International Fishmeal & Oil Manufacturers Association

DETERMINATION OF SOME FACTORS AFFECTING THE NUTRITIONAL AND BIOTOXICOLOGICAL VALUE OF FISH MEAL FOR USE IN FEED FOR SHRIMP CULTURE AND TO ESTABLISH QUALITY CONTROL NORMS

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S U M M A R Y

The shrimp industry world-wide has been plagued with disease and poor growth which has virtually brought productivity increases to a standstill. Intensive production, poor husbandry, poor feeds and pollution have been factors causing the difficulties. All have interacted; poor feeds, for example, limit growth and increase pollution. As fish meal and fish oil are major components of shrimp feeds, particularly for marine carnivorous shrimp, the quality of these ingredients is paramount, yet little was known what quality parameters should be set.

This project was designed to address this issue, particularly in connection with shrimp production in Mexico, the second biggest producer in Latin America.

Although little was known about fish meal quality requirements for shrimp, there is information available for marine carnivorous fish, in particular salmonids. On the basis of this, the following objectives were set for the project:-

1. Determine if raw material freshness or processing conditions damage the nutritional quality of fish meal used in shrimp feeds.

2. Evaluate gizzerosine effect on shrimp physiology.

3. Standardise an *in vitro* digestibility method with trypsin and determine its relationship with pepsin digestibility and the chromic oxide apparent digestibility.

4. Decrease environment pollution using more digestible and assimilated feeds to give better feed conversion ratios.

5. Determine the effects of rancidity of lipids in fish meal and fish oil when used in shrimp feeds.

6. Establish quality control norms for fish meals used in shrimp nutrition.

I EFFECT OF RAW MATERIAL FRESHNESS

Raw material spoilage of fish used to produce fish meal for shrimp feeds reduces feed intake and growth in shrimp. This was found working with Litope*naeus*

vannamei and Litopenaeus *stylirostris* at the Universidad Autonoma de Nuevo Leon (UANL) and with *Litopenaeus stylirostris and Penaeus monodon* in Tahiti (IFREMER). This was more pronounced with small shrimp (around 0.1g). Spoilage of raw material did not reduce protein digestibility. Studies with pure amines indicated these were not the cause of reduced growth but were useful indicators of raw material quality.

II GIZZARD EROSION PRODUCING FISH MEALS

Some Mexican tuna meals had been found to give poor growth when fed to shrimp. These same meals were also found to cause gizzard erosion in poultry. Studies in Japan have indicated the presence of toxins. These are produced in meals made from fish with relatively high histidine content when fine particles they contain are over-heated. One of these toxins, named gizzerosine, which has been shown to be particularly toxic to poultry, was fed to shrimp. It was found to be toxic, significantly increasing mortality.

For poultry, because of the difficulty in measuring gizzerosine and other toxic compounds, a test has been developed to measure gizzard erosion (biotoxicological) score *in vivo*. Using fish meals ranked in poultry as low, medium and high biotoxicological score, reduced survival was noted in small shrimp (66mg) with a medium score fish meal; detrimental effects were not obtained with larger shrimp fed another medium scored fish meal, or with small shrimp (70-100g) fed a high scored fish meal. The fish meal giving increased toxicity also had a high content of the amine histamine indicating it had been produced from spoiled raw material. Mortality was therefore attributed to the effects of raw material spoilage. It is recommended that fish meals with medium or high biotoxicological score, especially if produced from spoiled raw material, should not be fed to shrimp, particularly those weighing less than 1.5g. However, if It is necessary to use fish meals with a biotoxicological score, the mixing of one-third high scored fish meal with two-thirds of a normal one could be acceptable, provided that raw material freshness indicators in the fish meal do not indicate it is from the stale raw material (see section VI).

III *IN VITRO / IN VIVO* DIGESTIBILITY METHODS FOR SHRIMP

Working with aquatic animals to measure digestibility poses a major difficulty. Accurate feeding and collection of faeces is difficult; contact of feed and faeces with water can result in leeching which introduces large errors. The difficulties are even greater with shrimp. No widely accepted method has been established to measure digestibility in shrimp. Consequently, the first stage of this work involved both centres (UANL and IFREMER) working together to develop the methodology. This was then used subsequently for *in vivo* tests on fish meals of known protein digestibility in salmon and/or mink. Mink are known to correlate closely with salmon in terms of protein digestibility.

In the shrimp digestive tract the main enzyme involved in the digestion of protein (protease) is trypsin acting in the neutral or slightly basic pH of the shrimp digestive tract. This contrasts with land animals where pepsin is the main protease, acting under acid conditions following some degree of hydrolysis. As well as developing an *in vivo* digestibility procedure, an *in vitro* method was also developed using trypsin, and compared with a pepsin method.

Using *in vivo* procedures it was found that temperature exposure in the fish meal process, especially in the dryer, affected protein digestibility. More severe drying reduced digestibility; the gentle drying of so called 'low temperature' dryers achieved the highest protein digestibilities. Whilst two samples of fish meal produced to give wide differences in protein digestibility in mink (95% and 84%) were found to give similar differences determined in large shrimp (17g *P. stylirostris*) (94% v 81%) in smaller shrimp digestibilities were lower and the difference was much wider (1.2g *P. stylirostris*) (72% v 39%). Working with small shrimp (under 2g) protein digestibility correlated with that in salmon, but the correlation was not high. With mink digestibility there appeared to be no correlation, but there were problems in the sampling of the fish meals used in the mink test which may have invalidated this comparison.

Comparing *in vitro* methods with protease enzymes, an extract from shrimp hepatopancreas gave results which correlated well with *in vivo* results. However, a dilute pepsin (using 0.0002% concentration) procedure was also found to correlate well though values given were lower than values published in the literature. Nevertheless this is the *in vitro* method recommended on the basis of results obtained.

IV EFFECT OF DIGESTIBILITY ON SHRIMP GROWTH AND FEED CONVERSION RATE

Although the higher digestibility fish meals were expected to produce better growth and feed utilisation in shrimp, this was not demonstrated in either pond or tank trials for a number of reasons. First, the protein concentration in the diets, though set to literature recommended values, was probably excessive. Secondly, it was not possible to control other variables in the fish meals such as raw material type and freshness.

For the small *P. stylirostris* juveniles used in the growth studies, the protein concentration recommended in the literature is 35%. However, working with diets where most of the protein was supplied by fish meal a high quality and relatively high digestibility material, it was demonstrated that a lower concentration of protein (25% to 30%) is sufficient to obtain optimum growth. As well as reducing dietary costs this would also reduce nitrogen output.

Further work is required to compare growth achieved with fish meals of different digestibility working at lower dietary protein concentrations than those currently recommended.

V OXIDATIVE RANCIDITY

Highly oxidised (but stabilised) lipids in fish oils or meals fed to shrimp resulted in reduced feed intake, and as a consequence, reduced growth, but did not increase mortality. The status of the shrimp in terms of its vitamin E intake was found to be critical; where dietary vitamin E was low or not present, as would occur following any lipid oxidation occurring, mortality was increased. This was far more important (vitamin E status) than the degree of oxidation of the lipids. Indeed, in practice non-oxidised lipids were more likely to cause problems if they were not properly stabilised because of their tendency to oxidise and destroy vitamin E in the diet. The importance of using synthetic antioxidants in feed formulation must be stressed since they avoid the destruction of natural antioxidants, particularly vitamin E and vitamin C and carotenoid pigments. In the absence of vitamin E the feeding of fish lipids from meal or oil in an oxidised state increased mortality; this did not occur when dietary vitamin E concentration was adequate (met the published requirement).

VI FISH MEAL QUALITY NORMS FOR SHRIMP

1. Shrimp are sensitive to the freshness of raw material used in the production of fish meal; it is recommended that fresh raw material is used. A biogenic amine content (histamine + cadaverine + putrescine + tyramine) of below 3,000ppm and preferably below 2,000ppm is recommended, especially for small shrimp (below 1g).

2. The tendency of a fish meal to produce gizzard erosion in poultry (measured as biotoxicological score) can affect small shrimp (below 1.5g) adversely. This was found mainly where raw material was not fresh. If raw material freshness indicators are in an acceptable range (see 1 above) it is recommended that fish meals with low biotoxicological score are used for small shrimp (below 1.5g). Mixing a high score with low score meals can also avoid adverse effects. Larger shrimp seem less sensitive to biotoxicological score.

3. Whilst there is a nutritional case for using fish meals in shrimp feeds with a high digestibility of protein, methodology problems with both pond and tank studies prevent a recommendation being made from the results of this project based on growth trials. However, using a good quality fish meal these are indications that dietary protein concentration can be reduced. This should reduce nitrogen output.

4. In the presence of adequate vitamin E shrimp are not particularly sensitive to the oxidation of lipids in fish meal or fish oil though feed intake and growth will fall. However, in the absence of vitamin E, they become more sensitive. Avoiding oxidation of fish lipids, particularly in the shrimp diet where vitamin E would be destroyed as a consequence is paramount. The use of synthetic antioxidants in fish meal and fish oil, and in the feeds in which they are incorporated, is recommended, especially when using high quality non oxidised products which are rich in highly oxidisable polyunsaturated fatty acids.

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APPENDICES (reference papers *in extenso*)

Introduction

After a ten fold increase of its annual contribution to world shrimp production between 1982 and 1992, shrimp farming has since consistently produced about one fourth of the world production in recent years, i.e. 700,000 metric tons annually. Leading farmed shrimp producers are Thailand and Ecuador (210,000 and 130,000 tons produced in 1998 respectively), but there have been drastic changes in the ranking of Asian producers during the last ten years due to environmental and viral disease outbreaks (Rosenberry, 1998).

With 17,000 tons produced in 1998, México is the second largest American producer, and has not been exempt from temporary production stagnation, due to viral diseases. For example, production dropped from 15,492 tons in 1995 to 14,822 tons in 1996, due to the arrival of Taura syndrome (Reyes-Quintero, 1998).

Until 1996, shrimp stocking in Mexican farms was largely dependent on the supply of wild postlarvae, with the main cultured species being *Penaeus vannamei* (Pacific white shrimp). In 1997 and 1998, the proportion of farms stocked with *P. stylirostris* (Pacific blue shrimp) increased drastically (up to around 35 % in 1997 and perhaps 50% or more in 1998), due to the production of hatcheries using the Maritech SuperShrimp broodstock. Wild *P. stylirostris* broodstock is naturally resistant to Taura syndrome, but the SuperShrimp have the advantage of also being resistant to another viral disease (IHHN) which has caused heavy losses to blue shrimp farming in México since 1990.

Although farmers were very pleased with performance of Blue shrimp in 1997, they are now returning to *P.vannamei* because *P. stylirostris* yield in 1998 was far below their expectations, with insufficient growth at moderate to high densities and, above all, very high feed conversion ratios which made the cost of feeding prohibitive. The reasons for this could be less favourable climatic conditions in 1998 and a general drop in feed quality, which could be related to the increase in price of good quality fishmeal.

These circumstances highlight the different nutritional requirements of *P. stylirostris* and *vannamei*, so called carnivorous and herbivorous species respectively. Recommended protein content in grow-out feeds is 30 to 35% for the former and 25% for the latter. Another difference is a major dependence on artificial feed for *P. stylirostris*, this voracity being expressed by higher feed conversion ratios (FCR = amount of feed used per unit of live weight gain, generally greater than 2, and sometimes as high as 3.5) than those for *P. vannamei* stocked at the same density (expected FCR between 1 and 2). When

P. stylirostris was stocked at low density, generally mixed with a majority of *P. vannamei* postlarvae, it reached a larger final size (and a better price as a consequence), without modifying the *P. vannamei* stock yield. In contrast, when *P. stylirostris* is reared in monoculture at higher density, feed quality and price become critical parameters to obtain good growth and acceptable feed conversion ratios (FCR).

However, with feed conversion around 2, feed contributed for up to 42% of the variable costs and 30% of total costs for Mexican farms in the years 96-97-98, a contribution which could be reduced by improving natural food production (fertilisation), by monitoring water quality and feed consumption with precision (use of feeding trays), and by purchasing better quality feeds (Reyes-Quintero, 1998). Unfortunately, although Mexican farmer's skills improve with time, their production is greatly dependent on variables they can not control such as climatic conditions and often feed quality.

Traditionally, shrimp feeds include fish meal and fish oil at average inclusion levels of 25% and 3% respectively on a world basis (Chamberlain, 1993). As a consequence, shrimp feeds consumed about 24.3% of the 1.7 million tonnes of fish meal used for aquaculture in year 1995 (which in turn is 25% of the annual fish meal production). In year 2000, shrimp will consume 18% of the 2.0 million tonnes fish meal used in aquafeeds (Tacon, 1998). Fish meal provides high quality protein with an adequate balance of amino acids and fatty acids for fast growing marine carnivorous organisms, and the use of substitutes has been less successful in shrimp and salmonids than in terrestrial animals. Therefore, fish meal availability and quality are important limiting factors for good quality aquatic feeds. This problem has been recently understood in Mexico by feed manufacturing companies and by the shrimp farmers.

The quality requirements for fish meal, though, depend on the species to be fed. The fish should be tailored from the time of catching to the end of the manufacturing process to meet the requirements of the target species (Pike, 1998), but this is difficult to realise for shrimp since most of the investigations to date have been carried out on terrestrial animals and salmonids.

The objectives of the present project were to define the quality parameters as a guide to fish meal and oil producers, and those handling these products, in order to improve the results obtained by the shrimp farmers.

Selection of the quality parameters to be considered was oriented by the actual knowledge for terrestrial species and salmonids. The two main factors for fish meal to allow best growth in salmonids are raw material freshness at processing,

and low temperature and exposure time in dryer. More over, the combined effect of raw material spoilage in histidinic fish and overheating leads to the production of toxins (one of them gizzerosine), that provoke gizzard erosion in chicken. Due to the difficult isolation and quantification of gizzerosine by chemical means, Fundación Chile has developed a biotoxicological assay in chicken, which allows the certification of Chilean fish meals. Since gizzard erosion can occur when fish meal contains histamine at high concentration (as with fish meals produced from spoiled raw material), biotoxicological score is thus considered as a basic quality parameter for chicken and other terrestrial species, but has not been extensively tested in salmonids, and not at all in shrimp. Digestibility is another important fish meal quality parameter that is affected by high levels of ash in raw material (case of fish trimmings) and high temperature exposure in dryer, and is expected to have a strong influence on FCR in shrimp culture, conditioning its economical feasibility. Unfortunately, *in vivo* digestibility studies (using diets marked with chromic oxide as a tracer) are time consuming. *In vitro* methods are therefore commonly used in the feed industry to assess fish meal quality, provided that results were proved to be correlated with *in vivo* digestibility values. The official *in vitro* method uses pepsin to simulate the acidic digestion in vertebrate stomach. However, correlation *in vitro* and *in vivo* has not been established for shrimp, an invertebrate that lacks acidic digestion with pepsin, and which principal proteolytic enzyme in trypsin. Finally, lipid oxidation in fish meal or fish oil may affect shrimp due to their strict requirements for dietary polyunsaturated fatty acids, easily destroyed by oxidative rancidity.

These considerations led to the formulation of the following objectives for the proposed research (reproduced from the original proposal):-

1. Determine if raw material freshness or processing conditions damage the nutritional quality of fish meal used in shrimp feeds.
2. Evaluate gizzerosine effect on shrimp physiology.
3. Standardize an *in vitro* digestibility method with trypsin and determine its relationship with pepsin digestibility and the chromic oxide apparent digestibility.
4. Decrease environment pollution using more digestible and assimilated feeds to give better feed conversion ratios.
5. Determine the effects of rancidity of lipids in fish meal and fish oil when used in shrimp feeds.
6. Establish quality control norms for fish meals used in shrimp nutrition.

The present report consists of specific chapters for each of these objectives, with a brief description of:-

- the fish meal samples to be tested
- the experiments carried out to accomplish the objective
- the results

making reference to the scientific papers where details on these trials can be found (reference papers). Reference papers (rfp) are given *in extenso* as appendices 1 to 12.

Other papers cited in the text are referenced in an alphabetical list, including all communications generated by the present project. The latter are also classified by genus and chronological order in a comprehensive list.

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I. EFFECT OF RAW MATERIAL FRESHNESS

Objective 1: Determine if raw material freshness (deterioration), or processing conditions damage the nutritional quality of fish meal used for shrimp feeds

Introduction

Fish meals vary widely in their protein quality and nutrient composition depending on the freshness and type of the raw material, and processing temperature (drying process). Increasing production of farmed aquatic species which are sensitive to these parameters has created a demand for high quality fish meals (McCallum and Higgs, 1989; Barlow and Pike, 1990; Pike *et al.*, 1990; Hardy and Castro, 1994). "Special products", made from very fresh fish processed at low temperature, are now marketed principally for marine carnivorous fish feeds (Pedersen and Opstvedt, 1992; Romero *et al.*, 1994). It is not known yet if other farmed species of major economical importance, such as penaeid shrimp, are susceptible to the same quality factors.

The conditions and length of storage affect fish freshness before processing. From the time of catching, fish undergo changes, brought about by the action of the enzymes of the fish (autolysis) and also from the action of bacteria present on the surface of the fish and in the gut.

The evaluation of different fish meals by chemical parameters such as *t*otal *v*olatile *n*itrogen content in raw material, or biogenic amine content in fish meal has been paralleled with biological assays by several authors for different species. For chickens (Huisman *et al.*,1992) as well as for rainbow trout (Cowey and Cho, 1992) or salmon (Jensen, 1986; Anderson *et al.*, 1997), feed consumption and growth can be affected by the dietary inclusion of fish meals containing high levels of biogenic amines, i.e. fish meals made from deteriorated raw material.

The following experiments were performed to measure the effects of raw material freshness on growth of *Penaeus vannamei* and *P. stylirostris* juveniles, in terms of % weight gain, feed consumption, feed conversion ratio and survival. The aspects related to the processing conditions, particularly heat effects on digestibility, will be considered in part 2 of the present report.

Materials and Methods

Fish Meal Samples

Two sets of fish meals made from raw material with graded freshness, provided by IFOMA, were assayed in various growth trials on *P. vannamei* or *P. stylirostris*.

The freshness of the raw material used to manufacture these experimental fish meals can be quantified by means of their total biogenic amines content (sum of histamine + cadaverine + putrescine + tyramine) (table I.1).

Table I.1 Total amines contents (mg/kg) in the experimental fish meals for freshness experiments.

Raw material freshness	Fresh	Mod. fresh	Stale
1st set (anchovy)	114	3384	7873
2nd set (herring)	182	-	8819

Raw Material Freshness Experiments: F trials

A typical growth trial was carried out at UANL or IFREMER for the present study to test the effect of different fish meal samples on shrimp includes 2 steps. At first the fish meals to be tested were included at a dietary level between 30 to 50% among the other ingredients of a commercial like, practical diet; design (formulation) generally ensures a constant fish meal protein contribution in iso-proteic, iso-energetic diets; diets were manufactured by extruding the ingredients mix plus 30 to 40% water through 2 mm holes of a dye mounted on a meat grinder and drying the resulting spaghetti-like strands. Chemical analysis of the finished experimental diets allowed checking important mishandling in the diets manufacture process.

Secondly, the experimental diets were fed to replicated groups of shrimp, each group been housed in a fiberglass tank. Number of replicated groups (generally 3 to 6 per diet) and initial number of shrimp per group (from 3 to 15 in a 60 liters tank) is fixed for each growth trial depending on the availability of a homogeneous stock of animals (initial individual shrimp sizes in the shortest size class as possible). Weighed amounts of feed were given in each tank after siphoning the rests of the previous meal. Feeding *ad libitum* means in the present case that enough feed has been given to allow quantifiable, although minimum, feed remains to be recorded before next feeding. After two and four weeks of experiment, means for individual weight gain (expressed as a percentage of the initial weight) and individual feed intake were calculated for each replicated group as well as the survival rate (% survival) and the feed conversion rate (feed to gain ratio). Results are submitted to an analysis of variance, the statistical sample for each diet been the three (up to 6) replicate values.

Four growth trials were run at UANL, and three at IFREMER-COP with diets including the fish meals of the two sets mentioned above (Table I.1). General description of these trials is given in Table I.2; details of the method are available in the reference paper (rfp) mentioned on the right size of Table I.2. Reference papers are annexed *in extenso* to the report.

Table I.2 Growth trials to test raw material freshness effects on shrimp: F trials

Trial	Trial Description	Ref. paper
F1	*Chilean anchovy meals raw material freshness effect on small P. vannamei:* Growth trial by Isabel Abdo (UANL) on 3 anchovy meals (1st set). 3 diets were tested on small *P. vannamei* (0.9g initial weight), (15 days trial).	rfp 1
F2	*Chilean anchovy meals raw material freshness effect on small P. vannamei*: Growth trial by Isabel Abdo (UANL) with the same anchovy meals as for F1, but on larger *P. vannamei* (1.5g initial weight), (28 days trial)	rfp 1
F3	*Chilean anchovy meals raw material freshness effect on small P. monodon:* Growth trial by Aquacop (IFREMER-COP) on the 3 anchovy meals (1st set). 3 diets were tested on small *P. monodon* (2.5g initial weight), (30 days trial).	rfp 1
F4	*Chilean anchovy meals raw material freshness effect on medium sized P. stylirostris:* Growth trial by Aquacop (IFREMER-COP) on the 3 anchovy meals (1st set). 3 diets were tested on medium sized *P. stylirostris* shrimp (8.4g initial weight), (30 days trial).	rfp 1
F5	*Chilean anchovy meals raw material freshness effect on medium sized P. vannamei:* Growth trial by Aquacop (IFREMER-COP) on the 3 anchovy meals (1st set). 3 diets were tested on medium sized *P. vannamei* shrimp (7.6g initial weight), (28 days trial).	rfp 1
F6	*Norwegian herring meals raw material freshness and spiked amines effect on very small P. stylirostris:* Growth trial by Mireya Tapia-Salazar (UANL, Ph.D. thesis, in progress) on two herring meals (2nd set) plus four additional treatments including added cristalline biogenic amines: i.e. 6 diets tested on very small *P.stylirostris* juveniles (0.077 g average initial weight), (28 days trial).	rfp 2
F7	*Chilean anchovy meals raw material freshness effect on very small P. stylirostris:* Growth trial by Mireya Tapia (UANL) on the 3 anchovy meals (1st set): 3 diets tested on very small P. stylirostris (0.070 mg initial weight). (28 days trial).	rfp 3

A first series of growth trials on shrimp (F1 and F2 at UANL, and trials F3 to F5 at IFREMER) were carried out using iso-energetic compound diets, containing 30% of the experimental anchovy meals at UANL, and 40% anchovy meal at IFREMER. The fish meals were tested on *Penaeus stylirostris* (initial weight 8.4g), *P. monodon* (2.5g) and *P. vannamei* (0.9, 1.5 and 7.6g) (see Ricque *et al*, 1998 =rfp1 for details on the methods).

An experiment (F6) was carried out using crystalline biogenic amines added to a fish meal made from fresh herring, and comparing with a fish meal made from the same herring but spoiled (Tapia-Salazar *et al.*, 1999a = rfp2).

A last experiment (F7) has been carried out with the aim of testing again the toxicity of the meals made from stale anchovy (first set), this time on very young *P. stylirostris* (individual weight 1< 0.1g)(see Tapia-Salazar *et al.*, 1999b = rfp3 for details on the method), since toxicity was observed with such small organisms in trial F6 when using stale herring.

Results Compendium

Raw Material Freshness Effect on Different Shrimp Species and Sizes (Trials F1 to F5)

When compared to the control (fish meal made from very fresh anchovy), feeding the spoiled anchovy meal resulted in a reduced growth rate (15% less) for *P.stylirostris, P.monodon* and for *P.vannamei* of 0.9g. This difference in the growth rate was not noted in the 1.5g and 7.6g *P.vannamei*. The 8.4g *P.stylirostris* fed moderately fresh anchovy meals also exhibited a significantly reduced growth rate, thus displaying a higher sensitivity than the other two species.

It was concluded that raw material freshness, as indicated by TVN levels in raw material (less than 30mgN/100g) or by the sum of amine contents in the final product (less than 2000 mg/kg), is a quality parameter that should be considered when selecting fish meals for shrimp diets, particularly for very young juveniles and carnivorous species due to their susceptibility to fish protein degradation (Ricque *et al.*, 1998 = rfp1). However, further experiments were required to investigate the susceptibility of very small shrimp, since some mortality was seen in other experiments involving a fish meal with high biogenic amines content, run with very small shrimp (see trial G1, part 3 of the report).

Effects of Stale Herring vs Stale Anchovy on Very Young *P. stylirostris* (Trials F6 and F7)

With trial F6, it was confirmed that raw herring spoilage also affects feed consumption, growth and survival of very young *P. stylirostris*, although added cristaline amines do not. It was concluded that amines should be considered only as indicators, and not as factors of toxicity, which may be due to other substances like bacterial endotoxins (Tapia-Salazar *et al.*, 1999a = rfp 2). This work was the beginning of a PhD thesis for Mireya Tapia-Salazar, with the aim to confirm this hypothesis.

During trial F7, very small shrimp fed the meal from fresh anchovy expressed higher feed consumption and higher growth, as described in previous experiments, and a tendency to a better (lower) feed conversion ratio, but the stale anchovy meal had no effect on survival of the young *P. stylirostris*, although total levels of biogenic amines were similar both in stale anchovy and stale herring meals (Tapia-Salazar *et al.*, 1999b = rfp 3).

This difference in toxicity can be explained considering that a longer storage time (probably at least 10 days) was necessary for the herring stored in Norway to reach the

same level of biogenic amines that was obtained for the anchovy meal in Chile after only 36 hours, thus involving different bacterial development. It was thus confirmed that the effect of raw material deterioration on shrimp does not seem to be directly related to the level of amines, but rather to some microbial toxic compounds.

The first effect of these compounds would be a decrease in feed consumption, inducing decreased growth as the main consequence, mortality being obtained only at high doses or with very sensitive organisms (like very small shrimp). The consistent lack of effect on feed conversion weakens the first explanation based on protein alterations selectively affecting the carnivorous species.

However, biogenic amines determination still remains one of the best ways to estimate *a posteriori* the freshness of the raw material used for a given fish meal, even if their levels are not perfectly correlated with the level of the still hypothetical toxic compounds.

Conclusion

Raw material spoilage diminishes feed intake and growth in shrimp. This effect seems *more pronounced with* very small shrimp (around 0.1g) and *with P.stylirostris,* and when longer storage time allows increased spoilage of the raw material. Mortality occurred with herring when these three conditions were *combined,* suggesting the occurrence of a toxic factor. Feed to gain ratio was generally not affected, and further results on digestibility (see part 3 of the present report) suggest that raw material spoilage *does* not reduce significantly the protein quality for shrimp, confirming the probable toxic cause of the effect on shrimp growth. Results also prove that biogenic amines are not the toxic agent, and have to be considered only as useful indicators of the raw material spoilage (see part 5 of the present report for recommended concentrations).

Reference Papers (rfp) (these papers are annexed in extenso to the report)

rfp1 Ricque-Marie, D., Abdo-De la Parra, Ma.I., Cruz-Suárez, L.E., Cuzon G., Cousin, M., AQUACOP and Pike, I.H., 1998. *Raw Material Freshness, a Quality Criterion for Fish Meal Fed To Shrimp.* Aquaculture (Elsevier) 165 (2) 95-109.

rfp2 Tapia-Salazar M., Ricque-Marie D., Cruz-Suarez L.E., Opstvedt J., Nygård E. and Pike I.H., 1998c. *Reduced performance from fishmeal made from stale fish is not due to increased content of biogenic amines 2. Studies with blue shrimp (*Litopenaeus stylirostris *S).* Submitted to Aquaculture Nutrition in December 1998 (once IFOMA agreement was obtained).

rfp3 Tapia-Salazar, M., Ricque-Marie, D., Cruz-Suárez, L.E. and Pike, I.H., 1998e. *Effect of raw material deterioration on the nutritional value of anchovy meals for very small Penaeus stylirostris juveniles.* Submitted to Aquaculture (Elsevier) in June 1998.

Communications to the Scientific Community

(see references in alphabetical list of literature cited or chronological list of contributions issued from the project)

Results from the F1 and F2 trials were presented first as a poster at a joined meeting of the World Aquaculture Society (WAS) and European Aquaculture Society (EAS) (Abdo *et al.*, 1993), and then by oral presentations at various meetings (Cruz-Suárez & Ricque-Marie, 1995[a], 1995[b]; Cruz-Suárez, 1997[a], 1997[b]; Tapia-Salazar, 1997), and in a paper published in Aquaculture (Ricque *et al.*, 1998 = reference paper rfp 1).

Results of the three trials (F3, F4 and F5) run at IFREMER-COP, Tahiti with the Chilean fish meals (1st set) on *P. monodon, P. vannamei* and *P. stylirostris*, were joined with those of F1 and F2 in the paper published in Aquaculture (Ricque *et al.*, 1998 = rfp1).

Results from trial F6 were presented in Monterrey, Mexico (Tapia-Salazar *et al.*, 1997), in Las Vegas, Nevada (Tapia-Salazar *et al.*, 1998[a]), in Monterrey, Mexico, and in Las Palmas, Spain (Tapia-Salazar *et al.*, 1998[b]). A paper has been prepared for publication in Aquaculture Nutrition (Tapia-Salazar *et al.*, 1999a = rfp2)

Another paper has been prepared from the trial F7 results and sent to Aquaculture (Tapia-salazar *et al.*, 1999b = rfp3). These results were also presented at the WAS US Chapter meeting "Aquaculture America'99" in Tampa, FL, January 27-30, 1999 (Tapia-Salazar *et al.*, 1999 d).

II. GIZZARD EROSION PRODUCING FISH MEALS

Objective 2: Evaluate Gizzerosine Effect on Shrimp Physiology

Ten years ago, in a growth trial that was designed to evaluate the effects of increasing levels of a Mexican fish meal made from tuna offal, very poor growth and mortality led to suspect some problems of processing (overheating) associated with the histidinic nature of tuna flesh (Cruz-Suarez *et al.*, 1992). This finding gave rise to the present objective in the project.

The study of toxins formed when fish meals are overheated has been initiated as a consequence of the major economic losses experienced in the production of poultry due to an epidemic outbreak of gizzard erosion and black vomit in Japan. One of the agents responsible for these adverse effects in poultry is gizzerosine which is formed during inadequate fish meal processing and which is related to the quality of the raw product used (pressed or concentrated fish) and to the heating process.

It seems that this problem is confined only to anchovy meals and on occasion to mackerel meals (fish with a high red muscle content, so called "histidine fish") when processed in dryers where a small proportion of fine material is trapped and consequently over heated (100°C), thus leading to the formation of toxic gizzerosine (Pike, 1994). The gizzerosine is formed by precursors which develop during oxidative decarboxylation of histidine when fish spoils and consists of a compound between histamine and the epsilon radical of the amino acid lysine ligand bound to protein. This reaction is catalysed by high drying temperatures but also depends on the time the product spends in the dryer.

The gizzerosine content of fish meal can be quantified by liquid chromatography, though it is difficult to standardise because the compound is present only in small quantities. For this reason, there is no reliable standard chemical analysis at present for the routine classification and certification of the various toxicological characteristics found in fish meals. However, thanks to initial contacts with IFOMA and partial funding by Pesquera Iquique-Guanaye, a Chilean fish meal manufacturer, we were able to get the pure gizzerosine from Dr. Lermonth, CSIR division of Food Science, Pretoria, South Africa, and investigate if shrimp respond to it.

A new method to determine fish meal quality, called "biotoxicological analysis", has been developed in Chile (Casto-Campos, 1990). The method consists of growth trials in chicken to assess the various toxic effects that can be caused by fish meals. This analysis was initially developed to evaluate the degree of toxicity associated with the presence of gizzerosine, but it also detects evidence of ulcers associated with high levels of histamine and other substances causing gizzard erosion in chicken. This concept has made it possible to certify ingredients used not only by the poultry industry but also by the entire animal feed industry. Fundación Chile uses a highly standardised analytical technique to certify a large percentage of Chilean fish meals for local and foreign markets. The analysis consists of feeding one day old broiler chicken with a diet containing 50% of the meal to be analysed, determining gizzard erosion after 7 days and allocating an individual value of 0, 1, 2

or 3, which is then used to calculate the biotoxicological score for the tested fish meals. Fish meals can be classified into 4 different categories depending on their biotoxicological score (Table II.1). Note that biotoxicological score by Fundación Chile is not the mean of individual scores of the chicken used in the feeding trial, and correspond to the weighted incidence of moderate or severe lesions of congestion, erosion, haemorrhages, necrosis or ulceration of the gizzard epithelium (the number of chickens with a score of 2 is multiplied by that score (2) and those with a score of 3 by that score (3) and so on) (see Cruz-Suarez *et al.*, 1999[a] = reference paper 4, page 2, for details)

Table II.1 Classification of fish meals by Fundación Chile depending on their biotoxicological score.

Classification	Score
Normal	0.1 to 0.5
Low toxicity	0.5 to 1.0
Medium toxicity	1.0 to 1.5
High toxicity	1.5 to 3.0

The score values are used in the market to trade fish meal for different animal species according to the individual sensitivity of the species. Normal score meals (0.1 to 0.5) are used in Chile and other nations for the poultry and aquaculture markets; 0.1 to 0.8 score meals are exported to the South African poultry markets; low and medium score meals (0.8 to 1.5) are marketed for animal feeds in general but with some restrictions, while the high score meals can only be used when mixed with meals of lower scores to obtain marketable score meals.

It is therefore of prime importance to know the response of shrimp to this new classification criterion in order to facilitate trading and insure adequate quality of fish meal used in shrimp diets.

Material and Methods

Fish Meal Samples

Three different sets of Chilean fish meals classified upon their gizzard erosion score in chicken by Fundacion Chile (Table 3), were assayed on *Litopenaeus vannamei* in various types of trials. The first set was constituted of two normal fish meals (Na and Nb) and two fish meals of medium toxicity (Ma and Mb). The other two sets were constituted of four fish meals each, one in each category.

Table II.2 In Chicken Toxicological Scores of the Tested Chilean Fish Meals

	Toxicity Level			
	None	Light	Medium	High
1st. set	0.1 (Na) & 0.1 (Nb)	-	1.1 (Ma) & 1.4 (Mb)	-
2nd. Set	0.1	0.9	1.3	2.0*
3rd. set	0.1	0.8	1.3	2.3

*High scored sample obtained by heating the fish meal scored 1.3 of same set at 100°C for 5 hours.

Gizzard erosion experiments: G trials

Eleven trials of different types were carried out on *P.vannamei* juveniles with the gizzard-erosion-producing-fish meals (Table II.3). Growth trials were designed as typical growth trial at Programa Maricultura, already described for the study on the effects of raw material freshness (page 5, bottom). Digestibility trials are described in part 3 of this report. Digestive transit time trials were realised with artificially coloured diets by changing the colour during feeding and measuring time for the first emission of coloured faeces.

Table II.3 Gizzard erosion score trial: G trials

Trial	Trial Description	Ref. Paper
G1	*Normal and Medium scored fish meals and synthetic gizzerosine effect on very small P. vannamei* : Growth trial by Isabel Abdo (M.Sc. thesis) on 4 fish meals (1st. set) included at 30%, plus 4 gizzerosine supplement levels: 8 diets, initial shrimp weight 0.070 g. (42 days):	***rfp4***
G2	*Normal and Medium scored fish meals effect on P. vannamei juveniles digestive transit time:* Digestive transit time trial by Mario A. Morales-Loa (graduate thesis, 1996) on five fish meals: four from the 1st. set and also the PROESA2 fish meal (PROESA1 was assayed previously in a feeding trial for Dr. Opstvedt with 3 other Norwegians fish meals): 5 diets, initial weight 1.0g.	
G3	*In vivo digestibility of Normal and Medium score fish meals, and two Norwegian herring meals processed at low and high temperature:* Digestibility trial by Martha Nieto-Lopez (graduate thesis, 1995) on the 1st. set fish meals, plus IFOMA 163 and 539 herring meals, and Mexican PROESA2 fish meal: 8 diets including the reference diet without fish meal, initial shrimp weight .35, .45 and .89 g.	***rfp6***
G4	*Effects of Normal, Low and Medium score fish meals, and an overheated one, on small shrimp:* Growth trial by Isabel Abdo (M.Sc. thesis, 1994) on the 2nd. set fish meals (included at 30%): 4 diets, initial shrimp weight 0.260g. (28 days).	***rfp5***

Table II.3 Gizzard erosion score trial: G trials (Continued)

G5	*Effects of Normal, Low and Medium score fish meals, and a burnt one, on P. vannamei juveniles digestive transit time:* Digestive transit time trial by Mario A. Loa (graduate thesis, 1996) on the 2nd. set fish meals and PROESA2 fish meal: 5 diets, initial shrimp weight 1.1g.	
G6	*In vivo digestibility of Normal, Low and Medium score fish meals, and an overheated one*: Feces collection trial by Martha Nieto (graduate thesis, 1995) on the 2nd. set fish meals and reference diet (same reference diet as in G3): 5 diets, initial shrimp weight 0.88g.	***rfp6***
G7	*Effects of Normal to High score fish meals at a 50% dietary level on shrimp juveniles:* Growth trial by Mireya Tapia (M.Sc. thesis, 1996) on the 3rd. set fish meals: 4 diets, initial shrimp weight 1.04g. (28 days).	***rfp7***
G8	*In vivo digestibility of Normal to High score fish meals in juveniles:* Digestibility trial by Mireya Tapia-Salazar (1996) and other students on the 3rd set fish meals: 8 diets = 4 diets of G7 (fish meal inclusion level 50%) + 4 diets where the low toxicity fish meal (included at 30%) was replaced by the high toxicity fish meal at 0, 10, 20 and 30 % levels, were tested on shrimp juveniles (1.3 g initial weight).	***rfp7***
G9	*Effect of Normal to High score fish meals on very small shrimp:* Growth trial by Mireya Tapia (M.Sc. thesis, 1996) on the 3rd. set fish meals: 8 diets (same as G8), initial shrimp weight 0.17g.	***rfp7***
G10	*In vivo digestibility of Normal to High score fish meals in larger juveniles:* Digestibility trial by Mireya (M.Sc. thesis, 1996) on the 3rd set fish meals: 4 diets including 30% of the fish meals. Initial shrimp weight 2.5g.	***rfp7***
G11	*Effects of Normal to High score fish meals at a 30% dietary level on shrimp juvenile:* Growth trial by Mireya (M.Sc. thesis, 1996) on the 3rd set fish meals: 6 diets including 30% fish meal (4 diets with the different score fish meals and 2 diets where the 0.1 fish meal was partially replaced by the 2.3 fish meal included at 10 and 20%). Initial shrimp weight 1.2g.	***rfp7***

To investigate the effect of gizzerosine on *Penaeus vannamei*, a growth trial (G1) was carried out on very small shrimp (66mg) with diets containing increasing levels of synthetic DL-gizzerosine (1, 3, 6 and 9ppm).

To investigate the effect on shrimp of fish meals that cause gizzard erosion in poultry, 5 growth trials were carried out at U.A.N.L. (G1, G4, G7, G9 and G11), using three sets of commercial Chilean fish meals of different biotoxicological scores, selected and certified by Fundación Chile:-

1st set:	0.1a, 0.1b, 1.1 and 1.4 score fish meals	Trial G1
2nd set:	0.1, 0.9, 1.3 and 2.0 score fish meals	Trial G4
3rd set:	0.1, 0.8, 1.3 and 2.3 score fish meals	Trials G7, G9 and G11

The three sets of fish meals were evaluated by including them in iso-energetic compound diets at different inclusion levels (30 or 50%) and feeding them to *P.vannamei* of different size classes. Each set was evaluated in a separate growth trial over 28 days, using a different shrimp initial size for each trial. Only set 3 was evaluated on both small (0.17g) and large (1g or larger) shrimp.

Results Compendium

Effect of a Pure Gizzerosine Supplement in Shrimp Diets

The inclusion of gizzerosine significantly increased mortality even at low inclusion levels, thus proving the toxicity of this substance on shrimp. In view of this result, and also of the mortality observed with the two medium score fish meals tested in the same trial (G1, reference paper rfp 4), it was decided to investigate more extensively the toxicity of fish meals causing gizzard erosion in chicken.

Biotoxicological Score and Shrimp Nutrition

A comparison of the composition of the fish meals samples showed that those with the same gizzard erosion score displayed great variations in some of the chemical quality parameters, especially those indicating the degree of raw material freshness (histamine and free fatty acid contents).

Effect on Growth and Feed Conversion Ratio

Of the two fish meals with high score, only one (score 2.0 of set 2) led to significantly reduced growth (trial G4, rfp 5). This fish meal was produced by heating the 1.3 score fish meal of the same set at 100°C for 3 hours at laboratory. This treatment resulted in a significant reduction of the digestibility (trial G3, rfp 6) and an also significant and drastic increase of the feed conversion ratio (from 1.5 to 3.3)(trial G4, rfp 5). Therefore, protein degradation and thus reduced digestibility, due to overheating, was probably more responsible for the adverse effect on growth than biotoxicological score.

Of the 4 fish meals of medium score (1.0-1.5), only one (score 1.3 of set 2), led to significantly reduced growth (trial G4, rfp 5). This fish meal contained high levels of histamine (1425ppm) and free fatty acids (10.5%), an indication of reduced raw product freshness at fish meal processing, which is sufficient to explain reduced growth, as seen in part 1 of the present report. The three other medium score fish meals, tested in trials G1,G7,G9 and G11, had no effect on growth.

In shrimp fed low toxicity fish meals (scores 0.8, 0.9 and 1.1), a slight increase in growth (not significant) was observed when compared to the control (score 0.1). Moreover, a significant reduction in FCR (i.e. improvement) was found for low toxicity fish meals (trial G11, rfp 7).

Effect on Shrimp Survival

When viewing shrimp survival it can be seen that of the 5 growth trials carried out, only 2 trials, those using smaller shrimp (G1 and G9), displayed significant mortality before 28 days. These mortalities were observed for three medium score fish meals (of scores 1.1 and 1.4 in trial G1, included at 30% in the diet, and score 1.3 in trial G9, included at a 50% dietary level), not for the high score fish meals. Two of the fish meals involved with mortality (score 1.1 of set 1 and 1.3 of set 3) were characterised by high levels of histamine (4840 and 1897 ppm respectively) and free fatty acids (9.7 and 9.3% respectively). Again, these values indicate reduced raw product freshness at processing, which could be responsible of the mortality for the very small shrimp used in trials G1 and G9, as seen in part 1 of the present report.

Effect on Digestibility

Except for the overheated fish meal sample, no difference in digestibility was observed among the tested fish meals, and this laboratory treated sample constituted also the unique case of significantly poorer food conversion ratio than controls (normal score fish meal) among the three sets of fish meals causing gizzard erosion in chicken (Cruz-Suarez *et al.*, 1996[a], Cruz-Suarez *et al*, 1999b = rfp 5).

This demonstrates that the toxic effect in chicken is probably independent of the nutritional quality of the protein in fish meal (digestibility). This is in accordance with the theory on gizzerosine formation, which suggests that this compound is produced when pockets of fine fish meal particles are trapped in the dryer and overheated as a result; meanwhile the bulk remains intact.

The Effect of Replacing Normal Fish Meal (Score 0.1) with High Score Fish Meal (2.3) on *Penaeus vannamei*

Highest survival and best (lowest) feed conversion ratios were observed in shrimp fed the diet containing 20% of the score 0.1 fish meal and 10% of the one with score 2.3 (trials G9 and G11, rfp 7). In contrast, the conversion ratio increased when a larger proportion (20 and 30%) of the 2.3 score fish meal was included in the diet.

A possible explanation is that the proportion "2/3 normal score + 1/3 high score" tends to emulate a fish meal with low score, which proved to provide better feed conversion ratios and slightly better growth. However, this is in agreement with the general benefit observed when mixing various fish meals as the dietary protein source.

Conclusion

Except for one case where reduced survival in 66mg shrimp was obtained (1.3 score fish meal of set 1), no detrimental effect could be attributed to the fish meals of

medium and high score. Moreover, a slight benefit occurred when using a low score fish meal, or a mix of 2/3 normal + 1/3 high score fish meals.

It seems therefore reasonable to recommend for shrimp feed manufacturers the use of low biotoxicological score fish meals, or of a small proportion of medium or high score fish meal, provided that raw material freshness indicators have normal values.

Reference Papers

rfp 4 Cruz-Suárez L.E., Abdo-De La Parra Ma.I., Ricque-Marie D., Lara C. and Castro-Campos E., 1999a. **Gizzard erosion score in chicken as a quality criterion for fish meals fed to the American pacific white shrimp *Litopenaeus vannamei*: I.- Effect of medium scored fish meals and synthetic DL-gizzerosine.** Final report for research grant C11*-CT93-0300, Commission of the European Community, reference paper 4.

rfp 5 Cruz-Suárez L.E., Abdo-De La Parra Ma.I., Ricque-Marie D., and Galleguillos M., 1999b. **Gizzard erosion score in chicken as a quality criterion for fish meals fed to the American pacific white shrimp *Litopenaeus vannamei*: II.- Effect of low to medium scored fish meals and an artificially produced high scored fish meal.** Final report for research grant C11*-CT93-0300, Commission of the European Community, reference paper 5.

rfp 6 Cruz-Suárez L.E., Ricque-Marie D., Nieto-López, M.G, Mendoza-Alfaro R., and Galleguillos M., 1999c. **Gizzard erosion score in chicken as a quality criterion for fish meals fed to the American pacific white shrimp *Litopenaeus vannamei*: III Digestibility of low to medium scored fish meals and of a sample with artificially produced high score.** Final report for research grant C11*-CT93-0300, Commission of the European Community, reference paper 6.

rfp 7 Tapia-Salazar, M., Cruz-Suárez, L.E., Ricque-Marie, D. and Galleguillos, M. 1999c. **Gizzard erosion score in chicken as a quality criterion for fish meals fed to the American pacific white shrimp *Litopenaeus vannamei*: IV.- Evaluation of commercial samples of low to high scored fish meals on very small and small shrimp.** Final report for research grant C11*-CT93-0300, Commission of the European Community, reference paper 7.

Communications to the Scientific Community

Results from G1 were presented first at the WAS annual meeting in New Orleans (Cruz-Suárez *et al.*, 1994), and then mentioned in synthesis papers on this subject (Cruz-Suárez & Ricque-Marie, 1995[a], 1995[b]; Tapia-Salazar, 1997; Cruz- Suárez *et al.*, 1998b)

Results of trials G2 to G8 were presented in the WAS meeting AMERICA'96 (Cruz-Suárez *et al.*, 1996[a]), and also in synthesis papers (Cruz-Suárez & Ricque-Marie,

1995[a], 1995[b]; Cruz-Suárez *et al.*, 1997[a], 1997[b]; Tapia-Salazar, 1997; Cruz- Suárez *et al.*, 1998b).

Results from G9, G10 and G11 were presented as posters in two Nutrition meetings held at College Station, TX, or Monterrey, NL (Cruz-Suárez *et al.*, 1996[b] and 1996[c] respectively) and then in synthesis presentations (Cruz-Suárez, 1997 [a] and 1997[b]; Tapia-Salazar, 1997; Tapia-Salazar *et al.*, 1997).

Reference papers 4 to 7 have not been submitted for publication yet.

III. *IN VITRO / IN VIVO* DIGESTIBILITY METHODS FOR SHRIMP

Objective 3: Standardize an *in vitro* digestibility method with trypsin and determine its relationship with pepsin digestibility and chromic oxide apparent digestibility.

Introduction

Feed digestibility is clearly the optimal, empirical method for measuring nutrient availability of a feed (Schneider and Flatt 1975). A formulated feed can be well balanced and contain all of the dietary essential nutrients but still not produce good growth because the nutrients are not readily available. The true nutritive value of a formulated feed is ultimately dependent on the bioavailability of its nutrients and not simply its composition (Lee and Lawrence, 1997).

Considering how useful knowledge of the digestibility of the feedstuffs commonly used in shrimp feeds would be to crustacean nutritionists and feed companies, the determination of *in vivo* apparent digestibility values of these feedstuffs should be placed high on the list of fundamental research needed in crustacean nutrition.

Feed digestibility has become of even greater interest to aquaculturists recently due to the need for low pollution feeds (Cho *et al.* 1993, 1994; Lawrence and Lee, 1997). Strict environmental regulations on effluents and high treatment cost favor highly assimilable feeds that result in lower nitrogen and phosphate waste outputs. Production at intensive levels will not be possible in the future without carefully considering the environmental effects of feeds and feed management. The use of digestibility data will be important for the formulation of low pollution feeds by reducing the amount of empirical environmental chemistry required to certify a feed as a low pollution feed (Cho *et al.*, 1994). In fact, environmental regulations may have a greater effect in expanding aquatic feed digestibility research in the future than the needs of applied nutritionists.

The methodology used to obtain *in vivo* digestibility values has varied greatly, necessitating the establishment of some standards for the handling of the experimental animals and feed and fecal samples. On the other hand, feed manufacturers have to make decisions quickly when buying, combining or processing protein ingredients and will take actual digestibility results into account only if rapid analytical methods are available. This leads to the development of *in vitro* methods giving quick results, which have to correlate well with the *in vivo* figures in shrimp, thus justifying the present objective in the project.

In contrast to vertebrates, digestion in shrimp does not involve acidic hydrolysis with pepsin, and the main protease is trypsin, which is active at neutral or slightly basic pH. This fact seems important from nutritional and quality control points of view, since variations are expected with respect to the figures obtained for vertebrates.

The work on this objective was organised into two sections:-
(a) determination of *in vivo* apparent digestibility of a series of fish meal samples

(b) application of *in vitro* digestibility methods to the same samples (pH Stat method with shrimp enzymatic extracts and Torry-modified digestibility test with dilute swine pepsin). The relationship between the results obtained by these three different methods were then examined.

Material and Methods

(a) In vivo determination of apparent digestibility of fish meals in shrimp

The fish meal digestibility was determined according to the substitution principle applied to salmonids by Cho and co-workers (Cho and slinger, 1979). The test ingredient (fish meal) was substituted for 30% of a complete basal diet composed of practical feed ingredients (see table 3, in rfp 9 = Cruz-Suarez *et al.*, 1999d). Basal diet formulation was thus different from that proposed in the original project proposal (Akyama´s basal formula), which included more than 70% fish meal, and did not allow for the associative effects with the other ingredients present in a complete diet. Ideally, there should be no interaction between the test ingredients and the basal diet, and the results should be independent of the level of inclusion of the test ingredient, but a 30% inclusion level of test ingredient has been commonly been used in aquatic digestibility studies, in order to obtain a better representation of commercial feeds.

The apparent digestibility values of the basal and test diets are then determined *in vivo* by an indirect method using 1% chromic oxide as an inert marker which avoids the necessity of collecting all the faeces of the animals in experimentation.

The analytical methods for chromic oxide and protein determinations in shrimp faeces were adapted for small shrimp (1g individual weight). By means of a slight modification of the Kjeldahl micro-method using Tecator equipment, a single sample of only 30 mg dry matter is sufficient to measure protein and chromic oxide. In this way, *in vivo* digestibility figures can be obtained after a few days of faeces collection, even with relatively small shrimp, compared to the size commonly used for digestibility studies, *i.e.* 15-25g late juvenile shrimp. This analytical method is described in the reference paper rfp8 (Nieto *et al.*, 1999).

Shrimp (*P. vannamei and P. stylirostris*) were obtained from captive stocks located on the Mexican Pacific coast (Cib-Nor and Super-Shrimp) or in Tahiti (IFREMER-COP). The average initial weight ranged from 250 mg to 17 g. Shrimp were stocked in glass-fiber tanks as part of a closed recirculating system at UANL or an open system at IFREMER. Prior to each experiment, the shrimp were acclimated for at least 1 week until behaviour was normal. Before faeces collection, shrimp were fed the experimental diet (basal or test diet) for at least three days. Shrimp were then starved for 24 hours, the bottom of the tank cleaned thoroughly, and diets containing 1 % chromic oxide fed twice a day to the apparent satiety. Faeces were collected from one to three hours after feeding.

The *in vivo* method was first applied to the different sets of fish meals causing gizzard erosion. These digestibility trials have been classified already in the "gizzard

erosion" series (G3, G6, G8, and G10, see Chapter II of the present report, pp9-10) since they were principally designed to characterise fish meals producing gizzard erosion (Nieto-Lopez, 1995; Tapia-Salazar, 1996). However, trial G3 included two herring meals (539/93 and 163/94) specially selected to run in *vivo* digestibility studies in different laboratories.

A set of 12 fish meals (including 539/93 and 163/94) and 1 fish hydrolysate was sent by IFOMA to run *in vivo/in vitro* digestibility studies in shrimp, together with new samples of the 1st set of 3 fish meals used in the "raw material freshness" trials. These 16 samples, processed by commercial plants with different drying methods from differents raw materials (these factors presumably affecting protein digestibility), were thus tested on live shrimp in a digestibility trial (D1) and in a series of laboratory trials (D2) (Table III.1).

Table III.1 *In Vivo* and *In Vitro* Digestibility Trials to Test a Series of 16 Commercial Samples: D Trials

Trial	Trial Description	Ref. Paper
D1	*In vivo digestibility of 15 fish meals and one fish hydrolysate in very small white shrimp:* Digestibility trial by Martha Nieto (Ph.D. thesis) on 15 fish meals and 1 fish hydrolysate: 17 diets (16 test diets + 1 basal diet), tested on three replicate groups of *P. vannamei* juveniles (0.25g, 0.40 and 0.55 g average initial weights).	rfp 8+9
D2	*In vitro digestibility analysis of 15 fish meals and one protein hydrolysate:* 15 fish meals and one fish hydrolysate, and the resulting 17 diets were analyzed for their digestibility *in vitro* by the pepsin method (Torry modified), and by the pH-Stat method using a *P. vannamei* hepatopancreas crude extract of known proteolytic activity.	rfp 8+9

(b) *In vitro* digestibility methods: shrimp-pH Stat method and dilute-pepsin digestibility test

In the shrimp pHStat method, a sample suspension of fixed protein concentration (80mg/ml) is incubated for 30 min in the presence of the shrimp midgut gland extract, and the pHStat device automatically adds the sufficient volume of 0.1M NaOH to maintain the pH at 8.0. Total volume added is proportional to the number of peptide bonds hydrolysed, which is calculated as percentage of the total bonds in the sample, and named Degree of Hydrolysis (% DH). Since fish meals were found to be subject to auto-hydrolysis, the volume added to maintain the pH of the suspension in absence of shrimp extract is subtracted from the former to calculate a corrected value (% DHcor.) which is independent of the auto-hydrolysis capability of the tested product.

The dilute pepsin method (modified by the Torry laboratory, U.K.) uses a 1000 fold lower pepsin concentration than the AOAC method. 1g sample is incubated for 16 h in a weak acid solution and the reaction solution filtered to retain the insoluble part, which is then analysed by Kjeldahl. Insoluble protein is subtracted from the acid-insoluble protein (measured by a similar incubation in absence of enzyme) and the resulting estimation of soluble protein is expressed as a percentage of the acid-insoluble protein, giving the % acid-corrected pepsin digestibility. This procedure is useful to avoid an overestimation of the enzymatic action in fish meals rich in soluble protein.

Results Compendium

Results from D1 and D2 are presented in the reference paper 9 (Cruz *et al.*, 1999d).

(a) I*n vivo* Apparent Digestibility

Application of this method (*in vivo* method by Cho) to the diets containing fish meals causing gizzard erosion demonstrated that digestibility of these fish meals was not modified in medium and high score fish meals (see reference paper rfp 6), confirming the results of the growth trials (rfp 4, 5 and 7). Two trials on the digestive transit time (time of the first feces emission) obtained with the same fish meals (G2 and G5) indicated an inverse correlation with the digestibility figures. This is explained by the anatomy of the digestive tract in shrimp, since gross non-digestible particles are directed rapidly from the stomach to the posterior intestine and rectum by a sieve which allows only the fine particles (those which were reduced in stomach as in a chicken gizzard) to penetrate the hepatopancreas, where final digestion and absorption occur.

On the other hand, when comparing digestibility figures obtained by the *in vivo* method on such small shrimp, with figures obtained on 17g shrimp at IFREMER, Tahiti (experimental procedure presented in Cuzon & Aquacop, 1995, and results given as a personal communication), it appears that the range between low and high values is wider in young shrimp (1g, 0.4 or 1.2 g) than in older shrimp. This observation was made first on two herring meals (163/94 and 539/93) which were produced from the same raw material, but processed at high and low temperature respectively:

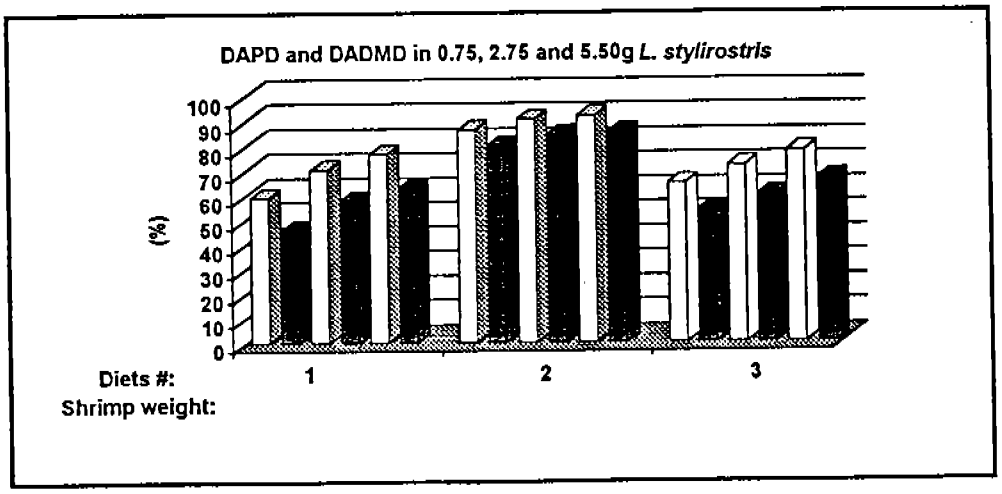
Table III.2 Apparent *in vivo* protein digestibility (%) of two fish meals on different species and sizes

	Fish meal 63	Fish meal 539
Mink (IFOMA)	84	95
P. stylirostris (17g) (IFREMER)	81	94
P. *vannamei* (1g) (UANL, trial G3)	41	83
P. vannamei (0.4g) (UANL, trial D1)	76	88
P. stylirostris (1.2g) (UANL, trial ED3)	39	72

A further trial carried out with commercial feeds (produced in a commercial plant but including 1% chromic oxide) demonstrated a clear effect of the shrimp size on *in vivo* digestibility: apparent digestibility was higher in larger *P. stylirostris* (Fig. III.1).

Results from the digestibility experiment D1 on 16 commercial fish meals and one fish hydrolysate showed that in terms of digestibility small shrimp are quite sensitive to alterations of the fish meal quality resulting from an excessive temperature exposure in the dryer. In contrast, raw material spoilage had no significant effect on fish meal digestibility in shrimp; protein digestibility increased slightly with the raw material spoilage, probably due to an increased soluble protein content, which in turn *caused* an overestimation of the apparent digestibility due to dietary leaching in sea water before ingestion by the shrimp.

Figure III.1 Apparent Protein and Dry Matter Digestibility of Three Commercial Feeds in Pacific Blue Shrimp Juveniles of Three Different Sizes (0.75, 2.75 and 5.5g initial weight)



DAPD = Dietary apparent protein digestibility (white)
DADMD = Dietary apparent dry matter digestibility (grey)

Fish meal apparent protein digestibility in shrimp (FMAPD) varied between 75.8 and 98.7% (Table 6 in rfp 9). Correlation between protein digestibility in shrimp and other species was found to be slightly significant in two of three trials for salmon, and not significant for the other trials on salmon, trout or mink. However, fish meal dry matter digestibility in shrimp (FMADMD) was significantly correlated with protein digestibility in salmon, when fish meal protein content was higher than 65% and ash content lower than 15% (Table 7' in rfp 9).

Lack of correlation of digestibility in mink with the other species was unexpected and has to be related to a bias which affected the sampling process: digestibility in mink was determined on a first series of samples (about 2 kg each) extracted from fish meal production batches of up to hundreds of tons, digestibility values in trout and salmon were then determined on another sample (about 200kg) extracted from the same production batches. But, since determinations were made on different samples

from sometimes very large batches, correlation between in mink and in salmonid values is therefore expected to be sub-estimated. Aliquots received at UANL were from the second sample as for trout and salmon determinations.

(b) *In vitro* digestibility: pH Stat method and Torry- pepsin digestibility test

Instead of using commercial bovine trypsin in an *in vitro* model based on the AOAC pepsin digestibility test (as stated initially in the project proposal) enzyme extracts from the shrimp hepatopancreas were used in a pH Stat method, which has already proved to give good correlation with the *in vivo* method in shrimp (Ezquerra *et al.*, 1997) or in salmon (Dimes and Haard, 1994).

The degree of hydrolysis (DH) using the shrimp pH-Stat method ranged from 13.6 to 35.4% (Table 8 in rfp 9), and did not correlate significantly with FMAPD in shrimp, unless low protein/high ash fish meals were excluded from the data base (Table 12 in rfp 9). For fish meals with a protein content was higher than 65% and ash content lower than 15%, a highly significant regression equation was obtained (R^2=0.64, p=0.0006, n=14) (see Fig. 5 a in rfp 9):

$$FMAPD = 1.3\ DH + 61$$

During the *in vitro* digestibility studies, fish meal samples were found to contain a wide range of soluble protein, from 17 to 42% as fed, with an exceptionally high content in the fish hydrolysate (64%)(Table 4 in rfp 9). The degree of hydrolysis of the fish meals were significantly correlated with their acid soluble protein content (Table 13 in rfp 9).

Uncorrected pepsin digestibility ranged from 42.7% to 86.3%. The fairly low results obtained when compared to the literature may be because the concentration of enzymes used in the other studies was higher, as it is possible that the enzyme activity stated on the container, was not the actual activity of the enzyme. The method of using a lower enzyme concentration (0.0002%) might lead to slightly lower results, but the method is also more sensitive. Indeed, uncorrected dilute pepsin digestibility correlation with FMAPD in shrimp was highly significant (R^2=0.67, p=0.00008, n=16). Moreover, the regression was not affected by the presence of low protein/high ash fish meals and therefore might be applied to any fish meal:

$$\%\ FMAPD = 59.6 + 0.373\ (\%\ \text{uncorrected dilute pepsin digestibility})$$

Correlation of the uncorrected value with apparent digestibility in shrimp was also better than for the corrected expression, perhaps because pepsin digestibility (*not* corrected for acid soluble protein) and FMAPD (*not* corrected for dietary leaching before ingestion) are both prone to overestimate digestibility of fish meals rich in soluble protein.

Conclusions

Fish meal protein digestibility in shrimp (FMAPD) is strongly affected by the temperature exposure in the dryer, but not by the raw material freshness, and it increases significantly with the content of soluble protein. Slightly higher readings obtained with the anchovy meal made from spoiled raw material may be explained by its higher content of soluble hydrolysed protein.

FMAPD in shrimp was barely correlated with FMAPD in salmon, less with trout's, and not at all with mink's, but in the latter case, insufficient sampling precision may explain this result.

In vitro digestibility with shrimp enzymes using pH-Stat was significantly correlated with FMAPD in shrimp for fish meals which protein content is higher than 65% and ash content lower than 15% i.e. by excluding from the data base two low protein/high ash fish meals (made from white fish offal and menhaden).

Dilute pepsin digestibility, without acid-soluble protein correction, gave unexpectedly a better correlation with FMAPD in shrimp than the pHStat method using shrimp enzymes. This highly significant correlation was not affected by the presence of high ash/low protein.

Uncorrected dilute pepsin digestibility therefore seems to be the method of choice to evaluate fish meal digestibility for the shrimp industry. However, care must be taken to confirm its correlation with apparent protein digestibility in shrimp, when the latter is corrected for the loss of protein before ingestion.

Reference papers

rfp 8 Nieto-López, M.G., Cruz-Suárez, L.E. and Ricque-Marie, D., 1999. Crude protein determination in wet acidic digestion samples prepared for chromic oxide assay. Final report for research grant C11*-CT93-0300, Commission of the European Community, reference paper 8, 4p.

rfp 9 Cruz-Suárez, L.E., Nieto-López, M.G. and Ricque-Marie, D., 1999d. *In vivo* digestibility of different quality fish meals in shrimp and its correlation with digestibility in salmonids and mink, and with *in vitro* digestibility (dilute pepsin and shrimp pH-Stat). Final report for research grant C11*-CT93-0300, Commission of the European Community, reference paper 9, 37p.

IV. EFFECT OF DIGESTIBILITY ON SHRIMP GROWTH AND FEED CONVERSION RATE

Objective 4: Decrease environment pollution using more digestible and assimilable feeds giving better feed conversion ratios.

Introduction

Digestibility is affected by two of the main quality factors to be considered for fish meals used in aquaculture feeds: raw material type, e.g. whole fish or trimmings; and processing temperature exposure (Pike, 1998).

Raw material type has a direct influence on ash content meal, which varies depending on the fish species and whether whole fish or trimmings was processed. Protein digestibility tends to diminish when ash content in fish meals increase (Romero *et al*, 1994). Fish meals made from whole fish generally have an ash content in the range of 10 to 20%. Meals made from trimmings that contains bones will generally have an ash content above 17%. If the ash content is above 23%, the amino acid profile of the protein is likely to be less favourable, due to different amino acid balance in bone tissue and muscle.

Another main concern about protein quality comes with application of excessive heat processing and drying, since protein quality may decrease due to the combined effect of temperature and the period of exposure (Pike and Hardy, 1997). Fair and average quality (FAQ) products (temperatures of up to 95°C) are well digested by pigs and poultry, but not necessarily by salmonids. A new process involving a drying process with indirect hot air has been developed to produce so called low temperature fish meals (LT), where the true protein digestibility determined in mink or rat would be over 90%, and the apparent protein digestibility in salmonids over 89%. A regular fish meal (FAQ) would have a true digestibility value approximately 5 units lower, and poorer fish meals which have been overheated could be 10 units lower (80% protein digestibility in mink). LT fish meals will give better growth. For example comparing a low temperature and regular fish meal, where the former was 5% units higher digestibility than the latter, growth of Atlantic salmon smolts was around 15% faster (Opsvedt, 1989, in Pike and Hardy, 1997).

The effects on growth of the factors that affect fishmeal protein digestibility have not yet been determined in shrimp species. Preliminary digestibility studies with shrimp have been made with two herring meals (539 and 163) differing by about 10 units in protein digestibility in mink, as a result of different temperature exposure. *In vivo* digestibility figures were obtained in large *P. stylirostris* (17g), and in small *P. vannamei* (0.4-1g). Results from large shrimp were similar to those from mink, while from small shrimp they differed largely, displaying a much larger difference between values obtained for the low and high digestibility fish meals (see Table III.2 on p17 of the actual report). Other preliminary studies have demonstrated that fish meal digestibility in shrimp is not affected by raw material freshness (Cruz-Suarez *et al.*, 1999d = rfp 9), presence of gizzerosine (Cruz Suarez *et al.*, 1999c = rfp 6) or

oxidation (Montaño-Aguillar *et al.*, 1998 = rfp 12), but diminishes in presence of an elevated ash content.

Material and Methods

A pond trial was carried out with the aim of comparing the growth and feed conversion ratios obtained with shrimp fed diets including fish meals of different digestibility (Table IV.1).

Table 1 Characteristics of the selected fish meals

Fish Meals	9274 B	NoTM	MOTM	539/93	163/94
Protein (% as fed)	67.2	59.4	64.7	74.2	74.0
Lipid (% as fed)	11.1	8.6	9.3	8.9	10.0
Ash (% as fed)	13.3	27.6	21.0	10.2	10.3
Apparent protein digestibility in shrimp (%)		75.1 ±2.1	84.8 ±5.0	88.2 ±0.7	75.8 ± 1.6

Two Mexican tuna meals were chosen (NOTM and MOTM). These meals were known to have been made from trimmings with a high proportion of bones in this raw material. Their digestibility was evaluated at our laboratory (see trial O4 in chapter V of the present report, rfp 12 = Montaño-Aguillar *et al.*, 1998) and found different due to a change of the bone proportion in two adjacent samples taken at the same manufacturing plant. Higher bones proportion in raw material is indicated by the higher ash content in fish meal identified as NOTM.

Herring fish meals 539/93 and 163/94 were also chosen for this trial due to their well known digestibility in mink, salmon, and shrimp (see chapter III of the present report). Fish meal 163/94 was processed in the same plant from similar raw material as 539/93, but with a stronger temperature exposure in dryer, which provoked a severe drop in protein digestibility.

Finally, a Chilean mackerel meal specially selected for shrimp by Inual-Tepual was used as a commercial reference for fish meals used in shrimp culture in Latin America.

Chilean and Norwegian fish meals were made from whole fish.

These five fish meals were included in iso-proteic practical diets, the formula for which was similar to that of a commercial diet. Dietary inclusion levels of the fish meals were fixed for each diet to allow the fish meal to contribute for 50% of the total dietary protein content. These diets were then tested on shrimp held in cages in a production farm pond or in tanks at laboratory (Table IV.2).

Forty eight 1m x 1m x 1.2m Netlon cages were constructed, and assayed in the Aquastrat farm, Escuinapa, Sinaloa in spring of 1997, with difficulties to control

mortalities, probably due to a low health status in the cultured stock (wild *P. stylirostris* stock) (trial ED1, see Table IV.1 hereafter). A further trial, using a selected stock commercialised under the name of Supershrimp by Maritech, was successful and constitutes the basis of a now satisfactory management procedure for cages in pond trials (trial ED 2). In this second trial, the cages were installed in a pond where shrimp size was enough to avoid entry of smallest ones inside cages, but not too large to avoid problems associated with the harvest proximity. Shrimp were selected from the same pond to allow a comparison between growth inside and outside of cages. Density inside cages was 20 shrimp / $m^{2,}$ half the estimated density in pond (40 shrimp/m^2).

However, in view of the pond trial results, an *in vivo* digestibility trial was carried out in tanks at laboratory in order to evaluate the diets digestibility and confirm the fish meals digestibility, under uniform environmental conditions (recirculated synthetic sea water system)(trial ED 3). A 6^{th} diet (basal diet) was formulated in order to allow the determination of digestibility values in fish meals. This basal diet included same ingredients as in the five practical diets, except fish meal. Other ingredients were in same relative proportion (average) as in the practical diets tested for growth, and included 1% chromic oxide. Methods for collecting faeces and analysing chrome and protein are described in detail in reference papers 8 and 9 (Nieto-Lopez *et al.*, 1999, Cruz-Suarez *et al.*, 1999d).

After confirmation of the differences in digestibility between the experimental diets, and in view of the absence of relationship between digestibility and shrimp performance in ponds, it was decided to re-evaluate the same diets in tanks in a controlled environment. The same diets were tested again for growth in two trials at laboratory (trials ED 4 and 5, Table IV.1). Methodology of this kind of growth trials has been described in detail in reference papers 1, 2 and 3.

Table IV.2. Effect of Digestibility on Shrimp Growth and Feed Conversion rate: ED trials

Trial	Trial description	Ref. Paper
ED 1	*First intent:* Growth trial in pond cages by Mario Novales (Graduate student) on 5 fish meals: five diets tested on *P stylirostris* of 17g in intensive shrimp farm Aquastrat. Trial stopped after 10 days due to high mortalities provoked by environmental stress.	
ED 2	*Effect of fish meals of different digestibilities on shrimp growth and feed conversion in pond cages*: Growth trial in pond cages by Mario Novales (Graduate student) on 5 fish meals (one Chilean jack mackerel meal, two Mexican tuna trimmings meals, and two Norwegian herring meals): five diets tested on *P stylirostris* of 6.5g in pond A1 at Aquastrat. (21 days).	rfp 10

Table IV.2. Effect of Digestibility on Shrimp Growth and Feed Conversion rate: ED trials (Continued)

Trial	Trial description	Ref. Paper
ED 3	*In vivo digestibility of one Chilean jack mackerel meal, two tuna by products meals and two herring meals (539 and 163) in small P. stylirostris juveniles:* Digestibility trial at laboratory by Mario Novales (Graduate student) and Claudio Guajardo (technical assistant) on 5 fish meals: 6 diets (5 test diets + 1 reference diet) tested in tanks (recirculating system) on *P. stylirostris* of 1 to 1.5g initial weight, with 3 replicates per diet.	rfp 10
ED 4	*Effect of 2 herring meals of different digestibility on shrimp growth and feed conversion in tanks, at laboratory:* Growth trial at laboratory by Luis Omar Pena (Graduate student) on 2 herring fish meals (539, 163): two diets tested in tanks (recirculating system) on *P stylirostris* of 0.125g initial weight with 5 replicates (28 days)	rfp 10
ED 5	*Effect of 5 fish meals of different digestibility on shrimp performance at laboratory:* Growth trial at laboratory by Luis Omar Pena (Graduate student) on 5 fish meals (539, 163): five diets tested in tanks (recirculating system) on *P stylirostris* of 0.5g average initial weight with 4 replicates .(28 days)	rfp 10

Results and methodology of trials ED1 to ED5 have not been published yet, and are presented in a preliminary report (Cruz-Suarez *et al.*, 1999e = reference paper rfp10).

Results Compendium

Pond trial

Main results of pond trial ED 2 are given in table IV.3. The growth in ponds was disappointing in that it didn't reflect the digestibility differences with the same raw material types. The reasons for this are believed to be due to a relatively high dietary protein, which exceeded the shrimp requirements and problems in pond with dissolved oxygen concentration due to a phytoplancton crash in the middle of the experiment. Consequently, emphasis has been placed on the growth results achieved in tanks in absence of reliable pond growth figures.

However, best growth for the herring meals was a common result in pond and both laboratory trials.

Table IV.3 Shrimp performance in pond cages

	DIET 1 Jack-mack. 9274B	DIET 2 Tuna NOTM	DIET 3 Tuna MOTM	DIET 4 herring 539 (LT)	DIET 5 herring 163	P (ANOVA)
Initial weight (g)	6.535 ± .056	6.486 ± .068	6.501 ± .094	6.538 ± .140	6.495 ± .146	.9376
Final weight (g)	9.37 ab ± .44	9.44 ab ± .03	9.08 a ± .15	9.65 b ± .45	9.83 b ± .23	.0363
% weight gain	43.4 ab ± 6.9	45.5 ab ± 1.9	39.7 a ± 3.5	47.7 ab ± 6.2	51.4 b ± 6.3	.0650
Feed conversion ratio	1.97 bc ± .38	1.82 abc ± .03	2.03 c ± .24	1.60 a ± .12	1.64 ab ± .17	.0494
Survival at 21 days (%)	82.5	91.25	90.0	90.0	85.0	.4551

Digestibility trial

Highly significant differences were obtained between the protein digestibility of the tested fish meals (Table IV.4).

Table IV. 4 Protein digestibility in fish meals

	DIET 1 Jack-mack. 9274B	DIET 2 Tuna NOTM	DIET 3 Tuna MOTM	DIET 4 Herring 539 (LT)	DIET 5 Herring 163	Diet 6 REFER. DIET	P (ANOVA)
IAPD	71.6 c ± 2.6	58.9 b ± 6.3	62.1 b ± 4.2	72.1 c ± 2.5	39.4 a ± 7.8		0.0001

IAPD = Ingredient's (fish meal) protein digestibility

Whole jack mackerel meal was found to have a high digestibility similar to the LT herring meal. Large difference between LT herring meal and its overheated homologue was confirmed, but the tuna NOTM with a high ash content was only slightly less digestible than MOTM, in contrast with previous result by Montaño-Aguillar *et al.* (1998)(Table IV.2).

Laboratory trials

The first growth trial at laboratory (ED4), with post larvae of 0.125 g initial weight, failed to separate diets 4 (herring LT) and 5 (herring, overheated) in terms of growth and feed conversion ratio, after 28 days of experiment (Table IV.5).

Table IV.5 Shrimp performance in a controlled environment (trial ED 4)

Performance parameters	DIET 4 Herring 539 (LT)	DIET 5 Herring 163 (STANDARD)	F. prob.
Initial weight g	0. 12 ±0.00	0.13 ±0.00	
Final weight g	0.72 ±0.04	0.75 ±0.07	
% Weight gain (28 days)	477 ±28	500 ±63	0.4839
Feed conversion ratio	1.7 ±0.1	1.6 ±0.2	0.6653
Survival %	100	100	

The second growth trial at laboratory (ED 5), with post larvae of 0.5g initial weight, also failed to show a significant difference between the high and low digestibility herring meals, although growth and feed conversion ratio were slightly better for diet 4 (LT herring meal)(Table IV.5). It also failed to separate significantly the high and low digestibility tuna meals, although MOTM (low ash content tuna meal) had slightly better results than NOTM, as expected. In contrast, the difference between fish meals of different types were highly significant, with lower growth and poorer feed conversion for diets 2 and 3 (tuna by-product).

Table IV.6 Shrimp performance in a controlled environment (growth trial DP5)

Performance Parameters	**Diet 1 Jackmackerel 9274B**	**Diet 2 Tuna NOTM**	**Diet 3 Tuna MOTM**	**Diet 4 Herring 539 (LT)**	**Diet 5 Herring 163**	**F. PROB.**
Initial weight g	0.51 ±0.00	0.51 ±0.01	0.51 ±0.00	0.51 ±0.00	0.51 ±0.00	
Final weight g	1.88 ±0.10	1.54 ±0.19	1.60 ±0.28	2.09 ±0.08	1.86 ±0.06	
% Weight gain (28 days)	268b ±19	203 a ±39	213a ±56	308b ±17	264b ±12	0.0025
FCR	1.7a ±0.08	2.2c ±0.33	2.1bc±0.41	1.5a ±0.08	1.8ab±0.06	0.0060
Survival %	90.0	87.5	87.5	92.5	92.5	

Conclusions

Shrimp growth and feed conversion were firstly related with the raw material type: fish meals made from whole fish, such as Chilean anchovy or Norwegian herring, performed significantly better than those made of tuna cannery by-products. The reason for this is probably that fish meals made from whole fish (herring or jack mackerel) had a more favourable amino acid balance to allow the diets to cover the shrimp amino acid requirements. It seems therefore that digestibility figures may not be useful to explain or predict shrimp growth and feed conversion when dealing with fish meals made from different raw materials.

For fish meals made from the same raw material fed to shrimp, growth would be expected to reflect differences in protein digestibility. This requires further confirmation in larger numbers of fish meals from the same materials. The present work failed to demonstrate this principle for the following reasons:-

- although LT herring meal had far better digestibility than its homologue processed at higher temperature, both gave similar growth and feed conversion because their dietary inclusion level (25%) was so high that even the low digestibility herring meal over-supplied the amino acid requirement in shrimp.

- although diets including 29-31% tuna meals achieved lower growth in tanks than those with herring meals (probably due some limiting amino acid), the difference in digestibility between high and low ash tuna meals was too low to provoke a significant difference in growth.

Care should be taken, especially when testing fish meals made from high quality raw materials with very high protein content, not to exceed the dietary amino acid requirement. As mentioned above, this error probably occurred in the present experiment, by using diets including 25% whole herring meals, leading to dietary protein concentration between 35% and 37%. Although 35% is currently accepted as the dietary protein requirement for *L. stylirostris* small juveniles, it should be noted that this has been demonstrated with semi-purified diets including vegetable protein which amino acid profile is not ideal for shrimp, and that supplementing by pure amino acid is inefficient due to their leaching in sea water before feed ingestion by shrimp. Indeed, actual dietary protein requirements in *L. stylirostris* small juveniles has been recently re-evaluated in our feeding trial facility with practical diets including the Chilean fish meal 9274B, and found to be between 25 and 30% (Cruz-Suarez *et al.*, 1999 f).

Reference paper

Cruz-Suárez L.E., Ricque-Marie D., Novales-Terreros M. and Peña-Ortega L.O., 1999e. **Effect of fish meals of different digestibility on *L. stylirostris* held in cages in a pond or in tanks in a controlled environment**. Final report for research grant C11*-CT93-0300, Commission of the European Community, reference paper rfp 10.

V. OXIDATIVE RANCIDITY

Objective 5: Determine the effects of fish meals and fish oils rancidity when used in shrimp feeds.

Introduction

For shrimp and other marine aquatic species, nutritional quality of lipids provided by fish meals or fish oil is a direct function of their content of poly-unsaturated fatty acids (pufas), especially those unsaturated in position n-3, which are part of their essential requirements.

The amount of these n-3 pufas depends on various factors:-

Raw material type: certain species like anchovy and menhaden are higher in ω3 pufas than others like herring or pout. Season is also important, since winter catch can be lower in ω3 pufas.

Two types of degradation occur during different steps of the fish meal and oil manufacture:-

Hydrolytic rancidity: Starts during the raw material storage. Enzymes present in fish tissues, and principally in bacteria, break fat to free fatty acids. This process practically stops after cooking the fish. Therefore, percent free fatty acids gives a crude measure of fish freshness.

Oxidative rancidity: Starts during the drying process. Combined actions of oxygen and heat produce fatty acid radicals, and then peroxides radicals, very active molecules which will propagate the reaction creating new fatty acid radicals, intermediate products like peroxides, or final products like ketones and aldehydes, known for their bad flavour. Peroxides quantification is useful to monitor oxidative rancidity in progress, while thiobarbituric acid (TBA) and anisidine values allow to measure the accumulation of final products. Addition of an artificial anti-oxidant (Ethoxyquin = ETQ) in drying process or immediately afterwards stops the oxidation.

There are few experiments on the effects of oxidative rancidity in feeds for shrimp. Liao (1992) in Taiwan has described a red discoloration disease where shrimp fed rancid trash fish present an atrophy of the hepatopancreas, before dying. In the Philippines, De la Cruz *et al.* (1989) studied with *Penaeus monodon* the consequences of feed oxidation during storage in tropical conditions. They stored a feed (particularly high in fat: 17%) during 10 weeks at 0, 10, 28 and 40 degree Celsius, and found that rancid feeds diminish the growth, and eventually provoke moralities associated with necrotic lesions in the hepatopancreas. Koshio *et al.* (1994a) showed that medium oxidised fish liver oil (peroxide value = 50 meq/kg) had little effect on *P. japonicus,* but a detrimental effect on fish growth, especially if the diet was deficient in vitamin E (Koshio *et al.*, 1994b).

Experiments described hereafter were designed to determine what would be the maximum acceptable level of oxidation in fish oil for shrimp, in presence of vitamin E and ETQ (natural and synthetic antioxidants), to avoid any effect on growth and feed conversion. Second initial objective was to relate the chemical indicators values (peroxides, TBA and anisidine) to some toxic effect in shrimp. The effect of oxidised fish oil on dietary digestibility was then examined.

A second series of experiments was designed to test the effect of oxidation on the nutritional value of a Mexican fish meal made from tuna cannery trimmings in terms of growth and digestibility.

Materials and Methods

Growth and digestibility trials were conducted with oxidised oils or fish meals (Table V.2).

In a first trial (O1), 3 degrees of oxidation were tested for the menhaden oil supplemented to the experimental diets: low, medium and high with peroxide values of 6, 50 and 100 meq/kg oil respectively. The fresh, medium oxidised or highly oxidised oil samples were included in diets which were supplemented or not with vitamin E (DL-α-tocopheryl acetate 100mg/kg diet). An additional variant was introduced for the diets containing the highly oxidised oil, taking out the dietary ETQ supplement (Table V.1) (see details in Ricque *et al.*, 1999 = reference paper rfp 11).

Table V.1 Diet Formulation for Trial O1 - Experimental Design

	Degree of Oxidation			
Supplement	**Nil**	**Med.**	**High**	**High**
VitE 100ppm	No Yes	No Yes	No Yes	No Yes
ETQ 130ppm	Yes	Yes	Yes	No
Diet	1 2	3 4	5 6	7 8

A second trial (O2) was carried out to establish *in vivo* digestibility values for the diets used in the first trial (O1), but digestibility was not affected by any of the factors mentioned above (Gonzalez-Valadez, 1998).

Samples of hepatopancreas, muscle and eyes were assayed for their glutathion peroxidase activity. This enzyme is a key element of the cellular detoxification system against rancidity. The technique was acquired at the CIAD laboratory (Centro de Investigación en Alimentos y Desarrollo), Hermosillo, Sonora, México, with Dr. Jane Whyatt., where preliminary assays demonstrated for *the* first time the presence of Glutathion Peroxidase in wild shrimp. The presence of glutathion

peroxidase was confirmed in shrimp, and its activity was studied (optimum temperature range, effect of sample or extract freezing)(Ponce-Ambris, M.Sc. thesis in progress).

Oxidative rancidity in fish meals was tested on samples of tuna meal (TM) made from cannery waste, which is readily available in México, and particularly prone to oxidation due to the high iron content in the red muscle (pro oxidative agent). O3 and O4 trials were carried out with samples of TM stabilised by a 500 mg/kg ETQ supplement immediately after production (Non Oxidised TM, with peroxide value PV=9.8 meq/kg) or after 180 days at 4°C (Med. Oxidised TM, PV=47.6 meq/kg) or after an additional period of 21 days at summer ambient temperature in Monterrey, N.L. i.e. 25-45oC (Highly Oxidised TM, PV=97.5meq/kg). A second source of variation was introduced fortuitously due to variable proportions of bone in the adjacent samples taken at the processing plant for the manufacture of non-oxidised TM (28% ash) and medium oxidised TM+ highly oxidised TM (21%). The tuna meals, and a Chilean fish meal as reference, were included at 20% in the experimental diets (see details in Montaño-Aguillar *et al.*, 1998 = rfp 12).

Table V.2 Experiments on the effects of fish oil and fish meal oxidation: O trials

Trial	Trial Description	Ref. Paper
O1	*Effect of oxidised menhaden oil, vit. E and ethoxiquin on P.vannamei growth and survival:* Growth trial by Pablo San Martin (M.Sc. thesis, 1995) with fish oils of graded oxidation levels, with or without dietary vit E or ethoxiquin: 8 diets, initial *P. vannamei* shrimp weight 0.250g. (56 days). Complementary analysis were obtained afterwards from Novus (St Louis, Mo) and Roche (by Willy Schuepp, Basel, Swiss) on samples of feeds and shrimp respectively.	rfp 11
O2	*Digestibility of diets supplemented with oxidised menhaden oil, vit. E and ethoxiquin:* Digestibility trial by Pablo Gonzalez-Valadez (graduate thesis, 1998): 8 diets (same as R1), on *P. vannamei* of initial weight about 1g.	
O3	*Effect of tuna by-product meal oxidation on shrimp growth and survival:* Growth trial by David Montaño-Aguillar (M.Sc. thesis 1998) with three tuna meals of graded oxidation levels and one Chilean (anchovy) fish meal: 5 diets (including ref. diet without fish meal), tested on *P. vannamei* juveniles (0.5 g initial weight).	rfp 12
O4	*Digestibility of tuna by-product meals with graded oxidation levels:* Digestibility trial by David Montaño (1998): 5 diet (same as R3), *P. vannamei,* initial weight about 1 g.	rfp 12

Results Compendium

Results from trial O1 (Ricque *et al.*, 1999 = rfp 11) suggested that: -

1) Only a high level of oxidation in fish oil reduces the feed consumption, and, as a consequence, the weight gain. This is probably due to the low palatability of the oxidation products
2) The lack of supplementary vitamin E is responsible for heavy mortalities (> 40%), whatever the oil oxidation level; more over, the use of a very fresh oil, containing high levels of poly-unsaturated fatty acids, tends to increase the requirement for Vitamin E
3) A dietary ethoxiquin supplement is useful to avoid mortality (as a dietary vitamin E protector) and to maintain feed consumption and weight gain (as a dietary poly-unsaturated fatty acid protector)
4) Dietary vitamin E supplementation correlates with high levels of vitamin E in the shrimp body and hepatopancreas

An hepatopancreatic atrophy was noted (significant) in shrimp surviving the treatments without supplemental vit.E, while shrimp fed oxidised oil in the presence of supplemental vit.E had increased hepatosomatic ratios. This suggests that disease and mortality associated with hepatopancreatic atrophy or lesions reported in case of rancid fish or feeds, is probably caused principally by vit.E deficiencies, not by the toxicity (unproved yet) of the oxidation products.

Tuna meal samples submitted to graded oxidation displayed no significant difference in terms of feed consumption, growth, feed conversion ratios and survival, although feed consumption and growth were slightly lower for highly oxidised TM, as expected (Trial O3). Shrimp growth and feed conversion rates were significantly better with the Chilean fish meal, and significantly poorer with the reference diet lacking a fish meal supplement (Montaño-Aguillar *et al.*, 1998 = rfp 12).

Unexpectedly, protein digestibility was significantly lower in the non oxidised tuna meal than in the moderate and highly oxidised ones, suggesting it was affected more by the ash content than by the degree of oxidation of residual fat (Trial O4). This particularly (low and high ash content in two samples of tuna meal made from raw material of same freshness) was the reason to use these samples in the previously described series of trials ED (see chapter IV of the present report).

Conclusion

These results confirmed that a reduced feed consumption would be the single effect to be expected when using highly oxidised (but stabilised) fish oils or meals in shrimp diets (high oxidation levels in commercial fish oils are unusual).

The importance of using synthetic antioxidants in feed formulation must be stressed, since they avoid the destruction of natural antioxidants like vitamin E, vitamin C and carotenoid pigments, the deficiency of which would produce high mortality,

especially when feeds are supplemented with high quality fish oils or fish meals, rich in highly oxidisable poly-unsaturated fatty acids.

It is believed that the importance of preventing oxidation in the feed is related to the need of the shrimp for vitamin E; in the absence of ETQ, some degree of oxidation occurring when feed is processed is inevitable and this will always destroy this vitamin E (inherent or supplemented) present in the feed formula, leaving the shrimp vulnerable to intracellular oxidation.

Reference Papers

rfp 11 Ricque-Marie D., Cruz-Suárez L.E., SanMartin-DelAngel P., Pike I.H., 1999. **Effect of oxidised fish oils and deficiency of vitamin "E" and/or artificial antioxidant in shrimp diets on *P.vannamei*.** Final report for research grant C11*-CT93-0300, Commission of the European Community, reference paper 11.

rfp 12 Montaño-Aguillar D., Cruz-Suarez L.E., Ricque D. and I. Pike, 1998. **Nutritional effect of tuna meal oxidation on Penaeus vannamei.** Poster presented at the WAS annual meeting, Las Vegas, Tx, USA Feb. 98. Abstract in the proceedings

Communications to the Scientific Community

(see references in alphabetical list of literature cited or chronological list of contributions issued from the project)

Results of experiment O1 have been extensively presented by oral communications on this single subject (Ricque-Marie & Cruz-Suárez, 1995; San Martin *et al.*, 1995; Ricque-Marie *et al.*, 1996; Ricque-Marie, 1997; Ricque-Marie *et al.*, 1997[b]) and also in synthesis papers (Cruz-Suárez, 1997[a], 1997[b]). Accord has been taken with Roche to publish together in a scientific journal.

Results from O3 + O4 were presented at the WAS 98 meeting (Montaño-Aguillar *et al.*, 1998).

VI. FISH MEAL QUALITY NORMS FOR SHRIMP

Objective 6: Establish quality control norms for fish meals used in shrimp nutrition.

Raw Material Freshness

Raw material spoilage leads to reduced feed consumption and growth in shrimp fed fish meals made from moderately fresh or stale fish, while feed conversion ratio does not show a definite tendency. No significant effect was observed on apparent *in vivo* digestibility.

The assessed species sensitivity to raw material freshness can be summarised as follows (Table VI.1): *Litopenaeus stylirostris* is very sensitive to moderately fresh raw material, from post-larval stage (70 mg) to late juvenile (8.4g), with significant growth reduction probably for all sizes, and mortality in post larvae. *Penaeus monodon* is also affected, but early post-larvae were not assayed. *L. vannamei* seems affected only at sizes under 1.5g, with a moderate growth reduction around 1 g, but significant mortality was observed on early post-larvae (70 or 170 mg). Note that the latter were fed fish meals that were also scored as of medium toxicity for their ability to cause gizzard erosion in chicken.

Raw material spoilage can be monitored by measuring total volatile *n*itrogen concentration(TVN) in raw material at process, or biogenic amines in the finished product. It has to be pointed out that, as far as the fish feed producers are concerned, they may not have access to the raw material TVN figures, but they will be able to monitor the amines. Since South American fish will produce predominately histamine and European fish predominately cadaverine, care should be therefore taken in interpreting histamine figures in isolation. Emphasis should be given to the sum of the amines (histamine and cadaverine) or better still histamine + cadaverine + putrescine + tyramine concentrations, when available.

In view of the results summarised in Table VI.1, it seems advisable to use "special" fish meals (PRIME, LT, etc.) made from fresh fish with TVN in raw material the nearest possible to 14 mg N / 100g and low histamine + cadaverine concentration in finished product, since first deleterious effects on shrimp growth or survival were observed already at 30 mg N / 100g raw fish or 2600 ppm histamine + cadaverine in fish meal.

Unfortunately we do not have enough samples of fish meals to give precise limits and there should be a qualification to distinguish between the sensitivity of the different species of shrimp and also the size of shrimp within a species.

Table VI.1 Summary of Trials concerning Raw Material Freshness Effects on Shrimp

Shrimp Characteristics and Observed Effects					Fish Meal Characteristics			
Species	**Weight g**	**Trial**	**Growth reduction**	**Mortality %**	**Raw Fish Species and Freshness Status**		**TVN in Raw Fish mgN/100g**	**Hist.+Cad. in Fish Meal ppm**
Stylirostris	0.077	F6	⇓ 10%	20%	Herring	Stale	-	5900
Stylirostris	0.072	F7	⇓ 33%	No	Anchovy	Mod. Fresh	30	2680
Stylirostris	8.4	F4	⇓ 8-13%	No	Anchovy	Mod. Fresh	30	2680
Monodon	2.5	F3	⇓ 20%	No	Anchovy	Mod. Fresh	30	2680
Vannamei	0.9	F1	⇓ 8%	No	Anchovy	Mod. Fresh	30	2680
Vannamei	0.070	G1	No	10-15%	Anchovy	Stale +score	50	8840
Vannamei	0.17	G9	No	22%	Jack – Mackerel	Mod. Fresh +score	-	2628
Vannamei	1.5	F2	No	No	Anchovy	Fresh-stale	14-50	79-6300
Vannamei	7.6	F5	No	No	Anchovy	Fresh-stale	14-50	79-6300

Biotoxicological Score

In a feeding trial carried out prior to the present project, growth reduction had been found in shrimp, which seemed related to that seen in chickens manifested in erosion of the gizzard. The degree of erosion of the gizzard has led to a scoring system in Chile under the name of "Biotoxicological Score". Gizzerosine, a compound of histamine and lysine formed at high temperature (more than 150°C which can occur if very fine particles get trapped in the dryer) is one of the factors which has been shown to be responsible for gizzard erosion in chicken, may therefore be a factor causing increases in mortality in shrimp under certain conditions.

However, although sensitive to synthetic gizzerosine (Trial G1), *P. vannamei* was not affected negatively by Chilean gizzard-erosion-producing fish meals (Trials G2 to G11) except in one case (Trial G1) were a contamination of the water source by the soluble synthetic gizzerosine was suspected. Medium or high biotoxicological scores had no effect on shrimp growth and feed conversion ratio, or digestibility. In contrast low scored fish meals (between 0.8 and 1.1) led to slightly better growth and feed conversion than normal score fish meals (0.1), and so did the mixing of 2/3 normal score and 1/3 high score.

Commercial fish meals, classified by their biotoxicological score, display considerable variations in some chemical quality parameters, especially those indicating the degree of raw product freshness (biogenic amines content, free fatty acids content). When the latter displayed increased values, fish meals with medium score were found to cause significant mortality in small *P.* vannamei (20 to 40 % after 28 days of feeding trial) but, since high scored fish meal had no effect on the same shrimp, mortality was attributed to the effects of raw material spoilage.

Sensitivity of other species remains to be assessed.

The use of low biotoxicological score fish meals, or equivalent mix, is therefore recommended, provided that raw material freshness indicators are not altered.

Digestibility

Original work done at IFREMER with 17g *L. stylirostris* showed that shrimp are at least as sensitive as salmon or mink to the factors which affect protein digestibility in fish meals, since digestibility values obtained for two of herring meals with low and high digestibility were in the same range as those obtained in salmon or mink.

Indeed, smaller shrimp, down to 0.5 g, displayed a wider range of protein digestibility for the same herring meal samples. The low digestibility herring meal, which had been overheated at drying, was less digestible for small *L. vannamei* used at UANL than for the large 17g *L. stylirostris* used at IFREMER. The higher sensitivity of small shrimp to fish meal quality in terms of digestibility was confirmed on a set of 12 other fish meals and one fish hydrolysate. However, correlation with digestibility figures in salmon was optimised by excluding fish meals with low protein (<65%) and high ash (>18%)

content, made from white fish offals or menhaden (see section III of the present report - Trial D1 = *in vivo* digestibility studies), which displayed out of range digestibility values in shrimp. *In vitro* digestibility values using shrimp enzymes (shrimp pH-Stat method) are significantly correlated with *in vivo* figures provide that same precaution is taken, i.e. excluding low protein/high ash fish meals. *In vitro* digestibility values using pepsin (AOAC, Torry modified, digestible protein expressed as a % of total crude protein) displayed an even better correlation with *in vivo* values, without the necessity to exclude any sample.

However, such important differences in fish meal quality in terms of digestibility had no effect on growth and feed conversion ratio when the same two herring meals were included at 25% in feeds containing 35 % crude protein fed to shrimp confined in cages located in a production pond or in tanks set up the laboratory (see section IV of the present report). The reasons for this are believed to be due to a relatively high dietary protein which exceeded the shrimp requirement and problems in pond with a low dissolved oxygen due to a phytoplankton crash.

In contrast, two fish meals made from tuna cannery trimming tested in the same conditions (iso-proteic diets with same basal formula) and having *in vivo* digestibility values intermediate between the high and low of herring meals, promoted slightly lower growth in the pond cages (barely significant), and poorer growth and feed conversion ratios in tanks (highly significant). Consequently, emphasis has to be placed on the raw material type when selecting fish meal for shrimp feeds.

In spite of the relative failure of experiments in section IV, there is an example in the present report that shows the effect of a low digestible fish meal on growth and feed conversion in shrimp. Trial G4 demonstrated that overheating a fish meal sample at the laboratory diminished drastically its digestibility and led to lower growth and poorer feed conversion ratio (see section II of the present report, pp. 11-12, and reference papers 5 and 6).

Finally, in view of the high sensitivity of shrimp to factors which affect fish meal digestibility, particularly temperature exposure in dryer, it seems evident that digestibility should be used to classify different batches of fish meals produced from the same type of raw material. Nevertheless, the definition of precise limits requires further confirmation using larger numbers of fish meals made from the same raw material.

Fish Meal and Fish Oil Oxidative Rancidity

The emphasis has to be put on the use of synthetic antioxidants in the feed formula, in order to control the oxidation during the feed process and storage. This avoids the subsequent destruction of dietary natural antioxidants (like vitamin E, but also vitamin C, and carotenoid pigments) which proved to lead to important mortalities, of at least 40% after a few weeks.

Provided that oxidation level in fish oil or in the lipids extracted from fish meal, do not exceed a medium value (Peroxide Value < 50 meq /kg, TBA < 8 mg Malonaldehide/kg, Anisidine Value < 52 Odx100), no adverse effect was expected.

With highly oxidised lipids (PV = 100 meq/kg, TBA = 15 mg Mal/kg, AV = 183 Odx100), feed consumption diminish, and, as a consequence, growth, but feed conversion ratio is not affected.

It is therefore recommended to avoid the use of highly oxidised fish oils in shrimp feeds, especially when feed has to compete with natural food in a pond environment.

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