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Title: Implications of Dietary Lipid and Carbohydrate Intake on the
Liver and the Susceptibility of Hatchery-reared Rainbow Trout (Salmo
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The hypotheses to be tested in this investigation are: 1) there
is a threshold level of liver lipid and glycogen content above which the
health condition of hatchery-reared fish is impaired, 2) fatty and high
glycogen livers impair liver function and structure, and 3) fatty and
high glycogen livers increase the susceptibility of fish to vibriosis.

Under the conditions of this study, levels of hepatosomatic index,
specific gravity, liver lipid and glycogen content sufficient to cause
pathologic conditions in rainbow trout were not established.

Blood parameters like hematocrits and mature red blood cell counts
were found to be normal. The condition factors (K) were indicative of
well fed fish. It was concluded that all the above parameters fell
within a normal range of values representative of healthy rainbow trout.

Determination of plasma alanine aminotransferase activity and
histological examination of the livers did not indicate any sign of
liver pathology. It was concluded that all the livers were normal and
healthy, even though the liver composition varied with each dietary
treatment.

The disease challenge did not reveal any difference in susceptibility to vibriosis as measured by cumulative mortalities and mean day to death. The agglutinating antibody titers against Vibrio anguillarum were similar in strength and might have been protective. It was concluded that susceptibility to disease was unaffected by the different liver composition observed for each dietary treatments.

Implications of Dietary Lipid and Carbohydrate Intake on the Liver and
the Susceptibility of Hatchery-reared Rainbow Trout (<u>Salmo</u> <u>gairdneri</u>) to
Vibriosis

by

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A mis queridos padres,

Jacobo y Lidia

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Implications of Dietary Lipid and Carbohydrate Intake on the Liver and the Susceptibility of Hatchery-reared Rainbow Trout (Salmo gairdneri) to Vibriosis

INTRODUCTION

In contemporary fish culture, improved water quality and hatchery practices permit increasingly intense feeding of fish held at high densities. In order to reduce feed costs, expensive protein is replaced by higher levels of lipids and/or digestible carbohydrates. This produces excellent growth rates in fish but may also cause fatty or high glycogen livers. The incidence of fatty and high glycogen livers in rainbow trout (Salmo gairdneri) has been reported to be responsible for high mortality and increased disease susceptibility in hatchery-reared fish. Enlarged and discolored livers often indicate problems with quality of feed. It is possible that abnormal accumulations of fat and/or glycogen in the liver impair liver metabolism and affect the performance of fish under hatchery conditions. It is not understood whether fatty and high glycogen livers impose physiological stress, suppress the immune response, or both, in hatchery reared fish. Because hepatocytes respond readily to nutritional factors, many studies in fish have been carried out to evaluate the effects of nutrition on hepatocytes. Most of these studies have been limited to clinical analysis of liver biochemistry, physiology, and histology, and thus provide little information on the overall performance capacity of the fish in hatchery environment. The addition of a disease challenge to the above assays may provide meaningful information on the fish health status. The experiments

reported here were designed: 1) to determine how the development of fatty and high glycogen livers in hatchery-reared rainbow trout are influenced by dietary lipid and carbohydrate intake, 2) to determine what level of fat and glycogen content in the liver causes hepatic damage, 3) to determine the relationship between the fatty or high glycogen liver and the susceptibility to vibriosis in rainbow trout, and 4) to determine the effect of fatty and high glycogen livers on the immune response in rainbow trout.

The hypotheses to be tested in this investigation are: 1) there is a threshold level of liver lipid and glycogen content above which the health condition of hatchery-reared fish is impaired, 2) fatty and high glycogen livers impair liver function and structure, and 3) fatty and high glycogen livers increase the susceptibility of fish to vibriosis.

Literature Review

Among teleost fish there are differences with respect to lipid and carbohydrate storage in the liver. These differences can be attributed to species, seasonal changes, temperature, age, reproduction and diet (Welsh and Storch, 1973). The deposition of large amounts of lipids in hepatocytes is normal for many marine teleosts, including cod, halibut, and haddock, as well as elasmobranchs such as dogfish shark. This phenomenon is less common, however, among freshwater teleosts and is pathological in warmblooded animals (Vague and Fenasse 1965; Bilinski 1974). In general, the liver is the major lipid storage site in sluggish, bottom-dwelling fish such as haddock, cod, etc., whereas skeletal muscle serves this function in more active species such as

rainbow trout, salmon, herring and mackerel (Vague and Fenasse 1965;
Tashima and Cahill 1965; Robinson and Mead 1973; Bilinski 1974;
Sargent 1976). Trout muscle contains more triglycerides than liver,
and the intestine contains even larger amounts of total lipid than
liver and muscle (Leger et al. 1981). Snieszko (1972) stated that wild
trout do not have fatty livers and that they are not fitted to utilize
fats which accumulate in the liver under hatchery conditions.

Fish contain relatively little carbohydrate in their body tissues.
In salmonids, most carbohydrate is stored as glycogen which is used as
an immediate energy reserve in liver and muscle (Hochachka and Sinclair
1962; Phillips 1969; Cowey and Sargent 1972). The natural diet of
trout contains little carbohydrate, and these fish are not fitted to
efficiently use large quantities of dietary carbohydrates (Phillips
1969). According to Storch and Juario (1983), livers of salmonids
primarily store glycogen, and contain little lipid under normal
conditions.

The Influence of Dietary Fats

Normal livers in freshly killed trout are light to dark reddish-
brown (Simon and Dollar 1967). Fatty livers are described as pale
yellow, swollen, and greasy in appearance (Phillips and Podoliak 1957).
Fatty infiltration of the liver may be diagnosed by observation of
intracellular fat droplets within hepatocytes of frozen liver sections
stained with Sudan Black B and examined by light microscopy (Post
1983). In advanced cases of fatty liver there is deposition of ceroid,
a yellow lipoid pigment (Wood and Yasutake 1956; Davis 1965). Fontaine
and Callamand (1953) observed that wild trout livers had 2% lipid. Lee

and Wales (1973) reported normal liver in rainbow trout to have 1.9 - 2.3% lipids.

Hewitt (1937a, b) studied the causes of fatty liver in hatchery-reared trout. He reported that in wild trout, total fats constituted 21.5% to 23.5% of liver dry weight. He also noted that livers of wild and hatchery trout were rarely similar in composition, chemically or structurally. Fatty degeneration could be observed in the livers of most hatchery trout which can be attributed to excessive feeding, feeding of diets high in fats and cholesterol, and to a dietary deficiency of vitamin C. Fry fed on liver 4 or 5 times a day developed fatty livers almost at once. Trout fed a diet low in vitamin C performed poorly, and fat content of their livers exceeded 30% dry weight. By contrast, trout fed a high vitamin C diet had a very low mortality rate, fast growth rates and livers lacking signs of fatty degeneration.

A chemical and histological study of hatchery trout by Wood et al. (1957) indicated that livers and viscera of these fish are more fatty than livers of wild trout. Phillips and Podoliak (1957) and Davis (1965) reported that fatty infiltration of the liver is caused by overfeeding and by diets high in fat. Extreme fatty infiltration of the liver has been responsible for anemia resulting in excessive mortality (Leitritz and Lewis 1980). Snieszko (1972) stated that trout raised in hatcheries are highly susceptible to liver damage caused by improper diets. Henderson and Sargent (1981) fed groups of trout diets containing 2%, 5%, or 10% fat in addition to 50% protein (as casein) and not less than 30% carbohydrate (as dextrin). They observed that livers of animals fed the 2% fat diet were generally

larger and had slightly higher lipid contents than livers of animals
fed the 5% and 10% fat diets. However, liver weight was not
significantly different among fish receiving the three diets. Reinitz
(1983) observed that fatty vacuolation of the liver increased with
feeding rates of diets that were either excessively high, or
excessively low, in fat and protein.

Two studies should be mentioned which failed to find pathological
changes in livers of salmonids fed high fat diets. Norris and
Donaldson (1940) reported that excessively fatty livers did not result
from feeding high (20%) fat and cholesterol diets to young chinook
salmon (Oncorhynchus tshawytscha). Hepatosomatic indices (liver
weight/body weight x 100) in these fish ranged from 0.75 to 2.02, and
liver lipid content varied between 8.2% to 17.7%, levels which these
researchers did not consider abnormal. Higuera et al. (1977) fed
rainbow trout a high fat diet (15%) ad libitum, and observed no
pathological changes in the livers. Microscopic observations showed
accumulation of lipid in the liver without any damage to the
hepatocytes.

Some studies have addressed the mechanism by which high fat diets
damage salmonid livers. Fowler and Wood (1966) reported that hard fats
(saturated) are metabolized very poorly in chinook salmon, and tend to
accumulate in liver cells as lipoprotein complexes. Prolonged feeding
of hard fats (saturated) also damages hematopoietic tissues, resulting
in anemia. Lin et al. (1977) studied the influence of dietary lipid on
lipogenic enzyme activities of coho salmon (Oncorhynchus kisutch).
Consumption of a high fat diet for 23 days or fasting for two days
decreased the in vitro and in vivo rates of fatty acid synthesis in the

liver. When fasted (48 hours) fish were fed a high carbohydrate diet for four hours the rates of hepatic fatty acid synthesis and lipogenic enzyme activity increased.

Several researchers have studied the effects on the liver of a deficiency of the essential fatty acids (EFA) $\omega3$ and $\omega6$. Poston (1968) fed trout a diet consisting of hydrogenated vegetable oil that are low in linoleic and linolenic acid and observed accumulation of lipid in the liver. In a similar study Castell et al. (1972) observed fin erosion, heart myopathy, shock syndrome, and fatty livers that were swollen and pale. Takeuchi and Watanabe (1979) observed higher hepatosomatic indices and liver lipid content in trout fed diets deficient in EFA.

Pfeffer and Barte (1980) studied the influence of the source of dietary fat and protein on fatty liver in trout. When 66% to 100% of dietary protein came from fish meal, livers contained an average of 2.6% fat and 17.7% nitrogen-free extract (NFE). When casein was the source of dietary protein, livers contained about 30% fat and 6% NFE. The influence of the source of dietary fat, however, were of minor importance.

The Influence of Dietary Amino Acids and Protein

Lee and Putnam (1973) observed that higher protein/calorie ratios were positively correlated with liver size, liver sugar content, percentage body fat, percentage body protein, and negatively correlated with percentage liver lipids. They stated that their correlations were observed for similar protein/calorie ratios regardless of the dietary levels of protein and lipid.

Walton et al. (1984) fed trout diets that contained approximately 20% starch, but which varied in lysine levels. They observed that liver lipid content was not significantly different in trout fed these diets, but the hepatosomatic index was higher in fish given low levels of dietary lysine.

The Influence of Dietary Carbohydrates

Fish livers that contain a high percentage of glycogen are swollen, glossy, pale and may be confused with fatty livers (Phillips et al. 1948). Hepatocytes contain large numbers of glycogen vacuoles that may be observed in liver sections stained with Best's Carmine (Post 1983). Normal liver glycogen levels in trout and salmon are reported to range from 0.5 to 3.0% of the liver weight, and glycogen levels greater than 15% in rainbow trout livers resulted in high mortalities and increased susceptibility to diseases (Post 1983).

A number of researchers have investigated the effects of high carbohydrate diets on salmonid livers. The early reports of Tunison et al. (1939, 1942) stated that brook trout (Salvelinus fontinalis) fed diets rich in cooked starch, sugar or dextrin developed enlarged livers with a high percentage of glycogen, but those fed raw starch or cellulose had livers comparable in size and glycogen content to trout fed meat alone. Phillips et al. (1948) and Phillips and Brockway (1956) reported that feeding carbohydrates over long periods of time may result in liver enlargement often followed by an increased mortality. Austreng et al. (1977) reported that trout fed high carbohydrate diets had heavier and discolored livers, which contained more fat and carbohydrate than those of trout fed moderate levels of

carbohydrate. Bergot (1979) fed trout diets containing varying levels of glucose. Animals receiving the highest (30%) levels of glucose retained higher energy reserves in the form of increased liver glycogen and greater visceral fat deposits.

Studies by Atkinson and Hilton (1981) showed that liver weight and liver glycogen increased, and liver protein and body lipid levels decreased in response to increased dietary carbohydrate. A similar study using glucose showed that liver:body weight ratios and glycogen content increased with increasing dietary glucose up to 15% of the diet (Hilton et al. 1982). Above 15% of dietary glucose no further increase in liver:body ratios and glycogen content was detected. Nigrelli and Jakowska (1961) and Hilton and Dixon (1982) reported that for salmonid diets, the maximum tolerable level of digestible carbohydrate is 20%. In a study by Hille et al. (1980), fatty degeneration occurred in rainbow trout fed to excess and in fish fed diet formulations high in carbohydrates and low in protein. Excess feeding of diets high in carbohydrates (36 and 53%) resulted in additional deposition of fat and necrotic changes in the liver.

The Influence of Dietary Vitamins

McLaren et al. (1947) reported that thiamine and p-aminobenzoic acid deficient trout had a fat content that was more than double that of controls. Both McLaren et al. (1947) and Halver and Coates (1957) found that choline was required for growth, efficient food conversion and prevention of fatty livers in both trout and salmon. A study by Halver (1972) linked fish diets containing excess levels of vitamin A to enlargement of liver and spleen, abnormal growth, and skin lesions.

The National Research Council (1973) reported that excess feeding (at the rate of 1%) of niacin caused increased levels of lipids in the liver of immature brook trout (<u>Salvelinus</u> <u>fontinalis</u>). Poston and McCartney (1974) observed abnormalities in fatty acid synthesis among rainbow trout deficient in biotin. Castledine et al. (1978) observed liver enlargement and excessive liver glycogen accumulation in trout fed diets deficient in this same vitamin. Barnett et al. (1979) showed that vitamin D3 (cholecalciferol) deficiency in rainbow trout was characterized by markedly decreased weight gains and feed conversion, and by increased lipid content of carcass, white muscle and liver.

The Influence of Feed Type

Miller et al. (1959) reported that the type of feed used can affect the livers of rainbow trout. Trout fed dry commercial pellets had higher glycogen levels in both liver and muscle than trout fed fresh beef liver diet. These investigators also observed that exercise did not reduce liver glycogen in trout fed pellets, but it did have this effect in trout fed liver. More recently, Hilton et al. (1981) observed that the hepatosomatic index and liver glycogen content were significantly higher in rainbow trout fed on extruded pellets compared to animals fed on steamed pellets. The extrusion process requires higher levels of heat and pressure than steam pelleting and consequently the former process increases the carbohydrate digestibility.

The Influence of Environmental Conditions

Water temperature has been shown to influence both the morphology and function of trout liver. Poston (1968) observed that deposition of glycogen accounted for most of the differences in liver among trout reared in colder water while both lipid and glycogen influenced liver size in fish reared in warmer water. Fish were fed the same diet at two different temperatures (8.3° and 12.4°C). Dean (1969) observed an overall trend toward higher levels of lipid and glycogen in red muscle, white muscle and in livers of cold-acclimated trout. Hazel and Sellner (1979) noticed enhanced rates of fatty acid lipogenesis in hepatocytes of cold-acclimated trout. However, it was observed that cold-acclimated trout have significantly lower levels of liver lipids than warm-acclimated fish. Hilton (1982) reported that trout reared at 10°C had higher liver weights and liver glycogen levels than trout reared at 15°C, regardless of whether they had been fed easily digestible or poorly digestible carbohydrates.

Saddler and Cardwell (1971) investigated the effect of tagging on fatty acid metabolism of pink salmon. Fish were anesthetized (MS 222) and tagged with Denison internal anchor tube tabs. The tag was inserted into epaxial muscle tissue just posterior and below the dorsal fin. They found that lipid in liver increased from 5.9% in untagged salmon to 10.4% in tagged salmon. These changes were also accompanied by increases in liver weight.

In a study designed to explain how environment controls annual cycles of fattening in fish, Vlaming et al. (1975) investigated the effect of prolactin on fish livers. Livers taken from fish late in the

photoperiod and incubated with prolactin had higher lipid levels than livers from control animals. The authors suggested that prolactin may stimulate lipogenesis or inhibit lipid autolysis.

The Influence of Rancid and Toxic Substances in Feed

The condition known as fatty or lipoid liver degeneration is considered to be an important nutritional disease in rainbow trout raised in hatcheries (Nigrelli and Jakowska 1961). It is characterized by accumulation of ceroid (insoluble fat) in the liver cells (Halver 1972). Ceroid deposition appears to be a general characteristic of teleosts, especially in fish kept in captivity, infected with parasites or exposed to toxic materials (Nigrelli and Jakowska 1961).

Ceroid deposition was first noted by Wood and Yasutake (1956) in an investigation of the effects of toxic materials upon rainbow trout. Adult trout suffering acute mortality caused by excreted metabolic products from too many fish in a limited water supply had moderately fatty livers which contained ceroid. Trout fed a diet contaminated with gossipol (yellow pigment from cotton seed) also showed excessive deposition of ceroid. These same researchers observed moderate to extremely fatty livers in rainbow trout fingerlings infected by bacterial disease (myxobacteria). They also reported signs similar to lipoid liver degeneration in adult brook trout infected with ulcerative disease (Hemophilus piscium). Ghittino (1965) and Rasmussen (1965) described signs of lipoid liver degeneration in rainbow trout infected with viral hemorrhagic septicemia (VHS).

Rancid feed with low antioxidant vitamins has also been shown to be a cause of fatty liver degeneration. Roberts (1978) and Smith

(1979) observed that fish exhibited fatty liver degeneration when fed diets deficient in vitamin C and E constituted from trash fish, or pelleted diets in which part of the lipid component had become rancid. Smith (1979) also reported that rainbow trout fed a rancid diet deficient in vitamin E and C showed growth depression, microcytic anemia and lipoid liver degeneration. Trout fed the same rancid diet but containing vitamins E and C did not develop these symptoms.

Other toxic substances may also cause pathological liver changes. Roehm et al. (1970) fed trout cyclopropenoic fatty acid (CPFA) and noted liver enlargement and microscopic examination of the liver showed large numbers of glycogen vacuoles. Halver (1972) described vacuolation of liver cells in fish exposed to high (1, 5, and 10 ppm) concentrations of copper. He also detected gross liver changes in fish fed a 1:1 mixture of trout pellets and bentonite for a three month period. Livers of juvenile rainbow trout became severely vacuolated after exposure to pesticides such as DDT (Halver 1972). Pfeifer et al. (1980) i.p. injected rainbow trout with carbon tetrachloride and observed that their livers became highly vacuolated with glycogen. However, he observed that rats exposed to this same chemical resulted in high deposition of triglycerides.

MATERIALS AND METHODS

General Design

The nutrition study was carried out in two experiments. Low fat diets 1 and 3 were assigned as the control diets to compare the effect of diets high in lipids and carbohydrates on the fish liver and overall performance. In Experiment I, each diet was assigned at random to two tanks of fish (Fig. 1). Diet 1 (control) was fed at restricted levels and Diet 2 (high fat) was fed ad libitum. Diets 1 and 2 were fed three times daily for 18 weeks (April-August). At the end of Experiment 1, one tank of fish per diet was sampled and waterborn challenged by Vibrio anguillarum. The remaining tanks of fish were maintained on their respective diets (1 and 2) for another three weeks. In Experiment II, the group fed Diet 1 was started on Diet 3 (low fat) and the group fed Diet 2 was started on Diet 4 (high fat) (Fig. 1). A third group (stock) fed Diet 2 ad libitum for 21 weeks was started on Diet 5 (25% dextrin). Diet 3 (control) was fed at restricted levels, Diet 4 and Diet 5 were fed ad libitum. Diets 3, 4, and 5 were fed three times daily for three weeks (August-September). At the conclusion of Experiment II all groups were sampled and waterborne challenged by Vibrio anguillarum. Feeding levels for Diet 1 and Diet 3 were adjusted every 14-18 days. The feeding level was decreased gradually from 5% to 2.9% of the body weight per day throughout the 24 weeks of feeding.

DURATION   DIETS

18 WEEKS

CONTROL
DIET 1
(10.9% fat)

DIET 2
(19.7% fat)

DIET 2
(19.7% fat)

a   b

a   b

a

SAMPLED &
CHALLENGED
EXP. I

SAMPLED &
CHALLENGED
EXP. I

3 WEEKS

CONTROL
DIET 3
(9.7% fat)

DIET 4
(28.1% fat)

DIET 5
(26.9% carbo-
hydrate)

3 WEEKS

SAMPLED &
CHALLENGED
EXP. II

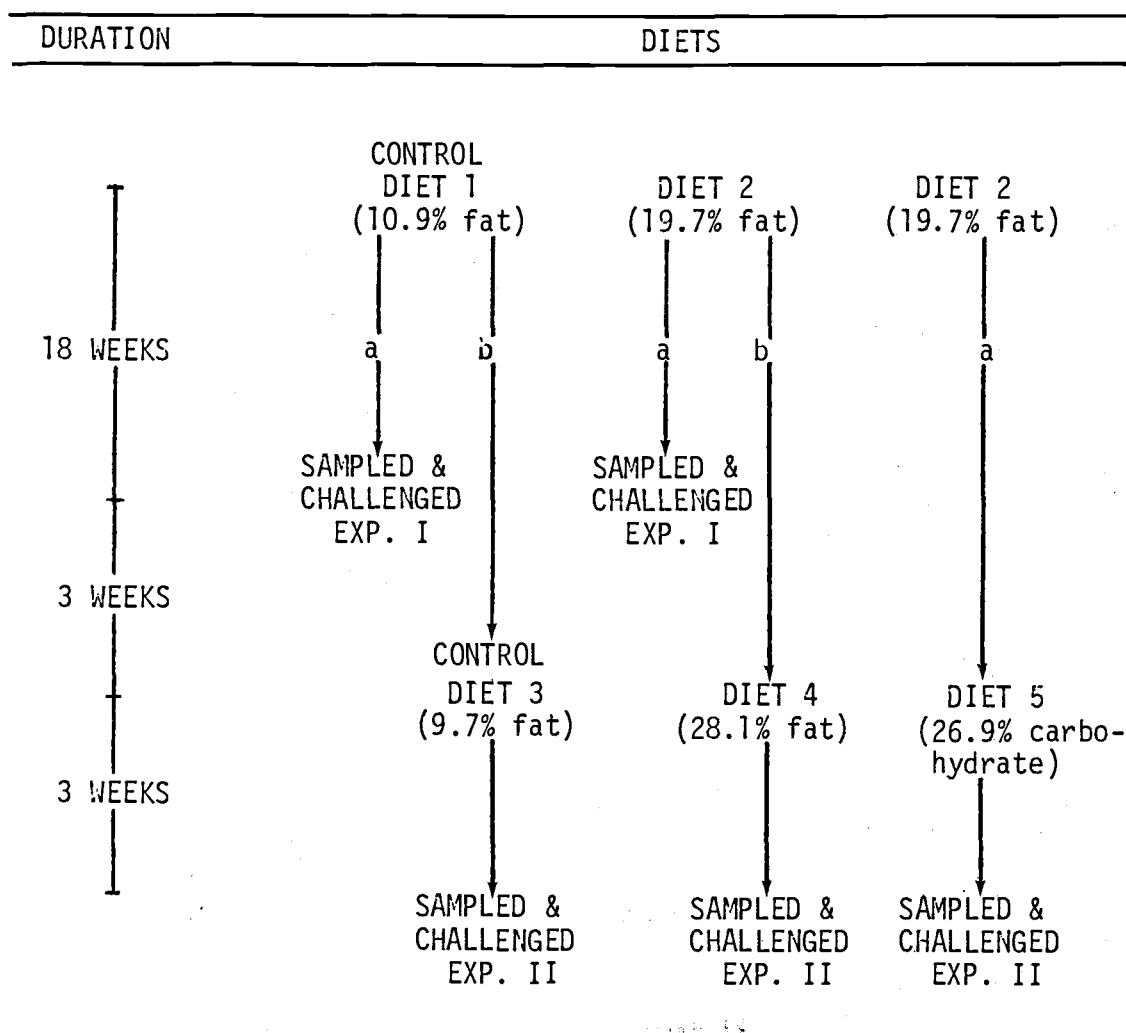SAMPLED &
CHALLENGED
EXP. II

SAMPLED &
CHALLENGED
EXP. II

Fig. 1.   Flow diagram of the feeding schedule.

Composition of Diets

Diets 1, 2, and 3 were commercial fish feeds (Silver Cup)
manufactured by Murray Elevator (Murray, Utah) (Table 1). Diet 4 (high
fat) was prepared by adding salmon oil to Diet 3 (commercial feed
Salmon No. 3) in the proportion 1:4 by weight. Diet 5 was prepared by
adding dextrin and salmon oil to Diet 3 in the proportion 2.5:1:6.5 by
weight. Salmon oil was used to help bind dextrin to the feed. All
diets were stored at -20°C. Proximate composition of all diets were
carried out at OSU Seafood Laboratory in Astoria, Oregon.

Experimental Fish - Care and Conditions

Rainbow trout fingerlings (average weight 1.4 g per fish) were
obtained from Rainbow Trout Gardens in Corvallis, Oregon. Fish were
transported in aerated containers to the Hatfield Marine Science Center
at Newport, Oregon. Fish were randomly assigned in groups of 200 to
round fiberglass tanks (100 liter capacity), and 140 fish were
transferred after nine weeks to larger tanks (600 liters capacity).
Fresh water supply from the city of Newport was dechlorinated on a
charcoal filter system and flow rate was adjusted to 4-5 liters/min per
tank. Throughout Experiment I and II, water temperature ranged from
11.5° to 18°C, and 18° to 19°C, respectively. Dissolved oxygen ranged
from 11.0 mg/liter to 8.2 mg/liter and pH averaged 7.34. White
fluorescent light in a windowless laboratory provided a photoperiod of
approximately 12 hours light. The mortality was less than 2% during
the feeding period in Experiment I, and dropped to zero in Experiment
II.

Table 1. Proximate analysis of experimental diets.

| Diet # | Percent Wet Weight | | | | | Energy[3] (kcal/g) |
	Moisture	Ash	Fat	Protein	Carb.[2]	
EXP I[1]						
1*	9.38	11.89	10.94	59.91	--	3.2
2	8.31	10.62	19.69	54.06	--	3.7
EXP II						
3*	10.07	9.30	9.70	46.91	--	2.6
4	8.06	9.43	28.09	47.59	--	4.1
5	7.33	7.97	17.10	40.75	26.85	4.3

[1]Experiment I: Diet 1, (Silver Cup, Trout No. 2); Diet 2, (Silver Cup, Salmon No. 2).
Experiment II: Diet 3, (Silver Cup, Salmon No. 3); Diet 4, (Silver Cup, Salmon No. 3 plus salmon oil); Diet 5, (Silver Cup, Salmon No. 3 plus dextrin).
[2]Carbohydrates estimated by % of difference.
[3]Based on 8 kcal/g of fat, 3.9 kcal/g of protein and 3.3 kcal/g of dextrin (National Research Council 1973).

*Control

Sampling Procedures

The sampling procedures carried out in Experiment I and II were as follows: fish were deprived of food for 24 hours and collected at random with a dip-net several at a time and placed in a small container with a water solution of unbuffered tricaine methane sulfonate (MS 222, approximately 40 mg/liter). The water solution (MS 222) was aerated and the temperature was similar to the fresh water supply. The anesthetized fish were killed by a sharp blow to the head. Fish were weighed on a top-loading balance (Mettler PL300) to the nearest 0.01 g and total length was determined to the nearest 0.1 cm. The caudal peduncle was cut off and blood was drawn into heparinized micro-hematocrit capillary tubes (75 mm long) and one end closed with

modeling clay. The capillary tubes were centrifuged within the hour at 5000 RPM for 15 minutes and kept in vertical position in refrigeration (4°C) overnight. The hematocrit was determined by means of a micro-hematocrit capillary tube reader. The plasma was kept for a maximum of 48 hours at 4°C and used for determination of alanine aminotransferase activity. The blood smears were prepared, air dried, fixed in methanol and stained with Giemsa to count mature red blood cells. Fish were dissected and the visceral cavity was examined for gross pathological symptoms. The liver was removed, placed in groups of 3 to 4, weighed on an analytical balance (Mettler) to the nearest 0.0001 g and the average weight was estimated. The hepatic volume was estimated by introducing a liver into the water column of a graduated glass syringe and reading the water displacement to the nearest 0.1 ml. Livers were then stored at -20°C until required for lipid and glycogen extraction. Other livers were frozen on dry ice and kept at -20°C for histochemical demonstration of lipids. Other livers were fixed in Bouin's solution and absolute ethanol:formalin (9:1, v/v) solution, until required for general histological observation and histochemical demonstration of glycogen, respectively.

Methods to Test Hypothesis 1: There is a Threshold Level of Liver
Lipid and Glycogen Content Above Which the Health Condition of the Fish
is Impaired

In Experiment I and II, the hepatosomatic index, specific gravity, lipid content and glycogen content of the liver were measured as indicators of pathological threshold level of lipid or glycogen in the liver. Hematocrits, mature red blood cell count, and condition factor (K) were determined to assess fish health condition. A pathological

condition was used as the rational for accepting or rejecting the first hypothesis.

The hepatosomatic index was calculated by the ratio of liver wet weight to body weight times 100. The specific gravity of the liver, i.e., density relative to water, was determined by the ratio of liver wet weight to hepatic volume. Liver lipid content, in percent of liver wet weight, was extracted by a combined method of Bligh and Dyer (1959), Kates (1972), and Lovell (1975) with tripalmitin used as a standard (Appendix A). Liver glycogen content, in percent of liver wet weight, was estimated by the method of Carroll et al. (1956) with glucose used as a standard (Appendix B).

Microhematocrit, the percentage of packed red blood cell volume, was carried out by the method of Snieszko et al. (1960). The mature red blood cells were differentiated by the Giemsa technique and the average percent was estimated after three counts of 100 cells per slide.

Condition factor (K), a mathematical relation between weight and length of fish, was calculated by the ratio of body weight to length cubed times 100 as explained by Piper et al. (1982). Total lengths, measured from the tip of the snout to the tip of the tail when spread normally, were utilized.

Methods to Test Hypothesis 2: Fatty and High Glycogen Livers Impair Liver Function and Cell Structure

In Experiment I and II, the plasma alanine aminotransferase activity and general histology of the liver were utilized to assess the possibility of liver damage (necrosis) and histopathlogy. A

histopathological condition and high plasma enzyme levels were used as the rational for accepting or rejecting the second hypothesis.

Alanine aminotransferase (glutamic pyruvic transaminase, GPT) was determined by the method in Sigma Technical Bulletin No. 505 with a few modifications as suggested by Pfeifer et al. (1977). The transaminase Sigma kit No. 505 was utilized and the enzyme activity was measured at 20°C with a spectrophotometer (Spectronic 70, Bausch and Lomb).

General histology of livers fixed in Bouin's fluid and liver sections (7μ in thickness) stained with hematoxylin and eosin was carried out. Livers fixed in ethanol:formalin (9:1, v/v) solution, and liver sections (7μ in thickness) were stained by the Best's Carmine technique for demonstration of glycogen in hepatocytes. Fresh frozen livers were sectioned (8μ in thickness) and stained with Sudan Black B for demonstration of lipid droplets in cytoplasm.

Methods to Test Hypothesis 3: Fatty and High Glycogen Livers Increase Susceptibility of Fish to Vibriosis

In Experiments I and II, a disease challenge by Vibrio anguillarum and determination of agglutinating antibody titers were utilized to evaluate the disease resistance and specific immune response in fish, respectively. A significantly higher mortality was used as the rational for accepting or rejecting the third hypothesis.

The disease challenge was conducted according to the method of Gould (1977). The Vibrio anguillarum (serotype I), a virulent strain, was obtained from the fish disease laboratory at Hatfield Marine Science Center. The waterborn challenge was run in triplicate of 20 fish each in tanks of 100 liter maximum capacity. The bacterial count of an overnight brain-heart infusion broth (BHI) (Difco) culture of V.

anguillarum was estimated photometrically at 525 nm on a Spectronic 20.
The appropriate inoculum was introduced into the challenge tanks to
make a final concentration of 1.6 x 10^5 bacteria per ml in Experiment I
and 10^5 bacteria per ml in Experiment II. Fish were transferred to
challenge tanks and after 15 minutes the water was turned back on at a
flow rate of 3-4 liters/min. The test was run for 10 days at 18.5°C
water temperature in Experiment I and 14 days at 18°C water temperature
in Experiment II. Mortalities were examined externally and internally
for gross pathological symptoms. Fish were disinfected externally by
swabbing with iodine-detergent solution. Aseptically, the fish were
dissected, an inoculum was taken from the kidney and streaked on BHI
agar plates for isolation of colonies. Presumptive tests for V.
anguillarum were as follows: gram negative bacteria, slightly curved
rods, and sensitive to novobiocin (Difco) and 0/129 (2,4-diamino-6,7-
di-isopropyl pteridine phosphate) sensitivity disks.

The agglutinating antibody titers were determined by the method of
Gould (1977). In Experiment I, the titers were determined in fish that
survived 24 days after being challenged at 18.5°C water temperature.
The test was run in triplicate. Blood from several fish from the same
diet was collected in tubes and allowed to clot at room temperature,
then placed at 5°C overnight. The tubes were centrifuged at 3000
R.P.M. (0°C) for 10 min. Serum samples of 50 μl were diluted in 50 μl
of phosphate buffered saline in a microtiter plate (round bottom) using
a microtiter diluter and calibrated dropper pipette. An equal volume
of V. anguillarum (serotype I) antigen was added to each dilution of
serum (1:2 to 1:64). Plates were incubated for 1 hour at room

temperature and then at 5°C overnight. Titers were read as the last dilution that displayed agglutination.

Statistical Analysis

In Experiment I, two sample t-tests, variance ratio tests and 2x2 contingency tables with the Yates correction for continuity were applied where appropriate (Zar 1974). In Experiment II, single factor analysis of variance, Bartletts' test, multiple comparisons among variances, Newman-Keuls test and 2x3 contingency tables were applied where appropriate (Zar 1974). The level of significance accepted was $\alpha=0.05$ in both Experiments I and II.

RESULTS

In both experiments I and II, fish fed a high fat diet were compared to those fed a low fat control diet. Additionally, a high carbohydrate diet was included in Experiment II. The comparisons included observations on the following variables.: Hepatosomatic index (Table 2); liver specific gravity (Table 3); liver lipid content (Table 5); liver glycogen content (Table 6); microhematocrit (Table 7); percent of mature red blood cells (Table 8); condition factor (Table 9); plasma alanine animotransferase activity (Table 10); histological examinatiion; disease challenge (Table 11); and agglutinating antibody titers.

In Experiment I, statistically significant differences were observed in the variances of hepatosomatic indices, and among the means of specific gravity and glycogen content of the liver, and condition factor. In Experiment II, differences in the variances of liver specific gravity, liver lipid content, and microhematocrits, and among the means of hepatosomatic indices, liver glycogen content, and condition factors were observed. However, the only variables that differed consistently in both experiments were the means of liver glycogen content and condition factors.

Plasma GPT activities were significantly different in both Experiments I and II. Observation of histological preparations of livers from Experiment I revealed little or no vacuolation in fish fed Diet 1 (low fat), and a low degree of vacuolation in fish fed Diet 2 (high fat) (Figures 2 and 3). Similarly, livers from Experiment II had little or no vacuolation in fish fed Diet 3, and moderate and high degrees of vacuolation in fish receiving Diets 4 and 5 respectively

(Figures 4, 5, and 6). In all liver sections observed the nuclei were uniform in size and shape, and the liver cells were arranged as a muralium (or wall) two cells in thickness. All livers observed were considered normal, even those with highly vacuolated hepatocytes (J.D. Hendricks, personal communication). The presence of glycogen granules was confirmed by treatment with amylase; granules were observed in untreated sections and no granules were observed in amylase treated preparations. However, the results of the glycogen granule and lipid droplet observations were inconclusive because cellular structure was severely damaged during tissue preparation.

Waterborne challenges with the common pathogen Vibrio anguillarum indicated that the changes in liver composition and structure did not increase susceptibility to vibriosis. In Experiment I, the fish fed Diet 2 (high fat) had a lower cumulative mortality (Fig. 7) than for Diet 1 (low fat). However, there was no significant difference in the cumulative mortality and mean day to death of fish fed Diet 1 and Diet 2 (Table 11). Similarly, in Experiment II the cumulative mortality was higher in fish fed Diet 4 (high fat) and Diet 5 (high carbohydrate) and lower for Diet 3 (low fat) (Fig. 8), but the cumulative mortality and mean day to death were again not significantly different in fish fed Diet 3, Diet 4 and Diet 5 (Table 11). Agglutinating antibody titers did not differ significantly between treatments in Experiment I.

Table 2. Hepatosomatic index (H.I.) (liver size, % of body weight).

Experiment I					Experiment II			
Diet #	N	Mean[1] H.I.	S.D.*	Var.[2]	Diet #	N	Mean[3] H.I.	S.D.
1	4	1.00	0.01	0.0001	3	4	0.93[a]	0.05
2	4	1.05	0.05	0.0025	4	4	1.02[a]	0.11
					5	4	1.40[b]	0.23

[1]Mean of 4 pooled samples of 4 fish per sample $\pm$ S.D.
[2]Variance = square of S.D., significantly different (0.02<P<0.05).
[3]Mean of 4 pooled samples of 3 fish per sample $\pm$ S.D. Values with
   different superscript are significantly different (0.0025<P<0.005)

*S.D.=Standard Deviation

Table 3. Specific gravity (g/ml) of liver (liver weight per liver
          volume).

Experiment I				Experiment II				
Diet #	N	Mean[1] Specific Gravity	S.D.*	Diet #	N	Mean[2] Specific Gravity	S.D.	Var.[3]
1	4	2.28	0.12	3	4	1.05	0.01	0.0001[a]
2	4	1.11	0.05	4	4	1.05	0.07	0.0049[b]
				5	4	1.08	0.05	0.0025[b]

[1]Mean of 4 pooled samples of 4 livers per sample $\pm$ S.D.,
   significantly different (P<0.0005).
[2]Mean of 4 pooled samples of 3-4 livers per sample $\pm$ S.D., not
   significantly different.
[3]Variance = square of S.D. Values with different superscripts are
   significantly different (0.025<P<0.05).

*S.D.=Standard Deviation

Table 4. Liver wet weight (g) and volume (ml).

	Diet #	N	Wet Weight Mean[1]	S.D.*	N	Volume (ml) Mean	S.D.
EXP. 1	1	4	0.2603	0.02	16	0.1141	0.02
	2	4	0.4600	0.07	16	0.42	0.12
EXP. 2	3	4	0.4303	0.03	16	0.41	0.05
	4	4	0.8500	0.09	12	0.81	0.12
	5	4	1.0180	0.20	12	0.94	0.24

[1]Mean of 4 pooled samples of 3-4 livers per sample $\pm$ S.D.

*S.D.= Standard Deviation

Table 5. Liver lipid content (% of liver wet weight).

Experiment I					Experiment 2				
Diet #	N	Mean % Lipid Content[1]	S.D.*	Var.[2]	Diet #	N	Mean % Lipid Content[3]	S.D.	Var.[4]
1	2	4.23	0.07	0.0049	3	2	4.54	0.23	0.05[a]
2	3	5.27	1.09	1.1900	4	2	7.05	2.27	5.15[b]
					5	2	2.79	0.01	0.0001[c]

[1]Mean of 2-3 pooled samples of 4 livers per sample $\pm$ S.D., not
    significantly different.
[2]Variance = the square of S.D., not significantly different.
[3]Mean of 2 pooled samples of 3-4 livers per sample, $\pm$ S.D., not
    significantly different.
[4]Values with different superscripts are significantly different
    (0.001<P<0.005).

*S.D.=Standard Deviation

Table 6. Liver glycogen content (% of total liver wet weight).

Experiment I				Experiment II			
Diet #	N	Mean % Glycogen Content[1]	S.D.*	Diet #	N	Mean % Glycogen Content[2]	S.D.
1	3	0.03	0.04	3	2	0.03[a]	0.08
2	3	0.36	0.17	4	2	1.41[a]	0.56
				5	2	5.77[b]	1.138

[1]Mean of 3 pooled samples of 3-4 livers per sample $\pm$ S.D., significantly different (0.02<P<0.05).

[2]Mean of 2 pooled samples of 3-4 livers per sample, + S.D. Values with different superscripts are significantly different (0.005<P<0.01).

*S.D.=Standard Deviation

Table 7. Microhematocrits (packed cells volume in %).

Experiment I					Experiment II				
Diet #	N	Mean Micro. (%)	S.D.*	Var.	Diet #	N	Mean Micro. (%)	S.D.	Var.[1]
1	16	50.81	4.67	21.80	3	15	51.20	4.02	16.16[a]
2	16	51.69	3.44	11.84	4	15	51.60	3.36	11.29a
					5	15	53.33	6.10	37.21b

[1]Variances with different superscripts are significantly different at $\alpha=0.10$ (0.05<P<0.10).

*S.D. = Standard Deviation

Table 8. Percent of mature red blood cells (RBC).

		Experiment I					Experiment II		
Diet #	N	Mean % mature RBC[1]	S.D.*	Var.	Diet #	N	Mean % Mature RBC[2]	S.D.	Var.
1	3	93.3	1.15	1.32	3	5	91.03	1.68	2.82
2	4	88.5	3.32	11.02	4	5	90.3	1.04	1.08
					5	5	89.3	1.44	2.07

[1]Mean of 3-4 averaged samples of 3 different counts per sample.
[2]Mean of 5 averaged samples of 3 different counts per sample.

*S.D. = Standard Deviation

Table 9. Condition factor (K), average body weight (g), and averaged total length (cm).

	Diet #	N	Body Wt.		Tot. Length		K Factor	
			Mean	S.D.*	Mean	S.D.	Mean[1]	S.D.
EXP I	1	20	25.02	3.83	13.1	0.84	1.1	0.07
	2	20	45.01	14.48	15.4	1.57	1.2	0.06
EXP II	3	18	45.94	4.95	16.2	0.64	1.1	0.05
	4	18	90.78	18.48	19.5	1.30	1.2	0.07
	5	18	75.32	16.09	18.2	1.52	1.3	0.15

[1]Mean values are significantly different (P<0.0005).

*S.D. = Standard Deviation

Table 10. Plasma alanine aminotransferase (GPT) activities (I.U./L)[1].

	Experiment I				Experiment II		
Diet #	N	Mean[2] GPT Activity	S.D.*	Diet #	N	Mean[3] GPT Activity	S.D.
1	2	13.38	0.77	3	2	7.86	0.25
2	2	7.32	0.51	4	2	6.06	0.25
				5	2	4.62	0.25

[1]One International Unit (IU) of enzyme activity is the amount that
    will convert one micromole of substrate per minute per liter of
    plasma.
[2]Mean of 2 pooled samples of 7-8 fish per sample $\pm$ S.D.,
    significantly different ($0.01<P<0.02$).
[3]Mean of 2 pooled samples of 7-8 fish per sample $\pm$ S.D.,
    significantly different ($0.0025<P<0.001$).

*S.D. = Standard Deviation

Table 11. Disease challenge of fish with <u>Vibrio</u> <u>anguillarum</u>
(Type I).

	Diet #	# of Fish Chall.	# of Deaths	# of Vibriosis Deaths[1]	% Mortality[2] by Vibriosis	Mean Day[3] to Death
EXP I	1	60(3)	34	34	56.7	4.17
	2	60(3)	29	24	43.6	4.27
EXP II	3	60(3)	18	12	22.2	5.0
	4	60(3)	21	19	32.8	5.23
	5	60(3)	20	18	31.0	5.42

[1]Number of deaths of combined replicates in parentheses. No
significant difference in Experiment I (χ^2 =1.46, 0.10<P<0.25) and
in Experiment II (χ^2 =1.72, 0.25<P<0.50).

[2]Percent equals deaths caused by vibriosis/number of fish challenged
minus non-specific deaths.

[3]Mean day to death of combined replicates in parentheses. There is
no significant percentage of mortality difference in Experiment I
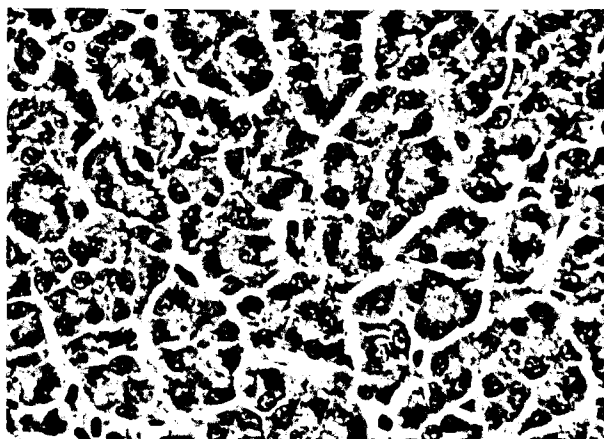(P>0.5) and Experiment II (P>0.25).

Fig. 2. Liver section showing low degree of vacuolation in fish fed Diet 1(10.9% fat), (H&E, X400).

Fig. 3. Liver section showing low degree of vacuolation in fish fed Diet 2(19.7% fat), (H&E, X400).
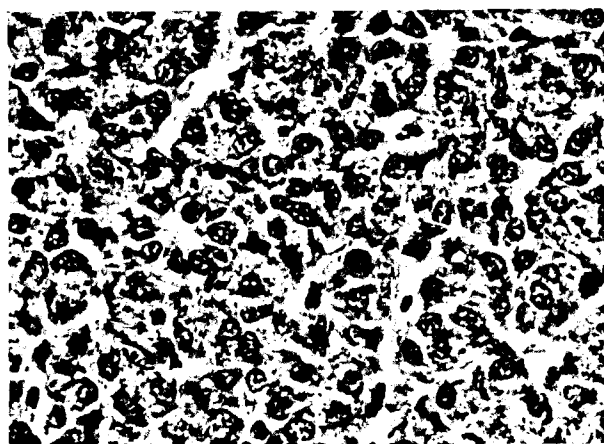
Fig. 4. Liver section showing low degree of vacuolation in fish fed Diet 3(9.7% fat), (H&E, X400).

Fig. 5. Liver section showing moderate amount of vacuolation in fish fed Diet 4 (28.1% fat), (H&E, X400).
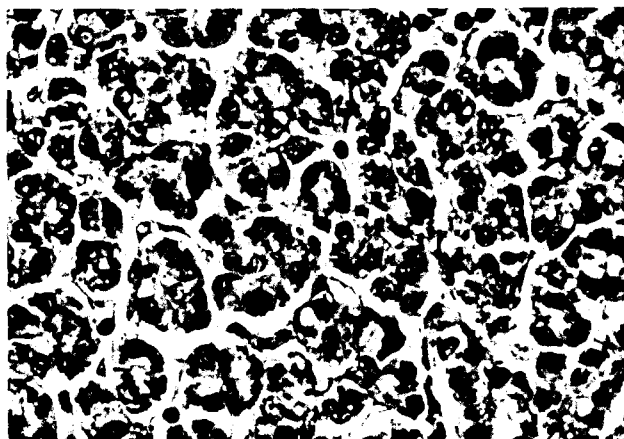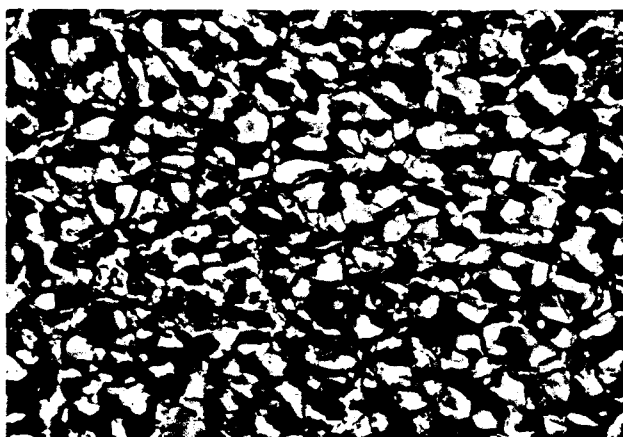

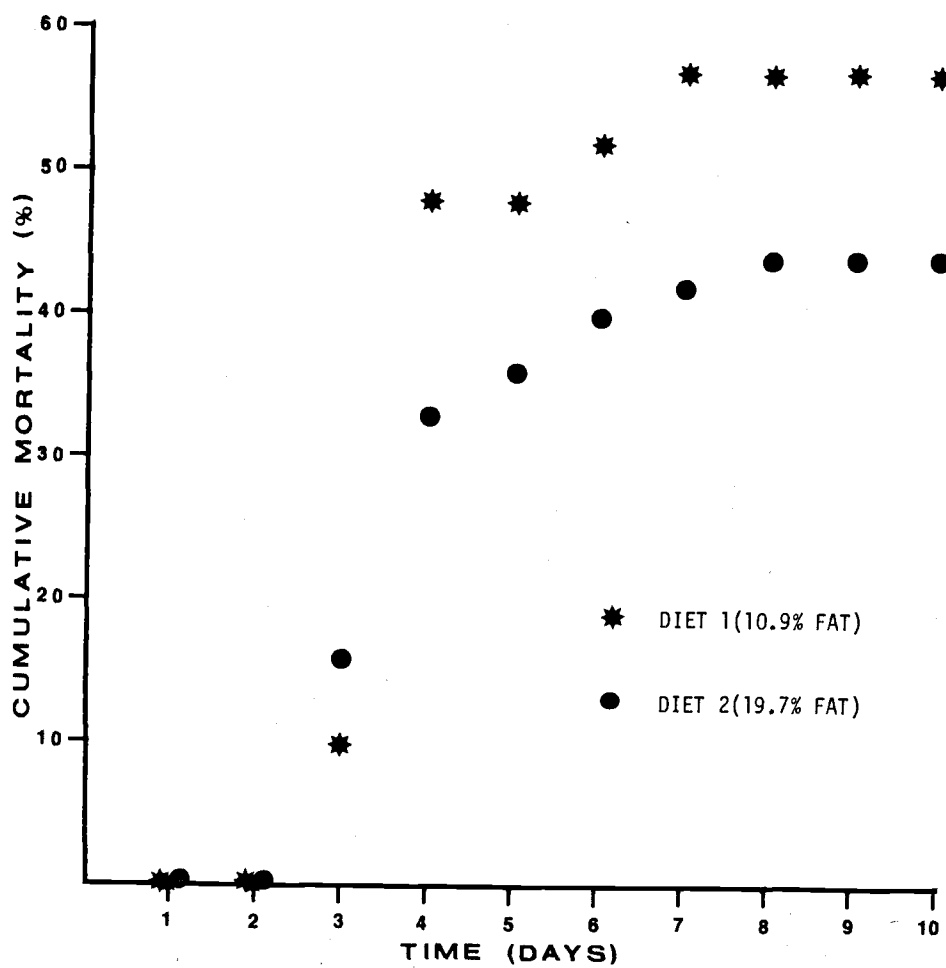Fig. 6. Liver section showing high degree of vacuolation in fish fed Diet 5 (26.9% carbohydrate), (H&E, X400).

Fig. 7. Cumulative mortality in the first challenge experiment.
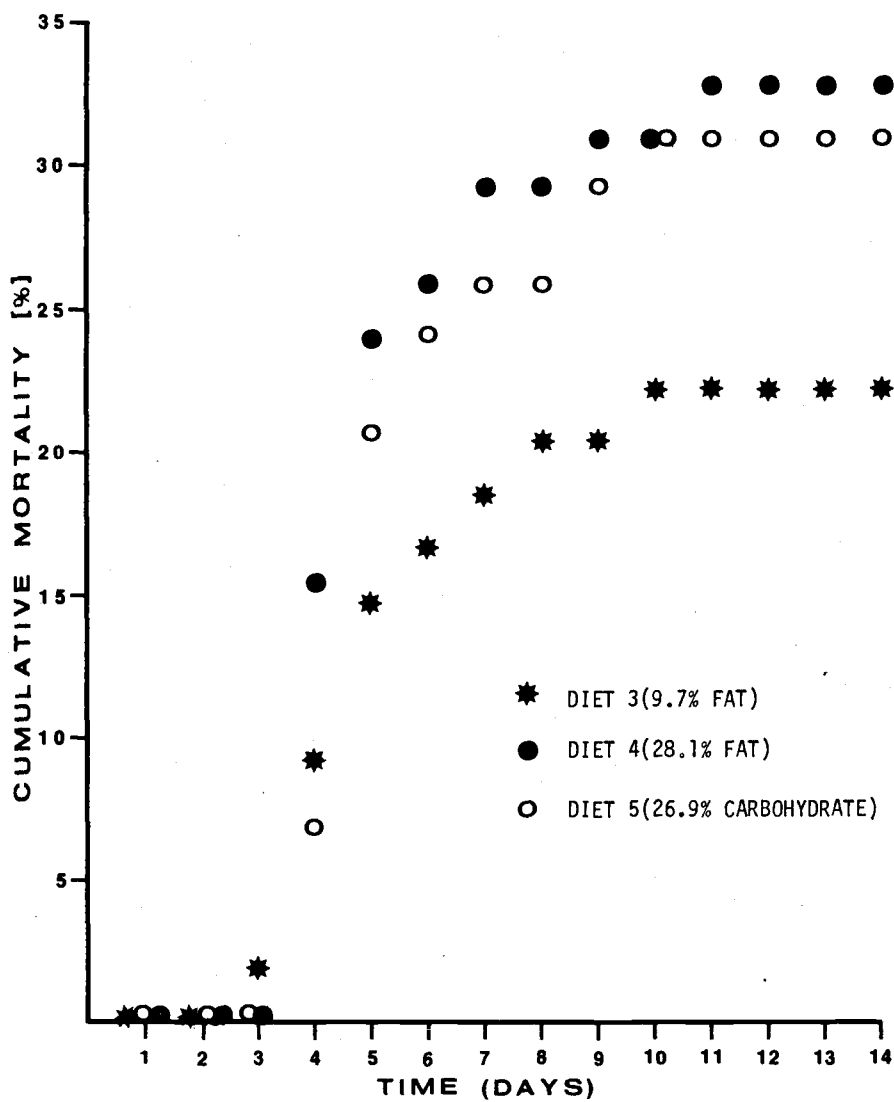
Fig. 8. Cumulative mortality in the second challenge experiment.

DISCUSSION

The levels of hepatosomatic index, liver specific gravity, liver lipid and glycogen content observed under the conditions of this study were not detrimental to the fish health condition. The levels of hepatosomatic index remained constant for fish fed low fat Diets 1 and 3 and was similar in fish fed high fat Diets 2 and 4. The index increased in the group fed Diet 5, because of the accumulation of glycogen in hepatocytes. This hepatosomatic index may be misleading, because the amount of fat present in the visceral cavity modifies the true body weight of the fish. Simon and Dollar (1967) and Post (1983) reported normal hepatosomatic indices in hatchery trout to be 1.1 - 1.7% and 0.8 - 1.5% respectively. It is concluded the hepatosomatic indices were normal.

The specific gravity of the liver in trout fed Diet 1 (low fat) was higher than in fish fed Diet 2 (high fat). No difference was observed in liver specific gravity in fish fed Diet 3 (low fat), Diet 4 (high fat), and Diet 5 (high carbohydrate). However, the liver lipid contents and glycogen contents were different in both experiments. These indexes could be explained by changes in the liver components other than glycogen and lipid reserves. Higuera et al. (1977) observed a lower soluble protein and water content in the livers of rainbow trout fed a high lipid diet (15.3%) and opposite effects in a group fed a low lipid diet (6.7%). Under the conditions of Experiments I and II, the specific gravity thus reflects changes in all liver components instead of changes in liver lipid content alone.

Liver lipid content in the fish groups fed low fat diets (1 and 3) was nearly constant. Henderson and Sargent (1981) observed that a dietary lipid level up to 10% does not depress fatty acid synthesis in the trout liver. Apparently, the newly synthesized fatty acids were not being accumulated in the liver of fish fed Diet 1 and Diet 3, and the newly synthesized lipids were being transported in the bloodstream to other tissues (Leger et al. 1981).

Liver lipid content in the group fed high fat diets (2 and 4) was higher than for Diet 1 and Diet 3. Lin et al. (1977) observed that hepatic lipogenic activities decreased significantly when the level of lipid in the diet increased in excess of 10% in coho salmon after 35 days of feeding. It has been observed that high levels of essential fatty acid (EFA   3) depressed lipogenic enzyme activities (Leger et al. 1981). The increase in liver lipid content in fish fed Diet 2 and Diet 4 was concluded to be a direct deposition of excess dietary lipid rather than newly synthesized lipids.

The group fed Diet 5 (high carbohydrate) had the lowest liver lipid content, which could be the result of depressed lipid synthesis in the liver or increased mobilization of lipids out of the liver. Lin et al. (1977) reported that feeding coho salmon with high carbohydrate diets for four hours after two days of fasting increased the rate of hepatic fatty acid synthesis. In the group fed Diet 5, it is probable that newly synthesized fatty acids were mobilized rapidly out of the liver as a source of energy, because trout are not able to utilize carbohydrate reserves readily.

The liver lipid levels in this study were lower than values reported by Higuera et al. (1977). They observed levels of 5.9% in

trout fed low lipid diet (6.7%) and 8.9% in trout fed a high lipid diet (15.3%) for six months ad libitum at 12°C. The low values found in this study could be explained by the higher water temperature (approximately 18°C) in which the fish were kept. The metabolic rates and consequently the utilization of energy reserves increased as the water temperature increased. Dean (1969) and Hilton (1982) observed higher levels of lipids and glycogen in the liver of cold acclimated rainbow trout in contrast to fish acclimated to warmer water.

Liver glycogen content apparently increased as dietary lipid levels increased (Table 6). However, these glycogen levels appeared to be below normal levels. The addition of Dextrin (starch) in Diet 5 rapidly increased the glycogen level in the liver to above normal levels. This result is consistent with observations of Tunison et al. (1939) and Phillips et al. (1948) who observed that trout fed high levels of digestible carbohydrate had large livers high in glycogen.

The low levels of liver glycogen observed in trout fed low fat diets (1 and 3) and high fat diets (2 and 4) were possibly the result of low levels of carbohydrates in diets, stress to chlorine, or warmer water. The free residual chlorine levels in the freshwater were 0.06-0.08 ppm for several days. However, no signs of distress were observed in fish. The upper limits for continuous exposure in salmonids is 0.003 ppm of total chlorine residuals (Thurston et al. 1979). Hilton (1982) reported that trout raised at 10°C had higher liver weight and liver glycogen than trout raised at 15°C on either high or low digestible carbohydrates. It was concluded that warm waters and consequently higher metabolic rates resulted in low glycogen content in the livers.

Blood parameters were found to be normal and characteristic of healthy rainbow trout. The microhematocrit readings were higher than reported values in healthy trout and similar among all diets. Wedemeyer and Yasutake (1977) reported readings of 24-43% in clinically healthy juvenile rainbow trout. High hematocrits could be the result of asphysia or stress from anesthesia. Unbuffered MS222 produces disturbances in metabolism, implying that the sulfonic moiety of MS222 is involved (Wedemeyer 1970). Unbuffered MS222 (40 ppm) was slightly acid (pH=6) and might be irritating to the gill membranes. Initial agitated swimming was observed when fish were put in unbuffered MS222 solution. It is concluded that the high readings resulted from the stress to the anesthesia rather than asphysia.

The mature erythrocyte counts were high and similar among all diets. Therefore, there was no indication of any abnormality affecting the maturation processes of red blood cells. Post (1983) indicated that fish affected by autoxidation products or rancid fat in fish feed will have lowered hematocrits and red blood cell counts, with the number of immature red blood cells more prevalent than older red blood cells. Whitmore (1965) looked at the effect of vitamin E deficiency on erythrocytes. Regardless of diet rancidity level, the percentage of immature red blood cells (29.7 - 34.8%) was above normal values (3.9 - 5.5%) in juvenile chinook. Vitamin E added to a rancid diet resulted in complete recovery of anemic chinook salmon. It is concluded that all diets were satisfactory because no pathology was observed.

The condition factors (K) were indicative of well fed fish and significantly different in every diet. Fish condition factors are sometimes used in hatchery management. A poorly fed fish typically has

a condition factor less than 1, a well fed fish has a condition factor approximately equal to 1, and an overfed fish has a condition factor greater than 1 (Piper et al. 1982). It was observed that fish fed Diet 5 containing high carbohydrate had greater fat deposition in the visceral cavity than for fish fed Diet 3 (low fat) and Diet 4 (high fat), and this is reflected in the condition factors for this treatment.

The plasma GPT activities are low and indicative of healthy livers. However, there are different GPT activities among all diets. These differences may be attributed to fish size, age, and diet composition. Trout fed Diet 2 (high fat) had the lowest activity in Experiment I. This result agrees with Sauer and Haider (1979), and Higuera et al. (1977), who noticed lowered GPT activities in rainbow trout overfed or fed on high lipid diets. The reasons for the apparent lower GPT activity in Diet 2 might be explained by the sparing action that high lipid levels have on protein. Less amino acids are catabolized for energy and consequently lowers the GPT activities in the liver and plasma. Trout fed Diet 5 (high carbohydrate) had the lowest activity. This result is consistent with observations made by Pieper and Pfeffer (1979), who noticed lowered GPT activities when about half of the dietary protein was replaced by gelatinized maize starch and not by sucrose. Sucrose has a lower digestibility, requiring a greater proportion of the amino acids to be catabolized for energy. It is concluded that both high lipid and carbohydrate in Diet 5 had a sparing action on protein. Trout fed low fat diets (1 and 3) resulted in different plasma GPT activities. The variable GPT activities probably result from many factors including diet, sex, age,

and time of the year (Gingerich and Weber 1979). Also, the methods for GPT determination in fish are adaptations from human clinical tests and these methods are not quite standardized for fish blood and biochemical parameters.

The histological examinations of liver sections show structurally normal liver cells and there is no indication of liver cell degeneration or necrotic tissues. However, differences are observed in the amount of vacuolation in hepatocytes among diets. The vacuoles observed in liver sections stained with hematoxylin and eosin (H&E) appeared to be typical glycogen vacuoles (J.D. Hendricks, personal communication). Fish fed Diet 4 (high fat) and Diet 5 (high carbohydrate) resulted in the most excessive vacuolation of liver cells. This observation is normal in hatchery fish according to J. Hendricks (personal communication). In conclusion, no pathology or increased susceptibility to vibriosis was observed in fish with different liver composition and cell structure.

The waterborne challenges revealed no difference in susceptibility to vibriosis among fish fed a low fat and high fat rations, and high carbohydrate ration. However, the liver composition was different in each dietary treatment. Apparently, the difference in liver composition was not great enough to upset the homeostasis in fish. The variation in liver composition was expected to modify the liver metabolism and consequently stress the fish. The bacterial concentrations used in the challenges are similar for Experiment I and II, and the cumulative mortalities are different between experiments. The challenge in the second experiment resulted in lower mortalities, and this outcome may be attributed to the fish size and age. As fish

grow older, the defense mechanisms become more developed and consequently renders a better protection against disease.

The agglutinating antibody titers produced against Vibrio anguillarum were of similar profile in fish fed Diet 1 (low fat) and Diet 2 (high fat) for Experiment I. Apparently, the production of agglutinating antibodies were not affected by different liver compositions, which were expected to produce changes in the liver metabolism, and consequently, injuries to certain aspects of fish immune defense mechanisms.

This study was unsuccessful in determining the threshold level of lipid and glycogen content above which the health conditions of the fish are impaired. One explanation for these results may be the warm temperature of the water during Experiments I and II. Juvenile fish metabolic rate and growth rate are higher in warm waters than in cold waters. Consequently a high utilization of energy reserves is required to meet these demands. An increased mobilization of lipids and glycogen out of the liver is expected at these high temperatures. Lipid reserves are utilized at a faster rate than glycogen reserves, because fish are better equipped to metabolize lipids. Overall, the liver energy reserves are expected to be reduced in warm waters.

It might be appropriate to consider that fatty livers and high glycogen livers are not necessarily detrimental or indicative of liver injury in fish. However, there may be other conditions that in association with fatty and high glycogen livers will produce liver injuries. Peroxidation of lipids in the liver may arise when diets have insufficient levels of antioxidants (vitamin E and C). Peroxidation of unsaturated lipids in the liver may be responsible for

the production of liver injuries or liver cell degeneration. In this study, all diets appear to have appropriate levels of antioxidants, because there are no signs of pathology associated with vitamin E and C deficiencies. Mechanisms associated with glycogen reserves that may produce liver injury are unknown. It is possible that liver function may be impaired by blocking or inhibiting processes in the liver.

The degree of liver injury may be questionable and it might not be detrimental to the overall health condition of the fish. Liver cell regeneration happens constantly and replaces dead cells or necrotic tissue. Thus, normal liver functions may not be affected unless the balance between liver cell regeneration and necrosis of the cells is upset in favor of the latter.

Under certain conditions, liver cells may have a maximum capacity for lipid or glycogen storage. Excess lipid and glycogen may be transported and stored in other tissue or the visceral cavity. Lipid reserves in the liver are not a product of fatty acid synthesis but a direct deposition of excess energy found in fish diets.

Rainbow trout in hatcheries are subjected to stressful conditions such as handling, crowding, confinement and transportation. Research in hatchery managment is needed to reduce the consequences of stressful conditions on survival, growth, and reproduction. The relationships between stress and fatty livers or high glycogen livers may provide important information for improvement of management techniques and definition of optimal rearing conditions.

SUMMARY AND CONCLUSIONS

Results from testing the implications of dietary lipid and

carbohydrate intake on the liver, and the susceptibility of hatchery-

reared rainbow trout to vibriosis are summarized below.

There is a Threshold Level of Liver Lipid and Glycogen Content Above
   Which the Health Condition of Rainbow Trout is Impaired (Hypothesis
   1)

In conclusion, Hypothesis 1 is rejected because no pathological

conditions were observed in the fish with respect to the levels of

hepatosomatic index, specific gravity, lipid content and glycogen

content of the liver observed under the conditions of this study.

Hematocrits and mature red blood cell counts were indicative of healthy

rainbow trout. Condition factors (K) greater than unity were

characteristic of well fed fish.

Fatty and High Glycogen Livers Impair Liver Function and Cell
   Structure (Hypothesis 2)

In conclusion, Hypothesis 2 is rejected because no liver damage

(necrosis) or histopathology was observed in the fish livers with

respect to the levels of lipid and glycogen extracted from the livers

in this study. Plama alanine aminotransferase (GPT) activities were

low and characteristic of normal livers, however significant

differences were observed among these values. Histological

observations of the liver revealed normal tissue, even those with

highly vacuolated hepatocytes.

Fatty and High Glycogen Livers Increase Susceptibility of Rainbow
  Trout to Vibriosis (Hypothesis 3)

In conclusion, Hypothesis 3 is rejected because no significant
increase in susceptibility to disease was observed with respect to the
levels of lipid and glycogen extracted from the livers in this study.
Waterborne challenges with <u>Vibrio</u> <u>anguillarum</u> revealed no difference in
cumulative mortalities and mean day to death even though liver
composition and structure were different among treatments.
Agglutinating antibody titers did not differ between diets in the first
experiment.

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APPENDICES

APPENDIX A

Lipid Extraction

Procedure

One gram of liver tissue is added to 1 1 of water and blended for one minute in a Potter-Elvehjem glass homogenizer with a motor drive teflon tip pestle. Three ml of methanol-chloroform (2:11, v/v) is added and homogenized for two minutes. The homogenate is centrifuged for ten minutes at 2800 RPM in refrigeration (4°C). The supernatant is decanted into a centrifuge tube with a rubber stopper and kept in a cold water bath. The residue is re-extracted twice with 3.8 ml of methanol-chloroform-water (2:1:0.8, v/v) as explained above. The supernatants are combined into one centrifuge tube, and 1 ml of chloroform is added and mixed. The mixture is centrifuged for ten minutes at 2800 RPM in refrigeration (4°C). The mixture is separated into three phases: the chloroform phase (bottom) containing lipids, the tissue phase (center), and the methanol-water phase (top). An aliquot (1 ml) from the chloroform phase is placed in a tared test tube (12 x 75 mm). Duplicate samples are prepared. The test tubes are placed in hot water bath (80°C) to evaporate the chloroform. After chloroform has evaporated, the test tubes are placed in a 65°C oven to dry for two hours. Test tubes are transferred to a dessicator to cool. The test tube and lipid are weighed and the test tube tared weight is subtracted to get the weight of lipid. Tripalmitin is used as a standard and it is recovered at approximately 91.5%. The lipid content of the sample is calculated as follows:

$$\text{Total lipid} = \frac{\text{weight of lipid aliquot x vol. of chloroform used}}{\text{volume of aliquot}}$$

$$\% \text{ lipid} = \frac{\text{total lipid}}{\text{weight of sample}} \times 100$$

APPENDIX B

Glycogen Extraction

Reagents

1. Anthrone reagent: mix 500 mg of purified anthrone, 10 g of thiourea and 1 liter 72% H_2SO_4. Warm mixture to 80-90°C, cool and store in refrigerator.

2. Standard glucose: (a) stock: mix 100 mg of glucose in 100 ml of saturated benzoic acid solution. (b) working solution: mix 5 ml of stock solution and 95 ml of saturated benzoic acid.

3. 5% trichloroaceticacid (TCA)

4. 95% ethanol

Procedure

To one gram of liver tissue, add two ml of 5% TCA and blend for three minutes in a Potter-Elvehjem glass homogenizer with a motor drive teflon tip pestle. The homogenate is transferred into a centrifuge tube and centrifuged for ten minutes at 2800 RPM in refrigeration (4°C). The supernatant is decanted into another graduated centrifuge tube with a rubber stopper. Two more extractions are made of the residue in the same manner as above to extract better than 97% of the glycogen present. All supernatants are combined into one centrifuge tube and the volume of extract is recorded. One ml of extract is pipetted into a 15 ml pyrex centrifuge tube. Duplicate samples are prepared. To each tube is added five ml of 95% ethanol with careful mixing. Tubes are capped with clean rubber stoppers and allowed to stand overnight at room temperature (or instead, the tubes are placed in a warm water bath at 37-40°C for 3 hours). The tubes are centrifuged for 20 minutes at 28 RPM in refrigeration (4°C). The

supernatant is decanted from the precipitated glycogen. The tubes are allowed to drain in an inverted position for ten minutes. Two ml of distilled water is added to each tube to dissolve the glycogen. A reagent blank is prepared by pipetting two ml of water into a pyrex centrifuge tube. A standard is prepared by pipetting two ml of working standard glucose solution. Ten ml of anthrone reagent is pipetted into each tube with vigorous blowing. Each tube is tightly capped with air condenser and placed in a cold tap water bath and cooled to room temperature. An aliquot of each tube is transferred to small tubes (12 x 75 mm) and read at 620 nm in a spectrophotometer (Spectronic 70, Bausch and Lomb). Calculation of glycogen is as follows:

$$DU \times 0.1 \times \frac{\text{volume of extract}}{\text{gram of tissue}} \times 100 \times 0.9 = \text{mg of glycogen/100 g of tissue}$$

DU = optical density of the unknown

DS = optical density of the standard

0.1 = mg of glucose in 2 ml of working standard solution

0.9 = factor for converting glucose to glycogen value