurs regardless of winter conditions and nutritive stress (Myint and Tyler, 1982). The accumulation of oil and subsequent disruption of hemocytic function may cause subtle changes that go unnoticed to the research biologist in measuring population changes. High winter casuality or low population recruitment may be determined long after the oil is no longer detectable.

EFFECTS OF OIL KNOWN FROM FIELD AND LABORATORY STUDIES

Petroleum and its specific constituents have been shown to cause structural and functional changes in cells, tissues, organs, and systems of marine organisms. In field studies gonadal and hematopoietic neoplasias, and cellular proliferative disorders (Brown, *et al.*, 1979; Mix, 1982; Yevich and Barszcz, 1976, 1977) of bivalves have been associated with oil contaminated water. A controlled field study, taking advantage of the *Amoco Cadiz* oil spill which resulted in heavy oil release into the water, showed that transplanted bivalves

had "clear histological and chemical differences" (Wolfe, *et al.*, 1981). Laboratory studies with crude oil or its components show structural and functional changes. The oil can disrupt or alter digestive diverticula cells (Lowe, *et al.*, 1981; Moore, *et al.*, 1978; Widdows, *et al.*, 1982; Wolfe, *et al.*, 1981). Physiological changes, enzyme changes, decreased growth and some mortality have been associated with bivalves and other marine organisms in petroleum contaminated water (Barry and Yevich, 1975; Dow, 1975; Heitz, *et al.*, 1974; Stekoll, *et al.*, 1980; Widdows, *et al.*, 1982). Immunological competency is depressed with benzo(a) pryene and other pollutants (Anderson, *et al.*, 1981), and phenol has been shown to alter *in vitro* phagocytic capacity as well as reduce hemocyte counts by selective cytolysis (Fries and Tripp, 1980). Phenol has also been reported to damage erythrocytes and leucocytes of fish (bream), resulting in a substantial decrease in red blood cells (Waluga, 1966). Soft shell clams (*Mya arenaria*) exposed for 28 days to high concentration of No. 2 fuel oil in seawater show generalized decreased numbers of hemocytes (Stainken, 1976). Metals found in crude oil or with oil exploratory operations, which include cadmium, chromium, iron, lead, nickel, zinc are implicated in carcinogenesis (Hodgins, *et al.*, 1977). Cadium is reported to interfere with the lysosomal breakdown of phagocytic ingestion of bacteria in fish. In summary, evidence suggests that internal structural changes and impairment of immune responses may result from petroleum contamination. Such changes and impairments may cause physiological stress that may accummulate over

time to decrease fitness against environmental stressors, to favor a pathogen, and to increase the incidence of infectious diseases (Hodgins, *et al.*, 1977).

STATEMENT OF APPROACH

Prudhoe Bay crude oil contains some phenol and a high percentage of aromatic hydrocarbons (Thompson, *et al.*, 1971). Mussels in low concentrations of Prudhoe Bay crude oil for an eight week exposure carry high body burdens of aliphatics and aromatics and show measurable physiological stress with increasing oil concentrations (Clement and Shaw, unpublished). Widdows, *et al.* (1982) have suggested that "the physiological and cellular condition of *Mytilus edulis* is in some way responding to the concentration or total content of aromatic hydrocarbons in the body tissues rather than to the concentrations in the environment." Widdows goes on to state: "There is no evidence of a gradual recovery, but there is a suggestion of a gradual deterioration in the general health and condition of mussels in response to chronic exposure." Clement and Shaw support the results of Widdows, *et al.* (1982) and show high concentrations of aliphatic and aromatic hydrocarbons in the animals.

Hemocytes may be directly affected by the oil or they may be responding to the general physiological stress of oil on homeostasis. In the present study, changes in hemocyte numbers and types as well as phagocytic function are examined as they may directly or indirectly be affected by crude oil after 4-5 weeks and 8-9 weeks exposures. This

study further examines internal structural changes associated with spawning, oil effects, and laboratory conditions and how these changes might be associated with hemocytes and their functions.

II. METHODS

EXPERIMENTAL CONDITIONS

Holding and Testing Facility. The experimental testing facility, described by Clement and Shaw (unpublished), was designed and operated by Clement for an Environmental Protection Agency funded research on metabolic effects. Fresh, dense, and cold seawater was drawn into the laboratory by a main pipe extending 900 ft. beyond the laboratory into Resurrection Bay, and 225 ft. below the surface to bring in subthermocline water. This frigid water was filtered and heated to 8 — 10°C in a large reservoir, warming the water to just below normal spawning temperature (12°C). From the reservoir the water flowed by gravity through the system, bringing a continuous flow of oiled water to test animals and non-oiled water to controls at a constant rate of one liter per minute. The study populations were housed separately in large glass culture dishes, each group being completely isolated from the others. Water was allowed to over-flow the dishes into a larger plastic container with a drain to channel waste water out. The dishes were cleaned once every week. Food was continually available to all animals, being introduced into the reservoir ahead of the oil. Food consisted of a mixed diet of cultured *Isochrysis galbana* and *Phaeodacytlum tricornutum* in concentrations of 10,000 to 12,000 cells per mL. These algae were grown in the laboratory under artificial light.

Oil Exposure. Fresh Prudhoe Bay crude oil was slowly and continuously injected into a long vertical glass tube 5 cm. in diameter. A large glass hypodermic needle was filled with oil and the plunger was mechanically pushed to inject oil at a constant rate to mix with fresh seawater flowing from the reservoir. A non-aerating glass stirrer mixed 2.6 µL of crude oil (per min.) with 50 mL. of fresh seawater (per min.). The resultant oil-in-water emulsion was channeled by water pressure and gravity through a series of glass tubes. Regulated fresh seawater from the reservoir diluted the oil-in-water mixture to an appropriate concentration.

Appropriate oil concentrations were obtained by a series of subsequent dilutions in a one way flow-through of oil water. The first dilution of oil resulted in an oil exposure concentration of 1000 nL/L. Part of this was channeled to the first glass container of animals to establish them as the high oil exposure population. The remaining oil-water travelled to another glass tube which received fresh seawater at a constant rate to dilute the 1000 nL/L oil-water to 500 nL/L. Part of this flowed to a second population, the medium oil animals. The remaining diluted oil then flowed to a third oil dilution and to a third population, but of none these animals were used in the present study. Fresh filtered seawater from the reservoir went to controls. All waste water flowing from the system was filtered by acrylic fiber floss to capture the waste oil. Flow rates for all dilutions and freshwater were adjusted daily. The oil receiving-mixing tube was cleaned weekly. Teflon and glass

stopcocks were used where flow rates were regulated. The syringe was refilled with oil weekly.

PRELIMINARY TESTS

The examination of test animals was preceded by a preliminary study. Twenty-five animals were examined to explore several different methods and techniques in approaching the problem of effects of oil on hemocyte numbers, kinds, and phagocytic response. Staining, enumerations, and time were variables to control for precision and quality in data gathering. The preliminary period allowed for the development of the necessary skill in obtaining hemolymph and in histological preparations of mussels. As a result, a rigid schedule and routine were adopted to assure consistency in technique and data gathering.

EXPERIMENTAL DESIGN

The study was designed to examine hemocyte changes occurring as a result of exposure time, oil concentration, and internal structural changes associated with spawning and tissue disturbances. The maximum duration of oil exposure was nine weeks, with population sampling in 4-5 weeks and 8-9 weeks. Forty-eight controls and forty-eight animals in medium oil (500 nL/L) represented the principal study populations, with ten animals in 1000 nL/L (high oil) to substantiate the effects identified in the medium oil population. Two to three experimental animals were examined in sequence to every one to two controls

examined. A total of twenty controls and thirty-three exposed animals were examined in a total of nine weeks. Histological examination was performed on all animals to determine spawning stage and the effects of oil and laboratory conditions in altering the structure of internal tissues.

The data were analyzed in terms of individuals and populations. The objective was to identify the effects on individuals to gain a better understanding of individual responses, including changes in hemocytes with tissue changes and with oil exposure, and then to examine the effects of oil on the population to determine a level of significance. Data on individuals were listed and described while data on populations were analyzed in terms of means and confidence limits and in terms of frequency distributions. Student's t-test and Chi-square statistic were applied in the analysis. A classification scheme for the grouping of individuals provided the means for determining comparative effects with respect to biological changes and variability, as suggested by Jeffries (1972).

EXAMINATION

Condition of animal prior to hemolymph extractions. As a result of the preliminary investigation, a standard and rigid laboratory procedure and time schedule were adopted. Each animal was maintained during fresh hemocyte counts in a culture bowl with its exposure water. Prior to hemolymph extraction, note was taken of the general condition and behavior of the animal, determined primarily by

observing the time to expose its foot in anticipation of reattachment, a requisite for survival under natural conditions. Appendix 1 shows the occurrence of the foot response. If no foot was seen within 20 min., note was made and other response signs observed such as filtering activity and tactile response.

Hemolymph extractions. Hemolymph was extracted from the posterior adductor muscle (Bayne, *et al.*, 1970). A notch was made on the dorsal edge of the shell in the region of the large adductor muscle, between the second and third growth rings from the posterior end (Figure 1). A 25 gauge needle was inserted until resistance of the muscle was felt. Hemolymph was withdrawn into a 2 1/2cc sterile disposable plastic syringe (Plastipak) containing 0.22 µL Millipore filtered sterile seawater (1:1). The extraction method was fairly consistent and reliable. The filled syringe was gently tapped to keep cells in suspension (Feng, 1965). The first three drops of hemolymph were discarded and each of two chambers of a dry, clean and sterile hemocytometer (Neubauer, model 4000, Clay Adams) was filled. Four drops of hemolymph from the syringe were added to precleaned glass slides for staining and enumeration of phagocytic response. Slides with hemolymph were placed in a moist, covered, 20°C chamber for a scheduled time as described for each parameter below.

Determination of total cell count. Hemocytes were allowed to settle for five minutes prior to making cell counts in the hemocytometer (Feng, 1965). All squares of both chambers of the

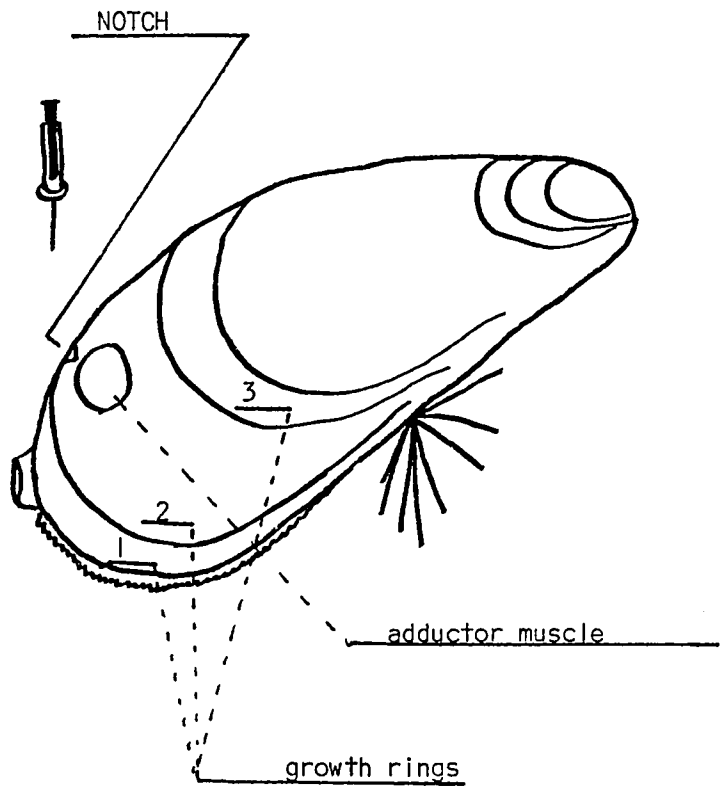

Fig. 1. Mytilus edulis to show location of the adductor muscle and the notch where needle was inserted to withdraw hemolymph.

hemocytometer (18 mm^2 in total area) were used in counting, beginning in the upper left hand corner, going across and down the squares according to hemocytological technique (Miale, 1981). Calculation for total cell count of hemocytes was made according to standard procedure. The general formula is given as follows:

$$\frac{\left(\begin{array}{c}\text{No. of cells} \\ \text{counted per } \mu L\end{array}\right) \text{ X (Dilution) X (Depth)}}{\left(\begin{array}{c}\text{No. of large} \\ \text{squares counted}\end{array}\right)} = \begin{array}{c}\text{No. of cells} \\ \text{per } \mu L\end{array}$$

Population data were analyzed statistically, measuring the differences in means and applying the Student's t-test. Because of the variability in hemocyte counts and the small sample size which produced large confidence intervals, a classification scheme was adopted to identify the frequency of individuals falling within defined hemocyte ranges of controls. The 2 X 2 contingency table for a 2-way classification of data allowed for testing the independence of two variables, controls vs. experimentals and hemocyte counts, using the Chi-square statistic ($X^2_{a\ .05}$) with the continuation correction factor (0.5) included for one degree of freedom (Zar, 1974).

Determination of differential cell count. Qualification and enumeration of each cell type was made while counting all hemocytes. Figure 2 shows the nine basic hemocyte forms identified in fresh hemolymph. Many of these forms were morphological variants of the

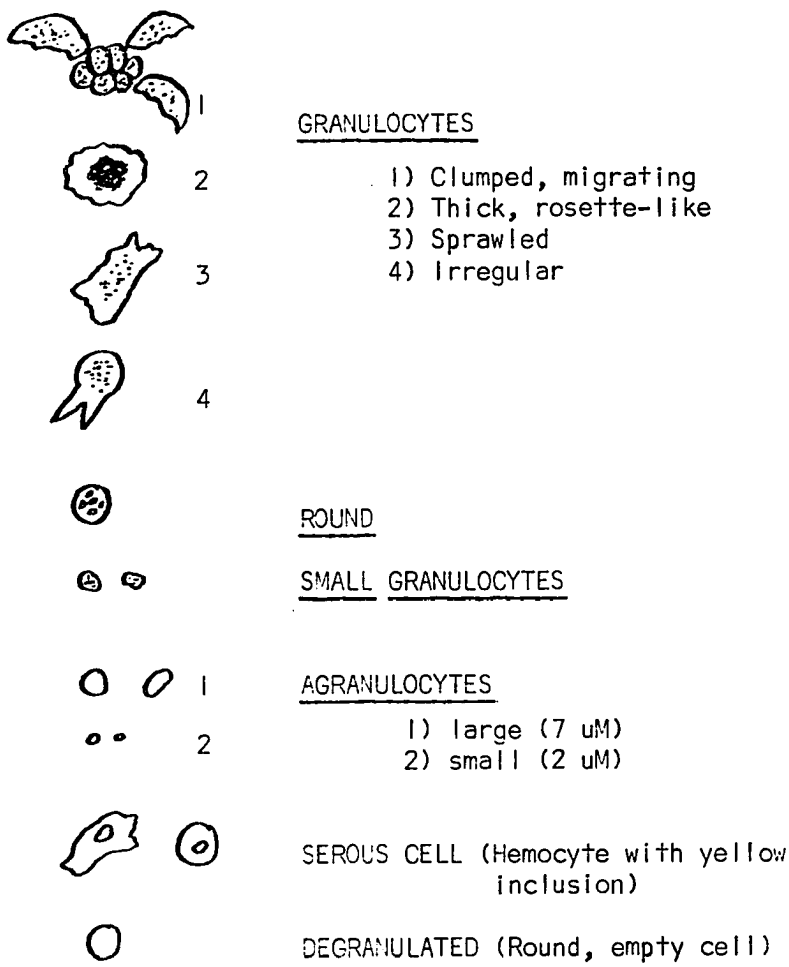

Fig. 2. Schematic drawings of different forms and types of hemocytes identified in fresh hemolymph samples under light microscopy.

same cell and required consolidation with others. As a result, six morphologically distinct groups are shown, each possibly representing functional or ontogenic differences of either two basic cell lines, granulocytes and agranulocytes (Cheng, 1981), or one cell line (Mix, 1976). Cheng notes that degranulated cells are granulocytes that have lost their granules; serous cells are hemocytes with accumulated intracellular refractive lipofusion. Without distinction to ontogenic cell lines, these six groups represent two major categories identified under regular light microscopy by Takatsuki (1934) and Cheng (1981). Takatsuki recognizes the morphologically variable and larger granulocytes which occur in greater frequency and the smaller less frequent and less variable agranulocytes which appear with few to no granules. In this study the granulocytes of *Mytilus edulis* contained varying percentages of granules, were highly mobile and pleomorphic while the agranulocytes were slightly opaque, non-mobile and non-pleomorphic, slow to spread, and were generally much smaller in size. Plate 1 shows the two forms counted and the second photo shows a larger agranulocyte.

Percent frequency of each cell type in the hemolymph of each animal was determined. Percent values were converted to a normal distribution by arc sin transformation to determine 95% confidence (Zar, 1974). Percent values are relative and may mislead (Miale, 1982); absolute values include total cells counted to identify if change was the result of one cell type increasing or decreasing, or if all cells were increasing or decreasing in the same relative proportions.

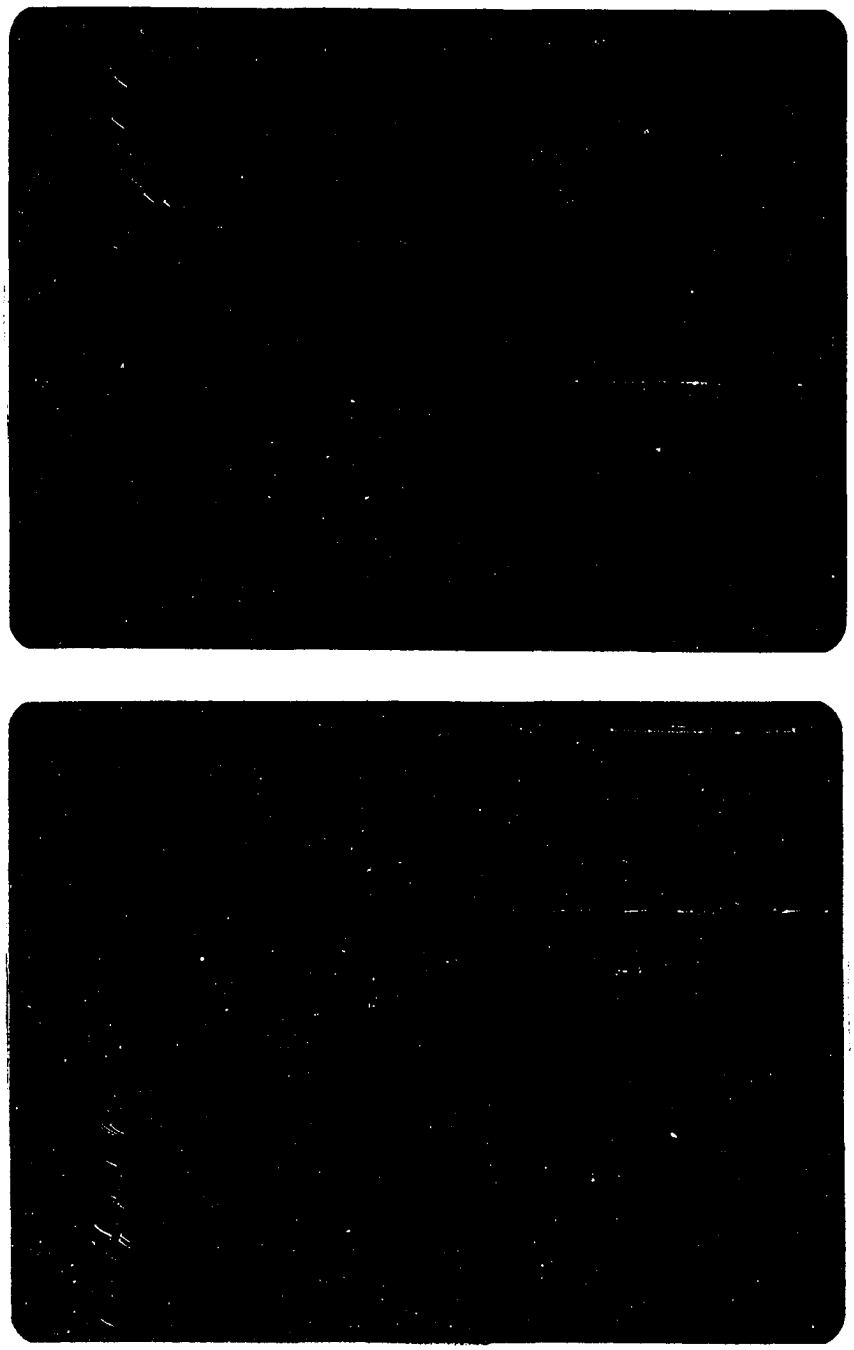

Plate I. Hemocytes in fresh hemolymph. (above) Granulocytes, the largest and agranulocytes, the opaque and smallest. (below) a larger agranulocyte. Unstained. 400X.

Stained hemocytes for nuclear/cytoplasm differentiation. Fresh hemolymph on slide preparations were stained for nuclear and cytoplasm details. The first slide was removed from the moist chamber after 15-20 min., the second after 40-50 min. Each was air dried and dipped into Wrights Fast Stain (Harleco) according to directions, then into distilled water, air dried and covered slipped.

Determination of phagocytic response. Four drops of fresh diluted hemolymph was added to each of three precleaned glass slides. To this, four to five drops of prepared yeast in concentrations of 5000 to 7500 years cells per µL were added and mixed by swirling the solution. The slides were put into a moist covered chamber; the first removed in 30 min. and stained in Wrights Fast Stain (Harleco) for cursory assessment of nuclear/cytoplasm details of phagocytes with yeast. The last two were removed in 55 and 65 min. respectively, for fresh sample enumerations of the phagocytic response. All slides were rinsed with Millipore filtered seawater prior to staining and counting to remove unattached hemocytes and unphagocytized yeast cells (Bayne, *et al.*, 1979; Fries and Tripp, 1980).

Yeast concentrations were prepared in the following manner. Dry granules of Baker's yeast were added to boiling distilled water for 10 min., then centrifuged. The supernatant was poured off and replaced with sterile Millipore filtered seawater to dilute the yeast concentration to approximately 5000 to 7500 yeast cells per µL. The yeast concentration was periodically checked during the study period to assure proper concentration and stored in a refrigerator.

Counts of hemocytes with and without yeast determined the phagocytic response. Counts were started at the upper right hand corner where the concentration of phagocytized yeast was evident, then proceeding down adjacent microscopic fields of view, to the left and back up the slide, counting 1000 hemocytes per slide. Hemocytes in clumps were counted as one, but distinct individuals with only slight attachment to the group were counted individually. Yeast cells were counted as phagocytized if they appeared "stuck" to the surface of the cell, the first step in phagocytosis (Bang, 1961). The second slide duplicated the procedure for additional assurance of the first count. The mean percentage and standard deviation for 1500 cells counted for all individuals are presented in Appendix 2.

Population differences were statistically analyzed. The percentage means converted to a normal distribution by arc sin transformation of the individual data then applying the Student's t-test to obtain the 95% confidence range (Zar, 1974).

Histological method. After hemolymph was removed, the animals in their exposure water had opened and were filtering and reattached indicating survival and responsiveness. In two to three hours after all counts were made, the adductor muscles were cut and each animal was immediately dropped into formol-calcium fixative (Luna, 1968). After several rinses, they remained in the final fixative until prepared for histology.

Histological methods followed standard procedures. The fixed animals were hand cut into three parts: a section from the posterior mantle for determination of spawning stage; two cross sections to

determine tissue and organ structural changes, one at the mid (posterior to the byssal thread) region and a second at the anterior. The anterior cut was made only for animals examined in the 8-9 week period when it became evident that oil accumulated there. The cuts were separated and rinsed in tap water then dehydrated by exposure to increasing alcohol concentrations. Two to three tissue sections (per animal) were separately blocked in paraffin. These paraffin blocks were then sectioned on a rotary microtome in 6-7 μM thickness, in continuous sequence but skipping 10 to 20 sections between slides. Three to six slides per block were made to total 6 to 18 slides per animal.

The sections were stained in duplicate by two different staining procedures to assure that any unusual feature was not the result of staining artifact. Procedures established by Luna (1968) were followed, with one set of slides stained in hemotoxylin and eosin and the second set in Papanicolaou (VWR). The papanicolaou staining procedure highlighted the presence or absence of lipid storage, the adipogranular cells of the mantle (Bayne, *et al.*, 1982). The number of slides analyzed were 132 for controls with 68 additional slides from 15 preliminary animals as control supplement; 147 slides for medium oil animals and 53 for high oil animals a totalling 395 slides.

Determination of spawning stage. The stereological technique (Lowe, *et al.*, 1982) was applied to mantle sections examined at 200X with an American optical microscope fitted with an occular eye piece graticule (Graticules, Ltd.). The graticule is a grid of staggered

lines bounded by statistically derived points from which to make point counts of tissue cells to determine percent changes in follicles and connective tissue as a result of spawning. Stereology is the extrapolation from two dimensional space to gain information on volume changes associated with gametogenesis (James, 1977). The technique used here closely followed that suggested by Wiebel, *et al.* (1966) and Briarty (1975) which consisted of placing the grid over randomly selected histological sections so that the bottom left edge of the grid fell over the bottom left section of the mantle. Counts were made and the section was moved to the non-overlapping adjacent field of view until ultimately the entire mantle tissue was counted. Differentiation of cell and tissue type was judged on the basis of defined criteria listed in Table 1. Counts from each of three mantle sections from two different histological slides provided statistically significant representation of each animal in determining the reproductive stage (Lowe, *et al.*, 1982). Cell percentages were averaged for the three mantle sections to provide the percent frequency of each cell type in the mantle (Appendix 3). The dominant percentage of follicles or storage tissue provided the basis for classifying animals into different spawning stages.

Spawning classification of *Mytilus edulis* was based on the knowledge that with advance spawning, gonadal tissue is replaced by vesicular and adipogranular storage cells as part of the gametogenic cycle (Bayne, *et al.*, 1982). Keiser (1978) described the gametogenic cycle of *Mytilus edulis* in Alaskan waters, and Lowe, *et al.* (1982)

Table 1

Criteria in identifying cells by stereological point count for spawning analysis of mantle tissue.

HEMOCYTE:

A free small round cell in the connective tissue, appearing light blue with Papanicolaou Stain, or red with Hemotoxylin and Eosin stain.

GAMETE:

Eggs or sperm within follicle.

FOLLICULAR SPACE:

Space between eggs or sperm cells but within follicle.

VESICULAR TISSUE (VT):

Angular, flat, recticular cells or elongaged spindle cells (fibrocytes) of follicular wall.

ADIPOGRANULAR CELL (AD):

Thickened lipid storage cells of mantle which take up the orange-red color in Papanicolaou Stain; occur outside of follicles.

GERM CELL:

Small basophilic cell on periphery of follicle wall.

SPACE:

Areas outside follicles where neither adipogranular nor vesicular tissue appeared in areas of the mantle.

quantified the changes during gametogenesis by applying the stereological technique. In determining spawning stage, the approach for gametogenesis used by Bayne, *et al.* (1982) was applied. Mid spawners were identified with 70% (or greater) volume fraction of ripe gametes and 15% or less storage; 70% and greater volume fraction of storage tissue with 15% or fewer gametes identified the resting stage of gametogenesis. The stages between Mid Spawning and Complete Spawning (Advance and End) represent the gradual percentage change in the spawning process. The percentage of criteria presented in Table 2 established the four spawning stages. Inter-stages represent border cases in which the percentage of gametes (follicles in general) or storage tissue (adipogranular and vesicular) might each qualify for different but adjacent spawning stages.

Spawning classification provided the basis for statistical analysis to measure changes occurring in spawning progression. Statistical differences in spawning progression between control and experimental populations as a result of time was determined by applying the Chi-square statistic ($X^2_a.05$) in a 2 X 2 contingency table to determine statistical differences in the number of indivduals within each spawning stage.

Determination of tissue condition. Examining structural details of particular organs, tissues, and cells of histological slides with an American Optical microscope, Series 200, at 300 and 600 magnification, provided the basis upon which to identify tissue condition. In order to establish whether control tissues were

Table 2

Criteria for percentage classification of mussels into post spawning stages by determining relative volume changes in follicles vs. storage cells of mantle tissue.

Mid Spawners:

Dominant percentage in gametes and follicles; >70% gametes and follicular space and <18% storage tissue; few if any developing gametes.

Advanced Spawners:

Reduced percentage dominance of gametes and follicles to slightly above or equal to storage tissue; 35-70% for follicle and 18-49% for storage tissue.

End Spawners:

Storage tissue increased percentage dominance; 15-35% follicles and 50-70% storage tissue.

Completed:

Storage tissue percentage dominates; <15% follicles and >70% storage tissue.

Interstages: (Mid-Advance; Advance-End; End-Complete)

Percentage values of two different stages to indicate transition. Could be one or the adjacent stage.

affected by laboratory conditions as compared to experimentals, or whether there were differences in the tissues and organs of oil stressed animals, all histological slide labels were covered, i.e., "blinded" and all slides were mixed for a systematic qualitative determination of each tissue slide. The criteria for this determination is listed in Table 3 to establish the guidelines for making a qualitative assessment of each section as either affected (T) or unaffected (C). Absolute rating of the criteria for quantification could not be determined due to the dynamic nature of spawning and resultant concurrent changes on the "Condition Factors" (i.e., adipogranular cells (AG), viscular tissue (VT), hemocyte infiltration, and disrupted viscera (digestive tubules). When all Condition Factors were considered together, the determination of the tissue quality for that section suggested a test (T) animal or control (C) animal. Figure 3 shows a schematic drawing to illustrate the qualitative condition upon which the Condition Factors were judged.

To evaluate the different Condition Factors, certain assumptions were made. Adipogranular cells (AG) required reference to the degree of spawning. When there was none or few adipogranular cells in the tissue sections of animals in Mid to early Advance spawning stages, the sample could be rated as "C", unaffected because little or no adipogranular cell development is expected. But with increased spawning the lack of adipogranular cells, the lack of its extensive development, or, if adipogranular cells were poorly formed or thin, "T" or affected tissue was suggested. Because of the importance of

Table 3

Condition Factors qualified for tissue quality determination. Selection T (affected), C (unaffected) or with *(C or T*) to show judgment is based on spawning stage.

Adipogranular Cells (AG)

1. Extensive and thick; well developed (C).
2. Extensive but thin (T).
3. Beginning-developing; VT is greater than AG, (T or C*).
4. Incipient; AG not extensive; hints of developing (T or C*).
5. Undeveloped, no AG has yet been layed down (T or C*).

Vesicular Tissue (VT)

1. Well formed; reticular and flat; few mantle holes in (C).
2. Reticular but some VT cells are small and compact, not regularly formed (T or C).
3. Large gaping holes in VT; thin cells (T or C*).
4. Small, compact, irregularly formed VT (T).

Hemocyte Infiltration:

1. Numerous: Hemocytes closely packed in single field of view (T).
2. Many: Hemocytes scattered throughout field of view (C).
3. Some: Hemocytes appearing throughout section sample but are not concentrated in single field of viewing (C).
4. Few: Hemocytes sparsely scattered throughout section; not easily found (T or C).
5. None: Hemocytes are not found in section (T or C).

Condition of Viscera:

1. Digestive tubule cells disrupted, vaculated, swollen; thin epithelium; lacking pale crystalline granuluation (T).
2. Stomach epithelium cells infiltrated by hemocytes; cells disorganized; basal membrane disrupted by numerous hemocytes; epithelium cells show granulocytmas, (T or C).

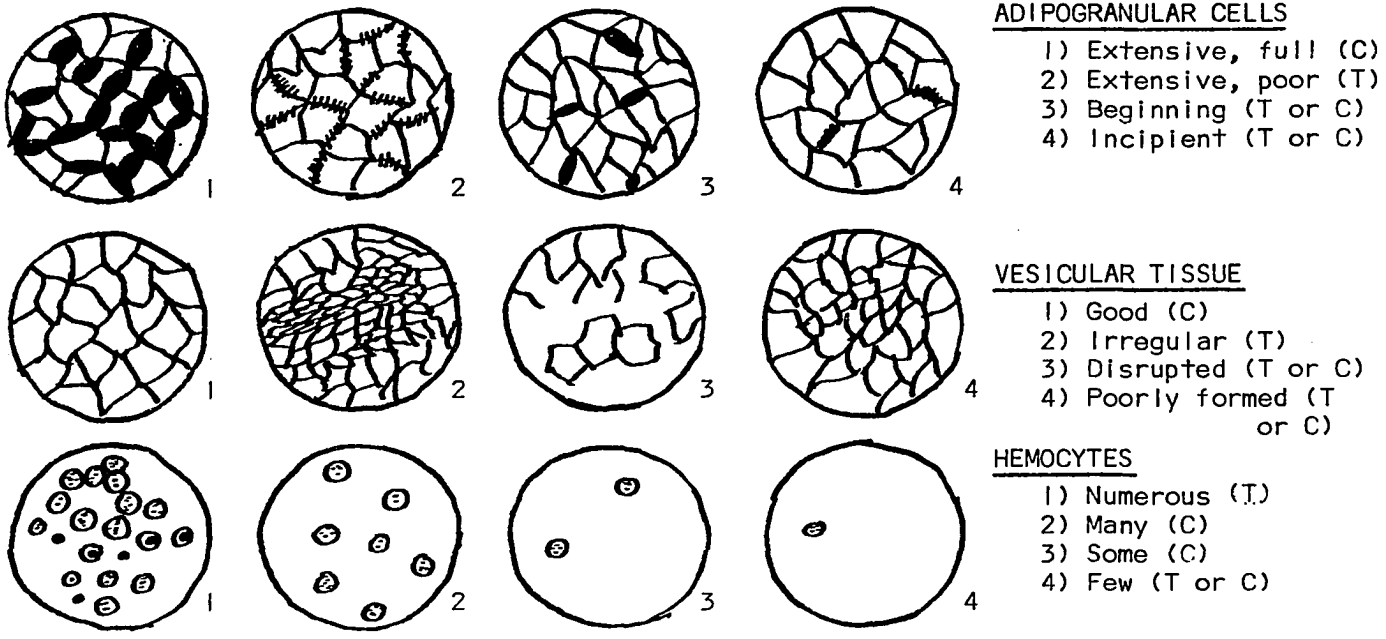

Fig. 3. Schematic drawings of three Condition Factors and their qualitative determination. C = unaffected; T = affected; T or C requires reference to spawning stage or other factors for better qualification.

adipogranular cells for winter survival and gametogenesis (Bayne, *et al.*, 1982), completed spawning was expected to contain extensive and full (not thin) AG cells.

The evaluation of vesicular tissue (VT) also required examination with reference to spawning stage. Although not verified, it appeared as though Mid to early Advance spawning may show disrupted VT and could be identified as either "C" or "T", possibly because of the release of gonadal material and the fixation of the animal prior to the repair process. But if VT showed poor development, irregular form, or thin in the later spawning stages, a "T" was given on the assumption that VT should appear well formed unless obscured by adipogranular cells.

Hemocyte concentration and types may also identify affected animal, however a better knowledge of their functional role in the spawning process is needed. It appeared that in areas where tissue disturbance was most evident, "numerous" mixed hemocytes appeared as identified in the literature (Cheng, 1981; Moore and Lowe 1977). Mixed hemocyte populations and dense concentrations suggested an affected (T) animal. In samples where "many" hemocytes were seen, the concentration was reduced and most were eosinophilic granulocytes. This was considered "C", unaffected, not representing an inflammatory reaction.

Other Condition Factors analyzed were unrelated to spawning. Digestive tubules change form as a result of feeding and submersion and show three cyclic phases (Langton, 1975), one (thin tubules) if

seen in high frequency is indicative of disturbance (Moore, *et al.*, 1978). These thin, round digestive tubules suggested an affected (T) animal, but some doubt could be expressed unless an adequate representative sample of the digestive tubules occurs in the histological section. If the digestive tubules were swollen, vaculated, or disrupted, "T" was suggested (Moore, *et al.*, 1978).

"Blind" analysis provided the technique for measuring the effects of oil on the Condition Factors discussed above and generally in identifying tissue quality. "Blind" means the masking of histological animal slide labels that identified the animal so that no prior knowledge could bias the judgment of observed features. Each of 395 slides was analyzed after all controls, preliminary animals, medium oil and high oil slide sections were thoroughly mixed together. A "T" was given for the slide section if the quality of the Condition Factors suggested a test animal having poorly formed adipogranular cells, hemocyte infiltration, or disrupted, vaculated, swollen or thin digestive tubules. For added assurance in the selective process, the over-all tissue sample was assessed as "Good", "Fair", or "Poor". After all slides were judged, the samples were identified. Each animal was represented by a series of slide evaluations as shown in Appendix 4. The series of evaluations provided the basis for establishing tissue condition for the animal. If the sum of the slide evaluations was judged "C" good-fair, the animal was classed in the Good category. If the sum of the slide evaluations showed a mixed response of T-Fair or C-Poor, or if there was about an equal number of

C-Fair and T-Poor sections to suggest that parts of the animal were affected and other parts were not, animal was classed Fair. If the sum of the slide sections was judged "T" and poor to bad in tissue quality, the animal was classed Poor. Thus the final analysis shows all animals in a category according to its tissue condition.

The percent frequency of animals in each tissue-quality category for each population and for each examination period provided the basis for suggesting qualitative changes in tissues as a result of oil exposure and as a result of exposure time to laboratory conditions.

III. RESULTS

DIFFERENTIAL CELL COUNT

Round granulocytes, small granulocytes, degranulated and serous cells each represent small percentages of the total (Appendix 5) for the individual study animals. No relationship in their occurrence could be established to identify or to correlate them with a particular function. These cells therefore are not included in the result analysis. Granulocytes and agranulocytes identified in this study for each animal represent the major kinds of circulating hemocytes (Appendix 5).

Granulocyte and agranulocyte percent means and 95% confidence interval for each study population are shown for two time intervals (Figure 4). Control percent means and confidence limits show a significant decrease in granulocytes ($P<.05$) with a significant increase in agranulocytes ($P<.05$) as a result of time in the laboratory. Experimental animals did not show these changes with time. In the 4-5 week period the medium oil population showed no difference in granulocyte percentages but the high oil population showed significantly ($P<.05$) reduced granulocyte percentage. With time, the medium oil population in the 8-9 week period showed no statistical percentage change for either granulocytes or agranulocytes, unlike the change noted for these cells in controls. The high oil population in the 8-9 week period showed granulocyte and agranulocyte percentages similar to the medium oil population, but significantly different from

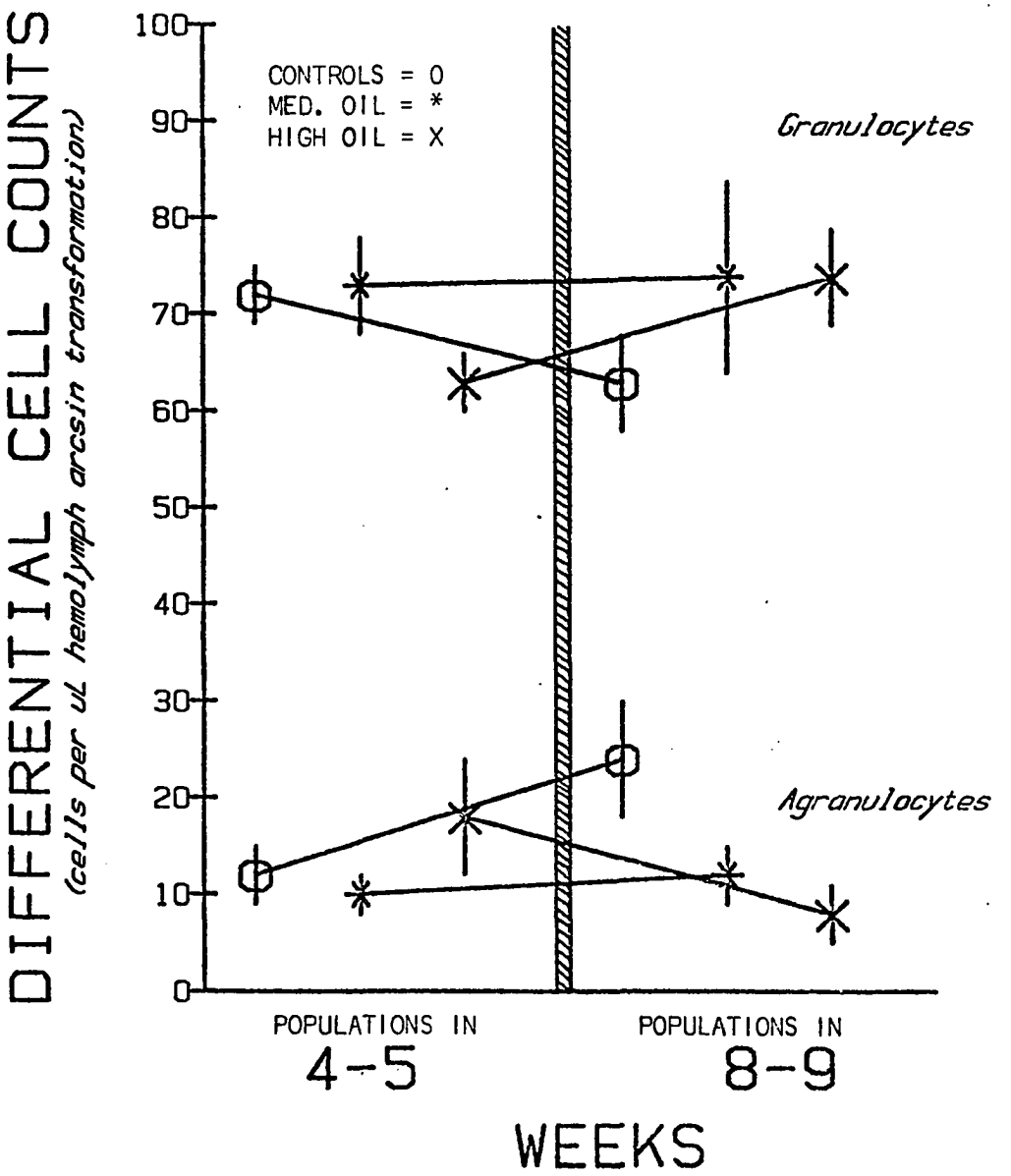

Fig. 4. Percentages of differential hemocytes as a result of time and exposure to 500 and 1000 nL/L of oil. Population means and 95% confidence for granulocytes and agranulocytes in 4-5 weeks and 8-9 weeks. Number of animals sampled: Controls = 9,11; Med. oil = 11,12; High oil = 4,5.

controls and directly opposite to its former 4-5 week value. These results showed that in the 4-5 week period 1000 nL/L of oil (high oil) appeared to decrease the number of granulocytes relative to control values. With time, oil affected both experimental populations with reduced percentages of agranulocytes and increased percentages for granulocytes.

The above percent values reflect relative change and require consideration in terms of total cell count. Absolute values clarify whether the relative changes in granulocytes and agranulocytes are the result of one particular cell type increasing or decreasing in abundance, or whether the change is due to increased numbers for all cells in the same relative proportions. Individual absolute values for four types of hemocytes are listed (Appendix 6), and population means, standard errors, and 95% confidence limits for granulocytes and agranulocytes are shown in Table 4. Mean absolute values in Figure 5 show that total cell count for controls had increased slightly with time due to an increase in agranulocytes, with little change occurring in the number of granulocytes. Unlike controls, both experimental populations showed a substantial increase in total cell count with time as a result of an increase in granulocytes; agranulocyte means increased only slightly with time for the medium oil population and decreased from higher values for the high oil population.

TOTAL CELL COUNT

Exposure to oil affected the number of circulating hemocytes. The total cell count for each individual is shown (Table 5) with

Table 4

Mean absolute values of granulocytes and agranulocytes for each study population for each time period. Includes population mean total cell counts and standard errors, with arc sin transformation of means and 95% confidence (Student's t-test).

					GRANULOCYTES				AGRANULOCYTES			
Time (weeks)	Group	No.	Total Cells (per µL) Mean	S.E.	Abs. Mean	S.E.	Arcsin Transf. Mean	95% C.L.	Abs. Mean	S.E.	Arcsin Transf. Mean	95% C.L.
4-5	Controls	9	2243	364	2243	347	72.13	69-75	105	33	11.89	9-15
	Medium Oil	11	1941	240	1768	240	72.93	68-78	53	10	10.23	8-12
	High Oil	4	1006	213	795	172	62.59	60-66	94	29	17.66	12-24
8-9	Controls	11	2426	332	1966	331	63.19	58-68	377	76	23.84	18-30
	Medium Oil	12	2867	313	2660	313	73.88	64-84	124	19	11.92	9-15
	High Oil	5	2614	615	2416	568	74.19	69-79	53	21	7.86	5-11

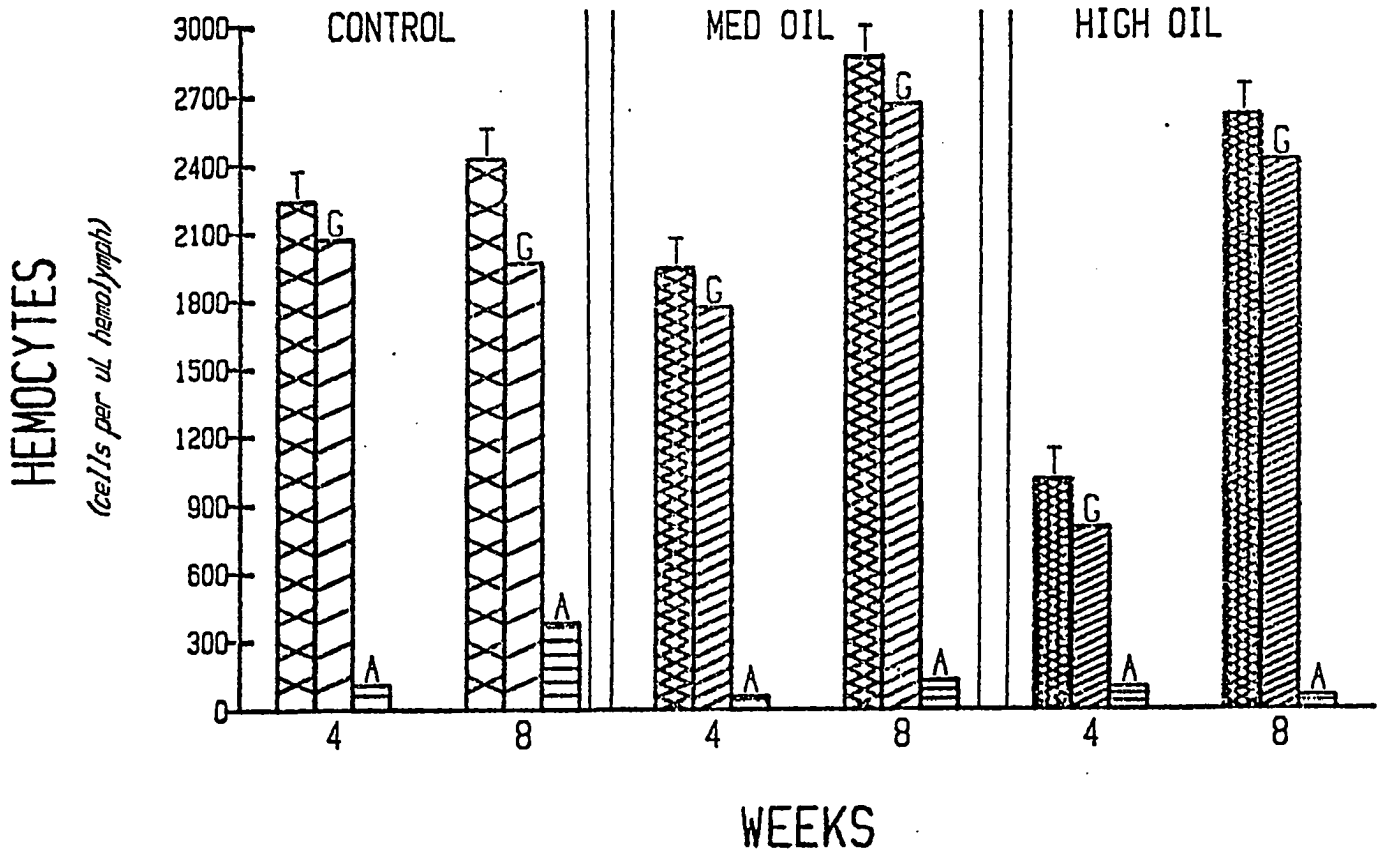

Fig.5. Population mean absolute values from differential cell counts to show changes in types as a result of time and exposure to 500 and 1000 nL/L of oil. Control, Medium oil and High oil mean total cell counts (T) compared to mean granulocytes (G) and agranulocytes (A) examined in 4-5 weeks (4) and 8-9 weeks (8).

Table 5

Total cell counts for individuals, with influencing environmental variables and with population means, standard error (S.E.) and 90% confidence limits (Student's t-test).

4-5 Week Period

		VARIABLES			POPULATION (Total Cells per µL)		
Animal	Sex	Temp (°C)	Size	Total Cells (per µL)	Mean	S.E.	90% C.L.
C-201	M	10.0	3.8	1597			
C-206	M	9.3	3.9	4042			
C-204	F	9.5	3.7	1544	CONTROLS		
C-210	F	9.0	3.5	875			
C-212	M	9.5	4.1	3388			
C-215	M	9.5	3.8	1744	2242	364	1565-2919
C-218	F	9.2	3.6	1622			
C-220	M	9.4	3.9	1908			
C-224	M	8.8	4.0	3464			
X-203	F	10.0	3.9	717			
X-205	M	9.7	3.8	993			
X-207	M	9.7	4.3	1844	MEDIUM OIL		
X-209	F	9.5	3.9	2102			
X-214	M	-	4.2	2142			
X-216	F	9.2	3.7	1260	1941	241	1504-2373
X-217	M	9.3	3.5	2051			
X-219	F	9.1	3.7	3337			
X-221	M	-	3.7	1600			
X-222	M	8.8	4.1	3071			
X-223	F	8.8	3.7	2235			
					HIGH OIL		
X-202	F	10.0	3.6	671			
X-208	M	9.2	3.6	1275	1006	213	505-1507
X-211	F	9.5	3.5	617			
X-213	F	9.3	2.9	1462			

Table 5 (Continued)

8-9 Week Period

VARIABLES					POPULATION (Total Cells per µL)		
Animal	Sex	Temp (°C)	Size	Total cells (per µL)	Mean	S.E.	90% C.L.
C-233	F	9.3	3.2	1534			
C-236	F	9.5	4.2	2528			
C-239	F	9.4	3.6	2115	CONTROLS		
C-244	F	9.5	3.4	2006			
C-248	M	9.5	3.9	1640	2426	344	1803-3049
C-252	M	-	3.5	1611			
C-253	F	9.7	3.9	4451			
C-254	M	9.7	4.7	3944			
C-256	M	10.2	3.9	3762			
C-259	M	10.4	3.5	1631			
X-237	M	9.3	3.8	1815			
X-238	F	9.3	4.7	4937			
X-241	F	9.3	3.8	1582	MEDIUM OIL		
X-242	M	9.5	3.8	3037			
X-243	F	9.5	3.9	1780			
X-245	M	9.2	3.8	3384	2867	314	2303-3431
X-246	F	9.3	4.0	1604			
X-247	F	9.3	4.0	2988			
X-249	M	9.3	3.6	2100			
X-250	M	9.3	4.3	3591			
X-257	M	10.4	3.6	3838			
X-258	F	10.5	3.8	3748			
					HIGH OIL		
X-230	M	9.5	3.4	451			
X-231	F	9.3	3.3	3717			
X-232	F	9.3	2.8	3126	2614	615	1303-3925
X-234	M	-	3.6	2093			
X-235	F	9.5	3.6	3682			

population means, standard errors, and 90% confidence limits (Student's t-test). Examining control means and 90% confidence limits (Figure 6) show that control cell counts changed little between the 4-5 and the 8-9 week periods. The high oil population, however, showed significantly ($P<.10$) reduced numbers of hemocytes in the 4-5 week period but values comparable to controls in the 8-9 week period.

The variability in population samples requires the examination of cell counts of individuals. Sixty percent of all controls for both time periods have total cell counts in the range of 1500 to 3000 cells per µL (Table 6). Table 6 shows the frequency of individuals with hemocyte counts that are in the high, average, and low ranges of controls. Identifying an average range for controls allows for the comparison of the number of experimental animals with hemocyte counts that fall outside the average range of controls. Table 7a shows a 2 X 2 contingency table to test the difference between the number of individual controls against the number of high oil animals in the same hemocyte ranges. The Chi-square statistic ($X^2_{\alpha.05}$) was applied to test the null hypothesis of difference. A significant number ($P<.05$) of high oil animals had hemocyte counts in the low range of controls in the 4-5 week period.

The jump from the low number of circulating hemocytes in the 4-5 week to the higher values comparable to controls in the 8-9 week period requires further examination. Using the 2 X 2 contingency table (Table 7b, c), the number of individuals with hemocyte counts in the low range against the number of individuals in the high range for

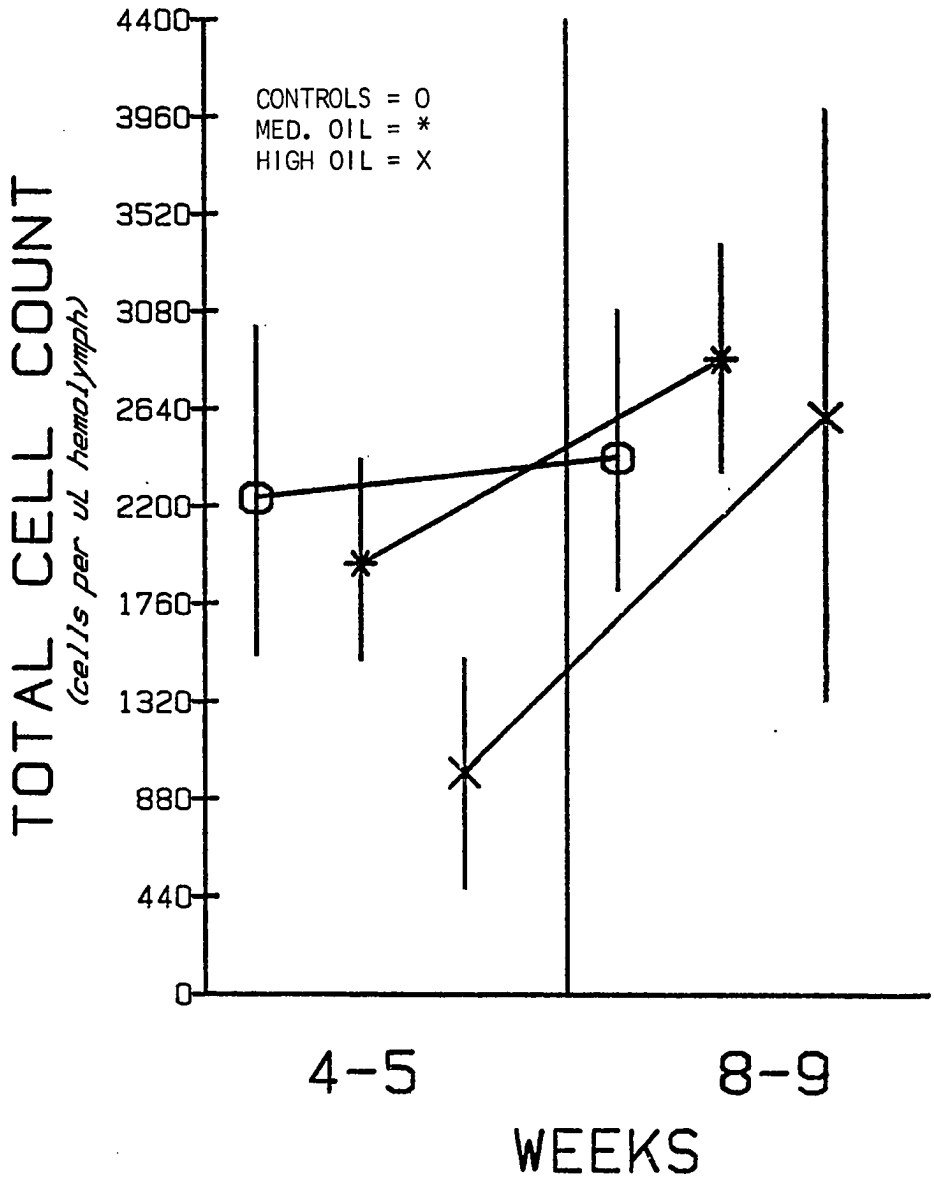

Fig. 6. Mean total cell counts and 90% confidence for each population in two time periods. Changes in total numbers of circulating hemocytes as a result of time and exposure to 500 and 1000 nL/L oil. Number of animals sampled: Controls = 9,11; Medium Oil = 11, 12; High Oil = 4,5.

Table 6

Classification of the number of circulating hemocytes, based on high, average, and low values of controls. Shows number of animals and percent frequency ( ) of population within hemocyte range.

4-5 Week Period

Hemocyte Count (cells per μL)	Group	Number and (Percent Frequency) Control	Medium	High
1-1499	low	1 (11%)	3 (27%)	4 (100%)
1500-3000	average	5 (56%)	6 (55%)	0
3000-plus	high	3 (33%)	2 (18%)	0

8-9 Week Period

1-1499	low	1 (9%)	0	1 (20%)
1500-3000	average	7 (63%)	6 (50%)	1 (20%)
3000-plus	high	3 (27%)	6 (50%)	3 (60%)

Table 7

2 x 2 contingency table to test different variables against high, medium and low hemocyte values.

A - Independence of controls from high oil animals of the 4-5 week period to show differences in the number of circulating hemocytes.

	Average Number Hemocytes	Low Number Hemocytes	
Controls:	7	3	10
High Oil:	1	3	4
	8	6	14

$X^2_{corr} = 7.09$ (calculated)

$X^2_{\alpha.05} = 3.841$ (critical)

B - Independence of the 4-5 from 8-9 week medium oil animals to show differences in numbers of hemocytes as a result of time.

	Low Number Hemocytes	High Number Hemocytes	
4-5 Weeks:	4	0	4
8-9 Weeks:	1	3	4
	5	3	8

$X^2_{corr} = 5.34$ (calculated)

$X^2_{\alpha.05} = 3.841$ (critical)

Table 7 (Continued)

C - Independence of 4-5 week 8-9 week high oil animals to show differences in the numbers of hemocytes as a result of time.

	Low Number Hemocytes	High Number Hemocytes	
4-5 Weeks:	3	2	6
8-9 Weeks:	0	6	6
	3	8	12

$$X^2_{corr} = 5.46 \quad \text{(calculated)}$$

$$X^2_{\alpha.05} = 3.841 \quad \text{(critical)}$$

two time periods are examined for each experimental population. The results showed that both the medium and high oil populations each had a significant ($P<.05$) number of individuals with hemocyte counts in the 4-5 week period unlike the hemocyte counts of individuals in the 8-9 week period. This indicates that the effect of oil initially was to cause a decrease in the number of circulating hemocytes for a significant number of individuals, but with time, possibly through feedback mechanisms, the production of hemocytes was stimulated to levels comparable to controls.

PHAGOCYTIC RESPONSE

Prudhoe Bay crude oil affected the phagocytic response in animals exposed to high oil after 8-9 weeks. The percent mean and standard errors for each population, with arc sin transformed means and 95% confidence levels are shown for each study period (Table 8). The data (Figure 7) showed a significant ($P<.05$) decrease in response for controls with time. There was no significant difference between controls and either experimental group in the 4-5 week period. The decrease occurring for controls in the 8-9 week period occurred in the medium oil population as well. However, the high oil population showed a significantly ($P<.05$) reduced response in the 8-9 week period in oil concentrations of 1000 nL/L.

SPAWNING ANALYSIS

Spawning stage was determined for all study animals by the dominance in percent frequency of follicle or storage tissue cells in

Table 8

Phagogytic response for each study population and time period.

				Arc Sin Transformation	
Animal	No.	Mean	S.E.	Mean	95% Confidence Limits
4-5 Week Period					
Controls	9	89	1.6	71	68 - 74
Medium Oil	11	80	3.6	64	58 - 70
High Oil	4	80	7.5	65	46 - 84
8-9 Week Period					
Controls	11	68	4.8	56	49 - 63
Medium Oil	13	67	5.8	55	47 - 63
High Oil	5	19	7.1	24	10 - 38

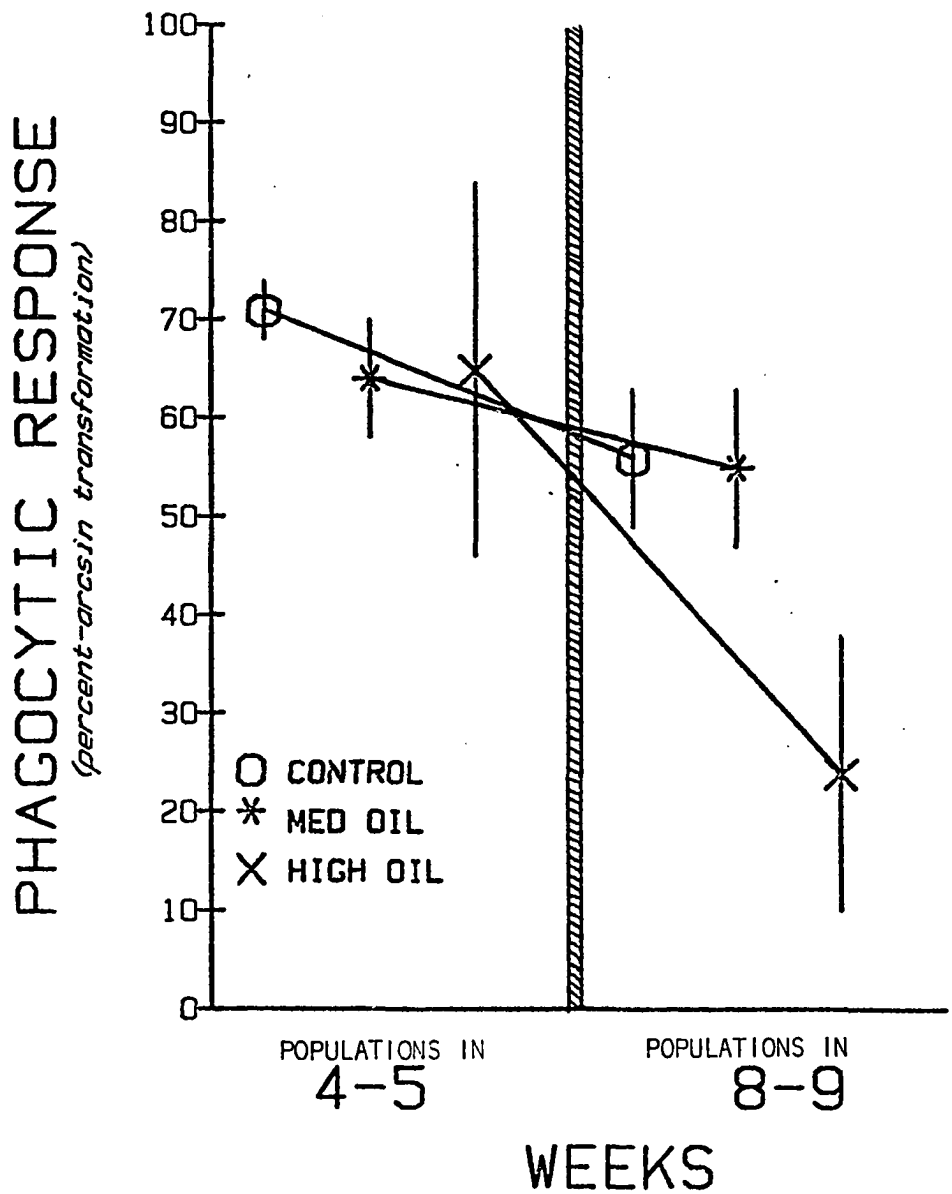

Fig.7. Phagocytic response to yeast. Population means and 95% confidence of percentage changes as a result of time and exposure to 500 and 1000 nL/L of oil. Number of animals sampled: Controls = 9,11; Med. oil = 11,12; High oil = 4,5.

the mantle, as presented in Table 9. As the spawning process produces dynamic changes in the mantle with time as a result of spawned gametes, reduced follicles, and the development of lipid storage cells (Bayne, *et al.*, 1982), it is expected that the mantle will show changes between the 4-5 week populations and the 8-9 week populations. Figure 8 shows the spawning changes of controls with respect to time. Controls in the 4-5 week period fall into the early spawning stages with 78% in Mid spawning. In the 8-9 week period none fall into Mid spawning and 100% fall into Advance to Completed spawning stages. For the experimental populations, this progression does not occur for all animals. Figures 9 and 10 shows the frequency in spawning stage for the experimental populations; about 30% of the animals in each experimental group still occur in Mid spawning. To test for significant differences between the spawning progression of controls and experimental animals, the 2 x 2 contingency table (Table 10) was applied. Using the Chi-square statistic ($X^2_{\alpha.05}$) to test the difference, there was no significant difference between controls and experimental animals in spawning behavior in the 4-5 week period, but a significant difference ($P<.05$) in the 8-9 week period. This analysis showed that the longer exposure to oil affected the natural spawning progression for a significant number of animals.

TISSUE CHANGES

The results of the histological analysis of tissue quality provided for the classification of animals into categories of Good,

Table 9

Spawning classification of individuals in each population by percent frequency of cell types mantle. (Space and hemocytes; in storage tissue not included, thus total not equal to 100%).

4-5 Week Period

Spawning Category	Animal	% Total Follicle (Gametes & Space)	% Total Storage Tissue (VT, AG & Space)
Mid Spawn	C-201	88	6
	C-204	86	9
	C-210	83	16
	C-212	79	16
	C-215	80	15
	C-218	93	6
	C-224	90	6
	X-203	81	18
	X-205	88	7
	X-209	80	11
	X-216	95	6
	X-219	90	6
	X-202	82	11
	X-208	80	8
Mid-Advance	X-223	71	28
Advance	C-206	66	28
	C-220	64	26
	X-214	44	49
	X-217	69	20
	X-221	63	23
	X-222	65	28
	X-211	70	20
End	X-207	34	57
Complete	X-213	7	82

Table 9 (Continued)

8-9 Week Period

Spawning Category	Animal	% Total Follicle (Gametes & Space)	% Total Storage Tissue (VT, AG)
Mid Spawn	X-241	79	15
	X-243	85	9
	X-247	80	14
	X-231	90	7
Mid-Advance	X-245	70	25
Advance	C-236	47	47
	C-240	38	47
	C-244	43	25
	C-259	68	30
	X-237	68	26
	X-257	*	
	X-230	62	30
	X-234	64	22
	X-235	69	25
Advance End	C-233	25	44
	C-253	40	55
	X-258	48	52
	X-249	19	38
End	X-239	33	56
	X-256	30	69
	X-238	35	52
End Complete	C-248	7	68
	X-246	15	65

* Classed according to observation rather than percentage.

Table 9 (Continued)

8-9 Week Period

Spawning Category	Animal	% Total Follicle (Gametes & Space)	% Total Storage Tissue (VT, AG)
Complete	C-252	8	80
	C-254	7	87
	X-242	14	75
	X-250	10	79
	X-255	2	91
	X-232	-	-

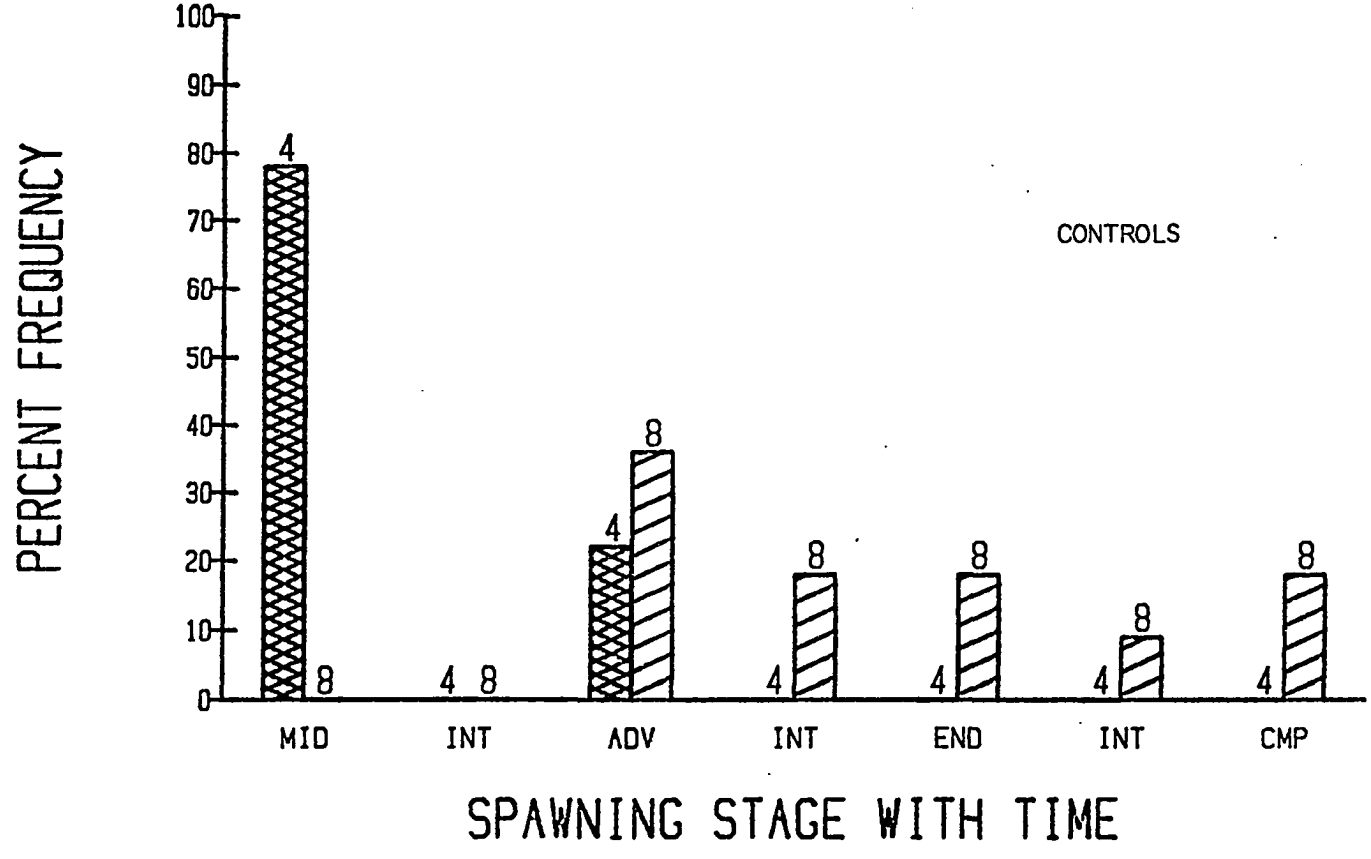

Fig. 8. Percent frequency of controls in spawning stages showing changes occurring with time. MID = Mid Spawning; INT. = Inter-stage; ADV. = Advance Spawning; END = Spawning; CMP = Completed Spawning. 4-5 week period (4) and 8-9 week period (8).

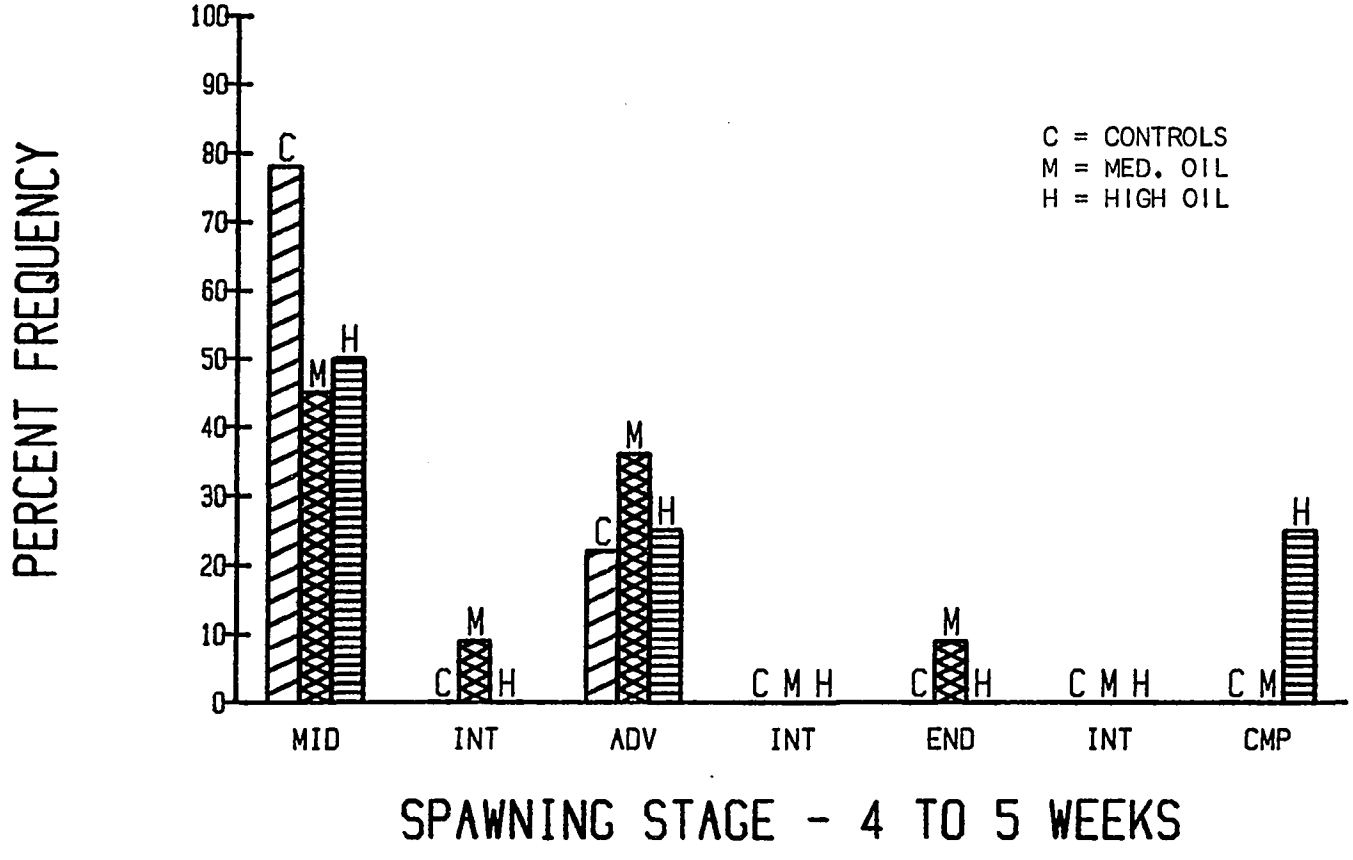

Fig. 9. Comparison of study populations to show frequency of animals in each spawning stage. MID = Mid Spawning; INT. = Inter-stage; ADV. = Advance Spawning; END = End Spawning; CMP = Completed Spawning.

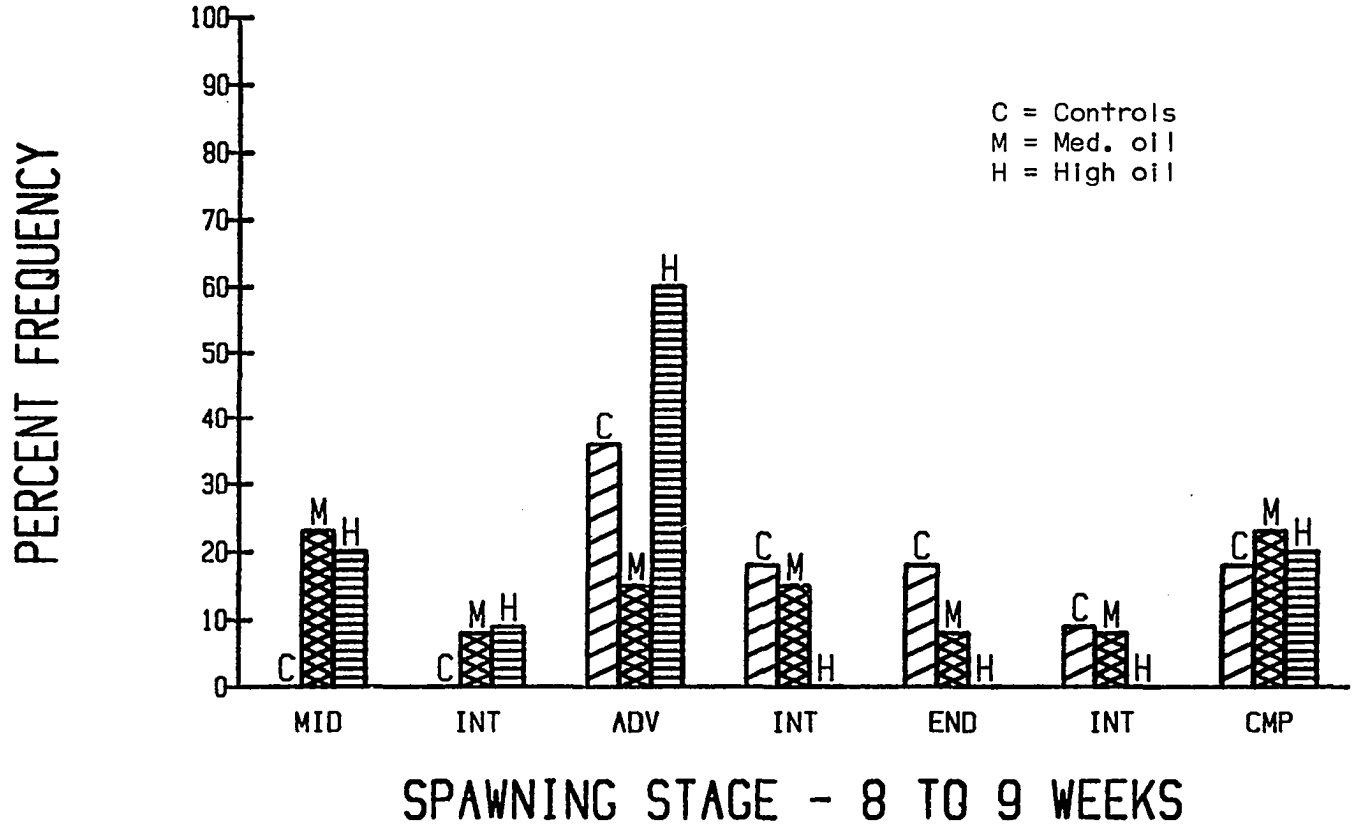

Fig. 10. Comparison of study populations to show frequency of animals in each spawning stage. MID = Mid Spawning; INT. = Inter-stage; ADV. = Advance Spawning; END = End Spawning; CMP = Completed Spawning.

Table 10

2 x 2 contingency table on spawning progression to show differences betweem controls and experimental animals.

4-5 Week Period

	Mid Spawn	Advance-Complete Spawning	
Controls	7	2	9
Experimentals	7	8	15
	14	10	24

$X^2_{corr} = 2.26$ (calculated)

$X^2_{a.05} = 3.841$ (critical)

8-9 Week Period

	Mid Spawn	Advance-Complete Spawning	
Controls	0	11	11
Experimentals	5	11	16
	5	22	27

$X^2_{corr} = 4.2$ (calculated)

$X^2_{a.05} = 3.841$ (critical)

Fair, and Poor (Table 11). The percent frequency of all animals in each condition for each population and time period are presented (Figure 11) to show that preliminary animals and controls had higher frequencies of animals in Good condition while the experimental populations each showed higher percentages in Poor condition. The results suggest that some controls were experiencing detrimental effects, as identified in structural changes, and that these changes were similar to those occurring in experimental animals. Structural changes in controls may have been caused by gas bubbles in the laboratory water as a result of heating cold seawater. Direct evidence of gas bubbles was apparent in only one control, C-244 which had a large bubble in its mantle. Its mantle tissue lacked adipogranular cells, had disrupted vesicular cells, large follicles with few gametes (eggs), few hemocytes except localized infiltrations, and large granulocytomas (Lowe and Moore, 1979) in the vesicular tissue of the viscera and in the stomach epithelium. Nevertheless, histological results showed that some controls were obviously unaffected. C-259, the last animal studied, showed very good condition, with very full and extensive adipogranular cells between full follicles and with no indication of either tissue or organ damage. Experimental animals, on the other hand, showed high frequency of animals in poor tissue condition. This could be indicative of the added stress of oil to gas bubble stress, resulting in tissue strain to the point of disruption. This added and cummulative strain disrupted tissues in a greater number of sections per animal and in a greater number of experimental animals.

Table 11

Classification of individuals by tissue quality and population. The percent frequency in Good, Fair and Poor are presented for each study population without consideration to time.

CONDITION	TIME		POPULATIONS				
	Weeks	Controls n=20	Pop. % Freq.	Medium Oil n=24	Pop. % Freq.	High Oil n=9	Pop. % Freq.
Good	4-5	C-201 C-204 C-206 C-220	44	X-203 X-216 X-223	27	X-208	25
	8-9	C-248 C-252 C-254 C-259	36	X-255	8		
	% of Total		40		17		11
Fair	4-5	C-210 C-212 C-215 C-224	44	X-209 X-214 X-205 X-219 X-222	45	X-202 X-213	50
	8-9	C-233 C-240 C-239	27	X-237	8	X-232	20
	% of Total		35		25		33

Table 11 (Continued)

CONDITION	TIME		POPULATIONS				
	Weeks	Controls n=20	Pop. % Freq.	Medium Oil n=24	Pop. % Freq.	High Oil n=9	Pop. % Freq.
Poor	4-5	C-218	11	X-217 X-221 X-207	27	X-211	25
	8-9	C-236 C-253 C-256 C-244	36	X-241 X-243 X-247 X-245 X-258 X-238 X-246 X-249 X-242 X-250 X-257	85	X-230 X-231 X-234 X-235	80
	% of Total		25		58		56

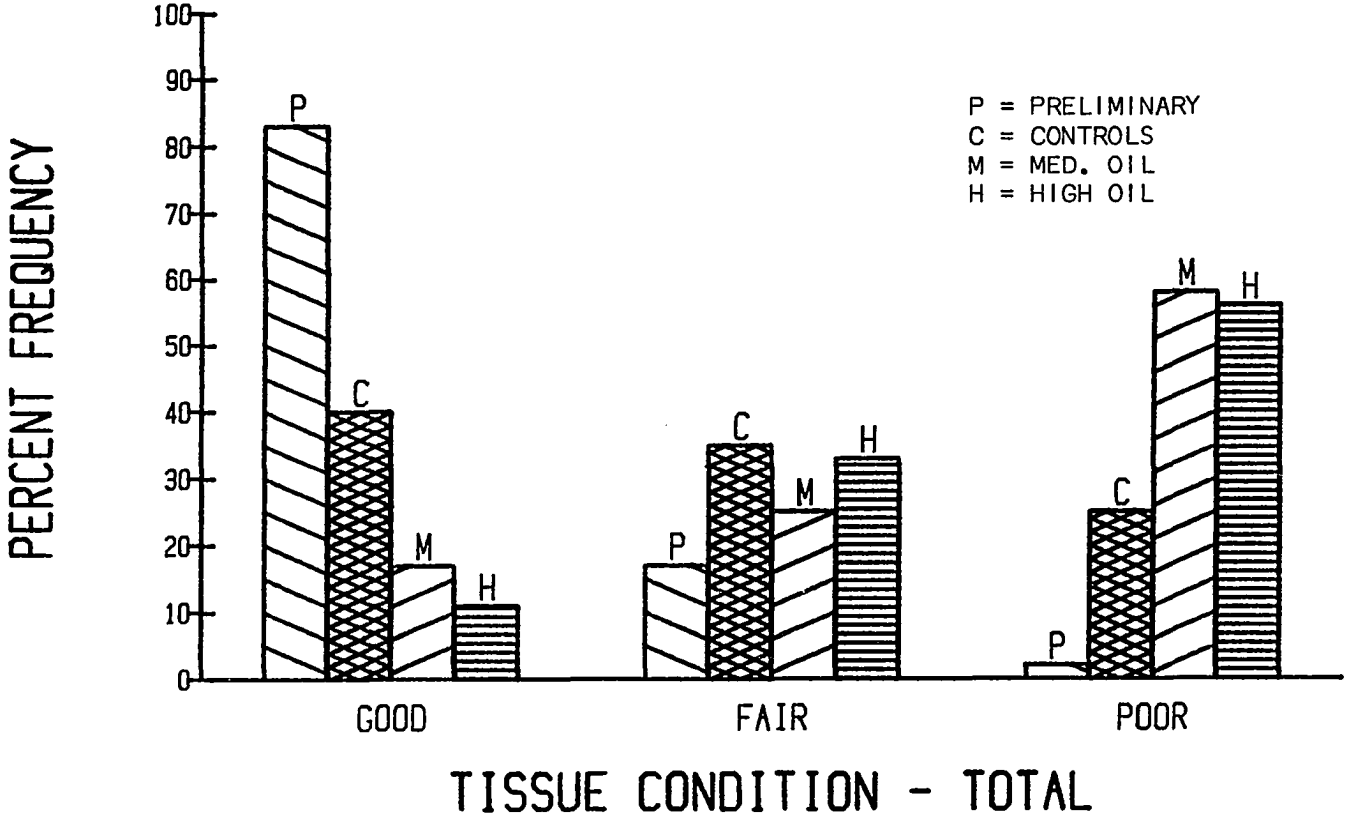

Fig. 11. Comparison of all study populations and the percent frequency of all animals classed as Good, Fair, or Poor condition.

The frequency of study animals in Good, Fair, and Poor condition for each time period (Figure 12) shows that in the 4-5 week controls, 88% of the animals were in good and fair condition. Those animals classed as Fair had tissues with some structural changes while other parts had little or no disturbance. In this time period only one control, C-218, was classed as Poor indicating that all or nearly all tissue sections showed disturbance. For experimental animals in the 4-5 week period, there were fewer animals in Good condition while there was a higher percentage (74%) in Fair and Poor condition. This indicates that more experimental animals were showing some sign of tissue change in parts of their bodies as determined by the high frequency in Fair condition and others classed as Poor were affected throughout their bodies.

Animals exposed 8-9 weeks to laboratory conditions and to oil showed increased detrimental effects, with higher frequencies in Poor condition. For controls, the effects of the laboratory water increased the frequency of animals in Poor condition to 36%. The frequency of experimental animals in Poor condition was 85% for medium oil animals and 80% for high oil animals. This clearly illustrates that the effect of oil was to increase the stress threshold to cause structural changes in far greater frequency and extent than observed for controls.

The results of analysis in determining tissue condition and oil effect require closer scrutiny of the selection process. One of the most indicative factors of tissue disturbance was the quality and

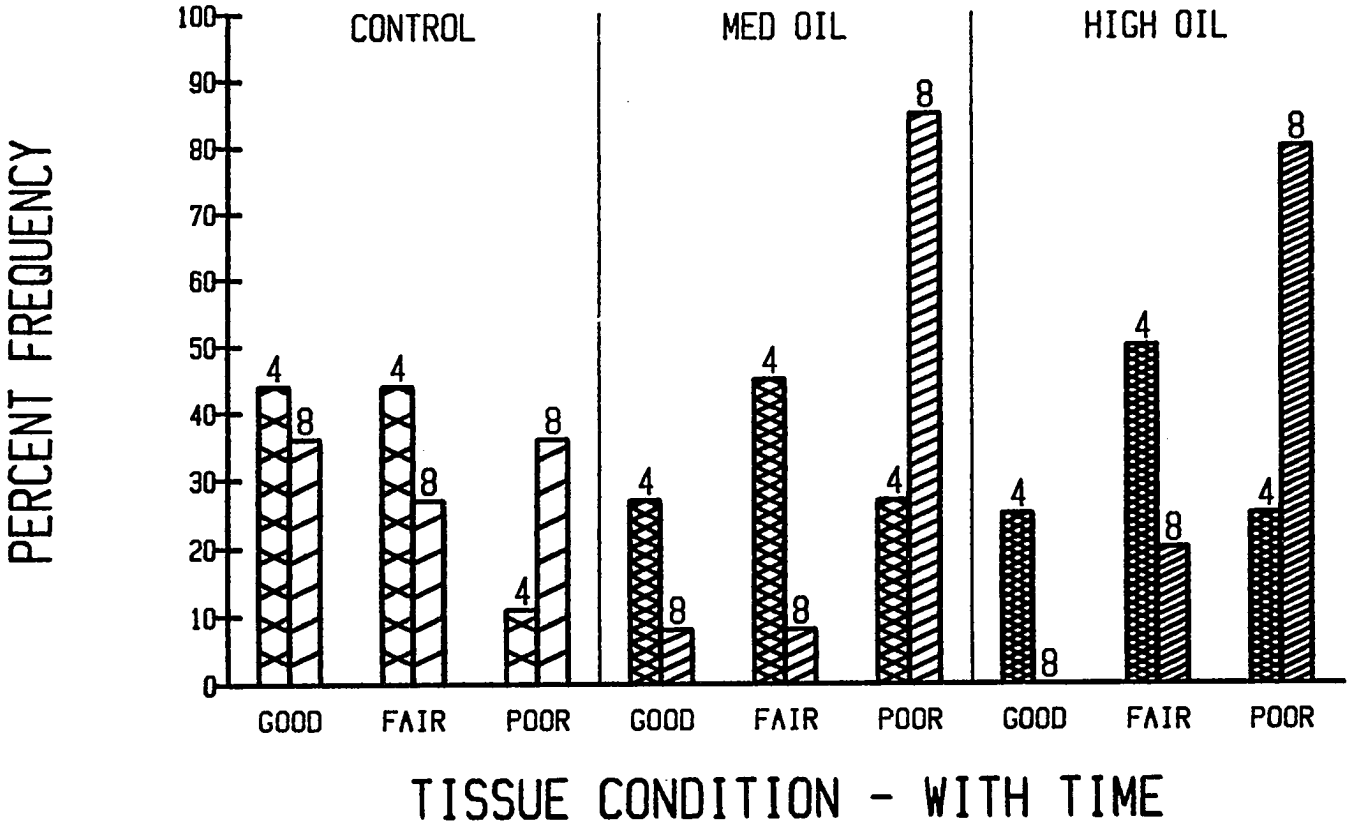

Fig. 12. Comparison of study populations to show percent frequency of animals in Good, Fair, or Poor condition in the 4-5 week period (4) and 8-9 week period (8).

extensiveness of adipogranular cells (AG). Plate 2 shows the difference in quality of good AG (red cells, top photo) and affected cells (bottom photo). Table 12 provides a descriptive summary of AG cells for each animal, with percentage quantification derived from stereological point count frequencies of the mantle cells and tissues. Controls classified as Good examplify expected field animals. They showed increased percentage of adipogranular cells with spawning progress and time. Although sample size was small, the 4-5 week Advance spawners showed less adipogranular cell development than those in the more advanced stages of spawning. This suggests that the more advanced spawners of controls were adding storage lipid reserve cells (AG) to mantle tissue with increasing length of time in the water as a result of continuous feeding. Controls in Fair condition showed increased adipogranular cell development with Advanced spawning but less extensively than those in Good condition. Controls classed in Poor condition showed a very obvious reduction or complete lack of adipogranular cell development, except C-253 which had 23% AG, although AG quality was poor and thin by comparison.

The effect of oil intensified the effect on the animal and on more animals. High oil animals showed a lack of AG development for all spawning stages in both time periods. Medium oil animals showed reduced percentage but not suppression, but AG quality of the AG appeared affected. One medium oil animal in Good condition, X-255, showed very extensive adipogranular development, with percentages comparable to Good controls. However, AG quality was poor and thin

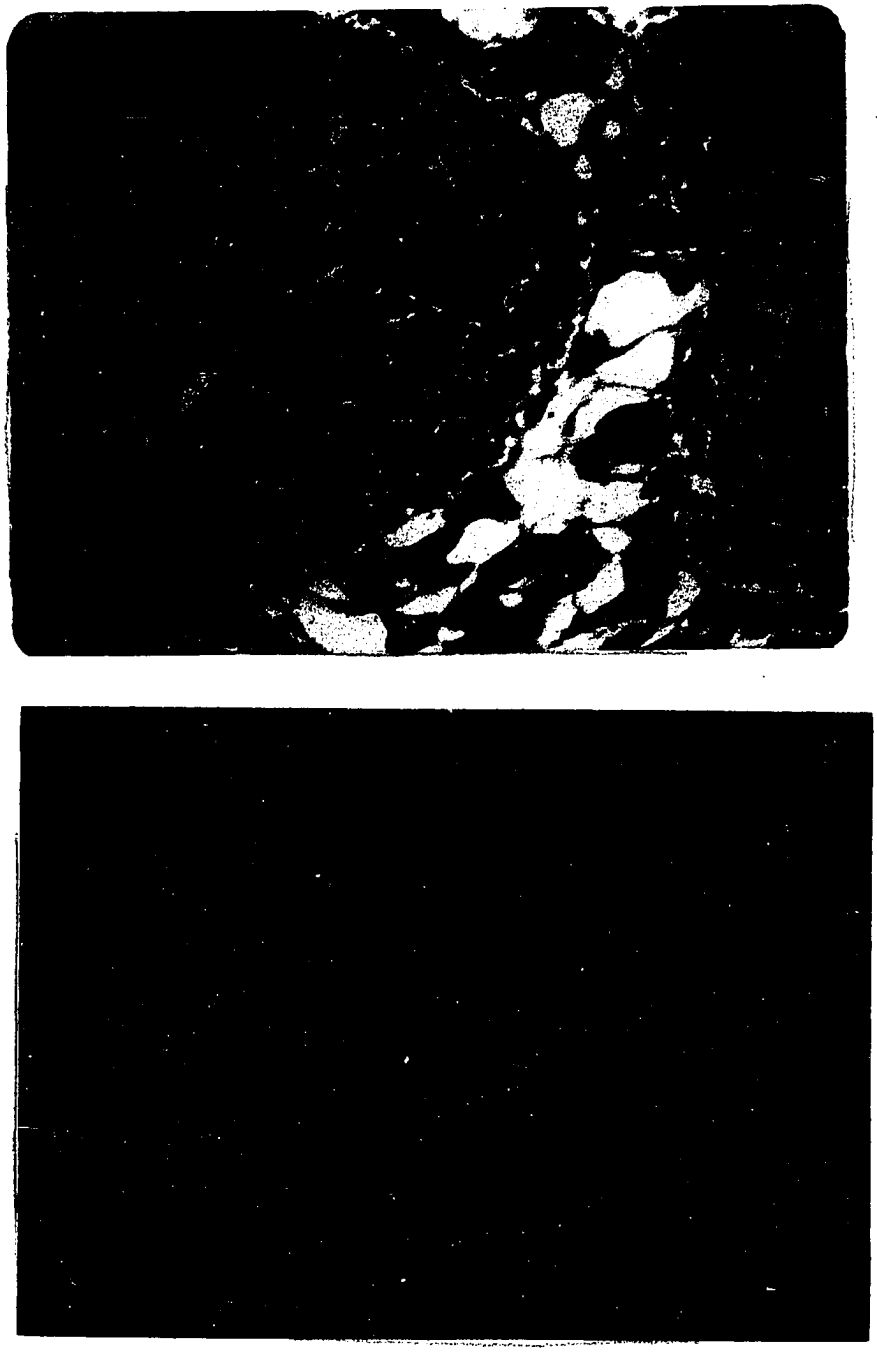

Plate 2. Adipogranular cells. (above) Full and fat. (below) thin and poor.

Table 12a

Adipogranular cell (AG) development according to tissue condition and spawning stage.

CONTROLS

Animal	AG Descriptive Quality	Percentage Point Count
	GOOD CONDITION	
Mid Spawners		
C-201	no development	0
C-204	no development	0
Advance Spawners		
C-206	extensive beginning	9
C-220	extensive beginning	10
C-259	extensive and full	13
End Spawners		
C-248	extensive and full	38
Complete Spawners		
C-252	extensive and fair (thin)	31
C-254	extensive and full (fat)	36
	FAIR CONDITION	
Mid Spawners		
C-210	incipient	2
C-212	developing	2
C-215	incipient	1
C-224	developing, full (thin)	10
Advance Spawners		
C-240	extensive and fat	25

Table 12a (Continued)

CONTROLS

Animal	AG Descriptive Quality	Percentage Point Count
Advance-End Spawners		
C-233	developing	23
End Spawners		
C-239	extensive, thin	22

POOR CONDITION

Mid Spawners		
C-218	no development	0
Advance Spawners		
C-236	incipient	4
C-244	none	0
Advance-End Spawners		
C-253	extensive thin	23
End Spawners		
C-256	none	0

Table 12b

Adipogranular cell development according to tissue condition and spawning stage.

MEDIUM OIL

Animal	AG Descriptive Quality	Percentage Point Count
	GOOD CONDITION	
Mid Spawners		
X-203	developing	3
X-216	no development	0
Mid Advance Spawners		
X-223	no development to incipient	2
Complete Spawners		
X-255	extensive and thin	33
	FAIR CONDITION	
Mid Spawners		
X-205	no development	0
X-209	no development	0
X-219	no development	0
Advance Spawners		
X-222	no development to incipient	0
X-237	developing	11
X-214	extensive and thin	14
	POOR CONDITION	
Mid Spawners		
X-241	no development	0
X-243	no development	0
X-247	incipient and developing	2

Table 12b (Continued)

MEDIUM OIL

Animal	AG Descriptive Quality	Percentage Point Count
Advance Spawners		
X-217	developing	3
X-257	no development	0
X-221	incipient and developing	6
Mid Advance Spawners		
X-245	incipient and developing	4
Advance-End Spawners		
X-258	incipient	14
X-249	incipient	2
End-Spawners		
X-207	incipient	0
X-238	developing	7
End Complete Spawner		
X-246	extensive and thin	27
Complete Spawners		
X-242	incipient/entensive (thin)	24
X-250	developing, thin (poor)	16

Table 12c

Adipogranular cell development according to tissue condition and spawning stage.

HIGH OIL

Animal	AG Descriptive Quality	Percentage Point Count
	GOOD CONDITION	
Mid Spawners		
X-208	incipient; extensive	0
	FAIR CONDITION	
Mid Spawners		
X-202	no development	0
Complete Spawners		
X-232	no development	0
X-213	no development	0
	POOR CONDITION	
Mid Spawners		
X-231	no development	0
Advance Spawners		
X-230	developing; condition	8
X-211	no development	0
X-234	no development - developing	1
X-235	no development	0

by comparison. In examining percentages only (Table 13), AG of high oil animals was affected compared to controls of the same spawning stage. Thus it is apparent that 1000 nL/L of oil may either interfere or surpress AG development. AG quality may be affected in animals exposed to medium oil concentrations (500 nL/L) in conjunction with bubble stress. The lack of AG in some controls of Poor condition suggest that bubble stress can also affect AG, but oil appears to act as an additive stress to affect more animals and to a far greater extent. In summary, oil stress appeared to add to general stress to interfere or to use up the energy stores available.

Examination of vesicular tissue (VT) also was indicative of oil stress. The normal appearance of vesicular tissue was a reticular net of flat cells, regular in size and form and with few large holes (Plate 3, above). Experimental animals showed an increase in disturbed vesicular tissue, identified as small irregular cells (Plate 3, below). Table 14 presents descriptive results to show VT development and condition of animals classed by tissue quality and spawning stage. In the 4-5 week examination period, VT was not a reliable indicator of oil stress but in the 8-9 week period poor quality was indicated by extensive irregular cells (Table 15). Oil may thus intensify the occurrence of irregular VT during the possibly critical VT restoration period after spawning as indicated by the increased percentage in the experimental populations (Figure 13a). VT may indicate stress in ways other than by disruption of structure. The vesicular cells may appear faint and thin with poor staining quality. Holes in the VT of the

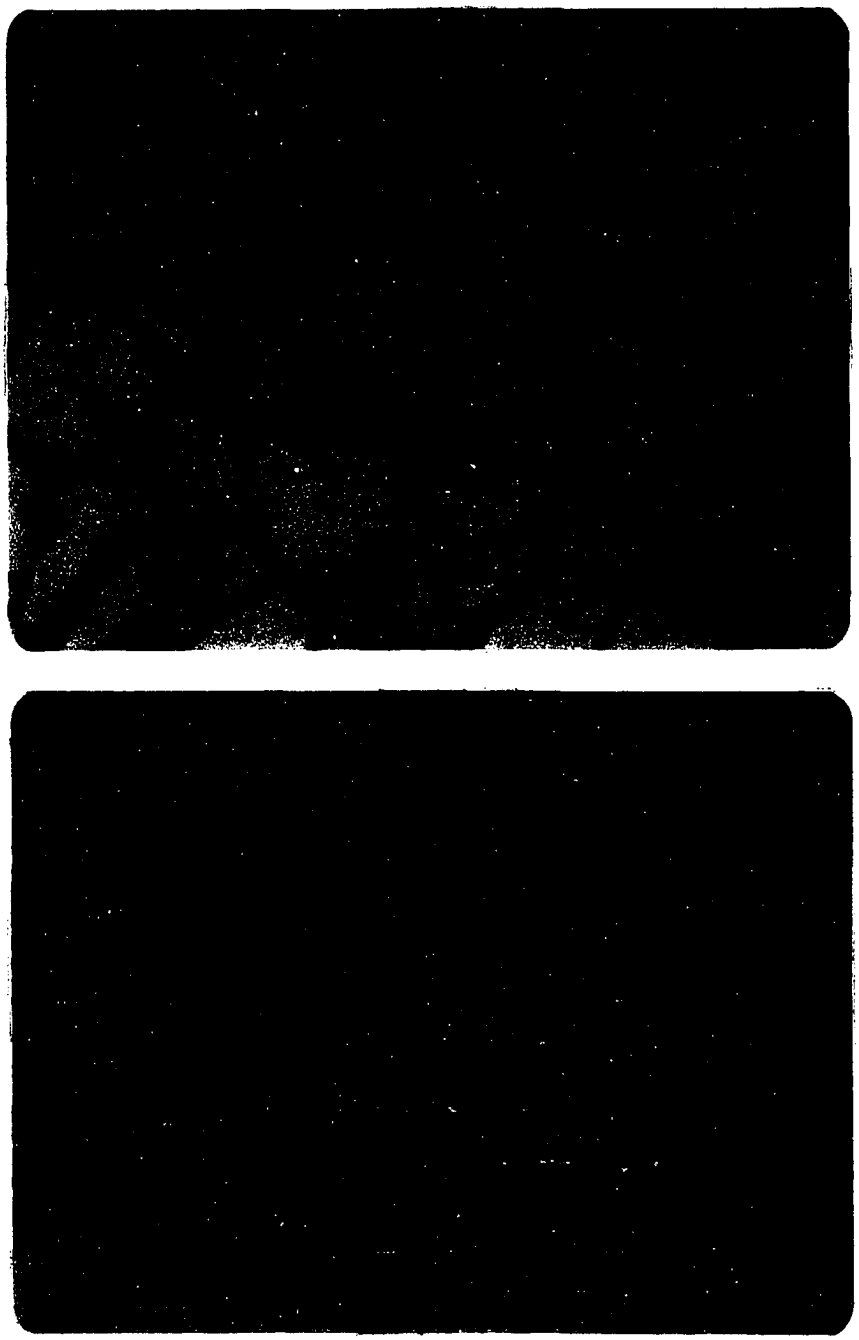

Plate 3. Vesicular tissue. (above) Good form. 400X. (below) Irregular and poor. 200X.

Table 13

Summary of adipogranular to compare all study animals AG percent frequency in mantle tissue.

4-5 Week Period

Controls	%AG	Medium Oil	%AG	High Oil	%AG
		GOOD CONDITION			
Mid Spawners					
C-201	0	X-203	3	X-208	0
C-204	0	X-216	0		
Mid Advance Spawner		X-223	2		
		FAIR CONDITION			
Mid Spawners					
C-210	2	X-209	0	X-202	0
C-212	2	X-205	0		
C-215	1	X-219	0		
C-224	10				
Advance Spawners		X-214	14	X-213	0
		X-222	0		
Complete Spawner		X-213	0		
		POOR CONDITION			
Mid Spawner					
C-218	0				
Advance Spawners		X-221	6	X-211	0
		X-217	3		
		X-207	0		

Table 13 (Continued)

8-9 Week Period

Controls	%AG	Medium Oil	%AG	High Oil	%AG
GOOD CONDITION					
Advance Spawner					
C-259	13				
End-Complete Spawner					
C-248	38				
Complete Spawners					
C-252	31	X-255	33		
C-254	36				
FAIR CONDITION					
Advance Spawner					
C-240	25	X-237	11		
Advance-End Spawner					
C-233	23				
End Spawner					
C-239	22				
End-Complete Spawner				X-232	0
POOR CONDITION					
Mid Spawners		X-241	0	X-231	0
		X-243	0		
		X-247	2		
Mid Advance Spawners		X-245	4		

Table 13 (Continued)

8-9 Week Period

Controls	%AG	Medium Oil	%AG	High Oil	%AG
Advance Spawners					
C-236	4	X-257	0	X-234	1
C-244	0			X-230	8
				X-235	0
Advance-End Spawners					
C-253	23	X-258	14		
		X-249	27		
End Spawners					
C-256	0	X-238	7		
End Complete Spawner		X-246	27		
Complete Spawners		X-242	27		
		X-250	16		

Table 14

Vesicular tissue descriptive condition of individuals listed by spawning stage and oil exposure.

4-5 Week Period

Controls	V.T. Condition	Medium Oil	V.T. Condition	High Oil	V.T. Condition
		GOOD CONDITION			
Mid Spawners					
C-201	undeveloped; small holes.	X-203	good; slight; irregular.	X-208	undeveloped; holes.
C-204	undeveloped.	X-216	fair.		
Mid-Advance Spawners		X-223	good.		
Advance Spawners					
C-206	some good; some poor.				
C-220	good; some small size.				

	Controls	V.T. Condition
Mid Spawners		
	C-210	good.
	C-212	good; small size.
	C-215	fair, some.
	C-224	poor-fair; large; hematoma.
Advance Spawners		
Complete		

Table 14 (Continued)

4-5 Week Period

Medium Oil	V.T. Condition	High Oil	V.T. Condition
FAIR CONDITION			
X-205	good; some.	X-202	undeveloped
X-209	thin; some holes.		
X-219	undeveloped.		
X-222	good; some holes.		
X-214	good; holes; some irregular.		
		X-213	good; some holes.

	Controls	V.T. Condition
Mid Spawners		
	C-218	big holes; poor.
Advance Spawners		
End Spawners		

Table 14 (Continued)

4-5 Week Period

Medium Oil	V.T. Condition	High Oil	V.T. Condition
POOR CONDITION			
X-217	good; holes.		
X-221	poor; big holes; irregular.	X-211	poor; thin.
X-207	fair; some holes.		

Controls	V.T. Condition
Advance Spawners	
C-259	little development.
End-Complete Spawners	
C-248	good; some holes.
Complete Spawners	
C-252	good; some holes; irregular.
C-254	good; some holes.
Advance Spawners	
C-240	good; some holes.

Table 14 (Continued)

8-9 Week Period

Medium Oil	V.T. Condition	High Oil	V.T. Condition
GOOD CONDITION			
X-203	good; slight.	X-208	undeveloped holes
X-255	fair; holes; irregular.		
FAIR CONDITION			
X-237	poor; holes.		

	Controls	V.T. Condition
Advance Spawners		
	C-236	poor
	C-244	bad
Advance-End Spawners		
	C-253	good; some. holes; irregular.
End Spawners		
	C-256	poor; holes.
End-Complete Spawners		

Table 14 (Continued)

8-9 Week Period

Medium Oil	V.T. Condition	High Oil	V.T. Condition
X-257	poor; big holes.	X-230	good; many holes.
		X-234	fair; holes; some irregular.
		X-235	poor; big holes.
X-249	poor; thin.		
X-258	poor; irregular.		
X-246	poor; irregular; thin; big holes.		

Controls	V.T. Condition
Advance-End Spawners	
C-233	good
End Spawners	
C-239	good; some thin; big holes.
Complete Spawners	
Mid Spawners	
Mid-Advance Spawners	

Table 14 (Continued)

8-9 Week Period

Medium Oil	V.T. Condition	High Oil	V.T. Condition
		X-232	fair
POOR CONDITION			
X-241	poor; irregular.	X-231	poor
X-243	poor; thin; irregular.		
X-247	poor; irregular.		
X-245	irregular; poor.		

Controls	V.T. Condition
Complete Spawners	

Table 14 (Continued)

8-9 Week Period

Medium Oil	V.T. Condition	High Oil	V.T. Condition
X-242	fair; irregular; holes.		
X-250	fair; irregular; holes.		

Table 15

Incidence of irregular vesicular tissue.

Group	Number in Population	Animals Affected	Week Examined	% Total Population
Controls	20	C-252	9	4
High Oil	9	X-234	8	11
Medium Oil	25	X-255	9	40
		X-241	8	
		X-243	8	
		X-247	9	
		X-214	5	
		X-221	5	
		X-245	9	
		X-258	9	
		X-246	9	
		X-242	8	
		X-250	9	

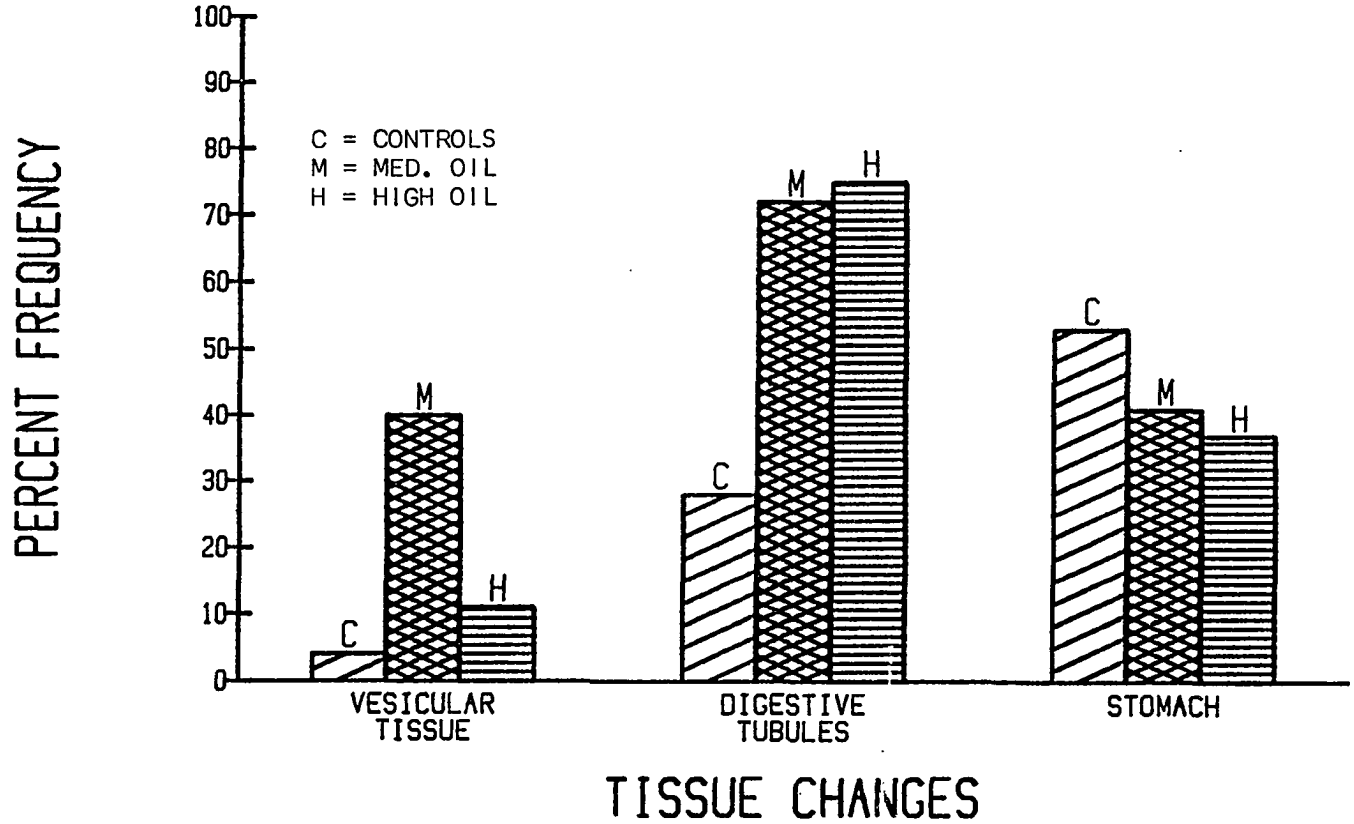

Fig. 13. Comparison of study populations and percent frequency of animals with structural disturbances to various tissues and organs.

mantle tissue were not necessarily good indicators of oil stress but more research into VT reconstruction after spawning could provide better insight.

An excessive number of hemocytes, as determined by observing numerous hemocytes in a single microscopic field of view, was not always a reliable indicator of oil effect in the identification process. In some highly disturbed animals (X-211, C-244) few hemocytes were seen in the mantle except in a few localized areas. However, the number of hemocytes in the tissue proved effective in conjunction with other indicators of stress effect such as organ structural disturbances where a localized response, indicated by an abundance of hemocytes, was associated with structural disturbance. The mixed types of hemocytes seen in an inflammatory reaction as well as the number may be more indicative of an effect. A better understanding of the role of the different types of hemocytes and their functions in the normal biology of the animal may provide a more accurate means for determining hemocyte infiltration and an inflammatory response. For example, a concentration of eosinophilic hemocytes may be observed in the mantle tissue, possibly indicating a function other than involvement in the inflammatory response. In this study, neither qualitative nor quantitative determinations of stained hemocytes in the tissue sections were made.

An effort was made, however, to estimate the observed numbers of hemocytes in each tissue section and to identify an average range for the number of hemocytes observed for the whole animal. "None", "few",

"some", "many" and "numerous" represent a relative estimate of the concentration of hemocytes in tissue sections based on defined criteria already presented. Generally, the concentration of hemocytes in the tissues was fairly consistent from slide to slide, but occasional concentrations (for reasons not always evident) may occur in localized areas. The relative persistent trend in the concentration of hemocytes allowed for the classification of each animal in terms of hemocyte number (Table 16). It thus was apparent that even though the hemocyte infiltration was not particularly instructuve in itself in distinguishing controls from experimental animals in each section analysis, the over-all results suggest that experimental animals, especially the high oil animals in the 8-9 week period, showed higher numbers of hemocytes in the tissues than controls (Table 16). Table 16 also compares the number of circulating hemocytes of the hemolymph with the number estimated in the tissues, but there appears to be no relationship. In summary, although hemocytic infiltration was observed to occur in tissues of both experimental animals and controls in the 4-5 week period, the 8-9 week period showed an increased concentration of hemocytes in the digestive tubule region of experimental animals.

A final factor in determining the effects of oil on the internal structure of animals was consideration of changes occurring in the viscera, especially in the stomach and digestive tubules. The percentages of animals with structural changes in the stomach and digestive tubules for each study population are shown in Figure 13b,c.

Table 16

Relative number of hemocytes observed in mantle and digestive tubules of individuals compared with total cell count.

NOTE:
none = 0
few = 1
some = 2
many = 3
numerous = 4

4-5 Week Period

CONTROLS				MEDIUM OIL				HIGH OIL			
Animals	D.T.	Mantle	Total Cell Count	Animal	D.T.	Mantle	Total Cell Count	Animal	D.T.	Mantle	Total Cell Count
C-201	2	2	aver	X-203	2-3	0-1	low	X-202	3	3	low
C-204	-	3	aver	X-205	1-2	1-3	low	X-208	2	2-4	low
C-206	3	3	high	X-207	-	2-3	aver	X-211	-	0	low
C-210	1	-	low	X-209	0	0	aver	X-213	3	0-4	low
C-212	2	2	high	X-214	4	1-2	aver				
C-215	3-4	2-4	aver	X-216	-	1-3	low				
C-218	-	4	aver	X-217	4	3	aver				
C-220	-	1-3	aver	X-219	1-2	2	high				
C-224	4	2	high	X-221	-	1-3	aver				
				X-222	3	0-1	high				
				X-223	0	1	aver				

Table 16 (Continued)

8-9 Week Period

CONTROLS				MEDIUM OIL				HIGH OIL			
Animals	D.T.	Mantle	Total Cell Count	Animal	D.T.	Mantle	Total Cell Count	Animal	D.T.	Mantle	Total Cell Count
C-233	2	2	aver	X-237	-	2-3	aver	X-230	4	2	low
C-236	3	2-4	aver	X-238	3-4	4	high	X-231	4	2	high
C-239	3-4	3	aver	X-241	3	3-4	aver	X-232	2	-	high
C-240	4	2	low	X-242	4	3	high	X-234	4	4	aver
C-244	2-3	1	aver	X-243	3-4	4	aver	X-235	3	2-3	high
C-248	2	1-2	aver	X-245	2	3-4	high				
C-252	1-2	2-3	aver	X-246	4	2-3	aver				
C-253	3	2-3	high	X-247	3-4	3	aver				
C-254	2-3	2	high	X-249	4	3	aver				
C-256	4	2-3	high	X-250	2	1	high				
C-259	1	1	aver	X-257	3	2	high				
				X-258	4	1	high				
				X-255	2-3	2	-				

The cells of digestive tubules of experimental animals were often seen as swollen and vaculated (Plate 4), with thin or broken basal cells. Experimental animals sometimes showed thin digestive tubules in higher frequency than the normal 10% noted by Langton (1975) in the disintegrating phase of unaffected digestion. Digestive tubule disruptions were also observed in Poor quality controls. Thus digestive tubules appear to be destabilized as a result of stress in general. Stomach disruptions occurred more frequently in controls than experimental animals (Figure 13c). The effects on the stomach were seen as disrupted epithelium or as hemocytic occulsions among epithelial cells. Subsequent break through into the lumen of the stomach was observed (Plate 5), similar to that described by Lowe and Moore (1979) for granulocytomas of hemolymph sinuses and for the connective tissue surrounding digestive tubules. Granulocytomas are lesions composed of hemocytes encapsulating fusiform granular hemocytes which break through the fusiform cell layer. Unlike above observations, the hemocytes disrupting the stomach region of controls in this study did not appear to encircle fusiform granular hemocytes but did disrupt the basal membrane and did appear in high concentrations.

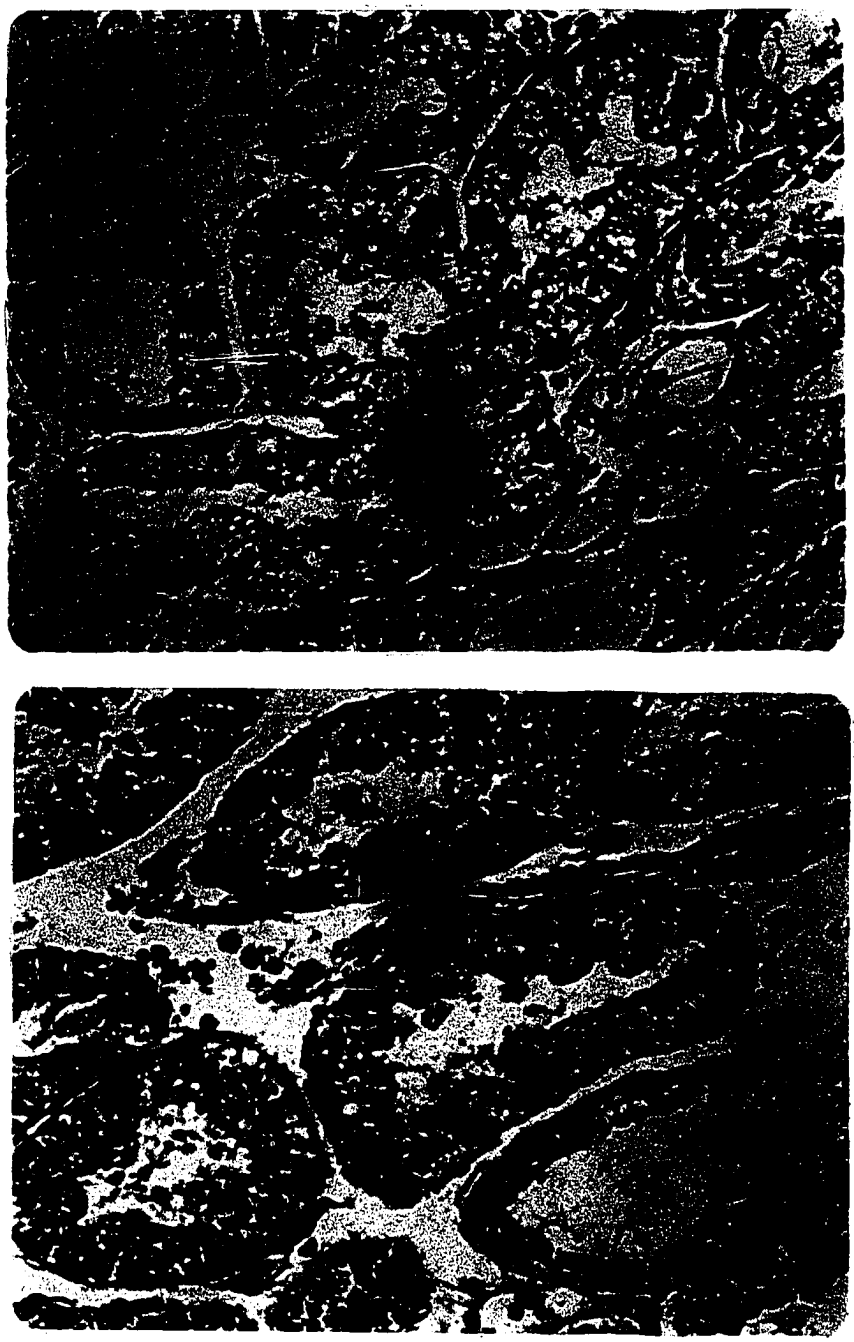

Plate 4. Digestive tubules. (above) Disrupted. (below) Good. 200X.

Plate 5. Stomach disrupted by hemocytes (ulcer) 200X

IV. DISCUSSION

The data suggest that continuous exposure to low concentrations of Prudhoe Bay crude oil emulsion during the post spawning stages of *Mytilus edulis* disrupts homeostatic mechanisms critical to gametogenesis and winter survival. The results of this study relate to and support those of the concurrent physiological research of Clement and Shaw (unpublished). Their study examined mussels taken at the same time from the same field population and exposed to the same experimental conditions for an eight week duration. The only difference between the animals was the smaller size of animals in the physiological study. Clement and Shaw show that long exposure to medium and high oil concentrations, as used in this study, resulted in metabolic changes. Oil concentrations of at least 500 nL/L of Prudhoe Bay crude oil, under laboratory conditions, caused a loss in weight, a reduction in shell growth, a reduced filtration rate, an increase rate of oxygen consumption, and a reduction in scope for growth, i.e., reduced available energy for growth. The results of both studies, independently derived, show agreement on the detrimental effects of Prudhoe Bay crude oil with 1000 nL/L concentration.

The changes in hemocyte numbers, types, and phagocytic response may reflect a direct effect of oil or a metabolic response to oil as a result of cummulative disturbances over time. It may be that a combination of these responses is occurring. It is known that the aromatic body burdens imposed on the animals were substantial with the

eight week exposure (Clement and Shaw, unpublished). The presented data therefore require closer examination in terms of the possible direct and indirect effects of oil and require a better understanding of normal biological changes.

CHANGES IN TOTAL CELLS AND DIFFERENTIAL COUNTS.

Results show that oil in 1000 nL/L concentration can decrease the total cell count after an initial 4-5 week exposure but with longer exposure, a significant number of individuals in both medium and high oil showed an increase in total cell count. This increase was due to an increase in granulocytes. Agranulocytes increased only slightly with time for medium oil animals; for high oil animals they decreased over time. These results suggest that Prudhoe Bay crude oil might be interfering with the production of agranulocytes and that granulocytes are occurring in greater number.

An increase in total cell count, an increase in granulocytes, and the lack of agranulocyte development in test populations may suggest that long exposure to oil may either be affecting hemocyte production or interfering with the capacity for hemocyte renewal. This calls for a reexamination of the different types of hemocytes in terms of ontogenetic lines. Cheng (1981) suggests the existence of two separate cell lines: granulocytes and hyalinocytes (agranulocytes) while Mix (1976) suggests a single cell line, a "cell renewal", as he calls it. If there are two lines then it may be considered that the

reaction to oil is to produce more granulocytes and fewer agranulocytes (hyalinocytes). If there is one cell line, then it may be considered that oil is interfering with the renewal of the cell line and fewer small agranulocytes are produced; the numbers of granulocytes increase as these mature cells accumulate (Sparks, pers. comm.).

INTERFERENCE OF HEMOCYTIC FUNCTION BY OIL.

Exposure to oil may affect important hemocytic function. The small agranulocytes which increased in controls in the 8-9 week population may be of importance in post spawning tissue reconstruction. The increase in agranulocytes in controls is associated with the advanced stages of spawning (Table 17). Experimental animals lack this increase in agranulocytes and oil inhibited spawning progression. Research is needed to ascertain the role of these small agranulocytes (hyalinocytes) and their possible connection with spawning and why agranulocyte production in experimental animals was not occurring. No relationship of agranulocyte number to tissue condition could be identified.

The increased number of granulocytes may signal a potential for infectious disease. Mix and Sparks (1980) correlated an increase in percentage of granulocytes in circulating hemocytes with fungal infection of the Tanner Crab *Chionocetes bairdi* Rathbun. Also, fewer agranulocytes were seen. Animals examined in the present study exposed to oil for 8-9 weeks had an increased percentage of granulocytes and

Table 17

Agranular/granular ratio of differential absolute count with spawning state showing increased ratio with spawning stage for controls but not test animals.

4-5 Weeks

Spawning Stage	Controls	Ratio	Medium Oil	Ratio	High Oil	Ratio
Mid Spawners	C-201	.02	X-203	.08	X-202	.17
	C-204	.04	X-205	.07	X-208	.07
	C-210	.08	X-209	.03		
	C-212	.09	X-216	.03		
	C-218	.08	X-219	.04		
	C-224	.09				
Mid - Average Spawners			X-223	.02		
Advance Spawners	C-206	.01	X-214	.01	X-211	.07
	C-220	.02	X-221	.06		
			X-222	.01		
			X-217	.01		
Advance-End Spawners			X-207	.02		
Complete Spawners					X-213	.17

Table 17 (Continued)

8-9 Weeks

Spawning Stage	Controls	Ratio	Medium Oil	Ratio	High Oil	Ratio
Mid Spawners			X-241	.04	X-231	.02
			X-243	.05		
			X-247	.04		
Mid-Advance Spawners	X-245	.02				
Advance Spawners	C-236	.60	X-237	.10	X-230	.02
	C-240	.17	X-257	.06	X-234	.01
	C-244	.21			X-235	.01
	C-259	.20				
Advance-End Spawners	C-233	.17	X-258	.05		
	C-253	.04	X-249	.13		
End Spawner	C-239	.09	X-238	.02		
	C-256	-				
End-Complete Spawners	C-248	.38	X-246	.01		
Complete Spawners	C-252	.63	X-242	.06	X-232	.04
	C-254	.18	X-250	.02		
			X-255			

a reduced phagocytic response. Although Foley and Cheng (1975) have shown that granulocytes are more phagocytic, this is not always true as the present study and the study by Mix and Sparks indicate. The reduced phagocytic response in granulocytes of animals exposed to oil may occur either as a direct effect of oil intercalating into membranes and interfering with surface recognition, or because mature granulocytes are less phagocytic. As a consequence, the animals are more susceptible to infectious diseases.

Other hemocytic functions may be affected. It is known that hemocytes function in digestion and food transport (Cheng, 1981 and others cited therein). The results of the physiological study by Clement and Shaw (unpublished) show that high oil animals did not grow nor was there energy available for their growth, as indicated by the negative "scope for growth" values. This may relate to hemocytic function. As nutrient digestion, transport and phagocytosis require similar mechanisms (Cheng, 1981), the reduction in phagocytic response may mean some interference with nutrition. If oil contamination affects hemocytes and their phagocytic and nutrient transport function during summer feeding, then the build up of lipid storage for winter may be affected, resulting in subtle changes not immediately detectable. Interference with nutrient tranpsort and storge can subsequently affect mussels during such life history events as gametogenesis, winter food storage, and tissue maintenance. A better understanding of hemocytic function in maintaining homeostasis and physiological needs is important to the understanding of sublethal

effects of oil. This study suggests a closer examination of changes in hemocytes during spawning or during other internal morphological changes so as to understand better the biological effects associated with oil pollution.

HEMOCYTE COUNTS, SPAWNING, AND TISSUE CHANGES.

No relationship or trend in control total cell counts could be identified with spawning stage of tissue/organ disruption, either individually or as a population. Thus it appears that the average number of circulating cells in controls is maintained despite morphological changes, although the types and functions may change. The higher and lower counts of some controls may reflect an individual response to immediate physiological events such as heart rate and feeding (Cheng, 1982; Feng, 1965; Huffman and Tripp, 1982). Variability in count may also result from sampling techniques (Miale, 1982). Thus population means for the average number of circulating cells are important in identifying trends associated with a particular pollutant.

HEMOCYTE COUNTS AND LOW AVAILABLE ENERGY FOR GROWTH.

The number of circulating hemocytes is maintained despite high aromatic and aliphatic body burdens and the low available energy for growth, as determined by Clement and Shaw. Dynamic processes such as metabolism and subsequent growth and movement of the animal depend on a supply of energy (Calow, 1981). Growth occurs when the amount of

food is more than enough to make up for losses incurred in the metabolic process. Oil exposed animals in this study had a continuous supply of food. Growth for them did not occur but high numbers of hemocytes had been maintained. This may possibly suggest a central importance of maintaining relatively constant numbers of circulating hemocytes at the expense of other physiological functions such as growth, assuming that hemocytes originate from two cell lines. If hemocytes arise from a single cell line and granulocytes represent the mature form, it may be that oil or low available energy is interfering with cell renewal, as suggested earlier. There are other possibilities. It may be suggested that the energy demanded for maintaining hemocyte production is slight, or that hemocytes may be gaining energy from other reserves. As there is a lack of adipogranular cell development in high oil animals, it may be that the hemocytes are utilizing this energy for immediate needs, sacrificing the reserves for gametogenesis.

HEMOCYTE TYPES AND POSSIBLE FUNCTIONS IN POST SPAWNING STAGES.

Little is known about the individual functions of different circulating cell types in the general biology of the animal. Circulating cells may either represent the specialized cells observed in tissues performing particular functions or they might be undifferentiated totipotent cells responding to particular conditions when the need arises. Although the literature shows little agreement on bivalve hemocyte classification (Huffman and Tripp, 1982), Cheng

(1981) concludes there are granulocytes and agranulocytes. Precursor hemocytes might arise from hematopoeitic sites, but no convincing evidence has emerged concerning the location of this site in bivalves (Cheng, 1981), although recent evidence might indicate otherwise (Sparks, pers. comm.). Others have stated that there is a genetic relationship between granulocytes and connective tissue cells (Liebman, 1946) and that hemocytes arise from connective tissue differentiation (Cheng, 1981 and other cited therein). Still others, in contrast, believe that hemocytes give rise to connective tissue (or adipogranular cells) (Lubet, *et al.* 1976).

In the present study, general observations using Papanicolaou's stain indicate that eosinophilic granulocytes of the mantle tissue may be an integral part of adipogranular and vesicular tissue development. Eosinophilic granulocytes (also described as macrophages) are found throughout the mantle (Plate 6), as others have observed (Moore and Lowe, 1977), and are associated with regressing gonadal material. The role of eosinophilic granulocytes in mantle tissue, however, might relate to cellular reconstruction of the mantle and lipid storage after spawning. It appears, upon close inspection of mantle cells, that some eosinophilic granulocytes may form vesicular and/or adipogranular cells functioning in the replacement of spawned gonadal tissue, as suggested by Lubet, *et al.* (1976). Plate 7 shows hemocytes in holes (spaces) of the mantle tissue and in intimate contact with vesicular cells. Stereotaxis rather than contact inhibition may direct cells to each other. When first examining clumps of fresh

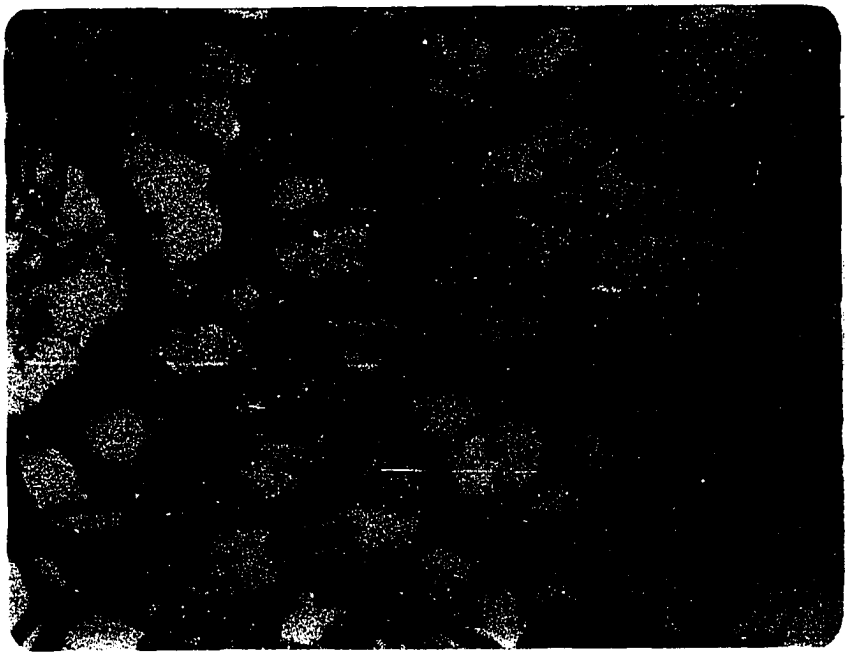

Plate 6. Hemocytes (blue and granulated) mesh with vesicular tissue. Papanicolaou stain. 200X

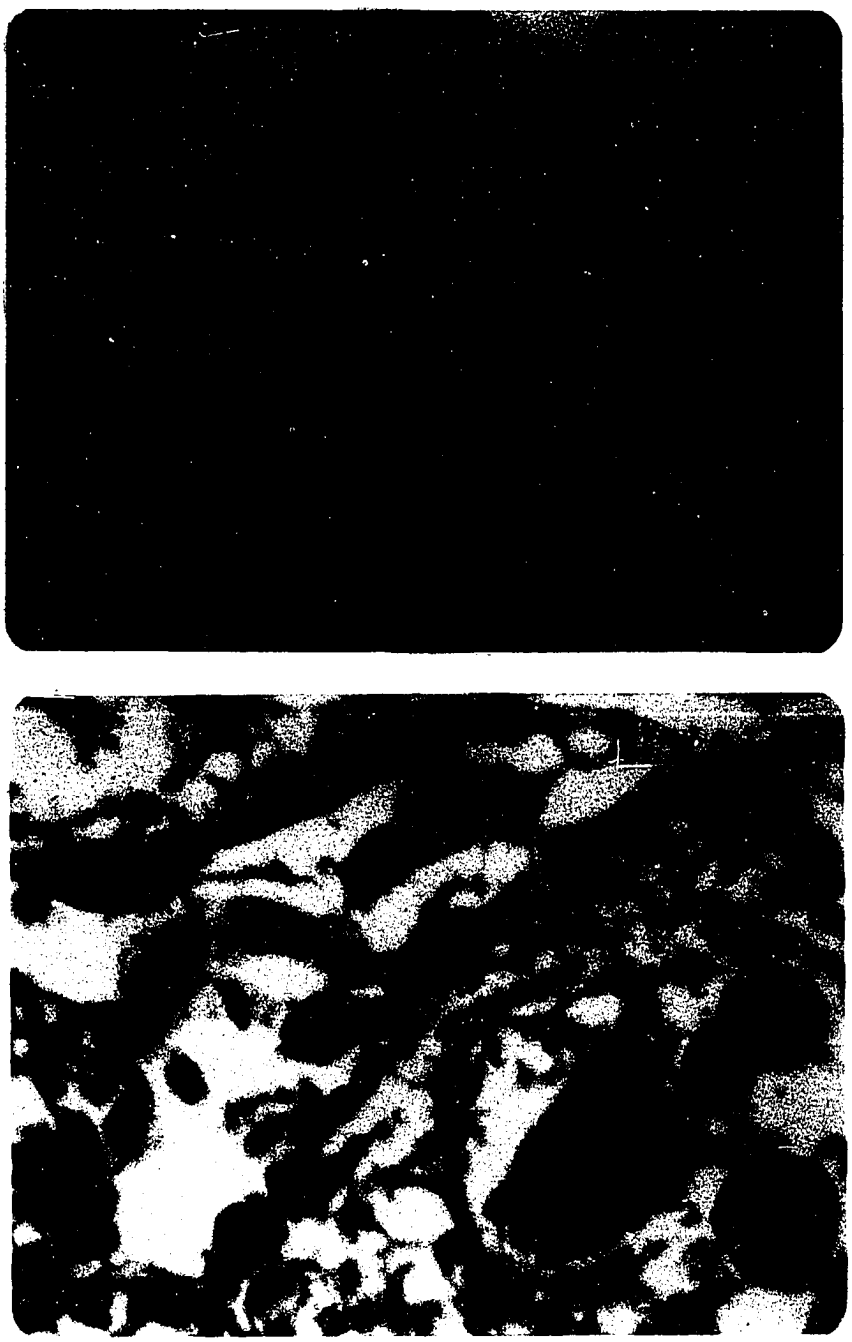

Plate 7. Filopodia extension, appearing to contact with vesicular tissue. (above) 1000X.. (below) 400X. Papanicolaou stain.

circulating hemocytes, there is a migration of some hemocytes away from the clump. At some distance from the clump the migrant (Plate 8) would form a single thickened filopodia-like extension of cytoplasm. This extension resulted in the relocation and reunion of the migrant with the clump, as the cytoplasmic extension contracted to draw the migrant cell back to the group. Hemocyte migration away from clumped hemocytes has been documented (Foley and Cheng, 1974), but there is no mention of the return of the migrant hemocyte to the group nor the possible significance of reestablishing contact.

Granulocytes may also function as adipogranular cells in lipid storage. Huffman and Tripp (1982) have shown that circulating cells show biochemical individuality (i.e., marked heterogeneity in hydrolytic lysosomal enzymes and in cytoplasmic glycogen and lipofuscin), and that approximately 2% of the granulocytes have positive reactions in periodic acid-Schiff stain to indicate the occurrence of glycogen. Bayne, *et al.* (1982) have shown that vesicular and adipogranular cells have glycogen. The hemocytes stained with Papanicolaou to accent adipogranular lipid cells normally have granulocytes with an affinity for the eosinophilic component of the stain (Plate 9, blue color of cells). However, some hemocytes have granulocytes taking up the eosinophilic stain and some that take up the orange G stain (orange-red color). The orange G stain is the dominate stain for adipogranular cells of the mantle tissue. More study is required to ascertain this relationship.

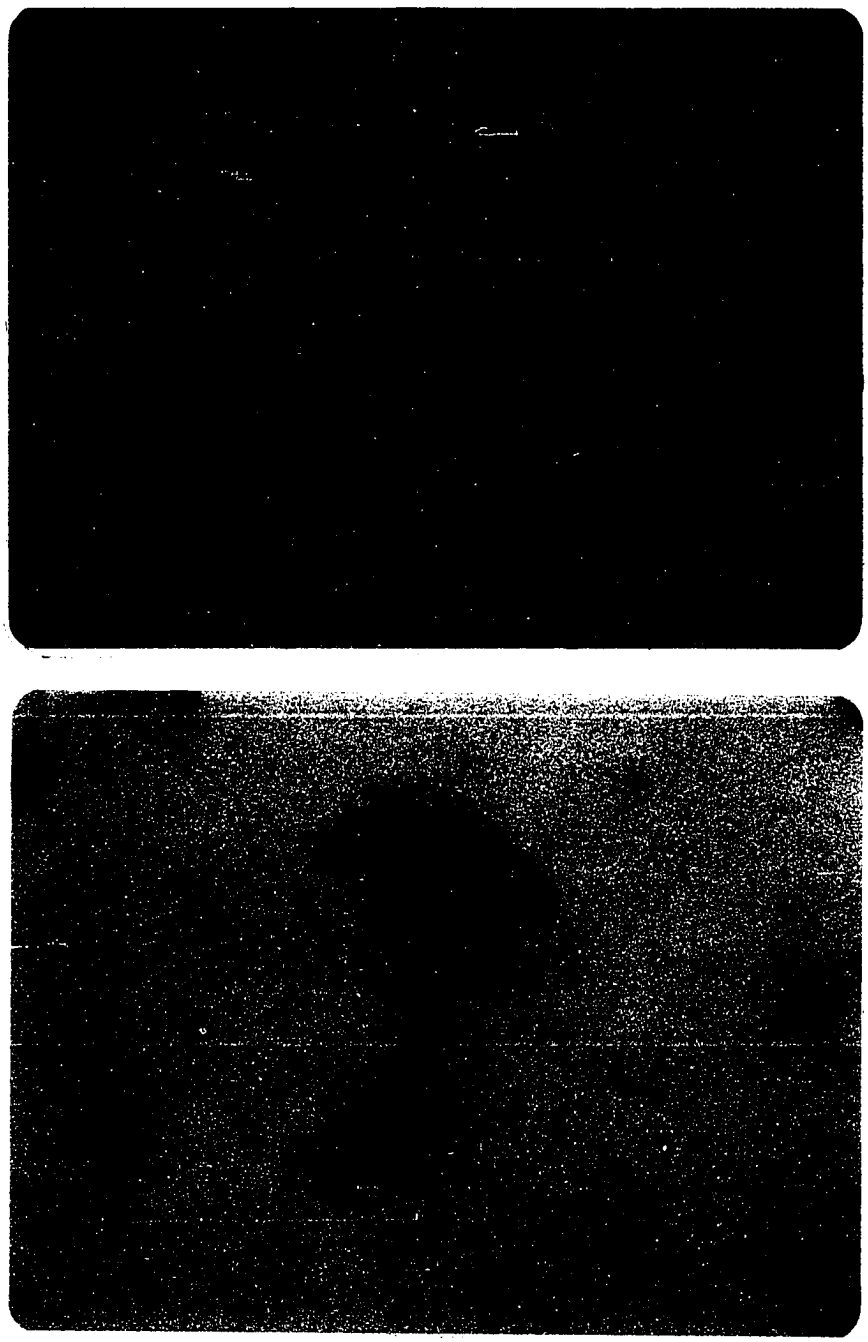

Plate 8. Hemocyte filopodia extension making contact with clump. (above) fresh, unstained. 400X. (below) Wright's stain. 400X.

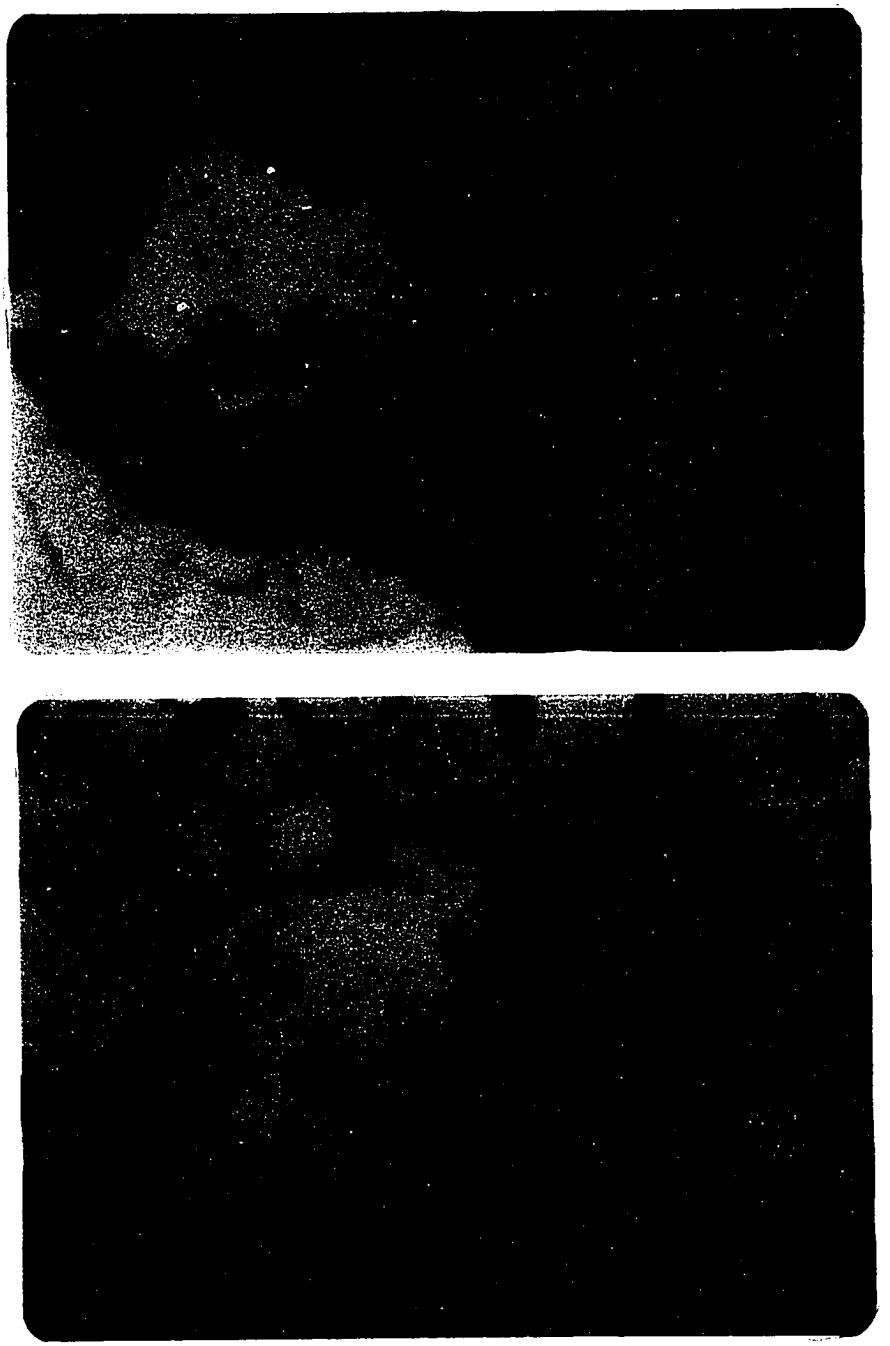

Plate 9. Granules of hemocytes taking up eosin stain (blue) and Orange G (red). Papanicolaou stain. 400X

The type, location, and functioning of hemocytes in tissues requires more careful study. In tissues, the hyalinocyte, (i.e., the small basophilic cells or agranulocytes with a large nucleus to cytoplasm ratio) and the larger basophilic and eosinophilic granulocytes have been identified by Moore and Lowe (1977). These three types of cells were also observed in the present study but not always together; basophilic granulocytes and small hyaline (agranulocytes) cells did not consistently appear, unlike the commonly distributed eosinophilic granulocytes. Nor did the small hyaline and the basophilic and eosinophilic granulocytes consistently appear with regressing follicles. Instead, degranulated cells of the follicular periphery, identified as fibrocytes (Cheng, 1981) and having cytoplasmic extensions into the lumen, appeared to be resorbing degenerate gonadal material, possibly through pinocytosis (Feng, 1965b) as a result of exocytosis of lysosomal enzymes (Cheng, 1981). In appearance, fibrocytes look like elongate vesicular cells, making stereological point count determinations difficult. In obviously disturbed areas of the mantle, there were mixed populations of hemocytes. This suggests that a closer examination and identification of hemocytes with tissue changes may provide more reliable clues as to their function in the biology of the animal, and a clearer understanding of functional roles in resorbing unspawned gametes.

Few studies have closely examined the means in which the follicles of *Mytilus edulis* might be receding when gametes are unspawned. Tranter (1958), examining resorption in the Australian pearl oyster,

believes that as the gonad empties and the follicular wall cells contract slowly, the adjacent follicles separate and are reorganized by vesicular tissue. Resorption of eggs occurs by hemocytes forming a sac around the residual oocyte, with subsequent ingestion. Wilson and Hodgkin (1967), examining *Mytilus edulis planulatus* found smaller hemocytes (4-6 μm) which formed aggregates within follicles containing few residual gametes. From observations in the present study of residual gametes, empty follicles often did show aggregates of small hemocytes similar to Wilson and Hodgkin's findings. Resorption of eggs by a sac formed by hemocytes, as observed by the finding of Tranter for oysters, was only occasionally seen. Unlike Tranter's findings, few if any hemocytes were consistently seen in full or near full follicles in the process of resorption as indicated by thin, lysed or degenerate-looking oocytes or by the occurrences of hemocytes at the follicular wall. Some peripheral hemocytes were often indistinguishable from vesicular tissue cells, follicular wall cells, fibrocytes, and adipogranular cells, and definitely appeared to be intruding into the follicle. As a consequence, it appeared that the follicle was receding (Plate 10). Storage tissue increased (Plate 11) and ultimately the resting stage in gametogenesis was established. Larger granulated and free hemocytes were not commonly seen within follicles. Hemocytes within the lumen of the follicle only appeared as very small clumped basophilic cells with poorly defined cytoplasm. In one highly disturbed control, C-218, however, numerous and large granulocytes were observed infiltrating full follicles, but numerous

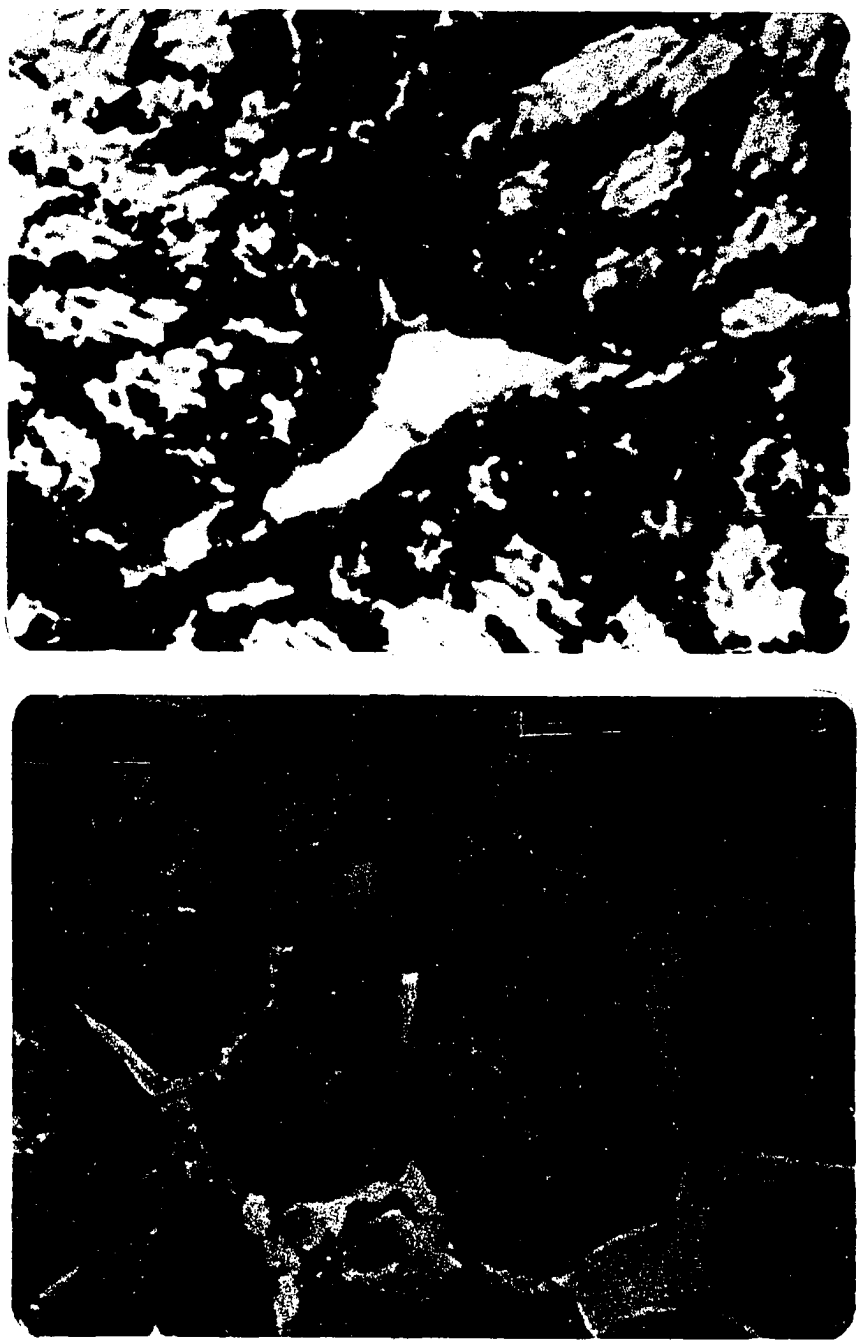

Plate 10. Hemocytes appearing to divide follicle into smaller area prior to spread of vesicular tissue. Papanicolaou stain. 400X

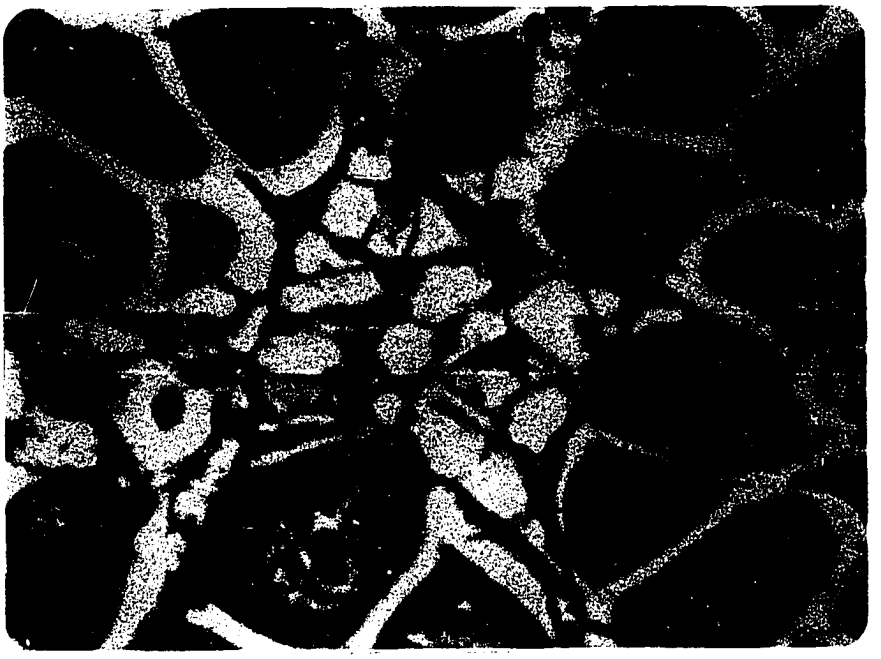

Plate II. Receding follicle as vesicular and adipogranular cells are in process of spreading. Papanicolaou stain. 200X

hemocytes were seen throughout the mantle and viscera of this animal, indicating a possible inflammatory response.

Fresh samples of hemolymph were stained. Hyalinocytes (small agranulocytes), basophilic and eosinophilic granulocytes were readily identified. No obvious dominance of a particular cell type in the peripheral hemolymph, based on cytoplasmic and nuclear staining characteristics, could be identified as a result of oil exposure. Degranulation of granulocytes as a consequence of drying, fixing and staining (Cheng and Foley, 1975) confounded the identity of granulocytes and agranulocytes, thus no quantification was made.

AGRANULOCYTES

The increased number of agranulocytes in controls in the 8-9 week population came from an increase in the number of small agranulocytes (see Figure 1). The staining character of this small cell was dense basophilic (indicating high nuclear content) and there was little to no cytoplasm. These small agranulocytes (1-3 μm in fresh samples) may fit the description of a young agranulocyte (hyalinocytes) proposed by Cheng (1981) for the existence of a hyalinocyte development series. Cheng hypothesizes the existence of a hyalinoblast which divides and differentiates. These then develop into young, small hyalinocytes which he designated prohyalinocytes and which represent the smaller hemocytes observed in samples. He characterized the prohyalinocyte as having a relatively large nucleus surrounded by a small volume of cytoplasm, an absence of cytoplasmic granules, few or no lobopodia, and essentially basophilic.

PHAGOCYTIC RESPONSE AND SPAWNING

The phagocytic response measures the ability of blood cells to recognize and engulf foreign cells and particles. Such recognition is an important function in bivalve immunological defense against infecting organisms such as bacteria. Such response, however, as recognized by Bang (1961) and others is not invariably certain for all hemocytes and the reasons for this have not yet been determined. The reduction in phagocytic response for controls between the 4-5 week and the 8-9 week period in this study could reflect post spawning changes. This suggests subtle differences in hemocytes and their functions with post spawning conditions, possibly making them less phagocytic and more susceptible to infectious disease. No distinction between the types of hemocytes with and without yeast was made in this study. This requires a better understanding of the different hemocyte types and their role in phagocytosis as studied by Foley and Cheng (1975). Examining phagocytic response with spawning stage (Appendix 7), the 4-5 week control population in early spawning stages had higher phagocytic counts than the 8-9 week population in Advance to End spawners. Complete spawning (or the gametogenic resting stage) hints of a return to higher phagocytic counts, but larger sample sizes are obviously required. It nevertheless might be hypothesized that the phagocytic response for controls is reduced during post spawning stages but resumes higher response during the resting stage of gametogenesis.

CONCLUSIONS

The results of this study show that Prudhoe Bay crude oil under laboratory conditions can affect hemocytes, reduce the immune capacity, disrupt the spawning, and interfere with lipid reserve build up for winter gametogenesis of *Mytilus edulis*. In conjunction with the results obtained by Clement and Shaw (unpublished) which show that the experimental animals accumulated high concentrations of hydrocarbons (at least a three-fold increase) above ambient exposures, the present study suggests that the hemocytes may be responding to the accumulated oil, directly or indirectly. However, for animals exposed to 1000 nL/L after 4-5 week exposure, the reduced number of circulating hemocytes may indicate that oil might act directly. Furthermore, as a result of oil, the natural spawning progression and the build up of adipogranular storage cells, which is critical for the initiation of gametogenesis, appeared to be affected. What connection the observed changes in hemocytes may have in post spawning conditions and gametogenesis is uncertain. The results of this study are not conclusive and reveal more questions than the one it meant to answer. It is apparent, however, that the study of hemocytes should not be made outside the context of the adaptive biology of the organism as hemocytes must be responding to physiological needs dictated by seasonal and immediate environmental conditions as well as to internal homeostasis. As animals accommodate to stressful conditions by utilizing energy in order to maintain homeostasis, the physiological response, with which hemocytes are intimately involved, may reach

thresholds that result in pathology or altered internal morphology. Our understanding is incomplete as to the many physiological mechanisms in which marine organisms react to changes in their environment, both naturally and human induced. However, it is becoming apparent that hemocytes must play an important role. How important can only be determined with more research and large sample sizes.

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APPENDIX 1

Mytilus edulis foot response prior to examination

CONTROLS			MEDIUM OIL			HIGH OIL		
Animal	Foot Extrude	Response time	Animal	Foot Extrude	Response Time	Animal	Foot Extrude	Response Time
C-201	yes	slow	X-203	yes	fast	X-202	yes	-
C-204	yes	fast	X-205	yes	fast	X-208	no	-
C-206	yes	slow	X-207	yes	slow	X-211	yes	fast
C-210	yes	fast	X-209	yes	slow	X-213	yes	fast
C-212	yes	fast	X-214	yes	slow			
C-215	yes	fast	X-216	yes	slow			
C-218	yes	fast	X-217	yes	fast			
C-220	yes	fast	X-219	yes	fast			
C-224	yes	fast	X-221*	no				
			X-222	no				
			X-223	no				
C-233	yes	fast	X-237	no		X-230	no	
C-236	yes	fast	X-241*	no		X-231	no	
C-239	yes	fast	X-242	yes	slow	X-232	no	
C-240	yes	fast	X-243	yes	slow	X-234	no	

APPENDIX 1 (Continued)

CONTROLS			MEDIUM OIL			HIGH OIL		
Animal	Foot Extrude	Response time	Animal	Foot Extrude	Response Time	Animal	Foot Extrude	Response Time
C-244*	yes	fast	X-245	no		X-235	no	
C-248	yes	fast	X-246	-	-			
C-252	yes	slow	X-247	yes				
C-253	yes	fast	X-249	no				
C-254	yes	slow	X-250	no				
C-256	yes	fast	X-255	no				
C-259	yes	fast	X-257	no				
			X-258	no				

*gas bubble seen

APPENDIX 2

Phagocytic Response

Weeks	Animal	Sex	Slide 1 %	Slide 1 S.D.	Slide 2 %	Slide 2 S.D.	Mean Total %	Mean Total S.D.
4-5	C-201	M	92	3	-	-	92	-
	C-204	F	85	8	93	5	89	5.6
	C-206	M	94	8	-	-	94	-
	C-210	F	82	8	82	1	82	0
	C-212	M	85	5	89	3	87	2.8
	C-215	M	92	3	90	9	91	1.4
	C-218	F	92	4	89	0	91	2.1
	C-220	M	94	3	94	3	94	0
	C-224	M	85	3	79	6	82	4.2
	X-203	F	59	7	58	5	59	.7
	X-205	M	67	22	75	3	71	5.6
	X-207	M	87	6	-	-	87	-
	X-209	F	79	9	86	4	83	4.9
	X-214	M	97	2	96	2	97	.7
	X-216	F	93	4	89	5	91	2.8
	X-217	M	69	11	71	6	70	1.4
	X-219	F	94	4	92	1	93	1.4
	X-221	M	80	6	81	6	81	.7
	X-222	M	71	8	70	12	71	.7
	X-223	F	77	4	74	7	76	2.1
	X-202	F	71	4	62	0	67	6.4
	X-208	M	97	2	-	-	67	0
	X-211	F	76	10	59	-	68	12.0
	X-213	F	90	4	88	5	89	1.4
8-9	C-233	F	72	9	71	11	72	.7
	C-236	F	52	9	78	6	65	18.3
	C-239	F	54	12	38	-	46	11.3
	C-240	M	60	9	59	16	60	.7
	C-244	F	82	6	74	8	78	5.6
	C-248	M	82	5	88	7	85	4.2
	C-252	M	72	7	-	-	72	-
	C-253	F	79	8	85	9	82	4.2
	C-254	M	92	2	89	3	91	2.1
	C-256	M	47	16	35	8	41	8.5
	C-259	M	46	10	63	19	55	12.0

Appendix 2 (Continued)

Weeks	Animal	Sex	Slide 1 %	Slide 1 S.D.	Slide 2 %	Slide 2 S.D.	Mean Total %	Mean Total S.D.
	X-237	M	73	5	73	6	73	0
	X-238	F	7	2	13	5	10	4.2
	X-241	F	90	3	92	5	91	1.4
	X-242	M	62	11	62	10	62	0
	X-243	F	70	6	-	-	70	-
	X-245	M	49	22	44	3	47	3.5
	X-246	F	70	15	-	-	70	-
	X-247	F	76	6	85	5	81	6.3
	X-249	M	85	5	83	3	84	1.4
	X-250	M	49	24	77	13	63	19.8
	X-255	M	73	19	91	2	82	12.7
	X-257	F	64	7	58	9	61	4.2
	X-258	F	84	4	75	6	80	6.3
	X-230	M	5	4	6	2	6	.7
	X-231	F	36	9	37	18	37	.7
	X-232	F	6	3	3	2	5	2.1
	X-234	M	35	13	-	-	35	-
	X-235	F	10	6	-	-	10	-

APPENDIX 3

Percent frequency of mantle tissue cells as determined by stereological points

Time (weeks)	Animal	FOLLICULAR TISSUE %Gam.	%Foll. Space	STORAGE TISSUE %VT	%holes	%A.G.
4-5	C-201	60	28	6	6	0
	C-204	50	36	9	7	0
	C-206	42	24	19	5	9
	C-210	57	26	16	2	2
	C-212	46	33	14	6	2
	C-215	52	28	14	4	1
	C-218	63	30	6	2	0
	C-220	27	37	16	10	10
	C-224	54	28	4	5	10
	X-203	52	29	15	0	3
	X-205	63	25	7	6	0
	X-207	17	17	57	7	0
	X-209	60	25	11	5	0
	X-214	26	18	35	7	14
	X-216	70	25	6	2	0
	X-217	43	26	17	9	3
	X-219	72	18	6	4	0
	X-221	45	18	17	11	6
	X-222	41	24	28	6	0
	X-223	49	22	26	1	2
	X-202	45	37	11	8	0
	X-208	40	40	8	6	0
	X-211	15	55	20	11	0
	X-213	0	7	81	8	1
8-9	C-233	1	24	44	0	23
	C-236	17	30	43	4	4
	C-239	2	31	34	7	22
	C-240	13	25	22	12	25
	C-244	18	25	25	29	0
	C-248	0	7	48	3	38
	C-252	0	8	49	8	31
	C-253	23	17	32	3	23
	C-254	0	7	51	7	36
	C-256	1	29	53	16	0
	C-259	61	7	17	1	13

APPENDIX 3 (Continued)

Time (weeks)	Animal	FOLLICULAR TISSUE %Gam.	%Foll. Space	STORAGE TISSUE %VT	%holes	%A.G.
	X-237	38	30	15	6	11
	X-238	6	29	45	8	7
	X-241	34	45	15	5	0
	X-242	0	14	51	7	24
	X-243	47	38	9	5	0
	X-245	55	15	21	3	4
	X-246	1	14	32	12	27
	X-247	40	40	12	5	2
	X-249	0	19	36	43	2
	X-250	0	10	63	8	16
	X-255	0	2	58	7	33
	X-257	-	-	-	-	0
	X-258	5	43	37	1	14
	(high oil)					
	X-230	30	32	22	7	8
	X-231	37	53	7	2	0
	X-232	-	-	-	-	0
	X-234	29	35	21	7	1
	X-235	25	44	25	5	0

APPENDIX 4

Results of Blinded Tissue Analysis Evaluation of Condition Factors for each slide analyzed for all animals. Identification of each section as "C" (control unaffected), "T" (test, affected) or "B" (undecided) and with an overall evaluation of the tissue quality as OK, fair, poor or bad.

PRELIMINARY ANIMALS:

P-1-3:	C, fair
P-100:	C, OK; C, fair; C, OK; C, OK
P-101:	C, OK
P-102:	C, OK
P-104:	C, OK; C, OK
P-119a:	T, poor; C, OK; C, OK; C, OK
P-120:	C, OK; C, good; C, fair; C, good; C, fair, C, OK
P-104b:	C, OK
P-123:	T, poor; C, OK; T, poor; C, good; C, fair; C, OK; B, fair; C, good; C, OK; C, OK
P-130:	T, poor; T, poor; T, bad; T, poor; T, poor; T, poor; t, poor
P-131:	C, fair; C, OK
P-132:	T, fair; C, OK; C, OK; C, fair; T, poor; C, fair; C, fair; C, fair; C, fair; C, fair; C, OK; T, poor
P-133:	C, OK; T, fair; C, good; C, OK; C, OK; T, fair; T, fair, C, OK; T, fair; C, OK
P-134:	C, good; C, good; C, good; C, fair; C, OK
P-150:	C, OK
P-151:	C, OK

CONTROLS:

C-201:	C, OK; B, fair; C, OK; C, good; C, fair; C, OK
C-204:	C, fair; C, OK; C, OK
C-206:	C, good; C, OK; C, OK; C, good; B, fair; C, OK; B, fair; C, OK
C-210:	B, fair; B, fair; B, fair-poor, C, OK; C, OK
C-212:	C, good; C, good; B, fair; T, poor-fair; B, OK; C, fair; B, fair
C-215:	C, OK; C, good; B, fair, T, poor; B, fair; T, fair; B, fair
C-218:	T, poor-fair; T, poor; T, poor; T, poor; T, poor
C-220:	C, good; C, OK; C, OK; C, fair; C, OK; T, fair
C-224:	C, OK; B, fair; T, fair; B, fair; C, OK; T, fair-poor; T, poor

Appendix 4 (Continued)

CONTROLS: Continued.

C-233: B, fair; C, OK; T, poor; C, fair; C, fair; C, OK; C, fair; C, fair
C-236: C, OK; B, fair; T, poor; T, poor; T, poor; T, poor; T, poor
C-239: T, fair; T, poor; T, fair; T, poor; T, fair-poor; T, poor; T, fair; B, fair
C-240: C, fair; C, OK; C, good; C, OK; C, OK; T, fair; T, poor-fair; C, fair
C-244: T, poor; T, bad; T, bad; T, poor; T, bad; T, poor; T, poor
C-248: C, good; C, fair; C, fair; C, OK; B, fair; C, OK
C-252: C, fair; B, fair; C, fair; C, fair; C, OK; T, poor-fair; C, OK; C, fair
C-253: C, OK; T, poor, T, poor-fair; T, fair, poor; T, poor; C, fair, poor
C-254: C, OK; C, fair; C, OK; B, fair; C, fair
C-256: T, poor; T, poor; T, poor; T, poor; T, poor
C-259: C, good, C, OK; C, good; C, good; C, good; C, good

EXPERIMENTS - Medium Oil:

X-203: C, good; C, good; C, OK; T, fair; C, fair
X-205: C, good; T, fair; T, fair; C, OK
X-207: T, poor; T, fair; T, fair; T, fair; T, poor
X-209: T, Poor-fair; T, poor; C, OK; T, fair-poor; T, fair-poor
X-214: C, good; B, OK; T, poor; T, poor; C, OK; T, OK; B, fair
X-216: C, OK; C, fair; C, OK
X-217: T, fair; T, poor; T, fair-poor; T, fair; T, fair-poor; T, poor
X-219: C, OK; B, fair; T, fair-poor; T, poor; T, poor-fair
X-221: T, poor; T, poor; T, poor; T, fair
X-222: C, OK; T, fair; C, OK; T, poor; C, OK; T, fair; C, OK
X-223: C, OK; C, good; C, OK; T, OK
X-237: T, poor; B, fair; T, fair; C, fair; T, OK; C, OK; C, OK
X-238: C, OK; T, poor; T, poor; T, poor; T, poor; T, poor
X-241: C, OK; t, poor; T, poor; T, poor; T, fair; T, poor; T, poor; T, poor
X-242: C, good; T, poor; T, fair-poor; C, fair; T, poor; T, poor
X-243: T, bad; T, fair; T, poor; T, poor; T, poor; T, poor; T, poor
X-245: C, OK; T, poor; T, poor; C, OK; T, poor
X-246: T, fair-poor; T, poor; T, poor

Appendix 4 (Continued)

EXPERIMENTALS - Medium Oil: Continued.

X-247: C, OK; T, fair; T, poor; T, poor; T, fair
X-249: T, bad; T, poor-bad; T, poor
X-250: T, fair; T, fair; T, poor-fair; T, poor-fair
X-255: C, fair; T, poor; C, fair; C, fair; T, poor
X-257: T, poor; T, bad; T, poor; T, poor; T, bad
X-258: T, fair; T, fair; T, poor; T, poor; T, fair

EXPERIMENTALS -High Oil:

X-202: B, fair; C, OK; C, OK; T, poor; T, poor; B, fair, C, fair

X-208: C, OK; B, fair; T, OK; T, fair; C, OK; C, fair
X-211: T, poor; T, bad; T, bad
X-213: C, OK; T, fair; T, poor-fair; T, fair; C, OK
X-230: T, poor; C, OK; T, bad; T, poor; C, OK; T, poor
X-231: T, fair, T, poor; T, poor; T, poor; T, poor; T, bad; T, poor; T, poor; T, poor
X-232: B, fair; C, OK; T, fair; T, poor-fair
X-234: T, poor; T, fair-poor; T, fair; T, poor; T, poor; T, poor; T, poor
X-235: T, fair; T, fair-poor; T, fair-poor; T, poor; T, bad; T, OK

APPENDIX 5

Differential Hemocyte counts with percent of total per uL of Hemolymph (* = high oil animals)

Time/ Expos. (Weeks)	Animal	Granulo-cytes	Round	Small G	Agranulo-cytes	Degranu-lated	Serous
4-5	C-201	1437(90)	58(4)	63(4)	31(2)	4(0)	0
	C-204	1421(92)	3(0)	45(3)	64(4)	9(1)	0
	C-206	3981(98)	10(0)	30(1)	22(1)	2(0)	0
	C-210	734(84)	20(2)	58(7)	59(7)	1(0)	1(0)
	C-212	3007(89)	23(1)	63(2)	270(8)	20(1)	0
	C-215	1568(90)	9(1)	100(6)	56(3)	6(0)	0
	C-218	1391(87)	24(1)	62(3)	114(7)	7(0)	2(0)
	C-220	1802(94)	8(0)	54(3)	38(2)	0(0)	1(0)
	C-224	3008(87)	41(1)	92(3)	294(8)	23(1)	1(0)
	* X-202	503(75)	1(0)	67(10)	89(13)	7(1)	2(0)
	X-203	620(87)	15(2)	21(3)	49(7)	9(1)	1(0)
	X-205	878(89)	9(1)	29(3)	66(7)	4(0)	3(0)
	X-207	1742(67)	3(0)	800(31)	57(2)	8(0)	0
	* X-208	1044(82)	10(1)	122(10)	80(6)	9(1)	1(0)
	X-209	1876(89)	16(1)	147(7)	59(3)	2(0)	0
	* X-211	494(80)	48(8)	28(5)	37(6)	7(1)	2(0)
	* X-213	1134(78)	6(0)	126(9)	183(13)	0	0
	X-214	2084(97)	9(0)	22(1)	17(1)	3(0)	0
	X-216	1197(95)	6(0)	10(1)	34(3)	11(1)	0
	X-217	1997(98)	14(1)	24(1)	12(1)	0	0
	X-219	3114(94)	41(1)	22(1)	132(4)	9(0)	0
	X-221	1424(90	30(2)	67(4)	73(5)	4(0)	0
	X-222	2960(97)	10(0)	52(2)	34(1)	8(0)	0
	X-223	1985(90)	19(1)	171(8)	36(2)	3(0)	0
8-9	C-233	1594(84)	28(1)	9(0)	260(14)	3(0)	0
	C-236	1495(63)	31(1)	20(1)	953(38)	24(1)	4(0)
	C-239	1753(83)	89(4)	80(4)	164(8)	22(1)	0
	C-240	1375(84)	7(0)	22(1)	235(14)	4(0)	0
	C-244	1487(75)	122(6)	31(2)	324(16)	33(2)	7
	C-248	1160(71)	11(1)	4(0)	444(27)	11(1)	2(0)
	C-252	962(60)	11(1)	13(1)	611(38)	13(1)	0
	C-253	4135(93)	56(1)	51(1)	200(4)	2(0)	7(0)
	C-254	3064(82)	51(1)	20(1)	569(15)	16(0)	0
	C-256	3462(92)	67(2)	53(1)	171(5)	7(0)	0
	C-259	1288(79)	20(1)	49(3)	256(16)	9(1)	0

Appendix 5 (Continued)

Time/ Expos. (Weeks)	Animal	Granulo-cytes	Round	Small G	Agranulo-cytes	Degranu-lated	Serous
*	X-230	1985(90)	19(1)	171(8)	36(2)	3(0)	0
*	X-231	4647(88)	41(1)	452(9)	118(2)	4(0)	6(0)
*	X-232	1935(91)	69(3)	31(1)	89(4)	22(1)	1(0)
*	X-234	1936(95)	53(3)	31(2)	22(1)	4(0)	2(0)
*	X-235	3580(97)	31(1)	40(1)	22(1)	4(0)	4(0)
	X-237	1740(89)	17(1)	17(1)	184(9)	2(0)	0
	X-238	4791(97)	13(0)	33(1)	98(2)	2(0)	0
	X-241	1320(83)	178(11)	18(1)	60(4)	4(0)	2(0)
	X-242	2735(90)	142(5)	16(1)	142(5)	2(0)	0
	X-243	1620(91)	60(3)	7(0)	84(5)	7(0)	2(0)
	X-245	3284(97)	18(1)	16(0)	56(2)	9(0)	2(0)
	X-246	1511(94)	40(2)	29(2)	24(1)	0	0
	X-247	2817(94)	13(0)	24(1)	120(4)	7(0)	2(0)
	X-249	1817(87)	9(0)	24(1)	231(11)	16(1)	0
	X-250	3456(96)	33(1)	11(0)	84(2)	7(0)	0
	X-257	3535(92)	27(1)	36(1)	224(6)	7(0)	0
	X-258	3615(92)	53(1)	49(1)	200(5)	4(1)	0

APPENDIX 6

Differential count absolute values

Animal	Total Cells (per uL)	Granulocytes	Round	Small Granulocytes	Agranulocytes
			4-5 Weeks		
C-201	1597	1437	64	64	32
C-204	1544	1420	0	46	62
C-206	4042	3961	0	40	40
C-210	875	735	18	61	61
C-212	3388	3015	34	68	271
C-215	1744	1570	17	105	52
C-218	1622	1411	16	49	114
C-220	1908	1794	0	57	38
C-224	3464	3014	34	104	277
* X-202	671	503	0	67	87
X-203	717	623	14	22	50
X-205	993	884	9	30	70
X-207	1844	1235	0	572	37
* X-208	1275	1046	13	128	77
X-209	2102	1871	21	147	63
* X-211	617	494	49	31	37
* X-213	1462	1140	0	132	175
X-214	2142	2078	0	21	21
X-216	1260	1197	0	13	38
X-217	2051	2010	21	21	21
X-219	3337	3137	33	33	133
X-221	1600	1424	32	64	80
X-222	3071	2979	0	61	31
X-223	2235	2012	22	178	45
			8-9 Weeks		
C-233	1564	1314	16	0	219
C-236	2528	1593	25	25	961
C-239	2115	1755	85	85	169
C-240	1435	1205	0	14	201
C-244	2006	1505	120	40	321
C-248	1640	1164	16	0	443
C-252	1611	967	16	16	612
C-253	4451	4139	45	45	178
C-254	3944	3234	39	39	592
C-256	3762	3461	75	38	188
C-259	1631	1288	16	49	261

Appendix 6 (Continued)

Animal	Total Cells (per uL)	Granulocytes	Small Round	Granulocytes	Agranulocytes
* X-230	451	406	4	36	9
* X-231	3717	3271	37	334	74
* X-232	3126	2845	94	31	125
* X-234	2093	1988	63	42	21
* X-235	3682	3572	37	37	37
X-237	1815	1615	18	18	163
X-238	4937	4789	0	49	99
X-241	1582	1313	174	16	63
X-242	3037	2722	91	30	152
X-243	1780	1620	53	0	89
X-245	3384	3282	34	0	68
X-246	1604	1508	32	32	16
X-247	2988	2809	0	30	120
X-249	2100	1827	0	21	231
X-250	3591	3447	36	0	72
X-257	3838	3531	38	38	230
X-258	3748	3448	37	37	187

APPENDIX 7

Phagocytic response percent means for control populations by spawning stage and time.

CONTROLS

Spawning Stage	4-5 Weeks Phagocytic Response			8-9 Weeks Phagocytic Response		
	N.	Mean	S.D.	N.	Mean	S.D.
Mid Spawners	7	88	4.2	-	-	-
Advance Spawners	2	94	0	4	65	9.8
Advance-End Spawners	-	-	-	2	77	7.1
End Spawners	-	-	-	2	44	3.5
End-Complete Spawners	-	-	-	1	85	-
Complete Spawners	-	-	-	2	82	13.4