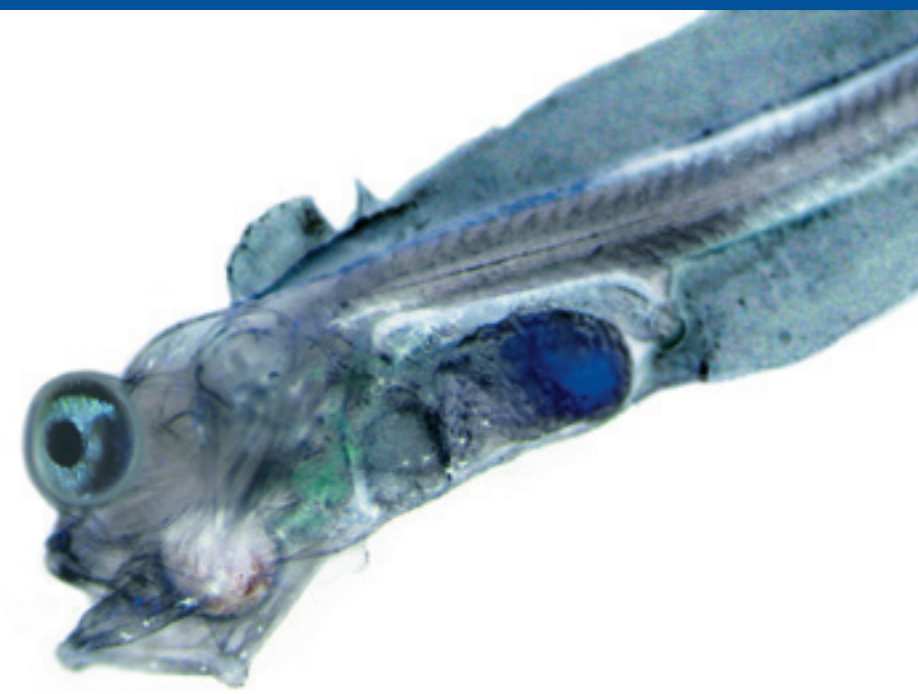
The Research Council of Norway

The fish larva: a transitional life form, the foundation for aquaculture and fisheries

Report from a working group on research on early life stages of fish

Large-scale Programmes
Aquaculture – An Industry in Growth



Large-scale Programmes

The RCN initiative to meet national research priorities

The Research Council of Norway

The fish larva: a transitional life form, the foundation for aquaculture and fisheries

Report on research on early life stages of fish

The Research Council of Norway
P.O.Box 2700 St. Hanshaugen
N–0131 OSLO
Telephone: +47 22 03 70 00
Telefax: +47 22 03 70 01
bibliotek@forskningsradet.no
www.forskningsradet.no/english

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Preface

Knowledge about early life stages in fish – eggs, larvae, fry and smolts – is critical for a number of high-priority areas in aquaculture research, both in Norway and abroad. Experience has shown that major bottlenecks occur in the early life stages, slowing efforts to commercialise new production species. As a rule, stringent requirements in terms of nutrition and physical/chemical/microbial environment must be met to ensure proper development and high survival rates during the early life stages. The causes of permanent malformations often arise in the early stages of development, to the detriment of fish welfare and performance in production. Regarding wild stocks, basic knowledge about early life stages may provide insight into how the fish adapt to their environment and survive under various conditions.

Several programmes at the Research Council are involved in research on the early life stages in fish, and knowledge in this area may benefit trade and industry as well as the government administration. The challenge to Norwegian researchers is to conduct research of high international calibre and to be considered attractive partners for collaboration in international research groups.

Against this background, the Aquaculture programme – in cooperation with the Oceans and Coastal Areas (HAVKYST) programme, the FUGE programme (Functional Genomics in Norway), and Independent Basic Research Projects (researcher initiated) in Biology and Biomedicine (FRIBIO) – appointed a working group to give an account of the state-of-the-art of research on the early life stages in fish. The working group is comprised of the following members:

Jon Vidar Helvik, University of Bergen (Professor; Committee Chair)
Kristin Hamre, National Institute of Nutrition and Seafood Research (NIFES) (Senior Scientist)
Ivar Hordvik, University of Bergen (Professor)
Terje van der Meeren, Institute of Marine Research, Bergen (Senior Scientist)
Helge Ressem, Norwegian Seafood Federation / Profunda AS (Manager)
Manfred Schartl, University of Würzburg (Professor)
Helge Tveiten, Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima), Tromsø (Senior Scientist)
Gunvor Øie, SINTEF Fisheries and Aquaculture, Trondheim (Senior Scientist)

Harald Sveier, Research Council of Norway (Committee Secretary)

The working group commenced its work in May 2008 and submitted its report in April 2009.

The working group's efforts are much appreciated.

Oslo, June 2009

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1 Mandate

Status of and strategies for research on development and function in early life stages of fish, and on the use of model species for mapping basic biological processes.

1.1 Mandate

In terms of thematic focus, knowledge about larvae, fry and smolts is critical for a number of prioritised areas within Norwegian and international aquaculture research. Experience with cod, halibut, sea bass, sea bream and other species shows that the early life stages represent a significant bottleneck in the work of bringing a new species to the point of commercialised production. For wild stocks, basic knowledge about early life stages will provide insight into how the fish adapt to their environment and survive under various conditions.

The early stages are normally characterised by strict needs regarding factors such as physical/ chemical environment, nutrition, and microbial environment for normal development and good survival rates. Often, the causes of permanent abnormal development occur in the early developmental stages, with consequences for fish welfare and performance later in the production process.

As a step in organising R&D activities on early life stages in fish, a working group has been appointed and charged with the following tasks:

- To describe the status of research on development and function through early life stages in fish (eggs, larvae, fry, smolts) in Norwegian aquaculture, in the key species found in Norwegian territorial waters, and in the species of greatest interest for international aquaculture.
- To assess the use of model species as a suitable method in studies of premature and abnormal development in fish during production.
- To identify the need for knowledge, both short and long-term, and advise on important priorities and strategies for research in this area in the coming years.
- To provide an estimate of the costs of conducting the research.

The working group is expected to start these tasks as soon as possible and to submit its report sometime around year's end (2008/2009).

1.2 The working group

Kristin Hamre, NIFES (Senior Scientist)
Jon Vidar Helvik, University of Bergen (Professor, Head of Committee)
Ivar Hordvik, University of Bergen (Professsor)
Terje van der Meeren, Institute of Marine Research (Senior Scientist)
Helge Ressem, Profunda /Norwegian Seafood Federation (FHL) (Manager)
Manfred Schartl, University of Würzburg (Professor)
Harald Sveier, RCN (Advisor NRC, Secretary of Committee)
Helge Tveiten, Norfima Tromsø (Senior Scientist)
Gunvor Øie, SINTEF Fisheries and Aquaculture (Senior Scientist)

1.3 External contributors

Howard Browman, Institute of Marine Research (Senior Scientist)
Lars Ebbesson, University of Bergen (Senior Scientist)
Jan Ove Evjemo, SINTEF (Scientist)
Trine Galloway, SINTEF (Research Director)
Synnøve Helland, Nofima Marin (Scientist)
Elin Kjørsvik, NTNU (Professor)
Anders Mangor Jensen, Institute of Marine Research (Group Leader)
Mari Moren, NIFES (Scientist)
Lisbeth Olsen, SARS (Associate Group Leader)
Ingrid Overrein, SINTEF (Scientist)
Ivar Rønnestad, University of Bergen (Professor)
Jorunn Skjermo SINTEF (Senior Scientist)
Øystein Sæle, NIFES (Scientist)
Bendik Terjesen, Nofima Marin (Senior Scientist)

2 Summary

The fish larva is a transitional life form that develops from the spawned egg through various embryonic stages, with yolk as its only nutrient and energy supply, until it finally hatches into a free-living larva able to catch and digest prey organisms. In nature, survival and success of the larva is mainly dependent on food supply and avoidance of predators, with only a few larvae surviving through metamorphosis to become juvenile fish that can be recruited into the fish stock. Under culture conditions, the success rate for fish larvae is much higher due to regulated food supply and absence of predators, but even under such conditions the mortality rate is high and can vary between batches. All organs and biological systems develop during the embryonic and larval stages, and how these systems are established during early development will influence how the fish performs later in life. As opposed to farm animals, whose most sensitive life stages occur inside the mother in a constant environment and with a steady supply of nutrition, fish in their early stages directly contend with a fluctuating, harsh environment.

Many of the challenges in fish aquaculture – not only raising larval survival rates, but also producing juveniles of high quality that are robust and will perform well later in life – are rooted in the larval stage. In response to this reality and the fact that allocation of research funding for early life stages of fish has been reduced dramatically in the last decade, the Aquaculture programme of the Research Council of Norway put together a committee of specialists with a mandate to review current knowledge on early life stages of fish species relevant for Norwegian aquaculture and fisheries. The committee was to examine the relevance of implementing studies of model species such as zebrafish, and recommend short- and long-term research strategies.

The strategy for making this report was as follows: First, the current knowledge of development of important biological processes was reviewed by leading specialists in Norway, whose contributions are listed as review papers at the end of the report. Secondly, knowledge of and challenges relating to aquaculture juvenile production were analysed together with the importance of understanding larval development in relation to natural ecosystems and recruitment. In addition, environmental effects such as nutrition, physical factors and anthropogenic pollution were discussed. All challenges were then considered within the context of available resources and infrastructure, and a new research strategy was recommended.

In general there is a lack of basic knowledge of biological processes important for larval development and performance. Often consequences of culture deficiencies manifest as low growth rate, abnormal development and high mortality, and with the current status of knowledge we are seldom able to pinpoint the mechanisms involved. Achieving predictable juvenile production of high-quality fish that perform well later in life requires a high level of control of various factors influencing normal development and growth, such as nutrition and physical environment. Optimising production will require detailed analysis and understanding of the underlying biological mechanisms.

Our understanding is also insufficient regarding larval life in relation to the natural environment, e.g. effects of oil pollution and potential effects of ocean acidification due to climatic changes. Our current knowledge gaps and lack of necessary tools may render us unable to see or measure important effects.

The current situation in Norway consists of fragmentary communities in research and industry that work on various aspects of fish larvae from different interests. Fish larval development is central to aquaculture research, fisheries and early life history research, and basic developmental biology research. There has been little investment in developmental research in Norway. This is

especially worrying since development is an important area of basic research internationally and is fundamental to Norwegian aquaculture and fisheries.

The HAVBRUK programme will benefit from basic knowledge continually generated by over 1,000 zebrafish laboratories worldwide, which will help in creating molecular tools that can bridge the information about model species to our key species (Atlantic cod, salmon, halibut, herring and wolffish) in order to obtain a deeper understanding of biological processes occurring in these diverse fish species during development and how they interact with the environment.

In general there is little focus on fish larval stages and metamorphosis, even by the international communities. In fact, the laboratories working on fish models (zebrafish and medaka) seek insight into human development and disease rather than basic problems related to fish biology such as larval life and transformation during metamorphosis. However, this also provides a great opportunity for Norway to use these models for basic research on more fish-related problems.

The working group's recommendations

The Research Council of Norway should establish a new strategic programme in fish larval research. This programme should finance the research on early life stages of fish, organising research activities into a single community/network working on fish larvae and comprised of researchers from basic developmental biology, aquaculture, ecology/early life history, toxicology and climate change.

- From 2010, establish a scientific board for fish developmental studies and organise a fish larval network/platform that includes common genetic resources. Establish common fish resources (embryos, larvae and juveniles). Initiate new research projects within two sub-programmes: Development of form and function and Nutrition and development.

- From 2011, increase the funding for existing activities and start a new sub-programme: Development and environment.

- From 2012, organise the activities into a new research programme: Fish development/fish larvae.

Such a programme would become a driving force for this important interdisciplinary area and secure long-term funding to build up the competence to make Norway an international leader in marine cold-water teleost developmental research and environmental impact. Such a programme would contribute to creating sustainable, successful aquaculture production and improved management of natural resources threatened by pollution and climate change.

3 Sammendrag

Fiskelarvestadiet representerer en midlertidig livsform som dannes etter den embryonale perioden hvor plommemassen fra morfisken har vært den eneste tilgangen på næring og energi. Ved klekking går larven over til et frittlevende stadium hvor den fanger og ernærer seg på byttedyr fra omgivelsene. I naturen er larvens overlevelse og videre suksess avhengig av tilgang på byttedyr og unnvikelse fra predatorer. Bare en liten andel av larvene overlever fram til metamorfose og blir yngel som senere rekrutteres inn i de naturlige fiskebestandene. I oppdrett er overlevelsen mye høyere enn i naturen siden larvene har god tilgang på føde og ingen predatorer finnes i systemet. Men selv under slike ideelle forhold er dødeligheten høy og svært variabel. I den embryonale og tidlige larvefasen dannes alle organer, og de fleste biologiske systemer blir etablert. Påvirkning i denne kritiske perioden kan derfor ha svært negative effekter og innvirke på fysiologien, funksjonen og veksten i senere stadier. I motsetning til dyr i tradisjonelt landbruk hvor alle tidlige og sensitive stadier utvikles inne i moren under konstant miljø og ernæring, møter fisk i tidlige livsstadier et variabelt og uforutsigbart miljø allerede fra gyting.

Mange av utfordringene i oppdrett av fisk – ikke bare å øke graden av overlevelse, men også evnen til å produsere yngel av høy kvalitet som er robust og voksevillig senere i livet – henger nøye sammen med påvirkninger under larvestadiet. Som en erkjennelse av denne sammenhengen og begrensede ressurser til forskning på tidlige livsstadier hos fisk, nedsatte Havbruksprogrammet i Norges forskningsråd våren 2008 en arbeidsgruppe for å evaluere forskningsinnsatsen på dette området med relevans for norsk havbruk og fiskeri, samt å se på hvordan en kunne implementere bruken av modellarter som zebrafisk, i videre forskning. Arbeidsgruppen skulle videre foreslå en plan for forskning innen fagfeltet "Tidlige livsstadier hos fisk".

Evalueringen fulgte følgende prosess: Først ble kunnskapsnivå i sentrale biologiske problemstillinger knyttet til tidlig utvikling hos fisk evaluert ved hjelp av sentrale nasjonale forskere på sine respektive områder. En detaljert gjennomgang av de ulike fagområdene er gitt i slutten av rapporten. Problemstillinger knyttet til yngelproduksjon og eksisterende utviklingsbiologisk kunnskap ble diskutert, samt viktigheten av å forstå fiskelarver i relasjoner til sitt naturlige miljø og rekruttering i ville bestander. I tillegg ble viktige ernæringsmessige, fysiske og andre miljøfaktorer som forurensning og havforsuring diskutert. Videre ble utfordringer for fagfeltet evaluert og sett i sammenheng med de muligheter som ligger i eksisterende ressurser og forskningsinfrastruktur, og lagt til grunn for arbeidsgruppens anbefalinger.

Generelt mangler det kunnskap relatert til basale biologiske prosesser som er viktige for forståelsen av marine fiskelarvers fysiologi, utvikling og funksjon. Konsekvensene i oppdrett manifesterer seg ofte i lav vekstrate, feilutvikling og høy dødelighet, og med dagens kunnskap er man sjelden i stand til å peke direkte på hvilke mekanismer som er relevante for å forklare de suboptimale resultatene. Stabil produksjon av yngel med høy kvalitet som vokser og utvikler seg optimalt, krever kontroll av viktige faktorer som kan påvirke normalutvikling, f.eks. ernæring og fysisk miljø. En optimalisering av yngelproduksjonen vil kreve mer detaljerte analyser og forståelse av underliggende biologiske mekanismer. Vår forståelse er også mangelfull i relasjon til fiskelarvers utvikling i det naturlige miljøet og hvordan miljøendringer som oljeforurensning og klimaendringer, inkludert forsurning, kan påvirke disse tidlige stadiene. Mangel på kunnskap og relevante verktøy kan hindre oss i å måle og oppdage viktige effekter som kan ha store konsekvenser for våre naturlige økosystemer.

Forskningsmiljøet på fiskelarver i Norge består av fragmenterte grupper med ulike interesser innen basal biologi, akvakultur og forvaltning. Tidlige livsstadier er en sentral del av akvakultur,

fiskeøkologi og forvaltning, og i grunnforskning innen generell utviklingsbiologi. Norge har investert lite i utviklingsbiologisk forskning, selv om dette er et stort og viktig fagfelt internasjonalt. Samtidig er det et viktig fundament for utviklingen av norsk havbruk og fiskeri.

Norsk havbruk drar nytte av all den basale kunnskap som stadig produseres i mer enn tusen zebrafisk- laboratorier verden over. Utfordringen er å utnytte best mulig den kunnskapen og teknologien som etableres her, og som blant annet omfatter grunnleggende molekylærbiologiske teknikker og relsultater innen genuttrykk. Å bygge bro mellom modellarter og våre nøkkelarter, som torsk, laks, kveite, sild og steinbit, vil være en viktig strategi for å øke forståelsen av fundamentale biologiske prosesser i disse artene som har en bred fylogenetisk opprinnelse og ulik tilpasning til det naturlige miljø.

Generelt er det lite fokus på fiskelarver og metamorfose i internasjonal basalbiologisk forskning. Laboratorier som studerer zebrafisk, er interesserte i grunnleggende molekylærbiologiske mekanismer relatert til human utvikelig og sykdom, og problemstillinger relatert til fiskelarvebiologi og metamorfose er derfor fraværende. Imidlertid bør dette være en mulighet for Norge til å fokusere på fisk som modellart innen tidlige livsstadier, både innen eksisterende modellarter fra ferskvann og nye arter fra det marine miljøet.

Arbeidsgruppens anbefalinger

Norges forskningsråd bør opprette et nytt strategisk program innen feltet fiskelarvebiologi. Programmet skal finansiere forskning på tidlige livsstadier hos fisk og organisere et felles forskningsnettverk bestående av en interdisiplinær samling av forskere fra basal utviklingsbiologi, havbruk, økologi, forvaltning, toksikologi og klima.

Følgende opptrapping foreslås:

- Fra 2010 etableres det en vitenskapelig komité for fiskelarveforskning og et forskernettverk/
  -plattform. Det etableres en struktur for genetiske ressurser og felles oppdrett/produksjon av biologisk materiale (embryo, larve og yngel) som analyseres i nettverket. Initielt startes to underprogrammer: ”Utvikling av form og funksjon” og ”Ernæring og utvikling”.
- Fra 2011 økes aktiviteten i det to eksisterende underprogrammene, og et nytt underprogram ”Utvikling og miljø” etableres.
- Fra 2012 tar en sikte på å samle og bygge opp aktiviteten i ett felles strategisk program.

Målet for programmet er å være en drivkraft for å etablere et interdisiplinært fiskelarvemiljø i Norge som er internasjonalt ledene på utvikling og forståelse av tidlige livsstadier hos marine fisk, inkludert laks, og hvilken effekt miljø og menneskeskapte parametere har på utviklingen i tidlige livsstadier. Et slikt program vil være sentralt for en bærekraftig og suksessrik oppdrettsnæring, samt for forbedret forvaltning av naturlige ressurser som er truet av forurensning og klimaendringer.

4 Vision

Most fish species go through a vulnerable larval period after the protected embryonic stage inside the eggshell and before they transform into more robust juvenile fish. Many of the problems related to juvenile fish production in aquaculture and understanding the natural variation in fish populations are linked to the larval stage. In contrast to livestock animals, which go through all vulnerable periods either inside the mother (pigs, sheep, etc.) or in large eggs (chickens), most fish species produce a huge number of small eggs that hatch early in development and are dependent on environmental conditions for normal development and survival.

The purpose of this evaluation is to focus on fish larvae and to draw needed attention to this critical life period so important for fish aquaculture and fisheries and for understanding anthropogenic impact on the ecosystem.

Several factors indicate that now is the time to investigate fish larval biology. Firstly, this field has become more accessible for experimental exploration thanks to the advances of recent years in culturing marine fish species. It is now possible to investigate natural larval responses under controlled laboratory conditions. Secondly, recent advances in developmental biology of fish using genetic model species such as zebrafish have amounted to a quantum leap in functional and mechanistic understanding of many biological processes, in particular embryonic development. Larval and adult development, however, have not been adequately addressed. Although an outstandingly useful toolbox has been assembled, it has hardly been utilised for addressing the urgent issues of larval biology outlined below. Thirdly, our ecosystems are threatened by climate change and pollution, and a deep understanding of fish larval biology is required to deal with these impacts.

The vision of this programme is to gain basic understanding of development of fish eggs and larvae, e.g. form and function and the influence of environment. Combining studies of salmon and marine fish species important for Norway with technology and advances in model species will create the needed platform necessary for streamlining the bottlenecks in aquaculture and understanding an ecosystem in transition.

5 Background

In recent years, there has been a reduction in the overall funding of research on the early life stages of fish (NIFU, STEP 2007 analysed the period 2001–2005), and this trend persisted in last year's allocations from the Research Council's Aquaculture programme. Funding for other aquaculture areas during the same period (2001-2005) increased 40 percent on average.

In the pioneering years of marine fish culture, research on early life stages was a foundation for the creation of production lines for the various species. Today the technological advances in this area have led to commercial production of several marine fish species. Experience has shown that marine fish larvae culture is complicated and requires the creation of a food web, including algae and live feed/zooplankton cultures. With the fish larvae culture systems up and running, the research focus changed to other areas such as disease before a biological foundation for sustainable production of marine fish was truly established. We still struggle with very high mortality and malformation, with little knowledge of what a high-quality juvenile fish actually is, i.e. one that will perform and grow optimally later in life. Early-life performance has effects on later growth and development, as shown in Atlantic cod where larvae fed natural zooplankton perform better in life than larvae fed rotifers and *Artemia*. In general, previous scientific investment has focused on creating production lines, but there has been little investment in further improvement and optimisation.

In the early period when the aquaculture wave hit Norway, there was very little fundamental knowledge of fish larval development beyond general descriptive studies of larvae sampled from the open sea. So basic biology became the starting point in trying to understand development and larval needs at various stages. Much of this initial work was done on larvae grown under suboptimal conditions; researchers were lucky if the fish survived an entire experiment. But progress in this area has made marine fish larvae accessible for all kinds of experimental studies.

In addition, interest in fish development has skyrocketed as fish have become a major model for understanding the development and function of biological processes in vertebrates. Today more than 1000 laboratories worldwide are using zebrafish or medaka as their model animal. This community's scientific output is amazing and has become the major source of information for understanding not only fish biology but also biology in general, with important implications for veterinary and human medicine.

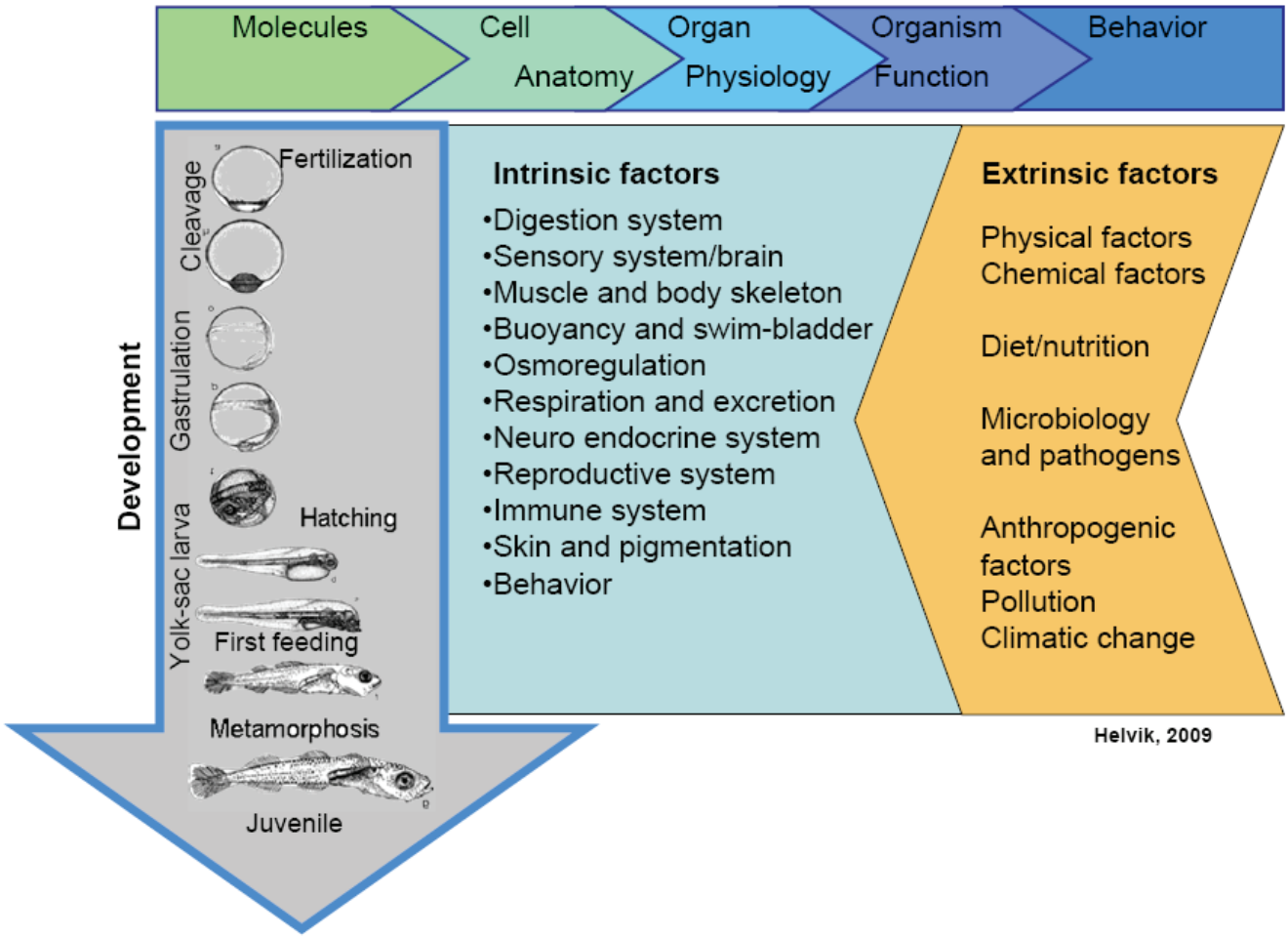

***Figure 1**. Development is a combination of the proliferation, migration and differentiation of cells into organs and a functional organism. All these processes are closely regulated by molecular mechanisms. Environmental conditions may influence development directly, by acting on the regulatory network and influencing the phenotype, or indirectly, through nutrition and factors that can damage developmental processes. Different environmental factors interact with the various intrinsic or biological processes.*

The aquaculture industry relies on advances in research and development, since the basis for production is the control and manipulation of complex biological systems. The progress of this industry therefore depends on recruiting the best candidates who can generate the knowledge needed for the future. But areas such as biomedicine and veterinary sciences compete for the same candidates, and their institutions can offer research profiles and programmes that are more long-term than what has been offered in aquaculture, where a short-term bottleneck approach has been the general rule.

6 Status and challenges

6.1 Juvenile production in aquaculture

Major improvements in juvenile production of Atlantic cod have been achieved in recent years, with production reaching 14 million in 2007. Production is expected to be 18-20 million juveniles in 2008. Most used is the intensive production approach, but both semi-intensive methods (plastic bag mesocosms) and extensive production in lagoons contribute to this production. However, the industry struggles with several issues that constrain juvenile production. These issues show similarities over a range of species, and will be presented here with mostly cod as an example. Input on the following issues has been collected from views expressed by contacts at several Norwegian cod hatcheries, among others.

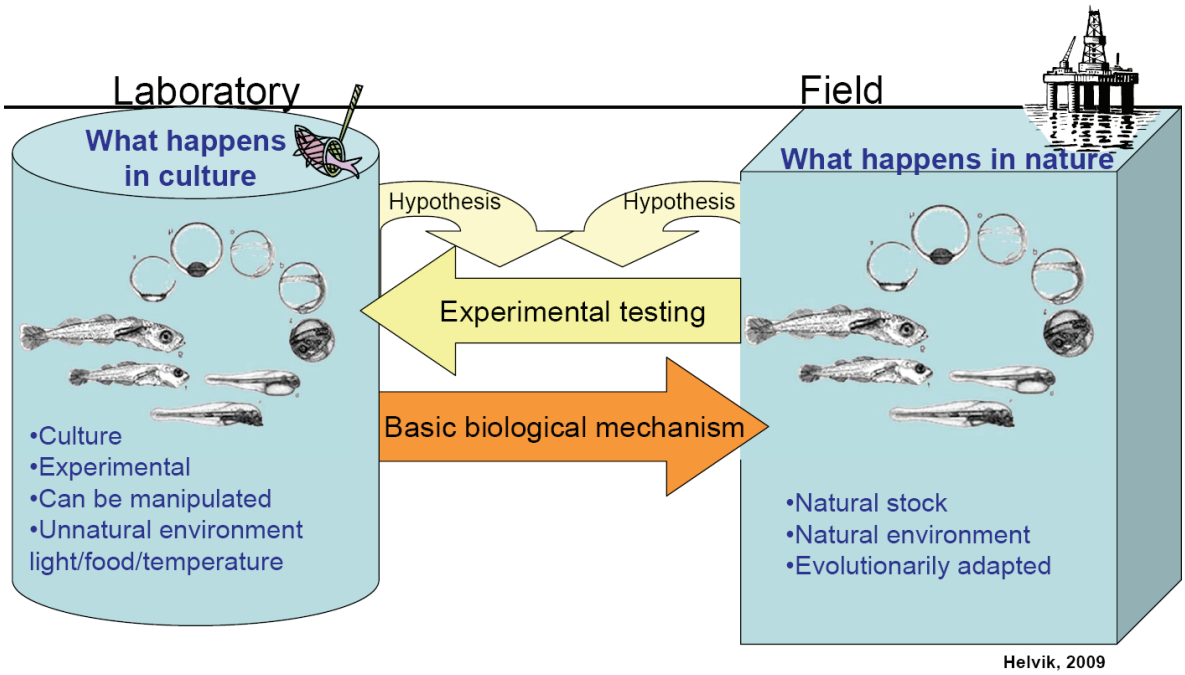

***Figure 2**. During the years of establishment of marine juvenile production, researchers applied much information and many ideas from the natural environment to which the various fish species and developmental stages are adapted. Culture situations allow a high degree of control and the ability to manipulate various factors that are important for better understanding the fundamental processes occurring in the natural environment.*

Quality of eggs and newly hatched larvae

In many hatcheries, egg availability and quality are key factors for stable juvenile production. The cod hatcheries express needs for parameters to assess "high egg quality" or "good larval quality" at the onset of feeding. Today, egg quality varies throughout the year, with variable hatching success and synchronisation. To obtain consistently high egg quality, it is important to give more attention to possible parental factors that can affect larval and juvenile quality. Broodstock welfare and nutrition are regarded as key factors, and the role of these on subsequent larval quality is largely unknown and needs to be addressed. Typical parameters for the evaluation of success in larval fish rearing are factors such as survival, growth, hatching rate, and behaviour related to foraging, the latter often evaluated subjectively based on experience of the staff operating the start-feeding units. However, these parameters are far from being adequate to explain causes of observed effects or incidents.

Larval nutrition, growth and survival

One of the most important bottlenecks in larval and juvenile production of marine fish is the live feed quality. It has become evident that larval fish have specific nutritional requirements that can be linked to the biochemical characteristics of their natural prey, namely the copepods. However, access to large-scale production of copepods is limited, and the nutritional requirements of fish larvae have induced an extensive international research effort to obtain a nutritionally adequate standard of the most commonly used prey in larval fish culture: rotifers (*Brachionus* sp.) and *Artemia*. Copepods harvested from ponds or intensively cultivated copepods are excellent prey for marine fish larvae, but harvested copepods may be a potential vector for parasites and pathogens. Further development of intensive copepod cultures may therefore be beneficial for future research in this area. Fish larvae produced on copepods have a substantially higher growth rate, lower mortality rate, and lower prevalence of bone deformations than fish larvae grown on rotifers and *Artemia*. It is therefore important to reveal the actual mechanisms that yield the observed benefits of copepods and transfer this to intensively produced live feed. In summary, the most important question related to nutrition, growth and survival is: W*hich nutritional and microbial characteristics of the prey and larval cultures create morphologically and immunologically robust, healthy, fast-growing, and viable larvae and juveniles?* Further improvements in this area can only be achieved by new knowledge-based advances in live feed production and larval rearing protocols and systems. Even small enhancements in larval growth and quality may create large improvements in the other end when it comes to fish size and juvenile quality at time of stocking into net pens.

Abnormal development

Besides problems with low growth rate and high mortality, some cod hatcheries struggle with high incidences of juvenile bone deformities. Deformities are undesirable in terms of fish welfare and represent a significant financial loss for the farmers, because deformed fish are graded out and discarded before vaccination. The variable quality of larvae and juveniles causes low predictability and profitability and may increase production costs to unacceptable levels. So there is great potential for improvements in cost efficiency by increasing growth and survival rates and reducing the prevalence of deformities. An example of such an improvement may be the problem with incomplete eye migration in Atlantic halibut, which was solved by simply starting to apply photoperiods during larval rearing. Up to 40-60% of halibut fry used to be discarded due to incomplete eye migration, but now this has been greatly improved. Bone deformities in juvenile marine fish such as cod can occur in a number of varieties, extending from the head to the tail regions. There is a striking difference in occurrence of deformities between intensively reared juveniles and juveniles reared in semi-intensive or extensive systems. However, it is not known to what extent the causative factors of these differences in deformity incidents are to be found in the environment or in the larval nutrition. Although some Norwegian cod hatcheries claim that bone deformities are insignificant, it is considered a major problem in other hatcheries where a substantial proportion of juvenile production must be discarded.

Rearing environment and water quality

There is a need for more detailed knowledge about how water quality and the rearing environment (e.g. light conditions, temperature, turbulence, noise, etc.) affect larval and juvenile performance and development. The intensive rearing situation is very different from the natural environment, and knowledge about how the physical environment influences larval and juvenile stress and physiology is needed to continuously improve the rearing systems and hence juvenile quality. This also includes issues on habituation and adaptation to the rearing conditions. Regarding water quality, more knowledge is required about how the microbial environment interacts with development of the immune system and gut functions in larval fish. Microbes are constantly presented to the digestive system of marine fish larvae by ingestion of feed and by drinking for

compensation of lost water due to osmotic stress. The use of sterilisation techniques (e.g. UV treatment) is common in commercial hatcheries with an open flow-through water supply, but the benefits of this as compared to other methods of water quality control (e.g. recirculation) need to be further investigated. Finally, obtaining knowledge about larval and juvenile tolerance in relation to metabolites and the physical environment in a welfare context must not be forgotten.

Long-term effects

Special attention should be drawn to developmental perturbations that extend into later life stages. Optimisation during the larval and juvenile stages will be reflected in the performance of the fish during ongrowing, which is the most cost-intensive phase of fish farming. Areas of particular attention here include, for example, issues related to control of maturation and reproduction, energy storage and metabolism, and behavioural aspects. These issues may potentially lead to large losses for the fish farmer.

Interactions with wild stocks

In contrast to salmonids, captive marine fish will spawn in the fish farms and release substantial amounts of fertilised eggs into the environment. For cod, control of maturation by use of light in outdoor cages has proven difficult. In addition, cod has shown "Houdini-like" behavior in escaping from net cages. Further, breeding programmes will quickly create a substantial genetic difference between wild and farmed fish. These issues all require control of reproduction in farmed marine fish. Solutions to the problem may be use of non-fertile fish (e.g. triploid fish) or mono-sex populations to prevent fertilisation of eggs. Both these aspects imply profound research challenges along the axis of broodstock-eggs-larvae-early juveniles.

6.2 The natural ecosystem

Recruitment and Management

A major impediment to understanding and modelling recruitment variability is the lack of information on biological and physical determinants of growth and survival during the early life history of fish species. One area where we have remarkably little information and understanding in the early life history of fish is natural mortality, from eggs through the pelagic phase, settlement and the nursery phase, to when a year-class recruits (which is species-specific). The mortality rates are high and minor changes can lead to large changes in the absolute number of survivors (recruits). At present we do not have good measures of the shifts in mortality rates through the life history nor a reasonable understanding of the processes that can be used to construct realistic models. Model predictions must be tested against process studies under natural conditions. This will involve the use of necessary tools and methods to characterise the state of individuals (age, size, condition and survival probability) in mesocosm and field settings and comparison of the results with model predictions. Some of the research areas that require investigation are:

- Egg/offspring quality in the context of spawner reproductive potential – leading to survival rates that are determined by the parents
- Predation rates and the identification of principal predators
- Principal prey, prey search and encounter rates and the potential effects of variable prey fields on either starvation or predation
- Transient impacts on life history processes (e.g. thin layers; turbulence; storms)
- Bridging the gap between the temporal and spatial scales on which the processes that affect early life stages occur, and sampling to assess these processes
- Swimming and orientation capacity in early life stages
- Metabolic costs of activity

- Temperature and size-dependent growth capacity and requirements (climate change context)
- Transport and connectivity between spawning and nursery areas, possibly within the context of the member/vagrant hypothesis, metapopulation dynamics and climate change
- Habitat quantity and quality in nursery areas and its impact on mortality rates; habitat destruction resulting from human activity
- Behaviours of young fish that are essential for survival or tipping points in the life cycle (e.g. settlement)

Anthropogenic factors

Waste products from humans can be found in every sea on earth. Most concern relates to biologically active chemicals with very low degradation rates, often because they are "artificial" and therefore not degraded by most natural enzyme systems. Such compounds can accumulate in the food web to levels that make marine animals unsuitable for human consumption, as these chemicals interact biochemically at very low concentrations. Long-living animals, usually top predators, are most to accumulating these pollutants. Since these chemicals often are lipophilic, they are transported along the marine food web by oil-rich algae, zooplankton, and fish. Larval fish is not at the top of the food web, but can be enriched maternally through the oil-rich yolk. In addition, larval fish undergo tremendous growth from hatching to juveniles, and consume large amounts of oil-rich zooplankton. Bio-active chemicals with shorter degradation times may have effects in coastal regions. It should be noted that fish stocks of major importance like cod and herring have their spawning grounds in near-shore waters. Furthermore, some chemicals have hormone-like functions and may interact with regulatory functions even at very low concentrations. Minor perturbations at any stage of larval development may potentially cause notable defects later, e.g. in the reproductive system as observed in freshwater fish in polluted rivers. Anthropogenic factors in the marine environment comprise a number of chemicals and compounds from various sources, as listed below:

- Chemicals from sewage (e.g. hormones, medicines, cosmetics, detergents)
- Petroleum industry discharges of oil and chemicals and effects of dispersion agents
- Antifouling agents
- Heavy metals
- Persistent organic contaminants (e.g. PCBs, chlorinated pesticides, dioxins, brominated flame retardants)
- Pesticides and fungicides

Other anthropogenic impacts on larval and juvenile fish may be related to specific activities or climate-related issues. Examples of such may be offshore petroleum exploration by use of seismics and acidification of the oceans by increased atmospheric carbon dioxide (CO_2) levels. The impact of CO_2 is global, and research on the indirect or direct effects of acidification on larval fish development is urgently needed.

6.3 Biological processes

The epigenetic changes and life history context is understood as the "setting" of physiological function by conditions during a sensitive developmental period to produce long-term effects on function and thereby on lifelong performance. A wealth of data for various species, from simple organisms to humans, supports the concept that environmental conditions in early life "programme" permanent changes in structure and function in the offspring.

The concept originates from the mammalian/human paradigm of *Developmental Origins of Health and Disease*, whose basic premise is that undernutrition, for example (or any other

treatment), during one stage in life can influence physiology, performance and susceptibility to disease later in life. The classical example is that people conceived during the Dutch winter famine of 1944-45 were not necessarily of smaller birth weight, but as adults they became prone to insulin resistance and obesity. Similar observations have been reported in sheep, in which periconceptual undernutrition has shown to reset the HPA axis (the HPA axis seems particularly prone to programming), and in rodents, where the conditions in which the preimplantation embryo develops will later influence fetal growth and postnatal phenotype.

Mechanistically and experimentally, there is an increasing body of knowledge showing that manipulation of the environment in the period extending from conception to infancy can be associated with permanent changes in physiology and/or structure. Such persistent effects of developmental plasticity, which are not determined by classical DNA sequence-related genetic mechanisms, have been proposed to help optimise the fitness of the organism to its predicted environment. Many of these changes are associated with permanent alterations in gene expression regulated by epigenetic factors such as DNA methylation and histone methylation / acetylation. In animal models, the HPA axis is altered following maternal undernutrition, glucocorticoid exposure in utero, or by manipulation of the neonatal behavioral environment. Alterations in the number of glucocorticoid receptors in regions of the central nervous system underpin these changes. Other examples include alterations in insulin secretion and insulin action, changes in hepatic enzymes regulating glucose metabolism, and endocrine systems with decreased growth hormone and IGF-1 activity. The central nervous system can also be targeted for developmental induction. Appetite, for example, and the neurotransmitters regulating it can be permanently altered by perinatal experimental manipulation in the rat. However, overnutrition, oxygen, and other environmental factors also appear to have programming effects.

The adaptive value of altered physiology (programming) depends on the mismatch between the anticipated and the actual mature environment the organism is exposed to – the greater the mismatch, the greater the risk of adverse consequences.

From a basic point of view, fish may be a good model to study such effects, since they have high fecundity, display large developmental plasticity and undergo a large part of their development outside the maternal body. Furthermore, if we put this into the context that farmed fish is "pushed" through different stages of early development, it is imperative to gain knowledge about what impact this may have on physiology, metabolic capacity and susceptibility to disease during subsequent life stages. For example, high temperature during incubation, inadequate start feeding, nutrient-dense feed, high feed intensity, elevated temperature etc. contribute to this pushing. The extremely efficient farming conditions as such may therefore induce physiological and metabolic changes, positive or negative, that may be manifested in the fish throughout the life cycle.

6.3.1 Early development, maternal effects and endocrinology

The earliest steps in embryonic development are dependent on and driven by maternal factors deposited in the oocyte during oogenesis. Maternal factors are stored in the form of specific mRNAs, proteins, hormones or any other biomolecule. At egg activation and fertilisation, such factors become available for embryogenesis, sometimes after a process of activation involving translation or protein modification. Very little is known about how these maternal factors influence embryonic and larval developmental capacity. There is a need to identify genes and maternal factors involved in early embryonic development and search for links between regulatory factors of maternal origin and "egg quality". Investigating the link between broodstock holding conditions, regulation of oocyte gene expression, and the developmental capacity of the resulting embryo and larva will be important.

After fertilisation, growth and ontogeny of the embryo and larvae follow a well-defined and genetically programmed sequence in which hormonal regulation is of critical importance. During the embryonic phase the endocrine organs develop, but are not functional, and hormones produced locally or of maternal origin may fulfil regulatory needs. Around the time of hatching there is a transition phase when larval endocrine organs begin to function but hormonal levels remain very low. In the post-hatch larva there is an accelerated activity and functionality of the endocrine organs which is accompanied by large fluctuations in hormone levels. The basic molecular mechanisms involved in functional maturation of the different endocrine axes in fish are far from understood and need to be elucidated. Little information is available about the influence of environmental factors, both exogenous and endogenous, on development and functionality of the different endocrine systems. Impaired development during embryonic and larval stages may have long-lasting effects and eventually influence the physiological performance of the adult fish.

6.3.2 Development and function of fish sensory system

A successful cultural system or production line for fish is dependent on our abilities to understand and implement the perceptual abilities of fish and how their sensory systems change during development. Such information also lends us the insight into adaptation to the natural environment and ecological interactions between the organisms and their communities. Central questions in sensory biology are how the organism senses the physical world, how this information is transmitted in order to perform certain tasks, and how particular environments may alter signals in ways that restrict the ability of animals to obtain information about potential prey, predators or mates.

Most comprehensive analyses of sensory systems in fish are done on zebrafish, including the photoreceptor system of the retina and pineal organ, the chemosensory system of the olfactory epithelium (smell) and taste buds, and the mechanosensory system of neuromasts, lateral line and ear. Among the key species of this evaluation, the salmonids are quite well studied, in terms of structure and changes in their visual system during smolt transformation as well as the olfactory system in relation to homing. Except for some solitary papers on marine fish species, mostly on the morphological structure of the sense organs, there exists little literature on functional analysis, and a deeper comparative understanding of differences among species and specialisation to environment is needed.

The sensory systems integrate the surrounding world and are critical for many internal processes such as circadian rhythm and behavioural decisions. Normal development and long-term performance depend on appropriate environment and stimulation during the critical early life stages of fish.

6.3.3 Brain and neuroendocrine system

The brain is the integrator of external and internal signals and its proper function is dependent on normal brain development. Present knowledge of morphological, genetic, physiological and behavioural processes in key target fish species during critical developmental periods is inadequate and often non-existent. Understanding brain development and the manifestations of “unnatural” or changing rearing environments will provide an important framework for evaluating impacts and attaining solutions associated with key critical phases in the culture of marine teleosts and salmon. The basis of knowledge of brain development in teleosts has been derived from comparative neuroanatomical approaches spread among a vast diversity of fish species, with the majority of the molecular regulation recently established in zebrafish. As with all vertebrates, general themes drive the moulding of the functional brain, including early neural developmental processes from fate maps to genetic control, neurotransmitter ontogeny, and environmental input and stimulation. In general, neural developmental processes are similar among vertebrates,

enabling one to draw knowledge from different model species. But without proper foundations within the target species, one cannot evaluate environmental impacts and long-term consequences on populations.

6.3.4 Behaviour

Behaviour is the manifestation of an organism's response to both internal (physiological) and external (environmental) signals. Observing how fish larvae behave under various environmental and feeding conditions provides information that is directly relevant to the development of appropriate culture protocols for fish.

Feeding fish larvae are more active and swim faster and longer than those that are not feeding. Issues frequently targeted in behavioural research are activity rhythms, swimming patterns, foraging and prey search, and prey capture efficiency. A detailed awareness of diurnal and seasonal activity patterns – and ontogenetic changes in them – will allow for the development of culture protocols that are better tuned to the basic biology of the species being reared. Behavioural observations of the facility with which larvae can locate, attack, ingest, and retain feed particles are also of central importance. Thus, identifying substances that can motivate the feeding response of marine fish larvae, and increase the probability that they will retain and digest it, holds promise for the rapid improvement of e.g. formulated feeds. Attractiveness, palatability, and retention of live and manufactured feed need specific attention, particularly at weaning from live to inert diets. Very little is known about the olfactory and gustatory responses of marine fish larvae. Electrophysiological and behavioural techniques can be used to generate concentration response curves for various substances and to characterise how the fish behaves in their presence.These techniques are akin to asking these animals which smells and tastes they can perceive, which they prefer, and perhaps more importantly, what they do not prefer. Direct observations of fish behaviour are therefore essential (e.g. during transitions such as the weaning period).

Behavioural indicators may also be used to assess state of stress and/or welfare. Until recently, very little information was available about the general behaviour, stress levels, discomfort/pain, anxiety/ fear, and "comfort" conditions of aquatic animals in culture situations. This kind of data is required to inform discussions and to guide policy development. There is virtually no information of this nature available about fish larvae and early juveniles.

6.3.5 Buoyancy and Osmoregulation

Eggs from various teleosts exhibit the same osmoregulatory traits of keeping the body concentrations fairly constant throughout development. In withstanding the osmotic pressure and protecting the egg cell from desiccation, the vitelline membrane has proven to be the most watertight biological membrane ever examined.

The low-osmolality body tissues of the developing eggs also serve another important feature – buoyancy. Since eggs have no option of locomotion, lift in the water must remain static by reduced mass. Osmoregulation and buoyancy regulation in eggs rely on membrane permeability and reduced ionic fluxes. Effects of environmental changes and impacts in connection to climate changes, anthropogenic pollution and petroleum activity may irreversibly affect egg homeostasis during the first fragile embryonic stages.

After hatching, the larvae in a marine environment develop further towards adult osmoregulation mechanisms that include drinking and salt secretion. In order to achieve a net gain of water by drinking, the larvae possess osmoregulatory mechanisms including salt secreting cells and chloride cells, normally associated with skin and gill filaments in young and older larvae, respectively. Insufficient osmoregulation may occur during intake of feed with low water content, or in unfavourable temperature conditions. These mechanisms have not been studied in detail.

At early larval stages, marine pelagic fish larvae develop large water-filled compartments along the body sides, called subdermal spaces. These contain low-osmo fluid that aids in achieving buoyancy. However, most teleost fish regulate their volume-specific mass with an air-filled swim bladder. The bladder may be closed (physoclist fish) or open to the oesophagus by a duct (physostome fish). First inflation of the swim bladder has been studied in a number of fish species, and there seem to be differences already at the larval stages. Some species have been shown to be dependent on atmospheric air from the surface for initial filling, while others seem to be able to secrete air into the bladder from the start. Failure of physostome initial inflation has been observed in connection with polluted surfaces. These mechanisms may be very important in future evaluation of e.g. oil spill risks.

6.3.6 Respiration and excretion

In fish early life stages, little convective gas transport occurs before circulatory function is established. In zebrafish oxygen receptors are found very early in larval development, although data from other species are few. Autonomic nervous systems controlling cardiac function, however, are only fully developed during later stages, but large species variation exists.
The concept of unstirred water boundary layers around the embryo and larva is important for interpretation of gas exchange in fish early life stages, since it is often considered to be one of the major resistance barriers to oxygen transport.

However, little is known about these aspects in our cultured marine species, and under industrial aquaculture conditions. For instance, the switch from cutaneous to gill gas exchange, and the molecular control mechanisms for this process, may be valuable to study in relevant species. Further, it has been reported that oxygen deficiency results in vertebral deformities in red sea bream, when embryos are exposed during somitogenesis. These findings warrant investigation in other species.

Recently, microarrays have been used to study a number of energy metabolic genes during development of sea bass, contributing to the understanding of preferred substrates for catabolism. However, compared to this species and zebrafish, relatively little is known about expression of genes involved in energy metabolism, and especially their control, during early development of Atlantic cod and halibut.

Considerable progress has been made in recent decades in the field of nitrogen metabolism and excretion in fish early life stages. One central rule is that the majority of nitrogenous end-products are in the form of ammonia, of which especially the gas NH_3 is toxic, dictating that this compound must be kept at low concentrations *in vivo*. Four major recent findings in the field of nitrogen excretion in fish in general are 1) the discovery of gill rhesus glycoprotein ammonia transporters, 2) the finding of urea transporters, 3) urea cycle activity in fish early life stages, and 4) the demonstration of occasional growth-promoting effects of ammonia.

Marine species such as Atlantic cod and halibut cover the bulk of their energy requirements from amino acids during certain periods of early development. This process generates toxic ammonia which is either excreted, detoxified, or stored safely in an acidic yolk sac. The current knowledge of these mechanisms is briefly discussed in the review.

Acute tolerance to ambient ammonia is substantial in fish embryos, compared to later developmental stages. Results from chronic exposure studies are few. During the yolk-sac stage and the first period of exogenous feeding, little is known about ammonia tolerance in our important cultured species. It is suggested that more detailed chronic ammonia exposure studies should be done, analysing different developmental stages and physiological situations.

6.3.7 Reproduction in fish: Focus on germ line and early gonadal development

Studies with zebrafish and medaka have revealed that they employ the preformation mode of germ line specification. The germ line is separated from soma by maternally deposited determinants that segregate with the germ line as a result of asymmetric cell divisions. Studies in model fish have led to the identification of some key players participating in germ line development and gonadal differentiation. Despite this, basic knowledge about germ line development remains elusive. We do not understand how germ cell fate is being controlled and maintained or how the somatic compartment of the gonadal anlage and germ cells obtain sexual identities. Furthermore we lack information regarding what kind of germ-soma interactions take place during gonadal differentiation.

An important issue for the aquaculture industry is to control sex differentiation and germ cell maturation in farmed fish species. Production losses due to precocious male puberty are a big problem for the aquaculture industry. In addition, escaped farmed fish represent a danger to the environment. In several fish species it is known that environmental conditions (e.g. high temperature) around the hatching stage/early larval stages lead to female-to-male sex reversal. Some fish species may even have a temperature-dependent sex determination or other forms of environmental sex determination. Basic knowledge about germ line development in model fish may lay the foundation for developing new tools and strategies for controlling germ line development in farmed fish species.

6.3.8 Development of the digestion system

Most of the studies on the digestive system in fish are on altrical fish larvae, such as cod and halibut. Since these larvae do not have a stomach at first-feeding, they have a reduced ability to digest conventional formulated diets, and are therefore given live feed. The morphological development of the digestive tract and associated organs such as liver, pancreas and bile bladder in these species is quite well described, also in cod and halibut larvae. The main developmental scheme is similar in different species, but the different events of development have species-dependent timing.

The functional properties of the digestive system have received less research interest. These comprise a range of closely integrated processes, including ingestion, secretion, digestion, absorption, motility, elimination, regulation and barrier function. The most studied topic is digestion, in particular protein digestion. Still, the developmental profiles are available for only some of the proteolytic enzymes in our target species, while incomplete information exists for the lipolytic enzymes. Digestion of other nutrients than protein and lipid has not been studied. There is very little information on what happens to the nutrients once they are inside the enterocyte and how they are transported further into the larval body.

The neuronal and hormonal regulation of the digestive system and processes such as appetite, secretion and reabsorption of fluids containing salts, digestive enzymes, bile etc., as well as gut motility and passage time is critical for homeostasis of an organism. Information on these regulatory mechanisms is available from mammals and the derived hypotheses are currently being tested on older fish. These processes are poorly described in fish larvae.

6.3.9 Muscle development

The fish meat, scientifically termed the axial swimming musculature, accounts for 40-60% of the total body mass in fish. Muscle grows by two mechanisms: formation of new muscle fibres (hyperplasia) and increase in size of already existing fibres (hypertrophy). Fish are different from birds and mammals in that their muscle grows by hyperplasia also after hatching/birth. The morphological development of muscle and mode of muscle growth during embryonic and larval stages in fish is quite well described and the regulation of growth through myogenic regulating

factors (MyoD, myf-5, myogenin, MRF4 and growth hormones) has been studied in adult fish. Only fragmentary information on the regulation of muscle growth exists for fish larvae.

Embryonic incubation temperature affects number, size and organelle composition of muscle fibres at hatching and first-feeding in cod, and larval whole body growth is closely related to white muscle hyperplasia rates in cod, but not in halibut. This should infer that environmental and nutritional effects on larval cod growth, which would be numerous, also should have an effect on myogenesis.

Embryonic and larval development of the swimming musculature has a great impact on the larva's swimming ability. Furthermore, early development of the swimming musculature influences growth throughout all subsequent life stages and potentially flesh quality at harvest. The swimming muscle also contains large amounts of connective tissue and is closely associated with the skeletal system – it therefore has a profound impact on the outcome of skeletal development. Further studies are needed, both on the regulation of embryonic and larval muscle and connective tissue growth, on how nutrition and the environment affect myogenesis and on how the development of musculature and bony structures affect each other.

6.3.10 Skin and pigmentation

The morphological development of skin and scales has been studied in zebrafish, but not in our target species. Very little is known about the regulation of skin and scale development in fish.

Due to problems with malpigmentation in farmed flatfish juveniles, considerable effort has been made to understand the development of adult pigmentation in these species. The migration of precursor cells from the neural crest to the skin has been described in a number of animal species, including zebrafish. Differences in proliferation and differentiation of these cells between the ocular and blind side of Japanese flounder have been studied in detail by morphological methods. It has also been shown that white skin on the ocular side in malpigmented fish has similar characteristics as skin on the blind side in normal fish, with regard to pigment cells, scales and mucus cells. However, how these processes are regulated is not known. Pigmentation is quite extensively studied in zebrafish, by knocking out genes involved in pigment cell development and function, so some knowledge can be extracted to work with our target species.

It is known that vitamin A and fatty acid composition in the feed and thyroid hormones affect pigmentation success in flatfish. However, an unbalanced fatty acid composition of the feed organisms used in intensive culture is the main reason for pigmentation errors of farmed flatfish. A fatty acid composition that yields malpigmented flatfish juveniles does not have a similar effect on cod. The thresholds for concentrations of the essential fatty acids for normal pigmentation in Atlantic halibut have been determined.

6.3.11 Development of bone

Different parts of the skeleton have different embryonic origins. The vertebra is formed from the inner part of the somites, the limbs from the lateral plate mesoderm, and the brachial arches and cranio-facial bones from the cranial neural crest. There are basically two types of bone: chondral bone formed from a cartilaginous template and dermal bone formed from dermal connective tissue. Cells that participate in bone formation and modulation in fish are chondroblasts, producing the cartilage bone model, osteblasts, which produce the bone matrix and deposit minerals, and osteoclasts, which absorb bone. The majority of teleosts do not have osteocysts which are embedded in cellular bone. Most of the current knowledge on bone development is from mammalian studies, but recent studies in zebrafish and medaka elucidate some of the processes in fish, including the main factors regulating bone growth and remodelling.

Development of craniofacial bones and vertebrae has been described morphologically in Atlantic halibut and in Atlantic salmon, respectively. The special case of eye migration, which involves dramatic remodelling of the frontal bone between the eyes, has been studied in detail in Atlantic halibut. However, the signalling responsible for regulation of bone growth is not described in our target species.

A large number of nutritional and environmental factors have been shown to affect bone development, both in fish and mammals, and the high ratios of bone deformities seen in intensive culture of fish may have many causes. Further studies are needed to identify the critical factors and the interactions between them and to understand their impact on the dynamic metabolism and interaction of chondroblasts, osteoblasts and osteoclasts in development of bone.

6.3.12 Development of the immune system in fish

Immune cells in fish develop from pluripotent hematopoietic stem cells. Initially, the stem cells develop into myeloid and lymphoid progenitor cells which subsequently give rise to different cell types, such as granulocytes, monocytes, macrophages, thrombocytes, erythrocytes, B-cells, T-cells etc. Although similarities are striking, some types of white blood cells in fish have retained primitive features which have been lost in mammals, e.g. the B-cells in fish, which have phagocytic and microbicidal abilities in contrast to their more specialised counterparts in mammals. There are also signifycant differences between fish and mammals with regard to the employment of different antibody classes.

Homologous genes of many transcription factors involved in mammalian hematopoiesis have been used to trace corresponding development in fish by *in situ* hybridisation. However, knowledge on the development of the immune system in fish is fragmented and limited compared to higher vertebrates. In some species, such as angelfish, initial hematopoiesis appears in yolk sac blood islands. In other species it appears in the intra-embryonic intermediate cell mass (ICM), as in zebrafish, or first in the yolk sac and later in the ICM, as in rainbow trout. Definitive hematopoiesis is established in the kidney, but an intermediate, larval site of hematopoiesis has been revealed in the tail of zebrafish. In recent years zebrafish has been established as the most important model for the development of the immune system in fish, and undoubtedly this common reference will facilitate future studies on other species.

6.4 Nutritional and environmental factors

6.4.1 Nutrition

Different stages of copepods comprise the main diet of marine fish larvae in the wild. The whole nutrient profile of copepods cultured in Norwegian pond systems has recently been characterised. The copepods have high levels of vitamins, minerals and n-3 fatty acids. The protein and free amino acid concentrations are also higher than in the intensively reared live feed organisms such as rotifers and *Artemia*, and the amino acid composition is balanced. Cod larvae reared on copepods grow better and develop less deformities than intensively reared larvae. The requirements for the different nutrients in marine fish larvae are not known, with a few exceptions, but one approach is to use nutrient levels in copepods as an indication of the requirements. However, these concentrations may vary according to species, food supply and environmental conditions, and it is not certain that marine fish larvae have requirements equivalent to nutrient concentrations in pond-cultured copepods. It is therefore advisable to conduct requirement studies with fish larvae where this is possible.

The nutrient profiles of rotifers (*Brachionus* sp.) and *Artemia* have been analysed for several decades. *Artemia* should be enriched with n-3 fatty acids, vitamin C, vitamin E, and thiamine. It also contains low levels of protein, iodine, and zinc compared to copepods. Otherwise *Artemia*

appears to be a good food item for fish larvae. The nutrient composition of rotifers is much more dependent on the rotifer diet, with potentially low levels of several vitamins and minerals, n-3 fatty acids, protein and certain amino acids. Pigments such as astaxanthin and cantxanthin are high in copepods and *Artemia*, respectively, but are low in rotifers. Physiological processes such as growth rate, satiation and starvetion also affect the chemical composition of feed organisms. Rotifers and *Artemia* can be enriched with many of the potentially limiting nutrients to controlled levels so that requirement studies can be conducted.

Compared to live feed, formulated diets most often result in reduced growth and survival when fed to marine fish larvae. The two most important reasons for this are probably nutrient leaching and that the nutrients, especially protein and lipid, are given in a form that is not easily digestible for the stomachless larvae. As a result, most marine fish larvae must be fed a live diet during the period after yolk absorption.

It is important that baseline studies on development of fish larvae are done on larvae that are fed the optimal diet, which at present is copepods. A strategy for the aquaculture industry could be to copy the copepod nutrient levels while enriching rotifers and *Artemia*, and adjust to actual requirements when these are available.

6.4.2 Microbiological factors

Intensively cultured fish are vulnerable to stressing conditions, especially during larval and juvenile stages. Microbial diseases induced by opportunistic pathogens (e.g. Vibrio spp., Francisella sp., IPN, Pasteurellosis) may cause high losses. For salmon, loss during the freshwater phase may be up to 25%, whereas for marine species up to 99% can be lost during the larval and juvenile stage. It is highly important to ensure optimal gut function and digestion of the feed. As the intestinal tract is a main entrance for microorganisms, the gut can get colonised by pathogens that can cause enteric diseases and eventually pass the epidermis into the blood and cause septicaemia. At larval and juvenile stages the fish has not developed a specific immune defence and cannot be vaccinated. In order to resist the pathogens, larvae and juvenile fish depend on non-specific mechanisms consisting of a protective microflora in the gut and a well functioning non-specific immune system. Much attention has been paid to the stimulation of these mechanisms, but there are still many uncertainties regarding their impact, and clearly more research is needed.

Stability in key environmental parameters such as water quality and the microbial environment is paramount for predictable production of high-quality juveniles. In the ocean, the natural habitat of fish larvae, many of the important environmental factors are stable partly due to low biomass load. Intensive aquaculture production methods, on the other hand, often lead to huge fluctuations in water quality due to direct perturbations and the enriched high biomass conditions. The water is heated, filtered, disinfected and aerated; both the live feed and the fish are fed and kept at relatively high densities, which may increase the bacterial load. Such perturbations may have negative impacts on fish performance, for example by jeopardizing normal, positive fish-microbe interactions. High and fluctuating organic load selects for opportunistic bacteria which may be harmful to fish larvae. At the same time stress caused by variations in water quality may weaken disease resistance of larvae and result in lower growth and survival rates. Microbial maturation of the culture water – allowing the microbial flora to stabilise and be dominated by mainly slower-growing specialists (K-selected types) as opposed to opportunistic types (r-selected types) – has been shown to benefit fish larvae. Recirculation seems to be a relatively simple and promising way to achieve such selection and stability, but is so far not documented sufficiently. In fact, little is known about how microbial communities develop in rearing systems. Thus, molecular methods for instantaneously surveying microbial composition and abundance in rearing systems would be of great advantage in future research and should be developed, particularly for the most common pathogens.

6.4.3 Physical factors

The environment and water quality may be different for fish larvae in aquaculture compared to fish larvae in the open sea. Although the environmental conditions may vary much for wild fish larvae, rearing environment and water quality between different hatcheries may vary even more. This is connected to the fact that various water treatment and rearing strategies (including recirculation) are applied and further developed by the hatcheries. In particular, a wide range of temperatures may be used, but except for having upper and lower lethal limits, temperature is a key regulator of metabolism and growth. Thus, more knowledge is needed of the temperature effects on larval fish physiology and biochemistry through ontogenesis. Similarly, the effects on larval fish of variations in salinity and oxygen content need further exploration. Salinity represents an osmotic cost while oxygen is essential for larval metabolism and growth. Potential benefits of better understanding the effects of temperature, salinity, and oxygen on larval fish performance may be significant.

Light is a strong biological regulator in fish, controlling feeding, digestion, and reproduction. Light probably also has an influence on the basic signalling systems in the fish brain, as all light-sensitive brain tissues, including the retina of the eye, contain serotonin, an important neurotransmitter. Light comprises different components such as quantity (light intensity), quality (spectral composition), light distribution (point source or evenly), and cycling (photoperiod and season). While outdoor light conditions are quite defined depending on latitude, season, depth, and algal blooms, hatchery conditions often provide very different conditions from natural systems. In particular, light intensity is lower, spectral composition is different with e.g. lack of UV radiation, and 24-hour light is commonly used. The latter will remove any diurnal signals from the fish larvae, with unknown impacts on larval development. Unfavourable light conditions that do not facilitate good feeding behaviour or that induce stress reaction may thus be one reason for poor larval conditions. Today there are no guidelines for optimal light conditions for larval and juvenile fish. Knowledge of how to optimize light conditions and regimes may have significant impacts, as exemplified by the improvements in eye migration by use of photoperiod in Atlantic halibut.

The fish density in intensive cultivation of larval fish is much higher than that in nature-like systems. Introduction of turbidity by adding particles to the water has proven beneficial in enhancing larval feeding, probably by reducing interactions among the individuals. Several hatcheries use a “green water technique” by adding algae or algal paste, but the positive effect of turbidity is also observed with the addition of inert particles such as clay. Turbidity may also modify the light conditions as well as improve the contrast conditions by making the prey more conspicuous. Particularly in small rearing systems, tank colour may influence the contrast conditions. Research to establish guidelines for optimal turbidity conditions for various species or stages of larval fish is needed. The issue of turbidity is further complicated as the particles used may be ingested by the larval food organisms and thus influence the nutritional value of the larval prey or modify the microbial conditions in the tank.

Use of air bubbling in larval fish rearing distributes larvae and prey in a beneficial manner for prey encounter and prevents sieves from being clogged. But it also creates turbulence at certain scales, with subsequent energy dissipation. Similarly, wind and tidal waves create turbulent conditions in the open sea, believed to have some impacts on prey encounter rates. However, in nature fish larvae can to some extent choose their turbulence exposure by evacuating from regions of strong turbulence close to the surface. This is less possible in a rearing tank. Turbulence generates shear forces that can provide drag on the larval body in different directions. Another creator of turbulence and very strong shear is the water inlet to the tank. The surface skimmer also adds shear on the young larvae. The effects of shear forces on the delicate larval body and larval

development are at present unknown. But a relationship between bone deformities and water current speed in the larval and juvenile tanks has been suggested.

Total gas saturation is influenced by heating of water or by air trapped in pipes under pressure. An increase of 1°C represents a 2% increase in gas saturation. Such increases in water temperature may frequently occur in larval rearing tanks for various reasons. Gas super-saturation has been blamed for over-inflation of the swim bladder, with subsequent eruption of normal vertebral development in the region above the swim bladder. However, the effects of gas over-saturation on larval development and physiology are subtle and call for more knowledge about this subject. With the use of hyper-oxygenation in high-density cultures, total gas saturation has had negative effects on juvenile fish when transferred to normal saturated water. Understanding the physiology of hypoxia and oxygen level acclimatisation in larval and juvenile fish of different species may prevent conditions that can lead to incidences of fish losses in intensive culture.

The sea is not a noise-free environment. Many larval fish have neuromasts freely exposed out into the water, with the possibility to detect various sound frequencies. It has been questioned if larval fish can "hear" the sound of their prey: swarms of grazing copepods. The sound environment of hatchery rearing tanks is very much different from the open sea. Flow of water through tubes and valves generates strong noise, as do pumps. The noise is carried effectively to the fish tanks through air, water tubes, and the ground. The effects of noise on larval and juvenile development and performance are unknown. Noise most likely represents increased stress, and noise level both at acute and chronic exposure needs attention.

Many larval fish species seem to be dependent on inflating their swim bladder through the water surface the first time, although this actually has to be verified for many species. The open sea surface is "cleaned" by means of wind, waves, and sun radiation. None of these are active in hatcheries, which face further pollution of the water surface by residues from enrichment of the live feed. Artificial surface cleaning by skimmers aided by air bubbling has been introduced, but detailed understanding of interactions between the surface layer properties and success of swim bladder inflation remains unknown.

6.4.4 Chemical factors

Metabolites may originate from the reared organism itself and from bacterial activity, mainly consisting of compounds such as ammonia (NH_3), nitrite (NO_2), nitrate (NO_3), and CO_2. In flow-through systems, metabolites are removed simply by balancing flow rate to biomass density. Larval fish rearing implies low biomass density, and metabolites are not regarded as a problem. However, in recirculation systems NH_3 is removed by bacterial aerobic nitrification to NO_2 and NO_3, which may be a slow process in low-temperature seawater. Build-up of all these metabolites may therefore have a potential effect, and knowledge of larval tolerance to chronic or acute is therefore needed. NH_3 is poisonous to fish, but dissociation into the more harmless ammonium ion (NH_4^+) in aqueous solutions enables considerable amounts of NH_3 to be solved in water. The equilibrium between NH_3 and NH_4^+ is dependent on pH, which again is dependent on CO_2. The knowledge base is limited for interactions between larval and juvenile physiology on one side and pH, metabolites, and physical factors such as oxygen content, temperature, and salinity on the other.

Recirculation also includes other water treatments than biofilters and degassing towers to remove NH_3 and CO_2, respectively. Protein skimmers are frequently used to remove small particles by foaming, and the process is enhanced by use of ozone (O_3), which also breaks down dissolved organic matter. However, O_3 increases the red-ox potential of the seawater and may also lead to accumulation of toxic bromate in the water if the O_3 concentration is too high. Knowledge is lacking on the effects of bromate on larval and juvenile fish development.

Degradation of organic matter (e.g. algal blooms, marine snow) may contribute to oscillations in the red-ox potential of the water. Hatchery managers have reported correlations between variation in red-ox potential and performance of the rearing systems. A number of exudates or toxins may originate from algal blooms, and potentially also from their degradation. Thus, more knowledge is needed on possible effects on larval and juvenile fish.

6.4.5 Antropogenic factors

Water-soluble pollutants may enter aquaculture systems and dissolved chemicals may be a potential risk for hatcheries. Due to filtration technology, pollutants accumulating in the oceanic biomass may be less important for reared larval fish, but the effects of alien compounds in the marine environment on larval fish need to be surveyed, as indicated in section 6.2.

7 Research resources

To obtain the goals for fish larvae research for the future, we have done some analysis on available tools, strategic investments and experimental set-up that are relevant for this area.

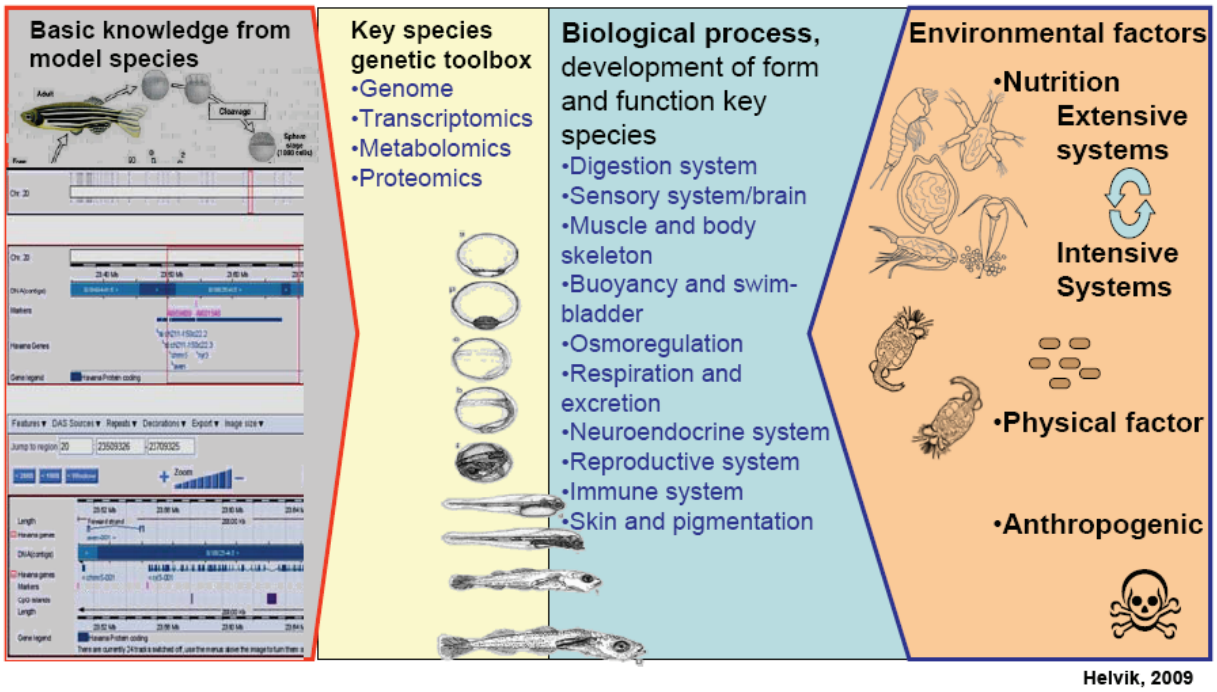

Figure 3. *Fish models have become an important approach for studying vertebrate/human development and disease. In these model species the mechanisms of many biological processes are known in great detail. Through aquaculture research and development we are now able to culture several marine fish. This opens up new research opportunities and the ability to analyse the effects of environmental factors such as food supply, physical conditions or toxic compounds. In this programme we want to link the detail knowledge and technology from model species with marine fish species in order to analyse environmental effects on a deeper biological level of various biological processes and systems. A key element in this approach is to use a molecular approach such as large shale sequencing of expressed genes (transcriptome) during development.*

7.1 Extensive/intensive systems

7.1.1 Production systems

Extensive systems for production of marine fish larvae are based on the use of natural zooplankton. In coastal lagoons and large natural enclosed systems (mesocosms) used for this purpose, larval fish are reared in an ecosystem consisting of a natural food web which includes several trophic levels. The natural plankton communities can be manipulated in order to increase the number of planktonic organisms that comprise the natural food for the fish larvae. This has been done by removing the natural predators or competitors, and stimulating primary production in the system by vertical mixing and additions of nutrients. Such systems will provide larval fish with their natural prey (copepods), which satisfy the nutritional requirements. Fish larvae in such systems will be exposed to variations in most physical and biological conditions experienced by wild larvae, including low density of conspecifics and prey organisms. However, as a production unit, extensive systems are only operable once a year. They are also characterised by low degree of control, but require low intensity of labour to operate.

In semi-intensive rearing systems, fish larvae are stocked in medium densities in large plastic bags or tanks with no self-sustaining zooplankton production. Thus, live feed must be supplied daily, which includes both copepods collected from extensive plankton production in ponds and intensive reared live prey such as rotifers or *Artemia*. Consequently, the organic load is higher than for extensive systems, but the benefits of a nutritious prey such as copepods may still be explored. Since most of the semi-intensive units are placed outdoor, they are subjected to all variations in the physical environment. Higher intensity and stronger demands of timing in live feed production are constraints that often lead to more variability in larval survival in such rearing systems.

Intensive production is the most common method for fish larval rearing today. The method is characterised by high larval density, and requires strict control of live feed production and different physical and chemical parameters. The hatcheries produce fish larvae in indoor tanks with the addition of "green-water" and intensively cultivated rotifers and *Artemia* as larval feed. These organisms are not the natural food for the fish larvae and have a lower nutritional quality compared with natural zooplankton. In order to improve the nutritional and microbial quality of the live feed, specific production and enrichment methods are being elaborated and developed.

7.1.2 Feed organisms

Rotifers

Rotifers from the genus *Brachionus* have been used in aquaculture since the early 1960s. The *Brachionus* complex is one of the most studied groups of rotifers; the taxonomy is still under constant review. Genetic analysis has indicated that *Brachionus plicatilis* is not a single species but is, in fact, a complex of at least 14 putative species. In Norwegian hatcheries two different types of rotifers are used. These rotifer types are *Brachionus plicatilis* (Nevada type) and *Brachionus ibericus* (Cayman type). Identifying the species used in experiments and hatcheries is a crucial step toward a more scientific approach to understanding rotifer biology. To elaborate, *B. plicatilis* was the subject of approximately 750 peer-reviewed articles between 1950 and 2000. Unfortunately, for the majority of these articles, we do not know which of the 14 putative species were used, limiting the utilisation of this large accumulation of knowledge. This is especially important, considering that various *Brachionus* sp. display different growth rate, lorica length, optimum for salinity and temperature, swimming activity, size preferences for food particles, and different nutritional content when they are offered the same feed type.

Different methods such as batch, semi-continuous, and continuous cultivation have been used for rotifer production. The tank volume and shape varies from indoor tanks of a few litres to outdoor concrete tanks of several hundred m^3. Production of high-quality rotifers requires strict control of physical parameters (oxygen, salinity, temperature, pH and ammonia), microbial content and nutritional value. The production is labour- and knowledge-intensive, and there is a need for more process control and automation. Rotifers can be cultivated in a wide range of feed types, and the nutritional value of rotifers reflects the enrichment or cultivation diet. Physiological processes such as growth rate, satiation and starvation also affect the biochemical composition.

Artemia

Populations of brine shrimp *Artemia* are found in more than 500 saline lakes all over the world and *Artemia* cysts are regularly harvested from inland saline lakes. Cysts are dehydrated, dried, and packed in oxygen-free conditions, and stored in boxes for sale. *Artemia* are mostly used as newly hatched nauplii or as short time enriched nauplii fed emulsified lipids for a period of 24h. Hatching procedures can be improved through decapsulation of the cysts, a process that improves the quality of poor hatching cysts, eliminates the hatching debris and reduces the bacterial load. On-grown *Artemia* (grown for 2 - 10 days) may also be used in first-feeding of some fish species, such as halibut.

Artemia can be cultivated in tanks of any possible shape and volume as long as the installed aeration ensures proper oxygenation and adequate mixing of food particles and nauplii. Optimum hatching requires control of pH, temperature, oxygen, and light. For use as feed for fish larvae, it is important to enrich the *Artemia* with polyunsaturated fatty acids such as DHA and EPA.

Copepods
Copepods may be cultured both in intensive tank culture and under natural conditions in lagoons or ponds as explained in section 7.1.1. In such extensive systems, copepods will feed on a variety of algal and protozoan species. Copepods are concentrated and harvested by filters, and specific size fractions matching the requirements of larval fish may easily be obtained. As most copepod species in the lagoons display dormant periods as resting eggs, synchronous hatching may easily provide large amounts of copepod nauplii at various times through the production season. In intensive production of copepods, it is possible to harvest copepod eggs or nauplii from different species. Eggs from some of the species can be stored for several months in refrigeration before hatching. The advantages of intensive produced copepods are their availability throughout the year, control of development stages and nutritional content. However, the production capacity of copepod eggs needs to be improved.

Numerous studies have demonstrated that copepods cultured under natural conditions have a higher nutritional value than rotifers and *Artemia*, as the nutritional profile of copepods appears to match better the requirements of marine fish larvae. In general, copepods from extensive systems are rich in free amino acids and have high protein content with what is assumed to be a good amino acid profile. They are rich in polar lipids, with high levels of DHA and EPA. Other characteristics are high amounts of astaxanthin, vitamins and minerals. Fish larvae fed copepods from ponds and lagoons have high growth rates, which also are observed for fish larvae reared on intensive cultured copepods. However, the nutritional profile of intensively cultured copepods will probably vary with diet composition. Intensively cultivated copepods may be available independent of season. Further elaboration of intensive copepod culture is expected.

In relation to larval feeding ecology, copepods move differently compared to rotifers and *Artemia*. This may explain the observed selection for copepod nauplii when offered together with rotifers. It also may explain observed improvements in larval swimming activity and other traits related to larval performance when copepods are used. Such observations need verification from more detailed behavioural studies.

7.2 Model species and marine fish development

In recent years, the zebrafish has become one of the most prominent vertebrate model organisms used to study the genetics underlying development, normal body function and disease. The growing interest in zebrafish research has been paralleled by an increase in tools and methods available to study zebrafish. The suitability of zebrafish as a model organism is due to the fact that zebrafish combine a number of key embryological and experimental advantages. They are easy to maintain and breed and the embryos are sturdy enough for experimental manipulation, such as microinjection and cell transplantation experiments. They develop very rapidly (embryogenesis takes only about 24h and organogenesis is largely complete after day 5 of development), enabling the observation of defined aspects of development as well as the completion of experiments within hours or days. Moreover, the transparency of the zebrafish shell and the translucency of its embryos and early larval stages allow easy visualisation of internal processes, such as the formation and function of internal organs inside the living animal. They also facilitate, for example, tracking the expression of fluorescently tagged transgenes and monitoring reporter gene activity (e.g. GFP and its derivatives). Zebrafish also have a large number of offspring compared to other model species, while their continuous production of eggs distinguishes them from most

aquacultural species. The constant supply of large numbers of offspring from defined pairs renders the zebrafish well suited to large-scale genetic approaches aimed at identifying novel genes and at uncovering their functions.

So far the aspect of larval development and metamorphosis has been neglected in the model organisms (major and minor models), zebrafish and medaka. But also in stickleback, tilapia, rainbow trout and carp, almost nothing has been studied. In fact, much can be learned from models (more easily and quickly) and then transferred to the important marine species. Studying larval development can create an interesting spin-off: so far all fish models for human diseases are using embryos, but human diseases are mostly diseases of the post-embryonic phase. Thus the disease models do not reflect the real situation. Larvae are certainly closer to the adult, and studying larval development may generate new and better-suited disease models.

As genomic resources for aquacultural species are increasingly being generated, a meaningful interaction between zebrafish researchers and aquacultural researchers now appears to be possible. In particular, research on nutrition and growth, stress, and disease resistance in the zebrafish can be expected to produce results applicable to the aquaculture fish. Forward and reverse genetics approaches in the zebrafish, together with the large genomic resources available for this species, offer the potential to identify and verify candidate genes for quantitative trait loci (QTLs) to be used in marker assisted breeding. Moreover, some technologies from the zebrafish field such as microinjection, morpholino knock-down and targeted induced local lesions in genomes (TILLING) may be directly transferable to aquaculture research and production.

Zebrafish is, however, less closely related to most fish species of aquaculture interest (salmonids, cod, sea bass, sea bream and flatfishes) than those fish are to each other, and it may be argued that a species more closely related to those used in aquaculture should be developed. The other model, medaka, is much more closely related to the aquaculture fish, so the knowledge gained from medaka can be used more efficiently for comparative embryology and genomics. The larval stages and the peculiarities of this period have not, however, been studied to any extent. One important issue is that the critical processes of sex determination happen in both models between hatching and metamorphosis. Is this also the case in aquaculture species?

The fish models established so far are all non-marine, so a marine model would be very desirable. Once established, it would allow better direct transfer of knowledge and research technology from the freshwater models, and from the new marine species to the aquaculture and fishery species. Developing a marine fish model would certainly create much attention, while its usefulness is beyond question. The central position of the Atlantic cod both in fisheries and aquaculture, together with its biology (high fecundity, egg and embryo translucency, sturdy eggs that can be manipulated and an embryonic and larval phase that is relatively short), make cod a promising candidate for a marine fish model. Some investment in genome sequencing has been established (see below).

7.3 GenoFisk: Genomic platform for cod and salmon

The Norwegian fish genomics consortium **GenoFisk** has been established as a national FUGE platform, consisting at the outset of five research groups, each with their own individual funding. GenoFisk is built on four main foundations: establishment of resources; technology development; building up of expertise, and national sharing of tasks and responsibilities (see “Norwegian marine genomics strategy plan”). The consortium’s prime objective is to enhance the quality and extent of functional genome research on fish and to strengthen Norway’s expertise and commercial activity in this field. The consortium will establish genomic information on Atlantic salmon and Atlantic cod by large-scale genome sequencing, transcriptome, proteome and

metabolome analysis, including microarrays and new technology sequencing. Genetic markers for selective breeding and management are a central goal of the programme.

Gene knockdown: A tool for gene function study in fish

There is a rapid increase in available DNA sequence information from different fish genome projects. However, the function of a gene cannot be deduced only by its DNA sequence or expression pattern. Therefore, a technique that can be used to investigate the function of the gene is needed. Gene knockdown, or antisense technology, is now being used as a powerful technique to study gene function in living organisms. Gene knockdown effects result from the introduction of an antisense molecule into living cells. The antisense agents bind, by their complementary nucleotide sequence, to target messenger RNA, thus inactivating the target gene expression. The inhibitory effects on protein production from the corresponding gene results in a phenotypic change and the function of the gene can be understood. To date, there are a number of antisense molecules that can mediate efficient gene knockdown in fish. These include antisense oligonucleotides, small interfering RNA (siRNA) and ribozymes, all of which cause specific gene inhibitor effects, but through different mechanisms. In fish, the most widely used antisense agent is morpholino phosphoroamidate oligonucleotides; siRNA has also been tested. Recently a new method for targeted knockout was established in zebrafish, using zinc finger nucleases that can be designed to cut the DNA at specific sites. Up to now, gene knockdown studies have almost exclusively been carried out in model species (e.g. zebrafish and medaka) so there is a need to develop this technique in aquaculture-relevant species as well.

Proteomics

Proteomics is a new analytical field aimed at simultaneously characterising entire protein repertoires of biological systems. Using proteomics, comprehensive lists of the protein constituents of cells and organisms can be established. It is important for developmental biology that changes in the relative amounts of the proteins and the patterns of their posttranslational modifications can be followed. For example, the complex temporal changes in protein expression patterns, such as those of a developing fish embryo and larvae, can be monitored and analysed. Thus, proteomics is emerging as one of the most powerful new tools for research in developmental biology. The advances in proteomics stem from the introduction of new instrumentation, methodologies and direct access to large gene and protein databanks. A precondition of conducting such analysis is the availability of genomic information from which the protein repertoire can be inferred for creating peptide databases and search algorithms for identifying the proteins.

Metabolomics

Metabolomics comprises the measurement of low molecular weight endogenous metabolites and can provide an overview of the metabolic status of a biological system. It can examine the physiological condition of a cell or organism and associate the metabolic changes to genetic or environmental modulation. Similar to the fields of genomics and proteomics, metabolomics can examine biological systems at several levels, including cellular, tissue, organ, or even whole organism in response to stressors, nutrients etc. The advantage of metabolomics is that it provides the most functional measure of cellular status and in principle can help to describe an organism`s phenotype. The metabolic “fingerprints” of an organism might be altered by genetic or environmental changes, which metabolomics records and then attempts to associate with biological functions. This approach is widely applied in the field of basic biology, but little used in aquaculture-related research.

8 Recommendations and strategies

The committee recommends that the Research Council of Norway establish a new research programme on fish larval development with the following goals, strategies and organisation.

8.1 Goal

The goal of the proposed programme is to characterise biological processes, nutrition, and environmental factors influencing normal development (embryo-larva-juvenile) of some cold-water teleosts important for Norwegian ecosystems, aquaculture industry and fisheries. Understanding the development of larvae and transformation into juveniles is the basis for solving problems in aquaculture. Further, it may add knowledge to gain a more comprehensive perception of recruitment in natural ecosystems, including potential effects of climate change and anthropogenic factors such as pollutants.

8.2 Strategy

The proposed programme has a highly interdisciplinary approach, bringing together classic developmental biology, nutrition, and early life history analysis. The programme connects studies and technology from model species (zebrafish and medaka) with key species for Norwegian aquaculture and fisheries (Atlantic salmon, cod, halibut, herring, and wolffish). These species represent evolutionary distances and life history strategies of many other species we find in our ecosystems. Most importantly, the established culture systems for these species, on both natural and laboratory scales, make this programme a unique combination on the international level as well.

The research programme should be organised into three sub-programmes:

Sub-programme I. Development of form and function
What are the form and function (biological processes) of various organ systems during development, and what type of specialisation and adaptation has evolution formed in the key species?

Sub-programme II. Nutrition and development
How does nutrition influence larval development, and what nutrients are critical for optimal growth and normal development?

Sub-programme III. Environment and development
How do environmental, microbial, anthropogenic (pollution) and climatic (acidification) factors influence larval development?

8.3 Organisation

A committee of highly rated scientists, both national and international, should be appointed to build up and manage this programme, and their backgrounds should reflect the interdisciplinary approach.

Funding should be allocated to organise a research community, by creating a common website/database (such as www.zfin.org) containing all common information, resources and findings. New findings should be organised and linked into the databases to allow scientists easy access, as well as hatchery managers and governmental officials, who may have less access to scientific literature.

Culturing larval fish is a complex and resource-demanding process, of which only a few institutions in Norway are capable. Therefore, resources should be used to produce larval material and create large-scale experiments that support all the users within the programme. This will provide a huge benefit of integrating and comparing the results, since scientists will work on the same biological material.

When studying development in the 21th century, access to gene/sequence information is essential. Some genome information is already available from Atlantic salmon and Atlantic cod, but little information exists about gene expression (transcriptome) during development of our key species. We therefore recommend that this programme build up EST and miRNA databases of the key species, using new technology to sequence a large number of genes from various stages of development and organ systems. Such sequence databases would be vital for *in silico* analysis of appearance of various biological processes, complexity and comparative analysis. Gene specific sequence information would facilitate the isolation of interesting genes for future analysis and reduce the gap between understanding biological processes in our key species and model species.

Research projects should be open in the three directions of this program: development of form and function, nutrition and development, and environment and development. Projects should be awarded as functional packages, including salary and operating costs, for 2 – 4 persons with a duration of 3 plus 2 years.

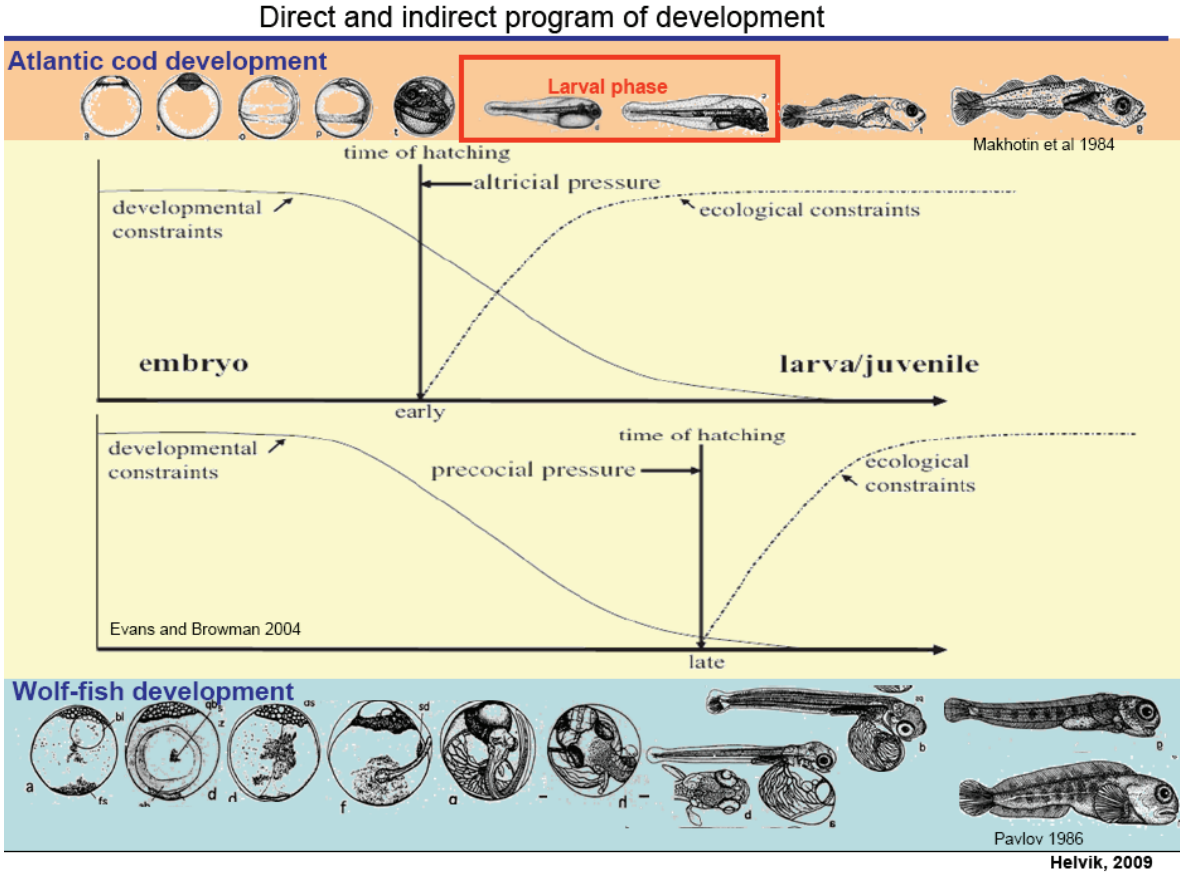

Figure 4. *Fish have various strategies of early development, either indirect with a long larval stage (herring, cod and halibut) and metamorphosis into the juvenile phase or more direct development (wolffish and salmon) in which the embryo hatches on a more advanced developmental stage, and the larval period is less pronounced. Detail analysis and comparison of the transcriptome of these species in a stage-specific manner will yield important information about complexity and similarity in the progress of the different developmental programmes creating these species that have such different life strategies. Such information will be the starting point to unwind specific biological processes and adaptations.*

8.3.1 First year

- Establish a scientific advisory board, containing central researchers in the field of development, aquaculture and early life history who are given the task of building up a fish developmental biology network/platform in Norway and who can advise the Research Council on further planning of a new research programme in this domain.
- Build up a transcriptome database of major developmental stages of Atlantic cod. The database should be based on one series of embryonic development, one series of cod larvae raised on natural zooplankton in a pond system, and one series fed cultured copepods in an intensive system under controlled conditions regarding rearing environment and water quality.
- Initiate sub-programmes 1 and 2
    - Research projects should be started in the areas of early development of Atlantic cod in the topics addressed by this evaluation.
    - Projects with an interdisciplinary approach (comparison model species – Atlantic cod/ culture versus natural environment) should also be included in order to establish the broad focus for this programme.
- Create a net-based marine larval fish/fish developmental biology community

8.3.2 Short-term (1-5 years)

- Build up a transcriptome database of major developmental stages of Atlantic salmon. The database could be based on the normal aquaculture production line. Detailed data on environmental conditions and nutritional status of the embryo/larvae/smolts should accompany the transcriptome data.
- Initiate sub-programme 3 and fully activate sub-programmes 1 and 2.
- Create the developmental baseline for staging and normal development of biological processes, functional properties, and organ systems, with organisms from the experiments listed above.
- Implement genomics and expression studies for comparative analysis between aquaculture species and fish models.

8.3.3 Long-term (1-10 years)

- Create similar transcriptome databases as that described above, for Atlantic halibut, wolffish and herring.
- Design experiments to vary key environmental and nutritional factors and monitor effects on gene expression and development of biological processes, also including the context of natural genetic adaptations within a species and genetic selection by breeding.
- Study long-term influences on growth and development during the life cycle, of environmental and nutritional variation during early life stages.
- Create comparative knowledge from key and model species of the form-function of organ systems and their biological processes to obtain a deeper understanding of the larval stages (direct/indirect development).
- Create functional analysis of biological processes by establishing the new tools of morpholino gene overexpression and zinc finger nuclease technology in the species of interest.

The programme will improve the understanding of development in early life stages of fish, and effects of environmental factors, including nutrition, on a deeper biological level. This knowledge is critical for understanding how fish larvae are adapted to their natural environment and how critical phases in development may be connected to problems of recruitment. Furthermore, the programme will generate new insight into how anthropogenic factors, including pollution and climate change, may affect normal development. Aquaculture will benefit from the programme by knowledge-based development of production lines and protocols to a much larger extent than at present.

9 Cost

We propose a programme over 10 years with an annual cost of NOK 90 million (Norwegian kroner). This represents about 0.2% of the annual value of Norwegian aquaculture and fisheries. Increased growth of fish juveniles due to better juvenile quality may balance this investment during a few years of production.

The initiation of this proposed fish larval programme starts with an annual cost of NOK 20 million for the first year to establish a fish development network and to initiate Atlantic cod as a marine fish model species (initiating sub-programme I, Development of form and function and sub-programme II, Nutrition and development). In the second year more is invested into these two sub-programmes in addition to initiating sub-programme III (Environment and development). From the third year the programme is up and running full-scale.

Since this programme is an interdisciplinary joint venture that naturally should be funded by different programmes of the Research Council of Norway and different ministries, one example of a funding plan is given below in order to underline the interdisciplinary nature of this proposed programme.

Activity	**2010**	**2011**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**
Collective recourses										
Culture of larval material										
Using natural zooplankton (lagoon)	2	5	5							
Using standardised plankton	1	5	5	10	10	10	10	10	10	10
Sequencing and bioinformatics	2	4	3	3	3	3	3	3	3	3
Database and network	1	1	2	2	2	2	2	2	2	2
Sub-programmes										
P I. Development of form and function	7	15	25	25	25	25	25	25	25	25
P II. Nutrition and development	7	15	25	25	25	25	25	25	25	25
P III. Environment and development		15	25	25	25	25	25	25	25	25
Estimate of cost in million NOK	20	60	90	90	90	90	90	90	90	90

Funding example for an interdisciplinary fish larval programme

Aquaculture	8	20	20	20	20	20	20	20	20	20
Natural populations	6	10	20	20	20	20	20	20	20	20
Basic biology and genomics	6	10	20	20	20	20	20	20	20	20
Effects of pollution		10	15	15	15	15	15	15	15	15
Effects of climatic changes		10	15	15	15	15	15	15	15	15
	20	60	90	90	90	90	90	90	90	90

10 Enclosure: Review of biological processes

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10.1 Early development, maternal effects and endocrinology

Helge Tveiten, Nofima Marin Tromsø

The earliest steps in embryonic development are dependent on and driven by maternal factors deposited in the oocyte during oogenesis. Maternal factors are stored in the form of specific mRNAs, proteins, hormones or any other biomolecule. At egg activation and fertilization, such factors become available for embryogenesis, sometimes after a process of activation involving translation or protein modification. Very little is known about how these maternal factors influence embryonic and larval developmental capacity. There is a need to identify genes and maternal factors involved in early embryonic development and search for links between regulatory factors of maternal origin and "egg quality". Investigating the link between broodstock holding conditions, regulation of oocyte gene expression, and the developmental capacity of the resulting embryo and larva will be important.

After fertilization, growth and ontogeny of the embryo and larvae follow a well defined and genetically programmed sequence in which hormonal regulation is of critical importance. During the embryonic phase the endocrine organs develop, but are not functional, and hormones produced locally or of maternal origin may fulfil regulatory needs. Around the time of hatch there is a transition phase when larval endocrine organs begin to function but hormonal levels remain very low. In the post-hatch larva there is an accelerated activity and functionality of the endocrine organs which is accompanied by large fluctuations in hormone levels. The basic molecular mechanisms involved in functional maturation of the different endocrine axes in fish are far from understood and need to be elucidated. Little information is available about the influence of environmental factors, both exogenous and endogenous, on development and functionality of the different endocrine systems. Impaired development during embryonic and larval stages may have long-lasting effects and eventually influence the physiological performance of the adult fish.

Maternal factors in early development

Maternal factors are essential for early animal development, including fish species. Maternal transcripts are produced and reversibly silenced during different stages of oogenesis. As the name implies, maternal genes are defined as those required in the adult female for the development of the offspring (Lyman-Gingerich and Pelegri 2007). The dependency on maternal genes derives from the fact that the earliest steps in embryonic development are driven by factors produced during oogenesis, which are stored in the form of mRNA, protein or any other biomolecule. At egg activation and fertilization, such factors become available for embryogenesis, sometimes after a process of activation involving translation or protein modification. Fish, as most animals, have a

substantial period of time in embryonic development which occurs prior to the activation of the zygote`s own genome. The initiation of the zygotic gene program, or embryonic genome activation (EGA), normally occurs during the blastula stages, at the so-called mid blastula transition (MBT). In zebrafish (*Danio rerio*) embryos, the only fish species studied in any depth, MBT occurs gradually, starting at cell cycle 9-10 (≈ 512-1000 cells) and ending at late cycle 13 (Pelegri, 2003). In rainbow trout (*Oncorhynchus mykiss*) EGA is assumed to be initiated at approximately 35 degree-days, which is equivalent to about 6 days post-fertilization at 6°C, whereas in Atlantic cod (*Gadus morhua*), for example, the 512-1000 cell stage is reached after about 36 hours post-fertilization at 7°C (10-11 degree-days) (Hall et al., 2004). However, no detailed study of EGA in Atlantic cod has been carried out.

Prior to the MBT, the entire developmental program depends solely on maternal products. The MBT constitutes a period of time during which the importance of maternal products gradually decrease and zygotic gene products become the primary factors driving development. However, in zebrafish, a role for maternal products, often in conjunction with zygotic products derived from the same gene, has also been demonstrated for a variety of developmental processes that occur after the MBT. The MBT marks the onset of zygotic transcription in the embryo, which is apparent by a dramatic increase in new RNA synthesis. Despite the "ball of cells" morphology in the blastula stage, a number of cell specification events have already taken place that profoundly influence subsequent differentiation and organ formation. Because the zygotic genome is transcriptionally quiescent for most of the blastula stage, the defining moments in the life of an organism occur without the benefit of transcription. Therefore regulation of maternal mRNA by maternal proteins plays a central role in the determination of cell fates and the establishment of embryonic axes (reviewed by Farley and Ryder, 2008). Additional cellular divisions, morphogenesis and cell differentiation processes result in the further regionalization of these structures and tissues. Genetic analysis has shown the

involvement of maternal factors in all of these processes (Pelegri 2003).

Translational regulation of mRNAs in the developing oocyte

The regulation of poly(A) tail length of translationally controlled mRNAs is a recurring theme in oogenesis and early development of many animal species (reviewed by de Moor et al., 2005). In most cases, long poly(A) tails (80-500A residues) correlate with activation of translation and short tails (20-50A) with repression of translation. Many of these regulated mRNAs are of maternal origin (de Moor et al.,2005). More specifically, studies on mammals and *Xenopus* indicate that the poly(A) tail at the 3′ end of each transcript has emerged as an important regulatory element for determining stability. Thus, cytoplasmic polyadenylation represents a key regulatory step in gene expression in early embryonic development (Gandolfi and Gandolfi, 2001). Further, oocyte germinal vesicle breakdown (GVBD) and meiotic resumption are preceded by a short burst of transcriptional activity. This transcription seems functionally important because its inhibition impairs oocyte maturation (Dalbies-Tran and Mermillod, 2003), and it may be hypothesised that this transcription is also of importance for subsequent embryonic development. The translational state of the mRNA must therefore be taken into consideration when such investigations are carried out.

Contribution of maternal mRNAs to embryonic patterning

A common regulatory theme in early development both in invertebrates and vertebrates is the specific localisation of maternal mRNAs. Beginning in oogenesis, mRNA encoding genes required for the specification of fundamental body axes as well as cell fates become enriched in specific regions of the oocyte. The resulting gradients of maternal mRNAs as well as the proteins they encode are crucial to patterning of the developing organism during embryogenesis (Farley and Ryder, 2008).

The maternal to zygotic transition

The final regulatory event in the life of maternal mRNAs is their degradation. Prior to the activation of zygotic transcription, maternally supplied mRNAs are degraded so that control over development is transferred to the zygotic genome. The timing and mechanism of this transition vary between model organisms. In Drosophila, Xenopus, and zebrafish, the transition from maternal to zygotic control occurs concurrently with the MBT, where synchronous cell cycles end and the individual blastomeres begin to grow.

Correctly timed clearance of maternal transcripts is critical for embryogenesis, as many maternal mRNAs encode proteins required for the establishment of early embryonic polarity, and their ectopic expression, can interfere with later events in embryogenesis. One strategy employed to selectively degrade maternal mRNA is the use of a zygotically transcribed selectivity factor that recognises specific maternal mRNAs and recruits degradation machinery to them. In zebrafish, the key selectivity factor is the micro-RNA miR-430 (Giraldez et al., 2006). Expression of miR-430 begins shortly after the MBT, and remains high through gastrulation (Giraldez et al., 2005). The ability to process miRNA is necessary for the degradation of at least 750 transcripts at the MBT as determined by microarray analysis (Giraldez, 2006). Recent genome-wide studies in Drosophila, C. elegans, and zebrafish demonstrate that approximately half of the genome is expressed in oocytes and early embryos (Tadros et al., 2007; Baugh et al., 2003; Giraldez et al., 2006). Moreoever, the expression levels of nearly half of these mRNAs change independently of zygotic transcription.

Is the presence of maternal regulatory factors and messengers important to embryonic development in finfish culture? Very few studies have been carried out, but in rainbow trout for example, post-ovulatory ageing of oocytes was associated with variations in the abundance of a number of transcripts (Aegerter *et al.,* 2004). Oocyte abundance of IGF-I receptor (IGFR Ib) and cyclinB transcripts correlated with morphological abnormalities at yolk sac resorption, while a maternal 'stockpile' of IGF-I, IGF-II and IGFR Ib mRNAs was positively correlated with embryonic survival. However, very little is known about the nature, and concentrations, of these regulatory factors in fish oocytes and early embryos. Further, there are very few studies on fish oocyte gene expression and mRNA deposition, their relationship to ovarian development, their involvement in early cell cleavage, and their possible importance in governing early development. There is therefore an urgent need to identify genes and maternal factors involved in early embryonic development, search for links between regulatory factors of maternal origin and egg quality, and investigate the influence of reproductive hormones on the regulation of gene expression in the oocyte and the embryo.

Developmental regulation prior to functional endocrine systems

Growth and ontogeny and the addition of new, improved physiological competence follow a well defined and genetically programmed sequence in which hormone regulation is critical (Brown and Bern, 1989). Strikingly, however, most embryonic

development occurs without mature endocrine tissues and functional endocrine axes. Actually, it is likely that development and maturation of the different endocrine systems are themselves under the control of factors synthesised outside a specific endocrine gland. Peptide hormones, usually considered to be endocrine factors responsible for communication between tissues remotely located from each other, are increasingly being found to be synthesised in developing tissues. Several hormones are now known to be produced in developing tissues that are unrelated to the endocrine gland of origin in the adult (Sanders and Harvey, 2008). These hormones are synthesised locally, and are active as differentiation and survival factors, before the developing adult endocrine tissues become functional. There is increasing evidence for paracrine and /or autocrine actions for these factors during development, thus placing them among the conventional growth and differentiation factors (Sanders and Harvey, 2008). For example, prior to the formation of the somatotropic (ST) axis, mRNA encoding for the growth hormone (GH) and insulin-like growth factor (IGF) isoforms and their receptors are present in piscine and mammalian oocytes and early embryos, and data suggest that the genes are actively expressed (Li et al., 2007). These findings have led to speculation about a role of these hormones in oocyte maturation and early cell cleavage of the embryo, and monitoring both maternal and zygotic GH and IGF and their receptor expression in early stages of rainbow trout embryos showed that mRNA encoding these genes occurred well in advance of the development of the pituitary gland or liver (Li et al., 2007). It has been proposed that the products of these genes play roles in the complex epigenetic processes that take place as the oocyte genome is down-regulated and the embryo genome is expressed.

Endocrinology

Principals of endocrine control

The release of hormones will often occur as a result of input signals received from the peripheral sense organs and transmitted to centres in the brain via the central nervous system. Within the brain there are connections to the hypothalamus, which is the major integrative centre controlling the release of a range of hormones. Stimulation of the hypothalamus initiates the synthesis and release of a series of hormones and hypothalamic messenger substances. These messengers are low-molecular-weight peptide molecules which, in turn, have effects upon the production and release of hormones from the pituitary gland. Subsequently, pituitary hormones travel in the circulation to their target organs where they exert their effects. The pituitary hormones may not only act upon the target organs, but can also have a direct feedback effect upon the hypothalamus, resulting in the inhibition of secretion of the releasing factor. Similarly, hormones from a peripheral target organ can provide the inhibiting feedback signal to the hypothalamus and/or the secretory cells of the pituitary itself.

Pituitary structure

The vertebrate pituitary gland, which is located ventral to the hypothalamus, consists of to major parts, the neurohypophysis (NH) and the adenohypophysis (AH), in which most hormone producing cells are located. As a central part of the hypothalamo-pituitary system (HPS), it constitutes a functional link between the nervous and the endocrine system to regulate basic body functions such as growth, metabolism and reproduction. As in mammals, HPS of teleosts is separable into three major parts: the hypothalamus, which is part of the diencephalon, the neurohypophysis, which derives from the ventral diencephalon, representing the neural compartment of the pituitary, and the adenhypophysis, which is the non-neural part of the gland (Pogoda and Hammerschmidt, 2007). The connection between the hypothalamus and the AH is either via a neuronal route or via a series of blood vessels that form localized portal systems similar to those seen in higher vertebrates. The teleosts, however, lack a hypothalamic-pituitary portal system, and the neurons from the hypothalamus project directly into the AH.

In contrast to mammals, where NH is a separate lobe located posterior of AH, the NH in teleosts interdigitates with the dorsal part of the AH. Studies of the pituitary of a range of fish species demonstrate that the AH consists of structurally separate parts; the rostral pars distalis (RPD), the proximal pars distalis (PPD) and the pars intermedia (PI). Recent studies on zebrafish indicate that AH develops from a primodeum of pituitary cells in the anterior neural ridge and moves inwards in the process of oral cavity formation (Herzog et al., 2003; Pogoda and Hammerschmidt, 2007).

Pituitary hormones

In teleosts, at least eight different AH cell types can be distinguished and chracterised by the hormones they make. Trophic peptide hormones of AH can be divided into three distinct chemical categories. The first category consists of prolactin (PRL) generated by lactotrophs, growth hormone (GH) generated by somatotrophs, and β- and α-somatolactin (SL) generated by somatolactotrophs. All of these are fairly large polypeptide chains with considerable structural and functional overlay (Pogoda and Hammerschmidt, 2007).

The second category of AH hormones is comprised of thyroid stimulating hormone (TSH) generated by thyrotropes, and follicle-stimulating hormone (FSH) and luteinizing hormone (LH) generated by gonadotrophs. As in mammals, the three glycoprotein hormones consist of a shared α-chain and a structurally distinct and hormone specific β-chain (TSHb, FSHb, LHb, respectively). In teleosts TSH appears to regulate growth of the thyroid gland and thyroxin production, while FSH and LH regulate gonadal development and sex hormone production (Pogoda and Hammerschmidt, 2007).

The third hormone category includes the smallest pituitary peptide hormones, which are generated via proteolytic cleavage of the common pro-hormone proopiomelanocortin (POMC). In the telost AH, at least two POMC products have been described so far, adrenocorticotropin (ACTH) and melanocyte-stimulating hormone (α-MSH), while other known POMC products, such as β-MSH, the lipotropins β-LPH and γ-LPH, and the opoid β-endorphin peptide have been less studied (Pogoda and Hammerschmidt, 2007).

The architecture of the fish AH, including the pattern of cellular arrangement and the timing and order of the first appearance of endocrine cells, varies among different groups and species (see below). However, in many fish species, somatotrophs, lactoprophs and somatolactotrophs are confined to the same structural area of the pituitary; somatotrophs are usually found in PPD, lactotrophs in the RPD and somatolactotrophs in PI.

The neuroendocrine part of the fish NH is made of neurosecretory nerve endings that have their cognate cell bodies in the hypothalamus. The NH of teleosts typically secretes two different nonapeptid hormones: a basic one called arginine vasotocin (AVT), and a neutral one called isotocin. The apparent main function of these two hormones is involvement in the regulation of water and salt balance.

Pituitary ontogeny

There have been few studies on the embryological development of pituitary hormone producing cells. During embryonic development in coho salmon (*Oncorhynchus kisutch*), PRL cells appeared first at 4 weeks post fertilization (eyed stage), whereas TSH, MSH, ACTH and GH cells were identified between week 5 and 6 (Mal et al., 1989). Immunoreactive GTH-I/FSH cells appeared shortly after hatching, whereas GTH-II/LH cells were not apparent at any stage of embryonic or larval development. In chum salmon (*Oncorhynchus keta*), five weeks after fertilization (eyed stage embryos), five cell types were detected in the AH anlage: PRL-, GH-, ACTH-, α-MSH-, and TSH-producing cells. The PRL-, GH- and ACTH-cells were relatively well developed as compared with MSH- and TSH-cells. Gonadotropes were not detected even three weeks after hatching (Naito et al., 1993). The NH began to grow around the eyed stage and neuroendocrine fibers from the tuberal hypothalamus and the preoptic regions reached the NH during the last week of embryonic life (Naito et al., 1993). In rainbow trout, Saga et al. (1993) reported that the pituitary anlage was first recognized at 18 days post-fertilization (dpf). At that stage, PRL and MSH cells were identified. Cells containing ACTH had appeared by 35 dpf. At the last stage before hatching (42 dpf) TSH cells were identified.

In Atlantic halibut (*Hippoglossus hippoglossus*), a marine fish which hatches at a less developed stage than salmonids, Einarsdottir et al. (2006) reported the distinction of the pituitary as a separate organ at 121 degree-days (D°), during the yolk sac stage, about 23 days post hatch. In feeding halibut larvae, from 360D° to the start of metamorphosis (around 600 D°) a clear differentiation of the pituitary into the PD, PI and NH occurs. In Atlantic halibut somatotrophs and somatolactotrophs are first identified at 121 D° (23 dph), whereas the first lactotrophs are found at about 134 D° (c. 25 dph). Scarce tyrotrophs are, however, not detected until stage 5 (first-feeding, 260-430 D°) (Einarsdottir et al., 2006). In sea bass, which is also a seawater teleost, ACTH-, MSH-, TSH- and GH-cells appeared the first day after hatching (Cambre et al., 1990). Cells which immunoreacted with PRL antiserum were detected between 9 and 15 days after hatching. Gonadotrophs could not be observed during the first 26 days after hatching (Cambre et al., 1990). Studies of other marine species revealed that in species with small pelagic eggs, somatotrophs and lactotrophs are detected shortly before the end of yolk sac absorption and at hatching in species with larger demersal eggs (Tanaka et al., 1995). To our knowledge, there is no detailed study on pituitary ontogeny of the Atlantic cod.

Development of functional endocrine systems

The presence of endocrine cells, and their successive increase in number during larval development, suggests that these hormones are important for embryonic and larval development. Nevertheless, the mere demonstration of endocrine cells does not constitute a full proof of endocrine regulatory activity, as it is not known if or when these hormones are being released into the circulation. For example, studies on the ontogeny of the hypothalamic-pituitary-interrenal (HPI) axis in rainbow trout show that the interrenal steroidogenic cells starts to express ACTH receptors before hatching (Barry et al., 1995a) but cortisol levels in

alevins are not increased by either cold or handling stress until about two weeks after hatching. This initial cortisol response to stress has been interpreted as evidence for the establishment of a functional HPI axis. The exact time of this cortisol response varies in different studies, depending on temperature and fish strain, and ranges from about the end of the second week post-hatch in fish reared at 10°C (Barry et al., 1995a,b) to 3-5 weeks after hatching in rainbow trout reared at about 5°C (Pottinger and Mosuwe, 1994). In trout the stress hypo-responsiveness period is not due to immaturity of the pituitary POMC producing cells, because corticotrophs have matured at hatching (Saga et al., 1993), nor due to non-responsiveness of the interrenal cortisol producing cells to ACTH because head-kidney tissue collected before and after hatching is responsive to ACTH in vitro. Also, in the zebrafish, the ability to synthesise cortisol, which occurs around the time of hatch, does not immediately give rise to stress-induced stimulation of cortisol production (Alsop and Vijayan, 2008). This lack of response was not likely to be related to ACTH receptor availability, as it was found to be strongly up-regulated at the time of hatch. Thus, although elevated ACTH receptor expression and basal production capacity at around the time of hatch, activation of the cortisol stress axis was delayed until just before exogenous feeding. Together, these studies may suggest that the final step in the maturation of the cortisol stress response is coordinated at the level of the brain. However, it is important to note that studies in tilapia (*Oreochromis mossambicus*) indicate the initial cortisol response may be regulated independently from corticotrophin-releasing factor (CRF) and ACTH (Pepels and Balm, 2004). Thus, the basic molecular mechanisms involved in functional maturation of the HPI axis, as well as other endocrine systems, are far from understood.

Information on development of the cortisol stress response during early development of marine fish larvae is almost absent. Atlantic cod larvae developed a measurable corticosteroid stress response within 8 days post hatch (at 10°C) (King and Berlinsky, 2006). However, also in cod larvae corticosteroids were found prior to (at hatch) the acquired ability to elicit a stress response (King and Berlinsky, 2006), but corticoid dynamics prior to hatch was not studied. In red drum (*Sciaenops ocellatus*), elevated cortisol in response to handling stress was first seen in 10 mm larvae (c.25 dph), whereas this response was not apparent in larvae of about 8 mm (c.22 dph) (Pérez-Domínguez and Holt, 2006), indicating rapid changes stress coping ability during larval development. Interestingly, in the latter study, small larvae had a 7-fold higher baseline cortisol concentration than larger larvae, indicating a role for cortisol during earlier stages of development. Interestingly, based on the demonstration of cortisol and its receptor mRNA in just-fertilized eggs, Shiraishi et al. (1999) suggested that the developing embryo of tilapia utilizes maternal cortisol as well as cortisol transcription factors before the commencement of its own cortisol production. Therefore it seems justified to suggest that cortisol signalling may be present in very early stages of fish, although this topic is basically unexplored.

Similarly, prior to the development of the larval thyroid gland, fish eggs, and subsequently, larval yolk sac contain significant amounts of thyroid hormones of maternal origin. (Kobuke et al., 1987; Tagawa and Hirano 1987; Brown et al., 1988; Greenblatt et al., 1989, Tagawa et al., 1990). This source of THs is likely to be of importance for the physiological regulation of growth, development and osmoregulation in larvae prior to the development of functional endogenous thyroid follicles. For example, in Atlantic halibut, larval THs (Einarsdottir et al., 2006) as well as their receptors (Llewellyn et al., 1999; Galay-Burgos et al., 2008) are present in advance of thyroid follicle development, suggesting a role of TH prior the formation of the embryo's own thyroid tissue.

Hormonal regulation of early development

Hormones are considered highly important for regulation of development and growth of larval fish (Tanaka et al., 1995). Thyroid hormones, cortisol, GH, IGF I and II, and PRL appear essential to these processes. Here we will briefly review their involvement during early stages of fish development. It is, however, important to emphasise that the focus put on the role of these hormones in no way precludes the involvement of other endocrine tissues and hormonal factors during early development.

Thyroid hormones

The importance of the thyroid hormones (THs) thyroxine (T4) and triodothyronine (T3) in vertebrate development is well established (Hadley 1992; Tata et al., 1993; Power et al., 2001). The activity of the thyroid gland is primarily regulated by TSH secreted from the thyrotrophs of the pituitary. Thus, TSH plays an important role in the endocrine regulation of fish larval development and the temporal and quantitative appearance of thyrotrophs may act as an indicator of the onset and degree of thyroid follicle activity. However, it is important to note that during early larval growth, thyroid follicular tissue and initial thyroid hormone production is independent of tyrotroph development and TSH (Alt et al., 2006). In fish, THs are involved in the transition from the larval to the juvenile stage, the most dramatic manifestation of which is flatfish metamorphosis. Elevated

endogenous TH concentrations have been observed during metamorphosis in the Japanese flounder (Paralichthys olivaceus), summer flounder (*Paralichthys dentatus*) and Atlantic halibut (Tagawa, 1990a, b; Schreiber and Specker 1998; Einarsdottir 2006). During metamorphosis in Atlantic halibut there is also a peak in TH receptor expression (Galay-Burgos et al., 2008). Further, TH treatments stimulated flatfish metamorphosis (Inui and Miwa 1985; Miwa and Inui 1987a; Solbakken et al., 1999) and the transformation from larvae to juvenile in round fish (Reddy and Lam 1992; de Jesus et al. 1998; Solbakken et al., 1999; Deane and Woo 2003). It is apparent that the THs play an important role in larval to juvenile metamorphosis. However, much information is lacking on specific modes of action, and the manner by which THs bring about their effect is less clear in fish than in other vertebrates.

Growth hormone and insulin-like growth factor

In fish, the hormones of the somatotropic (ST) axis, growth hormone (GH) and insulin-like growth factor-I and II (IGF-I and IGF-II), together with their receptors and plasma binding proteins, have been linked to cell proliferation and differentiation, regulation of growth, several aspects of behaviour, immune system function, intestinal tract growth and function, ionic and osmotic regulation, reproduction and smoltification (Canosa et al., 2007 and Li et al., 2007 for references). IGF-I receptor (IGFR-I) knockdown studies in zebrafish have established that the IGF pathway play a crucial role in embryonic development and growth in teleosts as well (Eivers *et al*., 2004). Targeted knockdown of the duplicate IGFR-Is in zebrafish embryos by use of antisense morpholinos resulted in dramatic developmental perturbations and eventual death. Morphant embryos exhibited marked growth retardation and compromised formation of many organs and tissues (e.g. eye, ear, heart, and muscle) (unpublished results referred in Wood *et al*., 2005). Together, this evidence suggests the distinct requirements for a functional IGF signalling during embryonic development. Although the ST axis is the primary source of circulating GH and IGF-I once the pituitary gland has developed and the liver is functional, genes encoding for these hormones are also present in many non-ST axis-related tissues of fish, as well as in embryos sampled prior to the appearance of the ST axis and liver development. These non pituitary (GH) and non-hepatic (IGF-1) sources of hormone appear to regulate a host of cellular events, probably through paracrine or autocrine interactions.

Prolactin

Prolactin is a versatile hormone with an amazing array of functions in different vertebrate classes (Boyle-Feysot et al., 1998). In freshwater and euryhaline teleosts, including some flatfishes, a major function appears to be osmoregulation (McCormick, 2001; Wada et al., 2004). Other functions of PRL in vertebrates include the regulation of growth, development, metabolism stimulation of endocrine glands, behaviour, reproduction and immune function (Bole-Feysot et al 1998; Forsyth and Wallis 2002). A recent study of early zebrafish development, using antisense morpholino oligonucleotide (MO) gene knock-down, showed that PRL MO-treated embryos showed defects in gas bladder inflation, reduced head end eye size, shorter body length and fewer melanophores than untreated controls (Zhu et al., 2007).

Somatolactin

Somatolactin has been shown to be involved in the regulation of adiposity and gonadal function in salmonids and sparid fish (Company et al., 2001), to have a hypercalcemic action in rainbow trout in fresh water (Kakizawa et al., 1993), proliferation and morphogenesis of chromatophore pigment cells in medaka (Fukamachi et al., 2004) as well as gas bladder development in zebrafish (Zhu et al., 2007).

Corticoids

Cortisol is the main product of the interrenal gland in teleosts, and has both gluco- and mineralcorticoid actions. The temporal changes in cortisol levels during the early life stages of teleosts are similar across a number of species. The initial maternal deposition of cortisol in the yolk is utilized during embryogenesis and reaches its lowest concentration around the time of hatch; then the larva begins to synthesise cortisol de novo (for references see Alsop and Vijajan, 2008). Cortisol has direct effects on development (de Jesus et al., 1990), metabolism (Mommsen et al., 1999), immune system (Bateman et al., 1989), and stress (Wendelaar Bonga, 1997). Cortisol may also interact with other hormones during development (e.g. THs, PRL and GH) and their interactions are often overlooked (Wada 2008 for references). More specifically, cortisol is elevated concomitant with metamorphosis and acts in concert with THs during larvae transformation. When treated with ACTH or cortisol in addition to THs, the combined treatments can elicit a more accelerated metamorphosis of fish larvae than THs alone, likely acting via a further elevation of thyroid hormones or increasing TH receptor levels (Wada, 2008). The development of the corticoid system during the early life stages of vertebrates, however, is not well understood. Specifically, little is known about the influence of maternally deposited cortisol on embryonic development, the timing of the activation of cortisol and receptor synthesis, or the molecular mechanisms involved.

Conclusion

Based on the reports cited above, three major phases appear to be involved in the development of endocrine functionality: 1.) the embryonic phase, during which the endocrine organs develop, but are not functional, and hormones produced locally or of maternal origin may fulfil regulatory needs, 2.) the transition phase, when larval endocrine organs begin to function but hormonal levels remain very low, and 3.) the transformation phase, when accelerated activity of the endocrine organs is accompanied by large fluctuations in hormone levels (see also diagram).

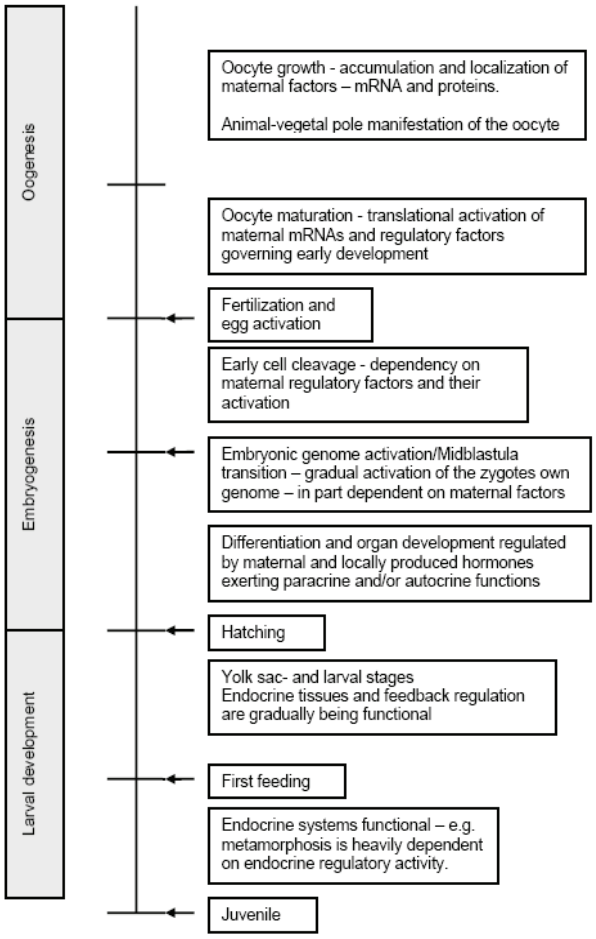

Diagram showing the timing of regulatory events during different stages of fish oocyte, embryonic and larval development

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10.2 Development and function of fish sensory system

Jon Vidar Helvik, Department of Biology, University of Bergen

A successful cultural system or production line for fish depends on our ability to understanding and implement the perceptual abilities of fish and how their sensory systems change during development. Such information also lends us the insight into adaptation to the natural environment and ecological interactions between the organisms and their communities. Central questions in sensory biology are how the organism senses the physical world, how this information is transmited in order to perform certain tasks, and how a particular environment may alter signals in ways that restrict the ability of animals to obtain information about potential prey, predators or mates.

Most of the comprehensive analysis of sensory systems in fish has been done on zebrafish, including the photoreceptor system of retina and pineal organ, the chemosensory system of olfactory epithelium (smell) and taste buds, and the mechanosensory system of neuromasts, lateral line and ear. Among the key species of this evaluation, the salmonids are quite well studied, both structure and changes in their visual system during smolt transformation and work done on the olfactory system in relation to homing. Except for some solitary papers on marine fish species, mostly on the morphological structure of the sense organs, there exists little literature on the functional analysis and a deeper comparative understanding of differences among species and specialisation to environment (see recent reviews in (Finn and Kapoor, 2008).

The sensory systems integrate the surrounding world and are critical for many internal processes such as circadian rhythm and behavioural decisions. Normal development and long-term performance depend on appropriate environment and stimulation during the critical early life stages of fish.

Chemoreception

The chemical world in the aquatic milieu is tremendously rich, and fish have evolved a sophisticated sensory system to decode this environment. There are three chemosensory systems in fish: 1) the sense of smell, received through the olfactory system localised on the peripheral organ on the snout; 2) the sense of taste, obtained through the gustatory system and localised in the oral cavity and body surface and 3) the less known solitary chemosensory cells (SCC), distributed on the entire body surface (see recent review (Døving and Kasumyan, 2008). Chemosensory system develops early in fish as in other vertebrates, and the sense of smell and taste do function at early larval stages.

Olfaction

High sensitivity to different types of odours is obtained by the olfactory system through a large number of sensory neurons with different odorant receptors. These receptors are often organised in lamella, creating a rosette that provides a large surface for odours/receptor interaction. To ensure ambient water transport over the organ, fish have developed sophisticated mechanisms based either on ciliary activity or pumping activity from accessory sacs in addition to water flow setup by the swimming activity. Chemical stimuli do not have a vector, so the fish must rely on other senses to orient towards water current and the chemical trail (Døving and Stabell, 2003). The fish olfactory epithelium transmits odour signals in three parallel pathways that merge in the olfactory bulb before they end up in different regions of telencephalon. Each partway transmits information, related to either food, pheromones or alarm substance; all elicit characteristic behaviour (Hamdani and Døving, 2005). The olfactory organs develop from ectodermal placodes in early embryos, in zebrafish, at the time when the optic vesicles evaginate from the diencephalon (Hansen and Zeiske, 1993). In zebrafish the olfactory placodes cells first appear as a mass of cells on the lateral edge of the anterior neural plate, organised in a pattern whose anterior-posterior locations give rise to specific regions of the developing olfactory organ. (Whitlock and Westerfield, 2000). As the olfactory pits develop, the covering epidermis separates and microvilli and kinocilia sensory cells become exposed to the environment. Axions from the olfactory epithelium creats pioneer neurons to the olfactory bulb. Ciliated and microvillus sensory neurons used this partway to create permanent connections and thereafter the pioneering neurons undergo programmed cell death (Whitlock and Westerfield, 1998).

In fish three distinct olfactory sensory cell types exist: ciliated, microvillous and crypt sensory neurons. Developmentally ciliated sensory neurons precede microvillous, and often dominate in the olfactory epithelium at larval stages. Fish have around 100 distinct genes encoding for the odorant receptors (Barth, et al., 1996) classified into three families: odorant receptors (OR) and two families of vomeronasal receptors (VR). The OR family are often found in ciliated sensory neurons and are

associated with perception of pheromones, while the two families of VR receptors (V1R and V2R) are expressed in microvillous sensory neurons and related to food search.

Table 1.. Overview of publications describing the sensory systems in key species for this evaluation

Biological process	Molecules	Cell	Organ	Organism	Behavior	Environmental factors
Chemoreception						
Atlantic cod					Migration (Saether, et al., 2007, Robichaud and Rose, 2002)	
Atlantic halibut					odor Sensitivity (Yacoob and Browman, 2007)	
Atlantic herring						
Atlantic salmon	Receptor (Wickens, et al., 2001, Dukes, et al., 2006, Dukes, et al., 2004)	Odorant stimulation (Moore and Scott, 1992, Moore and Scott, 1991, Lo, et al., 1991)	Olfactory bulb(Morin and Doving, 1992, Moore, 1994, Hara and Zhang, 1996)			
Sea bass			Olfactory structure (Diaz, et al., 2002) telencephalon (Cerda-Reverter, et al., 2001, Cerda-Reverter, et al., 2000, Batten, et al., 1993)		Stimulant feeding (Singh, et al., 2005)	
Sea bream					Feeding (Tanaka, et al., 1991)	
Mechanoreception						
Atlantic cod			Oticplacode(Miyake, et al., 1997)	Otolith and growth (Otterlei, et al., 2002, Nielsen and Munk, 2004, Hoie and Folkvord, 2006)		
Atlantic halibut			Neuromast (Blaxter, et al., 1983)			
Atlantic herring			Otolith (Brophy, et al., 2004, Burke, et al., 2008)			
Atlantic salmon			Otolith (Wright, et al., 2001, Veinott and Porter, 2005, Meekan, et al., 1998, Clarke and Friedland, 2004)			
Sea bass			Otolith (Secor and Rooker, 2000)			
Sea bream			Otolith (Tojima and Arai, 2000)		Sound learning (Anraku, et al., 1997)	

Photoreception					
Atlantic cod	Non-visual (Drivenes, et al., 2003)			Turbidity/ feeding (Meager, et al., 2005, Meager and Batty, 2007, Lokkeborg, 1998, Herbert, et al., 2004, Fiksen, et al., 1998)	Growth/ light environment (Browman, et al., 2006)
Atlantic halibut	Visual pigments (Helvik, et al., 2001a, Helvik, et al., 2001b)		Eye morphogenesis (Saele, 2006, Kvenseth, et al., 1996)	Optomotoric behavioiur (Helvik and Karlsen, 1996)	Diet/light (McEvoy, et al., 1998, Ronnestad, et al., 1998, Solbakken and Pittman, 2004)
Atlantic herring		Characterisation rod visual pigments (Jokela-Maatta, et al., 2007)			Diet (Navarro, et al., 1993, Mourente and Tocher, 1992), growth/light environment(Browman, Skiftesvik and Kuhn, 2006)
Atlantic salmon	Non-visual pigments (Soni and Foster, 1997, Philp, et al., 2000a, Philp, et al., 2000b)		Photoreceptor plasticity (Plate, et al., 2002, Kunz, et al., 1994, Flamarique, 2002, Cheng, et al., 2006, Browman and Hawryshyn, 1994a, Browman and Hawryshyn, 1994b)	Feeding/light (Fraser and Metcalfe, 1997)	Light environment (Migaud, et al., 2007)
Sea bass		Characterisation visual pigments (Singarajah and Harosi, 1992)	Melatonin rythms (Bayarri, et al., 2003, Bayarri, et al., 2002, Garcia-Allegue, et al., 2001, Sauzet, et al., 2008)		
Sea bream					

Gustation

The gustatory sensory system is involved in feeding behaviour, regulating food intake determined on palatability. The gustatory system is highly developed in fish and is more sensitive than is reported from higher vertebrates (Marui and Caprio, 1992). Both an oral and extra-oral locations of these taste buds are typical for fish, where the extra-oral play a prominent role in foraging behaviour. High densities of these taste buds are often found in regions of the body that stand a high probability of coming in contact with food items, such as the lower surface of the head, barbells, opercular, pectoral and ventral fins (Gomahr, et al., 1992). Fish taste receptors are innervated by one of three cranial nerves: facial nerve (VII) innervate taste buds on anterior oral cavity and lips, while glossophyngeal nerve (IX) and vagus nerve (X) innervate intra-oral taste buds.

Taste buds develop later than the olfactory organ and the solitary chemosensory cells. A typical taste bud is pear-shaped and contains from a few to several dozen elongated gustatory sensory cells (Reutter, 1992). Each sensory cell contains apical microvilli which together form the taste bud pore exposed to the environment. Vertebrates receive tastants, such as sugars, amino acids, and nucleotides, via taste bud cells in epithelial tissues. Two families of G protein-coupled receptors for tastants are expressed in taste bud cells: T1Rs for sweet tastants and umami tastants (l-amino acids) and T2Rs for bitter tastants. Zebrafish have three members of the T1R family and two members of the T2R family. All except T1R1 were found to be expressed in different subsets of taste buds (Ishimaru, et al., 2005).

Solitary chemosensory cells (SCCs)

All groups of fish possess SCCs, single spindle-shaped sensory cells with one or several microvilli that protrude out between epidermis. These sensory cells are evenly spread over the entire body, but with higher concentration on the head and trunk (Kotrschal, 1995). In zebrafish SCCs develop three days after fertilisation, which is two days before taste buds appear (Kotrschal, et al., 1997). Data concerning detail function and development of SCCs is lacking.

Except for a few studies in Atlantic salmon (see Table) few studies have analysed the development

and function of the chemoreceptor system in the fish species that are part of this evaluation.

Mechanoreception
The lateral line organs and the organs of the inner ear, the semicircular canals and otolith organs, are mechanical displacement detectors that share a common hair cell receptor structure (Flock, 1971, Blaxter, 1987). Mechanosensory organs, either in isolation or in conjunction with other sensory organs, are responsible for information about water currents (rheotaxis), schooling, prey, auditory perception and body posture. At early larval stages the mechanosensory systems are incomplete, but gradually change toward the adult conditions as the larva develops.

The mechanosensory receptor cell has an arrangement of displacement-sensitive "hairs", cells that project from the apical surface to form an asymmetric assemblage of stereocilia that progressively increase in length along an axis approaching a longer single and more rigid kinocilium (Flock, 1971), (Gibbs, 2004). A gelatinous mass, cupula, covers the sensory epithelium. The stereocilia-kinocilium axis gives the hair cells directional sensitivity, which relay signalling by either hyper or depolarisation of contacting neurons (Montgomery, et al., 2001).

Lateral line organ
The neuromasts of the mechanosensory lateral line have various locations, either superficially on epidermis, within epidermal pit or in dermis fluid-filled canals on the head and trunk (Gibbs, 2004). Fish have seven lateral canals on the head and trunk, with individual innervations and hydrodynamic contact with the environment through regularly spaced pores. Free neuromasts provide an important sensory role in rheotaxis by responding to water velocity over the skin. Canal neuromasts, on the other hand, respond to fine-scale water velocity stimuli of biological origin, such as prey and predators (Denton and Gray, 1988).

The inner ear
The paired organs of the inner ear, the semicircular canals and otolith organs, are endolymph-filled chambers on each side of the cranium. The semicircular canals (lateral, anterior and posterior) on each side of the head have sensory epithelium in a specific region detecting movement in the endolymph. In addition fish have three otolith organs located in the ventral portion of the inner ear: the utricule, sacculus and lagena (Lowenstein, 1971). The semicircular canals and otolith organs have the potential to detect body accelerations coincident with postural changes and swimming in addition to particle displacement component of the sound stimulus. Several studies have shown that there is a great variation in timing of auditory system development, but the pattern of development is highly conserved (Fuiman, 2004). The ears form from the otic placodes, one of several bilaterally paired localised thickenings of the dorsolateral embryonic ectoderm. The placodes grow into a solid ball of cells, then transform into a hollow sphere creating three pillars of tissue that later become the semicircular cannels (Bever and Fekete, 2002).

An interesting aspect of otoliths is that they grow incrementally on a daily basis, reflecting temperature- and food-dependent conditions. In addition the elemental incorporation in the otoliths reflects to some extent the ambient chemical composition in the water and food resources. Much effort has therefore been invested in analysing the otolith microstructure and microchemistry to trace the life history of harvested individuals prior to capture. Such studies are important for understanding mechanisms of growth, mortality, recruitment and individual origin. Combined with analyses of fish grown under controlled culture conditions, further insights on development and life history can be obtained by comparing offspring from culture conditions with offsping from natural conditions.

Except for some solitary articles and work in otolith microstructure, few studies have investigated the mechanosensory system in the key species. The free neuromasts system is important in early life stages and later, the lateral line develops, representing a different sensory input. Timing and function of these systems are important for understanding prey search and escape behaviour.

Photoreception
Vision is the major sensory apparatus to detect prey and to avoid predators. The vast majority of teleosts pass through a planktivorous larval phase where they are obligate visual diurnal feeders. The visual system in larvae as well as in adults should be adapted as regards to sensitivity (luminosity and chromaticity), structure (dioptric apparatus and retina) and analytic capabilities (brain).

Eye and visual pigments
There is variation of the overall structure of the teleostean eye, but anatomical structures are organised similarly in all vertebrates. The globe of the eye is formed by sclera, fibrous or partly cartilaginous structures, that inwards are covered by the pigmented and vascular choroid. Overlaying the pigment epithelium is the retina, consisting of photoreceptors and several neuronal layers. Axons from the neurons in the innermost retina converge over the retinal surface before leaving the eye in the optic disk and form the optic nerve, which projects

to several areas in the brain, including the tectum opticum. Light enters the eye through a transparent cornea and is focused on the retina by the lens.

Photoreceptors are the key element in vision, where light (photons) are transformed into neuronal signals in an intensity and spectral sensitivity manner. Retina contains an array of photoreceptor cells of two types: cones specialised for bright light and colour vision and rod cells specialised for vision in low-light environment. Photoreceptors achieve their spectra sensitivity by a photo pigment, opsin, that is encoded by opsin genes: a long- to middle-wave class (LWS; red-green region 490-570 nm), a middle-wave class (RH2; green region 480-535 nm), a short-wave class (SWS2; blue-violet region 410-490 nm) and a second short-wave class (SWS1; violet-ultraviolet 355-440 nm) in addition to a rod class pigment (RH1). (Bowmaker, 2008).

Characterisations of the photoreceptors are done directly by microspectrophotometric (MSP) analysis of the individual photoreceptors or indirectly by cloning and *in vitro* expression of the opsin genes in cell lines followed by spectral analysis of the purified protein. The number of different visual pigments and their distribution in retina determine ability of colour resolution and contrast sensitivity. The photoreceptors system has been extensively studied in some teleosts species, including how the system develops and transforms during different life stages (review see (Loew and Wahl, 2008). There is huge variation in the visual system of teleosts, from the upper dwelling species having all spectral classes of visual pigments, to the deep-water species with only rod opsins. For example, zebrafish have 8 cone visual pigments, where four belong to the green sensitive class (Chinen, et al., 2003), and their expression changes during development. Also in salmonids there is plasticity in the distribution of photoreceptors in retina, where UV-sensitive cones are lost during smoltification and regained in reproductive adults during the return to spawn in freshwater (Allison, et al., 2003).

Non-visual photoreceptors

Retinal photo detections with cones and rods are related to image formation and vision, but in addition other photoreceptors cells exist both in retina and brain. Their function is less studied but is clearly related to adjusting diurnal rhythm to photo environment. The pineal organ, closely linked to melatonin rhythm, contains several photoreceptors, where exo-rhodopsin is the most prominent (Bellingham, et al., 2003, Mano, et al., 1999). Several other less-known photoreceptors exist in fish, such as melanopsin, vertebrate ancient opsin etc., which are both expressed in deep brain and retinal neurons. Several light-sensitive biological processes have been reported in marine fish larvae, such as regulation of buoyancy and hatching in Atlantic halibut, at a stage prior to eye formation. This indicates the importance of light for various processes early on in development, and the lack of knowledge of the underlying mechanisms.

Recent studies have shown that almost all fish cells are photosensitive, and even cell cultures of fish cells respond to light in the incubators (Whitmore, et al., 1998). Little is known about the mechanism for this (Tamai, et al., 2007).

Unnatural light conditions, constant light or constant darkness are often used in culture of fish larvae, but we know little about the impact of this on a cellular level and long-term effects on development.

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10.3 Brain and neuroendocrine system

Lars O. E. Ebbesson, Unifob Environmental Research and Department of Biology, University of Bergen

The brain is the integrator of external and internal signals and its proper function is dependent on normal brain development. Present knowledge of morphological, genetic, physiological and behavioural processes in key target fish species during critical developmental periods is inadequate and often non-existent. Understanding brain development and the manifestations of "unnatural" or changing rearing environments will provide an important framework for evaluating impacts and attaining solutions associated with key critical phases in the culture of marine teleosts and salmon. The basis of knowledge of brain development in teleosts has been derived from comparative neuroanatomical approaches spread among a vast diversity of fish species, with the majority of the molecular regulation recently established in zebrafish. As with all vertebrates, general themes drive the moulding of the functional brain, including early neural developmental processes from fate maps to genetic control, neurotransmitter ontogeny, and environmental input and stimulation. In general, neural developmental processes are similar among vertebrates, enabling one to draw knowledge from different model species. Without proper foundations within the target species, however, one cannot evaluate environmental impacts and long-term consequences on populations.

General

The basis of knowledge of brain development in teleosts has been derived from comparative neuroanatomical approaches, with the majority of the molecular regulation established in zebrafish. As with all vertebrates, a general theme drives this early development, and the zebrafish has provided important foundations of early neurodevelopmental processes from fate maps to genetic control, neural plasticity, neurotransmitter ontogeny, and specific gene functions (through the use of expression manipulations). In general, neural developmental processes are similar among vertebrates, enabling one to draw knowledge from different model species. This does not, however, eliminate the need for adequate morphological, genetic, physiological and behavioural foundations in key target species at critical developmental periods, as these are not always represented in the life history of the model species. Understanding brain development and the manifestations of "unnatural" rearing environments will provide an important framework for evaluating impacts and attaining solutions associated with key critical phases in the culture of marine teleosts and salmon.

Early brain development

Ontogeny of the central nervous system is itself a highly complex morphogenetic process characterized by a strong aspect of spatiotemporal order observed similarly in all vertebrates during development. In teleosts, the zebrafish model provides the most comprehensive knowledge of early development and its regulation, demonstrating the similarity to higher vertebrates during this period. Briefly, the early vertebrate embryo exhibits three embryonic cellular sheets called germ layers, the most peripheral one being the ectoderm. Its dorsal portion represents the neuroectoderm (the future nervous system, which segregates early from the ventrolaterally lying general ectoderm (the future epidermal skin and its derivatives). Subsequently, the central main portion of the neuroectoderm, the neural plate, is swallowed by the general ectoderm through the process of neurolation. As a consequence, the neural plate separates from the general ectoderm and descends into the deep of the embryo, where it develops into a hollow neural tube surrounding a cerebrospinal fluid-filled ventricle. Meanwhile, the general ectoderm closes the temporarily opened backside of the embryo. The apparent formation of the three initial (forebrain, midbrain, hindbrain) and subsequent five brain vesicles (telencephalon, diencephalon, mesencephalon, metencephalon, mylencephalon) along the anteroposterior axis somewhat hides the parallel establishment of finer subdivisions called neuromeres (prosomeres in the forebrain and rhombomeres in the hindbrain. Next is the differentiation of functional subdivisions of the brain and spinal cord (e.g. the optic tectum/superior colliculus, a midbrain sensorimotor integration center, or the hypothalamus, the major control center of visceral processes (for reviews see Redies and Puelles 2001; Wilson et al III Neural development. This early development is strongly influenced by the genetic makeup and maternal influences (see Maternal effects) during this critical period. In the target species there are some papers describing the ontogenetic development of neurotransmitter systems (Amano et al., 1998; Amano et al., 2002; Amano et al., 2004; Amano et al., 2003; Amano et al., 2005; Becerra et al., 1995; Faraco et al., 2006; Moles et al., 2007; Parhar et al., 1995; Tollemar et al., 2003), whereas almost no data exists on the impacts of environmental or maternal factors on early brain development.

Neurondocrine development and critical period regulation

Critical periods allow the transition from one developmental stage in a specific environment to the next stage in a different environment, and include a migration between the two habitats. They correspond to drastic changes in body shape, physiology and behavior, and, unlike puberty, are encountered only in some phyla/species. In our target species these critical periods are represented by hatching, first-feeding, metamorphosis and smoltification (and although not presented here, puberty and sexual maturation). These life history stages are finely tuned and sensitive to environmental stimuli that synchronise the proper timing of the development to maximise survival. The environment (e.g. photoperiod and temperature) initiates these critical periods of development by strongly influencing hormonal regulation, which is conveyed through a light-brain-pituitary axis. Transient increases in specific hormones and their receptors trigger and regulate tissue development including the brain and neural plasticity that includes proliferation, differentiation and sprouting of neuron and glia cells, which establish new connections. Once connections are established, the survival of these new connections depends on the subsequent environmental stimuli molding the brain by keeping stimulated and losing unused connections, in order for the animal to progress into the next stage of life. The brain is under the developmental control of these hormones, especially thyroid hormones. Altered hormone levels during developmental periods have dramatic negative effects, resulting in impaired or abnormal transformation of the target tissues, which in turn dramatically affects development, growth and survival. Some of the molecular mechanisms by which these hormones influence gene expression and their impacts on the developmental processes are beginning to emerge in model species, whereas in the target species very little is known.

Environmental information is translated through sensory processes to neuroendocrine response in the brain, controlling pituitary function and ultimately physiological changes through different endocrine axes, targeting tissues and organs to transform. It has been shown in salmon smoltification that neural development during critical periods precedes the physiological changes that direct life histories and fitness (Ebbesson et al., 2003). At this point our understanding of larval and juvenile brain development in salmon is progressing, but the effects of undesirable environmental influences on brain development and smoltification during these critical periods in salmon are just beginning (Ebbesson et al., 2007; Kihslinger et al., 2006; Kihslinger and Nevitt, 2006). Among the other target species nearly no information on environmental impact is available. Endocrine surges occur around metamorphosis in flatfish yet no data is available on brain development associated with this period (Dufour and Rousseau, 2007). Understanding neuroendocrine development during this period will shed important light into the developmental trajectories and lead to a better understanding of how environment impacts this development.

In addition to neuroendocrine development, the integration and establishment of other functional neural pathways associated with sensory system development during critical periods are equally important for successful progression into the next developmental stage. Neuronal circuits are shaped by experience during critical periods of early postnatal life. Chemicals applied at particular times to a developing embryo produce specific malformations, with the most rapidly growing tissues being most sensitive to the change in conditions. The fish brain is especially sensitive, as proliferation is heightened throughout life relative to higher vertebrates (Zupanc 1999). Stimulated by the external world, the postnatal nervous system responds further to natural sensory experience. Time windows exist when brain circuits that subserve a given function are particularly receptive to acquiring certain kinds of information or even need that instructive signal for their continued normal development. Studies on the brains of teleosts have shown that the relative size of sensory brain areas reflects sensory specializations and the relative importance of a given sensory system. Moreover, the relative size of these brain areas can change in relation to ontogenetic shifts in habitat and feeding ecology (Lisney et al., 2007). A critical period is an extreme form of a more general sensitivity, when neuronal properties are particularly susceptible to modification by experience.

A number of facets of critical periods have been identified. First is functional competition between inputs. Genetic specification determines much of the basic structure and function of the nervous system. But, the environment and physical characteristics of the individual into which the brain is born cannot be encoded in the genome. A process by which neurons select their permanent repertoire of inputs (or maps) from a wider array of possibilities is required for proper brain function. Indeed, the tailoring of neuronal circuits custom fitted to each individual is the main purpose of critical periods that help define life strategies. Second is the particular role for electrical activity. Neurons communicate by the transmission of nerve impulses as a reflection of external stimuli or spontaneous, internal states. The various inputs from which the nervous system can choose during

the critical period are ultimately encoded in the discharge of action potentials. Most cellular models of plasticity are now based on the ability to potentiate or depress transmission at individual synapses through their pattern of activation. Under 'unnatural' conditions, these inputs may be abnormal in a particular system, thus disrupting its normal development. Third is a structural consolidation of selected pathways. Early experience specifies a neural commitment to one of a number of possible patterns of connectivity. The magnitude and permanence of anatomical changes (from dendritic spine motility to large-scale rewiring) distinguish developmental plasticity from adult learning. A critical period may be defined in systems where structural modification becomes essentially irreversible beyond a certain age. Although we assume that the critical periods in our target species entail heightened neural plasticity, there is little data available to confirm this except for salmon during smoltification (Ebbesson et al., 1996a; Ebbesson et al., 2007; Ebbesson et al., 2003; Ebbesson et al., 1992; Ebbesson et al., 1988; Ebbesson et al., 1996b; Stefansson et al., 2008). Fourth is the regulation of critical period onset and duration not simply by age, but also by experience. If appropriate neural activation is not provided at all, then developing circuits remain in a waiting state until such input is available, as demonstrated in salmon reared under continuous light (Ebbesson et al., 2007). Alternatively, enriched environments may prolong or enhance plasticity as seen in proliferation in the brain (Kihslinger et al., 2006; Kihslinger and Nevitt, 2006). In other words, the critical period is itself use-dependent. Understanding the cellular mechanism of this effect will greatly influence strategies for lifelong rearing conditions in teleost culture for aquaculture and enhancement. Fifth is the unique timing and duration of critical periods across systems. Not all brain regions develop with the same time course. There are both rostro-caudal gradients of maturation across modalities and hierarchical levels of processing within a given pathway. Intuitively, the critical period for one stage cannot begin unless its input from a preceding stage is ready. Cascades of critical periods and their cumulative sequence at different ages and levels of processing shape each brain function as the relevant neural pathways develop to a point where they can support plasticity.

The diversity among teleosts in their life histories and their ability to be plastic in response to their surrounding environment is highlighted by the diversity in brain morphology associated with the requirements of the individual (Kotrschal et al., 1998).

Environmental impacts on brain development

Investigations of environmental impacts on brain development in the targeted culture species are very limited. The majority of information comes from juvenile salmonids where photoperiod or enriched environment has been investigated. In fish, the size of neuroendocrine cells in the brain can be influenced by immediate environmental conditions such as social status and habitat stability (Lema and Nevitt, 2004a; Lema, 2006). While animals reared in captive or laboratory environments often exhibit neural phenotypes that differ from their wild counterparts (Plogmann and Kruska, 1990; Kruska, 1996), little attention has been directed towards understanding how captive environments proximately influence the development of the brain. Salmon and trout provide an excellent model system to study environmental effects on brain growth, both because hatchery environments differ from natural habitats, and because behavior and brain size can vary among hatchery-reared and wild-reared fish. In the wild, salmon spend the first portion of their life in dynamic fresh water streams. Eggs are laid in gravel nests (redds) and hatch into alevins. Alevins remain buried for a period of days to weeks before emerging to become free-swimming fish. In the hatchery, however, fish are reared in high densities in homogeneous concrete raceways with little environmental variability and are scatter-fed an artificial diet from the surface. Given these differences in rearing conditions, it is not surprising that hatchery fish show morphological and behavioral differences compared to their wild counterparts. For example, salmon and trout propagated in hatcheries often manifest growth and maturation patterns as well as anti-predator, feeding, and sexual behaviors that differ dramatically from wild fish (Gross, 1998; Flagg et al., 2000). Recently it was shown that hatchery reared rainbow trout have smaller forebrain structures (olfactory bulbs and telencephalons) relative to body size than wild fish (Marchetti and Nevitt, 2003). Scattered data have shown an influence of feed composition on cod behavior (Höglund et al 2005) and brain size (Furuita et al., 2003), emphasizing the importance of nutrition in brain development and indicating another area of research that needs to be addressed further.

Conclusion

Understanding brain development and the manifestations of "unnatural" rearing environments will provide an important framework to evaluate impacts and find solutions to key critical phases in the culture of marine teleosts and salmon. To date this knowledge is very limited, yet where it has been investigated it is clear that environmental conditions impact brain development. One advantage of looking at the brain is that it integrates

information from the whole body and therefore allows for a broader analysis of a given situation.

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10.4 Behaviour

Howard Browman, Anne Berit Skiftesvik and Terje van der Meeren, Institute of Marine Research, Austevoll

Behaviour is the manifestation of an organism's response to both internal (physiological) and external (environmental) signals. Observing how fish larvae behave under various environmental and feeding conditions provides information that is directly relevant to the development of appropriate culture protocols for fish.

Feeding fish larvae are more active and swim faster and longer than those that are not feeding. Issues frequently targeted in behavioural research are activity rhythms, swimming patterns, foraging and prey search, and prey capture efficiency. A detailed awareness of diel and seasonal activity patterns – and ontogenetic changes in them – will allow for the development of culture protocols that are better tuned to the basic biology of the species being reared. Behavioural observations of the facility with which larvae can locate, attack, ingest, and retain feed particles are also of central importance. Thus, identifying substances that can motivate the feeding response of marine fish larvae and increase the probability that they will retain and digest their feed holds promise for the rapid improvement of e.g. formulated feeds. Attractiveness, palatability, and retention of live and manufactured feed need specific attention, particularly at weaning from live to inert diets. Very little is known about the olfactory and gustatory responses of marine fish larvae. Electrophysiological and behavioural techniques can be used to generate concentration response curves for various substances and to characterize how the fish behaves in their presence. These techniques are akin to asking these animals which smells and tastes they can perceive, which they prefer, and perhaps more importantly, what they do not prefer. Direct observations of fish behaviour are therefore essential (e.g. during transitions such as the weaning period).

Behavioural indicators may also be used to assess stress and/or welfare state. Until recently, very little information was available on the general behaviour, stress levels, discomfort/pain, anxiety/fear or "comfort" conditions of aquatic animals in culture situations. This kind of data is required to inform discussions and guide policy development. There is virtually no information of this nature available for fish larvae and early juveniles.

The specific categories of behaviour that follow were selected – on the basis of experience working on marine fish and shellfish species – as being both highly relevant to aquaculture and most information-poor. In each case, we address explicitly how generating empirical observations of these behaviours can be of great practical significance in aquaculture. It is important to note that empirical information to address these research questions can only be obtained through direct, and often painstaking and time-consuming, behavioural observation.

Activity rhythms, swimming patterns, foraging and prey search, prey capture efficiency

Feeding fish larvae are more active and swim faster and longer than those that are not feeding (e.g. Browman and O'Brien 1992a,b; Skiftesvik 1992). There are often also pronounced diel patterns in activity; many fish species are most active during crepuscular periods (e.g. Browman & Marcotte 1986; Skiftesvik 1994; Reebs 2002). Swimming and feeding activity – and swimming patterns (e.g. cruise vs. pause-travel movements) directly affect metabolic costs and, thereby, the conversion of feed into growth (e.g. Anderson et al. 1997; Killen et al. 2007a,b; McCollum et al. 2006; Ruzicka & Gallager 2006a). Thus, a detailed awareness of diel and seasonal activity patterns – and ontogenetic changes in them – will allow for the development of culture protocols that are better tuned to the basic biology of the species being reared.

The volume of water contained in the visual perceptual field (VPF) of a 6 to 10 mm fish larva is approximately 0.8 to 1.0 ml (see Galbraith et al. 2004; Cobcroft & Pankhurst 2006; Chesney 2008). Thus, at an *absolute* prey abundance (AA) of 1000 l^{-1}, there would be only 0.8 to 1.0 prey items within the VPF at any given instant. The number of prey per VPF is the *visual* abundance (VA) and, from the perceptual perspective of the predator, VA, *not AA*, is the operational measure of prey availability. Thus, for this predator, AA would have to be > 2000 l^{-1} in order for VA to be > 1. These VA numbers illustrate the importance of knowing the details of the fish larva's predatory abilities as the basis for calculating something as critical as the abundance of feed particles to present them with. Similarly, it should be clear that behavioural observations of the facility (or lack thereof) with which larvae can locate, attack, ingest and retain feed particles is also of central importance.

Even small changes in light intensity and "quality" (i.e. spectral characteristics) can have significant effects on the feeding rate, survivorship and growth of marine organisms (e.g. Browman et al. 1994; Puvanendran & Brown 2002). Despite this, the choice of light environment in indoor intensive culture systems has, with few exceptions, been little more than guesswork. For example, fluorescent tubes are commonly used as light sources in such culture systems. The spectral emission of these tubes is narrow-band and centered on wavelengths that result in them looking white *to humans*. To marine organisms – whose visual systems are mostly sensitive at wavelengths different from that of humans – these lights will not look white at all, and they will not appear as intense to them as they do to us. In addition, unless we know the details of their spectral sensitivity, we are unable to evaluate – *a priori* – how easy (or difficult!) such lighting conditions might make it for them to detect food. Sensory biology and behavioural assays can be used to characterize the spectral sensitivity of marine organisms and this knowledge can be applied, using colour theory (see Wyszecki & Stiles 2000), to tailor the lighting conditions under which they are raised so as (for example) to maximize the contrast of prey against the background of the tank.

Attractiveness, palatability and retention of live and manufactured feed (particularly at weaning from live to microparticulate feed)

Intensive culture of marine fish larvae still depends upon live prey as the initial diet. Large-scale production of such prey is time-consuming and expensive. Thus, development of formulated microparticulate diets (MPD) that are readily consumed by larvae and juveniles is widely considered an essential step towards cost-effective farming of marine fish species.

Most of the research to develop MPDs has focussed on nutritional quality, digestibility, size, and texture (e.g. Cahu et al. 2001; Koven et al. 2001; Fletcher et al. 2007). However, knowledge about how various constituents of the feed will affect feeding behaviour is also important to develop a successful commercial diet. Certain substances might attract larvae, and motivate the feeding response. Such odours (and/or tastes) should be added to a formulated diet. Other substances might be repellent, and suppress feeding. Such odours/tastes should be avoided. To be anthropomorphic: if the food that you place on your children's plate looks or smells "wrong", they will not touch it, no matter how good it is for them. Why should fish be any different? Thus, despite significant effort, there has been limited success in producing MPDs upon which cold water marine species grow and survive as well as they do when fed live prey (e.g. Fletcher et al. 2007). Among the reasons suggested for the poor performance of MPDs are low palatability (and resultant ingestion rates) and low digestibility (e.g. Baskerville-Bridges & Kling 2000; Kolkovski 2001; Fletcher et al. 2007).

Several studies have evaluated the sensitivity of fish species to various amino acids: the olfactory and gustatory systems of even very young fish respond to a wide variety of such substances (Yacoob et al. 2004; Yacoob & Browman 2007a,b and references cited therein). Recent reports demonstrate that permeating formulated feeds with specific chemical odours can dramatically increase the growth rate of fish larvae (e.g. Kolkovski et al. 2000). Thus, identifying substances that can motivate the feeding response of marine fish larvae and increase the probability that they will retain and digest their feed holds promise for the rapid improvement of formulated feeds.

Very little is known about the olfactory and gustatory responses of marine fish larvae. Electrophysiological and behavioural techniques can be used to generate concentration response curves for various substances and to characterize how the fish behaves in their presence.These techniques are akin to asking these animals what smells and tastes they can perceive, which they prefer, and perhaps more importantly, what they do not prefer.

All of the preceding is particularly important in the context of the timing and protocols used at start feeding (see for example Yufera and Darias 2007) and to wean fish larvae from live feed to a MPD (e.g. Brown et al. 2003; Puvanendran et al. 2006. Several approaches to weaning have evolved. Some of these are: co-feeding of MPDs with live feeds, optimizing the age at which weaning begins and its duration, developing weaning tank systems and environmental conditions (e.g. temperature), modifying the physical and biochemical properties of MPDs, and choosing a live feed that facilitates early weaning (e.g. Opstad 2001; Puvanendran et al. 2006; Fletcher et al. 2007). The best weaning results reported to date for cod have been obtained using co-feeding protocols (see Callan et al. 2003; Fletcher et al. 2007). Thus, achieving results with MPDs that are equivalent to those obtained with live feed has been elusive. Direct observations of fish behaviour during the weaning period are, therefore, essential.

Behavioural indicators of stress and/or welfare state

Large-scale commercial production of aquatic animals has accelerated during the past decade, both in terms of total biomass produced and diversity of organisms cultured. As a result, the husbandry practices applied to these animals — and

the welfare states associated with their use — are emerging issues in national and international science programs, organizations concerned with the treatment of animals, and amongst consumers (see, for example, Conte 2004; Browman & Skiftesvik 2007). Until recently, however, very little information was available on the general behaviour, stress levels, discomfort/pain, anxiety/fear, "comfort" conditions, etc. of aquatic animals in these situations. This kind of data is required to inform discussions and guide policy development. There is virtually no information of this nature available for fish larvae and early juveniles. That said, it is unlikely that any meaningful indicator of stress or welfare state could be identified for the larvae of marine fish, since their development, and their mortality as a result of starvation or disease, occur on too short a timeline for any such indicator to be of practical use to hatchery operators. Thus, any effort in that regard should be applied to larger juvenile fish. Basic research on stress in the early life stages, and particularly on the sources of stress, are certainly appropriate.

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10.5 Buoyancy and Osmoregulation

Anders Mangor Jensen, Institute of Marine Research, Austevoll

Eggs from various teleosts exhibit the same osmoregulatory traits of keeping the body concentrations fairly constant throughout development. In withstanding the osmotic pressure and protecting the egg cell from desiccation, the vitelline membrane has proved to be the most watertight biological membrane ever examined.

The low osmolality body tissues of the developing eggs also serve another important feature – buoyancy. Since eggs have no option of locomotion, lift in the water must remain static by reduced mass. Osmoregulation and buoyancy regulation in eggs rely on membrane permeability and reduced ionic fluxes. Effects of environmental changes and impacts in connection to climate changes, anthropogenic pollution and petroleum activity may irreversibly affect egg homeostasis during the first fragile embryonic stages.

After hatching, the larvae in a marine environment develop further towards adult osmoregulation mechanisms that include drinking and salt secretion. In order to achieve a net gain of water by drinking, the larvae possess osmoregulatory mechanisms including salt secreting cells and chloride cells, normally associated with skin and gill filaments in young and older larvae, respectively. Insufficient osmoregulation may occur during intake of feed with low water content, or during unfavorable temperature conditions. These mechanisms have not been studied in detail.

At early larval stages marine pelagic fish larvae develop large water-filled compartments along the body sides, so-called subdermal spaces. These contain low-osmo fluid that aids in achieving buoyancy. However, most teleost fish regulate their volume-specific mass with an air-filled swim bladder. The bladder may be closed (physoclist fish) or open to the oesophagus by a duct (physostome fish). First inflation of the swim bladder has been studied in a number of fish species, and there seem to be differences already at the larval stages. Some species have been shown to be dependent on atmospheric air from the surface for initial filling, while others seem to be able to secrete air into the bladder from the start. Failure of physostome initial inflation has been observed in connection with polluted surfaces. These mechanisms may be very important in future evaluation of e.g. oil spill risks.

Buoyancy and osmoregulation in fish eggs and larvae

Marine fish eggs are shed into a saline environment from the isosmotic maternal ovary. Prior to spawning, the eggs swell into their mature size by influx of gonadal fluid as a consequence of increased osmolytes from protein breakdown (Finn et al, 2002). Eggs from teleosts exhibit the same osmoregulatory traits of keeping the body concentrations fairly constant throughout development.

When the eggs enter the hyper saline environment, their outer membrane, the egg shell, is freely permeable to salts and water, and leaves the osmotic barrier to the double bilayer vitelline membrane that surrounds the egg cell (Mangor-Jensen, 1986). To withstand the osmotic pressure and protect the egg cell from desiccation, the vitelline membrane has proved to be the most watertight biological membrane ever examined (Potts and Eddy, 1973). For comparison the anuran bladder tissue has a water permeability almost 1,000 times higher (Finkelstein, 1976). This low water permeability persists even after fertilization and secures the embryonic water supply throughout embryonic development.

The low osmo teleostean egg proves its heritage as an osmoregulating vertebrate. Measurements of cod, halibut and herring eggs all show the same patterns of osmoregulation by being nearly impermeable to water up to the point where the embryo is able to replace osmotically lost water by drinking (Mangor-Jensen and Adoff, 1987). In order to achieve a net gain of water by drinking, the young larvae need to possess osmoregulatory mechanisms including salt secreting cells and chloride cells, normally associated with gill filaments.

The low osmolality body tissues of the developing eggs also serve another important feature – buoyancy. Since eggs have no option of locomotion, lift in the water must remain static by reduced mass. A vast number of marine teleosts produce pelagic eggs that rely on its low density to sustain buoyancy. Pelagic eggs are in most cases hyaline, and almost invisible in the oceanic water. In addition to high water content, many eggs also have oil globules in the yolk sac to decrease weight. Typical examples of the latter are turbot and striped bass. Cod and halibut do not have oil inclusions in the yolk.

Although neutral buoyancy of a cod egg decreases during development (becomes heavier), no active regulation has been demonstrated. Nevertheless, the cod egg will remain lighter than sea water throughout embryonic development. At hatching the main heavy component – the egg shell – is lost, giving the newly hatched larvae increased lift (Mangor-Jensen and Huse, 1991). In hatcheries newly hatched cod larvae are found floating in the upper water layer. When halibut eggs are subjected to light, they respond by temporarily increasing their water permeability, allowing a small amount of water to be lost osmotically. This in turn increases the specific weight of the egg that sinks in the water column to a layer of higher salinity (Mangor-Jensen and Waiwood, 1995). This water loss is irreversible, but effective in bringing the halibut egg away from potentially harmful conditions on the surface. Halibut eggs are normally found in association with a pychnocline at depths of 50-150 meters.

In aquaculture halibut eggs need to be cultured in total darkness to avoid sinking in egg incubators. If halibut eggs are allowed to rest on the incubator floor, they will rapidly be infected by bacteria and die.

Osmoregulation and buoyancy regulation in eggs rely on membrane permeability and reduced ionic fluxes. Effects of environmental changes and impacts in connection to climate changes, anthropogenic pollution and petroleum activity may irreversibly affect egg homeostasis during the first fragile embryonic stages.

Larval osmoregulation and buoyancy

After hatching, the larvae develop further towards adult osmoregulation mechanisms that include drinking and salt secretion. Cod larvae have been shown to start exogenous uptake of water at very early larval stages (Mangor-Jensen and Adoff, 1987), but unlike with the adult fish, salt secretion is located to chloride cells in its outer membranes. At early larval stages marine pelagic fish larvae develop large water-filled departments along the body sides, so-called subdermal spaces, whichcontain low-osmo fluid that along with the small swim bladder aid in achieving buoyancy (Shelbourne,1956).

Most teleost fish regulate their specific weight with an air-filled swim bladder. This organ gives static lift that makes the fish closer to neutral buoyancy in the ambient water. The cost of swimming to achieve dynamic lift is thereby reduced. Some pelagic fish lack a swim bladder, relying on a constant forward and upward swimming behaviour to counteract sinking. Others again reduce their body mass by increasing the volume of body tissue with low density, such as fat. In the case of flatfish only the larval stages are pelagic, and often the SB is rudimentary and without function. In species that have a swim bladder, this structure develops at a very early larval stage and is easily recognized, resembling a small air bubble located above the gut in the transparent larvae (Goolish and Okutake, 1999).

The swim bladder develops as a pouch from the gut, and is thereby analogous to the mammalian lungs. In some fish the swim bladder has developed into functional respiratory organs, but for most species it is used as a hydrostatic organ for buoyancy regulation. Two main strategies in swim bladder inflation exist in fish. Due to its origin some fish have retained a connection between the esophagus and the SB. The difference is whether the bladder is closed (physoclist) or opens to the oesophagus by a duct (physostome). Cod is a typical example of a fish with a closed swim bladder. A closed swim bladder will only provide neutral buoyancy at a specific depth corresponding to its volume. Ascent or descent will change the gas volume in the bladder according to Boyle's law. A fish with closed swim bladder therefore has reduced ability to make quick vertical migrations. Experiments have shown that cod need more than 24 hours to adjust bladder volume to neutral volume after a pressure increase of 1 atm – corresponding to 10 meters. The physiological mechanism of inflating a physoclist swim bladder lays in the gas secreting ability of the gas gland that is located in the ventral part of the bladder. The gas gland extracts gas from the blood and pumps it into the bladder by locally decreasing the gas solubility with production of lactic acid. The lactic acid also acts to reduce the oxygen-carrying capacity of the haemoglobin, thus increasing the local partial pressure of oxygen.

The opposite reaction, where gas needs to be removed from the bladder upon ascent, is much faster and will bring the fish back to neutral buoyancy within a few hours given that the pressure reduction is within certain limits. First inflation of swim bladder has been studied in a number of fish species, and there seem to be differences already at the larval stage. Some species have been shown to be dependent on atmospheric air from the surface for intitial filling, while others seem to be able to secrete air into the bladder from the start. In cod there has been an ongoing discussion whether gas super saturation, and over inflation of the swim bladder may be part of the causality behind neck deformities often seen in intensively reared cod.

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10.6 Respiration and excretion

Bendik Fyhn Terjesen, Nofima Marine Sunndalsøra

In fish early life stages, little convective gas transport occurs before circulatory function is established. In zebrafish oxygen receptors are found very early in larval development, although data from other species are few. Autonomic nervous systems controlling cardiac function, however, are only fully developed during later stages, but large species variation exists. The concept of unstirred water boundary layers around the embryo and larva is important for interpretation of gas exchange in fish early life stages, since it is often considered to be one of the major resistance barriers to oxygen transport.

However, little is known about these aspects in our cultured marine species, and under industrial aquaculture conditions. For instance, the switch from cutaneous to gill gas exchange, and the molecular control mechanisms for this process, may be valuable to study in relevant species. Further, it has been reported that oxygen deficiency results in vertebral deformities in red sea bream, when embryos are exposed during somitogenesis, findings that should be investigated in other species.

Recently, microarrays have been used to study a number of energy metabolic genes during development of sea bass, contributing to the understanding of preferred substrates for catabolism. However, compared to this species and zebrafish, relatively little is known about expression of genes involved in energy metabolism, and especially their control, during early development of Atlantic cod and halibut.
Considerable progress has been made in recent decades in the field of nitrogen metabolism and excretion in fish early life stages. One central rule is that the majority of nitrogenous end-products are in the form of ammonia, of which especially the gas NH_3 is toxic, dictatating that this compound must be kept at low concentrations *in vivo*. Four major recent findings in the field of nitrogen excretion in fish in general are: 1) the discovery of gill rhesus glycoprotein ammonia transporters, 2) the finding of urea transporters, 3) urea cycle activity in fish early life stages, and 4) the demonstration of occasional growth-promoting effects of ammonia.

Marine species such as Atlantic cod and halibut cover the bulk of their energy requirements from amino acids during certain periods of early development. This process generates toxic ammonia which is either excreted, detoxified, or stored safely in an acidic yolk sac. The current knowledge of these mechanisms are briefly discussed in the review. Acute tolerance to ambient ammonia is substantial in fish embryos, compared to later developmental stages. Results from chronic exposure studies are few. During the yolk-sac stage and the first period of exogenous feeding, little is known about ammonia tolerance in our important cultured species. It is suggested that more detailed chronic ammonia exposure studies should be conducted to analyze different developmental stages and physiological situations.

Respiration

General introduction

Oxygen exchange is more critical for most water breathers than CO_2 exchange, since the bicarbonate buffer system acts to keep high CO_2 partial pressure gradients. Of course, CO_2 excretion is still a vital component, not only to avoid hypercapnia, but also to facilitate ammonia excretion. In general, the diffusion processes are the critical bottleneck. The major factors governing the rate of diffusion are described by a derivation of Fick's first law: $J = K*\Delta P*A/D$, where K is Krogh's diffusion constant (dependent on temperature), ΔP is the partial pressure gradient across a membrane, A is the area of the membrane, and D is the distance across the membrane.

In fish early life stages, little convective gas transport occurs before circulatory function is established. Autonomic nervous systems controlling cardiac function are only fully developed during later stages, although large species variation exists. Therefore, the driving force for gas transport is between environment and tissues, and not between blood and tissues. Indeed, vascular pO_2 is relatively low in salmonid early life stages [1]. Even in the comparably small zebrafish larvae, haemoglobin is only partially saturated [2]. Thus, cutaneous diffusion governs what oxygen the embryos/larvae will be able to extract.

The above concerns the physiological interpretation of respiration. The field of respiration also involves biochemical and molecular interpretation, such as energy metabolism and control. This review will briefly touch upon both interpretations of the field of respiration, but will focus on mechanisms that are considered by the author to be of most importance in an aquaculture setting.

Unstirred boundary layers and embryonic compartments

The concept of unstirred boundary layers (UBL) is important for interpretation of gas exchange in fish embryos and larvae. The UBL is one of the major resistance barriers to oxygen transport in salmonid embryos, and is 3-5 mm thick [3]. When trout embryos and larvae develop, the dissolved oxygen gradient and the thickness of the UBL increase [4]. Further, the oxygen concentration within the UBL decreases as the oxygen concentration in the free-stream water decreases. Finally, a decline in water flow rate increases the magnitude of the gradient and thickness of the boundary layer. Little is known about these aspects in our cultured marine species such as the Atlantic cod and halibut, under industrial aquaculture conditions.

It has long been discussed whether the chorion plays a role in gas transport. In a recent study, oxygen in the perivitelline fluid was at only 16±3.0% saturation [4], indicating that in this case the chorion and/or the perivitelline fluid was a significant barrier. However, others have shown little difference in oxygen consumption (MO_2) of trout embryos with or without chorion [5]. The contribution of the different areas of the larvae to total MO_2 shows that the yolk sac is a less efficient gas exchanger than the skin in salmon, despite its extensive vascularization [6].

Critical oxygen levels and other environmental factors

As for ammonia exposure, embryos appear to be more resistant to low oxygen levels than later developmental stages [7]. Hypoxia induces precocious hatching in several species [8], although it has only a minor role in regulation of, for instance, Atlantic halibut [9]. Of importance to commercial culture are the findings that O_2 deficiency results in vertebral deformities in red sea bream when embryos are exposed during somitogenesis [10].

Larvae respond to decreased pO_2 with increased ventilatory rate [11]. In zebrafish, oxygen receptors are present at 5 dpf, possibly associated with neuroepithelial cells (NECs) in the gill filaments [12]. Even earlier hypoxia reactions (2 dpf) may be induced by stimulation of 5-HT positive cells in the gill arches [12]. Central NMDA receptors are involved in ventilatory control from 8-13 dpf.

Concerning light, turbot yolk-sac larvae 9-12 dPF show a higher oxygen consumption in continuous light compared to darkness, although Q_{10} for MO_2 was not affected by light [13], unlike in cod [14]. The relationship between temperature effects on metabolic rate and the temperature effects on cardiac rate appears less tight in early developmental stages as compared to adults [7]. Further, a large variation has been found between species in terms of Q_{10} for MO_2 [15]. Zebrafish shows high Q_{10} values during early development [16], indicative of a stenothermic metabolism, while in Atlantic cod and turbot embryos, the Q_{10}s for MO_2 were more common at 2.4-2.6 [13, 14].

The relationship between body mass and metabolic rate

Intense research activity and discussions have surrounded the question of metabolic rate scaling with body mass in fish larvae. Scaling appears to be species-specific since some species show isometric or positive allometric scaling [17], while in others, like the Atlantic cod, oxygen consumption scales in a negative allometric fashion with body mass [14]. As suggested by Pelster et al (2008), this may be due to a species-specific reorganisation of tissues being more or less metabolically active over time.

Increased larval activity, measured in trout that were dechorionated, did not affect MO_2 significantly [5]. These authors [5] suggested that movement in fish early life stages has a trivial metabolic cost relative to the cost of growth. This does not appear be the case around hatching in Atlantic cod since [18] reported significantly higher MO_2 in active Atlantic cod larvae. In a recent work, maximal MO_2 was measured in three marine fish species over the entire life history, and while the aerobic scope was found to depend considerably on body size and developmental trajectory, it was very small during the early life stages. Still, this limited scope in larvae differed between the species. The authors suggested that a limited aerobic scope during early development could "affect the ability of young fish to 'multitask' physiologically demanding processes". Possibly, measurements of larval aerobic scope can be one of several indicators when evaluating candidate species for aquaculture purposes.

Metabolic modes, pathways, and fuel preferences

Several studies show that metabolism is fully aerobic during early development of turbot, halibut, and cod [19], although capacity for anaerobic energy dissipation does exist in many species [20]. Van der Meulen et al [21] assayed the expression of several genes involved in energy metabolism using immobile/mobile zebrafish larvae and concluded that metabolism was mainly geared toward providing energy for growth processes, while activity was of lesser importance.

Regarding fuel preferences, the main energy source in turbot and cod prior to gastrulation and epiboly is glycogen [22-24]. In rainbow trout, glucose transporters (GLUT1) are expressed already at the blastula stage, and *in situ* hybridization suggested

Table 1. Examples of research in the field of respiration during early development

Biological process	Species	Molecules	Cell	Organ	Organism	Behaviour	Environmental factors
UBL & embryonic compartments							
	Salmonids				UBL [4]; dechorionation [5]		pO_2, flow rate [4]
	Halibut						
	Cod						
Gill/cutaneous oxygen transport							
	Salmonids			Partitioning [6, 29]			
	Halibut						
	Cod			Morphology [30]			
Oxygen sensors							
	Zebrafish	Transmitters and receptors [31]	NEC [12]				
Critical oxygen levels							
	Red sea bream						Deformities [10]
	Zebrafish			Heart rate [16]		Ventilatory rate [12, 31]	Temp [16]
	Halibut				Hatching [9]		
	Salmonids			Heart rate embryos [7]	Hatching [8]		Temp [7]
Metabolic mode and mechanisms							
	Salmonids	Energy met mRNAs [32], GLUT [25]					
	zebrafish	Energy met mRNAs [21]					
	Halibut						
	Sea bass	Energy met mRNAs [33]					
	Cod	CK [34], orexin [35], GLUT [26]					Temp on CK mRNA [34]
Fuel preferences							
	Salmonids	Fuel classes [36]					
	Halibut	Fuel classes [27]					
	Turbot	Fuel classes [13]					Light, temp [13]
	Cod	Fuel classes [14, 23, 37]					Light, temp, fed, fasted [14]
Rates and scaling							
	Salmonids				O2 ates/scaling [38]		
	Turbot				O2 rates [13]		Light, temp [13]
	Halibut				Scaling, [27]		
	Cod				O2 rates [18], SDA [39, 40]; Feeding, scaling [14]	Cost of prey attacks [41]	Light, temp, [14], temp, swimming speed [42], anaesthesia [18],

that, at early developmental stages, GLUT1 was expressed in all cells [25]. GLUT are also present in Atlantic cod from fertilization and onward, but expression changed little after hatching, suggesting that transition from endo- to exogenous feeding had little impact on GLUT expression in cod [26]. The glycolytic pathway can be used by marine fish larvae, but activities through this pathway are likely less than that of TCA cycle enzymes, which are utilized for amino acid breakdown [14]. Indeed, based on stoichiometric and respiratory studies, it has been shown by several authors that free amino acids are a major substrate for energy dissipation during early development of marine species. Around hatching in the Atlantic halibut, amino acids contribute almost exclusively to energy dissipation [27]. In juvenile trout, the fraction of different energy sources changes with swimming speed [28], but these aspects are little known in fish early life stages. Finn and Rønnestad [13] did show, however, that light-exposed turbot larvae, presumably with a higher activity level than in darkness, had a higher NQ, and proposed that amino acids are a preferred fuel during exercise.

In trout, transcripts for several oxidative metabolism enzymes, such as citrate synthase, cytochrome oxidase and succinate dehydrogenase, are present in the whole myotome at hatching but later become concentrated in slow and in lateral fast fibres [32]. In cod, prepro-orexin, a hormone known to induce feed intake and metabolic expenditure, is expressed already at cleavage stage [35].

Recently, Darias et al [33] used microarrays to study a number of energy metabolic genes during development of sea bass, and found that the TCA cycle and neoglucogenesis gene expression were upregulated in early sea bass larval stages (7-13 dph) compared to later stages and other pathways. The authors concluded that this machinery catabolises amino acids for energy and provides glucose from amino acid precursors, and that metabolism is in general aerobic.

Areas in the field of respiration that should be prioritized in future research

What are the pO_2s across compartments in Atlantic cod and halibut embryos and larvae, in situations mimicking incubation in culture? What are the consequences of chorion bacterial growth? Experimentation on these small embryos will be a challenge.

How is the switch from cutaneous to gill gas exchange controlled in early stages of relevant cultured species, e.g. molecular control mechanisms, and rearing system and environmental impact?

Oxygen consumption rates for juvenile Atlantic cod, fed optimum rates and diet composition, as a function of body size and temperature. Such tables are very useful for farmers and scientists.

Compared to e.g. zebrafish and sea bass, relatively little is known about expression of genes involved in energy metabolism, and their control, during early development of Atlantic cod and halibut. Microarray approaches should be taken, coupled with detailed molecular studies of particularly interesting genes. It is important that such studies include, or are followed up with, experiments that can explain the functional importance of particular genes, especially by using treatments relevant to an aquaculture setting.

Nitrogen excretion

General introduction

Considerable progress has been made in the last decades in the field of nitrogen metabolism and excretion in fish. One central rule is that the majority of nitrogenous end-products are in the form of toxic ammonia, especially the gas NH_3, which dictates that this compound must be kept at low concentrations *in vivo* [43], although even this is under current scrutiny. Urea, which is the second most important in quantitative terms, is considerably less toxic [44]. Four major recent findings in the field of nitrogen excretion relevant to this review are: 1) the discovery of rhesus glycoprotein ammonia transporters, 2) the finding of urea transporters, 3) urea cycle activity, and 4) the demonstration of occasional growth-promoting effects of ammonia.

Ammonia toxicity

The toxic effects of ammonia on living tissues are manifold. High internal concentrations of NH_4^+ activate *N*-methyl-D-aspartate (NMDA) glutamate receptors in the central nervous system, resulting in a large Ca^{2+} and Na^+ influx that eventually can lead to ATP depletion, oxygen radical formation and cell death [45]. Ammonia interferes with normal cell metabolism by activation of glycolysis, while it affects the TCA cycle through inhibition of enzymes, and to some extent depletion of the intermediate α-ketoglutarate. Ammonia can cause alkalinisation of cells when NH_3 enters, since it consumes H^+ ions to form NH_4^+. The resulting change in H^+ concentration can affect important pH-dependent cellular functions, such as enzymes or signal cascades. Since NH_4^+ has a similar ionic radius to K^+, it can substitute for K^+ in ionic channels. Increases in extracellular NH_4^+ concentration can therefore cause membrane depolarisation.

Table 2. Examples of research within the field of nitrogen excretion and metabolism in fish early life stages.

Biological process	Species	Molecules	Cell	Organ	Organism	Behaviour	Environmental factors
Amm & urea compartments							
	Salmonids			Compartments [50]			pH, ionic strength, ambient ammonia [50]
	Halibut			Compartments [51]			
	Cod						
Urea transporters							
	Salmonids	UT [52]					
	Halibut						
	Cod						
Ammonia transporters							
	Salmonids	Rh glyco [53]					
	Zebrafish	Rh glyco [54]		Gill, yolk sac [54]			Salinity [54]
	Cod						
Ammonia exposure							
	Turbot	Ions, acute [47]			Mortality acute [47]		
	Cod	Juveniles, chronic, several compounds [55]			Acute on larvae [49]. Juvenile chronic mortality, growth [55]		
	Halibut	Embryos acute, several compounds [46]			Embryos acute, mortality, [46]		
	Salmonids	Sublethal acute [19]			Chronic [56] Sublethal acute [19]		
Pathways							
	Salmonids	Uricolysis [57], OUC [58], GSase genes [59]		Body/yolk [58]			Ammonia [58]
	zebrafish	Uricolysis [60]					
	Halibut	OUC, uricolysis [51]					
	Cod	OUC [61]					

When Atlantic halibut embryos were exposed to 15-264 μ*M* NH_3 for 6 days, no significant ammonia accumulation occurred during the first 2 days, and they maintained normal K^+, Cl^-, and Na^+ levels [46]. However, levels of K^+ in turbot and K^+, Cl^-, and Na^+ in rainbow trout yolk-sac larvae declined during NH_4Cl exposure [47, 48]. Thus, tolerance to ambient ammonia is substantial in Atlantic halibut embryos, as also observed in several other species during the embryonic stage compared to later developmental stages. During the yolk-sac stage and the first period of exogenous feeding, very little is known about ammonia tolerance in our important cultured species such as cod and halibut. However, preliminary data on ammonia tolerance in Atlantic cod larvae showed that with the exception of larvae of 1-1.7 mg dry matter, only 23% mortality was observed after 48 h exposure to up to 0.1. mg NH_3 [49]. A more detailed chronic ammonia exposure study should be conducted, analyzing a number of different developmental stages and physiological situations.

In juvenile rainbow trout, ammonia exposure has also been shown to increase growth [reviewed in 62]. These surprising findings are in contrast to the general consensus that ammonia is harmful to fish. If this hypothesis is further supported for other species and early life stages, it may have considerable impact on land-based aquaculture water quality management.

Ammonia handling by early life stages

The use of amino acids is vital in early life stages, and fish must have evolved efficient mechanisms for handling the substantial amounts of nitrogenous end-products that must be formed during their early development. The fates of ammonia generated by embryonic teleosts can be described by a simple model: a) it can be excreted through the perivitelline space, across the chorion, and then to the ambient water, b) it can be stored in an acidic yolk sac, or c) ammonia can be converted to other compounds, and subsequently stored or excreted [63].

Nitrogen excretory mechanisms

In adult teleosts, ammonia excretion can be facilitated by several mechanisms, including 1) NH_3 diffusion down a concentration gradient and entrapment as NH_4^+ due to concurrent H^+ or CO_2 excretion in the UBL, 2) through Na^+/NH_4^+ exchange, and 3) NH_4^+ diffusion. However, knowledge about the actual ammonia excretion mechanisms for embryonic teleosts is still limited. Rahaman-Noronha *et al*. [50] showed that an acidic UBL next to the chorion was present in rainbow trout, suggesting a linkage between NH_3 and CO_2 excretion also in embryos. In support of this, the rate of excretion from rainbow trout embryos was partly dependent on the NH_3 partial pressure gradient between the perivitelline space (PVS) and the UBL next to the embryo. Recent findings have shed light on the mechanisms in freshwater fish species, since rhesus glycoprotein genes were found to be expressed at the yolk sac and gills of zebrafish [54] and in trout embryos [53]. The latter authors proposed that early expression of Rh genes is critical for the elimination of potentially toxic ammonia from the encapsulated embryo, whereas retention of the comparatively benign urea molecule until after hatch is less problematic [53]. In early life stages of marine teleosts, however, nothing is known regarding mechanisms for ammonia excretion.

Regarding urea, it has only limited permeability across lipid bilayers. Thus, the presence of specific carriers is advantageous [64]. Mechanisms for urea excretion have been sparsely investigated in fish early life stages. However, in a series of experiments Pilley & Wright [52] showed that urea excretion in rainbow trout embryos is inhibited by urea analogues and by inhibitors of the UT-A family of urea transporters. Again, information for marine species is scarce.

Nitrogen accumulation

Several teleost species, especially marine, accumulate considerable quantities of ammonia during early development, concurrent to excretion. Fyhn [65] proposed that the acid yolk serves as a storage compartment during periods of ammonia accumulation in marine species with pelagic early life stages. In the Atlantic halibut, rates of whole-animal ammonia accumulation are higher than in the larval body, suggesting that the yolk is the major storage compartment for ammonia [51]. In contrast to ammonia, accumulation of urea in early fish life stages appears to be more evenly divided between the yolk and larval body, possibly reflecting the lower toxicity of urea. Rahaman-Noronha *et al*. [50] showed that ammonia was distributed according to transmembrane potential between yolk and the PVS in trout, but according to pH between the PVS and the UBL. Although yolk storage cannot eliminate ammonia, it may serve to separate this toxic compound from the nervous tissues of the developing embryo during times when apparently not all ammonia produced is excreted, and to keep NH_3 levels low due to the acid pH in yolk.

Detoxification to other nitrogen forms

Glutamine synthesis via GSase 1 and 3 [59] and the urea cycle are the only pathways thought to directly detoxify ammonia in fish embryos and larvae. Breakdown of nucleotides (uricolysis) also produce

urea, but cannot detoxify ammonia without previous purine synthesis. However, this pathway may have other functions as discussed by Andersen *et al.*, (2006). In most adult teleosts the activities of key OUC enzymes are low or below detection limits in the adult stage. Wright *et al*. [66] employed sensitive assay methods and detected central OUC enzymes from hatching and during a considerable period of rainbow trout development. A number of teleost species have been shown to express key OUC enzymes during early life stages, while in the adult state, only low levels or no activity can be detected. The location of the OUC to muscle in fish larvae is unique among vertebrates. One possibility is that anatomical constraints of fish larvae may dictate that muscle is primarily utilised for OUC expression, in contrast to liver [63, 67]. However, there are also other possibilities which should be further investigated. Studies should also focus on whether components of the fish OUC have links to glutamate family amino acid nutrition as in mammals.

Areas in the field of excretion that should be prioritized in future research

Chronic ammonia exposure studies and interaction of ammonia with other water quality parameters (e.g. CO_2, O_2) should be conducted to cover early life stages of Atlantic cod, halibut, and Atlantic salmon parr in freshwater at production temperatures.

Not only mortality/growth is important as indicators for ammonia stress: Markers should be developed that indicate when energy-consuming mechanisms are turned on during such exposure.

Urea cycle – amino acid nutrition: Do urea cycle components in fish larvae have vital functions in amino acid nutrition as in other animal groups?

Findings of a growth-promoting effect of ammonia within a certain concentration window should be verified for several species. If this is a general phenomenon, it will have considerable impact on land-based aquaculture water treatment.

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10.7 Reproduction in fish: Focus on germ line and early gonadal development

Lisbeth Charlotte Olsen, Sars International Centre for Marine Molecular Biology

Studies with zebrafish and medaka have revealed that they employ the preformation mode of germ line specification. The germ line is separated from soma by maternally deposited determinants that segregate with the germ line as a result of asymmetric cell divisions. Studies in model fish have led to the identification of some key factors participating in germ line development and gonadal differentiation. Despite this, basic knowledge about germ line development remains elusive. We do not understand *how* germ cell fate is being controlled and maintained or *how* the somatic compartment of the gonadal anlage and germ cells obtain sexual identities. Furthermore we lack information regarding what kind of germ-soma interactions take place during gonadal differentiation.

An important issue for the aquaculture industry is to control sex differentiation and germ cell maturation in farmed fish species. Production losses due to precocious male puberty are a big problem for the aquaculture industry. In addition, escaped farmed fish represent a danger to the environment. In several fish species it is known that environmental conditions (e.g. high temperature) around the hatching stage/early larval stages lead to female–to-male sex reversal. Some fish species may even have a temperature-dependent sex determination or other forms of environmental sex determination. Basic knowledge about germ line development in model fish may lay the foundation for developing new tools and strategies for controlling germ line development in farmed fish species.

Germ line development and migration

The unique feature of the germ line is its ability to form totipotent cells, which occurs when egg and sperm fuse to form a new zygote. A key problem in reproductive biology is to understand *how* a germ line has acquired this particular feature. Is this event linked to the early separation of germ from soma during embryonic development? Or can a germ line arise *de novo* in adult organisms?

Most metazoans form a germ line using either the preformation or the epigenesis mode of germ line specification (1). In the preformation mode, organisms rely on maternal-inherited determinants – also called germ plasm – for directing germ cell fate. In the epigenesis mode, germ line appears to be determined by inductive signals. It has been proposed that the epigenesis mode represents the ancestral mechanisms of germ cell specification.

Several groups have focused their studies on early germ line development using zebrafish and mekada as model organisms. Both these fish species use the epigenesis mode of germ line specification. In zebrafish and medaka, germ plasm components have been found and germ-soma segregation has been shown to be completed by the late blastula stage of development (2, 3). Among identified germ plasm components in fish are *vasa* and *nanos* RNA, both of which contain RNA localisation signals in their 3' untranslated regions (3' UTRs). These RNA localisation signals are now being used to label germ line cells transiently with fluorescent markers in different fish species during embryonic development (4). In addition, transgenic fish lines – including trout, medaka and zebrafish – expressing fluorescent marker protein specifically in the germ line under the control of regulatory regions of *vasa* have been established (5,6).

The migration route of primordial germ cells during embryonic development have been described for model fish. The G-protein coupled-receptor Cxcr4b and its ligand SDF-1 participate in guiding primordial germ cells (PGCs) to the region where the gonad will form (7, 8). Morpholinos have been successfully applied to assign biological function to some of the identified germ line factors in model fish. PGCs failed to colonise the gonadal anlage when morpholinos targeting translation of *nanos* RNA or *dead-end* RNA were used (9).

Sex determination and sex differentiation

When the PGCs reach the gonadal anlage, they respond to *unknown* signals to initiate gametogenesis. The somatic cells of the gonadal anlage that are in direct contact with the germ cells acquire specific differentiated sexual phenotypes. These cells develop into granulosa or Sertoli cells in female and male individuals, respectively. The somatic compartment of the gonad has a key role in controlling the differentiation of germ cells into mature eggs and sperm. Ablation experiments have shown that germ cells are important for sexual dimorphic development. Germ-cell ablated XX medaka fish, obtained by knocking down the function of cxcr4, undergo female-to-male sex reversal (10). Germ-cell ablated zebrafish, obtained by knocking down the function of the *dead-end* gene, developed into adults scored as phenotypical

males, based on skin color and mating behavior (11).

Fish use different sex-determining mechanisms. Sex can be controlled by genetic factors or environmental cues. Medaka has an XY sex-determining system while zebrafish lacks sex chromosomes. Some fish species have two distinct sexes, while others are hermaphrodites. In addition, some fish can change sex. The male-determining gene in medaka has been identified and shown to drive female–to-male sex reversal (12). Rainbow trout has a primitive XX/XY sex-determining chromosomal system. Spermatogonial stem cells isolated from testes of adult trout males – and used in transplantation experiments – differentiated into functional sperm or eggs depending on the somatic sex of the recipient (13). While transplantation experiments in trout indicate that soma controls sex differentiation of germ, ablation experiments in medaka and zebrafish show that absence of germ cells promotes male development. Thus, it appears that germ cells are required for controlling the sexual fate of the somatic tissue of the female gonadal anlage.

The Amh signaling pathway also plays an important role in regulation germ cell proliferation and sexual development. Amh is a member of the TGF-β signaling molecules and was first identified in mammals. Recent work from medaka has shown that this signaling pathway is important for both regulating proliferation of germ cells and sexual differention. In the *hotei* mutant, the affected gene encodes the receptor for Amh. Approximately 50% of the hotei XY mutants underwent male-to-female sex reversal (14).

Conclusions

During the last decade, considerable progress has been made in our understanding of reproductive biology in fish, particularly in model fish. Despite this, many important questions remain to be answered. How is germ cell fate being controlled and maintained? How is sexual dimorphic development initiated and which signaling molecules participate in this process? Through meiosis germ cells give rise to haploid gametes in both sexes. How do germ cells switch from mitosis to meiosis? How does the environment perturb normal sexual differentiation of fish species? A major problem for the aquaculture industry is how to control sex differentiation and germ cell maturations in farmed fish species.

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10.8 Development of the digestive system

Ivar Rønnestad[1], Sofia Morais[2], and Kristin Hamre[3]. [1]Department of Biology, University of Bergen; [2]CCMAR University of Algarve, Faro, Portugal; [3]NIFES, National Institute of Nutrition and Seafood Research

Most of the studies on the digestive system in fish are on altrical fish larvae, such as cod and halibut. Since these larvae do not have a stomach at first-feeding, they have reduced ability to digest conventional formulated diets, and are therefore given live feed. The general morphological development of the digestive tract and associated organs like liver, pancreas and gall bladder in these species is well described, also in cod and halibut larvae. The main developmental scheme is similar in different species, but the characteristic events of the development have species specific timing.

The functional properties of the digestive system have received much research interest, although with a limited focus. The digestive process comprises a range of closely integrated series of actions, including ingestion, secretion, digestion, absorption, motility, elimination, regulation and barrier function. The most studied topic in fish larvae is digestion, in particular protein digestion. Still, the ontogenetic profiles are available only for some of the proteolytic enzymes in our target species, while incomplete information exists for the lipolytic enzymes. Digestion of other nutrients than protein and lipid has not been studied. There is very little information on how the nutrients are processed once they are inside the enterocyte and how they are transported further into the larval body.

The neuronal and hormonal regulation of the digestion and related processes such as appetite, secretion and reabsorption of fluids containing ions, digestive enzymes, bile etc., gut motility and passage time is critical for optimising digestion. Information on these regulatory mechanisms is available from mammals and the mammalian models are currently being used as starting points for exploring these mechanisms on larger fish. These processes are poorly described in fish larvae.

A typical feature of altricial fish species with a distinct larval stage, such as Atlantic cod and Atlantic halibut, is that they lack a functional gastric stomach. Other altricial species, like zebrafish (*Danio rerio*), go through a larval stage but never develop a stomach. Although there are considerable differences in terms of the timing of the developmental events relative to each other, the basic ontogenetic patterns of the digestive tract in altricial fish larvae appear to be similar (Falk-Petersen, 2005). Basic studies that present an overall anatomical and histological overview of the digestive system during development exist for several species of fish larvae, while studies of the underlying molecular and environmental mechanisms are rare. Recent work on zebrafish by Wallace & Pack (2003) and Wallace *et al.* (2005) goes more into these details. Parts of the present contribution are based on a recent review by Rønnestad and Morais (2008).

Morphology

In general, the alimentary tract of first-feeding larvae is a simple, relatively undifferentiated tube with only one sphincter (intestinal-rectal valve) before the anal opening (Tanaka 1974). Some species have a rotated gut at the onset of exogenous feeding, while other species have a straight gut. After first-feeding, the subsequent development of the intestine involves for the most part quantitative changes, such as an increase in length and mucosal surface area, as well as an intensification of the activities of brush border enzymes (Segner *et al.*, 1994). The surface area will increase as a combined result of increased length (sometimes with increased number of rotated intestinal loops),an increase in the epithelial folding, and increased microvilli surface area (Wold *et al.,* 2008). The intestinal tube consists of a simple epithelium surrounded by connective tissue, smooth muscle, blood vessels and enteric nerves (Wallace *et al.*, 2005; Holmberg *et al.*, 2008). Different cell types that can be identified in the epithelial layer include enterocytes, single enteroendocrine cells (produce and secrete peptide hormones), goblet cells (mucus producing cells) and special enterocytes identified as “antigen presenting cells” (Wallace *et al.*, 2005). Goblet cells can be identified in all intestinal regions (Wallace *et al.*, 2005), while the distribution of the enteroendocrine cells varies between larval types (Kamisaka *et al.*, 2002; 2003; 2005, Rønnestad *et al.*, 2007). It is still debated to what extent enterocytes are physiologically functional at first-feeding (Segner *et al.*, 1994)

The stomach develops from a transitional region between the oesophagus and the intestine after peak metamorphosis (Rønnestad *et al.*, 2000a). All histological data demonstrate that marine fish larvae do not possess HCl-producing cells (gastric glandular acini) at the first-feeding stage. A study on Winter flounder (*Pseudopleuronectes*

americanus) demonstrated that the expression of proton pumping genes occurred at 20 days post-hatch and coincided with the appearance of gastric glands (Douglas *et al.*, 1999). After onset of metamorphosis, there is a steady fall in luminal pH (Walford & Lam, 1993; Rønnestad *et al.*, 2000a; Hoehne-Reitan *et al.*, 2001a). In Winter flounder, two forms of pepsinogen have been identified and the expression of one of them, pepsinogen IIb, is correlated with appearance of gastric glands and the initiation of pepsin activity (Douglas *et al.*, 1999), while pepsinogen IIa is activated approximately one week earlier. Even though morphologically a stomach appears to be present at 15 mm SL (approximately 30dph) in cod, (Pedersen and Falk-Petersen 1992), the specific activity of pepsin continues to increase until the larvae are at least 130 dph (Åsnes 2006). The pyloric cecae develop slowly and in parallel with the development of the stomach. (Pedersen and Falk-Petersen 1992).

The midgut appears to be alkaline throughout development (Walford & Lam, 1993; Rønnestad *et al.*, 2000a). Immuno-histochemical analysis for trypsin-like enzymes has demonstrated secretion into the gut lumen from first-feeding (Kurokawa & Suzuki, 1996) and it is likely that the production and secretion of pancreatic HCO_3^- occurs simultaneously. The hindgut is a separate compartment from the onset of first-feeding. In adult vertebrates the hindgut is mainly associated with final adjustments in the water and ion composition of the faeces. In larval stages of fish very little is known about these processes, although histological studies of the epithelial lining show frequent pinocytotic invaginations and intracellular vesicles (Kurokawa *et al.*, 1996).

The exocrine pancreas appears to be functional prior to the onset of exogenous feeding (Govoni *et al.*, 1986; Segner *et al.*, 1994; Kurokawa & Suzuki, 1996; Ribeiro *et al.*, 1999a). The secretory cells produce characteristic zymogen granulae that contain a series of proenzymes. In contrast to adults, the exocrine pancreas in teleost larvae is a compact and distinct organ - *pancreas compactum*. The adult *pancreas diffusum* is acquired during the late larval and early juvenile phases.

In terms of digestion, the main role of the liver is the production of bile. Studies have shown that the hepatocytes produce biliary salts before the onset of exogenous feeding. The liver and gall bladder are differentiated at hatching and functional before first-feeding in many fish species (Govoni *et al.*, 1986; Segner *et al.*, 1994; Kurokawa & Suzuki, 1996; Ribeiro *et al.*, 1999a). The gall bladder serves as a storage site for bile produced by the liver and is responsible for the release of bile into the midgut. Emptying of bile via the bile duct into the gut lumen is most likely controlled by neuronal and humoral factors (Rønnestad, 2002; Hoehne-Reitan & Kjørsvik, 2004; Kamisaka, 2005).

Many studies have been conducted on development of morphological and ultrastructural features of the digestive system in fish. The functional properties of digestion have also received research interest; albeit with limited focus. Digestion comprises a range of closely integrated processes, including ingestion, secretion, digestion, absorption, motility, elimination, regulation and barrier and self protection. Both environmental and dietary factors may influence the ontogeny and efficiency of the digestive functions. Knowledge on the details of many of these processes is virtually non-existent mainly due to experimental and methodological challenges related to the size of animals.

Ingestion, gut transit rates and elimination

Ingestion together with elimination determines the gut transit rate and ingestion is closely related to appetite. Based on the knowledge in mammals, a model for regulation of appetite via the neuroendocrine system in adult fish has been established (Volkoff *et al.,* 2005), and this model is currently being explored and tested. In fish larvae, however, knowledge of the factors that control food intake and their relationship with the digestive physiology is still largely incomplete.

A phenomenon that is frequently observed just after the onset of exogenous feeding of some species is that live food organisms pass undigested and sometimes even alive, through the digestive tract (Harboe *et al.*, 2009). This indicates that larvae, which are evolutionary adapted to the varying feed densities in the oceans, fail to control feed intake in intensive culture and continue to ingest prey despite a full gut. A number of studies suggest that a high density of feed organisms increases ingestion rate and transit time, but reduces digestion rate in marine fish larvae (Pedersen, 1984; Øie *et al.*, 1997, Morais *et al.*, 2006a). Accordingly, Tonheim *et al.*, (2005) showed that reduced digestion and absorption of a model protein was connected to a short transit time and early elimination of the diet in Atlantic halibut larvae. A short gut transit time may therefore cause incomplete digestion of nutrients and the larvae may also fail to reabsorb critical compounds such as bile acids, ions and water, secreted into the gut lumen during digestion. Recent studies suggest that some of these problems may be alleviated by pulse feeding and photoperiod regulation (Harboe *et al.*, 2009).

Secretion and re-absorption of secretes

Digestive enzymes, bile, ions and mucus are secreted in a watery solution into the lumen of the digestive tract as part of the digestive process. As

part of the mass balance a large amount of these secretes must be reabsorbed to save energy, water and ions. The secretion of these components is believed to be under both hormonal and neural control. In Atlantic herring larvae, Pedersen *et al.* (1987) and Pedersen & Andersen (1992) noted that the amount of pancreatic trypsin and trypsinogen secreted into the gut increased as the number of ingested preys increased, until a plateau was reached at high levels of food intake. The mass balance of water and ions in the digestive tract is critical in maintaining homeostasis in humans (e.g. diarrhea). Marine fish larvae live in a hyper-osmotic environment, which makes regulation of water and ions a demanding and critical task. Furthermore, bile is known to be reabsorbed and reused during the processing of a meal in mammals and one can speculate if a similar mechanism would be efficient in fish larvae, particularly during periods of high ingestion rates and short gut retention times. These issues have received little research attention.

Digestion

Proteins

Qualitatively, the digestive enzymes in fish are similar to those of higher vertebrates (Sire & Vernier, 1992). Altricial fish larvae lack HCl^- and pepsin-secreting cells, due to the lack of a stomach, and studies suggest lower rates of digestibility and assimilation of protein in pregastric larvae than in postmetamorphic fish (Tonheim *et al.*, 2004, 2005; Rust, 1995). The activity of pancreatic, proteolytic enzymes is generally found to be low when marine fish larvae commence exogenous feeding, but it rises as metamorphosis approaches (Hjelmeland, 1995; Cahu & Zambonino Infante, 2001). The activities of brush border membrane enzymes of the enterocytes in cod and halibut are low at first-feeding, but increase during larval development (Kvåle *et al.*, 2007). Cytosolic peptidases, proposed to participate in degradation of protein after pinocytoses, show high activities around first-feeding but tend to decrease as larvae develop, in some species (Cahu & Zambonino-Infante, 2001; Kolkovski, 2001). This picture is less clear in cod than in halibut (Kvåle *et al.*, 2007).

Several authors have suggested that fish larvae have a low endogenous proteolytic capacity and that the enzymatic content of their prey (exogenous source) contributes to the larval digestive process (Walford & Lam, 1993). However, considerable controversy exists around this notion as several other studies have quantified the contribution of live prey proteolytic enzymes to the overall digestive process and have concluded that this contribution is negligible (Pedersen *et al.*, 1987; Cahu *et al.*, 1995; Cahu & Zambonino-Infante, 1997; Kurokawa *et al.*, 1998; Perez-Casanova *et al.*, 2006).

Several studies have tried to quantify the capacity of fish larvae to utilise proteins. Results from these experiments suggest that the uptake of protein into the larval body is limited by proteolytic rather than by its absorptive capacity (Tonheim *et al.*, 2005), and agree with previous findings that indicated a rapid and efficient absorption of FAA, peptides and hydrolysed proteins in larvae (Rust *et al.*, 1993; Rust, 1995; Rønnestad *et al.*, 1999; 2000c; 2001; Rojas-Garcia & Rønnestad, 2003a). It is generally believed that the larval ability to digest intact protein increases gradually throughout ontogeny, in parallel with the increase in enzymatic activities, and different studies have tried to describe these changes (Rust, 1995; Tonheim *et al.*, 2005).

To compensate for the low proteolytic activities in early larvae, formulated diets are often supplemented with pre-digested (hydrolysed) proteins (Cahu *et al.*, 1999; Cahu and Zambonino Infante 2001; Kolkovski and Tandler 2000). The results from these studies are conflicting; for example, Atlantic cod larvae performed better with up to 40% hydrolysed protein in the diet, while Atlantic halibut larvae had reduced survival with more than 10% hydrolysed protein (Kvåle *et al.*, in press). The reasons for these discrepancies may be leakage of the hydrolysed protein, combined with slow versus rapid feed ingestion rates in halibut and cod, respectively. An optimal level of dietary hydrolysed protein has been found to promote maturation of the gastrointestinal tract in seabass larvae when intestinal maturation was estimated by comparing indicators for adult vs larval mode of digestion (Cahu *et al.*, 1997).

Lipids

Lipids are an important source of metabolic energy and of essential fatty acids (EFA), structural components of biological membranes and precursors of essential metabolites (e.g. eicosanoids) (Sargent *et al.*, 1989). Similarities have been found between the lipid digestive and absorptive processes of fish and mammals and it is presumed that the mechanisms are generally comparable (Honkanen *et al.*, 1985).

Two different neutral lipase activities have been identified in the pancreatic juice of higher animals – the classic pancreatic lipase or the pancreatic lipase-colipase system (bile salt inhibited and substrate specific) and the bile salt-dependent lipase (BAL) or carboxyl ester lipase (non-specific and bile salt stimulated) (Borgström, 1977). The exact nature of the neutral lipases found in fish is still not completely established as different studies of digestive lipolysis have yielded results that differ as to which types of lipases can be found. Tocher & Sargent (1984) reported that during lipid digestion in rainbow trout, TAG was rapidly hydrolysed to

FFA and 2-MAG, which may be further hydrolysed but only at a very slow rate, indicating a classical mammalian specificity for primary ester bonds. The enzymatic activity was dependent on the presence of bile salts, but the presence of a cofactor similar to the mammalian colipase could not be shown. Other studies have also found the activity of neutral lipase to be bile salt-stimulated (Lie & Lambertsen, 1985; Ozkizilcik *et al.*, 1996). In addition, the only lipolytic enzyme found in the pancreas and pyloric caeca of Atlantic cod was a pancreatic BAL, homologous to the mammalian one (Gjellesvik *et al.*, 1992). It is now commonly accepted that the major digestive lipase in teleosts, including larvae, appears to be bile-salt dependent and non-specific, producing mainly FFA and 2-MAG (Lie & Lambertsen, 1985; Lie *et al.*, 1987; Gjellesvik *et al.*, 1992; Koven *et al.*, 1994a; b; Olsen *et al.*, 1998; Hoehne-Reitan *et al.*, 2001b; c; Perez-Casanova *et al.*, 2006). BAL in fish appears to have a preference for PUFA as substrate (regardless of position), followed by monounsaturated FA (MUFA) and finally saturated FA (SFA). Within MUFA and SFA, digestibility appears to decrease with increasing chain lengths (Lie & Lambertsen, 1985, 1991; Gjellesvik, 1991; Koven *et al.*, 1994b; Lie *et al.*, 1987; Olsen *et al.*, 1998).

The digestion of dietary PL occurs entirely in the small intestine by pancreatic PLA_2, which acts at the *sn*-2 position to yield a lysophospholipid and a FA. PLA_2 is secreted as an anionic zymogen and is activated by tryptic cleavage in the presence of calcium ions. Its activity is also dependent on the presence of bile salts, at a 2:1 bile salt to phosphatidylcholine (PC) molar ratio for optimal activity (Nordskog *et al.*, 2001). PLA_2 activity has been found in several fish species, with similar activities and dependence characteristics as the mammalian form (Ozkizilcik *et al.*, 1996; Izquierdo & Henderson, 1998).

Several studies have shown significant levels of pancreatic lipases at the onset of exogenous feeding (Izquierdo *et al.*, 2000; Hoehne-Reitan *et al.*, 2001c) and have found lipid absorption capacities even before complete absorption of yolk reserves (Pedersen *et al.*, 1987; Kjørsvik *et al.*, 1991a; Segner *et al.*, 1994; Diaz *et al.*, 1997; Ribeiro *et al.*, 1999a;b). Morphological observations of the digestive tract just after the start of exogenous feeding revealed the presence of lipid vacuoles in the larval intestine of several species, e.g. Dover sole (*Solea solea*), summer flounder (*Paralichthyus dentatus*), gilthead seabream, and Senegalese sole, showing evidence of absorption of dietary FA (Boulhic & Gabaudan, 1992; Bisbal & Bengtson, 1995; Ribeiro *et al.*, 1999a). The expression and activity of lipases have been studied in many species of marine fish larvae and the common opinion is that fish larvae are capable of digesting and absorbing lipids from the start of exogenous feeding (Ribeiro *et al.*, 1999a; Lazo *et al.*, 2000; Hoehne-Reitan *et al.* 2001c; Izquierdo *et al.*, 2000; Ozkizilcik *et al.* 1996). However, most of these studies have their focus at the beginning of the larval stage and show only minor variation in lipase activities with time. Mollan *et al.* (2008) showed that Atlantic halibut at 40 days after first-feeding absorbed only 34% of tube-fed emulsified TAG and more than 90% of 2-MAG. In comparison, digestion and absorption of intact lipid in adult fish is most often more than 80%. Furthermore, Sæle *et al.* (unpublished) showed that the expression of both BAL and PLA_2 mRNA was low and stable in early larvae, but increased sharply from approximately 50 to 80 dph in cod larvae. This indicates that early cod and halibut larvae have limitations in the digestion of lipid.

The activity of BAL is induced by feeding and is low in unfed larvae (Hoehne-Reitan *et al.* 2001c; Kim *et al.*, 2001). Furthermore, diet quality in terms of total lipid level and fatty acid composition, appears to have a direct effect on the onset of the maturation processes of the digestive tract and the activity of lipases (Cahu & Zambonino-Infante, 2001; Izquierdo *et al.* 2000; Morais *et al.* 2004c). The existence of plateaus of lipolytic enzyme expression suggests that there is a maximal capacity for enzyme synthesis (Zambonino-Infante & Cahu, 1999; 2001).

Absorption

Protein

In quantitative terms it still remains to be shown whether the absorption of proteins in the hindgut (pinocytoses) is an important route of supplying amino acids to the growing tissue (Watanabe, 1984; Kurokawa *et al.*, 1996; Luizi *et al.*, 1999; Rønnestad *et al.*, 2001). The main absorption of dietary proteins, peptides and AA in larval fish has been proposed to occur in the midgut (Rønnestad & Conceição, 2005), as in other vertebrates. However, almost nothing is known regarding the mechanisms and ontogenetic changes of protein, peptide and FAA absorption in fish. In general, AA absorption involves many processes, some of them overlapping. Vertebrate enterocytes express a variety of transporters responsible for AA transport, but so far only one intestinal transporter has been described for marine fish larvae; the oligopeptide transporter 1 (PepT1). The presently available data suggests that the PepT1 transporter is expressed at hatching, both in zebrafish (Verri *et al.*, 2003) and Atlantic cod (Rønnestad *et al.*, 2007a; Amberg *et al.*, 2008). Also, transporters for basic amino acids have been identified before hatching in zebrafish (Narawane *et al.*, unpublished).

The methodological problems associated with *in vivo* analysis of absorption rates of AA in larval fish equals those that assess their digestibility. In a recent approach, *in vivo* tube feeding followed by hot chase has been used to acquire data on absorption rates and efficiency of various FAA, hydrolysates and whole proteins (Rust *et al.*, 1993; Rønnestad *et al.*, 2001). The data have shown that the absorption rates of a pepsin hydrolysed protein and a more extensively hydrolysed protein were 2.2 and 3 times faster than the intact protein, respectively (Tonheim *et al.* 2005).

Lipids
Until recently it was believed that the uptake of lipid digestion products by the enterocytes was through a passive diffusion process. However, several mammalian studies (reviewed by Nordskog *et al.*, 2001) have raised the possibility that some lipids may be taken up via energy-dependent carrier mediated processes and have indicated the existence of a FA binding protein (FABP) associated with the brush border membrane, but it is uncertain if this route is quantitatively important.

Once absorbed by the enterocyte, the products of lipid digestion migrate from the site of absorption to the smooth ER, where complex lipids are resynthesised, being deposited in large lipid droplets of the mucosal epithelial cells (Tso & Fujimoto, 1991; Nordskog *et al.*, 2001). The presence of a gene coding for a microsomal triglyceride transfer protein (MTP) large subunit similar to the human form has been recently described in zebrafish adults and larvae (Marza *et al.*, 2005). However, the many aspects of lipid resynthesis, intracellular transport and lipoprotein assembly are poorly described in marine fish larvae.

Larvae fed TAG rich diets commonly show an accumulation of lipid vacuoles in the basal zone of the enterocytes, which indicates that dietary TAG is digested and absorbed but that the transport capacity out of the enterocyte is low (Diaz *et al.*, 1997; Izquierdo *et al.*, 2000; Morais *et al.*, 2005b; c; 2006b). The accumulation of lipid droplets may be explained by limitations in lipoprotein synthesis due to deficiency of phospholipids for the lipoprotein surface, since supplementation of formulated diets with phospholipids alleviates this problem (Fontagné *et al.*, 1998). Iritani *et al.* (1984) reported that the □-glycerophosphate acyltransferase activity of fish is extremely low compared to other animals and it is now well established that marine fish, particularly in the larval stages, have a limited capacity for endogenous *de novo* PL biosynthesis. However, the causes and consequences of lipid accumulations in the enterocytes of fish larvae and the mechanisms involved are still not fully understood.

Digestion and absorption of micronutrients
Micronutrients often exist in several forms with different biological availability; some micronutrients are believed to be absorbed by diffusion while others have specific carriers. There is very limited information on the digestion and absorption of micronutrients in fish in general, and virtually no information on these processes and the ontogeny of them exists for marine fish larvae.

GI motility
Gut motility includes a range of movements based on smooth muscle contractions, including tonic (support and sphincters), phasic (move products), peristalsis (moves the chyme along the digestive tract) and segmentation (mixes the diet with secretions). There are almost no systematic studies on these properties in larval fish, although some authors comment on visual observations of muscular contractions. The few available quantitative data are from zebrafish (Holmgren and Olsen, 2008) and Atlantic halibut (Rønnestad *et al.*, 2000). Gut motility is mediated by circular and longitudinal smooth muscles in the intestinal wall and is under control of the enteric nervous system (Holmgren and Olsen, 2008). The central nervous system and peptide hormones, e.g. motilin, can also influence peristalsis.

Prey size has been hypothesised to affect intestinal peristaltic movements through an effect on the distension of the gut, with larger preys (such as adult copepods) inducing a more pronounced mechanical stimulation (Pedersen, 1984). This effect was postulated to be mediated by the autonomic nervous system of the fish (Pedersen, 1984).

Regulation of Digestion
Digestion is a complex and closely orchestrated process. In mammalian systems this has been extensively studied and the digestive process is known to be controlled and optimised by nervous and endocrine systems as well as by luminal factors, implicating neurotransmittors, hormones, paracrine-, signal transduction- and transcription-factors. The available data suggest that the regulative pathways and molecules are conserved among vertebrates, but specific responses in fish may be different from mammals (Buddington and Krogdahl 2004). There are few studies concerning the control systems of digestion in larval fish.

Cholecystokinin (CCK) is an important gastro-intestinal hormone and plays a key role in pancreatic enzyme secretion, gall bladder contraction, intestinal peristalsis as well as control of ingestion. It is uncertain if CCK is present in sufficient amounts to allow proper control of digestive functions at first-feeding (Kamisaka *et al.*,

2001; Rønnestad *et al.*, 2007). Several other peptide hormones, e.g. PYY, ghrelin, bombesin and motilin, are known to participate in regulation of digestion in other vertebrates, but so far, virtually no data exist for marine fish larvae.

From mammals it is known that regulation of digestion involves the central and enteric nervous systems with communication between the brain and the different regions of the digestive tract and associated organs, in addition to internal communication within and between the different parts of the digestive system. Very few data on these issues are available for fish in general and no data exist for larval stages.

Summary

The digestive system is the entrance of nutrients into the larval body, and it is therefore important to understand the functions and limitations in processing capacity during early life stages. The morphological development together with a few key factors such as digestive enzymes has been studied in several species, although information of the ontogeny of enzyme activities is not complete in our target species. There is very poor knowledge of the ontogeny of basic physiological mechanisms in fish larvae. We lack knowledge on all aspects of the digestive function, including how it is regulated, how different mechanisms interact and how it is affected by genetic, dietary and environmental factors. Compared with the mammalian literature, the knowledge on the digestive system in fish larvae is rudimentary.

Relevance for the aquaculture industry

A holistic understanding of the digestive functions is important for development of diets for use in larval culture and for adaptation of rearing conditions that meet the larval requirements for optimal ingestion, digestion and absorption of these diets.

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10.9 Muscle development

Trine Galloway, SINTEF Fisheries and Aquaculture

The fish meat, scientifically termed the axial swimming musculature, accounts for 40-60% of the total body mass in fish. Muscle grows by two mechanisms: formation of new muscle fibers (hyperplasia) and increase in size of already existing fibres (hypertrophy). Fish are different from birds and mammals in that their muscle grows by hyperplasia also after hatching/birth. The morphological development of muscle and mode of muscle growth during embryonic and larval stages in fish is quite well described and the regulation of growth through myogenic regulating factors (MyoD, myf-5, myogenin, MRF4 and growth hormones) has been studied in adult fish. Only fragmentary information on the regulation of muscle growth exists for fish larvae.

Embryonic incubation temperature affects number, size and organelle composition of muscle fibres at hatching and first-feeding in cod and larval whole body growth is closely related to white muscle hyperplasia rates in cod, but not in halibut. This should infer that environmental and nutritional effects on larval cod growth, which would be numerous, also should have an effect on myogenesis.

Embryonic and larval development of the swimming musculature has a great impact on the larva's swimming ability. Furthermore, early development of the swimming musculature influences growth throughout all subsequent life stages and potentially flesh quality at harvest. The swimming muscle also contains large amounts of connective tissue and is closely associated with the skeletal system – it therefore has a profound impact on the outcome of skeletal development. Further studies are needed on the regulation of embryonic and larval muscle and connective tissue growth, on how nutrition and the environment affect myogenesis, and on how the development of musculature and bony structures affect each other.

Knowledge status for cod, halibut and other fish species

The axial swimming musculature accounts for 40 to 60% of the total body mass in fish (Bone 1978). The muscle mass consists of a number of muscle fibre types with different functions; the predominant fibre types are red slow fibres, located in a narrow wedge at the level of the horizontal septum, and white rapid fibres, which make up the bulk of the musculature (> 90%).

Muscle growth involves two mechanisms: hyperplasia (formation of new fibres) and hypertrophy (increase in size of existing fibres). New muscle fibre formation is closely regulated by the myogenic regulating factors (MRFs) MyoD, myf-5, myogenin, MRF4 and growth hormones, all of which are expressed at various times throughout a fish's life. During the embryonic stage skeletal myotubes are formed by fusion of several myoblasts and are therefore multinucleated (Nag and Nursall 1972). They further differentiate into muscle fibres during or soon after formation, and produce contractile proteins which eventually constitute the bulk of the cell volume. Multiplication by simple mitotic division is therefore impossible. New muscle fibres in larvae, juveniles or adults arise from myogenic progenitor cells (MPCs) that originate in the embryo. MPCs are probably also the source of additional nuclei for hypertrophic growth of existing muscle fibres (Koumans and Akster 1995). Fish that grow to a large final size (typical of aquaculture species) are special in that they grow by hyperplasia during a large part of their adult life (Weatherley et al 1988), whereas in birds and mammals the number of skeletal muscle fibres is fixed shortly after hatching/birth (Goldspink 1974). Muscle fibre hyperplasia in fish with a large final size can be divided into three phases; embryonic, stratified (during late embryonic and early larval stages) and mosaic hyperplasia (during late larval stages and well into adult life).

Fish muscle growth and functionality shows a great plasticity to changing environmental conditions. Particularly temperature and dissolved oxygen concentrations determine the rate of myogenesis, the composition of sub-cellular organelles, patterns of gene expression and the number and size of muscle fibres in fish (Johnston 2006).

Embryonic and larval development of the swimming musculature has a great impact on the larva's swimming ability, the success of predator avoidance (in a captive environment, important in relation to cannibalism) and prey capture, and therefore ultimately on survival. Furthermore, early development of the swimming musculature influences growth throughout all subsequent life stages and potentially flesh quality at harvest. The swimming muscle is also closely associated with the skeletal system and therefore has, together with the connective tissues, a profound impact on the outcome of skeletal development.

There is a vast amount of literature on the effects of environmental factors on whole body growth in cod and halibut larvae, juveniles and adults, and growth is expressed in many different ways (wet weight, dry weight, total length, fork length, age, developmental stage, etc). There is also a large amount of knowledge available on mechanisms that regulate muscle formation and growth in fish in general, and how these are affected by environmental factors (reviewed in Johnston 2006). In cod, the embryonic temperature does not affect the expression of MRFs (Hall et al. 2003), which are responsible for the determination of stem cells to MPCs and the differentiation of MPCs to myoblasts and myotubes, respectively. However, embryonic incubation temperature does affect the number, size and organelle composition of muscle fibres at hatching and first-feeding (Galloway et al. 1998). The background of this apparent discrepancy, and its consequence for the cod farming industry in terms of growth potential in cod larvae and adults, should be studied further. The effects of rearing temperature and nutrition on larval cod muscle growth have also been studied (Galloway 1999, Galloway et al 1999a), and larval whole body growth rates are closely and positively correlated with white muscle fibre hyperplasia rates (Galloway et al 1999b).

In halibut, white muscle fibre hyperplasia rates have so far been shown to be independent of whole body growth rates (Galloway, unpublished results), but more such studies need to be conducted in order to elucidate the effects of early life growth on growth in later life in halibut. An interesting feature of halibut muscle development is that MyoD2 is expressed bilaterally asymmetrically in the developing embryonic somites (Galloway et al 2006), and this possibly results in a greater white muscle mass on the dorsal side compared to the ventral side in adult halibut (Hagen et al 2008). More such investigations of flatfish species could provide valuable information on how muscle-regulating mechanisms work in species with different anatomical, physiological and ecological traits. It is also interesting that adult female halibut have more white muscle fibres than males, due to a cessation of hyperplasia at a smaller body length in males (Hagen et al 2006; 2008). The determining factors for this sexual dimorphism could potentially be expressed in the early life stages, and an increased knowledge about the timing could be of great value to the halibut farming industry.

Use of model species

There are considerable advantages to studying muscle plasticity in model fish species, thanks to the extensive molecular and genetic resources available (Johnston 2006). However, most of the model species (*Danio rerio, Takifugu rubripes, Tetraodon nigroviridis, Oryzius latipes*) only grow to a small final size and are therefore not interesting from an aquaculture point of view since hyperplasia as a muscle growth mechanism ceases at or close to hatching in species with a final small size. Factors controlling growth and final size should therefore be studied for each commercially interesting fish species. In this aspect the cod and salmon genome projects may provide important molecular tools such as cDNA libraries, Expressed Sequence Tags and DNA microarrays.

Need for new knowledge

Since whole body growth is expressed in so many ways in the literature, it would be of great advantage to the farming industry to correlate different growth measurements and recommend one simple and practical way of determining early life growth.

The relative numbers of slow and fast growers between and within batches of fish larvae is a matter little studied. However, this is of major significance for commercial hatcheries. Fast growers may be preferred to increase productivity, but slow growers may be more resilient to stress and disease and have a better flesh quality at harvest. Growth dispersion may result in cannibalism in some species and will increase operational costs of grading procedures. The differential growth in a given batch depends on genetic factors as well as zootechnical conditions. The differences in growth mechanisms between slow and fast growers remain to be studied.

Much remains to be discovered about the regulation of embryonic and larval growth mechanisms in cod and halibut and effects of the rearing environment. For several fish species a connection between growth in early stages and later life has been shown, but this remains to be studied for cod and halibut. In this context there will be a considerable merit in adopting a systems biology approach, e.g predicting juvenile and adult growth rates based on genetics, early life stage physiology, environmental input and other relevant factors.

There is a close connection between musculature groups (swimming, jaws, fins etc.) and their associated skeletal systems, but virtually nothing is known about how the development of the musculature, together with its associated connective tissue, affects the outcome of skeletal development. A more holistic approach, i.e. investigating the development of several organ systems at the same time, will probably provide more practical answers than when only one organ system at a time is studied.

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10.10 Skin and pigmentation

Kristin Hamre, NIFES, National Institute of Nutrition and Seafood Research

The morphological development of skin and scales has been studied in zebrafish, but not in our target species. Very little is known about the regulation of skin and scale development in fish.

Due to problems with malpigmentation in farmed flatfish juveniles, considerable effort has been made to understand the development of adult pigmentation in these species. The migration of precursor cells from the neural crest to the skin has been described in a number of animal species, including zebrafish. Differences in proliferation and differentiation of these cells between the ocular and blind side of Japanese flounder have been studied in detail by morphological methods. It has also been shown that white skin on the ocular side in malpigmented fish has similar characteristics as skin on the blind side in normal fish, with regard to pigment cells, scales and mucus cells. However, how these processes are regulated is not known. Pigmentation is quite extensively studied in zebrafish, by knocking out genes involved in pigment cell development and function, so knowledge to work with our target species can be extracted.

It is known that vitamin A and fatty acid composition in the feed, and thyroid hormones, affect pigmentation success in flatfish. However, an unbalanced fatty acid composition of the feed organisms used in intensive culture is the main reason for pigmentation errors of farmed flatfish. A fatty acid composition that leads to malpigmented flatfish juveniles does not have a similar effect on cod. The thresholds for concentrations of the essential fatty acids for normal pigmentation in Atlantic halibut have been determined.

Skin and scale development

There is very little literature on skin and scale development in commercial aquaculture species, but these processes are well described at the morphological level in zebrafish (le Guellec et al., 2004; Sire and Akimenco 2004). Briefly, the epidermis in adult fish consists of three layers. The superficial stratum is a single layer of cells ornamented by microridges, which retain the mucus substances secreted at the skin surface. A layer with variable thickness, depending on species, constitutes the intermediate stratum and consists of several cell types, e.g. mucus cells and sensory cells, in addition to a pool of stem cells which will replace dead cells of different types. In the deep region of the epidermis there is a single layer of cells, whose main function is to keep the epidermis attached to the underlying dermis, but which also has important secretory and regulatory functions in development of the dermis and the scales. At 24 hph in the zebrafish, the epidermis consists of two cell layers (the intermediate stroma is absent) with an underlying basement-membrane. Development of the dermis starts with deposition of a collagenous stratum below the basement-membrane, probably synthesised by the deep epidermal layer (32hpf). The collagen layer gets larger and increasingly more structured and is eventually invaded by fibroblast (26dpf), which gradually takes over the synthesis of collagen (indicated by transfer of expression of type 1 collagen α2 chain). The epidermis still has two layers of cells at 10 dpf, which increases to four layers at 26 dpf (le Guellec et al., 2004). At 30 dpf, scale forming cells (fibroblasts?) are present in the upper region of the dermis. These cells accumulate in well defined regions of the skin and form the scale papilla, which later form the three distinct layers of the scale. As the scale grows, the anterior region sinks into the dermis and the posterior region protrudes into the epidermis, which forms a fold around it. The origin of the fibroblasts is unclear, but the authors propose that they migrate from the myosepta and that signalling from this tissue is responsible for the pre-patterning (indicated by the expression pattern of sonic hedgehog) of the skin which precedes the formation of scales (Sire and Akimenko 2004). The genetic regulation of the development of fish skin and scales is poorly described.

Pigmentation

Since pigmentation of flatfish has been a problem in aquaculture, work on this topic has been extensive in commercial species, compared to many other topics. This relates to the ontogeny of pigmentation and effects of environmental and nutritional factors on this process, but less so to the function of mature pigment cells.

The neural crest (NC) origin of pigment cells was first established by Du Shane (1935, 1936, 1938), in experiments with amphibians. Since then, the neural crest origin of pigment cells has been shown in birds (Dorris 1939; Eastlick 1939), in mammals (Rawles 1940, 1948), in lamprey (Newth 1956) and in zebrafish (reviewed by Eisen and Weston 1993). NC forms in the region between the neural ectoderm and the non-neural ectoderm and is later located in the dorsal region of the neural tube in gastrulated embryos. NC cells are pluripotent and

may form peripheral neurons, glia, connective tissue, bone, secretory cells and pigment cells, among other things (Gammil and Bronner-Fraser 2003). The cells must reach their final location by extensive migration. The migration routes of three specific chromatophores (melanophores, iridophores, xantophores) has been assessed in zebrafish (*Danio rerio*) (Raible *et al.* 1992, 1994; Schilling and Kimmel 1994; Dutton *et al.* 2001). Unlike in mammals, the chromatophores migrate along both the dorsolateral pathway, between the developing epidermis and dermomyotome, and the ventral-lateral pathway, between the dermomyotome and the neural tube.

Prior to metamorphosis in Japanese flounder (*Paralichthys olivaceus*), the majority of the pigment cells are melanoblasts and they are distributed evenly on both sides of the larvae. Some of these melonoblasts differentiate into larval melanophores which are evenly distributed on both sides of the larvae. Larval melanophores are generally larger than the adult type melanophores which appear later (Matsumoto and Seikai 1992). Concomitant with the migration of the right eye over the mid-dorsal ridge, there is a large increase in proliferation of melanoblasts on the dorsal side of normal larvae and stagnation in the proliferation of these cells on the blind side. On the dorsal side, pigment cell precursors differentiate into melanophores, xanthophores and iridophores, whereas on the blind side, these cells go through cytolysis, although a few differentiate to iridophores (Seikai *et al.* 1987a, b; Matsumoto and Seikai 1992, reviewed by Bolker and Hill 2000). The larvae possess no forms of scales, while the juvenile flatfish exhibit cycloid scales in the skin on the blind side and ctenoid scales on the ocular side (Matsumoto and Seikai 1992). In malpigmented larvae, dark pigmentation may be found on both sides, only in the cranial area or randomly distributed in the larval skin. White pigment develops in the remaining parts of the skin. Fish with impaired eye migration often become ambicoloured (Harboe and Hamre 2008), indicating that signals from the eye may be important in determining skin coloration. The type of scales normally found on ocular and blind side follows the pigment type, i.e. white pigmented skin on the ocular side display cycloid scales and dark pigmented skin on the blind side display ctenoid scales (Matsumoto and Seikai 1992). Further, the frequency of mucus cells is higher on the dorsal than on the blind side of normal juveniles, and in dark compared to white skin in malpigmented juveniles. The development of normal pigmentation in flatfish seems to be largely dependent on nutrition, since feeding copepods to flatfish larvae results in almost 100% correct pigmentation while rotifer- and *Artemia*-fed larvae often develop severe malpigmentation (Seikai 1985; Hamre et al., 2002). Further, temperature and UV light have been shown to affect pigment development in flatfish (Matsumoto and Seikai 1992; Aritaki and Seikai 2004), but the effects were much smaller than the nutritional effects found by Hamre et al. (2002) and Seikai (1985).

In 1987, Seikai *et al.* suggested that nutritional factor(s) present or deficient in *Artemia* nauplii could interfere with the development of adult-type melanophores, possibly through a modified tissue environment. Later, Matsumoto and Seikai (1992) hypothesized that pigmentation anomalies result from disruption of the mechanisms responsible for establishment of asymmetric skin structures and that this may be due to a blockade of the differentiation of melanoblasts or their precursors which become the adult type melanophores. An alternative hypothesis was presented by Kanazawa (1993), who held normally and abnormally pigmented Japanese flounder juveniles in a tank with a dark and light compartment and found that the normally pigmented fish would stay in the dark compartment in the day and in the light compartment at night. The malpigmented fish were randomly distributed between the two compartments during both night and day. He concluded that malpigmented fish had impaired vision. He claimed that nutrient deficiencies would interrupt visual stimulation of the nervous system due to incomplete rhodopsin formation. This might lead to lowered production of melanophore-stimulating hormone, which is necessary for development of pigment cells and synthesis of melanine. MSH producing cells are present in the pituitary of Japanese flounder one week after hatching and the ratio of MSH cell to pituitary volume was enhanced in flounder reared on a sandy, compared to glass bottom. However, MSH ratios in normal and pseudoalbinistic fish were similar (Estevez *et al*. 2001). The ventral skin produces Melanization inhibiting factor (MIF) (Fukuzawa and Ide 1988), which can override the stimulatory effects of MSH (Fukuzawa and Bagnara 1989). Therefore, melanoblasts which exist on ventral side, evidenced by their dendritic morphology and dopa content, fail to differentiate (Ohsugi and Ide 1983).

Malpigmentation in flatfish is a global problem, and has been linked to deficiencies of long chain n-3 fatty acids (Estevez and Kanazawa, 1995; Næss and Lie, 1998; Sargent et al., 1999; Shields et al., 1999; Hamre et al., 2007). A high level of vitamin A stimulates the development of adult type chromatophores in Japanese flounder (Takeuchi et al., 1995; Dedi et al., 1997; Haga et al., 2002) and thereby pigmentation. Thyroid hormone also modulates the rate of pigmentation in this flatfish

(Yoo et al., 2000) and iodine may be too low in *Artemia* to meet the requirement for thyroid hormone synthesis (Solbakken et al., 2003). However, the levels of vitamin A, which stimulates pigmentation, also yield skeletal deformities, indicating that they are toxic. Atlantic halibut larvae get sufficient vitamin A by converting cantaxanthin in *Artemia* (Moren et al., 2004 a,b) and enrichment of *Artemia* with iodine did not result in improved pigmentation (Moren et al., submitted). Thus, the dietary fatty acid composition seems to be the key to better pigmentation of Atlantic halibut (Hamre et al., 2007).

Recent results have shown some interesting effects of fatty acid composition on pigmentation and eye migration in flatfish. Estevez *et al.* (1999) varied the DHA:EPA ratio and the EPA:ARA ratio in live feed fed to turbot (*Psetta maxima*) larvae and found that increasing ARA level, and thus decreasing EPA:ARA ratio, reduced pigmentation success dramatically. This result has been confirmed in additional studies with turbot and halibut (McEvoy *et al.* 1998), in yellowtail flounder (*Limanda ferruginea*) (Copeman *et al.* 2002), in Senegalese sole (*Solea* Senegalese*ensis*) (Villalta *et al.* 2005a) and in common sole (*Solea solea*) (Lund et al., 2008). Furthermore, Villalta *et al.* (2005b) found a small, but significant, improvement of pigmentation in Senegalese sole in response to increasing levels of EPA in the diet, indicating that it is actually the EPA:ARA ratio that has an effect on pigmentation. In the study of Estevez *et al.* (1999), variation in the DHA:EPA ratio had no effect on pigmentation, contrary to the general opinion that this ratio is the most important factor influencing pigmentation success in flatfish.

The data indicate that eicosanoids are involved in development of pigmentation in flatfish, since ARA and EPA, which are the precursor of eicosanoids of the 2- and 3-series, respectively, have opposite effects on pigmentation and the ratios of these eicosaoids followed the ARA:EPA ratios and pigmentation success in Senegalese sole (Villalta et al., 2008). ARA supplementation, malpigmentation and PGE_2 concentration were also correlated in common sole (Lund et al., 2008). McEvoy et al. (1998) found that a brain EPA:ARA ratio of 4:1 yielded the best ratio of normal pigmentation while a ratio of less than 1:1 would produce 100% malpigmentation in turbot and Atlantic halibut. However, the best percentage of normal pigmentation found in Atlantic halibut was only 25%, while in turbot the best percentage was 86%. This may have been caused by Atlantic halibut's higher requirement for DHA compared to turbot. Hamre and Harboe (2008) found that an increase in DHA from 9.4 to 13.9% of fatty acids in *Artemia* at similar EPA:ARA ratios increased the rate of normal pigmentation in Atlantic halibut from 46±16% to 77±2%. The relatively small difference in fatty acid composition and the large effect on pigmentation indicates that 13.9% is near the threshold level of DHA for normal pigmentation in Atlantic halibut. In Senegalese sole, a low level of DHA (1.5% of fatty acids) does not seem to inhibit pigmentation (Villalta et al., 2005b), thus the requirement for DHA seems to be species-dependent.

In Atlantic cod larvae (2-28 dph), dietary ARA did not affect pigmentation, whereas green tanks led to darker pigmentation than grey tanks, combined with a higher level of whole body PGE_2. This effect was probably mediated by the effect of light on melanocytes already present in the skin, possibly by MSH (Bransden et al., 2005). Thus, the regulation of the development of pigment cells seems to differ between cod and halibut/flatfish.

It seems to be the induction of melanoblasts already in place in the dermis to proliferate and differentiate which is disrupted in intensively reared flatfish larvae fed *Artemia* or rotifers. The fact that double pigmentation appears only in fish with poor eye migration indicates that signals involved in axis formation also determine the fate of melanoblasts. In malpigmented fish, either these signals or the skin tissue, including scales, mucus cells and melanoblasts' ability to respond to the signals, may be disrupted. A hypothesis regarding the role of fatty acids, vitamin A and iodine in pigmentation of flatfish was proposed by Hamre et al. (2007), suggesting that these nutrients affect pigmentation through regulation of gene transcription by their nuclear receptors PPAR, RXR and TH.

The development of pigmentation has been extensively studied in several model teleost species, including zebrafish (Kelsh and Parichy 2008), where genetic information has been revealed from numerous pigment mutants. The knowledge base for studying these aspects in our target species is therefore extensive.

Relevance for the aquaculture industry

Pigmentation problems are currently small in culture of Atlantic halibut, due to improved enrichment products for *Artemia* which provide high and stable contents of DHA and a good EPA:ARA ratio. However, it is important to understand the mechanisms behind the development of the pigmentation pattern and effects of environmental and nutritional factors on these mechanisms, to prevent that changes in diets or rearing conditions accidentally lead to malpigmentation. The study of the development of the skin and scales is relevant for understanding the

process of wound healing, since this process greatly resembles the ontogeny of skin tissue.

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10.11 Development of bone

Øystein Sæle[1], Mari Moren[1], Synnøve Helland[2]; [1] NIFES, National Institute of Nutrition and Seafood Research, Nofima Marin

Different parts of the skeleton have different embryonic origin; the vertebrae are formed from the inner part of the somites, the limbs from the lateral plate mesoderm and the brachial arches and craniofacial bones from the cranial neural crest. There are basically two types of bone: chondral bone formed from a cartilaginous "model" and dermal bone formed from dermal connective tissue. Cells that participate in bone formation and modulation in fish are chondroblasts, producing the cartilage bone model, osteblasts, which produce the bone matrix and deposit minerals, and osteoclasts, which absorb bone. The majority of teleosts do not have osteocysts which are embedded in cellular bone. Most of the knowledge on bone development today is from mammalian studies, but recent studies in zebrafish elucidate some of the processes in fish, including the main factors regulating bone growth and remodelling.

Development of craniofacial bones and vertebrae has been described morphologically in Atlantic halibut and in Atlantic salmon, respectively. The special case of eye migration, which involves dramatic remodelling of the frontal bone between the eyes, has been studied in detail in Atlantic halibut. However, the signalling responsible for regulation of bone growth is not described in our target species.

A large number of nutritional and evironmental factors have been shown to affect bone development, both in fish and mammals, and the high ratios of bone deformities seen in intensive culture of fish may have many causes. Further studies are needed to identify the critical factors and the interactions between them and to understand their impact on the dynamic metabolism and interaction of chondroblasts, osteoblasts and osteoclasts in development of bone.

There are three distinct embryonic origins of the skeleton: *i*) the sclerotome (inner layer of the somites), which forms the axial skeleton (Inohaya *et al.*, 2007), *ii*) the lateral plate mesoderm, which forms the limb skeleton, and *iii*) the cranial neural crest that forms the branchial arches, the craniofacial bones and cartilage (Gilbert, 1997).
Furthermore there are two basic types of bones based on their further development: Chondral, or substitute bone, develops from a cartilaginous "model" or template with more or less the same shape as the future bone. Centres of ossification occur inside the cartilage (endochondral ossification), or in the perichondrum surrounding the cartilage (perichondral ossification). Eventually the cartilage will be completely replaced by bone.
Dermal bone originates in the ossification zones of the dermal connective tissue. It is derived directly from mesenchyme in the deeper layers of the dermis (intramembranous ossification). Intramembranous ossification appears to be more ancient than chondral ossification (Morriss-Kay, 2001). The frontal bones that play a key role in eye migration (Sæle *et al.*, 2006a, Sæle *et al.*, 2006b) are of dermal origin. The derivation of the frontals is not very clear, as they differentiate later than both cartilage and dermal bones of the viscerocranium (Morriss-Kay, 2001). Different avian studies conclude differently. Noden (1988) states the dermal skull roof to be of mesodermal origin whereas later research traces its origin to the neural crest (Couly *et al.*, 1993).

In chondral ossification, the mesenchymal cells (migratory cells of the meso- and ectoderm) will form a cartilage model for the future bone. The formation of the cartilage takes place in three stages: proliferation of the mesenchyme; condensation of the pre-cartilaginous mesenchyme; and finally, the differentiation of the chondrocytes. When the cartilage is formed, cells in the central part of the cartilage become larger and start secreting a matrix more susceptible to invasion by blood vessels. These cells are called hypertrophic chondrocytes. When the cartilage matrix degrades, the hypertrophic chondrocytes die (apoptosis) and are replaced by osteoblasts introduced by the blood (Gilbert, 1997).

However, the majority of teleost species have bone without osteocytes, termed acellular bone (Meunier and Huysseune, 1992), as is the case for Atlantic halibut and cod. In addition to the absence of osteocytes, acellular bone has smaller calcified crystals and a greater amount of organic substance, presumably collagen, than cellular bone (Moss, 1961). Studies have shown that there is no mix of cellular and acellular bone in one skeleton (Meunier and Huysseune, 1992). In general, cellular bone is found in less advanced groups of Teleostei and acellular bone is found in advanced groups.

Compact mesenchymal cells form a periosteum around the osteoblasts and their calcified matrix (hydroxyapatite salts), in both acellular and cellular bone. Cells on the inside of the periosteum become osteoblasts that deposit bone matrix in parallel with

the central calcified matrix, in this way building the bone layer by layer.

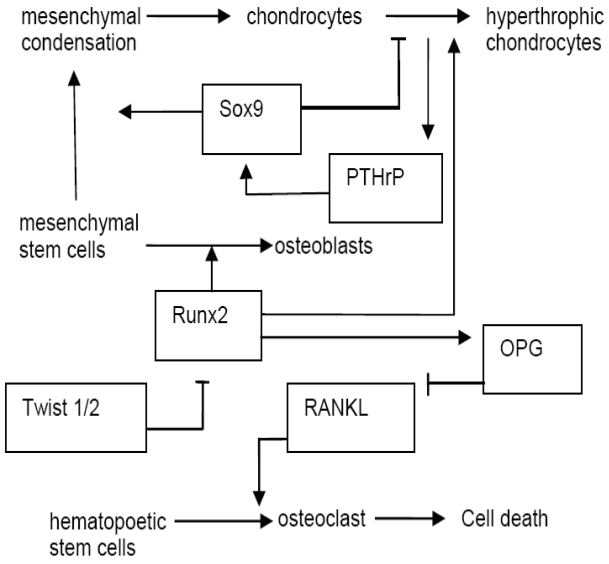
Remodelling of bone by osteoclastic activity is not only common in teleost fish, but necessary for growth (Witten and Villwock, 1997a, Witten and Villwock, 1997b, Witten *et al.*, 2001, Witten *et al.*, 2000).Tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CATK) are the main lytic enzymes that resolve minerals in bone. The osteoclast forms a sealed compartment between itself and the bone surface and the lytic enzymes are activated by the excretion of protons to the compartment. Mono- and multi-nucleated osteoclasts in teleosts have been shown to function in this same way (Persson *et al.*, 1999, Persson *et al.*, 1998, Persson *et al.*, 1995, Witten *et al.*, 2001, Witten *et al.*, 1999, Witten *et al.*, 2000).

Most knowledge about ossification today is based on mammals but increasing amounts of information on these processes in fish are emerging. In mouse Pax1 is essential for the development of the vertebral body and Pax9 for the neural arches but in Medaka both Pax1 and Pax9 are needed for neural arch and vertebral body development (Mise *et al.*, 2008).

Osteoclast proliferation and activation is controlled by the OPG/RANKL/RANK system. RANKL is the osteoclast differentiation factor expressed by osteoblastic stromal cells (Boyle *et al.*, 2003, Khosla, 2001) and chondroblasts (Komuro *et al.*, 2001). Retinoic acid directly up-regulates osteoclast activity, partly regulating bone formation and metabolism (Harada *et al.*, 1995) although the mechanism is unknown (Rohde and Deluca, 2003). This system is not described in fish yet.

Eye migration

Eye migration in flatfish is the most radical asymmetric development in any vertebrate. The complex remodelling of tissues is restricted to the area around the ethmoid plate and the eyes (Sæle *et al.*, 2006b). Other osseous asymmetries such as those in the jaw are not unifying in flatfishes in the same way as eye migration (Gibb, 1997). The complexity of eye migration is due to the large array of different tissues involved and comprises their intricate interactions. Most striking is the interaction between cartilagenous elements, perichondral and dermal ossification (Sæle *et al.*, 2006a). First sign of asymmetric growth is seen in the dorsal parts of the ethmoid plate and takes place around day 20-25 post first-feeding in halibut. We have demonstrated that there is a correlation between the position of the eyes and the osteoclastic modelling of the surrounding dermal bones (Sæle *et al.*, 2006a). Eye migration in Atlantic halibut is stimulated by application of a diurnal light regime compared to continuous light (Harboe et al., submitted).



Important factors regulating differiation and activity of chondrocytes, osteoblasts and osteoclasts. First described in mammals but have now been described in teleosts. Redrawn from Boyle et al., (2003) and Renn et al., (2006).

Effects of Nutrition

Several nutritional factors have been suggested to play a role in the skeletal development in fish (reviewed by Lall and Lewis-McCrea 2007), but few studies are conducted with marine fish larvae and the underlying mechanisms involving how these nutrients affect skeletal development are poorly understood (reviewed by Cahu, 2003).

Minerals constitute an important part of bone structures as well as participating in metabolic processes and signal transductions. Calcium (Ca), phosphorus (P), boron (B), zinc (Zn), copper (Cu), silicon (Si), vanadium (V), selenium (Se), manganese (Mn) and fluorine (F) are known to affect either bone formation or mineralization in terrestrial animals (reviewed by Beattie and Avenell, 1992). Comparing the mineral contents of enriched rotifers and *Artemia* with copepods has revealed that the diets used in commercial rearing may have significantly lower levels of P, Zn, Cu, Se, Mn as well as iodine (I) compared to copepods (Hamre et al, 2008; 2007), and further that Atlantic cod larvae fed copepods contain higher levels of Cu, Se, Mn and I compared to cod larvae fed enriched rotifers (Hamre, unpublished data). Roy et al (2003) showed that haddock juveniles fed a P-deficient diet (0.42% P in diet dry wt.) had an increased osteoclast activity and number as well as a lower number of osteoblasts compared to fish fed

a diet with sufficient levels of P (1.02% P in diet dry wt.). This effect may very well be valid for the larval stage of marine fish species. Riberio et al (unpublished data) showed that the skeletons of Senegalese sole juveniles were ossified to a higher degree if the larvae were fed an iodine-enriched diet compared to juveniles fed a normal diet. Phosphorus deficiency induces bone deformities in several fish species, such as Atlantic salmon, common carp, and haddock (Ogino and Takeda, 1976; Baeverfjord et al., 1998; Roy et al., 2002; Sugiura et al., 2004). In Atlantic salmon, P deficiency results in lowered whole body P and in poor mineralization of bones and scales, small and soft vertebrae, crooked spinal arches, and wrinkled and shortened ribs (Åsgård and Shearer, 1997; Baeverfjord et al., 1998; Vielma and Lall, 1998; Helland et al., 2005). Another important issue in nutritional effect on malformation in fish is the current use of more plant based ingredients in the fish feed and how these alter the mineral availability, and how this in turn affects the mineralisation of bones (Helland et al., 2006).

All the fat-soluble vitamins (A, D, E and K) are known to affect bone growth and development in fish as well as terrestrial animals. Vitamin A (VA) has received the bulk of the attention by larval researchers, probably because several studies have confirmed the teratogenity of excess VA (reviewed by Cahu et al. 2003). Immersing Japanese flounder larvae in relatively high concentrations of e.g. retinoic acid or retinol results in a high number of skeletal deformities (Takeuchi et al, 1998; Haga et al 2002).Villeneuve et al (2005a) fed European sea bass larvae increasing levels of VA and observed linear correlation between vitamin A level and malformation percentage. They linked this to alterations in the RXRα, RARα and RARγ expression patterns. Copepods and un-enriched commercial live feed contain insignificant levels of VA, if any (Hamre, et al. 2008; Moren, et al 2005), but both copepods and *Artemia* have high levels of carotenoids, which are precursors to VA (Moren et al 2002; 2004). Rotifers, on the other hand, contain little or no carotenoids (Hamre et al. 2008). Possible VA-deficient live prey is avoided by the added VA or its precursors in the commercial enrichments. Perhaps more interesting than the known teratogen effect of excess VA is that the dietary content of other nutrients can affect the expression of the VA activated nuclear receptors. Villeneuve et al (2005b) altered the expression of RARα and RXRα in European sea bass larvae by changing the phospholipid level and compostion in the diet. Mazurais et al (2007) fed European sea bass larvae diets with different amounts of vitamin mixture (both water- and fat-soluble) and found elevated PPARγ coinciding with low BMP-4 expression in larvae fed low levels of vitamin mixture. Further, these larvae exhibited a higher degree of deformities and a lower ratio of bone to cartilage compared to those fed higher levels, suggesting that high levels of PPARγ expression may have converted some osteoblasts into adipocytes and that this loss of osteoblasts caused the skeletal deformities. It remains to be shown which component or combination of vitamins caused this.

The amount, form (tri-, di- or monoglyserides, phospholipids), fatty acid composition (in particular the ratios between the ARA and EPA and DHA) and peroxidation of the lipids in the diet have proved to be important in relation to skeletal deformities in marine fish larvae (Cahu, 2003, Villeneuve et al., 2005b, Lall and Lewis-McCrea 2007). The runx family (runt-related transcription factors) are essential regulators in osteoblast and chondroblast differentiation and these are again regulated by the prostaglandin PGE_2, a derivative of arachidonic acid. Liu et al. (2006) discovered that the cox enzymes (converting ARA to PGE_2) of the brook trout had a much higher affinity towards ARA compared to the cox enzymes of human and ovine origin. An unbalanced diet (commercial live feed typically has a level of ARA that is too high compared to EPA and DHA) may produce too high PGE_2 levels. In rats, bone formation decreased with increasing PGE_2 levels (Watkins et al 2000).

There are a few published studies that correlate certain essential amino acids (arginine and lysine) to the synthesis of insulin-like growth factors and collagen in osteoblasts in terrestrial animals/cell cultures (Chevalley et al. 1998, Fini et al 2001). The main focus in studies with marine fish larvae has been bioavailability of dietary proteins and lately the involvement of cholecystokinin (Kvåle et al., 2002; Cahu, 2003; Cahu et al., 2004, Koven et al., 2002). Apparently, if the larvae receive insufficient amounts of proteins or an unbalanced amino acid profile, bone deformities appear. It is unknown whether this is due to impaired protein synthesis in general or is bone-specific.

Effects of environment

The morphology of some types of skeletal deformities is fairly well described (Bruno, 1990; Andrades et al., 1996; Madsen and Dalsgaard, 1999; Afonso et al., 2000; Boglione, 2001; Koumoundouros et al., 2002; Lewis et al., 2004; Fraser et al., 2004), while the information about causative factors is scarcer. Temperature, mechanical stress, water velocity, salinity and light intensity are some of the identified causative factors, and often there will be an interaction between various causative factors, complicating the picture. The symptoms and expressions of malformations are often similar across species.

Temperature affects the embryonic development as well as the developmental rate of the skeleton, and can affect the length of the fish, number of vertebra and prevalence of deformed vertebrae (Polo, A., 1991; Hansen and Falk-Petersen, 2001; Sfakianakis et al. 2004). Egg incubation temperatures outside the optimal range of the species can induce deformities in the vertebral column (Pavlov and Moksness, 1997; Baeverfjord et al., 1999; Baeverfjord and Wibe, 2003), deformities in the skull (pugnose, deformed jaws) (Bolla and Holmefjord, 1988; Lein et al., 1997a) and deformed opercula (Abdel et al., 2004). Experiments on egg rearing temperatures in Atlantic cod are inconclusive so far (Poppe, 2005; Fitzsimmons and Peruz, 2006).

Lordosis is characterized by V-shaped dorsoventral curvature of the body axis. Lordosis in the anterior part of the vertebral column in Atlantic cod (star gazers) can be induced by temperatures higher than the optimum during the first weeks of exogenous feeding (Grotmol el al., 2005, Lein et al., in prep). Also, strong water current during the period of ossification of the vertebral column induces haemal lordosis in sea bass, sea bream and Atlantic cod (Divanach et al., 1997; Kihara et al., 2002; Helland et al., in prep.). In a study on combined effects of temperature and swimming speed, Sfakinanakis et al. (2006) found that fish at low temperatures were more strongly affected by water current than fish at lower temperatures.

Presence of an oil film on the water surface can inhibit inflation of the swim bladder in physiostome fish. Pre-haemal lordosis was initially considered the most severe deformity in sea bass and sea bream, and is associated with non-inflation of the swim bladder (Boglione et al., 1995). The introduction of surface skimmers (Chatain, 1986, 1987, 1994, Chatian and Ounais-Guschemann, 1990) and the development of a simple technique of sorting by elutriation (Chatain and Corrao, 1992) lowered the frequency of this malformation.

Rearing salinities outside the optimum can induce deformities in stenohaline fish species. Deformity to the jaws at high or low salinities has been demonstrated in several fish species (Alderdice and Velsen, 1976; Santerre, 1976; Lein et al., 1997b). Light exposure during the early yolk sack stage induced jaw deformity in Atlantic halibut (Bolla and Holmefjord, 1988).

Recent biotechnological studies are looking into underlying mechanisms for development of bone deformities. Expression analyses of HSP70 have shown that embryos of Atlantic cod and Atlantic salmon are highly sensitive to temperature stress (Takle et al., 2005; Takle et al., in prep).

There is a need for further studies on the effect of single or combined causative factors for development of deformities in fish with regard to type and severity of the deformity, and on the underlying mechanisms.

Relevance for the aquaculture industry

Bone deformity is one of the most prominent challenges for the aquaculture industry, affecting most species of marine fish (Cahu 2003), including cod and halibut. In Atlantic cod juveniles produced in Norway in 2006, 30-80% had skeletal deformities (Lein et al., 2007). Skeletal deformities – although most probably present, as in other species – have not been registered systematically in Atlantic halibut, due to a focus on malpigmentation and impaired eye migration in this species. Errors in bone development seem to have multifactorial causes (Lall and Lewis-McCrea 2007) and both nutritional and environmental variables may stimulate development of malformations. To overcome this problem, it will be necessary to understand the mechanisms of bone formation and remodelling, and to identify the place, time and conditions under which errors are induced.

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10.12 Development of the immune system in fish

Ivar Hordvik, Department of Biology, University of Bergen

Immune cells in fish develop from pluripotent hematopoietic stem cells. Initially, the stem cells develop into myeloid and lymphoid progenitor cells which subsequently give rise to different cell types, as granulocytes, monocytes, macrophages, thrombocytes, erythrocytes, B-cells, T-cells etc. Although similarities are most striking, some types of white blood cells in fish have retained primitive features which have been lost in mammals, e.g. the B-cells in fish which have phagocytic and microbicidal abilities in contrast to their more specialized counterparts in mammals. There are also significant differences between fish and mammals with regard to the employment of different antibody classes.

Homologous genes of many transcription factors involved in mammalian hematopoiesis have been used to trace corresponding development in fish by *in situ* hybridization. However, knowledge on the development of the immune system in fish is fragmented and limited compared to higher vertebrates. In some species, such as angelfish, initial hematopoiesis appears in yolk sac blood islands. In other species it appears in the intra-embryonic intermediate cell mass (ICM), as in zebrafish, or first in the yolk sac and later in the ICM, as in rainbow trout. Definitive hematopoiesis is established in the kidney, but an intermediate, larval site of hematopoiesis has been revealed in the tail of zebrafish. In recent years zebrafish has been established as the most important model on the development of the immune system in fish, and undoubtedly this common reference will facilitate future studies on other species.

Introduction

Considerable progress has been achieved in fish immunology research during the last 20 years, characterizing and systemizing central immune genes, and revealing similarities and differences between fish and mammals. Although basic principles are conserved in the immune system of jawed vertebrates, there are gross differences in anatomical organization, and many central genes have been subjected to duplications and show differential gene expression patterns. Studies of the immune system in teleosts have been done on relatively few species, but even between these there are major differences. The immune system of cod, for example, appears to be different from that in salmon in key characteristics, such as antibody concentration in blood and the ability to raise specific antibodies after immunization (Magnadottir et al., 2001). Studies on the development of the immune system in fish are fragmented and it is very difficult to draw general lines (Zapata et al., 2006, and references therein).

Molecular components

Adaptive immunity in fish is based on molecular components with overall similarity to the mammalian counterparts, i.e. immunoglobulins, T cell receptors and MHC antigens, and a large number of accessory molecules (Sullivan and Kim, 2008). As in mammals, the T cells appear to be of either alpha/beta or gamma/delta type (Nam et al., 2003), and the T cell receptor is in a complex with CD3 molecules (Yun et al., 2008). However, after teleost fish made a separate branch on the phylogenetic tree, significant differences have been introduced between fish and mammals, both with respect to primary sequence of corresponding molecules and the introduction of new molecules by gene duplications. An example of this is the evolution of the immunoglobulins: Whereas IgM has been evolutionarily stable and maintained the primordial structure in all jawed vertebrates, the IgD gene has been evolutionarily labile and has been subjected to many internal duplications and deletions (Wilson et al., 1997; Hordvik et al., 1999; Stenvik et al., 2000; Ohta and Flajnik, 2006). The ancestral IgD gene probably arose very early in evolution, but it is likely that this molecule has a different function in fish and mammals. A newly discovered gene encoding an isotype named IgT/IgZ appears to be specific for teleost fish (Danilova et al., 2005; Hansen et al., 2005). On the other hand IgG, IgE, and IgA are isotypes present in mammals, but not in fish.

Immune cells

Leukocytes in fish comprise lymphocytes, thrombocytes, granulocytes, monocytes and macrophages. In contrast to mammals, thrombocytes and erythrocytes in teleost fish are nucleated. Thrombocytes and lymphocytes in teleost fish are not easily differentiated morphologically as both cell types are relatively small with little cytoplasm. Although thrombocytes function as part of the blood clotting system, additional roles have been described for this class of cells in fish, including phagocytosis. Whether thrombocytes are involved in antigen presentation is a matter of controversy (Köllner et al., 2004). Previously it was thought that phagocytosis was accomplished mainly by “professional” phagocytes, such as macrophages and monocytes. A recent report showed that B lymphocytes from fish have potent phagocytic and microbicidal abilities,

suggesting that fish lymphocytes have retained ancient features whereas the mammalian counterparts have lost this innate immune capacity and specialize in adaptive responses (Li et al., 2006). In fish, clusters of melanomacrophages are believed to form the teleost analogue to the germinal centres of mammalian lymph nodes, where specialized antigen-presenting dendritic cells interact with T cells, thereby initiating adaptive immune responses. It is assumed that melanogenesis is an important immune mechanism in fish (Haugarvoll et al., 2006). This feature seems to have been lost in mammals.

Early development and migration of immune cells

All immune cells and other blood cells derive from hematopoietic stem cells. The stem cells are self-renewing and give rise to progenitors that are committed to different blood cell lineages. Homologous genes of many transcription factors involved in mammalian hematopoiesis have been used to trace corresponding development in fish by *in situ* hybridization; e.g. Runx-1, Runx-3, hhex, cbfb, SCL, LMO-2, GATA-2, c-myb, Fli-1, Ikaros and GATA-1 (Meeker and Trede, 2008; and references therein). In some species, such as angelfish, initial hematopoiesis appears in yolk sac blood islands. In other species it appears in the intra-embryonic intermediate cell mass (ICM), as in zebrafish, or first in the yolk sac and later in the ICM, as in rainbow trout. Definitive hematopoiesis is established in the kidney, but an intermediate, larval site of hematopoiesis has been revealed in the tail of zebrafish. Early differentiation of zebrafish macrophages has been shown before the onset of blood circulation. These primitive macrophages can phagocytose and kill bacteria injected in the zebrafish embryos. In rainbow trout which have received intraperitoneal injection of carbon particles, phagocytic cells were mainly present in the integument, including the skin and gut and particularly the gills, 4 days post-hatching. As the fish ages (18 days to 8 months), the kidney and spleen become the major sites of phagocytic cells (Zapata et al., 2006; and references therein).

Organs involved in development and activation of immune cells

Some authors use the designation “kidney marrow” as the primary site for hematopoiesis in fish (versus the bone marrow in humans). The kidney is located in the body cavity in close contact with the osseous vertebral spine. An anterior segment contains predominantly hematopoietic tissue, and a middle and posterior segment are dominated by renal tissue but also contain hematopoietic tissue. Whereas the development of B cells occurs in the kidney, the mature T cells are generated in the thymus. The thymus is a paired organ in the dorsolateral region of the gill chamber, although the shape of the organ is not homogenous in all species. Involution of the thymus has been reported to be in correlation with sexual maturation, but further studies are required to reveal the generality of this finding (Bowden et al., 2005).

Other important organs in the immune system of fish are the spleen and mucosal tissues (gills, skin and gut). The spleen is usually located ventral and caudal to the stomach. In the spleen, kidney and liver, aggregations of pigmented cells are present – called melanomacrophage centers.

A lymphatic system sharing many morphological characteristics of the lymphatic vessels in other vertebrates is present in fish (Olson, 1996; Yaniv et al., 2006), but fish lack equivalents of the lymph nodes in mammals. However, lymphoid aggregates (consisting mainly of T cells) were recently detected in the gills of salmon (Haugarvoll et al., 2008).

Lymphocytes differentiate in lymphoid organs at different times relative to hatching. In freshwater fish the thymus is the first organ to become lymphoid, followed by the blood and head kidney and after some delay in the spleen. In marine fish species the order in which the major lymphoid organs develop is kidney, spleen and finally, thymus (Zapata et al., 2006).

Development of immune responsiveness

The specific antibody titres induced by immunization appear to increase with the age of the fish, and the duration of protection increases with age. Very early exposure to antigens can induce tolerance. Size might correlate better than age since development is influenced by parameters such as temperature (Zapata et al., 2006; and references therein). The onset of specific antibody production is dependant on the nature of the antigen (T cell dependent versus T cell independent).

The zebrafish model, molecular tools and future priorities

In recent years zebrafish has been established as the most important model on the development of the immune system in fish (Langenau and Zon, 2005; Meeker and Trede, 2008; and references therein), and undoubtedly this common reference will facilitate future studies on other species. The introduction of transparent zebrafish has offered biologists a new window into the development of the immune system in fish, with an exciting host of genetic tools. However, the research is seriously hampered by the lack of specific antibodies against cell markers. Tools to identify cells and molecules of the immune system have been an essential driving force in immunological research in humans.

Studies of leukocyte surface molecules have been organized through a series of international workshops known as the Human Leukocyte Differentiation Antigen (HLDA) Workshops. Characterized molecules CD1 to CD339 have been derived from these workshops. CD ("clusters of differentiation") antibodies are widely used for research, differential diagnosis, monitoring and treatment of disease. HLDA has now been succeeded by HCDM ("Human Cell Differentiation Molecules"), including studies of other cell types and intracellular molecules (Vidal-Laliena et al., 2005).

Since most cell markers diverge relatively rapidly, species-specific tools must be developed. In the characterization of cell markers and development of specific antibodies against them, commercial fish species have so far been ahead of zebrafish. Although this work requires time-consuming investigations, it will have a great impact on future research and will likely have spin-off effects as well, for example as tools to monitor immune cell responses during vaccination trials.

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10.13 Broodstock and larval nutrition

Kristin Hamre[1] and Elin Kjørsvik[2]. [1]NIFES, National Institute of Nutrition and Seafood Research, [2]Department of Biology, University of Trondheim

Introduction

Tissues under development need a balanced supply of nutrients, so that cells can proliferate and organs and tissues grow. Many nutrients also play important roles in cell signalling and regulation of the proliferation and differentiation processes of cells involved in ontogeny. It is therefore important to see development in the light of nutrition and to measure effects of nutrition on the biological processes, but up to now, developmental biology and nutrition have largely been two different fields of research with little overlap.

We know that the natural diet of marine fish larvae, which mainly consists of copepods, is extremely rich in most of the essential nutrients. *Artemia* and rotifers used in intensive culture contain much less of these components. Therefore, to avoid the study of ontogeny being done on fish larvae reared under sub-optimal conditions, the developmental baseline should be established using larvae fed on copepods. A possible strategy for aquaculture is to try to make the intensive feed organisms similar to copepods by enrichment. However, it is not certain that fish larvae have requirements which are equivalent to copepod nutrient levels and studies of requirements should be performed where this is possible. The present review will focus on the differences in nutritional composition between different larval feeds and possible effects on the fish larvae. An additional paragraph on broodstock nutrition is included because of possible relevance for the development of the oocyte and embryo.

Cod larvae on live feed

Cod larvae are 4-5 mm at first-feeding, so the feed particles must be very small for these larvae. In the wild, they prey mostly on copepod nauplii, while in intensive culture, cod larvae are fed rotifers until 20-30 days post hatch. Some hatcheries wean the larvae directly onto formulated feed, while others use a period where they feed *Artemia*, prior to weaning. Intensively cultured cod larvae grow at a much lower rate, (10 compared to 13->27% per day; McQueen Leifsson 2003; Otterlei et al., 1999) and develop more deformities (Imsland et al., 2006) than larvae cultured semi-extensively. Of the commercially produced cod in Norway in 2006, 30-80% had skeletal deformities (Lein et al., 2007). Intensive marine fish hatcheries worldwide have similar problems with deformities as we find for cod (Cahu et al., 2003). Sub-optimal nutrition in larvae fed rotifers is one possible reason for the inferior performance of intensively compared to semi-extensively reared larvae. The whole nutrient profile of both rotifers and copepods has recently been analysed (Srivastava et al., 2006; Hamre et al., 2008a; van der Meeren et al., 2008; Srivastava et al., unpublished). The protein contents and amino acid composition in several copepod species have also been published previously (Helland et al., 2003 a, b, c). There are numerous differences in nutrient composition between rotifers and copepods (see examples in Table 1), but many of the nutrients in rotifers can be manipulated by adjusting the rotifer culture and enrichment diets.

Table 1. Protein, taurine, phospholipid and mineral contents of rotifers grown on 4 different diets; Yeast and oil (YO), Yeast and Algamac 3000 (YA), Yeast and Chlorella (YC) and Culture Selco 3000 (CS), compared to copepod levels and the nutrient requirement in fish (NRC1993) (Srivastava et al., 2006; Hamre et al., 2008; van der Meeren et al., 2008).

	Rotifers				Copepods	NRC
	YO	YA	YC	CS		
Protein (%DW)	36.5	39.2	41.2	37.9	48-50	
Taurine (mg/g DW)	0.33	0.56	0.24	0.25	1.1-1.3	
Phospholipid (% total lipid)		40			57-58	
Phosphorus (µg/g DW)			9400±690		12400-15000	4500-6000
Iodine ”	5.8±3.8	3.2±3.4	7.9±2.3	3.0±0.5	50-350	0.6-1.1
Manganese ”	3.9±0.3	4.4±0.2	5.1±0.4	4.3±0.2	8-25	13
Copper ”	2.7±0.4	3.1±0.4	3.1±0.5	8.1±2.2	12-38	3-5
Zink ”	62±8	63±5	64±5	63±3	340-570	20-30
Selenium ”	0.087±0.015	0.077±0.012	0.080±0.017	0.093±0.015	3-5	0.25-0.3
Iron ”	88±17	84±14	114±23	57±7	85-371	30-150

The protein level in rotifers is lower than in copepods and at or slightly below the level required for maximal growth in cod postlarvae of 0.2 g startweight (Table 1; Hamre and Mangor-Jensen, unpublished). The aminosulfonic acid taurine is formed from methionine or cysteine via decarboxylation of cysteine sulphinic acid to hypotaurine with subsequent oxidation of the latter. Taurine has shown to be an essential nutrient for cats, and probably also for primates, especially during early development (Sturman 1993). Park et al. (2002) found that growth of juvenile Japanese flounder (*Paralichthys olivaceus*) increased with taurine present in the diet; they suggested that this flounder is unable to biosynthesise taurine from cystine. Taurine is not built into protein, but resides in the free amino acid pool and is used for osmoregulation and bile salt synthesis. Further, the ratio of polar to total lipid is lower in rotifers than in copepods (Table 1), but the ratio is dependent on the total lipid content in rotifers, since increase in total lipid is mainly an increase in neutral lipids in most animals. It is difficult to manipulate these nutrients in live feed, including rotifers, since lipid and protein are rapidly broken down in the gut of the prey organism. Both the protein and phospholipid levels as well as the amino acid composition of animals are genetically controlled. The rotifer will therefore build its own protein and the fatty acids from the phospholipids will be built into triacylglycerol, while free amino acids will stay in the free pool for a while and what is not built into protein will be catabolised. However, it is possible to almost stop the metabolism of rotifers by cooling them down to 6 °C and thereby keep at least som of these nutrients undigested in the gut.

Allthough numerous experiments have been performed that show that rotifers must be enriched with log chain n-3 fatty acids, the actual requirements for these fatty acids are not known for most marine fish larva species, including cod. A similar situation exists for vitamins, where it is assumed that rotifers should be enriched with vitamins C, E, A and B1 (Thiamine), but where the actual requirements are not known. The larval requirements for minerals have been given almost no attention, both in research and commercial culture. Table 1 shows that the minerals and trace element levels in rotifers, except calcium, magnesium and iron, are generally lower than in copepods. Magnesium and selenium are also lower in rotifers than the requirements of larger fish given by NRC (1993). In an experiment where rotifers were enriched with iodine and selenium up to copepod levels, there was a 32% increase in survival of the cod larvae (Hamre et al., 2008b). Furthermore, Ribeiro et al., (submitted) found goitre in Senegalese sole larvae reared on standard rotifers and *Artemia*, but not in those fed iodine-enriched live feed. These larvae were held in a recirculation unit where the bio available form, iodide, present in seawater, may have been oxidised to iodate. We do not know the actual requirements of minerals in marine fish larvae. It is assumed that fish larvae generally have higher requirements than larger fish, due to their high growth rates and the expenses connected with ontogeny and metamorphosis. It is also possible that fish larvae have adapted to their very nutritious natural feed. However, the nutrient levels in copepods vary and the actual requirements of the larvae may be lower than the levels found in copepods.

To measure the requirements by dose response experiments, it is necessary to be able to control nutrient levels in the rotifers. We have shown that there is a linear relationship between the concentrations of several vitamins and microminerals in the culture water and in the rotifers (Hamre et al., 2008b; Hamre et al., unpublished; Nordgreen et al, unpublished). We can also enrich rotifers with lipid spray beads with incorporated vitamins and minerals (Langdon et al, 2008). The total lipid level and the fatty acid composition can quite easily be manipulated in rotifers. It is therefore possible to run requirement studies with rotifers enriched with these nutrients.

Atlantic halibut larvae on live feed

Atlantic halibut larvae are larger than most other marine fish larvae at first-feeding, approximately 12 mm standard length, compared to 4-5 mm in for example cod (*Gadus morhua*). They are usually first-fed directly on *Artemia* and weaned onto a formulated diet 50-70 days post first-feeding. This means that the nutrient composition of *Artemia* is extremely important for larval development through metamorphosis. Our studies showed that Atlantic halibut larvae fed from first-feeding on *Artemia* may metamorphose into poor-quality juveniles compared to those fed copepods (Hamre et al. 2002). More than 90% of the larvae fed *Artemia* were malpigmented, whereas the number for copepod-fed larvae was 32%. Of the larvae fed *Artemia* and copepods, 90 and 12%, respectively, had impaired eye migration. This shows that the frequency of these two deformities at least in part depends on nutrition on the larval stage. Similar differences in development in response to diet have been found in other flatfish species (Seikai 1985). However, improvement of enrichment diets for *Artemia* has recently reduced malpigmentation in commercial culture of Atlantic halibut to a minimum and the thresholds of essential fatty acids for normal pigmentation of halibut have been

determined (Hamre and Harboe 2008). Furthermore, Harboe et al. (submitted) reared Atlantic halibut larvae to nearly complete eye migration using a photoperiod in contrast to constant light, which is common in halibut larvae culture.

Enriched *Artemia* has a lower protein content and higher lipid and glycogen contents than copepods (Hamre et al. 2002). The protein content of *Artemia* is lower than the assumed requirement of 580 mg g^{-1} dry diet, while carbohydrate is higher than the maximum tolerable level of 50 mg g^{-1}. This indicates that protein in *Artemia* may be too low while carbohydrate may be too high for Atlantic halibut larvae. The lipid level of *Artemia* varies with enrichment diet and method, but should be held between 50 and 250 mg g^{-1} (Hamre et al. 2003; Hamre et al. 2005a).

Of the water-soluble vitamins, only vitamin C and thiamine are lower in enriched *Artemia* than in copepods (Mæland et al. 2000), thus the other vitamins may be regarded as present in sufficient amounts. Merchie et al. (1997) have enriched rotifers and *Artemia* with vitamin C, using ascorbyl palmitate at 10 and 20% inclusion in the enrichment emulsion and obtaining 1-2 g kg^{-1} ascorbic acid in *Artemia.* When fed to shrimp and fish larvae, these feeds improved stress resistance. However, the authors' conclusion was that initial vitamin C levels of both rotifers and *Artemia* were sufficient to cover the larval vitamin C requirement. Thiamine is easily enriched in *Artemia*, and our studies indicated that increased growth of halibut larvae can be obtained by boosting *Artemia* with thiamine (Hamre et al., unpublished).

Of the lipid-soluble vitamins, vitamin A is absent in copepods and in *Artemia* not enriched with vitamin A (Moren et al. 2005). In addition, the use of pure oxygen in the enrichment tanks may lead to destruction of vitamin A supplemented to the enrichment emulsion, resulting in very low levels of vitamin A in *Artemia*. On the other hand, both *Artemia* and copepods contain ample amounts of canthaxanthin and astaxanthin, respectively. Both these carotenoids are converted to vitamin A in halibut juveniles (Moren et al. 2002) and appear to cover the requirement for vitamin A in Atlantic halibut larvae fed either *Artemia* or copepods (Moren 2004; Moren et al. 2004). Vitamin E is normally higher in *Artemia* than in copepods (Table 1), and can easily be enriched further by supplementation of the enrichment emulsion (Hamre et al. unpublished). Commercial emulsions are often very high in vitamin E, as it is used as an antioxidant due to the high levels of polyunsaturated fatty acids present in the product.

Except for iodine and zinc, the levels of minerals and trace elements in *Artemia* are generally similar to or higher than in copepods (unpublished results). Iodine is up to 700 times higher in copepods than in *Artemia* (Moren et al. 2006), allthough it is possible to enrich *Artemia*, either with iodine salt or a lipid-soluble iodine source, such as Lipiodol Ultra® (Moren et al. 2006). Zn is approximately two times higher in copepods than in *Artemia*.

It is difficult to get a good fatty acid profile in *Artemia* when enriching with oil emulsions, and the data in the literature give a variation in DHA (22:6n-3) content of 3-10% of total lipid or fatty acids (Estevez & Kanazawa 1995; Næss et al. 1995; Tocher et al. 1997; McEvoy et al. 1998; Shields et al. 1999; Hamre et al. 2002). An exception is Evjemo and Olsen (1997), who found 15-20% DHA in *Artemia* enriched with Super Selco™ and DHA-Selco™. Many strains of *Artemia* contain EPA (20:5n-3), and the catabolism of EPA in *Artemia* is lower than that of DHA (Evjemo et al. 2001). DHA and EPA are typically high in copepods, 20-40% and 15-20% of fatty acids, respectively (van der Meeren et al., 2008). ARA (20:4n-6) is found in un-enriched *Artemia* nauplii and can be quite high in enriched *Artemia* (>2% of fatty acids), even though the enrichment medium contains little ARA (Hamre et al., 2002). ARA is low in copepods (<1% of fatty acids, van der Meeren et al., 2008).

Thus, the fatty acid composition of *Artemia* enriched with lipid emulsions is generally unfavourable, with too low levels of DHA and EPA and too high levels of ARA. The levels of thiamine, zinc and iodine may be too low in *Artemia* and the macronutrient composition may be slightly imbalanced. Otherwise, *Artemia* seems to be a good source of nutrients for Atlantic halibut larvae.

Formulated diets for marine fish larvae

Marine fish larvae fed formulated diets before development of the stomach generally show lower growth and survival than larvae fed rotifers and *Artemia*. This is also the case with cod. MacQueen Leifson (2003) found growth rates of 10.7 and 7.2% per day, respectively, in cod larvae fed *Artemia* or a formulated feed from 24 to 36 dph. In another experiment, Callan et al. (2003) used a similar feeding regime from 22 to 64 dph and obtained growth rates of 7.1 and 5.1% per day, respectively.

One of the problems with formulated larval feeds is the high leaching rates. Measurements of leaching from two commercial and two experimental larval feeds showed that 18-42% of the protein leached from the feed within two minutes of hydration (Hamre 2006). This probably corresponds to the fraction of water-soluble protein in the feeds. Free

amino acids leach from formulated larval feeds at a higher rate than protein, as can be expected from their lower molecular weight. More than 50% of radio-labelled amino acids leached from a micro-bound diet after 1 min of hydration, and after 5 min less than 10% was left in the feed (Hamre 2006). Similar leaching rates have been found for water-soluble vitamins and minerals (Nordgreen et al., in press). Most commercial larval feeds are micro-bound, and similar leaching rates as those shown here may be expected.

Another problem is the form in which the nutrients are given. Formulated diets mainly contain intact protein and a large fraction is insoluble in water and therefore difficult to access for the larval digestive enzymes, when no acidification and pepsin activity is present in the stomachless larvae. In live feed, on the other hand, a large fraction of the protein has a low molecular weight and is water soluble (Carvahlo et al., 2003) and more digestible (Tonheim et al., 2007). A similar condition may be present with regard to lipid digestion in Atlantic halibut, since these larvae absorbed pre-digested fat at a much higher rate than intact fat (Molland et al., 2008). A solution to this problem is to supplement formulated larval diets with hydrolysed protein, to aid the larval digestion. Experiments attempting to find optimal levels of hydrolysed protein in diets for different larval species have yielded conflicting results (Day et al., 1997; Cahu et al., 1999; Kolkovski and Tandler 2000; Carvahlo et al., 2004; Kvåle et al., in press). A possible explanation for this is that protein hydrolysis makes the protein more water soluble and may therefore introduce a new leaching problem, resulting in reduced levels of protein in the feed that the larvae actually eat. Apparently, no studies have been done on feeding hydrolysed lipid to marine fish larvae.

Formulated diets for marine fish larvae should contain phospholipids. Larvae fed TAG rich diets generally show an accumulation of lipid vacuoles (steatosis) in the basal zone of the enterocytes, which indicates a reduced intestinal transport capacity of TAG (Diaz et al., 1997; Fontagné et al., 1998, 2000; Salhi et al., 1999; Izquierdo et al., 2000; Morais et al., 2005; 2006; Segner et al., 1993). The accumulation of lipid droplets may be explained by limitations in lipoprotein synthesis due to deficiency of phospholipids for the lipoprotein surface, since supplementation of formulated diets with phospholipids alleviates this problem (Fontagné et al., 1998). Iritani et al. (1984) reported that the α-glycerophosphate acyltransferase activity of fish is extremely low compared to other animals and it is now well established that marine fish, particularly in the larval stages, have a limited capacity for endogenous *de novo* PL biosynthesis (Tocher et al., 2008). However, the causes and consequences of lipid accumulations in the enterocytes of fish larvae and the mechanisms involved are still not fully understood.

Broodstock

Experiments with broodstock nutrition in cod have been performed on dietary carbohydrate exchanged with protein (Hemre et al., 1995), vitamin C (Mangor-Jensen et al., 1994) and the effect of dietary fatty acids on gonad fatty acid composition (Lie et al., 1991). The fatty acid composition of gonads was affected by the fatty acid composition of the diet (Lie et al., 1991), especially for arachidonic acid (ARA, 20:4n-6) that was greatly enriched in the PI fraction. On the other hand, an increase in dietary DHA (22:6n-3) and EPA (20:5n-3) yielded an increase in DHA and EPA in neutral lipids, but less so in phospholipids of the eggs. Experiments on the fatty acid composition of marine broodstock diets have focussed mainly on the requirement for n-3 highly unsaturated fatty acids (HUFA). HUFA are essential for marine broodstock, as deficiency in these fatty acids produces abnormal eggs of low hatchability and high larval deformities (Watanabe and Vassallo-Agius, 2003; Izquierdo et al 2001). Tuna orbital oil is high in all of DHA, EPA and ARA, and improves reproductive performance in European sea bass (*Dicentrarchus labrax*) broodstock, compared to a diet containing northern hemisphere oil, which has low levels of ARA (Bruce et al., 1999). In contrast, Furuita et al. (2002) found reduced egg and larval quality in eggs from Japanese flounder fed high levels of n-3 HUFA. Dietary ARA decreased as n-3 HUFA increased, and it is possible that the result was a consequence of an increased EPA:ARA ratio or decreased ARA. Fernandes-Palacios et al. (1995) found no effect of increasing dietary n-3 HUFA on egg quality of gilthead sea bream, but the larvae showed yolk sac hypertrophy and increased mortality. Supplementing extra vitamin E alleviated larval mortality and improved egg quality (Fernandes-Palacios et al. 1998).

There are also some recent and interesting reports on the need for ARA. ARA levels are higher in eggs and sperm from wild fish than from farmed fish, and in broodstock fed Mediterranean trash fish compared to those fed diets with northern hemisphere oils (Bell and Sargent 2003; Cejas et al, 2003). Supplemental ARA increases fecundity, egg quality and normality of larvae in Japanese flounder and Atlantic halibut (Furuita et al., 2003; Mazorra et al., 2003) and fish fed ARA-supplemented diets showed increased plasma levels of eicosanoids (Bell and Sargent 2003). EPA and ARA compete for the enzymatic pathways involved in eicosanoid syntheses, resulting in different eicosanoids with different activities. In mammals, the ARA derived

prostaglandin PGE2 stimulates ovarian synthesis of oestradiol-17b (E2; Abayasekara and Wathes 1999). E2 is the major female sex steroid in teleosts, and stimulates early stages in oogenesis as well as vitellogenin synthesis. It is also well known that eicosanoids are involved in regulation of ovulation, in both fish and mammals, where rupture of the follicle is stimulated by the prostaglandine PGF (Goetz and Garczynski 1997, Abayasekara and Wathes 1999). Apparently, progesterone, which is surged just before ovulation, stimulates eicosanoid synthesis, which in turn activates the mechanisms involved in ovulation (Goetz and Garczynski 1997). The dietary ratio of EPA/ARA will be mirrored both in the broodstock and in the eggs and sperm and may therefore affect the broodfish during maturation as well as ovulation, fertilisation and the development of the embryo (Izquierdo et al., 2001; Mazorra et al., 2003). The optimum supplementation of ARA is probably species-dependent (Bell and Sargent 2003). In an experiment with Atlantic cod, dietary ARA was varied between 0.5 and 4.0% of dietary fatty acids; there was increased fecundity at 1 and 2%, compared to 0.5 and 4% dietary ARA and the profiles of vitellogenin and steroid hormones through the year were influenced by ARA. No effects on fertilisation, survival or hatching of eggs were detected. The fish with the highest fecundity also had the highest mortality due to accumulation of eggs in the lumen of the gonad (Norberg et al., unpublished).

There are few studies on optimisation of the macronutrient composition of broodstock diets, but a screening of cod females from three different Norwegian hatcheries indicated that protein was the limiting macronutrient for building of gonads in these fish, while the lipid reserves in the liver were more than sufficient to supply the eggs with lipid (Hamre et al., unpublished). Furthermore, Bogevik (2003) showed that fecundity is dependent on feed intake in Atlantic halibut broodstock.

Eggs from Atlantic cod and Atlantic halibut fed trash fish and grow-out diets have lower levels of several vitamins than eggs from wild fish (Hamre et al., unpublished). This is especially the case for thiamine in broodstock fed trash fish, which is degraded by thiaminase, present in most fish species. In fish meal, on the other hand, thiaminase is inactive due to heating of the meal. Over time, broodstock fed raw fish may drain off their vitamin stores, and reproductive exhaustion may occur. The requirements for most vitamins in fish broodstock are not known, but it is assumed that they are higher than in the grow-out phase. At present, increased requirements for vitamins C and E have been established and implemented in commercial broodstock diets. In a project on cod, the whole nutrient profile in female cod broodstock from three different hatcheries in Norway was analysed and compared to three wild fish populations. The captive fish were fed specially designed broodstock diets. The results showed similar or higher levels of vitamins in captive fish compared to wild fish. Using the wild fish as a reference, most of the minerals also appeared sufficient in the captive cod (Hamre et al., unpublished).

Wild cod eggs contain approximately 7 mg/kg carotenoids (dry wt.), where 76% is astaxanthin. Farmed cod incorporate dietary astaxanthin and cantaxanthin into the eggs at similar levels as wild cod. Both are partly metabolised to other carotenoids (Grung et al., 1993). Watanabe et al. (1991) showed that red sea bream fed frozen krill or cantaxanthin have improved reproductive performance, and attributed this to the carotenoid supplementation. Further, astaxanthin and paprika powder supplementation improved both fecundity and hatching rates in yellowtail (Verakunpiriya et al. 1997; Vassallo-Agius 2002). Carotenoid could function as an antioxidant, quenching free radicals both in broodfish and eggs. Alternatively it works as a vitamin A source. Vitamin A is essential for embryonic development, where either deficiency or excess leads to malformations in the embryo. The optimal concentration of carotenoids and vitamin A in broodstock diets is not known, and different diets may contain different levels of these nutrients. Too low levels of astaxanthin may lead to drain-off of this nutrient in the broodstock. Vitamin A is often present in high amounts in the feed ingredients, so excess rather that deficiency of this nutrient may be expected.

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Knowledge about early life stages in fish is critical for a number of high-priority areas in aquaculture research, both in Norway and abroad. Experience has shown that major bottlenecks occur in the early life stages, slowing efforts to commercialise new production species. Regarding wild stocks, basic knowledge about early life stages may provide insight into how the fish adapt to their environment and survive under various conditions.

The Research Council of Norway
P.O.Box 2700 St. Hanshaugen
N–0131 OSLO

Telephone: +47 22 03 70 00
Telefax: +47 22 03 70 01
post@rcn.no
www.rcn.no

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