ifoma

International Fishmeal & Oil Manufacturers Association

FRESHNESS OF FISH MEAL ON PALATABILITY IN SALMON - TOXICOLOGICAL FACTORS IN FISH MEAL MADE FROM SPOILED FISH

by Norwegian Herring Oil and Meal Industry Research Institute, Kjerreidviken 16, N-5033 Fyllingsdalen, Norway

RESEARCH REPORT NUMBER: 1998-2

April 1998

STRICTLY CONFIDENTIAL

FRESHNESS OF FISH MEAL ON PALATABILITY IN SALMON - TOXICOLOGICAL FACTORS IN FISH MEAL MADE FROM SPOILED FISH

SUMMARY:

The aim of this investigation was to examine in which fraction of the fish meal produced from stale fish, i.e. presscake or solubles, a possible toxic factor affecting the performance of and pathogenicity to Atlantic salmon, reside. Five experimental fish meals were processed, each providing approximately 60 % of a basal experimental fish feed diet. FM 1 was made from herring in an absolutely fresh condition (TVN = 18 mg/100g) and FM 2 was made from herring stored until a TVN content of 130 mg/100 g was achieved. The amine content (cadaverine, histamine and putrescine) of the resultant meal was 4.68 g/kg (4680 ppm). One part of FM 2 was «extracted» with isopropanol in order to remove all soluble materials, thus making a third fish meal (FM 3). FM 4 was made from water-washed presscake from stale fish and FM 5 was made from the presscake of fresh fish to which was added the stickwater concentrate from the stale fish.

In a 12 week feeding trial with Atlantic salmon smolts (140 g), fish fed FM 2 suffered from impaired growth, reduced feed intake, reduced feed conversion, reduced protein digestibility (mink and salmon), increased mortality and histopathological changes, especially in the intestines. The combined results from the feeding trial indicate that the deleterious effect of fish meal made from stale fish are due to two factors. One factor is a toxic factor(s) residing in the water insoluble fraction of the presscake affecting feed conversion and the health status of the farmed salmon. The second factor residing in the water soluble phase has a detrimental effect on feed intake. It is concluded that compound(s) that are toxic to Atlantic salmon either are formed during unfavourable storage of fish, or during processing of stale fish.

Executive summary

- Herring was processed into 5 fish meals designated FM 1, FM 2, FM 3, FM 4 and FM 5, each providing approximately 60 % of a basal experimental fish feed diet. FM 1 was made from herring in an absolutely fresh condition (TVN = 18 mg/100g) and FM 2 was made from herring stored until a TVN content of 130 mg/100 g was achieved. One part of FM 2 was further «extracted» with isopropanol in order to remove fat and fat-soluble materials, thus making a third fish meal (FM 3). FM 4 was made from water-washed presscake from stale fish and FM 5 was made from the presscake of fresh fish to which was added the stickwater from the stale fish.

Overall results relative to the control diet - fresh fish, whole meal:

Diet	Fish meal	Growth (SGR)[1]	Feed intake	FCR[2]	Mortality %	GI tract[3] lesions
1	Fresh fish, whole meal	100^{a}	100^{a}	100^{a}	7.5	0
2	Stale fish, whole meal	70^{b}	69^{b}	121^{b}	17.8	9
3	Stale fish, IPA extracted	77^{bc}	79^{b}	117^{b}	10.6	15
4	Stale fish, PC meal	86^{d}	101^{a}	124^{b}	8.9	15
5	Fresh PC + stale stickwater	84^{cd}	75^{b}	97^{a}	8.1	0

(Different superscript letters indicate significant differences between dietary groups, $p < 0.05$)

[1] Specific growth rate
[2] Feed conversion ratio: feed/gain
[3] Number of fish showing morphological lesions (of gastrointestinal tract) out of 15

- All measured growth parameters were significantly correlated to the dietary levels of biogenic amines (cadaverine, histamine and putrescine), and thus the content of biogenic amines in the fish meals.

- Reduced feed intake was found in all groups fed diets which contained the whole or parts of the stickwater fraction of fish meal made from stale fish, i.e. FM 2, FM 3, FM 5. The feed intake further showed a close relationship to the amount of biogenic amines present in the experimental feeds.

- Reduced feed conversion was found in all groups fed experimental diets containing presscake from stored raw material, i.e. FM 2, FM 3 and FM 4. Feed conversion did not show any relationship to the dietary levels of biogenic amines or endotoxin in the fish meals.

- Results from the histopathological examination, showed a cytotoxic effect from the presscake made from stale fish. Although the degree of severity of the pathological changes varied, all fish fed presscake from stale fish, with or without the stickwater

fraction, suffered from histopathological changes, particularly in the intestines, and showed a small or significant infiltration of fat in the liver.

Total mortality, in general, was high for all dietary groups (7 - 10 %) and was significantly increased to approximately 18 % in the fish fed FM 2, i.e. whole fish meal made from stale fish, despite the fact that the histopathological changes showed that fish from this group were only moderately affected. This cannot be explained by some deleterious factors related solely to the presscake or stickwater fractions from stale fish, as mortality among fish fed FM 4 and FM 5 was similar to that found in the control diet. The reason for the high mortality in fish fed whole fish meal made from stale fish, may be the result of some combined, yet unknown negative effect of the two fractions, maybe through some kind of synergistic effect.

The combined results indicate that the deleterious effect of fish meal made from stale fish are due to two factors. One factor is a toxic factor(s) residing in the water insoluble fraction of the presscake, not yet identified, affecting feed conversion and the health status of the farmed salmon. The second factor residing in the water soluble phase has a detrimental effect on feed intake. Although the negative effect on feed intake seems to be correlated to the amount of biogenic amines in the experimental feeds, it has been shown in previous IFOMA studies that addition of crystalline biogenic amines did not affect feed intake in salmon. Thus, it may be concluded that the biogenic amines, while useful as indicators of raw material freshness, clearly cannot be the responsible factor for the reduced feed intake.

Introduction

The impaired performance of Atlantic salmon fed fish meal made from stale fish, was in a previous IFOMA study shown to be the result of a possible toxic effect leading to pathological changes in vital organs like the liver and the intestine (Opstvedt et al., 1996). The toxic effect seems to be correlated with the content of biogenic amines, while at the same time it was demonstrated that addition of crystalline biogenic amines (tyramine, putrescine, cadaverine and histidine) to fresh fish meal in diets for Atlantic salmon, did not affect growth rate or cause any histopathological changes in tissues. Thus it was concluded that compound(s) that are toxic to Atlantic salmon either was formed during unfavourable storage of fish, or during processing of stale fish. On this background, the fish meal industry was particularly interested to examine in which fraction of the fish meal, i.e. presscake or solubles, the toxic factor resides. If that was achieved, it was decided to provide this fraction(s) for further identification of the toxic factor(s). The ultimate aim, which was not a part of this project is to establish an analytical method that allows better quality control of fish meal than existing methods.

Experimental

Fish raw material and processing

Five different fish meals designated FM 1, FM 2, FM 3, FM 4 and FM 5 were produced from the same raw material. FM 1 was made from absolutely fresh herring (*Clupea harengus*) with 13.6 % fat, 27.2 % fat free dry matter and a Total Volatile Nitrogen (TVN) content of 18 mg/100 g. The fish was kept on ice (0 - 3 °C) and immediately processed upon arrival at the pilot plant 4-5 days after catch. Another sample of the same raw material was transferred to large containers, each of 350 kg herring, ice and water was carefully removed and the herring was stored at 17 - 18 °C for 8 days until a TVN content of 130 mg/100 g was achieved, before being processed. Whole fish meals were thereby made from fresh raw material (FM 1) and from stored raw material (FM 2) and the meals were processed according to the same cooking and pressing conditions. The fish were cooked in a conveyer belt cooker at a temperature of about 85 °C for 20 minutes before being pressed. Fish outlet temperature from the cooker was 60 - 65 °C, and from the press 62 °C. The presscake (PC) was thoroughly mixed and added 300 ppm ethoxyquin on a dry matter basis. Small volumes of the PC (7 - 8 kg) were put in plastic bags and quickly refrigerated on ice. The press liquor was desludged, separated into an oil and a water phase, and the water phase was concentrated in a falling film evaporator. The mixed presscake, sludge and concentrated stickwater was milled and dried in one operation in a pilot plant dryer. The meal was chilled immediately after drying, and stored in paper bags at ambient temperature.

FM 3 was produced by «extracting» or more correctly washing FM 2 with isopropanol (IPA) in order to reduce the lipid content. Batches of 35 kg FM 2 were

added to 45 l IPA and heated to 65 °C with periodic stirring. The heated blend was allowed to settle down prior to decanting of the IPA. This procedure was repeated three times. Following IPA extraction, the meal was allowed to dry in open air and thereafter in a pilot steam dryer at low pressure. Finally, the meal was spread out on a large plastic sheet, and residues of IPA were allowed to evaporate for 5 days before the fish meal was put in paper bags and stored at ambient temperature until used.

FM 4 was made from the water-washed presscake of stale fish. Thirty kg of the presscake was added to 50 l of water and heated under constant stirring until a temperature of 65 °C was reached. The blend was stirred for another 15 min., and allowed to settle down before decanting. Approximately 15 l of liquor was removed, and 20 l of fresh water was added before the mass was reheated to 65 °C. Water-washed presscake was filtered through a straining cloth following decanting. Excess water was removed in a small screw press. FM 4 was milled and dried according to the same procedure as described for whole fish meal.

The final fish meal, FM 5, was made from the presscake from the fresh raw material to which was added the stickwater concentrate produced from stale fish. The meal was milled and dried as described above. The proportions of the presscake and the concentrate were as for FM 2 on a dry matter basis.

Thus the experiment provided the following types of fish meal (FM):

FM 1 : Whole FM made from fresh herring, TVN = 18 mg/100 g.
FM 2 : Whole FM made from herring stored to 130 mg TVN/100 g.
FM 3 : FM 2 extracted by isopropanol.
FM 4 : Presscake FM made from water-washed presscake of stored herring.
FM 5 : Whole FM made from presscake from fresh herring to which was added the stickwater concentrate from stored herring at an equivalent amount to that present in FM 2.

The proximate composition, content of water-soluble protein, true protein digestibility (mink) and content of biogenic amines in the fish meals are shown in Table 1, together with the amount of biogenic amines in the experimental feeds. Fish meal made from stored raw material (FM 2) was characterised by a small reduction in crude protein content and a significant increase in the content of water-soluble proteins and biogenic amines (cadaverine, histamine, putrescine), thus indicating enzymatic and bacterial degradation of the fish. The IPA extraction of FM 2 to make FM 3, had only limited effects on the amount of water-soluble protein and biogenic amines, but the lipid content was reduced by more than 60 %, and the crude protein and ash content were slightly increased. The content of water-soluble protein and biogenic amines in FM 4 were approximately 1/3 of the respective levels found in FM 2. The content of biogenic amines in FM 5 was increased compared to that found in FM 1. The low content of water-soluble protein found in FM 5 compared to FM 2, may indicate that the stickwater concentrate was not added to FM 5 at an equivalent amount to that present in FM 2.

A small but statistically significant ($p < 0.05$) reduction in protein digestibility as determined in mink, was found in fish meal made from stale fish (FM 2) and in the IPA extracted fish meal (FM 3), as compared to fish meal made from fresh herring, FM 1 (Table 1). Analyses of the bacterial endotoxins in raw fish, fish meals, intermediate products and solubles, as well as in the experimental diets are given in Appendix 1. High endotoxin levels were detected in all soluble fractions produced from stale fish, and to a lower extent in the presscake made from either fresh or stale fish. High endotoxin levels were further detected in FM 3 and FM 5, which both contained soluble fraction of stale fish, but not in FM 2, which also contained the stickwater concentrate from stale fish. In the final experimental fish feeds, the content of endotoxins were low, and no differences between diets were found (Appendix 1).

Diets

One diet was made from each fish meal (FM 1, FM 2, FM 3, FM 4, FM 5). All diets were extruded (3.5 mm pellet) at fixed and defined conditions. In order to improve the binding power of the pellet made from the presscake (FM 4) and to a smaller extent from the whole fish meal made from the stale fish, the amount of water and the steam flow had to be increased during extrusion. Diet composition, and the nutrient and energy content of the experimental diets are shown in Table 2A and 2B. The difference in proximate composition in the presscake meal and the whole meals (Table 1) was balanced through different inclusion levels of soya-protein concentrate, NorSeaOil and Suprex maize in the diet (Table 2A). The fish meals were the main protein sources providing, on a dry matter basis, from 48 to 51 % of protein for Diet 1, 2, 4 and 5, and for Diet 3, 53.5 %. The small differences in protein contents were due to problems incurred in balancing the diets. All diets were isocaloric and the protein energy accounted for approximately 50 % of the total energy content in all diets, i.e. 11.7 - 12.1 MJ/kg. The chemical content and the amount of biogenic amines encountered in the diets, showed that the intended contents of nutrients and expected levels of biogenic amines were largely achieved (Table 1 and Table 2B).

Fish and handling

Atlantic salmon smolt were obtained from a commercial hatchery in April, and were fed a commercial diet throughout the acclimatization period until start of the experiment in June. The smolt were randomly distributed to fifteen 2x2 m glassfibre tanks (3.2 m^3), each of 120 fish, on 12th June 1997. The fish were fed one of the five experimental diets, in triplicate tanks, for a feeding period of 12 weeks until 4th September 1997. All tanks were equipped for continuous monitoring of feed refusals. Fish were fed to appetite by automatic feeders, and the daily feed rations were adjusted according to assumed fish biomass and feed intake. Feeding periods were of 20 second duration intervened by 300 seconds, and lasted from 12 p.m. to 01 a.m.

every day. The collected feeds were dried in an oven once a week, and the «true» amount of feed eaten was used for determination of feed intake and feed conversion. All tanks were supplied with running seawater taken from 50 m depth. Average water temperature was about 7.1 °C during the experiment, i.e. 6.7, 6.9 and 7.4 °C in June, July and August, respectively. Salinity was 32 - 33 ‰ throughout the feeding trial and the oxygen content in the outlet water from the tanks was approximately 8.5 mg/l. The fish were exposed to 24 hrs light during the experimental period. All fish were starved for three days prior to the start of the experiment in June, and prior to fish sampling at the end of the experiment at the beginning of September.

Sampling of fish

After a feeding period of 9 weeks, on 12th August 1997, five fish from each tank were killed and the livers immediately dissected. Small, equally positioned parts of the liver were fixed in neutralized formalin solution (4 %) for histological examination. Later, at the end of the feeding trial, 10 fish from each tank (fish no.1 - 10) were collected for evaluation of weight, length, liver weight, condition factor and hepatosomatic index (HSI). A morphological examination of the gastrointestinal tract was performed in 5 individual fish from each tank (fish no.1 - 5). Samples of liver and intestines from the same 5 fish were collected for histological examination. Blood samples were collected from *vena caudalis* from the other 5 fish per tank (fish no.6 - 10), and blood haemotocrit (hct), as well as serum protein, albumin and lysozyme were examined in individual fish (n = 5). Blood samples were withdrawn by a syringe, allowed to clot overnight (4 °C) and centrifuged at 3000 rpm for 10 min. Lipid content of liver was analysed in pooled samples of 5 fish per tank (fish no. 6 - 10), n= 3.

Digestibility trial

After finishing the feeding trial, apparent protein digestibility was determined in fish fed Diet 1 containing fish meal made from fresh fish, and Diet 2 containing fish meal made from stored fish. Chromic oxide was added to the feed as an inert marker at a level of 1 %, and a pooled sample of about 20 - 30 g of faeces were collected from each replicate tank by manual stripping of fish after a feeding period of 12 days. Chromium, Kjeldahl-N and amino acids were determined in the two experimental feeds and in pooled samples of faeces from each replicate tank (n = 3 per group).

Morphological and histological examination

The morphological examination of the gastrointestinal tract was performed following dissection from the oesophagus to the rectum. The intestines were clinically examined and evaluated for possible changes in colour and general appearance. Tissue samples of liver, and a small section of the intestines located approximately 1 cm posterior to the end of the pyloric caeca, were taken from each individual fish and fixed in 4 % formaldehyde in phosphate buffer. All tissues were embedded in

paraffin, sectioned to approximately 5 μm and stained with HES (Haematoxylin - Eosin - Safran). Histological examination was done by light microscopy at 100x and 400x magnification.

Analytical methods

Chemical analyses were carried out in duplicate by a laboratory accredited by the Norwegian National Accreditation body. Dry matter (ISO 6496-1983) and ash (ISO 5984-1978) content were determined gravimetrically after drying for 4 hrs at 105 °C and after combustion for 16 hrs at 450 °C, respectively. Crude protein (N x 6.25) was determined by the Kjeldahl method (ISO 5983 - 1979). The same method was applied for determination of water-soluble protein in the supernatant following extraction with boiling water (30 min) and filtering through black bond filter. Lipid content in the fish meals and diets were determined gravimetrically after petroleum ether extraction (Soxhlet technique), (AOCS Ba 3-38), and in fish meal also according to Bligh & Dyer (1959).

Lipid content in the fish livers were measured following extraction with etylacetat. Serum total protein and albumin were determined according to Sandnes et al. (1988). Total Volatile Nitrogen (TVN) was determined by distillation (AOAC, Methods of analysis, 1984, 2.065) and biogenic amines (i.e. cadaverine, histamine, putrescine) were measured by HPLC according to Mietz and Karmas (1978). Amino acids were analysed by the «Pico Tag» method described by Waters Chromatography Division, which involves precolumn derivatization with phenylisothiocyanate and separation by HPLC on a reversed-phase column (Water Pico Tag Column for total amino acids), (Aksnes and Brekken 1988). Chromium was determined by atomic absorption spectrophotometry after Kjeldahl digestion as described by Lied et al. (1982). True protein digestibility was determined in mature male mink as described by Skrede (1979). The Limulus Amebocyte Lysate gel clot test as applied to serial decimal dilutions of samples, is a semiquantitative method for detection of endotoxins (Levin et al. 1972). The test was performed according to the description enclosed to the test kit (PN 284: BioWhittaker), and conforms with the FDA guidelines.

Calculations

Growth, feed intake, feed conversion and apparent digestibility were determined according to the following formulas (BW_2 = final body weight, BW_1 = initial body weight):

Specific growth rate (SGR) = $(\ln BW_2 - \ln BW_1) \times 100 / \text{days}$

Thermal-unit growth coefficient (TGC) = $(BW_2^{1/3} - BW_1^{1/3}) \times 1000 / \Sigma (\text{temp.}(°C) \times \text{days})$
(Cho C.Y., 1992)

Daily feed intake per fish = g feed intake / days / no. fish

Daily feed intake in % of weight gain = g feed intake / days /((BW_2 + BW_1)/2 x 100) / no. fish

Feed conversion rate (FCR) = g feed intake / g live weight gain

Apparent digestibility = 100 - 100 x ((Cr_{Feed} x $Nutrient_{Faeces}$) / (Cr_{Faeces} x $Nutrient_{Feed}$)).

Statistical methods

Biological and analytical data were subjected to a one-way analysis of variance (ANOVA) and differences between means tested using Tukey's multiple range test (Sokal and Rohlf, 1981). The percentage of dead fish in each tank and number of affected fish in the histological examination were transformed to a zero to 1.0 scale prior to statistical testing. Analytical data measured in individual blood and serum samples, apparent protein digestibility as determined in fish and results from the histopathological examination were subjected to non-parametric test (Kruskal-Wallis One-Way Analysis of Variance (Chi-square approximation) and Mann-Whitney U-test). Pearson correlation analysis was used to examine possible relationships between feed parameters and dietary responses. All data were statistically treated according to SYSTAT 6.0 for Windows.

Results

Despite the low temperature in the sea water taken from 50 m depth, growth performance of the fish fed the control diet, FM 1, was satisfactory and about 80 % of that that can be expected under commercial conditions in the southern part of Norway. The experimental conditions were otherwise good, and the feeding trial was carried out without any major problems.

Growth, feed intake and feed conversion

Weight, growth, amount of feed eaten, feed conversion and mortality of the fish all showed distinct differences between dietary groups (Table 3). Impaired performance was found for all measured parameters in fish fed whole fish meal made from stale fish (FM 2). Compared to fish fed the control diet with fish meal made from fresh fish (FM 1), the relative weight gain in percentage of the control diet was 60, 69, 80, and 77 % for diet 2, 3, 4 and 5, respectively, thus indicating impaired growth in all experimental groups compared to the control group. Feed intake was reduced in the fish fed Diet 2, 3 and 5, which all contained soluble fractions from the stale fish, while the feed conversion was impaired in all fish groups fed diets containing presscake from stale fish, i.e. Diet 2, 3 and 4. The relative performance of the fish

fed the different experimental diets compared to those fed the control diet (FM 1) were as follows:

Relative performance of fish in percentages of the control group, FM 1

	FM 1	FM 2	FM 3	FM 4	FM 5
Weight gain	100	**60**	69	80	77
Final weight	100	**76**	82	88	87
SGR	100	**70**	77	86	84
TGC	100	**67**	74	84	82
g feed eaten/day/fish	100	**69**	79	101	75
% feed eaten/day/BM fish	100	**84**	92	110	85
FCR	100	**121**	117	124	97
Mortality	100	**237**	141	119	108

The impaired feed conversion found in all experimental groups fed diets which contained presscake from stale fish (FM 2, FM 3, FM 4), was combined with poor feed intake in the fish fed FM 2 and FM 3, which contained the soluble fractions of the fish meal, but was not present in the fish fed the water-washed presscake (FM 4). Following correction for actual differences in body weights between dietary groups, the relative feed intake compared to the control group (FM 1) was in fact 10 % increased in fish fed FM 4, thus indicating a compensatory effect related to the impaired growth performance. These results confirm that the negative effect on feed intake is exclusively related to the soluble fractions of fish meals. The negative effect on feed intake was also observed by the technical staff responsible for the feeding trial.

All growth parameters (final weight, weight gain, SGR, TGC), showed a significant correlation to the level of biogenic amines in the experimental feeds ($p < 0.001$) and in the fish meals ($p < 0.02$). Appetite measured by the amount of feed eaten per day per fish also showed a significant correlation to the level of biogenic amines in the feed ($p < 0.01$). Following correction for differences in weight between the dietary groups, and by measuring the amount of feed eaten in % of biomass, no significant correlation occurred ($p > 0.05$). However, the statistical significance levels indicated a close but non-significant effect for cadaverine ($p = 0.08$) and putrescine ($p = 0.09$), but not for histamine ($p = 0.21$). Statistical tests further showed that feed conversion was unaffected by the dietary levels of biogenic amines ($p > 0.05$).

Mortality

Total mortality was somewhat higher than normal in all tanks, but this can easily be explained by the handling at low temperature and the outbreak of «winter-wounds» following damages to the skin. Mortality among the fish fed the diet with the fish meal made from stale fish (FM 2) was more than twice as high compared to that of the fish fed the fish meal made from the fresh fish (FM 1), and was significantly

different from that of all the other dietary groups ($p < 0.05$). This cannot be explained by some deleterious factors related solely to the presscake or stickwater fractions from stale fish, as mortality among the fish fed FM 4 and FM 5 was similar to that found in the control diet (Table 3). The reason for the high mortality in the fish fed the whole fish meal from stale fish, may be the result of some combined, yet «unknown» negative effect of the two fractions; i.e. presscake and stickwater, maybe through some kind of a synergistic effect. While mortality was clearly reduced by 40 - 50 % in the last feeding period lasting from day 42 to 82 in fish fed Diet 1, 4 and 5, and by approximately 20 % in fish fed Diet 2, mortality of the fish fed the IPA extracted fish meal (Diet 3) showed an opposite trend (Table 3).

Condition-factor and liver hepatosomatic index

Weight and length of sampled fish resembled the mean body weights of the fish in all groups. The significant reduction in the condition factor found in the fish fed FM 2 compared to that of the fish fed FM 1 and FM 4 as the main protein sources, indicates a poorer nutritional status in fish fed whole fish meal from stale fish (Table 4). The explanation for the somewhat higher condition factor in fish fed fish meal made from presscake from stale fish (FM 4) relative to the whole fish meal fraction of stale fish (FM 2), may be explained by the lack of stickwater and the higher feed intake found in fish fed FM 4.

The hepatosomatic index was significantly increased in fish fed the IPA extracted fish meal (Table 4). Accordingly, the liver fat content in that group showed values more than twice as high as that of the control fish (Table 4). Fat infiltration was also present in fish fed the water-washed presscake (FM 4), although the hepatosomatic index was not severely affected. Liver fat content ranged from 2.1 to 2.2 % within all replicate tanks of fish fed Diet 1 and Diet 5, and ranged from 2.4 to 2.8 % in fish fed Diet 2. A tendency towards fat infiltration thus seems to be present also in the group fed whole fish meal from stale fish.

Blood haematocrit and serum protein, albumin and lysozyme

Serum protein and albumin levels were significantly reduced in the fish fed the fish meal from stale fish ($p < 0.05$), but serum lysozyme measured directly in the samples was not significantly affected ($p > 0.05$) compared to the control fish (Table 5). By measuring serum lysozyme activity related to the reduced serum protein levels, a weak stimulation of serum lysozyme activity seems to be present in the fish fed Diet 2 ($p < 0.05$). Blood dilution may also explain the tendency towards reduced blood hct levels in fish from all dietary groups as compared to the control fish. Due to large individual variation, no significant differences were found in blood hct levels between groups ($p > 0.05$).

Apparent protein digestibility in salmon

A small, but statistically significant reduction in apparent protein digestibility (Kjeldahl-N) was found in salmon fed FM 2, i.e. whole fish meal made from stale fish (app. prot. dig. = 89.8 ± 0.1 %) compared to salmon fed the control diet, FM 1 (app. prot. dig. = 91.0 ± 0.6 %). Dietary contents of amino acids showed some small changes which indicated microbial and enzymatic degradation during storage of the fish. The dietary levels of arginine, histidine, lysine, serine and thyrosine were slightly reduced in Diet 2 compared to Diet 1 (Table 6).

Histopathology

The results of the histopathological examination are shown in Table 7 and 8, and the clinical remarks and the pathologist's comments are given in Appendix 2. Histopathological changes were totally absent in the fish fed the fish meal made from fresh fish (Diet 1) and in the fish fed the fish meal made from fresh presscake combined with the stickwater concentrate from stale fish (Diet 5). In order of severely affected to less severely affected fish, the other diets ranked Diet 3 > Diet 4 > Diet 2. All these diets contained presscake processed from stale fish, but differed otherwise in type of processing and extraction of the meal. In general, the intestines seemed to be more affected than the liver for all examined fish. Fish fed FM 4, i.e. water-washed presscake from stale fish, showed more significant changes compared to those found in the fish fed the whole fish meal from stale fish, FM 2. As feed intake in the fish fed Diet 4 was approximately 30 % higher than that found in the fish fed Diet 2, the differences in degree of severity may be associated with actual differences in amount of feed eaten. The histopathological findings do not coincide either with the amount of biogenic amines present in the diets, or with the content of endotoxins, as measured by the Limulus Amebocyte gel clot test (Appendix 1), in fish meals.

All examined fish fed the IPA extracted FM 2, seemed to be significantly affected, most possibly due to toxins, according to the observed changes and pathologist's comments. Steps were taken to remove IPA from the fish meal prior to feed production. A weak odour of the fish meal and the diet, indicated however that small amounts of IPA were left after drying. Determination of the isopropanol (Propan-2-ol) content in Diet 3 after finishing the feeding trial, showed a dietary level of 60 ppm (Asplan & James Consultant Analysts, Cambridgeshire, UK). Thus, it has to be considered that the presence of IPA in the diet may itself have had a direct toxic effect or may have enhanced the deleterious effect of the toxic factor already present in the fish meal.

Discussion

The impaired growth and reduced performance found in salmon fed fish meal made from stale fish compared to that of fish fed fish meal made from fresh fish, confirms

previous findings in experiments conducted by IFOMA (Opstvedt et al. 1996). In the present study, growth of salmon measured by weight gain in the experimental period, was reduced by 40 %, while former studies have shown that growth was reduced by 53 and 43 % in trials with herring, and by 30 % in a trial with anchovy (referred to by Opstvedt et al. 1996). Differences in experimental conditions such as water temperature and fish size, as well as differences in degree of spoilage of stored fish, may very well explain this variation.

Fish meal quality is usually evaluated by protein digestibility, as measured in mink, showing an inverse relationship to the processing temperature in the manufacturing of the fish meal, and by the content of biogenic amines left in the fish meal, indicative of the raw material freshness. Reduced protein digestibility has been reported in some studies with salmonids and Atlantic halibut fed fish meal produced from stale fish (Aksnes and Mundheim 1997, Watanabe et al. 1983), while others have failed to demonstrate significant dietary effects of fish spoilage on protein digestibility of fish diets (Clancy et al. 1995; Opstvedt et al. 1996). In the present study, a small but significant reduction in protein digestibility as determined in mink, was found in fish fed whole fish meal produced from stale fish (FM 2). This result was confirmed in the digestibility trial with salmon, showing approximately 1 % reduction in protein digestibility as measured by faecal stripping. The inconsistent digestibility results may be explained by use of different experimental methods to obtain faeces, as discussed in the article by Aksnes and Mundheim (1997). The small reduction in protein digestibility of 1 - 2 % that is commonly found in spoiled fish, is not likely to explain the 30 - 50 % reduction in growth as reported in the above mentioned studies.

The technical equipment used for monitoring of feed refusals in the present study, allows a better control of feed intake in fish, and makes it more easy to distinguish between dietary effects on feed intake and feed conversion than previous experiments. We are thus able to distinguish the deleterious effect of fish meal made from stale fish into two factors, one factor having a detrimental effect on feed intake, residing in the water soluble phase, and a second cytotoxic factor residing in the water insoluble fraction of the presscake, reducing feed conversion and inducing histopathological and physiological changes in the tissues.

The negative effect on feed intake seems to be correlated to the amount of biogenic amines in the fish meals. In previous experiments conducted by IFOMA, however, it was concluded that addition of crystalline biogenic amines did not affect feed intake (Opstvedt et al. 1996). Further, in the present experiment, it was found that feed intake relative to the control group was slightly more reduced in the fish fed FM 5 compared to that of the fish fed FM 3, although the content of biogenic amines in FM 5 was about half of the level found in FM 3. It may be concluded that the biogenic amines, while useful as indicators of raw material freshness, clearly cannot be the responsible factor for the reduced feed intake, thus confirming results obtained in the IFOMA study conducted by Opstvedt et al. (1996). The negative factor affecting feed intake in fish may have been partly removed by the IPA extraction,

and thereby causing the slightly higher feed intake in the fish fed Diet 3 compared to that of those fed Diet 5.

In the present study, toxicological implications were clearly demonstrated in all fish groups fed the presscake from stale fish either as whole fish meal from stale fish (FM 2), IPA extracted whole fish meal (FM 3) or water-washed presscake fish meal (FM 4). The histopathological examination showed that the intestines and the alimentary canal were more severely affected than the liver for all examined fish. The increased mortality found in the fish fed FM 2, is not in accordance with the toxicological findings, ranging in order of severely affected to less severely affected; FM 3 > FM 4 > FM 2, and should be further investigated. The reduced feed intake in fish fed the whole fish meal with high dietary levels of cadaverine, may explain the slower development of degenerative changes in the tissue, as compared to the fish fed the presscake fish meal with low cadaverine levels. Solely in this latter group, a tendency towards a compensatory effect by increased feed intake in response to the reduced feed conversion of presscake appeared. In a recent publication in Aquaculture (Aksnes and Mundheim 1997), diets containing fish meal made from spoiled fish resulted in reduced lipid levels and protein retention in Atlantic halibut, concomitant with a higher content of lipid in the hepatocytes as measured by histological examination, and smaller, atrophic and pycnotic nucleus indicative of degenerative changes in the liver. It was suggested that the toxicological effects of spoiled fish was not caused by the biogenic amines, but «might be due to some other microbial metabolites, enzymatic activities or chemical reactions (e.g. oxidation) occurring during storage of the raw material» (Aksnes and Mundheim 1997). In the present study with salmon, accumulation of lipid in the liver was clearly demonstrated by chemical and histological analyses, thus confirming a disorder of the lipid metabolism promoted by feeding fish meal (i.e. presscake) produced from stale fish. In studies conducted with chinook salmon and rainbow trout, it was presumed that the significant reduction in digestibility of organic matter of fish meals produced from spoiled raw material, was probably caused by reduced lipid digestibility, as protein digestibility was more or less unaffected (Clancy et al. 1995).

Reduced fish meal quality due to spoiled raw material, as indicated by high dietary cadaverine levels, induces a very strong initial reduction in feed intake in several fish species (Aksnes and Mundheim 1997; Opstvedt et al. 1996). In Atlantic halibut it has been shown that the negative effect on feed intake related to raw material freshness disappears after an adaptation period (Aksnes A., personal communication). Evaluation of feed efficiency in experimental fish is complicated by the fact that any initial effect on feed intake measured as daily feed intake per day per fish, inevitably will lead to a calculated reduced feed conversion in experimental fish exposed to the negative factor. Even if fish overcome the negative effect after an adaptation period, feed conversion will be less efficient due to the lower body weights of fish recorded in this group as compared to control fish. Hence, by measuring daily feed intake in % of weight gain, it is possible to distinguish between initial and subsequent effects on feed conversion or feed efficiency. Unfortunately in the present study, we are not

able to do so, as no intermediate sampling and weight recording was performed due to the low water temperature.

Proposal for further studies

From this study it is concluded that compound(s) that are toxic to Atlantic salmon is located in the insoluble fraction of the presscake and is probably formed during unfavourable storage of fish or during processing of stale fish. It is of great importance to the fish meal industry to identify the toxic factor(s) and further to understand the underlying mechanisms for how it is formed and thereby preventing its formation. The ultimate aim is to establish an analytical method that allows better quality control of fish meal than existing methods.

References cited

Aksnes A. and Brekken B. (1988). Tissue degradation, amino acid liberation and bacterial decomposition of bulk stored herring. J. Sci. Food Agric. 45: 53 - 60.

Aksnes A. and Mundheim H. (1997). The impact of raw material freshness and processing temperature for fish meal on growth, feed efficiency and chemical composition of Atlantic halibut (*Hippoglossus hippoglossus*). Aquaculture. 149: 87 - 106.

Bligh E.G. and Dyer W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Cho C.Y. (1992). Feeding systems for rainbow trout and other salmonids with reference to current estimates of energy and protein requirements. Aquaculture. 100: 107 - 123.

Clancy S., Beams R., Higgs D., Dosanjh B., Haard N. and Toy B. (1995). Influence of spoilage and processing temperature on the quality of marine fish protein sources for salmonids. Aquaculture Nutrition. 1: 169 - 177.

Levin J., Poore T.E., Young N.S., Margolis S., Sauber N.P., Townes A.S. and Bell W.R. (1972). Gram-negative sepsis: Detection of endotoxemia with the Limulus test.
Ann. Intern. Med. 76: 1 - 7.

Lied E., Julshamn K. And Braekkan O.R. (1982). Determination of protein digestibility in Atlantic cod with internal and external indicators. Can. J. Fish. Aquat. Sci. 39: 854 - 861.

Mietz J.L. and Karmas E. (1978). Polyamine and histamine content of rockfish, salmon, lobster, and shrimp as an indicator of decomposition. Journal of the Association of Official Agricultural Chemists. 61: 139 - 145.

Opstvedt J., Mundheim H. And Nygård E.R. (1996). Project B 13A. Freshness of fish meal on palatability in salmon. SSF-report no. K-226. Contractor: IFOMA.

Pike I.H. (1993). Freshness of fish for fish meal - effect on growth of salmon. In: S.J. Kaushic and P. Luquet. (Eds.). Fish Nutrition in Practice. INRA. Paris, pp. 843 - 846.

Sandnes K, Lie Ø. and Waagbø R. (1988). Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon, *Salmo salar*. J. Fish. Biol. 32: 129 - 136.

Skrede A. (1979). Utilization of fish and animal bi-products in mink nutrition: IV. Fecal excretion and digestibility of nitrogen and amino acids in mink fed cod fillet or meat- and bone-meal. Acta Agric. Scand. 29: 241 - 257.

Sokal R.R. and Rohlf F.J. (1981). Biometry. W.H. Freeman and Co., New York, 859 pp.

Watanabe T, Nanri H., Satoh S., Takeuchi M. and Nose T. (1983). Nutritional evaluation of brown meals as a protein source in diets for rainbow trout. Bull. Jap. Soc. Scient. Fish. 49: 1083 - 1087.

Appendix 1.

Limulus Amebocyte Lysate gel clot test

Determination of endotoxin (lipopolysaccharide structures from Gram-negative bacterial cell walls) in experimental feeds, fish meals, intermediate products and solubles and in stored raw material (EU/ml).

Samples	Endotoxin EU/ml
Fish feeds	
Diet 1	12.5
Diet 2	12.5
Diet 3	12.5
Diet 4	12.5
Diet 5	12.5
Fish meals	
FM 1	125
FM 2	125
FM 3	1250
FM 4	125
FM 5	1250
Intermediate products and solubles	
Fresh presscake	1250
Spoiled presscake	1250
Press liquor	> 12500
Concentrate	> 12500
Water-washed presscake	125
Fish raw material, stored	1250

Appendix 2.

Pathologist's comments

Clinical remarks

There was one distinct clinical difference between fishes. In some fish the intestines were significantly hypertrophic and had a creamy white/yellow colour while the mucosa had a granular surface. The affected part was the section of the intestine between pylorus and rectum, and included the pyloric caeca. There seemed to be a clear difference between the different experimental groups and a high degree of conformity within different groups.

Histological examination

Changes observed

Intestine: Degenerative processes in the cylindrical epithelia cells of mucosa. The changes varied between mildly condensed nuclei to highly degenerative features with destroyed cell structure and pycnotic nuclei which seemed to have migrated downwards to the base of the cells. In some few tissues the mucosa epithelia seemed to have dissolved and fragments were found in the lumen.

Liver: A varying degree of lipid infiltration in the hepatocytes. In some of the tissues the infiltration was heavy and accompanied by occurrence of pycnotic nuclei.

In conclusion

Changes observed in many of the tissues might be associated with exposure to toxins.

Table 1.

Chemical composition (g/kg), water-soluble protein (% of total crude protein), true protein digestibility, % (mink) and content of biogenic amines (g/kg) in the different fish meals. The corresponding dietary levels of biogenic amines are given for each diet.

	FM 1 Diet 1	FM 2 Diet 2	FM 3 Diet 3	FM 4 Diet 4	FM 5 Diet 5
Fish meal					
Crude protein, g/kg	739	703	778	714	724
Lipid, g/kg	85	97	16	93	86
Water, g/kg	87	101	87	88	107
Ash, g/kg	99	96	111	87	91
Water-soluble protein, %	21.9	29.5	27.3	10.0	23.1
Lipid, g/kg (Bligh & Dyer)	118	132	47	136	119
Mink protein digestibility, %	96.3 ± 0.5^{a1}	94.5 ± 0.7^{bc}	94.1 ± 0.9^{bc}	95.4 ± 0.2^{ac}	95.4 ± 0.4^{ac}
Fish meal					
Cadaverine, g/kg	< 0.10	2.2	2.4	0.59	1.3
Histamine, g/kg	< 0.10	1.9	1.4	0.62	1.0
Putrescine, g/kg	< 0.10	0.76	0.88	0.20	0.47
Fish feed					
Cadaverine, g/kg	< 0.10	1.6	1.4	0.29	0.76
Histamine, g/kg	< 0.10	1.2	0.77	0.26	0.41
Putrescine, g/kg	< 0.10	0.55	0.52	0.11	0.27

[1] Different superscript letters indicate significant differences between dietary groups ($p < 0.05$).

Table 2 A.

Diet composition (%)

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
FM 1	61.9				
FM 2		62.8			
FM 3			62.8		
FM 4				61.1	
FM 5					62.8
Soyaprotein concentrate	2.8	4.7	0.2	6.0	3.0
NorSeaOil	21.1	20.3	25.4	20.7	21
Soyalecithin	0.5	0.5	0.5	0.5	0.5
Suprex maize	12.0	10.0	9.4	10.0	11.0
Vitamin mix[1]	1	1	1	1	1
Mineral mix[2]	0.5	0.5	0.5	0.5	0.5
Carophyll Pink	0.08	0.08	0.08	0.08	0.08
Inositol	0.03	0.03	0.03	0.03	0.03
Rovimix Stay-C	0.024	0.024	0.024	0.024	0.024
Betafin	0.04	0.04	0.04	0.04	0.04

[1] Vitamin mix (Hoffman La Roche) providing per kg feed:
Vitamin A, 3000 IE; Vitamin D_3, 600 IE; Vitamin E, 160 mg; Thiamin, 12 mg; Riboflavin, 24 mg; Pyridoxin, 12 mg; Ascorbic acid, 60 mg, Panthotenic acid, 48 mg; Biotin, 0.6 mg; Folic acid, 6 mg; Niacin, 120 mg; Vitamin B_{12} , 0.024 mg; Vitamin K_3 (MPB/K_3), 12 mg.

[2] Mineral mix providing per kg feed:
$MgSO_4.7H_2O$, 500 mg magnesium; $CuSO_4.5H_2O$, 5 mg copper; $MnSO_4.H_2O$, 10 mg manganese; KCl, 400 mg potassium; $ZnSO_4.7H_2O$, 80 mg zinc; $FeSO_4.7H_2O$, 50 mg iron.

Table 2 B.

Proximate composition (g/kg) and energy content (MJ/kg) of the respective experimental diets.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Protein, g/kg	507	495	514	494	507
Lipid, g/kg	268	271	260	258	268
Carbohydrate, g/kg	107	105	96	122	103
Water, g/kg	48	58	53	64	54
Ash, g/kg	70	71	77	62	68
Gross energy, MJ/kg[1]	24.4	24.2	24.0	23.9	24.3
Digestible energy, MJ DE/kg[2]	21.1	21.0	20.9	20.7	21.1

[1] Calculated according to the following caloric values (MJ/kg): Protein 23.7, fat 39.5 and carbohydrate 17.2.
[2] Calculated according to the caloric values and digestibility coefficients for nutrients as follows: Protein 0.87, fat 0.90 and carbohydrate 0.65.

Table 3.

Growth, feed intake, feed conversion and mortality in the experimental period, mean values ± standard deviation (n=3).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	ANOVA p[1]
Initial weight, g	139.9 ± 0.3	139.7 ± 0.2	140.2 ± 0.6	140.3 ± 0.2	140.0 ± 0.5	0.46
Weight gain, g	205.5 ± 11.6^{a2}	123.4 ± 10.5^{b}	141.7 ± 3.1^{bc}	165.3 ± 4.8^{d}	158.9 ± 8.6^{cd}	< 0.001
Final weight, g	345.4 ± 11.8^{a}	263.1 ± 10.6^{b}	281.8 ± 2.6^{b}	305.6 ± 5.0^{c}	298.9 ± 9.1^{bc}	< 0.001
SGR[3]	1.13 ± 0.04^{a}	0.79 ± 0.05^{b}	0.87 ± 0.02^{bc}	0.97 ± 0.02^{d}	0.95 ± 0.04^{cd}	< 0.001
TGC[3]	3.24 ± 0.14^{a}	2.16 ± 0.15^{b}	2.41 ± 0.05^{bc}	2.73 ± 0.06^{d}	2.65 ± 0.11^{cd}	< 0.001
g feed eaten/day/fish[3]	1.86 ± 0.10^{a}	1.29 ± 0.12^{b}	1.47 ± 0.08^{b}	1.87 ± 0.04^{a}	1.40 ± 0.02^{b}	< 0.001
% feed eaten/day/ biomass fish[3]	0.76 ± 0.03^{a}	0.64 ± 0.07^{b}	0.70 ± 0.04^{b}	0.84 ± 0.01^{a}	0.64 ± 0.01^{b}	< 0.001
FCR[3]	0.72 ± 0.02^{a}	0.87 ± 0.08^{b}	0.84 ± 0.08^{b}	0.89 ± 0.01^{b}	0.70 ± 0.03^{a}	< 0.01
Number of dead fish						
Day 1 - 41	18	36	16	20	18	
Day 42 - 82	9	28	22	12	11	
Total mortality, %	7.5 ± 2.5^{a}	17.8 ± 1.0^{b}	10.6 ± 4.2^{a}	8.9 ± 2.1^{a}	8.1 ± 1.7^{a}	< 0.01

[1] Probability for statistical significance.

[2] Different superscript letters indicate significant differences between dietary groups ($p < 0.05$).

[3] Determined according to the formulas given under Experimental

Table 4.

Weight (g), length (cm), condition factor, liver weight (g) and the hepatosomatic index (HSI) in fish after a feeding period of 12 weeks (n=3, each of 10 fish) and liver fat content in pooled samples of fish (n=3, each of 5 fish). Mean values ± standard deviations (n=3).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	ANOVA p[1]
Weight, g	358.0 ± 23.9^{a2}	286.3 ± 8.6^{b}	285.7 ± 7.3^{b}	326.7 ± 2.5^{ac}	307.1 ± 14.1^{bc}	< 0.01
Length, cm	30.8 ± 0.6^{a}	29.1 ± 0.4^{bc}	28.7 ± 0.1^{bc}	29.8 ± 0.1^{ac}	29.4 ± 0.6^{bc}	< 0.01
Condition factor[3]	1.22 ± 0.03^{a}	1.15 ± 0.03^{bc}	1.20 ± 0.03^{ac}	1.23 ± 0.01^{a}	1.20 ± 0.02^{ac}	< 0.05
Liver weight, g	3.8 ± 0.3^{a}	3.2 ± 0.2^{bc}	3.7 ± 0.1^{ac}	3.8 ± 0.3^{ac}	3.4 ± 0.2^{ac}	< 0.05
HSI	1.06 ± 0.02^{a}	1.12 ± 0.04^{a}	1.28 ± 0.07^{b}	1.14 ± 0.09^{a}	1.09 ± 0.02^{a}	< 0.01
Liver fat content, %	2.1 ± 0.1^{a}	2.6 ± 0.2^{a}	4.7 ± 0.4^{b}	4.2 ± 1.1^{b}	2.2 ± 0.1^{a}	< 0.01

[1] Probability for statistical significance.
[2] Different superscript letters indicate significant differences between dietary groups ($p < 0.05$).
[3] Condition factor = Body weight x 100/(LengthxLengthxLength)

Table 5.

Weight (g), blood haematocrit (hct) and the corresponding serum values of lysozyme (U/l), protein (g/l), albumin (g/l) and the albumin/protein ratio in fish after a feeding period of 12 weeks. Mean values ± standard deviations (n=3, each of 5 individual fish).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Variance analysis p[1]
Weight, g	353.0 ± 20.4[a]	281.0 ± 25.2[b]	285.7 ± 7.3[bc]	326.7 ± 2.5[ac]	307.1 ± 14.1[bc]	< 0.01
Hct, %	47.7 ± 1.1	42.8 ± 2.1	43.9 ± 1.1	42.7 ± 3.4	44.5 ± 0.9	0.06
Serum lysozyme, U/l	1227 ± 51[a]	1300 ± 45[a]				0.13
Serum lysozyme, U/mg prot	26.1 ± 1.4[a]	33.8 ± 3.5[b]				0.05
Serum protein, g/l	47.9 ± 0.9[a]	39.5 ± 3.3[b]				0.05
Serum albumin, g/l	22.7 ± 1.8[a]	17.2 ± 2.3[b]				0.05
Albumin/protein	0.47 ± 0.05[a]	0.44 ± 0.03[a]				0.27

[1] Probability for statistical significance according to non-parametric tests (Kruskal-Wallis One-Way Analysis of Variance).
[2] Different superscript letters indicate significant differences between dietary groups ($p < 0.05$).

Table 6.

Dietary contents of amino acids in the control diet produced with fish meal processed from absolutely fresh herring, TVN = 18 mg/100g, (Diet 1) and from herring stored to a TVN content of 130 (Diet 2).

Amino acids	Diet 1 Aa, g/100 g prot	Diet 2 Aa, g/100 g prot
ala	6.0	6.4
arg	5.9	5.7
asp	8.9	8.8
cys	-	-
glu	13.5	13.5
gly	6.3	6.3
his	2.1	1.8
ile	4.3	4.4
leu	8.1	8.2
lys	7.7	7.5
met	2.8	2.8
phe	3.8	3.9
pro	4.1	4.2
ser	4.2	4.0
thre	4.0	4.1
trp	-	-
tyr	3.0	2.8
val	4.8	4.8
Sum amino acids	90.4	90.1
Aa digestibility, %		

Table 7.

Organ histology in Atlantic salmon fed diets with fish meal produced from fresh (FM 1) and stale (FM 2) herring, and with different fish meal fractions, i.e. presscake and solubles (FM 3, FM 4, FM 5).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Variance analysis p[1]
Liver						
Number of affected fish	0 / 15[a2]	2 / 15[ab]	9 / 15[c]	4 / 15[bc]	0 / 15[a]	< 0.05
Lesion severity [3]:						
0	15	13	6	8	15	
1		2	7	3		
2			2	1		
3						
Intestines						
Number of affected fish	0 / 15[a]	4 / 15[b]	15 / 15[c]	7 / 15[b]	0 / 15[a]	< 0.01
Lesion severity [3]:						
0	15	8		8	15	
1		4	8	4		
2			7	3		
3						

[1] Probability for statistical significance according to non-parametric tests (Kruskal-Wallis One-Way Analysis of Variance).
[2] Different superscript letters indicate significant differences between dietary groups ($p < 0.05$).
[3] The clinical changes might be indexed as follows:
0 = Normal;
1 = Minor changes. Most probably reversible to normal;
2 = Significant changes. Most probably affecting the function of the organ;
3 = Strong changes; Irreversible. Necrosis.

Table 8.

Macroscopic evaluation of the gastroinstestinal tract in Atlantic salmon fed diets with fish meal produced from fresh (FM 1) and stale (FM 2) herring or with different fish meal fraction, i.e. presscake and solubles (FM 3, FM 4, FM 5).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Variance analysis p[1]
Gastrointestinal tract						
Number of affected fish	0 / 15[a2]	9 / 15[ab]	15 / 15[b]	15 / 15[b]	0 / 15[a]	< 0.05
Lesion severity [3]:						
0	15	6			15	
1		9		7		
2			15	8		

[1] Probability for statistical significance according to non-parametric tests (Kruskal-Wallis One-Way Analysis of Variance).
[2] Different superscript letters indicate significant differences between dietary groups ($p < 0.05$).
[3] The clinical changes might be indexed as follows:
0 = Normal appearance;
1 = Abnormal white/yellow colour;
2 = Distinct creamy white/yellow colour and clearly hypertrophic.