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Effects of DDT Upon the Hematology and Immunology of the Goldfish (Carassius auratus)

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EFFECTS OF DDT UPON THE HEMATOLOGY AND IMMUNOLOGY OF THE GOLDFISH (*CARASSIUS AURATUS*)

by

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Maurice G. Zeeman

TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (continued)

LIST OF FIGURES

NOTATIONS

DDT	1,1-bis-(p-chlorophenyl)-2,2,2-trichloroethane
DDE	1,1-bis(p-chlorophenyl)-2,2-dichloroethylene
DDD	1,1-bis(p-chlorophenyl)-2,2-dichloroethane
ppm	Parts per million
i.p.	Intraperitoneal injection
#RBC	Number of red blood cells (erythrocytes)
MH	Microhematocrit
Hb	Hemoglobin
ESR	Erythrocyte sedimentation rate
TPP	Total plasma protein
#WBC	Number of white blood cells (leukocytes)
%L	Percent lymphocytes
%N	Percent neutrophils
%M	Percent monocytes
%T	Percent thrombocytes
PBS	Phosphate buffered saline
SRBC	Sheep red blood cell(s)
PFC	Plaque forming cell(s)
BSA	Bovine serum albumin

ABSTRACT

Effects of DDT Upon the Hematology and Immunology of the Goldfish (Carassius auratus)

by

Maurice G. Zeeman, Doctor of Philosophy

Utah State University, 1980

Major Professor: Dr. William A. Brindley

Department: Biology

DDT can cause hematological and immunological alterations in mammals and birds. Interference with normal hematological and immunological defense systems has been strongly linked to increased incidences of disease and tumors. Fish exposed to pesticides have been noted to be more susceptible to disease. This study examined DDT effects on goldfish hematological and immunological parameters.

Adult goldfish were kept in 250 liter aquaria at 25 ± 1° C. Fish were dosed intraperitoneally with p,p'-DDT in corn oil. Doses ranged from 0 to 500 mg/kg for acute (96 hour) hematology studies to 0 to 50 mg/kg for sub-acute hematology and immunology studies.

The 96 hour LD_{50}'s increased from 213 to 480 to 579 mg/kg for groups of fish weighing approximately 20, 50 or 110 grams respectively.

In acute hematology tests, effects of DDT dose upon seven hematological parameters were determined. In general, numbers of

erythrocytes and leukocytes (and associated parameters) were decreased with increasing DDT dose. Hematological alterations were moderated as fish size increased.

In the sub-acute tests, effects of repeated sub-lethal doses of DDT upon the hematological parameters and immunological responses of medium-sized fish were investigated over a one to two month span. In general, some of the hematological parameters stabilized after increased duration of DDT exposure. Nevertheless, numbers of leukocytes (especially percent lymphocytes) often remained reduced after DDT treatment.

DDT treatment generally resulted in suppression of primary and secondary immune responses. Numbers of Jerne-plaque forming cells in anterior kidney and spleen were often reduced by DDT pretreatments, as were the serum titers of hemolysin, hemagglutinin and anti-bovine serum albumin. DDT also suppressed the normal rejection of scale-graft transplants (allografts). These results indicated that DDT interfered with the normal humoral and cell-mediated immune responses of this species and may help explain how pesticide exposure could result in increased susceptibility of fish to infectious diseases.

(142 pages)

INTRODUCTION

Pesticide contamination at sublethal levels constitutes a poorly understood hazard to fish health. (Meyer, 1979, p. 9)

DDT [1, 1-bis-(p-chlorophenyl) -2,2,2-trichloroethane] is a chlorinated hydrocarbon insecticide that can be lethal to both target and non-target organisms. The environmental persistence, biomagnification capabilities and carcinogenic potential of DDT resulted in cancellation of most of its uses in the United States in 1972 by the Environmental Protection Agency (EPA, 1975). Nevertheless, DDT still remains one of the world's most used insecticides and many less prosperous countries consider "DDT as the most important life-saver known to man" (Brooks, 1977). There are even calls for a cancellation of the EPA ban on the uses of DDT in the United States (Gray-Beatty, 1973).

Residues of persistent chlorinated insecticides, such as DDT, tend to accumulate in aquatic environments. The persistence and bioconcentration of DDT in aquatic environments has resulted in many fish species being contaminated with residues of DDT and its metabolites, especially DDE (Johnson, 1973; Hamelink and Spacie, 1977). Recent studies show that even with the 1972 ban and severe restrictions on the use of DDT in the United States, many fish sampled remain heavily contaminated with DDT and DDE residues (Butler and Schutzmann, 1978; McDermott-Ehrlich et al., 1978; Anon., 1979; Barber and Warlen, 1979; Smokler et al., 1979).

A great deal of research shows that non-lethal doses of DDT can have significant detrimental effects on fish and other animal growth, behavior, reproduction, and physiological responses (Johnson, 1968; Friend and Trainer, 1970; Holden, 1973; Kay, 1973; Fishbein, 1974; Ware, 1975). While the sublethal effects of DDT on fecundity, enzyme induction, blood cells or immunological resistance to disease are often not grossly observable, they may be of great significance to an animal's survival.

There is an intimate integration of fish (and other vertebrate) hematological and immunological systems. Areas of blood cell formation (hematopoiesis) are also critical for the generation of immune responses (Anderson, 1974). The circulating and fixed white blood cells (leukocytes) are important mediators of phagocytosis, inflammation, humoral antibody production and cell-mediated immune reactions (Finn, 1970; Corbel, 1975; Ellis, 1977; Ellis, 1978). The leukocytes, their effector proteins (antibodies) and various non-specific proteins (complement, properdin, interferon) essential to normal immune formation are transported in the blood. Thus, blood is integrated with and is also an essential part of vertebrate immune systems.

That DDT can affect hematological parameters and also alter immune responses has been widely demonstrated in birds and mammals (Hayes, 1959; Latimer and Siegel, 1974; Street and Sharma, 1975; Gadomska and Krechniak, 1976; Subba Rao and Glick, 1977; Iturri and Ringer, 1978; Wiltrout et al., 1978). Consequences of detrimental interference by DDT upon normal avian and mammalian hematological and immunological systems (immunosuppression) can vary from anemia

to increased susceptibility to disease and even possibly result in tumor formation (Hayes, 1959; Sanchez-Medal et al., 1963; Friend and Trainer, 1974; Thompson and Emerman, 1974; Traczyk, 1975; Fishbein, 1976; Kashyap et al., 1977; Vos, 1977; Iturri and Ringer, 1978).

In 1962, King suggested that exposure of fish to pesticides could cause increased sensitivity of these fish to infectious diseases. Within two years, three research articles were published which presented some evidence that fish exposed to DDT did indeed seem to show increased incidences of infectious disease (Allison et al., 1963; Schoenthal, 1963; Allison et al., 1964). How or why DDT caused these increased incidences in fish diseases was not explored.

The effects of DDT on fish hematology and immunology have not been studied in detail. This research examined the effects of DDT on several hematological parameters and immunological responses of goldfish (Carassius auratus). Lethal and sub-lethal doses of DDT altered many of the hematological parameters of goldfish and sub-lethal levels of DDT also disrupted some of their immunological responses.

REVIEW OF LITERATURE

The hematology and immunology of the goldfish are not as well known as in standard laboratory animals (i.e., mouse or chicken). Nevertheless, over the last half-century considerable data have been generated, and it is possible to say that many comparative immunologists now view the goldfish as their preferred test fish species.

Goldfish Hematology

> *The fully mature blood cells of the goldfish . . . often resemble the developing bone marrow cells of the equivalent mammalian cell types. (Weinreb, 1963, p. 224)*

Hematopoiesis. The kidneys and spleen are, respectively, the primary and secondary areas of hematopoiesis in the goldfish (Jordan and Speidel, 1924; Jordan, 1938; Watson et al., 1963a; Andrew, 1965; Neale and Chavin, 1971a). The kinds of blood cells produced in these organs are the erythrocytes and four types of leukocytes -- lymphocytes, granulocytes (neutrophils), monocytes and thrombocytes (spindle cells). All five kinds of blood cells are found in the two types of kidney, 1) anterior kidney (= head kidney = pronephros) and 2) body mesonephros, as well as the spleen. Davies and Haynes (1975) found goldfish mesonephric kidney to contain granulocytes and immature erythrocytes, which indicated to them that this organ was a center of erythropoiesis and granulocytopoiesis. Neale and Chavin (1971a) examined whole goldfish histologically and found that the major foci of lymphoid tissues occurred in the head kidney, mesonephric kidney and spleen.

In addition to the kidneys and spleen, other goldfish organs such as intestine, liver and gonads are (to a much lesser extent)

also involved in hematopoiesis (Watson et al., 1963a; Watson, 1969). Watson (1969) found that these hematopoietic organs were also the areas where injected antigen would localize in goldfish, and he thought it reasonable to assume they "may also be the major areas of antibody synthesis."

Weinberg (1975) showed that about 40% of the total number of cells found in the epithelial layer of the goldfish intestine were lymphocytes. After an antigenic stimulation, he also found plasma cells (antibody producing cells or B-cells) and suggested that the goldfish intestine might be considered a primitive equivalent to the avian bursa. Davies and Haynes (1975) also found that a layer in the goldfish intestine (tunica propria) contained "numerous granulocytes as well as lymphocytes."

The thymus of the goldfish consists predominantly of small lymphocytes (Marchalonis et al., 1977). While goldfish thymic lymphocytes are capable of binding foreign antigen, the cells are probably immune response helper cells (T-cells), as they do not seem capable of producing antibody (Ruben et al., 1977). In some fish, the thymus may be a special environment, as peripheral lymphocytes seem not to migrate through it and the traffic in thymic lymphocytes may be in only one direction, i.e. emigration (Ellis, 1977).

The erythrocytes and leukocytes are carried by the blood of fish to and from all organs of the body. Fish leukocytes can migrate directly into certain tissues. In plaice (Pleuronectes platessa) for example, lymphocytes migrate to appropriate environments in kidney and spleen (Ellis and de Sousa, 1974).

Fish leukocytes can also recirculate into the blood via the lymphatic system (Wardle, 1971).

The blood. The peripheral blood of goldfish consists of a suspension of erythrocytes and leukocytes in plasma. Goldfish erythrocytes are nucleated oval-shaped cells with dimensions of about 8 x 13 μ (Smith et al., 1952; Watson et al., 1963a; Weinreb, 1963). These cells are important in oxygen transport since their cytoplasm contains hemoglobin (Weinreb, 1963). The number of red blood cells per unit volume in goldfish blood varies with several factors, such as water temperature, fish diet, sex and age (Spoor, 1951; Linn, 1965; Summerfelt et al., 1967; Watson, 1969). The number of erythrocytes/mm^3 of goldfish blood is often from 1.5 - 2.0 x 10^6 (Hildemann, 1958; Watson et al., 1963a; Falkner and Houston, 1966; Watson, 1969).

The total erythrocyte count directly measures the numbers of circulating red blood cells/mm^3 of blood but there are also two other important indirect measures closely correlated with erythrocyte numbers and with each other (Summerfelt et al., 1967; Satchell, 1971). These measures are the microhematocrit and the hemoglobin levels which are often used because they are quicker and simpler to measure than is the total erythrocyte count.

The microhematocrit measures the percentage of the blood that is occupied by the erythrocytes. In centrifuged goldfish blood, this is normally from 25-45% of the blood volume (Saito, 1954; Falkner and Houston, 1966; Summerfelt et al., 1967; Hunn et al., 1968; Watson, 1969). Hemoglobin tests measure the amounts of this protein liberated upon lysis of the erythrocytes from a given volume

of blood. Goldfish hemoglobin levels are often from 7-10 g/dl of blood (Saito, 1954; Summerfelt et al., 1967; Hunn et al., 1968; Houston et al., 1976).

The blood plasma (or serum) of goldfish contains essentially proteins and electrolytes dissolved in water. Levels of total plasma proteins found in goldfish blood are commonly from 2.0-4.5 g/dl of blood (Linn, 1965; Summerfelt et al., 1967; Hunn et al., 1968; Watson, 1969). These levels can vary with the temperature or the season and/or the goldfish's sex and age (Houston and Fenwick, 1965; Linn, 1965; Summerfelt et al., 1967; Watson, 1969).

Plasma proteins have several functions. Proteins can act as buffers, help maintain osmotic pressure, supply nutritional requirements for many tissues, and transport ions, vitamins and hormones, while others are especially concerned with body defenses against many pathogenic organisms (Satchell, 1971). Some plasma proteins involved in immune functions are undoubtedly produced by the leukocytes of fish (Ellis, 1978). Fish leukocytes certainly produce antibodies, and alone, these proteins can account for about 5-10% of the total protein levels in plasma or serum (Ingram and Alexander, 1979).

Goldfish leukocytes are broadly divided into four groups of cells -- lymphocytes, granulocytes, monocytes and thrombocytes. These cells can be distinguished morphologically by certain characteristics -- such as cell size, shape, and the relative position and staining properties of the nucleus and cytoplasm. The following descriptions are based on research that used Wrights' or May-Grunewald or Giemsa stains (separately or in combinations) on

blood smears.

Goldfish lymphocytes are roundish cells that frequently can vary in diameter from about 6-11 μ (Loewenthal, 1927; Watson, et al., 1963a; Weinreb, 1963). Most of these lymphocytes are small in size and may be the more mature cells (Weinreb, 1963). Small lymphocytes appear as a rounded, darkly staining nucleus which is surrounded by only a narrow rim of basophilic cytoplasm. The nucleus of the larger lymphocyte is similar but eccentrically placed in a much more abundant cytoplasm. Weinreb (1963) reported that the fine structure of goldfish lymphocytes is similar to that of mammals.

In 1969 Weinreb and Weinreb reported that "the function of the lymphocyte in the goldfish has not been precisely defined . . ." These cells are undoubtedly involved in most of the immune functions in the goldfish.

Weinreb and Weinreb (1969) claim that lymphocytes are phagocytic, but Ellis (1977) suggests that the authors confused these cells with other phagocytic leukocytes. Studies on the rejection of foreign scale-grafts in goldfish suggest that the infiltration of the foreign scale tissues by host lymphocytes was actually an active cell-mediated immune rejection response (Goodrich and Nichols, 1933; Hildemann, 1956a and 1957). Several studies report goldfish can produce antibodies (Everhart and Shefner, 1966; Trump, 1967; Trump, 1970; Trump and Hildemann, 1970; Marchalonis, 1971; Everhart, 1972; Azzolina, 1978). It is quite likely that antigenic stimulation of the appropriate small lymphocytes resulted in increases in cell size and formation of antibody producing plasma cells (Neale and Chavin, 1971a; Chavin, 1973; Weinberg, 1975).

For several reasons, neutrophils (heterophils) will be the primary type of granulocyte described in detail here. The main reason is neutrophils were the only granulocyte identified in this study.

If present, other granulocytes should have granules that differ from neutrophil granules in size and staining properties. Two other kinds of granulocytes have been reported in goldfish blood -- eosinophils and basophils (Loewenthal, 1927; Saito, 1954; Watson et al., 1963a; Weinreb, 1963; Watson, 1969). Even in these studies, the neutrophils were by far the most common granulocyte found.

Other researchers have also not seen (or did not report) eosinophils and/or basophils in the blood of this species (Neale and Chavin, 1971a; Bennett and Neville, 1975; Davies and Haynes, 1975; Barber and Mills-Westerman, 1978).

These discrepencies could be due to any of several factors. First, even when found, eosinophils and basophils are rare and often comprise only about 3% of the total white blood cells. Second, granulocytes are fragile, degranulate easily and are often disrupted ~~which could~~ in blood smears (Watson et al., 1963a; Andrew, 1965). Third, various Romanowsky-dye stains were used in these studies which could account for any differences in granule staining ability. Fourth, heparin was often used as an anticoagulant, and this chemical can alter the staining characteristics of leukocytes (Schalm et al., 1975; Ellis, 1977).

Neutrophils of goldfish can vary in size from about 7-15 μ, are round to oval in shape, and have an eccentric nucleus that is variable in form and stains moderately to darkly purple in color (Loewenthal, 1927; Watson et al., 1963a; Weinreb, 1963). Nuclei of

neutrophils are most often round or oval in shape, but many neutrophils have a nucleus that is kidney-shaped and a few even have a nucleus divided into two or three lobes (Loewenthal, 1927; Watson et al., 1963a; Weinreb, 1963; Watson, 1969; Davies and Haynes, 1975). The cytoplasm of neutrophils often contains numerous small oval granules that do not stain markedly at neutral pH.

The neutrophils of goldfish are phagocytic (Watson et al., 1963a; Weinreb, 1963; Weinreb and Weinreb, 1969). Weinreb (1963) suggested that degranulation of goldfish neutrophils occurs during normal activity and during phagocytosis, which might be connected to release of lysosomal enzymes. In a later study, Weinreb and Weinreb (1969) found goldfish neutrophils were the cell most active in phagocytosis, suggesting neutrophils also play a role in inflammatory reactions.

Ellis (1977) described mammalian monocytes as large phagocytic cells circulating in the blood. These readily migrate from the blood to supply macrophages to the tissues when and where they are needed. Monocytes are up to 20 μ in diameter with a large moderately violet-staining nucleus. Their nucleus varies in shape from oval to horseshoe shaped and it takes up from 30-50% of the cell area. The cytoplasm can be slightly basophilic and often contains clear vacuoles. These cells normally constitute only about 5% of the total leukocyte population.

Research describing goldfish leukocytes often have descriptions of a cell type very similar to monocytes, but they were often called something other than monocytes. Loewenthal (1927) simply called them large mononuclears. Watson et al. (1963a) and Watson (1969)

said that no cells corresponding to the higher vertebrate monocyte were seen in goldfish blood smears, but their descriptions of goldfish macrophages and hemoblasts fit the above mammalian monocyte descriptions well. Weinreb and Weinreb (1969) reported that monocytes were not seen in C. auratus blood, yet described hemoblasts that had morphology and staining characteristics almost identical to those of mammalian monocytes (Ellis, 1977). Ellis (1977) believes that the above results are an example of a confusion in the fish literature and suggests that such cells be called monocytes.

Functionally, these goldfish monocytes (hemoblasts and/or macrophages) were actively phagocytic and could also have been involved in some inflammatory responses (Watson et al., 1963a; Weinreb, 1963; Watson, 1969; Ellis, 1977).

Thrombocytes in fish and in the other vertebrates are the functional counterparts to mammalian platelets. Structurally, goldfish thrombocytes are often similar in appearance to small lymphocytes. In fact, difficulty has been reported in telling these two kinds of goldfish leukocytes apart (Watson, et al., 1963a; Weinreb, 1963). Weinreb (1963) found such a striking similarity in the structures of the thrombocyte and lymphocyte that she discussed a possible direct relationship between these two cells.

This similarity notwithstanding, many researchers have not included the thrombocyte as a goldfish leukocyte. Loewenthal (1927) does not mention or describe a thrombocyte-like cell. Saito (1954) measured the numbers of goldfish "platelets" separately from the white blood cell counts and therefore the thrombocytes were not part of his differential leukocyte count. The same schema was used by

Watson et al. (1963a) and Watson (1969), who classified the thrombocyte not as a leukocyte, but as a non-erythrocytic blood cell. It seems likely that other studies did not even attempt to count the thrombocytes separately and probably characterized them as a small lymphocyte (Hildemann, 1958; Neale and Chavin, 1971a; Bennett and Neville, 1975).

In contrast, Weinreb (1963) and Weinreb and Weinreb (1969) include the thrombocyte as a goldfish leukocyte and in 1969 included it in their differential leukocyte counts. A recent comprehensive review on fish leukocytes definitely includes the thrombocyte as one kind of leukocyte (Ellis, 1977) and thrombocytes will be dealt with as such here.

Goldfish thrombocytes are the smallest leukocytes and vary from 3-5 μ in diameter (Watson et al., 1963a; Weinreb, 1963). The nucleus occupies most of the cell and stains very dark blue to violet. The thin layer of surrounding cytoplasm is faintly colored and when the cell is not spherical in shape, often projects from one end (fusiform shape) or both ends (spindle shape) of the cell (Watson et al., 1963a; Weinreb, 1963).

The spherical thrombocyte is most easily confused with a small lymphocyte. The possible confusion is reduced by comparing thrombocytes and lymphocytes for the following: thrombocytes tend to occur in groups, are smaller in size, have a nucleus which tends to stain more darkly, commonly have non-spherical shapes, and their cytoplasm is often sparser and almost colorless.

Functionally, goldfish thrombocytes are at least involved in coagulation of the blood (Watson et al., 1963a and b). Ellis (1977)

reports that in other fish species, the thrombocytes could also be phagocytic. Although he then dismisses this idea as unlikely, the thrombocyte has recently been found to be the most important phagocytic leukocyte in the chicken (Chang and Hamilton, 1979).

Goldfish blood has a much higher density of leukocytes than does the blood of mammals. The normal range in adult human leukocyte numbers is 4,500-11,000/mm^3 (Davidsohn and Nelson, 1974). Even ignoring numbers of thrombocytes (where counted separately) led to normal goldfish leukocyte values that ranged from 15,000-58,500/mm^3, with the most common average values between 20,000-35,000/mm^3 (Saito, 1954; Watson et al., 1963a; Linn, 1965; Watson, 1969; Neale and Chavin, 1971a). Goldfish thrombocyte numbers estimated separately varied from 11,888-20,400/mm^3 in Watson (1969), averaged 51,600/mm^3 in Watson et al. (1963a) and 89,000/mm^3 in Saito (1954).

The differential white blood-cell count estimates the proportions of various leukocyte types present in the blood. In every case except one (Saito, 1954), the lymphocytes were the predominant goldfish leukocyte, with proportions ranging from 71-97% (Watson et al., 1963a; Watson, 1969; Weinreb and Weinreb, 1969; Neale and Chavin, 1971a; Bennett and Neville, 1975). Neutrophils tended to be the next most common goldfish leukocyte, as their proportions varied from 3-18% (Watson et al., 1963a; Watson, 1969; Weinreb and Weinreb, 1969; Neale and Chavin, 1971a; Bennett and Neville, 1975). The only exception was again Saito (1954), who reported the neutrophils (polymorphs) were the predominant type of goldfish white blood cell. Monocytes were reported in a few studies, and their proportions

(1-7%) were always low (Saito, 1954; Watson, 1969; Weinreb and Weinreb, 1969). Only Weinreb and Weinreb (1969) included thrombocytes in goldfish differentials and they reported thrombocytes represented from 3-13% of the goldfish leukocytes.

Goldfish Immunology

Several basic aspects of fish immune systems (structures and functions) have been illustrated by research using the goldfish. Immunologically, the goldfish is one of the most studied fish species.

As leukocytes (and some of their products) are essential to normal functioning of immune systems, certain aspects of the goldfish immune system have already been mentioned in reviewing the hematology of this species. The organs and tissues where leukocytes were found, what kind and how many were present in the blood and some of their immune functions have already been briefly discussed.

Therefore, a complementary review on what else is known about this species' immune structures (the hematopoietic tissues, the white blood cells and the serum proteins these produce), non-specific immune functions (phagocytosis and inflammation) and specific immune functions (humoral antibody production and cell-mediated immune response) seems appropriate.

Immune structures. As already has been noted, the kidneys and spleen are the most important centers of lymphoid tissues in the goldfish (Jordan and Speidel, 1924; Watson et al., 1963a; Neale and Chavin, 1971a; Davies and Haynes, 1975). The thymus, intestine and other organs may also be of import in this regard (Watson, 1969; Davies and Haynes, 1975; Weinberg, 1975; Marchalonis et al., 1977).

The goldfish leukocytes have already been discussed. The cells involved in various aspects of the goldfish immune responses are the neutrophils (and other granulocytes), the monocytes (macrophages), and especially the lymphocytes (Ellis, 1977).

Fish leukocytes produce various serum proteins that are important in nonspecific and specific immune functioning. These proteins assist or heighten the normal immune responses of leukocytes. The proteins of non-specific activity found in fish serum are complement, lysozyme, properdin, interferon, transferrin, C-reactive protein and natural hemolysins and hemagglutinins (Cushing, 1970; Anderson, 1974; Corbel, 1975; Ellis, 1978).

Legler et al. (1967) found that goldfish serum contained complement and natural hemolysins against the erythrocytes of several foreign species. Heating goldfish serum at 50° C for 20 minutes inactivated complement activity. This activity was also labile to dilution or freezing. The natural hemolytic activity was increased if several fish sera were pooled. The authors said that "This indicates interaction between the natural antibody and other hemolytic components present in varying amounts in the constituent sera of the pool." Watson et al. (1968) and Watson (1969) report that C-reactive protein was found in goldfish sera. Normal fish had this protein and it was not increased after bacterial immunizations.

Non-specific immune functions. Non-specific immune capacities of fish are general reactions to injury and/or invasions by foreign organisms. Phagocytosis and inflammation are the two non-specific leukocytic responses that are common in fish (Corbel, 1975; McKinney et al., 1977).

Phagocytosis by leukocytes is an important part of inflammatory responses and a key step in the specific immune responses. Inflammation is one result of an accumulation of leukocytes attracted to an area of injury and/or infection.

Several leukocytes of the goldfish are phagocytic. Neutrophils (and eosinophils) are phagocytic towards bacteria and the endocytic agent thorotrast (Watson et al., 1963a; Weinreb and Weinreb, 1969). While Weinreb and Weinreb (1969) claim that certain blast cells and lymphocytes were also phagocytic towards thorotrast, Ellis (1977) feels that the blast cells described were actually monocytes and doubts that lymphocytes have this capacity. He suggests that the phagocytic lymphocytes were actually misidentified monocytes or even thrombocytes. Watson et al. (1963a) described macrophages (monocytes) which appeared to be phagocytic towards bacteria and cellular debris.

The same leukocytes also seem to be important in goldfish inflammatory responses. Janssen and Waaler (1967) injected silica intramuscularly into goldfish kept at 8° or 22.5° C and then looked for local inflammation. The cold fish died in five days and exhibited no inflammatory responses. The warm fish showed distinct inflammatory response with macrophages and lymphocytes infiltrating the area of injection. Weinreb (1963) and Weinreb and Weinreb (1969) suggest that the phagocytic properties of goldfish leukocytes (described above) may be part of an expected inflammatory reaction in this species. Mawdesley-Thomas and Bucke (1973) found acute and chronic inflammatory type cells (macrophages, lymphocytes and plasma cells) infiltrating into areas of damage to goldfish dermis and connective

tissue.

Specific immune functions. The specific immune responses of fish are essentially 1) a humoral antibody-mediated response produced against foreign antigens, and 2) a cell-mediated response demonstrated primarily by rejection of foreign tissue grafts and also by delayed hypersensitivity reactions. After second contact with foreign antigens, each of these specific sets of immune responses often shows the typical anamnestic (memory) characteristics of quicker, better, and more prolonged immune responses.

For three-quarters of a century scientists have known that fish are capable of producing specific serum factors, i.e. antibodies, that are generated in response to and will inactivate foreign pathogens. However, investigations of warm-water fish kept at sub-optimal temperatures and cold-water fish led some researchers to consider antibody production in fish to be vastly inferior to that of mammals. Neale and Chavin (1971b) state that fish show a low level primary antibody response to conventional antigens, such as bacterial vaccines, sheep red blood cells and bovine serum albumin. Heartwell (1975) said that the primary and secondary immune responses of fishes were often shorter, and fish secondary responses were more variable and weaker than in mammals.

Recent research using teleost species at optimal temperatures shows that some warm-water fish are indeed capable of primary and secondary responses that produce high titers of specific antibodies in relatively short periods of time (Avtalion et al., 1973; Heartwell, 1975; Sailendri and Muthukkaruppan, 1975; Collins et al., 1976). When tested, most fish species have also been shown capable of

producing antibodies against a wide range of naturally encountered and/or artificial antigens (Clem and Sigel, 1963; Ridgway et al., 1966; Corbel, 1975; Heartwell, 1975; Borysenko, 1976).

Goldfish also respond to various particulate and soluble antigens by producing antibodies. Carassius auratus is a warm water species but is also tolerant of cold water temperatures (Fry and Hart, 1948), which allowed some researchers to also examine the effects of temperature on production of antibodies in this species.

Watson et al. (1968) and Watson (1969) injected goldfish with live vaccines of either of two bacterial fish pathogens, Aeromonas liquefaciens or Streptococcus OX39. Antibody production was detected by a variety of methods, including bacterial agglutination. After the primary immunization with A. liquefaciens, the peak average agglutination titer was 1:1,024 on days 13 and 14. Measurable bacterial agglutination titers were detectable for at least 85 days. Secondary immunization resulted in a much higher peak titer and titers remained higher longer. Within a few days, secondary titers were much higher than peak primary titers. But these titers declined and then rose again to reach a peak average titer of 1:32,768 two weeks after the second immunization. Moderate agglutination titers were still found 65 days after the second immunization. A very similar pattern, but with slightly lower titers, was seen after the St. OX39 immunizations. Primary and secondary immunized goldfish were also challenged with known lethal doses of the respective bacteria, and the prior immunizations offered marked protection to these fish.

Neale and Chavin (1971b and 1973) immunized goldfish with an A. hydrophila protein complex (toxin). The protein was highly

antigenic and resulted in protection of the fish from lethal doses of the living bacteria within only 4-6 days. Multiple injections resulted in "a good titer of circulating antibody which selectively precipitated the soluble antigen, agglutinated the homologous bacteria, and conferred passive immunity to normal goldfish against challenge with living bacteria." They also could make mice passively immune to lethal doses of the living bacteria or their toxin by giving them injections of goldfish serum antitoxin.

These researchers also followed the primary antibody response in goldfish injected with heat-killed Salmonella typhi O-901 (Neale and Chavin, 1969 and 1971a). Two days after injection, antibody producing cells were found in lymphocytic tissues. Over time, the number of tissue leukocytes and antibody producing cells increased, but no direct bacterial agglutinating antibody was detectable in the fish sera. Use of a Coombs - type antibody allowed indirect detection of antibody in these sera by seven days after immunization. These titers increased throughout the 20-day experiment. Even the direct bacterial agglutinating antibody was detected after a second immunization.

Goldfish, at 4° or 22° C, were injected with a flagellar antigen from S. adelaide (Azzolina, 1978). The leukocytic actions that resulted in antibody production and titers of bacterial immobilizing antibody in the serum were then followed over the next five weeks. Kinetics of antibody formation were similar for both groups with peak titers reached by week three. Sera from warmer fish had higher titers at all times with a peak titer of around 1:4,000 versus 1:100 in the colder fish sera.

In contrast, Cushing (1942) found temperature did not have much of an effect on agglutinins made in goldfish injected with sea urchin sperm. Goldfish, at 14° or 28° C, were tested for these agglutinins for 25 days after they were immunized with a sea urchin sperm suspension. Antibody was detected by one week and reached peak titers (1:48) between weeks two and three. While sera from warm fish generally had detectable titers sooner, one cold fish had the highest titer measured.

Goldfish have also been immunized with the erythrocytes from humans or even from other goldfish. Rosen (1974) injected goldfish with human erythrocytes and measured the hemagglutinin titers induced. These erythrocytes were a much better antigen and resulted in higher antibody titers than when human saliva was used as an antigen. Warmer temperatures induced a quicker antibody response, but these antibodies were also removed from the fish's circulation quicker.

Hildemann (1956b) studied goldfish erythrocyte antigens by injecting individual fish several times with red blood cell suspensions from other goldfish. Hemagglutinin titers were detected in 12 days and peaked (at 1:1,024) in 25 days after the first immunization. Use of a similar protocol on a larger group of goldfish resulted in 30% of the fish developing agglutination titers $\geq$ 1:1,024 within three weeks of the first immunization (Hildemann, 1958).

Uhr et al. (1962) immunized goldfish with bacteriophage and in 2-4 weeks found about the same levels of neutralizing antibody as produced in analogously immunized mammals. They found higher levels of antibody in fish also immunized with Freund's adjuvant or kept

at elevated temperatures, but felt that repeated immunizations of this species did not elicit an anamnestic antibody response.

A commonly used soluble antigen with which goldfish are immunized is bovine serum albumin (BSA). Anti-BSA properties were examined after immunizations of goldfish with this antigen and an adjuvant (Everhart and Shefner, 1966; Everhart, 1971 and 1972). Goldfish anti-BSA was found to be at least as specific as rabbit anti-BSA in a gel double-diffusion precipitation reaction (Everhart and Shefner, 1966). Everhart (1971 and 1972) used the same antigen and also attached one of three haptens to BSA to immunize goldfish. He found that goldfish antibodies were very poor precipitins, yet the use of a sensitive passive hemagglutination technique allowed detection of antibody titers of up to 1:10,000. The antibody titers generated to these antigens in goldfish were similar to the titers found in immunized rabbits.

The passive hemagglutination test was also used to detect antibodies to BSA in goldfish given primary and secondary immunizations while kept at 20°, 25° or 30° C (Trump, 1967; Trump and Hildemann, 1970). Highest titers were often generated in fish kept at 25° C, but titers seldom were over 1:256. Nevertheless, higher temperatures generally resulted in a quicker response to antigens. No matter what the temperature, the secondary responses were also quicker forming than were the respective primary responses. In an additional study, Trump (1970) reported anti-BSA titers as great as 1:16,384 in goldfish given several booster injections of this antigen.

Other soluble protein antigens have also been found to cause an antibody response in goldfish. Rio and Recco (1971) found that

precipitins were formed in about two months in goldfish immunized several times with pooled human serum. Marchalonis (1971) immunized goldfish twice with specific human serum proteins (immunoglobulins G or M). Goldfish precipitins were able to distinguish among various classes of human immunoglobulins. Immunizations resulted in specific passive hemagglutination titers of up to 1:8,192.

Dryer and King (1948) immunized goldfish with horse serum or egg albumin. They then elicited anaphylactic responses when fish were given shocking doses of appropriate antigen at later times. The behavioral signs resulting from only the specific antigen first given the fish convinced the authors that a typical allergic anaphylactic reaction had occurred. The presence of allergic anaphylactic antibodies in goldfish is suggested by these results.

Evans et al. (1965) briefly mentioned their unpublished studies on goldfish immunized with keyhole limpet hemocyanin. Goldfish given primary and secondary doses of this antigen were found to produce precipitating antibodies. The abstract of a dissertation by Candiloro (1977) noted that goldfish kept at warmer temperatures had significantly higher antibody titers, but the antigens used were not specified.

Electrophoretic separation of sera from immunized fish has shown that antibody activity can reside in any of several fractions, but especially those analogous to the mammalian beta- and/or gamma-globulins (Corbel, 1975; Heartwell, 1975; Borysenko, 1976).

Uhr et al. (1962) found that goldfish anti-viral antibody activity was chiefly in the fraction corresponding to human gamma-globulin. Watson et al. (1968) and Watson (1969) found that

immunization of goldfish with bacteria resulted in increases in both the beta- and gamma-equivalent serum fractions and that absorption of this antibody activity resulted in decreases in both of these (but especially the gamma fraction). Marchalonis (1971) immunized goldfish with certain human serum proteins and also found that the resultant antibodies had electrophoretic mobilities of beta- or gamma-globulins.

Fish antibodies are the group of serum proteins called immunoglobulins (Ig). Fish antibodies are frequently described as of an IgM-like class because while some shark species appear to have a typical pentameric IgM, most fish species tested have a tetrameric IgM-like antibody molecule (Shelton and Smith, 1970; Acton et al., 1971; Klapper and Clem, 1972; Clem and McLean, 1975; Heartwell, 1975; Voss et al., 1978). There is some evidence for the presence of, or a shift to, a monomeric (IgG-like) antibody in fish (Uhr et al., 1962; Klapper and Clem, 1972). Although some fish have antibodies in their mucus and/or bile (Fletcher and Grant, 1969; Bradshaw et al., 1971; Spitzer et al., 1976; Lobb and Clem, 1979) and a few species have shown immediate hypersensitivity reactions (Dryer and King, 1948; Fletcher and Baldo, 1974; Baldo and Fletcher, 1976) other classes of immunoglobulins found in mammals (IgA, IgE or IgD) have not yet been demonstrated in fish.

Uhr et al. (1962) reported that goldfish immunized with bacteriophage shifted with time from production of heavier to lighter weight antibodies that were similar, respectively, to IgM or IgG in size. In contrast, Trump (1967 and 1970), Watson (1969), Trump and Hildemann (1970), Everhart (1971 and 1972) and Marchalonis (1971)

could find only one size of goldfish antibody, which Marchalonis (1971) said resembled the IgM antibodies of other vertebrates "in size and polypeptide chain structure." Reported immunizations of goldfish over periods of time of up to one year did not result in any shift in antibody size, but two populations of antibodies that differed in charge were detected by their different electrophoretic mobilities (Trump, 1967 and 1970; Trump and Hildemann, 1970; Everhart, 1971 and 1972). Watson (1969) reported that, "no component of C. auratus . . . was seen in the present study which had a migration rate or immunoelectrophoretic characteristics of IgG either in sera obtained from animals during the primary or anamnestic response in long-term immune studies, or in the serum of hyper-immunized fish." Marchalonis (1971) suggested that the IgG-sized antibody found by Uhr et al. (1962) was a degradation product due to the technique used.

Use of the Jerne plaque technique and other in vitro tests have shown that the cells producing antibodies in fish are located primarily in the anterior kidney and spleen. The number of fish species having one or more organ tested for antibody producing cells has increased rapidly and now includes the bluegill, Lepomis macrochirus (Smith et al., 1967), rainbow trout, Salmo gairdneri (Chiller et al., 1969a and b; Etlinger, 1975; Anderson, 1978; Anderson et al., 1969a, b and c), brown trout, S. trutta (Ingram, 1977), perch, Perca fluviatilis (Diener, 1970; Pontius and Ambrosius, 1972), black grouper, Mycteroperca bonaci (Ortiz-Muniz, 1969; Ortiz-Muniz and Sigel, 1971), gray snapper, Lutjanus griseus (Ortiz-Muniz, 1969; Ortiz-Muniz and Sigel, 1971), African mouth

breeder, Tilapia mossambica (Sailendri, 1973; Sailendri and Muthukkaruppan, 1975), rosy barb, Barbus conchonius (Rijkers and van Muiswinkel, 1977; van Muiswinkel et al., 1978), Crucian carp, Carassius carassius (Warr et al., 1977a) and goldfish (Neale and Chavin, 1969 and 1971a; Azzolina, 1978). Some of these studies also found lesser numbers of antibody producing cells in other organs such as the mesonephric kidney, thymus, liver, heart and peripheral blood. Lymphocytes and plasma cells were typically the leukocytes reported to produce antibodies.

An adaptation of the Jerne plaque technique was used by Neale and Chavin (1969 and 1971a) to detect antibody-forming cells in goldfish anterior kidney, body kidney and spleen. Fish were immunized with Salmonella typhi 0-901 and then sacrificed at selected times to obtain these potentially immunocompetent organs. Leukocyte syspensions from these organs were assayed for plaque (antibody)-forming cells (PFC) using sheep red blood cells (SRBC) sensitized with bacterial endotoxin. Increases in numbers of plaque-forming cells began in the kidneys on day two, peaked on day nine and continued through day 22. Antibody-forming cells were detected in the spleen on day five, peaked on day nine and returned to background levels quicker than did the kidneys. The authors were not surprised that the kidney was very active in the immune response as blast and mature cells of all the major blood cells were found in this organ. They felt that the longer induction of the spleen was due to the relative paucity of neutrophils in this organ resulting in slowed processing of antigen for subsequent induction of antibody formation. They also found that increases in numbers of leukocytes

in these three organs also paralleled the quicker and higher induction of plaque-forming cells in the kidneys versus the spleen.

Watson (1969) injected goldfish with radiolabeled phage and found that "the primary sites of antigen localization in C. auratus are also the principal areas of hemopoiesis in these animals, viz., anterior kidney or mesonphros, and to a lesser extent the liver, spleen, intestine and gonads." Watson considered it reasonable to assume that the principal sites of antigen localization could also be the areas of antibody synthesis. He suggested that future research in fishes attempt to correlate antigen localization with antibody production sites.

Since then, several studies have used the goldfish as a model examining aspects of Watson's idea. Azzolina (1975) said that it was possible that "the antigen binding cells are precursors of the antibody forming cells and the contact with the antigen triggers their proliferation and differentiation." He studied the antigen binding cells in spleen suspensions from goldfish immunized with flagellar antigen from S. adelaide. The antigen binding cells were small and medium-sized leukocytes. These cells increased in numbers and size within two days of immunization and then they probably emigrated from the spleen. Azzolina implied that the development of goldfish antibody forming cells was an open system which also involved the blood.

In a subsequent study, Azzolina (1978) once again immunized goldfish with S. adelaide flagellar antigen and isolated splenic antigen binding cells. He also used a bacterial adherance colony method to assay for antibody forming cells. The cells that bound

antigen had morphologies similar to medium and large lymphocytes. Lymphocytes from fish kept at 4° C bound antigen better than those from fish kept at 22° C, yet the number of antibody forming cells was always (non-significantly) higher in the warm adapted fish. Maximum numbers of antibody forming cells were found three or four weeks after immunization of the warm- or cold-adapted fish respectively.

Ruben et al. (1977) immunized goldfish with horse erythrocytes and then assayed anterior kidney, spleen and thymus lymphoid cells for antigen binding and for antibody production by using a rosette test. Immunization resulted in a several fold increase in numbers of both antigen binding and antibody producing cells within 6-10 days in anterior kidney and spleen but not in the thymus. Thymic antigen binding cell numbers doubled by 4-6 days after immunization, but antibody producing cells were absent at all times. A much lower dose of antigen resulted in even greater numbers of thymic antigen binding cells, but thymic antibody producing cells were still not evident.

DeLuca et al. (1978) immunized goldfish with three protein antigens and using immunofluorescence detected antigen binding cells in head kidney, spleen and thymus. Head kidney had the highest and thymus the lowest percentage of cells positive for antigen. All of the antigen binding cells had the morphology of lymphocytes.

In birds and mammals, humoral antibody-mediated and cell-mediated immune responses are due to the stimulation and/or interaction of the appropriate B (bursa derived) and T (thymus derived) lymphocytes. While some investigators are reluctant to accept the presence of T

and B cells in fish (McKinney et al., 1976; Sigel et al., 1978), several lines of research suggest that fish do possess two groups of lymphocytes acting in a fashion similar to the T and B lymphocytes of higher vertebrates (Marchalonis et al., 1978; Warr and Marchalonis, 1978; Lewis et al., 1979). Evidence for T and B cell co-operation is demonstrated by the following. First, a number of hapten-carrier effects have been noted in various fish species (Avtalion et al., 1975; Yocum et al., 1975; Ruben et al., 1977). Second, fish lymphocytes are composed of at least two populations, respectively stimulated by either T cell mitogens (phytohemagglutinin and concanavalin A) or B cell mitogen (lipopolysaccharide) (Lopez et al., 1974; Cuchens et al., 1976; Etlinger et al., 1976; Chilomonczyk, 1978). Finally, most of the fish species tested have lymphocytes with surface immunoglobulins. and goldfish lymphocytes have been examined in sufficient detail to demonstrate that the surface immunoglobins differ on the head kidney, spleen and/or thymus lymphocytes and that these also differ from the serum immunoglobulin (Warr et al., 1976a and b; Marchalonis et al., 1977; Ruben et al., 1977; Warr and Marchalonis, 1977; Warr et al., 1977b; DeLuca et al., 1978).

The serum immunoglobulin in the goldfish differs from those found on the surfaces of lymphocytes from the head kidney, spleen or thymus (Warr and Marchalonis, 1977; Warr et al., 1977a and b; Warr and Marchalonis, 1978). Serum immunoglobin was found to be a tetramer, while the immunoglobulins on lymphocyte surfaces were monomers with a heavy chain that differed in mass from the serum heavy chain. Warr and Marchalonis (1978) seemed to expect some

differences, "Such a distinction might be expected on purely thermodynamic grounds because serum immunoglobulins tend to be hydrophilic whereas surface immunoglobins exist in a fairly stable association with the plasma membrane lipid environment."

Surface immunoglobins have been found on a large proportion of the lymphocytes from anterior kidney, spleen and thymus of the goldfish (Marchalonis et al., 1977; Warr et al., 1977a). The immunoglobulin of the thymic lymphocytes seems to be endogenously produced and when removed is resynthesized and reexpressed on the cell surface (Warr et al., 1976a and b; Marchalonis et al., 1977). Non-lymphoid cells (erythrocytes and granulocytes) did not seem to have any immunoglobulin on their surfaces (DeLucca et al., 1978).

Goldfish cell surface immunoglobulins appear to function in the specific recognition of antigen. Antigen binding by these lymphocytes could be significantly inhibited by prior treatment with rabbit antibodies made against goldfish IgM (Ruben et al., 1977). When antigen was bound before anti-IgM treatment, the patches where antigen was bound coincided with the areas of anti-IgM (DeLucca et al., 1978). Ruben et al. (1977) felt that "Probably all lymphocytes in the goldfish express surface immunoglobulins which can function as receptor for antigen."

Goldfish lymphocytes seem divisible into classes which parallel the respective mammalian B and T cell functions of humoral antibody production and cell-mediated responses. The lymphocytes of the head kidney, spleen and thymus express cell surface immunoglobulins which differ in their mass, solubility and other characteristics (Warr et al., 1976a and b; Marchalonis et al., 1977; Ruben et al.,

1977; Warr et al., 1977a; Marchalonis et al., 1978). Thymus lymphocytes express only one kind of immunoglobulin, spleen lymphocytes express a different immunoglobulin and anterior kidney lymphocytes are a mixture of both kinds of cells (Ruben et al., 1977). The different surface immunoglobulins could be markers for the respective T- and B-like cells for this species. Thymus lymphocytes are suggested to be helper (T) cells and spleen lymphocytes to be antibody forming (B) cells (Ruben et al., 1977; Warr et al., 1977a; Marchalonis et al., 1978). B-type antibody forming cells were restricted to the spleen and anterior kidney and were not found in the thymus (Ruben et al., 1977; Warr, et al., 1977a). Marchalonis et al. (1978) felt that these and other results were "compelling support for the conclusion that separable populations of T-like and B-like cells occur in teleosts . . ."

The presence of T-cell-mediated immune responses in goldfish is also indicated by research examining rejection of foreign tissue (scale) grafts in this species.

Transplantation of scales between members of the same fish species (allografts) has been shown to be equivalent to skin graft transplantation in mammals. Several papers by Hildemann investigated the time relationships and temperature sensitivity of primary and secondary scale rejections and established the immunological basis of this cell-mediated response in the goldfish (Hildemann, 1956a, 1958, 1962 and 1970; Hildemann and Owen, 1956; Hildemann and Haas, 1960).

Goodrich and Nichols (1933) were among the first to transplant goldfish scales between individual fish. They were interested in the

resultant scale pigment changes seen in various color varieties of goldfish. Scales grafted onto a different area of the same fish (autografts) showed little change, while grafts made between different individuals of the same or other varieties degenerated rapidly. An initial inflammatory reaction was frequently seen shortly after grafting and was followed by loss of donor pigment cells. Abundant leukocytes were detected in the graft tissues. The authors suggested that these results were due to a "tissue incompatability" between individual fish.

Hildemann examined several aspects of the immunological basis of goldfish scale graft rejections. Goldfish were found to rapidly detect "not-self" scale tissue and respond with "an intense, local cellular reaction which mediates homograft [=allograft] destruction." (Hildemann, 1956a). The cellular tissues of the donor scales never persisted (Hildemann, 1957).

The sequence of events in rejection of foreign scale grafts was determined and the discrete pigment loss phenomena called "clearance" was used as a close approximation of graft survival end points (Hildemann, 1957). Clearance was the number of days it took for 100% loss of the orange pigment in a foreign scale. Individual clearance times were pooled and a median survival time estimated which was used to measure the degree of host immune responses against grafts from one or even several donors.

All of the foreign scales grafted caused a host immune response (Hildemann, 1956a, 1957 and 1958). A latent period after grafting was due to the time it took for host leukocytes to infiltrate and start phagocytosis of donor tissues. Host lymphocytes were found

to infiltrate donor tissue from all areas of contact. The presence of sensitized leukocytes was suggested as a reason for second sets of scale grafts being "destroyed more rapidly" than were first set of grafts.

Second set scale grafts survived only about 50-67% as long as first set grafts (Hildemann, 1957 and 1958). Third and fourth set grafts were not rejected significantly quicker than were second set scale grafts. Temperature had an effect on both first and second set rejection rates.

Keeping fish in water from 10° - 32° C showed that this immunological response was temperature dependent (Hildemann, 1957). Low temperatures greatly reduced any expected inflammatory reactions. High temperatures resulted in rapid rejection with cytoxic responses being so rapid at temperatures $\geq$ 28° C that conspicuous hemorrhage in contact areas was seen within one day of graftings.

Hildemann and Haas (1960) described their results in comparison to other animals and concluded that fish scale-graft responses may be "even more sensitive and vigorous than that of higher vertebrates." Goldfish destroyed skin (scale) grafts quicker than most other species tested and the simple techniques and equipment necessary offered several advantages over typical skin transplantation test methods.

DDT, Fish Hematology and Immunology

Few research studies have been primarily designed to examine the effects of DDT, or other insecticides on fish hematology or immunology. Most reports consist of little more than sidelights

scattered throughout the literatures of ichthyology or toxicology. Information gleaned from the literature is very suggestive of the possibility that fish hematological and immunological systems are adversely influenced by insecticides. Changes in the following structures and functions have been proposed as indicators of insecticide-caused alternations in fish hematology and/or immunology (Zeeman and Brindley, in press).

1. Structures
    a. Hematopoietic areas of the head kidney, mesonephric kidney, spleen or thymus.
    b. Numbers or types of circulating blood cells, or associated measures (i.e. hematocrit or hemoglobin)
    c. Serum (or plasma) protein levels.
2. Immune Functions
    a. Phagocytosis
    b. Inflammation
    c. Humoral antibody response
    d. Cell-mediated response
3. Immune Regulated
    a. Infection and disease susceptibility
    b. Tumor formation

Frequently all that has been reported after exposure of fish to insecticides were changes in serum protein levels, electrophoretic patterns, and/or disease or tumor incidences. Changes in these criteria are probably only marginal indicators of insecticide effects upon fish immune systems for three reasons. First, while antibodies are part of the proteins carried by the serum, they probably are no

more than 10% of the total. Second, fish antibody activity may reside in any of a variety of the serum globulin fractions. Third, disease or tumor incidence are also affected by sufficient contact with causative agents and by the "stress" caused by numerous environmental factors.

In contrast, more reliable indicators of changes in fish hematology and immunology may be the depletion of hematopoietic tissues or lymphoid elements from immunocompetent organs, decreases or increases in numbers or types of circulating blood cells (or associated measures), and especially any alterations in immune functioning.

As far as DDT is concerned, very little research has been done in most of these indicator areas.

Structures. A brief report of Shimada's research (1972) mentions that DDT caused damage and/or necrosis in several fish organs, including kidney and spleen. Walsh (1974) and Walsh and Ribelin (1975) reported that DDT and six other pesticides caused various pathological effects in organs of lake trout (Salvelinus namaycush) and coho salmon (Oncorhynchus kisutch). One organ consistently affected by pesticide treatment was the spleen. Two months of exposure to DDT caused reductions in the number of lymphocytes in the spleens of most fish. These spleens were also often significantly reduced in weight, and in 30% of the lake trout they were very small, shrunken, and pale in color. King (1962) also noted that the spleens of guppies (Poecilia reticulata) exposed to DDT for one month were generally shrunken in size and pale in color, which she attributed to absence of or reduction in

stored red blood cells.

For up to 20 months, Allison et al. (1963 and 1964) exposed cutthroat trout (S. clarki) to DDT in their food or water. Hematocrit was routinely measured and no differences between control and treated fish were seen. Cruea (1968) incubated DDT for 30 minutes in blood taken from carp (Cyprinus carpio). DDT caused the hematocrit and total plasma protein levels to decrease, but increased the hemoglobin level.

Immune functions. No research has been done in this area.

Immune regulated. DDT has been implicated in affecting diseases in fish. In 1962, King reviewed the histopathological effects of DDT upon fish and suggested that DDT could cause increased sensitivity of fish to diseases. Three research articles appeared shortly thereafter which presented evidence that DDT indeed did seem to affect incidence of fish diseases.

Schoenthal (1963) exposed several species of fish to DDT for two days and followed the resultant mortality for extended periods of time. As a side light, he noted that diseases were much more prevalent in the DDT treated fish. In mountain whitefish (Prosopium williamsoni), and to a lesser extent in trout, furunculosis and fungal diseases were more common in fish exposed to the insecticide. Similarly, Allison et al. (1963 and 1964) followed the mortality of cutthroat trout exposed to DDT. Fish were exposed to DDT-contaminated food or water at regular intervals over a 20-month period. All dying fish were examined for external symptoms of disease. The number of fish showing symptoms of disease was always greater in the DDT-treated fish. The incidence of disease tended to

increase with increased DDT dose.

A group of researchers recently noted a relationship between DDT and a fin erosion disease in Dover sole (Microstomus pacificus). Of the over 20,000 Dover sole collected off the Palos Verdes peninsula of southern California from 1972 to 1976, almost 40% showed a fin erosion disease (McDermott-Ehrlich et al., 1977). Due to a municipal wastewater discharge, the sediments in this area are contaminated with approximately 200 metric tons of total DDT residues (MacGregor, 1976; Mearns and Sherwood, 1977). Dry weight analyses of sediment samples often showed total DDT residues exceeding 100 parts per million (ppm). In the laboratory, healthy Dover sole were kept in aquaria with these contaminated sediments (Sherwood, 1976). Within 13 months, these fish showed early signs of fin erosion that greatly resembled that seen in fish caught off of Palos Verdes. Analysis of the DDT residues in healthy and diseased fish from the Palos Verdes area showed that DDT levels were significantly higher in the diseased fish (McDermott-Ehrlich et al., 1977; Sherwood and Mearns, 1977). While this evidence strongly infers that high levels of DDT affect this fin erosion disease, these sediments were also somewhat contaminated with PCBs, heavy metals, and other pollutants.

Several insecticides are suspected of causing tumors (and/or cancer) in mammals (Fishbein, 1976; Sternberg, 1979) and in fish (Brown et al., 1973; Kraybill, 1976). The association of fish tumors with suspected or actual environmental contamination with DDT (Dawe et al., 1964; Ljungberg, 1976) focuses attention upon this insecticide. DDT remains the only pesticide with experimental

evidence linking it to tumors in fish. High levels of DDT encorporated into the diet of rainbow trout resulted in increased incidence of liver tumors. Ashley (1970) gave trout a diet containing from 80 to 19,200 ppm of DDT. All doses, except the lowest, were lethal in two months. Low dose survivors were followed for up to 20 months, but less than 1% of the fish had hepatomas. The experiments of Halver (1967) and Halver et al. (1962) as elaborated upon in World Health Organization (1974), also exposed trout to DDT in their food for up to 20 months. A higher incidence of liver tumors was seen in the DDT groups. In one experiment, almost 37% of the trout given the higher dose of DDT developed hepatomas. The question remains as to whether DDT acts strictly as a tumor causing agent, or if it perhaps suppressed trout immune systems and caused increased susceptibility to other tumorigens.

Considering the fact that DDT has been such a widely used insecticide and that it still frequently contaminates the aquatic environment, it seems somewhat surprising that the effects of DDT on fish hematological and immunological systems has received so little attention. Considering that Johnson in 1968 reviewed the effects of DDT on fish disease resistance and said that "This may indicate a DDT effect on the ability of fish to produce antibodies, or interference in some other way with their natural, internal-defense mechanisms," it seems time to provide some evidence to support or refute those ideas.

OBJECTIVES

This research was intended to assist in filling in some of the gaps in our knowledge of the effects of DDT on normal fish hematological parameters and immunological functioning.

The first objective was to determine what levels of DDT given intraperitoneally (i.p.) were lethal to the goldfish. An acute (96-hr) LD_{50} study was performed. From this information, sub-lethal doses of DDT were estimated and used in the subsequent sub-acute studies.

A second objective was to determine the effects of DDT dose on normal goldfish hematology. Seven common hematological parameters were followed after an acute or sub-acute administration of this insecticide.

A third objective was to determine the numbers of immunocompetent cells found in various organs of this species. Effects of sub-acute administration of a sub-lethal dose of DDT on these cells was also to be determined.

The last objective was to determine a few normal humoral and cell-mediated immune responses of the goldfish and to see if sub-acute administration of sub-lethal doses of DDT would affect these immune functions.

MATERIALS AND METHODS

General

Adult goldfish were purchased (Ozark Fisheries, Inc., Stoutland, MO), shipped air freight to Salt Lake City and delivered here. To control external parasites, fish were dipped for one minute in water containing 50 ppm of malachite green oxalate (Baker Chem., Phillipsburg, NJ) and 250 ppm of 37% formalin (Fisher Sci. Co., Fairlawn, NJ). Fish were then distributed amongst six aerated 250 liter aquaria containing aged tap water filtered with Dynaflo Motor^{R} Filters (Model 430, Metaframe Corp. Paterson, NJ) containing aquarium-grade bone char charcoal and filter fiber. Fish were acclimated to the experimental temperature (25 ± 1° C) and photoperiod (10 hr light - 14 hr dark) for a minimum of two weeks before use in any experiment. Feed consisted of TetraMin^{R} large flake staple food given at a rate of approximately 0.1% of body weight/day when fish were simply being maintained and approximately 0.2% of body weight/day during an experiment.

The fish were anesthetized before almost all test procedures. A synergistic mixture of MS-222 (Tricane methanesulfonate; Crescent Research Chem., Inc., Paradise Valley, AZ) and quinaldine (Sigma Chem. Co., St. Louis, MO) was used for most test procedures (Schoettger and Steucke, 1970; Gilderhaus et al., 1973). A stock solution mixture of these two chemicals was added to a bucket of aged tap water to yield final concentrations of about 60 ppm MS-222 and 25 ppm quinaldine. This anesthetic mixture resulted in loss of the fish's righting response in 2-3 minutes. Shortly thereafter,

fish could be removed from the water and handled for approximately two minutes before having to be returned to water. Returning the fish to water resulted in relatively normal opercular movement and swimming in 3-5 minutes. Fish were often willing to feed about 10 minutes after being returned to water.

One experiment (scale-graft transplantation) required extended handling of fish. A solution of 70 ppm MS-222 alone caused sufficient but light enough anesthesia that fish could be kept in the anesthetic for over 30 minutes with no difficulty.

When necessary, fish were bled from the posterior dorsal aorta using disposable syringes and needles. If this technique did not result in sufficient blood, a fresh needle and syringe were used and cardiac puncture attempted. In rare cases this did not produce enough blood. If those fish were going to be sacrificed, the caudal peduncle was severed and blood collected from the posterior dorsal aorta into capillary tubes.

When serum was desired, no anticoagulant was used. When unclotted whole blood or plasma was necessary, needles and syringes were coated before use with appropriate amounts of sodium heparin (Control No. A942955, GIBCO, Grand Island, NY). Several anticoagulants have been used for fish blood (Klontz & Smith, 1968), but heparin is the most widely used and with carp blood caused the least problems in evaluation of normal hematological parameters (Hattingh, 1975; Smit et al., 1977).

LD_{50}

To determine how toxic DDT was to goldfish, six groups of six

fish each were given intraperitoneal (i.p.) injections of 99 + % p,p'-DDT [1,1-bis-(p-chlorophenyl)-2,2,2-trichloroethane; Aldrich Chemical Company, Milwaukee, WI] dissolved in corn oil at concentrations resulting in total body doses of 0, 10, 50, 100, 250 or 500 mg DDT/kg fish. As fish size influences toxicity of DDT (Marking, 1966; Buhler et al., 1969; Buhler and Shanks, 1970), 96 hour LD_{50}'s (doses causing 50% lethality in 96 hours) were determined using these doses on 36 small, 36 medium and 36 large fish (approximately 20, 50 or 110 grams respectively). Cumulative percentage mortality for each dose was recorded at 96 hours. Blood was drawn from all of these fish at 96 hours (or before, if moribund) for the acute hematology test.

Acute Hematology

Effects of DDT on blood were analyzed using a series of hematological tests (Table 1) that were run in the following manner. Up to 0.5 ml of blood was drawn from one fish into up to 0.025 ml heparin (135 U.S.P. units of heparin/ml blood) and the syringe gently rotated to mix its contents. Six to 10 Yankee Microhematocrit Tubes (Clay-Adams, New York, NY) were quickly filled to three-quarters of their capacity with this blood. Up to three of these tubes were plugged with Seal-Ease (Clay-Adams) and stood vertically for one hour to let the erythrocytes settle.

One unplugged tube of blood was touched to one end of Bev-1-edge^{R} slides (Propper Manufacturing Company, Inc., Germany) and blood smears were made. Smears were air dried and then fixed and dehydrated in absolute methanol for five minutes. The smears were

Table 1. Symbols for and units of the hematological parameters measured in the goldfish.

PARAMETERS	SYMBOLS	UNITS
Erythrocyte		
Number	#RBC	$10^6/mm^3$
Micro-Hematocrit	MH	%
Hemoglobin	Hb	g/dl
Sedimentation Rate	ESR	mm/hr
Plasma		
Total Protein	TPP	g/dl
Leukocyte		
Number	#WBC	$10^3/mm^3$
Differential		
Lymphocytes	L	%
Neutrophils	N	%
Monocytes	M	%
Thrombocytes	T	%

air dried again and stored in a slide box until stained. The differential leukocyte count was read at a later time.

Numbers of erythrocytes (#RBC) and leukocytes (#WBC) were counted in the same operation. A Sahli pipet was used to withdraw 20 µl of blood from a microhematocrit tube. The blood was pipetted into 1.98 ml of freshly mixed and filtered Yokayama's fluid (Yokayama, 1947). This was mixed gently and a sample pipetted into an American Optical Improved Neubauer Phase Hemacytometer. Cells were allowed to settle for a minute and numbers of cells counted at 450X using an American Optical Phase Contrast Microscope. Erythrocytes were counted in an area of 0.2 mm^2 and leukocytes in an area of 1 mm^2 (Hesser, 1960). Multiplication of the erythrocyte totals by 5,000 and the leukocyte totals by 1,000 gave their respective totals in 1 mm^3 of blood.

One-half tube of blood was put in an AO Hemoglobinometer (Buffalo, NY), the blood was laked with hemolysis applicators for 30 seconds and a hemoglobin (Hb) reading taken (g/dl). There was often enough blood so that this procedure could be replicated and an average taken.

Tubes that had been plugged and stood vertically for an hour were examined and the fall of the erythrocytes (erythrocyte sedimentation rate = ESR) measured (mm/hr). These tubes were then spun in a TRIAC centrifuge (Clay Adams) at 10,500-12,600 g (9,500-10,400 rpm) for three minutes. The tubes were removed and the microhematocrit (MH) read as percent packed cell volume on a Lancer Critocap Microhematocrit Tube Reader (Sherwood Medical Industries, St. Louis, MO). These tubes were then snapped just above the packed

cell-plasma interface and the plasma blown onto the reading surface of a National Clinical Refractometer (National Instrument Company, Inc. Baltimore, MD) and the total plasma protein (TPP) levels read (g/dl).

A few days after the blood smears were made, they were stained and covered with a slip. In order to stain smears, slides were individually covered with Wright's stain (Lot 6092G, Harleco, Gibbstown, NJ) for five minutes. Then, drop by drop, pH 6.4 Wright's buffer (Davidsohn and Nelson, 1974) was added and left for an additional two minutes. Slides were then rinsed lightly with distilled water and then were allowed to air dry. A fresh mixture of 98 ml of deionized water plus 2 ml of Giemsa stain (Log 6281G, Harleco) was made in a staining dish. Slides were put in stain for 20-30 minutes. Slides were then rinsed lightly with distilled water and again allowed to air dry. Slides were stored overnight in a slide box and the next day immersed in xylene for at least five minutes before covering with Coverboard^{R} adhesive (lot 6176 G, Harleco) and glass coverslips. Slides were allowed to set for a few days before differential leukocyte counts were made.

For the differential count, a slide from each fish was examined and as suggested by Schalm et al. (1975) duplicate differential leukocyte counts were made for each slide and the results averaged. Blood smears were examined under oil immersion (1,000 X) and 200 leukocytes were counted. The proportions of various leukocyte types (% L, N, M and T = % lymphocytes, neutrophils, monocytes and thrombocytes) were recorded.

When difficulty in determining nuclear structure, cytoplasmic

granules or other criteria was noted, phase contrast microscopy was used to differentiate between the types of leukocytes.

Sub-Acute Hematology

Fish were given multiple doses of three sub-lethal levels of DDT over a 56 day period in order to examine the effects of prolonged exposure to DDT on fish hematology. Thirty medium-size fish were divided into three groups of 10 fish each. These three groups of fish were given doses of 0, 10 or 50 mg DDT/kg fish on days 0, 26 and 52 respectively. All fish were sampled for blood 96 hours after the last dose of DDT, and the same hematological parameters were measured as in the acute hematology protocol.

General Immunology

Immunological tests using goldfish generally lasted from one to two months. Fish in these experiments were, on the average, restricted to medium size. As with the sub-acute hematology test, sub-lethal levels of DDT (0, 10 or 50 mg DDT/kg fish) were given in multiple doses over these extended test periods.

Jerne-Plaque Assay

Fifty-four control fish were given primary immunizations and 61 control fish were given primary and secondary immunizations with sheep red blood cells (SRBC). At specified intervals after immunizations, fish were sacrificed, and selected organs and tissues were tested to determine if leukocytes were present that were capable of making antibodies against SRBC. The relative numbers of these antibody forming cells and the times when they were present were

determined.

Effects of DDT on the numbers of these antibody forming cells were determined by pre-treating two other groups of fish with 50 mg DDT/kg fish two days prior to their primary, or primary and secondary immunizations with SRBC. There were 35 fish given DDT and used in the primary test, and 40 fish given DDT and used in the primary and secondary tests. These fish were also sacrificed at specified times, and their organs and tissues tested for the numbers of antibody forming cells.

Antigenic and antibody detecting SRBC were treated as follows. Fresh sheep blood was collected into EDTA (K_3)-containing B-D Vacutainer^R tubes. Within 30 minutes, fresh blood was pipetted into an equal volume of sterile Alsever's solution (Garvey et al., 1977), mixed gently, and stored at 4° C until used. This method permitted the storage of whole sheep blood for up to 10 weeks. Therefore, the cells from the same sheep could be used in immunization and also in the subsequent testing systems.

SRBC were prepared for use by discarding the Alsever's supernatant and washing the cells three times with sterile phosphate buffered saline (PBS) pH 7.2 (Herbert, 1973). Packed cells were then diluted to 30% (V/V) SRBC in sterile PBS.

A suspension of 30% of SRBC was used as the antigen and fish were immunized at a rate of 1 ml 30% SRBC/100 g fish, which resulted in an approximate dose of 5.9×10^9 SRBC/100 g fish. Primary and secondary immunizations were at this rate. Secondary immunizations were given 28 days after the primary immunization.

At appropriate times after immunizations, fish were bled and

the sera were saved for subsequent antibody (anti-SRBC) analyses. Usually these fish were then sacrificed and the following organs and tissues examined for antibody forming cells: anterior kidney, spleen, mesonephric kidney, liver, intestine and peripheral blood. Goldfish antibody forming cells (plaque forming cells = PFC) were detected using a Jerne-plaque technique (Jerne and Nordin, 1963) as modified by Cunningham and Szenberg (1968).

Organs or tissues were excised into sterile PBS containing 10% agamma calf serum (Control # R761419, GIBCO). Then most organs were gently rubbed against a sterile 25 mesh/cm stainless steel screen and the resultant cells and debris rinsed with PBS into sterile Syracuse dishes. Sterilized mini-funnels with small cotton plugs were then used to filter the debris.

This procedure was not necessary for the peripheral blood and was ineffective in separating cells from the intestine. Therefore, peripheral blood was collected into heparinized capillary tubes and diluted directly using 10% calf serum in PBS. A small section (about 2 cm) of intestine was rinsed with PBS and cut into smaller sections (about 2 mm) which were collected into 10% calf serum in a small blender and blended at 8,000 rpm for 15 seconds using a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, CN). Intestinal debris was filtered using a plugged mini-funnel.

The volume of each organ or tissue sample was adjusted to one ml using 10% calf serum. The leukocyte numbers/0.02 mm^3 were estimated by using a phase hemacytometer and a phase contrast microscope at 450 X. Multiplication by 50,000 gave the number of leukocytes/ml of sample. Use of phase contrast microscopy allowed

direct counting of viable ("bright") cells, as preliminary experiments demonstrated that phase contrast microscopy detected dead cells as well as the trypan blue exclusion method. In most cases, about 90% of the leukocytes were viable.

Samples were taken from each of the leukocyte cell suspensions for a Jerne-plaque assay. One-tenth of a ml of the suspension was mixed with 50 µl of 10% calf serum, 10 µl of 30% SRBCs and 10 µl of goldfish complement. The complement was made from unimmunized fish serum which had been adsorbed with 10 µl of packed SRBC at 4° C for 30 min. and then diluted 1:4 with PBS.

This mixture was pipetted into three chambers made by attaching three 22 x 22 mm glass coverslips to a slide by using Scotch Double Stick Tape (Cat. # 137, 3M Company, Minneapolis, MN). Total volume pipetted into the chambers averaged about 0.1 ml. The exposed edges of the chambers were sealed using a mixture of Vaseline^{R} and mineral oil. The slides made were then incubated 4 hours at 25 ± 2° C. Expanding circles of lysis (plaques) formed around those leukocytes producing antibody to SRBC. Numbers of plaques were counted at 0.5, 1, 2, 3 and 4 hours using a dissecting microscope at 20 X. Estimates of the number of leukocytes/ml and volume of mixture used allowed calculations of the numbers of plaque-forming cells (PFC)/10^{6} leukocytes (WBC).

Anti-SRBC Titers

Fresh sera from fish previously immunized with SRBC were kept separately in 0.5 dram vials and heated at 50° C for 20 minutes in a heat block. This regime has been shown to inactivate goldfish

complement (Legler and Evans, 1967). These sera were then frozen and kept at -20° C until analyses for a SRBC lytic antibody (hemolysin) and a SRBC agglutinating antibody (hemagglutinin) were performed.

Analyses for hemolysin and hemagglutinin were performed using a manual microtiter kit (Cooke Engineering Company, Alexandria, VA) for pipetting, diluting and mixing. Complement dependent hemolysin titers were determined using "U" bottom microtiter plates and complement independent hemagglutinin titers using "V" bottom microtiter plates. Procedures and titer endpoint criteria were those outlined in Conrath (1972).

Two-fold sequential dilutions of 25 µl of test sera were made in 25 µl of PBS. Then 25 µl of a 1% SRBC suspension in PBS was added and the plates agitated gently. Hemagglutinin plates were temporarily set aside.

For the hemolysin test, fresh sera from unimmunized goldfish must be used as the complement source. Preliminary tests using guinea pig complement showed this mammalian standard to be completely ineffective as a complement source for goldfish hemolysin tests. Fresh goldfish sera was adsorbed with packed SRBC to remove heterophile antibody. The supernatant sera was diluted 1:10 with PBS and 25 µl pipetted into each hemolysin well and the hemolysin plates gently agitated. All hemagglutinin and hemolysin plates were then covered with parafilm and incubated for 4 hr at 25° C before reading using a microtiter test reading mirror.

Anti-BSA Titers

To test the effects of DDT on the dynamics of antibody formation to bovine serum albumin (BSA) as a soluble antigen, four groups of six fish each were treated as follows.

Control fish were given neither BSA nor DDT. BSA control fish were given an i.p. dose of BSA (as prepared below) at time zero. The other two groups of fish were also given BSA at time zero, but, in addition were also given i.p. doses of either 10 or 50 mg DDT/kg fish one week before and four weeks after the BSA immunization. Starting one week after immunization, fish sera were collected via cardiac puncture at weekly intervals until week 10 and also at weeks 14 and 18. Sera were frozen and kept at -20° C until anti-BSA titers could be determined using a passive hemagglutination (PHA) technique described below.

BSA antigen was prepared as follows. Forty milligrams of crystallized, >96% pure BSA (Lot #44C-8000, Sigma Chemical Company, St Louis, MO) was dissolved in 10 ml of PBS. Five one-ml aliquots of this solution were injected sequentially (using a 1-ml syringe and 25 gauge needle) into 5 ml of Freund's Complete Adjuvent (Difco Labs, Detroit, MI) in a small sterile serum bottle. The 10 ml of resultant emulsion were further mixed and thickened by using a 2.5 cc syringe and working up to a 21 gauge needle for withdrawing and reinjecting the emulsion. This emulsion had a BSA concentration of 2 mg/ml and was injected i.p. at the rate of 0.25 ml/100 g fish. This procedure resulted in fish getting a total body dose of 0.5 mg BSA/100 g fish.

The anti-BSA titers were detected using a PHA technique modified

from that described by Herbert (1973). The detection system consisted of SRBC coated with BSA and thereby agglutinated in sera containing anti-BSA.

Fresh SRBC were collected in anticoagulant-containing vacutainers. The cells were washed three times with PBS and centrifuged. Six-tenths of a ml of packed SRBC were washed once more and resuspended in 10 ml of PBS. Ten ml of freshly made 1:50,000 tannic acid solution (tannic acid lot # 64C-0093, Sigma Chemical Co.) was added to the SRBC solution, mixed well and incubated at 37° C for 15 minutes. The tanned cells were centrifuged, washed once with 20 ml of PBS, centrifuged again and resuspended in 10 ml PBS. The SRBC were then coated with BSA by adding a BSA solution (20 mg BSA in 10 ml PBS), mixing gently and incubating at 37° C for 30 minutes. This mixture was gently shaken twice during incubation. The tanned and coated cells were then washed three times in 20 ml of 1% normal sheep serum in PBS (1 ml of complement inactivated and adsorbed sheep serum /100 ml PBS). The background sheep serum levels help prevent auto-agglutination of these tanned and coated cells.

A manual microtiter kit was used to determine anti-BSA titers. Only the "V" bottom microtiter plates were used. In a procedure similar to the hemagglutinin test, 25 µl of test serum was serially diluted in 25 µl of diluent (1% normal sheep serum in PBS) and 25 µl of BSA coated SRBC was added. Plates were gently agitated, covered with parafilm and incubated overnight (~ 18 hrs) before reading hemagglutination titers using a microtiter test reading mirror.

Scale-Graft Rejection

Rejection of foreign scales grafted between individual goldfish has been shown to be due to a host cell-mediated immune response to the foreign tissues of the grafts (Hildemann, 1957 and 1958). Techniques for transplantation and criteria for scale rejection (100% loss of orange pigment cells) were adapted from Hildemann (1957).

Three groups of five goldfish each were pretreated with 0, 10 or 50 mg DDT/kg fish two days before primary transplantation. Each group of five fish treated alike were grafted as follows. All five fish were anesthetized in about 8 liters of water containing 70 mg MS-222/liter. This concentration maintained a light anesthesia whereupon fish could be removed one at a time and the following done. Four alternative scales were plucked from the first row of scales above the lateral line just posterior to the operculum. These scales were placed in a diagonal array in spot plate depressions containing sterile PBS. The fifth and sixth alternate scales were then simply switched in positions, i.e. autografted, as controls. One at a time, the other four fish were treated in the same fashion. Then the first fish was again removed from the anesthetic solution and a linear array of scales (arranged so that it received one foreign scale from each of its four cohorts) were transplanted, i.e. allografted, into the four open scale sockets of this host fish. One at a time, the cohort fish were then allografted in the same fashion. Some foreign scales were too large to fit in the host scale socket space and were, therefore, trimmed with fine scissors at the edges and/or base of the scale in order to achieve a snug fit. Nevertheless, 15% of the

allografts were lost, most in the first day after transplantation. Only 0.6% of the autografts were lost.

Two days after primary transplantation, allografts were scored for percentage loss of orange pigment from each scale. The day when a foreign scale had lost 100% of the orange pigment was the day that scale was scored as "cleared" and ultimate rejection was assured (Hildemann, 1957). Each fish was examined daily until all remaining foreign scales were "cleared" -- a maximum of 10 days. Autografts never showed significant clearance.

Twenty six days after the primary dosing, the same fish were dosed again with either 0, 10 or 50 mg DDT/kg fish and two days later a secondary grafting was performed. This procedure was identical to the primary grafting except that the first row of scales below the lateral line were used and scoring for percentage clearance began one day after this grafting.

These primary and secondary procedures were replicated using three new groups of five fish each. The results from these replicates were pooled to bring the sample size for each treatment to 10 fish.

RESULTS

LD_{50}

DDT is relatively less lethal to larger size goldfish (Table 2). Linear regressions on the 96 hour cumulative percentage mortality data for the six doses of DDT used showed that the calculated LD_{50} increased with the size of fish. Correlation coefficients for the regression lines on dose-mortality data for small, medium and large fish were 0.79, 0.90 and 0.96 respectively. The reduction of DDT effects as fish size increases is also seen in the following acute hematology data.

Acute Hematology

In general, increase in the dose of DDT caused progressive changes in many fish hematological parameters (Tables 3 and 4). Often numbers of erythrocytes (#RBC), and the closely associated microhematocrit (MH) and hemoglobin (Hb) levels, decreased with increasing level of DDT. Increased DDT also elevated the erythrocyte sedimentation rate (ESR), often decreased total plasma protein (TPP) levels and frequently reduced the number of leukocytes (#WBC). Proportions of the remaining leukocytes changed, with percent lymphocytes (% L) decreasing and neutrophils (% N) and monocytes (% M) increasing in percentage.

Increase in fish size ameliorated the magnitude of effects of DDT on these hematological parameters. In general, the larger the fish, the less of an effect the same level of DDT had. Significant changes in small fish hematology were seen by doses $\geq$ 100 mg/kg while medium and large fish parameters were often significantly

Table 2. Acute intraperitoneal doses of DDT estimated to cause 50% mortality in 96 hours to three sizes of goldfish.

Descriptive Size	Average Weight (g) $\bar{X}$ ± S.E. (n)	Estimated LD_{50} (mg DDT/kg fish)	Regression* Coefficient
Small	19.4 ± 0.6(36)	213.3	36.0
Medium	49.1 ± 1.4(36)	480.5	43.2
Large	112.3 ± 3.0(36)	578.8	69.9

*Linear regression coefficients (log dose versus percentage mortality)

Table 3. Acute effects of intraperitoneal injection of DDT on the erythrocyte and plasma parameters of small, medium and large size goldfish.[a]

Fish Size	DDT Dose (mg/kg fish)	Erythrocyte				Plasma
		#RBC (10^6/mm^3)	MH (%)	Hb (g/dl)	ESR (mm/hr)	TPP (g/dl)
Small	0	1.71 ± 0.10	35.3 ± 1.4	8.16 ± 0.54	1.22 ± 0.18	4.91 ± 0.21
	10	1.75 ± 0.12	34.8 ± 1.6	8.30 ± 0.66	1.38 ± 0.16	4.61 ± 0.16
	50	1.50 ± 0.13	32.4 ± 2.1	7.63 ± 0.57	1.57 ± 0.26	4.64 ± 0.27
	100	1.26 ± 0.12*	30.1 ± 2.3	7.12 ± 0.41	1.81 ± 0.25	4.25 ± 0.27
	250	1.29 ± 0.15*	31.0 ± 2.3	7.21 ± 0.45	1.94 ± 0.28	4.32 ± 0.26
	500	1.18 ± 0.16*	29.3 ± 2.3*	6.84 ± 0.60	2.10 ± 0.29*	4.04 ± 0.32*
Medium	0	1.88 ± 0.10	37.1 ± 1.2	8.73 ± 0.34	1.19 ± 0.13	5.37 ± 0.14
	10	1.84 ± 0.14	36.6 ± 0.8	8.98 ± 0.29	1.44 ± 0.19	4.73 ± 0.06*
	50	1.65 ± 0.14	34.0 ± 2.8	8.17 ± 0.88	1.36 ± 0.16	5.49 ± 0.17
	100	1.80 ± 0.17	35.2 ± 1.6	8.34 ± 0.47	1.61 ± 0.36	5.33 ± 0.12
	250	1.74 ± 0.16	33.5 ± 2.2	7.53 ± 0.53	1.86 ± 0.30	5.14 ± 0.28
	500	1.66 ± 0.12	33.7 ± 1.2	7.63 ± 0.38	1.84 ± 0.27	4.94 ± 0.36
Large	0	2.30 ± 0.11	37.2 ± 1.1	8.78 ± 0.66	1.28 ± 0.17	5.70 ± 0.18
	10	1.97 ± 0.13	37.8 ± 1.4	9.26 ± 0.52	1.22 ± 0.10	5.21 ± 0.13
	50	1.99 ± 0.08*	37.3 ± 1.8	8.57 ± 0.76	1.39 ± 0.13	5.66 ± 0.28
	100	2.02 ± 0.13	36.3 ± 2.0	8.45 ± 0.49	1.56 ± 0.17	5.03 ± 0.23*
	250	2.06 ± 0.19	36.3 ± 2.2	8.40 ± 0.58	1.64 ± 0.22	4.82 ± 0.27*
	500	1.53 ± 0.21*	33.6 ± 3.3	7.00 ± 0.72	2.03 ± 0.12*	4.74 ± 0.32*

a = averages for six fish ($\bar{X}$ ± S.E.)

* = significantly different from respective 0 dose, t-test, $\alpha = 0.05$

Table 4. Acute effects of intraperitoneal injection of DDT on the leukocyte parameters of small, medium and large size goldfish.[a]

Fish Size	DDT Dose (mg/kg fish)	WBC ($10^3/mm^3$)	Differential Count			
			L (%)	N (%)	M (%)	T (%)
Small	0	34.5 ± 4.5	81.5 ± 1.9	2.50 ± 0.29	1.00 ± 0.37	15.0 ± 1.8
	10	27.7 ± 3.7	80.6 ± 2.3	1.67 ± 0.31	0.75 ± 0.34	16.9 ± 2.0
	50	29.0 ± 3.4	75.9 ± 3.9	3.83 ± 1.11	2.00 ± 0.83	18.3 ± 3.3
	100	19.7 ± 4.5*	72.8 ± 2.2*	4.00 ± 1.01	4.33 ± 0.49*	18.9 ± 1.4
	250	18.7 ± 3.6*	73.8 ± 3.2	6.25 ± 1.42*	4.00 ± 1.61	16.0 ± 2.3
	500	16.5 ± 4.1*	69.8 ± 3.1*	10.8 ± 2.34*	5.33 ± 1.14*	14.0 ± 2.4
Medium	0	39.0 ± 3.1	80.4 ± 3.2	4.83 ± 0.98	1.17 ± 0.40	13.6 ± 3.1
	10	34.5 ± 4.6	81.3 ± 2.5	4.17 ± 1.70	0.67 ± 0.31	13.8 ± 2.7
	50	40.3 ± 2.6	77.9 ± 3.4	3.92 ± 0.90	1.92 ± 0.81	16.4 ± 2.9
	100	34.5 ± 5.1	75.9 ± 3.7	4.92 ± 1.39	1.83 ± 0.48	17.3 ± 3.0
	250	20.5 ± 3.6*	75.7 ± 3.5	6.58 ± 2.38	4.17 ± 1.69	13.6 ± 1.6
	500	25.5 ± 4.6*	70.1 ± 6.7	13.2 ± 4.44	6.75 ± 3.15	10.0 ± 1.7
Large	0	54.5 ± 6.0	77.1 ± 2.5	3.58 ± 0.99	1.33 ± 0.44	18.0 ± 2.6
	10	51.2 ± 5.7	78.2 ± 2.4	4.08 ± 1.12	1.83 ± 0.51	15.9 ± 3.2
	50	40.0 ± 4.6	80.2 ± 2.8	3.00 ± 0.45	1.08 ± 0.27	15.8 ± 3.1
	100	42.5 ± 3.0	75.3 ± 2.6	4.33 ± 1.78	1.58 ± 0.35	18.8 ± 2.4
	250	43.2 ± 4.7	73.8 ± 3.8	4.58 ± 1.84	1.50 ± 0.43	20.1 ± 2.0
	500	26.2 ± 2.9*	68.4 ± 3.3	13.0 ± 3.20*	6.83 ± 1.57*	11.8 ± 3.6

a = averages for six fish ($\bar{X}$ ± S.E.)

* = significantly different from respective 0 dose, t-test, $\alpha = 0.05$

changed only by the 500 mg/kg dose of DDT.

The small fish erythrocyte parameters were progressively altered with increased DDT dose (Table 3). Numbers of RBC tended to decrease and were significantly decreased by doses ≥ 100 mg DDT/Kg fish. While the MH levels also decreased, a significant decrease was not seen until the highest DDT dose. Levels of Hb followed the same trend, but in this size fish (and also in medium and large fish) never decreased significantly. The ESR progressively increased with increased DDT dose, but only reached a significantly increased level at the highest dose.

In general, small size fish TPP levels decreased with increased DDT levels. Significant decrease was only found at the highest dose level.

Most leukocyte parameters of small fish were also progressively changed with increased DDT dose (Table 4). Numbers of WBC generally decreased with significant decreases found at doses of DDT ≥ 100 mg/kg fish.

Differential counts for small fish showed the following trends. Percent L generally decreased as DDT levels increased. Significant declines were seen at two of the three highest DDT doses. A compensation for this decrease was seen by increases in both % N and % M. Thrombocyte percentage (% T) was hardly altered at all.

Similar, but less significant, DDT dose effects were seen on the trends for medium size fish hematological parameters (Tables 3 and 4).

Numbers of RBC seemed to be slightly decreased with DDT treatment, but no consistent dose effect was evident (Table 3). As before, both MH and Hb levels generally decreased with increased DDT, but no

significant decreases were seen in either parameter. The ESR generally increased with increased DDT dose, but never increased to a significant level.

Medium fish TPP levels seemed also to decrease as DDT levels increased. However, the only significant decrease was at the 10-mg DDT level. This highlighted the curious fact that with all three sizes of fish, the 10-mg DDT dose had more of an effect on the TPP than did the 50-mg DDT dose.

Numbers of WBC were generally reduced by increased DDT dose. Significant decreases were found at the two highest DDT doses (Table 4).

As before, % L generally declined as DDT levels increased, % N and % M increased in compensation and % T were not much altered. In general, only the two highest DDT doses caused much effect.

In comparison to small (and even medium) fish, once again similar but less significant DDT effects were seen on large size fish hematological parameters (Tables 3 and 4).

Numbers of RBC seemed slightly decreased by all DDT doses except for a significant decrease at the highest dose level (Table 3). The decreased numbers of RBC were paralleled by declining MH and Hb levels, but no significant decreases were seen in either. The ESR only slightly increased as DDT increased, except for a significant increase seen at the highest DDT dose.

Large fish TPP levels generally seemed to decrease with increased DDT, but only significantly so at the highest doses of DDT.

As with the large fish #RBC, increased DDT dose only slightly decreased the #WBC, except for a significant decline at the highest

DDT level (Table 4).

Percent L did not start to decrease until DDT doses were ≥ 100 mg/kg. The largest, yet non-significant, decline was seen with the highest DDT level. Complementary trends were seen in the % N and % M as neither were much altered until significantly increased by the highest DDT dose. Percent T did not seem altered in any consistent fashion by DDT treatments.

Sub-Acute Hematology

Multiple low doses of DDT given over a more prolonged period of time also resulted in hematological alterations, but with some changes apparent when compared to the acute DDT results.

Numbers of RBC were again reduced by DDT treatments, but with only the 10-mg dose causing a significant decline (Table 5). In contrast, the MH and Hb levels increased with increase in DDT dose, the MH significantly so at the highest dose. Estimates of RBC cell volume and hemoglobin concentration showed that some acute DDT doses and both sub-acute DDT doses caused significant increases in mean corpuscular volume and hemoglobin levels (Appendix Tables 13 and 14). The ESR was elevated in both groups of fish given DDT, with the lower dose causing a significant elevation and the higher dose an almost significant elevation in ESR.

Also in contrast to the acute test results, the sub-acute TPP levels were not decreased by these DDT treatments. In fact, slight increases in TPP levels were evident.

As with the acute tests, numbers of WBC declined as DDT dose increased, with the 50-mg DDT doses causing a significant decrease

Table 5. Sub-acute effects of intraperitoneal injections of DDT on days 0, 26 and 52 on the erythrocyte and plasma parameters of medium size goldfish.[a]

DDT Dose (mg/kg fish)	Erythrocyte				Plasma
	#RBC (10^6/mm^3)	MH (%)	Hb (g/dl)	ESR (mm/hr)	TPP (g/dl)
0	2.16 ± 0.18	35.4 ± 0.7	8.43 ± 0.31	1.30 ± 0.08	5.13 ± 0.21
10	1.54 ± 0.11*	36.3 ± 1.0	8.52 ± 0.27	1.82 ± 0.17*	5.18 ± 0.28
50	1.77 ± 0.14	39.5 ± 1.5*	8.94 ± 0.27	1.80 ± 0.35	5.32 ± 0.18

a = averages for 10 fish ($\bar{X}$ ± S.E.) on day 56

* = significantly different from 0 dose, t-test, $\alpha = 0.05$

Table 6. Sub-acute effects of intraperitoneal injections of DDT on days 0, 26 and 52 on the leukocyte parameters of medium size goldfish.[a]

DDT Dose (mg/kg fish)	#WBC (10^3/mm^3)	Differential Count			
		L (%)	N (%)	M (%)	T (%)
0	47.4 ± 3.1	81.9 ± 1.9	5.15 ± 1.04	1.40 ± 0.50	11.6 ± 0.8
10	37.8 ± 4.2	82.0 ± 2.7	2.80 ± 0.48	0.45 ± 0.14*	14.6 ± 2.4
50	31.5 ± 4.4*	74.6 ± 2.4*	4.10 ± 0.60	1.10 ± 0.27	20.1 ± 2.1*

a = averages for 10 fish ($\bar{X}$ ± S.E.) on day 56

* = significantly different from 0 dose, t-test, $\alpha = 0.05$

(Table 6).

Differential % L were only decreased by the 50-mg DDT doses, while in contrast to acute results, % N and % M were not increased in compensation, but the % T were increased (significantly so at the highest DDT level).

Jerne-Plaque

Plaque-forming cells (PFC) were found at some time in all of the goldfish organs and tissues assayed. In this species the most important PFC areas were the anterior kidney and spleen. PFC responses of the mesonephric kidney, intestine, liver and peripheral blood were mostly of secondary importance and as the control information for the primary response of these four areas was quite incomplete, the unanalyzed information for these four tissues was put in Appendix Tables 15 and 16. Nevertheless, the trends seen in the following data for anterior kidney and spleen seem also to be found in the mesonephric kidney, intestine, liver and peripheral blood data.

The primary PFC responses of the anterior kidney and spleen were very similar, with the anterior kidney reaching higher numbers of PFC at all times (Figure 1 and Table 7). Detectable levels of PFC were found in both organs four days after the primary immunization. Numbers of PFC increased rapidly and peaked on day seven with anterior kidney and spleen averaging 142.7 and 33.3 PFC/10^6 WBC respectively. Numbers of PFC in both organs declined fairly quickly to zero within 10 days of their peak responses.

Second injections of SRBC resulted in distinct enhancements (over

Figure 1. Primary PFC responses in anterior kidney and spleen of goldfish immunized with SRBC on day 0.

Figure 2. Secondary PFC responses in anterior kidney and spleen of goldfish immunized on days -28 and 0 with SRBC.

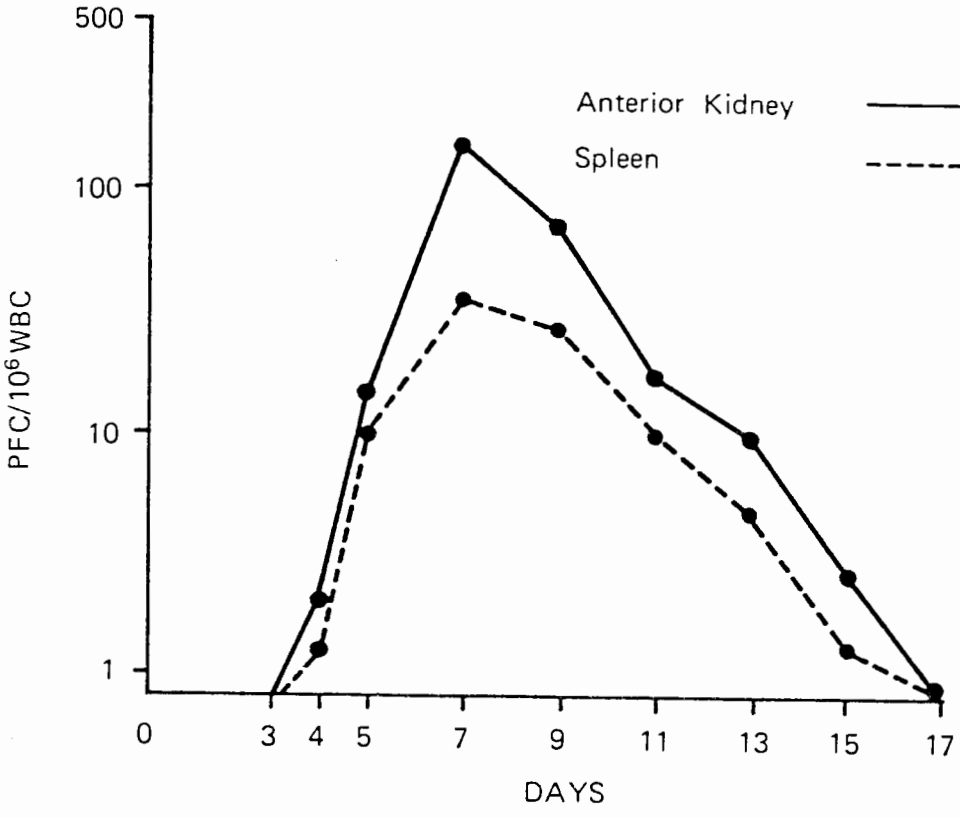

Figure 1.

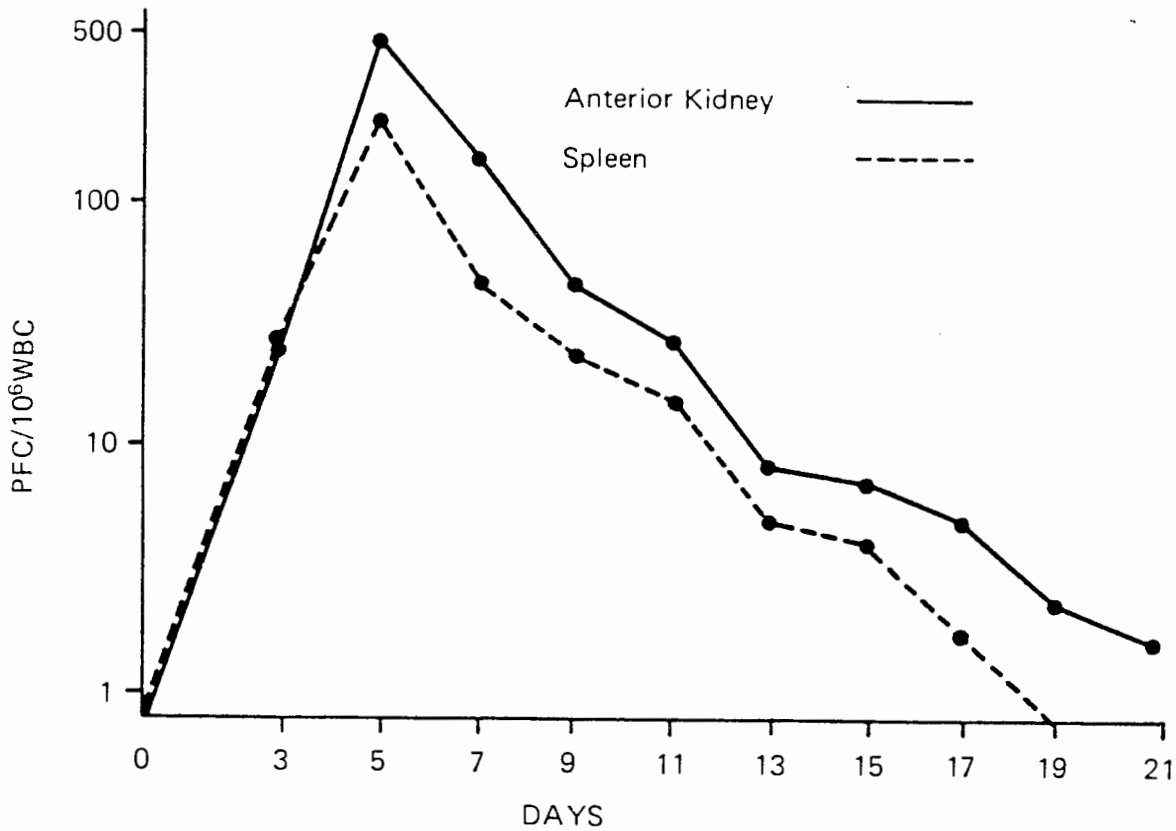

Figure 2.

Table 7. Primary and secondary PFC responses in anterior kidney and spleen of goldfish immunized with SRBC.

Days After Primary Immunization	n	PFC/10^6 WBC ($\bar{X}$ ± S.E.) Anterior Kidney	Spleen
0	5	0.0 ± 0.0	0.0 ± 0.0
3	5	0.0 ± 0.0	0.0 ± 0.0
4	5	2.0 ± 1.0	1.2 ± 0.8
5	6	14.3 ± 4.4	10.0 ± 3.7
7	7	142.7 ± 35.7	33.3 ± 8.5
9	6	66.5 ± 15.2	26.6 ± 6.9
11	6	16.3 ± 4.0	9.6 ± 3.8
13	5	9.2 ± 4.1	4.4 ± 2.5
15	5	2.4 ± 1.4	1.2 ± 0.8
17	5	0.0 ± 0.0	0.0 ± 0.0
Days After Secondary Immunization			
0	6	0.2 ± 0.2	0.0 ± 0.0
3	6	24.0 ± 6.6	25.1 ± 7.9
5	6	446.8 ± 82.1	209.6 ± 47.8
7	6	142.3 ± 27.2	45.2 ± 9.6
9	6	45.1 ± 10.6	23.5 ± 6.8
11	6	26.5 ± 6.5	15.0 ± 4.1
13	5	8.1 ± 2.3	4.8 ± 1.8
15	5	7.0 ± 2.1	4.1 ± 1.6
17	5	4.9 ± 1.7	1.7 ± 0.9
19	5	2.3 ± 0.9	0.2 ± 0.1
21	5	1.6 ± 0.8	0.0 ± 0.0

primary results) in both the anterior kidney and spleen PFC performances (Figure 2 and Table 7). For both anterior kidney and spleen secondary responses, PFC appeared a day sooner and reached much higher peak numbers (446.8 and 209.6 PFC/10^6 WBC respectively) two days sooner than in the primary response. The secondary responses were also more prolonged, as detectable numbers of PFC appeared to persist for at least 14--16 days after secondary peak responses.

In general, DDT pretreatment did not seem to significantly influence the primary or secondary times for PFC to appear or times to reach peak numbers or the general shape of the primary or secondary curves. Nevertheless, peak numbers of PFC were often quite reduced in fish given DDT (Figures 3 and 4, Table 8). Perhaps in compensation, DDT treatment tended to cause a prolongation in PFC persistance times after peak numbers were reached.

The primary PFC responses of the anterior kidney and spleen in DDT pretreated fish (Figure 3) were similar in timing and shape to the primary controls (Figure 1). Initiation of DDT primary PFC responses started at about the same time and peaked as quickly as the controls. In comparison to controls, however, DDT did cause primary anterior kidney and spleen peak PFC responses to be quite reduced (54.4 and 18.1 PFC/10^6 WBC respectively). Numbers of PFC in both anterior kidney and spleen of DDT treated fish declined less rapidly than in controls and did not reach zero by 10 days after peak responses. In spite of similarities to controls, primary anterior kidney PFC response was overall significantly reduced by DDT treatment (completely randomized design (CRD) ANOV, arranged

Figure 3. Primary PFC responses in anterior kidney and spleen of goldfish pretreated with 50 mg/kg DDT two days prior to immunization with SRBC on day 0.

Figure 4. Secondary PFC responses in anterior kidney and spleen of goldfish pretreated with 50 mg/kg DDT two days prior to immunizations on days -28 and 0 with SRBC.

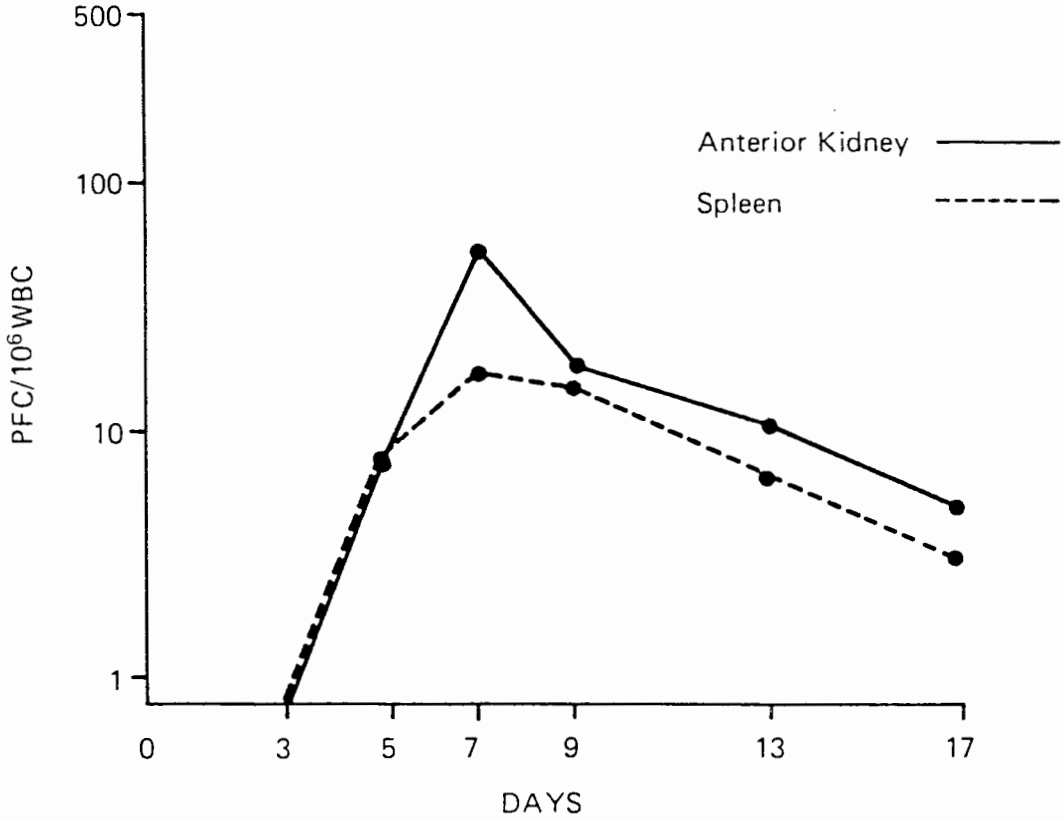

Figure 3.

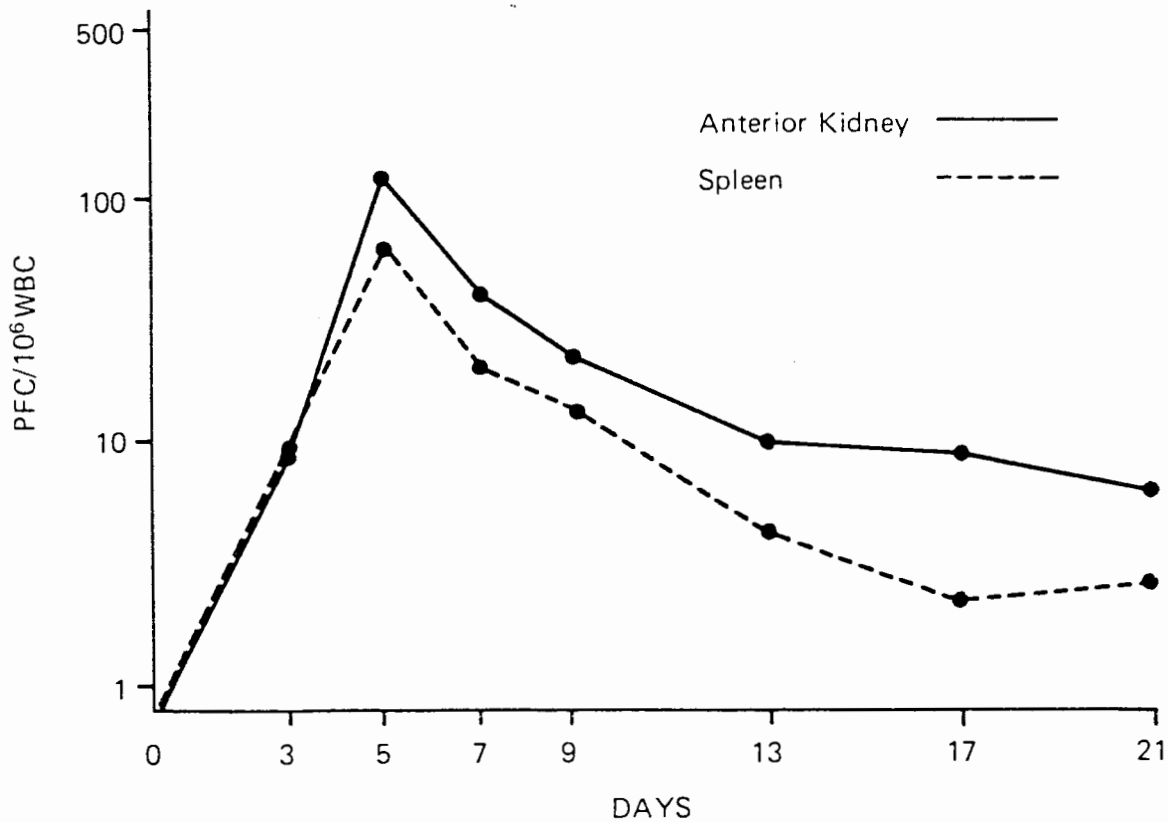

Figure 4.

Table 8. Primary and secondary PFC responses in anterior kidney and spleen of goldfish pretreated with 50 mg/kg DDT two days prior to immunizations with SRBC (n = 5 for all).

Days After Primary Immunization	PFC/10^6 WBC ($\bar{X}$ ± S.E.)	
	Anterior Kidney	Spleen
0	0.0 ± 0.0	0.0 ± 0.0
3	0.0 ± 0.0	0.0 ± 0.0
5	6.8 ± 5.0	7.9 ± 4.9
7	54.4 ± 31.9	18.1 ± 8.2
9	19.5 ± 9.1	15.7 ± 5.1
13	11.0 ± 3.9	6.7 ± 4.6
17	4.9 ± 1.5	3.1 ± 2.0
Days After Secondary Immunization		
0	0.6 ± 0.2	0.3 ± 0.2
3	8.6 ± 3.6	8.7 ± 4.5
5	119.8 ± 31.5	63.3 ± 15.3
7	39.4 ± 16.7	20.1 ± 8.1
9	22.3 ± 7.1	13.3 ± 4.7
13	9.7 ± 3.2	4.1 ± 2.8
17	8.7 ± 5.1	2.1 ± 1.2
21	5.8 ± 2.6	2.5 ± 1.0

factorially, $\alpha = 0.05$, $F = 4.93$) while the primary spleen response was non-significantly decreased ($F = 1.48$).

As with the DDT primary PFC responses, the secondary PFC responses of the anterior kidney and spleen in fish pretreated with DDT (Figure 4 and Table 8) were similar in timing and shape to their controls (Figure 2 and Table 7). Initiation of DDT secondary PFC responses started and peaked at the same time as controls. As with the DDT primary responses, DDT treatments caused secondary anterior kidney and spleen peak PFC responses to be very reduced (119.8 and 63.3 PFC/10^6 WBC respectively) in comparison to controls. Once again, numbers of PFC in anterior kidney and spleen declined less rapidly in DDT treated fish and here had not reached zero by 16 days after peak responses. Nevertheless, both anterior kidney and spleen secondary PFC responses were overall significantly reduced by the DDT pretreatments ($F = 18.6$ and 10.6 respectively).

Hemolysin and Hemagglutinin

Hemolytic and hemagglutinating antibodies were detected in goldfish sera in response to SRBC immunizations. The PFC were undoubtedly responsible for production of, at least, the lytic antibody. Therefore, it was expected that times of initial increase and peak serum levels of hemolysin (and perhaps hemagglutinin) would generally lag a few days behind the respective initiation and peak primary or secondary PFC responses. At any one time, hemolytic antibody titers were usually higher than the hemagglutinin titers.

Slight increases in $\log_2$ titers of both hemolysin and hemagglutinin were seen by five days after the primary SRBC

immunization (Figure 5 and Table 9). Peak primary titers of both hemolysin and hemagglutinin were reached by day 13 and were $\log_2$ 5.29 and 4.19 respectively. Titers of both hemolysin and hemagglutinin quickly declined, but hemolysin levels were not back down to background levels by day 21, while hemagglutinin titers were.

Secondary immunization with SRBC resulted in enhanced (over primary) hemolysin and hemagglutinin serum titers levels (Figure 6 and Table 9). Increase in secondary hemolysin titer was detected two days sooner than in primary response and by day nine reached much higher peak levels ($\log_2$ 7.94) than in the day 13 peak primary response. Increase in secondary hemagglutinin titer was also detected by day three, but secondary hemagglutinin levels did not reach a much improved peak titer ($\log_2$ 4.80) and this only two days prior to the peak primary hemagglutinin response. Both secondary hemolysin and hemagglutinin titers remained elevated for a longer time than did their respective primary responses.

DDT pretreatment caused primary hemolysin and hemagglutinin titer increases (Figure 7 and Table 10) to delay two days over the primary controls (Figure 5). As in the primary controls, peak DDT primary hemolysin and hemagglutinin levels ($\log_2$ 4.73 and 2.47 respectively) were reached on day 13. Titers of the DDT primary hemolysin and hemagglutinin sera did not seem to decrease quite as quickly as in the primary controls. Neither hemolysin nor hemagglutinin titers were down to background levels by day 21. Overall, the primary responses of both hemolysin and hemagglutinin were non-significantly decreased by the DDT treatment (CRD ANOV, arranged factorially, $\alpha = 0.05$, $F = 0.25$ and 0.48 respectively).

Figure 5. Primary hemolysin and hemagglutinin titers in sera of goldfish immunized with SRBC on day 0.

Figure 6. Secondary hemolysin and hemagglutinin titers in sera of goldfish immunized on days -28 and 0 with SRBC.

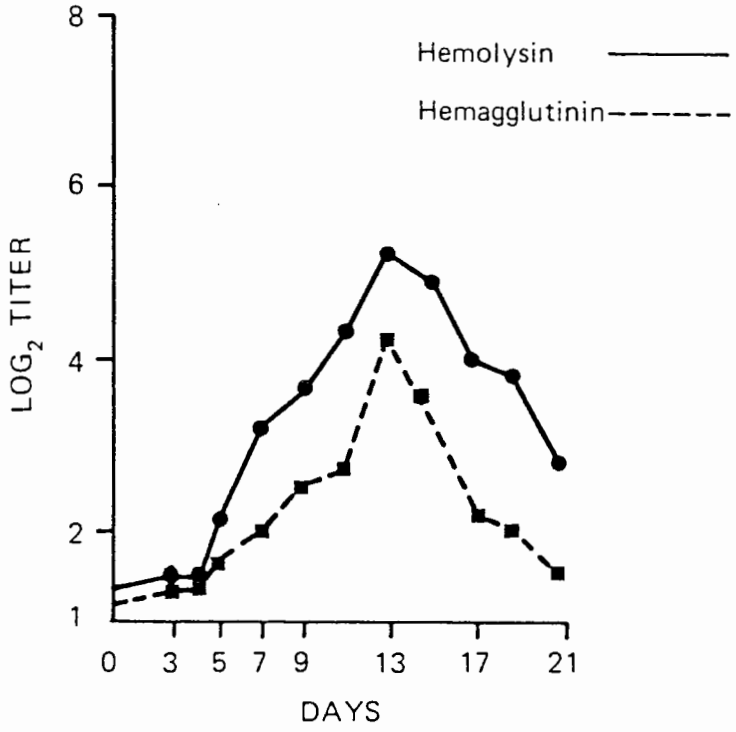

Figure 5.

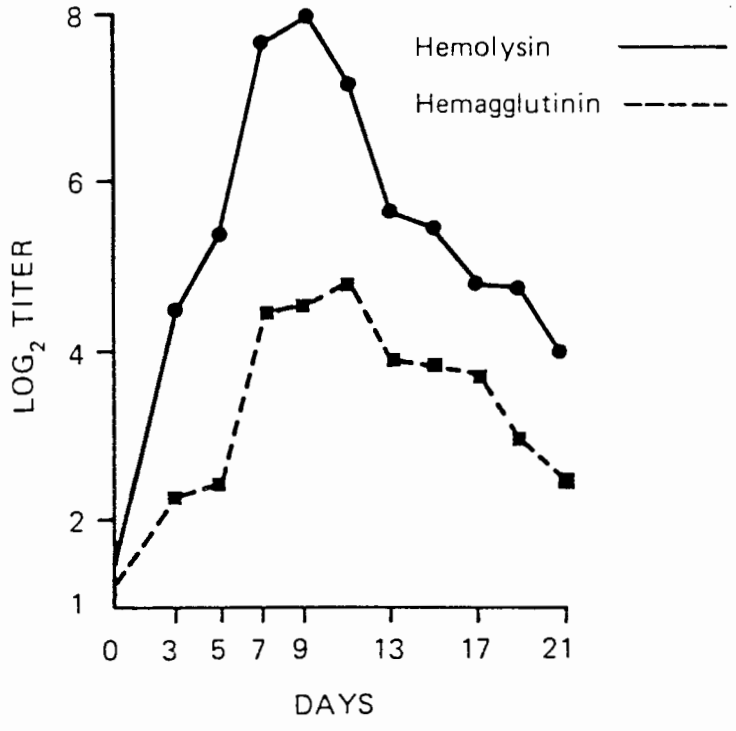

Figure 6.

Table 9. Primary and secondary hemolysin and hemagglutinin titers in sera of goldfish immunized with SRBC.

Days After Primary Immunization	n	Log_2 Titer ($\bar{X}$ ± S.E.) Hemolysin	Hemagglutinin
0	7	1.33 ± 0.42	1.14 ± 0.27
3	7	1.43 ± 0.37	1.24 ± 0.29
4	7	1.43 ± 0.27	1.29 ± 0.32
5	7	2.14 ± 0.46	1.67 ± 0.28
7	7	3.24 ± 0.23	2.05 ± 0.33
9	7	3.62 ± 0.39	2.62 ± 0.43
11	7	4.38 ± 0.44	2.81 ± 0.32
13	7	5.29 ± 0.35	4.19 ± 0.42
15	6	4.94 ± 0.28	3.22 ± 0.24
17	6	4.06 ± 0.23	2.22 ± 0.20
19	6	3.89 ± 0.43	2.06 ± 0.38
21	5	2.93 ± 0.57	1.47 ± 0.29
Days After Secondary Immunization			
0	6	1.44 ± 0.20	1.22 ± 0.31
3	6	4.50 ± 0.35	2.33 ± 0.37
5	6	5.22 ± 0.51	2.50 ± 0.44
7	6	7.67 ± 0.46	4.39 ± 0.34
9	6	7.94 ± 0.63	4.44 ± 0.47
11	5	7.13 ± 0.65	4.80 ± 0.50
13	5	5.53 ± 0.60	3.87 ± 0.78
15	5	5.33 ± 0.50	3.67 ± 0.67
17	5	4.73 ± 0.37	3.60 ± 0.36
19	5	4.67 ± 0.23	2.87 ± 0.31
21	5	3.93 ± 0.38	2.53 ± 0.54

Figure 7. Primary hemolysin and hemagglutinin titers in sera of goldfish pretreated with 50 mg/kg DDT two days prior to immunization with SRBC on day 0.

Figure 8. Secondary hemolysin and hemagglutinin titers in sera of goldfish pretreated with 50 mg/kg DDT two days prior to immunizations on days -28 and 0 with SRBC.

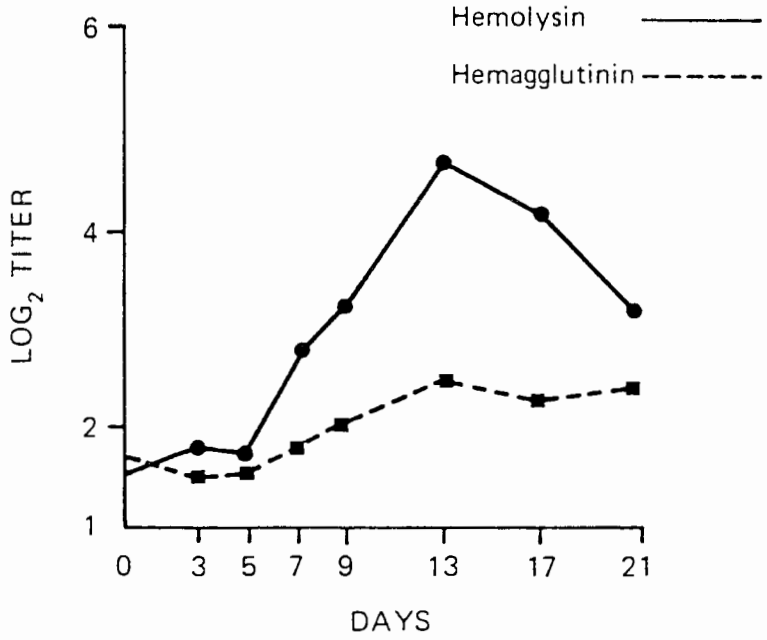

Figure 7.

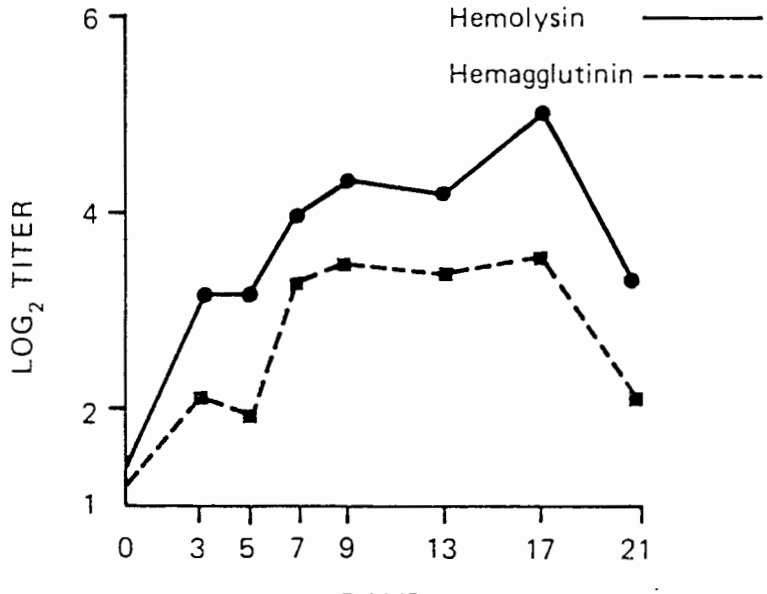

Figure 8.

Table 10. Primary and secondary hemolysin and hemagglutinin titers in sera of goldfish pretreated with 50 mg/kg DDT two days prior to immunizations with SRBC (n = 5 for all).

Days After Primary Immunization	Log_2 Titer ($\bar{X}$ ± S.E.)	
	Hemolysin	Hemagglutinin
0	1.53 ± 0.23	1.67 ± 0.28
3	1.80 ± 0.37	1.47 ± 0.29
5	1.73 ± 0.32	1.53 ± 0.27
7	2.80 ± 0.29	1.80 ± 0.37
9	3.27 ± 0.25	2.07 ± 0.07
13	4.73 ± 0.58	2.47 ± 0.43
17	4.20 ± 0.20	2.27 ± 0.19
21	3.20 ± 0.38	2.40 ± 0.45
Days After Secondary Immunization		
0	1.40 ± 0.25	1.20 ± 0.20
3	3.13 ± 0.22	2.07 ± 0.29
5	3.13 ± 0.22	1.93 ± 0.07
7	3.93 ± 0.52	3.27 ± 0.77
9	4.33 ± 0.49	3.47 ± 0.47
13	4.20 ± 0.29	3.40 ± 0.41
17	5.07 ± 0.30	3.60 ± 0.68
21	3.33 ± 0.69	2.07 ± 0.34

The secondary hemolysin and hemagglutinin responses in fish pretreated with DDT (Figure 8 and Table 10) were markedly altered from their secondary controls (Figure 6). Whereas increases in DDT secondary hemolysin and hemagglutinin titers were started at the same time as in secondary controls, the titers did not rise nearly as quickly, nor peak nearly as high as in the secondary controls. Peak DDT secondary hemolysin and hemagglutinin titers were only $\log_2$ 5.07 and 3.60 respectively and were not reached until day 17. After that, the DDT secondary hemolysin and hemagglutinin titers seemed to decrease just as quickly as in the secondary controls. DDT pretreatment caused the overall secondary responses of both hemolysin and hemagglutinin to be significantly decreased ($F = 49.9$ and 4.64 respectively) from the secondary controls.

DDT seemed to cause more suppression of secondary than of primary anti-SRBC responses. A similar pattern was seen in enhanced suppression, by DDT, on secondary versus primary PFC responses.

While after-peak declines in the primary and secondary PFC responses were prolonged by DDT treatments, this was not seen to be followed by a concomitant prolongation in serum titers of hemolysin (or hemagglutinin).

Anti-BSA

Goldfish will produce antibodies against BSA (Figure 9 and Table 11). Fairly high background control levels in unimmunized fish sera were the result of the sensitivity to autoagglutination of the tanned and coated SRBC. Nevertheless, dramatic increases in anti-BSA titers were seen after immunization of the other three groups of

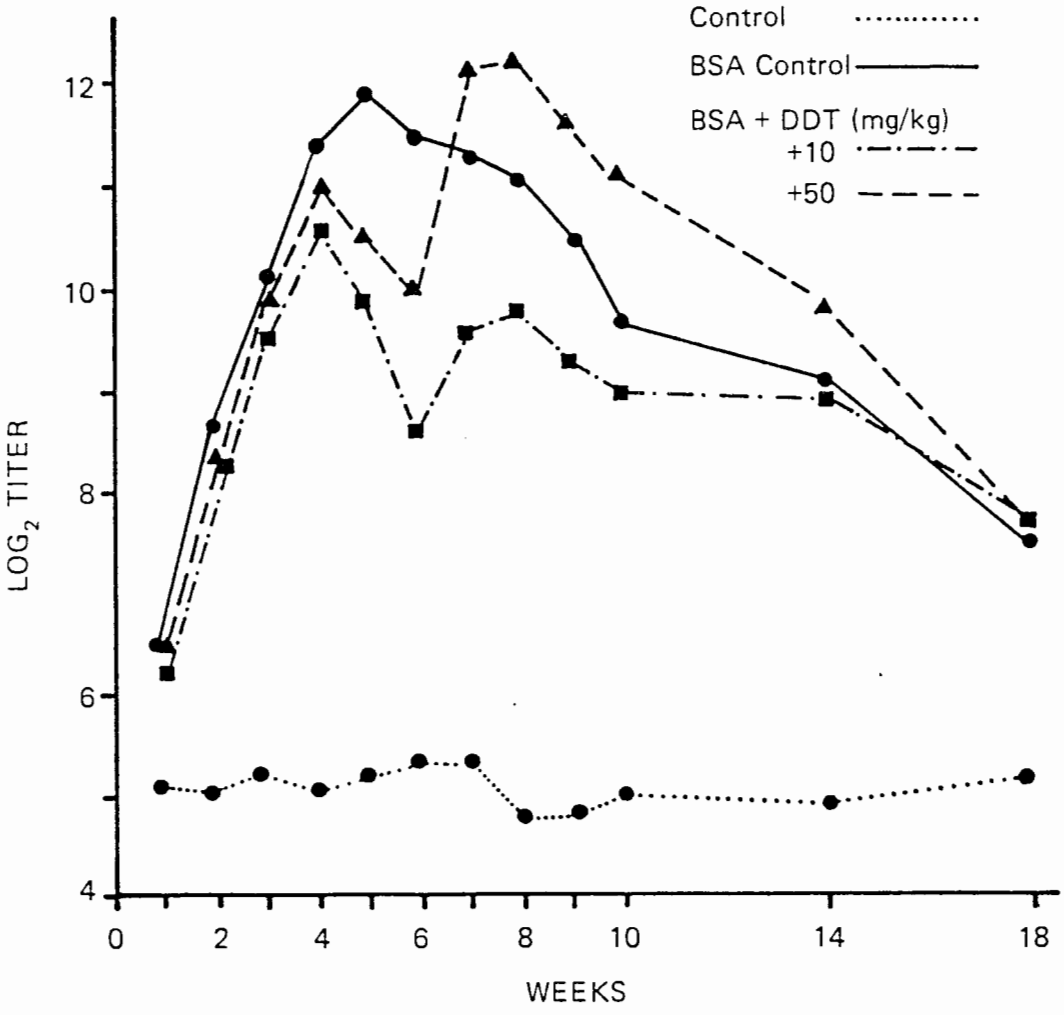

Figure 9. Primary anti-BSA titers in sera of goldfish given no treatments (Control), BSA only (BSA Control), or BSA and DDT doses (BSA + 10 mg DDT/kg fish, BSA + 50 mg DDT/kg fish) one week before and four weeks after BSA dosing at time 0.

Table 11. Primary anti-BSA titers in sera of goldfish given no treatments (Control), BSA only (BSA control), or BSA and DDT (10 or 50 mg/kg).

Weeks After Immunization	Log_2 Titer ($\bar{X}$ ± S.E.(n))			
	Control	BSA Control	BSA + 10 mg/kg DDT	BSA + 50 mg/kg DDT
1	5.11 ± 0.28(6)	6.56 ± 0.27(6)	6.22 ± 0.41(6)	6.56 ± 0.32(6)
2	5.06 ± 0.29(6)	8.67 ± 0.60(6)	8.45 ± 0.39(6)	8.45 ± 0.89(6)
3	5.22 ± 0.29(6)	10.11 ± 0.63(6)	9.67 ± 0.53(6)	10.00 ± 0.72(6)
4	5.06 ± 0.22(6)	11.40 ± 0.63(5)	10.56 ± 0.48(6)	11.17 ± 0.78(6)
5	5.17 ± 0.14(6)	11.87 ± 0.44(5)	9.94 ± 0.53(6)	10.50 ± 0.51(6)
6	5.33 ± 0.19(6)	11.53 ± 0.40(5)	8.61 ± 0.55(6)	10.07 ± 0.69(5)
7	5.33 ± 0.21(5)	11.33 ± 0.53(5)	9.56 ± 0.51(6)	12.17 ± 0.80(4)
8	4.78 ± 0.31(6)	11.07 ± 0.66(5)	9.84 ± 0.67(6)	12.25 ± 0.83(4)
9	4.83 ± 0.25(6)	10.53 ± 0.89(5)	9.28 ± 0.68(6)	11.67 ± 0.59(4)
10	5.00 ± 0.23(6)	9.73 ± 1.10(5)	9.00 ± 0.62(6)	11.17 ± 0.63(4)
14	4.94 ± 0.29(6)	9.07 ± 1.04(5)	8.87 ± 0.62(5)	9.78 ± 0.78(3)
18	5.17 ± 0.24(6)	7.53 ± 0.71(5)	7.73 ± 0.29(5)	7.67 ± 0.88(3)

fish with BSA and is a clear indication of anti-BSA response capability in this species.

For the first four weeks, anti-BSA titers in the two groups pretreated with DDT were only slightly below the BSA control titers. Second DDT doses were given at week four, just prior to expected peak anti-BSA titers and caused significant declines in anti-BSA titers for at least the next two weeks in the 10-mg DDT fish group (Student's-t, $p < 0.05$) and almost significant declines for two weeks in the 50-mg DDT group ($0.05 < p < 0.10$). These antibody titer levels then rebounded to near, or even above, the BSA control levels. The enhanced rebound seen in the highest DDT dose group was due, at least in part, to the death of the two poorest anti-BSA responding fish in this group just prior to the noted rebound.

As seen in some of the hematological results, the 10 mg DDT dose seemed to have more of an effect than did the 50-mg DDT dose. The two 10-mg DDT/kg fish doses caused an overall significant decline in anti-BSA titers (CRD arranged factorially, $\alpha = 0.05$, $F = 15.2$) while the enhanced anti-BSA rebound in the 50-mg DDT/kg fish group negated previous declines and overall, resulted in a non-significant treatment effect ($F = 0.33$).

Scale-Graft Rejection

The average time for primary control scale-graft rejections (100% clearance of the orange pigment) was slightly less than a week (Table 12). Average secondary control values were over two days quicker and indicated a typical enhanced secondary immune response.

Both DDT doses interferred with (suppressed) the normal time for

Table 12. Sub-acute effects of intraperitoneal injections of DDT on the rejection rates of scale-graft transplants in goldfish.

DDT Dose (mg/kg fish)	#Days for Rejection ($\bar{X}$ ± S.E.(n)[a])	
	Primary Transplants	Secondary Transplants
0	6.43 ± 0.28 (37)	4.21 ± 0.21 (33)
10	7.74 ± 0.23*(34)	5.57 ± 0.17*(37)
50	7.32 ± 0.27*(31)	5.12 ± 0.21*(33)

a = 10 fish/group. Four foreign scales/fish = 40 scales transplanted/group. (n = # scales left to score clearance on).

* = significantly different from respective 0 dose, t-test, $\alpha = 0.05$.

rejection of foreign scale-grafts. Both primary and secondary responses were significantly slowed by either DDT dose. Doses of 10- or 50-mg DDT/kg fish caused significant interference with the expected times to primary scale rejection (Student's t, $\alpha = 0.05$, $p < 0.001$ or 0.05 respectively) and also to secondary scale rejection ($p < 0.001$ or 0.01 respectively). Once again, the 10-mg DDT dose had more of an effect than the 50 mg dose upon both the primary and secondary responses. In the primary and secondary experiments the 10-mg DDT dose caused from a 1.3-1.4-day delay in rejection and the 50-mg dose caused a 0.9-day delay in both primary and secondary responses.

DISCUSSION

LD_{50}

Holden (1973) felt that most research suggests that fish susceptibility to pesticides decreases with increasing fish size. He further suggested the decreasing sensitivity with increasing fish size could be due to experimental difficulties in exposing fish using static systems and might be resolved by using flow-through systems with regulated concentrations of toxicants. In contrast, Tucker and Leitzke (1979) felt that the flow-through design may not best represent the natural environmental exposure of fish. Due to differential body metabolism, respiration rates, and surface areas of absorptions, neither the static nor flow through approaches solve the problem of giving different size fish the same dose of a pesticide.

While Cope (1969) found that in using a static test there was little difference in DDT susceptibility in various size (0.5-40g) rainbow trout, Buhler et al. (1969) and Buhler and Shanks (1970) gave salmon DDT in a contaminated diet and found that salmon size played a significant role in sensitivity. Smaller fish were considerably more susceptible to DDT. Due to differences in feeding rates, the dose of DDT given to different size fish was difficult to control and doses given individual fish were relatively uncontrolled.

One advantage of the i.p. injection technique used here was that dose levels given individual fish were certain and made dose responses comparable irrespective of inherent size differences. The data presented in this research clearly indicate that goldfish

show a decreased sensitivity to DDT with increasing size.

As goldfish size increased, proportionately larger doses of DDT were usually necessary to cause the same effects (i.e., % mortality or change in hematological parameter). Buhler et al. (1969) and Buhler and Shanks (1970) suggested that mechanisms for relatively increased tolerance of larger fish to DDT might include differences in body metabolism, storage, and detoxication. Increased metabolic rates of smaller fish could make them accumulate DDT quicker and therefore be more vulnerable to disruptions in nervous condutions. Larger fish often have relatively increased lipid deposition and lipid could readily serve as storage depots for DDT residues (Buhler and Shanks, 1970). It is possible that larger, more mature fish may also have an increased detoxication capacity (Tucker and Leitzke, 1979) or be more tolerant to the debilitation and exhaustion resulting from DDT toxicity.

As goldfish size increased, the 96-hr LD_{50} for DDT increased proportionately. The DDT LD_{50} values determined here were considerably higher than found by Bathe et al. (1976) for the closely related Crucian carp, could be much lower than in carp tested by Loeb and Kelly (1963), and might be close to goldfish values given by Ellis et al. (1944).

Bathe et al. (1976) gave i.p. injections of p,p'-DDT (WHO standard) to Crucian carp weighing 25-50 g and kept at 14 ± 2° C. He found the seven day LD_{50} was 6.5 mg/kg. This value is considerably below the LD_{50} values of the present study and likely reasons for this discrepency between our results include differences in time, temperature and species.

A seven-day rather than four-day observation period after injection obviously resulted in additional opportunity for mortality. The longer the time from exposure to end point, the better the chances for formerly sub-lethal doses to finally result in some lethal effect.

Temperature difference between Bathe's and my study (14° versus 25° C) could have had a considerable impact upon DDT mortality. While many pesticides seem to cause increased effects with temperature increase, DDT has been found to do just the opposite (Johnson, 1968; Holden, 1973; Tucker and Leitzke, 1979). A temperature of 14° C could therefore also have contributed to the increased susceptibility of Crucian carp to DDT.

Even though closely related, the goldfish may be an inherently more DDT tolerant species than is the Crucian carp. Whenever the goldfish and rainbow trout were tested using comparable methods, goldfish were always considerably more tolerant to DDT (and several other insecticides) than were rainbow trout (Marking, 1966; Macek and McAllister, 1970; Ingham and Gallo, 1975). In Bathe's study by comparison, rainbow trout were just as tolerant as Crucian carp to DDT.

In three trials, Loeb and Kelly (1963) gave carp, weighing an average of three pounds and kept at 18.5° C, per os doses of p,p'-DDT from 98-240 mg/kg and were unable to observe any mortality over the 24-, 72-, or 96-hr observation periods.

Again, size of fish may have been an important factor. Although goldfish and carp are also closely related species, when DDT sensitivity of these two species was measured in fish groups of

comparable weights (1-2 g), the goldfish was always the more tolerant species (Marking, 1966; Macek and McAllister, 1970). It seems probable that larger fish size is one cause for the enhanced carp tolerance to DDT. Perhaps goldfish as large as these carp would be even more tolerant than carp to DDT.

Ellis et al. (1944) incorporated DDT into the diet of goldfish weighing from 6-10 g. Fish mortality correlated roughly with the dose size and total mortality was about 55% for single doses ranging from 63-200 mg/kg. Time of fish deaths ranged from 1-6.5 days. Although "total mortality" is difficult to interpret, if doses causing about 50% mortality were near the top of this dose range, these doses would be quite close to the DDT LD_{50} estimated here for small goldfish.

DDT Pharmacodynamics

The mode of administration of DDT can have a significant influence upon the effects of this insecticide. Even though sparingly soluble in water, minute amounts of DDT in water can be lethal to fish. The LC_{50} for goldfish varies from about 10-180 mg/L, depending on the fish size, water temperature, or time of static exposure (Odum and Sumerford, 1946; Henderson et al., 1959; Marking, 1966; Macek and McAllister, 1970; Ingham and Gallo, 1975).

When in the water, DDT is rapidly taken into fish through contact with their gills. As fish pump large volumes of water over their gills for respiratory purposes, relatively large amounts of DDT can be quickly removed from the water, transferred to the gills

and from there move directly into the blood. Administration of DDT in this manner may be somewhat analgous to an intravenous infusion.

Once in a fish's blood, DDT is rapidly and almost completely absorbed by the plasma lipoproteins. Then within the next few hours DDT is quickly distributed throughout the body (Phillips, 1970; Dvorchik and Maren, 1972 and 1974; Darrow and Addison, 1973; Pritchard et al., 1973; Plack et al., 1979). The initial distribution of DDT to the sites of highest affinity is reached in a few days to a week and while this distribution is not always correlated with the percentage lipid content of each specific organ, DDT is often found concentrated in body areas containing the largest proportions of total body lipids (i.e., muscle, liver, fat, gonad, nerve, etc.) (Grzenda et al., 1970; Macek et al., 1970; Phillips 1970; Dvorchik and Maren, 1972 and 1974; Darrow and Addison, 1973; Pritchard et al., 1973; Jarvinen et al., 1977). Lipid can be an important storage site for DDT (Ohmiya and Koga, 1978).

Once in a fish, DDT often tends to stay there. DDT is only slowly metabolized, eliminated and/or excreted by many fish. Due to its redistribution, storage, and (even when available in the plasma) its relatively complete binding to plasma proteins, DDT is not readily available for either diffusion at the gills or filtration by the kidneys (Pritchard et al., 1973 and 1977; Dvorchik and Maren, 1974). Sequestration in tissue lipids and the low rates of metabolism or conjugation also prevents significant excretion of DDT in fish bile (Dvorchik and Maren, 1974; Statham et al., 1976).

DDT has a residue half-life of about 20-30 days in goldfish (Gakstatter and Weis, 1967; Grzenda et al., 1970; Young et al., 1971).

Goldfish appear to be one of the metabolically more capable fish species (Hinz and Matsumura, 1977). DDT was fairly quickly metabolized by goldfish to DDE and even perhaps DDD (Grzenda et al., 1970; Young et al., 1971). Studies reporting DDD had fed goldfish DDT in their diet (Grzenda et al., 1970; Young et al., 1971) and fish intestinal microflora can be capable of considerable metabolism of DDT to DDE and DDD (Wedemeyer, 1968; Cherrington et al., 1969).

Dosing fish with DDT via their diet has additional complications. DDT may be relatively poorly absorbed or assimilated by the digestive tract of fish. Shimizu et al. (1978) gave carp DDT in their food for up to 20 days and found that this insecticide was not easily absorbed by carp intestinal tract. Values of DDT storage or retention of about 30% of the total dietary dose are common in fish (Buhler et al., 1969; Macek et al., 1970; Mitchell et al., 1977; Warlen et al., 1977). Young et al. (1971) found that goldfish retained about 40% of the DDT (as DDT and metabolites) given in food over a 20-day feeding period.

Significant portions of the dietary DDT could be absorbed and distributed by the lymphatic system. Sieber et al. (1974) and Sieber (1976) found that DDT given in ethanol to rats introduodenally was selectively absorbed by the intestinal lymphatic system. When administered in a corn oil carrier, even greater amounts of DDT were found in the lymph. Within one day, about 40% of the DDT dose was found in the body fat, and about 15-30% of the DDT given was found in the lymph. Approximately 90% of the DDT in lymph was carried in its lipid fraction (chylomicron).

Iatropoulos et al. (1975) found similar results with rats given

hexachlorobenzene or dieldrin via stomach intubation. Relatively little hexachlorobenzene was absorbed by stomach or duodenum. The major part was absorbed by the intestinal lymphatic system and reached an equilibrium between the lymph and adipose tissue. Although dieldrin was absorbed well from the stomach region and transported to the liver, it was redistributed within two days in the adipose tissue. With dieldrin, the lymphatic system was also a major transport pathway with an equilibrium reached between the adipose fat and the lymph.

Intraperitoneal injection of DDT is a method that allows accurate dosing of fish and avoids the possibility of poor absorption by the digestive tract or metabolism by intestinal microflora. This mode of administration is very common in immunizing fish with antigen and results in the quick distribution of foreign materials (Watson, 1969). Beasley (1967) injected goldfish i.p. with diquat and found that this herbicide was transported via the plasma and distributed into tissues and organs within one to two days. Ellis et al. (1976) injected plaice i.p. with colloidal carbon. Within one hour, some free carbon particles were detected in spleen and kidney. Ellis et al. (1976) felt that the free carbon particles probably gained access to the blood and lymph by mesenteric blood vessel absorption from the peritoneal fluid.

Hematology

Increasing the acute dose of DDT given i.p. often caused progressive changes in many goldfish hematological parameters.

Several of the blood parameters tended to decrease with increased DDT dose (# RBC, # WBC, MH, Hb, TPP). DDT may have caused decreases in numbers of circulating blood cells (and associated parameters) by interfering with blood cell production or causing cell lysis or hemodilution or stress effects.

Whether DDT directly interferes with production of blood cells is arguable. There are studies that say DDT can cause or is associated with various forms of blood cell anemias (Case, 1945; Wright et al., 1947; Karpinski, 1950; Sanchez-Medal et al., 1963; Traczyk, 1975; Iturri and Ringer, 1978; Iturri et al., 1978) and these are countered by reports claiming no such effects or associations have been seen or proven (Mastromatteo, 1964; Laws et al., 1967; Hayes et al., 1971; Hayes, 1975; Morgan and Lin, 1978). The effect of DDT on fish hematopoietic organs were briefly reviewed on pages 34-35.

DDT may bind directly to the erythrocytes of some fish species. As in mammals, there are probably species differences in the capacity of fish erythrocytes to bind pesticides (Yang et al., 1975). Dvorchik and Maren (1974) found that the erythrocytes from dogfish (Squalus acanthias) exposed to DDT intravenously did not contain significant levels of DDT, though Plack et al. (1979) found that rainbow trout erythrocytes accumulated about 5% of the DDT incubated in trout whole blood.

Though some pesticides have been found to cause erythrocyte lysis via membrane damage (Kumar et al., 1975), O'Brien and Hilton (1978) found that DDT and its metabolites actually protected human erythrocytes from lysis. Therefore, even if DDT binds to goldfish

erythrocytes, it seems likely that it may not induce lysis.

A hemolytic anemia may be induced by repeated exposure to a pesticide. Hamilton et al. (1978) found that frequent exposure to dieldrin was the most likely cause of a case of human immunohemolytic anemia. Anti-dieldrin antibodies were detected on the erythrocytes and in the serum and were probably responsible for the anemia.

Antibodies to DDT were not very likely to have been an influence in this study. Not only is it difficult to get DDT to act as an immunological hapten (Haas and Guardia, 1968; Centeno et al., 1970), but the acute hematological depressions seen after just four days was simply not enough time for a significant immunological response to have been mounted against DDT.

The term "stress" has a variety of interpretations (Fraser et al., 1975). Regardless of interpretation, stress is an inclusive term and in regard to DDT expresses numerous possible mechanisms for the effects of DDT upon goldfish hematological and immunological parameters. A current discussion on the effects of stress on fish hematology may be found in Hlavek (1978).

Some toxic agents can be stressful to fish and act through Selye's general adaptation syndrome (Selye, 1973) to cause interactions between fish endocrinological, hematological, and immunological systems (Wedemeyer et al., 1976; Sindermann, 1979). Stress in fish often results in hypersecretion of corticosteroids (primarily cortisol) and catecholamines (primarily adrenaline or nonadrenaline) from adrenal equivalent cells (Mazeaud et al., 1977; Strange et al., 1977) or decreased prolactin secretions by the hypophysis (Spieler and Meier, 1976). In freshwater fish, stress

can result in a hemodilution due to hormonal disturbances in mineral balance and osmoregulation. Hemodilution probably occurs because increased adrenaline levels increase fish gill permeability to water, and decreased prolactin levels disrupt osmoregulatory capacity (Bern, 1975; Mazeaud et al., 1977).

The hemodilution and any direct corticosteroid actions on leukocytes will probably result in hematological changes (Mazeaud et al., 1977; Wedemeyer and Yasutake, 1977). Water influx would cause decreased numbers of erythrocytes per unit volume and would lower associated parameters (MH and Hb), increase the ESR, and decrease the TPP and total numbers of leukocytes. Soivo and Oikari (1976) and Casillas and Smith (1977) found stress to cause some of these effects in freshwater fish hematological parameters, but others did not find such effects of stress on various red blood cell parameters of freshwater fish (Vaala, 1971; Kirk, 1974; McLeay and Gordon, 1977). Time of sampling after stress could have been a factor in such differences in results (Hlavek, 1978).

Numbers of fish leukocytes are rather consistently decreased by stress perhaps partly due to hemodilution, but also because the corticosteroid hormones released may cause direct lysis of fish lymphocytes (Wedemeyer, 1970; Wedemeyer et al., 1976; McLeay and Howard, 1977).

Interference in fish leukocytic parameters can have serious implications for suppression of fish immune responses and decreased disease resistance (Snieszko, 1974; Esch et al., 1975; Wedemeyer, et al. 1976; Mazeaud et al., 1977). Ellis (1977) said that in relation to fish, "Particular attention should be given to the effects of stress

on the functioning of leukocytes" because ". . . little is understood of the induction and effect of stress on the general immune mechanisms."

The effects of stress on goldfish hormones include increased serum levels of adrenocorticotrophic and corticosteroid hormones (Spieler, 1974; Fryer, 1975; Singley and Chavin, 1975) and decreased levels of prolactin (Spieler and Meier, 1976).

DDT and other chlorinated hydrocarbon insecticides can damage fish adrenal equivalent tissues (interrenal and/or chromaffin cells) and also may change serum cortisol levels (King, 1962; Grant and Mehrle, 1972; Grant and Schoettger, 1972; Shimada, 1972; Walsh, 1974; Walsh and Ribelin, 1975). A DDT analog (o,p'-DDD) interfered with the normal stress - cortisol reaction in Tilapia aurea (Ilan and Yaron, 1977). DDT (50 mg/kg) given i.p. to goldfish altered levels of brain and eye neurotransmitters (McDonald and Fingerman, 1979; McDonald, 1980). Levels of noradrenaline and other transmitters were changed because DDT decreased monoamine oxidase and increased catechol-O-methyl transferase activities.

DDT also inhibited various membrane ATPases in fish brain, muscle, kidney, liver, intestine, and gill (Cutkomp et al., 1971; Janicki and Kinter, 1971; Gruppuso and Kinter, 1973; Leadem et al., 1974; Desaiah et al., 1975). ATPases are important in the active transport of ions in several of these tissues. Inhibitions of these enzymes has been suggested as another mechanism for DDT interfering with the osmoregulatory capabilities of freshwater and marine fish (Janicki and Kinter, 1971; Leadem et al., 1974; Waggoner and Zeeman, 1975; Coleman et al., 1977; Miller and Kinter, 1977; Haux and

Larsson, 1979).

Weisbart and Feiner (1974) exposed goldfish to sublethal levels of DDT of up to 35 ppb in their water and then followed osmotic and ionic regulation in exposed fish. DDT treated fish almost always showed a trend towards hemodilution with lower plasma osmotic and sodium concentrations. These alterations were often modified with increased times of exposure to DDT. The authors were surprised at the small magnitude of the alterations seen and concluded that sublethal concentrations of DDT appeared to have little effect on goldfish osmoregulatory ability.

The sublethal levels of DDT used by Weisbart and Feiner (1974) were probably below the sublethal levels of DDT used here. It seems reasonable that the larger acute DDT doses given here would have caused increased levels of stress and/or disruption in osmoregulatory ability of goldfish. The effects of stress and/or osmoregulatory disturbance could account for the acute DDT hematological alterations seen in my study.

Some of the acute DDT trends continued in the sub-acute test for DDT effects on goldfish hematology. Numbers of RBC, WBC and the ESR showed the same trends as in the acute study, but MH, Hb and TPP returned to or exceeded normal levels. Over time, some accomodation in many erythrocytic or plasma parameters was reached. This acclimation to DDT effects may be a reflection of the decreased effects (over time) of DDT on goldfish osmoregulation seen by Weisbart and Feiner (1974).

While some of the sub-acute DDT effects on leukocytic parameters returned to near control levels, the total numbers of WBC and the % L

were considerably depressed after the third sub-acute DDT dose. These results suggest that while the effects of DDT in osmoregulation may have ameliorated somewhat with time, there probably was a continued sensitivity of lymphocytes to the repeated stress effects caused by DDT.

Immunology

Neale and Chavin (1971b) and Heartwell (1975) caution that, in some cases, fish do not demonstrate effective immune responses. One reason for choosing the goldfish for this study was that this species had previously demonstrated an effective immune responsiveness at warm water temperatures (Hildemann, 1957 and 1958; Marchalonis, 1971; Neale and Chavin, 1971a; Everhart, 1972; Azzolina, 1978).

The test temperature used here (25° C) was selected as most compatible with test criteria for bioassay of aquatic pollutants with warmwater fish (Sprague, 1973) and was also near the preferred temperature (28° C) for this species (Ferguson, 1958; Covert and Reynolds, 1977).

The goldfish used in my study demonstrated an effective primary and enhanced secondary immune responsiveness to SRBC, BSA and allograft scale transplants. Goldfish PFC, hemolysin, hemagglutinin, anti-BSA and scale-graft rejection performances in this study were generally superior to or on a par with those seen in other fish species (Hildemann and Haas, 1960; Clem and Sigel, 1966; Smith et al., 1967; Chiller et al., 1969b; Diener, 1970; Hildemann, 1970; Pontius and Ambrosius, 1972; Avtalion et al., 1973; Sailendri and Muthukkaruppan, 1975; Anderson et al., 1979d and e).

Pretreatment of goldfish with 50 mg/kg DDT depressed the peak numbers of primary and secondary PFC to SRBC found in anterior kidney and spleen. This action was generally paralleled by a DDT-induced depression in the primary and secondary hemolysin and hemagglutinin antibody titers. Doses of 10 or 50 mg/kg DDT also resulted in at least temporary declines in goldfish anti-BSA titers. Finally, these two DDT doses also interfered with the goldfish primary and secondary cell-mediated immune response to scale allografting.

The effects of DDT on PFC responses to SRBC has been examined in mice and chickens. Seto (1975) gave mice various doses of DDT orally and found no effects on their secondary PFC response. Wiltrout et al. (1978) found that the numbers of mice PFC were always decreased by acute oral doses of 30 or 300 mg DDT/kg mouse, but that dose timing was very important, as the only significantly depressed PFC response was found when the highest DDT dose was given two days after SRBC immunization. When mice were given the 30 mg DDT/kg dose daily for 8 or 28 days prior to immunization, the PFC responses were only slightly decreased or increased, respectively. Glick (1974) dosed chickens with 0 or 500 mg/kg of DDT in their feed for five weeks and found a non-significant decline in the PFC response of the DDT treated birds.

The influence of DDT upon serum hemolysin and/or hemagglutinin titers to SRBC-immunized rabbits or poultry has been investigated. Street and Sharma (1975) found that in rabbits, exposure to DDT levels of up to 150 mg/kg in the feed for 43 days had no effect upon either their hemolysin or hemagglutinin titers. Wassermann et al. (1971) gave rabbits 200 ppm of DDT in their drinking water for 38

days and subsequently found out that hemagglutinin titers were 40% of control titers, yet were non-significantly decreased by DDT. Kosutzky et al. (1974) fed ducks and chickens up to 100 mg of technical DDT/kg feed for up to 11 months and found that, over time, the hemagglutinin titers were, at first, non-significantly higher, and subsequently, non-significantly lower than in poultry not fed this insecticide.

Effects of DDT on antibody responses to BSA have been followed in rats and chickens. Lukic et al. (1973) gave rats orally 40 mg technical DDT/kg/day for 60 days. They found that DDT potentiated the humoral immune response to BSA. Glick (1974) fed chickens for several weeks on a diet containing up to 1600 mg/kg of DDT and he found that the anti-BSA titers were often (but not consistently) depressed by the DDT treatments. Latimer and Siegel (1974) gave chickens a feed with up to 625 mg/kg of technical DDT for several weeks and they also noted inconsistent effects of DDT on BSA antibody titers.

Immunosuppression and DDT

DDT could have interfered with fish immune responses by several mechanisms. Any hemodilution would probably dilute serum antibody levels and any stress-related corticosteroid lysis of lymphocytes would interfere with the key immune functions performed by these cells. The acute DDT exposure part of this study would tend to support the possibility of hemodilution. The sub-acute exposure part might tend to support a lymphocyte lysis mechanism, as several of the hematological parameters seemed to stabilize, yet

the # WBC and % L were still depressed by DDT.

DDT seems capable of interfering with inflammatory responses (Askari and Gabliks, 1979; Gabliks and McLean, 1979; Gabliks and Utz, 1979) and antibody production (Vos, 1977; Wiltrout et al., 1978) and has also been shown to have direct inhibitory effects on other aspects of regular immune functioning.

Often foreign antigens must first be encountered, engulfed, and digested by phagocytic leukocytes. DDT has been shown to reduce the migratory and phagocytic abilities of mammalian leukocytes (Evdokimov, 1974 and 1975; Traczyk et al., 1976; Lis and Mierzejewski, 1977). Certain lymphocytes must then be able to respond properly to an antigenic stimulation, but DDT has been found to depress lymphocyte metabolism and normal blastogenic transformation (Subba Rao and Glick, 1977; Kannan and Sharma, 1979; Lee and Park, 1979).

Fish seem as likely to be susceptible to DDT-caused immunosuppression as are the other vertebrates. The mechanisms for such effects need to be explored further.

Anomalous Dose-Responses

In certain sections of the results presented in this dissertation, the lowest dose of DDT had an enhanced effect over some of the higher doses. This nonlinear dose-response effect may be partially explained by the following. Bliznakov (1977) found that agents that modify the immune response can exhibit dual effects (stimulation and depression) depending on the dose level. He found that these dose-response relationships were "not only nonlinear and nonmonotonic, but generally W- or M-shaped". This idea is partially supported by

a study (Carmines et al., 1979) in which Kepone caused changes in the phagocytic ability of macrophage-like tissue culture cells. Various doses of Kepone switched in their stimulatory or inhibitory effects on phagocytic ability with increased time of exposure. A nonlinear dose-response relationship was also seen in radiation effects on mouse spleen PFC responses to SRBC (Anderson and Lefkovits, 1979). Small doses of radiation augmented the PFC response, while the higher doses suppressed this response.

Despite a few anomalous results, my research has shown that DDT can cause significant alterations in some hematological parameters and immunological responses of the goldfish. Admittedly the doses used here were large, but then the goldfish is a hardy species, and rather high levels of DDT (and metabolites) have been found in several species in nature. As DDT is not by any means the only chemical of environmental concern that can interfere with vertebrate hematological parameters and immunological responses, perhaps this work can stimulate others to explore the effects of other environmentally persistent agents on fish hematological parameters and immunological responses.

SUMMARY

DDT has been and remains an important insecticidal chemical. Fish have been commonly exposed to DDT and even concentrate DDT residues. There are few data available on effects of DDT upon fish blood and immune responses. This study was designed to examine some effects of lethal and sub-lethal doses of DDT on various hematological and immunological criteria.

Doses of DDT causing 50% mortality in a group of goldfish in 96-hr increased as fish size increased. Increased doses of DDT generally caused numbers of erythrocytes and leukocytes (and associated hematological parameters) to decline. The hematological alterations seen tended to moderate as fish size increased.

DDT treatments often resulted in suppression of goldfish primary and secondary immune responses. DDT reduced the number of antibody-forming cells found in anterior kidney and spleen, and DDT also often reduced the titers of antibodies found in the serum. DDT suppressed the normal immune rejection of foreign scale-grafts. The insecticide interfered with the normal humoral and cell-mediated immune responses in the goldfish.

Mechanisms probably inducing these hematological and immunological changes may have been a general hemodilution caused by inhibited osmoregulatory abilities and could also involve a stress-induced hormonal interference in normal leukocyte functioning.

This research attempted to provide a framework of information about effects of an insecticide on the blood and immune responses of a fish. Hopefully others will build on this framework. Comparisons

between my results and those of others testing additional species of fish and other chemicals of environmental concern would probably result in increased understanding of the mechanisms involved.

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APPENDICES

Table 13. Acute effects of intraperitoneal injection of DDT on mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)[a] of small, medium or large size goldfish.[b]

Fish Size	DDT Dose (mg/kg fish)	MCV(μ^3)	MCH(pg)	MCHC(%)
Small	0	209 ± 7.1	47.9 ± 1.5	23.1 ± 1.0
	10	201 ± 6.3	47.3 ± 1.1	23.7 ± 0.9
	50	218 ± 8.2	51.2 ± 1.6	23.5 ± 0.4
	100	243 ± 14.5	58.1 ± 4.3*	24.0 ± 1.2
	250	236 ± 14.3	57.7 ± 3.6*	23.4 ± 0.9
	500	258 ± 14.4*	59.9 ± 2.9*	23.3 ± 0.6
Medium	0	199 ± 8.8	46.8 ± 2.3	23.5 ± 0.3
	10	203 ± 11.6	49.7 ± 2.8	24.5 ± 0.4
	50	208 ± 11.4	49.1 ± 3.0	23.7 ± 1.1
	100	203 ± 17.4	48.1 ± 4.2	23.8 ± 1.1
	250	197 ± 11.2	44.2 ± 2.7	22.5 ± 0.3*
	500	209 ± 18.7	47.3 ± 4.4	22.7 ± 1.2
Large	0	163 ± 7.3	38.3 ± 2.6	23.4 ± 1.2
	10	195 ± 10.4*	47.3 ± 1.1*	24.5 ± 0.7
	50	188 ± 4.9*	42.8 ± 2.7	22.8 ± 1.4
	100	180 ± 4.7	42.0 ± 1.6	23.4 ± 0.8
	250	180 ± 10.7	41.5 ± 2.3	23.3 ± 1.7
	500	228 ± 16.4*	47.1 ± 2.7*	20.8 ± 0.6

a = MCV, MCH and MCHC calculated as described on p. 123 in Davidsohn Nelson (1974)

b = averages for six fish ($\bar{X}$ ± S.E.)

* = significantly different from respective 0 dose, t-test, $\alpha = 0.05$

Table 14. Sub-acute effects of intraperitoneal injections of DDT on days 0, 26 and 52 on the mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC)[a] of medium size goldfish.[b]

DDT Dose (mg/kg fish)	MCV(μ^3)	MCH(pg)	MCHC(%)
0	173 ± 12.6	41.0 ± 2.9	23.7 ± 0.5
10	248 ± 22.6*	58.5 ± 5.9*	23.5 ± 0.3
50	235 ± 21.3*	52.9 ± 3.8*	22.8 ± 0.8

a = MCV, MCH and MCHC calculated as described on p. 123 in Davidsohn and Nelson (1974)

b = averages for 10 fish ($\bar{X}$ ± S.E.) on day 56

* = significantly different from 0 dose, t-test, $\alpha = 0.05$

Table 15. Primary and secondary PFC responses in the mesonephric kidney, intestine, liver and peripheral blood of goldfish immunized with SRBC.

Days After Primary Immunization	PFC/10^6 WBC ($\bar{X}$ ± S.E.(n))			
	Mesonephric Kidney	Intestine	Liver	Peripheral Blood
7	5.84 ± 0.0 (1)	-	1.95 ± 1.95(2)	9.39 ± 0.0 (1)
9	9.39 ± 7.41(2)	-	0.0 ± 0.0 (2)	2.25 ± 2.25(2)
11	1.28 ± 1.28(2)	-	-	-
Days After Secondary Immunization				
0	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)
3	9.47 ± 3.33(6)	5.59 ± 1.94(6)	2.34 ± 1.26(6)	1.55 ± 0.98(6)
5	107.6 ± 26.6(6)	32.5 ± 20.0(6)	15.2 ± 4.65(6)	6.63 ± 2.51(6)
7	19.0 ± 6.19(6)	4.44 ± 1.77(6)	1.54 ± 1.00(6)	2.07 ± 0.98(6)
9	7.25 ± 2.47(6)	1.63 ± 0.78(6)	0.0 ± 0.0 (6)	0.57 ± 0.57(6)
11	5.58 ± 2.06(6)	0.39 ± 0.39(6)	0.0 ± 0.0 (6)	0.0 ± 0.0 (6)
13	3.53 ± 1.69(5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)
15	3.67 ± 1.34(5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)
17	2.51 ± 1.02(5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)
19	0.63 ± 0.26(5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)
21	0.08 ± 0.08(5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)	0.0 ± 0.0 (5)

Table 16. Primary and secondary PFC responses in the mesonephric kidney, intestine, liver and peripheral blood of goldfish pretreated with 50 mg/kg DDT two days prior to immunizations with SRBC (n = 5 for all).

Days After Primary Immunization	PFC/10^6 WBC ($\bar{X}$ ± S.E.)			
	Mesonephric Kidney	Intestine	Liver	Peripheral Blood
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
5	2.63 ± 2.42	0.0 ± 0.0	1.68 ± 1.10	0.0 ± 0.0
7	6.51 ± 4.36	0.0 ± 0.0	1.29 ± 1.29	1.65 ± 1.65
9	1.75 ± 0.88	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
13	0.86 ± 0.56	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
17	0.10 ± 0.10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Days After Secondary Immunization				
0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	2.73 ± 0.99	2.57 ± 1.09	2.27 ± 1.03	1.84 ± 1.14
5	53.4 ± 31.0	13.4 ± 5.13	8.54 ± 2.96	4.23 ± 1.95
7	12.2 ± 5.28	4.97 ± 2.77	2.87 ± 1.33	2.02 ± 1.30
9	15.9 ± 7.77	2.20 ± 2.20	1.03 ± 0.66	0.63 ± 0.63
13	6.12 ± 1.67	0.51 ± 0.51	1.52 ± 0.95	0.0 ± 0.0
17	3.17 ± 2.15	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
21	1.92 ± 0.90	0.0 ± 0.0	0.56 ± 0.56	0.0 ± 0.0

VITA

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