Control of malformations in fish aquaculture

Science and practice

'FINEFISH' is a European project that has examined how to improve the sustainability of European fish aquaculture by a better control of the incidence of malformations in juvenile fish.

The project consortium has prepared a 'Diagnostic Manual on Malformations in Fish' and this publication on the 'Control of malformations in fish aquaculture: Science and practice'.

This manual targets the operators and managers of the hatcheries that raise the major species that are produced in European aquaculture, although there are many protocols and recommendations that are applicable to the many 'minor' species that are also the subject of professional attention.

It aims be a practical tool that provides efficient and reliable protocols, combined with professional advice and recommendations, for the reduction of malformations and best hatchery practice. The manual is the result of nearly 4 years of research, case studies and communication, applying the knowledge and experience of scientists and hatchery operators within and outside of the project consortium.

Its goal is to assure awareness of the different issues and conditions encountered within this specialised and important component of European fish farming and to promote best practice throughout the sector.

'FINEFISH' - 'Improving sustainability of European fish aquaculture by control of malformations' – has received European funding under the 6th Framework programme (Project 012451), under the Horizontal Research Activities involving SMEs (Collective Research)

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www.finefish.info

Foreword

European Union fish farming (EU27) increased its production from 60,000 tons in 1970 to more than 600,000 tons in 2000 (source Fishstat+ (FAO)), to 620,000 tons in 2004 (FEAP report[1]) and is now around 650,000 tons (FEAP estimate).

This dynamic sector is now facing a period of consolidation after many years of growth. The fish farming industry thus contributes more than 20% of fisheries landings in the European Union which, in the face of declining wild fisheries, has increased its imports from 45% to 65% in the last decade (source AIPCE[2]). In the face of increasing market competition, European fish farming has to become increasingly productive while respecting the objectives of sustainable development, as outlined in the Communications on Aquaculture of the European Commission (COM(2002) 511 and COM(2009) 162 final).

Within Europe as a whole, more than 1.6 million tons are produced, where Norway is a major contributor with more than 860,000 tons of salmon and trout. Additional salmon production is significant in the Faeroe Islands (35,000 tons) while Turkey has developed both its marine (>75,000 tons) and freshwater production (>40,000 tons). A large percentage of this production targets the European market.

At the source of every major sector of European fish farming (e.g. salmon, trout, seabass, seabream, carp) are the hatcheries, which maintain the broodstock and practise the controlled reproduction of these valuable fish. Their products – fertilised eggs, larvae, fry and juveniles – represent the starting materials for the majority of European fish farmers.

Problems with malformed fish were identified as a major obstacle for the industry at the PROFET workshop on hatchery technologies (Bordeaux January 2004). The PROFET workshop initiative was organised by the Federation of European Aquaculture Producers (FEAP) and its Member Associations, as a forum for the identification of RTD needs of the European fish-farming sector.

As a growing food supply industry, aquaculture has the potential to supply the European consumers with high-quality, healthy aquatic foods in a future where fisheries on wild stocks appear to be increasingly limited. The markets, however, demand quality, where this term pertains to the product itself and, increasingly, also relates to the social, environmental and economical soundness of the industry. An improved image will be of key importance to the future increase in markets for aquaculture products, where the issues of quality and welfare are increasingly referenced. The traceability of products, enabling the consumer to trace the fish backwards from fork to farm, is a new element in aquaculture that is being stressed, in parallel to other food producing sectors. It is meant to give the consumer confidence in the products, and to assure the consumer that the marketed fish has been reared and treated using the highest standards throughout the production cycle. In this situation, the fact that malformation problems persist in farmed fish is an indication that the industry has lacked the knowledge concerning the welfare and production standards that are required to make buyers (e.g. the processors and the retail sector) and the consumer content.

[1] FEAP = the Federation of European Aquaculture Producers (www.feap.info)

[2] AIPCE = the European Association of Fish Processors (www.aipce-cep.org)

The welfare of all livestock reared for food production is a key concern for the consumer and producers. Within the EU, minimum standards were covered by Council Directive 98/58/EC while the Council of Europe's Standing Committee of the European Convention on the Protection of Animals kept for Farming Purposes made 'Recommendations for Farmed Fish' that came into force on 5th June 2006. In addition, the World Organisation for Animal Health (OIE) adopted Guiding Principles for an Aquatic Animal Health Code in 2008.

In the Final Report of the PROFET project and the Minutes of the meeting in Bordeaux, two key priorities in the identified needs for better hatchery technologies were

1. Develop strategies to decrease malformation and deformities in juveniles and
2. Develop a scheme for "Best Hatchery Management Practices".

Malformations remain today as an unwelcome but inherent aspect of aquaculture production. Individual malformed fish appear in variable and unpredictable numbers in farmed stocks in both warm and cold waters, and entail severe losses to the production sector. The similarity of malformation symptoms across fish species and culture environments implies that there is a general causal effect within the rearing conditions of farms, and a wide scope research effort was seen as being required when seeking to identify this or these causes.

An important approach to this issue was the development of the FINEFISH Collective Research project - 'Improving sustainability of European fish aquaculture by control of malformations' – which has received European funding under the 6th Framework programme (Project 012451).

During the negotiation of the project with the European Commission, it became evident that much background and benchmarking information is either missing or has not been established. For example, the rates of malformation or the type or degree of malformation represent.

In addition to this manual on the 'Control of malformations in fish aquaculture: Science and practice', the project consortium has prepared species specific 'Diagnostic Manuals on Malformations in Fish' that will be continuously updated and are available on the website www.finefish.info. The diagnostic manuals were, together with the new research results, presented on the species specific training courses and on the final workshop that were arranged as parts of the FINEFISH dissemination.

The project consortium has produced a wide range of ground-breaking results within the major key topics of early rearing temperature, abiotic factors and nutrition, and how these affects normal and abnormal skeletal development.

This manual targets the operators and managers of the hatcheries that raise the major species that are produced in European aquaculture, although there are many protocols and recommendations that are applicable to the many 'minor' species that are also the subject of professional attention.

It aims to be a practical tool that provides efficient and reliable protocols, combined with professional advice and recommendations, for the reduction of malformations and best hatchery practice. The manual is the result of nearly 4 years of research, case studies and communication, applying the knowledge and experience of scientists and hatchery operators within and outside of the project consortium.

Acknowledgements

'FINEFISH' - 'Improving sustainability of European fish aquaculture by control of malformations' – has been achieved with financial support of the European Union's 6th Framework programme, under the theme of Horizontal Research Activities involving SMEs, using the instrument of Collective Research Projects (Project 012451).

The FINEFISH Consortium recognises the support and assistance accorded by the EC Project Officer, Mr. Fernando Trabada Crende, during the project.

The project comprised 20 partners, from different parts of Europe, each making its own specific contribution to the project's realisation, being the Industrial Associative Grouping (FEAP) that represents the professional sector, the SMEs – for whom the research was achieved, and the Research Institutions.

The project was coordinated by the Federation of European Aquaculture Producers, which is a Federation of National Associations responsible for professional fish farming in Europe. The coordinator was assisted by Francesca Margiotta (2009) and Margreet van Vilsteren (2005-2008) of the FEAP Secretariat; additional organisational support was provided by Catherine Pons.

Expert sectoral advice and inputs were provided by personnel representing the Member Associations of the FEAP, notably Kjell Maroni (Norwegian Seafood Federation [FHL]), Stefan Hofer (German Freshwater Fishfarming Association [VDBi]) and Cristobal Aguilera (Spanish Association of Marine Fish Farmers [Apromar]).

The professional hatcheries represented were the following, divided by species:

Trout

- Aquasearch Ova – Denmark – represented by Torben Nielsen
- Browwell Fisheries – United Kingdom - represented by Jonathan Jowett
- Viviers de France – France - represented by Marie Forraz and Fred Cachelou

Salmon

- Bolaks AS – Norway – represented by Erik Sørheim

Cod

- Profunda AS – Norway – represented by Helge Ressem

Seabass & seabream

- Andromeda SA – Greece – represented by Panos Kolios and Nikos Katribouzas
- Ferme Marine de Douhet SA – France – represented by Jean-Sebastien Bruant
- Panittica Pugliese Srl. – Italy – represented by Massimo Caggiano
- Tinamenor ASA – Spain – represented by Soledad Álavarez Guerra and Carlos Mazorra
- Viveiro Vilanova AS (2005-2007) – Portugal – represented by Joana Amaral

The Research institutes were:

- Nofima Marin AS (formerly AKVAFORSK) – Norway – which provided the Project's Technical Manager, Dr. Grete Bæverfjord. Additional key personnel were Dr. Synnøve Helland, Dr. Ingrid Lein, Dr. Harald Takle, Ms. Kirsti Hjelde and Ms. Elisabeth Ytteborg
- University of Patras – Greece – represented by Prof. Giorgos Koumoundouros
- Ifremer – France – represented by Dr. Beatrice Chatain
- INRA (National Institute for Agricultural Research) – France – represented by Prof. Sadasivam Kaushik and Dr. Stephanie Fontagné
- Royal Veterinary College – United Kingdom – represented by Prof. Neil Stickland
- Oceanographic and Limnological Research – Israel – represented by Dr. Amos Tandler and Dr. Bill Koven
- CCMAR (Centre of Marine Science) of the University of the Algarve – Portugal – represented by Prof. Deborah Power
- HCMR (Hellenic Centre for Marine Research) – Greece – represented by Dr. Pantelis Katharios and Prof. Pascal Divanach
- UMR Nuage (IFREMER-INRA-University of Bordeaux) – France – represented by Dr. José Zambonino

As the project developed, a focus was given to more efficient and effective data collection on malformations and a company specialised in data management systems, Pepite SA [Belgium] joined the project consortium – being represented by Philippe Mack and Benjamin Stevens to assist the development of this project component.

Acknowledgements

Achievement of this complex project could not have been made without the valuable inputs, professionalism and enthusiasm of these different project partners.

The Manual on the 'Control of Malformations in Aquaculture: Science & Practice' has been prepared on the basis of the research and experience of the hatcheries, individual experts and the research institutes that have worked together in the FINEFISH project.

It has been written by the authors cited for each section, under the coordination of Synnøve Helland and Courtney Hough, who also prepared the layouts for printing. Editing was made by Grete Baeverfjord, Synnøve Helland and Courtney Hough

Its goal is to assure awareness of the different issues and conditions encountered within this specialised and important component of European fish farming and to promote best practice throughout the sector.

Courtney Hough
Finefish project coordinator

October 2009

Representatives of the FineFish Consortium

The Hatchery Managers—their role in FINEFISH

Concluding opinion of the FINEFISH hatchery managers:

"We have learned much about how to prevent malformations in fish, but further focus on causal factors are needed"

The following text is the result of personal interviews with the hatchery managers of the SMEs which participated in Finefish and have been summarised by Dr. Ingrid Lein

Introduction

FineFish was a Collective Research project; a specific type of European collaborative project that aimed to address problems that are common for an industrial sector. This means that the interests of the hatchery sector were the prime targets when conceiving and executing this project, and consequently, that the hatcheries were required to make significant contributions, both through practical work and through discussions and knowledge development within the project Consortium. The nine FineFish hatcheries are a heterogeneous group of companies, spread throughout seven countries and producing five different fish species between them. Despite these basic differences between the companies, the SME representatives have been an invaluable part of the project's work and achievements, not least in challenging the scientists to address the right issues, and to develop scientific results into practical recommendations that have an immediate effect in operational conditions.

In the following section, some comments and opinions are presented from four of the FineFish industry partners .

"Tinamenor"—Seabass and seabream hatchery

Grupo Tinamenor S.L. (Spain) is an integrated company producing sea bream and seabass in the installations located in the north of Spain and in the Canary Islands. Their motivation for becoming part of the FineFish project was to reduce the incidence of malformations in their production and thereby improve the overall economy of the company.

Carlos Mazorra is the hatchery manager of Tinamenor in Pesues in Cantabria (northern Spain). He states that, like all producers of sea bass and sea bream, Tinamenor puts a lot of effort into sorting of juveniles at 1 gram size. Mazorra says that the cooperation between the industrial partners in Fine Fish has been important, but also very challenging due to long distances for travel/ communication and very busy days 'in the office'.

The most relevant results from FineFish for Tinamenor were those obtained within the field of nutrition. The results have been difficult to implement directly in the production scenario, but they have given the producers important information and knowledge that is useful in discussions with feed suppliers for recommendations on formulations.

Tinamenor encouraged all the relevant feed producers to join the Fine Fish Workshop in Gent in September 2009 so as to discuss the results and recommendations with the scientists involved in the project.

With regard to the benchmarking programme that has been developed in the project, Mazorra is awaiting the final outcome of the work done at the model farm and that, in any case, he feels that the benchmarking program need further development before it can work as a functional tool for commercial producers.

Mazorra further expresses the need for a stronger link between science and industry in aquaculture, for example in form of a technology centre where new methods or equipment can be tested before implementation at full scale. As a final conclusion, Mazorra says that although a lot of work has been done to solve the problems of malformations in farmed fish during the three year project period, there is still need for more focused research work to be done so as to eliminate this problem in European aquaculture.

Sorting juvenile fish with malformations

"Ferme Marine de Douhet"—Seabream hatchery

Ferme Marine de Douhet (FMD) (France) is a sea bream hatchery located on the island of Oleron on the west coast of France. FMD has been heavily involved in the development of the benchmarking system developed during FineFish and has been used extensively as a model farm in this part of the Fine Fish project.

The Hatchery Managers—their role in FINEFISH

Jean-Sébastien Bruant is the executive director of FMD and has represented the interests of the company during the FineFish project. In his opinion, the bench-marking system has a potential to be an exemplary future tool for monitoring and controlling hatchery production, but that more work is needed to reach the desired goal of providing a valid predictive and statistical tool for the producers.

Bruant states that he has found the cooperation with all participants of the Fine Fish project to be very fruitful, both from the industrial and RTD sectors, and that FMD is motivated for further cooperative work within the consortium. According to him, FMD has implemented knowledge from the Fine Fish project in their production protocols, especially those aspects concerned with larval nutrition. FMD were able to use the results directly, because they produce their own enrichment diets for the live feed.

During the project period, FMD has managed to reduce considerably the rejection of malformed fish at 1 gram individual size.

Bruant concludes that the philosophy of the Fine Fish project was good, but, similarly to Carlos Mazorra, he states that continued efforts are needed to fulfil the goal of low prevalence of malformations.

"Profunda" - cod hatchery

Profunda A.S. (Norway) is a hatchery producing Atlantic cod juveniles at Barstadvik on the west coast of Norway. The company decided to join the FineFish project because it wanted to reduce the prevalence of malformations in their production, which was very high, and to understand more about what causes malformations in farmed fish.

Helge Ressem, who is the manager of Profunda, says that their participation in the Fine Fish project has resulted in a new and wider focus on the effects of tank environment on protocols for cod production, in particular the need to control water current. This development came as a result of discussions with scientists and farmers involved in seabass and sea bream production, and realisation that the knowledge generated for these species was applicable also to cod.

During the project period, Profunda has focused on water circulation and aeration of incoming water, with the intention of achieving a calmer environment in the larval and juvenile tanks. Produnda made significant material investments in the hatchery to this purpose. As a result of this work, Profunda has managed to lower the incidence of lordosis considerably, resulting in much lower percentages of malformed juveniles.

With regard to the benchmarking program, Profunda agrees with FMD that this program has considerable future potential, but that further development is needed before it can become a useful tool for the industry. In agreement with other participants, Ressem has also found that the contact with the other companies and scientists has been fruitful, not only the formal meetings but also the informal discussions held around these. Ressem also found that all the visits to the different hatcheries were useful.

During the project period Profunda developed a close cooperation with Nofima and its personnel, and Ressem says that they were very lucky to have their national RTD institute located geographically close to them, and thinks that this contributed significantly to the success of the cooperation.

"Viviers de France" - trout hatchery

Viviers de France S.A. (VDF) (France) is a fully integrated fish farming company that produces rainbow trout in freshwater. VDF has several operational locations, mainly in the south-west of France in the Landes region.

Frédéric Cachelou is the President of Viviers de Sarrance and also works as a consultant for VDF. He says that VDF appreciated the construction of the FineFish project with eight different scientific institutes cooperating on the same topic and also the involvement of industry working on different fish species.

During the project period VDF became more focused on the effects of temperature during the early life stages on the development of malformations. They plan to implement this knowledge and develop their juvenile production, considering the knowledge about temperature effects. VDF has a long tradition for cooperation with the national RTD institutes INRA and Ifremer, who were within FineFish, and as the project developed, close cooperation was developed with Nofima, concerning the topics of temperature and disinfection.

VDF is not so optimistic with regard to the future of the benchmarking programme and its suitability for salmonid juvenile monitoring. Cachelou says that the applications of the programme might be better suited for the marine fish species reared in aquaculture and recommended that, in any case, the ranges and type of data that are used in monitoring should be revised in the future.

Glossary of terms

adipocytes

- Also known as lipocytes and fat cells, are the cells that primarily compose adipose tissue, specialised in storing energy as fat.

caudal

- At or near the tail

collagen

- The main protein of connective tissue in animals and the most abundant protein in mammals. Collagen tissues consists of a glycoprotein matrix containing densly packed collagen fibres.

cranial

- On the head area

deformity

- A deformity, dysmorphism, or dysmorphic feature is a major difference in the shape of body part or organ compared to the average shape of that part. Often referred to a change in a structure that is already formed.

enrichment

- Improving the nutritional composition of live feed, i.e. with rotifers or Artemia

euthanised

- Ending of life in a painless manner

haemal arch

- A bony arch on the underside of a tail vertebra of a vertebrate.

hyperoxic

- Elevated concentrations of oxygen. The result of breathing elevated concentrations of oxygen is **hyperoxia**, an excess of oxygen in body tissues.

hypoxic

- reduced concentration of oxygen. is a pathological condition in which the body as a whole (generalized hypoxia) or a region of the body (tissue hypoxia) is deprived of adequate oxygen supply.

kyphosis

- axial /\ - shaped deviation seen from a lateral view, a dorso-ventral bending of the spine.

lateral

- Of or pertaining to the side

lordosis

- axial \/ - shaped deviation seen from a lateral view, a dorso-ventral bending of the spine.

malformation

- a deformity in the shape or structure of a part, especially when congenital. Often induced during embryonic and early juvenile development as the structures are formed.

morphogenesis

- the development of the form or structure of an organism during the life history of the individual

notochord

- the longitudinal axial support (skeleton) of the embryos of all chordates, which lies ventral to the nerve cord and dorsal to the alimentary canal. Remnants of the notochord usually remain in the adult between the vertebrae, which come to surround it.

ossification

- the process of the synthesis of bone from cartilage. There are two types of ossification—intramembranous and endochondral ossification.

osteoblast

- a mononucleate cell that is responsible for bone formation. Osteoblasts produce osteoid, which is composed mainly of Type I collagen. Osteoblasts are also responsible for mineralisation of the osteoid matrix. Bone is a dynamic tissue that is constantly being reshaped by **osteoblasts**, which build bone, and **osteoclasts**, which resorb bone.

Glossary of terms

platyspondyli

- Flattened and compressed vertebrae often found in the tail region.

protein skimmer

- or **foam fractionator** is a device used to remove organic compounds from the water before they break down into nitrogenous waste.

pughead

- Various degrees of a malformation of the snout in fish where there is a reduction of the snout leaving the lower jaw protruding beyond the upper jaw. Reduction of the front skull and upper jaw bones, anterior – posterior compression of etmoid region and upper jaws, malformation in the maxillaries, pre-maxillaries, parassphenoid and etmoid plate.

scoliosis

- Axial deviation, S shaped vertebral curvature from a dorsal view, sideways bending of the spine.

specific growth rate (SGR)

- SGR (%)=100*(ln. final weight of fish – ln. initial weight of fish)/ number of days

stargazer

- Axial deviation,\/ shaped curvature in the neck

supernumerary

- Too many, often referred to number of vertebrae in the spinal column or fin rays

surface skimmer

- Ddevice for removal of organic film in the water surface

weaning

- Gradual transfer from live feed to formulated feed (pellets)

Diagnostics - Basic concepts of fish radiography

Kirsti Hjelde & Grete Bæverfjord

Introduction

Radiography, or the use of X-rays for analysis, is the preferred method for fish skeletal deformity diagnostics. X-rays have enough energy to penetrate soft tissues, but not bone and other hard substances. Radiography thus allows the creation of a negative image of the skeletal structures of the fish, which allows the evaluation of the development and identification of pathology in the bones — without cutting into or even killing the fish. However, fish radiography is not a common procedure and, therefore, it represents some challenges for those who wish to use this diagnostic technique.

Small structures, weak contrast

The major limiting factors in fish radiography are **object size** and **low bone density** of the fish skeleont. These limitations are challenged constantly by the need or request for early diagnostics, leading to the sampling of very small fish.

In hatchery-size fish (i.e. juveniles), each structure of the skeleton is very small compared to standard radiography objects. The vertebrae in a salmon of 8 cm length will be less than 1 mm long; this is very small, even when compared to the bones in a kitten leg or other small mammalian objects. The mineral content of the developing fish bone is also low compared to mammalian counterparts. The consequence is that use of standard X-ray equipment, e.g. in a veterinary clinic, may produce images of too low contrast and/or resolution to be of any use for diagnostic procedures in small fish. In larger fish, on the other hand, such equipment can be more than adequate.

Mineral deposition in skeletal structures increases rapidly with fish age and size, after hatching and through first feeding. However, the time course and rate of mineralization also varies with species. Thus, even though an X-ray image of a 1g salmon may be of very little diagnostic value, an image of a sea bass of corresponding size may be fully adequate for evaluating skeletal deformities. It is therefore necessary to take both species and size into account when developing a diagnostic project based on radiography (Figures 1a & 1b).

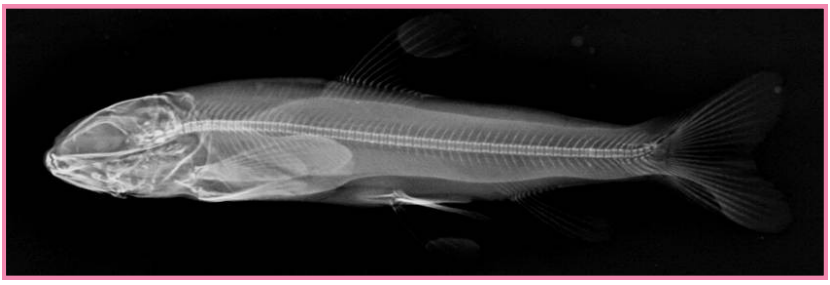


Figure 1a: Radiographic image of Atlantic salmon (Nofima Marin)

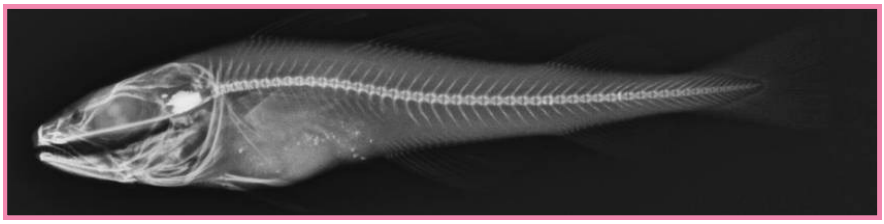


Figure 1b: Radiographic image of Atlantic cod (Nofima Marin)

The figures 1a and 1b are radiographic images of Atlantic salmon (1a) and Atlantic cod (1b), both of 1g size. The x-rays were taken with the same equipment and settings, illustrating the differences in development of skeletal structures between two species of the same weight (Photographs: Nofima Marin AS)

Analogue and digital radiography

Standard radiography images are made by exposure of a film, which is covered by a silver salt emulsion to X-rays. The image is subsequently created by developing the film. Undeveloped films are light sensitive, and development of the exposed film requires either a dark room or automated developer devices, as in traditional photography. When developed, the image exists on the film-foil only, and must be digitalized in a scanner or photographed in order to be processed further. These processes will commonly result in a certain loss of detail.

During the last decade, digital radiography has gradually taken over a significant part of the radiography market. Both direct digital systems and semi-digital systems are available. In direct digital systems, there are no films, and the image is recorded directly by a sensor. The image is subsequently created in the computer and displayed on a monitor. This technology does not yet give a good picture quality for fish, and it is still rather expensive. The semi-digital systems consist of a reusable image plate (substituting the film) and a plate reader, as well as the computer system. This system gives an image quality comparable to the traditional film-foil systems, even of small fish if one uses equipment used for mammography .

Both direct digital and semi-digital systems use the same type of radiography sources as analogue radiography.

The advantages of digital radiography, compared to analogue, are easier image storage and handling, resulting in images that can be subjected to analysis without loss of information. The digital systems may also provide automated procedures for image processing, e.g. contrast enhancement and edge sharpening.

Diagnostics - Basic concepts of fish radiography

Generally, digital systems also allow achievement of the same results - with lower radiation doses to the patient than in analogue radiography. This might not be so relevant for the fish but may still be of importance to the safety of the operator.

The main drawback of digital radiography compared to high quality analogue images is that you will get a limit in picture quality if you want to look closely at details, because a digital image is built up by pixels. Thus, the resolution is determined by pixel size.

There are also risks of creating artefacts in the computer, e.g. during contrast enhancement or as the result of unpredicted variation in greyscale. The use of automated image processing will also hamper the potential for comparing different groups of fish, since the image enhancement may treat different images in different ways. This variation is comparable to variation in image quality induced by the different settings (mAs, kV, film-focus distance) in analogue radiography.

X-ray sources

The challenge in working with small fish bones is that standard radiography systems give too strong radiation even at the lowest settings. Mammography, or soft tissue X-ray, was developed to detect small deviations in the density of human mammary gland tissue and, in mammography, special sources with low dosage are used.

Setups for mammography radiography, therefore, are generally much closer to the ideal for achieving X-rays of small fish, as opposed to standard equipment, and should be the preferred choice for any fish < 100g.

Mammography can also be used in bigger fish, eventually to image skeletal details when the body size limits imaging of whole fish (see Figure 2). In fish >100g, standard radiography will usually give acceptable results.

Figure 2: Mammography of four fused vertebrae in a 3kg Atlantic salmon. The level of detail is high, and the changes in the vertebrae adjacent to the fusion are clearly visible. (Photo:

The difference in image resolution between standard radiography and mammography can be considerable; for instance, using 10 pixels/mm in a standard semi-digital system and 20 pixels/mm in a corresponding mammography system (Fuji Medical systems). This difference reflects a double reading (from both sides) of the mammography image plates, which are transparent on both sides of the absorbing material ("film"), compared to standard plates who are transparent on one side only.

Analogue radiography films also present a variety in film qualities, depending on the area of use. Mammography films are generally small in size but give good image quality with high resolution. Standard radiography films are delivered in several sizes designed for different body parts, and give a sufficient quality for mammalian structures and for bigger fish.

Technical x-ray is a tool designed for studying inanimate objects rather than animals, and has the potential of giving a very high image quality. This can be used for dead animals with good results, but is generally more expensive than standard films, and may require more adjustments to obtain the full potential from the film.

Mammography equipment is stationary, whereas standard X-ray sources come in a variety of different scales, also as mobile units that can be easily transported.

Standard radiography setups are commonly available in veterinary clinics and similar. To find mammography equipment, it is usually necessary to identify human radiography laboratories, either in hospitals or clinics.

Settings and adjustments

There are three main settings to balance in order to obtain the best results, and for any setup, it will be necessary to adjust the equipment before use. It is strongly advised to engage a skilled radiography technician in this process.

1. kV (kilovolt) describes voltage
2. mAs (milliampere x seconds) electron current against the anode x exposure time in seconds.

   Note: The combination of kV and mAs defines the radiation dose.
3. The third variable is film-focus distance.
   In a typical standard radiography setup, film-focus distance can be adjusted within a wide range. In most mammography setups, the film-focus distance is fixed.

The relations in terms of the effects of radiation dosage is highly variable with different equipment, and it is difficult to provide universal guidelines.

Diagnostics - Basic concepts of fish radiography

These general considerations apply:

- Generally, fish radiography requires lower radiation doses (lower kV and mAs) than comparable setups for mammalian radiography in order to produce an acceptable image.
- Increased mAs gives less noise in the image and better contrast; an increase in mAs is a good option for improving image quality.
- Both increased kV and increased mAs give a darker image.
- Increased kV gives poorer contrast (greyer image) but better penetration (e.g. in thick(er) objects).
- Increased film-focus distance means that fewer x-rays reach the film since the rays spreads obliquely from the radiation source. Increasing this distance will allow you to have a larger exposure area (bigger film, more fish in one exposure), but the image quality may be somewhat reduced.

In the Nofima Marin fish X-ray laboratory, which is a semi-digital setup, common settings are 22 kV/50 mAs in mammography and 35 kV/50 mAs in standard X-ray.

Fish material for radiography

Fresh, newly killed fish is always preferable for radiography (Figure 3), but fish may be radiographed live or dead, fresh, frozen or fixed, and still give acceptable image quality.

Radiography is also less sensitive to sample freshness than most other analyses, as bones deteriorate much slower than soft tissues, and acceptable radiographs may be obtained of fish long after they have been rejected for other analytical purposes.

The best results are, however, obtained in fish that are sampled fresh and prepared for radiography. Ideally, and following a simple rule of thumb for radiography, fish should be placed sideways down, in a relatively straight posture.

When working with live fish for X-ray, sedation is necessary and must be sufficient to keep the fish still during exposure. For live radiography, it is important to sedate only a few fish at a time in a relatively strong sedative.

Use a few fish, strong sedation, short time in the air and then back to aerated clean water, rather than many fish, weak sedative and long time in the sedation.

The fish use oxygen from the water if they struggle for minutes before they become calm, and the sedative solution soon becomes less efficient.

For fish welfare purposes, and to prevent unnecessary mortalities, it is strongly advised to pay sufficient attention to this point, and preferably have a designated and skilled person to handle the sedation.

Figure 3: Atlantic salmon (3.5 kg) euthanised and ready for radiography. Photo: Nofima Marin AS

For dead fish, it is equally important to pay attention to posture. It can be difficult to predict the onset of rigor mortis and, once the fish has entered rigor in a bent or skewed posture, it may be impossible to obtain a good image. If the fish is euthanised, it is still important to have aerated sedation and a sufficiently strong solution.

If there are too many fish in poorly oxygenated water, they will not be able to utilise the sedative because of lack of oxygen, and they will die from asphyxiation. In addition to the very poor welfare aspect of this, some of the fish will die gasping for oxygen. X-rays of gasping fish are useless for neck and, to some part, head diagnostics, and represent a visual disturbance to the evaluation in general.

Freezing

For X-ray purposes, fish must be frozen individually.

This is easily obtained by placing the fish side by side, in the right position, on a non-adherent, hard and smooth surface, e.g. a plastic covered board, and freeze in a standard freezer. When the fish are frozen through, they can be placed in plastic bags or boxes for storage.

Diagnostics - Basic concepts of fish radiography

Small fish <5g

Small fish will defrost quickly or can break even if they are well packaged.

This situation makes fixation of the fish specimens in 4% formaldehyde solution a better alternative. The fish are euthanised by an overdose of sedative and put into formalin solution. These are kept in the solution until properly fixed, when they get a “rubbery” consistence.

For histological purposes, the volume of fixative should be ten times that of tissue. If the fixation is for radiography only, a 5:1 ratio is sufficient.

The fish can then either be sent for analysis in the formalin solution in water proof containers, or rinsed in ethanol and sent in sealed plastic bags after removal of excess fluid.

Due to the known effects of local irritation and the possible carcinogenic effect of formalin, gloves should be used when handling the solution. A ventilation chamber is also necessary.

Large fish >1 kg

With large fish, the volume and weight alone can represent a challenge for freezing, handling and storage. Bigger fish can be filleted to save space, and to make transport easier and cheaper. Make sure, however, that the filleting knife does not cut into the spine. The spines can then be sent fresh on ice or frozen in a flat and straight position. Filleted spines on ice stays fresh for days and are easy to handle.

For evaluation purposes, it is a huge advantage that all fish are uniformly placed for imaging, e.g. all fish right side down, all dorsal fins towards the top, and all heads pointing to the left. This condition applies whether the material consists of single-fish images or if there are multiple fish per image.

Although having no effect on image quality per se, experience is that this simple measure eases the evaluation process and detection of deviations and differences between fish.

Labelling

Labelling is a commonplace, but very important issue if the fish are to be transported or stored, both for diagnostics and for experimental purposes. Confusion about the identity of the objects can make even the most perfect radiographic images worthless. The need for labelling may vary. In most cases, a group identity (ID) is sufficient, but in some cases there is a need to ID individual fish, which may be more challenging.

Groups can be labelled on the wrapping with a waterproof pen or pencil. It is a good rule to label groups very neatly - particularly if they are being sent to someone else; what may be logical to the sender may be completely confusing for the receiver. It is much better to write too much than not enough!

It is also important to remember that plastic bags may break when frozen, and that ink may dissolve if it gets wet, and to take due precautions. It is a good idea to duplicate the label information on a piece of paper, put it in a sealed plastic bag, and put it into the wrapping with the fish.

Tagging

There are numerous ways of tagging individual fish but only two are mentioned here:

1. Tagging of live fish with PIT-tags (see Figure 4). These tags are glass-encapsulated with a unique ID-code, and are placed in the abdominal cavity. This procedure is easily done on live, sedated fish > 10 grams. The tag remains in the abdomen for the entire life span of the fish, enabling identification with a PIT-tag reader. This tag type is commonly used for experimental purposes and in breeding programs, and allow for repeated retrieval of the same fish.

2. Dead fish can be labelled with a note of waterproof paper stapled to the tail fin (see Figures 4 and 5) or put under the operculum. Avoid putting notes in the fish' mouth as that forces the head back and the jaw open.

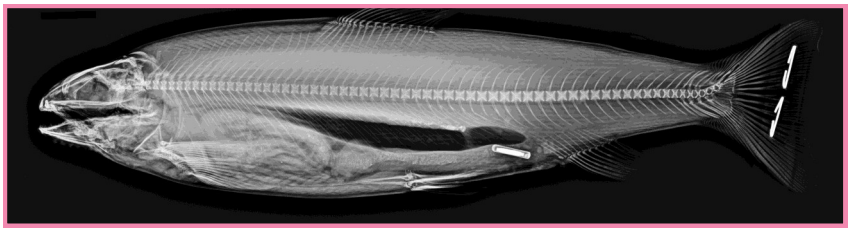


Figure 4. Atlantic salmon (150g) with PIT-tag in the abdomen (ID tag) and staples on the tail fin (external labelling). Radiograph taken by mammography. Photo: Nofima Marin AS

Diagnostics - Basic concepts of fish radiography

Figure 5: Salmon smolt euthanised, labelled and ready for freezing on a plastic covered board. Photo: Nofima Marin AS

Evaluation of radiographic images

The evaluation of fish radiographic images (Figure 6) is a science that remains under development. There are no reference laboratories for the evaluation of fish skeletal malformations, but numerous publications that describe skeletal pathology of different types and causalities exist. Also, in some sectors of aquaculture production, there is considerable competence within companies on how to read and interpret the images.

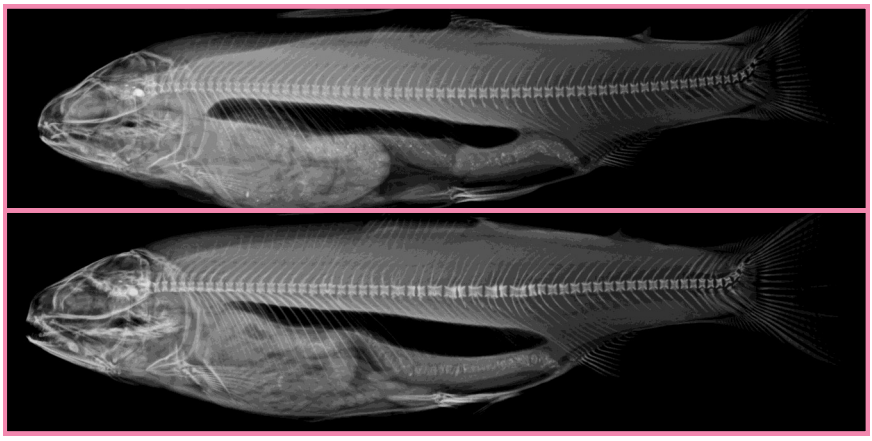


Figure 6. Rainbow trout (20 g) illustrating normal (top) and deformed (bottom) spinal columns. Radiograph taken by mammography. Photo: Nofima Marin AS

Guidelines for classification of skeletal malformations

Within the Fine Fish project, manuals for classification of skeletal deformities have been prepared for some of the most common aquaculture species.

- The combined sea bass and sea bream manual was prepared by the University of Patras (Greece), and presents extensive documentation of the most common malformations seen in these species, as well as current knowledge on causative factors.
- Manuals for Atlantic salmon, rainbow trout and Atlantic cod were compiled by the Nofima Marin (Norway) skeletal deformities research group.

Much less is published on skeletal malformations in these species, but these manuals provide examples and suggest some basic guidelines for classification.

These publications will be continuously updated. Please refer to the project website, www.finefish.info, for the latest versions of these manuals.

Diagnostics - Staining protocol of cartilage & bone

Synnøve Helland

General information about the staining of cartilage and bone, and the clearing technique

There are a lot of methods available, but these all follow the same basic principle of killing, fixation, staining, clearing, and preservation. It is possible to do double staining, i.e. both cartilage and bone staining. The following is a method for staining for bone and cartilage mainly based on Potthof, 1984, and adapted by S. Helland, Nofima Marin, based on experience in using this and other staining and clearing methods.

- For double staining follow all steps, 1 to 9.
- For cartilage staining follow steps 1 to 6, and then step 8 and 9.
- For bone staining follow steps 1 and 2, and then step 5 to 9.

List of Chemicals	
MS222	Hydrogen peroxide (H_2O_2)
4% phosphate buffered formalin	Potassium hydroxide (KOH)
96% Ethanol	Distilled water
Absolute ethanol	Trypsin powder
Acetic acid	Alizarin Red S
Alcian Blue 8GX	Glycerine
Sodium borate (Borax, Di sodium tetraborate decahydrate)	Thymol crystals (optional)

List of Materials		
Glass containers	Forceps	Sieve

Method for cartilage and bone staining, and clearing – Steps 1 to 9

1. Killing

Use a lethal dose of MS222

2. Fixation

Fixation in 4% phosphate buffered Formalin (10:1 ratio of formalin:fish).

- Fish standard length 10 to 80 mm: 2 days
- Fish standard length 80 to 100 mm: 3 days
- Fish standard length >100 mm: 5 days or more (remove some flesh on left side)

3. Staining of cartilage

Dehydration

A.) Rinse thoroughly with fresh water

B.) Transfer to 50% ethanol.

- Fish standard length 10 to 20 mm: 1 day
- Fish standard length 20 to 80 mm: 2 days
- Fish standard length 80 to 200 mm: 3 days
- Fish standard length >200 mm: 5 days

C.) Transfer to absolute ethanol *(95% ethanol may also be used)*

- Fish standard length 10 to 20 mm: 1 day
- Fish standard length 20 to 80 mm: 2 days
- Fish standard length 80 to 200 mm: 3 days
- Fish standard length >200 mm: 7 days

Staining with Alcian blue

⇒Fish standard length 10 to 80 mm: use *Solution A*

⇒Fish standard length 80 to >500 mm: use *Solution B*

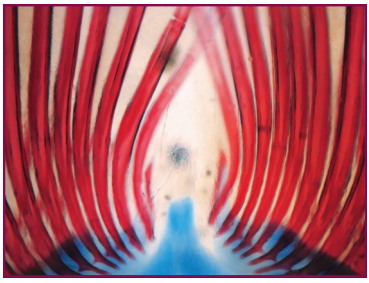
Diagnostics - Staining protocol of cartilage & bone

Solution A

70 ml absolute ethanol, 30 ml acetic acid, 20 mg Alcian blue.

Solution B

60 ml absolute ethanol, 40 ml acetic acid, 30 mg Alcian blue.



A.) Mix the ethanol and the acetic acid and then add the Alcian blue. Place on stirrer until dissolved. Filter the solution. The staining solution can be used twice.

B.) Soak the fish in the staining solution

- Fish standard length 10 to 80 mm: max 1 day (Solution A)
- Fish standard length 80 to 500 mm: 1.5 days (Solution B)
- Fish standard length >500 mm: 2 days (Solution B)

C.) Monitor the staining process by taking the specimen and examine through a stereoscope, do not overstain the specimen. Staining can not be removed from the cartilage by any of the chemicals that are used later in the clearing and staining process.

4. **Neutralisation** (to prevent calcium loss during bleaching)

Move the fish directly from the staining solution to a saturated sodium borate solution

- Fish standard length 10 to 80 mm: 0.5 day
- Fish standard length 80 to 500 mm: 2 days (change solution after 1 day)

5. Bleaching (optional)

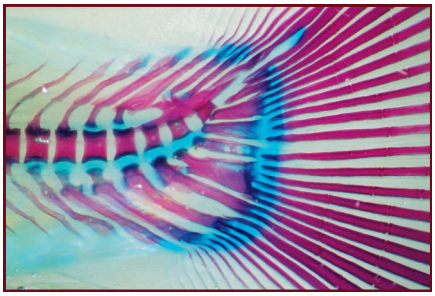
Bleaching solution

15 ml of 3% hydrogen peroxide (H_2O_2) with 85 ml of 1% potassium hydroxide (KOH)

Be very careful and do not bleach for too long, observe continuously! If done for too long, gas bubbles will form within the skeleton, e.g. inside the vertebrae. Change to 1% KOH immediately if bubbles are formed and repeat changes until no bubbles are formed.

6. **Clearing** (trypsin digestion)

Clearing solution



35 ml saturated sodium borate, 65 ml distilled water, trypsin powder

Keep the fish in the clearing solution until about 60% clear, change solution at least every 10th day. Illumination speeds up the clearing process.

The amount of trypsin powder must be evaluated - based on the amount of tissue surrounding the fish; for small fish, no trypsin is needed.

7. Staining of bone

Fish that comes directly from formalin (if step 3 and 4 are not used) should first be washed with running tap water and then pre-soaked in 1% KOH.

- Fish standard length 10 to 80 mm: 1 day
- Fish standard length 80 to 200 mm: 2 days (change solution after 1 day)
- Fish standard length >200 mm: 4 days

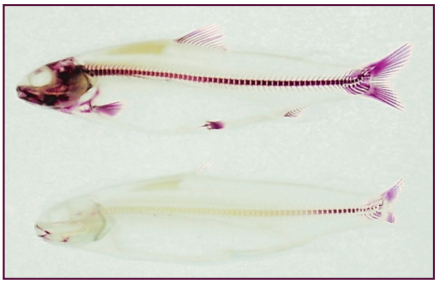
Staining solution

100 ml 1% KOH solution, 1 mg Alizarin Red stain

Mix the KOH and the Alizarin Red stain. The solution will now turn purple due to the alkaline pH. Place on stirrer until fully dissolved. Filter the solution.

- Fish standard length 10 to 80 mm: 1 day
- Fish standard length 80 to 200 mm: 2 days (change solution after 1 day)
- Fish standard length >200 mm: 4 days

If possible the staining should take place on a rotor at room temperature. If not, stir regularly. The containers should be kept in darkness (ex. covered with aluminium foil).

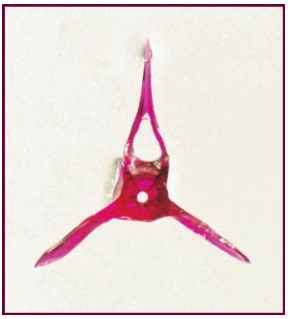


Diagnostics - Staining protocol of cartilage & bone

8. Destaining

Destaining solution 1.

35 ml saturated sodium borate solution, 65 ml distilled H2O, trypsin powder



Destaining solution 2.

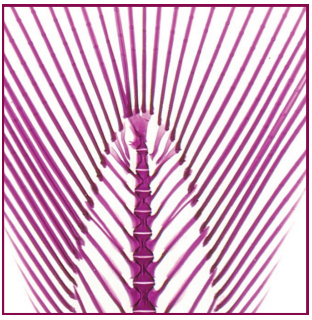
1% KOH

- Fish standard length 10 to 20 mm: 2 days
- Fish standard length >20 mm: Change to fresh solution every 10 days until solution remains unstained and specimen is clear.
  Alternate between destaining solution 1 and 2.

9. Preservation

Preservation solution 1.

30% glycerine and 70% KOH



Preservation solution 2.

60% glycerine and 40% KOH

Preservation solution 3.

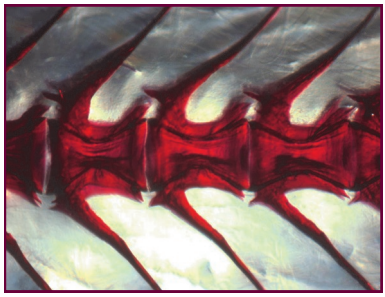
100% glycerine (with thymol as a final preservative for long time storage)

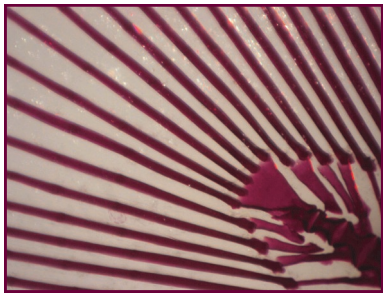
Place the fish in the preservation solutions 1, 2, and 3, and change of solution first after the fish has sunk to the bottom of the container. They are then ready for the next solution in the series. Direct sunlight and glycerine helps to clear and destain difficult specimens.

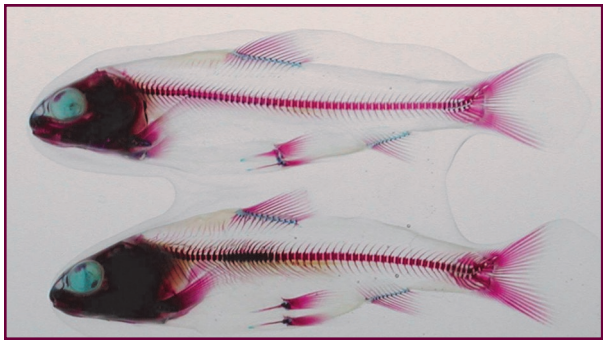
- Fish standard length 10 to 20 mm: 1 week
- Fish standard length 20 to 100 mm: 2 weeks
- Fish standard length >100 mm: 4 weeks

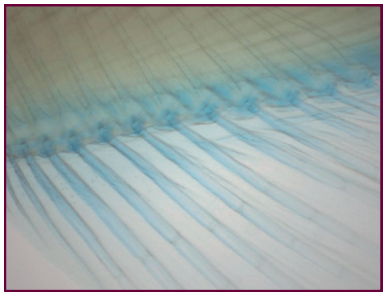
Most often the preservation step takes less time than foreseen

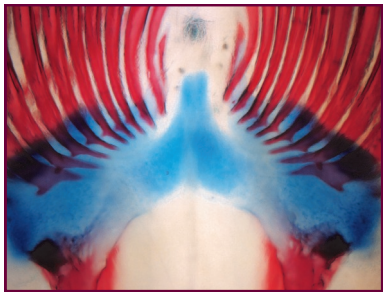
Some examples of staining using this protocol











Diagnostics - Staining protocol of cartilage & bone

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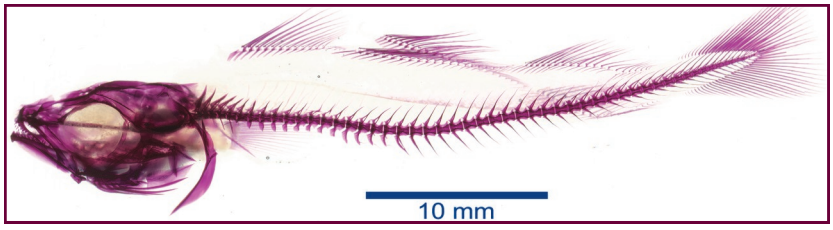

All photographs taken by Nofima Marin AS

Temperature effects on malformations in trout (O. mykiss)

Ingrid Lein, Synnøve Helland, Kirsti Hjelde & Grete Bæverfjord

Introduction

Rainbow trout is the most universal of the fish species reared in European aquaculture. It is raised from the North to South, East to West, in freshwater and seawater. It is harvested at different sizes, and is the basis of a range of different finished products. It is also the "oldest" of the fish species used for intensive farming in Europe, with a history in aquaculture dating as far back as the 1800s.

Despite the long experience with this species in aquaculture, malformations, and skeletal malformations in particular, are an important problem in modern rainbow trout aquaculture, irrespective of the rearing environment. In rainbow trout, malformations cause economical losses both in the hatchery sector and the on-growing sector. In addition, high numbers of malformed fish represent an ethical problem for the industry as a whole.

The causes of skeletal malformations in rainbow trout are probably as diverse as in other species, and one of the known causal factors is temperatures during egg incubation. In a previous study, the relation between temperature during embryonic development and malformations was established. The study showed that the best results were obtained when eggs were incubated at 10°C, and that relatively small effects on malformations were observed between 8°C and 12°C.

This study was, however, done with Norwegian eggs, and an important objection from producers from other geographic areas was that eggs from Norwegian stocks were not necessarily representative for other geographic strains of the species. In addition, it was noted that a significant proportion of the European production was done on triploid individuals, and specific information on the temperature tolerance of triploids compared to diploids was requested. Thus, as part of the FineFish project, an experiment was designed to address these issues.

The present study aimed to investigate the temperature tolerance of rainbow trout eggs of different geographic origins (north-south) and to investigate whether diploid or triploid rainbow trout have different temperature tolerance with regard to the development of skeletal malformations. In addition to these issues, it was decided to expand the range of test temperatures in the lower range, as there were indications from commercial production that there might be a lower limit for egg incubation temperatures in rainbow trout.

Experimental setup

Pooled egg groups originating from three different genetic strains (1, 2 and 3) were fertilised in their hatcheries of origin and transported to the experimental hatchery of Nofima Marine in Sunndalsøra, Norway for incubation.

Strain 1 consisted of eggs from Aqua Gen in Norway, the other two strains from southern altitudes. After fertilisation, half of the eggs from strain 3 were pressure treated (200 bar for 10 minutes at 10°C) to produce triploid fish.

The egg transport was done by courier, and was standardized as far as possible, so that all egg groups reached the destination within 24 hours post-fertilization.

The egg groups were split into small batches and incubated at 6, 10 or 14°C in triplicate small insulated units equipped with individual inlets and outlets (Figure 1).

The temperatures were kept stable from fertilisation to first feeding.

Figure 1. Experimental units for hatching of rainbow trout eggs. The units have individual inlets and outlets and, each unit is insulated to insure stable temperatures.

From first feeding, all fish were reared at 12°C, until termination of the experiment at approximately 30-40g.

Experimental Results

Survival after hatching

Embryonic temperature did not influence the survival rates from hatching to first feeding (range 67.6-78.1 %). However, one strain had an approximately 20% lower survival than the two others. Triploid groups had significantly lower survival than diploid groups from the same strain (38.4% and 61.3%, respectively).

Skeletal malformations

The most common skeletal malformations were fused or compressed vertebrae (see Figure 2).

The lowest prevalence of spinal malformations was obtained at 10°C, and fish originating from the 6°C eggs were comparable to those from 14°C eggs. There were also differences in number of fish with spinal malformations between the three geographic strains. Triploid groups had, not unexpectedly, a higher prevalence of fish with spinal malformations than diploid groups from the same strain. To a large extent, differences in prevalence were reflected also in the severity of lesions, so that the fish groups with the highest number of affected fish also had the most severe lesions.

Temperature effects on malformations in trout (O. mykiss)

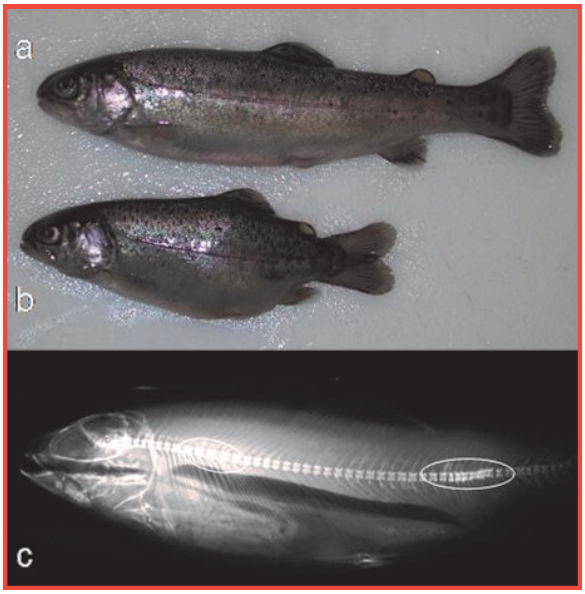

Figure 2. Rainbow trout with a normal vertebral column (a) and with a severe compression of the vertebrae in the caudal area (b). The radiograph (c) shows examples of compressed

To give a better description of these observations, a "severity index" was calculated as:

% of affected fish x number of malformed vertebrae per affected fish (see Figure 3)

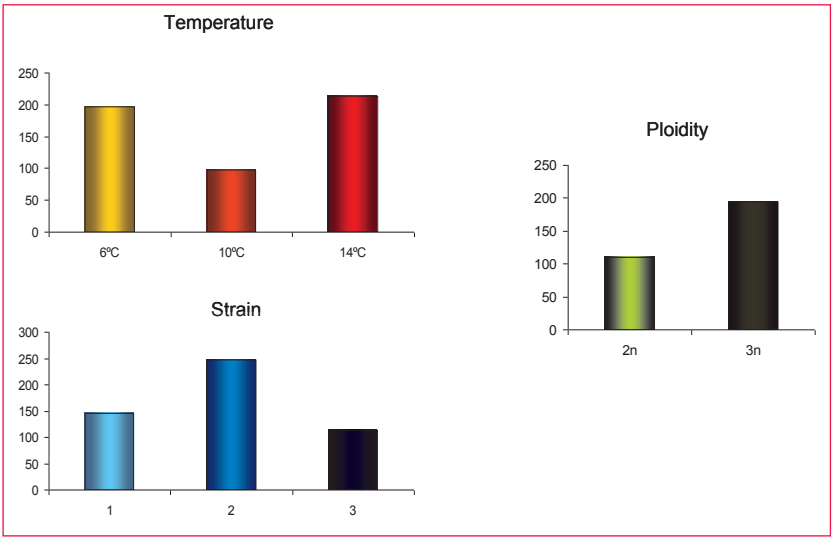

Figure 3. Effect of embryonic temperature, geographic strain and triploidy on severity of vertebral malformations, calculated as prevalence *number of affected vertebrae per fish with malformations.

Other malformations displayed a more variable pattern, but in sum, the results presented in Figure 3 are fairly representative of the overall results, in that

- eggs incubated at 10°C gave fish with the lowest rate of malformations and
- the results with incubation at 6°C and 14°C were inferior both in diploids and triploids.

Some strain specific malformations were observed in low numbers, and some malformations were more common in triploids, but these effects were of minor importance compared to the temperature effects.

Specific growth rate

The specific growth (SGR) rate from first feeding to approximately 40 g had a converse relation to increasing embryonic temperatures. Thus, the best growth rates in the juvenile stage were obtained in groups given the coldest water and longest development time during egg incubation.

It is important to note that from first feeding and onwards, all fish were reared at a common temperature (12°C), so that any differences in growth rate can be attributed to effects induced prior to first feeding.

When comparing strains, there were significant differences between them. Ploidity, on the other hand, did not affect the SGR (Figure 4).

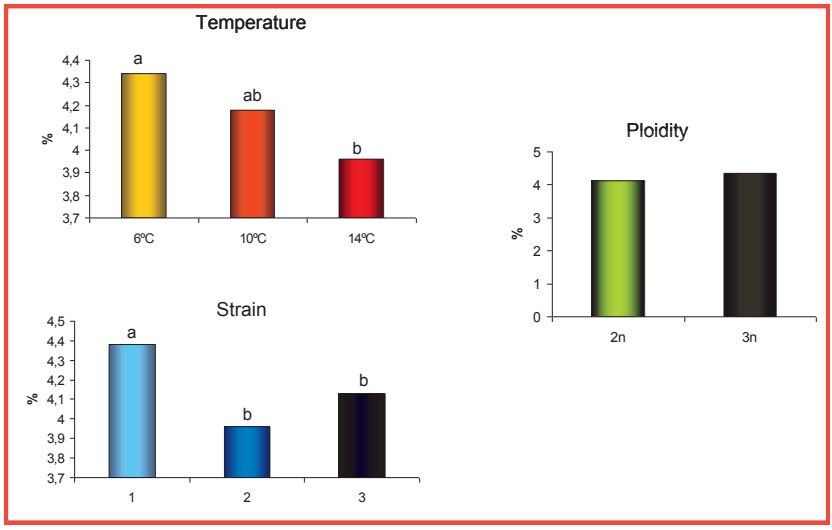

Figure 4. Effect of embryonic temperature, geographic origin and triploidy on the specific growth rate (SGR) from first feeding to approximately 40 g size. Different letters means statistically significant differences.

Temperature effects on malformations in trout (O. mykiss)

Conclusions and recommendations

The results confirm previous findings that the optimal temperature for incubation of rainbow trout eggs and yolk sac fry is 10°C, and this conclusion seems to be valid for each of the three European strains that were tested, despite the different geographic origins.

The study clearly showed that to achieve a normal development in rainbow trout an incubation temperature of 14°C is too high while 6°C is too low.

When combined with results from previous studies it can be concluded that rainbow trout should be incubated at temperatures between 8°C and 12°C, and that 10°C seems to be optimal for this species.

One of the strains generally showed inferior results compared to the other two strains. This may, however, be due to the fact that these eggs were stripped at the end of the local spawning season and also were obtained from females that had been stripped repeatedly. Thus, this is likely to be a result of low egg quality, rather than a specific strain effect. Other than that, there were some strain-specific effects on malformations, but the temperature response was the same for all three strains, and also for the triploid groups.

Triploid groups generally displayed higher malformation rates than diploid groups, which is in accordance with earlier studies. Thus, the obvious advantages of using triploids must be weighted against problems associated with the commercial production of all-female triploid females for each production area and market.

High embryonic temperature (14°C) resulted in a low SGR during first feeding, but the SGR did also differ between strains. This is could be due to a different genetic background with regard to different genetic improvement intensity (breeding). There was no difference in SGR between diploid and triploid groups.

Recommendations:

The egg incubation temperature for rainbow trout, both diploid and triploid, should be controlled between 8 and 12°C to control malformations, irrespective of the individual genetic strain.

Temperature effects on malformations in cod (Gadus morhua)

Ingrid Lein, Lars Thomas Poppe, Yoav Barr, Kirsti Hjelde, Synnøve Helland & Grete Bæverfjord

Introduction

Nofima Marin AS has made two experiments studying effects of temperature, during start feeding, on skeletal deformities on cod. The first was done outside of the FineFish project in 2005 (together with 'Troms Marin Yngel' in Norway (a commercial partner)), and the other was a follow-up and was achieved with one part funding from FHF and one part within the FineFish project.

The results of these experiments show a clear relationship between temperature and bone malformation, especially with the abnormal curvature in the neck area, often referred to as "stargazers".

Experimental set-up I

Three temperature regimes were tested out in small scale units (90L tanks) with four replicates per treatment (Figure 1). The cod were either start fed at a constant 12°C or 8°C, or they were subject to a gradual increase from 6°C to 12°C during a seven week period. The fish from the second regime were kept at 6°C for 16 days after start-feeding before the water temperature was increased by one centigrade per week until it reached 12°C.

This latter regime is based on the ambient temperatures in Lofoten during the start feeding period. The experiment was ended when the cod were about 50g, and all fish were radiographed using mammography.

All deviations were registered, classified and axis deviations were graded on a scale from I to IV, where grade I constitutes small deviations, and grade IV the severe deformations.

Figure 1. Experimental start feeding units with water and air temperature control.
Photo: Nofima Marin AS

Results of the first Experiment

The results showed a clear effect of start feeding temperature on the presence of malformations, where the highest number of malformed cod was found in those which were start fed at the highest temperature. A similar picture was seen for the abnormal curvature in the neck (Figure 2), where cod start fed at 12°C had the highest, and cod with a gradual increase from 6°C to 12°C had the lowest level of abnormal curvature of the neck (Figure 3).

At the end of the experiment there was no difference in weight between fish from the constant 12°C and the regime of gradual increase from 6°C to 12°C, while fish from the constant 8°C had a lower final weight.

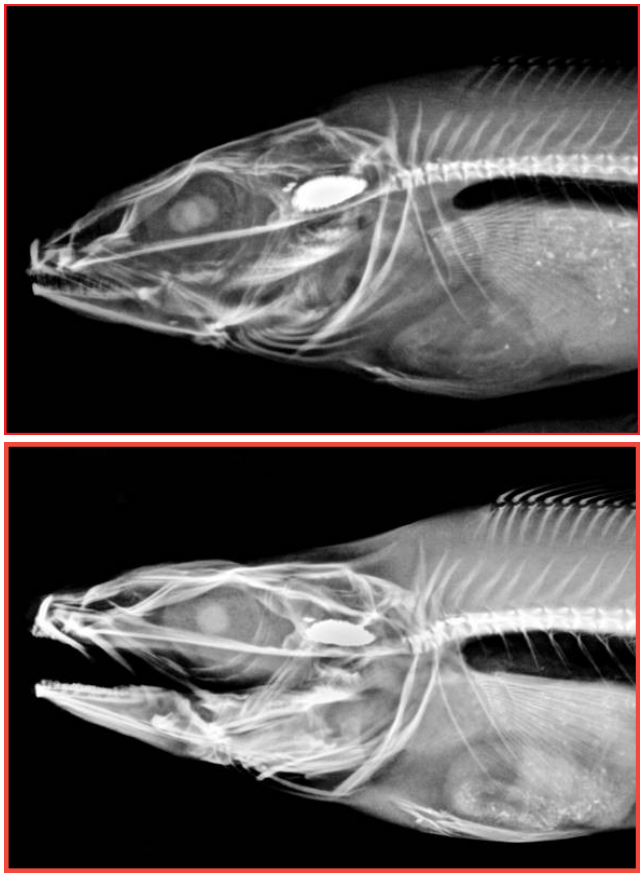

Figure 2. Radiographic image of Atlantic cod juvenile with normal (upper image) and an abnormal (lower image) curvature of the neck (grade II). Photo: Nofima Marin AS

Temperature effects on malformations in cod (Gadus morhua)

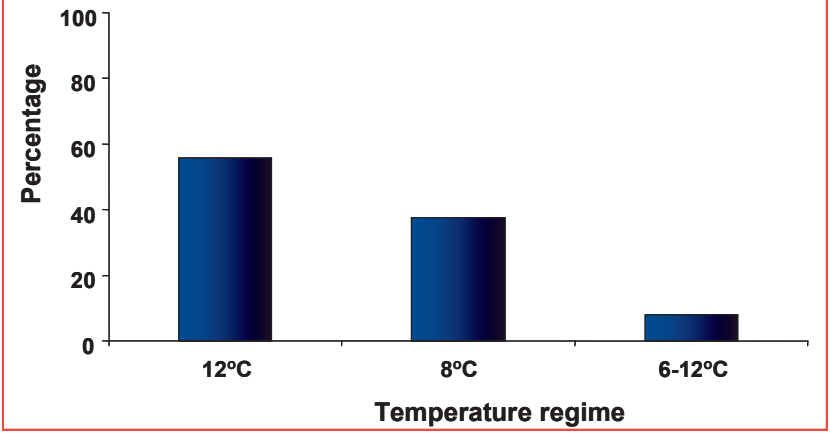

Figure 3. Proportion of Atlantic cod juveniles with abnormal curvature of the neck "stargazers" start fed at three temperature regimes; Constant 12°C, constant 8°C, or a gradual increase from 6°C to 12°C during a seven week period.

Experimental set-up II

Two temperature regimes were tested, both with a gradual increase from 6°C to 12°C. In the control group the increase was done during seven weeks, and it the other experimental group a more rapid increase of five weeks (fast temperature increase) was tested. Otherwise the set-up was the same as in the above described experiment.

Results of the second Experiment

No difference in weight was found between the control group and the 'fast temperature increase' group when the experiment was finished at a final weight of 2 gram fish. However, the fish from the most rapid increase were longer that the fish from the control group.

A higher incidence of total number of deformed fish, and of abnormal curvature of the neck, was found in the group start fed at the fastest temperature increase (see Table 1).

Table 1. Total percentage of deformed fish, abnormal curvature of the neck of Atlantic cod juveniles

Atlantic Cod juveniles	Control group	Fast Temperature Increase
Deformed fish %	13.1%	16.7%
Abnormal curvature of the neck	17.9%	21.9%

Conclusions and practical recommendations

The bone formation and the mineralisation processes take place during the start feeding period, and the rearing temperature during this stage therefore has a strong effect on formation and/or malformation of the vertebral column. Skeletal deformities can be induced by several factors. In the cod that were start fed at the temperature regime with the best results from these two experiments, there are still a high numbers of malformations. Thus, it is likely that there are abiotic or biotic factors that are sub-optimal.

Based on the present studies, the temperature regime with the lowest number of malformations, but with no negative impact on final weight, was the gradual increase from 6°C to 12°C during a seven week period. Therefore, it appears that temperature must be controlled, and that it is important not to push growth by increasing the temperature too fast during this early live stage.

Temperature limits for gilthead sea bream and sea bass

Pantelis Katharios, Pascal Divanach, E. Georgakopoulou & Giorgos Koumoundouros

Introduction

The rearing temperature during the early stages of the life cycle is one of the most acknowledged factors implicated in the development of malformations of gilthead seabream and European seabass (Polo et al., 1991; Sfakianakis et al., 2004; Sfakianakis et al., 2006; Georgakopoulou et al., 2007). The mechanisms causing these deformities remain unclear, but it is known that hyperthermia alters numerous functions, including metabolism, respiration, membrane function, and it is involved in dramatic changes in the expression of specific genes (Podrabsky and Somero, 2004).

The objective of this study was to identify the effect of water temperature during the early stages of gilthead sea bream and sea bass on rearing performance and deformation response in fine-tuned large scale experiments.

Methods

The experiments were performed at the hatchery of the Institute of Aquaculture (Crete) using 12 500-L tanks. The ambient conditions were electronically controlled and adjusted to the desired levels. The rearing prot°Col followed the methodology described in Divanach et al (1997) and Papandroulakis et al (2001).

The experimental set up for sea bass called for using three schemes (in duplicates) with different temperatures at the autotrophic and exotrophic stages (e.g. fish starting at 15°C and going up progressively to 18°C at first feeding, from 18→21°C and from 21→18°C), and three schemes with constant temperature (15, 18 and 21°C) for both autotrophic and exotrophic stages which served as reference controls. The same set up was also used for sea bream although the temperatures were set one degree Celsius higher (16→19°C, 19→22°C, 22→19°C and three groups with constant temperatures 16, 19 and 22°C) (Table 1). The fish of all treatments were reared in the same temperature after they reached the size of approximately 16 mm total length. The experiment was finished when the fish had reached 1 g in weight. Six samplings were made for each treatment at certain developmental stages.

Analysis of deformities was performed at the end of larval rearing phase and at the juvenile stage, by means of double staining and x-rays, respectively.

Results and Discussion

In sea bass the best growth rates were recorded in the fish which were either constantly reared in 21°C or they started at 18°C and moved to 21°C, followed by the fish which were reared in 18°C, 15→18°C and 21→18°C.

Scheme for Seabream	16°C	19°C	22°C
Scheme for Sea bass	**15°C**	**18°C**	**21°C**
A	Autotrophic phase	Exotrophic phase	
B		Autotrophic phase	Exotrophic phase
C		Exotrophic phase	Autotrophic phase
D	Autotrophic & exotrophic		
E		Autotrophic & exotrophic	
F			Autotrophic & exotrophic

Table 1. Experimental set up for sea bass and sea bream

The lowest growth rate was recorded at the fish which were reared constantly in 15°C. In terms of survival, the best scheme, until the fish were put in common conditions (16-18 mm), was the one that used 15°C as a starting temperature and 18°C for the exogenous feeding, while after the fish went to the common conditions the best survival was achieved by the fish which were constantly grown in 15°C.

Two severe and one light (with no effect on the external phenotype) types of skeletal deformities were detected in experimental populations of sea bass. Severe deformities consisted of the haemal lordosis and the shortened upper jaw (pugheadness), light deformity was the presence of urinary calculi. Results clearly demonstrated a significant effect of rearing temperature on all the types of skeletal deformities. Our results verified those of previous studies (Sfakianakis et al. 2006), clearly indicating that any deviation (temperature increase) from an early water temperature of 15 °C results to elevated incidence of haemal lordosis. However, we have to underline that the incidence of pugheadness was elevated at 15°C (as in the case of branchiostegal deformities, Georgakopoulou et al. 2007), thus underlining the need of future research on the combined effects of temperature and other factors important for the normal bone development (e.g. nutrition, Georgakopoulou et al. 2007). Concerning the presence of urinary calculi, this was elevated at the higher temperatures tested.

Temperature limits for gilthead sea bream and sea bass

For gilthead sea bream the best growth rates were recorded in the fish which were reared at the highest temperatures (22, 19→22, 19, 16→19) while the highest survival was recorded in the fish which started at 16°C and progressively moved to 19°C and the lowest in the fish that started at 22°C and moved down to 19°C.

In gilthead sea bream, two severe and five light (with no effect on the external phenotype) types of skeletal deformities were detected. Severe deformities consisted of the inside folding of the gill-cover and of haemal lordosis. Results clearly demonstrated a significant effect of rearing temperature on gill-cover deformities, but not on lordosis. Interestingly, gill-cover deformities were proven to develop up to 12 mm TL, while in the next stages their incidence was significantly decreased (probably due to a higher mortality of the affected fish).

Current results therefore recommend the range of 19-22°C (especially during the feeding larval phase) as being safe temperature limits for early development of sea bream.

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Molecular pathology of temperature-induced vertebral deformities

Elisabeth Ytteborg, Grete Baeverfjord, Jacob Torgersen, Kirsti Hjelde & Harald Takle

Introduction

In cold water aquaculture, an efficient way of increasing rate of growth and development is to apply elevated temperatures. Unfortunately, in Atlantic salmon there is a clear relation between high temperatures in juvenile stages and increasing frequency of skeletal deformities. However, we still do not know why deformities develop, and the molecular pathways involved are still far from understood.

In order to elaborate the mechanisms involved in temperature induced deformities, we designed an experiment where Atlantic salmon was exposed to two different temperature regimes, designated high and low temperature group. Our first aim was to analyze and compare normal (non-deformed) spinal columns from a high and low intensive temperature regime in order to see if there were any underlying differences between the two groups associated with higher risk of developing deformities. The second aim was to describe the fusion process in detail to get more knowledge of the underlying mechanisms involved.

Material and methods

Fish from the high intensive and low intensive temperature groups were subjected to radiographic screening. Spinal columns of interest were sampled at two developmental stages (2 and 15g) and studied by a variety of histological and gene expression analysis techniques (Fig. 1).

Real time RT-PCR analyses and *in situ* hybridization (*ISH*) are two methods used to quantify and locate gene expression, respectively (Fig. 2).

Thus, these analysis gives the ability to quantify and locate the level at which a particular gene is expressed within a cell, tissue or an organism.

Gene expression is the process by which information from a gene is used to make functional gene products (proteins). Gene regulation gives the cell control over which proteins that are to be made, hence structure, function, differentiation and the ability to adapt to stimuli. To find genes of interest we analyzed pathways involved in bone and cartilage development that are known from mammalian studies. For example, the mesenchymal cells (MSC) are stem cells that can differentiate into osteoblasts (bone forming cells), chondrocytes (cartilage forming cells), adipocytes (fat cells) and myotubes (muscle cells). What kind of cell type they will turn into is decided by a number of regulatory proteins, e.g. the transcription factors.

Some of the key transcription factors in bone metabolism include Runx2 and Osterix, which are involved in differentiation and maturation of osteoblasts that express bone matrix (collagen1a) and matrix mineralizing (osteocalcin and osteonectin) genes.

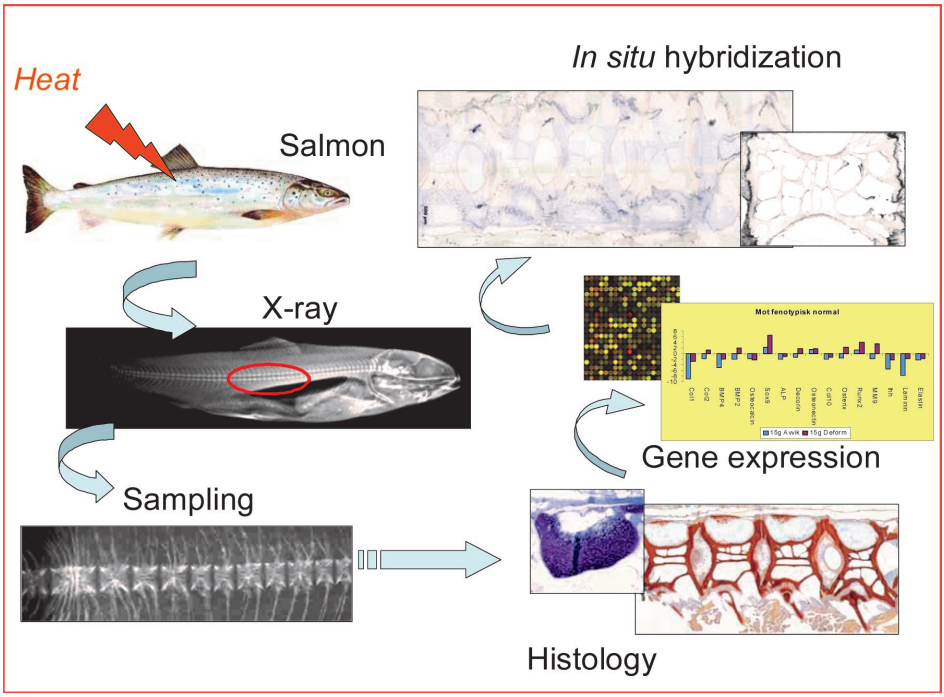

Figure1. Overview of the experimental pipeline used to study bone development and vertebral deformities in Atlantic salmon (*Salmo salar*). Fish was exposed to a high and low intensive temperature regime from fertilization till 20g. During the experiment, fish were sedated and radiographed at 2g, 15g and 60g. From the live-radiography, normal, aberrant and deformed spinal columns were sampled and used for histological analysis, real time RT PCR and *in situ* hybridization. This methodological approach has given new insight into the underlying mecha-

Mineralisation is the process where minerals are incorporated into the bone matrix, hence hardening of the tissue. In comparison, early chondrocyte differentiation is controlled by sox9, which regulates transcription of collagen2a, the major extracellular matrix (ECM) component of cartilage. Further, mef2c assures that chondrocytes further mature into collagen10a producing hypertrophic cells.

Both mineralized bone and cartilage is remodeled through the activity of osteoclasts (bone resorbing cells). These cells provide an acidic environment, through the expression of a number of proteins (cathepsins, TRAP (Tartrate Resistant Acid Phosphatase) and Mmps (Matrix metalloproteinases)), where mineralized matrix may be broken down. By analyzing pathways like these, we ended up with a “molecular toolbox” containing 22 of the most important genes involved in skeletogenesis and a number of histological staining methods, including immunostaining of specific proteins.

Molecular pathology of temperature-induced vertebral deformities

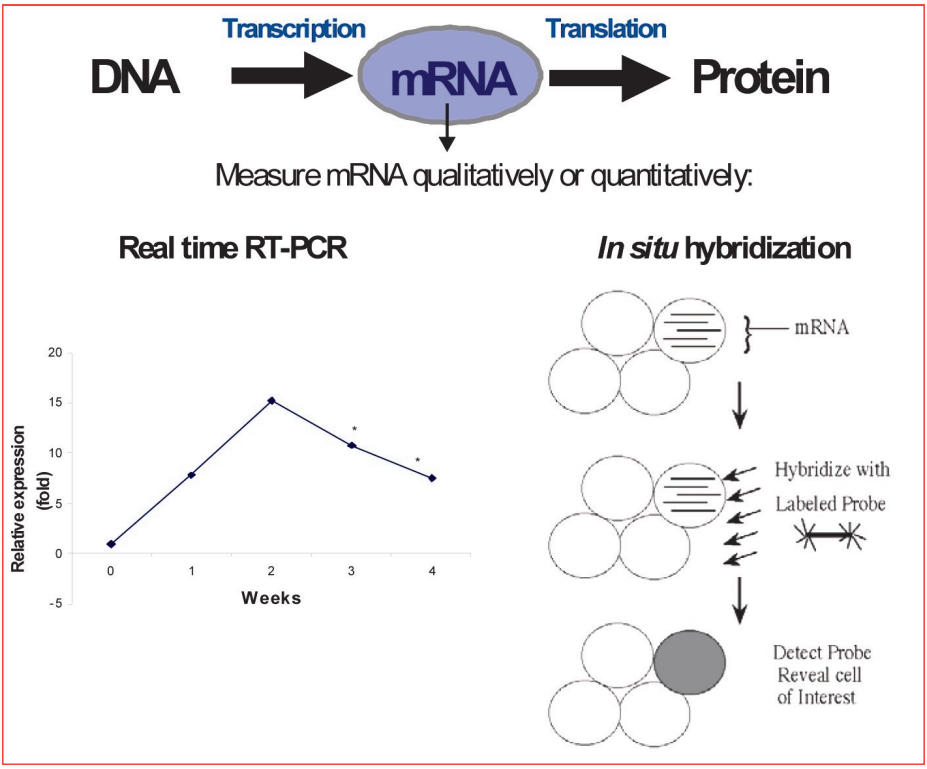

Figure 2. Genes from a DNA template are transcribed into mRNA, carrying the coding information that can be translated into proteins. Two methods used to measure mRNA transcription are real time RT-PCR and *in situ* hybridization. Real time RT-PCR is used to quantify a target mRNA, whereas *in situ* hybridization use labeled probes to localize the mRNA of interest in a tissue or a section of the tissue.

Results

Radiological comparison of non-deformed vertebral columns indicated that Atlantic salmon juveniles from the low intensive temperature group displayed denser and more regular vertebral bodies throughout the vertebral column than those from the high intensive temperature group. Correspondingly, a significant higher number of deformities were found in the high intensive temperature group. The number of deformities increased with time, at 2g size approximately 10% showed deformed spinal columns, at 60g size almost 30%.

Most deformities were of the fusion type. As expected, the fish reared at a high temperature regime grew much faster than those reared at low temperature, e.g. fish reared at high temperatures reached 2g in 6 weeks compared to 11 weeks at low temperature and 60g in 7 months compared to 10 months.

Molecular analysis of non-deformed vertebrae revealed that most genes were differently expressed in the high temperature group compared to the low temperature group.

The expression of genes involved in matrix production and mineralization showed significantly less activity (down-regulation) in the high temperature group at both 2g and 15g. Expression of *col1a1, col2a1, osteocalcin, decorin*, and *osteonectin* were reduced in all individuals from the high temperature group. In addition, *ISH* with these genes showed a more restricted area of expression. The down-regulation of the genes encoding structural proteins taking part in the bone matrix supported the tendency of weaker radiodensity in the high temperature group and indicated that fast growth gives lower mineralization of the vertebral tissue. Through *ISH* we also identified expressional similarities of the ECM components in Atlantic salmon to other vertebrates.

It is apparent that most of the factors and pathways that control bone formation are highly conserved in vertebrates. During optimal conditions, chondrocytes in the areas connecting the arches to the spinal column are lined up in three distinct and well organised bands. Detailed examination of these areas in fish from the high temperature group showed that the chondrocytes had a more distorted pattern. We also observed an increased expression of genes involved in the final maturation of chondrocytes, *mef2c* and *collagen10a*. Also from *ISH*, we observed an expanded area of *collagen10a*, indicating an increased area of hypertrophic chondrocytes in the cartilage. Chondrocyte hypertrophy is the final stage prior to endochondral ossification, a process where cartilage is replaced by bone. In addition, we did not find positive TRAP activity and real time expression data showed that *mmp9* and *mmp13* were both down-regulated. TRAP and Mmps are needed in bone remodelling by the osteoclasts to bring about the final steps in endochondral ossification. Our findings strongly indicates that temperature induced fast growth is severely affecting gene expression in osteoblasts and chondrocytes; hence change in the vertebral tissue structure and composition. The findings further indicated that bone in salmon developed at high temperature have a “softer” bone phenotype, and the higher percentage of deformities in this group may be linked to a reduced resistance to withstand mechanical pressure from the high muscle mass in farmed salmon.

To further understand the mechanisms involved in the development of vertebral deformities, we established a model for studying the pathogenesis of vertebral fusions in Atlantic salmon by using live radiography to identify fish at an intermediate and terminal stage of the fusion process, respectively. We confirmed by molecular tools that the calcification of the heterotrophic intervertebral cartilage and its subsequent remodeling into bone facilitates the fusion of vertebral bodies. Analyzing deformed spinal columns by *ISH* revealed an increased amount of cells expressing mixed signals of genes involved in both osteoblasts and chondrocytes, supporting the hypothesis that formation of chondroid bone (resembling both bone and cartilage) is facilitated during fast growth and pathological conditions.

This ectopic bone formation appears to be the basic mechanism in development of vertebral fusions. V*imentin*, producing a protein that makes chondrocytes more resistant to withstand mechanical pressure, was also down-regulated during the fusion process. In addition to having a "soft" bone phenotype, this low vimentin transcription may further reduce the ability to withstand mechanical forces during fast growth. Of the 22 genes we analyzed, structural genes (*osteocalcin, decorin, vimentin* and the collagens) and genes involved in differentiation *(pdgfrb, bmps* and the *hh*) were down-regulated in deformed spinal columns compared to non-deformed. Further, deformed spinal columns were characterized by an increased cell proliferation simultaneous with an increase in cell death, results reflecting a malignant potential of spinal fusions. The mechanisms behind these processes have not been described in detail.

Cells in culture:
A potential replacement for *in vivo* experiments

To study gene function it is an advantage to isolate the systems of interest and thereby have a more simplified model to work with. Thus, we have started developing an osteoblast cell culture for Atlantic salmon. This cell culture system has subsequently been used to study the effects of factors that influence bone formation in Atlantic salmon. Studies have shown that differentiating osteoblasts are highly sensitive to increased temperature at early stages of differentiation, results that support our *in vivo* observations in the spinal column. In line with our findings above, a long-term heat exposure to the cells resulted in decreased expression of *alp*, *col1a1* and *osteocalcin*, indicating that the model system is suitable for study osteoblast biology.

Conclusion

Cultured Atlantic salmon is bred for rapid growth, and the industry will aim at obtaining the optimal growth rate by optimizing e.g. diets and environmental factors accordingly. Therefore, it is important to understand the molecular and cellular events in bone development in salmon, in order to deal with any problems with skeletal development that arise as a result of intensive rearing conditions.

By combining molecular tools with targeted radiography based sampling and histology, we are now able to describe a more complete picture of how spinal deformities in Atlantic salmon develop. Importantly, management control of deformities and health in general demands precise tools and knowledge to depict any problem as early as possible in the production line. The reliable correlation between defined skeletal markers and the risk of developing vertebral deformities found in our temperature experiments indicates that these genes can be developed as prognostic markers. Further, our skeletal tool-box can be used to investigate how the progression of skeletogenesis is modulated in response to other stimuli.

Effects of water speed on lordosis & heart ventricle weight in cod

Synnøve Helland, Ingrid Lein, Kirsti Hjelde & Grete Bæverfjord

Introduction

Intensive farming of Atlantic cod is a rapidly growing industry in several of the countries bordering the North Atlantic. One of the major obstacles in the intensive production of cod has been the high degree of malformation, and this study addresses lordosis, a condition which encompasses the abnormal ventral curvature of the vertebral column—see Figure 1.

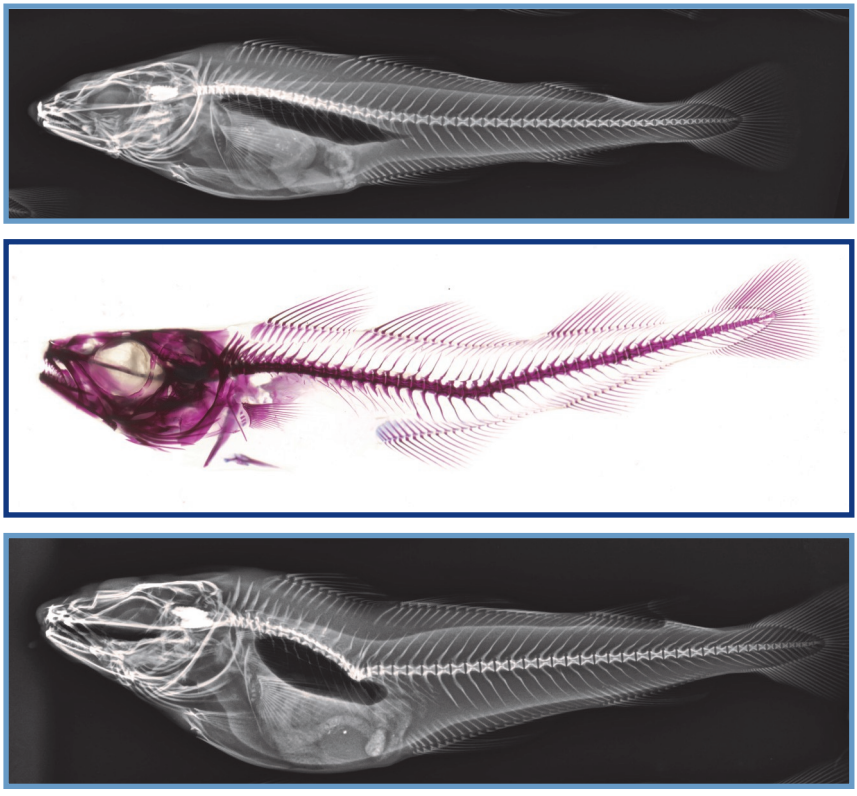

Figure 1. Cod juveniles (Gadus morhua) with

a) normal vertebral column (radiographic image),

b) lordosis (Alizarine staining) and

c) severe lordosis (radiographic image).

Photos: Nofima Marin AS

The motivation for choosing this approach to establish causative factors of lordosis was based on the findings from some of the researchers, working within FINEFISH, and their experiences with sea bass and sea bream.

The combined effect of early rearing temperature and swimming speed of European seabass (*Dicentrarchus labrax*) was studied in the EU-funded project entitled "Optimisation of rearing conditions in sea bass for eliminated lordosis and improved musculoskeletal growth" (ORCIS— EU project QLRT-2000-01233).

Eggs and larvae of European seabass had been reared at either 15 or 20ºC—until metamorphosis. After metamorphosis, they were reared at the same temperature. The researchers found that lordosis was more frequent in fish from the 20ºC protocol, independent of the water current applied (Sfakianakis et al., 2006). Furthermore, they found a clear effect of water current velocity on lordosis development in populations coming from both developmental temperatures.

The objective of the present study was to test if high water speed would also induce lordosis in the skeleton of cod juveniles.

Experimental set-up

Three days prior to hatching, eggs from Atlantic cod (*Gadus morhua*) were disinfected and transferred to a 1200L tank where the eggs hatched and the cod larvae were grown until day 60 after hatching. The temperature at incubation was 6^{o}C and, from start feeding, this was increased to 12^{o}C—during a period of 7.5 weeks. The cod juveniles were then transferred to eight 90L tanks.

At start of the experiment (day 60 post hatch), four tanks were rigged so that there was minimal water speed in the tanks (control) and the fish had arbitrary swimming directions. Four other tanks were rigged so that the fish was able to swim against the water current between 5 and 10 sec., before the fish changed behaviour to again swim against the current (Figure 2).

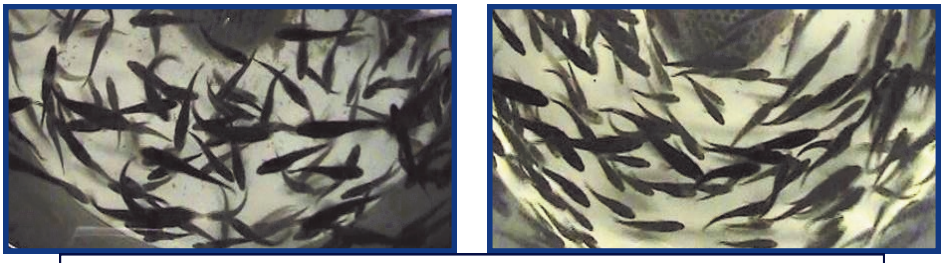

Figure 2. Atlantic cod juveniles in control tanks with arbitrary swimming directions (left image) and high water speed tanks and cod swimming against the current (right image). Photos: Nofima Marin AS

The water speed was based on swimming behaviour, which was checked and regulated twice a week . This meant that with increasing swimming ability, an increased water speed was applied.

The water exchange rates were the same in all eight tanks.

Effects of water speed on lordosis & heart ventricle weight in cod

At 4 and 13g average size 50 and 130 fish, respectively were sampled for radiographic images. At the last sampling, the heart ventricle was dissected from the fish and weighed for calculation of cardio somatic index (CSI= (weight of cardiac ventricle*100/body weight), as an indicator for cardiac development.

Results

The weights of the cod at the end of the experimental period of four months were the same for the control and the high water speed treatments (13.1 g). Water speed did however have an effect on the fish total length, where the cod from the high water speed treatment (22.9 cm) were longer than the control treatment (20.8 cm).

As a consequence, the condition factor differed between the treatments, being highest in the control treatment.

The weight of the heart ventricle was higher in the cod from the high water speed (0,019 g) treatment than the control (0,024 g), and also the calculated cardio somatic index.

There was a higher incidence of lordosis in cod at 13 gram size reared with a high water speed than low water speed (control), and a higher incidence of lordosis at 13 than at 4 gram size within treatment (Figure 3).

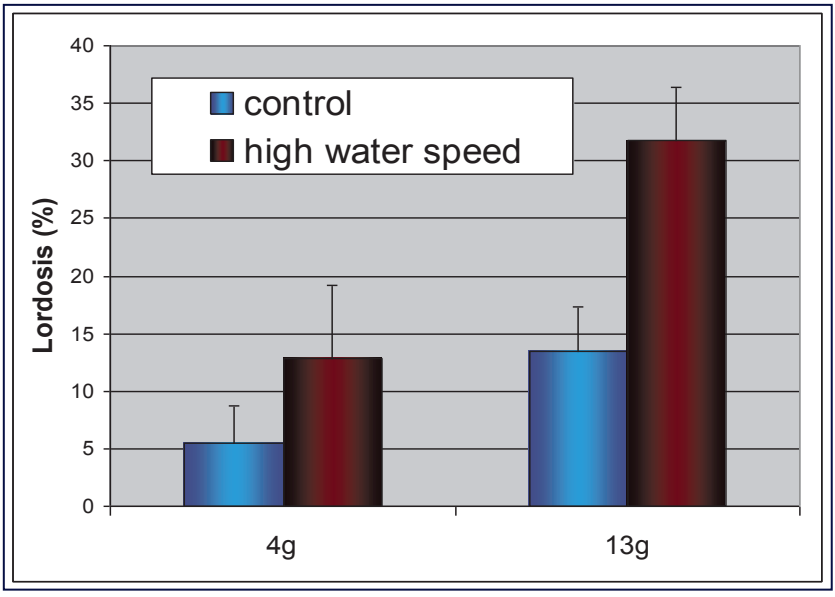

Figure 3. Incidence of lordosis in Atlantic cod (*Gadus morhua*) at 4 and 13 grams size (radiographic evaluation) reared with low water speed (control) or high water speed.

The vertebra in the centre of the lordosis axis break was calculated (average of all fish that were diagnosed with lordosis), and this was vertebrae number 18 counted from the cranial end.

Normal fish were randomly selected and the angle was measured from the centre point of vertebrae number 18 and four vertebrae towards the cranial and caudal ends.

Similarly, the angle from the lordotic centra in fish with lordosis was measured.

The average backbone angle was 174 and 143 degrees of normal and lordotic cod, respectively.

Neither platyspondyli, fusions, nor axis deviations in the neck of the cod were affected by high water speed.

Conclusions/practical recommendations

The systems used for cod rearing are partly based on knowledge and experience obtained from the salmon and the sea bass/bream industries. This knowledge transfer has given a head start for the development of the intensive cod aquaculture industry. Nonetheless, such transfer of knowledge from other species not only has advantages but, also, limitations.

It is common in studies of the Mediterranean fish species to differentiate between pre-haemal and haemal lordosis since these seem to have different causal factors.

The pre-haemal lordosis is associated with the lack of a functional swim bladder, and is thought to be an effect of a compensatory swimming behaviour due to a lack of buoyancy (Chatain 1994; Chatain and Dewavrin, 1989). The cod in the present study had no problem with non-inflated swim bladder or pre-haemal lordosis.

Haemal lordosis is attributed to the swimming effort of sea bass *Dicentrarchus labrax* juveniles and of red sea bream *Pagrus major* with inflated swimbladder (Divanach et al. 1997; Kihara et al., 2002).

An effect of early developmental temperature on the severity of deformity, both in respect to the angle of the lordosis and the number of affected vertebrae, was found by Sfakinanakis et al. (2006) in *Dicentrarchus labrax*. From the same experiment, the allometric studies revealed that an inappropriate force results in vertebral adaptations in the form of lordosis (Kranenbarg et al., 2005).

Similarly to the studies made on sea bass, the present study shows that high water speed induced lordosis in cod.

Lordosis appeared in the present study at a higher degree at 13g than at 4g size,. Thus it is likely that lordosis can be induced at quite a late developmental stage (i.e. after 4g size). It may also be that ‘damage’ made at an early stage manifests itself at a later stage.

The main reason for having a high water speed is to increase self cleaning of the fish tanks. Therefore, the safe water current velocity at the various life stages of cod should be tested further , since self cleaning in the tanks strongly reduces the labour needed for manual tank cleaning , the stress that this may cause on the fish and improves the tank water quality.

Effects of water speed on lordosis & heart ventricle weight in cod

Increased exercise is one of several long term environmental parameters that are known to alter the fish cardiac physiology and anatomy (Gamperl and Farrell, 2004). In their review, the hypothesis is put forward that training-induced cardiac growth occurs predominantly in the compact myocardium, receiving oxygen-rich coronary arterial blood. The increased CSI found in the present study is likely to be an effect of increased exercise/swimming activity in the tanks with high water speed.

It is concluded that high water speed in the rearing tanks induces lordosis, reduced condition factor and increased cardio somatic index in Atlantic cod juveniles.

Therefore, swimming speed must be controlled through the control of water speed in tanks for juvenile Atlantic cod. Until further knowledge is available, water speed should be reduced as much as possible while allowing for the water speed that is required for obtaining the self-cleaning properties desired in the tanks.

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Salinity & temperature effects on seabream (Sparus aurata)

Amos Tandler & Bill Koven

Introduction

This section reports on the effects of salinity and temperature, during larval rearing, on the incidence of deformities in juvenile gilthead seabream (Sparus aurata). The experiments described were on specimens reared in the Red Sea (Israel).

It is well documented that providing the correct nutrition and environment improves growth and survival during the larval rearing of commercial teleost fish , factors which ultimately determine both production levels and quality.

However, environmental factors such as temperature and salinity can also have far reaching effects on the quality of juveniles produced, in terms of skeletal and cranial deformities. In fact, morpho-anatomical abnormalities can reach 90% of hatchery production, which can impact profoundly on the quality of marketed fish.

A study was made on gilthead seabream so as to determine the effect of larval rearing temperature protocols, at two different salinities (25 and 40 ‰), on the incidence of skeletal deformities that were screened at a later time in juvenile development.

Methods and Materials

The temperature protocols were employed throughout larval rearing and consisted of (1) constant 19 °C, (2) the NCM standard, which is an incremental increase from 19-24 °C ,and (3) constant 22 °C.

Each of the 6 larval rearing protocols (3 temperatures at 2 salinities) was replicated in 6 tanks.(Figure 1).

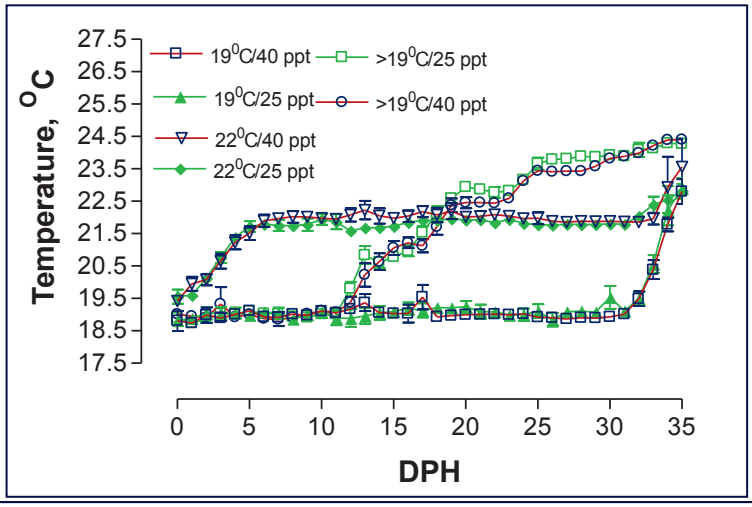

Figure 1: The experimental temperature/salinity (green always depicts the 25 ppt treatments) regime to which seabream larvae were exposed to measure its effect on deformities

Since rearing larvae at different temperature regimes would also mean different developmental rates, the end of larval rearing was determined at 1060 degree days.

At this stage samples were taken to determine swim bladder inflation, wet weight and length. The fish skeleton was not dense enough to be X-rayed so samples were stained with Alcian blue/Alizarin red in order to look at anomalies in bone and cartilage development. This procedure gives a very detailed and impressive result but, when fish are over 20 mm long, this approach demands much larger quantities of stain and fixing the tissues can take considerable periods of time.

After larval rearing, the fish were maintained in their separate treatments in the nursery but all were fed the same food until about 6 months after hatching. Once the fish were over 45 mm, they were X-rayed for skeletal deformities.

Results and discussion

The treatments had no significant effect on notochord length or biomass gain at the end of larval rearing (1060 degree days). However, larvae reared at the higher salinity (40 ‰) showed markedly poorer swim bladder inflation that their cohorts at 25 ‰ in all three temperature regimes (Figure 2).

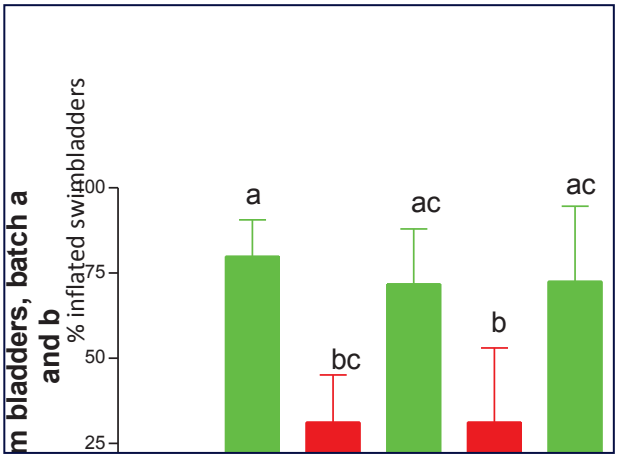

Figure 2: The effect of temperature (19, 22, NCM) and salinity (25, 40 ‰) rearing protocol on swim bladder inflation. Percent values having different letters were significantly (P<0.05) different (green always depicts the 25 ppt treatments).

There was also a tendency for better survival in the lower salinity treatments, which was significant in fish reared at 22 °C. In fact, juvenile fish from the 40 ‰ salinity treatments exhibited the highest incidence of skeletal malformation (mainly lordosis, kyphosis and vertebral compression) but no clear effect on cranial deformities when compared to fish reared at 25 ‰ at the end of the study (Figure 3).

Salinity & temperature effects on seabream (Sparus aurata)

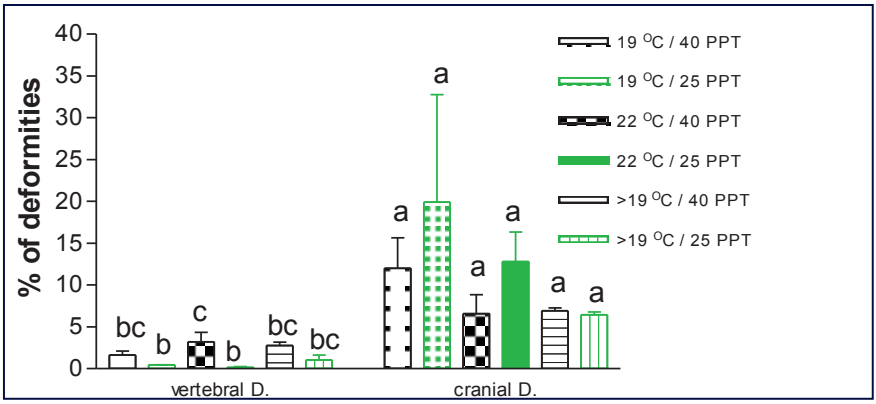

Figure 3: The combined effect of temperature/salinity regime (green always depicts the 25 ppt treatments) on the percentage of seabream having vertebral or cranial deformities

It is interesting to note that among fish lacking a swimbladder (Figure 4) the incidence of vertebral deformities was very high in all treatment combination except for the 22ºC/25 ppt. Furthermore, pug headedness was minimized at a salinity of 25 ppt, independently of the temperature regime.

Regime / Deformity	19-40	19-25	22-40	22-25	NCM-40	NCM-25
Vertebral	70.2	75.0	75	0	96.3	43.3
Pug head	27	0	27.6	5.1	23.3	0

Figure 4: Percent of deformity type found in fish lacking swim bladders

This difference was particularly conspicuous in the constant 22°C temperature rearing regime. Fish that are lacking a swim bladder must expend considerably more energy by excessive swimming in order to maintain their position in the water column.

This increases the stress load in the tail region which causes the vertebrae to respond by increasing bone volume, flattening dorsal zygapophyses and growing extra lateral ridges which decrease the strain from the faster tail beating but frequently results in haemal lordosis.

However, this study did not show a significant effect of larval rearing temperature on skeletal deformity. This observation is at odds with studies in other species, such as the European sea bass (*Dicentrarchus labrax*), which showed a very clear effect of early developmental temperature on the incidence of lordosis. Moreover, this deformity increased in severity if coupled with increasing critical swimming speed.

In fact, the overall incidence of skeletal deformity in fish from our study was conspicuously low (1.7%) when compared to gilthead sea bream culture in Europe.

This observation may be tied to broodstock selection from the warmer seawater of this region (Red Sea). Conceivably, this results in the progeny exhibiting lower levels of abnormality when challenged with higher temperature rearing conditions.

Taken together, this suggests that genetics is also an important factor, apart from environmental influences, in explaining varying levels of deformity among different geographical populations of gilthead sea bream.

Recommendations (for gilthead seabream)

1. **For better seabream larval survival, it is recommended to rear at lower salinities (tested salinity was 25 ppt)**
2. **In order to minimize vertebral deformities (1.7%), perform your larval rearing at a salinity of 25 ppt**
   The incidence of skeletal deformities was independent of the rearing temperature within the range of 19-25ºC
3. **For best rearing results, in terms of malformation and growth, rear at 22ºC and 25 ppt for the entire larval period of 35 DPH (770 degree days)**

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Water quality and skeletal deformities: What are the critical factors?

Grete Baeverfjord, Åsa Espmark, Kirsti Hjelde and Synnøve Helland

Introduction

This article covers skeletal deformities in Atlantic (A.) salmon, which is a complex issue, and it is clear that no single factor can explain all cases. Unfavourable rearing temperatures and inadequate mineral supply have been established as causal factors for skeletal deformities in salmon, and the symptoms and developmental mechanisms related to these factors are approaching a more complete understanding (see other chapters in this publication). But when these factors are ruled out, there are still a number of cases that remain unexplained. Among the topics discussed most often in the more obscure cases are issues related to water quality.

Water quality of intensive smolt production

Water quality of intensive smolt rearing is, like skeletal deformities, a complex topic. It has to be noted that, within the Norwegian industry, increasing numbers of smolts are being produced at fewer sites than before, and this trend is expected to continue. A similar trend is being observed for hatchery production in the Mediterranean—for seabass and seabream. Thus, available water resources at each specific site may become a limiting factor for production.

The preference of bigger smolt size at seawater transfer adds to this challenge, through increasing the standing biomass during critical periods prior to seawater transfer. The usual way of coping with limited water supply is to decrease the specific water flow (measured in litres kg^{-1} min^{-1}) to the tank, and add oxygen. The resulting water quality, typical for intensive smolt rearing in flow-through installations, is characterized by high levels of dissolved CO_2, low pH and fluctuating levels of O_2, including the risk of periodic or fluctuating O_2 supersaturation. Accumulation of organic compounds is no real issue in flow-through systems for salmon.

Despite extensive research efforts, few (if any) clear limits have been developed for the individual water quality parameters in A. salmon. Thus, the current regulatory guidelines given by the Norwegian Food Authority specify a set of water quality criteria that are based on a “best choice” of experiences and scientific results from a number of sources, and with no particular reference to skeletal deformities.

Nevertheless, anecdotal evidence from practical smolt production indicate that some aspect of the intensive water quality may be of relevance to vertebral pathology, in particular in cases where the water quality parameters were suboptimal and unstable.

CO_2, pH and O_2

Previous research on the relation between water quality and skeletal deformities has given some indications on the nature of these effects, but so far no reliable explanation. In Nofima Marin, this has been a research topic of high importance. Results from some experiments awaiting publication are summarised below (Baeverfjord et al, unpublished results):

In a series of two experiments, the water quality of intensive smolt rearing was modelled under experimental conditions. In these experiments, biomass was built gradually as the fish grew, under constant water supply but with increasing O_2 supplementation, similar to a production situation. The controls were kept in flow through without O_2 supplementation, at a much lower fish density. In the first of these experiments, water quality was well controlled and no adverse effects on skeletal development were identified even at relatively high levels of CO_2. In the second experiment, the rearing period was characterized by a series of events leading to O_2 and CO_2 fluctuations, periodic O_2 supersaturation, and eventually a crisis induced by severe hypoxia due to a pump breakdown.

In these fish, long term effects on skeletal development were identified, as more than 20% of fish from these groups developed severe platyspondylia when they were close to harvest size. However, due to the irregular nature of the events leading to these observations, no conclusion as to the nature of the specific causal factors was possible.

In another experiment, oxygen was supplemented at different levels through various stages of parr rearing and smoltification, with particular attention to the effect of O_2 supersaturation. In this experiment, platyspondylia pre-stages were observed in vertebra of post-smolts, following a four month rearing period in seawater. The effect was prominent in fish exposed to hyperoxic water during smoltification, compared to controls reared in normoxic water during the same period. However, as the experiment was terminated at a fish size of approximately 400g, the issue of further development of the condition remained unanswered.

Hyperoxia and fish density

The potential effect of hyperoxia on vertebral deformities was pursued as part of the FineFish project. An experiment was designed, addressing two experimental factors, each at two levels and in combination, in a 2x2 design.

The factors were fish density (high and low), expected to produce differences in tank water CO_2 and pH, and tank water oxygen content (normoxic and hyperoxic). The experimental period was through smoltification, that is, 6 weeks of photoperiod treatment initially, followed by 6 weeks of continuous light.

Water quality and skeletal deformities: What are the critical factors?

The high density groups were > 100 kg m3^{-1} towards seawater transfer, compared to less than half in the control groups. The tank O_2 level in hyperoxic groups varied between 100% and 130% saturation. The fish were individually tagged, and were X-rayed repeatedly. Following seawater transfer, the fish were placed in a common sea cage and reared to a size of 2,1 kg, at which time they were killed and X-rayed.

In this experiment, there were no differences in the prevalence of vertebral deformities at the end of the seawater-rearing period between treatment groups, neither in response to the fish density nor to the hyperoxia. The most notable effect in the experiment was a severe growth depression in the high density groups compared to the low density groups, prior to seawater transfer. This effect was not present at harvest, but should nonetheless serve as an indicator that Atlantic salmon may be particularly sensitive to high density rearing conditions.

In summary, these experiments support the hypothesis that there is a link between water quality conditions during freshwater rearing and skeletal deformities, but they also confirm that this relationship is less straightforward than, for instance, with rearing temperature. Thus, no single parameter or set of parameters could be defined, despite extensive research efforts. On the other hand, reports from commercial production indicate a reduction of skeletal deformities, as well as a general improvement of results, in response to efforts to improve water quality.

Recommendations

On a practical level, the following recommendations can be extracted:

- ☑ *Control biomass through controlling growth rates*
- ☑ *Avoid the higher levels of fish density (>50 kg m^3)*
- ☑ *Comply with the limitations provided by the Norwegian Food Authorities:*
  - ☑ *O_2 saturation in tank water <100%*
  - ☑ *Dissolved CO_2 in tank water < 15 mg l^{-1}*
- ☑ *Keep water quality controlled and stable*
- ☑ *Pay particular attention to O_2 saturation in the final weeks of smoltification*

The effects of minerals on juvenile Atlantic salmon

Grete Baeverfjord, Kirsti Hjelde, Synnøve Helland and Ståle Refstie

Introduction

Previous research has identified restricted dietary level of digestible phosphorus (P) as a causal factor for vertebral deformities in Atlantic salmon. In these experiments, effects on skeletal development were seen with dietary levels of P which were in line with or higher than publishes dietary requirements, indicating that requirement estimates in this species were due for revision. The most prominent effects from a long term subclinical P deficiency were a thick and distorted lower jaw, a break in the tail axis, and compressed and short vertebrae, in particular in the tail (Figure 1 a, b & c). These were, however, long term effects, which were expressed primarily in big fish (>1kg), even though some of the affected fish were exposed to the restricted P diets only in juvenile stages (0-20g).

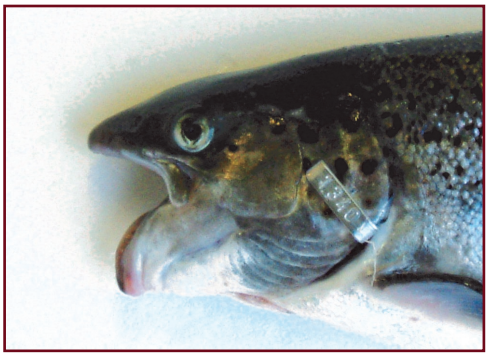

Figure 1a . Pathology of long term, subclinical P deficiency in A. salmon: a) Thick and distorted lower jaw ("screamer disease") in harvest size salmon,

In early juvenile stages, Atlantic salmon grows very fast. Under commercial conditions daily growth rates (SGR) is commonly 5-7% per day, combined with a low feed conversion ratio (FCR, kg feed fed/per kg gain in body weight). A FCR of 0.6 is not uncommon, and means that 600g feed is required to produce 1 kg of fish. Thus, the nutritional quality of the feed becomes a critical factor. Recent research has demonstrated that P bioavailability from common fishmeal sources may be low and unpredictable, and the introduction of vegetable meals in feeds for juvenile fish represents an additional challenge, as soybean meal and some of the other vegetable meals contain phytic acid which may impair mineral absorption. Thus, the P content of the feed per se is an inadequate indicator for dietary supply, and the most reliable reference is mineral content of fish.

Mineral content of salmon is commonly expressed as whole body content on a wet weight basis. Whole body analyses are the easiest to standardize, and for juvenile salmon there are reliable reference values to compare with (Shearer et al., 1994). A juvenile salmon with normal mineralization of skeletal structures should contain 4000 mg kg-1 or more of both Ca and P, and the level of Ca should be equal to or higher than the level of P. In fish from commercial production, low levels of P are frequently found, indicating that not all batches of commercial diets supply adequate amounts of P.

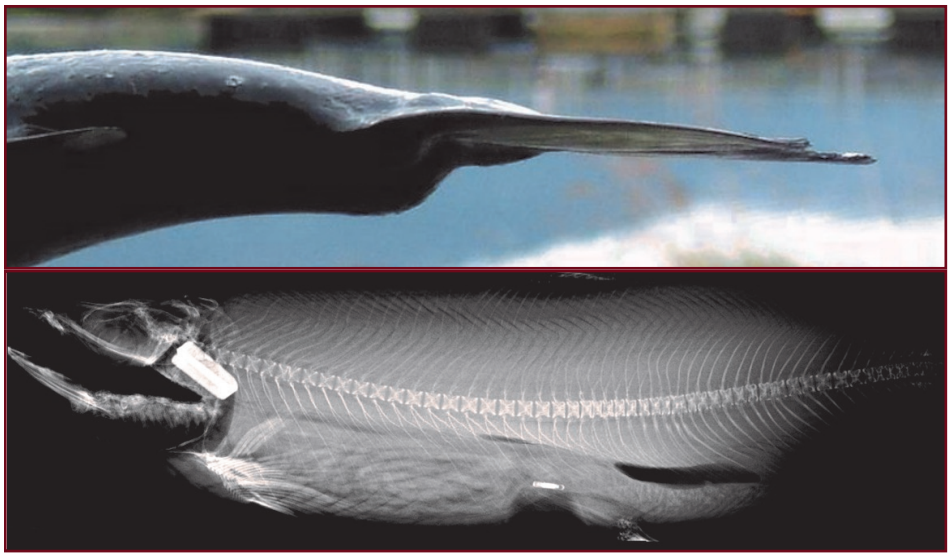

Figure 1b & 1 c . Pathology of long term, subclinical P deficiency in A. salmon: b) broken tail axis induced by low P in juvenile rearing, c) X-ray image of 4 kg salmon fed a diet deficient in P and Zn in freshwater rearing.

Low zinc (Zn) was identified as an additional risk factor for impaired skeletal mineralization. Analyses of commercially reared fish demonstrated that whole body levels for this element commonly were low, with values typically seen in the 30-40 mg kg-1 range compared to reference values of 50-60 mg kg-1, and with values below 30 mg kg-1 occurring sporadically. In previous experiments, the effects of low Zn levels were examined in combination with low P, and low Zn was found to aggravate the symptoms of P deficiency. In the FineFish project, an experiment was done addressing the supplementation of P, Zn and Mg in starter and juvenile diets more specifically. Mg was included as an experimental factor in addition to P and Zn, due to observed variable levels of this element on whole body analyses both in experimental groups and in commercially reared fish.

Material and methods

There were four experimental diets with differing dietary mineral contents, and each diet was fed to triplicate groups of fish. The diets were:

1. Control diet (1,6-1,7g P kg^{-1}, 190-200mg Zn kg^{-1}, 2,4g Mg kg^{-1})
2. Low P diet (1,3g P kg^{-1})
3. Low Zn diet (85mg Zn kg^{-1})
4. Low Mg diet (1,8-1,9g Mg kg^{-1})

The effects of minerals on juvenile Atlantic salmon

Each diet was formulated into a series of particle sizes suitable from first feeding up to fish size >30g.

Each group consisted of 400 first feeding fry. The fish were reared in circular fibre-glass tanks (Æ 0.5m) with flow-through water supply, and each tank was fitted with an overhead light source. The fish were reared under continuous light supply, and feeding was 24 hours per day, in excess. The fish were weighed and sampled at start (0,2g), 1g size, 5g size and 15g size. At termination, approaching 30g size, individual weights and lengths of 50 fish per tank were recorded.

Feeds and fish were analysed for mineral contents. At 5g and at termination, fish were sampled for radiography. The X-ray equipment used was a semi-digital system, using a mammography X-ray source, and reusable image plates for mammography with a final image resolution of 20 pixels/mm.

Duration of the feeding trial was 20 weeks. Following termination of the experimental treatments (30g size), a selection of fish from each tank was tagged, all fish were mixed in a pooled group, and subsequently reared through smoltification and transferred to a sea cage. Following 8 months seawater rearing, the fish were killed and examined again at approximately 2 kg size, to be able to describe any long term effects from different dietary treatments in early life.

Results

Mortality throughout first feeding and the rest of the experimental period was low, 5-10% per tank, and no differences were noted between dietary treatment. Overall growth rates were comparable to growth tables (Club N, Skretting AS). At the end of the experimental period (30g size), fish fed the low P and low Zn diets weighed significantly more than those fed the control and low Mg diet. The low Zn fish had significantly higher condition factors than fish on the other diets.

The mineral content of the fish showed relatively small differences in elemental composition of fish, except the low Zn fish, which were down to levels <20 mg kg^{-1}. The low P fish were low in P and Ca at 1g size, but not particularly so at later samplings. On radiographs, however, both the low P and the low Zn fish displayed significant changes from normal skeletal morphology. The low P groups had a significant number of fish with high density (HD) vertebrae (Helland et al., 2006). The low Zn fish had a high number of fish displaying a specific condition in which the larger part of the spinal column was affected. These spines were characterized by a lack of inter-vertebral space, and compression of the vertebral bodies. No specific effects were seen in the low Mg fish.

The long term effects recorded at 2 kg size were prominent; in fish with juvenile Zn deficiency, vertebrae were narrow and distorted. There were, however, few external signs of the impaired skeletal development in the same fish. Fish diagnosed with HD vertebrae at seawater transfer followed one of two developmental patterns; approximately half of the HD vertebrae were not identifiable at 2 kg size, whereas the other half had developed into fusions. There were no differences in weight between the dietary groups at the final sampling.

Discussion

The experiment was successful in distinguishing between the specific effects of a subclinical P deficiency and a corresponding Zn deficiency. Analyses of commercially reared fish demonstrate that these two elements may be low, either one or both at the same time. Thus it is helpful for future diagnostics to be able to identify early signs and to distinguish between the subclinical pathology related to these elements.

The high density vertebrae associated with subclinical P deficiency are comparable to the changes described by Helland et al (2006), who hypothesised that this pathology could be read as an early sign of subadequate mineralization. The level of P deficiency observed in this experiment was, however, very moderate, compared to the vertebral pathology induced in other experiments with low P diets, and the typical platyspondylia in big fish associated with juvenile P deficiency was not observed.

The Zn-deficiency induced compressed vertebrae lacking intervertebral space may be compared to the “short body dwarfism” described by Murai and Andrews (1978) in catfish, in response to riboflavin deficiency. The results from this study indicate that this type of pathology relate to Zn deficiency specifically also in Atlantic salmon, and may be used as a diagnostic indicator.

The results confirm the importance of proper mineralization of skeletal structures in the early juvenile stages for prevention of skeletal deformities.

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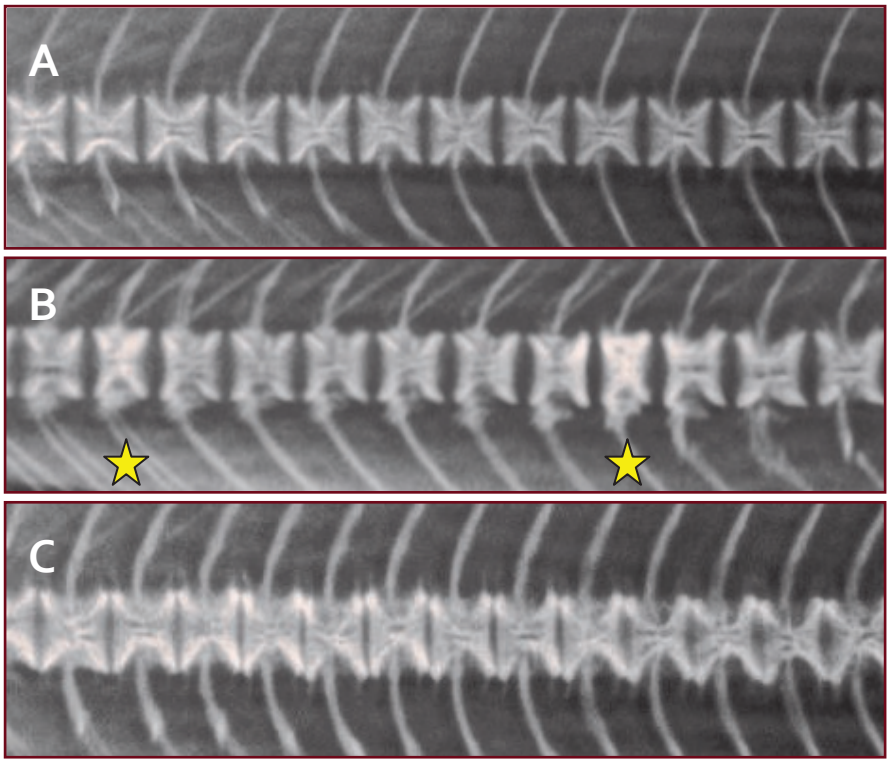

Figure 2. Detail of X-ray, A. salmon parr at 30 g size, following an experimental period with diets differing in dietary mineral content: a) normal vertebrae (control), b) high density (HD) vertebrae (*) in fish fed low P diet, c) compressed vertebrae in fish fed low Zn diet.

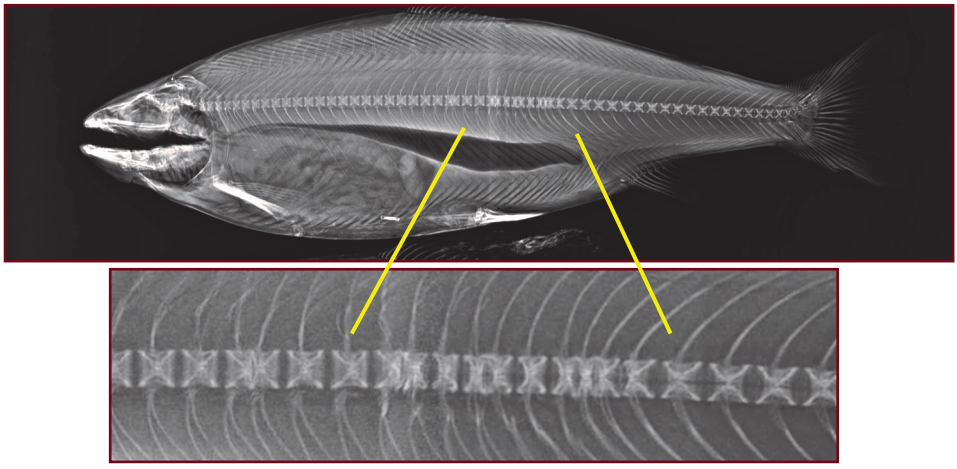

Figure 3. Zn-deficiency induced vertebral pathology in A. salmon. Exposure to deficient diet from first feed-

The impact of nutritional components on rainbow trout

Stephanie Fontagné

1. Introduction

Skeletal deformities have been recognized as a recurring problem in salmonids, especially in rainbow trout. A wide range of risk factors for development of skeletal abnormalities have been identified, including infections, toxins, genetics, environmental and nutritional factors. Inadequate feeding, especially during early development, has been reported to induce malformations in fish. Almost all nutrients can affect skeletal development of fish. However, three focus groups of nutritional factors have been identified for rainbow trout:

- Minerals, particularly phosphorus and calcium, as these key minerals are directly involved in the development and maintenance of the skeletal system and their availability in fish feeds can vary depending on the formulation with plant or marine ingredients.
- Vitamin A, as this vitamin plays a central role in many essential biological processes such as vision, immunity, growth and development. Vitamin A is also required for reproduction. However it can be teratogenic through its active metabolite, retinoic acid.
- Lipids, as significant amounts are present in skeletal tissues including collagen.

Polyunsaturated fatty acids which are characteristic of fish tissues are particularly susceptible to lipid peroxidation which is known to cause several pathologies including the development of malformations. Phospholipids also have been reported to influence skeletal development and mineralization.

2. Impact of dietary minerals on rainbow trout development

Two feeding trials were performed to assess:

1. the influence of dietary calcium and phosphorus on bone formation and mineralization in rainbow trout,
2. the influence of dietary phosphorus deficiency on bone formation and mineralization in rainbow trout fry and the impact on the later development,
3. the influence of triploidisation on the impact of dietary phosphorus deficiency in rainbow trout fry.

Experimental set-up

Feeding trial 1

Six semi-purified diets were tested on swim-up rainbow trout fry for 12 weeks at water temperature of 17°C. The basal diet A contained only phosphorus supplied by casein (0.5%) and other diets were supplemented with 0.4, 0.8, 1.2 and 1.6% of highly available phosphorus supplied as a mixture of sodium and potassium phosphates. These five diets were supplemented with 1% available calcium whereas another diet F, supplemented with 0.8% phosphorus (considered as the control level), was calcium-free.

Feeding trial 2

Half of the eggs from a common batch from 8 rainbow trout females and 6 males was triploidised by hyperbare pressure. The two groups, diploid and triploid rainbow trout fry, were then fed from first-feeding onwards the two semi-purified casein based diets C and A supplemented or not with 0.8% P. The feeding trial with the two experimental diets lasted 12 weeks and then the four groups of rainbow trout fry were fed a common commercial diet containing 1% P for another 12 -week period.

Results

Feeding trial 1

There was no effect of dietary phosphorus or calcium level on growth.

A lower survival was observed in rainbow trout fry fed the high phosphorus diet E (Figure 1) whereas a lower skeletal mineralization was seen in rainbow trout fry fed the diet A with a low phosphorus level (Figure 2). This group of fish also displayed higher incidence of abnormalities with irregular placement of vertebrae with twisted arches at later developmental stages. So, both dietary deficiency and excess of phosphorus are detrimental to rainbow trout development.

Concerning the impact of dietary calcium level, the results were less obvious. However, a lowered or delayed skeletal mineralization with also a higher incidence of skeletal abnormalities such as kyphosis or fused vertebrae was observed in fish fed the low calcium diet F.

The impact of nutritional components on rainbow trout

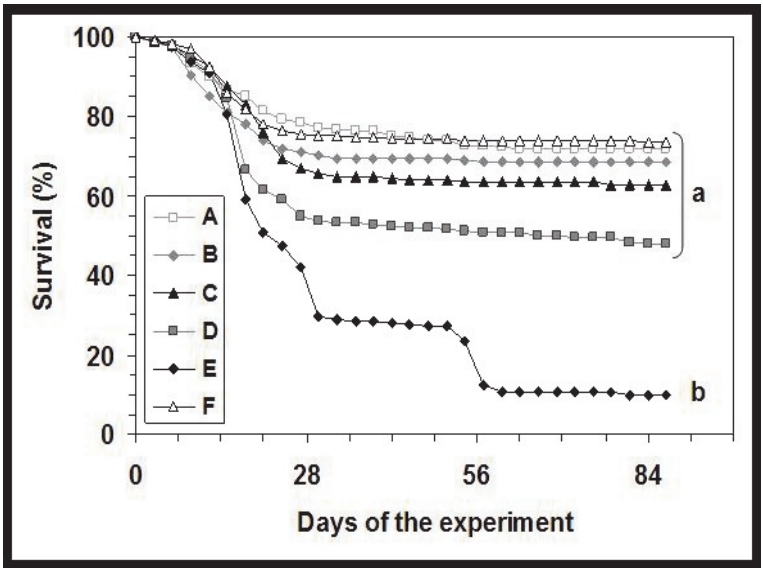

Figure 1. Survival of rainbow trout fry fed the six diets supplemented with different levels of phosphorus (0, 0.4, 0.8, 1.2 and 1.6%) and calcium (0 and 1%).

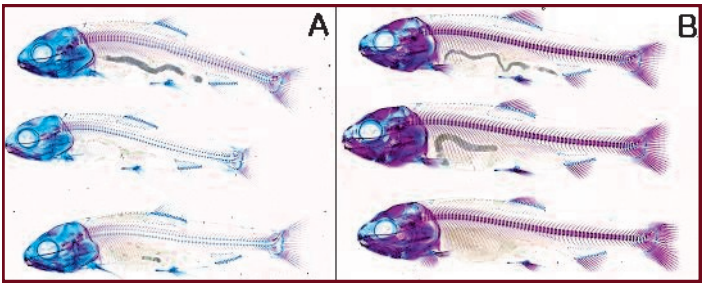

Figure 2. Rainbow trout fry sampled at day 28 of the experiment, fixed in 10% formalin and stained with Alcian blue for cartilage and Alizarin red for calcified bone and fed the P-deficient diet A (A) or the P-control diet F (B).

Feeding trial 2

No difference of survival and growth was noticed between the four groups. At the end of the first part of the trial, rainbow trout fry fed the phosphorus-deficient diet A displayed lower whole body phosphorus content (Figure 3) and were less ossified. There was no difference between diploid and triploid fish. At the end of the second part of the trial, the whole body phosphorus content of fish fed diet A was restored (Figure 3) and no external malformation was detected.

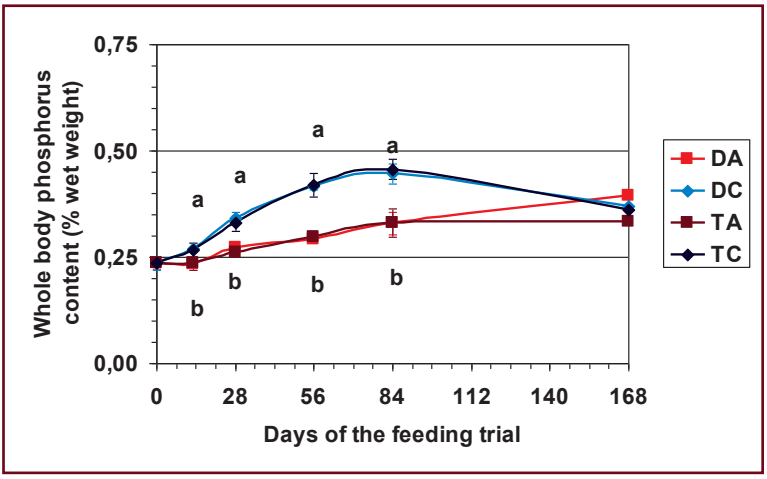

Figure 3. Whole body phosphorus content of the four groups of rainbow trout fry. DA: diploid fish fed the P-deficient diet A, DC: diploid fish fed the P-control diet C, TA: triploid fish fed the P-deficient diet A, TC: triploid fish fed the P-control diet C.

Conclusions

This study highlights the importance of dietary mineral supply for adequate skeletal mineralization. A special attention should be paid to the level and availability of phosphorus in the formulation of diets for normal vertebral development during early ontogeny of rainbow trout. The dietary available phosphorus requirement is estimated to be approximately 1% diet for adequate bone mineralization of rainbow trout fry.

3. Effect of feeding vitamin A to rainbow trout broodstock on skeletal development of the offspring

The experiment was set up to assess the influence of vitamin A level in broodstock diets on rainbow trout fry development. Vitamin A or retinol is essential for reproduction but high levels appear to be detrimental for larval development of many fish species.

Experimental set-up

Three groups of 15 rainbow trout broodstock females and 5 males each, individually tagged, were reared for 6 months before spawning at water temperature of 7° C.

The impact of nutritional components on rainbow trout

They were fed practical diets A1, A2 and A3 supplemented with different levels of vitamin A supplied as retinyl acetate: 0, 20 and 200 IU/g diet (1 IU = 0.3µg retinol), leading to a dietary level of 20, 40 and 200 IU/g diet. The first two levels correspond to the common levels found in commercial feeds for broodstock.

Results

The best fecundity, early growth and potential of muscle development were noticed in the group fed the highest level of vitamin A (Figure 4). Eggs from this group contained higher levels of retinyl palmitate, a storage form of vitamin A. However, no difference of retinoic acid level, the active metabolite of vitamin A involved in morphogenesis and early development, was recorded between eggs obtained from the 3 different groups. Likewise, no external malformation of fry originating from the 3 groups (from swim-up stage to the end of the 3-month feeding trial) was detected, only a small delay in mineralization processes was recorded in rainbow trout fry originating from the group fed the highest level of vitamin A.

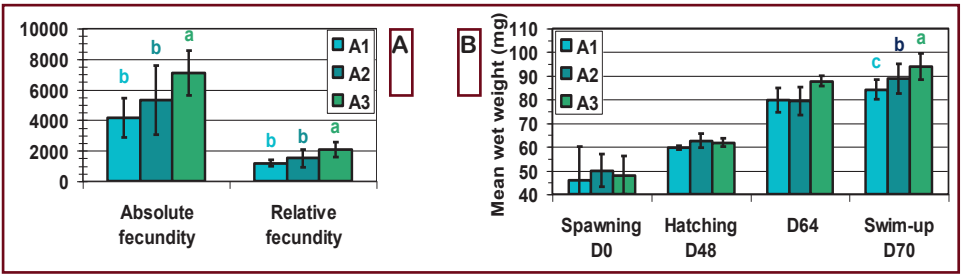

Figure 4. Fecundity of rainbow trout broodstock fed diets A1, A2 and A3 with different levels of vitamin A (A) and early growth of offspring from rainbow trout broodstock fed diets A1, A2 and A3 with different

Conclusion/Recommendations

For rainbow trout, quite high levels of vitamin A are recommended for broodstock nutrition (around 200 IU/g diet). The supplementation of 20 IU/g diet that is usually used in commercial diets for salmonids might not be enough to fulfill the vitamin A requirement of rainbow trout.

High dietary levels of vitamin A are beneficial for reproduction and early growth and no effect on skeletal development was noticed in comparison to other fish species. This difference might be due to the fact that the level of retinoic acid, the active metabolite of vitamin A, appears to be well controlled in eggs of rainbow trout.

4. Impact of dietary lipids on rainbow trout development

The experiment was set up to assess the impact of dietary lipids on rainbow trout development as previous studies in several fish species, especially in marine fish, have highlighted the influence of dietary lipids on the incidence and development of malformations. This study focused on the influence of the dietary phospholipid content and level of lipid peroxidation.

Experimental setup

Six semi-purified diets were tested on rainbow trout fry for 4 weeks at water temperature of 17°C at two different developmental stages: either from first-feeding onwards (trial 1) or 8 weeks after this stage (trial 2). Diets D1, D2, D3 and D4 contained 6% phospholipid supplied either as soybean lecithin or egg lecithin. Diets D5 and D6 were phospholipid-free and contained 6% soybean oil. Diets D2, D4 and D6 were supplemented with 12% oxidised fish oil whereas diets D1, D3 and D5 contained fresh fish oil.

Results

Dietary supplementation with oxidized lipid resulted in depressed growth in both early and late developmental stages of rainbow trout fry. In trial 1 with first-feeding fry, an improved growth was noticed in fish fed diets with phospholipids.

No external malformation was noticed. The skeletal development of fish was not affected by dietary oxidized lipid whereas a lower mineralization was noticed in rainbow trout fry fed diet D5 without phospholipid in trial 1 with swim-up fry.

Dietary control of antioxidant enzymes and vitamins was low in 4-week rainbow trout fry whereas increased activities of antioxidant enzymes and decreased vitamin E contents were noted in 12-week rainbow trout fry fed oxidized lipid compared to fish fed fresh lipid. This resulted in higher content of lipid peroxidation products in 4-week rainbow trout fed oxidized lipid compared to fish fed fresh lipid.

Conclusions

The results suggest that compared to late developmental stages, early stages are more susceptible to dietary oxidative stress, possibly due to lower response of endogenous antioxidant defense system.

This study has highlighted the importance of the control of lipid peroxidation in fish feeds for normal growth of rainbow trout fry. A correct supply of antioxidants (such as vitamin E or C) should be provided in fish feeds to protect polyunsaturated fatty acids from lipid peroxidation.

The results of this study have also highlighted the importance of dietary phospholipid supply for early growth and adequate skeletal mineralisation.

The impact of dietary minerals on rainbow trout development

Deborah Power

1. Introduction

The minerals calcium (Ca) and phosphorus (P) are important for the development of the skeletal system and maintain its stability as they are deposited in the bone. Fish can obtain Ca from the diet and bathing water but the only source of P is the diet. In adult fish an inadequate supply of dietary P leads to poor growth, reduced feed efficiency and skeletal deformities, poor bone mineralization, low ash and high lipid content in the whole body.

A number of mechanisms regulate the uptake and availability of Ca and P in order to ensure a constant supply of these minerals and maintain their balance in the body even when fluctuations in dietary supplies occur. The endocrine system, a series of glands which synthesize and release regulatory factors (hormones) has a key role in mineral balance. The hormones directly implicated in calcium balance in fish are stanniocalcin (STC), parathyroid hormone related protein (PTHrP) and calcitonin (CT). So far no endocrine factors have been directly linked to P balance in fish, although recently a new group of factors, the phosphotonins were identified in mammals.

The impact of modified dietary availability of Ca and P during early development when the skeleton and associated tissues are developing is unreported. It was hypothesized that the less developed endocrine system during early fish ontogeny may make them more vulnerable to fluctuations in dietary minerals. Particularly since this is a time when the rapid development of the skeleton and other crucial tissues means requirements for Ca and P are high.

2. Impact of dietary minerals on rainbow trout development

A feeding trial was performed to assess:

1. The consequence of early dietary restriction on the ontogeny of the skeleton in early stages, the characteristic of the skeleton and incidence of malformations in juvenile fish.
2. If a functional endocrine system exists in early developmental stages which may contribute to overcome dietary deficiencies in essential minerals.

Experimental set-up

Feeding trial

See the previous chapter (Impact of nutritional components: Stephanie Fontagné) for details of the feeding trial.

Sample analysis

To assess the status of cartilage and bone development, intact specimens were stained with alcian blue and alizarin red which stained cartilage blue and bone red, respectively. To assess the status of the skeleton in older fish which cannot be readily observed using staining methods they were subject to radiography and X-rays evaluated using an image analysis program.

The existence of a functional endocrine system was determined by immunohistochemistry. In this method a probe (specific antibody) is used to localize the hormone of interest and using stereology tissue activity is assessed.

Results

Ontogeny, characteristics and malformations of the skeleton

Reduction in the availability of Ca or P in the diet significantly delayed the formation of the endochondral skeleton (cartilage→bone) compared to trout fed diets with an adequate mineral content. Diets low in P had a more pronounced effect on the delay in skeletal formation than diets low in Ca.

To establish if a connection exists between the supply of Ca and P during skeletal development and the characteristics of the vertebral column, the vertebra were examined in juvenile trout (figure 1). Reduced dietary Ca led to significantly smaller vertebra but did not reduce the bone density. In contrast, reduced dietary P did not modify the size of vertebra but significantly reduced the density of the bone (figure 1). The incidence of malformations of the vertebral column leading to deviations in the body axis was greatest in trout maintained on a low Ca diet. Trout maintained on a low P diet had a high number of modified vertebra but they did not cause deviations in the body axis.

Identification of a functional endocrine system

Corpuscles of Stannius (CS) which produce stanniocalcin (ST) were identified in the dorsal part of the trunk kidney in all developmental stages of trout analyzed (swim-up onwards). The cells of the CS were rounded in shape, had a large nucleus and appeared in clusters of variable sizes and were active (figure 2). Trout fed a low P diet had significantly more and bigger CS than trout fed adequate diets, suggesting that this component of the endocrine system is already functional in early development.

The impact of dietary minerals on rainbow trout development

The ultimobranchial gland (UBG) which produces calcitonin (CT) was identified in the transverse septum, immediately beneath the circular musculature of the oesophagus in all developmental stages of trout analysed (figure 3). No clear link was observed between UBG activity and dietary mineral availability.

Conclusions

This study indicated that an inadequate supply of dietary Ca and P leads to a delay in skeletal development and a modification in the form and density of bones in juvenile stages. The modification in skeletal development caused by inadequate dietary minerals during development is associated with an increased incidence of skeletal malformations in juveniles. The endocrine glands, CS and UBG, the products of which regulate mineral uptake and balance are active early in trout development (swim-up onwards) and probably contribute to ameliorate the effects of reduced dietary Ca and P. Further studies will be required to establish the long-term impact of poor diets during development on performance of juvenile and adult stages.

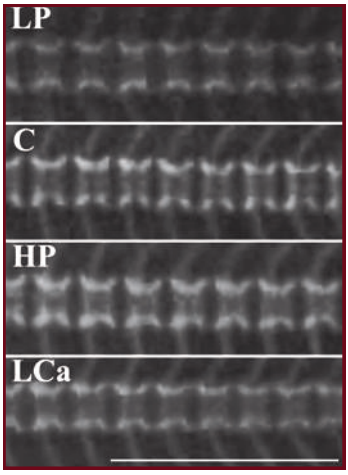

Figure 1. Soft X-ray of vertebra depicting the differences in morphology (form) and degree of calcification (intensity of white) of trout fed diets low in P (LP), low in Ca (LCa), high in P (HP) and adequate levels (C).

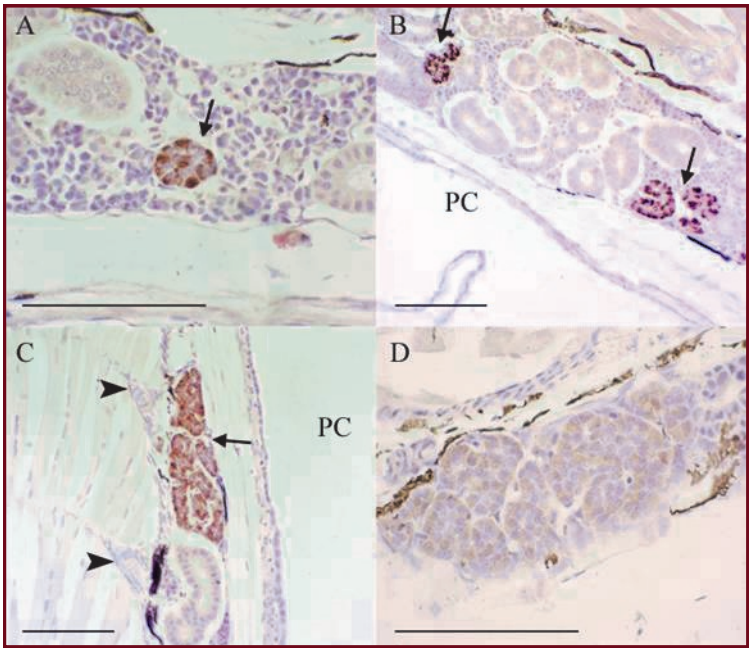

Figure 2. Corpuscles of Stannius corpuscles (arrows) in the trout kidney stained with stanniocalcin antisera. A- A single CS; B - The distribution of CS in the kidney; C –larger corpuscles are located in the anterior part of the kidney, between the pleural ribs (arrow heads) and the peritoneal cavity (PC); D – Very large, globular CS. Scale bar 100 μm.

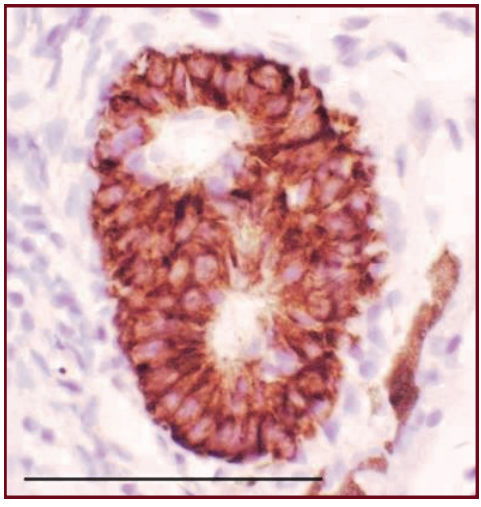

Figure 3. Ultimobranchial gland in a 79 dpf trout, stained with calcitonin antisera. Scale bar 100 μm.

The influence of nutrition at the larval stages in marine fish

José Luis Zambonino-Infante, Giorgos Koumoundouros, Amos Tandler

1. Introduction

Marine fish larvae are very immature at hatching and undergo major developmental changes during the larval period. The most visible changes concern not only the morphology of the larvae, which acquire progressively the aspects of the juvenile stage, but also other crucial changes that occur at the tissular and cell levels (maturation of the digestive functions, onset of the immune system, settlement of metabolic pathways..). A description of most of these tissular and metabolic changes is reported in Zambonino-Infante et al. (2008).

Several parameters could influence these developmental processes and therefore could negatively affect the quality of the larvae with, in particular, the appearance of malformations. Fish deformities have the more detrimental effect on the consumers' image of aquaculture and, therefore, also on the market value of the juvenile fish.

Larval deformities are mostly induced at the hatchery stagge by several environmental factors (abiotic factors), diseases and some dietary components.

In the present work, we chose to investigate the potential influence of some dietary vitamins in the appearance of malformations. We evaluated the impact of the dose of the vitamin mix recommended by NRC93, and we more particularly studied three vitamins: Vitamin A (retinol acetate), vitamin D (1,25 dihydroxycalciferol), and vitamin C (ascobyl polyphosphate).

Two marine fish species were considered in the present work: European sea bass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*). The recent development of an appropriate microparticulated compound diet (Cahu et al. 2003) allowed for more precise investigations of the influence of nutrients on fish larval morphogenesis than studies based on the use of live prey. Such inert feed was used in sea bass larvae experiments from mouth opening, with a total replacement of live prey, which resulted with good survival and growth. Seabream experiments were mainly based on the classical live prey feeding sequence (rotifers and Artemia). In order to better characterise malformations, sea bass and seabream juveniles were kept until they reached 2 g weight or 3 months age respectively..

2. Why dietary vitamins?

Marine fish larvae feeding sequence contained significant amounts of lipophilic vitamins like vitamin A and D, and hydrophilic vitamins like vitamin C. Besides their strict nutritional role, these vitamins can have physiological effects.

- Vitamin A is involved in night vision and is an antioxidant. It is associated with cell differentiation, and controls the expression of many genes involved in morphogenesis (it can be teratogenic)
- Vitamin D is an hormone that maintains calcium homeostasis and directly acts on bone cells.
- Vitamin C is hydrophilic, and is largely used in marine fish feeding sequence. It is an antioxidant, essential for collagen synthesis, and participates in several metabolic processes.

In consequence, we have considered essential to assess the impact of the dietary dose of these vitamin during the developmental process of marine fish larvae.

3. Effect of the dietary vitamin mix on larval morphogenesis

The vitamin mix used in larvae diets is 8 times more concentrated than the vitamin mix 762 (NRC 93 recommendations) established for juveniles. Is it appropriate?

Experimental set-up

Six replicated groups of sea bass larvae were reared at 20°C and fed, from day 6 until day 38 post-hatching, micro-particle diets incorporating 0.5%, 1.5%, 2.5%, 4 % (the standard level), 5% and 8% of the 2 times concentrated vitamin mix.

Results

The NRC standard vitamin mix incorporated into larvae feeds at 8 times the content recommended for juveniles gave the best results in terms of growth, survival, and also morphogenesis (Figure 1).

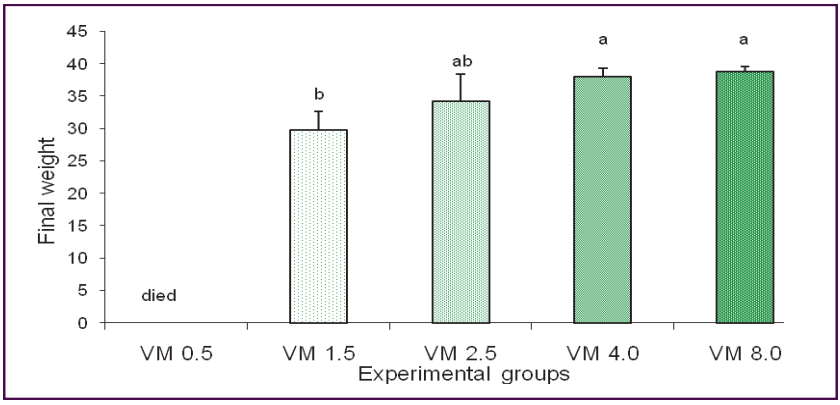

Figure 1 : Final weights of 38 days-old seabass larvae fed the different experimental diets containing graded levels of vitamin mix.

Conclusions

Our results showed that low dietary vitamin levels disrupted a temporal sequence of co-ordinated growth factor expression, involving different genes controlling the differentiation of osteoblasts ; part of the osteoblast is then converted into adipocytes and this led to the appearance of deformities. The results have been published (Mazurais et al. 2008).

The influence of nutrition at the larval stages in marine fish

Recommendations

The standard vitamin mix level induced a significant percentage of head and column deformities (Figure 2) showing the need to further refine the proportions of certain vitamins (particularly those known to be involved in bone and collagen synthesis, i.e. Vitamin A, D and C).

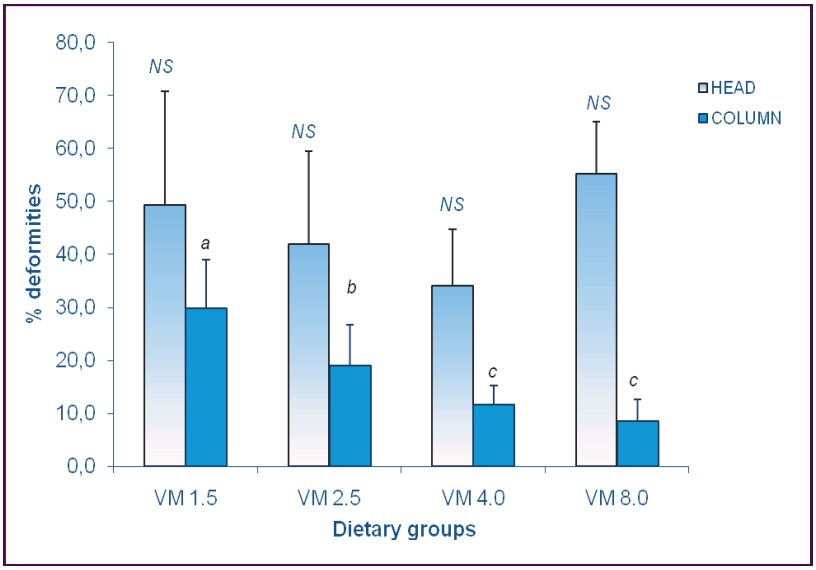

Figure 2 : Percentage of head and column deformities in 38 days-old sea bass larvae fed the different experimental diets containing graded levels of vitamin mix.

Avoid the incorporation of high HUFA levels in larvae diets (i.e. > 2g EPA+DHA/100g DW) during early stages to prevent the conversion of osteoblasts into adipocytes.

4. Effect of the dietary vitamin A on larval morphogenesis

Sea bass larvae:

Experimental set-up

Seven replicated groups of sea bass larvae were reared at 20°C and fed, from day 6 until day 42 post-hatching, microparticulated diets incorporating 0, 5, 10, 15, 25, 35 and 70 mg retinol acetate (RET)/kg of the diet and corresponding to 0, 16600, 33200, 50000, 83300, 116600, 233300 IU/ kg diet.

Results

The best growth and survival were observed in the larvae groups fed RET 10 and RET 15 diets, the other groups exhibiting a significant lowering in growth and survival. An analysis of malformations indicates that the optimum levels of dietary retinol for reduced deformities incidence depend on the malformation type considered; the structures that develop earlier are less affected by the low vitamin A levels. The best compromise in retinol acetate (vit A) level has to be comprised between 5 and 15 mg/ kg diet, which is lower to the recommended level of vitamin A.

The better results in ossification were also observed for vit A comprised between 5 and 15 mg/ kg diet; this dietary vit A levels also represented the better compromise for reduced deformities incidence. Our gene results strongly suggested that Vitamin A may regulate ossification/mineralisation processes and probably influences patterning of different cell types. These data have been published (Mazurais et al. 2009).

Seabream larvae:

Experimental set-up

3 groups of seabream larvae were fed with a classical live prey sequence, i.e. rotifers from day 4-19 and Artemia from day 20-34. Live preys were enriched or not with different levels of retinol. Group 1 received enriched rotifers, group 2 received enriched Artemia, group 3 received enriched rotifers and Artemia.

Results

High dietary vitamin A levels mainly affected the growth of seabream larvae at the earlier age, up to day 19, and induce a high percentage (50%) of cranial deformities (Figure 3).

At a later age, high dosage of vitamin A dramatically induced a high percentage of vertebral deformities (Figure 4).

Conclusions/recommendations:

Similar effects of the dietary levels of vitamin A were observed in sea bass and sea bream larvae. High dietary vitamin A levels strongly affect early larval development (before day 20); the effect is less marked after this stage. The dietary vitamin A level should be then changed during larval development in order to minimize the appearance of deformities: The optimal dose for the early developmental stage should be ~ 15 µg/g DW until day 20 post-hatching. A higher dose ~ 45 µg/g DW could be used afterwards.

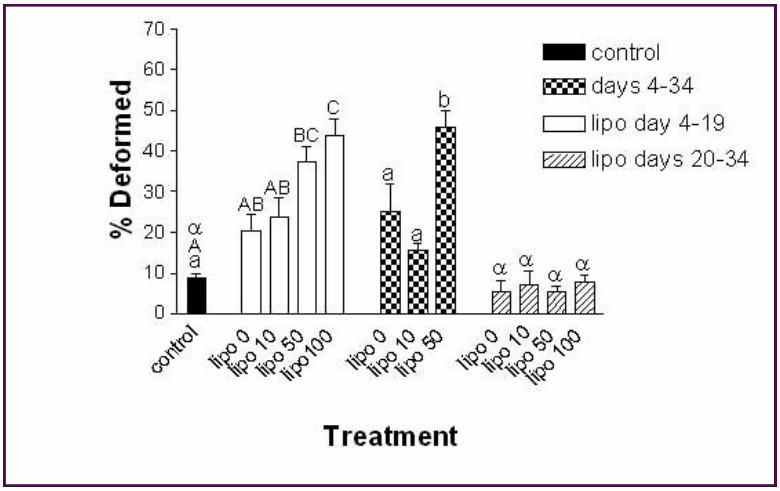

Figure 3 : Percentage of cranial deformations in 120 days-old seabream fed the different experimental feeding sequences during the larval period.

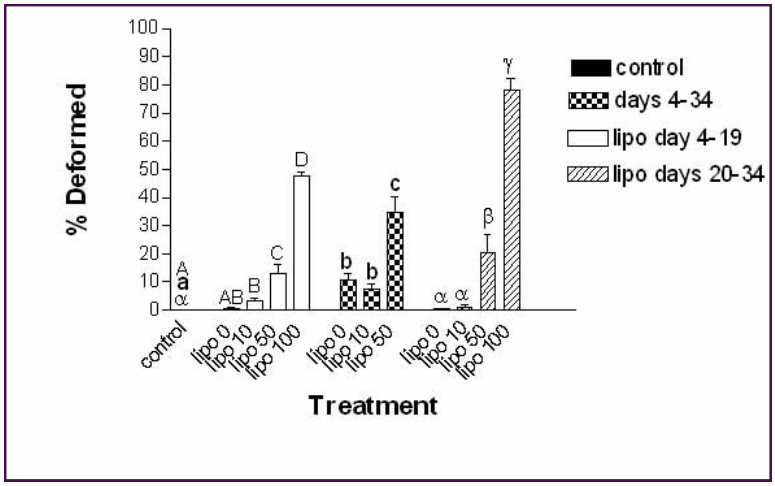

Figure 4 : Percentage of vertebral deformations in 120 days-old seabream fed the different experimental feeding sequences during the larval period.

5. Effects of the dietary vitamin D and C on larval morphogenesis

Experimental set-up

Four doses of dietary vitamin D: 11.2 (VD-0), 27.60 (VD-1), 42 (VD-2) and 120 (VD-3) IU VD_3 per gram of diet, and six doses of vitamin C: 0, 5, 15, 30, 50, 400 mg vit C/Kg diet were tested from day 6 until day 45 post-hatching.

Results

The lower dietary dose of vitamin D induced a poor mineralization, with many types of deformities (pugheadness, vertebral and caudal deformities). A disruption of intestinal calcium absorption was noted (lower expression of TRPV-6 gene). The 2 highest vitamin D doses induced a delay in the larvae mineralization process and the appearance of vertebral deformities (Figure 5).

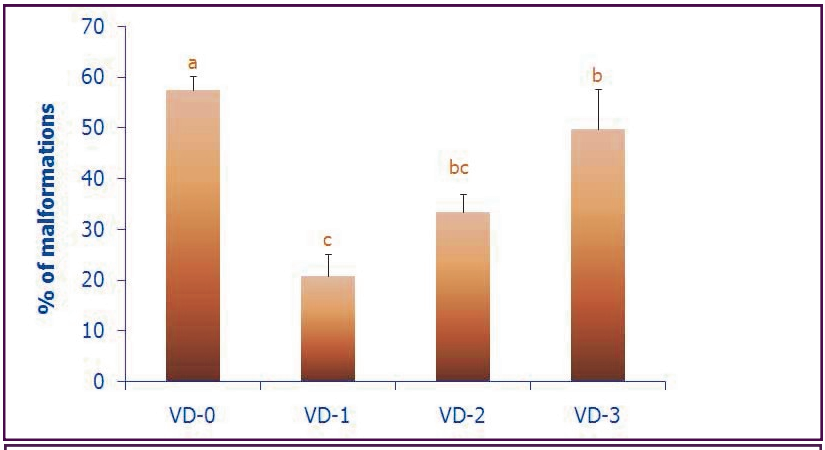

Figure 5: Percentage of deformities in 45 days-old seabass larvae the different experimental diets containing graded levels of vitamin D.

Larvae fed VIC0 and VIC5 did not survive after day 30 post-hatching. Dietary doses in vitamin C lower than 30mg/kg diet were associated with a poor mineralization in larvae, deformities in the head (pugheadness) and vertebral (one vertebra loss) areas. The highest dose of vitamin C also induced a poor mineralization and deformities in the head and vertebral (supernumerary vertebra) areas. The abnormalities observed with the low and high vitamin C levels were associated with a disruption of intestinal vitamin C transport (lower expression of SVCT-1 gene) and with a modulation of genes involved in lipid metabolism.

The influence of nutrition at the larval stages in marine fish

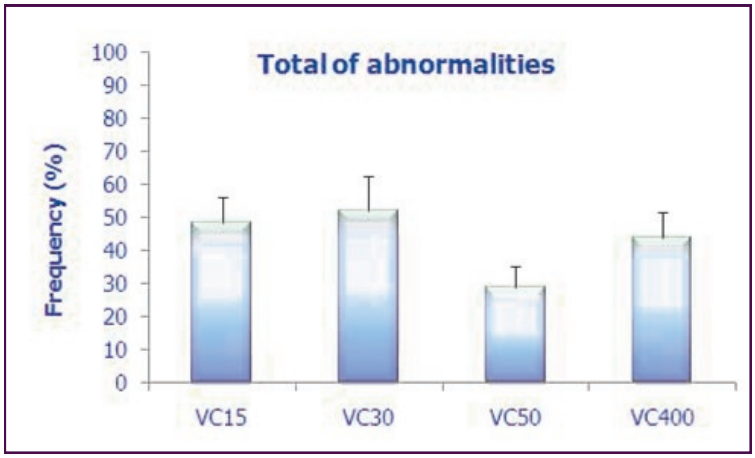

Figure 6: Percentage of deformities in 45 days-old seabass larvae the different experimental diets containing graded levels of vitamin C.

Conclusions

Low vitamin D levels in feeds could represent a risk for larval development; however this risk is unlikely considering the amount of vitamin D present in the live prey enrichments or in larval feed incorporating marine products.

The level of vitamin C in the feeds has to be accurately defined concurrently to the dietary lipids, in order to prevent a poor mineralization linked to a disruption of bone formation induced by the metabolism of lipids.

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The effect of PC/PI ratio on malformations in Gilthead seabream

Amos Tandler & Bill Koven

Introduction

Malformation in commercially raised fish, such as cranial, skeletal and gill cover deformities is a major factor reducing their market value (Koumoundourous et al., 1997). Although these deformities are most apparent in the juvenile and adult stages they may originate from suboptimal nutrition during the critical larval rearing stage. Previous research has hypothesised that dietary phosphatidylinostol (PI) was more effective in reducing deformities than the main membrane phospholipid phosphatidylcholine (PC) (Geurden et al., 1997, 1998). Consequently, the aim of this study was to test the effect of different dietary ratios of PC and PI fed to the gilthead sea bream (Sparus aurata) larvae, on developmental performances in juvenile fish in terms of survival, growth and malformation rate.

Materials and Methods

Four microdiet (MD) treatments, that differed in their PC/PI ratio and replaced 75% of the normal Artemia ration (wt/wt), were fed to 20-34dph (days post hatching) sea bream larvae. In addition to the high PC/PI or low PI containing MD control, a commercial reference treatment (100% Artemia ration) was given. The dietary PC/PI ratio in the experimental treatments is outlined in Table 1. *The composition of the microdiets is given at the end of this section.*

Treatment	A	B	C	D	Art
(g/100 g dry diet)	25% Artemia +75% MD[1]	25% Artemia +75% MD[1]	25% Artemia +75% MD[1]	25% Artemia +75% MD[1]	100% Artemia
Phospholipids					
Phosphatidylcholine (PC)	5.7	4.54	4.42	3.88	4.42
Phosphatidylinositol (PI)	1.86	1.95	2.76	3.04	1.78
PC/PI in total diet	**3.07**	**2.32**	**1.6**	**1.28**	**2.48**

Table 1: The dietary PC/PI ratio in the experimental treatments

At 40 dph, the fish were graded in all treatments into small (<1.3mg dw larva^{-1}) and large (>2.9mg dw larva^{-1}) larvae, in order to test if growth rate influenced treatment effect throughout development to 141 dph.

The experiments were carried out in four replicates and results analyzed by a one-way ANOVA followed by the Tukey-Kramer Honestly Significant Difference (HSD) multiple range test ($P<0.05$).

Results

There was no marked (P>0.05) treatment effect on growth rate in 40 dph larvae although larvae fed the MDs were significantly (P<0.05) smaller then the commercial reference treatment (Art) larvae.

On the other hand, in later juvenile development (67 dph), decreasing dietary PC/PI ratio contributed to a significantly (P<0.05) better growth and non-significant (P>0.05) higher survival (Figure 1).

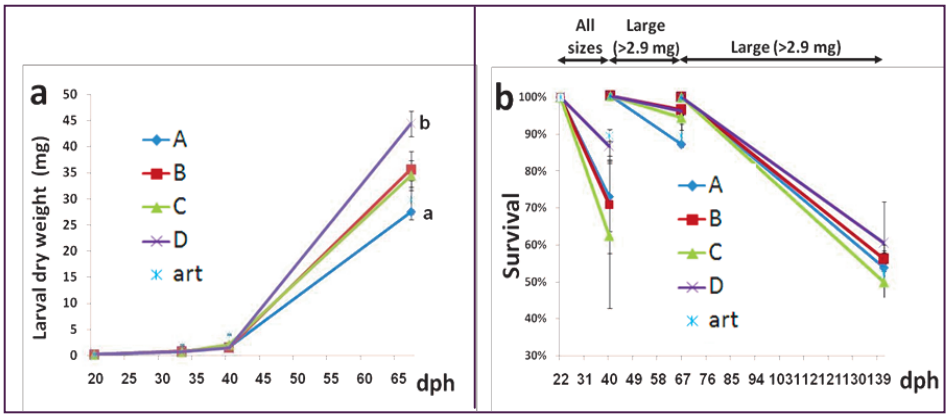

Figure 1: The effect of dietary PC/PI ratio on large (>2.9mg dw larva^{-1}) larvae (a) dry weight (20-67dph) and (b) survival (22-141dph).

Moreover, reducing dietary PI markedly (P<0.05) increased jaw (cranial) deformity in both size groups at 67dph, which may have adversely affected their feeding once weaned completely on to a dry hard starter feed. This is suggested since fish fed the high PC/PI ratio (low PI) diet demonstrated poorer growth at 67dph.

Conversely, increasing dietary PI (reducing PC/PI ratio) showed a non-significant trend of increased skeletal deformity which was markedly (P<0.05) higher in faster growing larvae in all MD treatments (Fig. 2). Interestingly, both Cranial and skeletal malformations increased throughout development (67-141dph) in most of the treatments ,suggesting that they were not deleterious.

A possible explanation for the jaw-skeletal contradiction is by the Osteocalcin (BGP) mRNA levels. High level of PI contributed to higher BGP levels, which reduced significantly (P<0.05) the jaw deformity levels while simultaneously elevated the skeletal deformities due to over mineralization.

Although there was no clear affect of PC/PI ratio on gill cover deformity rate, there was a size dependent susceptibility to this deformity where smaller larvae showed the highest incidence of this malformation.

The effect of PC/PI ratio on malformations in Gilthead seabream

Moreover, throughout development (67-141dph), gill cover deformity generally decreased, suggesting the possibility of operculum regeneration or that the exposure of the gills was deleterious with age.

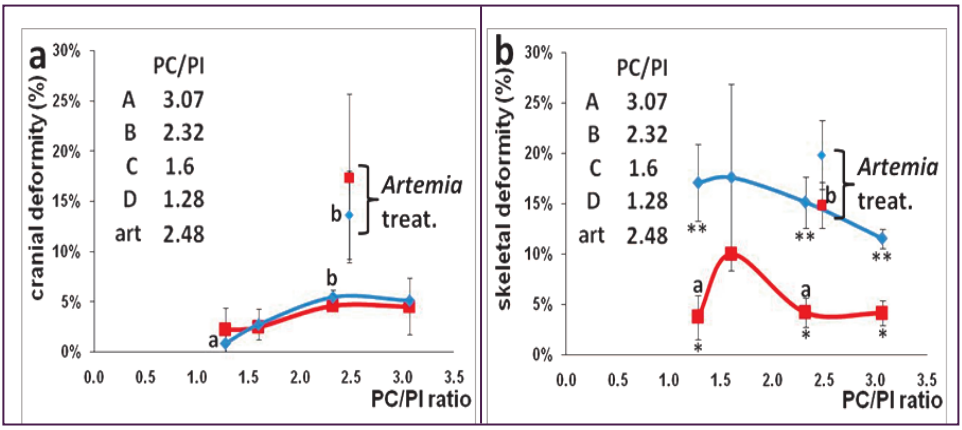

Figure 2: The effect of dietary PC/PI ratio on (a) cranial and (b) skeletal deformities level (%) in small (<1.3mg dw larva^{-1}) and large (>2.9mg dw larva^{-1}) juveniles at 67 dph.

This work has demonstrated an effective dietary PC/PI ratio of 1.28 for the sea bream larvae, effecting positively on jaw (cranial) deformity rate, growth and survival in juvenile fish.

Conclusions

Decreasing the dietary PC/PI ratio (5% total phospholipid) during larval rearing significantly increased juvenile growth. This may have resulted from a reduced incidence of jaw deformity which leads to more effective pellet feeding. More-over, the decreased rate of cranial deformity may be due to PI enhancing osteocalcin synthesis and normal jaw development. The results conclude that a PC/PI ratio of 1.28 or a PI level of 3.04g.100g^{-1} DW diet gave the best larval and fry performance.

Recommendations (seabream)

1. **For best larval growth performance, when microdiets are used, never exceed the ratio of 5 between PC/PI in the phospholipid fraction.**
2. **The best PC/PI ratio is 1.28 and a PI dietary level of 3.04g 100g^{-1} diet (DW)**

Composition of microdiets

Microdiets	A	B	C	D
Formulation (g/kg^{-1}) Basal diet [2]	950	950	950	950
De-oiled Soybean Lecithin [3]	0	20	40	50
Phospholipon 80 H [4]	50	30	10	0

[1] Produced at KARMAT Coating Industries Ltd., Israel.

[2] Per kg diet: 559.5 g fish meal; 44 g gelatin; 6 g acacia; 63 g fish oil (Matmor Feeds, Israel); 27 g EPAX 1050 (EPAX AS, Aalesund, Norway); Coating amino acids: 20 g glycine ; 20 g arginine; 20 g alanine and 20 g betaine (Sigma, St. Louis, USA); 0.5 g vitamin C (Stay C, Hoffman LaRoche, Switzerland); 40 g mono calcium phosphate (Fluka, Buchs, Switzerland); 10 g vitamin and mineral premix; 10 g choline chloride (Sigma, St. Louis, USA); Supplemented amino acids: 10 g valine; 10 g isoleocine; 10g tryptophan; 20 g phenylalanine(Sigma, St. Louis, USA).

[3] Deoiled soybean lecithin (Enzymotec, Migdal HaEmeq, Israel): 70.7% total PL; 23.9% phosphatidylcholine; 20.2% phosphatidylinositol; 14.5% phosphatidylethanolamine; 6.2% phosphatidic acid; 5.2%

[4] Phospholipon 80 H (Phospholipid GmbH, Köln, Germany): hydrogenated PC; 60%, Lyso PC: 10%.

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Protocol for sampling live feeds for chemical analysis

Ingrid Lein and Synnøve Helland

1. Introduction

One of the most important bottlenecks in the larval and juvenile production of marine fish species is assurance of the nutritional quality of the live feed. It is evident that there are species and life stage specific nutritional requirements of marine fish larvae and juveniles. The nutritional requirements of marine fish larvae has induced an extensive international research effort to obtain nutritionally adequate standards for the live prey organisms most commonly used in commercial hatcheries; these are rotifers (Brachionus sp.) and Artemia sp. (the most commonly used is Artemia franciscana) (Figure 1). Even small differences in the nutritional quality of the live feed organisms can have a large impact on the quality and robustness or the fish at later stages.

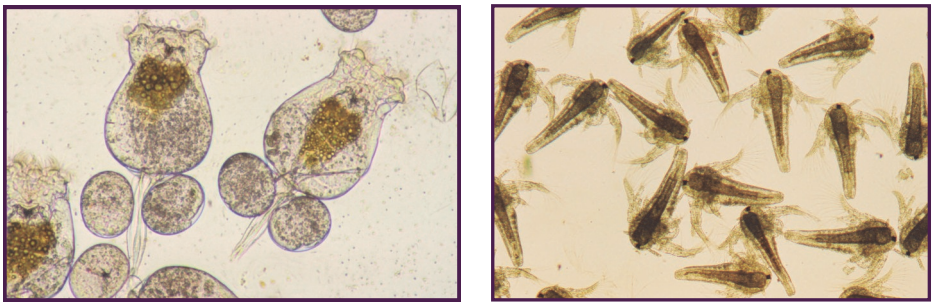

Figure 1. The most commonly used life feed organisms; the rotifer (Brachionus sp., left image) and the Artemia sp (right image). Photos: Nofima Marin AS

There are several methods employed for producing rotifers such as batch, semi-continuous, and continuous cultivation. Also, the scale of production varies from small indoor tanks to large outdoor tanks.

Artemia are bought as dried cysts that must be hydrated and hatched, and the cyst shells are often chemically removed before hatching, a process that also reduces the bacterial load. Most often Artemia are fed as newly hatched nauplii or they are enriched for about 20 to 24 hours.

High quality live feed production requires strict control of the nutritional quality of the enrichment mediums, the microbial environment and of the physical parameters like oxygen, temperature, salinity, pH, and of the turbulence created during mixing.

This protocol is a general method for sampling and transport of samples of live feed organisms for chemical analysis. For specific analyses there may be other protocols and, therefore, the protocol must be discussed with the laboratory that will analyse the samples—before sampling and shipping of samples— whether it is a commercial laboratory or a collaborating research and development partner.

2. Sample size and number of samples:

Before starting the sampling of the different live feeds, the required amount of rotifers or Artemia per analysis must be defined.

In order to reflect variations in different production batches, it is a good idea to take samples over several days. These may be analysed as one pooled sample or as separate samples, which mainly will be a question of cost. The same tube might also contain live feed samples from several days sampling.

Most relevant parameters to analyse:

- Dry matter
- Energy
- Total lipids and fatty acids
- Lipid classes
- Total protein
- Vitamins
- Minerals and trace elements

Sampling:

- Harvest the decided amount (wet weight in grams) of enriched rotifers or Artemia on a sieve. Rinse well with fresh water (temperature ~20-30°C) in order to remove all enrichment products on the surface of the live feed. *This is very important, because the results will be completely masked if some enrichment medium remains on the surface of the live feed .*
- Dry the underside of the sieve with a dry paper cloth to remove as much water as possible.
- Carefully transfer the sampled live feed to a vial with a small clean spoon or spatula.
- Label the samples with a PENCIL.— Ballpoint or markers may be unreadable if a sample leaks.
- Freeze the samples. Store them at least as cold as –20°C, preferably as cold as -80 for lipid analysis.
- Do not fill the vials completely, they will burst when freezing!

Protocol for sampling live feeds for chemical analysis

3. Transport/Shipping:

- Put samples in ice packs (ice elements) in at least –20°C.
- Just before sending: wrap the frozen vials with insulating plastic foil around the ice elements. You can use tape to stick the plastic foil to itself (do not freeze the plastic foil)
- Put everything in a Styrofoam box
- Leave the Styrofoam box in the refrigerator until the transport company picks it up.
- Make sure that the transport box has been properly labelled with the name and address of the receiver
- Include an overview of the samples together with the shipment and also send this by e-mail when you notify the receiver about the shipment
- It is very important to notify the receiver before sending the samples!

Labelling:

Include on all labeling, the following information

- Company name
- Name of contact person
- Address
- Appropriate telephone number
- “Biological material – for science only”

Mineral content in salmonids—sampling & analysis

Grete Baeverfjord, Synnøve Helland & Torbjørn Åsgård

1. Introduction

The role of minerals in skeletal pathology was recently reviewed by Lall and Lewis-McCrea (2007). In the review, the impact of dietary calcium (Ca) and phosphorus (P) was addressed. The importance of these two elements for normal skeletal development, especially in salmonids, is further supported by activities in the FineFish project. In addition to Ca and P, zinc (Zn) should be given attention. This element has shown variable and low values in whole body analyses done on farmed salmonids within the last years, and have previously been associated with skeletal malformations in salmonids.

Monitoring of mineral status

The challenges associated with establishing mineral requirements in fish were reviewed by Lall (2002). Test methods that monitor specific biological functions are sparse in fish, and future development of methods that allow for more refined evaluation of mineral homeostasis is anticipated. Some studies related to expression of genes involved in P uptake have been presented (Sugiura et al., 2007), and this field is expected to expand rapidly.

In previous studies on mineral requirements, the recommendations were based on a range of different criteria (Rodehutscord, M., 1996, Vielma and Lall, 1998, Maage et al., 2001). In view of recent results that identify the link between suboptimal mineralization of bone and deformities, the response variables used to monitor mineral status should include some measure of mineral contents of bone.

The most relevant and reliable measure for mineral content of fish appears to be whole body mineral content (Shearer, 1984). The methodology related to sampling and analysis contains few sources of error, and gives data that can be compared across sampling points and fish sizes. For Atlantic salmon and rainbow trout, relatively complete sets of reference values for different stages of the life cycle exist (Shearer, 1984, Shearer et al., 1994).

In summary, based on the literature, whole body elemental concentration is suggested as the standard response parameter in Atlantic salmon and rainbow trout. There is a strong correlation between mineral content in bone and whole body levels (Baeverfjord et al., 1998), there are absolute reference values to compare with, and the methods for sampling and analysis are relatively foolproof. The value given should be mineral content in wet weight, as it was demonstrated by Shearer (1984) that values given on a dry weight basis is too much influenced by variation in lipid content. The main weakness is that the fat and muscle content of the fish will have some influence on the result, but in commercially farmed salmonids, this variation is not expected to be very high.

The most important limitation is fish size, as the method is best suited for salmon in freshwater stages (< 500g). The experience is, however, that the juvenile stages are the most vulnerable, so the method is well suited for the most relevant fish sizes.

Reference values

Reference values for whole body content of elements in various life stages of Atlantic salmon and rainbow trout were given by Shearer et al (1994) and Shearer (1984). With present knowledge, the most relevant parameters are whole body P, Ca and Zn.

For practical evaluation of Atlantic salmon, the following values can be used as a rule of thumb:

- P ≥ 4000 mg kg^{-1}
- Ca ≥ 4000 mg kg^{-1}
- Ca ≥ P, i.e. Ca:P ratio ≥ 1
- Zn: 40-60 mg kg^{-1}

In commercially farmed fish, values tend to be somewhat lower. For P, low mineralization should be considered as a risk factor for skeletal deformities when whole body levels are reduced by 10% or more, i.e when P is 3600 mg kg^{-1} or lower. Ca levels decrease faster than P levels, so in this situation, Ca<<P. P levels between 3600 and 4000 mg kg^{-1} are in a grey zone. The values are expected to decrease somewhat with fish size, and adequate Ca and P levels are more critical for fish in the juvenile stage than in the grow out phase.

In farmed fish, Zn levels are commonly between 30 and 40 mg kg^{-1}, which also should be considered as a grey zone. Zn is a risk factor for skeletal deformities when whole body levels are in the 20-30 mg kg^{-1} range or lower.

Procedures for sampling and analysis

The most common approach is analysis of elements in a pooled sample of fish. Individual analyses can be done, but in most commercial and experimental settings, pooled samples will give an adequate answer. A common sample size is 10 fish per group, and should be no less than 6 fish per group.

Suggested points for sampling in salmonids may be 1g or 5g, 20g and 100g (alternatively sea water transfer), but sampling can be done at any fish size. Whole body analysis can be done also on bigger fish, but homogenization will then represent a challenge.

Mineral content in salmonids—sampling & analysis

Sampling procedures:

1. Fish should be fasted overnight before sampling. If necessary, place sampled fish in separate tank with running water overnight
2. Take a random sample of 10 fish
3. Kill the fish by overdose of anaesthetic (preferred) or another non-invasive method. Avoid bleeding.
4. Let the water run off, but do not dry fish with paper. Remove internal or external identity tags.
5. Place the fish in one or more plastic bags, and seal with as little air in the bag as possible.
6. Label the bags well; company, date, location, fish size, group id etc. (Tip: Place an extra label (pencil on waterproof paper) inside the bag)
7. Freeze at -18ºC until transport/analysis.

Laboratory procedures (Nofima Marin):

1. Frozen, whole fish must be homogenized and mixed well. If necessary, thaw fish slightly and cut half defrosted fish into pieces for better result.

2. Approximately 1 g of homogenate is sampled (avoid the first part of the homogenate)

3. Sample is dried (105°, over night or until stable weight). Record weight before and after: Dry matter %

4. The dried sample is ashed (550°C, over night or until stable weight). Weigh the ash for determination of ash%

5. The ash is hydrolyzed in acid (e.g. HCl + HNO3) and then dried slowly until stable weight. The dry matter is again dissolved in 5 % HCl for the lab to have a known acid concentration

6. Element concentration in hydrolysate analysed by ICP mass spectroscopy (ICP -OES Optima 5300 DV, Perkin Elmer, USA)

7. Calculate concentration of elements in mg kg^{-1} (ppm) of wet weight

Details of laboratory procedures and instrumentation may vary.

Figure: Two Atlantic salmon parr (approximately 5g) with different status of mineralization. Top, fish fed diet with adequate amounts of P and Zn, bottom, fish fed diet with suboptimal level of P and Zn. Note extreme difference in deposition of minerals in skeletal structures in fish of same size.

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Moving from data collection to improving performance

Philippe Mack, Francesca Margiotta & Courtney Hough

Introduction

The core objective of the FineFish project was to generate new practical knowledge on how to reduce the incidence of malformations in the major species used in European Aquaculture and apply this to the professional sector. While preparing the project's workplan at the start, some very simple questions were posed such as 'What is the current level of malformations in hatcheries?', 'How much do malformations cost the sector?' and 'How can you measure improvements?'

During the project start-up period it was quickly realised that the monitoring procedures within the different hatchery activities covered by the project (salmon, trout, cod, seabass, seabream) were all different and not standardized. It was also observed that none of the hatcheries used proprietary software for following production profiles, most using spread-sheet software for data entry and analysis.

Generally, there is a lack of publicly available data and information on hatchery performance in all of the sectors covered, making comparative work extremely subjective. This is for evi-dent professional reasons concerning corporate performance.

In addition, appreciation of malformations has been very subjective, with diagnostics being limited to the nature of the malformation in question – being measured as severe rather than on a scale of influence (e.g. 1-5).

Finally, from an analytical point of view, the complexity of the different influences on malfor-mation incidence – ranging from diets and nutritional components, through the complexity of in-tank abiotic factors, to temperature profiles at different stages, while not forgetting ge-netic aspects – means that there is no simple single and evident answer to monitoring.

To respond to this position, within Finefish, 3 main questions were posed

- ✧ How to measure technical and economic performance improvements?
- ✧ How to understand the underlying causes of malformation problems?
- ✧ How to transform new knowledge into best practice?

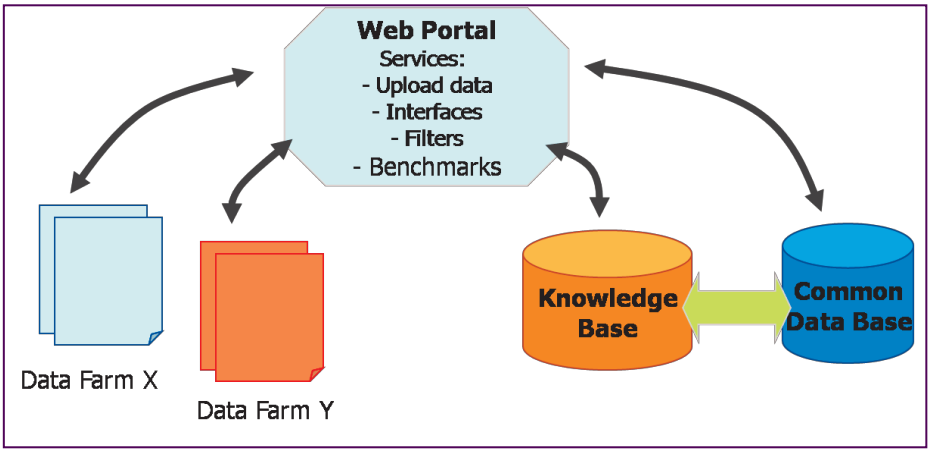
It was thus that FineFish examined how to organise the systematic collection of hatchery data and its analysis with regard to the incidence of malformation in hatcheries and commercial fish farms, the ultimate target being to help aquaculture operators in:

- ✧Benchmarking their activities so as to measure variations
- ✧Being able to share data and knowledge without compromising confidential information;
- ✧Implementing good practices and new knowledge

Approach

The data collection for benchmarking started by using relatively simple worksheets for follow-up and reporting. However, it was rapidly realised that this approach did not meet the expectations of the SMEs and that a rather professional approach was necessary. Software exists on the market that can recover and analyse production parameters but each SME has a different programme that is adapted to its own operations. Consequently, it was decided to develop a **common platform** that could be applied for all hatchery operators.

The basic concept is that individual hatcheries would prepare data in a standardised form that is submitted to a database via the internet (a web portal). This data can then be retrieved by the hatchery for benchmarking and monitoring purposes of the hatchery's performance. Obviously, this information is confidential to the hatchery in question and no other operator would be able to access this.

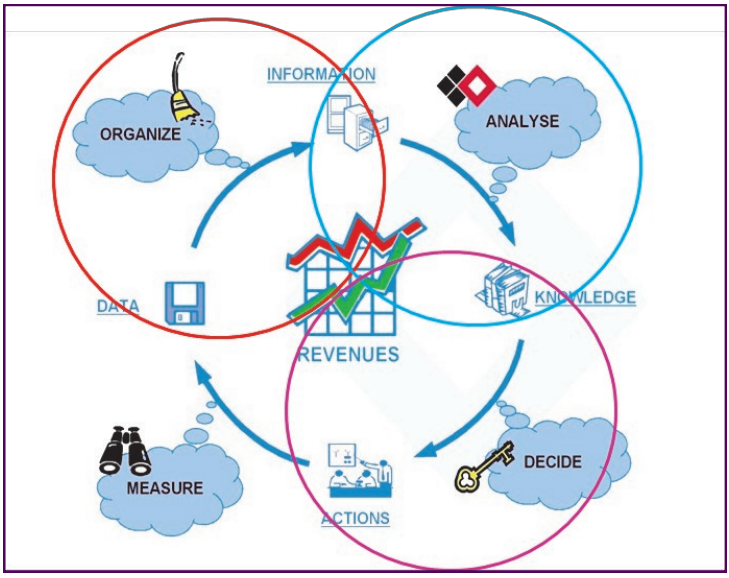


However, the database would thus contain information of high value for analytical purposes if many different hatcheries provide data and which would not breach the conditions of confidentiality. For example, if one wanted to compare the incidence malformations in sea-bass vs. temperature, or whether lighting regimes have an effect, or whether specific nutritional profiles reduce malformations….

By having the maximum amount of data stored within a single, standardized database one would enable the comparison and benchmarking of data on production methodologies applied in different hatcheries.

Moving from data collection to improving performance

The subsequent analysis of the data will then enable the extraction of useful information that can be applied to the improvement of current practices. The main analytical goal is to identify key factors affecting production performance and the underlying causes of the onset of malformations.



When examining the issues of analysis – and recognizing the enormous array of factors that could be causal to malformation incidence – the possibility of applying **data mining** technology appeared as a highly interesting option for application.

DATA MINING is "the science of **extracting implicit, previously unknown, and useful information from large data sets or databases**". It can also be defined as "the **process of discovering meaningful new correlations, patterns and trends** by sifting through large amounts of data stored in repositories, using **pattern recognition technologies** as well as **statistical and mathematical techniques**."

The benefits of this approach include the use of a systematic and smart way of looking at data and the application of a methodology that is able to extract - from very large databases - information that is:

- ✧ Previously unknown
- ✧ Valid
- ✧ Understandable
- ✧ Useful

There are also a wide range of tools available to the users (i.e. the hatchery) which include visualization of results, statistical analysis of data. A newer tool is the fact that automatic learning can be generated within the system, allowing the application of prediction or forecasting models – which can also give guidance through decision trees within a process.

In summary, the concept proposed is to develop and provide a comprehensive service that will allow better use of the data 'assets' that, in many cases, are already collected at the individual hatchery level, enabling the individual operator to

✧Understand the past

- Explain the behaviour of key performance indicators [KPI] (e.g. malformation rates, growth rates…)
- Transform **implicit knowledge** into **rules of procedure** (protocols)
- Identify the past conditions that improved production performance (so as to reproduce these consistently)
- Identify process weaknesses and root causes of failure

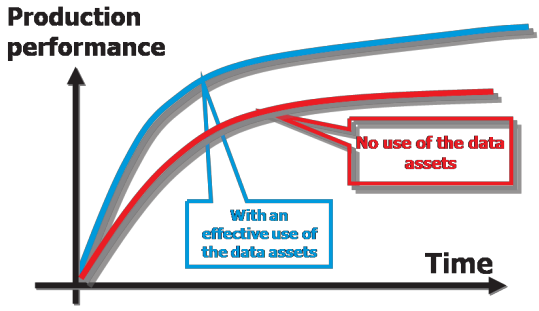
✧Address the present

- Take decisions based on reliable KPI
- Track process drifts (early detection of abnormal fluctuations in malformation rates, production performance)

✧Foresee the future

- Predict process states or KPI values – ideally develop a "predictive model of malformation rate"
- Predict maintenance actions and
- Predict actions to improve performance

Understand the past → **Address the present** → **Foresee the future**



Moving from data collection to improving performance

In a broad sense, **a key performance indicator (KPI) is a tool that is used for business improvement, focusing upon significant measurements made within a company's activities that indicate success or failure of that particular business.**

Following consultation with the farm managers and technicians in the hatcheries within FineFish, the main KPI identified was:

MALFORMATION RATE x BATCH

A KPI is a main component of a measurable objective, which is made up of:

a direction, KPI, benchmark, target, and time frame.

An example of a measurable objective for a hatchery would thus be *"Decrease malformation rate per batch from 30% to 20% by 2010".* In this case, 'the malformation rate per batch' is the KPI.

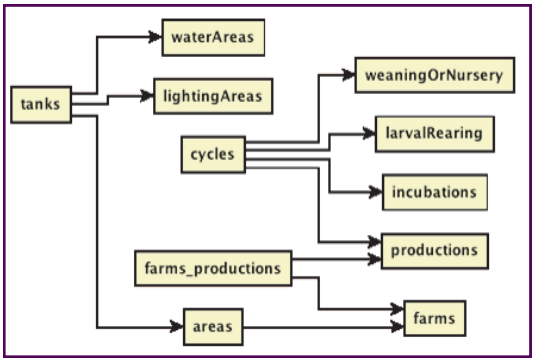
A KPI is therefore a composite of the following:

- a **measure of the performance** of specific goals that a business has defined to be of critical importance to their success à **malformation % x batch**
- a **target** (or targets) à **set of a threshold value > than 10 %**
- an **action** resulting from that measurement and leading to corrective actions if the value is exceeded

The FineFish Database system — "FindIT"

After a period of observation and interviews in a test hatchery (Ferme Marine de Douhet) and detailed analysis of different hatchery structures and procedures, including interactions of the various areas (water area, tanks, lighting area…) within the hatchery, the logical relationships between these components were used to **design a data model to be implemented in a RDBMS.**

RDBMS is a **relational database management system** that is based on a relational model – data is stored in form of tables and the relationship(s) between the data is also stored in form of tables. An example of table structure is given below.



This procedure allowed the modelling of the hatchery production process, an audit of the data collection procedures on site and led to the design of a data model. This design was implemented within a data warehouse (Data warehouse is a repository of an organisation's electronically-stored data. Data warehouses are designed to facilitate reporting and analysis)

While the first model was based on the test farm "FMD" it is adaptable to all farms, because it is a model of the real functional world and its relationships. Knowledge based logic is inherent to the system.

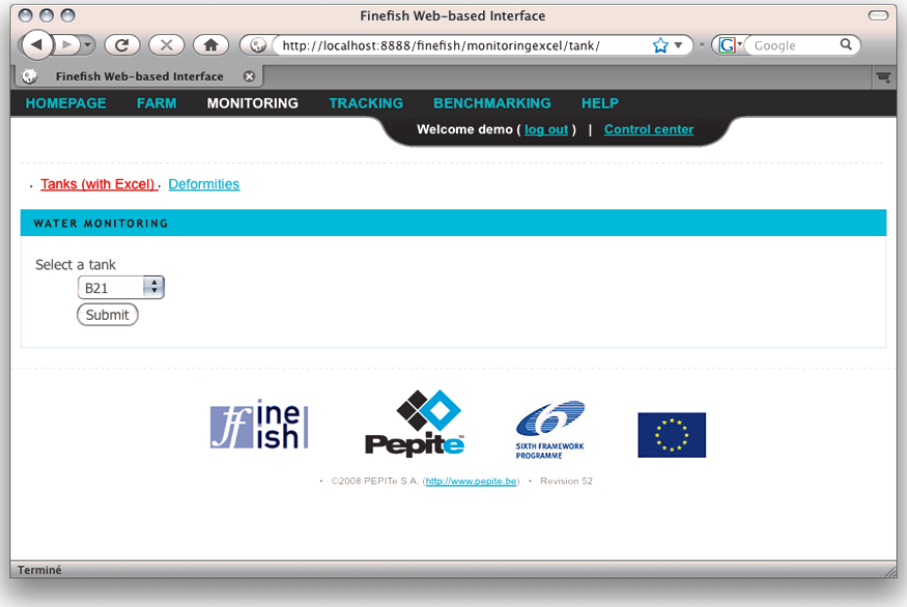
Using 'FindIT'

The first stage is configuration of the 'hatchery', which means providing generic information on the operations, physical structure and materials of the installations, which is achieved through the web-portal (see example below).

- ✧Generic farm information
- ✧ Farm production profile (i.e. species)
- ✧ Farm 'area' conditions
  e.g. Water parameters, lighting
- ✧ Tanks, ponds (materials, volume, depth...)
- ✧ Production cycles
- ✧ Feeds....(Feed identification, linked to ingredients)

Once this stage is completed, monitoring data can be included, such as the parameters of single units (tanks, ponds)... pH, Temperature, O2, salinity, water flow rates...

This is then supplemented by data on the types of malformation and the number of malformations/batch.



Moving from data collection to improving performance

As all livestock managers know, stocks are moved regularly and it is essential to follow their origin. Allowance is therefore made for identification of broodstock and their offspring batches, setting their position (in tanks) and their position in cycle parameters (i.e. nursery, larval rearing…). Data is included on broodstock and batch movements, broodstock food/ diet and batch food/diet.

Benchmarking

With the information entered into the database, it is possible to start benchmarking activities, which can be simple or more complicated.

As examples, one can benchmark the farm's structure (i.e. tank volumes, colour, depth) or any of the relevant monitoring data entered (e.g. temperature).

Output

The main outputs are tables that are made in an organised structure. These can range from, for example, a simple report of the daily temperatures for the month or the growth rates per batch using different diets within a defined production cycle. However, all of these outputs depend on a data request or query.

Queries are precise requests for information retrieval within a database and information system and their structure depends on what information is required. Their structure is quite complicated and requires specialist assistance if the hatchery does not employ an IT expert.

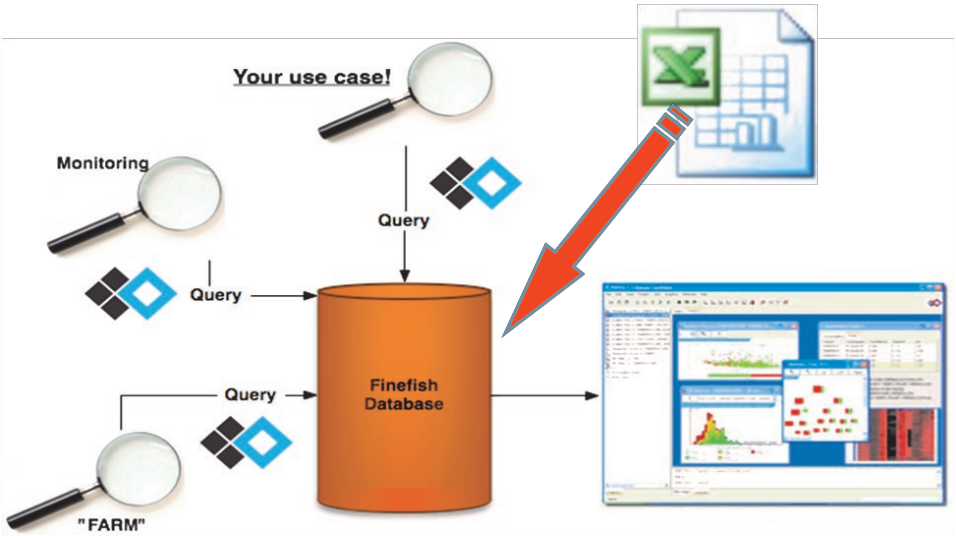
Examples for regular monitoring could be the growth and malformation incidence of a specific batch, requiring access to all stock movements and batch tracking, in addition to malformation assessment and measurement.—as indicated below

identifier	tank_departure	tank_destination	cycle_from	cycle_to
batch one	C1	B110	Incubation	Larval rearing
batch two	C7	B107	Incubation	Larval rearing

As a result, the data gathered through such monitoring provides new information tables as outputs.

This new data can then be used for the application of data mining - using a dedicated IT tool available at the FineFish web portal.

The diagram below summarises the operations. Data (as a worksheet file) is uploaded into the Database. Queries also can be made—which generate new tables. These new tables can then be analysed within PEPITo—the data mining tool



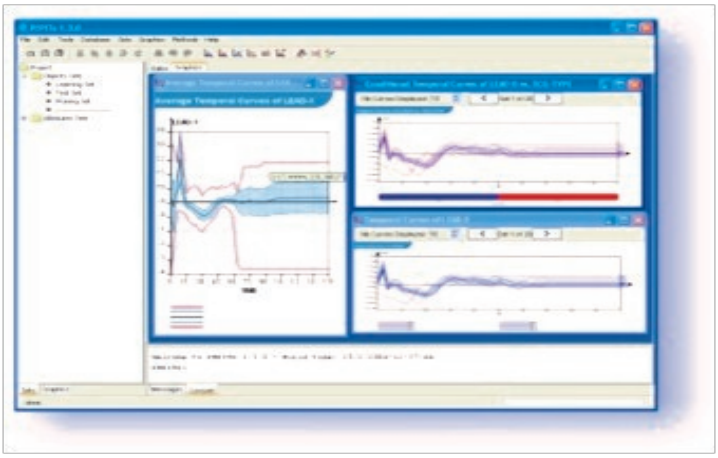
This tool is the proprietary software 'PEPITo' which accomplishes the following advanced tasks:

- ✧Data validation and filtering
- ✧Data transformation: FFT (Fast Fourier transform, sampling...)
- ✧Data visualisation, using
  - distribution plots, scatter plots, temporal curves,...
- ✧Statistical analysis:
  - analysis of variance, correlation analysis,...
- ✧Predictive analysis:
  - neural networks, decision trees, association rules,...

The core interest in this application is access to a comprehensive data treatment system that provides both predictive and root cause analysis.

Moving from data collection to improving performance

Examples of Data and Information Graphic plots within PEPITo



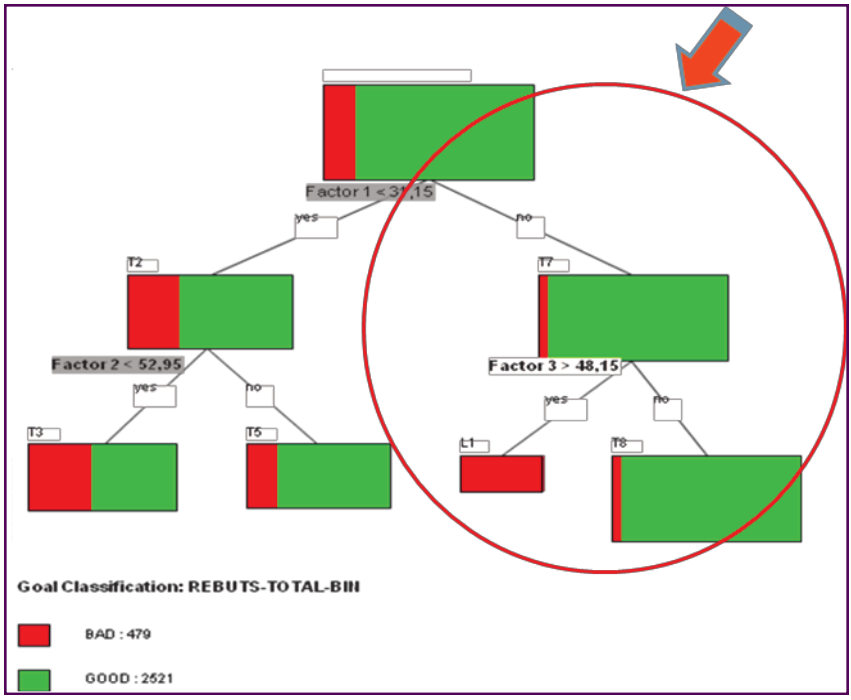
Examples of Decision Tree creation

Once enough data is stored in the system (a combination of the database and the newly created data information tables), it is then able to apply predictive analysis tools (such as decision tree models) to detect the root causes of malformation rate. Evidently, without adequate data entry no analysis can be achieved.

Possible analysis could be:

- ✧to detect in an individual farm the parameters that explain a malformation rate drift between two production cycles (in this case, the conclusion would probably be **specific to the hatchery**)
- ✧ to detect in the complete set of data within the database why the malformation rate is higher in a range of farms' production (e.g. in the Mediterranean on seabream); in this case, one can expect that the conclusion(s) would be broader, and that improvement actions could be applied to **every hatchery** within a range of conditions.

Consequently, the benefits of providing data to such a system are applicable to both the data provider and the sector as a whole.



This example of a decision tree shows the influence of 3 factors within a process, where decisions have to be taken so as to obtain the highest ratio of "good" (green) vs. "bad" (red). The absence of factor 3 is indicative of the best result (vs. factor 2). This type of predictive analysis should be able to help production and process planning

Moving from data collection to improving performance

Current situation

5 hatcheries have configured their structural inputs and are registering their production cycle data and PEPITe and FEAP are working in synergy with these farms:

✧Giving individual support and training to upload data and perform analysis.

✧Using the experience and knowledge of their technical and scientific staff so as to improve the system

Through farm feedback it will be possible to:

- Improve the web interface and make it more user-friendly and responsive to farmer's needs and requirements
- Identify possible bugs in the database and correct these in order to enable and facilitate the correct and easy input of data
- Modify the database and data organisation so as to enable the creation of a range of specific queries to be analysed through the data mining tool.

Identified needs

"we want to be able to keep track of parameters such as temperature, pH, salinity, food quality and quantity (fed to fish larvae) per tank in time."

"we want to be able to keep track of different light regimes in tanks over time"

"we want to keep track in time of the different treatments reserved to fish in hatchery tanks. "

All of these are simple to achieve with the current version of FindIT.

✧ The database maintains records of all fish movements within a hatchery/farm as well as the different conditions of each tank over time

✧ For each batch of fish produced within a hatchery, one can extract historical data, over time, for

- Location/position within the farm (tank/pond id)
- Treatments (e.g. chemical treatment, antibiotics…)
- Monitored parameters (T°, pH, light…)
- Feeding regimes(rotifers, algae, different feeds..)

It is therefore possible to integrate all of the variable factors that are believed to have an influence on malformations as well as FCR and growth performance, linking all of the fixed operations of the hatchery to operational variables.

Potential and future of 'FindIT'

The function and expectations of using this tool are the following:

- To discover unexpected correlations between parameters
- To benchmark production patterns within different hatcheries and identify the best practices (BMP)
- To verify *ad hoc* knowledge with historical data so as to detect abnormal situations early within a production cycle
- To expand the system to other KPIs (identify new KPI on the basis of analysis)
- To include genetic information in the system (relating to broodstock)
- To automate the creation of reports
- To automate the import of data into the system which is collected using other proprietary production monitoring software

It has not been possible to complete the full development of 'FindIT' within the FineFish project, partially because it was an unexpected project component but also because it requires more work on rendering the application to be user-friendly and applicable to a wide variety of situations. At the present, data is being entered by Finefish partners and it is hope to extend this to RTD institutes and companies operating within the Larvanet COST action (see http://www.larvanet.org/)

It is evident that the complexity of preparing automated queries that provide the anticipated answers needs thorough investigation and preparation. Easy data uploading and analytical procedures are the first priorities in the development of this aspect.

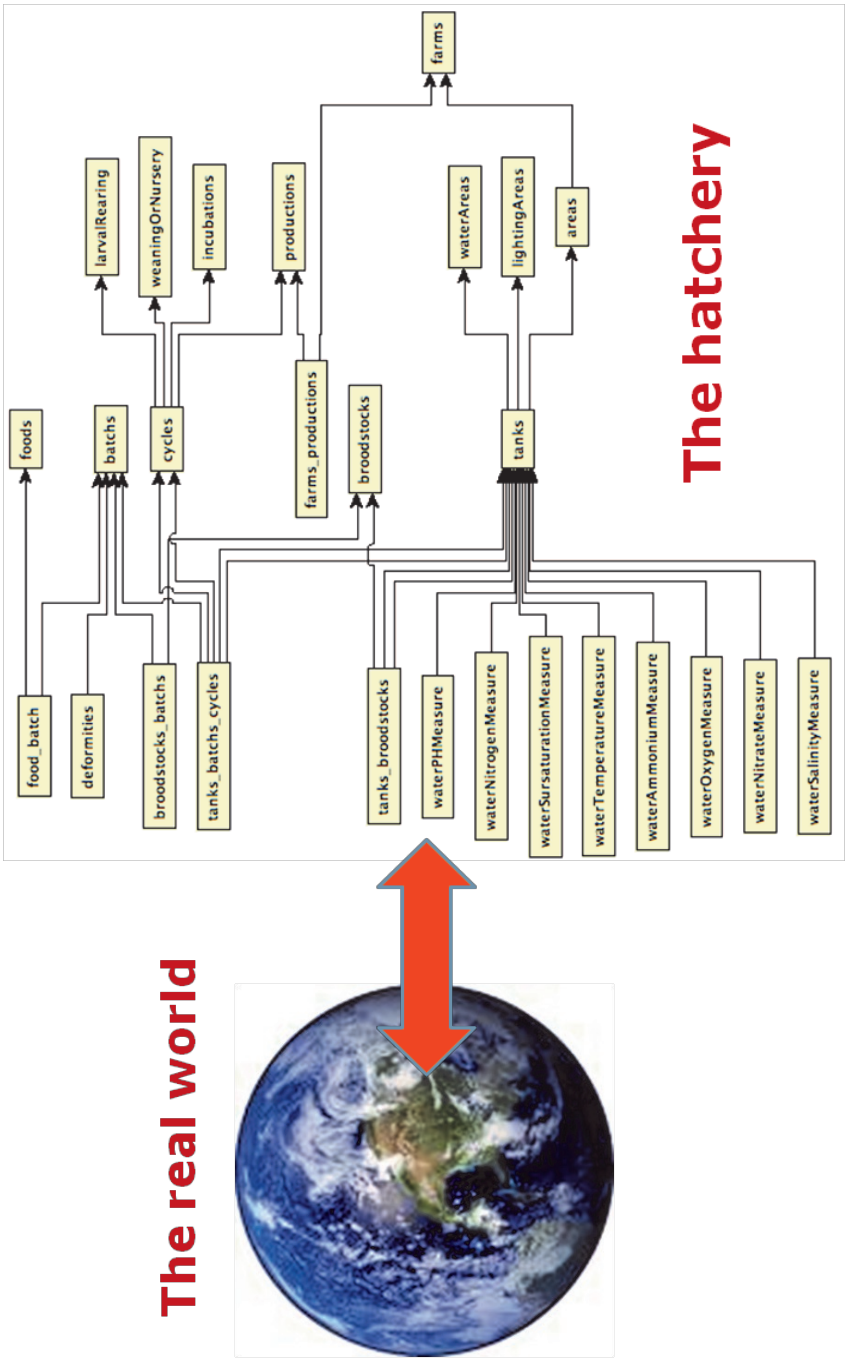
The FEAP, Pepite and other FineFish project partners are examining the means of achieving this so as to progress 'FindIT' to being able to answer the expectations developed within FineFish .

It is clear that this experience is a fine example of a new cross-cutting and innovative approach to monitoring and forecasting.

Companies or research institutions who wish to know more about the progress of 'FindIT' and/or who would be prepared to provide data should contact the FEAP Secretariat at secretariat@feap.info.

Moving from data collection to improving performance

FindIT's components and operation is structured to reflect reality



Best Management Practice - Cod (*Gadus morhua* L.)

Synnøve Helland, Ingrid Lein, Yoav Barr, Bendik Fyhn Terjesen & Grete Baeverfjord

1. Introduction

The Intensive farming of cod (Figure 1) is still a young industry that has had a very rapid growth. One of the major obstacles has been the high degree of malformation within the young livestock. In order to improve the sustainability and profitability of the cod farming industry, the hatcheries aim to produce robust cod juveniles, with low levels of deformities, good growth capacities, and to achieve these characteristics with a high degree of predictability. This BMP has been tested to achieve this and is based on the following procedural components:

- Low temperature on the brood stock during egg production
- Low temperature during the egg stage
- Disinfection of eggs after fertilisation and before hatching
- A moderate pace of temperature increase during the larval stage
- Sufficient water treatment
- Good tank hygiene
- Careful handling and slow moving water
- Fresh live feed organisms

Figure 1: Juveniles of Atlantic cod of +/-15 grams Photo: Nofima Marin AS

2. General remarks

Several studies, both on cod and Atlantic halibut, have shown that better growth and larval development are obtained when the larvae are fed zooplankton harvested from the sea as opposed to feeding *Artemia* and/or rotifers (Næss et al., 1995; Shields et al. 1999).

The specific cause(s) for these superior results, however, has/have still not been ascertained.

The temperature and feeding regime presented here is quite conservative compared to that used by several cod hatcheries. The idea is that, because rotifers and *Artemia* have a nutritional inadequacy in supporting the same growth as that obtained with wild-caught zooplankton, growth should not be stressed at this life stage where so many organs are forming and become functional. Thus, the regime presented below allows a slow development that secures, as much as possible, that the requirements of the larvae are best met, thereby reducing malformation risk and eventual negative impact(s) on later life stages.

Other important factors in reducing deformities for cod larvae, which are discussed in this Best Management Practice, are

- brood stock management
- handling
- water treatment
- water speed
- tank hygiene

This production protocol has been optimised for use by personnel working at the research units at Nofima Marin Sunndalsøra Research Station.

The use of this protocol has resulted in the production of cod juveniles that have low levels of deformities, about 3% at 20g size.

Furthermore, the fish grew similar or better than the information presented in the table of specific growth rates (SGR) provided by BioMar (2008).

4. Some aspects of broodstock management

Broodstock temperature

The earliest steps in the embryonic development are dependant and driven by maternal factors deposited in the oocyte during oogenesis. Very little is known about how these maternal factors influence the embryonic and larval development. However, it is known that exposing cold water broodstock to elevated temperatures affects the reproductive endocrinology of fish (Van der Kraak and Pankhurst, 1997), resulting in increased abnormal cell cleavage in early embryonic stages and to reduced survival (Tveiten et al., 2000, 2001).

It is thus important that the temperature is low and stable during oogenesis and spawning. Normal temperature for cod broodstock is a maximum of 6°C.

Spawning

The most common way of obtaining the cod eggs for production is by communal spawning of broodstock (see Figure 2) and the collection of eggs from the water surface using specially-designed egg collectors.

It is important that the egg collector is designed so that all eggs get an ample supply of well oxygenated sea water and are not damaged mechanically .

Another method used is to strip the eggs and the milt from the broodfish, followed by fertilisation. This technique is used by the professional breeding companies since they need to maintain control of the ancestral history and, of course, it can be used if there are special qualities that one wants to cross in the process.

In any case, it is very important to keep a high focus on hygiene and not to stress the broodstock.

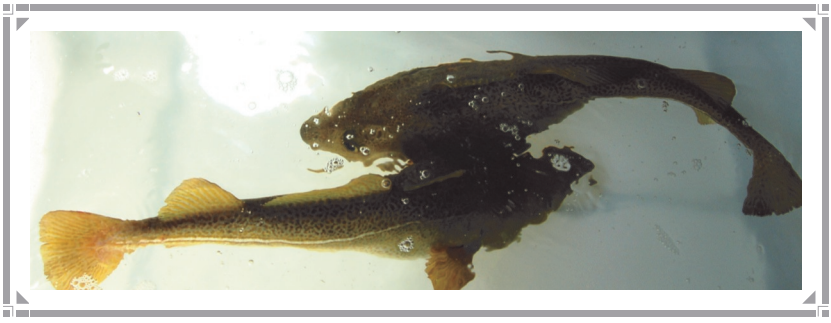


Figure 2: Broodstock of Atlantic cod Photo: Nofima Marin AS

Egg disinfection

A recent study shows that

- one disinfection of the newly fertilised eggs and
- repeated two days prior to hatching

were able to prevent the vertical transfer of the bacteria *Francisella noatunensis* from broodstock to eggs (Midtlyng et al. 2009).

The disinfectant used was glutaraldehyde (25% glutaraldehyde, 8 ml per 10 L sea water, for a duration of 9 minutes).

This disinfection will also remove other microorganisms, and the bacterial load at hatching will be less.

5. Sea water treatment

Temperature

Temperature controls the speed of many biological processes, such as growth and other developmental processes in fish larvae (see separate section on temperature).

Experiments have shown that a constant temperature of 6°C at the egg stage (see Figure 3 below) followed by a gradual increase from 6° to 12° C during a 6 week period, raising the temperature by one degree per week (Figure 4), results in cod larvae with few malformations *(for more information see the section on temperature effects*).

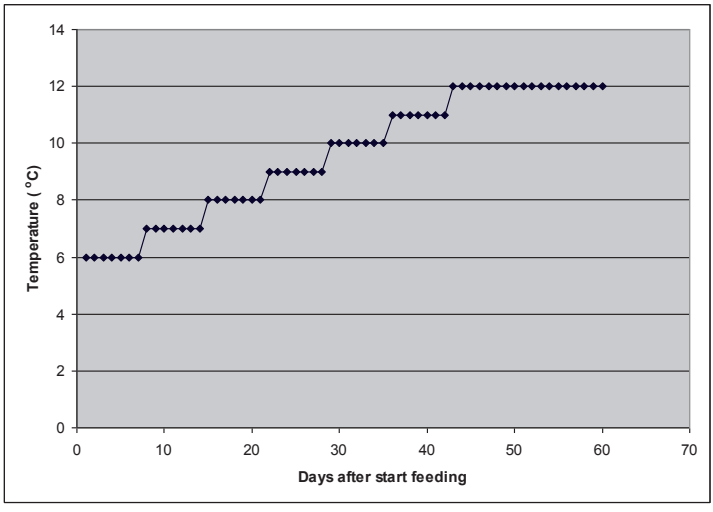


Figure 3. The control temperature regime from start feeding (day 0) used at the Nofima Marin Research station at Sunndalsøra.

Best Management Practice - Cod (*Gadus morhua* L.)

Water exchange/water flow

An ample supply of water is important in order to keep the level of dissolved gasses and excretory products within the biological levels for good growth and welfare of the fish larvae.

An unnecessary high rate of water exchange may be expensive if the water is temperature regulated, and also algae and live feed may be removed from the tank too fast.

A too low water exchange may result in poor tank hygiene and an accumulation of live food organisms with a reduced nutritional status.

Figure 4: Eggs of Atlantic cod, prior to hatching.
Photo Nofima Marin AS

At an initial larval density of 150 larvae per liltre, a tank water exchange rate —before hatching - of 10 to15 times per day works well BUT it is important to reduce the flow when the first signs of hatching appear. The preference is that one day prior to expected hatching exchange rate is reduced to the same level as used during the start feeding with rotifers— this is about 7 times (exchanges) per day.

However, one should consider increasing the flow up to 10 exchanges per day if there is a reduced O_2 level during the *Artemia* feeding period.

During weaning: 10 exchanges per day is recommended.

After weaning the flow is to be regulated according to the O_2 levels in the tanks.

Filtration, protein skimming, degassing and UV

Depending on the quality of the intake water, there are several methods that can be used to treat the water. Most cod hatcheries are based on flow-through (no recirculation) and it is common to filter the intake water to various degrees, depending on the life stages.

The technique used at the research station, and also common in the cod culturing industry, is to filter the sea water to 1 µm during the egg, hatching and often also the start feeding stages.

It is also common to use a protein skimmer, with or without ozone, to remove particles, a vacuum degasser, eventually with O_2 addition for adjusting the dissolved gas saturation, and UV radiation before the water enters the fish tanks.

As the fish grows, the required amount of water increases substantially and the fish is also more robust. The level of water treatment, and type of treatment needed changes and is seasonally dependent.

At the research station we commonly use 10 µm filtered sea water in the formulated feed phase, and continuously evaluate whether the sea water needs to be oxygenated.

Oxygen saturation

One study has been done that showed a positive effect on growth in cod raised in hyperoxic conditions (140 to 150% saturation) compared to cod raised in normoxic conditions (85 to 95% saturation) (Helland et al., unpublished data).

The fish from the hyperoxic tanks had a larger ventricle (heart) size, and it appeared that hyperoxia was not a causative factor for lordosis.

More studies are needed however before any safe conclusions on tank oxygen level can be made. Until further studies are done, the tank oxygen level should be adjusted to just below 100% saturation in the outlet.

Nitrogen saturation

Even a small supersaturation of nitrogen has a strong impact on cod larvae and juveniles.

The larvae will position themselves in the upper or lower areas of the tank and the appetite will be strongly reduced.

In case of higher nitrogen saturation the cod larvae and juveniles will die.

Total gas saturation must be checked regularly, and it is important to be very careful in mixing two water sources of different temperatures.

In case of N_2 super saturation all the sea water or a part of the water can be vacuum degassed, preferably with addition of oxygen. If a part of the total water flow is vacuum degassed make sure that the water is well mixed before it enters the fish tanks.

6. Tank rigging

Tank water speed/water velocity

Resent research shows that high water speed in the tanks induces lordosis in cod *(see section on abiotic factors, water speed for more information).*

One way to regulate the water speed is by regulating the water inlet. There are water inlet systems that have been specially developed to reduce the water speed.

Another technique is to reduce the water speed by adjusting the positioning and shape of the water inlet.

The water speed should be kept to a minimum during the egg, larval and early juvenile stages. However, it is very important also to maintain good tank hygiene, and the minimum water speed necessary to facilitate a good tank environment ought to be set for each rearing facility.

Best Management Practice - Cod (*Gadus morhua* L.)

Surface skimmers

Surface skimmers are to be used from the start of rotifer feeding until weaning. It is important that any eventual bio-film on the water surface is broken for the cod larvae to be able to fill the swim bladder with air.

Aeration

Use an aeration rod or a centre aeration ring from egg transfer until weaning so as to keep the eggs, larvae and the live feed organisms in the water column.

Use only sufficient aeration, aeration that is too strong may easily damage the larvae and malformation or mortality may occur.

Light

A pilot study has been done showing that light regimes (e.g. light:dark, 18:6) or the use of a single light source on the tank edge had no effect on skeletal deformities (Lein et al., unpublished data).

However, growth rates were reduced when introducing a night period, and weight was more affected than length. Growth was also reduced by the single light source.

Until further information is available, it is recommended to use standard ceiling light 24h a day.

This will also help to maintain good tank hygiene, rapid spotting of filamentous bacteria and facilitates good observation conditions of the state of the cod larvae and juveniles.

Algae

The addition of algae in the diet of Atlantic halibut larvae (*Hippoglossus hippoglossus* L.) has been shown to have a positive effect on feeding incidence, survival and growth rates during *Artemia* feeding.

No direct nutritional value of the algae on the halibut larvae was found (Naas et al. 1992), but it appears that these positive effects may be linked to factors such as turbidity and effects on the light regime, and also the eventual leaking of feed attractants like free amino acids.

Most cod hatcheries use algae paste that has been mixed with sea-water and then added to the tanks. Normally this is done three to four times a day, resulting in clearing of the tank water in some periods during the day facilitating tank cleaning and observation.

7.Feeding regime

Live feed phase

Cod larvae are start fed when the yolk sac is almost empty (3 or 4 days after hatching). The following feeding regime is currently the control protocol used at the research station (Figure 5).

The cod larvae should be fed solely on rotifers until day 25 (4 rotifers per ml).

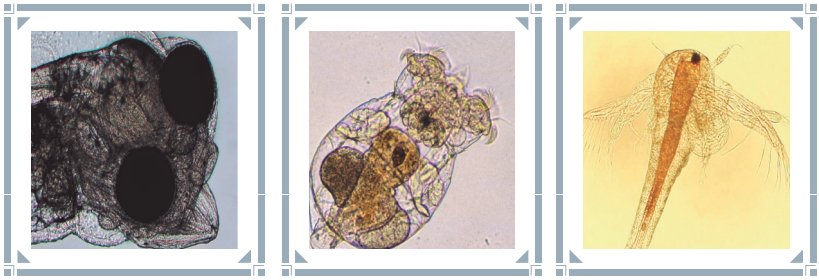


Figure 5. Atlantic cod larvae at start feeding (left), *Brachionus spp.* rotifer (middle) and *Artemia franciscana* (right) Photo: Nofima Marin AS

It is important to observe the appetite and adjust the feeding strength accordingly.

It is important to remember that old prey have a lower nutritional level that fresh prey, so avoid congestion of rotifers in the tank. Also, think about tank hygiene. After day 25, the cod larvae are co-fed rotifers and *Artemia* 50/50 (2 rotifers and 2 *Artemia* per ml), and after day 32 they are fed only *Artemia* (4 per ml) for one week.

Weaning phase

After day 39, co-feed *Artemia* and formulated feed for 1 week, during which the amount of *Artemia* is gradually reduced and the amount of formulated feed is increased.

Do look at the larvae regularly to check that they are eating the formulated feed.

Growth expectancy

In 2008 ,Nofima Marin produced cod families for MarineBreed, a commercial breeding company in Norway (F2 generation). The growth of these families has been followed for more than one year from 1.4 gram size, and the SGR is similar or above that of the BioMar SGR table of 2008. The fish have been given commercial diets and have been reared at ambient temperature.

Bjørnsson et al (2007) made a growth model for cod, showing the effects of temperature and body weight on growth rate. These models are based on wild fish, while most Norwegian hatcheries use fish from one of the two breeding programs. This growth model tends to underestimate the growth obtained in some of the cod hatcheries.

8. Concluding remarks

For all animals, there are biological constraints to physical development and crossing these limits may lead to malformation, malfunction, and may even be lethal.

In the rearing of cold water marine fish larvae, the mortality rate is still high and the predictability of results is low.

This implies that the production is on, or crossing, the biological tolerance limits. With the rather easy measures described in this BMP for Atlantic cod, one remains within the biological limits that we know of today for the prevention of malformation .

Development of the Best Management Practice is, however, a continuous process, where new technology, new feeds etc.. would necessitate an ongoing revision.

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Recommendations on prevention of malformations in rainbow trout

Grete Baeverfjord

1. Introduction

For rainbow trout, the recommendations given in the following section are based on results obtained from work made within the FineFish project and some closely related activities.

2.Temperature in early life stages

The effect of temperature exposure on malformations in rainbow trout has so far been studied only in fish exposed to different temperatures during egg incubation and up to the stage of first feeding. In summary, the results confirm the hypothesis that rainbow trout can tolerate temperatures that are somewhat higher than salmon at similar life stages and that the early life stages of rainbow trout, unlike salmon, are vulnerable to low temperatures (< 8°C).

Data from salmon indicate that temperature is an issue for the induction of malformations in salmonids also beyond first feeding,. Similar experiments for rainbow trout are strongly demanded by the profession.

The existing recommendations can be summarised as follows:

- The optimal incubation temperature for rainbow trout eggs is 10°C
- The tolerance range is 8°C to 12 °C, i.e. that no effect or minor effects on malformations are expected when applying temperatures within this range
- Temperatures > 12°C and < 8°C are likely to induce skeletal malformations
- Rainbow trout eggs should not be subjected to cooling regimes
- Temperature sensitivity remains also beyond eyed egg stage
- Temperature recommendations are the same for diploids and triploids

These temperature recommendations are valid for the different geographic strains which have been examined until now

1 More information on temperature effects during egg incubation can be found in Chapter 3 of this book.

3.Impact of nutritional components

The impact of nutritional components on development of malformations was addressed in Chapter 5 of this publication. Based on these data, some recommendations can be given:

- The importance of dietary mineral supply for adequate skeletal mineralization is confirmed. Special attention should be paid to the level and availability of phosphorus in the formulation of diets for normal vertebral development during the early ontogeny of rainbow trout.
  The dietary available phosphorus requirement is estimated to be approximately 1% diet for adequate bone mineralization of rainbow trout fry
- For rainbow trout, quite high levels of vitamin A are recommended for broodstock nutrition (around 200 IU/g diet). The supplementation of 20 IU/g diet that is usually used in commercial diets for salmonids might not be enough to fulfil the vitamin A requirement of rainbow trout
- High dietary levels of vitamin A are beneficial for reproduction and early growth and no effect on skeletal development was noticed in comparison to other fish species. This difference might be due to the fact that the level of retinoic acid, the active metabolite of vitamin A, appears to be well controlled in eggs of rainbow trout
- Experimental results suggest that compared to late developmental stages, early stages are more susceptible to dietary oxidative stress, possibly due to lower response of endogenous antioxidant defence system
- The importance of the control of lipid peroxidation in fish feeds for normal growth of rainbow trout fry was highlighted. A correct supply of antioxidants (such as vitamin E or C) should be provided in fish feeds to protect polyunsaturated fatty acids from lipid peroxidation

The importance of dietary phospholipid supply for early growth and adequate skeletal mineralization has to be highlighted

4.Water quality

Presently, little data on effect of water quality on malformations in rainbow trout is available from scientific studies, and more studies in this field are clearly warranted. Some limited and rough advice can be given, based on a field experiment done in a commercial hatchery. The study tested the effect of dissolved CO_2 at levels between 3 and 30 mg L^{-1}, and a dose-response relation between CO_2 level and skeletal malformations was indicated during early juvenile rearing.

Recommendations on prevention of malformations in rainbow trout

Control levels of dissolved CO_2 in rearing water for juvenile rainbow trout. Until further data are presented, it is suggested to use the corresponding limit in Atlantic salmon as a guideline, which is too maintain dissolved CO_2 <15 mg L^{-1}.

Figure 1 : Rainbow trout (approximately 50g wt.) with severe shortening of the body (top) caused by fusions of vertebrae, compared to normal (bottom)

Recommendations for malformation control in Atlantic salmon juveniles

Grete Baeverfjord, Ingrid Lein, Kirsti Hjelde, Harald Takle & Synnøve Helland

1. Introduction

The manner in which Atlantic salmon is produced is diverse, and there is a range of different production systems and strategies in operation in the industry. Thus, a standard protocol for the production of Atlantic salmon juveniles and smolts would be of limited relevance. In the following sections, a set of recommendations is presented which summarises experimental results and experiences from commercial production on how to prevent malformations in the professional production of Atlantic salmon. Some of these recommendations are justified by scientific documentation, whereas others are less well substantiated. Thus, not all the recommendations meet the standards expected from a standardised protocol as such. Nevertheless, these should be regarded as advice to be considered for both problem solving and production planning.

2.Background

For Atlantic salmon, malformations in freshwater juvenile production are difficult to detect and, as an additional challenge, malformations continue to develop throughout the rearing period. In commercial production, a key issue is that the full extent of malformations may not be detected until fish are approaching harvest size.

Nonetheless, a number of studies show that a significant proportion of problems may be induced in early rearing, i.e. during egg incubation and in juvenile rearing. Thus, a strategy of sorting and rejecting juveniles with deviations at an early stage, comparable to the position experienced with sea bass and seabream production, is not feasible for Atlantic salmon. The salmon markets are also increasingly sensitive to ethical issues and the prevention of malformations through biological improvements must be the preferred overall strategy.

Interaction between environmental factors giving rise to malformations is a known fact in practical production, although such phenomena are difficult to analyse, both in retrospect and in predictive models. Thus, anecdotal reports exist of successful production under environmental conditions that conflict with the recommendations that follow. Conversely, reports exist of severe malformations in fish batches produced in compliance with the same recommendations.

It is acknowledged that the knowledge on causal relations for inducing malformations in Atlantic salmon is still fragmentary and far from complete. There are, in all probability, causal relations of importance that have not yet been identified and addressed. In particular, the impact of rearing conditions in the marine environment during the grow-out stage has been studied little . Nevertheless, case reports also exist that claim significant improvements in the rate of malformations following implementation of some of the recommendation presented here.

3.Recommendations

The recommendations can be summarized as follows:

- **Keep freshwater rearing temperatures moderate and stable**
- **Control freshwater growth rates at a moderate and stable level, and avoid periods of maximized growth**
- **Monitor and stabilize the water quality**

Feedback from commercial production implies that, by following these key principles, while freshwater production rates may be held back the long term productivity will gain - as a result of less deformities, a sustained long-term high growth rate and, possibly, lower mortalities also .

1. Temperature

Atlantic salmon is a cold water species. Although development and growth can be accelerated by the use of warmer water, side effects will include increased number of fish with skeletal malformations if the temperature charge exceeds some biological limits.

The most vulnerable period is embryonic development, i.e. egg incubation. At this stage, even a short episode of temperature stress can produce lasting effects. At later stages, the effects will generally depend on the magnitude of the temperature charge, i.e. *the magnitude of the temperature increase x duration of the episode*. Thus, even though juvenile salmon may tolerate 16°C well for a limited time and without consequences for skeletal development, the same temperature applied in the long term may give severe malformations in return.

a) Egg incubation and yolk sac stage

- **Temperature during egg incubation must be controlled at 8°C or lower**
- **The early stages of egg incubation (before eyeing, 300-350 d°) are more vulnerable to temperature stress and requires strict temperature control**
- **Avoid rapid temperature fluctuations also when operating below 8°C. As a rule of thumb, temperature changes of 2,5°C or more within 24 hours may be expected to have impact on skeletal development**
- **Cooling of eggs towards freezing (<1°C) should be avoided during the first 100-150 d°**

The effects of increasing temperatures to 9°C or 10°C during the yolk sac stage are not expected to have dramatic consequences, but will increase the risk of inducing fusion type pathology by influencing skeletogenesis. Thus, control the temperature to 8°C as far as possible towards first feeding.

b) First feeding and parr rearing

- **Temperatures at 12°C and lower are considered optimal for skeletogenesis in first feeding fry and parr rearing**
- **At 14°C, increased incidence of pathology, primarily fusion type, is expected**

Between 12°C and 14°C, detailed temperature recommendations can not be provided at the present time . A dose-response relation is expected, i.e. increasing incidence with increasing temperature charge.

In industrialised smolt production, this rearing period is normally achieved under conditions of temperature control and using heated water - at least until biomass increase outweighs an available warm water supply.

Thus, during this stage of the production, the juveniles are at the producers' mercy with regards to temperature. However, during summer conditions, the situation may be the reverse, as ambient temperatures may reach levels of 16-20°C at some sites, without the possibilities of access to cooling water.

Optimal temperatures for growth during the juvenile stages are 15-16°C, and the use of heated water or ambient warm water to maximize growth gain is an obvious option. However, the documentation showing that this situation will increase the susceptibility to skeletal deformities is so strong that the recommendation to hold back on temperature during this period is very clear.

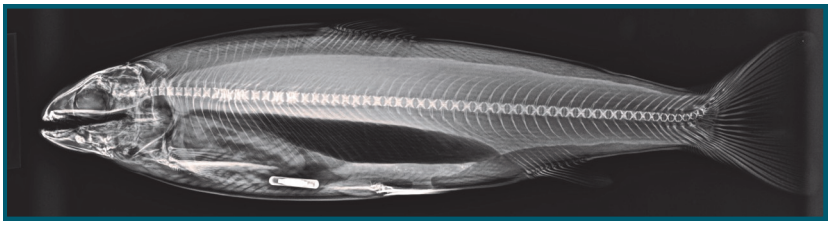

Figure 1: Radiography of Atlantic salmon parr (70g) with severe vertebral fusion, induced by high rearing temperature in early developmental stages.

c) Smoltification

In yearling production, temperatures during smoltification are not expected to represent a problem when ambient water supply is used during a natural smoltification period for the species.

In underyearling production, smoltification must be induced — through photomanipulation or alternative procedures — and the process takes place at a different time of the year than the normal biological programming implies. Thus, in underyearling batches, smoltification is induced by giving a "winter signal", typically during late summer, when ambient temperatures are at the highest.

In a typical photomanipulation program (6 weeks 12D:12L followed by 6 weeks 24L), the recommendation is to avoid a temperature drop from the first to the second period.

d) Seawater rearing

There are no clear recommendations that can be given for temperature control during seawater rearing at this time. Seawater rearing is mainly done in open cages with ambient water supply, and in most cases there are no possibilities for temperature control. The temperature effects on induction of skeletal deformities during on-growing in seawater warrants further investigation.

2. Nutrition

Although the number of nutritional factors that may have an impact on skeletal development is high, the most robust indications point towards inadequate dietary mineral supply as the most relevant nutritional risk factor. In particular, the evidence is strong against the impact of suboptimal phosphorus (P) and zinc (Zn) supply.

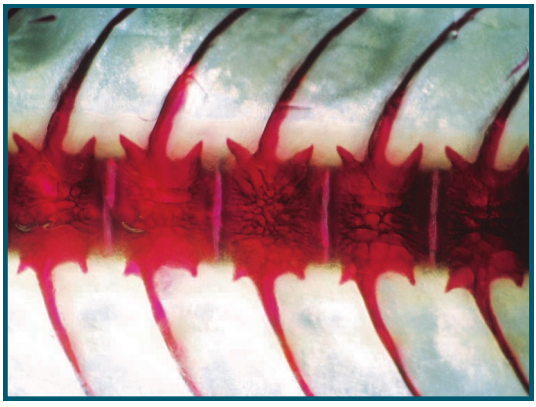

Figure 2: Hyper dense (HD) vertebrae in spinal column of salmon parr. HD vertebrae in juvenile salmon are indicative of disturbed mineralisation. (Alizarin Red staining)

Recommendations for malformation control in Atlantic salmon juveniles

In salmon farming, nearly 100% of feeds are commercial products. Thus, the nutritional quality of the feeds is to a large extent a matter of confidence and trust between feed supplier and customer, i.e. the fish producer.

The critical factor is mineralisation of skeletal structures. When mineralisation is inadequate, the actual cause can be one of several factors: Dietary supply of minerals depends not only on the concentration of the element in the feed, but also on bioavailability of the element and of the feed conversion ratio (FCR, kg feed given per kg of weight gain). The product of these three factors determines to a large extent the resulting concentration of the element in the fish, and thus the susceptibility to skeletal deformities induced by suboptimal mineral supply.

When choosing feeds:

- **Request documentation on performance ability of feeds under commercial conditions, with respect to mineralisation of skeletal structures**
- **If necessary, analyse fish mineral content and skeletal development through appropriate diagnostic procedures**

Mineralisation and development of skeletal structures can be evaluated by:

- **Analysis of mineral content of fish**
    - The most reliable parameter for evaluation on P and Zn status are whole body analyses
    - Please refer to chapter 6 in this booklet for guidelines on monitoring of mineral content in fish
- **Skeletal diagnostics**
    - X-ray diagnostics of freshwater salmon
    - Whole mount staining techniques using Alizarin Red
    - Please refer to specific guidelines for diagnostic criteria (available at www.finefish.info)

3. Growth rates

Growth rates in juvenile salmon are generally high. Measured as specific growth rate (SGR, % daily weight increase), values may be as much as 7-10% in early juvenile stages - when growth rates are maximized.

The most important determinant for growth rate in freshwater salmon rearing is temperature. Consequently, the specific effects of growth rate on skeletal development is difficult to separate from temperature effects under conditions that are relevant for commercial production.

Some producers maintain that SGR is the most important control parameter for skeletal malformations. Although this argument cannot be substantiated, experience from both research and commercial production support the theory that skeletal development benefits from a moderate long-term growth rate, without extreme periods, giving a smooth growth curve.

The effect may well be that a controlled growth rate will ease the pressure on nutritional balance. No matter what the mechanisms are, the best way of achieving a controlled and moderate growth rate is through temperature control.

- **Control growth rate through control of rearing temperature**
- **Avoid periods of maximized growth rates**

4. Water quality recommendations

The water quality of intensive smolt production is a complex issue (see chapter 4 in this publication). Variation in tank size, quality of raw water and technical solutions for water treatment adds to the complexity. Based on a 'best choice' principle, some general recommendations are given:

- **Control biomass through controlling growth rates**
- **Avoid the higher levels of fish density (>50 kg m^3)**
- **Comply with the limitations given by the Norwegian Food Authorities:**
    - O_2 saturation in tank water <100%
    - Dissolved CO_2 in tank water < 15 mg L^{-1}
- **Keep the water quality controlled and stable**
- **Pay particular attention to O_2 saturation in the final weeks of smoltification**

Recommendations for malformation control in Atlantic salmon juveniles

There are other aspects of malformation control in production of Atlantic salmon juveniles that are not addressed by these recommendations, in particular related to vaccination strategies and procedures.

Please refer to other sources for further information.

Project Partners & Contact Points

Industrial Associative Grouping—Project Coordinator

Federation of European Aquaculture Producers

rue de Paris 9, B-4020 Liège, Belgium

- Courtney Hough – secretariat@feap.info
- Francesca Margiotta – francesca@feap.info

www.feap.info

SME partners

Profunda AS

N- 6174 Badstadvik, Norway

- Helge Ressem— profunda@online.no

Ferme Marine de Douhet S.A.

Port du Douhet, F-17840 La Bree les Bains 4, France

- Jean-Sebastien Bruant – bruant@douhet.com

www.douhet.com

Aquasearch Ova

Siljeager 1, DK-7300 Jelling, Denmark

- Torben Nielsen – torben@aquasearch.dk

www.aquasearch.dk

Tinamenor A.S.A.

Marisma de Pesues s/n, E-39548 Pesues-Cantabria, Spain

Carlos Mazorra – carlosmazorra@tinamenor.es

www.tinamenor.es

Bolaks A.S

Lambaneset, N-5640 Eikelandsosen, Norway

- Erik Sorheim — bolaks@bolaks.no

http://bolaks.no

Viviers de France S.A.

Route de Taller, F-40260 Castets des Landes, France

- Fred Cachelou— sarrance@akersea.fr

www.viviersdefrance.com

Brow-well Fisheries Ltd

Phoenix Cottage, Hebden Skipton, New Yorkshire, BD23 5DH United Kingdom

- Jonathan Jowett - jonathanjowett@pcm-online.co.uk

www.brow-well.co.uk

Andromeda S.A.

Ortos-Kato Kastritsi, GR-26500Rio Achaia, Greece

- Panos Kollios
  pkollios@andromeda-aquaculture.gr

www.andromeda-aquaculture.gr

Panittica Pugliese SpA

Strada comunale del Procaccio, I-72010 Torre Canne di Fasano, Italy

- Massimo Caggiano – panpug@libero.it

www.panittica.com

Pepite S.A.

3 rue Paul Devaux, B-4000 Liege, Belgium

- Philippe Mack – ph.mack@pepite.be

www.pepite.be

RTD partners

Nofima Marin AS

Sjolseng, NO-6600 Sunndalsøra, Norway

- Grete Bæverfjord – grete.baverfjord@nofima.no
- Synnøve Helland – synnove.helland@nofima.no
- Ingrid Lein – Ingrid.lein@nofima.no

www.nofima.no

Postboks 5010,NO-1432 Ås, Norway

- Harald Takle – harald.takle@nofima.no

UMR NuAGE (INRA-IFREMER-University of Bordeaux)

INRA - Hydrobiology research unit, Quartier Ibarron, F-64310 Saint Pee sur Nivelle, France

- Sadasivam Kaushik - kaushik@st-pee.inra.fr
- Stephanie Fontagné - fontagne@st-pee.inra.fr

www.inra.fr

IFREMER—Z.I. Pointe du Diable B.P. 70, F-29280 Plouzane, France

- José-Luis Zambonino – jlzambon@ifremer.fr

Project Partners & Contact Points

IFREMER

Chemin de Maguelone 34250 Palavas-les-Flôts France

- Beatrice Chatain – bchatain@ifremer.fr

www.ifremer.fr

Centre of Marine Sciences - University of Algarve

Universidade do Algarve, Camous de Gambelas 8005-139 Faro, Portugal

- Deborah Power – dpower@ualg.pt

www.ccmar.ualg.pt

Hellenic Centre for Marine Research

Sounio Av, PO Box 712, GR-19013 Anavissos Attica, Crete, Greece

- Pantelis Katharios - katharios@her.hcmr.gr

www.hcmr.gr

National Centre for Mariculture (of IOLR)

Tel- Shikmona, P.O.B. 8030, Haifa, 31080 Israel

- Amos Tandler - tandler@agri.huji.ac.il
- Bill Koven - koven@agri.huji.ac.il

www.ocean.org.il

Royal Veterinary College

Royal College Street, NW1 0TU London, United Kingdom

- Neil Stickland - nstickland@rvc.ac.uk

www.rvc.ac.uk

University of Patras

University Campus, Panepistimioupolis-Rion, GR-26500 Patras, Greece

- Giorgos Koumoundouros - koumound@upatras.gr

www.upatras.gr