13

BARD

FINAL REPORT

PROJECT NO. I-497-82

**The Use of Feed Additives to Modulate Hormonal
Balance in Fish in Order to Improve the Utilization
of Dietary Protein for Growth**

B. Hepher, A. Gertler, A. Madar, R.T. Lovell

66
630.72
BAR/HEP -

2nd copy

1987

29408

000000011518

24408 0

Date: June 30, 1987.

BARD
P.O. BOX 6
BET DAGAN, ISRAEL.

BARD PROJECT No. I-497-82

THE USE OF FEED ADDITIVES TO MODULATE HORMONAL BALANCE IN FISH IN ORDER
TO IMPROVE THE UTILIZATION OF DIETARY PROTEIN FOR GROWTH.

INVESTIGATORS' NAMES

Dr. B. Hopher
Prof. A. Gertler
Dr. Z. Madar
Prof. R.T. Lovell

INVESTIGATORS' INSTITUTIONS

Agricultural Research Organization
Hebrew University of Jerusalem,
Faculty of Agriculture
Auburn University, Department of
Fisheries and Allied Aquaculture

Project Starting date: September 25, 1983

Type of Report: Final

B. Hopher

Signature
Principal Investigator

State of Israel
Ministry of Agriculture
AGRICULTURAL RESEARCH ORGANIZATION
The Volcani Center Bet-Dagan

G. Gelsenstein

Signature
Institution's Authorizing Official

630.72

BAR | HEP

2nd copy

: 639.3: 636.087;

636.089.240.5;

636.089.239.6;

636.089.239.8

TABLE OF CONTENTS

Abstract.....	3
Objectives of the original reseasch proposal.....	4
Final report.....	6
A. Metabolic hormonal activity and its modulation.....	6
1. General methods - description and development.....	6
2. Effect of starving or feeding with high and low protein diet on metabolism of cabohydrate and amino acids.....	14
3. The effect of cobalt on metabolic paramenters, plasma components and growth of carp.....	16
4. The effect of chromium on metabolic parameters, plasma components and growth of carp.....	20
5. The effect of metformin on metabolic parameters, plasma components and growth of carp.....	21
6. Conclusions.....	23
7. References.....	24
8. Tables.....	25
9. Figures.....	40
B. Protein and energy requirement for maximum growth.....	46
10. Objective of the research.....	46
11. Objective 1: Protein and energy requirements for maximum growth of channel catfish and the availability of gross energy in dietary ingredients for channel catfish.....	46
12. Objective 2: Protein sparing effect of carbohydrates in channel catfish.....	49
13. Objective 3: Metabolic fate of carbohydrates fed to channel catfish.....	50
14. Objective 4: Effect of dietary addition of anabolic hormones on protein gain.....	50
15. Tables.....	52
16. Figures.....	60
Description of cooperation.....	67
Evaluation of research achievements.....	67
List of publications.....	68

ABSTRACT

The study on improving the efficiency of carbohydrate utilization by fish proceeded on two different lines. The first approach was to study the metabolic hormonal regulation of the fish and try and modulate it, especially that of insulin, by additives and drugs. For that, a number of methods have been developed. Antibodies serum to bonito insulin has been prepared by immunization of mice against the insulin and inoculating them with sarcoma cells which induced ascites fluid with a strong anti insulin activity. These antibodies showed a high cross reaction with carp insulin as determined by immunocytochemical studies on common carp Brockman bodies. A radioimmunoassay (RIA) has been developed for carp insulin. Another method developed to determine gluconeogenesis using ^{14}C -alanine and determine radioactivity in the glucose fraction. Also a method to determine protein synthesis in vivo has been developed using ^3H -leucine and measure its incorporation into muscle and liver proteins.

Starved fish and fish fed low and high protein diets were tested for glucose tolerance. This showed that common carp behave like diabetic animals by having a low glucose disposal rate. Starving increased gluconeogenesis as did feeding with high protein diet.

Intraperitoneal injections of cobalt, chromium, and to some extent the drug metformin, increased glucose clearance from the blood and decreased gluconeogenesis. However, the effect on protein synthesis was not decisive. Adding cobalt (as CoCl_2) to the diet affected significantly, in one experiment growth rate, feed conversion and protein efficiency ratio. However, these results could not be repeated in subsequent experiments. Nor did the other additives (chromium, nickel and metformin) affect the above parameters. An interaction with other factors such as absorption of the additives by the fish is suspected.

The second approach was to determine the dietary protein and energy requirement for maximum protein synthesis, the availability of gross energy in dietary ingredients and the effect of anabolic hormones on this process. Digestibility and digestive energy (DE) values were determined for various feed ingredients. These varied from nil for cellulose to 8.6 kcal/g for lard. Cooked starch produced 3.4, 3.0 and 2.8 kcal/g (DE) for the inclusion in the diet at levels of 20, 30 and 40%, respectively. For small fish (1-10g) food and protein intake decreased as DE increased. Weight and protein gain were as good on the low protein diet (27%) as on the high protein diet (39%) when energy level was low and allowed a high rate of food (protein) consumption. In larger fish (20-50g) protein intake decreased but protein gain increased with increasing dietary energy, which indicates a protein sparing effect of the non protein energy. This effect decreased somewhat in fish of 100 to 250g.

Daily protein and energy requirement for maximum protein gain were estimated in small channel catfish (1-3g) at 1.64g protein and 16.8 kcal DE per 100g BW, while for fish grown from 100 to 260g, a minimum of 0.43g protein and approx. 5.0 kcal DE/100g BW/day was required. At lowest added energy level starch and fat produced about an equal protein sparing effect.

Addition to the diet of 11-ketotestosterone at a rate of 2 mg/kg improved growth rate of channel catfish over that of the control, but higher levels reduced growth rate. All levels of 17-methyltestosterone caused a reduction in bone mineralization.

OBJECTIVES OF THE RESEARCH PROJECT

The objective of the research was to improve the utilization of carbohydrates as an energy source to spare protein for growth, through modulation of hormonal activities (especially insulin) which influence energy metabolism. Ultimately, these findings will be applied in practical fish feeds to reduce the protein percentage.

While the final target of the study was to improve the profitability of fish farming by improving the efficiency of protein utilization, it was intended also to provide the following scientific information:

By the Israeli research team:

- (a) energy metabolism in fish
- (b) mechanisms of hormonal control of protein and carbohydrate metabolism in fish
- (c) insulin activity in common carp (Cyprinus carpio) and channel catfish (Ictalurus punctatus).
- (d) effects of dietary application of pharmaceutical agents and minerals on regulation of hormonal balances
- (e) development of methodology for research with fish, such as radioimmunological techniques for determination of hormone activity, cannulation procedures for in vivo studies with hormone, and improvements in fish tissue and organ culture.

By the American research team:

- (1) Dietary protein and energy requirements for maximum protein synthesis for channel catfish of various sizes, and the availability of gross energy in dietary ingredients for channel catfish.
- (2) Protein sparing effects of carbohydrate in channel catfish diets.
- (3) Metabolic fate of carbohydrate fed to channel catfish.
- (4) Effects of dietary additions of anabolic hormones on protein gain in channel catfish.

The last mentioned objectives will be specifically referred to in the body of the report by the American research team.

FINAL REPORT

The study was carried out in two distinct lines: The study of the metabolic hormonal activity and the attempt to modulate it was carried out in Israel at the Department of Biochemistry, the Faculty of Agriculture of the Hebrew University and at the Fish and Aquaculture Research Station - Dor, The Agricultural Research Organization. The study, by the more conventional nutritional ways, to utilize better the carbohydrate was carried out in the USA, at the Department of Fisheries and Allied Aquaculture, Auburn University. The following is the final report of all three institutions.

A. METABOLIC HORMONAL ACTIVITY AND ITS MODULATION

1. General methods - description and development.

1.1. Animals

Common carp (Cyprinus carpio) were used as the experimental animals. The fish were obtained from the Aquaculture Research Station, Dor, Israel. Experiments were performed with fish of both sexes. The fish, weighing 150 - 250 g were maintained in 100-liter, well-aerated, freshwater tanks at a temperature of 21° - 24°C. The photoperiod for the fish was 12 h light and 12 h dark. All fish went through 10 days acclimation period prior to any treatment. The experiments were carried out with starved or fed fish that received low or high protein diets. Fish were fed once daily at approximately 3% of their body weight/day. During feeding experiments, fish were fasted 12 hr prior to the actual sampling. Pulse or chronic injections were performed by intra-peritoneal injections. Blood was drawn via the caudal vein by syringes rinsed in 400 U/ml solution of heparin and then immediately centrifuged. The plasma was frozen at -20°C.

1.2 Anaesthesia

Injections and sampling were performed under anaesthesia. The fish were transferred to a tank containing a solution of 150 ppm tricaine

methane sulfonate (MS 222, Sigma, U.S.A.). MS-222 was chosen because of its minimal effect on glucose levels and stress reaction as compared to other anaesthetics. Using these concentrations, induction time was 60 - 90 seconds and recovery time was one minute. After treatment, fish were returned to the experimental tanks.

1.3 Glucose loading tests

Experiments were performed 12 hours after the last feeding. Two methods were used: 1. Single blood sampling 2. Repeated sampling from the same fish. The single sampling method employed several groups of fish, each sampled only once, at different intervals after the glucose loading. The disadvantage of this method results from having only one control group. Furthermore, the method requires a large number of fish in order to acquire data on as many time intervals after loading as possible. The advantages of this method appears to be a reduction of stress reaction which may appear during repeated sampling of the same fish.

Much less fish are required when repeated sampling is employed during the experiment. Also the control group, which was not loaded with glucose, is sampled each time. Increasing the time intervals between sampling and reducing sampling frequency minimizes the stress which may affect the levels of some metabolites in the blood.

D-glucose was injected into the peritoneal space of anaesthetized fish. The fish then were returned to their original water tanks and blood samples were drawn as previously described. Plasma was separated by centrifugation at 3000 g for five minutes. Glucose levels were determined by glucose-oxidase method using a Beckmann Glucose-Analyzer.

1.4 Gluconeogenesis

The fish received an intraperitoneal injection of radioactive alanine while under anaesthesia and were returned to the experimental tanks. Time studies of the formation of radioactive glucose in the carp indicated that maximum conversion of ^{14}C -alanine occurred approximately 90 minutes after injection. Therefore, 90 minutes post injection, carp were anaesthetized with MS-222 and blood was collected with heparinized syringes. The blood was centrifuged at 3000 g for seven minutes and the plasma sample

deproteinized by the method of Somogyi (1945). The soluble fraction was alkalinized by the addition of 1 M glycine buffer (pH 10.5) and further passed through columns (2 ml) of resin (Dowex X-8 anionic form, Sigma, U.S.A.) which were previously washed with 0.1 M Na-glycine buffer, pH 10.5.

The sample applied to the columns was then eluted with glycine buffer, pH 10.5 (elution of glucose), with 0.1 M Hepes buffer pH 5.8 (elution of alanine) and with 0.02 M hydrochloric acid, pH 2, (elution of the organic acids such as pyruvic and lactic acids).

The eluted fractions were collected in scintillation vials and their radioactivity measured by adding a 1 ml samples to 12 ml of scintillation fluid. Radioactivity was determined on a liquid beta scintillation spectrometer (Beckmann).

Total glucose concentrations in the samples were determined as described in the former paragraph.

The amount of ^{14}C -Alanine converted to glucose was calculated from the following equation:

$$\text{Amount of dose incorporated into glucose (\%)} = \frac{\text{body weight (g)} \times \text{glc diffusion space} \times \text{plasma glucose (dpm/ml)}}{\text{radioactive Ala injected (dpm)}} \times 100$$

The glucose diffusion space constant was assumed to be 20.7% of the body weight as determined by Mazeud (1973) and the specific activity of glucose is presented as dpm/umole.

To validate the method, we added labelled alanine, pyruvate and glucose to fish plasma and after deproteinization, recovered the radioactivity by fractionation on a Dowex* X-8 column. As shown in Table 1, the recovery was excellent. Time course of appearance and disappearance of labelled glucose pyruvate and alanine in plasma was assayed in three fish and is presented in Figure 1.

As shown, the values of glucose reach their highest level and then taper off after 180 min while the values of pyruvate/lactate and alanine were maximal after 30 min. We decided to choose 90 min as the assay period since the levels of glucose were almost maximal while the levels of pyruvate/lactate were still quite high.

1.4 Incorporation of labelled amino acid into tissue fractions

Fish were anaesthetized with MS-222 before intraperitoneal injection of L - (4,5 ^3H) leucine (specific activity ~ 45 Ci/mmole), dissolved in 0.75% NaCl. A delivery volume providing 12 uCi/200 g was injected to pre-weighed fish. Maximum incorporation of the labelled amino acid to the fish muscle protein was observed 16 - 24 hr after injection of the labelled amino acid. The fish were sacrificed by a cranial blow 18 hr following injection of ^3H -leucine in all incorporation experiments. Blood samples were drawn from the caudal vein using disposable syringes flushed with heparin. Excised livers and portions of white muscle dissected from the fish were quickly placed into vials in ice. The samples were transferred for storage at -20°C prior to analysis.

After removing the blood cells by centrifugation, plasma was deproteinized with 3 vol of 5.5% sulfosalicylic acid and centrifuged at 2000 g for 10 min. The plasma precipitates were solubilized in 2 ml of KOH 33%. One ml sample was counted in 14 ml of a toluene scintillation cocktail containing PPO and POPOP. Samples were counted in a Beckman Beta Counter. 250 mg samples of muscle and 200 mg samples of liver were homogenized in 0.75% ice-cold saline using a teflon-glass homogenizer. The total volume of homogenates was 5 ml. Samples were precipitated with 10% TCA and subsequently washed with 5% TCA followed by repeated centrifugation at 2000 g for 10 min. The supernatant contained an unbound, non-protein ^3H -leucine. Aliquots of 1 ml were added to 14 ml of toluene cocktail for liquid scintillation as previously described. The precipitates were solubilized overnight at 50°C in 2 ml of KOH 33%. Aliquots of 1 ml were added to 14 ml of toluene cocktail for liquid scintillation. Samples were counted for 10 min and results are presented as dpm incorporated per milligrams of wet tissue.

1.5. Determination of insulin and cortisol in the plasma.

1.5.1. Preparation of iodinated bonito insulin.

Bonito insulin (14.5 u/mg) was received from Dr. M. Furuichi, Kyushu University, Japan. Porcine insulin and bovine insulin were obtained from Sigma Chemical Co. (St. Louis, USA). The insulin was dissolved in a small quantity of 0.1 N HCl and then diluted with 0.1 M Hepes buffer (pH 7.4).

Further dilutions were made with the same buffer containing 0.25% bovine serum albumin. Iodination of Bonito insulin with Na-¹²⁵[I] (Amersham Inc.) was performed by the chloramine-T method (Greenwood et al., 1963) as modified in our laboratory (Gertler et al., 1984). The specific activity of the labelled insulin was 90 - 118 uCi/mg with labelling efficiency of 60% - 70% incorporation.

1.5.2. Preparation of antibodies to bonito insulin

Five BALB/C mice (female, six weeks old) were each immunized with 100 ug of insulin in their footpads (FP) and intraperitoneally (IP) using complete Freund's adjuvant (CGA). Two, four and six weeks later, the mice were given booster injections IP using 100 ug of insulin mixed with incomplete Freund's adjuvant (InCFA).

After three injections, mice were bled and the serum was examined for anti-insulin activity. Those animals showing positive anti-insulin reaction were injected once again with insulin followed 48 h later by inoculation of 0.2 ml of 10% suspension of sarcoma cells - "Krebs" to induce ascites fluids in the immunized animals. Ten and thirteen days later, the ascitic fluid was drawn, pooled and centrifuged at 2000 rpm for five minutes; the supernatant was tested for anti-insulin activity. As a negative control, non-immunized mice were injected with the same amount of sarcoma "Krebs" cells. The ascitic fluid was collected as described above.

1.5.3. Radioimmunoassay of insulin

The incubation mixture consisted of 400 ul of 0.1 M Hepes buffer pH 7.4, 100 ul of ascitic fluid containing antibody, that was diluted in Hepes buffer, containing 0.25% bovine serum albumin, to a final dilution range between 1:4000 - 1:8000, 50 ul of ¹²⁵[I]-Bonito insulin (12,000 - 20,000 cpm per tube) and 50 ul of standard insulin sample or fish plasma. No changes were found either when the insulin samples were diluted in insulin-free plasma (after charcoal treatment) or in Hepes/BSA buffer. The reaction mixture was incubated for 24 h at 4°C and then again for a further 24 h at 4°C following addition of 400 ul water suspension of Protein A (0.2% w/v), that contained 0.1% Na-azide (BioYeda, Rehovot, Israel). At the end of this period, the tubes were centrifuged at 7000xg for 25 min; the supernatant was aspirated and the pellets were counted in a gamma counter for 2 min. All standards and samples were assayed in triplicate. The

maximum binding of ^{125}I -Bonito insulin to the antibodies (without addition of unlabelled insulin) was 12 - 28%. The logit-log method was used for calculation of the standard curve.

Figure 2 shows the results obtained from four different dilutions of antibodies when varying amounts of unlabeled insulin were added. The binding fraction varied inversely with the antibody dilutions. When 1:3000 dilution was used with 1.4 uU of unlabeled Bonito insulin added to the tracer, the displacement was insignificant. At 1:6000 dilution with the addition of 1.4 uU of unlabelled Bontio insulin to the tracer, the displacement was 17% and with 1:12000 and 1:18000 dilutions, the displacement was 29 and 32%, respectively. A dilution of 1:6000 was found suitable for assaying 2 - 100 uU of insulin per tube, which is in the range of insulin plasma levels described for most of the fish.

In order to compare the immunological differences between fish and mammalian insulins, bovine and porcine insulin were incubated with antibodies raised against bonito insulin. The results shown in Fig. 3 indicate that porcine insulin had only a slight displacement activity. Bovine insulin, on the other hand, was capable of displacing the bonito insulin, but a 10-fold higher concentration (as compared to unlabelled bonito insulin) was required to achieve 50% displacement. Scatchard analysis revealed that the affinity constant for Bonito insulin is 5.0×10^9 l/mol and for bovine insulin, 1.2×10^9 l/mol.

Other polypeptide mammalian hormones, such as bovine and human growth hormone and ovine prolactin did not exhibit any cross-reactivity with anti-Bonito insulin ascitic fluid.

To test the validity of the assay for determination of carp plasma insulin, increasing amounts of plasma obtained from six different fish, were tested. As shown in Fig. 4 at the range of 50 - 200 ul plasma and the displacement curves were parallel to the standard curve. At higher or lower concentrations (not shown), the curves were not parallel. The low sensitivity of the assay was 1.5 uU/tube or 2.5 uU per ml plasma.

1.5.4. Radioimmunoassay of cortisol

The radioimmunoassay was performed using the kit for cortisol RIA purchased from Amersham, according to the instructions of the producer.

1.6. Immunocytochemical studies

In order to examine the specificity of our antibody to insulin, we examined the immuno reactions between the anti-bonito insulin and tissue sections from carp endocrine pancreas. The immunocytochemical tests were performed on both light and electron microscope levels. Altogether, five adult specimens (four males and one female) of Cyprinus carpio were used for these examinations.

For the light microscope study, Brockmann bodies from the carp were fixed for 1h in Bouin solution, dehydrated and embedded in Paraplast + (at 56°C) or in paraffin (at 60°C), for not longer than one h for the three changes of the embedding agent. Mounted blocks were cut 6 µm thick. For the electron microscope investigation, the tissue was fixed with Karnovsky's (1965) fixative or with a 2% cacodylate buffered glutaraldehyde solution. The tissues were embedded in 'LR White' (London Resin Co., Basingstoke, UK) at +50°C or in Lowicryl K4M (Lowi, Waldkraiburg, FRG) at -35°C. In preliminary experiment embedding was made with Epon or Spurr. Thin sections were cut with a LKB ultratome (Sweden) mounted on Nickel or gold grids and viewed with a CX 100 JEM electron microscope.

For the LM immunotechniques, we used as a secondary antibody, biotinylated anti-mouse IgG, and biotinylated horseradish peroxidase mixed with avidin (Vector, Burlingame, USA). As a peroxidase substrate, we used either 3-amino-9-ethylcarbazole or diaminobenzidine (Sigma, Israel). For the EM immunotechniques, we used protein-A gold prepared in our laboratory by reduction of chloroauric acid according to the technique of Roth (1982), or used commercially available protein-A gold emulsion (Janssen, Beerse, Belgium).

Immunocytochemical studies (Fig. 5) indicated that cells of the endocrine and exocrine pancreas of Cyprinus carpio form tissue aggregations which surround the gall bladder duct. Endocrine islets may occur as isolated "unit-islets" of about 150 cells per section, or the unit-islets may fuse to larger endocrine systems containing up to 20 unit-islets per section. Islet tissue consists mainly of cells reacting with antibody to insulin; aside insulin reactivity somatostatin, and glucagon cells were also present, the former between the insulin cells, while glucagon reactivity was observed at the periphery of the unit-islets. About 10 - 15% of the cells did not react to any of the antibodies tried until now,

which included commercially available antibodies to pancreatic polypeptide, and vasoactive intestinal peptide, among others.

1.7. Fish growth studies

The effect of feed additives, which may modify the hormonal regime of the fish, on growth and protein utilization was studied in a series of tank experiments performed at the Fish and Aquaculture Research Station, Dor (ARO). One experiment was carried out in a series of commercial ponds on two fish farms.

The tanks were equipped with a self-cleaning drainage system, especially developed for the present study, which was found to be very efficient. Average growth rates of common carp achieved in the tanks, for the weight range of 100-400g, was 4-5g/fish/day, as compared to less than 1g/fish/day usually obtained in these tanks before installing the self-cleaning drainage system.

All tanks were stocked with an equal number of common carp. The stocking rate varied somewhat between the different experiments, according to the average initial weight of the fish, at a range of 19-25 fish/tank. The average weight of the fish also varied between the experiments at the range of 92-125g. Mortality during the experiments was very small, if at all. However, few fish were removed from each tank (at equal numbers) during the experiments for the physiological and histological analyses. All fish of each tank were weighed every two weeks as a group and the average weight per fish was calculated. Feeding rates (3 - 4% of body weight per day - see different experiments) were adjusted according to the average weight, separately for each tank. The daily food ration was divided into two meals (0800 and 1400 hours). The removal of fish from the tanks for analyses during the experiment varied somewhat the average weight of the fish remaining in the tanks. The average weight could not be used, therefore, for construction of growth curves and the average gain per fish has been used instead.

Since one aim of the study was to determine the effect of the additives on the protein utilization, all tank experiments were factorial. One variable was the protein content of the diet and the other the feed additive(s). Two levels of dietary protein were tested: (a) high protein diet (HP), which during the first year (1985) contained 40.7% protein and

during the second year - 38%; (b) low protein diet (LP), which during the first year contained 27.5% protein and during the second year was reduced to 23%. The basic compositions of these diets (before additive inclusion) are given in Table 2.

Four "additive" treatment were tested in each tank experiment. They consisted either of graded levels of the same additive as in the first two experiments with cobalt and metformine, or different additives tested simultaneously in order to compare their effect, as in the last two experiments. Each treatment was carried out in triplicates (2 diets x 4 treatments x 3 replicates = 24 tanks).

2. Effect of starving or feeding with high and low protein diet on metabolism of carbohydrate and amino acids

2.1 Glucose tolerance test and insulin plasma levels.

The glucose tolerance test is a meaningful tool for determination of hormonal effect on glucose clearance in mammals. Since glucose applications per os in fish are problematic, we decided to apply the glucose both by oral and intraperitoneal (IP) injection. fish were injected with 0.5 - 2.0 g glucose per Kg body weight. Samples of blood were withdrawn after 2, 4, 6 and in some cases, 12 or 16 h and analyzed for glucose content with a glucose analyzer. To calculate the disposal rate, the glucose values were plotted on a semi-logarithmic plot as a function of time. The disposal rate constants were calculated from the slope ($k = 2.303 \times \text{slope}$) and the half-lives $t_{1/2}$ from equation 2:

$$(2) \quad t_{1/2} = \frac{0.693}{k}$$

The results of six experiments given in Figure 6 and Table 3 indicate that although starving decreased the basal glucose level, the rate of disposal was similar in both fed and starved fish. It should be noted that these values are much lower than in normal mammals and are characteristic of the diabetic state. Another parameter indicating the diabetic-like state in carps was obtained by measuring the compilation of glucose levels 2, 4 and 6 h after injection. All values, except in Exp. 1 (in which a very low

level of glucose was given) are higher than 500 mg %, a value indicative of the diabetic state in man.

To assess the feasibility of our method of estimating insulin levels in carp, we measured the plasma levels of insulin in fed and starved fish and compared them to the respective glucose levels. As shown in Table 4 a good correlation between the nutritional state of the fish and the levels of glucose and insulin in the plasma was found. Both insulin and glucose levels were doubled in fed as compared to the starved fish and the difference was statistically significant.

2.2 Gluconeogenesis

The process of gluconeogenesis is common to all animals and its main purpose is to supply glucose to peripheral tissues. In view of the fact that glucose and glycogen utilization is impaired in fish, the importance of gluconeogenesis is even more pronounced. To follow this process, we have developed a detailed protocol (see paragraph 1.3) in which conversion of ^{14}C -alanine to glucose is determined. Two experiments were carried out to determine the effect of starving and protein level in diet. The results shown in Table 5 indicate that starving increased the gluconeogenesis. Interestingly, as shown in Table 5 the increase of gluconeogenesis in starved fish was paralleled by significant decreases in insulin and a dramatic increase of cortisol in blood. High protein diet (Table 6) dramatically increased the conversion of alanine to glucose and the specific radioactivity of glucose.

2.3 Determination of protein synthesis

Since impaired glucose utilization led to increased rate of gluconeogenesis, a question was raised whether protein synthesis was also affected. To test this, we assayed incorporation of ^3H -leucine into white muscle and liver TCA-insoluble fraction (protein). As shown in Table 7, starvation significantly decreased the rate of incorporation in the muscle but not in the liver. Time course analysis revealed that maximal values of incorporation were obtained after 8 h in the liver and 16 h in the white muscle.

3. The effect of cobalt on metabolic parameters, plasma compounds and growth of carp

Two sets of experiments were performed on the effect of cobalt on the metabolic parameters and protein utilization: (a) laboratory experiment in which cobalt was injected intraperitoneally and its effect on blood glucose and glucose tolerance, gluconeogenesis and protein synthesis was determined; (b) tank and pond experiments where cobalt was added to the feed and its effect on growth rate and protein utilization was determined. During each tank experiment and at its end fish were taken for the determination of glucose, insulin levels in the blood serum, and test were carried out on the incorporation of ^3H -leucine into the muscle.

3.1. Laboratory studies

3.1.1 Blood glucose and glucose tolerance test

Three experiments of glucose tolerance test were carried out. As shown in Table 8. Only in one experiment with fish supplemented with cobalt did the glucose level register lower values, 6 h after loading.

3.1.2 Gluconeogenesis

The effect of cobalt supplementation for 15-30 days prior to the test, on gluconeogenesis was determined in three experiments. Although the basal glucose level in cobalt supplemented diet was lower in only one experiment, the conversion of labelled alanine to glucose and the specific radioactivity of glucose (see Table 9) was lower in all three experiments. In experiments one and three, the differences were statistically significant.

3.1.3 Protein synthesis

The effect of cobalt on protein synthesis in the liver and the white muscle of fish fed a low (23%) protein diet is summarized in Table 11. Two experiments in which two levels of cobalt were applied by intraperitoneal injection were performed and the results indicate lack of any remarkable effect on incorporation of labelled leucine into the protein (TCA-insoluble) fraction in both organs. In one experiment, the level of

non-protein label in the white muscle fraction was lower in fish given cobalt.

3.2. Tank and pond experiments

3.2.1 Growth and protein utilization

The first tank experiment, carried out during 1985 intended to determine the effect of increasing graded levels of cobalt, added to the feed as $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, on growth and protein utilization. The control diet did not contain cobalt (HP0 and LP0), while the treatments contained the following levels: 2mg Co/kg feed (HPCo2, LPCo2) - a concentration used successfully in the Soviet Union during the sixties and subsequently recommended as standard additive to the diet; 10mg Co/kg feed (HPCo10 and LPCo10) - a concentration used successfully in Yugoslavia; and 15mg Co/kg feed (HPCo15 and LPCo15).

Tanks were stocked with 22 common carp of an average weight of 92g. The experiment continued for 65 days and the results are presented in Table 11. Analysis of variance shows a significant effect of cobalt on growth rate, food conversion ratio and protein efficiency ratio. Duncan's multiple comparison tests ($p < 0.05$) showed that without cobalt the fish grew better on the high protein diet (HP0) than on the low protein diet (LP0), but the difference was not statistically significant. The addition of cobalt to the high protein diet at a rate of 15mg/kg feed increased growth. The difference from HP0 was again not significant statistically, but it made the difference from the LP0 statistically significant. A more striking effect was that of adding the cobalt, at all levels, to the low protein diet. Growth rate obtained with cobalt supplemented low protein diet was higher than that obtained by the high protein diet, and the difference from LP0 was statistically significant. Similar results have been obtained with feed conversion ratio and protein efficiency ratio. Both were improved by the addition of cobalt to the low protein diet, but had a lesser, or no effect when added to the high protein diet. This has been taken as an indication to a better utilization of the protein for growth, especially when protein is limited.

Experiments carried out during 1986 intended to varify the above results and also compare the cobalt to other additives. Similar to the 1985 experiments, also the 1986 experiments tested the effects of the additives

in a factorial layout at two levels of protein - 38% (HP) and 23% (LP). For the composition of these diets see Table 3. The additive treatments consisted of: (a) control with no supplementation (HP0, LP0); (b) cobalt, added as CoCl_2 - 2mg Co/kg feed (HPCo, LPCo); (c) chromium, added as CrCl_3 - 2mg Cr/kg feed (HPCr, LPCr); and (d) a mixture of cobalt and chromium - 1 mg of each/kg feed (HPCC, LPCC).

The experiment lasted for 85 days (April 6, 1986 - June 29, 1986). 25 common carp of an average weight of 116.7g were stocked into each tank. Feeding rate was 4% of their body weight per day, adjusted every two weeks. The results are given in Table 11.

It can be seen that the rate of growth in all treatments was again very good, but contrary to the results in the previous experiment, there was no significant effect of the added cobalt (nor of the other additives) on the parameters determined (growth rate, FCR and PER). Again, in contrast to the previous experiment, fish fed on low protein control (LP0) grew somewhat better than fish fed the high protein control (although the difference was not statically significant). This may have been caused by either of the following: (a) the lower protein level is sufficient and the high protein level is excessive, which does not seem to be likely in view of the many studies on protein requirement of common carp and the results of the previous experiment; (b) excessive feeding level for the conditions in the tanks. Indeed some other indications, such as large amounts of uneaten residues and the resulting low O_2 concentrations, showed that this may have been the case.

In view of the above the experiment was repeated again, with reduced feeding rate to 3% of the body weight per day, while the concentration of the additives was increased to 5mg/kg feed. Another variation was the inclusion of nickel, added as $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ instead of the mixture of cobalt and chromium. The second 1986 experiment started (after adaptation of the new batch of fish to the tank conditions) on July 30, 1986 and continued through September 28, 1986 (i.e. it lasted 60 days). The results are presented in Table 13.

Like in the first experiment with cobalt low protein diet resulted in lower growth rate (although, again, the difference is not statistically significant). However, the addition of cobalt did not have a significant effect on any of the parameters determined.

3.2.2 Pond experiments

The effect of cobalt on yield and protein utilization was also studied under pond conditions in commercial fish farms. Commercial fish feed, containing 18 or 25% protein was supplemented with 2 mg Co/kg feed. The feed was prepared especially for this study by "AMBAR" Feed Mill, to whom our thanks are due. This feed was given to fish in 7 ponds, 2 to 4.3 hectares in area each, at two commercial fish farms: Mishmar Hasharon and Ma'abarot. Yield, FCR and PER were recorded and calculated for the experimental ponds and compared to similarly managed adjacent ponds and to the same ponds in the previous year.

All ponds were under a polyculture management system and were stocked simultaneously with common carp (Cyprinus carpio), tilapia (Oreochromis niloticus x O. aureus), grey mullet (Mugil cephalus) and a hybrid between silver carp (Hypophthalmichthys molitrix) and bighead carp (Aristhychthys nobilis). In order to compare yields obtained in ponds of different area and during a different culture periods, the unit of yield chosen was kg/ha/day. The number of days taken into account for the above calculation was only those during the growing season - March 15 to November 15. For the calculation of FCR and PER the yield of the hybrid silver carp x bighead carp was excluded since being a filter feeder it does not consume pelleted feed. The results of the experiment are given in Table 14.

At Mishmar Hasharon both FCR and PER in the ponds where fish have not received cobalt were worse in 1986 than in 1985. In the ponds where fish have received cobalt in 1986, the decrease in performance was less pronounced. However, factorial analysis of variance have shown this interaction to be statistically not significant. At Ma'abarot, small differences in yield, FCR and PER in favour of the ponds where the fish received cobalt were noticed but also these were not statistically significant.

3.2.3 Blood glucose tests

The effect of cobalt supplementation in diet on the basal glucose levels in fish fed low (27.5%) and high (40.7%) protein diet for 65 days is summarized in Table 15. As shown in the low protein diet, the basal glucose levels were significantly lower in fish supplemented with cobalt while at a high protein diet, no effect of cobalt supplementation was found. This was most likely because fish fed a high protein diet, without

cobalt supplementation exhibited lower glucose levels than those fed the low protein diet. The test was repeated two months later with no differences in the basal glucose level between the different treatments.

3.2.4 Plasma insulin levels

The effect of cobalt supplementation was tested at two protein levels in fish fed low and high protein diets for 65 days. As shown in Table 16, no significant differences were noted.

3.2.5 Protein synthesis

Protein synthesis was also tested in fish fed low (27.5 %) and high (40.7 %) protein diets for 65 days. Results shown in Table 17 indicate the rate of ^3H -leucine incorporation, with one exception, not to be affected.

4. The effect of chromium on the metabolic parameters, plasma components and growth in carp

4.1. Blood glucose and glucose tolerance test

As shown in Table 18, fish treated with chromium and fed a low protein diet had lower basal glucose levels but only in Exp. 1 were the differences statistically significant. In all three experiments, chromium supplementation increased the clearance of the glucose from plasma. The differences between fish treated with chromium and non treated fish, observed after three hours, was still not significant. However, after six hours, the decrease was remarkably and statistically significant in all three experiments.

4.2 Gluconeogenesis

As shown in Table 19, the treatment with chromium resulted in two significant changes: (a) it significantly decreased the plasma glucose levels and (b) it significantly decreased the gluconeogenesis as expressed by slower conversion of labelled alanine to glucose and lower specific activity of labelled glucose.

4.3 Protein synthesis

As shown in Table 20, treatment with chromium had no effect on protein synthesis as evident from the values of the TCA-insoluble fraction in white muscle. In the liver of chromium treated fish, the values were somehow lower but the difference was not statistically significant. In the first experiment, TCA-soluble fraction levels, which are associated with free amino acids and peptides, were, for unexplained reason, significantly lower in the muscle and higher in the liver.

4.4. Growth and protein utilization

The effect of chromium added to the diet on growth and protein utilization was studied in two tank experiments during 1986. The chromium was supplemented as chromium chloride at a rate of 2 and 5 mg Cr/kg feed at the first and second experiment, respectively. In the first 1986 experiment it was also tested in combination with cobalt, each added at a rate of 1 mg/kg feed. The results are presented in Tables 12 and 13. The addition of these levels of chromium to the diet did not affect growth rate or protein utilization by the common carp.

5. The effect of metformin^{*} on metabolic parameters, plasma components and growth in carp

5.1. Laboratory experiments

5.1.1 Blood glucose and glucose tolerance test

Intraperitoneal injections of metformin at the range of 1.6- 80 mg/kg body weight had no significant effect on the basal glucose levels in fish fed low protein diet (see Tables 21 and 22). The effect on glucose disposal using glucose tolerance test was less clear and therefore, six experiments were carried out. These results are summarized in Table 21 and indicate that six h following loading in five out of six trials, the levels of

* Thanks are due to "ABIC" Chemical and Pharmaceutical Ind. for supplying this drug gratis.

glucose in fish pretreated with metformin were lower. In three cases, where fish received high dose (80mg/kg body weight intraperitoneally, the difference was statistically significant.

5.1.2 Gluconeogenesis

The effect of pretreatment with metformin on the gluconeogenesis was tested in two experiments. As shown in Table 18, metformin decreased the rate of gluconeogenesis as exposed by suppression of alanine-glucose conversion and lower specific radioactivity of plasma glucose.

5.1.3 Plasma insulin and cortisol levels

As shown in Table 23, metformin treatment had no effect on insulin level but significantly increased the level of cortisol.

5.1.4 Protein synthesis

The effect of metformin on protein synthesis in white muscle and liver was tested in six experiments. In all cases, pretreatment (intraperitoneal injection) with metformin increased the rate of labelled leucine incorporation into protein fraction in the white muscle (Table 24) but the difference was statistically significant only in two trials. The effect on synthesis of liver protein was less clear and only in one case, the increase due to metformin treatment was statistically significant.

5.2. Tank experiments

5.2.1 Growth rate and protein utilization

The effect of diet supplementation by graded amounts of metformin on growth rate, FCR and PER were tested during 1985 in the 24 tanks, similar to the experiments described above for cobalt. The high protein diet contained 40.7% protein and the low protein diet contained 27.5%. Feeding rate was 4% of body weight per day, adjusted every two weeks. The levels of metformin added to these diets were: 0 (HP0, LP0), 10 (HPM10, LPM10), 20 (LPM20, LPM20), and 40 (HPM40, LPM40). The experiment was 60 days long. The results of this experiment are presented in Table 25. The highest weight gain was obtained by fish fed the unsupplemented low protein diet (LP0), which contradicts the results of the previous experiment, and may be an indication of some disturbance in the experimental conditions (excess

feeding?). The diets containing 40 mg metformin/kg feed showed statistically significant retardation of growth, worse food conversion and protein efficiency ratios (The last parameter only in the low protein level). The lower levels of metformin supplementation did not show any positive effect on any of the determined parameters.

5.2.2 Blood glucose level

Basal blood glucose levels were somewhat lower in fish receiving the low protein level receiving metformin. Only in one case the difference was significant statistically.

5.2.3 Protein synthesis

Two experiments were performed in fish fed low (27.5%) and high (40.7%)— protein diets supplemented with metformin. As shown in Table 27, in experiment two, supplementation with 1.6 mg/kg/bwt resulted in a small but significant increase in TCA-insoluble fraction (protein) in fish fed both low and high protein diets. No effects were found in lower doses of the drug and in experiment one.

6. Conclusions

From the results given above it seems that common carp, like other fish species behave like diabetic animals. Glucose disposal rate after loading was much slower than in normal mammals. There was no difference in disposal rate between starved and fed fish nor between different loadings of glucose, which indicates a diabetic-like state. Starving increased gluconeogenesis in the common carp and in parallel decreased insulin level and increased cortisol level in the blood. Gluconeogenesis was also higher in fish fed high protein diets, as compared to those low protein diet.

Intraperitoneal injections of cobalt and chromium, and to some extent also of the drug metformin, increased glucose clearance from the blood after glucose loading and decreased gluconeogenesis. The effect of these injections on protein synthesis was, however, small and usually not significant statistically.

Lesser effects were observed with the adding of the above compounds to the diet on growth rate, feed conversion and protein utilization. In one experiment the addition of cobalt at 2-15 mg/kg feed did affect

significantly the above parameters, but these results could not be repeated. the effect of cobalt in the subsequent experiments was small. Also other additives such as chromium, nickel and metformin had a small and statistically insignificant effect.

It seems that the effect of the additives on protein synthesis, growth and protein utilization for growth is compounded with other unknown factors which should be studied in the future. Thus, one explanation on the variable affect of the additives on protein utilization is reduced assimilation of these chemicals which tend to form insoluble compounds. Food ingredients such as phosphates or calcium may have reacted with the cobalt and chromium to form insoluble and therefore unassimilated compounds. The development of organic complexes could remedy this and should be examined in the future.

7. References

- Greenwood, R.C., Hunter, W.M. and Glover, J.S. (1963), The preparation of ^{125}I -labelled human growth hormone of high specific radioactivity. *Biochem. J.* 89 114-123.
- Karnovsky, M.J. (1965), A formaldehyde - glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.*, 27, 137A-138A.
- Mazeaud, F. (1973), Recherches sur la regulation des acides gras libres plasmatiques et de la glyceruie ches le poissons. Ph.D. thesis, Univ. of Paris.
- Roth, J. (1982), The protein-A gold technique. Aqualitative and quantitative approach for atigen localization on thin sections. In: *Techniques in Immunocytochemistry*; ed. G. Bullock and P. Petrusz, Vol. I, pp. 107-133, Academic Press, London.
- Somogyi, M. (1945), Determination of blood sugar. *J. Biol. Chem.*, 169: 69-75.

Table 1. RECOVERY TEST OF ^3H -GLUCOSE, ^{14}C -ALANINE AND ^{14}C -PYRUVATE AND THEIR MIXTURE IN FISH PLASMA

Isotope tested ¹	Total dpm applied on the columns	Dpm eluted at pH:			Total recovered (dpm)	% recovered
		10.2	5.8	2.0		
^3H -Glucose	80801	74912	0	0	74912	99
^{14}C -Alanine	22011	0	23444	0	23444	100
^{14}C -Pyruvate	33000	0	0	32868	32868	99
Mixture of all three						
^3H	80801	71430	0	0	71430	89
^{14}C	55011	0	15843	39270	55113	100
Mixture of all three						
^3H	242403	195899	0	0	195899	81
^{14}C	165033	0	49581	123324	172905	100

¹ The tested radioisotopes were mixed with 0.5 ml of carp plasma, deproteinized and applied on the columns

Table 2. COMPOSITION OF THE DIET USED IN THE TANK EXPERIMENTS DURING 1985 AND 1986

INGREDIENTS in % (% Protein)	HIGH PROTEIN (HP)		LOW PROTEIN (LP)	
	Year:		Year:	
	1985	1986	1985	1986
Protein content:	40.7%	38%	27.5%	23%
Fish meal (72)	38.7	31.7	19.3	12.7
Soybean meal (44)	20.3	20.0	10.2	7.0
Wheat, whole-milled (14)	38.6	46.0	68.1	78.0
Vitamin mix(1)	0.5	0.5	0.5	0.5
Mineral mix(2)	1.9	1.8	1.9	1.8
	100.0	100.0	100.0	100.0

(1) Vitamin mix contained (per kg feed): Riboflavin 4mg; Pyridoxine 6mg; Pantothenic acid 30mg; Niacin 30mg; Inositol 350mg; Cholin 1400mg; Biotin 1mg; Vitamin A 6500 IU; Vitamin E 17 IU.

(2) Mineral mix contained (per kg feed): $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ 0.3g; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.06g; $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ 0.32; KI 0.018g; NaH_2PO_4 17g; $\text{ZnSO}_4 \cdot \text{H}_2\text{O}$ 0.41g.

Table 3. GLUCOSE TOLERANCE TEST IN STARVED AND FED CARP

Exp. no.	Glucose dose ¹ (g/kg b.w.)	Physiological state	Basal glucose level (mg/dl)	Disposal rate ² (h ⁻¹)	Compiled glucose levels ³
1	0.5	starved	95	0.33	453
2	1.0	"	57	0.38	553
3	2.0	"	38	0.13	696
4	2.0	"	65	0.33	1097
5	1.0	fed	75	0.37	787
6	2.0	"	90	0.34	1379

¹Given intraperitoneally

²for calculation of this rate see text (p. 11).

³Compilation of glucose levels at 2, 4 and 6 h after injection

Table 4. PLASMA GLUCOSE AND INSULIN LEVELS IN CARP UNDER DIFFERENT NUTRITIONAL STATES

	No. of fish	Weight ^a (g)	Plasma Glucose ^a mg/dl	Plasma Insulin ^a uU/ml
Fasted ^b	6	270 ± 10	67 ± 6	72 ± 13
Fed	7	241 ± 15	105 ± 6*	158 ± 27*

^a mean ± SEM, * - significantly different from the fed fish, P < 0.01

^b for 7 days.

Table 5. THE EFFECT OF STARVING ON THE CONVERSION OF ^{14}C -ALANINE TO GLUCOSE OR ORGANIC ACIDS AND ON THE INSULIN AND CORTISOL PLASMA LEVELS.

Exp. no.	Parameter tested	Feeding status ^a	
		Starved ^b	Fed
1	% glucose formed	68.5 $\pm$ 6.5 (2)	34.5 $\pm$ 2.4 [*] (4)
	% organic ac. formed	20.8 $\pm$ 2.8 (2)	53.0 $\pm$ 1.7 [*] (4)
2	insulin (ng/ml)	5.0 $\pm$ 0.96 (7)	10.9 $\pm$ 1.94 [*] (5)
	cortisol (ng/ml)	759 $\pm$ 102 (7)	94 $\pm$ 36 [*] (5)

^a mean $\pm$ SEM, the numbers in parentheses indicate number of fish,

^{*} - significantly different from the starved fish, $P < 0.01$.

^b for 7 days.

Table 6. THE EFFECT OF PROTEIN LEVEL ON INCORPORATION OF ^{14}C -ALANINE INTO GLUCOSE AND ON PLASMA GLUCOSE (MEAN $\pm$ SEM).

Level of diet protein	No. of fish tested	Plasma glucose (mg/dl)	% incorporated into glucose	Specific activity of glucose
Low ^c	12	101.5 $\pm$ 5.1	1.62 $\pm$ 0.15 ^b	0.15 $\pm$ 0.02 ^a
High ^c	10	110.0 $\pm$ 3.5	7.94 $\pm$ 2.37	0.55 $\pm$ 0.17

^a $P < 0.05$, as compared to high protein diet.

^b $P < 0.02$, as compared to high protein diet.

^c For composition of the diets see Table 2.

Table 7. TIME DEPENDENT INCORPORATION OF ^3H -LEUCINE INTO WHITE MUSCLE AND LIVER PROTEINS OF STARVED AND FED FISH

Organ tested	Feeding status	Incorporation of ^3H -Leu (dpm/mg tissue)			
		8h ^a	16h ^a	18h ^b	24h ^a
White muscle	Starved	5.1±1.6	9.7±1.6	8.7±1.0	9.5±0.4
	Fed	12.5±2.3*	24.7±7.4*	21.0±5.3*	24.0±2.3*
Liver	Starved	234±29	172±10	159±28	165±9
	Fed	140±41	194±21	131±11	165±26

^a mean ± SEM, * - significantly different from the starved fish, P < 0.01,
^b

Table 8: THE EFFECT OF COBALT ON UTILIZATION OF GLUCOSE DURING GLUCOSE TOLERANCE TESTS AND ON BASAL GLUCOSE LEVELS.

Exp. no.	Cobalt ug/kg.bwt	No. of fish	Glucose injected (g/kg/bwt)	Glucose level after (mg/dl)		
				0 h	3 h	6 h
1	None	7	2.0	123 ± 11	578 ± 11	499 ± 30
	51	9	2.0	108 ± 8	496 ± 21	326 ± 63*
2	None	14	1.7	152 ± 8	440 ± 15	289 ± 33
	402	14	1.7	128 ± 9	445 ± 26	259 ± 39
3	None	10	1.7	119 ± 5	436 ± 20	224 ± 21
	402	10	1.7	103 ± 6	436 ± 28	227 ± 28

^a All results given as mean ± SEM. Results marked * differ significantly (p<0.05) from the respective controls.

1 Intraperitoneally

2 Orally

Table 9: THE EFFECT OF COBALT ON PLASMA GLUCOSE LEVELS AND GLUCOSE FORMATION AFTER INJECTION OF ^{14}C -ALANINE

Exp. ^a No.	Cobalt added ug/kg bwt	No. of fish	Plasma glucose (mg/100 ml)	% Incorporated into glucose	Specific Activity of glucose
1	None 5 ¹	3	130 $\pm$ 13	2.06 $\pm$ 0.46	0.22 $\pm$ 0.02
		3	114 $\pm$ 5	0.97 $\pm$ 0.12	0.13 $\pm$ 0.02*
2	None 5 ¹	4	113 $\pm$ 3	1.45 $\pm$ 0.23	0.20 $\pm$ 0.03
		4	85 $\pm$ 4*	0.99 $\pm$ 0.22	0.18 $\pm$ 0.03
3	None 40 ²	12	101 $\pm$ 5	1.62 $\pm$ 0.15	0.15 $\pm$ 0.013
		10	93 $\pm$ 5	0.87 $\pm$ 0.08*	0.08 $\pm$ 0.009*

^a All results given as mean $\pm$ SEM. Results marked * differ significantly (p<0.02) from the respective controls.

1 Intraperitoneally

2 Orally

Table 10. THE EFFECT OF COBALT ON INCORPORATION OF ^3H -LEUCINE INTO MUSCLE AND LIVER OF FISH FED LOW PROTEIN DIET

Exp. no.	Cobalt added ug/kg bwt	No. of fish	Incorporation of ^3H -Leu (dpm/day, mean $\pm$ SEM ³)			
			White muscle		Liver	
			TCA- insoluble fraction	TCA- soluble fraction	TCA- insoluble fraction	TCA- soluble fraction
1	None 5 ¹	10	30.3 $\pm$ 2.8	17.8 $\pm$ 1.0	257 $\pm$ 14	33 $\pm$ 2.8
		7	23.7 $\pm$ 4.1	66 $\pm$ 9.3 ^b	215 $\pm$ 7 ^a	24 $\pm$ 1.8 ^a
2	None 60 ²	10	26.5 $\pm$ 3.2	16.9 $\pm$ 0.4	122 $\pm$ 9	26 $\pm$ 2.0
		10	29.1 $\pm$ 2.1	16.2 $\pm$ 0.5	122 $\pm$ 9	24 $\pm$ 0.8

³ a - P < 0.02, b - P < 0.001

1 Intraperitoneally

2 Orally

Table 11. THE EFFECT OF DIFFERENT LEVELS OF COBALT ADDED TO THE DIET ON GROWTH RATE, FEED CONVERSION RATIO (FCR) AND PROTEIN EFFICIENCY RATIO (PER).

TREATMENT	WEIGHT GAIN (g)	FCR*	PER**
HPO	146.0abc	1.52ab	1.62a
HPCo2	146.6abc	1.51ab	1.63a
HPCo10	139.0bc	1.62a	1.52a
HPCo15	161.6a	1.47b	1.68a
LPO	131.6c	1.64a	2.23b
LPCo2	157.7ab	1.43b	2.54c
LPCo10	149.3abc	1.46b	2.49c
LPCo15	152.1ab	1.44b	2.53c

* $FCR = \frac{\text{Feed (g/fish)}}{\text{Gain (g/fish)}}$

** $PER = \frac{\text{Gain (g/fish)}}{\text{Protein fed (g/fish)}}$

Table 12. THE EFFECT OF VARIOUS FEED ADDITIVES ON THE GROWTH RATE, FEED CONVERSION RATIO (FCR) AND PROTEIN EFFICIENCY RATIO ON COMMON CARP IN TANKS

TREATMENT	WEIGHT GAIN (g)	FCR*	PER**
HPO	213.6	2.2	1.1a
HPCo	211.5	2.3	1.1a
HPCr	218.3	2.2	1.1a
HPCC	228.2	2.1	1.2a
LPO	217.0	2.2	1.6b
LPCo	208.0	2.3	1.5b
LPCr	195.7	2.4	1.5b
LPCC	199.3	2.3	1.5b

* ** see footnotes to table 11

Table 13. THE EFFECT OF VARIOUS FEED ADDITIVES ON THE GROWTH RATE, FEED CONVERSION RATIO (FCR) AND PROTEIN EFFICIENCY RATIO ON COMMON CARP IN THE SECOND 1986 TANK EXPERIMENT

TREATMENT	WEIGHT GAIN (g)	FCR*	PER**
HP0	277.1a	1.09a	1.84ab
HPCo	271.0ab	1.06ab	1.87ab
HPCr	234.0abc	1.18ab	1.70a
HPNi	265.3ab	1.15ab	1.98b
LP0	225.6abc	1.22ab	2.72c
LPCo	217.0bc	1.22ab	2.77c
LPCr	221.3abc	1.25ab	2.71c
LPCC	201.8c	1.32b	2.55c

* ** see footnotes to table 11

Table 14. THE EFFECT OF COBALT SUPPLEMENTED TO COMMERCIAL DIET (2mg Co/kg feed) ON YIELD, FEED CONVERSION RATIO (FCR) AND PROTEIN EFFICIENCY RATIO (PER) OF FISH IN FARM PONDS.

A. MISHMAR HASHARON

POND (Growth cycle)	1 9 8 5			1 9 8 6		
	YIELD (kg/ha/day)	FCR	PER	YIELD (kg/ha/day)	FCR	PER
<u>No cobalt added in any of the years</u>						
Ma'agar (long cycle)	24.1	1.28	3.31	40.1	2.41	1.98
4 (1st cycle)	29.1	0.80	6.09	32.5	1.11	4.49
4 (2nd cycle)	49.0	0.65	5.36	37.1	1.75	2.28
8 (1st cycle)	37.3	0.65	6.15	43.2	1.28	3.78
8 (2nd cycle)	27.4	2.64	1.51	28.7	3.36	1.19
Average	33.4	1.21	4.49	36.3	1.98	2.74
<u>Cobalt added in 1986 only</u>						
3 (1st cycle)	31.7	1.73	2.72	35.4	1.41	3.65
3 (2nd cycle)	27.9	1.38	2.91	48.2	1.91	2.16
6 (1st cycle)	32.5	1.24	3.46	24.5	2.05	1.97
7 (1st cycle)	26.7	1.40	3.66	31.4	1.36	4.02
7 (2nd cycle)	37.4	1.17	3.31	28.5	2.53	1.58
Average	31.24	1.38	3.21	33.6	1.85	2.68

B. MA'ABAROT

cobalt added in 1986 only

7 (long cycle)	27.3	1.41	3.67	40.0	1.36	3.90
8 (long cycle)	24.0	2.37	2.12	27.8	2.32	2.51
9 (long cycle)	28.8	1.90	2.74	30.0	3.21	1.71
12 (long cycle)	31.8	3.31	1.50	27.0	2.27	2.44
Average	28.0	2.25	2.51	31.2	2.29	2.64

Table 15: THE EFFECT OF COBALT SUPPLEMENTATION ON PLASMA BASAL GLUCOSE IN FISH FED DIFFERENT LEVELS OF PROTEIN IN THEIR DIET

Dose of Co++ in food mg/kg feed	Basal plasma glucose (mg/dl) ^a	
	Low protein diet	High protein diet
0	105.7 ± 5.4	91.7 ± 6.4
2	90.1 ± 4.4*	82.4 ± 3.5
10	75.2 ± 2.9*	96.2 ± 5.2
15	88.4 ± 3.4*	91.1 ± 4.3

^a mean ± SEM, n = 15, * - significantly different from the control, P < 0.01,

Table 16: THE EFFECT OF COBALT SUPPLEMENTATION ON PLASMA INSULIN LEVELS IN FISH FED LOW OR HIGH PROTEIN DIET

Dose of cobalt mg/kg feed	Plasma Insulin (ng/ml, mean ± SEM)	
	Low Protein Diet	High Protein Diet
0	9.6 ± 1.0 (15) ^a	11.6 ± 1.3 (12)
2	13.3 ± 1.5 (11)	11.0 ± 1.2 (13)
10	11.4 ± 1.7 (14)	12.6 ± 1.3 (14)
15	10.4 ± 1.9 (9)	10.3 ± 0.9 (13)

^aThe number in parentheses indicate no. of fish.

Table 17: THE EFFECT OF COBALT SUPPLEMENTATION TO TWO FISH DIETS ON THE INCORPORATION OF ^3H -LEUCINE (dpm/mg) INTO WHITE MUSCLE^a

Low Protein Diet				High Protein Diet		
Dose of cobalt mg/kg feed	No. of fish	TCA-insoluble fraction	TCA-soluble fraction	No. of fish	TCA-insoluble fraction	TCA-Soluble fraction
0	15	48.8 $\pm$ 2.4	6.3 $\pm$ 0.3	12	43.2 $\pm$ 2.7	7.4 $\pm$ 0.6
2	11	60.1 $\pm$ 3.6*	6.6 $\pm$ 0.4	13	42.7 $\pm$ 4.9	5.6 $\pm$ 0.3
10	14	49.4 $\pm$ 3.7	6.1 $\pm$ 0.3	14	43.9 $\pm$ 2.7	6.0 $\pm$ 0.3
15	9	49.8 $\pm$ 3.7	7.0 $\pm$ 0.4	13	47.6 $\pm$ 3	6.5 $\pm$ 0.4

^a All results given as mean $\pm$ SEM. Results marked * differ significantly (P<0.02) from the respective controls.

Table 18: THE EFFECT OF CHROMIUM ON POST-PRANDIAL GLUCOSE UTILIZATION OF GLUCOSE DURING GLUCOSE TOLERANCE TESTS AND ON BASAL GLUCOSE LEVELS*

Exp. no.	Chromium ug/kg bwt	No. of fish	Glucose injected (g/kg/bwt)	Glucose level after: (mg/100 ml)		
				0 h	3 h	6 h
1	None	7	2.0	123 $\pm$ 11	528 $\pm$ 11	500 $\pm$ 30
	2.5 ¹	9	2.0	100 $\pm$ 6 ^a	540 $\pm$ 45	328 $\pm$ 46 ^b
2	None	14	1.7	152 $\pm$ 8	440 $\pm$ 15	289 $\pm$ 33
	40 ²	11	1.7	131 $\pm$ 10	394 $\pm$ 40	145 $\pm$ 30 ^c
3	None	10	1.7	119 $\pm$ 5	437 $\pm$ 20	224 $\pm$ 21
	40 ²	10	1.7	107 $\pm$ 5	382 $\pm$ 24	144 $\pm$ 19 ^a

* All results given as mean $\pm$ SEM. Results marked with a letter differ significantly from the respective controls: ^a - P < 0.02, ^b - P < 0.01, ^c - P < 0.001

¹ Intraperitoneally

² Orally

Table 19. THE EFFECT OF CHROMIUM ON PLASMA GLUCOSE LEVELS AND GLUCOSE FORMATION AFTER INJECTION OF ^{14}C -ALANINE*

Exp. ¹ No.	Chromium added ug/kg.bwt	No. of fish	Plasma glucose (mg/100 ml)	% Incorporated into glucose	Specific Activity of glucose
1	None	3	130 $\pm$ 13	2.06 $\pm$ 0.46	0.22 $\pm$ 0.02
	2.5 ¹	3	92 $\pm$ 3 ^a	0.84 $\pm$ 0.19 ^a	0.13 $\pm$ 0.02 ^a
2	None	4	113 $\pm$ 3	1.45 $\pm$ 0.23	0.205 $\pm$ 0.03
	2.5 ¹	4	85 $\pm$ 4 ^c	0.74 $\pm$ 0.01 ^a	0.149 $\pm$ 0.005
3	None	12	101 $\pm$ 5	1.62 $\pm$ 0.15	0.147 $\pm$ 0.013
	40 ²	11	76 $\pm$ 2 ^c	0.86 $\pm$ 0.009 ^c	0.093 $\pm$ 0.009 ^b

* All results given as mean $\pm$ SEM. Results marked with a letter differ significantly from the respective controls: a - P < 0.05, b - P < 0.01, c - P < 0.001

¹ Intraperitoneally

² Orally

Table 20. THE EFFECT OF CHROMIUM ON INCORPORATION OF ^3H -LEUCINE INTO WHITE MUSCLE AND LIVER

Exp. no.	chromium added ug/kg/bwt	No. of fish	Incorporation of ^3H -Leu (dpm/day, mean $\pm$ SEM)			
			White muscle		Liver	
			TCA- insoluble fraction	TCA- soluble fraction	TCA- insoluble fraction	TCA- soluble fraction
1	None	7	23.7 $\pm$ 4.1	66.3 $\pm$ 9.3	215 $\pm$ 7.0	23.8 $\pm$ 1.8
	2.5 ¹	11	24.0 $\pm$ 2.6	15.2 $\pm$ 0.9	198 $\pm$ 15.0	99.1 $\pm$ 14.0
2	None	2	29.1 $\pm$ 2.1	16.2 $\pm$ 0.5	122 $\pm$ 9.0	24.0 $\pm$ 0.8
	40 ²	1	27.0 $\pm$ 1.7	17.0 $\pm$ 0.4	103 $\pm$ 6.0	24.0 $\pm$ 1.2

Table 21. THE EFFECT OF METFORMIN ON UTILIZATION OF GLUCOSE DURING GLUCOSE TOLERANCE TESTS AND ON BASAL PLASMA LEVELS.

Exp. no.	Metformin mg/kg bwt	No. of fish	Glucose injected (g/kg.bwt)	Glucose level after (mg/dl)		
				0 h	3 h	6 h
1	None	5	2.0	118 ± 6	454 ± 103	304 ± 43
	80 ¹	6	2.0	169 ± 15	630 ± 63	128 ± 9 ^b
2	None	6	2.0	122 ± 12	596 ± 47	349 ± 72
	1.6 ²	5	2.0	120 ± 8	496 ± 45	218 ± 40
3	None	7	1.0	102 ± 6	329 ± 49*	84 ± 2
	80 ¹	7	1.0	106 ± 11	292 ± 26	90 ± 6
4	None	10	2.0	148 ± 6	594 ± 7	539 ± 21
	80	12	2.0	138 ± 9	572 ± 10	448 ± 17 ^b
5	None	7	2.0	155 ± 9	533 ± 37	428 ± 49
	1.6 ²	9	2.0	144 ± 6	552 ± 46	384 ± 37
6	None	7	2.0	123 ± 13	578 ± 11	499 ± 30
	80 ¹	9	2.0	143 ± 13	543 ± 26	357 ± 39 ^a

* All results given as mean ± SEM. Results marked with a letter differ significantly from the respective controls: ^a - P < 0.02, ^b - P < 0.01,

¹ Intraperitoneally

² Orally

Table 22: THE EFFECT OF METFORMIN ON PLASMA GLUCOSE LEVELS AND GLUCOSE FORMATION AFTER INJECTION OF ¹⁴C-ALANINE*

Exp. no.	Metformin added mg/kg.bwt	No. of fish	Plasma glucose (mg/100 ml)	% Incorporated into glucose	Specific activity of glucose
1	None	3	130 ± 13	2.06 ± 0.46	0.22 ± 0.02
	80	3	149 ± 7	1.27 ± 0.12	0.128 ± 0.02 ^b
2	None	4	113 ± 3	1.45 ± 0.23	0.205 ± 0.03
	80	3	123 ± 2 ^a	1.34 ± 0.19	0.13 ± 0.018

* All results given as mean ± SEM. Results marked with a letter differ significantly from the respective controls: ^a - P < 0.05, ^b - P < 0.02.

Table 23: THE EFFECT OF METFORMIN ON PLASMA INSULIN AND CORTISOL LEVELS IN TREATED FISH*

Exp. No.	Dose of Metformin (mg/kg.bwt)	No. of fish	Plasma insulin (ng/ml)	Plasma cortisol (ng/ml)
1	None	5	10.0 $\pm$ 1.9	94 $\pm$ 36
	45	7	6.5 $\pm$ 1.1	522 $\pm$ 107 ^b
2	None	8	4.1 $\pm$ 0.4	176 $\pm$ 46
	75	9	6.1 $\pm$ 0.7	338 $\pm$ 54 ^a
3	None	9	8.5 $\pm$ 0.8	623 $\pm$ 61
	80	9	8.9 $\pm$ 1.2	1070 $\pm$ 92 ^b

* All results given as mean $\pm$ SEM. Results marked with a letter differ significantly from the respective controls: ^a - P < 0.05, ^b - P < 0.01.

Table 24. THE EFFECT OF METFORMIN ON INCORPORATION OF ³H-LEUCINE INTO TCA-FRACTIONS PF WHITE MUSCLE AND LIVER*

Exp. no.	Metformin added mg/kg bwt	No. of fish	Incorporation of ³ H-Leu (dpm/mg)			
			White muscle		Liver	
			TCA-insoluble fraction	TCA-soluble fraction	TCA-insoluble fraction	TCA-soluble fraction
1	None	5	21.0 $\pm$ 5.0	9.4 $\pm$ 0.4	131 $\pm$ 11	36 $\pm$ 7
	45	7	32.0 $\pm$ 5.0	14.6 $\pm$ 0.8 ^b	115 $\pm$ 26	39 $\pm$ 3
2	None	8	12.4 $\pm$ 2.2	17.7 $\pm$ 1.2	151 $\pm$ 13	30.5 $\pm$ 1.8
	75	9	20.6 $\pm$ 4.4	32.8 $\pm$ 1.9	126 $\pm$ 11	37.6 $\pm$ 2.8 ^a
3	None	7	33.2 $\pm$ 4.1	6.7 $\pm$ 0.5	130 $\pm$ 7	21 $\pm$ 2
	80	7	43.4 $\pm$ 7.1	11.8 $\pm$ 1.7 ^a	120 $\pm$ 17	22 $\pm$ 2
4	None	9	18.7 $\pm$ 2.3 ^a	9.2 $\pm$ 0.5	189 $\pm$ 15.7	ND
	80	9	25.7 $\pm$ 2.4	14.4 $\pm$ 0.9 ^c	235 $\pm$ 12.1	ND
5	None	11	21.7 $\pm$ 2.3	9.5 $\pm$ 0.7	ND	ND
	80	11	40.3 $\pm$ 3.1 ^c	8.3 $\pm$ 0.5	ND	ND
6	None	7	23.7 $\pm$ 4.1	66 $\pm$ 9	215 $\pm$ 7	23.8 $\pm$ 1.8
	80	11	29.3 $\pm$ 3.4	99 $\pm$ 10 ^a	251 $\pm$ 14 ^a	31.4 $\pm$ 1.6 ^b

* All results given as mean $\pm$ SEM. Results marked with a letter differ significantly from the respective controls: ^a - P < 0.05, ^b - P < 0.01, ^c - P < 0.001

ND - not determined.

Table 25. The effect of metformin, supplemented to the diet at increasing levels, on growth rate, feed conversion ratio (FCR), and protein efficiency ratio (PER) of common carp in tanks

TREATMENT	WEIGHT GAIN (g)	FCR	PER
HP0	152.14 _{abc}	1.84 _a	1.06 _a
HPM10	175.80 _{ab}	1.78 _{ab}	1.09 _a
HPM20	169.82 _{abc}	1.73 _{ab}	1.12 _a
HPM40	148.26 _{bc}	1.86 _a	1.05 _a
LPO	184.27 _a	1.62 _b	1.72 _b
LPM10	154.62 _{abc}	1.81 _{ab}	1.59 _c
LPM20	157.61 _{abc}	1.78 _{abc}	1.63 _{bc}
LPM40	135.60 _c	1.83 _a	1.57 _c

Table 26. THE EFFECT OF METFORMIN SUPPLEMENTATION OF PLASMA BASAL GLUCOSE IN FISH FED DIFFERENT LEVELS OF PROTEIN IN THEIR DIET

Dose of metformin (mg/kg feed)	Basal glucose blood (mg/100 ml)*	
	Low protein diet	High protein diet
0	130 ± 8 (10)	126 ± 6 (15)
10	120 ± 8 (14)	134 ± 6 (15)
20	103 ± 5 ^b (15)	110 ± 4 ^a (15)
40	117 ± 7 (15)	123 ± 6 (15)

* All results given as mean ± SEM, the number in parentheses indicate number of fish per treatment. Results marked with a letter differ significantly from the respective controls: a - P < 0.05, b - P < 0.01.

Table 27. THE EFFECT OF METFORMIN SUPPLEMENTATION ON THE INCORPORATION OF ³H-LEUCINE INTO WHITE MUSCLE FRACTIONS (dpm/mg) IN FISH FED LOW (27.5%) OR HIGH (40.7%) PROTEIN DIET FOR 60 DAYS*.

Exp. no.	Dose of metformin (mg/kg feed)	No. of fish	Low protein diet		High protein diet		
			TCA-insoluble fraction	TCA-soluble fraction	No. of fish	TCA-insoluble fraction	TCA-soluble fraction
1	0	9	57.9 ± 1.6	6.9 ± 0.4	11	44.7 ± 2	7.0 ± 0.3
	10	15	55.1 ± 2.7	7.5 ± 0.3	13	51.4 ± 3	6.3 ± 0.2
	20	14	52.2 ± 1.9	7.0 ± 0.4	14	50.4 ± 2.5	6.4 ± 0.4
	40	12	60.0 ± 2.5	6.0 ± 0.3	14	46.8 ± 2	6.7 ± 0.4
2	0	10	27.2 ± 1.3	6.9 ± 0.4	15	26.7 ± 1.2	7.7 ± 0.4
	10	14	28.8 ± 1.3	7.2 ± 0.5	15	23.1 ± 1.4	9.7 ± 1.0
	20	15	31.0 ± 1.6	6.5 ± 0.3	15	23.4 ± 1.4	7.4 ± 0.4
	40	14	31.8 ± 1.4 ^a	7.3 ± 0.4	15	28.6 ± 1.2 ^b	9.6 ± 0.8 ^a

* All results given as mean ± SEM. Results marked with a letter differ significantly, (within the same experiment) from the respective control without metformin: ^a - P < 0.05, ^b - P < 0.01,

Fig. 1. The radioactivity in glucose (—), organic acids (---), and alanine (-o-), after the injection of ^{14}C - alanine to starved carps.

Figure 2: Effect of ascitic fluid dilutions on ^{125}I -Bonito insulin binding in the presence of different amounts of unlabelled Bonito insulin. 1:3000 - (o); 1:6000 - (o); 1:12000 - (Δ); 1:18000 - (Δ).

Figure 3: Standard curves for Bonito (o), bovine (□) and porcine (□) insulins, using ^{125}I -Bonito insulin as the tracer.

Figure 4: Dose response relationship of six different plasma from common carp.

Figure 5: (a) Medium sized endocrine pancreas island from a carp Brockman body. Insulin cells react with the Bonito insulin antibody (reacting cells appear dark); x 210. (b) As in 5a at higher magnification: x 900. (c) Electromicrograph of an Epon embedded carp insulin cell (IN) without immuno treatment; ns - neurosecretory axon; arrows point to a synaptoid thickening between the axon and the insulin cell; nu - nucleus; x 16700. (d) Carp insulin granules (IN) after reaction with anti-bonito insulin diluted 1/100 and Protein-A gold. The size of the gold particles is 10 nm; x 25000. (e) Insulin granules treated with the antibody diluted 1/20 and goat anti-mouse IgG+IgM with 15 nm sized gold particles; ns - neurosecretory axon; NR - cell with granules not reacting to insulin antibodies; x 16000.

Figure 2: (a) Medium sized endocrine pancreas island from a carp Brookman body. Insulin cells react with the positive insulin antibody reacting cells appear dark; (b) A normal size islet of Langerhans magnification x 800. (c) Electronmicrograph of an Epon embedded carp insulin cell (IM) without immunoprecipitation; no - neurosecretory axons are pointing to a synaptic clefting between the axon and the insulin cell; no - antibodies; x 16700. (d) Carp insulin granules (IM) after reaction with anti-positive insulin diluted 1:100 and Protein-A gold. The size of the gold particles is nm; x 16000. (e) Insulin granules treated with the antibody, diluted 1:20 and post-embedding with IgG+Mg with 1% as fixed gold particles; no - neurosecretory axons; IR - cell with granules reacting to insulin antibodies; x 16000.

Figure 6. Glucose disposal rate during glucose tolerance test.

B. PROTEIN AND ENERGY REQUIREMENTS FOR MAXIMUM GROWTH

10. OBJECTIVES OF THE RESEARCH:

1. Determine dietary protein and energy requirements for maximum protein synthesis for channel catfish of various sizes. Determine availability of gross energy in dietary ingredients for channel catfish.
2. Determine protein-sparing effects of carbohydrate in channel catfish diets.
3. Determine metabolic fate of carbohydrate fed to channel catfish using carbon-14 labeled glucose.
4. Determine effects of dietary addition of anabolic hormones on protein gain in channel catfish.

BODY OF REPORT:

11. Objective 1:

Because of lack of information on energy requirements of channel catfish for maximum protein synthesis, the initial study involved determining available energy values of selected dietary ingredients for channel catfish; then, determining the optimum amount of available energy per gram of protein required in the diet for maximum rate of protein accretion in fish of various sizes fed to satiety. Effects of varying the dietary ratio of energy to protein on composition of fish gain were also investigated.

11.1 Methods

Digestibility of energy in cooked corn starch, gelatin, casein, fish flour, fish oil, and animal fat were determined by substitution of the test ingredient into a standard diet formula at the rate of 30% of the formula. The test diets were fed to fish in metabolism chambers. A chamber was designed and built to measure metabolizable energy (ME) in the experimental diets for channel catfish (Figure 1). The chamber functions satisfactorily in allowing separate and complete collection of fecal, gill and urinary excretions. However, ME values could not be determined because the fish regurgitated part of the diet. Digestible energy (DE) values were determined, however, which were consistent among replicate measurements. The procedures have been described in detail by Mangalik (1986). Resulting DE values for individual ingredients were used to formulate the eight diets shown in Table 1 and these diets were subsequently evaluated for DE in the metabolism chambers.

Eight semipurified diets containing three levels of protein at three energy planes (Table 1) were fed to triplicate aquariums of channel catfish of initial weight of 1 gram for 10 weeks. Subsequently, similar diets were fed to the same number of channel catfish of initial size of 10 grams for 10 weeks. A third feeding

trial was conducted with 100-gram channel catfish. The fish were fed to satiety twice each day. Weekly weight gain and food consumption were measured. Fish samples were analyzed to measure composition of gain (protein, fat, gross energy). The data were used to determine DE and protein requirements for channel catfish of various sizes for maximum protein synthesis.

11.2 Results

The digestibility trials revealed DE values (kcal/g) for the diet ingredients as follows:

cellulose	0
gelatin	4.7
casein	4.4
fish flour	3.9
fish oil	8.5
lard	8.6
cooked starch:	
20% of diet	3.4
30% of diet	3.0
40% of diet	2.8

Digestibility of gross energy was determined in each of the experimental diets, using the same procedure. Digestibility coefficients increased in diets 1-4 and in 5-8 as starch and lard replaced cellulose (Table 2). The estimated DE values for each diet agreed very closely with the determined DE values, indicating that digestibility coefficients for the individual ingredients presented above are reliable when the ingredients are used in combinations. It is of interest that starch (cooked), at 20% of the diet, has a relatively high DE value, 3.4 kcal/g.

Results of the feeding experiments are summarized in Figures 2-7. For the small fish (1-10 g) food and protein intake decreased as digestible energy in the diet increased. The higher protein diet depressed food intake more than the lower protein diet. Weight and protein gains decreased as energy content increased in the low protein (27%) diet but not in the higher protein (39%) diet. Weight and protein gain were as good on the low protein diet as on the higher protein diet when the energy level was low and allowed a high rate of food (protein) consumption.

In the fish grown from 20-50 grams, both energy and protein in the diet depressed food consumption. At the higher protein level, protein intake decreased with a rise in dietary energy, but protein gain increased with increasing dietary energy. This indicates a protein sparing effect of nonprotein energy. There was less sparing in the low protein diet. The responses of the largest fish (100-250 grams) were similar to those of the medium-size fish except that protein sparing in the high protein diets was not as remarkable.

Results of the three feeding experiments show that the rates of food consumption and growth decrease markedly as fish size increases. Because of this, protein and energy requirements change as size increases. Data from the three experiments were used to predict daily protein and energy requirements for maximum growth rate, measured as protein gain, for channel catfish up to 266 g in size (Table 4). These requirements were based on the minimum daily protein intake that produced maximum growth of fish at various sizes. Small channel catfish, grown from 1 to 3 g, required a minimum of 1.64 g of protein and approximately 16.8 kcal of DE per 100 g BW/day for maximum growth while fish grown from 100 to 266 g required a minimum of 0.43 g protein and approximately 5.0 kcal of DE/100 g BW/day.

Decreasing protein intake below the amounts presented in Table 4 will decrease growth rate; increasing protein intake beyond these amounts without increasing DE will not increase growth rate. Decreasing or increasing DE from these quantities, without changing protein percentage will reduce the rate of protein gain; however, increasing DE may not cause a reduction in the rate of weight gain because of increased fat synthesis.

The energy required per gram of protein in the diet, or the energy-protein ratio, for maximum growth did not change with fish size as much as absolute requirements for protein and energy. The optimum ratio of DE to protein for channel catfish grown from 1 to 3 g in size is 10 kcal/g protein, and for channel catfish grown from 24 to 266 g, the ratio is 11 kcal/g protein.

Small channel catfish (1 to 24 g) are able to obtain their daily protein and energy requirements for maximum growth from lower protein diets than larger channel catfish (24 to 260 g), provided the fish are allowed to feed to satiation and the diet is relatively low in DE. This is partially because of the lower DE requirement per g of protein for small channel catfish but primarily because they consume more food in proportion to body weight. Larger channel catfish (100 to 266 g) can meet their protein requirement from low protein, low DE diets but they cannot obtain their energy requirement.

The ratio of DE to protein in the diet is important in channel catfish diets when the fish are fed to satiation. As DE concentration in the diet increases, the amount of diet and, thus, protein consumed decreases, which can suppress growth rate. Also, as DE concentration in the diet increases, fat synthesis increases.

Weight gain is a deceptive evaluation of growth in channel catfish, even in very small channel catfish. Data from this study indicated that among all sizes of fish, weight gains could be equal among diets while protein gains were different because the higher energy diets produced more fat.

Efficiency of conversion of diet protein to fish gain decreases with increases in protein in the diet and with protein consumption,

but not with the fish size. Protein efficiency (g weight gain/g protein consumed) at maximum growth rate was 3.30:1 for fish grown from 1 to 24 g, and 3.03:1 for fish grown from 100 to 266 g. Protein efficiency improves as dietary DE increases. Protein efficiency was markedly lower for fish consuming protein for maximum rate of protein gain than for fish consuming lower concentrations of protein.

Feeding channel catfish at restricted rates has practical advantages in that uneaten feed is minimized and efficiency of utilization of feed, protein and energy are increased. However, for all sizes of channel catfish in this study, growth rate in restricted fed fish was less and generally proportional to the percentage of satiation that they were fed.

This study has provided minimum protein and optimum DE quantities for daily consumption by channel catfish, up to 266 g, for maximum growth. Because channel catfish are not fed ad libitum, as are livestock or poultry, to achieve maximum growth, it is difficult to recommend specific dietary percentages or concentrations of protein and DE because daily food intake will vary with management and environment. Thus, the practical nutritionist, in designing production diets for maximum growth for channel catfish, must have an estimate of food intake for the fish to be fed, then design the diets to provide the required quantities of protein and energy.

12. Objective 2:

Protein-sparing effect of carbohydrates was compared with fat on a DE basis for growth by channel catfish.

12.1 Methods

Fat and carbohydrates were each added at various DE levels to a protein-based diet that was otherwise nutritionally complete. Starch (cooked) and glucose each replaced fat (lard) on an equal DE basis, based on previously determined digestion coefficients. Experimental diets are shown in Table 5. Triplicate aquaria of 10-g channel catfish were fed each diet to satiation for 10 weeks. Weight, protein, energy and fat gains were determined. Protein-sparing effect was measured as additional weight on protein gain caused by a unit increase in nonprotein energy in the diet.

12.2 Results

Table 6 shows the protein and fat gains of channel catfish fed increasing increments of digestible energy, as either carbohydrates (cooked starch or glucose) or fat, added to a low energy, protein-based diet. For the two carbohydrates, increasing DE actually reduced protein and fat gain. This was probably because increasing levels of dietary DE reduced food and protein intake. Increasing energy level as fat, however, did not reduce calorie intake. Starch seemed to suppress energy intake more than glucose.

At the lowest added energy level, starch and fat produced about the same amount of additional growth (above the basal). This indicates equal protein sparing effect of starch and fat when digestible calorie intake by the fish is equal. Glucose appears to have less protein sparing action than starch. At each energy level, more protein was consumed by the fish fed glucose but less protein was gained, compared to the starch fed fish.

13. Objective 3:

The previous two experiments showed that carbohydrate does have protein sparing effect. A budget for absorbed glucose will be made by injecting carbon-14 labeled glucose into channel catfish and measuring labeled carbon in CO₂, urine, fat and nonfat tissue component. This study did not begin until May 1987 because of remodeling of the radioisotope laboratory at Auburn University which delayed this project about 18 months. It will be completed by August 31, 1987.

14. Objective 4:

Synthetic and natural compounds with hormonal activity have been fed to livestock and fish to increase rate of protein accretion. Several steroids and recombinant DNA-produced growth hormone have increased efficiency of protein synthesis in salmonid fishes. Favorable and adverse effects of diet administered hormones on channel catfish were studied.

14.1 Methods

Channel catfish fingerlings were fed diets containing the following compounds in aquariums for 10 weeks: three levels (2, 8 and 32 mg/kg) of each of 17-methyltestosterone (MT) and 11-ketotestosterone (KT), one level of keto-isopropionic acid (KIC), two levels (5 and 50 mg/kg) each of ethylestrenol (EE) and of 3,5,3-triiodothyroxine (T₃). Channel catfish were fed production diets in ponds for 6 months which contained 2.5 and 10 mg/kg of 17-methyltestosterone. Each diet was fed to four groups of fish in the aquarium and pond experiments. Weight gain, protein gain, feed efficiency, protein efficiency, histopathology of various tissues, and bone quality were measured in the experimental fish.

Analytical procedures included determination of Kjeldahl nitrogen for protein analysis; sectioning and staining tissues for histopathology; measuring breaking strength of bones with a penetrometer; and determining calcium, phosphorus and magnesium in bones.

14.2 Results

Weight gain data are presented in Table 7. The lowest level of KT (2 mg/kg) improved growth rate over that of the control fish ($P <$

0.05). The two highest levels of MT reduced growth rate ($P < 0.05$). The lower level of T_3 improved growth rate slightly ($P < 0.10$) and the higher levels of ethylestrenol and T_3 reduced growth rate ($P < 0.05$). All other treatments had no significant effect on growth rate. MT fed in ponds at both levels (2.5 and 10 mg/kg) suppressed rate of gain.

All levels of MT caused a reduction in bone mineralization in the fed fish. The spines on the catfish, normally very sharp, were soft and covered with skin. While skinning and beheading the fish, the backbones broke easily. Laboratory assay for bone quality has not been completed, but results thus far indicate clearly that this androgen reduces bone mineralization. This might indicate an interference by MT of the calcium mobilizing hormone 1,25-dihydroxycholecalciferol. MT and KT both caused severe hypertrophy of male gonadal tissue but not female.

Most of the hormones evaluated have increased growth or protein accretion rate in salmonids. KIC has increased protein synthesis in livestock. Possibly the levels of the compounds fed in this study were high. The fact that the lowest levels of MT and KT gave the best results suggests this.

Table 1. Percentage composition of diets for digestibility studies

Diet	Glucose level	Uncooked starch	Cooked starch	Standard Reference ¹	Supplements ²
1	12.5	-	-	77.0	10.5
2	25.0	-	-	64.5	10.5
3	50.0	-	-	39.5	10.5
4	-	12.5	-	77.0	10.5
5	-	25.0	-	64.5	10.5
6	-	50.0	-	39.5	10.5
7	-	-	12.5	77.0	10.5
8	-	-	25.0	64.5	10.5
9	-	-	50.0	39.5	10.5
10	-	-	-	89.5	10.5

¹Standard Reference diet contained fishmeal and soybean meal in the ratio of 1:3.

²Supplements: complete vitamin premix, 1.5%; binder (CMC), 4.0%; mineral premix, 4.0%; chromic oxide, 1.0%.

Table 2. Ingredient composition and estimated crude protein (CP) and digestible energy (DE) contents of eight experimental diets fed to channel catfish in experiment 1

Item	Diets							
	1	2	3	4	5	6	7	8
Ingredient Composition:	%	%	%	%	%	%	%	%
Casein (vitamin-free)	18.6	18.6	18.6	18.6	27.2	27.2	27.2	27.2
Gelatin	4.6	4.6	4.6	4.6	6.8	6.8	6.8	6.8
Fish flour	9.3	9.3	9.3	9.3	13.6	13.6	13.6	13.6
Cooked starch	4.0	23.5	35.2	44.8	2.0	13.4	23.0	34.2
Fish oil	2.4	2.4	2.4	2.4	2.0	2.0	2.0	2.0
Lard	1.3	4.6	8.6	12.6	0.0	1.5	4.7	8.7
Hydrolyzed celufil	42.1	29.3	13.6	0.0	49.9	28.0	15.2	0.0
CMC	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Vitamin mix ¹	1.4	1.4	1.4	1.4	1.4	1.4	1.4	1.4
Vitamin C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral mix ²	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Nutrient Content:								
Estimated CP (%)	26.0	26.0	26.0	26.0	38.0	38.0	38.0	38.0
Estimated DE (kcal/g)	2.2	2.8	3.4	4.0	2.2	2.8	3.4	4.0
DE/CP ratio (kcal/g)	8.4	10.7	13.0	15.3	5.7	7.3	8.9	10.5

¹Vitamin mix (amount of active vitamin/50 kg diet): vitamin A, 200,000 IU; vitamin D-3, 100,000 ICU; vitamin E, 2,500 IU; menadione-Na-bisulfite, 500 mg; thiamin, 1,000 mg; riboflavin, 1,000 mg; niacin, 7,500 mg; pantothenic acid, 10,000 mg; folic acid, 250 mg; pyridoxine, 1,000 mg; choline-chloride, 100,000 mg; vitamin B-12, 1 mg; biotin, 100 mg; inositol, 20,000 mg; ethoxyquin, 10,000 mg; cellulose, 500 g.

²Mineral mix: Williams and Briggs salts, 5 lb mix with $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2232 g, $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$, 0.2958 g; Na_2SeO_3 , 0.0112 g.

Table 3. Estimated versus determined values for crude protein (CP) and digestible energy (DE) for experimental diets fed to channel catfish in experiment 1

Diet No.	Estimated ¹		Determined ²	
	CP (%)	DE (kcal)	CP (%)	DE ³ (kcal/g)
1	26	2.2	27.1	2.21
2	26	2.8	27.0	2.77
3	26	3.4	27.3	3.48
4	26	4.0	27.3	4.01
5	38	2.2	39.7	2.31
6	38	2.8	39.9	2.64
7	38	3.4	39.3	3.27
8	38	4.0	40.1	3.88

¹Based on computation from digestibility coefficients of ingredients.

²Means based on duplicate analyses.

³Means based on computation from determined digestibility coefficients.

Table 4. Protein and digestible energy (DE) requirements by various sizes of channel catfish for maximum protein synthesis

Fish size (g)	Protein (g/100 g fish/day)	DE (kcal/100 g fish/day)	DE/protein ratio (kcal/g)
3	1.64	16.8	10.2
10	1.11	11.4	10.3
56	0.79	9.0	11.4
198	0.52	6.1	11.7
266	0.43	5.0	11.6

Table 5. Percentage composition of diets

Ingredients	Control	Cooked starch			Dextrose			Lipid		
		1	2	3	4	5	6	7	8	9
Fish flour	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Casein	20.96	20.96	20.96	20.96	20.96	20.96	20.96	20.96	20.96	20.96
Gelatin	5.24	5.24	5.24	5.24	5.24	5.24	5.24	5.24	5.24	5.24
Cooked starch	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Lipid	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Added cooked starch	0.00	12.00	24.00	36.00	0.00	0.00	0.00	0.00	0.00	0.00
Dextrose	0.00	0.00	0.00	0.00	11.79	22.02	30.84	0.00	0.00	0.00
Added lipid	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.10	9.53	13.35
Vitamin premix	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40
Vitamin C	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Mineral	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Binder (CMC)	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Cellulose	53.80	41.80	29.80	17.80	42.01	31.78	22.93	48.70	44.27	40.45
Estimated crude protein	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00
Estimated DE (kcal/gm)	1.62	2.03	2.39	2.69	2.03	2.39	2.69	2.03	2.39	2.69
DE/CP	6.49	8.13	9.55	10.77	8.13	9.55	10.77	8.13	9.55	10.77

Assume: DE of animal fat = 8.6 kcal/gm
 DE of dextrose = $3.67 \times 92\% = 3.46$ kcal/gm
 DE of cooked starch: = 3.4 kcal/gm (12%)
 = 3.2 kcal/gm (24%)
 = 3.0 kcal/gm (36%)

Table 6. Additional protein and energy gained from addition of starch, glucose or fat to protein basal diet¹

Diet	Added energy (kcal/g)	Consumed per fish		Additional gain above basal	
		Energy (kcal DE)	Protein (g)	Protein (g/fish)	Fat (g/fish)
Basal	0.00	24.0	3.7	-	-
Starch	0.41	31.4	4.6	3.5 a	2.0 a
	0.77	28.1	3.5	0.3 c	1.8 ab
	1.07	27.5	3.0	0.0 d	1.5 ab
Glucose	0.41	37.1	5.6	1.7 b	1.2 bc
	0.77	33.4	4.3	0.5 c	0.7 bcd
	1.07	30.9	3.6	-0.6 d	0.4 d
Fat	0.41	34.8	4.9	2.1 b	2.3 a
	0.77	34.1	4.2	1.0 bc	2.3 a
	1.07	53.6	5.9	3.5 a	4.2 e

¹Means in column with same letter are not different at $P < 0.05$.

Table 7. Average weight gain by channel catfish fed diets containing various levels of 17-methyltestosterone (MT) or 11-ketotestosterone (KT) in aquariums or ponds¹

Aquariums			Ponds		
Hormone	Dose (mg/kg)	Weight gain per fish (g)	Hormone	Dose (mg/kg)	Weight gain per fish (g)
MT	2	91.3 b	MT	2.5	352 a
MT	8	73.5 c	MT	10	263 c
MT	32	60.6 c			
			0 (control)		485 b
KT	2	109 a			
KT	8	102 ab			
KT	32	87 b			
0 (control)		97 b			

¹Means in columns with same letter are not different at $P < 0.05$.

Table 8. Average weight gains by channel catfish fed diets containing various levels of ethylestrenol (EE) and 3,5,3-triiodothyroxine (T₃), and one level of keto-isopropionic acid (KIC) in aquariums¹

Compound	Dose (mg/kg)	Weight gain per fish (g)
EE	5	19.9 a
EE	50	18.0 a
T ₃	5	22.7 a
T ₃	50	17.6 a
KIC	2.5	19.0 a
Control		20.1 a

¹Means in column with same letter are not different at $P < 0.05$.

Scale : 0.28 cm = 1 cm.

Figure 1. Longitudinal Section of Metabolism Chamber.

Figure 2. Regression of Daily Consumption of Food (A), Crude Protein (B), and Digestible Energy (C) on Dietary Concentrations of Digestible Energy (DE) at Two Concentrations of Crude Protein (CP), by Channel Catfish Fed at Satiation (S) Feeding Rate in Experiment I.

Figure 3. Regression of Gains in Weight (A), Protein (B), Fat (C), and Energy (D) on Dietary Concentrations of Digestible Energy (DE) at Two Concentrations of Crude Protein (CP), by Channel Catfish Fed at Satiation (S) and Restricted (R) Feeding Rates in Experiment I.

Figure 4. Regression of Daily Consumption of Food (A), Crude Protein (B), and Digestible Energy (C) on Dietary Concentrations of Digestible Energy (DE) at Two Concentrations of Crude Protein (CP), by Channel Catfish Fed at Satiation (S) Feeding Rate in Experiment II.

Figure 5. Regression of Gains in Weight (A), Protein (B), Fat (C), and Energy (D) on Dietary Concentrations of Digestible Energy (DE) at Two Concentrations of Crude Protein (CP), by Channel Catfish Fed at Satiation (S) and Restricted (R) Feeding Rates in Experiment II.

Figure 6. Regression of Daily Consumption of Food (A), Crude Protein (B), and Digestible Energy (C) on Dietary Concentrations of Digestible Energy (DE) at Two Concentrations of Crude Protein (CP), by Channel Catfish in Experiment III.

Figure 7. Regression of Gains in Weight (A), Protein (B), Fat (C), and Energy (D) on Dietary Concentrations of Digestible Energy (DE) at Two Concentrations of Crude Protein (CP), by Channel Catfish in Experiment III.

DESCRIPTION OF COOPERATION

The two research teams, the Israeli and American, tried to tackle the main objective of the project - a more efficient utilization of carbohydrate by fish - from two different angles. The Israeli approach was the less conventional, trying to modulate the hormonal regulation of carbohydrate metabolism. The American team's approach was more conventional, trying to find the best conditions and food ingredients for maximum carbohydrate utilization. The two approaches complement each other. It was ment that any food additive which can affect carbohydrate utilization found by the israeli team will be applied to channel catfish at the conditions found best by the American team. Prof. A. Gertler visited prof. R.T. Lovell at Auburn University to discuss the two approaches and their final conversion.

While much has been learned by the Israeli team on the hormonal control and the effect of food additive on it, this has not culminated by finding an applicable feed additive that could be used on channel catfish in the USA. It is clear that more work must be carried out to achieve this goal, as indeed it is planned to do (outside the framework of BARD). The israeli team plans to continue testing new additives (including organic complexes that will be absorbed better by the fish). Cooperation between the teams also did not end with termination of the present project since Dr. Hepher plans to spend most of his Sabbatical leave with prof. Lovell at Auburn University trying the more promising food additives, including those which will be developed in the meantime at the Hebrew University) on young channel catfish. Thus, cooperation will continue beyond the scope of the present project.

EVALUATION OF RESEARCH ACHIEVEMENTS

The research project contributed to the knowledge of the normal metabolic hormonal activity in common carp and on the effect of certain compounds on this activity. New methods of investigation in this field have been developed such as RIA for carp insulin and methods to determine gluconeogenesis and protein synthesis in fish. In this aspects the research

project fulfilled all its stated objectives. As for the use of food additives to modulate the hormonal activity. this proved to be a more difficult task than anticipated, but much has been learned on this aspect which will, no doubt, enhance further work on these lines.

The main contribution of the work in the United States is the development of digestible energy-protein ratios of growth by channel catfish. This information has not been available in technical literature and practicing nutritionists have no basis for setting energy allowances in commercial fish feeds. The data should be generally applicable to other warmwater species. Also, determination that carbohydrate (starch) has significant protein-sparing energy value for channel catfish is important. Carbohydrate has probably been underestimated as an energy source in fishes previously.

LIST OF PUBLICATIONS

Hertz, T., N. Epstein, M. Abraham, Z. Madar, B. Hopher and A. Gertler.
(sent for publication) Radioimmunoassay for carp (Cyprinus carpio)
insulin with antibodies raised against bonito insulin.

Mangalik, A. 1986. Determination of protein and energy requirements of
channel catfish. Ph.D. Dissertation, Auburn Univ., Alabama, USA.