HEALTH RISKS OF FEEDING OF RUMINANTS WITH FISHMEAL IN RELATION TO THE RISK OF TSE

Opinion of the Scientific Panel on Biological Hazards

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Opinion of the European Food Safety Authority on the assessment of the health risks of feeding of ruminants with fishmeal in relation to the risk of TSE[1]

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SUMMARY

Since Bovine Spongiform Encephalopathy (BSE) was reported for the first time in 1986 in the UK, the European Commission (EC) has developed a comprehensive set of risk reducing measures on transmissible spongiform encephalopathies (TSEs) in order to protect human health from BSE and to control and eventually eradicate TSEs in animals. This Regulation (EC No 999/2001) of the European Parliament and of the Council lays down rules for the prevention, control and eradication of certain TSEs.

One of the most effective risk reducing measures consisted of a total EU wide ban on the use of processed animal protein in feeds for any animal farmed for the production of food, with some exceptions (*e.g.* use of fishmeal in non-ruminants). A temporary EU-wide ban on the use of fishmeal in ruminant feed has been in place since 2001 (EC 2000/766 and 2001/9). In 2003, the temporary ban was transferred into permanent measures within the TSE regulations (EC 999/2001). Fishmeal was banned because of the difficulties of detecting small amounts of ruminant proteins in feed containing fishmeal. Feed microscopy is currently the only method officially endorsed by the European Commission to test for the presence of animal protein in feeds. A revision of Regulation (EC) No 999/2001 is foreseen and the current draft revision allows feeding young ruminants with fishmeal and introduces a tolerance level for fishmeal in feed for adult cattle under strict conditions. Consideration to any lifting of this temporary ban on fishmeal use in ruminant diets should be supported by (1) a scientific risk assessment indicating if a risk of spreading BSE to ruminants through fishmeal is existing or not and (2) the development of a validated method allowing detection, identification and discrimination up to the mammalian species level of the presence of mammalian Meat and Bone Meal (mMBM) in ruminant feeds even in the presence of fish meal in the same feed.

In October 2004 the European Parliament adopted a resolution where it calls on the Commission to withdraw its Draft Regulation amending Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards animal nutrition (SANCO/3027/2004) on the consideration that the feeding of fishmeal to ruminants is not consistent with the duty imposed on the Community by Article 152(1) of the EC Treaty and that the level of public health protection can not be lowered. The European Food Safety Authority (EFSA) and its Scientific Panel on Biological Hazards was invited by the European Parliament to provide an opinion on the state of play as regards the health risks of the feeding of ruminants with fishmeal in relation to the risk of TSE and if this could it have negative consequences in terms of public health.

The experts of the Scientific Panel on Biological Hazards concluded that if there is any risk of TSE in fishmeal, this could arise from the mammalian feed being fed to this fish or through fishmeal contaminated by Meat and Bone Meal (MBM). If and when fish meal would be allowed back into the feed chain, in terms of Public Health, the concerns remain at the level of the prevention of cross contamination with MBM. The risk of TSE in fish, either being fed directly or by amplification of infectivity is remote. Much progress is made in tests used for the detection of MBM in feed using PCR for the detection of species specific DNA in heat treated animal proteins. This progress in tests developed and the combination of different tests now allow better detection and differentiation of MBM up to the species level, however, there is still no 100% guaranteed method available. Following these conclusions, also a number of recommendations were made for further research.

KEY WORDS: TSE, fishmeal, ruminants, Meat and Bone Meal, discriminatory test evaluation

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1. INTRODUCTION

Since Bovine Spongiform Encephalopathy (BSE) was reported for the first time in 1986 in the UK, the European Commission (EC) has developed a comprehensive set of risk reducing measures on transmissible spongiform encephalopathies (TSEs) in order to protect human health from BSE and to control and eventually eradicate TSEs in animals. That legislation has continuously been reviewed in the light of new scientific evidence, the evolution of the TSE situation and the practical implementation in the field.

The key piece of legislation to protect human and animal health from the risk of BSE and other TSEs was adopted on 22 May 2001. This Regulation (EC) No 999/2001 (EU, 2001b) of the European Parliament and of the Council lays down rules for the prevention, control and eradication of certain TSEs.

One of the most effective risk reducing measures consisted in a total EU wide ban on the use of processed animal protein in feeds for any animal farmed for the production of food, with some exceptions (*e.g.* use of fishmeal in non-ruminants).

Since the implementation of the TSE Regulation in 2001, more than 50 million of adult bovine animals have been tested across the EU and around 7000 cases have been detected. A constant decline (about 35% per year) in the number of cases has been recorded: from 2167 cases in 2001 to around 520 cases in 2005. Out of this only 11 cases were related to animals born after the start of the total feed ban as mentioned above.

In referring to fish meal specifically, a temporary EU-wide ban on the use of fishmeal in ruminant feed has been in place since 2001 (EU, 2000 and EU, 2001a). In 2003, the temporary ban was transferred into permanent measures within the TSE regulations (EU, 2001b). The ban was introduced as part of emergency BSE control measures. It has been generally thought up to now, that the role of fish and fishmeal in the transmission of BSE to ruminants is little or non-existing (SSC, 2003). Fishmeal was banned because of the difficulties to detect small amounts of ruminant proteins in feed containing fishmeal. Feed microscopy is currently the only method officially endorsed by the European Commission to test for the presence of animal protein in feeds. This official method is capable of discriminating between fish protein and mammalian protein, but its sensitivity in detection of mammalian processed animal proteins decreases in the presence of significant amounts of fishmeal. Moreover, the test can not discriminate within the mammalian species, up to the species level.

A revision of Regulation (EC) No 999/2001 is foreseen for the end of 2006; the current draft revision allows feeding young ruminants with fishmeal and introduces a tolerance level for fishmeal in feed for adult cattle under strict conditions.

Consideration to any lifting of this temporary ban on fishmeal use in ruminant diets should be supported by:

1. A scientific risk assessment reviewing all scientific data available supporting the earlier conclusion (SSC, 2003) that the risk of spreading BSE to ruminants through fishmeal is negligible or non existing

2. The development of a validated method allowing detection, identification and discrimination up to the mammalian species level of the presence of MMBM in ruminant feeds even in the presence of fish meal in the same feed.

These two conditions are reviewed and an update based on scientific data is provided in this document. More specifically for the second condition, a more sensitive analytical method has now been developed, based on the EU existing official method, which clearly identifies the presence of meat and bone meal in animal feed, depending on concentration, even in the presence of fishmeal. The EU official microscopic method which shows after revision an improved performance profile (Commission Directive 2003/126/EC) allows for the detection of meat and bone meal in animal feed, also in the presence of fishmeal. However, at trace levels of MBM in the range of 0.1% the presence of fishmeal could lead to an increased ratio of false negative results. In addition, classical microscopy does not allow for animal or species specific differentiation of MBM.

2. TERMS OF REFERENCE

The European Food Safety Authority (EFSA) and its Scientific Panel on Biological Hazards is invited by the European Parliament to provide an opinion on:

- *The state of play as regards the health risks of the feeding of ruminants with fishmeal in relation to the risk of TSE*
- *Could it have negative consequences in terms of public health?*

3. BACKGROUND TO THE MANDATE

3.1. Scientific knowledge and former SSC opinions

The Opinion of the Scientific Steering Committee (SSC, 2003) on "*The feeding of wild fishmeal to farmed fish and recycling of fish with regard to the risk of TSE*", addressed the question whether the historical practice of feeding Mammalian MBM and other mammalian products and the common practice of intra-species and intra-order recycling via feed could enable mammalian TSE agents to establish themselves in fish and for species adaptation of such agents to occur. This could lead to the development of a TSE in fish that might lead to a TSE epidemic in fish and/or create a health risk for the consumer or for susceptible ruminants if fed with contaminated fishmeal.

The former Scientific Steering Committee (SSC) was invited:

1. To advise whether the feeding of wild fishmeal to farmed fish presents any risk to animal or human health vis-à-vis TSEs;
2. If appropriate, to suggest examples of conditions under which intra-species or intra-order recycling of fish could be allowed.

The opinion was largely based on various SSC opinions and reports of the TSE/BSE *ad hoc* group related to animal waste disposal and intra-species recycling, on elements from the report of the Scientific Committee on Animal Health and Welfare on "*The use of fish waste in aquaculture*" and on the interim results of the FAIR CT97 3308 project entitled "*Separation, identification and characterization of the normal and abnormal isoforms of prion protein*

from normal and experimentally infected fish". Some recent publications were also taken into account in that opinion, *i.e.* that a homologue to prion-protein was identified in the pufferfish (*Fugu rubripes)*, showing high homology with mammalian PrP sequences and that the normal isoform of prion protein (PrP) was described in brains of spawning salmon. The main conclusions in this opinion were that, from the limited available research results, scientific literature on TSEs in fish and routine examinations of fish brain in the course of fish disease diagnosis, it could be concluded that there is no evidence that a natural TSE exists in fish and that there are no indications of replication of scrapie or BSE agent in experimental transmission studies. On the question *whether the feeding of wild fishmeal to farmed fish presents any risk to animal or human health vis-à-vis TSE's*, the SSC therefore concluded that there was currently no evidence of any such risk existing. However, some theoretical risks could exist, linked to feeding possibly TSE-contaminated feeds to animals currently believed to be not susceptible, including fish. These risks include the possible build-up of a pool of infectivity in animals that do not develop disease but may potentially be able to harbour the agent as residual infectivity in the digestive system and/or replicate the agent. The latter risk is higher when intra-species recycling is practised due to the absence of a species barrier. Also the risk of adaptation of the agent to hitherto non-susceptible hosts should be considered. Regarding the request to, *if appropriate, suggest examples of conditions under which intra-species or intra-order recycling of fish could be allowed*, the SSC therefore considers in general that fish should not be fed with potentially TSE infected feed and that sourcing of fish by-products (including for their use in fish-derived feed) should not be performed from fish that have been exposed to potentially infected feed.

With regard to the appropriate treatment of fish materials, further background information is provided in the Opinion on 'Fallen stock' by the Scientific Steering Committee (SSC, 1999) and the Scientific Committee on Animal health and Welfare (SCAHAW, 2003).

3.2. Justification of the request for a scientific opinion

In October 2004 the European Parliament adopted a resolution where it calls on the Commission to withdraw its Draft Regulation amending Annex IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council as regards animal nutrition (SANCO/3027/2004) on the consideration that the feeding of fishmeal to ruminants is not consistent with the duty imposed on the Community by Article 152(1) of the EC Treaty and that the level of public health protection can not be lowered. It is recognised that the ban on the feeding of animal meal to ruminants has produced most results in combating TSEs. It appears that there is still insufficient research on the possible infectivity of meat products derived from ruminants fed with fishmeal. There is also a zero-tolerance on contamination with bone spicules in ruminant feed because it was not clear in how far the tests available could differentiate fish bones from ruminant bones.

Under the terms of reference of the mandate, the European Food Safety Authority (EFSA) is invited to provide an opinion on the assessment of "What is the state of play as regards the health risks of the feeding of ruminants with fishmeal in relation to the risk of TSE? Could it have negative consequences in terms of public health?"

4. Risk Assessment

4.1. *Preamble*

This assessment takes account of the general control measures in place and assumes effectiveness of these controls avoiding any possible cross-contamination, willingly or unwillingly. The implementation of the feed ban and national feed ban controls are audited by the Food and Veterinary Office of the European Commission documented in their reports. The Rapid Alert system for Food and Feed (RASFF) reported a number of cases where processed animal proteins and/or bones were discovered in animal feed (Table 1).

Table 1: **Reported cases in EU of feed contaminated by animal proteins and/or bones during the period 01/01/2002 - 30/11/2006**

Year	Ruminant feed samples*	Non-ruminant feed samples*	Feed samples*	N° of reported cases***
2006	**	**	**	6
2005	**	**	**	17
2004	**	**	**	5
2003	19112	15410	10049	10
2002	26106	17053	7910	12
2001	24102	14751	2315	

*Source: European Commission DG Sanco
**No exact data available but numbers have increased as compared to previous years
***Source: Rapid Alert system for Food and Feed - EU

4.2. *Introduction*

Since the former Opinion of the Scientific Steering Committee (SSC, 2003) on "*The feeding of wild fishmeal to farmed fish and recycling of fish with regard to the risk of TSE*", new scientific information became available as well as research results carried out on different tests which claim they can be used to differentiate fish bone spicules from mammalian bone spicules.

The assessment of the health risks of feeding of ruminants with fishmeal in relation to the risk of TSE is based on data of new, published or ongoing experimental studies since the former opinion of March 2003 (SSC, 2003) and takes account of the knowledge on recently developed differentiating tests. It also takes into consideration the important aspect that cross contamination of fishmeal with mammalian (ruminant) meat and bone meal might be the most probable, if not only, cause of infectivity in fishmeal, if BSE/TSE in fish is inexistent.

The relationship between PrP^{sc} and infectivity is clearly a fundamental issue which is still subject of continuing scientific debate. According to the prion hypothesis, PrP^{sc} is an infectious protein and the causative agent of TSEs (Prusiner, 1982). In TSEs, the accumulation of PrP^{sc} in tissues of infected individuals is correlated with the presence of infectivity (McKinley *et al*., 1983; Race *et al*., 2001). While titration of infectivity through bioassay remains the only effective tool for quantifying the TSE agent, the development of

sensitive PrP measurement tools, combined with the use of recombinant PrP as external standard, has allowed a robust quantification of PrP^{sc} in various tissues (Gatti, *et al.,* 2002, Andreoletti *et al.,* 2002, Andreoletti *et al.,* 2004). In recent studies, the PrP^{sc} quantities (after PK digestion) were compared to infectious titre as assessed in a transgenic (VRQ PrP protein) ovine mouse model (Andreoletti *et al.,* 2004) and an apparent linear relationship was established over a limited range of PrP concentrations. In this experiment infectious titre could still be detected in the absence of a PrP^{sc} positive signal (~102 LD50 per g).

Not surprisingly the biochemistry of PrP varies with the prion strain or type of disease. This can be illustrated by recent data obtained on atypical scrapie in sheep. Le Dur *et al.,* titrated a “discordant” case of sheep TSE in tg338 mice over-expressing the VRQ allele of ovine prion protein (Le Dur *et al.,* 2005) They found high levels of infectivity (> 108 LD50 per g) in brain with a very low content of protease-resistant prion protein. In a recent field trial, no PrP^{sc} signal could be detected below 1/500 dilution by any rapid tests in cerebral cortex of Nor98 atypical cases (EFSA, 2005a, b).

Taken together these data appear to indicate:

- The correlation between the PrP amount and infectivity depend on the type of TSE agent
- Sensitivity of abnormal PrP biochemical detection is still lower than most efficient bioassays: absence of abnormal PrP does not mean an absence of infection in a tissue.

In Annex 1, background on the production and use of fishmeal is provided.

4.3. Analysis of new scientific information

The RTD supported project FAIR-CT-1997-03308 (RTD, 1997) was unable to find any evidence of the replication of TSE in fish.

RTD currently supports one project relating to TSE in fish (QLK5-CT-2001-00866). This project is carrying out a long term infection study in sea bream, bass and trout, to investigate the transfer of prions at the level of the gut and examines the molecular biology of fish prion protein homologues. Further in the report, details are given on the findings from this work.

In the first call of FP7, it is foreseen to include a topic on "Protecting animal and human health from prions in food, feed and the environment" however, the topic is focused on infection from ruminants.

4.3.1. Update on recent research on Prion Proteins in fish

Fish may play a role in the transmission of prions to mammals in 2 ways:

1. by amplifying infectivity and passing fish prions on to mammals

2. by passing on infectivity after having been fed with contaminated animal meal

4.3.1.1. Research on the potential transmission of Prion diseases to fish

During the past few years farmed fish production in five EU countries has increased more than 10 times to over 90 tons per year. All these fish receive commercial food containing 40 - 55% protein. Animal proteins other than fishmeal, although forbidden, may also be present and the occurrence of orally transmitted encephalopathies cannot be excluded until experiments currently underway are completed.

Within the last 3 years attempts were made in order to address the question and evaluate the possibility of possible transmission of transmissible spongiform encephalopathies (TSEs), scrapie and BSE, in fish. The nature of the TSEs agent is thought to be the pathological isoform of normal cellular prion protein that can change conformation. TSEs have been studied in higher organisms including primates and rodents, but little is known about TSE pathogenesis in fish. Such knowledge is important however, as fish farming is becoming a very important industry and provides high protein nutrition. Animal proteins may also be present in fish feed and the occurrence of the pathological protein cannot be excluded. Alternatively farmed fish exposed to these pathogens might be potentially introduced to animal feeding as well. The primary objective of the contacted research was a much-needed determination if TSEs (BSE and scrapie) can be transmitted to fish.

During this period TSE transmission studies got underway with the force-feeding of natural scrapie and control sheep brain homogenates to sea bream and sea bass, while the force-feeding of BSE and control cow brain homogenates to these species was completed later. Since then, and for at least 3.5 years clinical evaluations of all challenged fish have been made on a daily basis and the routine sampling of these fish has been carried out at selected time points. Histopathological examination of tissues is ongoing, as are immunohistochemical and western blot analyses of PrP^{Sc} in the sampled tissues.

Complementary experiments evaluating the possibility of intestinal binding/absorption in trout *in vitro* are detailed further.

The second avenue of research undertaken by us and other research groups was the identification of PrP and PrP-like proteins in fish genomes. This line of the research was deemed necessary as the PrP gene in fish had not yet been identified at the project's outset. During this period PrP and PrP-like proteins from Atlantic salmon, zebrafish, carp, sea bass, sea bream and trout have been identified (see molecular studies mentioned below). Further analysis of the identified prion gene sequences and their promoters, as well as the proteins they encode, will continue.

Polyclonal or monoclonal antibodies are raised against recombinant PrP-like proteins from pufferfish, trout and more recently against PrP1 and PrP2 from zebra fish (data not published). All generated antisera are currently used looking for novel histopathological hallmarks in fish tissues that have been collected during the post challenge period (time points at 0, 3, 6, 12, 18, 24, 36 months). The ultimate proof for prion transmissibility to fish would be the detection (if any) of fish PrP^{Sc} depositions with detectable resistance to proteinase K, a characteristic property of mammalian prions.

In general, the actions outlined here will be continued until summer of 2007 when the financial support to this project will be terminated. Fish already challenged with natural scrapie has been recently boosted with mega doses of infectious material and they should be

monitored both by daily clinical examination and by histopathological, immunological and biochemical evaluation of sampled tissues. Additionally, tissue samples will be taken from these fish at specified time points (from day 1 to day 30) for histopathological and immunological evaluation, as well as for studies of potential TSE transmission to mice. A tissue bank has also been established.

Construction and breeding of transgenic mice carrying a fish PrP gene has been initiated as well as transgenic zebra fish carrying mouse PrP. It is expected that challenge of these transgenic mice with mouse scrapie homogenates will provide additional information concerning homologous or heterologous transmissibility.

The evaluation of intestinal binding/absorption *in vivo* and *in vitro* of natural and mouse-adapted scrapie in fish should also be continued.

Clearly, the groups who will receive the greatest benefits from the results of this research are fish farmers, the consumers of farmed fish, and the prion scientific community. The major social and economic relevance of the results will be for aquaculture, which is growing in importance as a tool for fish production.

4.3.1.2. Prion infectivity in fish, (passing fish prions on to mammals)

The critical pathogenetic event in TSE diseases is the conformational change of the physiological host prion protein (PrP^{c}) into an insoluble form (PrP^{Sc}), able to provoke the pathognomonic brain lesions and death. Although mammalian proteins are banned from commercial fish feed in most of the countries, these may be present, and the occurrence of TSE infected material cannot be excluded. In other words, after eating PrP^{Sc}, fish might, possibly, be a reservoir of the infectious agent, even if not undergoing any disease (Race and Chesebro 1998).

The need to give an answer to public concern about safety of food possibly contaminated with TSE agents prompted was the leading thread to set up an experiment that uses fish as recipient of a scrapie agent (mouse-adapted 139A strain).

As gastrointestinal tract may represent a first target for PrP^{Sc}, after oral ingestion (Palmer *et al.,* 2000), both the "*in vitro*" and “*in vivo*” ability of PrP^{Sc} to cross the intestinal tissues of Rainbow trout (*Oncorhynchus mykiss*) were challenged by adding 139A respectively, to the mucosal side of everted or statically perfused intestine, and to the homogenate utilised for the forced feeding.

In the “*in vitro*” experiments, to investigate whether trout intestine binds PrP^{Sc} and transfers it to the serosal side where it may spread to the lymphoid tissues and, eventually, into the CNS, everted trout intestine immersed in a solution containing PrP^{Sc} (scrapie 139A) were used. On the other hand “not everted” sea bream intestine filled with a physiological solution containing PrP^{Sc} were also tested. It was observed that PrP^{Sc} slightly absorbed to the mucosal intestinal layer as shown by a low, yet detectable signal at the Western blot, but it was not detected in the serosal layer. When statically perfused intestine was used, it was possible to detect the presence of PrP^{Sc} by immunohistochemistry in the *stratum compactum* both in the trout intestine and in the pyloric caeca. The absence of signal for PrP^{Sc} at the western blot in the solution fluxed into the serosal side of the everted intestine excludes, at least in this

experimental setting, an active secretion of PrP^{Sc} from one side to the other side of the intestinal tract.

In the "*in vivo*" trials, the distal intestine showed the signal of immunolabelling up to 7 days post forced feeding, as visible after silver enhancement. Signal was detected in pyloric caeca too, although only up to 3 days. In both cases, PrP^{Sc} localization occurred in *stratum compactum*, juxtaposed to *stratum granulosum* within the mucosa. No immunological reaction was shown in *lamina epithelialis* and *lamina propria*, as well as in more external tissues when starting from the gut lumen, i. e. *tunica muscularis* and serosa. Immunolabelling of control sections from non infected forced feed meal labelled with Mab SAF 83 and control samples from tissues of fish fed with PrP^{Sc} but labelled with monoclonal anti human AGP (α_1-Acid glycoprotein), produced only a low unspecific background, after silver enhancement. After oral inoculation of scrapie prions to fish, a mouse bioassay became positive only with a single fish intestinal sample taken from the fish one day after its infection, while all other fish organs were unable to contaminate the mouse even up to 90 days after the fish inoculation.

These experimental procedures led to the detection of PrP^{Sc} both in pyloric caeca and descending intestine, though giving no evidence of PrP^{Sc} crossing through the intestinal barrier.

In conclusion, the work performed on prion infectivity in fish is not exhaustive and the research has to be extended to seawater species. Marine fishes are living in a hyperosmotic environment and their intestine plays a fundamental role as osmoregulator organ. Marine fishes drink salt water to balance the loss of organic fluids, and therefore expel salts with a specific apparatus. The influence of the water flow from the intestinal lumen through intestinal tissues on different strains of PrP^{sc} absorption should be investigated in further works.

4.3.1.3. Current knowledge on Prion protein genes in fish

To date, genes coding for homologues to the mammalian Prion protein genes have been identified in a number of fish species (Japanese pufferfishes *Fugu rubripes* and *Tetraodon nigroviridis*, Atlantic Salmon *Salmo salar*, zebrafish *Danio rerio*, common carp *Cyprinus carpio*, stickleback *Gasterosteus aculeatus*, rainbow trout *Oncorhynchus mykiss*, Medaka *Oryzias latipes*, Fathead Minnow *Pimephales promelas*, Japanese seabass *Lateolabrax japonicus*, gilthead seabream *Sparus aurata*, Japanese flounder *Paralichthys olivaceus* (Oidtmann *et al*. 2003, Rivera-Milla *et al*. 2003, Liao *et al*. 2005, Cotto *et al*. 2005, Maddison *et al*. 2005, Rivera-Milla *et al*. 2006, Strumbo *et al*. 2006).

The homologues display almost all typical features described for mammalian Prion proteins: N-terminal signal sequence, GP-rich repeat region in the N-terminal domain, followed by a hydrophobic region, Cystein residues that could form a disulfide bridge in the C-terminal domain, a GPI-anchor and Asparagin residues that may be used as glycosylation sites.

In contrast to mammals, fish genomes encode for more than one PrP homologue. Two distinct cDNAs / genes have been identified in most species investigated to date. These additional PrP genes are the result of genome duplication events that have occurred after the ray fin fish line diverted from the common ancestors of mammals, birds and reptiles. A further genome

duplication event has been identified in some salmonid and cyprinid species, which could mean that 4 instead of 2 PrP genes could be present in those species.

Research on related prion protein homologues or homologies between fish and mammalian PrP^{s}, expression patterns and analysis of 3D structures;

Sequence identities between fish PrPs and human or cow PrP on amino acid level in the PrP core region (corresponding to aminoacids 90-230 of the human PrP) range from 12-22%.

Investigations into the expression of PrPs in various tissues have revealed similar patterns for PrP1 in Atlantic salmon (Oidtmann *et al*. 2003, m-RNA levels, protein expression unpublished) and rainbow trout (Maddison *et al*. 2005 and unpublished results) compared to expression patterns found in mammals. High levels of expression are seen in brain, medium level expression in heart muscle tissue and gill, and relatively low expression in muscle tissue.

Expression patterns of carp PrP2 mRNA was markedly different from those in salmonid PrP1s: Expression levels in skeletal muscle tissue were at the same level as in brain, suggesting that relevant levels of PrP2 expression may be expected in skeletal muscle tissue.

Expression of PrP in skeletal muscle tissue of mammals is low. Expression patterns of duplicated genes often evolve away from that of the father/mother gene. Therefore, relevant expression levels of duplicated PrP genes may be found in skeletal muscle of fish, which could have implications for the likelihood of fish to potentially serve as a source of PrP^{Sc}. However, if sequence homologies between yet to be identified fish PrPs and mammalian PrPs are similarly low to those identified to date, the likelihood of bridging the species barrier appears to be very low.

Attempts to analyze the 3-dimensional structure of recombinant fish PrPs have not yet been successful. Therefore predictions about the likelihood of mammalian PrP^{Sc} to impose a PrP^{Sc} conformation on fish PrPs are difficult to make.

Sequence identities between mammalian and fish PrPs are relatively low (maximum 22% on protein level), suggesting a substantial species barrier. However, fish PrP genes with higher homologies may be detected in the future.

4.3.1.4. PrP and PrP-related genes in lower vertebrates

The first PrP sequences reported from lower vertebrates were from a reptile and an amphibian species (Simonic *et al*. 2000, Strumbo *et al.* 2001). Despite remarkable differences among vertebrate PrP amino acid sequences, both their main structural motifs and their 3D structures of the C-terminal domains are well conserved from amphibians to mammals. On the contrary, ability of non-mammalian vertebrate PrPs to undergo conformational changes is still unknown and questionable. Moreover, the existence of species barriers inside mammals is a strong argument to suggest that productive interactions between proteins of even more distant species and exhibiting remarkable different primary structures are very unlikely.

Moreover, the existence of species barriers inside mammals suggests that it would be very unlikely a productive interaction between proteins exhibiting remarkable different primary structures.

The same conformation is shared by the mammalian PrP-related protein Doppel, which exhibits very different expression pattern and physiological role as well as inability to adopt the pathological isoform.

Searches for PrP in fishes led to the identification of many different fish proteins with some similarities, but always significantly longer or shorter than mammalian PrP. They can be divided into two groups. The first comprises two long molecules (PrP1 and PrP2), which have more or less the same structural motifs of the mammalian PrPs but their sizes and primary structures are considerably different; furthermore, their identity range inside fish species is very wide (35 - 85%). The second group comprises three short PrP-related molecules called PrP-like protein, Shadoo2 and Shadoo, which retain some, but not all, the structural motifs of the mammalian PrP. Out of these five proteins, Shadoo is unique since it is very well conserved in the whole vertebrate lineage. The other four proteins are fish-specific and, among them, PrP1 and/or PrP2 are the best candidates as PrP orthologue(s) although they have diverged significantly and may have evolved to different specialized roles. They also show different expression patterns. Therefore the above hypothesis has to be supported by further studies, especially on their folding and misfolding abilities.

In conclusion, our knowledge about fish prion greatly advanced in the last years; nevertheless identity of a PrP orthologue is not still conclusive.

4.3.2. Analysis of the research on the evaluation of different discriminatory tests

Various methods exists which can be used to differentiate fish material from mammalian material in animal feed and thus for the detection of meat and bone meal in animal feed. Within the European Union classical microscopy needs to be applied in the frame of official control as specified in Commission Directive 2003/126 EC (European Union, 2003). This Directive also contains a detailed method description. The Directive states that with this method, very low amounts of MBM (< 0.1%) in animal feed can be detected. However, the actual limit of detection could be different or even higher depending on various factors (e.g. the bone fraction of the MBM, the presence of fishmeal) Nevertheless, the limit of detection of 0.1% is set as benchmark against which the suitability of other methods can be measured. Recent interlaboratory studies (von Holst *et al*. 2006) confirmed that microscopy is capable of detecting 0.5% of MBM in animal feed that also contains 5% of fishmeal indicated by a very high ratio of correct positive results (sensitivity about 95%). Furthermore, sensitivity drops to about 70% when the same feed only contains 0.1% of MBM. In another interlaboratory study, a better sensitivity was obtained on feed samples, also containing 5% of fishmeal and 0.1% of meat and bone meal (van Raamsdonk and van der Voet 2003; van Raamsdonk *et al*., 2004). However, the results from these studies are not directly comparable (e.g. differences in material used).

Inherently, the discriminative power of classical microscopy towards a higher taxonomic level (e.g. mammalian MBM from poultry MBM) is limited, thereby requiring the development and validation of alternative techniques such as polymerase chain reaction (PCR). European legislation (European Union, 2003) allows the *additional* use of such methods to gain more information on a sample. In principle, PCR is capable to differentiate ruminant MBM from non-ruminant MBM or more generally even to identify its species composition, provided that some precautions are taken in the test design due to the severe heat treatment MBM has undergone. A study conducted in 2003 (Gizzi *et al*., 2004) revealed that almost all PCR methods failed to detect in animal feed a level of 0.1% MBM treated according to EU

legislation (steam pressure sterilisation at 133^{0}C, 20 min and 3 bars). In a recent paper (Fumière *et al*., 2006) on specific real time (RT)-PCR it was shown that by using short DNA targets (below 100 bp) ruminant MBM treated according to European legislation could be detected when present in compound feed at 0.1%. In addition, it was shown that the sensitivity of the method is dependent on the rendering process: MBM treated at lower temperatures are easier to detect than those treated at higher temperature (Chiappini *et al*., 2005). This PCR method and two other RT-PCR methods were also evaluated in a recent inter-laboratory study (Prado *et al*., 2006) in which blind compound feed samples containing 0.1% of cattle MBM and various ingredients including fishmeal and porcine MBM were sent to the laboratories concerned for subsequent analysis. In this exercise the laboratories applied their own methods which focused on different DNA targets (ruminant, cattle, sheep and pig). *All* methods were capable of detecting the 0.1% of cattle MBM, either via ruminant or cattle DNA targets. This held true, *regardless* of whether the compound feed contained fishmeal or not. All blank compound feed samples for cattle were correctly classified as negative, but some of the blank animal feed samples for pig were wrongly classified as positive for cattle/ruminant MBM. One reason could be the presence of bovine fat which is a legal feed ingredient and which could contain enough bovine DNA traces to be detected by the PCR methods. In recent years, the application of PCR method on heat treated material has been largely improved. Therefore, in order to increase reliability of the final result of analysis, the outcome from both classical microscopy measurement and PCR has to be taken into account. In this context other techniques such as the combination of microscopy with near Infrared spectroscopy will contribute significantly to future control strategies (Baeten, 2005). Presently the CRL-AP is organizing an inter laboratory trial with two aims: one part is dedicated to check the proficiency of the National Reference Laboratories in detection of processed animal proteins (presence/absence) while another part is designed to assess in how far the reference method is able to quantify fishmeal in feed. Results are expected for early spring 2007.

5. CONCLUSIONS

5.1. Conclusions on the TSE risks including public health of feeding of ruminants with fishmeal

a. PrP homologues in fish have been described in considerable number of fish species, some of these relevant for human consumption (salmon, trout, sea bass, sea bream, turbot)

b. The range of sequence homology for PrP between fish and mammalian species is lower than 40%, suggesting a high species barrier for transmission of mammalian prions to fish. However, the range of sequence homology between fish varies considerably and data obtained in a particular species cannot be applied directly to other fish species.

c. Currently there are no indications that natural TSEs occur in farmed or wild fish. However, this is based on very limited examination data both in terms of fish species examined, number of samples and detection methods applied.

d. In on-going feeding experiments in sea bream and sea bass, no clinical signs or pathological lesions were shown after 3.5 years post-exposure.

e. Up to today, current research does not indicate that mammalian prions induce formation and replication of fish prions.

f. Feeding experiments in rainbow trout indicate that PrP^{sc} does not remain longer than 15 days in the fish intestine and does not cross the intestinal barrier either. This experimental work shows that there is a potential hazard of residual TSE infectivity in fishmeal produced from fish recently fed with TSE contaminated feed.

g. If there is any risk of TSE in fishmeal this could arise from the mammalian feed recently being fed to these fish or through fishmeal contaminated by MBM or fishmeal contaminated by MBM.

5.2. Conclusions on detection methods

a. Recent studies with at least three specific PCR methods show sensitivity to detect 0.1% heat treated MBM in feed even in the presence of up to 5% fishmeal, which is an improvement compared to the classical microscopy.

b. The detection of heat treated MBM in the presence of fishmeal has been considerably improved using the combination of both RT-PCR and classical microscopy.

c. PCR has the advantage over the microscopic method of detecting species specific DNA in heat treated MBM allowing to identify potential intra-species recycling.

d. Authorised ingredients (such as tallow, milk) used in animal feed can produce positive PCR results as they might be sources of DNA too. Therefore, in the presence of a positive PCR test with negative results for several other techniques to detect MBM (e.g. immuno-assay, microscopy, FTNIR-microscopy), it should be considered that the PCR result might be due to DNA originating from authorised feed ingredients.

e. It should be stressed that the reference method as well as the newly developed methods are primarily qualitative methods. Their extent to quantitative purposes has not yet been assessed in depth.

f. Due to the complexity the material in MBM in feed, all the different test results have to be taken into consideration in order to come to a final conclusion.

6. REPLY TO THE TERMS OF REFERENCE

1. If there is any risk of TSE in fishmeal, this could arise from the mammalian feed being fed to this fish or through fishmeal contaminated by MBM.

2. If and when fish meal would be allowed back into the feed chain, in terms of Public Health, the concerns remain at the level of the prevention of cross contamination with MBM. The risk of TSE in fish, either being fed directly or by amplification of infectivity is remote. A huge progress is made in tests used for the detection of MBM in feed using PCR for the detection of species specific DNA in heat treated animal proteins. This progress in tests developed and the combination of different tests now allow a better detection and differentiation of MBM up to the species level, however, there is still no 100% guaranteed method available.

7. RECOMMENDATIONS

1. Due to a potential TSE infectivity in fishmeal, either prepared from fish recently fed with MBM or fishmeal contaminated by MBM, a quantification of this risk should be considered.
2. It is recommended to consider the following topics for potential future research, focussing on the assessment of the likelihood that mammalian prions can replicate in fish and possible quantification of this amplification if any.
   a. Testing for any transmissibility of a “silent” prion disease of fish to both normal mice and transgenic mice carrying the relevant fish PrP gene(s) with tissues (spleen, brain and thymus) collected at various time points of primary transmissions.
   b. To check for residual infectivity and PrP^{sc} in different tissues using appropriate sensitive tests in combination with time-course titrations. This would allow comparison with previous tests and quantification of the residual infectivity. Time course should be done with several edible fish types, including both marine and freshwater fish.
   c. Further development of Abs specific against fish PrP^{s}
   d. Further research on alternative feeding strategies in fish avoiding TSE risks.
3. It is recommended that testing should rely on a combination of different methodologies because of the complexity of the composition of MBM (bones) or feed (tallow) in general, (*e.g.* various fractions of bones from different origins in the presence of allowed DNA from bovines. Sensitivity of the different tests should be further improved.

8. DOCUMENTATION PROVIDED TO EFSA

Letter from Mr. Borrel, President of the European Parliament dd.26.10.2005 (RE 205049) including the request and justification

Relevant SSC opinions

- Scientific Opinion of the Scientific Steering Committee on the risks of non conventional transmissible agents, conventional infectious agents or other hazards such as toxic substances entering the human food or animal feed chains via raw material from fallen stock and dead animals (including also: ruminants, pigs, poultry, fish, wild/exotic/zoo animals, fur animals, cats, laboratory animals and fish) or via condemned materials. Adopted by the Scientific Steering Committee at its meeting of 24-25 June 1999
- Scientific Opinion of the Scientific Steering Committee on The risk born by recycling animal by-products as feed with regard to propagating TSE in non-ruminant farmed animals. Adopted by the Scientific Steering Committee at its meeting of 17 September 1999
- Scientific Steering Committee Opinion on the Scientific basis for import bans proposed by 3 member states with regard to BSE risks in France and the Republic of Ireland; on the Scientific basis for several measures proposed by France with regard to

BSE risks and on the Scientific basis for banning animal protein from feed for all farmed animals, including pig, poultry, fish and pet animals. Adopted by the Scientific Steering Committee at its meeting of 27-28 November 2000

- Scientific Committee on Animal Health and Animal Welfare (2002). Draft report on "The use of fish waste in aquaculture."
- Opinion of 6-7 March 2003 the Scientific Steering Committee Opinion on the feeding of wild fishmeal to farmed fish and recycling of fish with regard to the risk of TSE. Adopted by the Scientific Steering Committee at its meeting of 6-7 March 2003

***Research projects**:*

Results of the FAIR CT97 3308 project entitled "*Separation, identification and characterisation of the normal and abnormal isoforms of prion protein from normal and experimentally infected fish*"

Results of the RTD shared cost 2002-2007 QLK5-2002-00866 entitled "*Evaluation of the possible transmission of prions (scrapie and BSE) to different fish species"*

Result of the G6D-2000-CT-00414 project entitled *"Strategies and methods to detect and quantify mammalian tissues in feedingstuffs"* (acronym: STRATFEED).

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10. ANNEX 1: BACKGROUND ON FISH MEAL PRODUCTION AND USE FOR FEEDING

Feeding of farmed fish

Since the end of the Second World War, the rate of growth of marine fisheries has been consistently somewhat higher than the rate of growth of the world's human populations. It has therefore been much higher than the rate of growth of agricultural food production. In fact, since the 1950's, practically each year's world fish catch has set a new record.

Aquaculture is defined as the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants. Farming implies some intervention in the rearing process to enhance production, such as regular stocking, feeding and protection from predators.

Artificial feeding of fish is one of the principal ways of increasing production in fish farming. In intensive fish farming artificial feeding is essential for growth and, even in extensive farming, some artificial feeding is usually required. The majority of fish farmed in intensive aquaculture systems in the EU are carnivorous, having a high requirement for protein in their diets. Generally, fishmeal is used as the major source of protein in feeds formulated for cold-water fish rations.

Because many species of fish which are farmed are carnivorous, by nature they feed on other species of fish and crustaceans. Consequently, the feed of farmed marine and freshwater fish is mainly composed of re-cycled dead fish in the form of fishmeal and fish oil. The fishmeal is predominantly produced from a variety of ocean-caught marine fish.

Farmed and wild fish also often have particular dietary requirements in relation to fats and amino acid requirements. The salmonids have a requirement for omega-3 (n-3) fatty acids of longer chain lengths and certain amino acids. Consequently, the most important ingredient in the diets of farmed fish is fishmeal.

Mammalian-derived materials have also been used, to some extent, as an ingredient for feeding farmed marine and freshwater fish. For example, up to recently, blood meal was used in fish feeds. However, because of EU legislation banning such ingredients, it is no longer used.

Fishmeal is obtained from whole dead wild caught fish or trimmings of such fish after filleting for human consumption. The most widely used technique for fish meal processing is the wet reduction process, which is operated continuously and requires large amounts of raw material. The fish is steam cooked and pressed. The pressing of the cooked fish results in a protein fraction called press cake, and a mixed water and oil fraction with suspended and soluble protein. Oil and the water fraction with proteins are separated. The stick water is concentrated through evaporation. The temperature used, particularly at the drying stage, should be hot enough to kill any bacteria but not so hot that it denatures the protein. A drying temperature of 15-80°C is usually considered optimum.

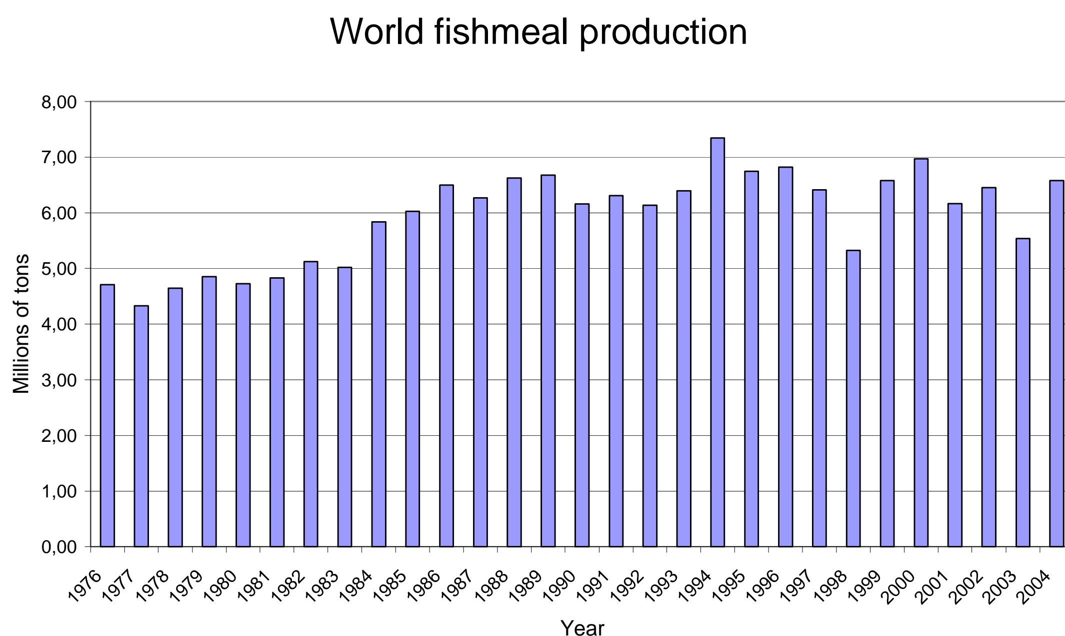
The feeding with fishmeal raises the question of intra-species or intra-order recycling of fish tissues. Generally, although recycled fish in the form of fishmeal is the principle ingredient of food for farmed fish, recycled farmed fish tissues are not used as an ingredient of fishmeal produced for fish feeds and moreover this latter practice is no longer allowed according to the

animal by-products regulation 1774/2002. Even if intra-species recycling of fish tissue did occur, the heat and drying treatment used to produce fishmeal should be sufficient to destroy any conventional fish or human pathogens, but not TSE agents if present.

Fishmeal production

The world-wide annual production of fish meal is about 6.5 millions of tons per year (Fig. 1).

Figure 1: **World annual fishmeal production in millions of tons**
Source: FAO FishStat Plus - Commodities Production and Trade 1976 - 2004



In 2004 the total world production was 6.58 millions of tons and the 10 main world producers accounted for 85% of that (Table. 1)

Table 1: **Fishmeal production of the 10 main world producers in 2004**
Source: FAO FishStat Plus - Commodities Production and Trade 1976 - 2004

Country	Production (millions of tons)	% of total production	Cumulative %
Peru	1,97	30,0%	30,0%
Chile	0,99	15,1%	45,0%
China	0,84	12,7%	57,8%
Thailand	0,42	6,4%	64,2%
Denmark	0,30	4,6%	68,8%
United States of America	0,26	3,9%	72,7%
Iceland	0,25	3,9%	76,6%
Japan	0,23	3,5%	80,1%
Norway	0,21	3,2%	83,3%
South Africa	0,11	1,7%	85,0%

The fishmeal production of the countries which are part of the current EU25 it's approximately 0.6 millions of tons (Fig. 2) and accounts for about 10% of the total world production (Fig. 3).

Figure 2: **European fishmeal production**
Source: FAO FishStat Plus - Commodities Production and Trade 1976 - 2004

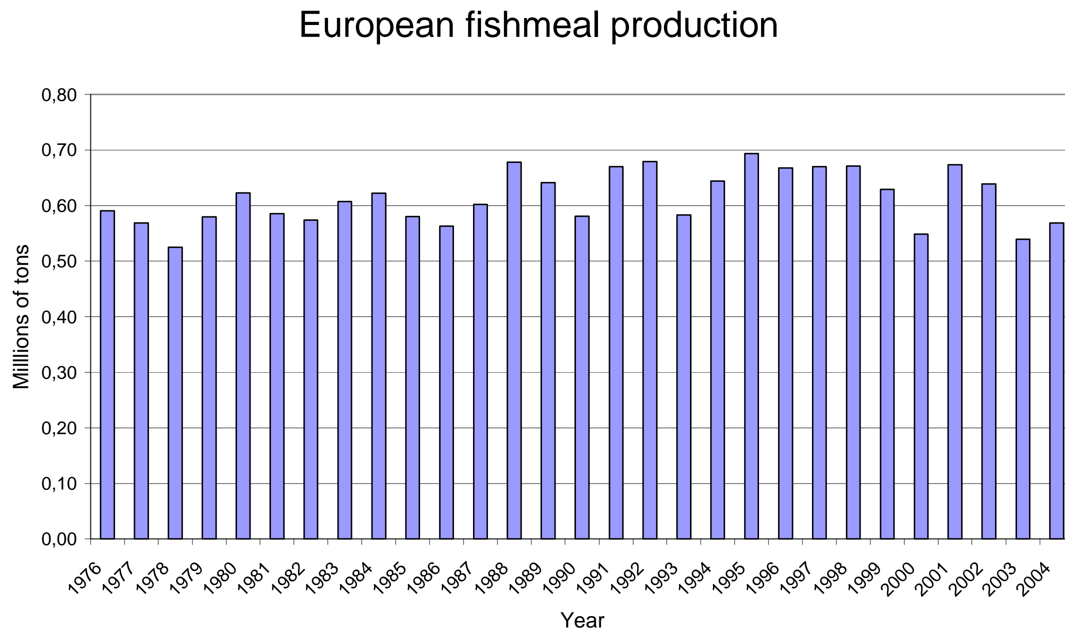
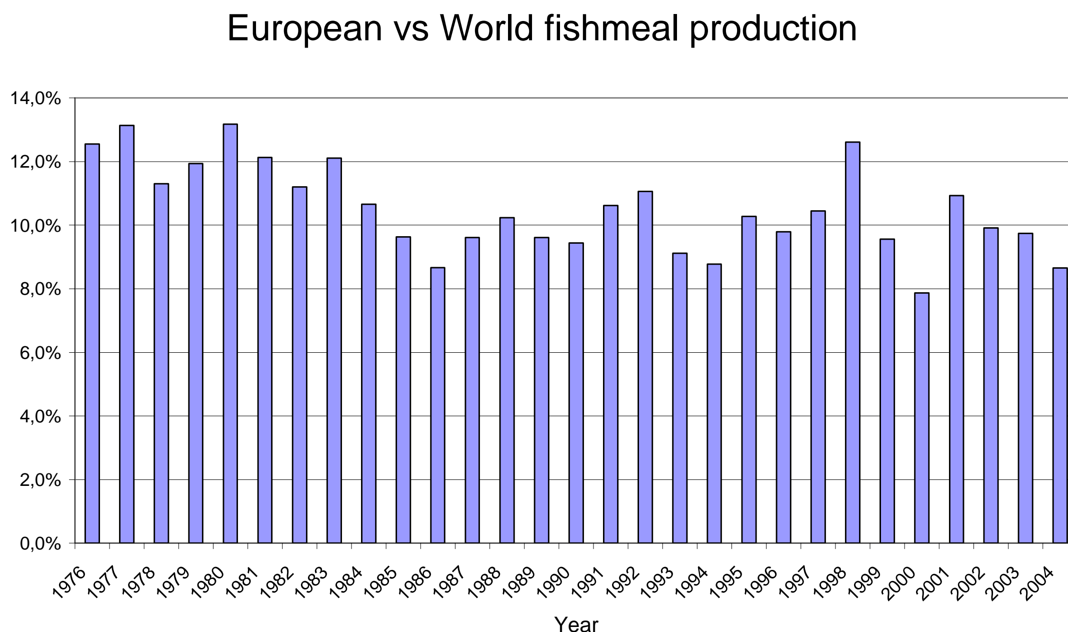


Figure 3: **European % fishmeal production compared to the total fishmeal production**
Source: FAO FishStat Plus - Commodities Production and Trade 1976 - 2004



The fishmeal import, export and apparent consumption of the countries which are part of the current EU25 are shown in Fig. 4, 5 and 6.

Figure 4: **European fishmeal import**
Source: FAO FishStat Plus - Commodities Production and Trade 1976 - 2004

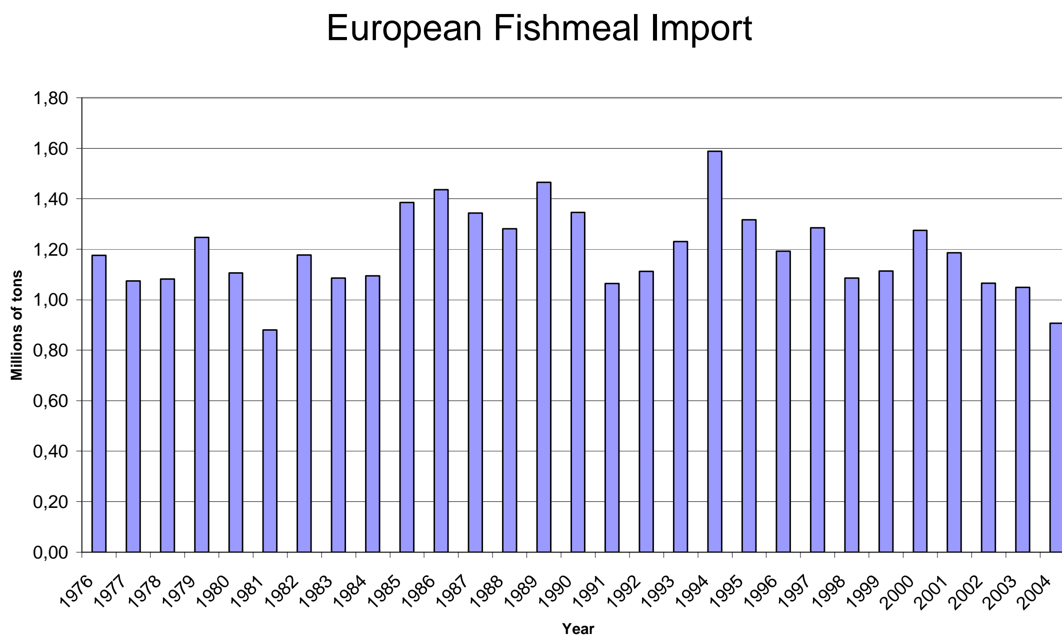


Figure 5: **European fishmeal export**
Source: FAO FishStat Plus - Commodities Production and Trade 1976 - 2004

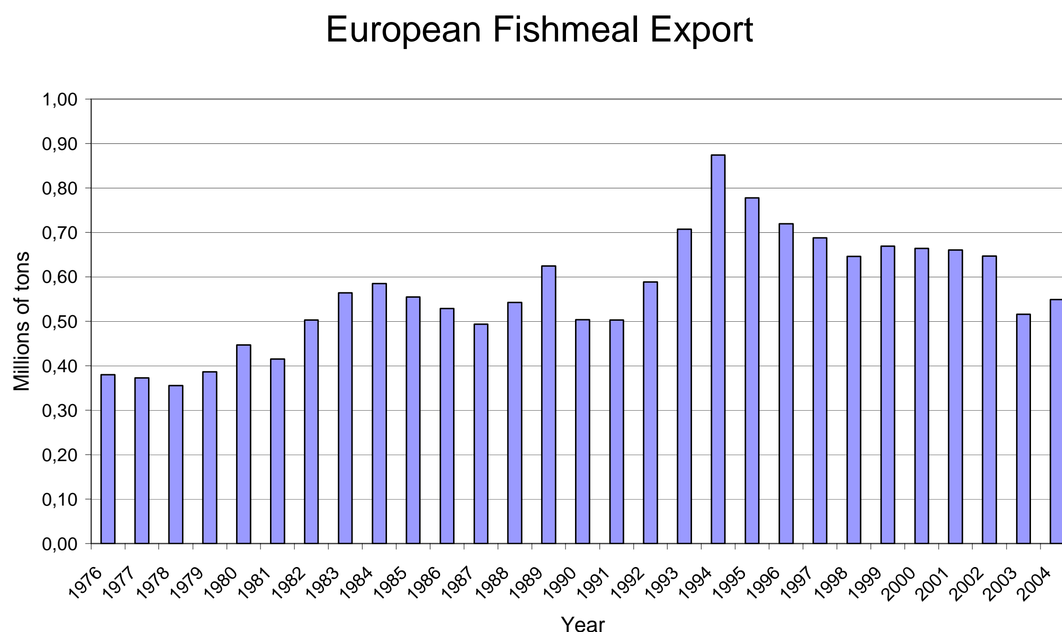
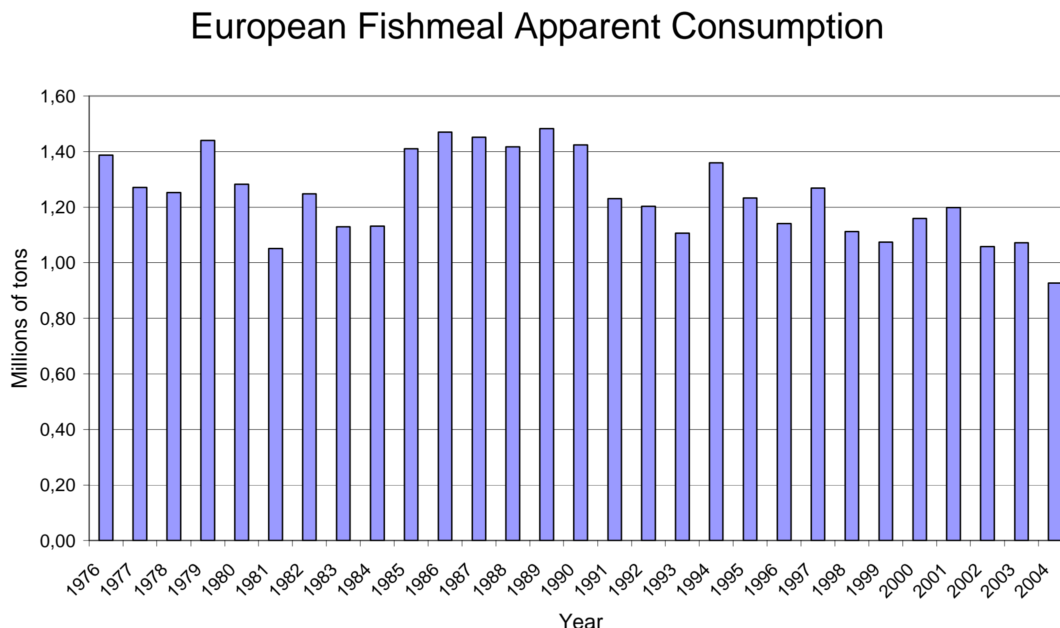


Figure 6: **European fishmeal apparent consumption**
Source: FAO FishStat Plus - Commodities Production and Trade 1976 - 2004
Apparent Consumption calculation= production+import-export



Fishmeal utilization in Europe

Within the EU in 2002 the total fishmeal consumed was used in the sectors of aquaculture, pigs, poultry and other (including pets) respectively for 33, 32, 29 and 6% (European Parliament 2004, http://www.europarl.europa.eu/EST/download.do?file=8814#search=%20fish%20meal%20oil%20industry%20).

The typical inclusion rates for fish meal in animal diets are around 2-10% for terrestrial animal species, but can to rise to in excess of 40% for fish diets (European Parliament 2004, http://www.europarl.europa.eu/EST/download.do?file=8814#search=%20fish%20meal%20oil%20industry%20).