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United States - Israel Binational Agricultural Research and Development Fund

FINAL REPORT

PROJECT No. I-141-79

Study of Systemic Granuloma, a Diet Related Disease of the Cultured Marine Fish *Sparus Aurata*

I. Paperna, L. Reshef, M. Landolt

1984

BARD
P.O.B. 6
Bet Dagan, 50250 ISRAEL

BARD Research Proposal No. 1-141-79

Title of Proposed Research

Study of systemic granuloma, a diet related disease of the
cultured marine fish Sparus aurata.

Name and Address of Investigators

Principal Investigator: Dr. I. Paperna, Dr. L. Reshef

Cooperating Investigator(s) Dr. M. Landolt

Name and Address of Affiliated Institutions

Principal Institution: Hebrew University of Jerusalem

Cooperating Institutions(s): University of Washington, College
of Fisheries

Project's Commencement date 1980

Type of Report: Final

Signature

Principal Investigator

Institution's Authorized Official Dr. I. Paperna, Dr. L. Reshef
(signature and official stamp)

M. Landolt
Cooperating Investigator

Dr. M. Landolt

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1. ABSTRACT

The objectives of the study of systemic granuloma in cultured Sparus aurata were aimed at investigating the dietary factors involved in the disease aethiology, understanding the biochemical-metabolic background for this disease and to describe the pathological process of the disease. Results obtained by the completion of this three year study can be outlined as follows:

Adequate biochemical micromethods were developed for determination of tyrosine and assay of enzymes. At all conditions Sparus aurata kidney was found to concentrate tyrosine at 13 fold the blood level. Thus the tyrosinaemia which has been found to develop in all fish with acute SG was a sufficient cause for the deposition of crystalline tyrosine and urolites in the kidney with the subsequent nephropathic effects. The other sparid susceptible to SG is Acanthopagrus bifasciatus which, like S. aurata, concentrates tyrosine in its kidney, while Diplodus noct which concentrates less tyrosine in the kidney is not susceptible to SG. Tyrosinaemia subsides and crystalline tyrosine deposits in the kidney disappear when the disease progresses to its subsequent chronic granulomatous phase. A terminal condition was identified in 2-3 year old fish with characteristic emaciation and severe degeneration of the hepato pancreatic system. Dietary experiments demonstrated a positive correlation between food intake and SG incidence, but failed to yield an answer as to the aethiological origin of the disease. Study of the enzymatic catabolic system of tyrosine - tyrosine amino transferase and p-Hydroxyphenypruvate oxidase did not evidence any discrepancies in the activity level between healthy and sick fish. With these conclusions dietary overload was tested as a possible origin of tyrosinaemia and to consequent deposition of crystalline tyrosine. Out of 6 fish fed on trout pellets with 10% added tyrosine, 5 developed characteristic signs of acute systemic granuloma.

2. FINAL SCIENTIFIC REPORT - SUMMARY OF THE RESEARCH RESULTS

In the final report is summarized the most important data resulting from the study. A detailed description of the methodology used in this research and of the biochemical and histopathological findings were presented in the comprehensive report submitted at the end of the second year of the project (1982).

The objectives of this study were aimed at (1) Describing the pathology of systemic granuloma (SG); (2) Optimizing dietary conditions to examine these associations with the disease, and (3) Studies to understand the biochemical basis of the disease.

2.1 Dietary studies

Feeding trials in the years preceeding this project (1976-1978) provided evidence of the dietary origin of systemic granuloma in S. aurata. At the same time it also became evident that due to the apparent inconsistencies in the SG inducing potency of the tested diets and the highly individual variability in the susceptibility to SG among fishes (Paperna, 1979, Proc. World Symp. Finfish Nutr. Fishfeed Tech. 2, 369-376), the aethiological origin of SG cannot be identified through empirical dietary experiments.

Consequently our research efforts were shifted to biochemical studies aimed at identifying the background

and causes for the aberrations in the tyrosine metabolism characteristic to the SG condition in the fish. Nevertheless, in the absence of a standardized SG inducing diet, the need for SG affected fish required repeated empirical tests for high SG inducing feeds, as well as optimization of feeding conditions and fish maintenance in order to increase incidence of SG in the experimental fish.

Data obtained via these empirical feed tests confirmed earlier data on the gradual loss of SG inducing potency in stored feeds, even if deep frozen (at -18°C) (Table 1) (first year report, 1981). The selected working diet with high SG inducing potency was a commercial grade II shrimp feed (JSF), Japanese product containing 60% protein, in 1980/81 trials yielding 50-70% incidence of SG (with one instance of 100% in test fishes (first year report, 1981). This diet lost its potency towards 1982, while another available commercial shrimp feed (grade III) was of low SG inducing potency.

Results of the dietary trials aimed to the optimization of SG inducing capacity were presented in the first year report, and can be summarized as follows: Incidence in test groups increases with the increase in the efficiency of feeding, e.g., by increase in the overall level of daily food intake by the fish (approximately 6-8% per body weight, per day) and reducing individual variability in

food intake between the fishes within tested groups by eliminating competition for food. This was achieved by excessive feeding two and three times a day. No cases of SG occurred in groups of fish fed at a rate of 2% of body weight per day. This confirmed earlier observations on fish held in sea cages (Paperna, 1979, cited above).

The number of fish per tank was of no importance (up to 1.7 g biomass/lit), so far as high food intake was achieved. No significant correlation could be demonstrated between incidence of SG in the tested groups and initial or final stocking biomass, growth rate or condition factor, of individual fish in each test-group or means for groups exposed to the different test diets.

Additional dietary tests were done with SG inducing diets treated by autoclaving and ethanol extraction. Neither of these treatments reduced the diets' SG potency. Tyrosine loading:

In consequence to the results obtained showing no decline in the activity of the tyrosine catabolic enzymes in the affected fish it became relevant to test the impact of dietary overload by tyrosine on the blood tyrosine levels and whether such elevation will result in deposition of tyrosine in the kidney and the consequent pathological changes of SG. This experiment was performed in the final year of the project when only a few small fish were available and even these were already over one year old (stunted fish)

thus obtained results are only indicative and need confirmation by repeated experiments with optimal number of fish and adequate controls.

The test diet prepared by re-pelleting crushed trout pellets with added 10% crystalline L- tyrosine:

Crushed trout pellets:	-87.2%
Attractant (Mytilus extract containing 0.43 mg/g tyrosine (Mackie, A.M. et al., 1980, J. Fish Biol. 16, 701-708)	- 1.8%
Fish oil	- 1%
Tyrosine	-10%

The pellets were commercial product of "Milobar" containing 45% crude protein.

The addition of tyrosine to feeds appears to reduce their palatability to the fish. This was now further confirmed with currently tested fish meal formulations (in experiments still not completed by the time of writing the report), nevertheless in spite of the lower than expected food intake, even on twice to three times daily feeding of the 5 tested fish, 3 died with positive findings of heavy deposits of tyrosine; 3 were autopsied after 13 and 45 days of feeding respectively. In 2 heavy deposits of tyrosine were located in the kidney and histopathology demonstrated typical condition of systemic granuloma. In the third fish only residual deposits of tyrosine were detected and mild histopathological changes in the 2d proximal tubules. (The remaining 2 fish died and were

eaten up by the other tested fish.) For control we used other fish of the same age and size fed on trout pellets (2 fish), "Oregon" fish meal supplemented with 1% each of the following: Fish oil, vitamin mix ("Milobar commercial product") and Mytilus attractant (2 fish) and another 5 fish in parallel experiments fed on casein test diet.

2.2 Clinical and Pathological Processes in Systemic Granuloma

2.2.1 The First Phase-- The Acute Condition

Gross pathology confirmed by histopathology clearly evidenced that the pathological process initially develops in the trunk kidney. Heavy crystalline tyrosine overloads the tissue and large tyrosine urolites accumulate in the uriniferous ducts and the urinary bladder. Histological study evidences progressing degeneration and necrosis of the epithelial cells lining the second proximal tubule leading to eventual collapse of the affected sector of the tubule and obliteration of the uriniferous lumen (for detailed histopathological description see comprehensive report, 1982). The distal segments of the renal tubules become gradually obstructed by necrotic substance and cellular debris, however the epithelial lining remains intact. Severe damage in these tubules becomes evident only when they become obstructed with tyrosine urolites. Glomeruli at the initial phase of the process remain intact, however, with the advent of the pathological process, thickening of the basal membranes of the capsule (hyalinisation) occurs followed by degeneration of the cells (epithelial as well as endothelial) of the capsule.

Granulomatous process, e.g., macrophage proliferation, initiates around the degenerating tubules, the site of the degenerating tubule is gradually infiltrated by monocytic cells and a nodule of proliferating macrophages

are eventually formed in the site of the collapsed segment of the second proximal tubule. Proliferative changes also develop around the distal tubules obstructed by tyrosine urolites. The crystalline tyrosine seen in fresh tissue impression was dissolved in the process of histological preparation, thus we were unable to locate the exact location of the crystals in the tissue nor to evaluate the tissue response to these crystals. Examination of impressions of fresh tissue did suggest a formation of granuloma foci around aggregates of crystals.

The degenerative changes in the renal tubules with the subsequent formation of granulomata progress gradually and assume multifocal pattern.

The course and severity of the clinical-pathological process was primarily related to the SG inducing potency of the tested diets. With highly potent diets (Japanese shrimp feed - JSF) severe pathology was already evident after 5-10 days of feeding schedule, resulting in morbidity and mortality. External symptoms were evident only in the terminal state - hemorrhages around the urinary pore and frequently also dropsy; autopsy demonstrated severe edema (hemorrhagic or non hemorrhagic) of the viscera and severe hemorrhagic inflammation of the distal part of the kidney and the urinary bladder as a direct effect of

the presence of urolites in the uriniferous ducts. Diets with lower potency (Ewos, South African and Norwegian fish meals) induced more gradual clinical and pathological changes. Evident gross and micropathological signs of SG were detectable often only following 15-30 days of feedings. morbidity was low and most affected fish survived phase and developed chronic granulomatous conditions.

2.2.2 The second phase - proliferative-chronic granuloma

In fishes surviving the first acute phase, the burden of the pathological process is shifted from the renal tissue to the hematopoietic tissue. The transition from the acute to the chronic phase may occur within 10 to 30 days of feeding on potent diets and may last for several months to over a year. The timing of transition depends on the potency of the diet but is also subject to wide individual variability among the exposed fishes. This phase is the most obvious condition in SG affected fish, as being the most prolonged and most evident by gross pathology and thus was already investigated in details before the onset of the reported project (Paperna et al., 1980, J. Fish Disease, 3, 213-221).

The histopathological process at this phase is characterized by extensive formation of granulomata while the degenerative process in the renal tubules slows down and intact segments appear to resume normal excretory function. This coincides with the gradual clearance of the tyrosine crystalline deposits and urolites from the kidney (and biochemical

regression of the tyrosinaemic condition-see vide). Proliferating macrophages initially accumulating around necrotic residues of the renal system spread and replace the hemato-poietic system. Secondary proliferation foci are formed in the sites of the macrophage centers in the kidney, the head kidney and subsequently in the spleen allipsoids. The core of the granulomatous tissue usually degenerates and becomes gradually necrotic. The proliferative process consequents major increase in tissue volume resulting in evident gross hypertrophy of the kidney, head kidney and spleen. The necrotic cores provide this hypertrophic tissue with a typical white nodular texture.

Another characteristic change occurs gradually in the eyes, necrosis of the iris results in displacement of the pupil with subsequent exophthalmia and gradual degeneration of the entire eye. The process occurs unilaterally and serves as a reliable external gross pathological diagnostic feature.

In extreme conditions, granulomata spread to other visceral organs - liver, mesenteries and interstine wall and the skin dermal tissue, but maintain a focal rather than diffuse pattern.

Morbidity and mortality at this stage of the disease may be either sparse or intense, but nevertheless incidence among the fish spreads over a prolonged period and therefore often remains unnoticed until the greater part of the stock perishes.

2.2.3 The third phase - Latent or regressing granuloma

This condition was seen in fish one to three years old. The proliferative process gradually regresses and the kidney and spleen resume their normal or slightly enlarged size. The extent of regression is variable ranging from complete regeneration of the renal and hematopoietic tissue leaving only a few fibroblasts encapsulated necrotic foci, to numerous scattered necrotic (soft coagulative or hardened hyalinized) foci. In the regression process macrophages were at times replaced by fibroblasts and consequently deposition of collagen which caused severe fibrosis of the kidney. Regression was more evident in the kidney and less evident in the spleen. In some apparently recovered fish, spleens remained grossly enlarged, completely necrotic or heavily loaded by hyalinized necrotic foci. Also irreversible were the changes in the eyes, leaving fish either blind, due to total regression of the eye, or exophthalmic due to extreme hypertrophy of the sclera.

2.2.4 The fourth phase - terminal granuloma

This condition occurred in 2-3 year old fish, utilized as spawners. Fish gradually lost weight, became lethargic, heavily pigmented, skin became abraded and eventually died. Many of these fish were unilaterally or bilaterally blind with degenerated eyes. All dying fish had large encapsulated necrotic nodules in the kidney, spleen and occasionally also in the liver. Kidney was atrophic, often dark due to a heavy load of melanin (seen also in healthy fish), and

loaded with hyalinised nodules. Spleens were either normal or grossly enlarged and necrotic. In the kidney, and to a lesser extent, in the spleen there was evidence of still active pathological processes. The histopathological picture varied considerably from fish to fish but evidenced active macrophage proliferation, proliferation of giant cells, and/or degenerative process in part of the renal tubules. Heavy infiltration by eosinophils was evident in all visceral tissues; in the kidney eosinophils often replaced the residual hematopoietic tissue. The most evident pathological changes were in the liver and the exocrine pancreas: livers had grey green coloration. Large patches of hepatocytes in the liver were either atrophic or in a state of hydropic degeneration or loaded with lipofuscin globules, while others still retained their globular vacuolar appearance due to storage of lipids. Macrophage centers were also heavily loaded with lipofuscin (in some fish also in the spleen and the kidneys). The acinar cells of the pancreas were atrophic, without or with depleted quantities of zymogen granules, containing varying numbers of necrotic cells and lipofuscin deposits. In some fish the biliary ducts were obstructed by ground substance and the lining epithelium was in a varying degree of degeneration, proliferation and sloughing.

2.3 Biochemical studies

Amino acid analysis employed in the first stage of our study (first year report, 1981) was subsequently substituted by a method utilizing HPLC column which allowed for the separation of tyrosine at a discrete peak. By this method, described in detail in the comprehensive report (1982), we were able to estimate tyrosine in small aliquots of serum (30-100 µl) and small amounts of tissue (4-8 mg) obtained from young (5-30 g) fish.

2.3.1 Amino acid analysis in sera of *S. aurata*:

Amino acid analysis in sera of *S. aurata* yearlings (150-200 mm long) cultivated in Eilat, fed on formulated feeds (IOLR formulation for *S. aurata*, Tandler et al., 1982) and whole blood* of wild caught *S. aurata* from Bardawil lagoon (North Sinai, Egypt) about two years old (200-250 mm in length) were quite comparable except for taurine. High levels of taurine were also detected in whole blood samples of cultivated fish from Eilat. The amino acid profile of a fish with chronic SG (of same size and age) did not diverge from the obtained from healthy fishes (Table 2).

2.3.2 Tyrosine serum levels in SG affected fish:

Tyrosinaemia consistently accompanied the acute phase of SG in young of the year *S. aurata*. Values of 1.2 - >2.0 mM of tyrosine were consistently found in affected fish compared to <0.4 mM in unaffected fish (Table 3). Tyrosine levels in sera of fishes fed on SG potent diet (JFS) which failed to develop disease conditions, remain within the

*(There were no facilities on site to centrifuge the blood.)

normal range. Tyrosine levels in the blood of both normal and tyrosinaemic fish were not affected by a lapse between the time of food intake and the time of bleeding (24, 48 and 72 hours).

The level of tyrosine in the blood of normal fish of similar age estimated by the amino acid analysis and by HPLC differed by about 3 fold. Furthermore tyrosine determinations in various samples from normal fish using the HPLC method yielded different estimates for levels of tyrosine. These discrepancies were limited to serum and blood samples containing low level tyrosine. The differences resulted from a lower degree of resolution reached with the specific HPLC column that we used. As a result the peak tyrosine had to be corrected for contribution by materials separating close to it. This interference was limited to serum samples from normal fish containing low level tyrosine. In samples containing high levels of tyrosine (from acute SG fishes) the tyrosine peak was very apparent and was therefore minimally interfered by neighbour peaks. Samples from kidneys and muscles did not contain such interference.

2.3.3 Concentration of tyrosine by the kidney:

Tyrosine is concentrated by the kidney of S. aurata about 13 fold compared to serum concentration. This function occurs under all circumstances in healthy as well as in SG affected fish. It appears that in affected fish under conditions of tyrosinaemia this maintained ratio is sufficient

to result in the precipitation of tyrosine in the kidney. Tyrosine is similarly concentrated, close to 13 fold, in the kidneys of Acanthopagrus bifasciatus, a sparid fish from the Red Sea which was found susceptible to systemic granuloma, while kidneys of another sparid from the Red Sea - Diplodus noct not susceptible to SG concentrated tyrosine only at a ratio of 8.7 to blood levels (Table 3).

Similar and even more extreme ratio of blood-kidney tyrosine levels was found in 2 year old S. aurata unaffected and with chronic and terminal granuloma. Nevertheless in the absence of tyrosinaemia there was no development of kidney tyrosinosis. Tracing injected ^{14}C -tyrosine into both normal and acute SG affected fish further confirmed tyrosine accumulation in the kidneys of both healthy and affected fish (Table 4. see comprehensive report, 1982). Active concentration of tyrosine was also evident in the liver, however crystalline deposits were never traced in fish suffering acute SG. Muscles, however, do not concentrate tyrosine above blood levels.

2.3.4 Tyrosine Amino Transferase:

Two isoenzymes of tyrosine amino transferase, a cytosolic and a mitochondrial are recognized in animals. In the rat liver, more than 90% is cytosolic while in humans over 2/3 is mitochondrial. Similar distribution is described also for phosphoenlpyruvate carboxykinase in the rat and human liver respectively. Moreover, in a case of hereditary tyrosinemia reported in humans (Kennaway N.G.

and Buist, N.R.H. 1971, *Pediat Res* 5, 287-297, normal activity of the mitochondrial with complete absence of the cytosolic form was reported. We therefore determined the distribution of the activities of these two enzymes in the liver of S. aurata (Table 5).

It is interesting that the distribution of the two enzymes differs in Sparus aurata, while most of the activity of phosphoenolpyruvate carboxykinase is of the mitochondrial isoenzyme (like that found in avians) tyrosine amino transferase activity is mostly of the cytosolic type, which is very similar to the rat. Moreover the activity of the enzyme in adult S. aurata is relatively high and equals that found in rats only when under induced conditions (Ernest, M.J.; Feigelson, P., J. 1978, *Biol. Chem* 253, 319-322; Nogouchi, T., Diesterhaft, M. and Granner, D., 1978, *J. Biol. Chem* 253, 1332-1335; Hofer, E. and Sekeris, C.E., 1978, *Europ J. Biochem.* 86, 547-554). Clearly, in the adult fish no deficiency of tyrosine aminotransferase exists.

Considerable variation in the activity of tyrosine amino transferase is noted in fish younger than a year. However it is not significantly altered in fish with acute SG, although in both groups of young fish, the activity of the enzyme is somewhat lower than that in adult fish (compare data with Table V, Comprehensive report).

2.3.5 P-Hydroxyphenylpyruvate Oxidase:

Recently we improved the assaying conditions of the activity of the enzyme (Fig. 1). Separation of substrates and products at termination of the reaction is much better with homogentisic acid well separated from either p-hydroxyphenylpyruvate and from p-hydroxyphenyllactic and acetic (Fig. 2). Enzyme activity assayed in young fish both healthy, and those with acute SG, demonstrated no deficiency of this activity (Table 6). Moreover the activity of the enzyme assayed at 25°C was reduced by only 33% (Table 6) compared to tyrosine amino transferase which retains at 25°C only 32-42% of the activity assayed at 37°C (see Table IV Comprehensive report). Clearly, these two activities are not deficient in fish with acute SG. We, of course, cannot rule out inhibition of tyrosine metabolism in situ. However, this requires another experimental approach, as discussed later.

2.3.6 Conversion of Phenylalanine to tyrosine, as discussed in the Comprehensive report, 20-40% of C^{14} injected phenylalanine to 0-aged fish, converted to tyrosine within 20 mins. This must be considered an important active endogenous supply of tyrosine in these fish.

2.3.7 Clearance of C^{14} tyrosine - Clearance experiments are discussed in length in the Comprehensive report (1982). We were able to determine quite precisely the clearance rate in adult fish. Difficulties were encountered with similar experiments in young fish, susceptible to the

disease, yet they do suggest ~~the possibility~~ of delayed clearance of tyrosine in fish with acute SG. On the basis of the more recent findings that the activities of the two key enzymes in liver tyrosine metabolism are not deficient during the acute phase of the disease, attempts should be made in the future to carefully estimate tyrosine clearance in young fish. It should especially be attempted to estimate tyrosine excretion and its metabolism in the kidney.

3. DISCUSSION AND CONCLUSIVE REMARKS

Tyrosinaemia during the acute phase of systemic granuloma, the indication of a slowed tyrosine clearance in sick fish and the possibility of producing SG by diets enriched in tyrosine, strongly suggests a limitation of the catabolism of tyrosine in Sparus aurata. Effort should be directed to evaluate this limitation:

Studies in the liver - although maximal activities of the key enzymes indicate that these are not deficient, one lacks evidence on the metabolism of tyrosine in situ either in the liver or in the kidney. The technique developed to assay pHydroxyphenylpyruvate oxidase activity by separating substrates and products, is also a suitable method to identify tyrosine metabolites in the liver. Two experimental approaches should be adopted (a) in vivo experiments - analysis of tyrosine metabolites after pulse injection of C^{14} tyrosine to fish fed diets enriched with tyrosine; (b) metabolism of tyrosine in liver organ culture in vitro - both approaches should be aimed at identifying a possible limiting step in tyrosine catabolism in the liver. The in vivo system may of course be used to determine the distribution of tyrosine in other organs and its excretion.

Clearance experiments should manipulate a technique of incubating young fish in a small volume for a relatively longer period of time (hours). This would allow to estimate tyrosine excretion as well as its distribution in the body.

It remains an open question why the kidney concentrates tyrosine. Alternative tyrosine metabolism or its excretion by the kidney should be considered as it lacks tyrosine amino transferase. One possibility is the utilization of tyrosine for the synthesis of melanine and /or its excretion. If these are affected during the acute SG, it would result in tyrosinaemia. We do not know as yet anything about tyrosine metabolism in the kidney.

The ability of S. aurata to concentrate tyrosine appears to be a primary factor in the susceptibility of this fish species under culture conditions to systemic granuloma. Interspecific variability among sparid fish in susceptibility to SG was found directly correlated with the levels of tyrosine concentrated in the kidney by individual species. Thus any increase in free tyrosine levels in the blood, from whatever aethiological cause, is sufficient to result in precipitation of this low soluble (0.045 gr/100 ml, at 25°C) amino acid in the kidney and consequently initiating the nephropathic process. Our preliminary results of inducing SG by diets overloaded with tyrosine need further confirmation and at this stage can serve only as a working hypothesis for the aethiological origin of the induced tyrosinaemia. Formulated feeds (of 45-50% crude protein) of cultured fish including Sparus aurata already may contain excessive levels of tyrosine and phenyl alanine, well above (30-40 g/kg diet) the known

requirement levels for salmonids, Japanese eel and carp (21-29 gr/kg diet) (Cowey, C.B., et al., 1974; Br.J. Nutr., 31, 297-306; Cowey, C.B., 1979, Proc. Wld Sym. Finfish Nutr. Finfish feed tech., 1, 3-16; Higgs et al., 1979, Ibid., 2, 191-218). In fish meals combined levels of tyrosine and phenylalanine range from 45 to 60 g/kg. Fish meals and feeds containing high levels of protein (JSF) were the best SG inducing diets in our experiments. Incidence of SG also increased with the optimization of feed intake by the fish. This could suggest that transition from wet natural feeds to dry pelleted, formulated feeds in culture increases feeding efficiency and exposes S. aurata to excessive levels of tyrosine while this particular species has limited capacity for tyrosine catabolism.

Natural feeds of S. aurata in Bardawil lagoon consist predominantly from Molluscs but also from annelids, while very young fish consume copepods, among mollusc, Mytilus edulis contains high levels of tyrosine-phenyl alanine, 55 gr/kg (dehydrated tissue), but Ostrea edulis and several others contain less than 40 gr/kg, of the other organisms. Tubifex contain 29.1 g/kg and some copepods 33.5 g/kg.

This nevertheless does not explain the inconsistencies and variations in SG inducing potencies in feeds during sotrage, the individual variability among fish in susceptibility to SG and the apparent decline in susceptibility

to SG with age.

Even less understood is the nature of the pathological process after the acute phase, when blood tyrosine levels regress to normal and the nephropathy is overwhelmed by granulomatous chronic inflammation and the causes for the terminal condition of the atrophy of the liver tissue and the pancreas. The progressive nodular and proliferative granuloma, expanding from the kidney to the other visceral organs is reminiscent of chronic granuloma induced by hypersensitivity reaction known in high animals to foreign bodies or chemical excitant (residual crystalline tyrosine?). Close analogy can be drawn between systemic granuloma and nephrocalcinosis or Visceral granuloma of salmonids where initial nephropathy excited by deposited calcium is subsequented by chronic granuloma of the viscera (Dunbar, C.E. and Herman, R.L., 1971, J. Nutr., 101(11):1445-1451; Landolt, M.L., 1975, In Pathology of Fishes, ed., W.E. Ribelin and G. Migaki, The Univ. Wisconsin Press, 793-801.).

4. EVALUATION OF THE RESEARCH ACHIEVEMENTS WITH RESPECT TO THE ORIGINAL RESEARCH PROPOSAL

Results obtained by the completion of this three year study provide us with a better understanding of the metabolic aspects of the pathogenic process in systemic granuloma and the course of the pathological process from the initial lesions in the renal tubules through the stage of proliferative granuloma, to the terminal degenerative state of the liver and the pancreas (objective No. 3). Adequate biochemical micromethods were developed and standard methods in current use in mammalian biochemistry were adjusted for work with material obtained from fish (objective No. 2). Sparus aurata kidney was found to concentrate, at all conditions, tyrosine at 13 fold the blood level. Consequently any elevation in blood levels of tyrosine, a low soluble amino acid, is likely to result in its deposition in the kidney. Thus it has been established that the tyrosinaemia which was determined in all tested fish with acute SG is the direct cause for the deposition of crystalline tyrosine and formation of urolites and the consequent nephropathy and the chronic inflammation. In examination of the susceptibility to SG of other species of sparid fishes (objective No. 6) the susceptibility to SG was found to be closely linked with the kidney species' nature to concentrate tyrosine. Thus, Acanthopagrus bifasciatus, whose kidneys concentrate tyrosine at the same rate as

S. aurata, was susceptible to SG while the other two sparilids concentrated considerably less tyrosine in their kidneys and were also found non susceptible to SG.

The clinical chemical parameter of tyrosinaemia and the gross pathological feature of crystalline deposits and unpolites may be used as a reliable diagnostic criteria for acute systemic granuloma. However, these two parameters disappear in the chronic phase of the disease and reliable diagnosis can be performed only through micropathological examination for differential diagnosis from chronic bacterial, parasitic and fungal infections that induce granulomata and chronic lesions in the viscera (objective No. 2). The aethiological background for the advanced chronic pathological process remains unknown and calls for further investigation.

Dietary experiments conducted during the first year of the project with a wide range of diets in an attempt to screen out diets with high SG inducing potency, did not yield the expected answers on the aethiological origin of SG, nor did treatments of diets by alcoholic extraction or heat affect their inducing potency (objective No. 1). These experiments were relevant only as a means for obtaining suitable diets for obtaining SG affected fish for biochemical and histopathological studies. Through these experiments it has been established, however, that high

incidence of SG can be induced only when a high consistent food intake by the fish is maintained, and there is an evident individual variability among fish in their susceptibility to SG under identical feeding conditions and an apparent decline in susceptibility as they approach their first year of life.

In an attempt to identify the causes for the occurrence of tyrosinaemia, and hence the aetiological link between potent diets and the occurrence of the disease (objective No. 5) priority was given to enzymatic studies over analysis of feed components, which at that stage of our knowledge had to be entirely empirical.

Study of the enzymatic catabolic system of tyrosine (Tyrosine amino transferase and pHPP oxidase) did not evidence any discrepancies in their activity level between healthy and sick fish. With these conclusions, dietary overload by tyrosine was suspected to be the direct cause for the tyrosinaemia. Due to time limits and shortage of fish for experiments during the third project year only a few fish were tested for dietary tyrosine overload. Four out of five fish fed on a diet overdosed with tyrosine developed kidney tyrosinosis with characteristic pathology of systemic granuloma. However, these findings require further experimental confirmation. If dietary overload by

tyrosine is proven to be the aethiological origin, then the subsequent step will be analysis of the feeds used for cultured S. aurata to determine the causes for accumulation of tyrosine.

This line of research assumes more relevance considering the fact that most formulated diets contain tyrosine and phenyl alanin in excess to the approximate necessary dose metabolically required by the fishes. A further aspect of the study will be to investigate the factor (metabolic or genetic) involved in the inherent variation in the susceptibility to SG of individual fish, as well as the causes of the decline in susceptibility with age.

5. DESCRIPTION AND RESULTS OF THE COOPERATION

Dr. Marsha Landolt was asked to serve as co-operator on this project because of her previous research experience with diet related diseases in salmonids and her present research interest in experimental toxicology of fishes. The location of her laboratory in the School of Fisheries at the University of Washington was of particular advantage to this cooperative program. The University incorporates teaching and research programs in fish nutrition and genetics, and Dr. Landolt's laboratory collaborates closely with the histopathology laboratory at the University of Washington Medical School. Dr. Landolt's laboratory also maintains close association with the Western Fish Disease Laboratory (U.S. Wildlife Service), the Northwest Alaska Fisheries Center and fish farms in the Seattle area. Through this wide spectrum of personnel and laboratories engaged in fish diseases and fish nutrition, valuable advice and feedback for our research program and results were obtained. Dr. Landolt was continuously briefed via regular correspondence. Dr. Paperna visited Seattle for a period of three weeks (funded by the Hebrew University Sabbatical funds) in February, 1981; in October 1982 (funded through this grant) Dr. Landolt visited the laboratories at Eilat and Jerusalem for two weeks in April, 1982. During both visits results of the research were reviewed and annual reports

and future work plans were formulated, prepared, and discussed.

While in Seattle Dr. Paperna presented research data for discussion (through formal seminars) to the staff of the School of Fisheries and the School of Medicine.

Conferences were arranged with the following persons:

Dr. Emil Chi, University of Washington, Department of Pathology - on histologic and electron microscopic techniques; arrangements were made for technical assistance on these aspects of the work.

Dr. John Halver, University of Washington, School of Fisheries - on design of dietary experiments and review of the biochemical data obtained.

Dr. Tosh Jasutake, National Fisheries Research Center, Western Fish Disease Laboratory - on fish kidney pathology, aspects of chronic inflammation in fish and dietary pathology. Relevant microscopic material on diet related pathology and kidney diseases was compared with material from S. aurata.

Dr. Gary Wedermeyer, National Fisheries Research Center, Western Fish Disease Lab - on physiological aspects of diet related diseases.

Dr. Lee Harrel, Northwest and Alaska Fisheries Center - on comparative aspects of diet related diseases of salmonids (gall bladder disease) and systemic granuloma in S. aurata which apparently have similar aethiology.

Dr. Richard Kocan, University of Washington, School of Fisheries - on aspects of fish toxicology and in vitro assays of toxins.

During Dr. Landolt's visit in Eilat, she was introduced to the mariculture facility at Eilat, where S. aurata is cultured, and discussed aspects of fish breeding, spawning and nutrition with the scientific staff of the laboratory.

A subsequent visit of Dr. Paperna in Seattle was in October, 1982. In addition to joint review of the research results and setup of the data for the final report, Dr. Paperna, in collaboration with Dr. E. Chi of the University of Washington, Pathology Department, and Dr. R. Kocan of the University of Washington School of Fisheries, processed histological material with specific methods suitable for metacrylate embedding and made autoradiographies of the histologic material marked by ^{14}C -tyrosine. Explant culture methodology was evaluated for use as a toxicologic assay method in our studies. Future aspects for collaboration were discussed and joint research programs are planned for submission to the Bi-National U.S.-Israel funding agencies.

6. BENEFIT TO AGRICULTURE

Systemic granuloma disease is a severe setback to the mariculture of Sparus aurata. Losses at the acute phase of the disease reached in some years up to 50% of some culture lots in ponds, sea cages and circular trophs. The disease is not unique to S. aurata cultured in Eilat, it occurs also in the same fish cultured in Spain. Disease appears to persist in a chronic state in ongrowing fish often causing unilateral or bilateral blindness. Severe morbidity, and subsequent mortality in brood stock was identified to result from advanced chronic and terminal state of this disease. Affected fish were severely emaciated and lethargic and became useless for breeding, thus comprising total loss of threes invested in growth and maintenance.

Through this three year research we achieved better understanding of the physiological-biochemical and pathological aspects of the disease, through which we developed reliable diagnostic means to identify the different stages of the disease. Determination of the relationship between the disease and the tyrosine metabolism opens new avenues for identification of the aethiological causes in feeds and thus eventually advice for the nutritionists in formulating diets with no risk of inducing SG (see further in Discussion and Conclusive Remarks p. 21).

Table 1. Summary of 1980 dietary experiments.

Diet	Test date	Feeding Days	Fish size (L or W)	Survival	%SG	No. fish examined
WF	July	13-16	(L) 60-83mm	80%	46%	24
	November	7	(W) 16-24 gr	100%	0%	10
Ewos	July	13-21	(L) 60-77mm	96%	7%	60
	August	16-24	(W) 5-15gr	100%	0%	15
HFM	July	30	(L) 55-75mm	83%	8%	25
JSF (grade II)	July	9-22	(L) 50-75mm	78%	34% (9-60%)	44
	August	16-41	(W) 9-18gr	100%	26% (0-100%)	56
		* 7-18	(W) 9-16gr	100%	0%	30
		**5-18	(W) 6-16gr	90%	22%	27
	September	5-9	(W) 4-40gr	88%	43% (16-80%)	44
		***6	(W) 3-6gr	100%	46%	30
	October	5-8	(W) 4-10gr	85%	4%	24
	November	7-8	(W) 18-25gr	95%	5%	20
	controls for experiments	-Same as relevant tests-		84%	3%****	171

WF - minced mackerals stored in -18°C since 1977. In 1978 induced 11-73% SG.

Ewos - commercial feed containing 45-50% protein, stored in 4°C since 1977, in 1978 yielded 43-44% SG.

SAF - pelleted South African fish meal (1977 stock) +1% Vit. mix, in 1978 yielded 0-26% SG.

HFM - pelleted herring fish meal (Norwegian, 1977 stock) +1% Vit. mix, in 1978 yielded 25-47% SG.

JSF II - commercial Japanese shrimp feed, stock from 1980, contains 60% protein.

FF - minced fresh caught fish (atherinids mainly), stored at -18°C since July 1980.

* -at 2% body weight daily ratio, ** -autoclaved, *** - Eton extracted, **** -low incidence occurred already in stock fish from the ponds.

Table 2. Analysis of Amino Acids

	Eilat Cultivated Fish-Serum (4)	<u>Sparus aurata</u>		<u>Carp</u>	
		Chronic SG Serum (1)	Bardawil Wild Fish Whole Blood (14)	Kidney (1)	Serum*
		μmol/ml		μmol/g	μmol/ml
Aspartic	0.084±0.036	0.120	0.092±0.020	0.338	Tr
Threonine	0.486±0.064	0.523	0.335±0.090	0.317	0.3-0.8
Serine	0.430±0.044	0.323	0.216±0.025	0.371	0.12-0.21
Glutamic (acid)	0.240±0.070	0.298	0.348±0.53	1.586	Tr
Proline	0.133	0.216	0.223±0.032	0.269	
Glycine	0.730±0.050	0.665	0.664±0.051	0.564	0.11-0.56
Alanine	0.710±0.070	0.708	0.712±0.062	1.472	0.14-0.7
Valine	0.430±0.085	0.605	0.328±0.031	0.298	0.24-0.5
Methionine	0.270±0.028	0.227	0.267±0.037	0.209	
Isoleucine	0.240±0.046	0.347	0.310±0.051	0.285	0.17-0.34
Leucine	0.340±0.064	0.510	0.371±0.028	0.578	0.3-0.66
Tyrosine	0.350±0.036	0.227	0.222±0.016	0.185	0.06-0.10
Phenylalanine	0.280±0.028	0.190	0.216±0.017	0.178	0.05-0.10
Gln + Ash	1.62±0.230	0.947	(3)1.240±0.230	2.154	
Citrulline	0.48±0.084	0.778	0.483±0.068	-	
α-amino-n butyric	0.11±0.043	0.336	(3)1.240±0.230	0.124	
Taurine	1.38±0.215	1.163	13.240±1.053	11.000	

Sparus aurata whether wild or cultivated in Eilat are about 3 years old.

No. in parenthesis indicate number of fish.

* After Zebian, M.F. and Y. Creach, 1979, Proc. Wld Symp. Finfish Nutr. and fishfeed Tech. 2, 531-544.

Table 3. Tyrosine concentration in serum and kidneys of various fishes.

<u>Fish species</u>	<u>Age</u>	<u>No.</u>	<u>Diet*</u>	<u>Condition</u>	<u>Serum</u> mMol/ml	<u>Kidney</u> mMol/g tissue	<u>Kidney/ serum</u>
<u>Sparus aurata</u>	0+	6(a)	FF	Normal	0.045±0.008	0.589±0.116	13.1
	0+	7(a)	JSF	Acute SG	1.313±0.076	>6-crystals**	4.6**
	0+	9(b)	FF	Normal	0.163-0.480		
	0+	6(b)	JSF	Acute SG	1.08-1.85		
	0+	2(b)	JSF	Normal	0.104, 0.117		
	2+	1(a)	T***	Normal	0.095	2.154	22.7
	2+	4(b) [†]	T***	Normal	0.350±0.036		
	2+	3(a)	T***	Chronic SG	0.106±0.016	1.512±0.078	14.3
	2+	1(b) [†]	T***	Chronic SG	0.227		
<u>Acanthopagrus bi-fasciatus</u>	1+	4	T***	Normal	0.043±0.008	0.545±0.234	12.7
<u>Diplodus noct</u>	>1+	3	T***	Normal	0.044±0.013	0.369±0.139	8.4
<u>Rhabdosargus haffara</u>	>1+	3	T***	Normal	0.066	0.337±0.155	8.4

* Refers to Table 1.

** Actual level considerably higher, since part of the crystals failed to dissolve.

*** Trout feed routinely used in S. aurata culture.

[†] Refers to amino acid analysis, Table 2.

Table 4. Free C^{14} tyrosine and its concentration in tissues 20 min after injection to control and acute systemic granuloma fish.

Tissue	Control		Acute systemic granuloma	
	c.p.m.	μmol^{**} /g tissue	c.p.m.	μmol
*Serum	59500	0.144	52200	1.134
liver	157143	--	196000	--
muscle	26888	0.154	27491	0.798
kidney	106300	0.880	422300	6.100

* c.p.m./ml serum

** tyrosine amount was determined by dianizidine in the corresponding tyrosine fractions eluted from HPLC. (See methods).

Table 5. Activities of tyrosine aminotransferase and phosphoenylpyruvate carboxykinase in liver cytosol and mitochondria of adult Sparus aurata.

Enzyme Activity (37°C)

	Cytosol	Mitochondrial
	Unit/g tissue	
Tyrosine amino transférase (3)	10.2 ± 0.92	0
Phosphoenylpyruvate carboxykinase (5)	0.57 ± 0.13	2.27 ± 0.31

Table 6. Activity of P-Hydroxyphenylpyruvate oxidase in young healthy and acute SG fish.

		<u>Enzyme Activity</u>	
		37°	25°
		unit/g liver	% activity of that at 37°
Young healthy fish	(7)	0.91±0.12	67%±6% (6)
Acute systemic granuloma	(3)	2.41±0.74	

Figure 1.

PhPP oxidase (activity with increasing amount of liver cytosol)