SUCCESS FACTORS FOR FISH LARVAL PRODUCTION

EDITED BY
LUÍS E.C. CONCEIÇÃO AND AMOS TANDLER

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1

Introduction

As fish are efficient protein producers, in fact the most efficient farmed animal, aquaculture has been recognized as a key activity in terms of food security worldwide. Europe imports a substantial fraction of its fish consumption. Currently, the European aquaculture industry produces about 2.3 million tonnes of finfish per annum (FAO 2016), equal to one-third of the EU fishery market value, while representing only 20% of its volume! The Food and Agriculture Organization (FAO 2016) estimates that in order to achieve the per capita contribution of fisheries to the 2030 per capita consumption, the yearly global aquaculture production needs to grow by 27 million tonnes.

In order to meet the challenge of a steadily growing global aquaculture sector, there is a need to assure a steady supply of high numbers of high-quality fish larvae. Furthermore, in terms of future feed conversion efficiency, reduced malformation rates and the efficient conversion of feed to high-quality fish, quality fingerlings are of paramount importance for environmentally and economically sustainable aquaculture growth. However, aquaculture currently suffers from poor-quality fingerlings in terms of their future efficiency in converting food to fish meat, which affects aquaculture economics and its impact on the environment. Despite considerable progress in European aquaculture in the past 20 years, for example with production of over 1 billion seabass and seabream fry in 2012, high mortality during larval production and variable fry quality still plague the industry. This is exacerbated by an increasing need for diversification into new species, where these problems are even more acute. Therefore, there is still a significant amount of research to do to make the industry more cost-effective and sustainable.

The lack of a predictable supply of high-quality fish juveniles is largely attributed to uncontrolled environmental and nutritional factors during the larval rearing phase as well as the lack of tools for early prediction of larval quality in terms of phenotype and performance. There is thus a clear need for improvement of the scientific knowledge base that will support sustainable development of aquaculture. In addition, the well-documented environmental impact of factors such as climate change on fish production will place even greater demands on the application of an integrated multidisciplinary approach to improve larval performance and juvenile quality in the European aquaculture industry. This refers essentially to all non-salmonid fish species, as salmon and trout do not have a true larval stage, and most of the problems described for these species throughout this book are already solved or have a lower impact.

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Maximizing fish production requires in-depth knowledge of biological, ecological and abiotic mechanisms, which affect the developing organism prior to reaching the grow-out farms. This is further exacerbated by the fact that the aquaculture industry is based on a multitude of species. So for instance, first feeding diets given to larvae have been identified as a determining factor for the quality of the juvenile phenotype in a number of species. This stems from the fact that various nutrients act on gene regulation of major physiological functions and thus should be an important feature of stage- and species-specific diet formulation but this has been largely ignored so far. While waterborne components such as endocrine disruptors have been well investigated for their effects on fish reproduction, there is almost no research on their effects on the larval to juvenile transition, despite the well-documented important role of hormones, and the endocrine system in general, in this process. The integration of molecular, nutritional and morphophysiological results is of paramount importance, as the influences on juvenile fish quality are multifactorial. Epigenetic research, for example how early environmental and nutritional impact can affect the phenotype later in life and even in the next generation(s), is relatively 'new' within research on farmed animals, including fish, although basic research in this area has been ongoing for several decades. The new tools which become available within this field will probably revolutionize the possibilities for juvenile quality prediction. Thus, in order to achieve a quality and sustainable aquaculture in Europe, there is a clear need for investment in fish larval research, to improve its scientific knowledge basis.

In order to tackle the aforementioned challenges, LARVANET, a network of researchers and producers working with fish larvae, was started in 2008. LARVANET was supported by a COST Action (FA0801). As a forum for constructive dialogue between stakeholders and researchers, LARVANET aimed to directly co-ordinate and build the know-how necessary to promote sustainable development and competitiveness at a basic level, and contribute to the cost-effective production of quality juveniles. It intended to integrate knowledge obtained in national and European research projects, and practical experience, in order to look for knowledge gaps on the way to improve quality of fish larvae used in aquaculture. It facilitated international co-operation, exchange of scientists and students, and efficient use of resources at all levels, and intended to exercise a lobby to influence long-term policy in the area of edible species larval research as a means to dramatically influence the resulting EU aquaculture efficiency, product quality and environmental and societal impact.

Reference

FAO (2016) *The State of World Fisheries and Aquaculture 2016. Contributing to Food Security and Nutrition for All*. Food and Agriculture Organization, Rome.

2

Gamete Quality and Broodstock Management in Temperate Fish

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Executive Summary

Background

The ability to fully control sexual maturation and spawning and produce large numbers of high-quality seeds 'on demand' (i.e. all year long) is a primary requirement for the successful development of aquaculture. This relies on optimal broodstock management practices based on extensive knowledge of the nutritional and environmental requirements of fish in captivity. However, for many established, emerging and new farmed fish species, such knowledge is limited or not available yet. The level of domestication also plays an essential role as stocks with improved traits in farming conditions are selected. Importantly, reliable indicators of egg quality are still lacking as in many farmed fish species hatcheries still rely on wild harvested broodstocks. These key challenges must be addressed urgently to ensure the sustainable development of the European fish farming sector.

Principal Findings

The growth of the aquaculture industry depends to a large extent on the ability of hatcheries to supply good-quality eggs with selected traits, as required by the grow-out farmers. However, this remains problematic in many species, especially emerging new species selected for domestication for the diversification of the aquaculture industry. These often suffer from high variability in egg quality among stocks and parents. Therefore, more basic and applied research is required on all aspects of broodstock management including, *inter alia*, nutrition, environmental effects, genetics, gamete quality and preservation. This includes the definition of optimal egg quality at the genomic, proteomic and physiological levels in fish and the translation of this basic knowledge into a set of robust, reliable markers/analytical tools that can provide early confirmation of quality parameters for commercial hatcheries. A better understanding

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of the process of postovulatory ageing in fish broodstock is also required. The nutritional requirements of fish broodstock for optimal gametogenesis and egg/larvae quality and development (such as reduced deformity, etc.) must be defined, and sustainable, species-specific feed formulations developed.

The development of domestication/selective breeding programmes for emerging and new aquaculture species is critical to select the best strains, stocks and families for a range of traits of interest. Knowledge-based breeding programmes should be developed to minimize the effects of inbreeding on fertility, fecundity and egg/larvae quality traits (survival, growth, malformation). Research should also focus on gaining a better understanding of the environmental conditions that promote spontaneous, out-of-season spawning and good egg quality in established and new candidate species. Finally, the roles of maternally transferred mRNA, proteins and any other biomolecules on egg and larvae quality/performance should be studied and how broodstock conditioning/management can influence such epigenetic processes.

Scientific Significance

This review gives an overview of methods to assess egg/sperm quality and many of the most important factors impacting on gamete production and quality, including broodstock nutrition, environmental and spawning induction protocols, and genetic factors for broodstock management, gamete preservation and new reproductive strategies. From this review, a list of key gaps in knowledge has been identified as critical for a sustainable growth of the European fish aquaculture sector.

Practical Application

Challenges associated with the supply of seeds are amongst the most important constraints on the development of aquaculture. Scientific knowledge on optimal conditions for captive fish spawning and a set of parameters/methods that define gamete quality will be essential for the scaling up of many commercially important aquaculture species. Egg quality biomarkers could serve as predictors of fish quality to avoid occupying hatchery facilities with what may turn out to be unproductive batches of eggs.

Introduction

Aquaculture production has continued to grow at an ever-increasing rate from <1 million tonnes in the 1950s to 55 million tonnes in 2009 increasing at three times the rate of world meat production (2.7% from poultry and livestock together) with an average annual growth rate of 8.3% worldwide (FAO 2010). Much of this increase has occurred since the mid 1980s with the vast majority of the production being from Asia and the Pacific rim, particularly China. Farmed and managed seafood now accounts for 50% of global consumption. It is estimated that in order to maintain the current level of *per capita* consumption, global aquaculture production will need to reach 80 million tonnes by 2050. The main species farmed in Europe for human consumption are salmonids (Atlantic salmon, *Salmo salar* and rainbow trout, *Oncorhynchus mykiss*), bass and bream (mainly sea bass, *Dicentrarchus labrax* and sea bream, *Sparus auratus*),

flatfish (mainly turbot, *Scophthalmus maximus* and halibut, *Hippoglossus hippoglossus*), Atlantic cod (*Gadus morhua*), carp (common carp, *Cyprinus carpio*, grass carp, *Ctenopharyngodon idella* and silver carp, *Hypophthalmichthys molitrix*), and emerging species such as sole (*Solea senegalensis* and *S. solea*), meagre (*Argyrosomus regius*), amberjack (*Seriola dumerili*) and percids (mainly Eurasian perch, *Perca fluviatilis* and pikeperch, *Sander lucioperca*).

Difficulties in the supply of seed are amongst the most important constraints to the development of aquaculture. For many farmed species, production is totally dependent on the harvest of broodstocks or seeds from wild populations. Therefore, the ability fully to control sexual maturation and spawning and to produce high quality seed is a primary requirement for a successful aquaculture production. Egg quality, defined as those characteristics of the eggs that determine its capacity to survive, is a significant problem for many of the species currently being farmed and is almost certain to be a problem for the culture of any new species. In general for many marine species, e.g. bass, bream, turbot and halibut, the mortality rate for eggs is very high with survival of larvae post-weaning often being <5–10%. Only the salmonids exhibit better egg and larval quality with survival being >50%. Little is still known about the determinants of egg quality, although many factors have been implicated as possible causative agents including broodstock nutrition, genetics, environmental conditions and any stress factors such as handling and spawning induction. Crucially, there is little agreement regarding reliable methods for the assessment of quality, an essential prerequisite if any firm conclusions regarding the factors that determine egg and larval quality are to be reached.

The aim of this article is to review the state of knowledge on methods to assess egg/sperm quality and broodstock management of key commercially important temperate fish species in Europe, focusing on the nutritional, genetic and environmental factors. The subsequent goal is to identify gaps in knowledge and research needs for the sustainable development of a growing fish farming industry.

Egg and Sperm Quality and Assessment

The control of gamete quality is a major issue for the aquaculture industry. This is especially true in the context of global environmental changes and the current increase in the number of aquaculture species (Chevassus-au-Louis & Lazard 2009) for which the success of reproduction can be a major issue.

The quality of a gamete can be defined as its ability to fertilize or to be fertilized, and subsequently develop into a normal embryo (Bobe & Labbe 2010). The identification of predictive estimators or markers of gamete quality would have major applications in research and industry. However, to date, it seems clear that no effective predictive marker of gamete quality exists even though non-viable gametes can sometimes be identified in some species, through the assessment of simple parameters such as buoyancy, appearance, or motility (Bobe & Labbe 2010). Thus, apart from markers of extremely low quality, it is still very difficult accurately to assess the quality of the gametes prior to fertilization. In contrast, a thorough analysis of developmental defects/failure or success can be extremely valuable for deciphering the cause of poor gamete quality. Given the increasing number of species that will be raised for aquaculture, the current challenge

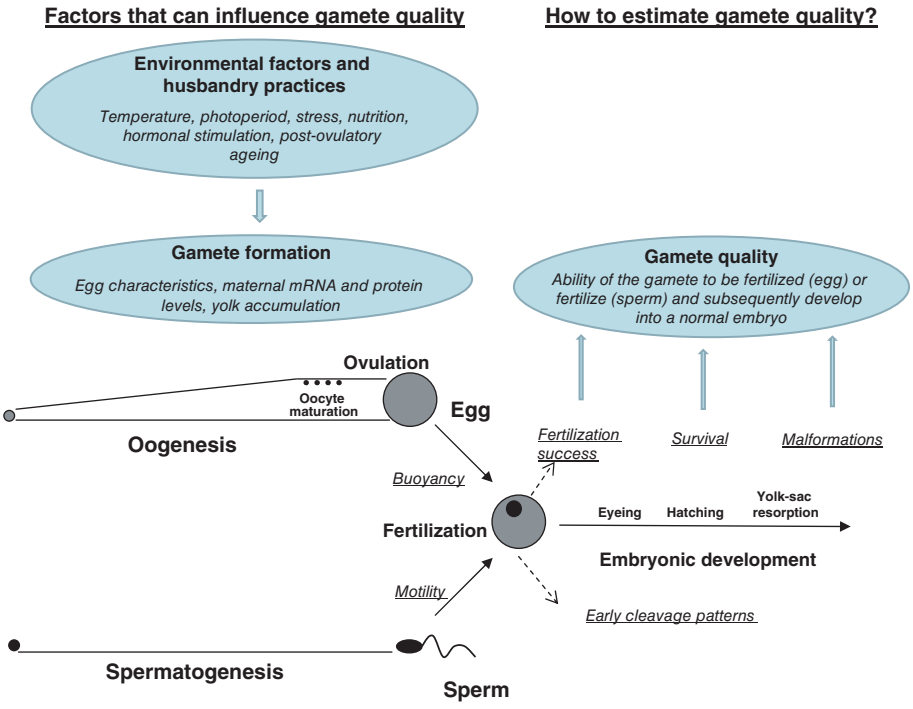

Figure 2.1 Main factors that can influence gamete quality in fish and main parameters that can be recorded to fully characterize gamete quality.

is to understand how environmental factors and rearing practices can impact gamete quality. Similarly, a better understanding of the mechanisms of gamete production during gametogenesis will be of great interest so as able to control, *in fine*, the quality of the gametes produced. Here we summarize the parameters that can be used to estimate or describe gamete quality and gamete characteristics and review new advances made in commercially important species (Fig. 2.1).

Egg Quality

As indicated above, fish egg quality, also known as oocyte developmental competence, can be defined as the ability of the egg to be fertilized and subsequently to develop into a normal embryo.

Prior to fertilization, it is extremely difficult to predict the success of development. As documented previously, the size of the egg is not always linked to its quality and eggs of varying size can exhibit similar developmental competence, as shown in trout (Bromage *et al.* 1992) and sea bass (Cerda *et al.* 1994a,b). Similarly, it is not possible to use morphological or macroscopic parameters to predict subsequent developmental success. Some parameters, such as sinking eggs in marine fish or white eggs in salmonids, can be used to identify non-viable eggs. The use of lipid distribution, that has been proposed to eliminate non-viable eggs in salmonids, is limited under normal hatchery conditions and the lack of a consistent relationship between the distribution of lipid droplets and egg quality has been stressed by other investigators (Ciereszko *et al.*

2009). A correlation exists between buoyancy and development such that buoyancy of pelagic eggs is often better in egg batches that develop normally as shown in the red sea bream (*Pagrus major*; Sakai *et al.* 1985) and other species (Kjörsvik *et al.* 1990), even though this does not hold true for all species (Brooks *et al.* 1997).

In species that produce transparent eggs, the shape of the first embryonic cells (blastomeres) and the patterns of cell division can be assessed to identify abnormal development during early embryogenesis (Shields *et al.* 1997; Kjørsvik *et al.* 2003; Avery & Brown 2005). This was, however, recently challenged by a study demonstrating that an abnormal cleavage pattern does not necessarily result in embryonic failure (Avery *et al.* 2009). In favour of this second hypothesis would be the 'checkpoint' set up by the developing embryo at the time of zygotic genome activation at mid-blastula stage (Kane *et al.* 1992; Kane & Kimmel 1993).

Survival at a specific embryonic stage is one of the most common and relevant ways of characterizing the ability of the fertilized egg to develop successfully. Survival can thus be assessed at specific stages such as the eyed stage, hatching and yolk sac resorption stage, which can be monitored in most fish species. It is also noteworthy that monitoring survival at successive developmental stages can be extremely valuable for characterizing the timing of embryonic mortalities that can significantly differ between experimental treatments or rearing conditions (Kopeika *et al.* 2003; Bonnet *et al.* 2007a). Similarly, monitoring embryonic and/or larval malformation can be useful for characterizing the developmental competence of the egg and to decipher potential causes of developmental failure. In rainbow trout, some malformations are specifically induced by environmental factors or husbandry practices while other malformations are female dependent and can be observed regardless of the life-history of the female broodstock (Bonnet *et al.* 2007a,b).

In the past few years, significant research efforts have been devoted to the study of the molecular mechanisms that are responsible for good or bad egg quality. Several types of genomic approaches such as transcriptomics (Aegerter *et al.* 2005; Bonnet *et al.* 2007b) and proteomics (Crespel *et al.* 2008; Ziv *et al.* 2008) have been used to decipher the mechanisms involved in oocyte developmental competence acquisition during oogenesis. Even though these studies have been successful in pointing out the specific molecular pathways possibly involved in the control of egg quality, the molecular picture of the good quality oocyte remains fuzzy. Further analyses, using for instance next-generation sequencing, are required to better understand what makes a good egg. The transcriptomic analyses carried out using eggs of varying quality strongly suggest that the maternal mRNAs provided to the embryo to support early development are important for obtaining good quality eggs. The influence of environmental factors on egg quality can also be mediated through epigenetic changes, such as DNA methylations, in male and female gametes. While the link between epigenetics and gamete quality has received no, or little, attention in fish, several recent studies have, however, revealed very specific methylation patterns of several gene promoters, including striking differences between male and female gametes (Marandel *et al.* 2012a,b,c).

Sperm Quality

Sperm quality can be defined as its ability successfully to fertilize an egg and subsequently allow the development of a normal embryo. In addition to the evaluation of embryonic success detailed above it is also relevant to estimate sperm quality by the

analysis of several sperm characteristics as reviewed by Cabrita *et al.* (2008). Sperm quality can be assessed by its constituents: seminal plasma and spermatozoa. Standard analysis may include parameters such as spermatozoa concentration, motility, sperm volume, seminal plasma osmolarity and pH. Basic studies on seminal plasma constituents and its variation such as enzymes (lytic, oxidative, metabolic), metabolites, sugars, vitamins, amino acids, fatty acids and other inorganic compounds can provide very useful information on sperm status (Rurangwa *et al.* 2004; Cabrita *et al.* 2008). These analyses allow identification of the loss of specific compounds as well as alterations in cell integrity and metabolism. Sperm cell function has also been the focus of attention as a marker for sperm quality. Motility has been the most used parameter through subjective evaluation methods. Nowadays, spermatozoa motility can be well characterized in terms of velocity and motility patterns using computer assisted sperm analysis (CASA; Kime *et al.* 2001; Cabrita *et al.* 2008). Data produced by CASA systems can be individually analysed showing the existence of sperm subpopulations in terms of motility characteristics, which inform more precisely about the quality of a given sample than the average values of motile parameters (Martinez-Pastor *et al.* 2008; Beirão *et al.* 2011a).

Other cellular characteristics should also be evaluated in order to assess the fertilization ability of milt. Most assays describing cell viability or mitochondrial status are currently performed with the use of fluorescent probes combined with microscopy or flow cytometry. The evaluation of antioxidant status through the determination of ROS (reactive oxygen species) levels or by TBARS has been applied recently to fish sperm (Martínez-Páramo *et al.* 2012). Oxidative events taking place during sperm ageing promote changes in membrane fluidity, protein damage, mitochondria impairment, DNA fragmentation and consequently, a decrease in spermatozoa functions (Sanocka & Kurpisz 2004). Sperm DNA damage assessment is one of the most recent focuses, linked with sperm fitness and offspring quality. Methods to evaluate chromatin integrity include the comet assay (single cell gel electrophoresis), TUNEL (terminal deoxynucleotidyl transferase-nick-end-labelling), SCSA (sperm chromatin structure assay) and the analysis of specific DNA sequences using qPCR (Zilli *et al.* 2003; Cabrita *et al.* 2005, 2011; Pérez-Cerezales *et al.* 2010a, 2011). All these techniques, although very useful in the evaluation of sperm quality and offspring viability, are still on a laboratory scale and need to be adapted for industry. Table 2.1 shows current applications of sperm analysis in most common commercial species.

The choice and combination of sperm quality parameters depend on the objective of the evaluation, as well as on the available equipment and expertise. Quality assessment should be relatively simple for routine analysis in fish farms. It is also important to consider that sperm is not a homogeneous mixture of cells and plasma, but a pool of cells with different genotype, maturation stage and characteristics, and assessment would therefore benefit from the analysis of spermatozoa subpopulations (Martinez-Pastor *et al.* 2008; Beirão *et al.* 2011a,b).

Germ Cell Preservation

Eggs

Gamete storage at low temperature could be a valuable tool in aquaculture. Unfortunately, in fish, it is currently not possible successfully to freeze/thaw mature eggs due to

Table 2.1 Application of sperm analysis in most common commercial species.

Species	Analyses	Application	References
Brown trout (*Salmo trutta*)	Sperm motility, cell viability, hatching rate, progeny analysis (microsatellites)	Cryopreservation for research and gene banking	Martínez-Páramo *et al.* (2009)
Rainbow trout (*Oncorhynchus mykiss*)	Sperm motility, cell viability, DNA integrity, hatching rate, progeny analysis (gene expression)	Cryopreservation for research and gene banking	Labbé *et al.* (2001); Pérez-Cerezales *et al.* (2009, 2010a,b, 2011)
Atlantic salmon (*Salmo salar*)	Sperm motility, hatching rate, progeny performance	Cryopreservation for research	Dziewulska *et al.* (2011)
Common carp (*Cyprinus carpio*)	Sperm motility, hatching rate, larval malformations	Cryopreservation for production	Horváth *et al.* (2007)
Gilthead seabream (*Sparus aurata*)	Sperm motility, DNA integrity, hatching rate	Cryopreservation for research and production	Cabrita *et al.* (2005)
European sea bass (*Dicentrarchus labrax*)	Sperm motility and viability, DNA integrity, lipid and protein oxidation, antioxidant enzymatic activity, membrane lipids composition	Cryopreservation for research; male quality	Zilli *et al.* (2003); Martínez-Páramo *et al.* (2012)
Senegalese sole (*Solea senegalensis*)	Sperm motility and concentration, cell viability and functionality, DNA integrity	Broodstock improvement	Beirão *et al.* (2011a,b)
Turbot (*Scophthalmus maximus*)	Motility analysis, hatching rate	Cryopreservation for research and production	Suquet *et al.* (2008)

their high vitellogenic content. It is, however, possible to store unfertilized fish eggs at low temperature prior to fertilization (Bobe & Labbe 2008). The maximum storage time is dependent on the species as the post-ovulatory decrease of egg quality occurs in a species-dependent manner. For some species, egg storage can be carried out in coelomic (also known as ovarian fluid) or for short period of times in artificial medium. The use of chilled storage is possible at least in cold-water species. In that case, chilled storage will improve storage timing in comparison with ambient or 'normal' water temperature (e.g. 10–12°C for salmonids). For warm-water species, chilled egg storage is an issue as they normally reproduce in a totally different range of temperatures. For those species, optimal storage temperature, time and conditions (e.g. oxygenation) have to be investigated specifically in each species. Finally several practical details such as holding conditions, fertilization procedures, collection time and transport conditions could also play a significant role in the overall success of the storage procedure.

Sperm Storage and Management

Sperm cryopreservation protocols have been developed for a range of farmed species and optimized for different purposes (Cabrita *et al.* 2008), from gene banking of interesting species or strains to routine use in species requiring artificial fertilization. However,

implementation of this technology within the industry has only been made in a few species (e.g. salmon, turbot).

It is consensus that only good quality sperm can be used for cryopreservation because susceptibility of sperm to cryodamage increases in samples of suboptimal prefreezing quality. In general, most of the sperm quality indicators are reduced after cryopreservation as reviewed by different authors (Rurangwa *et al.* 2004; Cabrita *et al.* 2008; Bobe & Labbe 2010) and DNA integrity, on which we will focus, is not an exception. Several reports described DNA fragmentation and nucleotides oxidation in rainbow trout, gilthead seabream and European sea bass post-thaw sperm (Zilli *et al.* 2003; Cabrita *et al.* 2005, 2011; Pérez-Cerezales *et al.* 2009, 2010a). The degree of DNA fragmentation is different according to the species, going from high levels in rainbow trout (Pérez-Cerezales *et al.* 2010a) to non-significant effects in gilthead seabream (Cabrita *et al.* 2005). Sperm selection mechanisms before fertilization are quite simple in fish, and cells with at least 10% of fragmented DNA are able to fertilize the oocytes in rainbow trout (Pérez-Cerezales *et al.* 2010b). Pérez-Cerezales *et al.* (2010b) demonstrated the capacity of the zygote to repair low levels of DNA damage, as well as a significant increase in development failure after fertilization with sperm carrying high levels of damaged DNA. No effects on the progeny are expected when DNA integrity is preserved, but the use of DNA damaged sperm could result in poor survival and affect offspring performance, as demonstrated by Pérez-Cerezales *et al.* (2011) when analysing some gene expression patterns in rainbow trout fry. The evaluation of the progeny obtained with fresh and frozen semen has been carried out focusing on different aspects: Labbé *et al.* (2001) and Young *et al.* (2009) did not report differences in growth, survival rates or juvenile performance in rainbow trout larvae. However, Hayes *et al.* (2005) observed in the same species abnormal growth and changes in cortisol response to acute stress in juveniles from some batches fertilized with frozen sperm. Horváth *et al.* (2007) reported increased rates of abnormal karyotype in carp produced from cryopreserved semen, but no differences in genetic variability of brown trout progeny obtained with fresh and frozen sperm were described by Martínez-Páramo *et al.* (2009). These different results could be due to the different degree of DNA damage caused to the sperm during cryopreservation and to species-specific resistance to cryodamage. To counteract these effects, an appropriate selection of samples before freezing, as well as the use of optimized protocols, is required. DNA fragmentation has been significantly reduced in rainbow trout when egg yolk low density lipoproteins were added to the extender (Pérez-Cerezales *et al.* 2010a) and more benefits are expected with the progress made on the study of appropriate antioxidants (Cabrita *et al.* 2011).

Sperm cryopreservation, when performed in a well-controlled manner, is a safe method to preserve male genetic material. Its use should benefit the fish farm industry at different levels, from management of reproduction to genetic selection by producing overlapping generations for monitoring genetic changes, exchange of genetic resources and gene banking. However, efforts are required to transfer current technologies and make sperm banking accessible for fish producers through national and international networks.

Other Sources of Germplasm: Undifferentiated Germ Cells and Surrogate Production

Recent advances in reproductive biology have provided new and interesting sources of genetic material, other than gametes, useful for reproduction, broodstock management

and gene banking in aquaculture. Cells from the germinal line at early stages (e.g. primordial germ cells (PGCs) and spermatogonia type A) can be isolated from the genital ridges of larvae or from the gonads of juveniles, and used as sources of germplasm for surrogate production. These cells, once transplanted to a recipient, have demonstrated their ability to divide and differentiate into gametes according to the sex of the recipient (Kobayashi *et al.* 2007; Yano *et al.* 2008). The resulting gametes (spermatozoa or oocytes) are produced by the host species, carrying the genetic information from the donor (Yoshizaki *et al.* 2010).

Grafting of PGCs and spermatogonia has been performed between individuals from the same species, but also between different species. Yoshizaki *et al.* (2010) reported the first trout fries obtained by artificial fertilization of gametes collected from triploid Masu salmon (*O. masu*) after microinjecting trout PGCs from a p*vasa-Gfp* line in the larvae coelomic cavity. Recently fertile sperm from a tilapia strain have been obtained in males from a different line after injection of spermatogonia directly into the adult testes (Lacerda *et al.* 2010), overcoming the difficulties and long delay that represent the transplantation to earlier life stages. In marine species, preliminary experiments using Nibe croaker (*Nibea mitsukurii*) as the donor, demonstrated the ability of mackerel to support colonization and proliferation of grafted xenogenic germ cells from donor species, encouraging further experiments with larger pelagic species, such as tuna or drum, difficult to maintain in captivity (Yazawa *et al.* 2010; Yoshizaki *et al.* 2010).

Cryopreservation of undifferentiated germinal cells provides good survival rates (Kobayashi *et al.* 2007; Cabrita *et al.* 2010) and gives a source of male and female gametes, overcoming the limitations of long term preservation of oocytes and embryos. Several techniques have been used to store this material: cell suspensions frozen in encapsulated systems, testes fragments containing spermatogonia A or genital ridges containing PGCs, both cryopreserved in cryovials (Kobayashi *et al.* 2007; Cabrita *et al.* 2010; Riesco *et al.* 2012). However, the implementation of cryopreservation techniques and further transplantation of germ cells will require significant research efforts before it can become a commercial reality.

Knowledge Gaps and Research Needs

As discussed above, the influence of external factors and husbandry practices on gamete quality has been well established. The mechanisms mediating the effect of those factors remain, in contrast, poorly understood. In the context of aquaculture diversification, one of the main challenges that we are facing is the identification of key molecular mechanisms at gene, protein and epigenetic levels that would be shared by a large number of species within teleost fish. This stresses the need for evolutionary-oriented genomic studies designed to identify common molecular traits of the fish oocyte. In addition, there are clear gaps in knowledge for defining optimal egg quality at genomic, proteomic and physiological levels in fish and translation of this basic knowledge into a set of robust, reliable markers/analytical tools that provide early confirmation for commercial hatcheries. The establishment of robust and reliable markers will also require extensive validation, not only within a species for different factors but also before transfer to evolutionary distant species.

Importantly, the assessment of sperm and oocyte quality cannot rely on one single parameter but the analysis of several parameters using simple methods to more sophisticated approaches, none of them alone predicting accurately the reproductive success.

The range of optimal indicators should be defined according to the species, sperm fate or reproductive strategy: artificial fertilization, cryopreservation, gene banking or mass production. Basic research in this field is helping to develop appropriate quality evaluation schemes and early biomarkers of reproductive success, bearing in mind transfer to industry.

Management of fish reproduction depends on the use of the best breeders. It should thus be stressed that improving egg and sperm quality is important to determine potential selection differences in a breeding programme. Indeed, reproductive success is often not used for the definition of genetic indexes in selection schemes. In the long term, this could induce significant problems in the success of reproduction, as observed for dairy cows in the past.

Broodstock Nutrition

Background

The production of larval fish and their subsequent growth, development and health, and also their potential productivity as future broodstock are highly dependent on the quality of eggs available to the industry (Bromage *et al.* 1992; Bromage & Roberts 1995). Since egg quality, in terms of the macro- and micronutrients they contain, is dependent on nutrient delivery from the female it is vital that broodstock nutrition is optimized to ensure good larval survival and early development (Izquierdo *et al.* 2001). Thus, broodstock diets should be formulated to ensure all essential nutrients requirements are met for the species being cultured. Therefore, the development of broodstock feeds rests with the hatcheries and they have little time to spend on research, which can be very expensive when considering controlled broodstock trials.

Lipids are the most studied macronutrient in terms of broodstock nutrition. Most fish species preferentially utilize lipids to provide energy for somatic growth but they are also a source of essential fatty acids (EFA) required for the formation of cell membranes that are vital for successful larval development (Sargent *et al.* 2002). It is therefore important that the correct EFA are provided, in excess of requirement levels, to allow the production of robust and healthy larvae. This means that the female broodstock must accumulate sufficient energy-providing fatty acids and EFA from their diet to fuel growth and deliver the essential long-chain polyunsaturated fatty acids (LC-PUFA) that are required for successful larval production. In most cases the nutritional requirements for salmonid growth are similar for wild and farmed salmon since most farmed stocks are, for the most part, not significantly deviant from wild stocks. Thus, while farmed fish may have access to higher energy feeds, which may allow more rapid growth, the dietary components used for farmed salmonids are largely similar to those that would be available to wild stocks, in terms of fatty acid, vitamin and micronutrient levels. The data provided by Almansa *et al.* (1999) on seabream would suggest that no significant detriment to egg quality was apparent over the spawning period.

This short review will present recent developments in lipid nutrition in broodstock and their effects on egg and larval quality and will also consider other nutrients including vitamins and carotenoids that can impact on larval success. This section is structured according to species with a focus on salmonids, bass and bream, cod, flatfish (mainly Japanese flounder, halibut, sole and turbot), eel and carp.

Salmonids

Salmonids have an advantage over most marine species in that their egg size, being much larger, can store more nutrients than most marine eggs and as a result salmonids are easier to culture. For that reason the literature on salmonid eggs and broodstock is much less than that for marine species. However, when developing broodstock diets for any fish species it is useful to analyse the fatty acid composition of wild eggs as these provide useful information for optimizing dietary formulations (Tocher & Sargent 1984; Bell & Sargent 2003; Salze *et al.* 2005). Table 2.2 shows the fatty acid compositions of Atlantic salmon eggs collected from wild and cultured stocks in Scotland as well as cultured Chilean salmon. This suggests that salmon are fed a diet containing fish oil (FO), as is the case for the cultured stocks, while the wild stocks will have consumed prey from the North Atlantic/North Sea, which will comprise, for the most part, pelagic

Table 2.2 Fatty acid compositions (% weight) of farmed and wild Scottish salmon and farmed Chilean salmon.

Fatty acid	Scottish wild[a]	Scottish wild[b]	Scottish farmed	Chile farmed
14:0	1.36 ± 0.03^{c}	1.45 ± 0.10^{c}	2.35 ± 0.13^{a}	1.95 ± 0.07^{b}
15:0	0.24 ± 0.00^{b}	0.27 ± 0.02^{ab}	0.28 ± 0.02^{a}	0.24 ± 0.01^{ab}
16:0	12.67 ± 0.01^{b}	14.32 ± 0.51^{a}	13.35 ± 0.07^{ab}	12.42 ± 0.62^{b}
18:0	5.55 ± 0.04^{b}	5.88 ± 0.70^{ab}	5.12 ± 0.57^{b}	6.88 ± 0.05^{a}
Total saturates	19.82 ± 0.03^{b}	21.95 ± 1.31^{a}	21.10 ± 0.48^{ab}	21.83 ± 0.76^{ab}
16:1n-9	0.45 ± 0.25	0.50 ± 0.08	0.53 ± 0.03	0.36 ± 0.04
16:1n-7	5.08 ± 0.40	5.21 ± 0.70	4.62 ± 0.04	5.09 ± 0.30
18:1n-9	26.31 ± 0.42^{a}	23.99 ± 1.04^{b}	20.15 ± 0.46^{c}	15.11 ± 0.11^{d}
18:1n-7	3.53 ± 0.55	3.62 ± 0.48	3.02 ± 0.06	3.66 ± 0.04
20:1n-9	2.29 ± 0.03^{a}	1.42 ± 0.26^{b}	2.56 ± 0.04^{a}	0.48 ± 0.06^{c}
22:1n-11	0.26 ± 0.02^{ab}	0.18 ± 0.09^{b}	0.33 ± 0.01^{a}	n.d.[c]
Total monoenes	38.21 ± 0.22^{a}	35.37 ± 0.89^{b}	31.51 ± 0.49^{c}	26.12 ± 0.41^{d}
18:2n-6	1.39 ± 0.03^{c}	1.11 ± 0.14^{c}	3.17 ± 0.40^{a}	2.31 ± 0.27^{b}
20:2n-6	0.21 ± 0.00^{b}	0.18 ± 0.01^{b}	0.32 ± 0.05^{a}	0.25 ± 0.06^{ab}
20:3n-6	0.26 ± 0.02^{b}	0.32 ± 0.09^{b}	0.51 ± 0.08^{a}	0.29 ± 0.01^{b}
20:4n-6	1.23 ± 0.00^{b}	1.24 ± 0.19^{b}	1.08 ± 0.05^{b}	1.68 ± 0.08^{a}
Total n-6	3.09 ± 0.03^{c}	3.02 ± 0.10^{c}	5.08 ± 0.48^{a}	4.52 ± 0.41^{b}
18:3n-3	0.66 ± 0.02^{b}	0.52 ± 0.22^{b}	1.01 ± 0.03^{a}	0.69 ± 0.04^{b}
18:4n-3	0.26 ± 0.00^{b}	0.24 ± 0.05^{b}	1.05 ± 0.19^{a}	0.43 ± 0.06^{b}
20:4n-3	2.05 ± 0.02^{b}	1.46 ± 0.35^{c}	2.53 ± 0.22^{a}	2.23 ± 0.11^{a}
20:5n-3	8.36 ± 0.05^{b}	10.02 ± 0.36^{b}	9.89 ± 0.35^{b}	13.88 ± 1.27^{a}
22:5n-3	6.14 ± 0.02^{b}	6.10 ± 0.19^{b}	4.50 ± 0.15^{c}	8.13 ± 0.56^{a}
22:6n-3	19.88 ± 0.10^{bc}	21.30 ± 0.80^{a}	22.02 ± 0.81^{a}	18.94 ± 0.28^{b}
Total n-3	37.47 ± 0.15^{a}	39.67 ± 0.72^{b}	41.12 ± 1.39^{b}	44.29 ± 0.57^{a}

Values for Scottish wild ([a]River Tay; [b]River Don) and farmed Scottish and Chilean eggs are mean ± SD, $n = 3$. Values assigned a different superscript letter are significantly different ($P < 0.05$).

and demersal fish from that region. Thus, there are only minor differences between the Scottish wild and cultured eggs and the resulting impacts on component compositions are small, largely involving the PUFA 18:2n-6 (derived from plant meals/oils in the diet) which is a relatively minor component in both wild and farmed fish. Provided 18:2n-6 levels do not exceed 20% of the total fatty acids in the diet formulations for farmed salmon there is no evidence for health or growth functions in farmed salmon fed such diets (Torstensen *et al.* 2008; Bell *et al.* 2010). However, when considering the functionally important fatty acids for membrane development, especially in neural and endocrine tissues and immune regulation, etc. (Sargent *et al.* 1995, 2002), namely arachidonic (ARA), eicosapentaenoic (EPA) and docosahexaenoic acids (DHA), there are no differences between Scottish wild and farmed eggs. However, comparing Scottish eggs with Chilean eggs showed that ARA and EPA are significantly increased, while DHA is significantly reduced in Chilean eggs. These reflect Chilean fish being fed southern hemisphere FO rich in EPA and ARA, compared with northern fish oil. Thus, the ratios of EPA/ARA and DHA/EPA are altered in Chilean salmon which may affect egg and larval development given the preference for DHA and ARA for membrane functions and immune/stress function, respectively (Cowey *et al.* 1985; Sargent *et al.* 2002). However, the changes in fatty acid compositions between northern and southern hemisphere fish oils are relatively minor and would have no significant impact on fish growth, health and survival. In a study by Pickova *et al.* (1999) two wild Swedish landlocked salmon stocks were compared with cultured eggs. The two landlocked strains had lower EPA compared with the cultured fish (6% vs 13%), while ARA showed the opposite effect with values of 6.5% vs 2.4% for the landlocked and cultured fish, respectively. However, despite significant differences in DHA intake the cellular values were the same between both stocks. Pickova *et al.* (1999) suggested that the lipid source during gonadal maturation can alter egg fatty acid composition and this could disturb subsequent embryonic development. The evidence above suggests that matching fatty acid intake to values in wild eggs should be adopted to maintain egg and larval quality in salmonid culture. Maintaining high levels of fishmeal and fish oil may prove more difficult in the future as new 'alternative feeds' with higher levels of plant proteins and oils are introduced. However, as egg and larval success is paramount, the formulations should attempt to reproduce the compositions of wild eggs, in terms of LC-PUFA and amino acid balance, to reduce any negative outcomes in terms of larval survival and quality.

Bass, Bream and Related Sparids

European sea bass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) are currently the most cultured marine fish species in southern Europe and are sold widely across Europe and beyond. In most marine fish the egg size is small compared with salmonids with most being ~1 mm in diameter compared with 5–6 mm in Atlantic salmon (Moffett *et al.* 2006). Thus, the egg composition needs to contain all the essential nutrients required for rapid, early development so that the yolk sac larvae have enough energy and EFA, amino acids (AA), vitamins and minerals to allow successful first feeding.

Sea bass, as with all major cultured marine species, is unable to synthesize the long chain EFA, EPA, DHA and ARA from shorter chain C_{18} precursors, 18:2n-6 and 18:3n-3 (Sargent *et al.* 1995, 2002). Thus, it is vitally important that these EFA, as well as the saturated and monounsaturated fatty acids required for energy production, are provided to the broodstock in sufficient quantities to allow optimal transfer to the developing

gonads. It is estimated that in juvenile marine fish between 0.5% and 1.7% of dry diet should be long-chain n-3 fatty acids (n-3 LC-PUFA; Sargent *et al.* 1995, 2002), although given the rapid growth of larvae and the high neuro-somatic index (high brain and retinal tissue) in small larvae the requirements may exceed these values. Deficiencies of n-3 LC-PUFA in early larval feeding can cause developmental defects in the neural system that can affect visual function and prey capture (Bell *et al.* 1995a,b).

Early broodstock diets for bass rely heavily on wet fish diets that carry a risk of disease transfer. However, a study comparing a wet fish diet from bogue (*Boops boops*) with two formulated diets showed superior egg and larval quality with the wet fish diet although this may have been due to the high inclusion of corn oil in the dry diets (Thrush *et al.* 1993; Bell *et al.* 1997; Navas *et al.* 1997). Eggs from this study showed higher levels of both ARA (threefold) and DHA (38% higher) in bass fed the wet fish compared with those fed the corn/fish oil blend (Bell *et al.* 1997). In a subsequent feeding study, northern fish oil (NFO) and tuna oil diets were compared with the wet fish diet used previously in 2 year old bass broodstock of farmed origin (Bruce *et al.* 1999). The EPA levels in the three diets were in the range 5.6–6.7% of total fatty acids but the ARA and DHA contents were different, being 0.4% and 1.4% and 4.6% and 7.8%, 19.5% and 22.1%, respectively, for the NFO, tuna oil and wet diets (Bruce *et al.* 1999). Spawning performance with the tuna oil diet was superior to the NFO diet and similar to that in the wet fish diet, suggesting that the increased ARA and DHA in the tuna diet had beneficial effects on egg and larval quality. In summarizing this study we would suggest that it is most important to meet the broodstock requirements applicable to the species being studied, in this case gilthead seabream. Thus, the wet fish diets provided more of the ARA and DHA that are essential for the early development of eggs and larvae in marine species such as seabream, while NFO is less able to provide enough of these EFA to meet requirements for growth and development. Clearly the high levels of ARA and DHA in the wet diet provided the best growth and survival for seabream, while the use of vegetable oils for larval marine fish is not generally advised. However, it should be pointed out that dry diets can provide the same or better nutrition levels for marine fish broodstock than wet diets which can be prone to disease risk and deterioration of feed quality (Thrush *et al.* 1993; Bell *et al.* 1997; Navas *et al.* 1997; Bruce *et al.* 1999).

The beneficial effects of optimizing n-3 LC-PUFA in diets for gilthead seabream were first reported by Rodríguez *et al.* (1998) who fed an n-3 LC-PUFA deficient diet compared with a diet with 1.8% n-3 LC-PUFA. The higher n-3 PUFA level in the supplemented diets resulted in higher EPA and DHA in the polar and neutral lipid fractions of the n-3 PUFA supplemented tissues, resulting in increased egg quality evidenced by higher levels of fertilized and hatched eggs. In a later study with seabream broodstock, improved egg viability, reduced abnormal eggs and non-fertilized eggs were observed when broodstock were fed more than 1.6% n-3 LC-PUFA, although at the highest n-3 level (3.15%) decreased fecundity and yolk sac hypertrophy were observed. The egg n-3 LC-PUFA content was positively correlated with the n-3, mainly EPA, content of eggs (Fernández-Palacios *et al.* 1995). A study conducted by Almansa *et al.* (1999) compared seabream fed a control diet with n-3 LC-PUFA supplied by cod liver oil with an n-3 LC-PUFA deficient diet, containing olive and linseed oils. Although early spawning eggs were not affected by diet, mid and late season eggs showed reductions in n-3 LC-PUFA in the deficient group, although no data on egg and larval quality were presented.

A study with white seabream (*Diplodus sargus*) investigated fatty acid compositions of ovaries from wild fish with ovaries and eggs from cultured fish (Cejas *et al.* 2003). There

were no differences between ovarian DHA levels in wild and cultured fish, although EPA was increased and ARA decreased in cultured fish compared with wild, giving EPA/ARA ratios of 5.45 in the former and 1.61 in the latter. Given the importance of ARA in reproductive processes (Bell & Sargent 2003) and the influence of both ARA and EPA on tissue eicosanoid production it is likely that maintaining both n-6 and n-3 LC-PUFA at values close to wild values will be of benefit to subsequent egg and larval success.

The common dentex (*Dentex dentex*) has been investigated as a potential species for aquaculture development as it has a high market value and growth rate. However, problems with a lack of juveniles for on-growing and solutions to variable egg quality are the subject of investigation. In a study conducted by Gimenez *et al.* (2006), hatching rate, mortality at 3 and 5 dph and day of total mortality were investigated in two groups of common dentex broodstock. A comparison was made between low and high quality batches (low quality: mortality at 3 dph >35%; high quality: mortality at 3 dph <10%). No differences were observed between batches for lipid content, lipid class and fatty acid compositions, although the high-quality batches had higher levels of neutral lipids.

Atlantic Cod

In the past decade significant advances have been made in Atlantic cod (*Gadus morhua*) culture techniques and the closure of the life cycle has reduced dependence on wild broodstock (Brown & Puvanendran 2002; Brown *et al.* 2003). However, as with many marine species the consistent production of good quality eggs and larvae for on-growing has been problematical, especially from second generation farmed broodstock that can have variable fertilization rates and high larval losses compared with wild eggs (Brown *et al.* 2003). Nutrition has a clear influence on egg quality and it is known that fecundity in wild populations is linked to broodstock liver oil levels (Marshall *et al.* 1999). It is also recognized that poor egg and larval quality can be linked to broodstock diet and that optimizing the intake of n-3 and n-6 LC-PUFA can improve fecundity, egg quality, hatching success and the incidence of deformities (Sargent *et al.* 1999a,b; Pavlov *et al.* 2004).

In a study conducted by Salze *et al.* (2005), the composition of eggs from wild broodstocks, wild broodstock fed a formulated feed and farm-produced eggs was compared in terms of lipid content, lipid class, fatty acid and carotenoid pigment concentrations. This study found no difference between eggs from wild fish or wild fish held in captivity and fed a commercial formulated diet, but there was a significant reduction (66%) in farm reared eggs in terms of fertilization rate and cell symmetry score (40%; Salze *et al.* 2005). However, there were no differences in egg lipid content of which 95% was from four lipid classes namely phosphatidylcholine (PC; ~40%), phosphatidylethanolamine (~15%), triacylglycerol and cholesterol (~20% each). The only differences seen between the different broodstocks was increased PC in farmed eggs compared with wild eggs and a significant difference between all three treatments in phosphatidylinositol (PI) which was highest in wild and lowest in farmed eggs. As phosphatidylinositol is where ARA is concentrated, lower levels of this lipid class might impact on eicosanoid production and fish health (Sargent *et al.* 2002).

Flatfish

Flatfish culture has expanded along with a general increase in aquaculture globally over the past 10 years with the range of species cultured increasing as a result. Studies with

Japanese flounder *Paralichthys olivaceus* fed diets containing 0.4%, 0.8% and 2.1% of dry diet as n-3 LC-PUFA for 3 months before and during spawning were performed (Furuita *et al.* 2002). The results showed that the percentage of normal larva survival at 3 dph and the starvation tolerance index correlated positively with dietary n-3 LC-PUFA intake, while higher ARA also correlated with improved egg quality. In a later trial using the same species, fish were fed higher levels of n-3 LC-PUFA (2.1%, 4.8% and 6.2% of dry diet) for 2 months before and during spawning (Furuita *et al.* 2002). Egg production was highest in the fish fed the highest level of n-3 LC-PUFA, although egg quality parameters including the percentage of floating eggs, hatching rate and a percentage normal larvae were highest in the group fed the 2.1% n-3 LC-PUFA diet (Furuita *et al.* 2002). The results suggest that 2.1% n-3 LC-PUFA as percentage of dry diet may be the optimal level for Japanese flounder and that higher concentrations may be detrimental. A lack of antioxidant protection was raised by Lavens *et al.* (1999) who observed reduced egg quality in turbot fed n-3 LC-PUFA. This position was supported as addition of vitamins E and C improved the hatching rate of turbot fed high n-3 LC-PUFA (Lavens *et al.* 1999). In Japanese flounder fed ARA enriched diets (0.1%, 0.6% and 1.2% of dry diet) for 3 months before and during spawning, the highest egg production was seen in fish fed the 0.6% diet and lowest in the 1.2% diet (Furuita *et al.* 2003). Increased dietary ARA reduced the EPA content of eggs and this may have been a factor explaining the decline in egg quality in fish fed 1.2% ARA. Evidence shows that EPA and ARA are in direct competition for the *sn*-2 position in tissue phospholipids such that an excess of one will displace the other (Bell *et al.* 1989; Sargent *et al.* 2002).

Early culture of Atlantic halibut (*Hippoglossus hippoglossus*) used wet fish diets which, while sometimes successful, were difficult to store and risked disease transmission from the trash fish. Two formulated diets supplemented with either krill meal or tuna orbital oil, rich in DHA and ARA, were compared with a traditional wet fish diet (Mazorra *et al.* 2003). The results showed that the two formulated feeds gave a similar performance to the wet fish diet in terms of relative fecundity and fertilization rate. In a second trial the spawning quality and egg performance were compared in broodstock fed two diets that differed only in their ARA contents which were either 0.4% and 1.8% of total fatty acids, conducted over two successive spawning seasons. The higher ARA concentration resulted in a significantly higher fertilization rate (59%), blastomere morphology score (14.2%) and hatching rate (51%) compared with the 0.4% ARA group (31.0, 12.5, 28.0, respectively; Mazorra *et al.* 2003).

A recent study with common sole (*Solea solea* L.) compared egg fatty acid compositions and egg quality parameters in wild caught and cultured fish (Lund *et al.* 2008). Eggs from the cultured stock had higher levels of 18:2n-6, 18:3n-3 and 20:1n-9, while the wild eggs were higher in 16:1n-7, 20:4n-6 and 20:5n-3, due to dietary input. Larval growth was compared between wild and cultured groups and while larval growth was not linked to broodstock origin, the fatty acid composition, egg or larval size and larval survival were much lower in cultured larvae (Lund *et al.* 2008).

While limited new data are available on turbot broodstock nutrition the studies conducted by Lavens *et al.* (1999) indicated that supplementation, for 2–3 months before the reproductive season, with n-3 and n-6 HUFA resulted in increased egg diameter, oil globule diameter and fertilization rate. Broodstock were also supplemented with vitamins C and E resulting in an increased oil globule volume compared with fish fed no vitamin supplement.

Carp

A number of studies on grass carp *Ctenopharyngodon idella* and common carp *Cyprinus carpio* (Manissery *et al.* 2001; Khan *et al.* 2004) have shown the benefits of optimizing protein content on egg and larval performance. Broodstock grass carp were fed formulated diets with protein contents of 20%, 25%, 30%, 35% and 40%. The highest weight gain was seen in the 30% and 35% protein diets, although values for gonadosomatic index, fertilization and hatchability rates were similar to fish fed the 25% protein diet (Khan *et al.* 2004). Compared with studies on protein nutrition and in comparison with other fish species, very little work has been done on optimizing lipid nutrition in carp. In a more recent study with the Indian major carp *Catla catla*, a control diet devoid of any LC-PUFA was compared with an experimental diet supplemented with 10% fishmeal and 1% fish oil over a 2 year period (Nandi *et al.* 2007). The spawning response was higher in the supplemented fish (96%) compared with 76% in the control, and egg and larval quality was improved by lipid supplementation as evidenced by an increased fertilization rate and larval survival in the supplemented group (Nandi *et al.* 2007).

Knowledge Gaps and Research Needs

There is clearly still much to be done in the development of broodstock feeds for the growing number of cultured fish species. However, conducting trials with broodstock is difficult and expensive, especially when replication is required to provide accurate and statistically significant data. For feed companies there is little interest in conducting such trials as the volumes of feed specifically for broodstock are very small compared with on-growing diets. Despite this, benefits are being shown with manipulation of broodstock diets especially with respect to lipid and fatty acid compositions and ratios as well as vitamins and carotenoid pigments. If we are to produce benefits in egg and larval quality it is vital to conduct such trials as well as to improve our knowledge of wild fish compositions as these often provide key information for formulators of specialized broodstock diets. We also need to be aware of the possible implications of reducing fishmeal and fish oil in broodstock diets on the success of egg and larval production. As broodstock feeds are produced in relatively small amounts and the importance of marine raw materials in providing essential nutrients for health and development is well known, major changes to broodstock feeds should be prevented if at all possible. However, some studies should be conducted, in a number of cultured species, to investigate the possible effects of diets with higher levels of plant meals and oils and to ascertain whether they might be detrimental to egg quality and larval production since these alternative diets are likely to be introduced for some species in the near future.

Applications of Genetics and Genomics to Broodstock Management

General Considerations and New Advances

Genetic management and genetic mismanagement tend to occur at or around the time of spawning. It is the hatchery manager's role to utilize techniques that produce quality seed with the correct characteristics for the on-grower, but also to replace the existing broodstock with equally good or better fish for the future. The genetic improvement or genetic degradation of farmed stocks occurs at the point of spawning and depends on

decisions as to what fish are mated and why. This decision needs to be based on basic genetic principles and information regarding the pedigree and performance of individuals or their relatives. Advances in aquaculture genetics have given us a range of new tools that are making the task of managing and improving fish easier, and these have been reviewed by a number of workers (Hulata 2001; Dunham 2004; Gjedrem 2005). The main applications include selective breeding, single sex production, chromosome set and sex manipulations and genetic engineering. To realize the full potential of the available aquatic genetic resources, the industry will need to domesticate many species through long-term selective breeding programmes and apply techniques, to generate single sex or sterile offspring that enable these fish to achieve their full growth potential. There has been an increase in the number and sophistication of breeding programmes in fish and shellfish and a growing recognition that combining the skills of quantitative and molecular geneticists with reproductive biologists early in the domestication process can result in immediate and more sustainable improvement in the productivity of farmed stocks. Major resources have been invested in the development of the facilities and tools needed for the genetic management and improvement of farmed fish; some of these are discussed in the following sections.

The biology of aquatic organisms offers many opportunities to farmers and geneticists to improve the efficiency of production and the rate of genetic gain in ways not available to those involved in terrestrial animal production. The high fecundity and external fertilization normal in most aquatic organisms enable large numbers of gametes to be collected, manipulated and fertilized under controlled hatchery conditions. The large family sizes result in more accurate estimates of genetic parameters, higher selection intensities and direct transfer of improvements to the farmer without a multiplication stage. Many species being essentially wild have high levels of genetic variation so measured heritabilities (h^2) for many traits are medium to high, further making selection worthwhile.

Aquatic organisms are cold-blooded and phenotypic plasticity is common, meaning that any given genotype can be modified by the environment and potentially has many possible phenotypes. It is possible to change the phenotypic sex of many aquatic organisms by changes in the rearing temperature or by administering hormones during the sexually labile period of development. This enables single sex stock to be generated directly or through controlled breeding. The high fecundity also means that techniques such as chromosome set manipulations and transgenesis, that can cause high mortality at the egg stage, can be used to generate unique and highly valuable genotypes in a cost effective way for the industry (e.g. YY males).

Selective Breeding Programmes

It is critical for the long-term development of aquaculture that we improve the quality of the strains we farm. Selective breeding has been applied successfully to all of the major agricultural animal and plant species and has proved highly efficient in improving the yield and quality of the food we eat today. The widespread uptake of selective improvement by the fish and shellfish industries was disappointingly slow and often has had to be supported by national or international initiatives by government agencies until the industry was able to adopt the programmes. Globally <10% of farmed fish come from scientifically managed breeding programmes (Gjedrem 2005). Europe probably leads the world in the number and diversity of breeding programmes (Table 2.3).

Table 2.3 Present status of breeding programmes in some European species. Some of this information is derived from an AQUAbreeding Survey on the breeding practices in the European aquaculture industry (up to 2009) and the various species reviews available at www.aquabreeding.eu.

Species	No. breeding programmes	Countries	Traits	Selection method (generations)	Management tools	Genomic tools
Salmo salar	7	Norway 4 Ireland 1 Scotland 1 Iceland 1	Growth/processing yield Disease resistance Product quality Maturity Morphology/deformity	Combined selection (>8)	Triploidy Genetic fingerprinting Marker assisted selection	Genetic and physical map Genome being sequenced Microarrays
Oncorhynchus mykiss	10	Norway 2 France 4 Denmark 1 Finland 1 UK 2	Growth/processing yield Disease resistance Product quality Maturity Morphology/deformity	Individual and combined (>8)	All-female Triploidy Genetic fingerprinting	Genetic map Genome being sequenced Microarrays
Sparus aurata	5	France 1 Greece 3 Israel 1	Growth Disease resistance Product quality Morphology	Combined within family Crossbreeding (>3)	Genetic fingerprinting	Genetic map
Dicentrachus labrax	3	France 1 Greece 1 Spain 1	Growth Disease resistance Product quality Maturity	Individual Within family (>3)	All-female Genetic fingerprinting	Genetic map

Gadus morhua	2	Norway 2	Growth/processing yield Disease resistance Product quality Maturity	Combined (>3)	Triploid Genetic fingerprinting	Genetic map Genome being sequenced
Scophthalmus maximus	2	France 1 Spain 1	Growth Morphology	Individual Combined	All-female Genetic fingerprinting	Genetic map
Salmo trutta	1	France 1	Growth Product quality Morphology	Individual	All-female triploidy	Genetic map
Solea solea	1	Holland 1	Growth Morphology Maturity	Combined	Genetic fingerprinting	Genetic map
Argyrosomus regius	1	France 1	Growth	Individual		
Cyprinus carpio	5	Russia Bulgaria Czech Rep Hungary Poland	Growth Morphology Disease resistance	Individual Family Combined Crossbreeding		Genetic map

Traditional domestication approaches in fish have had a disastrous impact on many farmed strains. The problems often relate to the high fecundity and relatively small number of broodstock required to produce the necessary egg or fry in production. When this is compounded with difficulties in tagging individual fish and the inevitability of related mating in hatchery populations, it can result in the accumulation of inbreeding and a reduction of the viability of the stocks. One of the most detailed studies, by Kincaid (1983), showed a loss of performance across a wide range of commercial traits at different levels of inbreeding in rainbow trout. The management of the effective population size (N_e) is critical to the long-term viability of any strain, particularly in the absence of a pedigree based approach to broodstock replacement. The various strategies for maximizing N_e, to minimize the risks of inbreeding and genetic drift, are detailed in the book by Tave (1992). Today the availability of better tagging systems, such as PIT tags, and a variety of genetic markers makes the management of farmed fish stock much easier.

Selective improvement programmes established after the Second World War in many Eastern European countries resulted in common carp strains being developed to improve the yield from semi-intensive and intensive pond culture systems across eastern Europe and Russia (reviewed by Kirpichnikov 1981, 1987; Hulata 1995). Recent carp improvement programmes find many of these original strains lack the additive genetic variation needed for continued selection for growth, probably because of the extended period of unscientific domestication. The preferred strategy uses crossbreeding between the many existing strains to develop new synthetic populations or to include wild isolates as the starting point for family based selection. Such programmes have been documented from Israel (Wohlfarth & Moav 1990), Vietnam (Nihn *et al.* 2011) and the Czech Republic (Vandeputte *et al.* 2008).

It was the development of the salmon industry in Norway and the almost simultaneous establishment of a breeding programme by AKVAFORSK at Sunndalsøra in 1971 that was the model adopted for many advanced fish breeding programmes around the world (Gjoen & Bentsen 1997). Today nearly all farmed salmon are sourced from a relatively small number of breeding companies that utilize the original Norwegian material (Gjedrem *et al.* 1991) or a mixture of local and imported strains in Scotland, Chile and Iceland. Similar procedures are used to improve rainbow trout and Pacific salmon. The design of the salmonid programmes generally utilizes a family unit to on-grow individual families to size when a representative number in each family can be tagged, usually PIT tags, so that individual and family performance for a range of traits can be tested under commercial or disease challenge conditions. Some incorporate the use of pedigree assignment by microsatellite markers to undertake trials on commercial farms without the need for physical tagging (Guy *et al.* 2006). Some of these companies are now applying the latest genomic techniques to identify quantitative trait loci (QTL) to enhance the speed of improvement in disease resistance and post harvest traits (Houston *et al.* 2008, 2010).

In other species, particularly marine species, the application of genetics to broodstock replacement and improvement has been slow because the spawning behaviour and more complicated life-cycles have made pedigree based breeding using separate family rearing difficult. Recent advances in DNA marker technology, covered later, allow the offspring from known genotyped parents to be assigned to a family at any point in the life-cycle, making it possible retrospectively to reconstruct pedigrees from fish grown communally in large commercial populations. This technology can be used to

assess production traits under commercial conditions without the risk of tags entering the human food chain. Data on the commercial performance and post harvest traits are being used to generate breeding values for siblings held in the breeding nucleus. Markers can also be used to identify breeding candidates directly from production animals. High performing fish can be biopsied for genotyping and PIT tagged for later identification as potential breeding candidates. The added advantage of fish grown communally is that phenotypic differences in performance should correlate well with genetic breeding values, because all fish have been exposed to a common environment for their whole life. Many of the marker systems developed in these emerging species have been through collaborations between university research groups and small private farming companies funded by EU or national research funds. There are microsatellite protocols available for a number of species, including halibut (Jackson *et al.* 2003), sea bream (Brown *et al.* 2005; Castro *et al.* 2007), cod (Herlin *et al.* 2007), sea bass (Novel *et al.* 2010) and turbot (Borrell *et al.* 2004).

Genetic Markers

The continuous development of genetic markers since the 1960s and their application in aquatic organisms have been reviewed by a number of authors (Liu & Cordes 2004; Liu 2007). The development of marker technology has been critical to our better understanding of the population structure and evolution of aquatic organisms and will underpin future developments in aquaculture genetics and genomics. Genetic markers have been used for a range of management purposes such as assessing levels of genetic variation in farmed vs wild stocks, species purity in species prone to intentional and unintentional hybridization, pedigree assignment in large commercial populations for broodstock replacement or selective breeding purposes, and increasingly markers are used to characterize species/strains so they can be traced throughout the supply chain (Liu 2007).

It was the identification and mapping of large numbers of highly variable microsatellite markers in the 1990s that radically improved our ability to manage farmed fish. These markers enabled parentage assignment and application of pedigreed breeding programmes in many new species in which individual family rearing units were impracticable because of the biology of the species or the size of the industry did not justify the cost of such facilities. The application of markers in some species such as halibut (Jackson *et al.* 2003) and cod (Herlin *et al.* 2007), two marine species with low egg and larval survival, showed that F1 fish from wild parents, potential replacement broodstock, comprised relatively few large families that could lead to inbreeding without pedigree information.

With the growing number of highly variable genetic markers it was possible to build recombinant genetic maps and to identify associations between markers and commercially important traits known as QTL within the context of a pedigreed stock. Increasing marker density associations between alleles at a given locus and important traits, such as IPNV resistance in salmon, became highly significant and could be used to speed up the rate of improvement for this trait in a commercial salmon strain (Moen *et al.* 2009; Houston *et al.* 2010).

Next-generation sequencing (NGS) technologies have dramatically increased the volume and reduced the cost per base of sequencing and have resulted in a new class of

marker, single nucleotide polymorphisms (SNP), becoming easier to identify and verify in any species. The development of SNP in the past required well-developed genome resources for the species involved and multiple sequences of the same gene, but these do not exist in the majority of emerging farmed species. The NGS technique, known as restriction site associated DNA sequencing (RAD seq), does not require any prior knowledge of the genome of the species. The DNA is cut up with at least one restriction enzyme and the associated fragments are end labelled with adapters and sequenced. The system enables the DNA from different individuals to be coded with different adapters and sequenced together. The data are presented as 'stacks' of identical or specified levels of mismatch in the sequenced fragments. Because all fragments are coded, a sequencing error within an individual or the presence of a SNP within the population can be identified accurately. The large amount of sequence data generated by this approach enables genetic maps of RAD sequences for new species to be generated quickly if a small number of families are analysed and adequate bioinformatics capacity is available.

SNPs are very common in all genomes and will saturate genetic maps with many new markers, so increasing the probability of an association between a marker and a trait. The application of SNP technologies within breeding programmes, although expensive, is well established in terrestrial species to speed up selection. The main limitation with the application of SNP technology within aquaculture will be the development of cost effective SNP genotyping protocols. The technology does lend itself to automation but the cost of individual SNP chips needed to adopt this approach is still relatively high compared with the value of individual fish.

Functional Genomics

Alongside the development of new markers, most large fish genomic projects have included the sequencing of cDNA derived from the mRNA of genes expressed in a number of different tissues in a range of species under different environmental conditions. These are generally called expressed sequence tags (EST) generated by single pass 3′ sequencing of cDNA derived from mRNA isolated from tissue libraries. The resultant sequence is then BLAST searched against all existing genetic databases, hopefully identifying homology of the sequences to known genes in other species. The EST can be mapped using a variety of techniques such as recombination linkage mapping, radiation hybrid panels or probing end sequenced bacterial artificial chromosome libraries (BAC) of the species of interest. As type 1 markers, known genes, they can be used in comparative genomic studies with other species (Liu 2007).

The other driver for the discovery of ever more ESTs is the construction of gene expression microarray chips that contain cDNA clones or more specific oligonucleotide sequences derived from critical parts of the genes in question. These microarray chips can contain thousands of ESTs derived from the whole animal or more focused subsets of genes specific to a tissue or a biological function, such as response to disease. The correct application of this technology enables the comparison of gene expression between populations being subjected to a range of different environments and/or challenges. The work in salmonids has progressed the furthest in this field and a description of the development of the genetic resources, including the various microarrays and the protocols used in these types of study, is provided by Rise *et al.* (2007).

Microarrays can be used to study a wide range of potential traits of interest to the aquaculture sector. Disease and immune related gene expression responses to a variety

of pathogens and parasites have been analysed (Rise *et al.* 2004; Ewart *et al.* 2005; Morrison *et al.* 2006). Other traits of interest include growth responses in transgenic salmon (Rise *et al.* 2006) and stress responses associated with handling (Krasnov *et al.* 2005) and temperature (Vornanen *et al.* 2005) and recently HUFA lipid metabolism under different diets (Morais *et al.* 2011).

With large numbers of genes being monitored for their expression under a range of different conditions, it is clear that this type of study may identify potential candidate genes that have large effects on the phenotype and may be of commercial importance. In the absence of a full genome and a high density genetic map the integration of expression and QTL approaches increases the probability of identifying expression patterns that correlate with candidate genes for the traits being studied (Haley & de Koning 2006). The NGS techniques (RNA-seq) are making it feasible to sequence the whole transcriptome of the rainbow trout (Salem *et al.* 2012) or a particular tissue such as nucleated erythrocytes and their role in mounting an immune response (Morera *et al.* 2011) rather than just the subset of genes present on a microarray. There is a growing interest in epigenetics; the processes that modify genome function that can result are changes in the phenotype without any change in the DNA sequence. There are many examples of phenotypic plasticity in fish related to changes in the rearing environment that might have implications for the aquaculture industry, such as the changes in muscle fibre number related to the development temperature and its potential for growth enhancement in Atlantic salmon (Johnston *et al.* 2003). Many of the processes involved in pre- and post-translational modification, such as methylation and the role of the many non-coding RNAs in modifying gene expression and possible splice variants, can now be monitored in ways not previously possible using different NGS techniques.

The limitations with NGS will lie in developing the bioinformatics platforms and skills to analyse these large data sets and to annotate the sequences so that we can identify all the genes being expressed.

Chromosome Set Manipulation

The plastic phenotype displayed by many aquatic species and the lack of genetic imprinting in many lower vertebrates and invertebrates enable a range of genetic and environmental manipulations to be undertaken in these species that can have profound effects on their subsequent performance. These chromosome set manipulations are not classed as genetic modification, as many of the outcomes have been observed to occur naturally in wild fish populations.

The most widely applied technique is the production of individuals with three sets of chromosomes sets known as triploids (3n). If eggs are fertilized with normal sperm and are then exposed to an appropriately timed temperature or pressure shock, the second polar body at the second meiotic division is retained and will generate an embryo that contains two maternal and one paternal chromosome (Dunham 2004; Piferrer *et al.* 2009). Triploidy appears to have no significant impact on somatic growth in some species (e.g. salmon; Taylor *et al.* 2012) but the unbalanced chromosome number does seriously interfere with the meiotic divisions associated with the production of gametes and the development of the gonads. In females few, if any, ova are produced so the ovaries remain small and secrete little hormone, so the fish show few signs of sexual maturation. In males spermatogenesis still occurs and few, if any, viable sperm are

produced but the testicular tissue secretes enough hormones for the fish to show the unwanted symptoms of sexual maturity. In the production of large rainbow trout 3n production is normally only undertaken in all-female strains.

Triploidy has been used to advantage in aquaculture species in which sexual maturity can reduce the potential size and quality of the production fish. This technology has been applied to the production of larger rainbow trout (Bye & Lincoln 1986), channel catfish (Wolters *et al.* 1982) and in other aquaculture species in which the onset of maturity in diploid strains slows growth during on-growing and affects product quality at harvest. In some species costly manipulations of day length are needed to delay the onset of maturity until after harvest (salmon, cod, sea bass). Day-length control is very difficult to manage effectively in large floating cages. Single sexed triploid female stocks would go a long way to solving the maturity problem as well as avoiding potential problems of genetic introgression from escapes. It can also be used as a biological control mechanism to stop spawning in exotic species, such as grass carp used for the control of aquatic weeds (Watterdorf 1986). To reduce the risk of introgression of genes from stocked or escaped farmed strains into native populations of the same species (e.g. farmed Atlantic salmon in Europe and eastern USA and Canada; restocking of brown trout in the UK and rainbow trout and cutthroat trout in the USA and Canada; Kozfkay *et al.* 2006), sterility through triploidization has been put forward. It would also provide a potential means to stop the introgression of genes from transgenic fish strains that might escape into the wild (Piferrer *et al.* 2009).

One of the most powerful chromosome set manipulations is the ability to induce parthenogenesis and to derive offspring from wholly maternal (gynogenetic) origins (see Dunham 2004; Komen & Thorgaard 2007 for reviews and details of these technologies). There are a number of potential outcomes from using these techniques: haploid (n) embryos, although they usually die before hatching, make a good resource for gene-mapping as they are effectively large single gametes; meiotic gynogenetic offspring are on average homozygous at 50% of their loci because they retain a pair of chromosomes, sister chromatids, which have just undergone recombination and are useful in gene-mapping and the genetic analysis of complex traits; mitotic gynogenetic offspring are 100% homozygous because they arise from the duplication of a single maternal chromosome set and are described as double haploid (DH) individuals. Wholly paternal (androgenetic) individuals are produced when eggs treated with UV or gamma radiation, to destroy their maternal nuclear DNA, are then fertilized with a normal sperm to produce a haploid embryo that can be made diploid by disrupting the first mitotic division to produce an androgenetic DH that is 100% homozygous. Double haploid individuals from either a gynogenetic or androgenetic background are homozygous at all loci and can be used to generate DH isogenic lines after a second round of parthenogenesis. It is therefore possible to generate isogenic lines in as little as two generations for any particular species or strain of fish. Isogenic lines have been particularly useful as experimental organisms in disease resistance (Quillet *et al.* 2007) and vaccine studies (Purcell *et al.* 2006).

Although gynogenesis and androgenesis are rarely used directly for production purposes, they are used to generate genotypes valuable for aquaculture. The techniques can be used to speed up and reduce the cost of developing broodstock with the homogametic sex chromosome sets needed to produce single sex progeny. The route adopted will depend on the sex determination system of the species and the preferred

sex for production (Dunham 2004). The development of the YY male tilapia system used to produce all-male XY tilapia (XX female × YY male) can be greatly speeded up by producing them directly using androgenesis, as any male androgenetic offspring produced in *O. niloticus* will have the YY genotype and saves the several generations of breeding and progeny testing needed in the sex reversal and breeding route (Myers *et al.* 1986). In the silver barb (*Puntius goniontus*), females are the preferred sex and all-female meiotic gynogenetic offspring can be produced in large numbers and can be sex-reversed to generate neomales without the need for costly progeny testing (Pongthana *et al.* 1995, 1999). These examples can be used as models greatly to reduce the time taken to develop the homozygous sex genotype needed to develop single sex production systems in new species or to replace systems that rely on direct hormone treatment of production fish.

Gene Transfer Technologies

The work by Palmiter *et al.* (1982) and Palmiter and Brinster (1986) showed huge growth improvements in mice, induced by the integration of growth hormone (GH) constructs that stimulated much of the early work in fish transgenesis. The development of piscine gene constructs resulted in growth enhancement in a number of commercial species including rainbow trout (Penman *et al.* 1990), common carp (Zhang *et al.* 1990; Chen *et al.* 1993), channel catfish (Dunham *et al.* 1992) and tilapia (Rahman *et al.* 1998). These studies resulted in a wide range of gains in growth performance, between 10% and 500%. Some of the most dramatic work was on salmonids and when successfully integrated, these piscine constructs resulted in a significant elevation of circulating growth hormone levels (40-fold), particularly in younger fish (Devlin *et al.* 1994).

It is now clear that fish species respond very differently to the effects of growth hormone; salmonids appear to be very sensitive and this is probably related to their physiology. The increased level of growth hormone in transgenic salmon allows them to grow rapidly in the colder winter months when natural GH levels are low. This early advantage is magnified as these fish smolt earlier and gain a further advantage with the associated boost in growth that can be obtained during the subsequent grow-out phase compared with the non-transgenic controls.

Warm water species such as tilapia and carp can grow rapidly throughout the year and are probably not as reliant on growth hormone regulation (Devlin *et al.* 2001). Transgenic individuals derived from wild and selected domesticated rainbow trout strains showed a very different response. The wild transgenic fish showed a 17-fold improvement over the wild fish, but they did not grow better than the fast growing non-transgenic domestic trout. Introducing the gene into the domesticated strain only improved overall performance by 4.4%.

Although some see transgenesis as a shortcut to improve strains, it can take 4–5 generations of breeding to develop a stable transgenic fish line in order to obtain a single step improvement in growth performance. It may also take a very long time to get such lines licensed as in the case of the Aquabounty AquaAdvantage salmon (1995–2010).

The large amount of work going into identifying QTL and the rapid advances in functional genomics and sequencing technology will identify genes, especially in the area of improved disease resistance, that might be useful transgenic candidates in the future (Dunham 2009). Increased resistance has been observed in channel catfish and medaka

transgenic for the lytic peptide cecropin B. Pleiotropic effects on disease resistance have been observed in fish transgenic for non-disease constructs (Dunham 2009).

The demand for transgenic fish is likely to remain weak because of public perceptions, particularly in Europe, and because many of the existing technologies with measurable gains, such as selective breeding and single sex populations, have yet to be implemented widely.

Knowledge Gaps and Research Needs

The shortages in skilled manpower for managing and improving farmed fish are being overcome as the young scientists needed in genetics, genomics and bioinformatics are trained. The development of scientifically based breeding and improvement programmes for all farmed species is critical so as to avoid the genetic degradation of farmed stocks through inbreeding and genetic drift. The application of the various technologies described, either singly or in combination, will help to improve the efficiency and resilience of the industry and will ensure that aquaculture continues to grow its contribution to global food security. The recent developments in NGS technology will dramatically enhance our ability to develop the tools needed to domesticate the new species of the future. At this stage in the development of the industry, as scientists we need to collaborate and develop partnerships with industry if we want to see rapid uptake and advances that these technologies are capable of achieving.

Broodstock Environmental and Hormonal Manipulations

General Concepts

Fish display a multitude of reproductive strategies that have been shaped through the 500 million years of evolution to the diverse environments occupied by vertebrates during that time. These strategies are essential for the survival of the species and allow fine tuning of the timing of reproduction with the seasonal environmental changes. Indeed, most temperate teleosts are seasonal spawners with the release of gametes programmed so that the progeny is produced when conditions are most favourable. The entrainment of such reproductive cycles is therefore of paramount importance. A range of environmental (light, temperature, salinity), nutritional (feed quality, quantity) and social (sex ratio, size structure, dominance/hierarchy) factors play important roles in the synchronization of broodstock spawning. Differences seen today between temperate fish species in terms of seasonality of reproduction, fecundity or spawning type are, to a large extent, the consequence of rapid adaptation to particular photic and thermal niches into which groups have been pushed by a variety of unrelated selection pressures. The sole purpose of seasonal reproduction is to improve progeny survival through the co-ordination of larval abundance with favourable environmental conditions and an abundant food source.

The challenge in aquaculture is first to ensure 'normal' reproductive behaviour (e.g. as in the wild) and the production of high quality gametes in artificial, enclosed rearing systems and then, when successful, alter the time course of reproduction to fit aquaculture needs (all year round production). However, due to the difficulty in recreating ambient natural habitats, rearing facilities are most likely perceived by fish (especially

wild caught broodstock) as 'alien' environments that can be inadequate to the species requirements (environmental seasonal change, physical constraints of the enclosed system, social structure) and subjected to anthropomorphic perturbations (e.g. noise, handling). In such conditions, fish can fail to reproduce and spawn normally, despite the simulation of seasonal changes in photoperiod and temperature. Most common problems are impaired reproduction (either no initiation or regression, as seen in F1 male *Solea senegalensis*), lack of spontaneous spawning (as seen in halibut, turbot), poor egg quality (seen in most species) and lack of fertilization. It is especially true in novel candidate species for aquaculture in which little domestication has been done yet and production relies on limited numbers of broodstock harvested from the wild. It is only through research that these problems can be solved and aquaculture can grow. Importantly, the large diversity of reproductive tactics and control means that problems are very often species specific. In species where domestication and even selective breeding have been running for some time (e.g. salmon), problems of egg quality and fertilization can still remain despite the many husbandry and technological advances made. To our knowledge, the most recent review published on the environmental control of fish reproduction in broodstock was done by Bromage *et al.* (2001) and was almost exclusively focused on photoperiodic manipulations in salmonids with little reference to other factors and species, while another review focused on the control of puberty during on-growing (Taranger *et al.* 2010). It is therefore now time fully to re-evaluate the entrainment of reproduction in a range of species by bringing together new advancements and past and current theories and to define future challenges.

Entrainment of Reproduction: Proximate Factors

Success in breeding of most temperate species in artificial rearing conditions relies on knowledge gathered from wild stocks, especially the environmental regulation of reproduction (i.e. seasonal changes in abiotic factors). As with other seasonally breeding animals, fish rely on cues from the external environment to entrain and synchronize their reproductive cycle to the changing season. These cues have been defined as 'proximate' or determining cues in opposition to 'ultimate' or 'modulating' cues that can adjust the latter stages of the reproductive cycle and spawning such as temperature, stress or energy storage (Bromage *et al.* 2001; Migaud *et al.* 2010; Wang *et al.* 2010). Importantly, the nature of the proximate factors can differ between species according to their reproductive strategy, phylogenetic history and environmental conditions. As such, modulating factors in some species can become determining in others. A number of environmental factors have been implicated as possible proximate cues in fish including photoperiod, temperature, rainfall, food supplies and pheromones. All such factors can provide timely indications of pending conditions and are often used in conjunction to provide the animal with specific entrainment to the prevailing local conditions. However, research over the past decades has shown that it is the seasonally changing pattern of day length and temperature that is responsible for the cueing and timing of reproduction in the majority of fish species in Europe (Migaud *et al.* 2010). It is commonly believed that the daily changes in light intensity and the concomitant seasonal changes in day length provide the most consistent 'noise free' signal perceptible in almost all environments (Migaud *et al.* 2010). Such fluctuations in photoperiod are a result of the tilted axis of the earth relative to the sun, and become more pronounced as the distance

from the equator increases. Hence, in middle to high latitudes, light is the principal exogenous synchronizer (*zeitgeber*) that regulates many of the daily and seasonal biological rhythms (Foster & Hankins 2002), including reproduction (Bromage *et al.* 2001), feeding and growth (Sánchez-Vázquez & Tabata 1998) or smoltification in salmon (Duston & Saunders 1992).

Salmonids are by far the best studied model among commercially important temperate fish species with environmental manipulation used throughout the production cycle. Hence, there is a natural tendency to generalize results found in salmonids to the whole teleost phylogenic class. However, the control of reproduction in salmonid species is very different from that of other species and should not be used as a systematic reference. Salmonids even appeared to be unique among teleosts for their acute responsiveness to lighting regimes (Bromage *et al.* 1993, 2001), their circadian organization exclusively based on the pineal gland and their lack of pacemaker activity (Ekstrom & Meissl 2003; Falcon *et al.* 2010; Migaud *et al.* 2010) and overall their life cycle. Nevertheless, photoperiod is the principal determinant of maturation, not only in salmonids but also bass, bream, gadoids and flatfish that collectively comprise the major intensively farmed species in Europe (Migaud *et al.* 2010). The strongest evidence for photoperiod being the dominant environmental zeitgeber in many temperate fish is that even when the photo-thermal cycles are out of phase, reproduction can still be entrained to the photoperiodic signal (Davies & Bromage 2002; Norberg *et al.* 2004). Similarly in European sea bass, Atlantic cod, turbot and Eurasian perch, the application of long day photoperiods in advance or after day lengths have increased or decreased will also inhibit maturation even under suitable temperatures (Zanuy *et al.* 2001; Imsland *et al.* 2003; Begtashi *et al.* 2004; Migaud *et al.* 2004; Davie *et al.* 2007b). These studies clearly demonstrate open phases also called windows of opportunity in the reproductive cycle which can be closed, delayed or advanced by photoperiodic manipulations irrespective of the thermal regime.

Crucial to the success of these 'windows of opportunity' is the coincidence of the environmental signals with a permissive physiological gate, which in temperate fish species would appear to be based around suitable nutrition and/or energy status (Thorpe *et al.* 1998; Migaud *et al.* 2010). These periods are deemed 'critical' because they correspond to an open phase in the yearly cycle, as proposed by gating models (Duston & Bromage 1988). An effective approach to examining and defining seasonal windows of reproduction is to classify fish species according to that conventionally used in mammalian and avian models, on the basis of when reproductive cycles are actually initiated with respect to photoperiodic history and the direction of change (Foster & Kreitzman 2005). In this context species can be divided into two simple classifications of 'initiating' window: those that recruit under increasing day length stimuli such as the salmonids (Taranger *et al.* 1999), Atlantic halibut (Norberg *et al.* 2001) and turbot (Imsland *et al.* 2003; comparable to 'long day' breeders in mammals such as the Syrian or Siberian hamsters and Japanese Quail) and those that recruit under shortening day length stimuli such as Atlantic cod (Davie *et al.* 2007b), haddock (Davie *et al.* 2007a), Eurasian perch (Migaud *et al.* 2004) and sea bass, *Dicentrarchus labrax* (Felip *et al.* 2008; comparable to 'short day' breeders in mammals such as sheep). It is evident that not only do fish utilize a recruitment window but there is also a need for a 'completion' window whereby the animal can ensure that it completes gonadogenesis and spawns at the appropriate time, e.g. shortening days for salmonids

(Migaud *et al.* 2010) and increasing days in gadoids (Davie *et al.* 2007a,b). Within these photo-responsive windows fall the 'permissive physiological gates' whereby the reproductive cycle will proceed only if certain criteria are met (e.g. nutritional status). To initiate a reproductive cycle following the correct photoperiodic signal within the right window, a threshold of size (for first time spawners, e.g. puberty), growth rate and/or energy storage (for repeat spawners, e.g. broodstock) must be surpassed during this critical period for sexual maturation to initiate and succeed as observed in mammals (Thorpe *et al.* 1998; Taranger *et al.* 1999). This concept is clearly evident in the salmonids, although less well characterized in other temperate species (Begtashi *et al.* 2004), whereby individuals assess themselves on the basis of whole body lipid. Feeding a larger ration or higher energy diets is known to increase fecundity (Shearer & Swanson 2000), while feed deprivation a year ahead of spawning reduces both fecundity and the maturation rate (Bromage *et al.* 1992). The process is a kinetic phenomenon under strict endocrine control through the brain–pituitary–gonadal (BPG) axis (Zohar *et al.* 2010). This axis is organized around a series of hormones that are produced, released into the blood circulation and act on target tissues within the brain, pituitary, liver and gonads to initiate and control sexual development (Zohar *et al.* 2010). It is organized around: (i) the hypothalamus of the brain which releases neuropeptides and neurotransmitters that innervate and influence directly, (ii) the pituitary (gonadotroph cells), which synthesizes and releases gonadotropins (follicle stimulating hormone, FSH; luteinizing hormone, LH) which are transferred through the bloodstream and stimulate (iii) the gonads' steroidogenic cells (Sertoli/Leydig cells in testes and follicular cells in ovary) to produce sex steroids (androgens, oestrogens and progestagens) necessary for gametogenesis and positive/negative feedback regulation of reproduction (Fig. 2.2). All three major regulators of the BPG axis integrate with growth/energy pathways (e.g. leptin, growth hormone, Igf1) to regulate reproductive processes in synchrony with life stage and the surrounding environment to ensure spawning in favourable conditions (Migaud *et al.* 2010).

Photoperiod Regimes Used in Aquaculture

For most fish species, broodstocks exposed to phase-shifted simulated natural photoperiod and temperature regimes of 12 months duration have been the common practice of hatcheries to produce eggs all year round. Consequently, the set-up of three groups exposed to 1 year-long environmental regimes shifted by 3, 6 and 9 months will probably be enough to satisfy the ever growing industry demand for year-round production of eggs and juveniles (Carrillo *et al.* 1993; Bromage & Roberts 1995; Taranger *et al.* 2010). In their simplest sense, seasonal photoperiod cycles could be viewed as the continuous gradual transition between long (summer) and short (winter) day lengths. Therefore, the use of constant day length photoperiods was first investigated in rainbow trout culture in the early 1980s. Combinations of short days (6 h light:18 h dark, 6L:18D) and long days (18L:6D) were able to control spawning, providing advanced and delayed spawning times in a range of salmonids, including masu salmon (Takashima & Yamada 1984), Atlantic salmon (Taranger *et al.* 1998, 1999), brown trout (Bromage *et al.* 1990) and rainbow trout (Bromage *et al.* 2001) as well as Atlantic cod (Norberg *et al.* 2004), European sea bass (Carrillo *et al.* 1993, 1995), halibut (Norberg *et al.* 2001) and Eurasian perch (Migaud *et al.* 2004; Fontaine *et al.* 2006).

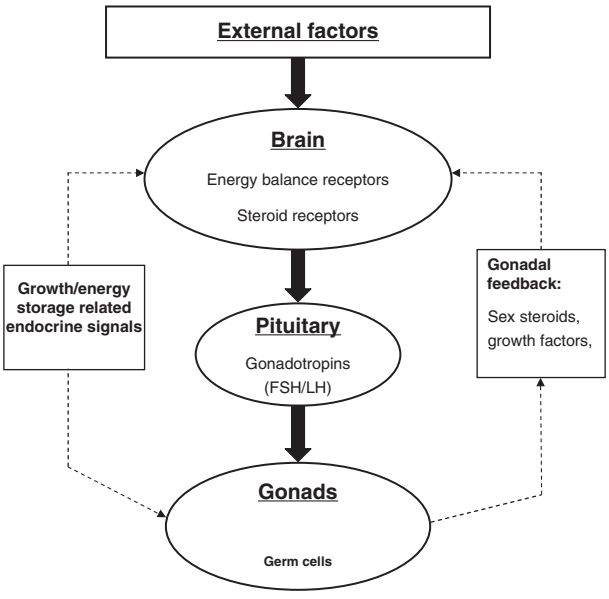

Figure 2.2 Schematic representation of regulatory pathways in the BPG axis during puberty in teleosts (adapted from Migaud *et al.* 2010; Taranger *et al.* 2010).

Although the start of a reproductive cycle can be induced easily in these species, given the correct photoperiod signal, successful completion of gametogenesis and subsequent spawning is determined by numerous ultimate (or modulating) factors. Diet composition provided to the broodstock (Carrillo *et al.* 1995; Bell *et al.* 1997; Navas *et al.* 1997) or temperature alterations used as ultimate factors may therefore have a strong influence on fecundity, egg quality and viability of the progeny (Zanuy *et al.* 2001). Of key importance in many flatfish species is the maintenance of broodstock below threshold temperature levels to maintain gamete quality (Bromage & Roberts 1995; Brown *et al.* 2006). A number of flatfish species, for example Atlantic halibut and turbot, do not naturally spawn in captivity which makes it necessary to strip fish manually and fertilize using a wet technique of activating the sperm in sea water before mixing with eggs. It should be noted that recent research in Senegal sole has identified an underlying rhythm in ovulation patterns synchronized with the lunar phases, with spawning production peaking at the new moon (Oliveira *et al.* 2009). The entrainment to lunar cycles is more common in marine teleosts that inhabit lower latitudes, but as they are not recreated in enclosed broodstock facilities their importance in other species should not be ruled out (Takemura *et al.* 2010).

Temperature as an Ultimate Factor

While it is commonly accepted that temperature can greatly influence the quality of the gametes and the subsequent development of embryos by acting as an 'ultimate' factor, there is also a case to be made for its 'proximate' role in controlling the timing of, and commitment to, spawning. While reviewing the photothermal entrainment of reproduction in temperate fish, Wang *et al.* (2010) identified three groups of entrainment.