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HISTOLOGICAL AND PHYSIOLOGICAL EFFECTS OF DIESEL OIL FRACTIONS ON *AMBYSTOMA TIGRINUM*

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled HISTOLOGICAL AND PHYSIOLOGICAL EFFECTS OF DIESEL OIL FRACTIONS ON *AMBYSTOMA TIGRINUM*, submitted by Margaret E.C. Haag in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Ten day incipient LC_{50} were determined on *Ambystoma tigrinum* larvae treated with concentrations of light, heavy, and cracked diesel oil fractions, at 7°C and 15°C. Larvae were exposed to a sublethal concentration (0.4 toxic unit) of light, heavy, and cracked diesel oil fractions and tested for possible oxygen consumption and histological alterations.

Larvae in the light fraction treated groups showed little variation from control groups in oxygen consumption experiments. Oxygen consumption rates were significantly lowered in larvae after seven days' exposure to both the heavy and cracked diesel oil fractions. Electron microscopy studies of the larvae in the heavy and cracked treated groups showed possible structural modification of the mitochondrial cristae. This could have a direct effect on cellular metabolism, thus lowering the overall oxygen consumption levels.

Behavioral changes observed during exposure to the diesel oil fractions could have an additional effect on lowering oxygen consumption rates, especially in those experiments carried out at 15°C.

Exposure to the heavy and cracked diesel oil fractions also caused an apparent increase in permeability to water, with increases up to 6% in body weight. The frequency of the intercellular bridges between the stratum corneum and the stratum granulosum, as revealed by electron microscopy studies, were reduced in all groups, especially the heavy and cracked, thereby facilitating a possible path for water uptake into the intercellular spaces.

Histological studies revealed no alteration in cellular structure in skin, gill, and lung tissues taken from larvae treated with 0.4 toxic unit of the light or heavy fractions. However, larvae treated with 0.4 toxic unit of the cracked fraction showed an increase in the number of Leydig cells in the gill and skin epidermis.

Gas chromatographic analysis of the three diesel oil fractions was carried out. Although measurement of the qualitative and quantitative changes in the diesel oil fractions after seven days' exposure to the larvae was unsuccessful, gas chromatograms of control samples of each fraction are compared to show the hydrocarbon distribution and boiling point range.

Hydrocarbon determinations of the three diesel oil fractions showed the per cent composition of aromatics, olefins, and saturates. Although the LC_{50}'s for the individual components of the diesel oil fraction implied that the best correlation of toxicity existed in the olefin compounds, it was found that the fractions containing a greater composition of aromatics caused greater toxic effects.

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INTRODUCTION

With the advent of supertankers and overland oil pipelines, there has been an increased concern about the possibility of major oil spills, resulting in oil pollution. Oil spills at sea have received the most attention because large areas are quickly contaminated and because of the resultant adverse effects on birds, coastal animals, and shorelines (Wein and Bliss, 1973). Thus much of the oil pollution research has dealt with the contamination of intertidal zones and marine ecosystems (Carthy and Arthur, 1968; Cowell, 1971; Nelson-Smith, 1972), while few studies have dealt with the adverse effects of oil pollution on fresh water habitats (Bury, 1972).

The serious problems associated with oil pollution are caused mainly by the "persistent oils" including crude, residual fuel, and lubricating oils (Nelson-Smith, 1970). The light fuel oils, kerosene, gas oils, and gasolines, while not damaging the physical quality of the shore[illegible]es, have been found to have adverse effects on both animal (Chipman and Galtsoff, 1949; Tagatz, 1961; Bury, 1972) and plant life ([illegible], 1970).

Hydrocarbons are by far the most important component of any petroleum. Crude oils contain as much as 98% hydrocarbons, while petroleum gases and gasolines are almost pure hydrocarbons (Sachanen, 1954). Four major classes of petroleum hydrocarbons have been described: the paraffins (alkanes) consist of stable, saturated, branched or straight chain compounds; the naphthenes (cycloparaffins) constitute the saturated ring structures; the olefins (alkenes) are composed of unsaturated compounds; the aromatics are made up of unsaturated, cyclic compounds based on the benzene ring, with resonating double bonds

(Morrison and Boyd, 1968). The chemical composition of the particular oil has a direct bearing on its toxicity and changes which it might undergo after exposure to the environment (Bury, 1972)

Bury (1972) has shown that oils contain toxic components which, in general, are stable compounds and remain in an ecosystem for relatively long periods of time. Two months after contamination of an intertidal area by a fuel oil spill, the relatively unchanged fuel oil was being released from bottom sediments (Blumer et al., 1970). Blumer et al. (1970) found slightly altered fuel oil in *Crassostrea virginica* and *Aequipecten irradians* two months after exposure. They reported that although straight chain and branched chain hydrocarbons were modified, the more toxic aromatic hydrocarbons remained unchanged within the organisms.

While the aromatic components of oils are by far the most toxic to an organism (Havis, 1950; Blumer et al., 1970; Nelson-Smith, 1970; Morrow, 1974), it has been shown that if an oil or petroleum derivative is emulsified in water by chemical or physical means its toxicity is greatly enhanced (Tarzwell, 1970). The emulsified petroleum exerts its adverse effects by affecting a much greater volume and hence comes into contact with a greater variety of organisms.

The detrimental effects of oils on aquatic organisms have been reported in the literature for some time. McKee (1956) summarized the deleterious effects of petroleum products on aquatic organisms as follows: a) free oils and emulsions may act on the epithelial surfaces of fish, thereby interfering with respiration, or may coat and destroy algae and plankton, which removes sources of food; b) oily substances that settle to the bottom may coat and destroy benthic organisms and

interfere with spawning areas; c) soluble and emulsified oils may be ingested by fish, thereby tainting the flavor of the flesh, or water-soluble compounds may have a direct toxic action on aquatic life.

Chronic exposure to low concentrations of some hydrocarbons, especially benzene, has been found to cause bone marrow aplasia, leukopenia, aplastic anemia, chromosome aberrations, and leukemia in several aquatic organisms (Finkel, 1960).

Aromatic constituents of oil are known to cause copious secretions of mucus when applied to fish gills, interfering with respiration and water-salt balance (Nelson-Smith, 1972). Erosion of gill epithelia has also been reported when fuel oils were emulsified and applied to trout gills (Mironov, 1970).

Polycyclic aromatic compounds cause chronic injury to cells by penetrating the plasma membrane, thereby displacing the normal units of the membrane (van Overbeek and Blondeau, 1954). Diesel oils have been found to contain quantities of polycyclic aromatic hydrocarbons (PAH). Certain algae (i.e. *Chorella*) are known to synthesize PAH from petroleum compounds (Borneff et al., 1968). It has been suggested that changes in the cell membrane due to hydrocarbons, more specifically PAH, may play an important role in causing malignancies in animals (Goldacre, 1968). Sonneborn (1964) has shown that it is possible for membrane changes to be inherited without any disruption of the cell nucleus; hydrocarbon carcinogens could then cause permanent changes in the cell membrane structures.

The anesthetic property of oils has been demonstrated in oysters with resulting effects on the ciliated gill epithelium, thereby decreasing the normal pumping rate (Galtsoff et al., 1935; Chipman and Galtsoff,

1949; Lunz, 1950). Marsland (1933) has shown that paraffin compounds have narcotic effects on *Amoeba dubia*. Goldacre (1968) has extended Marsland's work to include cycloparaffins and aromatic compounds, producing a similar anesthetic response in the amoeba. It is thought that this response may be due to the petroleum derivative's effect on the plasma membrane. As the concentration of the cycloparaffins and aromatics reached the anesthetic concentration, the plasma membrane was shown to increase in thickness, eventually withdrawing from the granular cytoplasm (Goldacre, 1968).

Interference by the hydrocarbons with the chemical senses and neuromuscular control has been reported in various fish (Nelson-Smith, 1972). Deleterious effects of oils are also known to interrupt the hatching and development of fish eggs (Nelson-Smith, 1972). It is thought that the aforementioned responses are due to similar effects on membrane surfaces as reported by Goldacre (1968).

Although certain pelagic microorganisms are affected by oil pollution (Mironov and Lanskaja, 1967), the reproductive rate of most pelagic species is such that productivity has outweighed the long-term damage by the pollutant (Glover et al., 1970).

The primary effects of oil on marine birds has been to penetrate or cling to their plumage (Nelson-Smith, 1970). This coating of the feathers has resulted in reduced buoyancy and insulative properties of the feathers (Hartung, 1967; Goethe, 1968). Ingestion of oil by birds during preening has caused severe intestinal irritations, lipid pneumonia, fatty changes in the liver, necrosis, and adrenal enlargement (Hartung and Hunt, 1966). Rittinghaus (1956) also reported reduced hatching rates in terns after exposure to low concentrations of diesel fuel.

When coated with various oil products, marine mammals, like marine birds, tend to lose the insulative property of their fur (Peller, 1963). The lethal effects of the oil are usually secondary, with the primary reason for mortality being pneumonia. Spooner (1967) reported damage to seals after the "Torrey Canyon" incident, with severe eye irritations being the most noticeable effect.

Marine birds, mammals, and many mobile aquatic organisms, tend to avoid oil contamination if possible, thus eliminating most chances of damage. The major impact of oil spills has been to the more sedentary animals (Crapp, 1971a,b), to aquatic vegetation (Baker, 1970; Cowell, 1969), and to pelagic larva (Mironov and Lanskaja, 1967; Glover et al., 1970; Nelson-Smith, 1970).

Thus a great many studies and observations have been made on the adverse effects and toxicity of petroleum oils and their derivatives on aquatic organisms. However, nearly all these are field studies to confirm that oil pollution exists and to measure the harmful effects, or they are short-term studies of acute toxicities. There have been few studies which indicate safe levels of petroleum products under continuous or long-term exposure. Even fewer studies have been undertaken to determine whether or not sublethal changes over long periods of time reduce the chances of the animal being successful in its environment (Sprague, 1971).

Many pollutants, even when present in the water in concentrations well below lethal levels, may cause changes in the physiology and behavior of an organism (Anderson, 1971). Understanding the physiological action of a potential toxicant is the key to predicting its subsequent sublethal effects (Sprague, 1971). Cairns (1966) has claimed that measuring oxygen consumption is one of the best sublethal assay methods, while Sprague

(1971) has stated that the most widely used tool for determining the mode of action of any pollutant is via histology. The purpose of the present study was to utilize the two disciplines of physiology and histology in determining some of the sublethal effects of three petroleum fractions of diesel oil on the neotenic form of the tiger salamander, *Ambystoma tigrinum* (Green).

The larval *Ambystoma tigrinum* was chosen as the experimental animal in this study because it is an aquatic organism known to utilize more than one mode of respiration. Thus, the effects of oil on cutaneous, branchial, and pulmonary respiration could be investigated.

The diesel oil fractions used in this study were refined from Great Canadian Oil Sands (GCOS) crude. This so-called synthetic crude is separated from the tar sand by steam and subsequently put through a coking process. The resulting cracked stock is subjected to distillation procedures and refined into "light," "heavy," and "cracked" fractions. The chemical composition of the diesel oil can be varied from high boiling point kerosenes to low boiling point gas oils, depending on the proportion of the three fractions blended (Rossini and Mair, 1959). It should be noted that the diesel oils refined from the tar sand crude are composed of a larger proportion of naphthenic and aromatic compounds than conventional crude oils (Imperial Oil Ltd., pers. comm.). This aspect is reflected in the relative toxicity of the diesel oil fractions.

The first part of this study was designed to establish the sublethal concentrations for the three diesel oil fractions used throughout the histological and physiological experiments on the *Ambystoma tigrinum* larvae. Median tolerance limits to diesel oil vary according to the organisms being tested (Sprague, 1971), and the oil fraction being utilized.

Gutsell (1921) found that gasoline has a toxic effect on rainbow trout, *Salmo gairdneri*, at 100 parts per million (ppm). McKee and Wolf (1963) reported that agitated solutions of gasoline at concentrations of 100 ppm and jet aviation fuel at 500 ppm were lethal to fingerling salmon, *Oncorhynchus* sp. Zahner (1962), on the other hand, found the median tolerance limits to diesel oil to be 300—400 ppm in *Salmo gairdneri*, whereas Tagatz (1961) determined these limits to be 167 ppm when testing juvenile shad, *Alosa sapidissima*. In 24-hour LC_{50}'s, Shaw (pers. comm.) found values of 200 ppm for light fraction of diesel oil, 80 ppm for the heavy fraction, and 20 ppm for the cracked fraction in experiments using juvenile rainbow trout. These three fractions are similar to those utilized in the present study.

The second objective of this study was to determine the effects of sublethal concentrations of the diesel oil fractions on oxygen consumption rates in the larvae. The effects of petroleum derivatives on respiration are controversial. Some authors have reported an increase in oxygen consumption (standard oxygen consumption in the sunfish, Cairns and Scheier, 1962) due to the possible uncoupling of oxidative phosphorylation (Beevers, 1953). Other authors have reported decreases in oxygen consumption rates (Galtsoff, 1936; Hood et al., 1960) which may be due to disruption of the mitochondrial membrane (Baker, 1970). Marchetti (1962) has shown that increases or decreases in oxygen consumption are due to the nature of the specific petroleum product being investigated, some acting as stimulators and others acting as depressants.

The third objective of this study was to do histological examination of the gill, skin, and lungs of larvae treated with sublethal concentrations of the diesel oil fractions to determine whether modification of

cellular structure occurred. Schmidt and Mann (1961) and Scheier and Cairns (1966) have reported the erosion of gill epithelia and destruction of mucous cells in sunfish, *Lepomis gibbosus*, treated with sublethal concentrations of phenols. It is also evident that fuel oils cause extensive damage to the gills of trout, with extensive erosion of the mucous membranes (Reichenbach-Klinke, 1962; Maláćea et al., 1964). Exposure to 10 ppm of the light fraction of diesel oil at 7°C caused a sloughing off of gill and skin epithelium of rainbow trout (personal observation).

The final objective of this study was to determine, using gas chromatographic techniques, whether changes occurred in the diesel oil fractions after seven days' exposure to the larva in the experimental tanks. It is known that, initially, the lower boiling point fractions will evaporate. Later, the successively higher boiling point fractions will evaporate (Berridge et al., 1968). Gas oils, kerosenes, and gasolines disperse and evaporate much more readily than crude oils (Nelson-Smith, 1970). As evaporation takes place, the specific gravity and viscosity of the mixture are altered (Berridge et al., 1968). One might expect that there are initial changes in the composition of the oil being tested during exposure in specific environments. If after this time the oil is still toxic, then perhaps the chemical nature of the toxic components of the oil can be determined.

In addition to physical alterations of the composition of the oils, there are also possible modifications resulting from microbial action. Stone et al. (1942) have shown that light to medium weight fractions of oils were subject to attack by gram-negative bacteria (i.e. *Pseudomonas* sp.) more so than the heavy, viscous fractions. Davis and Hughes (1968)

reported that individual aliphatic, olefinic, and naphthenic compounds are most susceptible to attack by microorganisms. Work by Jobson et al. (1972) has shown the preferential use of n-saturate (paraffinic and cycloparaffinic) fractions of crude oil by microbes. Long-term exposure tests with *Ambystoma tigrinum* in water containing oil fractions may result in the microbial alteration of the fraction. Therefore, it seemed important to analyze the fractions, before and after testing, by gas chromatographic procedures to determine any qualitative and quantitative changes over time.

METHODS AND MATERIALS

Collection and Storage

Larval tiger salamanders, *Ambystoma tigrinum*, were collected from Tyrrell's Lake (Sec. 17, 18 and 19, Twp. 5, Rge. 17, W4th; Sec. 24, Twp. 5, Rge. 18, W4th), a highly alkaline (pH 9.1—10.2; total alkalinity 636.2 mg/litre, see Appendix I), irrigation-fed lake, four miles east of New Dayton, Alberta. Collecting trips were made monthly throughout the summer of 1973, from June through September, with two subsequent trips in May and early October of 1974. The most productive collecting periods were in mid-May and late September, coinciding with the new moon or just thereafter. Since the larvae seemed to display a diurnal, vertical migration pattern, all collecting was carried out between 11 p.m. and 3 a.m.

All collections were made at the northwest corner of the lake. This area proved to be the most productive as well as the most accessible. A 12.2 m x 1.8 m x 1.8 m bag seine (Whale Enterprises, Inc.), with 2.2 kg weights attached at the lower edge every 1.5 m along its length and with a 45.8 m length of rope attached to each end, was used for collecting. The seine was rowed out 45.8 m from the shore and extended parallel to the shoreline. It was then drawn back to shore by means of the 45.8 m ropes attached at its ends. The larval salamanders were removed from the net and placed in 30-litre plastic containers holding lake water, for transport back to the laboratory.

Four 1-litre water samples were brought back to the laboratory for analysis (see Appendix I).

In the laboratory, the animals were kept in 313-litre flow-through tanks at 5°C ± 1°C, using chilled, dechlorinated, aerated water. Additional cooling units (Frigid Unit, 1/3 hp) were used to maintain proper water temperature control. Utilization of such low temperatures made feeding unnecessary, as well as retarding any fungal or bacterial infection.

The animals were allowed to acclimate to laboratory conditions for at least two weeks prior to any experimental procedures. During this initial period of acclimation, three fractions of diesel oil (Great Canadian Oil Sands, Imperial Oil, Ltd.) were obtained through the Energy Resources Conservation Board of Alberta. The three diesel oil fractions were designated "light," "heavy," and "cracked" depending upon the position of removal from the distillation column during processing at the Imperial Oil Refinery, Edmonton, Alberta; the "light" fraction being first removed, followed by the "heavy" fraction, then the "cracked" fraction. It is the blend of varying proportions of these fractions that determines the "weight" (summer or winter), and thus the pour point of the resulting refined diesel oil (Rossini and Mair, 1959).

A test for the various types of hydrocarbons in the individual diesel oil fractions was performed by a fluorescent indicator adsorption method (ASTM Standards, D-1319, 1972). The hydrocarbons were separated, according to their adsorption affinities on activated silica gel, into aromatics, olefins, and saturates (paraffins and cycloparaffins), the boundaries of which were visible under ultraviolet light. The volume percentage of each hydrocarbon was calculated from the length of each zone in the column.

LC_{50} Determinations

In order to determine "safe" or sublethal concentrations (as defined by Sprague, 1970) of the three diesel oil fractions, incipient LC_{50} (the lethal concentration for 50 per cent of individuals on long exposure, greater than 96 hours) were determined for each fraction at experimental temperatures of 7°C and 15°C. First preliminary tests were performed to determine the range of concentrations of each fraction that should be utilized in the extensive tests. The upper concentration limits of these preliminary tests were estimated using the results of the previously described hydrocarbon fluorescent indicator method. The more aromatics per fraction, the greater the toxicity. Thus, knowing the per cent aromatics per fraction, the upper concentration limits could be established. The method for selection of specific concentrations, based on progressive bisection of intervals on a logarithmic scale, was adopted from Taras et al. (1971).

For these experiments, 10 animals were weighed (mean weight 115 grams) and placed in individual 30-litre, glass aquaria containing aerated, dechlorinated water with one gram per litre sea salts (Utility Seven Seas Marine Mix). An additional four animals were weighed and placed in similar tanks to serve as controls. This procedure was repeated for each fraction tested.

Two series of experiments were carried out at each of the experimental concentrations: one with animals that had been acclimated for 14 days at 7° ± 0.5°C and the other with animals that had been acclimated for 14 days at 15° ± 0.5°C. The 7° temperature was chosen so that comparisons could be made with diesel oil toxicity tests previously done

with rainbow trout, *Salmo gairdneri* (Shaw, pers. comm.), whereas the 15° temperature corresponded to the average lake temperature at the time of collection of the larvae.

At the completion of the temperature acclimation period the animals were removed and fresh water, at the prescribed temperature, was added to each tank. The animals were replaced into their respective tanks, with a total of 10 animals being used for each fraction tested at each of the two experimental temperatures. The diesel oil fractions were measured using a 100 µl syringe and administered to each tank, 10 cm below the water surface. Animals were checked for mortality every three hours. Ten-day incipient LC_{50} were then calculated according to Sprague (1969) and from this, estimates of the sublethal, and presumably "safe" concentrations of the fractions were established.

Histology

Tissue samples were taken from representative animals from each of the above experimental conditions. Tissue samples from those larvae treated with lethal concentrations of the diesel oil fractions came from animals that were found dead in their experimental tank (death having occurred since previous check for mortality) or were so close to death (only a slight heartbeat could be detected) that they were killed in Tricaine methane sulfonate, 15 g/litre. The first primary gill filament, a 5-mm portion of the anterior area of the tail, and an entire lung sac were taken and placed in Bouin's fixative for a minimum of 72 hours. Prior to removal of the lung sac it was injected with one ml of Bouin's fixative. Samples were then individually dehydrated, infiltrated, and embedded according to procedures in Humason (1967). Seven micron sections

were stained using the Masson-Trichrome method (Humason, 1967).

Oxygen Consumption Experiments

Oxygen consumption measurements were made on animals before and after 10 days' exposure to sublethal concentrations of the three different diesel oil fractions. Individual animals were placed in 30-litre glass aquaria, as previously described, and acclimated for two weeks at the two experimental temperatures prior to oxygen consumption measurements.

Oxygen consumption experiments were carried out in 30.5 cm x 10.2 cm x 15.2 cm plexiglass respiration chambers (see Appendix II), designed in such a way that half the internal volume was filled with water, while the other half remained as an air space (Cormie, 1975). Such a design allowed the *Ambystoma tigrinum* larva to utilize one or all three modes of respiration -- cutaneous, branchial, or pulmonary. The respiration chambers and experimental set-up have been completely described by Cormie (1975). Animals were weighed and then placed in one of the four respiration chambers. The four chambers were sealed and set in a 66.1 cm x 150.0 cm x 30.5 cm thermostatically controlled waterbath which regulated temperature at 7° ± 1.0°C or 15° ± 1.0°C, depending on which series was being tested.

The chambers were connected to a series of manometers; the manometers, in turn, were connected to a compensation chamber. Each chamber was fitted with water inlet and outlet tubing, as well as a 10-ml glass syringe with which water samples could be taken.

Once the animals were placed in their respective chambers, the chamber-manometer system was opened to the room air and the system was

allowed to equilibrate for two hours. In initial experiments, the air space within the chambers was flushed with oxygen, 2 psi. This step proved to be unnecessary once relative oxygen consumption values were determined because the $ml O_2/hr/g$ (dry weight) consumed by the larva were relatively low. When air, 2 psi, instead of oxygen was used to flush the air space in the chambers, the animals did not appear to be stressed during the five hour test period. In fact, when dissolved oxygen levels were determined in the water samples, there was less variation among samples tested. Thus, in all subsequent experiments, air was used instead of oxygen.

At the end of the two hour equilibration period, three 10-ml water samples were taken from each chamber prior to sealing the chambers to establish a closed-system respirometer. The chambers remained closed for a period of five hours. During this time, air pressure within the chambers changed as the oxygen was being utilized by the larvae. These changes were recorded on the manometers and nitrogen was injected to re-establish pressure equilibrium (Cormie, 1975). At the end of five hours, three additional 10-ml water samples were taken from each chamber. In all cases, water samples were immediately tested for dissolved oxygen content by a modification of the Winkler Iodometric method devised by Burke (1968). The difference between the initial and final dissolved oxygen concentrations, plus the total volume of nitrogen injected, in millilitres, equalled the amount of oxygen utilized by the larvae over the five hour period. The volume of oxygen consumed was then determined as $ml O_2/hr/g$ (dry weight), corrected to STP.

After the oxygen consumption measurements on untreated, temperature acclimated animals, oxygen consumption measurements were repeated on

each animal, 24 per each fraction tested, after seven days' exposure to a sublethal concentration of the individual diesel oil fraction. During this seven day exposure period, the animals were removed daily, weighed, and then replaced in their respective experimental tanks. At the end of the oxygen consumption measurements the animals were killed using Tricaine methane sulfonate, 15 g/litre. Histological samples were taken as previously described.

Dry weights for each larva were determined after final oxygen consumption measurements. Pre-weighed animals were killed, as previously described, and set on filter paper in drying ovens at 80°C. In the first series of experiments, the dried larvae were weighed daily until the dry weights did not vary from the previous day's measurements. The maximum time for this to occur was four days. In all experiments thereafter, the larvae were weighed after a minimum of four days in the drying ovens.

Electron Microscopy

During the seven day exposure to the diesel oil fractions, there was a substantial increase in weight of the larvae, especially those exposed to the "heavy" and "cracked" fractions. In several cases when the body cavity was opened to remove a lung sac for histological study, it was noted that the body cavity was full of fluid. The larvae were not fed during testing, thus it was assumed that any weight gain was due to an increase in water. Several investigators (Currier and Peoples, 1954; van Overbeek and Blondeau, 1954; Goldacre, 1968) have reported an increase in cell membrane permeability to water at low concentrations of various oil products. Since no cellular damage was apparent on most slides prepared for standard histological examination, the investigator

considered that a change in cell membrane structure, and thus a mechanism for increased water permeability, might be revealed by electron microscopy. A small scale study was carried out to test this hypothesis. A total of six larvae were treated with the sublethal concentration of each of the diesel oil fractions, at 15°C. An additional two animals served as controls. At the end of seven days, a 1-mm section was removed from the tail region of the larva and fixed in a 2.5% glutaraldehyde solution (pH 7.6). At first, a single, secondary gill filament was chosen for electron microscope studies. This structure proved to be too difficult to work with, thus a section from the tail region of the larva was used. The tissues were post-fixed in 2% OsO_4 (pH 7.2) and subsequently dehydrated in an ethanol series (35—100%). Each sample was then embedded in araldite and sectioned with a Porter-Blum MT-2 Ultramicrotome. Sections 50—60 nm thick were mounted on 200-mesh coated grids and stained with saturated, aqueous uranyl acetate, followed by 10% lead citrate. Samples were examined using a Phillips 200 Electronmicroscope, and photographs were taken.

Oil Fraction Analysis

Diesel oil fractions were analyzed by gas chromatographic techniques with the aim of determining any quantitative changes in the petroleum hydrocarbons in these fractions after seven days' exposure to the larvae in the experimental tanks. Diesel oil fractions were extracted from the water by repeated washings with n-heptane, 2 ml n-heptane/800 ml water-oil mixture. Gas chromatographic separation of a 0.2 µl sample of extract was achieved by using a Hewlett-Packard Gas Chromatograph model 5830A (HP terminal 188508) with a flame ionization detector and a 100-ft SCOT

(supported-coated-open-tubular) capillary column, OB-1. The instrument was programmed as follows: linear temperature program 35—230°C; rate of programming, 2°C per minute; injection block temperature, 300°C; hydrogen flow rate, 15.0 ml/min; air flow rate, 300 ml/min.; calculation of area under each hydrocarbon peak, based on previously determined retention times. Each water-diesel oil fraction was tested and compared with control samples of each diesel oil fraction.

RESULTS

Hydrocarbon Determinations

Results from hydrocarbon determinations via the fluorescent indicator method (ASTM, 1972) (Table 1) showed that the light fraction contained 14.6% aromatics while the heavy and cracked fractions contained 59.0% and 72.0%, respectively. Per cent olefins were low in all fractions, while the per cent saturates (paraffins and cycloparaffins) were 84.6% in the light fraction, 40.0% in the heavy fraction, and 26.8% in the cracked fraction.

LC_{50} Determinations

Ten-day incipient LC_{50} estimates were determined by plotting cumulative per cent mortality after ten days versus the concentration in parts per million (ppm) of each of the diesel oil fractions (at the two experimental temperatures) (Table 2). A best-fit relationship of the data transformed from probits to log, was determined by linear regression analysis, and the incipient LC_{50} concentrations were read directly from the graph (Figs. 1, 2 and 3).

The incipient LC_{50} values at 7°C for the light, heavy, and cracked fractions were 125 ppm, 100 ppm, and 62 ppm, respectively (see Figs. 1, 2 and 3). At 15°C, the estimates were slightly less with values for the light fraction being 110 ppm, and the heavy 90 ppm, and the cracked 50 ppm (See Figs. 1, 2 and 3). The incipient LC_{50} of each fraction tested equals 1.0 toxic unit (Sprague, 1970). In all experiments the sublethal concentration used was 0.4 toxic unit. At this level there were no obvious detrimental effects to the larva.

TABLE 1

HYDROCARBON COMPOSITION OF THE THREE DIESEL OIL FRACTIONS BASED ON FLUORESCENT INDICATOR ADSORPTION (ASTM, D-1319, 1972)

	(Per cent composition)			
Fraction	Aromatics	Olefins	Saturates	Total
Light	14.6	0.8	84.6	100
Heavy	59.0	1.0	40.0	100
Cracked	72.0	1.2	26.8	100

TABLE 2

CUMULATIVE PER CENT MORTALITY AFTER TEN DAYS FOR DIFFERENT CONCENTRATIONS OF DIESEL OIL FRACTIONS FOR DETERMINATION OF TEN DAY INCIPIENT LC_{50}s

LIGHT FRACTION

7°		15°	
ppm	% mortality	ppm	% mortality
32	0	32	0
56	5	56	10
75	10	75	20
100	30	100	40
135	60	135	70
240	95	240	100

HEAVY FRACTION

7°		15°	
ppm	% mortality	ppm	% mortality
32	0	32	0
56	5	56	10
75	30	75	30
100	50	100	60
135	80	135	90
180	95	180	98

CRACKED FRACTION

7°		15°	
ppm	% mortality	ppm	% mortality
32	1	32	5
42	10	42	30
56	40	56	70
75	70	75	95
100	95	100	100
135	100	135	100

Figure 1. *Regression lines relating the cumulative per cent mortality to the concentration of light fraction (ppm) for determination of ten day incipient* LC_{50}.

LC_{50} at 7°C (————)

LC_{50} at 15°C (--------)

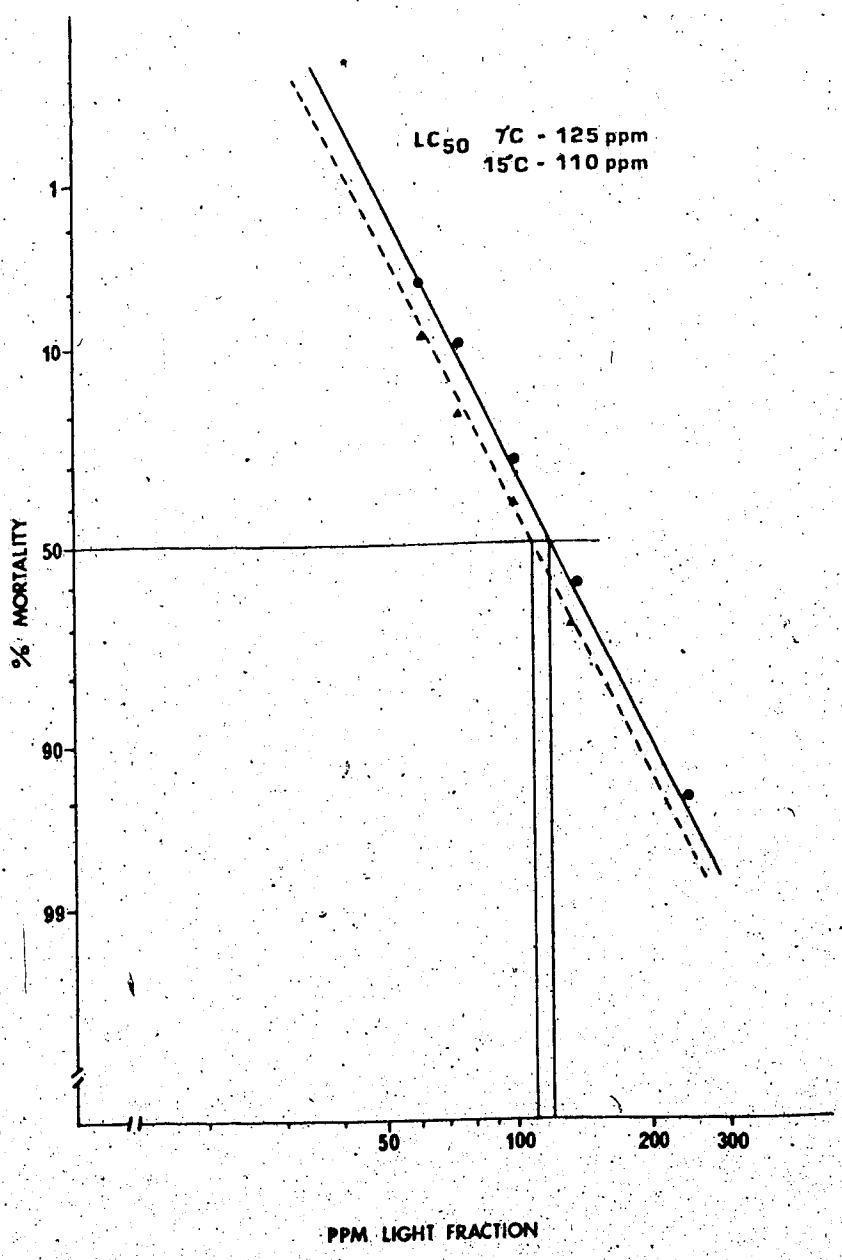

LC50 7C - 125 ppm
15C - 110 ppm
1
10
50
90
99
% MORTALITY
50
100
200
300
PPM LIGHT FRACTION

Figure 2. *Regression lines relating the cumulative per cent mortality to the concentration of heavy fraction (ppm) for determination of ten day incipient* LC_{50}.

LC_{50} at 7°C (————)

LC_{50} at 15°C (-------)

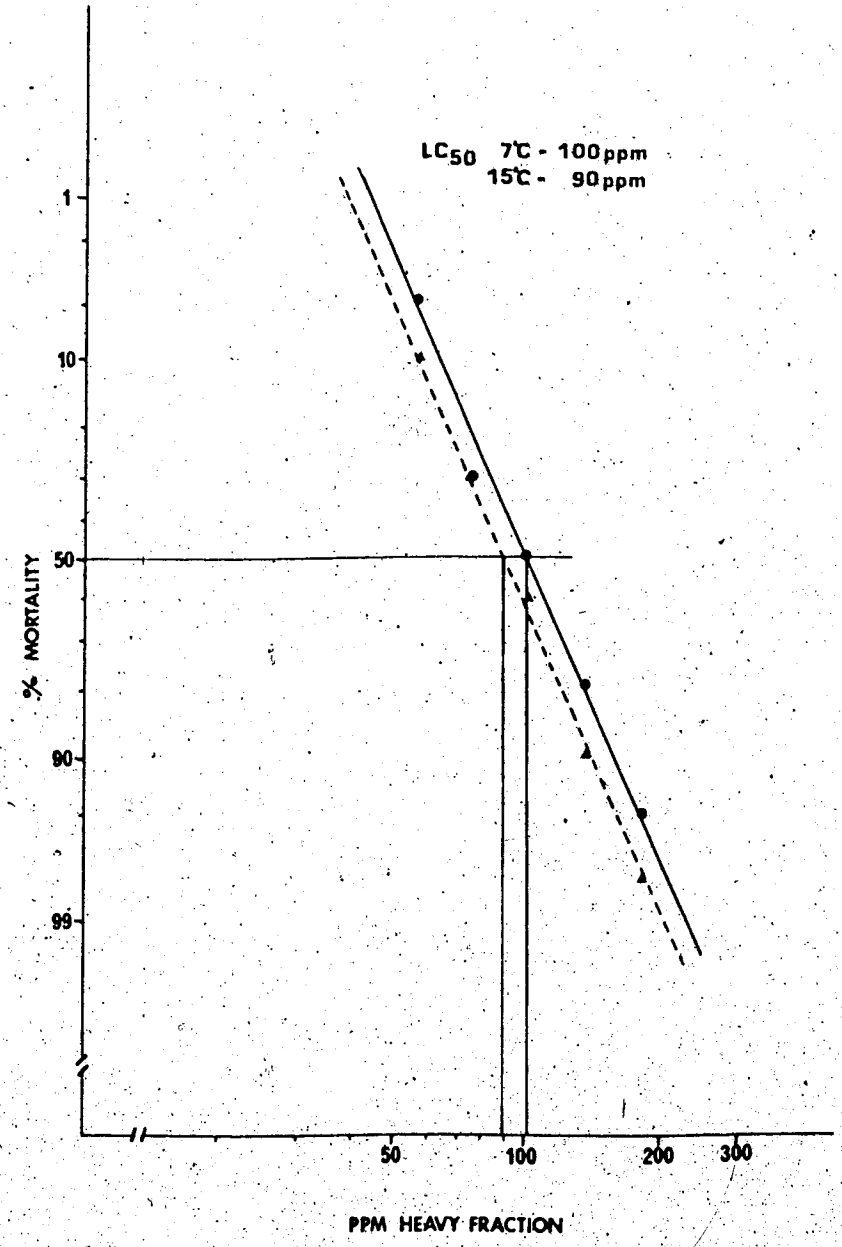

LC50 7°C - 100ppm
15°C - 90ppm
1
10
50
90
99
% MORTALITY
50
100
200
300
PPM HEAVY FRACTION

Figure 3. *Regression lines relating the cumulative per cent mortality to the concentration of cracked fraction (ppm) for determination of ten day incipient* LC_{50}.

LC_{50} at 7°C (————)

LC_{50} at 15°C (-------)

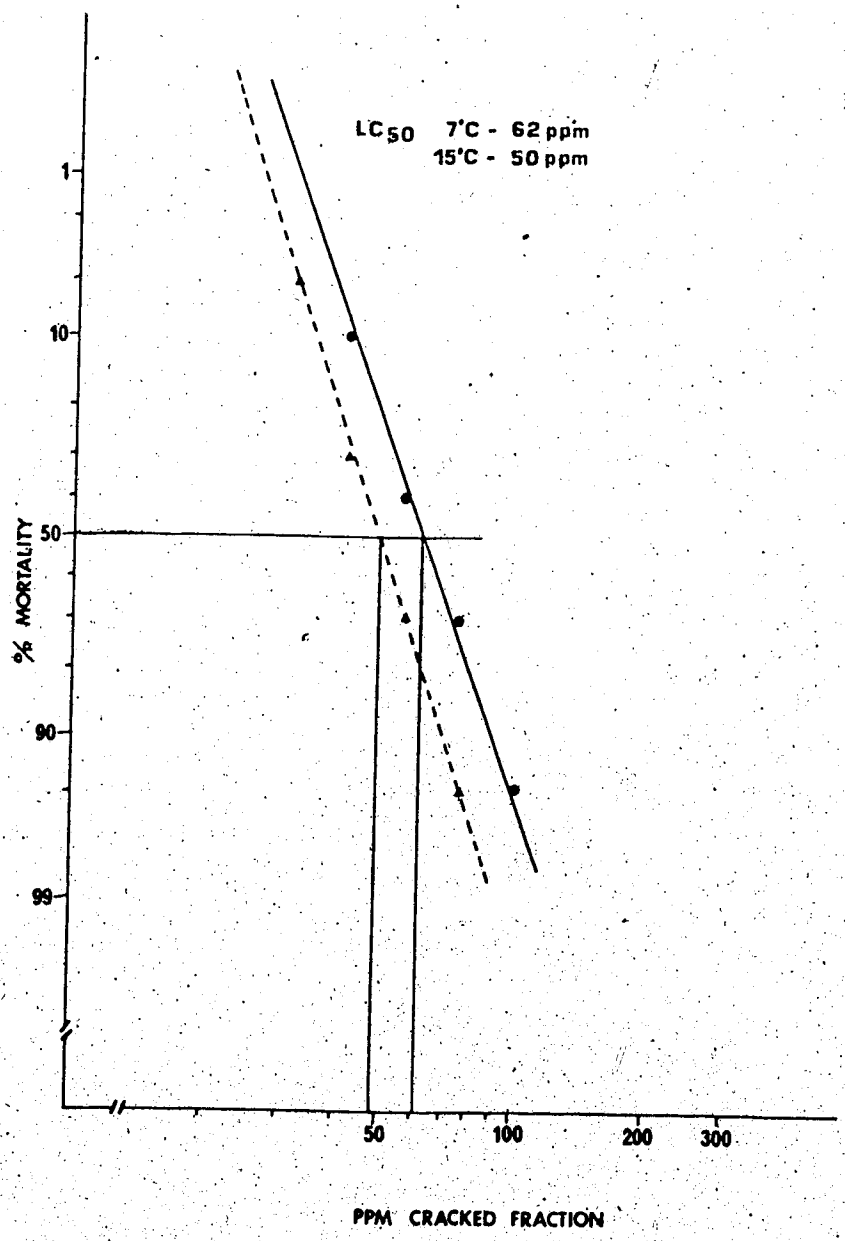

LC50 7°C - 62 ppm
15°C - 50 ppm
1
10
50
90
99
% MORTALITY
50
100
200
300
PPM CRACKED FRACTION

TABLE 3

LC_{50} FOR EACH COMPONENT (AROMATICS, OLEFINS, AND SATURATES) CALCULATED FROM THE TEN DAY INCIPIENT LC_{50} FOR EACH FRACTION BY USING THE PER CENT COMPOSITION OF THE THREE MAJOR COMPONENTS IN THE THREE DIESEL OIL FRACTIONS. TAKEN FROM TABLE 1 AND FIGS. 1, 2 AND 3

Fraction	LC_{50}	Aromatics	Olefins	Saturates
		(ppm LC_{50} at 7°C)		
Light	125	18.25	1.00	105.75
Heavy	100	59.00	1.00	40.00
Cracked	62	44.64	0.74	16.62
		(ppm LC_{50} at 15°C)		
Light	110	16.06	0.88	93.06
Heavy	90	53.10	0.90	36.00
Cracked	50	36.00	0.60	13.40

The LC_{50}'s for the major components (aromatics, olefins, and saturates) of the three diesel oil fractions were calculated (Table 3) from the ten day incipient LC_{50} for each fraction (see Figs. 1, 2 and 3) by using the per cent composition of each of the three major components in the diesel oil fractions (see Table 1).

Whenever the diesel oil fractions were administered to the larvae in the experimental tanks, certain behavioral changes were observed. Prior to treatment the larvae would swim freely within the tanks, occasionally rising to the surface to gulp air, and also would rest periodically on the bottom of the tank. In the control tanks this pattern persisted. However, after the diesel oil fraction was placed in the tank, the larvae began swimming erratically and bumped into the sides of the tank. Frequent gulps of air were taken during this initial period. The oil appeared to be an irritant to the animal. After 30 or 40 minutes this behavioral pattern ceased, and thereafter the larvae usually remained on the bottom of the tank. Rarely was any further gulping of air observed. This response was characteristic for the majority of the animals tested at the sublethal concentrations (0.4 toxic unit) of the diesel oil fractions. At higher concentrations of the fractions, the larvae would float aimlessly at the water surface after four days in the test sample. Death usually followed.

Histology

The histological structure of the respiratory organs (gill, lung, and skin) of the larvae was examined for any modification and/or destruction. Examination of the tail skin of the larvae treated with 0.4 toxic unit of the light fraction of diesel oil revealed no observable

changes from control samples (Plates 1 and 2). The cellular arrangement observed in the tail skin of the control and light fraction treated samples was as follows:

The primary layer, the stratum corneum, was composed of thin cuticle. Beneath the stratum corneum was a simple, unstratified layer composed of transitional polyhedral cells known as the stratum granulosum and the stratum spinosum (Weiss and Ferris, 1954; Patt and Patt, 1969). These cellular layers rest upon a germinative layer of low columnar cells known as the stratum germinativum which, in turn, rests upon a thin basement membrane. Within the stratum granulosum and stratum spinosum large spherical cells known as Leydig cells (Weiss and Ferris, 1954; Andrew and Hickman, 1974) are seen. These are secretory in function. In most cases, multicellular glands of the mucous and serous types were apparent within the stratum germinativum. The smaller mucous glands are composed of low columnar epithelial cells, while the larger serous glands are composed of irregular shaped, granular cells (Andrew and Hickman, 1974).

The epidermal layer lies directly on a dermal layer composed of a fibrous connective tissue, overlying a more dense connective tissue. The junction of the epidermal and dermal layers is heavily marked with branched chromatophores of varying types. The dermal layer also contains a large number of blood vessels, which can be clearly seen. There are numerous nerves located in this dermal region but they are not clearly visible with this specific staining method.

Examination of the secondary gill filaments from the control and light fraction treated larvae again show no observable differences in cell structure (Plates 3 and 4). The cellular layers of the gill

PLATE 1

Section through the tail skin from control *Ambystoma tigrinum* larva under light microscopy.

SC - stratum corneum

SG - stratum granulosum

SS - stratum spinosum

SGr - stratum germinativum

BM - basement membrane

V - venule with erythrocytes

CT - connective tissue

Ch - chromatophores

L - cells of Leydig

MG - multicellular glands

Er - erythrocytes

PLATE 2

Section through the tail skin from *Ambystoma tigrinum* larva treated with 0.4 toxic unit of light fraction diesel oil.

SC - stratum corneum

SG - stratum granulosum

SS - stratum spinosum

SGr - stratum germinativum

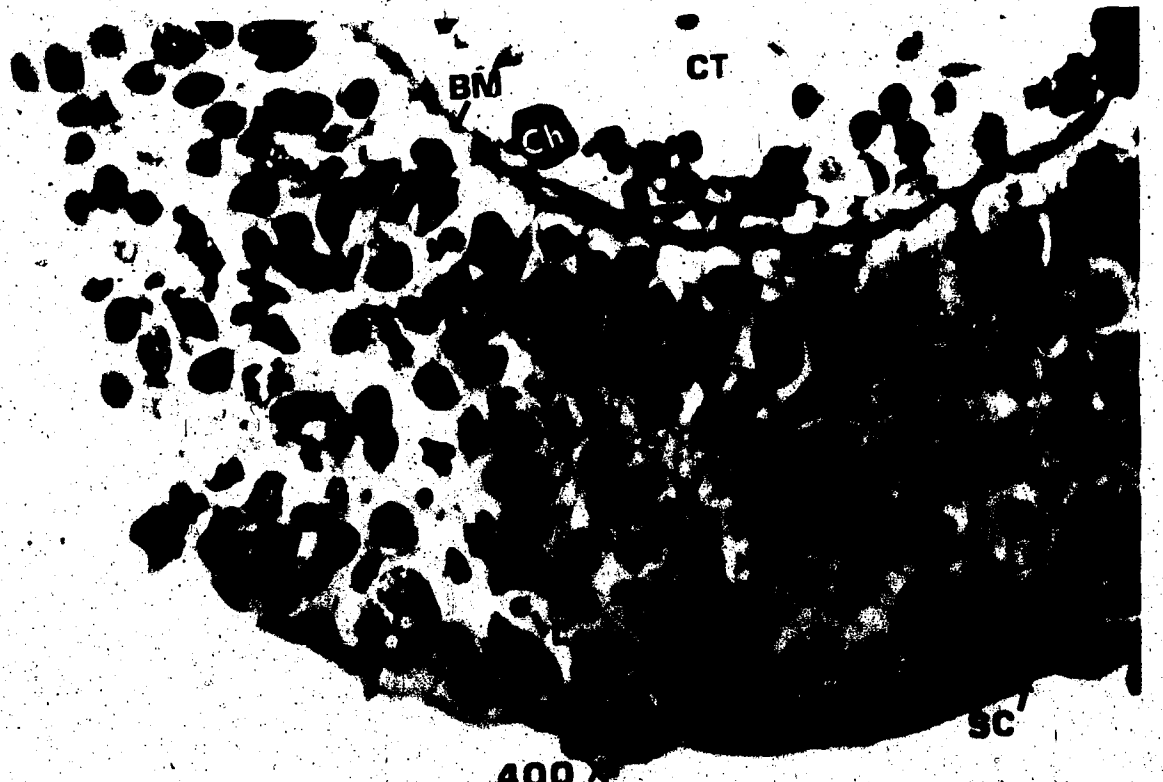
BM - basement membrane

CT - connective tissue

Ch - chromatophores

L - cells of Leydig

200X



400 X

PLATE 3

Section through the secondary gill filaments from control *Ambystoma tigrinum* larva.

E - epidermis

De - dermis

Ch - chromatophores

BV - blood vessel

PLATE 4

Section through the secondary gill filaments from *Ambystoma tigrinum* larva treated with 0.4 toxic unit of light fraction diesel oil, under light microscopy.

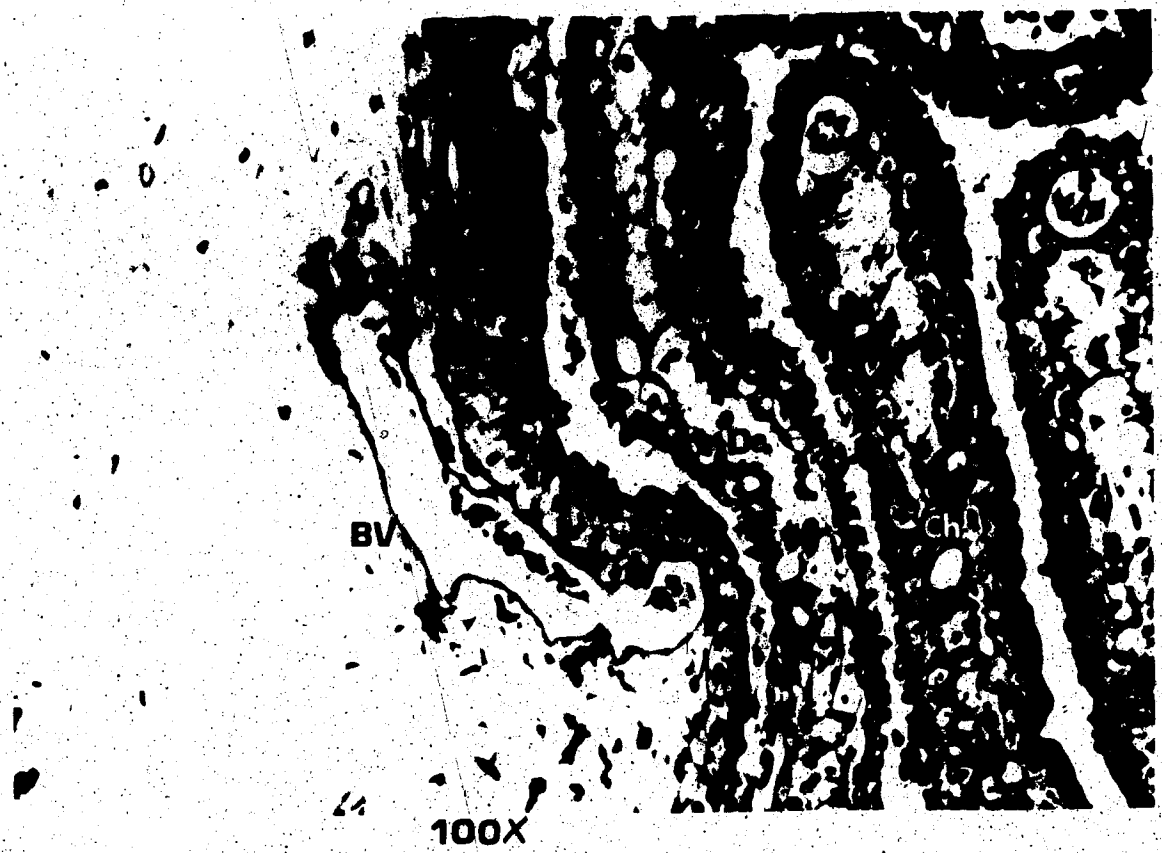
E - epidermis

De - dermis

BV - blood vessel

Ch - chromatophores


De
Ch
BV
100X


BV
Ch
100X

epithelium are similar to those described for the integument. The epidermal layer, however, is much thinner and the cells themselves appear much smaller. The abundance of mucous glands within the dermal layer is clearly evident, as well as an increase in the number of branched chromatophores within the gill filaments. Numerous capillaries containing several nucleated erythrocytes are visible. Where the gill filament attaches to the main body of the gill arch, larger blood vessels are evident as well as large bundles of skeletal muscle fibers located throughout the dermal layer.

Amphibian lungs vary in structure according to the degree of function in the specific organism being observed (Noble, 1954). The variation in structure is correlated with the amount of folding of the epithelial lining and the degree of vascularization within the lung sac (Noble, 1925). It is evident from examination of the lung sacs from control and light fraction treated larvae (Plates 5 and 6) that there is an appreciable amount of folding of the epithelial lining. The lung sac is divided by many septa which are composed of simple ciliated, low columnar epithelium. The infundibula are divided by many smaller septa. The large spaces leading from the infundibula to the smaller divisions, the alveoli, are known as alveolar ducts. Both the alveolar ducts and the alveoli are characteristically lined with a single layer of squamous epithelium. The cell membranes of these epithelial cells are extremely thin and allow the diffusion of oxygen into the thin-walled capillaries which lie in the connective tissue adjacent to the epithelium. Also lying within the connective tissue beneath the epithelium are numerous lymphatic vessels. Larger arterioles and venules are visible and contain numerous erythrocytes.

PLATE 5

Section through the lung sac from control *Ambystoma tigrinum* larva, under light microscopy.

SE - squamous epithelium

SCE - ciliated low columnar epithelium

CT - connective tissue

Er - erythrocytes

Cp - capillary

Al - alveolus

Ad - alveolar duct

PLATE 6

Section through the lung sac from *Ambystoma tigrinum* larva treated with 0.4 toxic unit of light fraction diesel oil, under light microscopy.

SE - squamous epithelium

SCE - ciliated low columnar epithelium

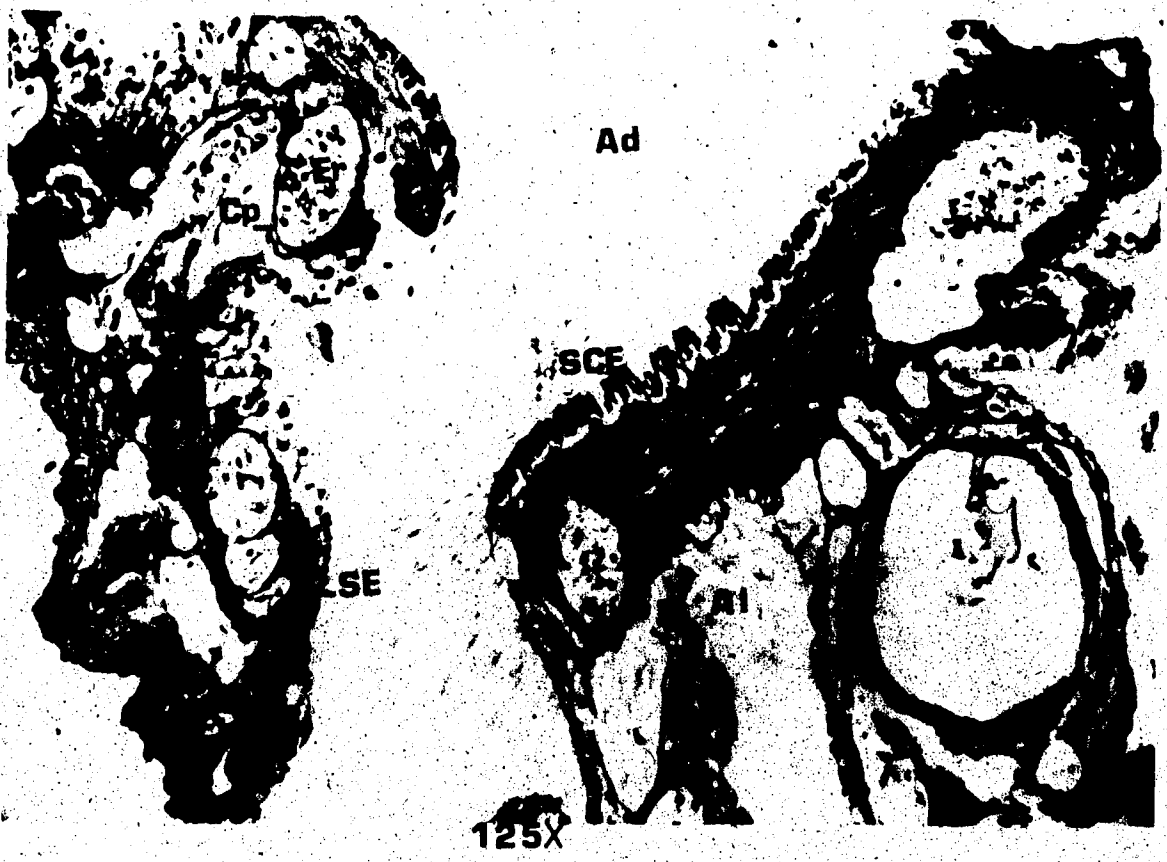
CT - connective tissue

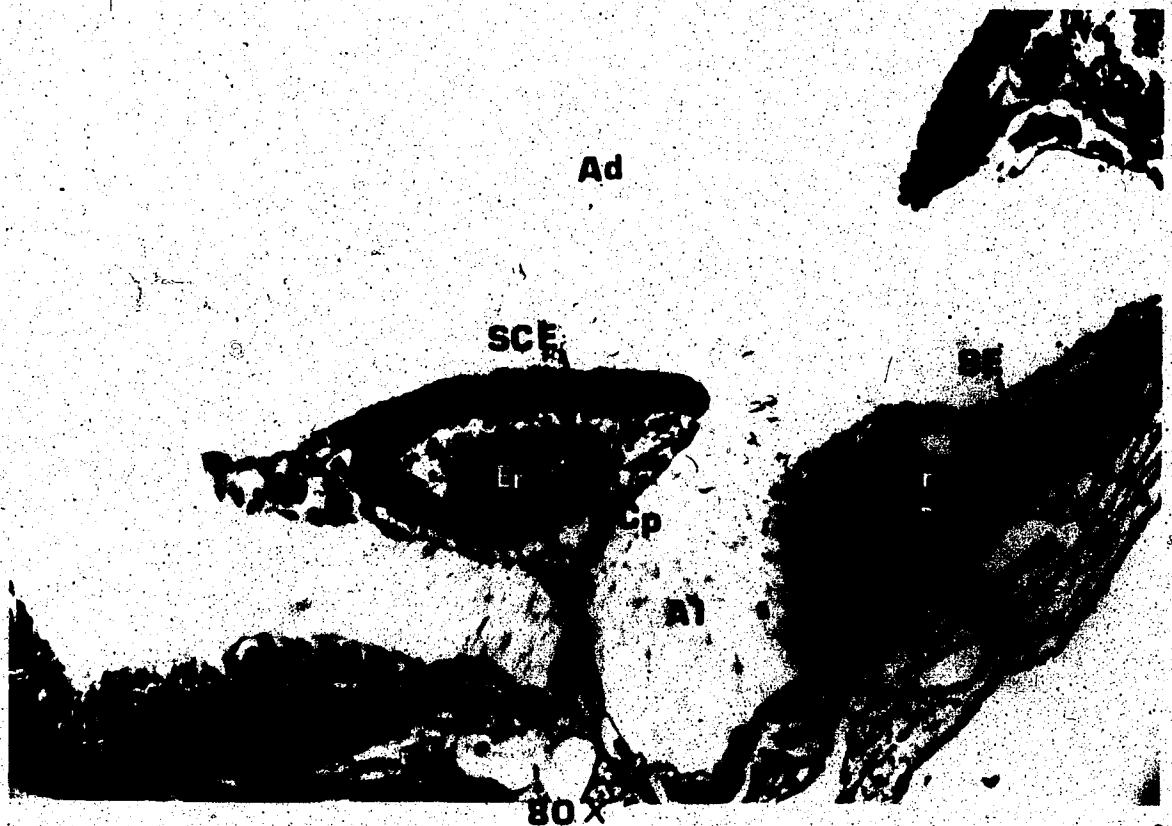
Er - erythrocytes

Cp - capillary

Al - alveolus

Ad - alveolar duct


Ad
Cp
SCE
LSE
Al
125X


Ad
SCE
Cp
Al
80X

The tissues from the larvae treated with 0.4 toxic unit of the heavy and cracked fractions of diesel oil are similar to those from the control and light treated groups in most respects (Plates 7, 8, 9, 10, 11 and 12). In the larvae treated with the cracked fraction, there appears to be an increase in the number of unicellular secretory glands, the Leydig cells (see Plate 8). Also, in a few instances, there appears to be some sloughing off of epithelial cells in both the gill and skin of cracked fraction treated samples (see Plates 8 and 10). At sublethal concentrations this sloughing off was not extensive and only occurred in a few specimens.

One noticeable change in both these experimental groups (heavy and cracked fraction) was the degree to which the tissues took up the histological stains. In the Masson-Trichrome method (Humason, 1967) one step in the procedures involves the use of picric acid to differentiate the tissues previously stained with hematoxylin. This differentiation should occur within three to five minutes (Seward, pers. comm.). Tissues from larvae treated with the heavy and cracked fractions required about three minutes, whereas tissues from the control and light fraction treated larvae took four and a half minutes to reach the same color intensity. For comparative purposes, all further tissues were differentiated with picric acid for four and a half minutes, as exemplified by controls. Any differences in the staining intensity were noted.

If tissues from larvae treated with 0.4 toxic unit of any of the three diesel oil fractions are compared with tissues from larvae treated with 1.2 toxic units (from LC_{50} determination tests), very distinct differences are seen: Lethal concentrations cause destruction of the

PLATE 7

Section through the tail skin from *Ambystoma tigrinum* larva treated with 0.4 toxic unit of heavy fraction diesel oil.

SC - stratum corneum

SG - stratum granulosum

SS - stratum spinosum

SGr - stratum germinativum

BM - basement membrane

CT - connective tissue

Ch - chromatophores

PLATE 8

Section through the tail skin from *Ambystoma tigrinum* larva treated with 0.4 toxic unit of cracked fraction diesel oil.

SC - stratum corneum

SG - stratum granulosum

SS - stratum spinosum

SGr - stratum germinativum

BM - basement membrane

V - venule with erythrocytes

CT - connective tissue

Ch - chromatophores

L - cells of Leydig


SC
CT
BM
SG
Ch
300X


SC
CT
SG
BM
160X

PLATE 9

Section through the secondary gill filaments from *Ambystoma tigrinum* larva treated with 0.4 toxic unit of cracked fraction diesel oil, under light microscopy.

E - epidermis

De - dermis

Ch - chromatophores

L - cells of Leydig

PLATE 10

Section through the secondary gill filaments of *Ambystoma tigrinum* larva treated with 0.4 toxic unit of heavy fraction diesel oil, under light microscopy.

E - epidermis

De - dermis

BV - blood vessel with erythrocytes

Ch - chromatophores

SM - skeletal muscle fibers

L - cells of Leydig

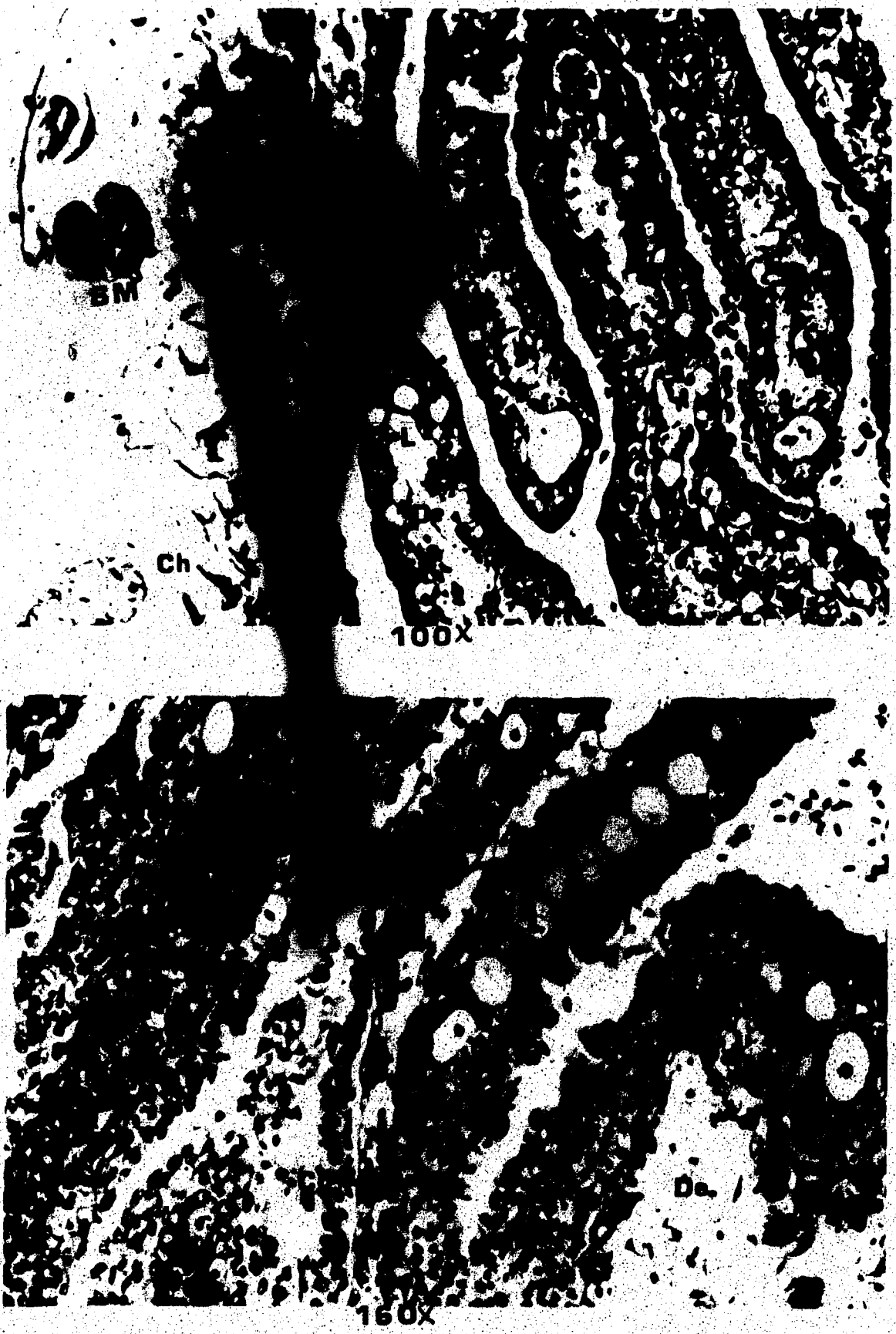

BM
L
Ch
100X
De
160X

PLATE 11

Section through the lung sac from *Ambystoma tigrinum* larva treated with 0.4 toxic unit of heavy fraction diesel oil, under light microscopy.

SE - squamous epithelium

SCE - ciliated low columnar epithelium

Cp - capillary with erythrocytes

Al - alveolus

Ad - alveolar duct

PLATE 12

Section through the lung sac from *Ambystoma tigrinum* larva treated with 0.4 toxic unit of cracked fraction diesel oil, under light microscopy.

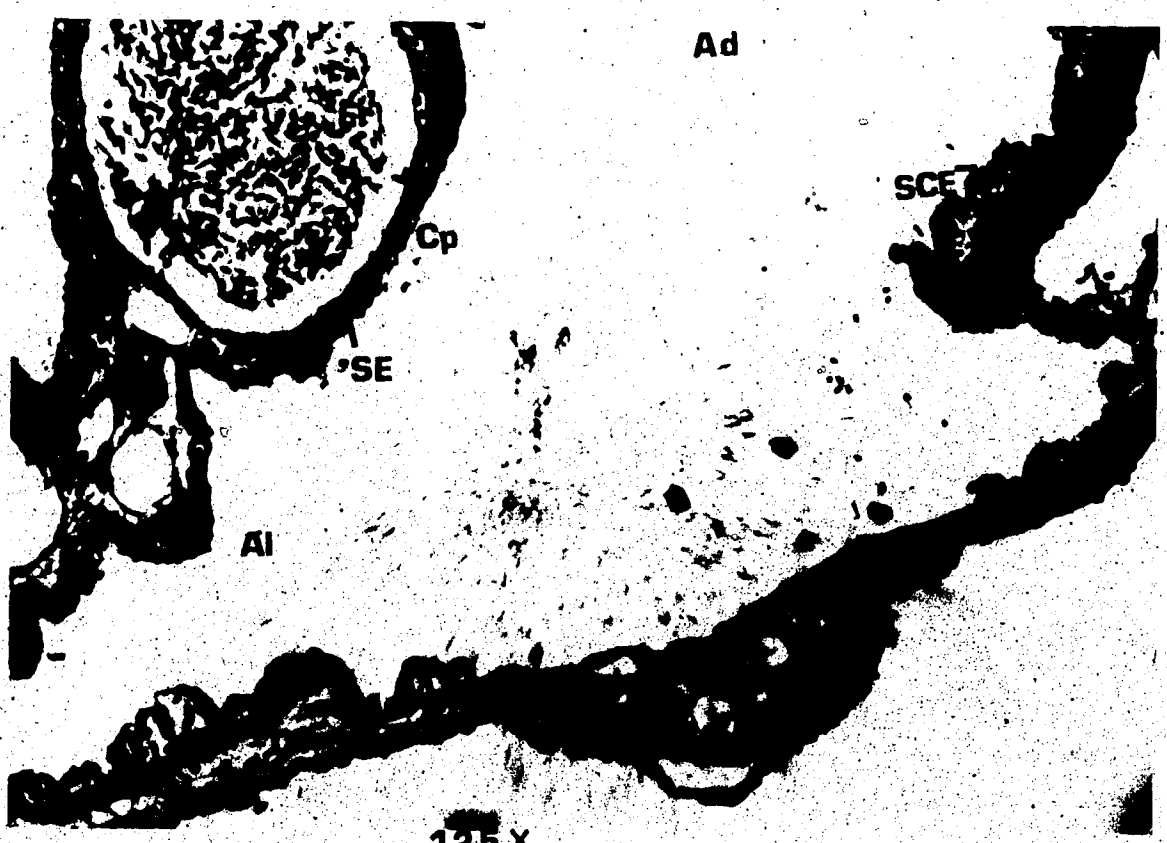
SE - squamous epithelium

SCE - ciliated low columnar epithelium

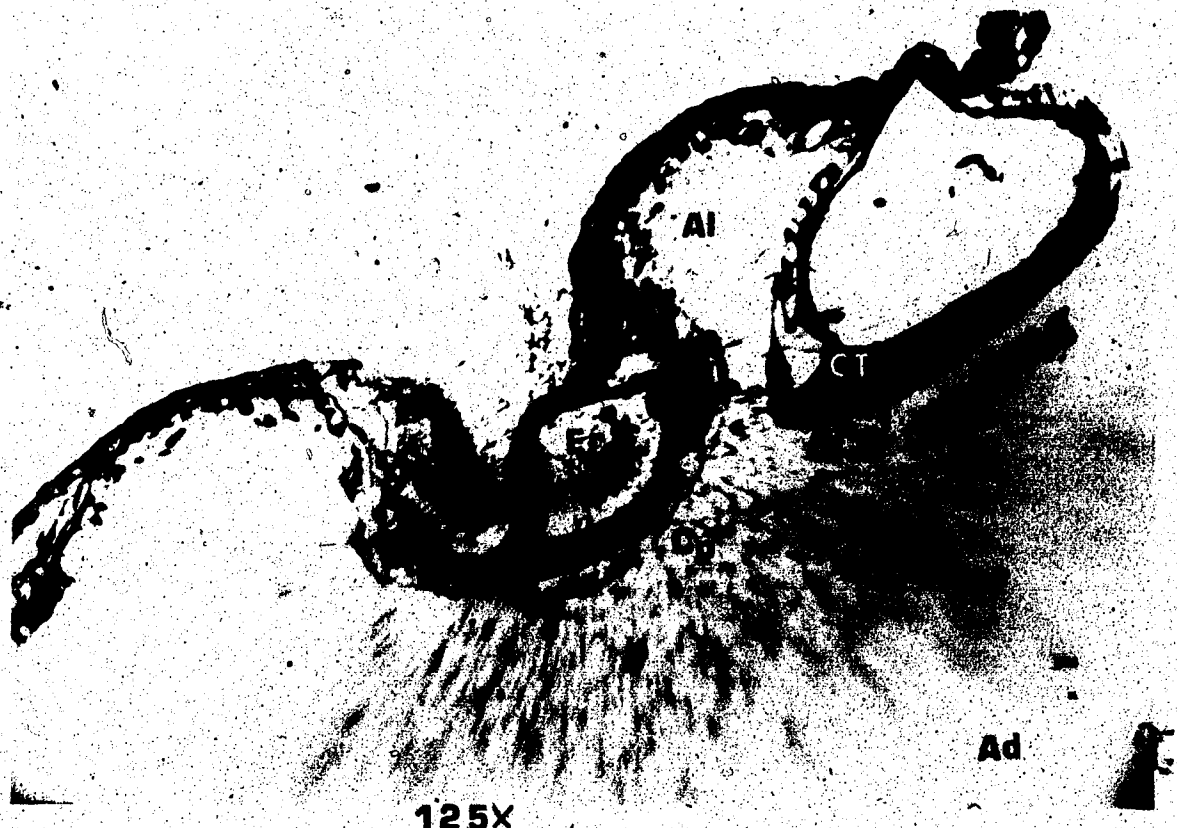
Al - alveolus

Ad - alveolar duct

CT - connective tissue



125 X



125X

intercellular attachments or bridges of the epithelium (Plates 13, 14, and 15) thus causing the sloughing of these cells from the basement membrane. Not only are the cellular layers destroyed, but individual cells show disruption of their cell membranes. Unlike the tissues taken from larvae treated with 0.4 toxic unit of the cracked fraction which showed an increase in the number of Leydig cells in the skin epidermis there was no apparent increase in these cells in the larvae treated with 1.2 toxic units of any of the fractions (Plate 15; see Plates 13 and 14).

In some samples, especially in the gills, the basement membrane appears damaged (see Plate 14), with large areas broken apart exposing the structures within the dermal layer. Although the walls of the gill blood vessels do not appear to have been disrupted, it is quite evident that there has been extensive alteration of many of the erythrocytes within (see Plate 14). Many of these erythrocytes appear to be denucleated with the nuclei floating freely within the [illegible]l. Many of the intact erythrocytes appear to be misshapened.

Damage to the epithelial layers of the integument treated with 1.2 toxic units of any of the diesel oil fractions (see Plate 15) does not appear to be as extensive as in the gill tissues. However, large sheets of damaged cells have pulled away from the basement membrane. In many cases, total disruption of the epithelial cells has occurred.

No apparent differences were detected in lung tissues treated with 1.2 toxic units of any of the diesel oil fractions (Plate 16).

Oxygen Consumption

Oxygen consumption rates as mls O_2/hr/g (dry wt.) were determined

PLATE 13

Section through gill filament from *Ambystoma tigrinum* larva treated with 1.2 toxic units of cracked fraction, under light microscopy.

E - epidermis

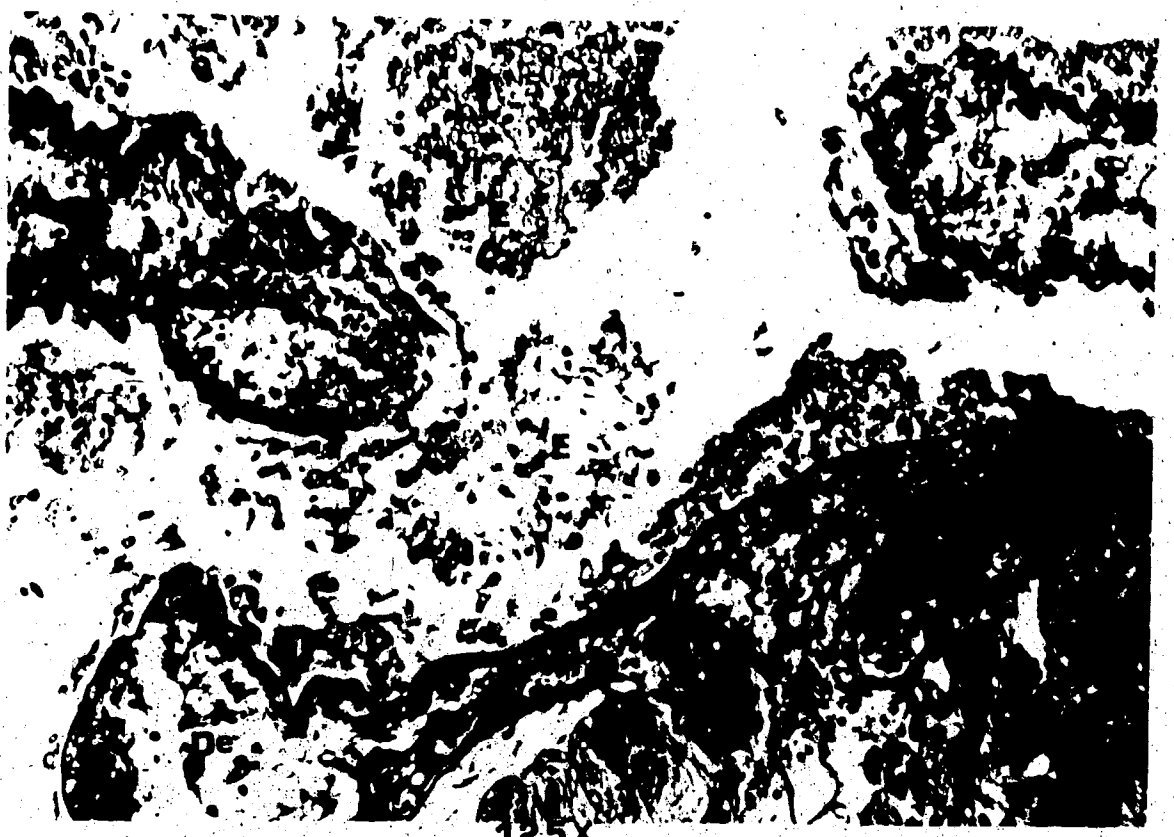
De - dermis

PLATE 14

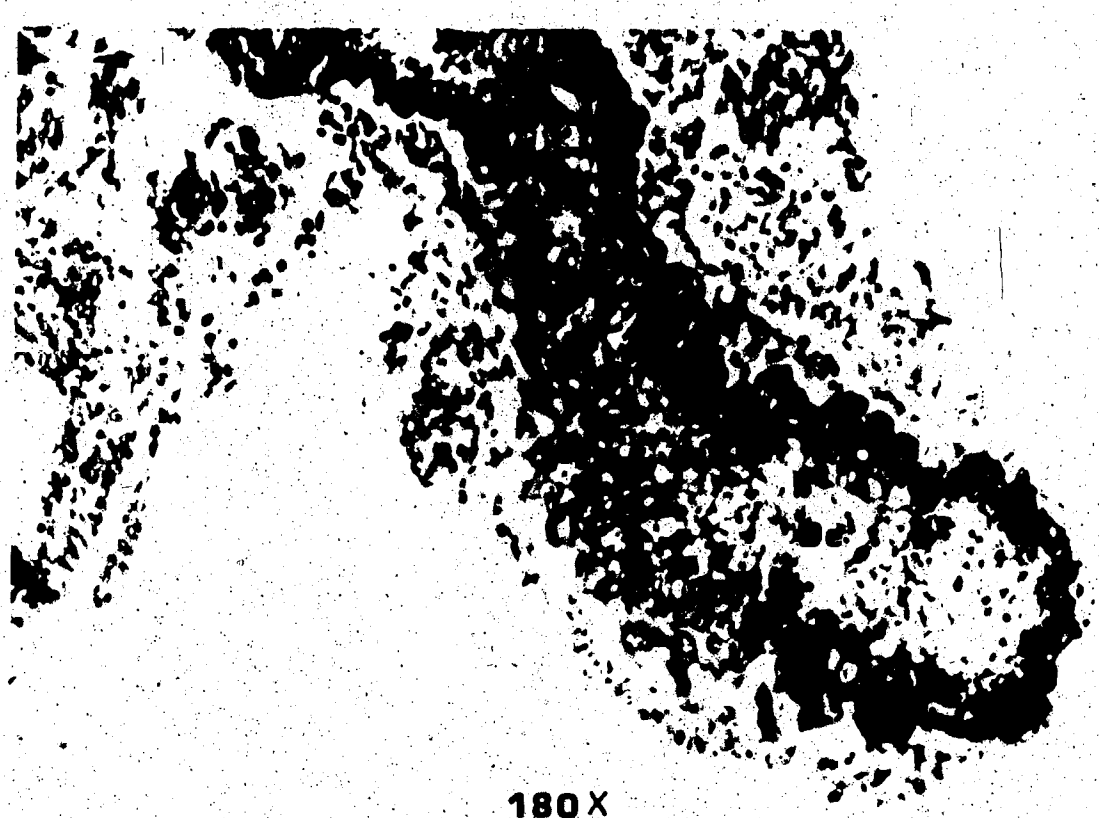
Section through the gill filament from *Ambystoma tigrinum* larva treated with 1.2 toxic units of cracked fraction, under light microscopy.

E - epidermis

De - dermis



125X



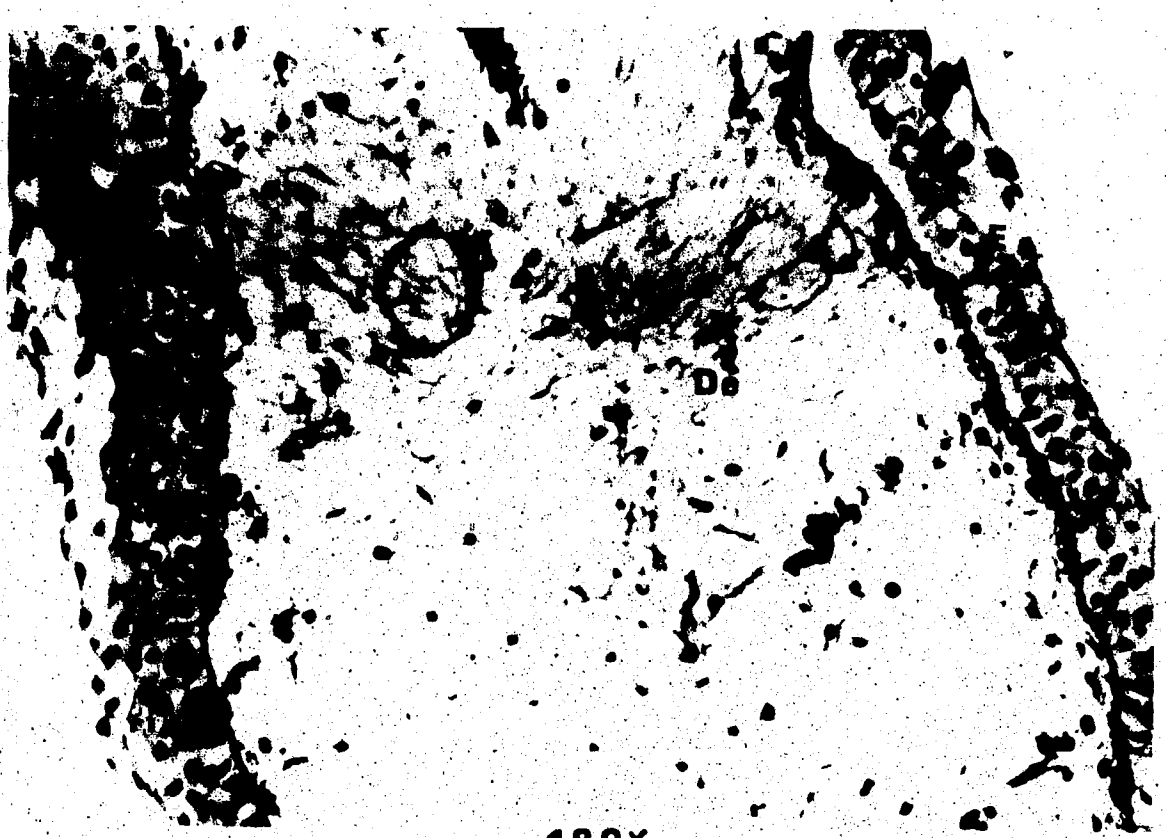
180X

PLATE 15

Section through the tail skin from *Ambystoma tigrinum* larva treated with 1.2 toxic units of cracked fraction, under light microscopy. Note sloughing of epithelial cells.

E - epidermis

De - dermis



180X

PLATE 16

Section through the lung sac from *Ambystoma tigrinum* larva treated with 1.2 toxic units of cracked fraction, under light microscopy.

SE - squamous epithelium

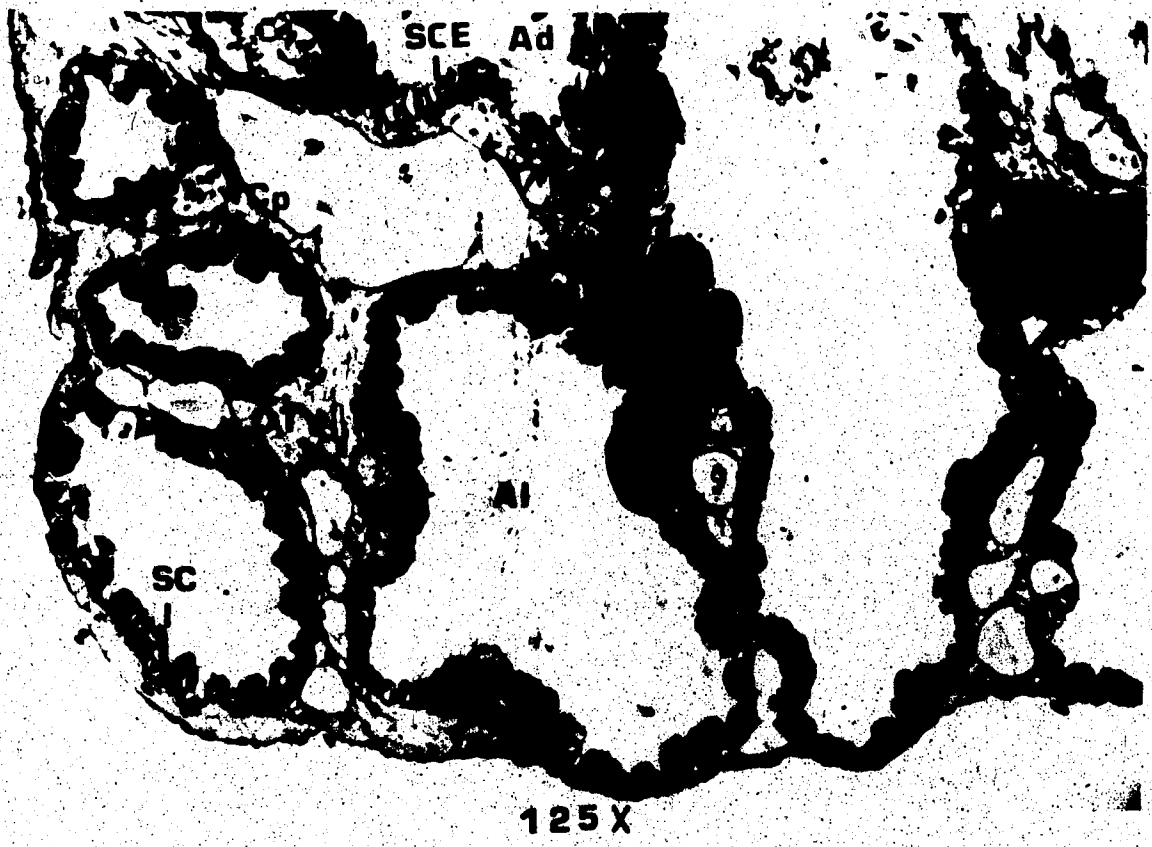
SCE - ciliated low columnar epithelium

Al - alveolus

Ad - alveolar duct

CT - connective tissue

Cp - capillary with erythrocytes


SCE
Ad
Sp
AI
SC
125 X

for pre- and post-oil treated larvae (Appendix III). Paired observations were made for each of the three test groups using 24 larvae per diesel oil treatment. Dry weights for all pre-oil tested animals were estimated from the wet weights of control and light groups (both in oxygen consumption experiments and LC_{50} experiments) by using a wet weight-dry weight regression based on the known corresponding wet and dry weights of these larvae (see Appendix IV) (Fig. 4; taken from Tables 1, 2, 5 and 6 in Appendix IV).

Control larvae, at 7°C, showed little difference in oxygen consumption (mls O_2/hr/g [dry wt.]) between the initial rates and those seven days later. Although individual rates fluctuated slightly, mean rate (mls O_2/hr/g [dry wt.] ± 1 S.E.M.) changes were not significant (Before = 0.1454 ± 0.006; After = 0.1452 ± 0.006; df = 23; t = 0.3691) (Table 4 and Fig. 5).

A similar pattern was observed in oxygen consumption rates determined on pre- and post-oil treated (0.4 toxic unit of the light diesel oil fraction) larvae at 7°C. Again, individual variation between before and after treatment oxygen consumption rates were observed, but mean rate changes were not significant (Before = 0.1361 ± 0.0006; After = 0.1359 ± 0.006; df = 23; t = 0.3677) (see Table 4 and Fig. 5).

Significant decreases in oxygen consumption rates at 7°C were observed for larvae tested after seven days' exposure to 0.4 toxic unit of either the heavy or cracked diesel oil fraction (see Table 4 and Fig. 5). Larvae in the pre-heavy fraction treated groups had mean initial oxygen consumption rates of 0.1414 ± 0.0036. Rates for this group dropped to a mean value of 0.1117 ± 0.0036 after seven days' exposure to the oil sample. This decrease is significant at $p < 0.001$

Figure 4. *Regression line relating the wet weight of control and post-light treated larvae (from oxygen consumption experiments and LC_{50} experiments) to dry weight.*

For the equation $Y = A + BX$,

where

$A = 0.14454$

$B = 6.8790$

$$B = \frac{\Sigma XY - \frac{(\Sigma X)(\Sigma Y)}{N}}{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}$$

$$A = \frac{(\Sigma x)(\Sigma xy) - (\Sigma y)(\Sigma x^2)}{(\Sigma x)^2 - N(\Sigma x^2)}$$

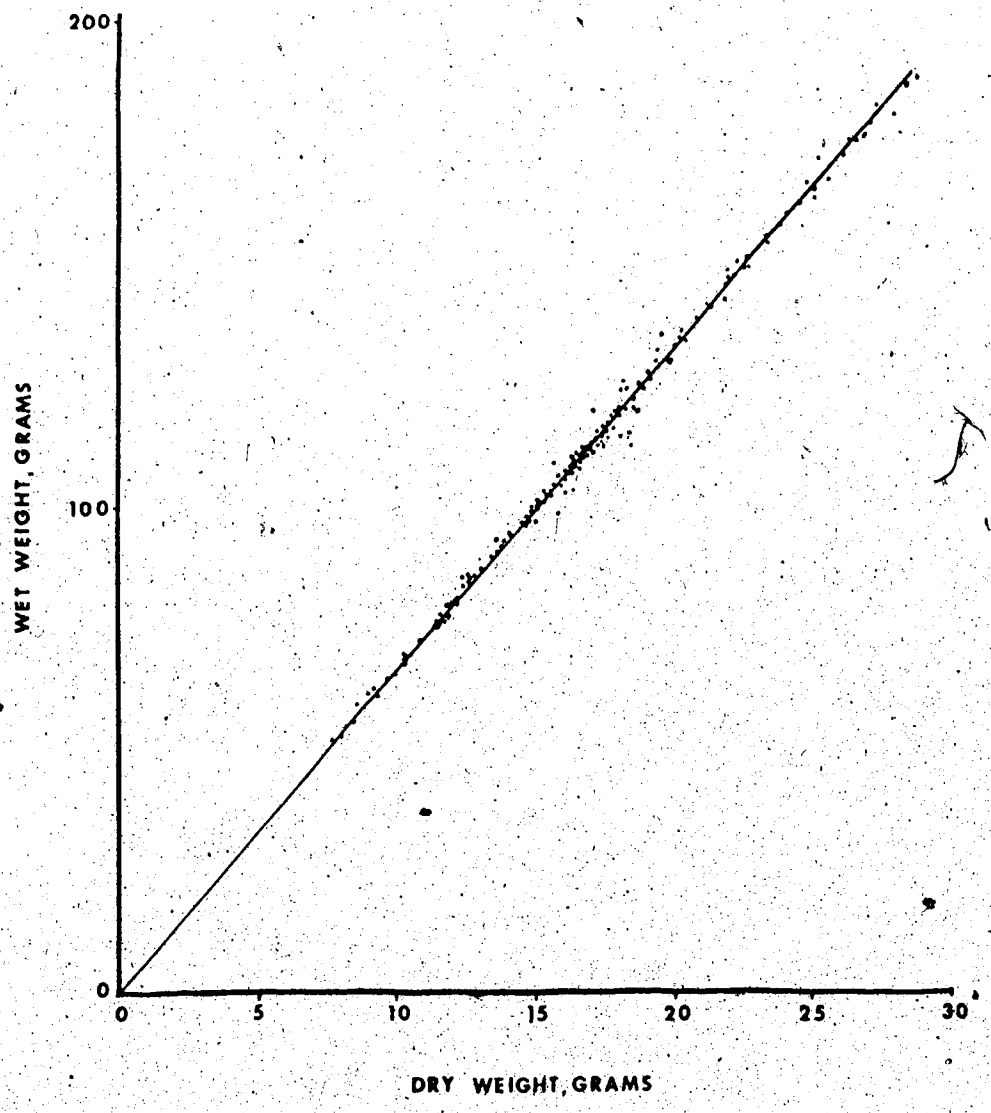

200
100
0
WET WEIGHT, GRAMS
0
5
10
15
20
25
30
DRY WEIGHT, GRAMS

TABLE 4

OXYGEN CONSUMPTION VALUES (± S.E.M.) FOR *AMBYSTOMA TIGRINUM* LARVAE TESTED AT 7°C AND 15°C

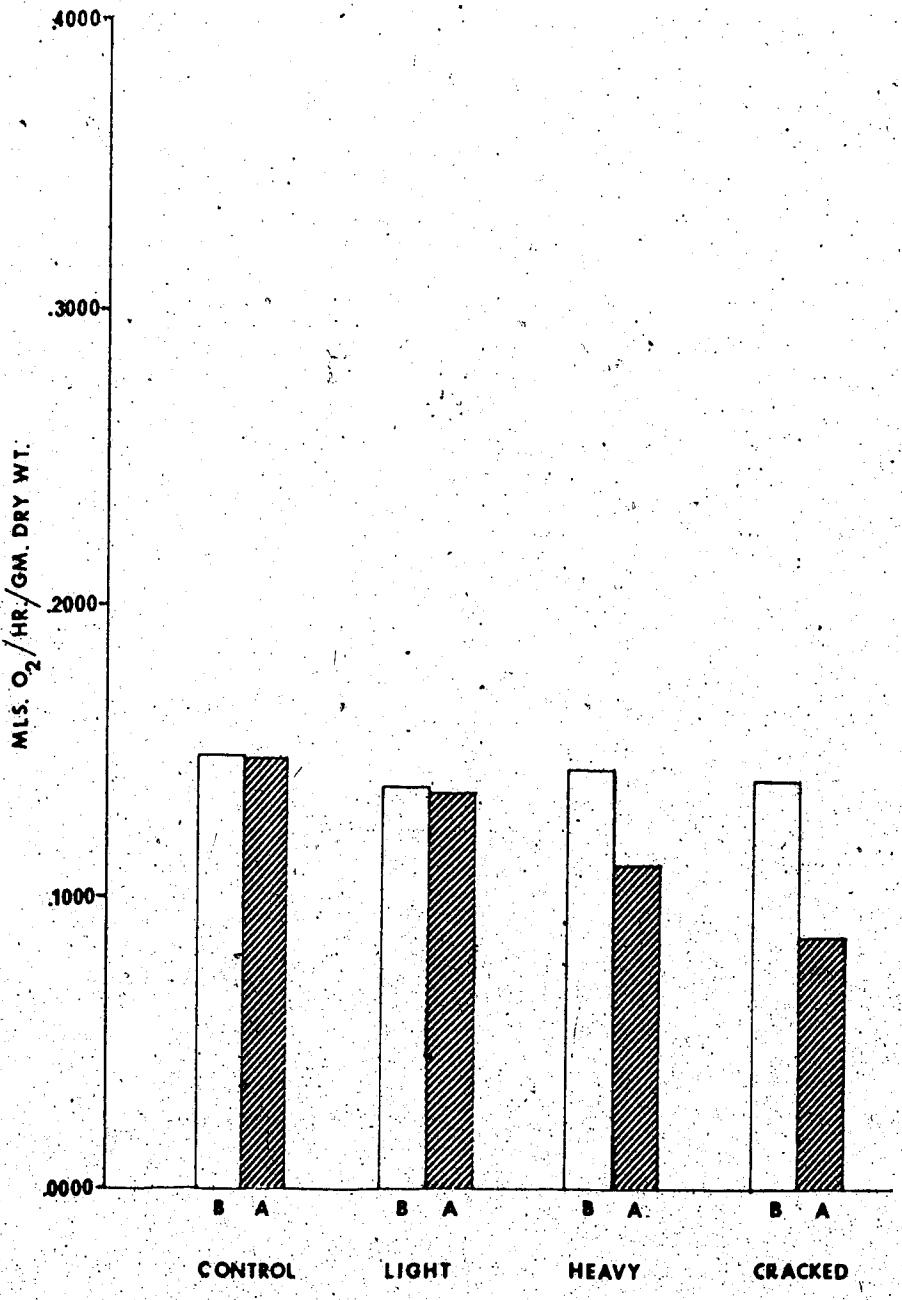
Fraction	Temperature (°C)	Number	mls O_2/hr/g (dry wt.) Pre-oil	mls O_2/hr/g (dry wt.) Post-oil	
Control	7	24	0.1454 ± 0.0006	0.1452 ± 0.0006	NS
Light	7	24	0.1361 ± 0.0006	0.1359 ± 0.0006	NS
Heavy	7	24	0.1414 ± 0.0036	0.1117 ± 0.0036	**
Cracked	7	24	0.1398 ± 0.0018	0.0838 ± 0.0018	**
Control	15	24	0.3022 ± 0.0056	0.2989 ± 0.0056	NS
Light	15	24	0.3054 ± 0.0014	0.3025 ± 0.0014	*
Heavy	15	24	0.2666 ± 0.0022	0.1533 ± 0.0022	**
Cracked	15	24	0.2709 ± 0.0063	0.1465 ± 0.0063	**

* significant difference at $p < 0.01$
** significant difference at $p < 0.001$
NS no significant difference

Figure 5. *Oxygen consumption values for* Ambystoma tigrinum *larvae measured at 7°C, 24 larvae per fraction.*

B - before oil treatment

A - after oil treatment (0.4 toxic unit) for seven days


.4000
.3000
.2000
.1000
.0000
MLS. O_2/HR./GM. DRY WT.
B A
B A
B A
B A
CONTROL
LIGHT
HEAVY
CRACKED

(df = 23, t = 8.2291). A more pronounced decline in oxygen consumption rates was evident from larvae treated with 0.4 toxic unit of the cracked fraction. Mean initial values of 0.1398 ± 0.0018 are significantly different ($p < 0.001$) from final mean oxygen consumption rate of 0.0838 ± 0.0018 ($p < 0.001$; df = 23; t = 30.9009) (see Table 4 and Fig. 5).

Oxygen consumption measurements made at 15°C showed a slightly different overall pattern. Initial values for controls and experimental groups alike showed approximately an 80 per cent increase in rate, compared to those at 7°C. This is to be expected for an 8°C increase in temperature. A mean value for control animals was 0.3021 ± 0.0056 before and 0.2989 ± 0.0056 after seven days. This difference is not significant (df = 23; t = 0.5893) (see Table 4).

Larvae treated with 0.4 toxic unit of the light oil fraction at 15°C for seven days showed a slight decrease in oxygen consumption rates as compared with their pre-oil tested values. The mean pre-oil treated value was 0.3054 ± 0.0014 compared to the post-oil treated value of 0.3025 ± 0.0014; this is significant at $p < 0.01$ (df = 23; t = 2.0833) (Fig. 6, and see Table 4).

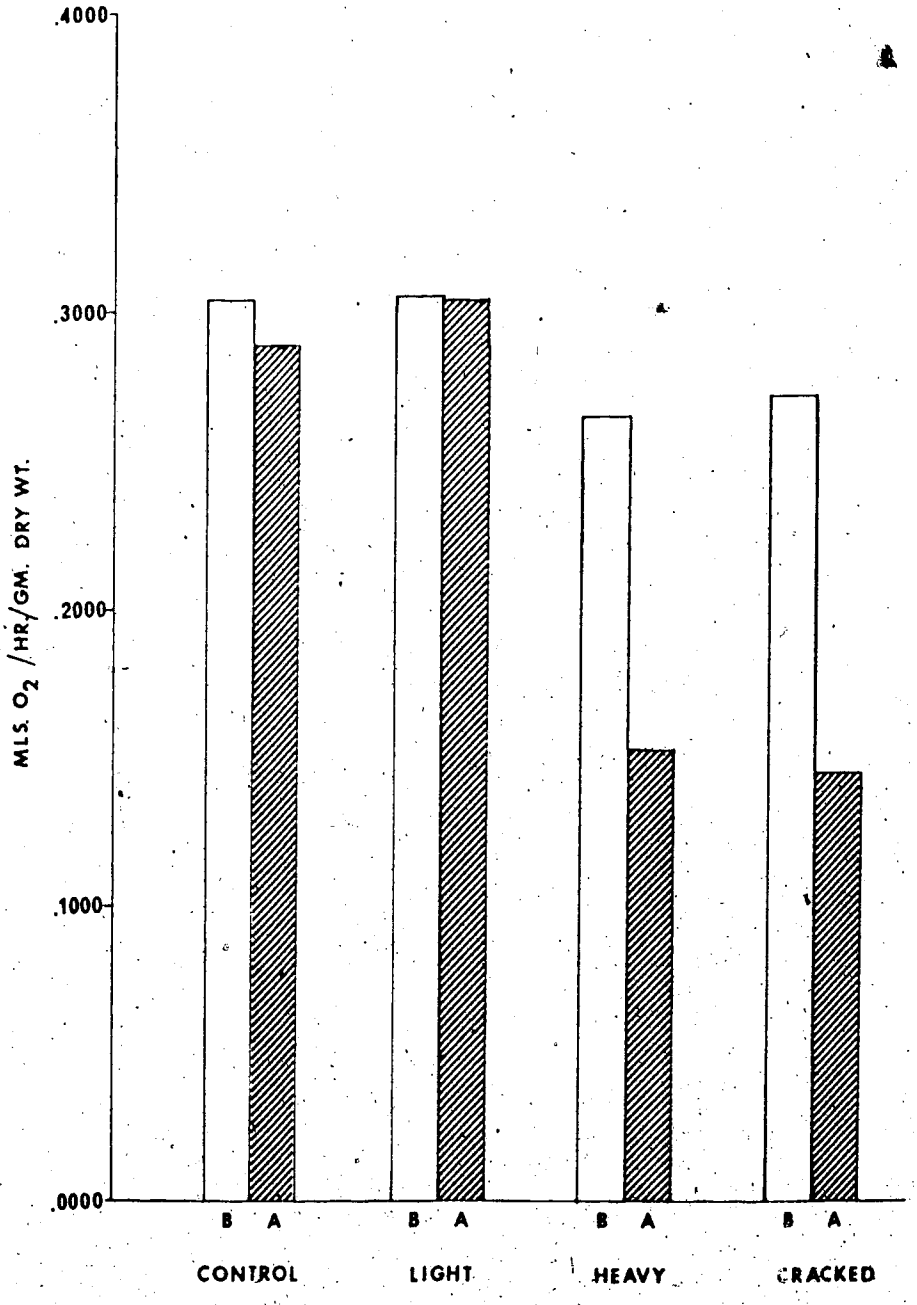
Again, the greatest changes in oxygen consumption rates were in the heavy fraction and cracked fraction treated groups. Mean values for the heavy and cracked pre-oil treated groups were 0.2666 ± 0.0022 and 0.2709 ± 0.0063, respectively. These oxygen consumption rates dropped significantly to a mean final value for the post-heavy treated group of 0.1533 ± 0.0022 and a mean final value for the post-cracked treated group of 0.1465 ± 0.0063 ($p < 0.001$; df = 23; t = 13.3161 for the heavy group; t = 10.2185 for the cracked group (see Table 4 and Fig. 6).

Larvae from the heavy and cracked treated groups showed a wet

Figure 6. *Oxygen consumption values for* Ambystoma tigrinum *larvae measured at 15°C, 24 larvae per fraction.*

A - before oil treatment

B - after oil treatment (0.4 toxic units) for seven days


.4000
.3000
.2000
.1000
.0000
MLS. O_2 /HR./GM. DRY WT.
B A
B A
B A
B A
CONTROL
LIGHT
HEAVY
CRACKED

weight increase after seven days' exposure to 0.4 toxic unit of either oil fraction. This increase occurred regardless of whether 7°C or 15°C was implemented for the oxygen consumption experiments (see Appendix IV).

In experiments carred out at 15°C, mean initial weight for the pre-heavy treated larva was 103.7 ± 0.45 (Table 5). After oil treatment, this weight increased significantly to a value of 107.6 ± 0.45 ($p < 0.001$; $df = 23$; $t = 5.88$). A greater increase in weight occurred in the cracked treated group with a mean initial weight of 100.4 ± 0.45 and a mean final weight of 105.7 ± 0.45 ($p < 0.001$; $df = 23$; $t = 12.28$) (Table 5).

At 7°C the weight increases in these two experimental groups were also evident. A mean weight in the pre-heavy treated group was 114.1 ± 0.70 as compared to a mean final weight of 118.2 ± 0.70 ($p < 0.001$; $df = 23$; $t = 8.80$) (see Table 5). Similarly for the cracked treated group there was a significant increase ($p < 0.001$; $df = 23$; $t = 12.01$) from the initial mean weight of 116.4 ± 0.46 to the mean final weight value of 122.1 ± 0.46 (see Table 5).

As previously mentioned, throughout the seven day test period the larvae were not fed. If the estimated dry weight values for the pre-heavy and pre-cracked treated groups at 7°C and 15°C are compared to the actual dry weight values determined on these animals, the values remain almost constant. This would lead one to conclude that the weight increase in these two experimental groups is due to an increase in body water. Calculating these weight increases as a per cent water increase relative to the initial wet body weight of each larva tested, the average per cent increase is 4.9 at 7°C and 5.3 at 15°C. These weight differences were

TABLE 5

MEAN WET WEIGHTS (grams) BEFORE AND AFTER SEVEN DAYS' EXPOSURE TO DIESEL OIL FRACTIONS

Fraction	Temperature (°C)	Number	Initial Wet Wt.	Final Wet Wt.	% Increase H_2O
Control	7	24	115.9 ± 0.18	116.0 ± 0.18	0.0
Light	7	24	116.8 ± 0.22	116.6 ± 0.22	0.0
Heavy	7	24	114.1 ± 0.70	118.2 ± 0.70	4.6*
Cracked	7	24	116.4 ± 0.46	122.1 ± 0.46	5.2*
Control	15	24	107.2 ± 0.33	107.2 ± 0.33	0.0
Light	15	24	103.8 ± 0.56	103.9 ± 0.56	0.0
Heavy	15	24	103.7 ± 0.45	107.6 ± 0.45	4.6*
Cracked	15	24	100.4 ± 0.45	105.7 ± 0.45	6.1*

*Significant difference at $p < 0.001$

not evident in control or light-treated groups (see Table 5).

Electron Microscopy

Electron microscopic studies of the integument from the tail did not reveal any conclusive evidence regarding modifications of cell membrane structure, as hypothesized by van Overbeek and Blondeau (1954), after exposure to 0.4 toxic units of any of the diesel oil fractions. Examination of several sections of cell membranes from heavy fraction treated larvae suggested that there might be a slight "opening up" (van Overbeek and Blondeau, 1954) of the unit membrane structure by the hydrocarbons, but this could not be fully substantiated.

Although alterations in the cell membrane structure itself were not observed, there was considerable cellular modification between cells within the experimental samples which was not evident in light-microscopy studies. In control skin samples there are numerous regions of membrane fusion, or occluding zonules (Farquhar and Palade, 1965) (Plate 17), which are regularly seen between the adjoining cells in the stratum corneum. Attaching the stratum corneum to the stratum granulosum these elements are known as "composite desmosomes" or zonules occludentes (Farquhar and Palade, 1965) (Plate 18). Within the stratum granulosum these elements are seen as complete desmosomes or macula adherens (Farquhar and Palade, 1965) (see Plate 17). Desmosomes are a bipartite structure consisting of local differentiations of the opposing cell membrane (Bloom and Fawcett, 1970). The desmosomes lie midway on the protruding structures known as intercellular bridges. The spaces between the intercellular bridges are termed interfacial canals (Farquhar and Palade, 1965) (Plate 19).

PLATE 17

Electron micrograph of a one millimeter section of tail skin from control sample of *Ambystoma tigrinum* larva.

SC - stratum corneum

SG - stratum granulosum

N - nucleus

D - complete desmosome

IB - intercellular bridge

IS - intercellular space

PLATE 18

Electron micrograph of a one millimeter section of tail skin from light-fraction treated larva (0.4 toxic unit) of *Ambystoma tigrinum*.

SC - stratum corneum

SG - stratum granulosum

N - nucleus

IB - intercellular bridge

IS - intercellular space

CD - composite desmosome


N
N
18,000 X


N
B
B
N
18,000X

PLATE 19

Electron micrograph of a one millimeter section of tail skin from control sample of *Ambystoma tigrinum* larva showing components of a typical desmosome.

T - tonofilament

D - desmosome

IB - intercellular bridge

IS - intercellular space

IF - interfacial canals

S - secretory granule


IF
IS
S
20,000 ×

PLATE 20

Electron micrograph of a one millimeter section of tail skin from heavy-fraction treated larva (0.4 toxic unit) of *Ambystoma tigrinum*.

SC - stratum corneum

SG - stratum granulosum

N - nucleus

IB - intercellular bridge

IS - intercellular space

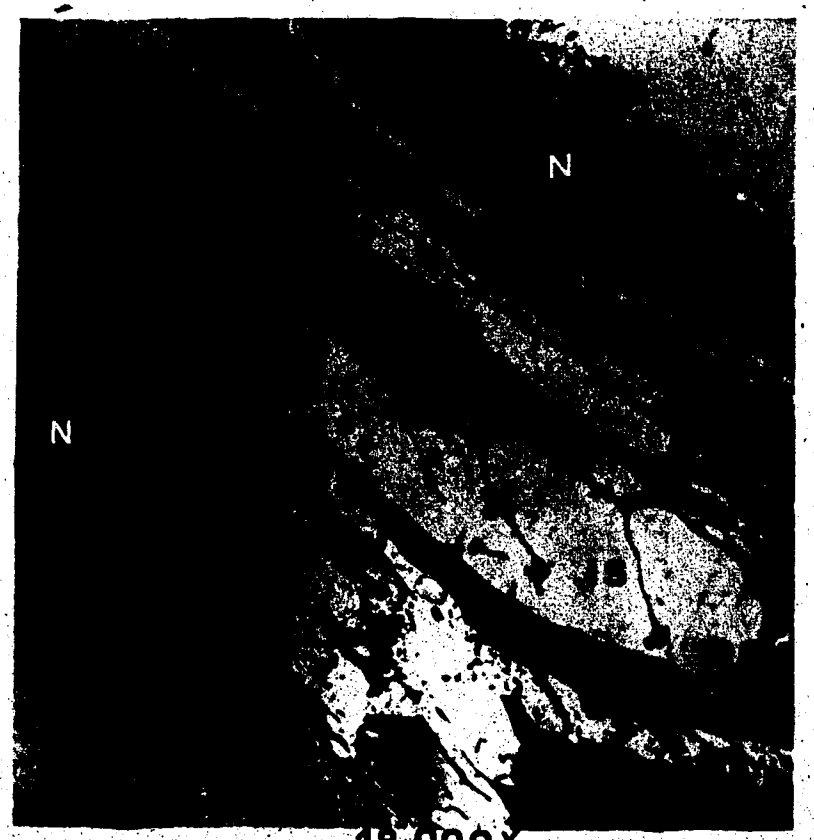
PLATE 21

Electron micrograph of a one millimeter section of tail skin from cracked-fraction treated larva (0.4 toxic unit) of *Ambystoma tigrinum*.

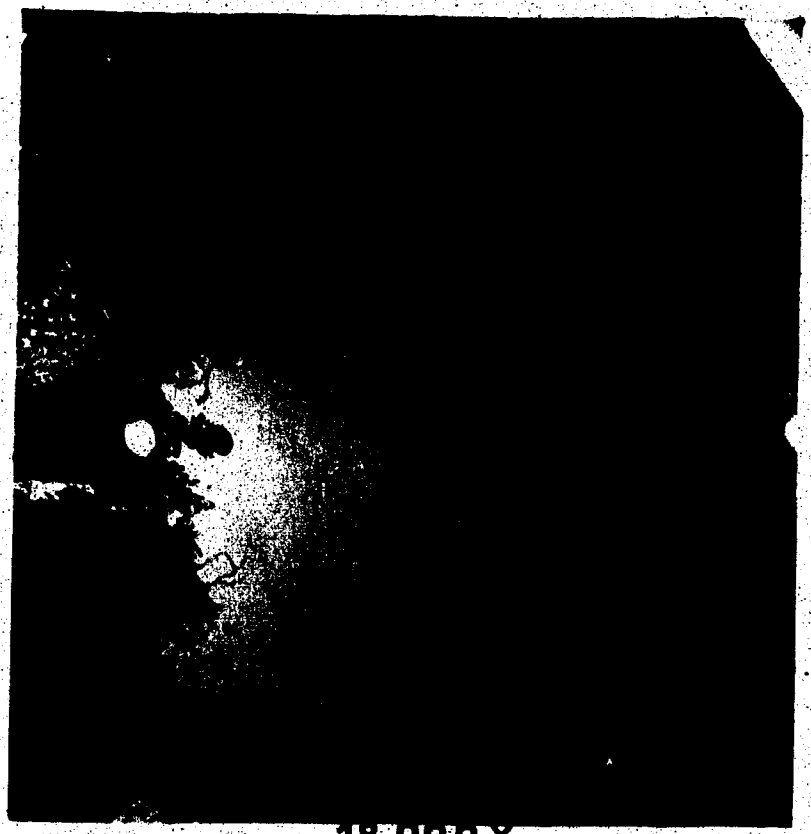
SC - straum corneum

SG - stratum granulosum

IS - intercellular space



18,000X



18,000X

When compared with controls, the skin from the larvae treated with 0.4 toxic unit of the light fraction appeared to have fewer "composite" desmosomes between the cells of the stratum corneum and stratum granulosum (Plate 18). There are also fewer complete desmosomes or macula adherens in the stratum granulosum (see Plate 18). The intercellular bridges, when present, are considerably longer and thinner than those of controls.

The degree of destruction or alteration of the composite and complete desmosomes is magnified in the skin of the heavy and cracked treated larvae. In the heavy fraction treated larvae, the intercellular bridges are more elongated (Plate 20), whereas in the cracked fraction treated larvae, they are almost totally absent (Plate 21). With a decrease in the occurrence of these cell junctions, the intercellular spaces of the epidermal cells are increased substantially. In control samples, these intercellular spaces form a largely continuous network which is closed to the external environment but open to the dermal interstitium (Farquhar and Palade, 1965) (see Plate 17).

In most skin cells examined from the oil treated larvae, mitochondria were found that appeared to be damaged. Mitochondria in the light and heavy fraction treated tissues had gaps inside the mitochondria where the folded cristae lie (Plates 23 and 24). At the same level of magnification, in control samples, these gaps were not apparent (Plate 22). In fact, the cristae were not clearly distinct at this magnification in most samples studied. Mitochondria in skin cells of cracked fraction treated larvae showed very distinct gaps close to the unit membrane surface (Plate 25). The unit membrane of the mitochondria, however, appeared to be unaltered.

PLATE 22

Electron micrograph of a one millimeter section of tail skin from control sample of *Ambystoma tigrinum* larva showing mitochrondria.

SC - stratum corneum

SG - stratum granulosum

M - mitochondria

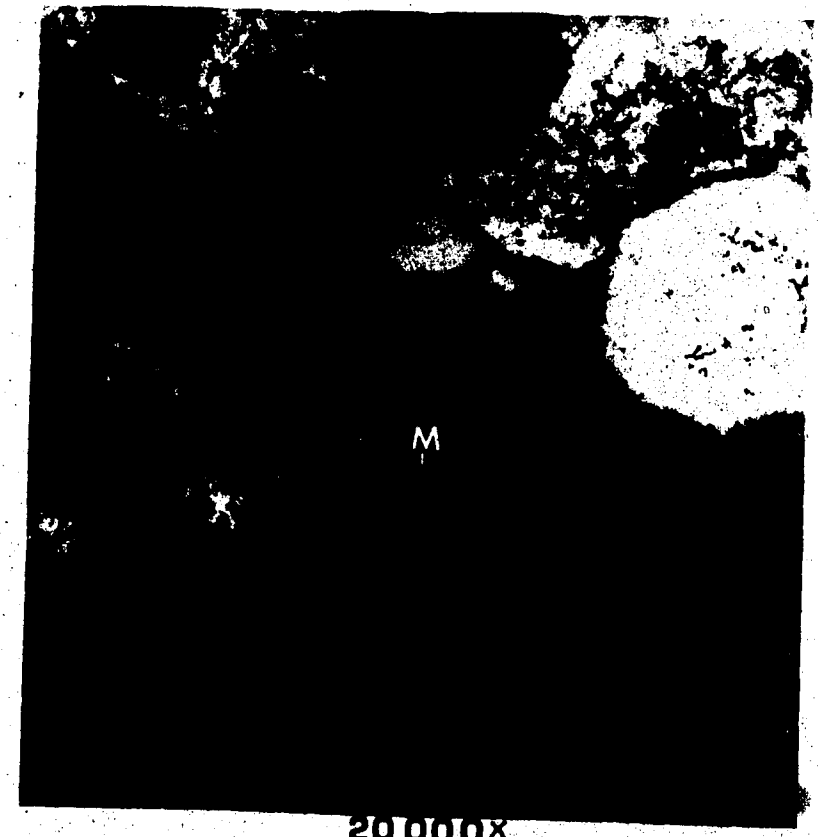
PLATE 23

Electron micrograph of a one millimeter section of tail skin from light-fraction treated *Ambystoma tigrinum* larva showing mitochondria.

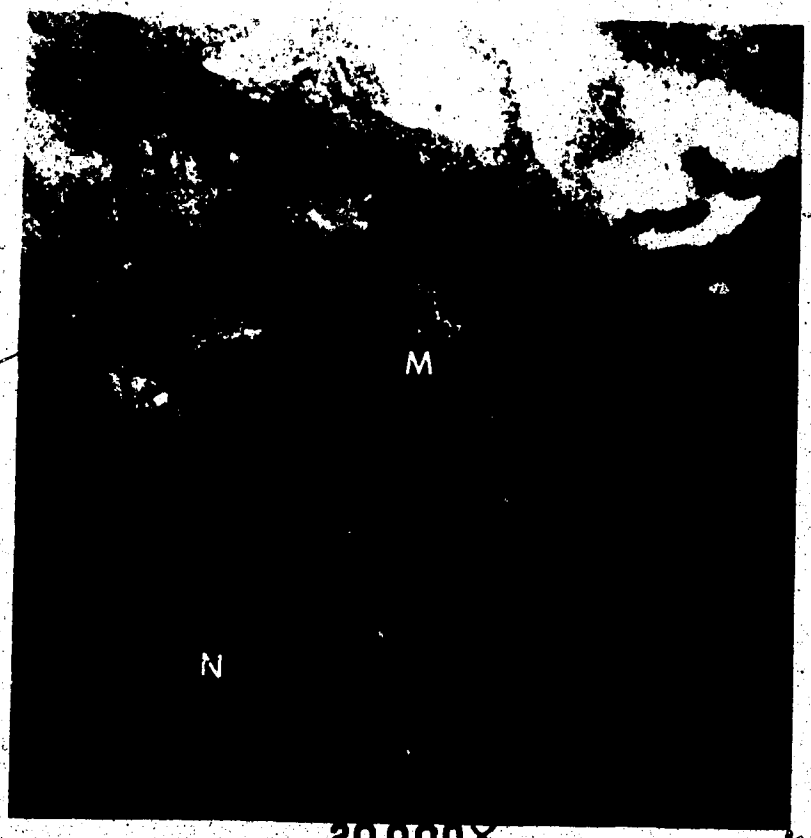
SG - stratum granulosum

M - mitochondria

N - nucleus



20,000X



20,000X

PLATE 24

Electron micrograph of a one millimeter section of tail skin from heavy-fraction treated *Ambystoma tigrinum* larva showing small gaps within the cristae of the mitochondria.

IS - intercellular space

IB - intercellular bridge

M - mitochondria

PLATE 25

Electron micrograph of a one millimeter section of tail skin from cracked-fraction treated *Ambystoma tigrinum* larva showing large gaps within the cristae of the mitochondria.

SG - stratum granulosum

M - mitochondria

20,000X

24,000X

Oil Fraction Analysis

Quantitative analysis of any changes in diesel oil fractions by gas chromatography proved unsuccessful. The sublethal concentrations of each fraction analyzed were too small for any changes in the hydrocarbon constituents to be clearly determined by the gas chromatograph. Also, the fact that each oil fraction was recovered from a water-oil mixture by repeated washings with n-heptane provided a large margin of error in obtaining quantitative results.

Gas chromatograms of each diesel oil fraction are thus included merely as a means of identifying the major hydrocarbon groups of each fraction used throughout this research project (see Figs. 7)

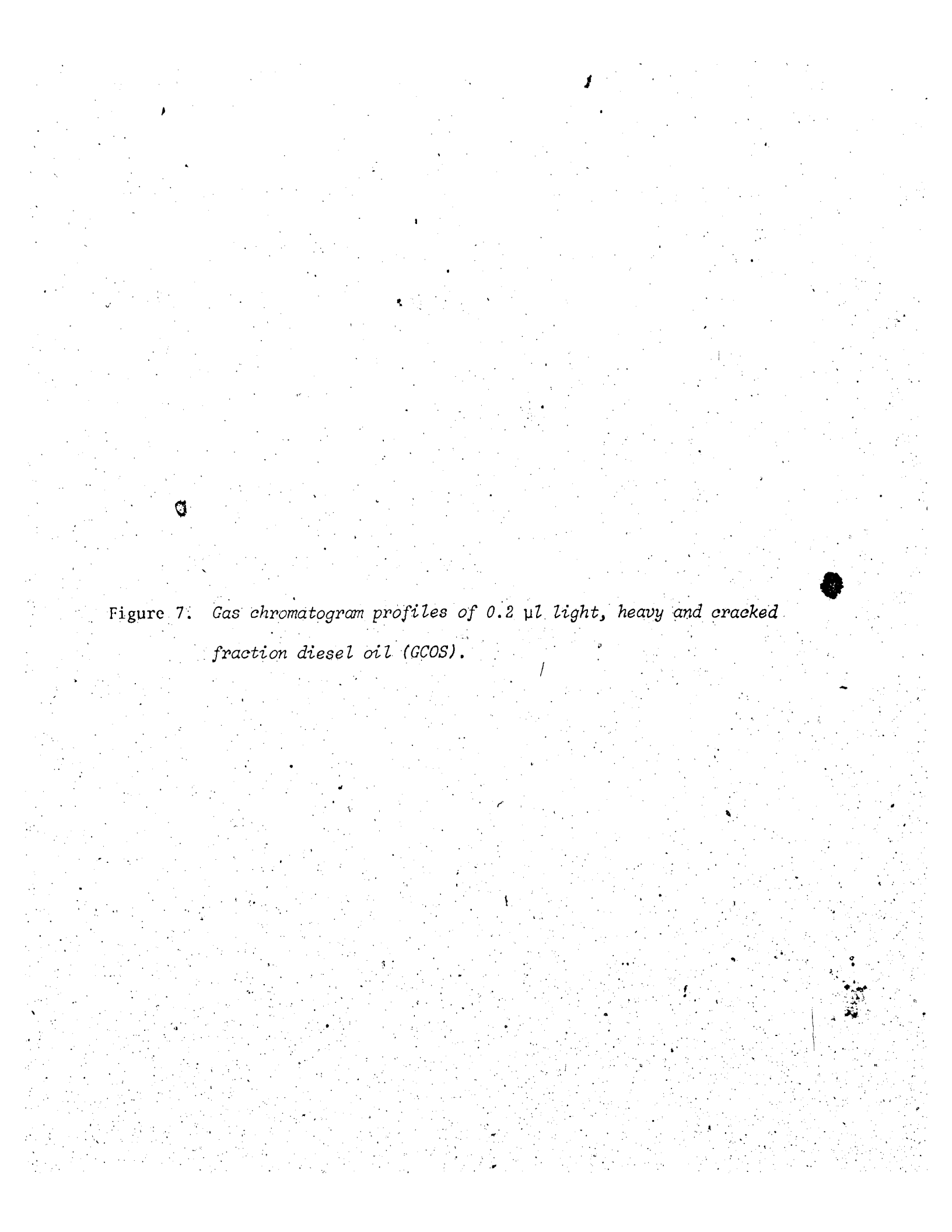
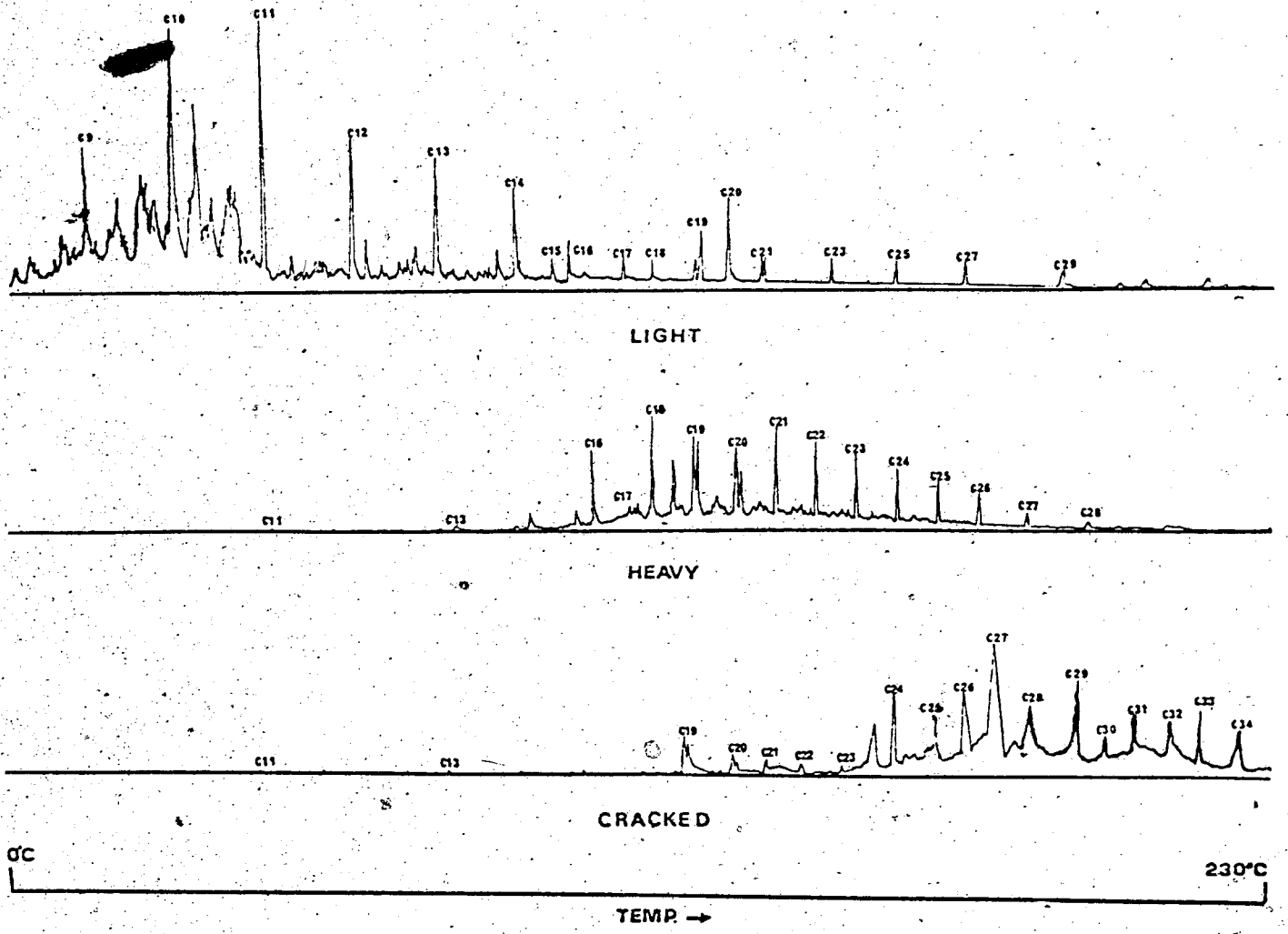


Figure 7. *Gas chromatogram profiles of 0.2 µl light, heavy and cracked fraction diesel oil (GCOS).*


LIGHT
HEAVY
CRACKED
0°C
230°C
TEMP. →

DISCUSSION

Results from both histological and oxygen consumption experiments indicate that the sublethal concentrations of two of the three diesel oil fractions, at 0.4 toxic unit, may be detrimental to the *Ambystoma tigrinum* larvae. Oxygen consumption rates were significantly lowered after seven days' exposure to both the heavy and cracked fractions at 7°C and 15°C. Exposure to these fractions also caused an apparent increase in permeability to water, with increases of up to 6% in body weight.

Those larvae treated with the cracked fraction, and to some degree the heavy fraction, showed an increase in the number of Leydig cells within the epidermal layer of the integument and gills. At the ultrastructural level, the frequency of intercellular bridges between the cells of the stratum corneum and the stratum granulosum were reduced in all groups, especially in the heavy and cracked groups. Some disruption of the mitochondrial cristae was also evident.

In all experiments, a basic pattern was established regarding the effects of the sublethal concentrations of the diesel oil fractions. The results from all tests using 0.4 toxic unit of the light fraction varied little from controls, while those utilizing 0.4 toxic unit of the heavy and cracked fractions showed significant changes from control groups.

Three possible explanations for the toxic effects exerted by the diesel fractions are implicated from the calculated LC_{50}'s for each component (aromatics, olefins, and saturates) (Table 3): 1) the best correlation of toxicity exists in the olefin components; 2) the aromatics of the light fraction appear to have a greater toxic effect

than the aromatics of the heavy and cracked fractions; 3) there is the possibility of synergistic effects between the aromatic and saturate components, especially in the light fraction. However, to discuss these results in this manner, one must first assume that the three major components of the three diesel oil fractions are, in fact, the same in each of the fractions and, second, that the olefins are more toxic than either of the other two components.

To suggest that the olefins are the most responsible for the toxic effects of these diesel oil fractions on the larvae seems inconclusive in light of the known toxic effects of pure hydrocarbons on plant and animal cells. There is agreement among many authors that toxicity increases along the series paraffins (saturates), olefins (unsaturates), cycloparaffins (saturates), and aromatics (unsaturates) (Havis, 1950; van Overbeek and Blondeau, 1954; Baker, 1970). Within each series, the smaller molecules were found to be more toxic than the larger molecules. Van Overbeek and Blondeau (1954), and later Baker (1970), have shown that although 12-carbon olefins were slightly toxic to several different plants, 12-carbon aromatics at the same concentration were even more so. Dodecane, a 12-carbon paraffin, was found to be almost nontoxic.

In view of the difference in the hydrocarbon distribution of these fractions, as shown by the gas chromatograms (Fig. 7), one cannot assume that each of the aromatic, olefin, or saturate components of the three diesel oil fractions are the same. The major carbon range of the light fraction is C_9 to C_{20}, while in the heavy the range is C_{16} to C_{26}, and in the cracked, C_{24} to C_{34}. The boiling point range of these hydrocarbons further substantiates the fact that the components must be different.

Thus, it would seem that other factors must be taken into account before making predictions regarding the cause of the toxicity exerted by these fractions. The solubility in water has been shown to decrease with increasing carbon numbers (Scarrat, 1974). Baker (1970) has found that the toxicity of a hydrocarbon group is inversely proportional to its solubility in water. Low boiling point, short chain aromatics are very soluble and are the most toxic, causing acute effects in most animals (Scarrat, 1974). Low boiling point, saturated hydrocarbons are less soluble and show little toxicity, whereas low boiling point olefins occupy an intermediary position between the aromatic and saturate components (Scarrat, 1974). The larger polycyclic aromatic molecules, while being relatively less soluble than the other components, have been found to cause chronic effects in some animals and may be carcinogenic (Nelson-Smith, 1972; Scarrat, 1974).

The possibility of synergistic action occurring between the aromatic and saturate components, especially in the light fraction, cannot be ruled out. However, the toxicity of these three diesel oil fractions appears to more closely correspond to the relative abundance of aromatic compounds. The heavy and cracked fractions which contained over 50% aromatics consistently showed greater toxic effects (lower LC_{50}'s and oxygen consumption rates as well as some histological alterations) on the *Ambystoma tigrinum* larvae than the light fraction which contained only 14.6% aromatics.

Goldacre (1968) has found that low concentrations of aromatic hydrocarbons increased surface irritability in *Amoeba dubia*. As the concentration of the aromatics was increased, this irritability was replaced by anesthesia, swelling of the plasma membrane, contraction of the granular

cytoplasm, and finally the bursting of the cell, resulting in death. Goldacre (1968) proposed that the swelling of the plasma membrane, and thus the increase in permeability, was due to the solubility of the hydrocarbons in the lipid phase of the unit membrane.

Van Overbeek and Blondeau (1954) have found that aromatic compounds increase the uptake of water in plant cells. They have indicated that this increase may be due to an increase in permeability of the plasma membrane, facilitated by the "opening up" of the membrane by the hydrocarbons. The hydrocarbons would solubilize the plasma membrane, thus displacing the lipid molecules. Aromatic compounds, or any foreign substance which alters the dynamic structure of the membrane, will cause "leaks" in it (van Overbeek and Blondeau, 1954). Van Overbeek and Blondeau (1954) have shown that slight structural damage may increase permeability under certain conditions which may, in turn, result in water uptake. Morrow (1974) using coho and sockeye salmon, has also shown that aromatic compounds may increase cellular permeability.

Results obtained from this study support this increased permeability hypothesis. The larvae treated with the heavy and cracked fractions, which are composed of over 50 per cent aromatics, showed what appeared to be a substantial increase in the uptake of water.

The mechanism by which an increased permeability is mediated is questionable. Electron microscope studies of the tail skin did not substantiate earlier theories regarding alteration of the internal plasma membrane structure thereby increasing cellular permeability. At 20,000X magnification there was no clear evidence of the hydrocarbons penetrating the protein-lipophilic-protein membrane structure resulting

in an expansion of the membrane, as proposed by van Overbeek and Blondeau (1954).

The failure to obtain conclusive evidence regarding alteration of the membrane structure may be due in part to the fixation technique with OsO_4. Sjostrand (1959) has reported varying thicknesses of the unit membrane, from 30—105 Å. Robertson (1961) has concluded that these variations in thickness are probably due to OsO_4 fixation failure which could prevent the resolution of the unit membrane structure in various areas of the sections. Farquhar and Palade (1965) have found inadequate contrast in the outer leaflets of the cell membrane after OsO_4 fixation alone. They have reported that complete fixation with OsO_4 followed by staining with $KMnO_4$ or phosphotungstic acid is necessary during dehydration procedures to enhance the contrast of the membrane structure (Farquhar and Palade, 1963a,b; 1964). Thus, with some modification in the fixation-staining procedures, and a much larger sample size, it may be possible to show alterations in the plasma membrane of the *Ambystoma tigrinum* larval cells.

While the cell membrane expansion hypothesis cannot be ruled out, it cannot be interpreted as the only means by which an increase in water uptake is facilitated. Frog integument has been widely investigated as a biological membrane, especially in the studies of transport functions of epithelia (Ussing, 1960; MacRobbie and Ussing, 1961; Dainty and House, 1966; Machin, 1969; Biber and Curran, 1970). It is known to exhibit a unilateral osmotic response (MacRobbie and Ussing, 1961). In the light of these physiological findings, Farquhar and Palade (1965), using electron microscopy along with physiological studies of the frog integument,

have suggested that the duffusion of water, ions, and small water soluble molecules is impeded along the intercellular spaces of the epidermis by the zonulae occludentes (composite desmosomes). It is then facilitated from cell to cell within the epidermis by zonulae and maculae occludentes (composite and complete desmosomes). Any disruption of the zonulae occludentes would then allow water and ions to diffuse unimpeded into the intercellular spaces of the amphibian integument. Results from electron microscopy in this study indicated a disruption and decreased frequency of the zonulae occludentes between the stratum corneum and stratum granulosum, thereby opening up a pathway for the diffusion of water into the intercellular spaces of the larval epidermis. An increase in permeability via this pathway could result in an increase in total body water. This increase in total body water could be reflected in increases in body weight, as shown by the present study.

The literature on the effects of oils on respiration is controversial. Beevers (1953) has shown that dinitrophenol increases oxygen uptake by uncoupling electron transport from phosphorylation; electron transport is increased while the energy released is lost as heat. Nozzolillo and Helson (1959) have also reported an increase in oxygen uptake in plants after exposure to various hydrocarbons. Slight increases in cellular respiration in sunfish were found after exposure to sublethal concentrations of crude oils (Cairns and Scheier, 1962), while Hood *et al.* (1960) have shown a decrease in respiration in brine shrimp held under similar experimental conditions. Several authors have reported decreases in shell movements associated with diminished pumping action in oysters after exposure to water soluble oil constituents

(Galtsoff *et al.*, 1935; Chipman and Galtsoff, 1949; Lunz, 1950).

These conflicting results may be due to several reasons. Baker (1970) has reported the effects of different oils on cellular respiration in plants. She has shown that oils high in aromatics reduce or stop cellular respiration causing widespread cellular injury, while nonherbicidal oils generally increase cellular respiration rates. Ottway (1971) and Nelson-Smith (1972) have stated that differences in the overall effects of oil compounds are obtained not only because of their chemical nature but also due to the way in which the "pollutant" is applied, the life stage of the organism, and the season.

The effects of oils on oxygen uptake have been thought to be indirect with the oil film decreasing gas exchange at the water-oil surface, thereby decreasing the oxygen tension of the water. Roberts (1926), however, has reported that gaseous exchange at the water-oil surface was 75 per cent that of the controls. Other authors have more recently substantiated these results showing that gaseous exchange is not severely diminished by oil at the air-water surface (Boswell, 1950; Brown and Reid, 1951).

Exposure to diesel oil fractions caused a lowering of oxygen consumption rates in the groups reported in this study. These decreases are likely due to direct effects on gaseous exchange. Oil products, especially crude oils, have been known to act as "mechanical blankets" covering the gills and skin of aquatic organisms, thereby blocking gas exchange (Nelson-Smith, 1972). However, it has been found that diesel fuel oils do not cling to aquatic organisms unless the oils are emulsified (Rushton and Jee, 1923; Tarzwell, 1970). Mironov (1970) coated

trout gills with droplets of fuel oils and found an inhibition of gas exchange. Examination of gill and skin tissues taken directly from the larvae treated with the diesel oil fractions showed no visible signs of coating under light microscopy. Thus "mechanical blanketing" does not appear to be inhibiting oxygen uptake in experimental larvae.

Manwell and Baker (1967) have found that crude oil interfered directly with the activity of enzyme systems in both plants and animals. Dissolved or dispersed hydrocarbons are thought to interfere with the structure of the intercellular membranes which regulate essential metabolic processes in plants (Nelson-Smith, 1972). Results from the present study have shown alteration of mitochondrial cristae in skin cells of larvae treated with diesel oil fractions. Enzymes involved in the tricarboxylic acid cycle (TCA) and in oxidative phosphorylation are located in the mitochondria. When the membranous organelles of the mitochondria are disrupted, the ability to respire aerobically is lost (Baker, 1970). The mitochondrial membranes could be damaged enough to inhibit the TCA cycle and oxidative phosphorylation, thus lowering oxygen uptake (Baker, 1970). If at low concentrations of oils, as used in this study, the membrane is "opened" sufficiently to allow the influx of hydrocarbons within the mitochondria (van Overbeek and Blondeau, 1954), then perhaps alteration of the mitochondrial cristae would occur. Street (1963) speculated that oil can possibly penetrate the mitochondria and cause alteration internally without disrupting the mitochondria completely. This could have a direct effect on cellular metabolism thus lowering the overall oxygen consumption rates as reported in this study.

Ultrastructural modifications of cellular components reported in this study are not quantitative. A more extensive investigation of desmosomal and mitochondrial alterations must be made in order to substantiate physiological findings. Although the "structural" cause and the "physiological" effect detailed thus far from this study seem plausible, one cannot rule out additional indirect effects of the oil fractions on oxygen consumption. As noted previously, after exposure to any of the diesel oil fractions certain behavioral changes took place. Although the larvae initially gulped air they remained at the bottom of the test tank for the remainder of the experiment. In other words, they were relying on external gas exchange (skin and gills) and not internal gas exchange. At lower temperatures, i.e. 7°C, this behavioral change in itself should not substantially lower oxygen consumption rates. Whitford and Hutchison (1965) and others (Whitford and Hutchison, 1967; Lenfant and Johansen, 1967; Whitford and Sherman, 1968; Guimond and Hutchison, 1968, 1972, 1973) have shown that at low temperatures amphibians utilize their skin almost totally for gas exchange, especially for carbon dioxide elimination. Although the gills, skin, lungs, and buccopharyngeal regions may all be functional in aquatic, neotenic amphibians (Noble, 1925), at lower temperatures the skin provides enough oxygen for metabolic demands (Guimond and Hutchison, 1973). Czopek (1962) has found that 50 per cent of the respiratory capillaries are located within the skin, thus providing a large enough surface area for gas exchange at lower temperatures.

However, at higher temperatures, i.e. 15°C and above, the [redacted] a greater oxygen consumption due to the Q_{10} effect. Pulmonary

respiration thus assumes a greater role at higher temperatures to meet metabolic needs (Guimond and Hutchison, 1973); this has been demonstrated in experiments using neotenic *Ambystoma tigrinum* (Whitford and Hutchison, 1965). In *Noturus maculosus*, Guimond and Hutchison (1973) have also shown that the gills assume a greater responsibility for increased oxygen uptake at 15°C, while in *Siren lacertina* the lungs supply increased surface area for oxygen exchange. If at 15°C the metabolic demands cannot be met, because the larvae are continuously submerged and using only external respiration, then one would expect oxygen consumption rates to be lower in these animals. Whitford and Hutchison (1967) have shown that if oxygen uptake through salamander skin and pulmonary surfaces is not sufficient to supply the required oxygen, then the metabolic rate would not increase at higher temperatures. Thus the decrease in oxygen consumption rates at 15°C may be due, in part, to the structural changes within the mitochondria, as well as being due to the inability of the larvae to meet increased oxygen demands without additional pulmonary contributions.

It should be restated that oxygen consumption changes reported in this study reflect the mean differences in rates between pre-oil treated and post-oil treated groups for the individual fraction tested only. No attempt was made to test significant changes in each experimental group (before and after) with control groups (before and after). At 7°C oxygen consumption rates in all "before" groups were not significantly different from each other. However, at 15°C there was considerable variability in oxygen consumption rates among the "before" groups. Whitford and Sherman (1968) have reported that 15°C appears

to represent the optimal temperature for activity in *Ambystoma tigrinum*. They have found variability within test groups similar to that reported in this study. They have also noted that no correlation exists between body weight and oxygen consumption rates at 15°C (r = 0.06), apparently related to the great variation in metabolic activity at this temperature. This fact, too, was brought out in the present study.

Thus the effects of oil on oxygen consumption show how each individual fraction affects the oxygen consumption of the larvae. The results clearly emphasize basic patterns in terms of these effects: treatment with the light fraction, at either experimental temperature, does not alter oxygen consumption significantly, while treatment with either the heavy or cracked fraction results in highly significant changes in the oxygen consumption rates.

The findings from the present study indicate that presumably "safe" or sublethal concentrations of diesel oil fractions alter both behavior and cellular structure of the larvae to the point that significant physiological changes occur. Certain forms of cellular modification may leave the larvae more susceptible to further injury or disease. Changes in cutaneous permeability exert additional pressures upon the animal by altering ionic and osmotic equilibrium, thus providing an additional stress to the larva. The effects of the oil fractions on metabolic processes such as lowering oxygen consumption rates provides less energy for normal activity. In nature these stresses could have serious, long-term effects on the *Ambystoma tigrinum* larvae by lessening their ability to catch and assimilate food, to grow, and to escape from predators.

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APPENDICES

APPENDIX I

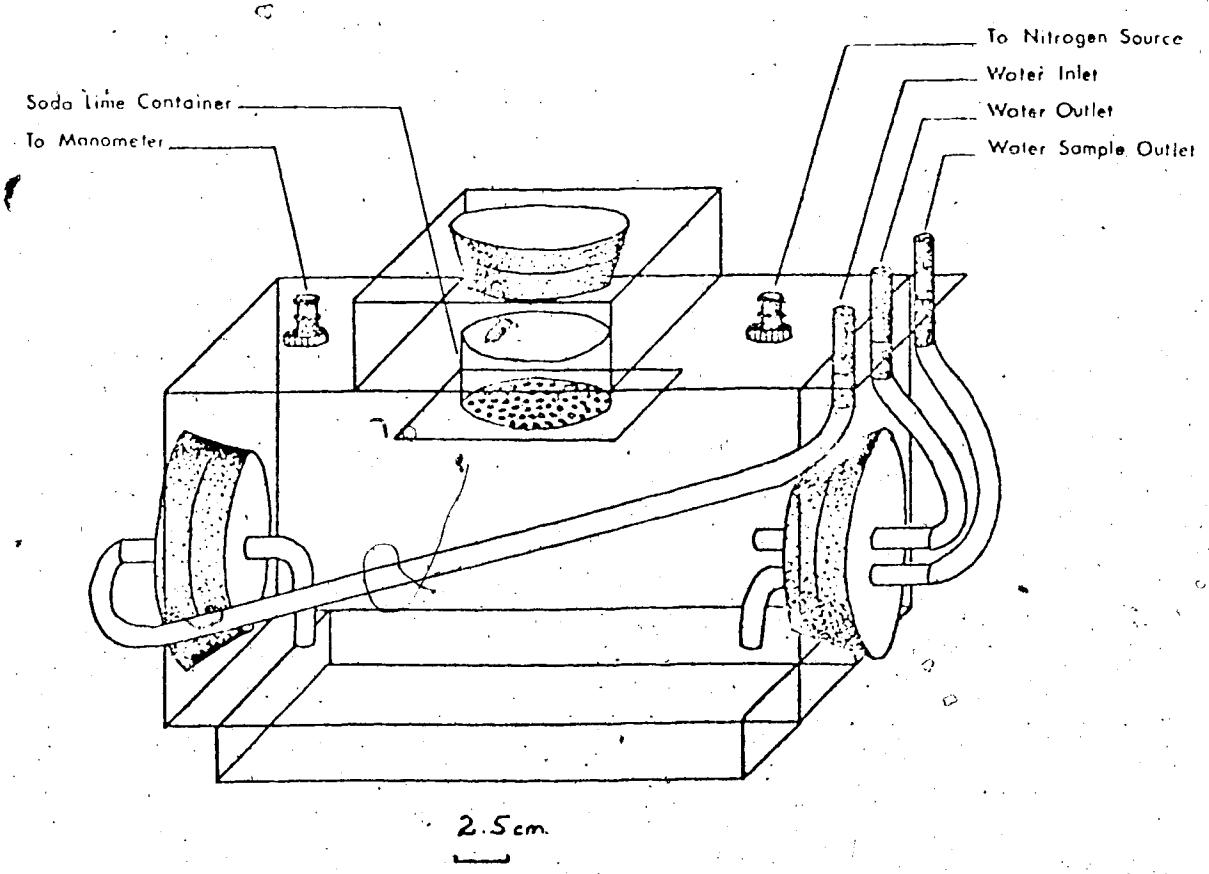
I. Water Analysis

A. Source of sample: Tyrrell's Lake

Date analyzed: June 19, 1973

Alkalinity, Phenol as $CaCO_3$	76.5	mg/l
Alkalinity, Total as $CaCO_3$	636.2	mg/l
Color	47.0	
Hardness, Ca (as $CaCO_3$)	746.9	mg/l
Hardness, Total as $CaCO_3$	872.0	mg/l
Iron	0.24	mg/l
pH	9.10 (10.2 Sept.1973)	
Phosphate, Ortho	0.29	mg/l
Silica	1.16	mg/l
Specific Conductance	8000	micromhos
Sulfate	3700	mg/l
Total dissolved Solids	5433	mg/l
Turbidity	12	JTU
Chloride	156.6	mg/l

APPENDIX II



Plexiglass Respiration Chamber used in Oxygen Consumption Experiments (taken from Cormie, 1975).

APPENDIX III

Individual Oxygen Consumption Rates at 7°C

ml O_2/hr/g (dry wt.)		ml O_2/hr/g (dry wt.)	
Control	Control	Pre-light treated	Post-light treated
0.1562	0.1580	0.1101	0.1138
0.1601	0.1582	0.1036	0.1046
0.1647	0.1709	0.0972	0.0920
0.1623	0.1600	0.1233	0.1289
0.1113	0.1102	0.1251	0.1243
0.1302	0.1293	0.0968	0.0928
0.1479	0.1495	0.0824	0.0812
0.1460	0.1440	0.1465	0.1466
0.1613	0.1575	0.1046	0.1006
0.1387	0.1399	0.0638	0.0665
0.1692	0.1726	0.0987	0.0914
0.1697	0.1708	0.1704	0.1720
0.1226	0.1200	0.1597	0.1584
0.1452	0.1423	0.1581	0.1585
0.1169	0.1159	0.1813	0.1821
0.1541	0.1568	0.1499	0.1479
0.1871	0.1861	0.1501	0.1516
0.1699	0.1642	0.1409	0.1400
0.0932	0.0903	0.1685	0.1697
0.1264	0.1289	0.1762	0.1766
0.1465	0.1497	0.1453	0.1463
0.1354	0.1350	0.1890	0.1872
0.1450	0.1424	0.1759	0.1804
0.1325	0.1330	0.1494	0.1480
n = 24		n = 24	

APPENDIX III

Individual Oxygen Consumption Rates at 7°C

ml O_2/hr/g (dry wt.)		ml O_2/hr/g (dry wt.)	
Control	Control	Pre-light treated	Post-light treated
0.1562	0.1580	0.1101	0.1138
0.1601	0.1582	0.1036	0.1046
0.1647	0.1709	0.0972	0.0920
0.1623	0.1600	0.1233	0.1289
0.1113	0.1102	0.1251	0.1243
0.1302	0.1293	0.0968	0.0928
0.1479	0.1495	0.0824	0.0812
0.1460	0.1440	0.1465	0.1466
0.1613	0.1575	0.1046	0.1006
0.1387	0.1399	0.0638	0.0665
0.1692	0.1726	0.0987	0.0914
0.1697	0.1708	0.1704	0.1720
0.1226	0.1200	0.1597	0.1584
0.1452	0.1423	0.1581	0.1585
0.1169	0.1159	0.1813	0.1821
0.1541	0.1568	0.1499	0.1479
0.1871	0.1861	0.1501	0.1516
0.1699	0.1642	0.1409	0.1400
0.0932	0.0903	0.1685	0.1697
0.1264	0.1289	0.1762	0.1766
0.1465	0.1497	0.1453	0.1463
0.1354	0.1350	0.1890	0.1872
0.1450	0.1424	0.1759	0.1804
0.1325	0.1330	0.1494	0.1480
n = 24		n = 24	

APPENDIX III (Cont'd)

Individual Oxygen Consumption Rates at 7°C

ml O_2/hr/g (dry wt.)		ml O_2/hr/g (dry wt.)	
Pre-heavy treated	Post-heavy treated	Pre-cracked treated	Post-cracked treated
0.0692	0.0436	0.1496	0.1076
0.1329	0.1092	0.1512	0.1020
0.1133	0.0788	0.1406	0.0968
0.1456	0.0929	0.1365	0.0724
0.1481	0.1041	0.1427	0.0841
0.0990	0.0692	0.1522	0.0904
0.0863	0.0687	0.0946	0.0438
0.0988	0.0564	0.1343	0.0719
0.1270	0.0840	0.1638	0.1080
0.1342	0.1026	0.1114	0.0583
0.1309	0.1166	0.1373	0.0784
0.1421	0.1430	0.1489	0.0793
0.1565	0.1552	0.1531	0.0815
0.1723	0.1250	0.1084	0.0592
0.1797	0.1221	0.1691	0.1011
0.1609	0.1317	0.1442	0.1035
0.1481	0.1015	0.1283	0.0710
0.1442	0.1006	0.1387	0.0890
0.1723	0.1463	0.1325	0.0726
0.1691	0.1357	0.1074	0.0512
0.1566	0.1491	0.1626	0.0922
0.1672	0.1265	0.1822	0.1353
0.1839	0.1509	0.1292	0.0762
0.1548	0.1663	0.1355	0.0843
n = 24		n = 24	

Page 3
APPENDIX III (Cont'd)

Individual Oxygen Consumption Rates at 15°C

ml O_2/hr/g (dry wt.)		ml O_2/hr/g (dry wt.)	
Control	Control	Pre-light treated	Post-light treated
0.5879	0.5706	0.1472	0.1469
0.1507	0.1694	0.1743	0.1731
0.1557	0.1723	0.2396	0.2479
0.3383	0.3134	0.4517	0.4502
0.2396	0.2360	0.2385	0.2393
0.4377	0.3742	0.1729	0.1734
0.3855	0.3567	0.4284	0.4268
0.3328	0.3015	0.3306	0.3319
0.1307	0.1590	0.3170	0.3065
0.1963	0.2137	0.3959	0.3901
0.3122	0.3278	0.4861	0.4769
0.2836	0.2946	0.1552	0.1568
0.4535	0.4419	0.4943	0.4837
0.5378	0.5313	0.3121	0.3090
0.2383	0.2769	0.1578	0.1352
0.3169	0.3256	0.1449	0.1440
0.2101	0.2168	0.3263	0.3273
0.1754	0.1895	0.3372	0.3384
0.2930	0.2435	0.3951	0.3855
0.4764	0.4189	0.3100	0.3092
0.3507	0.3790	0.4435	0.4386
0.2835	0.2851	0.2854	0.2931
0.1926	0.1925	0.2903	0.2928
0.1740	0.1829	0.2947	0.2830
n = 24		n = 24	

Page 4
APPENDIX III (Cont'd)

Individual Oxygen Consumption Rates at 15°C

ml O_2/hr/g (dry wt.)		ml O_2/hr/g (dry wt.)	
Pre-heavy treated	Post-heavy treated	Pre-cracked treated	Post-cracked treated
0.2276	0.1840	0.2793	0.1026
0.1735	0.0951	0.2617	0.1308
0.3115	0.2164	0.2689	0.2140
0.2338	0.1501	0.2872	0.1157
0.3186	0.2009	0.2564	0.1099
0.2940	0.2017	0.3669	0.1390
0.4156	0.2945	0.3713	0.2001
0.2221	0.1263	0.1498	0.0663
0.2973	0.1979	0.2684	0.1949
0.2460	0.1567	0.1586	0.0741
0.1598	0.0818	0.1541	0.0898
0.1727	0.0996	0.1936	0.1362
0.2864	0.2143	0.2977	0.2579
0.2362	0.1379	0.4220	0.2264
0.1331	0.0784	0.3283	0.1355
0.2419	0.1791	0.1513	0.0422
0.3267	0.2040	0.1978	0.1196
0.2640	0.1136	0.3119	0.1407
0.2316	0.1002	0.3028	0.1569
0.1573	0.1175	0.5078	0.2545
0.2460	0.1857	0.4053	0.2970
0.1639	0.1239	0.1735	0.0729
0.1712	0.1258	0.2220	0.1383
0.1477	0.0943	0.1642	0.0999
n = 24		n = 24	

APPENDIX IV

TABLE 1

WET WEIGHT AND DRY WEIGHT FOR CONTROL LARVAE

ACCLIMATED AT 7°C

Pre-control		Post-control	
Wet Wt. (g.)	*Dry Wt. (g.)	Wet Wt. (g.)	Dry Wt. (g.)
107.8	16.1	107.1	16.0
93.6	13.9	94.2	13.8
86.5	12.9	86.4	12.9
80.0	11.9	80.9	11.9
147.0	21.9	147.3	21.8
122.4	18.3	122.9	18.1
116.8	17.4	116.4	17.3
109.1	16.3	109.1	16.5
86.5	12.9	86.6	12.5
140.7	21.0	141.0	21.9
79.2	11.8	79.5	11.7
182.3	27.2	181.7	27.4
146.9	21.9	146.8	21.8
112.0	16.7	112.1	16.9
155.3	23.2	155.3	23.2
107.5	16.0	107.4	16.1
68.2	10.2	69.2	10.2
81.4	12.1	80.5	12.0
165.6	24.7	165.5	24.8
133.7	19.9	133.6	19.9
109.8	16.4	109.9	16.4
116.1	17.4	116.0	17.4
110.9	16.6	111.1	16.4
124.0	18.5	124.0	18.7
$\bar{x}$=115.9	$\bar{x}$=17.3	$\bar{x}$=116.0	$\bar{x}$=16.9

*Estimated dry weight, from Figure 4

TABLE 2

WET WEIGHT AND DRY WEIGHT VALUES FOR PRE- AND POST-LIGHT FRACTION TREATED LARVAE ACCLIMATED AT 7°C

Pre-oil		Post-oil	
Wet Wt. (g.)	*Dry Wt. (g.)	Wet Wt. (g.)	Dry Wt. (g.)
151.7	22.64	142.5	21.30
156.3	23.32	147.4	22.00
172.5	26.96	172.6	25.80
148.5	22.16	149.1	22.30
138.8	21.68	144.2	21.50
169.0	26.40	168.3	25.50
146.7	21.90	148.7	22.50
104.5	15.60	104.5	15.61
153.5	22.93	156.4	23.40
148.6	23.22	148.4	24.48
165.6	24.72	168.9	25.20
80.2	11.97	81.3	12.50
92.8	13.85	92.7	13.84
93.9	14.01	94.0	13.82
70.1	10.46	70.0	10.14
97.5	14.55	96.9	14.46
90.0	13.43	91.2	14.03
110.3	16.46	109.7	16.13
82.1	12.25	81.8	12.39
78.9	11.78	79.1	11.98
109.2	16.29	110.3	16.96
65.4	9.76	62.4	9.45
78.5	11.72	77.9	11.45
98.3	14.67	98.6	14.94
$\bar{x}$=116,8	$\bar{x}$=19.28	$\bar{x}$=116.6	$\bar{x}$=15.70

*Estimated dry weight, from Figure 4

APPENDIX IV (Cont'd)

TABLE 3

WET WEIGHT AND DRY WEIGHT VALUES FOR PRE- AND POST-HEAVY FRACTION TREATED LARVAE ACCLIMATED AT 7°C

Pre-oil		Post-oil	
Wet Wt. (g.)	*Dry Wt. (g.)	Wet Wt. (g.)	Dry Wt. (g.)
199.6	29.79	205.5	29.06
121.8	18.18	116.5	17.92
151.1	22.55	154.5	22.60
110.3	16.56	111.7	16.37
120.0	17.90	132.2	17.75
172.9	25.80	179.2	25.60
175.5	26.19	175.5	25.90
172.7	25.78	175.6	24.80
129.0	19.25	138.3	19.09
144.1	21.50	148.4	21.21
120.4	17.97	119.9	17.83
105.2	15.70	109.5	15.60
91.7	13.69	92.8	13.71
69.4	10.36	72.4	10.41
79.8	11.91	83.9	11.88
83.2	12.42	89.9	12.39
99.0	14.77	104.2	14.83
100.6	15.01	107.0	15.06
81.7	12.19	85.9	12.21
88.6	13.22	93.4	13.81
90.8	13.55	96.8	13.47
74.1	11.06	79.7	11.10
60.7	9.06	65.6	9.12
95.5	14.25	98.0	14.30
$\bar{x}$=114.1	$\bar{x}$=17.02	$\bar{x}$=118.2	$\bar{x}$=16.89

*Estimated dry weight, from Figure 4

APPENDIX IV (Cont'd)

TABLE 4

WET WEIGHT AND DRY WEIGHT VALUES FOR PRE- AND POST-CRACKED FRACTION TREATED LARVAE ACCLIMATED AT 7°C

Pre-oil		Post-oil	
Wet Wt. (g.)	*Dry Wt. (g.)	Wet Wt. (g.)	Dry Wt. (g.)
115.2	17.1	121.8	17.5
94.3	14.1	99.3	14.2
112.7	16.8	120.1	16.5
122.7	18.3	124.5	18.1
105.3	15.7	111.7	15.5
90.6	13.5	96.4	13.8
162.5	24.2	169.2	24.5
126.6	18.8	134.9	18.5
90.4	13.5	90.0	13.9
110.5	16.4	117.6	16.0
135.3	20.1	140.3	19.7
110.4	16.4	119.2	16.8
92.3	13.7	99.8	13.9
158.1	23.5	163.4	23.0
88.7	13.2	89.1	13.1
100.2	14.9	109.6	14.9
149.4	22.3	154.9	22.4
127.6	19.0	133.2	18.5
125.3	18.7	127.6	19.0
154.2	23.0	161.0	23.1
80.1	11.9	82.9	11.9
69.5	10.4	73.2	10.5
141.4	21.1	145.7	21.0
130.8	19.5	135.8	19.4
$\bar{x}$=116.4	$\bar{x}$=17.4	$\bar{x}$=122.1	$\bar{x}$=17.3

*Estimated dry weight, from Figure 4

TABLE 5

WET WEIGHT AND DRY WEIGHT VALUES FOR CONTROL LARVAE

ACCLIMATED AT 15°C

Pre-control		Post-control	
Wet Wt. (g.)	*Dry Wt. (g.)	Wet Wt. (g.)	Dry Wt. (g.)
84.5	12.4	84.1	12.5
229.2	34.2	228.9	34.1
129.7	19.3	129.8	19.4
118.7	16.6	119.1	17.7
79.7	11.9	79.6	11.8
91.4	13.6	91.6	13.7
75.0	11.1	75.3	11.2
66.6	9.9	66.5	9.9
203.3	30.3	203.8	30.4
113.9	17.0	113.0	16.8
101.4	15.1	100.9	15.0
63.4	9.4	64.2	9.5
75.9	11.3	76.1	11.4
57.2	8.5	57.0	8.5
99.7	14.8	99.2	14.8
65.8	9.8	65.9	9.8
67.1	10.0	66.3	9.9
148.5	22.16	148.4	22.1
118.9	17.7	119.2	17.8
77.9	11.6	77.3	11.5
61.5	9.2	61.7	9.2
63.8	9.5	63.5	9.4
187.6	27.9	186.9	28.8
194.6	29.0	194.0	28.9
$\bar{x}$=107.2	$\bar{x}$=15.9	$\bar{x}$=107.2	$\bar{x}$=16.0

*Estimated dry weight, from Figure 4

APPENDIX IV (Cont'd)

TABLE 6

WET WEIGHT AND DRY WEIGHT VALUES FOR PRE- AND POST-LIGHT FRACTION TREATED LARVAE ACCLIMATED AT 15°C

Pre-oil		Post-oil	
Wet Wt. (g.)	*Dry Wt. (g.)	Wet Wt. (g.)	Dry Wt. (g.)
136.2	20.8	136.1	20.9
147.1	21.9	146.9	21.9
79.6	12.0	80.2	12.2
75.2	11.2	75.3	11.1
69.4	10.4	69.4	10.4
151.1	22.7	150.8	22.8
93.5	13.6	93.7	13.8
100.2	14.8	100.4	14.9
112.9	16.9	113.0	16.9
84.7	12.8	84.6	12.8
89.3	13.4	89.0	13.3
162.8	25.1	162.5	25.0
59.0	8.6	59.1	8.8
103.6	15.3	103.5	15.4
129.4	19.7	129.6	19.6
170.5	25.3	171.0	25.5
108.0	16.2	108.2	16.4
96.5	14.6	96.6	14.9
84.9	12.6	84.8	12.7
99.2	15.1	99.1	15.0
90.1	13.9	90.2	13.7
67.6	10.5	67.8	10.3
61.5	9.4	61.3	9.4
119.9	18.5	119.7	18.4
$\bar{x}$=103.8	$\bar{x}$=15.6	$\bar{x}$=103.9	$\bar{x}$=15.7

*Estimated dry weight, from Figure 4

TABLE 7

WET WEIGHT AND DRY WEIGHT VALUES FOR PRE- AND POST-HEAVY FRACTION TREATED LARVAE ACCLIMATED AT 15°C

Pre-oil		Post-oil	
Wet Wt. (g.)	*Dry Wt. (g.)	Wet Wt. (g.)	Dry Wt. (g.)
54.2	7.9	59.8	8.0
143.0	20.8	149.3	21.3
50.5	8.4	55.1	7.9
97.7	14.6	99.2	14.7
65.0	9.8	68.0	9.7
63.5	9.4	67.3	9.3
65.3	9.7	69.4	9.8
61.7	9.3	67.2	9.2
112.1	16.7	119.7	16.8
60.9	9.0	65.6	9.2
85.0	12.6	87.1	12.8
173.6	25.9	179.2	25.6
51.6	7.7	53.0	7.7
50.9	7.5	50.8	7.8
71.6	10.6	75.4	10.4
56.5	8.4	57.3	8.5
199.6	29.7	205.6	29.9
121.8	18.7	126.9	18.7
151.1	23.6	156.8	23.8
110.3	17.5	118.3	17.4
120.0	17.9	124.2	17.9
172.9	25.8	176.4	25.6
175.5	26.2	175.9	26.4
172.7	25.8	175.8	25.6
$\bar{x}$=103.7	$\bar{x}$=15.6	$\bar{x}$=107.6	$\bar{x}$=15.6

*Estimated dry weight, from Figure 4

TABLE 8

WET WEIGHT AND DRY WEIGHT VALUES FOR PRE- AND POST-CRACKED FRACTION TREATED LARVAE ACCLIMATED AT 15°C

Pre-oil		Post-oil	
Wet Wt. (g.)	*Dry Wt. (g.)	Wet Wt. (g.)	Dry Wt. (g.)
111.2	16.6	118.3	16.9
63.1	9.4	69.4	9.5
80.5	12.0	82.6	12.6
71.5	10.8	78.7	10.7
90.5	13.5	95.5	13.4
61.7	8.8	69.8	8.9
65.5	9.8	69.1	10.0
137.4	20.5	143.0	20.1
54.8	8.2	57.2	8.2
129.2	19.3	136.9	19.8
192.7	28.8	198.6	28.9
117.8	14.7	121.3	14.6
97.7	14.6	99.2	14.7
57.5	8.6	61.0	8.8
60.6	9.0	67.9	9.3
200.3	29.9	210.1	30.1
139.1	20.8	142.7	21.4
104.1	15.5	111.1	15.7
61.3	9.1	68.5	9.2
59.7	9.2	63.6	9.2
75.2	11.0	79.5	11.1
140.3	20.9	148.0	20.8
69.5	10.4	73.7	10.6
168.0	25.0	172.0	25.0
$\bar{x}$=100.4	$\bar{x}$=14.9	$\bar{x}$=105.7	$\bar{x}$=14.9

*Estimated dry weight, from Figure 4