

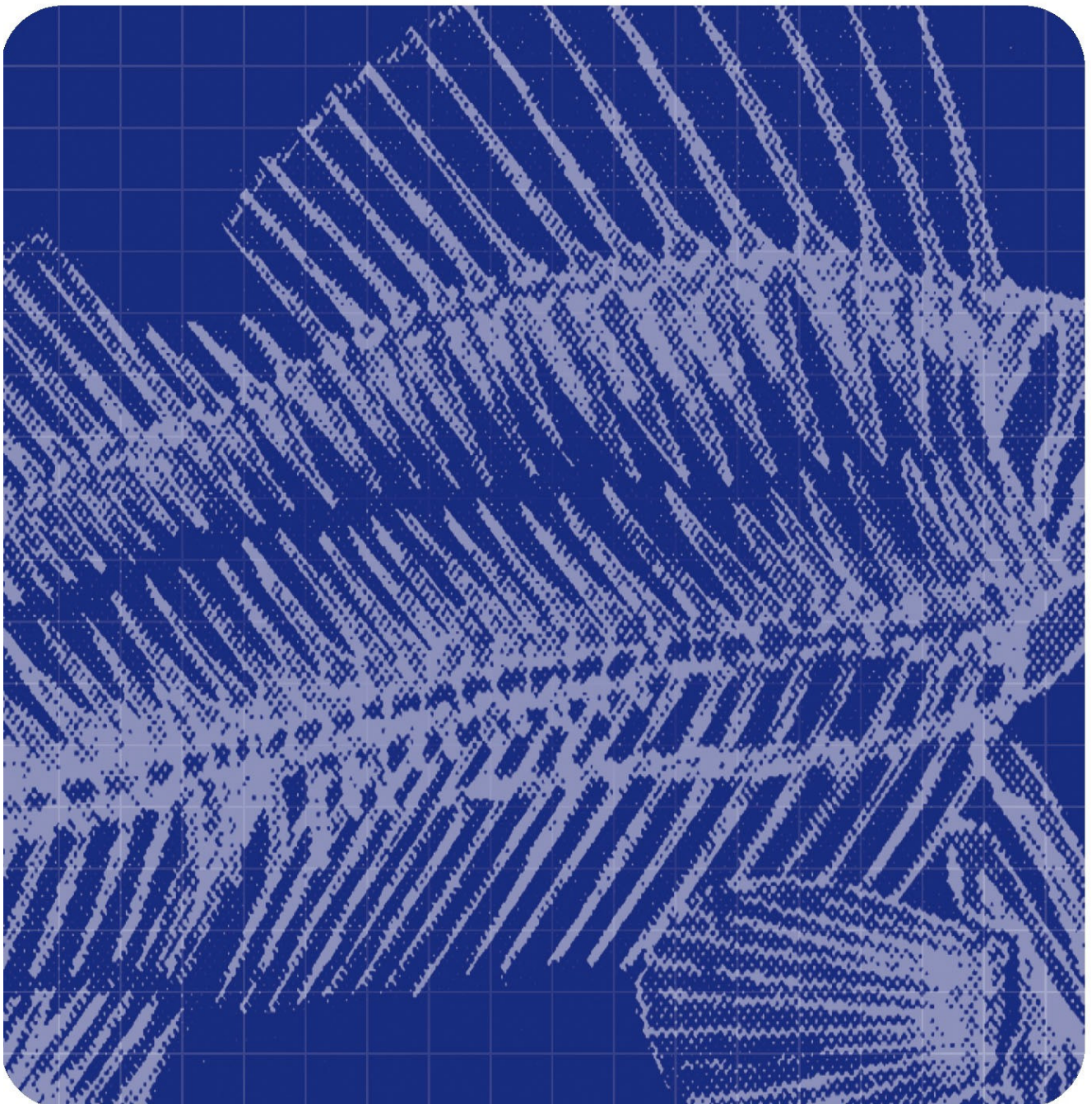


Fiskeriforskning

Report 20/2005 • Published December 2005

Melanin in Farmed Cod

Marie Cooper BSc. PhD.





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REPORT

 ISBN-13 978-82-7251-570-5
 ISBN-10 82-7251-570-9

 Report no:
 20/2005

 Accessibility:
Open
Tittel:
Melanin in Farmed Cod
Date:

December 5th 2005

Number of pages and appendixes:

19

Director of Research:
Even Stenberg
Author (s):

Marie Cooper BSc. PhD.

Project no:

20161

By agreement with:

Innovasjon Norge

Employers ref.:
3 keywords:

Copper, mineral mix, cod

Sammendrag: (maks 200 ord)

Melanin hos oppdrettstorsk: Denne rapporten beskriver en 6 måneders pilot-studie der effekter av kopper og andre spormetaller på utvikling av svarte blodårer i oppdrettstorsk er undersøkt. Torsk ble overført på fire ulike testdietter umiddelbart "post weaning". Første uttak ble gjort etter to måneders fôring og deretter hver måned. Resultater oppnådd frem til uttaket i slutten av november viste at både tilsatt kopper alene og tilsatt mineralmiks hadde klar innvirkning på utvikling av svarte blodårer i oppdrettstorsken.

English summary: (maks 100 ord)

We have conducted a six month pilot study to determine the effects of added copper and minerals on the development of black blood vessel in farmed cod. Cod introduced to the trial immediately post weaning were placed on one of four experimental diets. Fish were sampled after two months and every subsequent month. Up to the end of November our results show that both added copper alone and added mineral mix strongly influence the development of black blood vessel in farmed cod.

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1 INTRODUCTION AND BACKGROUND

Fiskeriforskning's objective is to both assess and improve the quality of farmed cod. We have been examining the causes of black blood vessels which appear as black stripes in the fillets of farmed cod. During these investigations we have discovered a possible link between dietary copper intake and melanin production. To confirm this relationship we have performed feeding trials on farmed cod using diets containing known amounts of copper, without added copper and without mineral supplements. As we do not know how early in the life of the fish the black blood vessels begin to appear we have attempted to take these trials from the earliest possible stage of development. Melanin deposits in white muscle represent a potentially serious quality problem for farmed cod. Black lines of deposited melanin are associated with blood vessels and consist of layers of melanin filled cells. Despite numerous reports of black stripes in farmed cod no detailed investigations into the underlying mechanisms have been undertaken. In our previous studies we have confirmed that melanosis is present in both farmed and wild cod. However, the level in wild fish is much lower than in farmed fish. In addition we have demonstrated that tyrosinase; a copper dependent enzyme which is the primary enzyme of melanin synthesis is present in cod tissues. Our measurements have shown that copper levels are significantly different in the white muscle of farmed (0.5 ± 0.03 mg/kg wwt) and wild (0.34 ± 0.01 mg/kg wwt) cod $p < 0.05$ and in the blood vessels of farmed (2.23 ± 0.37 mg/kg wwt) and wild (0.32 ± 0.02 mg/kg wwt) cod $p < 0.05$. We have measured similar differences in the levels of tyrosinase activity in both muscle and blood vessels. Melanised tissues contain higher copper concentrations than tissue containing little or no melanin. We have also shown that addition of copper to protein extracts from fish muscle causes an increase in tyrosinase activity in vitro. Despite the visual impact of black blood vessels the phenomenon appears to have no impact in terms of fish health or welfare. Indeed farmed cod with black vessels show no signs of distress or disease and heavily affected animals can only be distinguished post-mortem. The fish grower will, therefore, have no pre-slaughter indication that the product might be unacceptable to consumers. The possible function of melanin associated with blood vessels is not clear from the proposed roles of melanin in normal physiology. In the context of metal ion homeostasis the capacity of melanins in the eye to absorb metals and protect the organ has been well documented (Bowness et al. 1952a, Bowness and Morton 1952b, Bowness and Morton 1953) in mammals, fish and amphibians. It is widely assumed that binding of heavy metals and iron by melanins

in the nervous system of mammals functions as a detoxification mechanism protecting these tissues from toxic effects, but the physiological basis and regulatory mechanisms for this function of melanin remain enigmatic (Nicolaus 2005). Almost no information is available regarding the needs of marine species for mineral supplements in the diet. Fish can easily absorb minerals from seawater (Lall 1979, Love 1980) and as a consequence it may be that mineral supplements are completely unnecessary for marine fish species maintained in normal sea water. Nonetheless we have measured levels of copper, zinc, iron, magnesium and calcium in commercial cod feeds that demonstrate clearly that minerals are added using standard mineral mixes. Our results suggest that excess copper in commercial feeds leads to increased levels in the fish which in turn causes increased melanin synthesis via enhanced tyrosinase activity (Cooper and Midling in press).

2 EXPERIMENTAL DESIGN

The trial has been designed in the following stepwise fashion:

Initial introduction of fish at the weaning stage. Fish were supplied by the breeding programme in Tromsø. An assumption of 90% mortality was made at the outset of the experiment with the intention of having 800 survivors entering the feeding trial. However, the mortality among the weanling fish in 2005 was higher than expected resulting in 400 fish of approximately 0.75g in weight entering the trial post-weaning.

Fish were divided into eight groups of approx 50 fish per group. Four diets were obtained from Fiskeriforskning Bergen. The only difference between the diets was in the amount of added minerals. All of the diets also contained 1% vitamin mix giving final concentrations per kg of feed of 3000 I.E. vitamin D3, 160 mg vitamin E, 20 mg thiamin, 30 mg riboflavin, 25 mg pyridoxine HCl, 200 mg vitamin C, 60 mg calcium pantothenate, 1mg biotin, 10 mg folic acid, 200 mg niacin, 0.05 mg vitamin B12 and 20 mg menadione bisulphite.

Fiskeriforskning in Bergen formulate their own mineral mix from highly purified ingredients. The standard mix is added to diets at 0.4% by weight to give final concentrations per kg of feed of 500 mg magnesium, 400mg potassium, 80 mg zinc, 50 mg iron, 10 mg manganese and 5 mg copper.

The experimental diets were formulated as follows.

Diet 1: Mineral mix without copper.

Diet 2: Mineral mix resulting in 5mg of added copper per kg of feed.

Diet 3: Mineral mix resulting in 10mg of added copper per kg of feed.

Diet 4: No mineral mix.

The composition of the diets is shown in tables 1 and 2

Fish were maintained in tanks at Fiskeriforskning's land based facility at Kråknes. Water temperature, water quality and oxygen saturation were monitored routinely throughout the experiment. Mortality rates and condition were reported weekly. Weight, length and a record of the appearance of each fish was taken every four weeks during the trial.

Each diet was fed to two tanks of fish. The diets were fed via automatic feeders to replicate groups of fish to eliminate the possibility that the location of the tank or the conditions in any individual tank might influence the outcome of the experiment.

At intervals eight fish were removed from each tank, measured, photographed and tissue samples were taken for analysis.

| Component | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|------------------------------|---------------|---------------|---------------|---------------|
| Fish Meal (FM88/04) | 75.2 | 75.2 | 75.2 | 75.2 |
| North Sea Oil (06/04) | 8.7 | 8.7 | 8.7 | 8.7 |
| Wheat Meal | 14.3 | 14.3 | 14.3 | 14.7 |
| Vitamin Mix | 1.0 | 1.0 | 1.0 | 1.0 |
| Betafin (T505) | 0.4 | 0.4 | 0.4 | 0.4 |
| Inositol | 0.03 | 0.03 | 0.03 | 0.03 |
| Selected Mineral Mix | 0.4 | 0.4 | 0.4 | 0.0 |

Table 1. Composition of the experimental diets. Fish meal is the only protein source in these diets. Betafin is an osmoprotectant and chemoattractant developed for use in the salmon farming industry, it is commonly used in cod feeds. Values are given as percent of diet by weight (g/100g).

| Component | Diet 1 | Diet 2 | Diet 3 | Diet 4 |
|---------------------|---------------|---------------|---------------|---------------|
| Protein | 54.7 | 54.7 | 54.7 | 54.8 |
| Lipid | 15.0 | 15.0 | 15.0 | 15.0 |
| Carbohydrate | 11.0 | 11.0 | 11.0 | 11.3 |
| Ash | 11.7 | 11.7 | 11.7 | 11.3 |
| Water | 7.8 | 7.8 | 7.8 | 7.9 |
| Energy MJ/kg | 20.9 | 20.9 | 20.9 | 20.9 |

Table 2. Nutrient composition of the diets. Values are given as percent of diet by weight (g/100g). Energy values are given as Megajoules per kilogram.

3 METHODS

3.1 Sampling of Fish

Animals were transported a short distance to the laboratory in groups of four to minimize waiting times and stress. Animals were killed by a blow to the head, weighed and measured. Every animal removed for sampling was assigned a unique identifying number and photographed using a digital camera. The images were analysed using Scion Image image density analysis software. Samples of muscle, skin, liver, stomach and gut were collected from each animal.

3.2 Black blood vessels

To quantify blood vessel melanosis a scoring system to count black blood vessels was introduced. The system involved opening the fish via a straight line incision parallel to the spine and separating the muscle to reveal the black lines in the tissue (see figure 1(a)). The score consists of the number of black blood vessels clearly visible in the first twenty muscle segments counted from as near the head as possible and progressing towards the tail. The score was taken on one side of each fish. To utilise this system with the very small animals involved in the early stages of the trial a magnifier was used.

3.3 Metal analysis

In addition to tissues removed from the experimental animals samples of all feeds were taken for metal analysis. Trace element analysis was conducted by atomic absorption spectrophotometry. Samples for metal analysis were prepared according to the method recommended by the Norwegian committee on food analysis. Briefly, samples were weighed into Teflon tubes containing 4ml 25% Hydrochloric acid (Merck) and 2ml 30% H₂O₂ (Sigma) and digested by boiling in a Perkin-Emer multiwave microwave oven. Extracts were removed from the Teflon tubes and filtered through ashless filter circles. Metal concentrations were determined using a Perkin –Elmer Protein 3110 flame atomic absorption spectrophotometer and a standard curve using Tritisol (Merck) metal standard solutions diluted in extraction

solution was performed with each analysis. Extractions were performed in duplicate. Blanks containing extraction solution without sample and samples of a certified reference material were extracted in parallel with experimental samples and measured in the course of each assay.

3.4 Statistical Analysis

Data from blood vessel scoring are shown in binary format, no black blood vessels is shown as 0 and all positive observations are recorded as 1. This format was selected to prevent excessive influence of one heavily affected individual.

Quantitative data are expressed as means \pm sem. Statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by Tukeys test as the *post hoc* test of statistical significance (MINITAB statistical software). Data were deemed significantly different for p values less than or equal to 0.05.

4 RESULTS

Table 3 shows a summary of data obtained from measuring the mineral content of the experimental feeds directly by atomic absorption spectrophotometry. The table also shows the levels of the same minerals from the diet with no added mineral mix. This represents the minerals available from the raw ingredients. Interestingly the addition of purified salts of both manganese and magnesium results in a reduction of the amount present in the final feed. It is well understood that mineral salts are not chemically inert and that many mineral salts interact both with each other and the biological components in the feed mixture. Competition for binding sites and the presence of lipid in the feed matrix are two factors that would contribute to explaining these measurements. In table 4 similar data obtained for commercially produced feed and the muscle of farmed and wild adult cod are shown. The most important aspects of these data are:

1. That the amount of added copper and other trace minerals found in commercial diets far exceeds the estimated requirements of the fish and the normal body content of a wide range of marine fish and shellfish that would normally comprise the cod diet (ref).
2. Increased intake of minerals in the diet is reflected in increased levels in the muscle of the fish. Earlier results from our laboratory have also shown increases in mineral levels in other tissues of the fish, particularly those of the gut where the minerals are absorbed from the diet.

In figure 1 the results of applying the black blood vessel scoring system to adult fish are given. Clearly there are far more and darker, visible black blood vessels in farmed cod than in those caught in the wild. Similar data for the fish in the copper controlled feed trial are shown in figure 2. The number of black blood vessels reported by two independent scorers was much lower in those fish on a no added copper diet and no incidence of black blood vessels was observed in the samples taken in either October or November when the fish had been on the experimental diets for 12 and 16 weeks respectively.

Mortality has been reported weekly throughout the course of the trial and no significant differences in mortality rates have been observed. Interestingly, whilst not statistically significant a trend towards lower mortality in the groups without added copper or without any added minerals has been seen. Water temperature and oxygenation have also been monitored throughout the trial. In figure 1 temperature and oxygen saturation are plotted over time. No

differences were seen between tanks and both oxygen levels and temperatures have been stable over time.

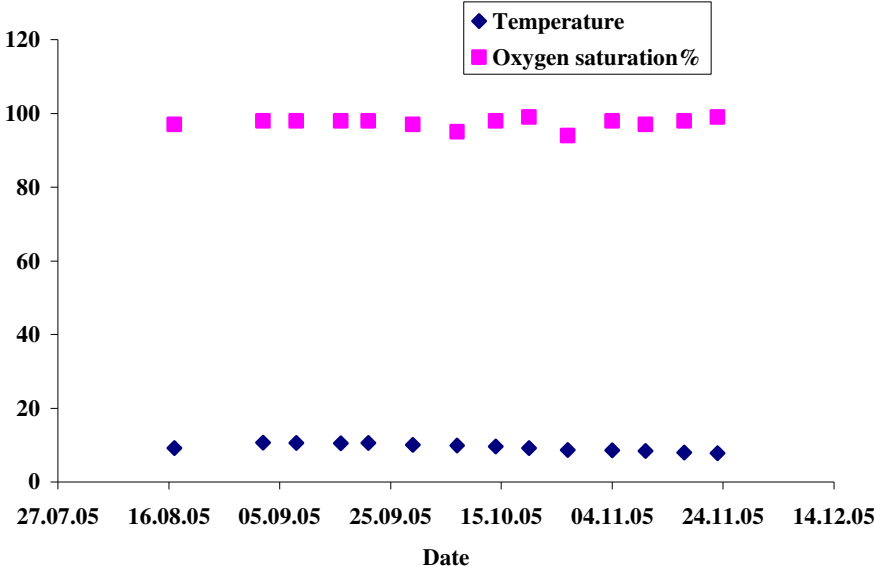


Figure 1. Temperature in degrees centigrade and oxygen saturation % over the course of the study

| Diet | Specified Copper Added | Copper mg/kg | Zinc mg/kg | Iron mg/kg | Manganese mg/kg | Magnesium mg/kg | Calcium mg/kg |
|------|---|--------------|------------|------------|-----------------|-----------------|---------------|
| 1 | none | 3.13 | 157.47 | 140.33 | 1.84 | 615.62 | 13202.4 |
| 2 | 5 mg/kg | 8.28 | 160.57 | 210.64 | 2.25 | 637.6 | 13508.0 |
| 3 | 10mg/kg | 14.89 | 164.42 | 127.17 | 1.89 | 627.19 | 13170.3 |
| 4 | no mineral mix | 3.22 | 42.1 | 99.52 | 10.23 | 635.63 | 14742.7 |
| | normal mineral mix ¹ | 5.0 | 80.0 | 60.0 | 10.0 | 600.0 | 18.9* |
| | estimated requirement ² | 3.0 | 20.0 | 30.0 | 3.0 | 400.0 | unknown |
| | available in seawater ³ mg/l | 0.0003 | 0.01 | 0.01 | 0.002 | 1350.0 | 450.0 |

* Added as chalk

¹ Information provided by Fiskeriforskning, Bergen

² Taken from Nutrient Requirements of Fish, National Academies Press 1993

³ Wilson, T.R.S. 1975. Salinity and the major elements of sea water. In J. P. Riley and G. Skirrow (eds.), Chemical Oceanography, Academic Press, London, 1: 365-413.

Table 3. The concentrations of a range of minerals measured in the experimental feeds. Also shown are the levels added in mineral mix and estimates for the requirements of fish as well as levels available in seawater.

| Sample Type | Copper mg/kg | Zinc mg/kg | Iron mg/kg | Magnesium mg/kg | Calcium mg/kg |
|---|---------------------------|------------|------------|-----------------|---------------|
| Commercial Feed (15 variations from 5 sources) | 18.46 (range 12 to 32) | 223.4 | 282.8 | 15344.3 | 1009.3 |
| White muscle farmed cod (n=20) | 0.5 | 11.6 | 10.9 | 247.5 | 580.7 |
| White muscle wild cod (n=10) | 0.3* | 4.4* | 11.9 | 204.0 | 128.7* |

* significantly different $p < 0.05$

Table 4. Concentrations of minerals measured in the muscle of farmed and wild cod and in commercial feeds for cod farming. The feed samples were drawn from variations produced by four feed producers and included weaning feed, growing on feed and feed for adult fish.

Despite the very high level of iron found in commercial feeds the fish appear able to regulate body iron content more precisely than other metals. It is, however, very difficult to completely exclude blood from a muscle sample and the similarities in iron levels may reflect contamination of the samples with blood.

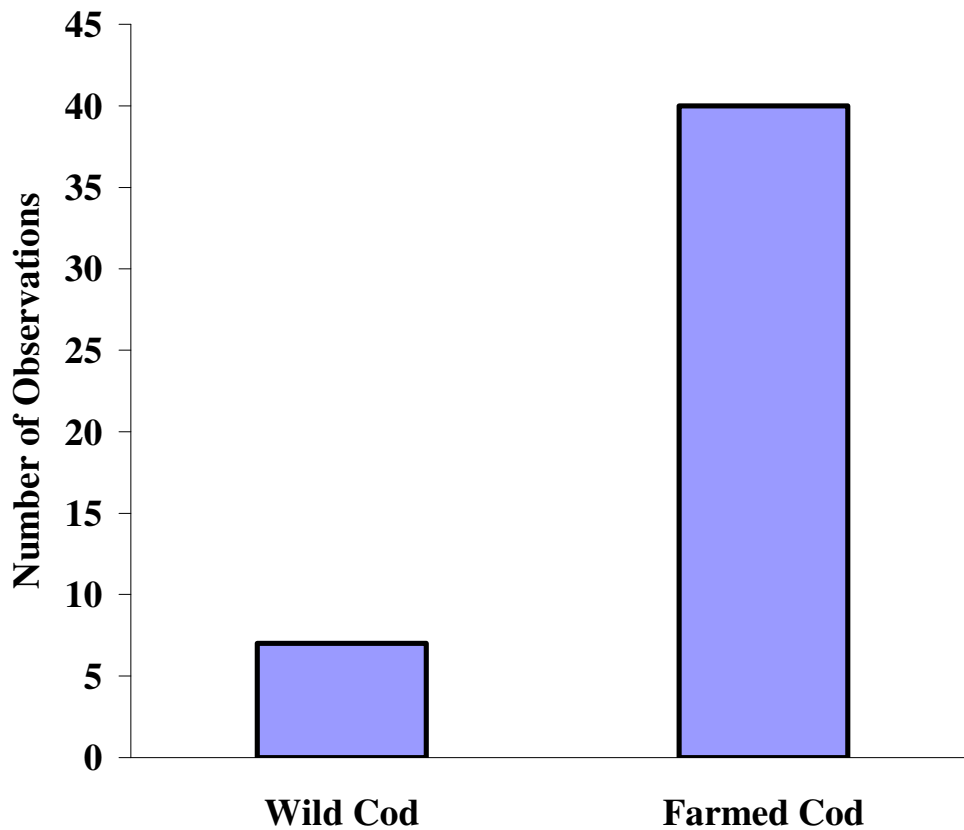


Figure 2. The results of the blood vessel scoring system applied to adult (1.5 -2.5 kg) farmed and wild cod (2 – 10 kg). Thirty fish from each group have been added to the scoring system over a period of one and a half years. The number of black blood vessels in individual wild caught cod is also significantly lower than that for farmed cod and the intensity of colour is also less pronounced.

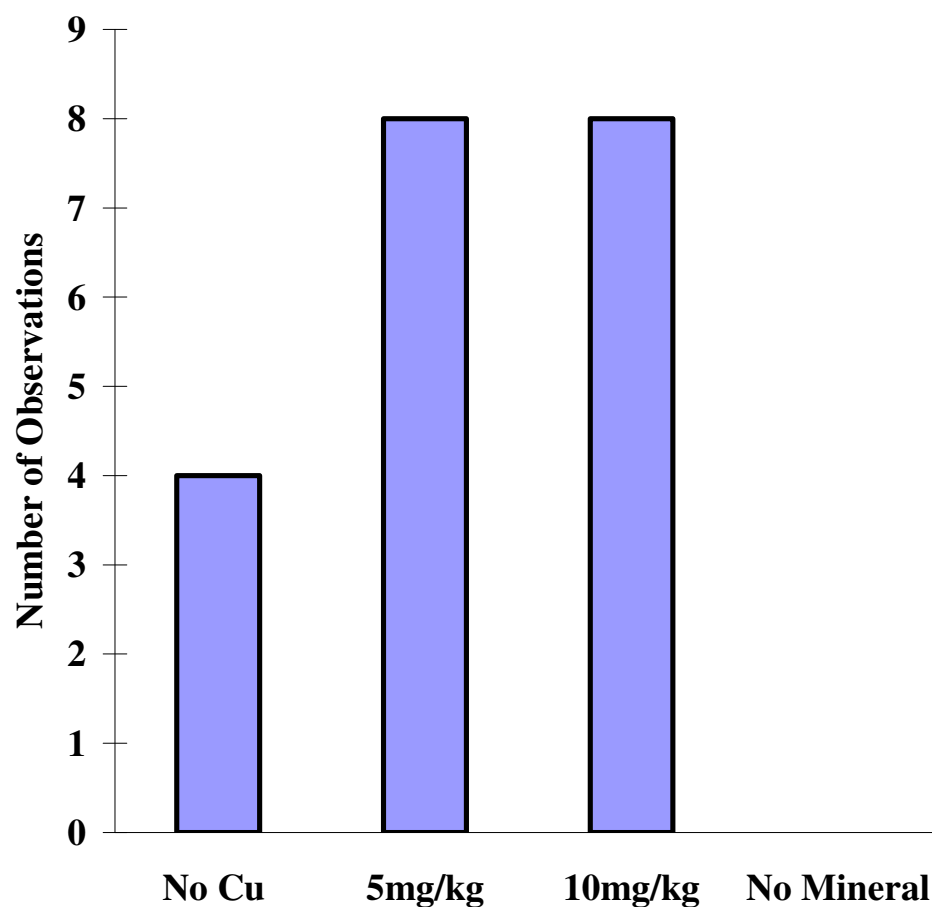


Figure 3. Results of the scoring system applied to fish from the copper and mineral supplement controlled feed trial. The data are derived from the samples taken in October and November of 2005 and comprise the total number of observations in each feed group. Thirty two individuals were examined in each feed group. Half of the number of fish in the no added copper diet had black blood vessels and none of the fish in the no mineral supplement diet had any observable black blood vessel.

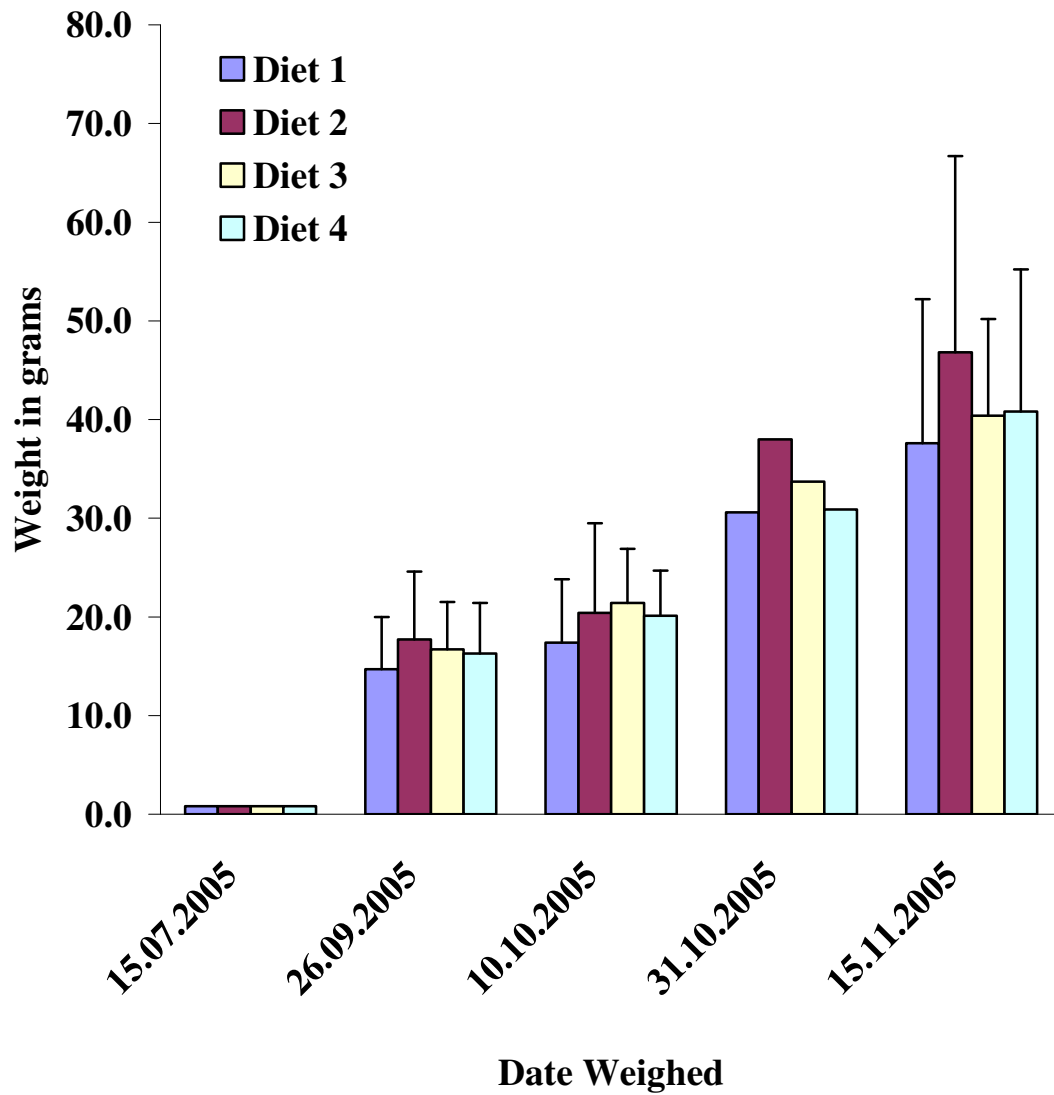


Figure 4. Mean total body weight of 16 fish from each feed group. There are no significant differences between total weight or weight gain. Body weight varied considerably between individuals and reflects both the size of the fish and the amount of food in the gut

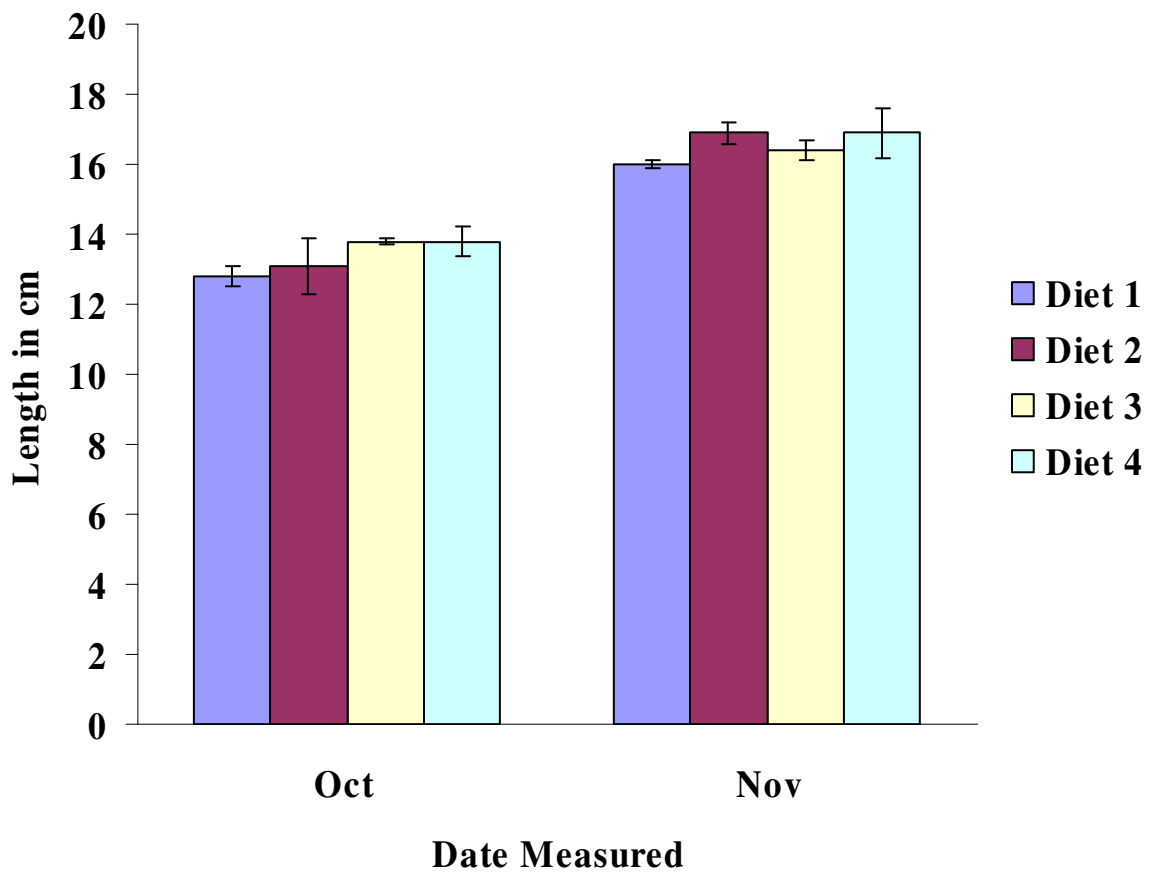


Figure 5. Shows the changes in body length measured at the two sampling periods separated by four weeks. The data are less variable than those for total body weight and with smaller fish represent a more reliable index of growth. There are no differences between feed groups. Sixteen individuals were measured in each group at each sample taking period.



Figure 6a. Example of a sampled fish from the November sampling period. The number displayed is the fish unique identifier. This animal had been fed on a diet containing no mineral supplement from the beginning of August 2005.

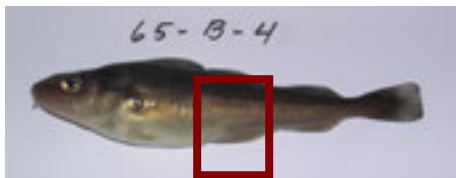


Figure 6b. This fish was drawn at the same time from the group receiving the diet with 10mg/kg of added copper in addition to normal mineral supplement. Note that the darkest areas on the two fish are similar in colour. However, skin pigmentation is observed over a larger proportion of the body surface in fish 65-B-4. The area selected for image analysis is outlined in red.

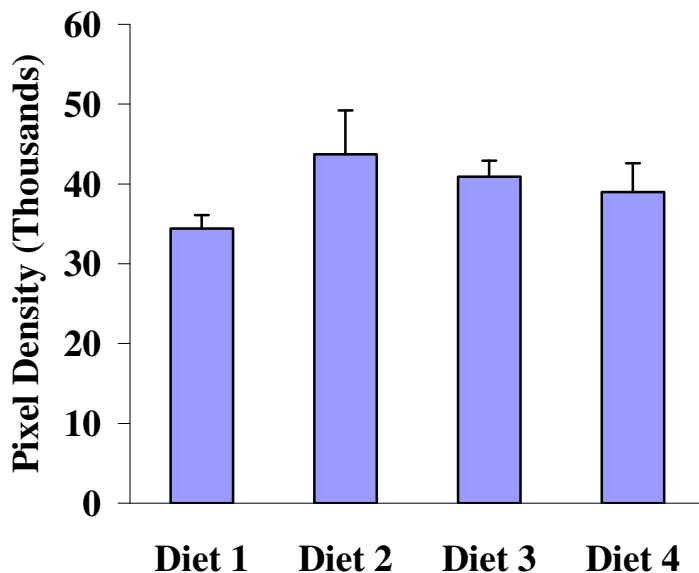


Figure 6c. Graph of data derived from image analysis. The subjective impressions of the observers were confirmed using image analysis. However the measured differences in skin colour are not great. There were also large individual variations.

5 DISCUSSION AND CONCLUSIONS

Our data up to the middle of November 2005 show that copper loading in the diet has a profound influence on the development of black blood vessels in cultured cod. However, the results from the group without any mineral supplement clearly show that the development of black stripes is not only dependent on copper loading, but is also influenced by other components of the mineral supplement. The interactions between metal ions in biological systems can be multilayered and complex. It is known that copper is required for iron uptake and transport of both metals takes place via some of the same proteins. Zinc is known to compete with copper for uptake and is given as a therapy to people who have copper handling defects such as Wilson's disease in order to prevent the build up of copper in their tissues. To determine precisely the components of mineral supplement that are required for the development of black blood vessel would require experiments designed to test these multifactorial interactions. Nonetheless, the results from this simple study are clear. The presence of mineral supplements in the diets of these cultured cod has had no obvious positive benefit. The removal of both supplemental copper and all mineral supplements has had, for the period of the experiment, no obvious negative effects on growth, mortality or condition of the fish. We have observed no cataract development or skeletal defects. Secondary to our observations on blood vessels we have seen some interesting effects on the overall colouration of the fish. The skin colour and peritoneal lining have also shown indications of reduced melanin production in low or no copper supplemented diets and the skins of animals fed a completely unsupplemented diet were clearly lighter than those receiving mineral supplements.

Our measurements of the mineral content of unsupplemented feed ingredients coupled with the little data we have been able to accumulate in respect of the requirements of marine fish for minerals clearly indicate that mineral levels in commercial feeds for farmed cod are far higher than necessary. Clearly the lack of knowledge with regard to the real needs of these animals has led to a degree of over compensation.

Copper is an essential micronutrient and a cofactor for a number of important enzymes including super oxide dismutase, cytochrome oxidase, lysyl oxidase and tyrosinase. It is estimated that cod, like most mammals, birds and those fish for whom the dietary requirement has been determined experimentally, will have a requirement in the range 2 – 5 mg/kg of dry diet (Shiau and Ning 2003, Gatlin and Wilson 1986). Nonetheless our measurements show

that commercially formulated feeds for farmed cod contain 12-32 mg Cu/kg dry diet, a range confirmed by the Norwegian National Institute of Nutrition and Seafood Research (Julsham et al 2004). On the assumption that the dry diet is formulated to reflect the levels available in wild prey and those normally found in cod flesh it would be reasonable to expect the levels in the dry material to be higher than in the equivalent wet material. However, the copper concentrations in many fish are within the range 0.1 – 0.3 mg/kg wet weight (Teeny et al. 1984), equivalent to 1- 3 mg/kg dry weight, which fails to correlate with levels in dry diets. Whilst the levels in crustaceans which also constitute part of the wild cod diet have been reported at between 0.5 – 1 mg/kg. Furthermore, there is little indication from the experience of cod farming that the feeding of a dry diet with a high caloric value results in reduction in the volume of food consumed by cod. Cod are active predators that experience variable access to food in the wild and hence will eat to satiation when food is available regardless of the composition of the feed offered.

Indeed this characteristic is an important factor in the enhanced growth rates observed in farmed cod. It is also a factor that would contribute to increasing the final doses of copper received.

The physiological effects of chronic, sub lethal, excess copper exposure in cod have not been previously been investigated. It has been shown that copper levels in the tissues of various species of fish frequently reflect those in the feedstuffs provided for them (Handy et al. 2000, Kamunde et al. 2000, Sagiura et al. 1998). This suggests that copper from feed is readily biologically available and will accumulate in tissues during chronic exposure. There is clearly relationship between copper availability from the diet and concentrations found in the bodies of cod. In juvenile rockfish Kim and Kang (2004) demonstrated an order of tissue accumulation with the liver being the most important storage organ and the muscle the least, this does not appear to be the case in Atlantic cod. Whilst mechanisms exist in some fish species to regulate copper uptake across the gut into the circulation (Handy et al. 2000) it has not been shown that mechanisms exist to completely exclude copper when physiological demand has been fulfilled and adequate stores are available. Our investigations imply that continuous exposure and therefore continuous uptake result in the recruitment of additional storage capacity, in this instance, in the form of melanin deposition.

6 FURTHER INVESTIGATIONS: THE NEXT STEP

During the course of 2006 to 2008 a major study, funded by Norgesforskningsråd will be conducted to confirm the data derived from this pilot study and to widen the scope of the investigation to include other components of the mineral mix. In addition to this study, if funding is made available, we wish to test whether the reduction in mineral content of the diet fed to adult fish with black blood vessels can reverse melanin deposition and result in the removal of black blood vessel. In many ways this is the most important experiment. As mentioned earlier in this document the current situation means that the fish farmer has no means of knowing, prior to slaughter, whether the fish will be saleable or acceptable to the customer. If we are able to produce fish without black blood vessel from a starting population with the problem then we can offer the fish farmer a way to ensure a marketable product without wasting valuable resources. This study would involve the introduction of approximately 600 cod weighing between one and one and a half kilograms into tanks. The fish would be divided into six groups of 100 and fed either the same feed they have consumed up to their introduction to the trial, a feed containing no added copper or a feed containing no added mineral mix. Two groups to be fed each diet. Twenty fish from each group would be removed, slaughtered and examined at the outset of the trial to establish the black blood vessel status at the start. A further twenty fish from each group would then be removed and examined every twelve weeks. The trial would last approximately one year enabling us to determine:

1. The reversibility of black blood vessel by manipulation of diet mineral content.
2. The time course of changes
3. Any short term or medium term negative effects of mineral limitation.

7 ACKNOWLEDGEMENTS

The author wishes to gratefully acknowledge the financial support for this project received from Innovation Norway.

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ISBN-13 978-82-7251-570-5
ISBN-10 82-7251-570-9
ISSN 0806-6221