Genetic management of Atlantic cod (*Gadus morhua* L.) hatchery populations

By

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DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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Abstract

Intensive aquaculture of Atlantic cod is fast developing in both Northern Europe and Canada. The last six years have seen major improvements in the larval rearing protocols and husbandry techniques for this species. Although breeding programmes are currently being developed by both governmental and private institutions in the main cod producing countries (i.e. Norway, Iceland and Canada), most hatcheries still rely on the mass spawning of their own broodstock.

Mass spawning tanks are complex systems where fish are left to spawn naturally and fertilised eggs are collected with the overflowing water, with little or no control over the matings of the animals. Few published studies in other commercial marine species (i.e. turbot and sole) have attempted to analyse the output from such systems using microsatellite markers and several parentage analysis software programs. A review of these publications exposed a lack of consistency in the methods used to analyse such complex datasets. This problem was addressed by carrying out a detailed comparison of two analytical principals (i.e. assignment by strict exclusion and assignment by probabilities) and four parentage software programmes (i.e. FAP, VITASSIGN, CERVUS and PAPA), using the DNA profiles, at 5 loci, from 300 cod fry issued from the mass spawning of a large hatchery cod broodstock tank (consisting of 99 fish). This study revealed large discrepancies in the allocation outcomes between exclusion-based and probability-based assignments caused by the important rate of typing errors present in the dataset. Out of the four softwares tested, FAP (Taggart, 2007) was the most appropriate to use for handling such a dataset. It combined the most conservative method of assignment with the most informative output for the results displayed.

In an attempt to study the breeding dynamics in a cod commercial hatchery, parental contributions to five groups of 300 fry (from five single days of spawning

and from two commercial mass spawning cod tanks) were analysed, based on the genotyping data from eight loci. The parentage results from the exclusion-based analyses revealed that, on a single day, at least 25 to 30% of the total breeding population contributed to fertilised eggs that resulted in viable offspring at 50 and 83 days post-hatch. Family representations were highly skewed - with the marked dominance of a few males - and effective breeding populations were consistently low (approx. 5% of the total breeding population). Parental contribution to a group of 960 codlings - produced following intensive commercial practices (i.e. including successive size gradings and mixing of batches) and belonging to a single graded group - was also analysed, based on the genotyping data from eleven loci. The effective breeding population size of the juvenile batch (c. 14% of the total broodstock population) was two to three times greater than the effective size observed on a single day of mass spawning. The per-generation rate of inbreeding was however relatively high, for this batch alone, at 2.5%. Based on these results, suggestions were made to manage hatchery cod broodstock populations and implement genetic selection.

Early maturation of farmed cod in sea cages (at two or three years old) is a major concern for ongrowers. Understanding the mechanism(s) behind sex determination in cod would probably help the development of a method to control sexual maturation. In an attempt to elucidate sex determination in cod, a protocol to induce gynogenesis was developed. Gynogenetic fish were successfully produced by irradiating cod milt with UV and applying a cold shock (at -6^{o}C) to newly fertilised eggs. However, due to poor survival during larval rearing, only one gynogenetic fish survived long enough to be sexed; not enough to conclude anything on the sex determination mechanism(s) in cod.

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I would like to dedicate this Thesis to Mr. Gary Van Ree.

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Glossary of common and Latin names of fish species

Atlantic cod	*Gadus morhua*
Atlantic halibut	*Hippoglossus hippoglossus*
Atlantic salmon	*Salmo salar*
barramundi	*Lates calcarifer*
brine shrimp	*Artemia franciscana*
channel catfish	*Ictalurus punctatus*
common carp	*Cyprinus carpio*
European seabass	*Dicentrarchus labrax*
gold fish	*Carassius auratus*
haddock	*Melanogrammus aeglefinus*
herring	*Clupea harengus*
Mozambique tilapia	*Oreochromis mossambicus*
Nile tilapia	*Oreochromis niloticus*
Pacific cod	*Gadus macrocephalus*
pollack	*Pollachius virenscontains*
pollock	*Theragra chalcogramma*
rainbow trout	*Oncorhynchus mykiss*
gilthead seabream	*Sparus Aurata*
Senegalese sole	*Solea senegalensis*
turbot	*Scophthalmus maximus*

Glossary of abbreviations and acronyms

ABI	Applied Biosystems
AFLP	Amplified fragment length polymorphism
approx.	approximately
am	"ante meridian" (Latin)
ANOVA	analysis of variance
AS	"aksjeselskap" (Norwegian), translates to "stock company"
bp	base pairs
c.	"circa" (Latin) translates to "about"
CCD	charge coupled device
cf.	"confer" (Latin), translates to "consult"
cm	centimetre
cum.	cumulative
^{o}C	degree centigrade / degree Celsius
^{o}C days	degree days
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dph	days post hatch
EDTA	ethylene diamine tetra acetic acid
e.g.	"exempli gratia" (Latin) translates to "for example"
etc	"et cetera" (Latin) translates to "and other things"
et al.	"et alii" (Latin) translates to "and others"
FAO	Food and Agriculture Organization
FAP	family assignment program
FCR	feed conversion ratio
Freq.	frequency
F1	first generation
F2	second generation
g	gram
HCl	hydrochloric acid
i.e.	"id est" (Latin) translates to "in other words"
Inc.	Incorporation
IoA	Institute of Aquaculture, University of Stirling
ID	identification
KCl	potassium chloride
$KHCO_3$	potassium hydrogen carbonate
kg	kilogramme
L / l	litre
M	mole / molar
m	metre
MAS	marker-assisted selection
MERL	Machrihanish Marine Environmental Research Laboratory
mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre
mm	millimetre

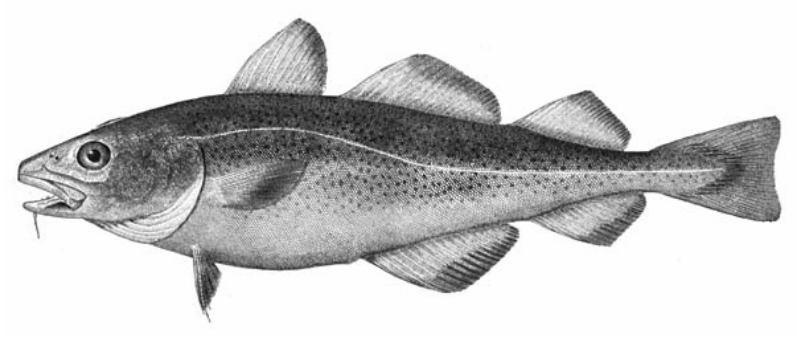
mM	millimolar
MMF	Machrihanish Marine Farm Ltd
MS222	tricaine Methanosulfonate
mtDNA	mitochondrial DNA
n, No or N	number
NCBI	national center of biotechnological information
ng	nanogram
nm	nanometre
Ltd.	Limited
P	probability
PAPA	package for the analysis of parental allocation
PCR	polymerase chain reaction
PF	post-fertilisation
PIL	personal license
PIT	passive integrated transponder
ppm	parts per million
QTL	Quantitative trait locus
Rnase	ribonuclease
rpm	revolutions per minute
s	second
SD	standard deviation
SE	standard error
Spz	spermatozoa
T	T-test value
TAE buffer	tris aetic acid EDTA
Taq	*Thermus aquaticus*
TE buffer	tris EDTA
Tris buffer	Tris(hydroxymethyl)aminomethane buffer
UER	uniform error rate
UK	United Kingdom
URL	uniform resource locator
UV	ultra violet
USA	United States of America
VCR	video cassette recorder
vs.	versus
µg	microgram
µl	microlitre
µm	micrometre
µM	micromolar
µW	microWatt

Chapter 1. General Introduction: Aquaculture of Atlantic cod and molecular tools available for its genetic management

1.1. The Atlantic cod and its potential for the aquaculture industry:

1.1.1. Biology and Geographical Distribution

The Atlantic cod, is a finfish which belongs - like haddock and pollack - to the Gadidae family. Gadoid fish are characterised by an elongated body, three dorsal fins and a barbel located under the jaw (Figure 1.1).

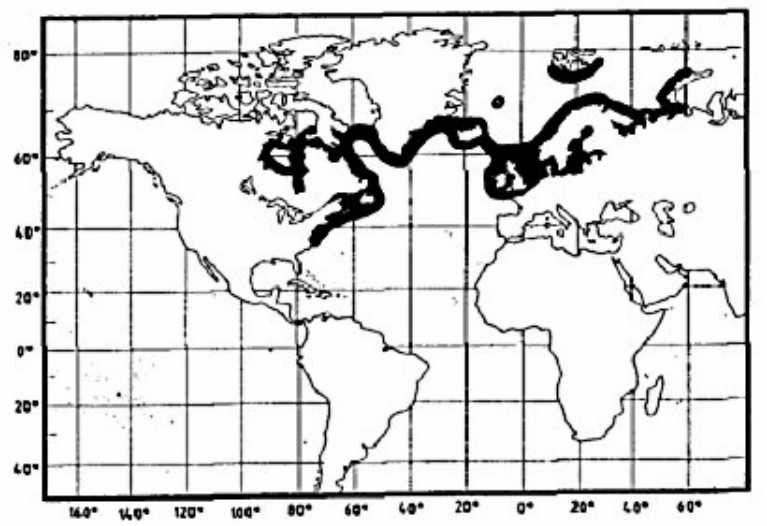


Note: Figure from http://www.biologie.de (2007)

Figure 1.1. Atlantic cod (*Gadus morhua*).

Cod are benthopelagic brackish and marine fish widely distributed in the North Atlantic Ocean, the Baltic and the Barents seas (Walden, 2000). They are naturally encountered at depths of 1-600 m (FishBase, 2003) and are adapted to a temperate climate, with a temperature tolerance between 0 and 20°C (Walden, 2000).

Atlantic cod is a very adaptable species with different stocks present in a large variety of habitats, from immediate shorelines to continental shelves. Cod stocks display annual migration patterns which coincide with both the onset and the end of the spawning season (Lawson and Rose, 2000). Migratory routes followed by fish shoals vary depending on geographic location and life history of stocks. Spawning grounds are mostly located in the shallow waters surrounding the Lofoten Islands, Norway, Greenland, Iceland and Newfoundland (Brander, 1994; see Figure 1.2).



Note: Figure adapted from Choa (2004)

Figure 1.2. Global distribution of Atlantic cod (indicated by ■).

Cod females are extremely fecund. Individual productions per spawning season range from hundreds of thousands to several millions of eggs (Hutchings et al., 1999). Cod females are batch spawners, releasing 15 to 20 batches of eggs throughout a given spawning season (Walden, 2000), every 2 to 6 days (Hutchings et al., 1999). In the wild, females usually mature at 6 years old (when approx. 40-60 cm in length) while males have a tendency to mature at a younger age and smaller size. Cod eggs and larvae are extremely small and fragile. Survival rates, in the wild, are very poor: about 1 egg out of a million succeeds in completing the life cycle (Ryan, 1996).

Cod are omnivorous. They naturally feed on a variety of fish and invertebrates. Growth rates remain highly heterogeneous between cohorts / individuals.

1.1.2. Exploitation of the stocks by the fisheries

Atlantic cod fishery history began around the 10th century, coinciding with the first Viking explorations into the North seas (Gallagher, 2003). Cod fishing became a

real international industry during the 16th century as European countries like Spain, Portugal, France and England started fishing annually for cod on the banks off Newfoundland (Ryan, 1996). This activity reached its golden age almost a century later as the demand for salted dried cod significantly increased worldwide.

During the 19th century, major technical improvements were achieved and resulted in the steady increase of cod landings, until the 1970s, with an annual record of three million tonnes (Globefish, 2003b; Dybdal and Kennedy, 2003). Since then, catches have decreased dramatically over the years, as a direct consequence of overfishing activities. Overall, the total cod catch has fallen by two thirds in 30 years (Globefish, 2003b) and currently, most cod stocks are considered to be under threat of extinction (Walden, 2000; Svåsand et al., 2004). In several countries, measures have already been taken to reduce fishing pressure. Canada closed fisheries off Newfoundland as early as 1992 while Europe progressively introduced fishing quotas which are under review each year.

Those measures became, over the recent years, a source of major political, economic and scientific debates. While scientists claim that cod is still overexploited in many areas, politicians are concerned about the threatened fishery economic sector if further cod fisheries are to be closed down (Esmark, 2004).

1.1.3. Current status of the wild populations

The pressure exerted by the fisheries, over the past decades, seems to have had detrimental and irreversible effects on most wild cod populations. Cod stocks in the North Sea, the Baltic Sea, the Irish Sea and the West of Scotland are reported to be below safe biological limits. In 2002, CITES (the Convention on International Trade

in Endangered Species of wild fauna and flora) threatened to add Atlantic cod to the list of species which can only be traded internationally under restrictions or tight control.

As a result of years of overexploitation, it appears already that important biological parameters such as the age / size at first sexual maturity (Saborido-Rey and Junquera, 1999) or the proportion of recruit spawners have been shifted in several natural stocks. For example, in Norwegian cod stocks, both age and size at first maturation have decreased significantly (Godø and Haug, 1999) and, in the North Sea, the number of sexually mature cod has fallen by more than 90% (Globefish, 2003a-b). Surveys of the Arcto-Norwegian cod stock underlined an important decline in the average age of the population accompanied by an increase in the percentage of first time spawners, from 20 to 80%, between 1930 and 1980 (Larsen, 2002).

1.1.4. Market for cod in the Northern Europe

Despite the alarming situation of the natural resource, the worldwide market for Atlantic cod remains as strong as ever. It is fuelled by a strong market demand originating, for a large part, from Europe (Hjaltason, 2003). In 2006, global catches of cod accounted for 831 000 tonnes (Sackton, 2006) of which 60% were sold in Europe.

The United Kingdom is marketing about 100 000 tonnes of cod each year (Solsletten and Cameron, 2002; Globefish, 2007). A high proportion (~80%) is in fact imported from Iceland, Russia and Norway. The range of marketed cod products is extremely wide but the leading product remains frozen cod fillet (Globefish, 2007). Cod is generally regarded as a convenient family dish and does not suffer from any seasonality in its consumption (Hamnvik, 2004). Due to the collapse of the major cod

fisheries, the last few years have seen a significant increase in the retail prices of cod products (Walden, 2000; Globefish, 2003a-b). Indeed, the retail price of fresh cod fillet in the UK has risen to about £10/kg, which is more expensive than the price of a fresh salmon fillet (~£9/kg). The market size for cod varies between 2.5 and 5 kg.

Two alternatives are currently being exploited in order to supply the cod markets without increasing the fisheries pressure. The first alternative consists in the partial substitution of the lost Atlantic cod catch by Pacific cod. The second and more promising alternative is the development of Atlantic cod commercial aquaculture (Globefish, 2003b).

1.1.5. Aquaculture prospects: brief history, current situation and challenges

Atlantic cod is considered by many aquaculture specialists as a serious candidate for intensive aquaculture in northern Europe and Canada. The recent renewal of interest in cod has been driven primarily by market considerations (Brown et al., 2003) and the opportunity to make cod aquaculture a profitable business.

The first cultivation experiments with cod started as early as in the 1880s when Norwegian and Canadian fishermen were performing artificial fertilisation and incubation onboard of fishing vessels and releasing yolk-sac larvae to stock the sea. The first production of cod juveniles in an enclosed system was performed at the Fløviden Research Station, in Norway, in 1886 (Moksness et al., 2004). Semi-intensive and intensive cod cultivation trials, at a commercial level, were initiated at the end of the 1980s, again in Norway. However, these attempts to start cod aquaculture failed in 1993 due to both the insufficient number of juveniles produced

and a sudden fall in cod market price caused by an unexpected increase in the supply of wild caught fish (Walden, 2000; Rosenlund and Skretting, 2006).

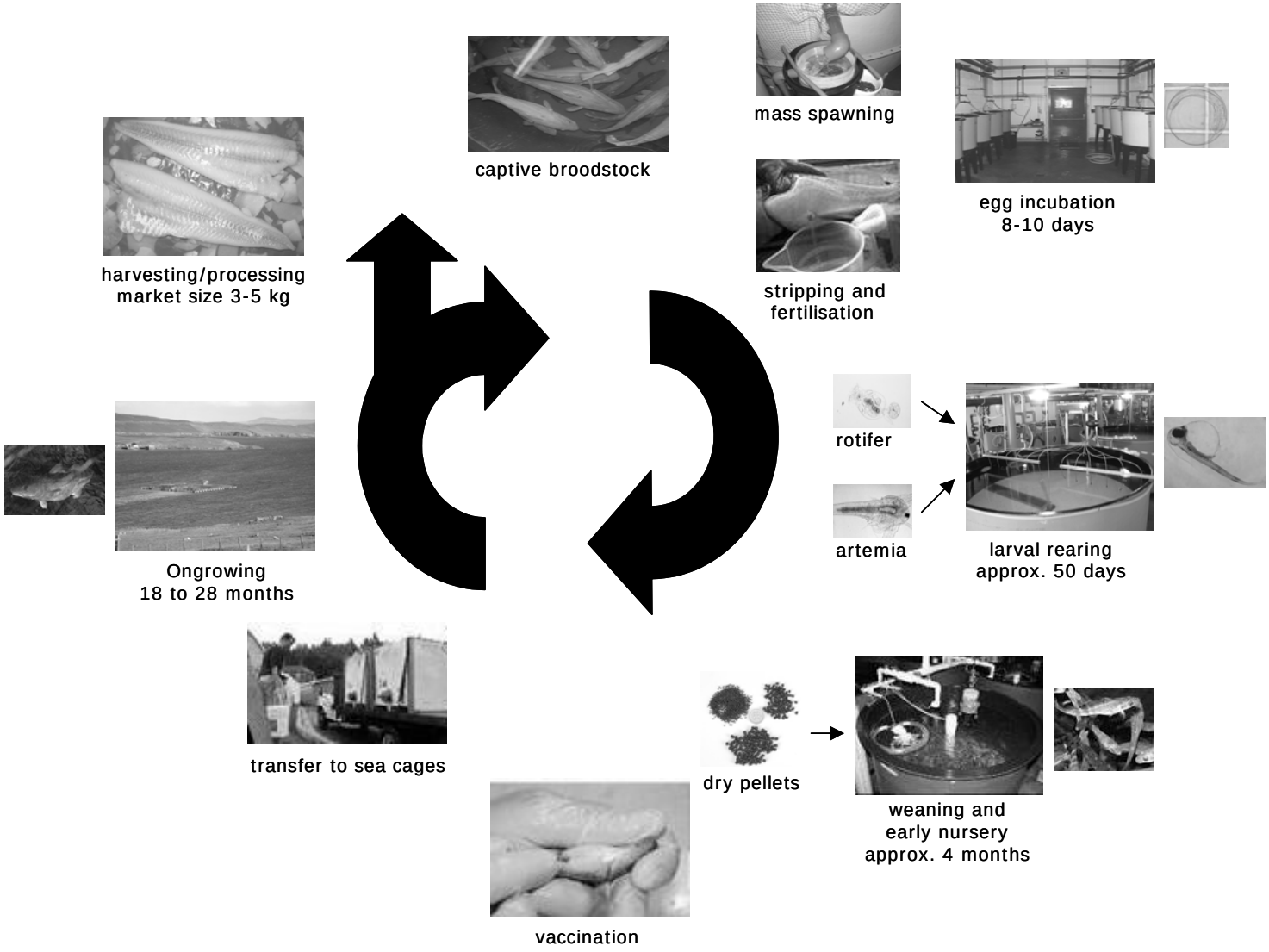
However, since 2000, interest in Atlantic cod aquaculture has been revived worldwide. This time, the context seems to be more favourable for the cod aquaculture industry to finally take off. The considerable progress made in terms of hatchery techniques (thanks to the experience gained with both seabass and seabream mariculture over the preceding ten years) was decisive. The technology required to rear cod juveniles in intensive systems is now available even if scope for improvement still remains in areas such as larval nutrition and health management. Considerable investments have taken place over the last 6 years in Iceland, Norway, Canada and Scotland and rapid development of cod farming is underway.

In Europe, Norway currently leads the cod aquaculture industry. More than 300 licences for commercial farming were issued in 2005 by the Norwegian Directorate of the Fisheries (Standal and Bouwer Utne, 2007). In 2004, Norway produced 3200 metric tonnes of 2.5-5 kg farmed cod (Björnsson et al., 2005). This figure more than doubled two years later (i.e. 5 500 tonnes in 2005). The national production is expected to increase, in the years to come, to approx. 20 000 tonnes (Standal and Bouwer Utne, 2007). Optimistic projections for 2010 envisage a production of 150-200 000 tonnes worldwide (Standal and Bouwer Utne, 2007).

However, despite a promising future, many challenges are still ahead for cod farming. To further develop, the aquaculture sector primarily requires further financial support and, in some countries like Norway, the task has been rendered more difficult since the beginning of the farmed salmon crisis in 2001. In terms of rearing

technologies, and despite the recent research inputs in larval rearing techniques, producing cod larvae is still expensive. Major costs are attributed to the production of enriched live feed (rotifers and brine shrimp) to rear cod larvae during the first few weeks post-hatch (Figure 1.3). Poor larval survival rates, high rates of larval deformities and cannibalism are other areas of concern when producing codlings. Cod ongrowers are also already facing difficulties associated with *Vibriosis* outbreaks, cod escapes and early maturation in net pens.

Commercial cod aquaculture is still at an early stage of development which means that the industry is focusing mainly on rearing protocol "adjustments", which are of immediate application. Long term management of the production, through genetic selection and improvement, starts to stir a lot of interest. Breeding programmes for cod were established in the last five years in Norway, Iceland and Canada (Björnsson et al., 2005; see also section 1.2.3). However, hatchery broodstock populations still almost exclusively consist of captured wild fish (Pavlov et al. 2004).



Note: Figure inspired by www.codgene.ca/latest.php (2007)

Figure 1.3. Intensive cod aquaculture: an overview of the commercial production cycle.

1.2. Genetic management in aquaculture

1.2.1. Domestication of fish species and management of genetic changes

Although the cultivation of aquatic organisms (including fish, shellfish, algae and other aquatic organisms) began about 4 000 years ago in China, the domestication of fish - with humans controlling the entire life cycle of animals from selective breeding in hatcheries to feeding, growout and harvest - is a fairly recent phenomenon (Balon, 1995). Indeed, apart from common carp and gold fish which have both been domesticated for centuries (Balon, 1995; Suquet et al., 2004), the domestication of aquaculture species has, at most, 40 years of history.

The process of domesticating a live organism begins with the establishment of a base population (initially sampled from the wild): both the source of the stock(s) and the initial population size define the framework in which domestication will take place. Then, as time goes on, inevitable genetic changes will affect the genetic make up of this base population (Vandeputte and Launey, 2004; Goodrich and Wiener, 2005). The genetic mechanisms which accompany domestication are of four different types: 1) inbreeding / genetic drift, 2) inadvertent genetic selection, 3) relaxation of natural selection / emergence of selection in captivity and 4) artificial selection (Vandeputte and Launey, 2004).

Both inbreeding and genetic drift arise from the breeding of small isolated / captive populations. Genetic drift represents the gradual changes which occur in the genome pool of a small population when bred repetitively. Losses of “rare” alleles and changes in allelic frequencies are among the most immediate consequences of breeding from a small number of parents. In extreme cases (i.e. only a few fish

contributing to the spawning), important changes in the genetic make up of a hatchery population can occur in a single generation of breeding (Gheyas, 2006).

1.2.1.1. Inbreeding

Inbreeding arises from the breeding of close relatives. Recurrent matings between related parents progressively increase the frequency of homozygous genotypes among offspring. Severe inbreeding rates in fish populations pose the risk of impairing the overall fitness of the stock in terms of survival, growth performance, disease resistance, etc. (Fessehaye et al., 2007). These deleterious effects due to inbreeding are also known as "inbreeding depression". Inbreeding depression has been previously described in sub-populations of farmed Atlantic salmon (Rye and Mao, 1998) and rainbow trout (Su et al., 1996).

1.2.1.2. Inadvertent genetic selection

Inadvertent genetic selection refers to the selection which occurs "unintentionally" in a hatchery environment (Vandeputte and Launey, 2004). Unintentional selection in commercial hatcheries arises as a direct consequence of both hatchery designs and management decisions. Unintentional selection may either have positive or detrimental effects to the performances of a captive stock. For example, Brummett et al. (2004) showed how errors made while measuring fish growth could greatly influence the genetic composition of a small tilapia hatchery broodstock population.

1.2.1.3. Relaxation of natural selection

The rearing conditions experienced by captive fish are usually very different from the rearing conditions experienced by their wild counterparts. Selection

pressures operating in both environments can therefore differ greatly. The relaxation of natural selection corresponds to the reduction in selection pressure, which takes place in captivity, for traits of otherwise great importance in the wild. It can affect traits directly related to body colour, feed intake, reproductive and social behaviours (Vandeputte and Launey, 2004; Mignon-Grasteau et al., 2005). In the long term, the gene pools of wild and cultured livestocks are likely to become very distinct (Mignon-Grasteau et al., 2005).

1.2.1.4. Artificial selection

Artificial selection refers to the process which consists in deliberately selecting for traits of commercial interest among hatchery broodstock populations. The most common traits currently being selected for, in commercial hatcheries, include improved growth rate, resistance to disease and delayed maturation (Su et al., 1999; Henryon et al., 2005; Quinton et al., 2005; Gheyas, 2006). Over time, intensive unidirectional selection is likely to lead to a significant reduction in genetic diversity (Gheyas, 2006). In order to maximise genetic gains while mitigating inbreeding, artificial selection is often implemented as part of a monitored genetic selection programme (Figure 1.4). Genetic selection programmes can address a wide range of "breeding goals" depending on the nature of the aquaculture production (i.e. ornamental fish, fish for human consumption or fisheries restocking). They can be undertaken at various industrial levels: from programmes developed and run by private hatcheries (or group of hatcheries) to "national" programmes developed and run by governmental research institutes (Ponzoni et al., 2007).

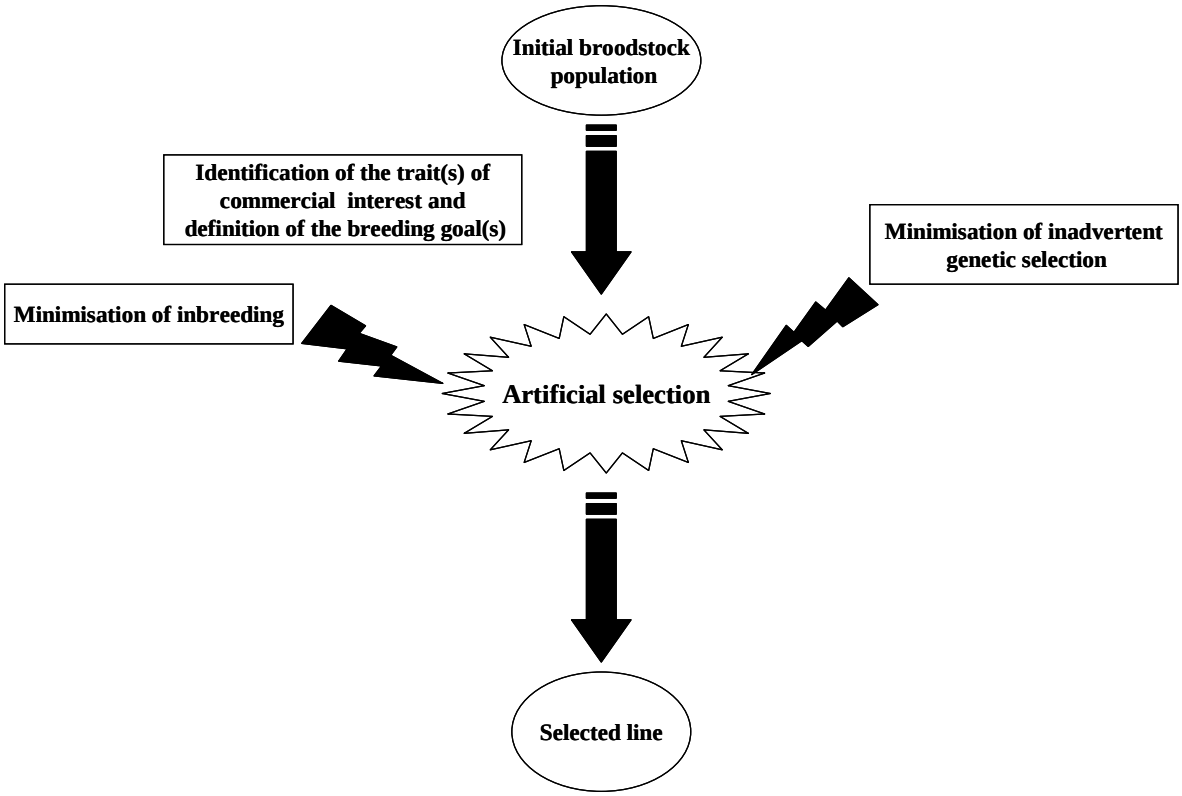


Figure 1.4. Diagram representing the components involved in a genetic selection programme.

1.2.2. Risks associated with poorly managed farmed stocks

Virtually all decisions related to either broodstock management or broodstock replacement are likely to impact the genetic make-up of a given captive fish population. Being misguided and / or oblivious to the consequences of these decisions may ultimately result in the deterioration of the performances of the fish produced (Penman, 2005). Indeed, a poorly managed selection programme can be at least as detrimental to the hatchery production as a "non genetically informed" broodstock management (Sonesson, 2007). For example, in Vietnam, a genetic programme for growth improvement in carp recently had dramatic genetic impacts on the selected stock, after only six generations of mass selection (Penman, 2005). Apart from improving the growth rate of fish by 33% (compared to the base population), the

selection process also resulted in a drastic reduction of the realised genetic heritability (i.e. from 0.20-0.29 to nearly zero).

1.2.3. Progress in the genetic selection of Atlantic cod

Cod aquaculture is still a young industry (see section 1.1.5) which explains why very limited work on cod genetics has been yet published. However, research is currently being carried out, in three countries (i.e. Norway, Canada and Iceland). In East Canada, a three years research programme, co-financed by the Canadian government and several private aquaculture companies, started in 2006 (Codgene, 2007). In Iceland, a national breeding programme ("Icecod"), founded by the Icelandic Marine Research Institute and three private companies, has been running for four years. Finally, in Norway, two breeding programmes have been operating since 2002. The first programme is being carried out at the Fiskeriforskning Institute (Tromsø) and is financed by the Norwegian Ministry of Fisheries and Coastal Affairs. This programme has currently produced about 200 fullsib families from three distinct wild coastal and Arctic cod populations. Early results from the rearing of these families suggest a high heritability for fish growth and a moderate heritability for resistance to vibriosis (Kjersti Fjalestad, personal communication). A second programme is operated by Marine Breed AS (Sunndalsøra), a commercial breeding company partly owned by Akvaforsk Genetics Center AS (Sunndalsøra). Early published results suggest potential for genetic improvement in both growth performance and spinal deformities of Atlantic cod (Kolstad et al., 2006; Imsland and Jónsdóttir, 2002; Imsland and Jónsdóttir, 2003).

1.3. The use of DNA microsatellite markers in the management of aquaculture stocks

1.3.1. DNA profiling

Multilocus DNA microsatellite profiling is a powerful molecular tool which can be used to generate individual-specific genetic fingerprints in both humans and animals (Wright, 1997). It has a large spectrum of applications from species genome mapping to parentage analysis. This technique relies on the existence, throughout the genome, of highly polymorphic, non-coding, short DNA sequences (Park and Moran, 1995; Wright, 1997). Among these polymorphic DNA fragments, some repetitive DNA short sequences containing tandemly repeated nucleotides have been isolated. They are referred to as both "simple sequence repeats" or "microsatellite DNA" (Park and Moran, 1995) for a wide range of animal and plant species. They constitute reliable genetic markers to generate DNA profiles.

1.3.2. Parentage analysis

DNA profiling allows for retrospective determination of parents in systems where products from different crosses are reared together. Parentage analysis, using multilocus DNA profiles, relies on the inheritance principle of alleles which was first uncovered by Mendel in 1865. By conducing various experiments on plant hybrids in the late 19th century, Mendel defined the concept of alleles as being alternative versions of a given gene / locus. He also demonstrated that diploid organisms have two alleles (identical or different) for each gene / locus they possess, each allele being directly inherited from either of both parents. Parentage analysis consists in matching offspring and parents based on their allelic arrangements.

The molecular analyses necessary to built DNA profiles have the enormous advantage that they can rely on non-destructive sampling methods. In aquaculture, such molecular techniques are already routinely used for several freshwater and marine fish species such as salmonids, carps, tilapia, catfish, seabass or halibut (Park and Moran, 1995; Fishback et al., 1999; Neff et al., 2000; Campos Ramos, 2002; Mickett et al., 2003).

1.3.3. Other applications

1.3.3.1. Population genetics

DNA microsatellites are increasingly used in relatedness studies of natural and captive fish populations (Chistiakov et al., 2005). On several occasions, they proved more efficient than allozymes and / or mitochondrial DNA (mtDNA) in detecting genetic variations between fish stocks (Gheyas, 2006). In Atlantic cod for example, analyses of mtDNA between North American wild cohorts concluded on the existence of a "unique" stock. However, further analyses using microsatellite loci, revealed higher levels of genetic variation between cohorts, suggesting the existence of distinctive inshore and offshore wild cod populations (Beacham et al., 1999; Knutsen et al., 2003).

1.3.3.2. Linkage mapping of Quantitative trait loci (QTLs)

Most of the performance traits of commercial importance in aquaculture (i.e. growth, resistance to disease, feed conversion efficiency, etc.) are controlled by the combined expression of several genes and are inherited as quantitative traits (Liu and Cordes, 2004; Zimmerman et al., 2005). Historically, selecting for these complex traits required breeding the best performing individuals / families for generations, which ultimately represented large inputs in time, space and money. The development

of DNA-based genetic markers recently offered new tools to facilitate the selection of quantitative traits. In agriculture, marker-assisted selection (MAS) recently saw a large uptake in a wide range of crop species (including maize, beans and tomato) but still has limited applications in the commercial production of both terrestrial and aquatic livestocks (Ruane and Sonnino, 2007). One essential prerequisite for implementing MAS lies in the identification of loci / genes linked to the quantitative trait of interest (QTLs). To localise QTLs in the genome of plant or animal species, geneticists must resort to linkage mapping. DNA microsatellites are used - often alongside other types of genetic markers - to generate such maps. Genetic maps are available for several fish species including Nile tilapia, channel catfish and rainbow trout (Gheyas, 2006) but they are still of relatively low density (Sonesson, 2007).

1.3.4. Use of DNA microsatellites in cod aquaculture

Over the last ten years, a large number of microsatellite DNA markers were isolated from Atlantic cod DNA (Dahle 1994; Miller et al., 2000; O'Reilly et al., 2000; Jakobsdóttir al., 2006; Wesmajervi et al., 2007). However, only a few of these markers were published at the start of this PhD project. Recent work carried out in Norway, resulted in the production of cod DNA profiles from a pentaplex assay (i.e. simultaneous amplification of 5 DNA microsatellites) (Delghandi et al., 2003; Delghandi et al., 2004). This standardised multiplexing technique was extensively used in the early stages of the Norwegian breeding programmes to solve the parentage of cod juveniles cohorts (Delghandi et al., 2004; Kolstad et al., 2004). In 2006, a research project aiming to construct a first genetic high density linkage map in Atlantic cod began at Fiskeriforskning (Madjid Delghandi, personal communication). As a first step of a project on "Disease resistance in Atlantic cod: construction of a genetic map, QTL mapping and implementing QTLs in a genetic improvement

programme", new DNA microsatellites (approx. 100) were isolated and sequenced (no published data yet available).

1.4. Addressing the problem of early maturation in farmed cod

1.4.1. Current situation

Early maturation constitutes a major problem for cod ongrowers. In net pens, virtually all cod mature at 2 years old (Chambers and Howell, 2006). Fast growing individuals reach sexual maturity at both lower age and shorter length. An increased growth rate, due to good nutritional status, is likely to promote early maturation (Godø and Haug, 1999). This may explain why farmed cod mature earlier than their wild counterparts. Other farmed species such as tilapia, salmon or halibut also mature early when farmed. In cod, it was reported that both males and females down to few hundred grams could mature in commercial seacages (Davie et al., 2007a). Sexual maturation is associated with major losses in body weight (up to 40%) (Hansen et al., 2001; Davie et al., 2007b). Although, to some extent, compensatory growth takes place after the spawning season, both condition factor and FCR of mature fish are poor (Taranger et al., 2004). This increases the time necessary to bring the fish to market size and therefore represents a large loss in terms of profit (Hansen et al., 2001). Early maturation and spawning activity potentially taking place in net pens raise also the problem of farmed egg release in the wild environment. In the near future this issue could be used against the cod farming industry (Esmark, 2004).

1.4.2. Perspectives for improvement

1.4.2.1. Delaying cod maturation using continuous light

There is no obvious external sexual dimorphism in cod, either among maturing or non-maturing individuals when fish are kept under natural conditions (Karlsen and Holm, 1994). However, experimental trials, using photoperiod as a mean to control maturation, suggested the existence of a sex-specific growth pattern. Immature females, kept under those artificial conditions, grow significantly faster than males (Davie et al., 2007b). Good results in delaying maturation were obtained, in tank trials, by applying continuous light (Davie, 2005). However, transposing this technique to seacages is proving extremely challenging and is not efficient.

1.4.2.2. Culture of sterile fish

Ploidy manipulation in Atlantic cod currently stirs a lot of interest. Inducing triploidy proved, over the years, to be an effective way to achieve sterility in several cultured fish species such as Atlantic salmon, rainbow trout, European sea bass and turbot (Ojolick et al., 1995; Cal et al., 2006). However, reported growth performances of triploids greatly vary between species (Peruzzi et al., 2007). A protocol to induce triploidy in cod was recently developed by Peruzzi et al. (2007). High triploidization rates (87%) were achieved by applying a 20 minutes heat shock treatment, at 20^{o}C, on fertilised cod eggs, 20 minutes after fertilisation (Peruzzi et al., 2007). An ongrowing trial is currently underway with the aim to assess growth performances and confirm the sterility of cod triploids (Stefano Peruzzi, personal communication). If cod triploids prove of economic value and can be produced at a commercial scale (despite the technical problems which are likely to accompany the stripping of broodstock), they will be of major interest for cod ongrowers.

1.4.2.3. Monosex culture

Monosex culture presents a clear economic interest not only in the production of fish species with sex-specific growth patterns but also in the production of fish susceptible to reproduce during the grow out period (as it alleviates the occurrence of reproduction altogether). In Nile tilapia, monosex male production significantly improves harvest yields (Mair et al., 1997) while in rainbow trout, monosex female production is economically more advantageous as it stops male from maturing (Galbreath et al., 2003). In cod, scientific evidence supports the existence of differentiated growth rates between sexes (Davie et al., 2007b; see also section 1.4.2.1). Cultivating all sterile (triploid) females might well represent a way forward for cod aquaculture.

1.4.2.4. Breeding programmes

Progressively selecting fish for delayed maturation / increased weight can also prevent sexual maturation from occurring during ongrowing: fast growing fish reach market size earlier, before maturation occurs. This is already the case for Atlantic salmon (Fjalestad et al., 2003) and might also become a reality for cod.

1.5. Introduction to the Thesis: aims and structure of the project

As time will go on, cod breeding programmes are likely to further expand and improve the quality of cod strains dedicated to intensive rearing. As a result, more opportunities will be created for commercial hatcheries to purchase seeds from these specialised breeders. However, it seems reasonable to assume that a significant number of farms will choose to carry on managing their own broodstock.

Most of the cod juveniles currently produced by intensive hatcheries, in Northern Europe, originate from the mass spawning of captive wild broodstock populations (see section 1.1.5). In this system, fish reproduce spontaneously and fertilised eggs are collected at the tank overflow. In this case, very limited control over the reproduction dynamics of the stock can be exerted. Essential parameters - such as the effective breeding population size (Ne), the range of individual contributions to a spawning event, the mating success and the reproduction dynamics in force during a spawning season - are difficult to assess. Without this precious information, farmers are unable to conduct an "informed" broodstock management programme.

In this context, the present research project primarily investigated the genetic transfers which take place in this type of hatchery. To do so, the following questions were addressed:

1/ What is the parental contribution to single (daily) batches of fry produced by a mass spawning broodstock tank?

2/ What are the effects of grading and mixing, during hatchery rearing, on family composition and genetic diversity of cod fry batches?

3/ Which recommendations can be made regarding the development of a genetic management / selective breeding programme for this type of hatchery?

4/ Early maturation of farmed cod in sea-cages raises concern among ongrowers (see section 1.4). Fundamental research is needed to elucidate the sex determination mechanism(s) operating in this species as it, in turn, will inform on the

best maturation control method to apply at a commercial level. In an attempt to study sex determination in cod, this project will investigate the induction of gynogenesis.

Chapter 2. General Materials and Methods

2.1. Collection of biological samples

2.1.1. Presentation of the Broodstock populations studied

Three distinct commercial broodstock populations were studied over the course of this research project. The choice of these stocks was made for both practical and technical reasons (i.e. possibility to access / sample the fish and feasibility to process all the samples).

The first population, referred to as "Norwegian broodstock", was held in a Norwegian intensive commercial cod hatchery near Bergen (Grieg Marine Farms AS, Nedstrand). The study focused on a breeding tank (5 m diameter x 1.5 m deep with a water capacity of about 27 m^3) which contained 99 brood fish of wild origin (coastal North Sea around Rogaland). The fish were introduced into the hatchery in August 2002 at about 2-3 kg weight (age uncertain but estimated to be around three years old). At the time of the study, they had been held in captivity for about a year and progressively introduced to an artificial photoperiod regime to advance the spawning season to early winter.

A second population, referred to as "farmed Scottish broodstock" consisted of 249 broodfish which were bred and reared at Machrihanish Marine Farm Ltd., an intensive commercial cod hatchery located on the west coast of Scotland. According to farm records, the studied fish belonged to the second generation of farmed fish bred from originally less than ten wild cod captured in the coastal Scottish waters. This farmed F2 population supplied fertilised eggs to the hatchery for two years (between 2001 and 2003) before being killed and replaced by wild caught fish due to poor reproductive performances.

The third population, referred to as "wild Scottish broodstock", was made up of 141 broodfish recruited from the wild, in 2003, by Machrihanish Marine Farm Ltd. These fish were caught in Scottish waters, in the Firth of Clyde (West coast). They were transferred to a commercial hatchery broodstock tank (5 m diameter x 2 m deep with a water capacity of about 35 m^3) during the winter 2003. They first spawned in captivity the following spring and were progressively introduced to an artificial photoperiod which brought forward the 2005 spawning season by few months (from the end of January to the end of March).

2.1.2. Sampling of cod juveniles for parentage analysis

Overall, three juvenile sample sets were collected and analysed for parentage during the course of this research project.

The first set of offspring collected consisted of three hundred fry which originated from a single day of spawning (19/11/03) from the "Norwegian broodstock" tank held at Grieg Marine Farms AS. At the time of the sampling, the fry were 83 days post hatch. They already had been size graded (at 15 mm), as part of routine hatchery procedures, and belonged to the smallest graded group.

The second set of fry collected consisted of four separate batches of three hundred fish taken at 50 days post hatch. The fish sampled originated from the mass spawning activities of the "wild Scottish broodstock" tank held in Machrihanish Marine Farm Ltd (MMF). The four fry batches each belonged to a single day of egg collection from the 2005 winter spawn (from the 21st of January to the 31st March). The fry were sampled before any size grading had taken place.

The third and final sample set consisted of 960 fin clips from codling (approx. 20 g) held in a nursery tank at MMF. All the juveniles from the sampled tank originated from the "wild Scottish broodstock" 2005 winter spawn and had been reared under commercial intensive conditions (i.e. involving frequent size gradings and mixing of batches from different spawning dates). As a result, at the time the sampling took place (in July 2005), this nursery tank may have contained codlings from up to six different spawning dates (including the four dates previously sampled).

2.1.3. Fish handling, collection of tissue samples

Experiments involving fish handling complied with the Home Office Animals (Scientific procedures) Act (personal licence No. PIL 60/9637; project licence No.60/2911). Samples of commercial origin were provided by the commercial hatcheries.

2.1.3.1. Anaesthesia

Prior to any physical handling, the fish were systematically anaesthetised. Tricaine Methanesulfonate (MS222) or Benzocaine were both used on large cod. The juveniles however were only anaesthetised using MS222 since the use of benzocaine was reported to cause unexpected mortalities (William Roy, personal communication) in case of specific environmental conditions (i.e. high water temperature, low dissolved oxygen level). The anaesthetic dosage used for both benzocaine and MS222 was of 100 ppm. Minor adjustments to that dosage were made if necessary with large fish.

2.1.3.2. PIT-tagging

Fish kept at the Marine Environmental research Laboratory (MERL) were each injected with a 11.5 x 2.1 mm glass encapsulated alphanumeric transponder supplied by Trovan® (Identify UK Ltd., UK). The transponders (PIT tags) were injected in the muscle located under the first dorsal fin. The ten digits alphanumeric tag codes were read using a Trovan® GR-250 High- Performance portable reader (Identify UK Ltd., UK).

At the time the project started, commercial hatchery broodstock populations had already been tagged with PIT tags or T-bar Floytags, as part of routine procedure.

2.1.3.3. Sampling for DNA analysis

Non-invasive DNA samplings were performed, under anaesthesia, when the fish were of sufficient size (i.e. >5 g). For each individual, a piece of dorsal fin (< 0.5 cm^2) was removed with dissecting scissors and placed in a 1.5 ml Eppendorf tube filled with 95% ethanol.

For parentage analysis studies, batches of fifty and eighty days post hatch cod fry, sampled in 95% ethanol, were provided by the hatcheries.

2.1.3.4. Monitoring of the broodstock populations

The broodstock populations were regularly monitored as part of routine hatchery procedures. The fish were individually looked at on average twice a year: two months before and two months after the spawning season. Prior to the spawning season, each fish was individually sexed (using ultrasound scanning technology), weighted and, for the Norwegian stock only, measured. Complementary observations

on the general health status were also recorded. Following the spawning season, the fish were checked for physical damages and, in case of high stocking densities, the smallest males were culled.

For better clarity throughout the reading of this thesis, the key information on each of the three broodstock population studied is summarised in Table 2.1.

Table 2.1. Summarised information on each of the three broodstock populations studied during the course of this research project.

	"Norwegian"	"wild Scottish"	"farmed Scottish"
Origin	coastal North sea	Firth of Clyde	F2 of less than 10 fish of wild origin
Hatchery	Grieg Marine Farms	Machrihanish Marine Farm	Machrihanish Marine Farm
Number of broodstock	99	141	249
Holding tank	one tank of $27m^3$	one tank of $35m^3$	two tanks of $35m^3$
Mean age (years)	4+	4+	4
Mean weight (kg)	6.3	4.6	NA*
Spawning period	October-December	January-March	NA
Number of loci analysed (broodstock)	8	11	5
Number of fingerlings sampled	300	four sets of 300	0
Sampling dates	19/11/2003	04/02/2005, 18/02/2005, 21/02/2005 and 26/02/2005	--
Age of the fingerlings at sampling	83 days (size graded)	50 days (not size graded)	--
Number of loci analysed (fingerlings)	8	8	--
Number of juveniles sampled	0	960	0
Sampling date	--	July 2005	--
Age of the juveniles at sampling	--	5 months	--
Number of loci analysed (juveniles)	--	11	--

*NA: information not available

2.2. From fish tissue samples to genetic profiling

2.2.1. DNA extraction

Overall, four different DNA extraction techniques were tested on cod samples of various natures (i.e. fin clip, cod larvae and cod eggs). Three techniques (i.e. phenol-chloroform, Chelex and Dynabeads) were chosen based on the published literature available on Atlantic cod (Miller et al., 2000; Clemmesen et al., 2003; Delghandi et al., 2003). The fourth technique (using the REAL pure extraction kit) was tested on cod samples after good results were obtained "in-house" for tilapia, salmon and carp fin samples.

For a given type of cod tissue sample, the average yield of DNA extracted and the level of purification were not consistent between the four techniques tested (see Table 2.2).

The following paragraphs describe each of the four techniques tested on cod samples and mention their main advantages and weaknesses.

Table 2.2. Assessment of four DNA extraction techniques on various types of cod samples.

DNA extraction method		Sample type				Maximum duration of storage at 4°C (to perform PCR amplification)
		broodstock fin clip	50dph* fry	egg	juvenile fin clip	
Phenol Chloroform	quality of the genomic DNA extracted	very good	not tested	not tested	very good	several months
	average quantity of genomic DNA extracted	12.50 µg ± 1 µg	--	--	7.20 µg ± 1 µg	
	suitability for singleplex PCR amplification	✓	--	--	✓	
	suitability for multiplex PCR amplification	✓	--	--	✓	
Chelex	quality of the genomic DNA extracted	poor quality	not tested	not tested	poor quality	2 weeks
	average quantity of genomic DNA extracted	no data	--	--	10.20 µg ± 3 µg	
	suitability for singleplex PCR amplification	X	--	--	X	
	suitability for multiplex PCR amplification	X	--	--	X	
REAL pure extraction kit	quality of the genomic DNA extracted	very good	not tested	not tested	very good	several months
	average quantity of genomic DNA extracted	11.00 µg ± 6 µg	--	--	8.70 µg ± 4.5 µg	
	suitability for singleplex PCR amplification	✓	--	--	✓	
	suitability for multiplex PCR amplification	✓	--	--	✓	
Dynabeads Universal extraction kit	quality of the genomic DNA extracted	very good	very good	very good	very good	4 weeks
	average quantity of genomic DNA extracted	1.00 µg ± 0.5 µg	2.40 µg ± 1 µg	0.05 µg ± 0.03 µg	0.12 µg ± 0.1 µg	
	suitability for singleplex PCR amplification	✓	✓	✓	✓	
	suitability for multiplex PCR amplification	✓	✓	X	X	

*dph: days post hatch

2.2.1.1. Phenol-chloroform extraction

Considered by many geneticists as the standard procedure for extracting DNA from any organic tissue sample, this technique relies on the ability of neutralised phenol and chloroform to separate proteins from nucleic acids when they both coexist in solution. Phenol-chloroform extraction was previously successfully performed on adult cod fin clips by Clemmesen and colleagues (2003).

This extraction protocol consisted of six consecutive steps and was designed to be carried out in 1.5 ml Eppendorf tubes. A small piece of tissue sample (approx. 0.25 cm^2) was first digested overnight, at 55°C, by 12 µl of proteinase K (20 mg/ml) in the presence of 340 µl of 0.2 M EDTA. The digested sample was further treated with 10 µl of RNase (2 mg/ml) for one hour at 37°C. DNA was then extracted using 340 µl of pure phenol followed by 340 µl of pure chloroform. The aqueous phase containing the DNA was separated from the organic phase containing the rest of the organic material by centrifugation at 1200 rpm for 10 minutes. The upper aqueous phase, containing the DNA, was then pipetted out and placed in a new 1.5 ml Eppendorf tube. The DNA was consecutively precipitated by adding 900 µl of 92% ethanol and shaking vigorously. A washing step using 70% ethanol was then performed before the DNA was finally resuspended in 0.1x TE buffer (1x TE buffer is 10 mM Tris, 1 mM EDTA, pH 8.0).

2.2.1.2. Chelex extraction

Chelex is a chelatin resin which acts as a powerful adsorbent for separating charged ions or molecules from proteins. This property was used to develop an extraction protocol for DNA (Walsh et al., 1991; Estoup et al., 1996; Yue and Orban, 2001) which is regarded as a quick and inexpensive method to obtain genomic DNA

from any organic substrate. Miller et al. (2000) reported using this technique to extract DNA from cod samples of blood, heart and muscle fibres.

The following protocol was first designed to extract DNA from salmonid fin clips in 0.2 ml PCR 96 well plates (John Taggart, personal communication) and was later transposed to cod.

A small piece of fin tissue (approx. 0.25 cm^2) was digested overnight at 55°C, by 3 µl of proteinase K (10 mg/ml) in the presence of 100 µl 10% Chelex solution. Centrifugation (1200 rpm for 1 minute) at the end of the incubation achieved the separation of the proteins adsorbed onto the Chelex beads from the upper aqueous phase containing the DNA.

2.2.1.3. DNA extraction using the REAL pure DNA extraction kit (Thistle Scientific, UK)

This DNA extraction kit was specifically designed for the extraction of high quality genomic DNA from a wide variety of tissue and fluid samples. It was successfully tested on salmon, tilapia and carp fin samples in the Institute of Aquaculture (IoA) molecular biology laboratory. The kit included three solutions: a cell lysis buffer, a protein precipitate solution and a DNA resuspension buffer.

The following protocol was adapted from the manufacturer's instructions to perform extractions in 0.2 ml PCR 96 well plates (Ninh Huu Nguyen, personal communication). About 0.25 cm^2 of tissue was digested overnight, at 55°C, in 3 µl of proteinase K (10 mg/ml) and 75 µl of cell lysis solution (part of the extraction kit). 3 µl of RNase was then added to the digested sample and the solution was further incubated for 1 hour at 37°C. Protein residues were precipitated by adding 45 µl of

the protein precipitate solution and then centrifuging at 4100 rpm for 20 minutes. 50 µl of the supernatant, which contained the DNA, was then transferred to a new PCR well filled with 75 µl of pure isopropanol. Centrifugation at 4100 rpm for 10 minutes followed in order to precipitate the DNA from the aqueous phase (it formed a pellet at the bottom of the tube). The isopropanol was then washed off with 150 µl of 70% ethanol. A last centrifugation at 4100 rpm for 5 minutes was carried out before entirely removing the ethanol from the well (by turning the plate upside down). The DNA pellet was finally resuspended in 15 to 105 µl of 0.1x TE buffer depending on the nature of the sample.

2.2.1.4. DNA extraction using magnetic beads (Dynabeads® genomic universal DNA kit, Invitrogen, UK)

Dynabeads® are uniform superparamagnetic monodisperse polymer particles which were designed to adsorb DNA molecules to their surface. This technique was previously reported to successfully extract DNA from Atlantic cod preserved tissue samples : blood, fertilised eggs and larvae (Delghandi et al., 2003). The following protocol was adapted to suit 0.2 ml 96 PCR well plate extraction (Madjid Delghandi, personal communication).

Up to 0.25 cm^2 fish tissue was digested by 4 µl of proteinase K (10 mg/ml) in the presence of 96 µl of Dynabeads slurry at 55°C for 4 hours (total volume of digestion buffer = 100 µl). The DNA/Dynabeads® complex was washed twice using the buffer provided in the extraction kit. The DNA was then separated from the magnetic beads by incubation at 60°C for 15 minutes in 10 to 40 µl of 0.1x TE buffer depending on the nature of the sample.

2.2.1.5. Selection of the best suited DNA extraction method for large scale genotyping

Out of the four DNA extraction methods described above, the technique using magnetic beads (Dynabeads® DNA Direct™ universal kit, Invitrogen, UK) and the REAL pure extraction kit (Thistle Scientific, UK) were selected for large scale genotyping on cod larvae and fin clip tissue samples.

The phenol-chloroform method, despite achieving high yields of very good quality DNA, could not be adapted to suit DNA extraction in 96 well plates. The Chelex extraction method failed to deliver consistently good quality DNA which was required in order to successfully perform multiplex polymerase chain reactions.

The shelf life of the extracted DNA varied depending on the extraction method used. DNA molecules extracted by the Chelex method could be stored at 4^{o}C for up to two weeks. However, when using both the phenol-chloroform and the REAL pure DNA extraction kit, the storage of extracted DNA at 4^{o}C would lasts several months (see Table 2.2).

2.2.2. DNA quantification

The quantity and the purity of total genomic DNA extracted were assessed using a nanodrop ND-1000 spectrophotometer (Labtech International, UK). The nanodrop ND-1000 is a full spectrum (220-750 nm) spectrophotometer which operates by measuring the concentration of nucleic acids in 1µl samples. It also assesses the purity of DNA by measuring the ratio of sample absorbance at 260 and 280 nm. A ratio which equals to 1.8 indicates that the extracted DNA is pure while a ratio below this value indicates that protein residues or other contaminants are

present in the sample. Figures 2.1 and 2.2 show the concentration and purity results for DNA extracted from cod juvenile fin tissues, using both phenol-chloroform and Chelex. The DNA samples extracted with Chelex beads (Figure 2.2) had a ratio of absorbance (260 nm/280 nm) of 1.51 suggesting that the purity of the sample was poor.

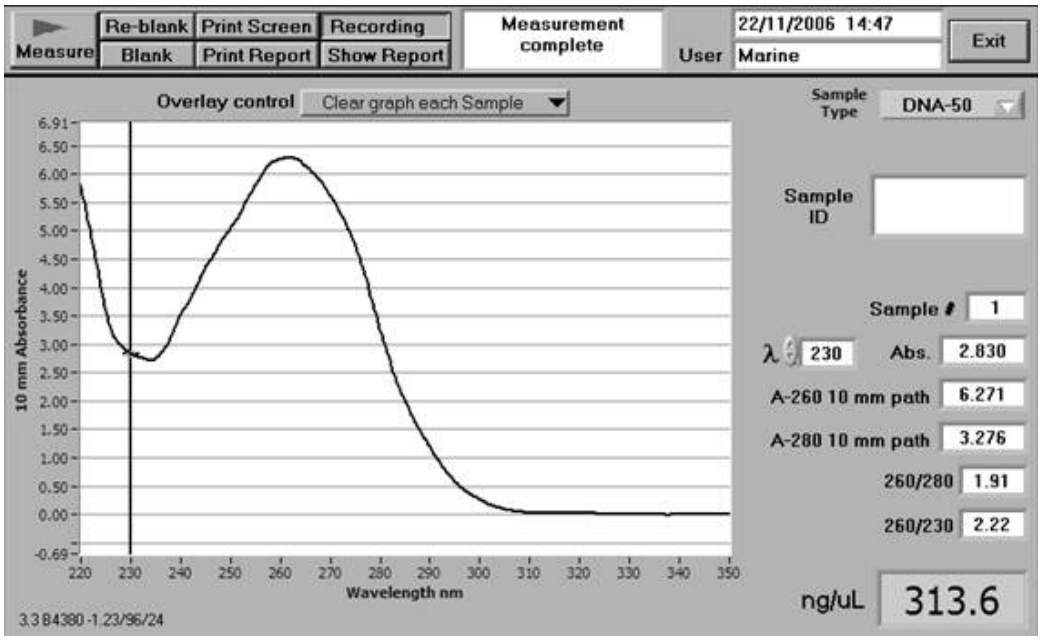


Figure 2.1. Concentration and purity of DNA extracted from cod juvenile fin tissue using phenol-chloroform.

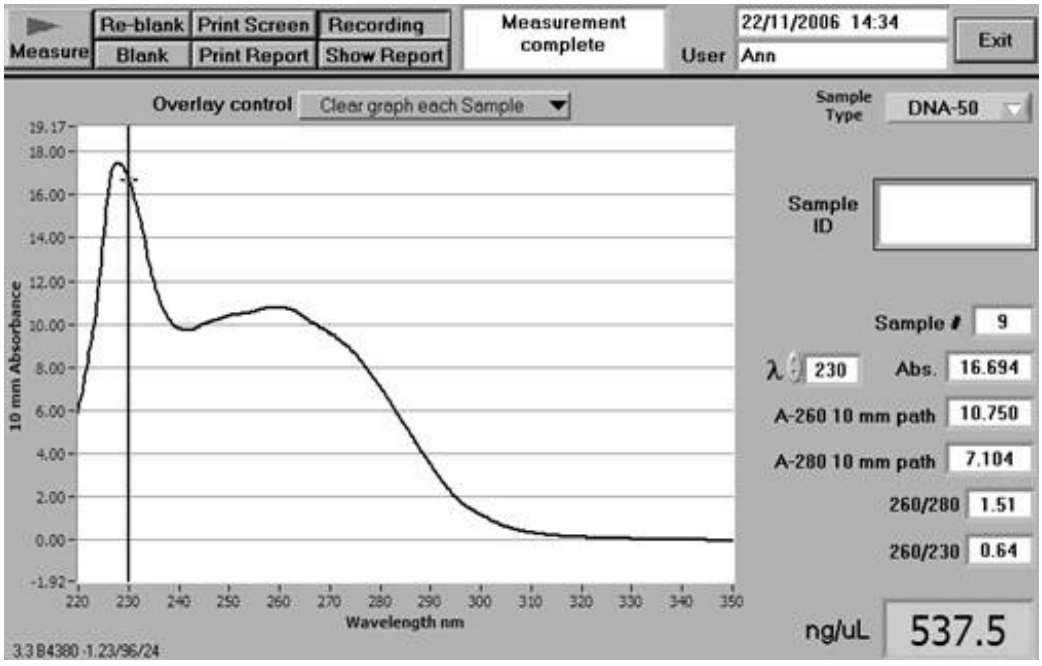


Figure 2.2. Concentration and purity of DNA extracted from cod juvenile fin tissue using Chelex.

2.2.3. Polymerase chain reaction

The polymerase chain reaction or PCR is a molecular biology technique in which numerous copies of targeted DNA segments are synthesised using DNA polymerase. This technique was used to amplify polymorphic DNA microsatellite markers.

2.2.3.1. DNA microsatellites used in the multilocus DNA profiling of cod samples

When this project initially started, only seven polymorphic DNA microsatellites had been readily made available for Atlantic cod (Miller et al., 2000). Thirteen additional markers sequenced from the genomic DNA of walleye pollock were also reported to successfully cross amplify with Atlantic cod DNA (O'Reilly et al., 2000). Subsequently, a DNA profiling protocol, using a combination of six of these published markers, was developed and tested on small breeding cod populations (<20 individuals) by Delghandi et al. (2003). Following this study, Wesmajervi and colleagues (2006) solved the parentage of 2336 cod juveniles from a hundred potential parents, based on the genotyping data from 5 loci (i.e. one locus from the previously published hexaplex assay (Delghandi et al., 2003) was dropped). The rate of unambiguous allocations reported, with this pentaplex assay, was 91.2% using the parentage program PAPA (see section 2.4.2.1.).

Towards the end of this project, Delghandi and collaborators reported the isolation of 105 new microsatellite sequences from genomic Atlantic cod DNA (Madjid Delghandi, personal communication) some of which were posted on the Genbank database in August 2006 (NCBI webpage, 2007).

In the first instance, the five DNA microsatellites used by Wemajervi et al. (2006), were employed to solve the parentage of cod fry (see Chapter 3). Then, as the project progressed, new markers were included in the genotyping analysis until a total of eleven markers were used to allocate juveniles to a single pair of parents. The list and description of the markers used in this research project are provided in Table 2.3. Either the forward or the reverse primer, depending on the marker, was fluorescently labelled for detection of PCR products on an automated fragment analyser (see section 2.2.4.2).

Part of the genotyping analyses were realised at the molecular biology laboratory in Fiskeriforskning, during two visits of 4 weeks (November 2004 and February 2007). All the DNA samples were processed and analysed by myself.

Table 2.3. Description of the polymorphic microsatellite markers used in the DNA profiling of Atlantic cod samples.

Microsatellite Name	Primer Sequence (5'-3')	Tandem repeat (bp)	Allele size range (bp)	Annealing temperature (^{o}C)	Reference
*Gmo*8	F: GCA AAA CGA GAT GCA CAG ACA CC R: TGG GGG AGG CAT CTG TCA TTC A	4	126-322	50	Miller et al. (2000)
*Gmo*19	F: CAC AGT GAA GTG AAC CCA CTG R: GTC TTG CCT GTA AGT CAG CTT G	4	120-224	50	”
*Gmo*35	F: GGA GGT GCT TTG AAG ATG R: CCT TAT CAT GTA CGT TGT TAA C	3	113-146	55	”
*Gmo*37	F: GGC CAA TGT TTC ATA ACT CT R: CGT GGG ATA CAT GGG TAC CT	4	236-320	46	”
*Gmo*3	F: AGG CAC GCA GGT GGA CAG GAA C R: GCA GCA CGA GAG AGC TAT TCC TC	4	182-211	46	”
*Gmo*34	F: TCC ACA GAA GGT CTC CTA A R: GGT TGG ACC TCA TGG TGA A	4	89-117	50	”
*Gmo*36	F: ACC GCA TGC CCT TTT CA R: GGT GAT GGA GGC TCT AGT	4	182-202	50	”
*Tch*11	F: ATC CAT TGG TGT TTC AAC R: TCG AGT TCA GGT GGA CAA	4	118-218	50	O'Reilly et al. (2000)
*Gmo*C18	F: AAGCATGCGTTTGTGTTATTAC R: ATCTGTTCTCGCTTTCCTTCATT	3	140-182	49 53	Stenvik et al (2006)
*Gmo*C20	F: CTGCCAAAGCCTGTGACG R: GATGGTGGTGTTGATTGTGGTTGT	3	104-167	52 57	”
*Gmo*C42	F: CCCCCTTGATCCTGTAGACGGTTAT R: GTTTCGGAAGACGGATGGTG	3	155-176	59 54	”
*Gmo*C52	F: ATAACCCCATAGCTCCACGAAAACC R: GATAAGGCACCAGCAGGGAGACGA	3	283-304	60 62	”
*Gmo*C71	F: TGACGATACATTCAAGAGCACCAC R: GTTTCTTAAGAACCAGCACACGATTTGACA	2	203-233	56 56	”
*Gmo*C80	F: ACAACGCTGCTGAAGGAGGAA R: GTTTCTTACCCCGACACGTCAATCACC	2	126-213	56 57	”
*Gmo*C88	F: CAGCAATAGGACTGTTCACTGGAA R: GTTTCTTCACAAGATCAACCTGCATTTACCT	2	181-203	55 54	’’
*Gmo*C90	F: TCC CCT GGC CCG TTG ATG TA R: GTT TCT TAC CAG GCG TGA TTG TGA TTA GCA G	2	252-342	56	Unpublished*

*Unpublished: Delghandi (personal communication)

2.2.3.2. Multiplex PCR protocols

This technique refers to a type of PCR which consists in co-amplifying several DNA microsatellites in a single reaction. Several multiplex assays were successively used for genotyping cod DNA samples. Three multiplex assay protocols were borrowed from both published and unpublished research works by Dr Madjid Delghandi and colleagues, when working at the Fiskeriforskning Institute (Norway, Tromsø) in November 2004 and February 2007. Three other multiplex assays (one tetraplex and two duplexes) were further developed at the IoA.

*Gmo*8, *Gmo*19, *Gmo*35, *Gmo*37 and *Tch*11 were coamplified as a pentaplex assay as described by Wesmajervi et al. (2006). A tetraplex using the markers *Gmo*8, *Gmo*19, *Gmo*35 and *Gmo*37 was adapted from this pentaplex to suit the automated detection of up to four markers on the Beckman CEQ8800 (*cf.* section 2.2.4.2.1). The 10 µl reaction contained 10-30 ng of DNA template, 1x PCR-buffer (10 mM Tris-HCl; pH 8.3, 50 mM KCl), 400 µM of each dNTP, 1.5 mM $MgCl_2$, 0.4 µM of each primer (i.e. forward and reverse) for *Gmo*19 and *Gmo*35, 0.2 µM of each primer for *Gmo*8, 0.3 µM of each primer for *Gmo*37 and 0.5 U of *Taq* polymerase (ABgene, UK). The PCR amplification program was: initial denaturation at 95°C for 5 minutes, 34 cycles of 95°C for 35 seconds, 57°C for 35 seconds, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes.

*Gmo*3, *Gmo*34, *Gmo*36 and *Tch*11 were coamplified as two duplexes. The 10 µl PCR reaction for the coamplification of the markers *Gmo*3 and *Gmo*34 contained 10–30 ng of DNA template, 2x PCR-buffer, 150 µM of each dNTP, 1.5 mM $MgCl_2$, 1.3 M Betaine (Sigma Aldrich), 0.3 µM of each *Gmo*3 primer, 0.6 µM of each *Gmo*34 primer and 0.5 U of *Taq* polymerase (ABgene, UK). The

PCR amplification was: initial denaturation at 95°C for 2.5 minutes, followed by 34 cycles of 95°C for 50 seconds, 48°C for 50 seconds, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. The coamplification protocol for markers *Gmo*36 and *Tch*11 was identical to that for *Gmo*3 and *Gmo*34 except that all four primers were at a concentration of 0.6 µM and the annealing temperature within the PCR cycle was 50°C.

*Gmo*C18, *Gmo*C20, *Gmo*C42, *Gmo*C52, *Gmo*C71, *Gmo*C80, *Gmo*C88 and *Gmo*C90 were coamplified as two tetraplex PCR reactions developed at the Fiskeriforskning Institute. Both 10 µl PCR reactions contained 10–30 ng of DNA template, 1x QIAGEN Multiplex PCR Master Mix (Qiagen, UK) and 0.1 µM of each *Gmo*C18, *Gmo*C20, *Gmo*C42 and *Gmo*C52 primers (or *Gmo*C71, *Gmo*C80, *Gmo*C88 and *Gmo*C90 primers). Both PCR amplifications were: initial denaturation at 95°C for 15 minutes followed by 32 cycles of 94°C for 30 seconds, 56°C for 90 seconds, 72°C for 1 minute, and a final extension step at 72°C for 30 minutes.

PCR reactions were carried out on 96 well plate ThermoCyclers (T-gradient 96 ThermoCycler, Thistle Scientific; Applied Biosystems 2720, Applied Biosystems).

2.2.4. DNA fragment analysis

Amplification of DNA fragments was checked on Ethidium bromide stained agarose gels before being analysed using an automated DNA sequencer.

2.2.4.1. Agarose gel electrophoresis

For a medium size gel (i.e. 35 ml), 0.42 g of agarose powder was diluted and boiled in 35 ml of 0.5x TAE buffer (1x TAE buffer = 40 mM Tris, 1 mM EDTA,

20 mM Acetic acid, pH 8.5). While the gel mix was still liquid, 0.7 µl of 10 µg/ml Ethidium bromide was added (in a fume cupboard). The gel was then poured in a casting tray with a comb inserted in one end and left to set. About 1.5 µl of PCR products and 3.5 µl of 3x Bromophenol blue dye (1x Bromophenol blue dye = 25 mg/ml Ficoll 400, 83 µg/ml Bromophenol blue, 83 µg/ml Xylene cyanol FF) was loaded per well. The DNA samples were run against a DNA ladder (Phi X 174, digested with HaeIII, 100 µg/ml) in order to control the size of the fragments amplified (1 µl of the DNA ladder and 4 µl of 6x Bromophenol blue dye per well). After electrophoresis migration in 0.5x TAE buffer, PCR products could be visualised using a UV transilluminator (UVIdoc, Thistle Scientific, UK) as shown in Figure 2.3.

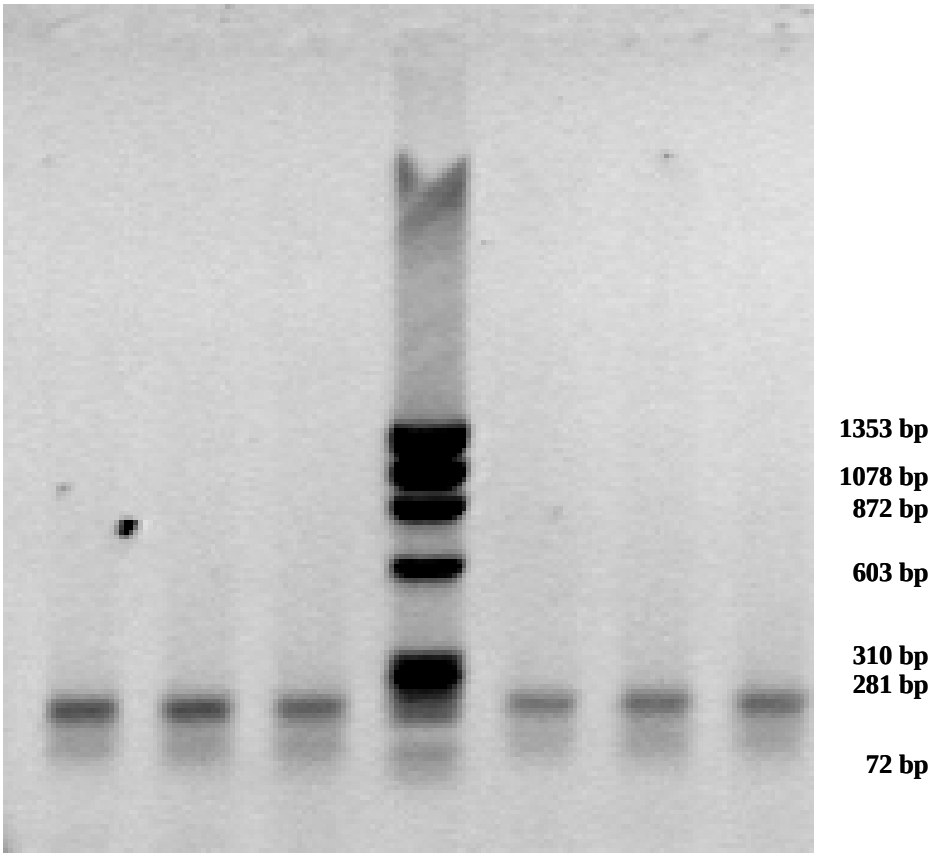


Figure 2.3. Image of a 1.2% agarose gel showing multiplex PCR products (tetraplex of *Gmo*8, *Gmo*19, *Gmo*35 and *Gmo*37) and the Phi χ 174 DNA ladder.

2.2.4.2. Genetic analysis systems

The PCR amplified DNA fragments were processed on two different laser-based capillary electrophoresis instruments: an ABI 310 Avant Genetic analyser (Applied Biosystems) and/or the CEQ ^{TM} 8800 Genetic Analysis System (Beckman Coulter). Some markers were analysed using both the ABI 310 and CEQ 8800 for cross-calibration between the two instruments (see Chapter 4).

2.2.4.2.1. Beckman-Coulter CEQ ^{TM} 8800

The CEQ 8800 is fitted with a laser sensitive to the detection of four fluorescent wavelengths. Primers used in the synthesis of DNA fragments - for subsequent analysis on the CEQ 8800 - were extended with WellRED dye terminators (Beckman Coulter, UK). PCR products were processed on 0.2 ml 96 well-plates, each well containing 0.8 µl of undiluted PCR products, 28 µl of formamide solution, 0.25 µl of labelled size standard (60 bp - 400 bp) and a drop of mineral oil (all chemicals supplied by Beckman Coulter, UK). Allele sizes of the amplified DNA fragments were called using the CEQ 8800 data analysis software (see Figure 2.4).

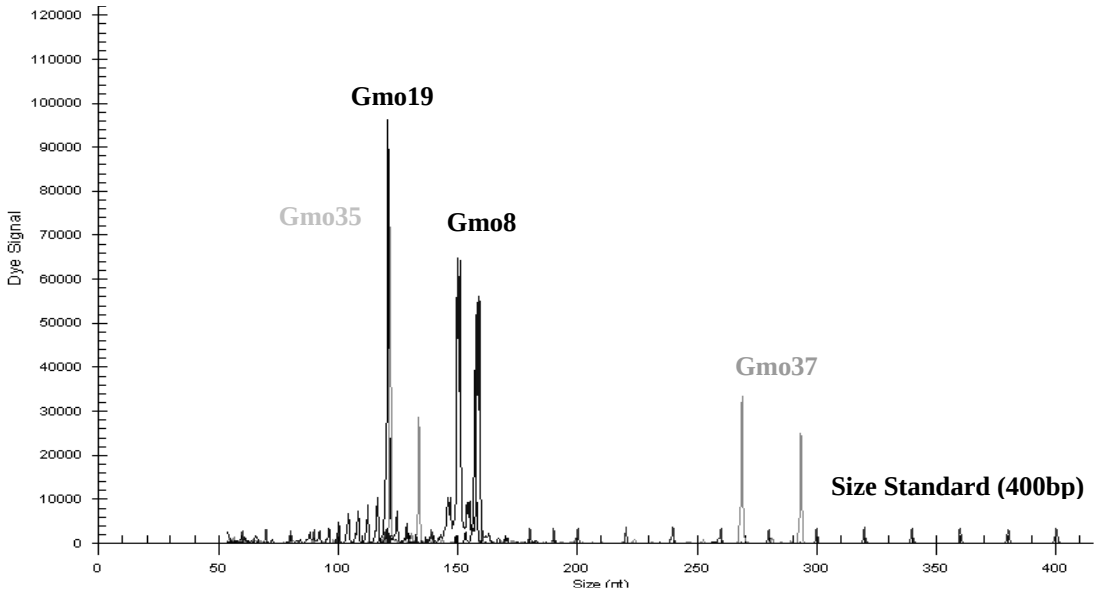


Figure 2.4. Genotyping traces of tetraplex PCR products analysed with the Beckman CEQ8800 automated sequencer.

2.2.4.2.2. ABI 310 Avant

Up to five fluorescent wavelengths could be detected by the ABI laser, thus allowing for the microsatellite primers to be labelled with up to four different dyes: 6-Fam, Vic, Ned and Pet (Applied Biosystems, Norway). PCR products were processed on 0.2 ml 96 well-plates, each containing 3 µl of 20x diluted PCR products, 8 µl of formamide solution and 0.08 µl of GS500liz size standard (35 bp – 500 bp). Allele sizes were analysed using the software programs Genescan 3.7 and Genemapper 3.7 from Applied Biosystems.

2.3. Characterisation of DNA microsatellites

The DNA microsatellites used in this research project (see Table 2.3) were characterised by parameters which contributed to their suitability for being included in parentage analyses. Those parameters also provided a mean of comparison between the three broodstock populations studied.

2.3.1. Verification of Mendelian inheritance

Two test crossings were set up at MERL, alongside the gynogenesis experiments (see Chapter 6), to confirm the inheritance of the sixteen microsatellite markers used for parentage analysis in this research project. The first test crossing was set up during the 2004 spring spawning season to verify the Mendelian inheritance of the microsatellite markers *Gmo*8, *Gmo*19, *Gmo*35, *Gmo*37, *Gmo*3, *Gmo*34, *Gmo*36 and *Tch*11. A second test crossing was carried out during the spring season 2006 to verify the Mendelian inheritance of the markers *Gmo*C18, *Gmo*C20, *Gmo*C42, *Gmo*C52, *Gmo*C71, *Gmo*C80, *Gmo*C88 and *Gmo*C90. The two test crossings followed the same experimental protocol. One male and one female, from the cod broodstock kept at MERL (wild origin, 4+ years old in 2004, natural photoperiod), were stripped for eggs and milt. They were also fin clipped (for subsequent DNA analysis). About 1000 eggs were artificially fertilised by 3 ml of undiluted milt in a 25 ml universal vial containing 15 ml of seawater. The fertilised eggs were incubated in a 500 ml glass beaker (as described in Chapter 6, section 6.2.3.1). Ten hatched larvae were sampled in 95% ethanol for subsequent DNA analysis. DNA profiles from both parents and offspring were analysed (see section 2.2) and compared. All the 16 microsatellite markers complied with the Mendelian inheritance law (i.e. each offspring inherited one allele from its father and one allele from its mother; see Table 2.4).

Table 2.4. Results of the test crossing to confirm Mendelian inheritance for seven microsatellite markers.

	*Gmo*8	*Gmo*19	*Gmo*35	*Gmo*37	*Gmo*3	*Gmo*34	*Gmo*36
Female	150/295	121/196	128/137	269/293	195/195	101/105	194/201
Male	150/158	121/157	122/134	269/293	195/195	110/110	194/201
Offspring1	150/150	121/121	122/137	269/293	195/195	105/110	201/201
Offspring2	150/158	121/157	122/128	269/293	195/195	101/110	194/201
Offspring3	158/295	121/157	134/137	269/293	195/195	105/110	194/201
Offspring4	158/295	121/157	128/134	269/293	195/195	101/110	194/201
Offspring5	150/295	121/121	122/137	293/293	195/195	105/110	194/194
Offspring6	150/150	121/121	122/137	269/293	195/195	105/110	194/201
Offspring7	150/295	121/121	128/134	293/293	195/195	105/110	201/201
Offspring8	150/295	121/196	122/128	293/293	195/195	105/110	201/201
Offspring9	158/295	157/196	122/137	269/293	195/195	101/110	194/201
Offspring10	150/158	121/121	128/134	269/293	195/195	105/110	194/201

Note: allele sizes are expressed in base pairs.

2.3.2. Polymorphism and allelic frequencies

The concept of allelic polymorphism is synonymous to the number of alleles (n) encountered at a single locus. Allelic frequencies (F) were calculated, for a given fish population, using both the parentage analysis program VITASSIGN (Vandeputte, 2006) and the population genetics program GENEPOP (Raymond and Rousset, 1995).

If "A", "B" and "C" stand for the three different alleles encountered at a particular locus and if "F(AA)", "F(AB)", "F(AC)", "F(BB)", "F(BC)"and "F(CC)" represent the genotype frequencies for each possible allelic combination, then the frequency of allele "A" is:

$$\mathbf{F(A) = F(AA) + 0.5F(AB) + 0.5F(AC)}$$

with $\mathbf{F(AA) + F(AB) + F(AC) + F(BB) + F(BC) + F(CC) = 1}$

2.3.3. Expected and observed heterozygosity

Both the expected and the observed heterozygosity were calculated for each marker used to build the genetic profiles of the three broodstock populations studied. Both parameters were used in the first instance to assess and compare the genetic diversity of each stock (see Chapter 4). They also proved extremely useful in spotting high frequencies of typing errors (i.e. large allele dropouts and allele miscallings) in the Norwegian study case (see Chapter 3).

The observed heterozygosity (Ho) for a given diploid population equals:

$$\mathbf{Ho = \sum^{n}_{i=1} (1 \text{ if } A_{i1} \neq A_{i2}) / n}$$

with "n" the number of individuals in the population and "A_{i1}", "A_{i2}" the alleles possessed by the individual "i" at the locus of interest.

The expected heterozygosity (He) for a given diploid population equals:

$$\mathbf{He = 1 - \sum^{m}_{j=1} (f_j)^2}$$

with "m" the number of alleles at the locus of interest and "f_j" the frequency of the j^{th} allele.

Both observed and expected heterozygosity were calculated using features of the parentage analysis software program CERVUS.

2.3.4. Effective number of alleles

Effective numbers of alleles (AE) - at a given locus - were calculated from the expected heterozygosity values (Hexp) given by CERVUS in the "allele frequencies" analysis output:

$$AE = 1 / (1 - Hexp)$$

2.4. Parentage assignment

In total four different parentage assignment software programs were used and compared (see Chapter 3) during the course of this research project. They can all be downloaded or directly requested from the authors free of charge.

2.4.1. Exclusion-based programs

2.4.1.1. Family Assignment Program

FAP (Family Assignment Program) was developed "in-house" at the Institute of Aquaculture (Taggart, 2007). This program offers two complimentary functions: 1) a predictive mode to calculate the resolving power of specific parental genotypic data sets for unambiguously discriminating among families / groups of families (achieved by complete enumeration of all possible genotypic combinations); and 2) an assignment mode to identify all possible parental combinations for each offspring based on the exclusion principle. The latter mode includes the option to allow a specified level of allele mismatch tolerance to accommodate / identify potential genotype scoring errors. Three classes of assignment are possible: "single-match" – where only one parental-pair is allocated; "multi-match" – where two or more potential parental-pairs are identified (all are listed); and "no-match" – where all potential parental-pairs are excluded (indicating errors in data provided). When

single or multiple allocations occur with mismatches, the program identifies the relevant problematic locus / loci. The program was primarily designed for a "closed" environment, i.e. all potential parents are known (genotyped).

2.4.1.2. VITASSIGN

VITASSIGN (Vandeputte, 2006) also allocates offspring to pairs of parents using the exclusion principle. Overall, the features offered by VITASSIGN are very similar to the ones provided by FAP. In assignment mode, VITASSIGN can take into account allelic mismatches in the analysis and in case of "multi-match" outcomes provides a list of the matching families. Like FAP, when allocations occur with allele mismatches, VITASSIGN identifies the problematic locus/loci. VITASSIGN also includes two additional features compared to FAP: it can generate a mating matrix based on the allocation results and provide a summary of allele frequencies for each analysed locus. Lastly, VITASSIGN can be used to run simulations of allocation based on the genotypes of the putative parents. The program first generates a given number (fixed by the operator) of offspring genotypes based both on the "declared matings' matrix" and the parents' genotypes. The "created" offspring are then assigned by the program and the rate of single-matches is calculated (i.e. corresponds to the predicted rate of successful allocation).

2.4.2. Probability based programs

2.4.2.1. PAPA

PAPA v2.0 (Package for the Analysis of Parental Allocation; Duchesne et al., 2002) uses likelihood scores to allocate parental-pairs. For each offspring a 'breeding likelihood' (Sancristobal and Chevalet, 1997) is calculated against each potential parental-pair. The pair with the highest likelihood is assigned parentage. Offspring

are not allocated when either all parents show zero likelihood ('null likelihood') or where two or more parental pairs share the highest positive likelihood (an 'ambiguity'). In both the simulation and the allocation modes, a degree of transmission error (i.e. allele mistyping and / or genetic mutation) can be accommodated. This transmission error rate can be either uniform (all errors assumed to be equally likely) or non-uniform (to reflect greater mis-scoring between alleles of similar mobility). Simulations run using the chosen error model / value can be used to evaluate the likely power of the allocation and provides a computed measure of 'correctness', i.e. the level of confidence / accuracy that can be expected from actual assignments. The program can run sexed/unsexed predictions and allocations in both "closed" and "open" systems (where only part of the parental genotyping data is available).

2.4.2.2. CERVUS

CERVUS 2.0 is a paternity /maternity allocation program (c.f. parental-pair allocation approach used by FAP and PAPA), which relies on likelihood-based assignments. It was originally designed to infer paternity in natural Scottish red deer populations (Marshall et al., 1998; Slate et al., 2000). The program derives likelihood ratios for paternity / maternity for each offspring which, taken with population allele frequency data, is used to define a statistic for allocating, with confidence, the most likely parent. CERVUS was originally designed for solving the parentage in a closed system where one parent is known (e.g. mother/offspring relationship). The program allocates one parent at a time. In studies where both parents need to be resolved, two consecutive allocations need to be performed. The first allocation attempts to find the most likely (and statistically robust) parent in the entire broodstock population (male or female). A second allocation can then be performed to assign the second parent

(informed by the now known genotype of the allocated first parent). For this study the default confidence levels were used for allocations i.e. 95% strict, 80% relaxed. Where applied, a genotyping error rate of 1% was assumed (the default suggested in user manual).

2.5. Indicators to assess the degree of genetic diversity within a given population

2.5.1. Hardy-Weinberg test

Hardy-Weinberg test corresponds to a Chi-square test which compares the observed and expected allele frequencies within a given population and determines whether there is a statistically significant difference between the two. The model is based on five basic assumptions: 1) the studied population is large, 2) there is no gene flow between populations, from migration or transfer of gametes, 3) mutations are negligible, 4) individuals are mating randomly; and 5) natural selection is not operating on the population. If the test shows that there is no significant difference between the observed and expected allele frequencies, then the population is said to be at Hardy-Weinberg equilibrium (i.e. both its genotypes and allele frequencies will remain unchanged over successive generations).

The web-based population genetics software GENEPOP (Raymond and Rousset, 2000) was used to performed Hardy-Weinberg and population differentiation tests. The genotyping data was submitted online following the authors' instructions and the results were returned, via the web browser, as electronic mail.

2.5.2. Effective breeding population size

To estimate the effective breeding population size (Ne) of a given population of cod fry or juveniles, an assumption of unequal individual contributions was made. Results from the parentage analyses were used to calculate Ne as follow:

$$\mathbf{Ne = 1 / [2 \times (0.5 \times \sum_{parents} C_i^2 - 0.25 \times (1 / 2N_m)^2 - 0.25 (1 / 2N_f)^2)]}$$

(Brown, 2003)

where C_i stands for the fractional contribution of parents, N_m is the number of contributing males and N_f the number of contributing females.

2.5.3. Per-generation rate of inbreeding

The per-generation rate of inbreeding ΔF was also calculated following the formula given by Brown (2003). An assumption of population propagation in discrete generations - following random selection from a single broodstock population - was made:

$$\mathbf{\Delta F = 0.5 \times \sum_{parents} C_i^2 - 0.25 \times (1 / 2N_m)^2 - 0.25 (1 / 2N_f)^2}$$

Note: with **Ne = 1 / (2ΔF)**

where C_i stands for the fractional contribution of parents, N_m is the number of contributing males and N_f the number of contributing females.

2.6. Statistics

The statistical analyses were performed using the statistical package SPSS 14.0.

Before performing a statistical analysis, the data was tested for both normality and homogeneity of variance. The Kolmogorov-Smirnov test was used to verify the

data followed a normal distribution while a F-test was performed to verify the hypothesis of homogeneity of variance was true.

2.6.1. T-test

The unpaired T-test was used to compare the means from two independent, random populations (assuming they followed a normal distribution). The null hypothesis tested was that the means of the two populations were equal. Assuming equal variances, the test statistic was calculated as follow:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

with

$$s^2 = \frac{\sum_{j=1}^{n_1}(x_j - \bar{x}_1)^2 + \sum_{i=1}^{n_2}(x_i - \bar{x}_2)^2}{n_1 + n_2 - 2}$$

where x bar 1 and x bar 2 are the sample means, s^2 is the pooled sample variance, n_1 and n_2 are the sample sizes and t is a Student t quantile with $n_1 + n_2 - 2$ degrees of freedom.

When the samples were dependant a paired t-test was used. Assuming equal variances, the test statistic was calculated as follow:

$$t = \frac{\overline{X}_D - \mu_0}{s_D/\sqrt{N}}.$$

where X_D and S_D are the average and standard deviation for the paired observations. Under the null hypothesis, the constant μ0 equals zero and the degree of freedom used is N-1.

2.6.2. Chi square test

The Chi square test was employed to compare the frequency distribution of certain events observed in a sample with the frequency distribution of a particular theoretical model. The null hypothesis for this test was that the frequency distributions of the sample and theoretical model were equal. The Chi-square statistic was calculated as follow:

$$\chi^2 = \sum_{i=1}^{n} \frac{(O_i - E_i)^2}{E_i}$$

where O_i is an observed frequency, E_i is an expected frequency (from the theoretical model) and n the number of possible outcomes of each event.

The Chi-square statistic was then used in a p-value statistical test to compare the value of the Chi-square statistic with a Chi-square distribution.

2.6.3. Analysis of variance (ANOVA)

To compare the means between two or more groups / treatments, a one way ANOVA test was performed. This test was used providing that the following assumptions were met: 1) response variable were normally distributed, 2) the samples were random and independent and 3) the variance of the populations were equal. The null hypothesis for this test was that the means between the different groups / treatments compared were equal.

Chapter 3. Analysing the parentage of a complex genotyping dataset

3.1. Introduction

3.1.1. Multilocus DNA microsatellite profiles for parentage analysis

DNA microsatellites are being increasingly used in both parentage allocation and relatedness studies of natural and captive fish populations (Chistiakov et al., 2005). Initial fisheries-related publications reported on resolving parentage issues in situations where limited numbers of families were involved (e.g. Ferguson et al., 1995; Herbinger et al., 1995; O'Reilly et al., 1998). However more complex systems are now being studied (e.g. mass spawning tanks containing a large number of fish in commercial hatcheries), where very large numbers of potential families need to be resolved. Recent publications on Senegalese sole, turbot and Nile tilapia have focused on describing the output of such large breeding populations (Borrell et al., 2004; Fessehaye et al., 2006; Porta et al., 2006). Using only five loci, Fessehaye (2006) and Porta (2006) successfully traced the pedigree of offspring produced from the mass spawning of 10 x 10, 20 x 20 and 25 x 12 crosses. In both case studies, the parentage allocation program PAPA was used and 90 to 98% of the offspring analysed were unambiguously allocated to a single pair of parents. In the study published by Borrell et al. (2004), the genotyping data from eight loci allowed CERVUS to solve the parentage of offspring issued from the mass spawning of 25 to 60 parents with a success rate of 70%. The authors suggested that both allele mutations and null alleles were responsible for most of the allelic mismatches observed between parents and offspring.

3.1.1.1. Number and choice of microsatellite markers

Recently a number of questions have been raised regarding the number of microsatellite loci required, the optimal level of variability at each locus and the

management of typing errors in parentage analyses (Castro et al., 2004; Pompanon et al., 2005). A common trend appears to be recommending the screening of a minimum number of markers (i.e. 4 to 6), in order to limit costs (e.g. Fessehaye et al., 2006; Porta et al., 2006). By reducing the number of markers used to compile a genetic profile, Borrell et al. (2004) have also argued that the occurrence of genotyping errors should also be reduced.

3.1.1.2. Calling allele sizes and building pedigrees

Characterising allele sizes is of paramount importance when performing parentage analysis. Indeed, the success of an allocation exercise relies largely on both the accuracy and the consistency of allele size calling. The tedious task of interpreting chromatogram traces can be assisted by using semi-automated detection software programs such as Genemapper (Applied Biosystems). Providing allele detection thresholds and bin sizes are carefully customised, the program will automatically generate genetic pedigrees. Relying on semi-automated detection of alleles has the advantage to readily build consistent parent and offspring profiles. However, it requires careful handling as it could also represent a source of errors (Pompanon et al., 2005). This is illustrated by the case study presented in this Chapter.

3.1.2. Parentage analysis

3.1.2.1. Methods of allocation

There are two major methods of conducting parentage analyses: exclusion and likelihood-based approaches (Jones and Ardren, 2003). The exclusion principle relies solely on Mendelian genotypic incompatibilities between potential parents and offspring to filter out false parents / parental pairs. Where more than one set of non-excluded parents remain, likelihood approaches may be applied to select the most

probable parent / parental pair (e.g. Meager and Thompson, 1986; Sancristobal and Chevalet, 1997). There are advantages and disadvantages to using either allocation method. The exclusion method is conceptually simple and transparent but is particularly sensitive to typing errors and / or allele mutations. However, provided the locus set has a high assignment power (>99%) and error rates are low (less than 4%), accurate allocation is feasible using software that can accommodate occasional mismatched alleles (Vandeputte et al., 2006). Likelihood computations allow for a less rigid approach to parental assignment, which often results in more apparent assignments from less genotypic data. The algorithms applied usually incorporate a means for dealing with some degree of transmission error and missing data. However, the relationships among 1) the mathematical models implemented, 2) the error level set by the user for running the allocation and 3) the resultant sensitivity / accuracy of the assignment are more difficult to predict, and extra care is needed when interpreting the outcomes.

3.1.3. Aims of the study

During a parentage assignment exercise involving cod fry from a commercial mass spawning broodstock tank, discrepancies were noticed between exclusion- and likelihood-based assignment outcomes. This prompted a more detailed comparison, reported in this Chapter. The aims for comparing these two allocation methods were 1) to study and understand the origin of the discrepancies observed when assigning offspring using likelihood vs. exclusion, 2) to decide on the best method / software program to assign offspring issued from the mass spawning of a large number parents and 3) to study the influence, on the allocation results, of adding/removing loci from the analysis. Using a genotype dataset based on five multiplexed loci (Delghandi et al., 2003; Wesmajervi et al., 2006), we compared assignments produced by four

freeware parental assignment programs (CERVUS, Marshall et al. 1998; PAPA, Duchesne et al., 2002; VITASSIGN, Vandeputte et al., 2006 and FAP, Taggart, 2007), already used in aquaculture contexts.

3.2. Materials and methods

3.2.1. Presentation of the genotyping datasets

3.2.1.1. Origin of the samples

The study focused on one breeding tank from a Norwegian commercial cod hatchery (Grieg Marine Farms AS, Nedstrand). This broodstock population was chosen at the time (i.e. back in 2003) because sufficient number of eggs were produced on a daily basis to stock hatchery tanks with single batches of fry. This was not the case at the Scottish hatchery (to stock a hatchery tank, fry issued from several consecutive days of spawning were pooled).

Farm records showed that the Norwegian cod population was made up of 38 males, 54 females and 7 unsexed fish (see also Table 2.1). The fish had been sexed (using ultrasound technology) two month prior to the spawning season and PIT-tagged. A fin tissue sample from each fish was taken for DNA analysis. The fin samples were stored in 95% ethanol at 4°C until being processed.

Three hundred cod fry originating from a single day of spawning (19/11/03) were sampled. At the time of the sampling the fry were 83 days post-hatch. They had been size graded but kept as a single batch. The sampled fry, which were a random sample from the smallest size graded group (being the only group kept as a single batch after the grading took place), were stored in 95% ethanol at 4°C until analysed.

3.2.1.2. Genotyping datasets

The samples were processed at the Fiskeriforskning Institute, in Tromsø. Both the broodstock and the offspring were genotyped using five published DNA microsatellite markers (see Table 3.1) combined in a pentaplex assay, as described by

Wesmajervi et al. (2006). Three datasets, derived from the same genotyping data, were analysed with four parentage assignment programs (see Chapter 2). The first dataset is referred to as "five loci raw". It corresponds to the genotyping data from five markers (*Gmo*8, *Gmo*19, *Gmo*35, *Gmo*37 and *Tch*11) obtained following automated allele scoring using Genemapper 3.7 (Applied Biosystems). Analytic parameters included the selection of the default advanced algorithm for allele peak detection and the cubic spline method for calling sizes. Bin sizes and allelic thresholds were both customised using advanced options in Genemapper. For trinucleotide tandem repeats the bin size was set to ± 1.45 base pairs of the actual allele size and for tetranucleotide tandem repeats, it was set to ± 1.90 base pairs. In order to discriminate between non specific amplification or gel artefacts and actual alleles an intensity threshold was applied which automatically disregarded any peak less than a third as intense as the prevalent allele. The second dataset, "five loci corrected" was obtained following rigorous manual scrutiny of chromatograms and adjustment of accompanying genotype output files, informed by initial allocation results produced by FAP (see Results section). The third dataset "four loci corrected" corresponds to the corrected dataset minus the data from one marker (*Gmo*37, a moderately informative marker as 13 distinctive alleles were encountered within the Norwegian broodstock population).

These three datasets did not include genotypes from the seven unsexed fish in the parental files, since they were shown not to contribute to the offspring sampled (see Chapter 4). The fry which were not fully genotyped successfully for all the five markers (22 out of 300) were also removed from the offspring dataset.

Table 3.1. Description of the polymorphic microsatellite markers used in the present study.

Microsatellite Name	Allele size range (bp)	Number of alleles identified in the Norwegian broodstock population (N = 99)	Reference
*Gmo*8	126-322	22	Miller et al. (2000)
*Gmo*19	120-224	20	”
*Gmo*35	113-146	7	”
*Gmo*37	236-320	13	”
*Tch*11	118-218	19	O’Reilly et al. (2000)

3.2.2. Parentage analysis programs

The genotyping datasets were analysed using each of the four parentage programs presented in Chapter 2 (i.e. FAP, VITASSIGN, CERVUS and PAPA). FAP, VITASSIGN and PAPA were relatively easy to operate providing the recommendations and examples given by the authors were followed (*cf.* user guides for each program). Therefore, no further details will be provided in this chapter concerning those three programs.

The use of CERVUS, however, proved to be more challenging. Since the program only solves a parent at a time, two successive allocations were performed for each analysed dataset. The first allocation aimed to attribute, to each analysed offspring, the most likely parent (male or female). Based on the results of this first allocation, new input files were generated for the second parental allocation. Those files took into account the identity of the first allocated parent, its sex and its attributed confidence level of allocation (i.e. 95%, 80% or “relaxed” confidence). In essence, the second allocation consisted in searching for the second most likely parent of opposite sex, knowing the identity of the first parent. A global confidence level for

the chosen family was then deduced from the confidence levels of both parental allocations.

3.2.3. Simulated offspring datasets

A set of 296 offspring genotypes was computed from the "5 loci corrected" broodstock data. Offspring genotypes were randomly generated - from the genotyping data of 24 parental pairs (so it matched the actual FAP results) - using a macro built in Windows Excel. The simulated offspring file was designed so that it reflected the FAP allocation results of the "5 loci corrected" dataset (both the contributing families and the percentages of contribution were identical). Genotyping errors were subsequently added to the simulated offspring file. Two error models were implemented: 1) a "real error" model which reflected the typing error levels encountered in the "5 loci raw" dataset (i.e. 17.5% error for *Gmo*19, 7% for *Gmo*8, 61% for *Gmo*35, 2% for *Gmo*37 and 15% for *Tch*11) and 2) a "10% typing error" model which was characterised by 10% typing errors for 3 out of 5 markers (i.e. *Gmo*19, *Gmo*35 and *Tch*11). Results from these two simulated datasets were compared to allocation outcomes obtained with FAP and PAPA (see section 3.3.1.6) using the "5 loci corrected" parental files.

3.3. Results

3.3.1. Comparison between FAP and PAPA parentage allocations

3.3.1.1. Predictions and parentage allocations of the "five loci raw" genotyping dataset

The "five loci raw" dataset initially generated by automated genotype scoring was first analysed using both FAP and PAPA. The parentage analysis using PAPA was carried out allowing for a uniform error value of 0.02 (the default value for uniform error rate in PAPA). A one allele mismatch tolerance was allowed for the FAP allocation. Despite predicting a similar assignment power for the five locus set (c. 90%), the two procedures showed striking differences in the proportions of offspring successfully allocated to a single family (Table 3.2). PAPA allocated a parental pair to 98% of offspring (with an expected "correctness" of > 0.98) whereas FAP only allocated 41% to a unique, single family. In all cases the exclusion-based allocations concurred with the likelihood assignments. Despite allowing for up to one allele mismatch in the exclusion-based allocation, 37% of the offspring were not matched to any family (Table 3.2). This suggested that a significant number of typing errors were present in the dataset (potentially from both offspring and parental data).

Table 3.2. Comparison of the prediction and allocation results for 278 cod offspring given by PAPA and FAP using the "five loci raw" dataset (automated genotype scoring).

Assignment outcome	Predicted assignment		Actual assignment	
	PAPA	FAP	PAPA [1] 0.02 uniform error	FAP 1 allele mismatch
Single-match	94.0%	90.0%	273 (98.2%)	114 (41.0%)
Multiple-match	6.0%	10.0%	5 (1.8%)	60 (21.6%)
No-match	--	--	0	104 (37.4%)
Contributing families	--	--	74	29

[1] 'Correctness' computed as 0.98 (allocation and production error model values = 0.02)

3.3.1.2. Dataset cleaning and origin of the genotyping errors

Hardy-Weinberg tests (using Genepop; Raymond and Rousset, 1995) were performed on the raw parental genotypic data. These revealed highly significant excesses of homozygotes at most loci, but particularly for *Gmo*19 and *Gmo*35, which suggested at least one potential source of error, i.e. large allele dropout (Table 3.3). Consequently, all chromatograms were manually checked and many genotyping errors were identified (Table 3.4). Overall, *Gmo*35 genotypes were subjected to the most corrections (24% of the parental and 68% of the offspring genotypes). The main, but not only, source of error encountered for most loci was "technical" large allele dropout – i.e. where weakly fluorescing larger allelic PCR products were not detected because the peak detection threshold was set too high in the automated scoring macro employed. Another relatively common error encountered was the miscalling of allele size due to size standard calibration problems. Overall 400 changes were made, a detected error rate of 10.8%.

Table 3.3. Genepop analysis on the "5 loci raw" parental dataset showing the expected number of homozygotes vs. the observed number of homozygotes for each of the five loci genotyped.

Locus ID	Number of fish genotyped	Number of observed alleles	Expected number of homozygotes	Observed number of homozygotes
*Gmo*8	92[1]	23	10	14
*Gmo*19	92	20	8	23*
*Gmo*35	92	7	22	49*
*Gmo*37	92	13	15	18
*Tch*11	92	20	7	17*

[1] the seven unsexed broodstock were not included in the analysis
* Difference statistically significant (Chi square test with two-tailed P value < 0.05)

Table 3.4. Summary of the manual corrections made to the parental and offspring genotypes originally generated by automated allele scoring. Both the number (and percentage) of allele designations that were changed at individual loci are given.

	Number of genotypes corrected				
	*Gmo*8	*Gmo*19	*Gmo*35	*Gmo*37	*Tch*11
Parental alleles	3 (3.3%)	3 (3.3%)	22 (23.9%)	4 (4.3%)	14 (15.2%)
Offspring alleles	21 (7.6%)	54 (19.4%)	188 (67.6%)	7 (2.5%)	42 (15.1%)

3.3.1.3. Predictions and parentage allocations of the "5 loci corrected" genotyping dataset

In order to evaluate the impact of the corrections made to the original dataset, Hardy-Weinberg tests (using Genepop; Raymond and Rousset, 1995) were run on the corrected parental genotypic data (Table 3.5). These showed that reviewing the chromatograms resulted in reducing by 50% the number of homozygotes observed for *Gmo*35 (Table 3.5). However, the difference between the "expected number of homozygotes" and the "observed number of homozygotes" was still significantly different for two loci out of five (i.e. *Gmo*19 and *Tch*11; see Table 3.5).

Table 3.5. Genepop analysis on the "5 loci corrected" parental dataset (genotypes manually corrected) showing the expected number of homozygotes vs. the observed number of homozygotes for each of the five loci genotyped.

Locus ID	Number of fish genotyped	Number of observed alleles	Expected number of homozygotes	Observed number of homozygotes
*Gmo*8	92	22	10	15
*Gmo*19	92	20	7	22*
*Gmo*35	92	7	18	25
*Gmo*37	92	13	14	18
*Tch*11	92	19	6	11*

* Difference statistically significant (Chi square test with two-tailed P value < 0.05)

The "five loci corrected" dataset was then reanalysed by both FAP and PAPA (Table 3.6). The corrections led to a similar increase in predicted power of assignment for the five loci (from c. 90% to c. 98 %) calculated by the two softwares. The percentage of offspring successfully allocated to one family, using FAP (allowing for up to one allele mismatch per assignment), increased from 41% to 77%. No-matches decreased from 37% to 14%. Both results suggest that the corrections made to the genotyping data improved the quality of the allocation analysis. Of the single family allocations identified, 24 were assigned on the basis of one allele mismatch. Those 24 families all had a predicted assignment success of at least 78% according to FAP (Table 3.7). Re-examination of chromatogram traces from these allocations did identify further genotyping errors as predicted, or involved cases of suspected large allele dropout / null allele (i.e. either one of the parents or the offspring was homozygous for a small sized allele at the mismatched locus). Comparison between the percentages of homozygotes among the matched and non-matched offspring suggested that genotyping errors were likely to remain in the dataset - especially for the markers *Gmo*8 and *Gmo*19 - despite the extensive corrections undertaken (Table 3.8).

In contrast the major corrections implemented made little difference to the overall allocations produced by PAPA. Predicted accuracy of assignments was virtually unchanged ("correctness" = 0.99). Perhaps somewhat counter-intuitively, the proportion of offspring successfully allocated to a single pair of parents actually decreased slightly (from 98% to 96%), while the number of ambiguous matches doubled to 3.6%. The number of offspring not matched to any family remained at zero. As with the "five loci raw" dataset, all FAP single family assignments concurred with PAPA allocations. Nevertheless, conspicuous differences still remained between the two analyses. PAPA identified more than double the number (54) of contributing families compared to only 24 single-match families with FAP (Table 3.6). Although the latter figure was derived from 54 fewer assignments, one or more of these 24 families were also potential contributors in 18 of the additional 24 multiple matches identified by FAP.

Table 3.6. Comparison of the prediction and allocation results for 278 cod offspring given by PAPA and FAP using the "five loci corrected" dataset.

Assignment outcome	Predicted assignment		Actual assignment	
	PAPA	FAP	PAPA [1] 0.02 uniform error	FAP 1 allele mismatch
Single-match	98.4%	97.0%	268 (96.4%)	214 (77.0%)
Multiple-match	1.6%	3.0%	10 (3.6%)	24 (8.6%)
No-match	--	--	0	40 (14.4%)
Contributing families	--	--	54	24

[1] 'Correctness' computed as 0.99 (allocation and production error model values = 0.02)

Table 3.7. FAP predicted assignments for the 24 contributing families identified by FAP (using the "5 loci corrected" dataset with up toone allele mismatch allowed).

Contributing family	Percentage of predicted assignments by FAP
F49xM10	0.984
F40xM10	1.000
F25xM14	0.789
F40xM14	0.938
F19xM14	0.969
F63xM14	1.000
F58xM15	1.000
F29xM30	0.938
F22xM30	0.902
F40xM30	0.938
F40xM38	1.000
F51xM42	0.969
F49xM42	0.918
F82xM42	0.953
F29xM42	0.936
F19xM42	0.930
F44xM54	1.000
F51xM65	1.000
F22xM65	0.969
F29xM65	1.000
F40xM65	0.984
F51xM72	0.969
F19xM72	0.969
F51CxM83	1.000

Notes: the percentage of predicted assignments is deduced from the identification and the counting of all possible shared genotypes for each pairwise family combination (Taggart, 2007).

Table 3.8. Comparison of the percentages of homozygotes between the allocated and the non allocated offspring using the "5 loci corrected" dataset and based on FAP results (one allele mismatch).

Offspring group	*Gmo*19	*Gmo*8	*Gmo*35	*Gmo*37	*Tch*11
Offspring not matched	35.9	21.9	10.9	34.4	9.4
Offspring matched to a single family	13.6	7.5	7.0	34.1	12.6

3.3.1.4. Conservativeness of the allocation results

After correcting the genotyping data, 49 offspring (17.6%) were allocated to a different family by PAPA, while only 4 (1.4%) of offspring were similarly reassigned with FAP. The number of contributing families found using PAPA was also more heavily influenced by the corrections made to the genotyping dataset. The number fell from 74 to 54, while with FAP it only decreased from 29 to 24.

3.3.1.5. Predictions and parentage allocations of the "4 loci corrected" genotyping dataset

In order to explore the consequences of using fewer loci, the assignments were recomputed (Table 3.9) with a reduced "four loci corrected" dataset. A moderately informative locus, with apparently low error rate (i.e. *Gmo*37), was removed from the dataset to intend estimating, at best, the influence of a single locus on the overall assignment success achieved by each of the four assignment programs studied. With only 4 loci, predicted assignment values fell by c. 12% for FAP, and by c. 6% for PAPA. The "correctness" estimate (allocation accuracy) for the latter remained high (c. 97%), similar to that computed for the full "five loci corrected" dataset. Omitting this locus only reduced the number of "successful" allocations computed by PAPA by 11. The power of actual assignment was more significantly reduced for the FAP analysis, with 28 fewer assignments being made. In terms of conservativeness, PAPA parental-pair allocations changed for 34 (12%) of offspring while no allocations changed using exclusion-based analysis.

Table 3.9. Comparison of the prediction and allocation results for 278 cod offspring given by PAPA and FAP using the "four loci corrected" dataset. (i.e. *Gmo*8, *Gmo*19, *Gmo*35 and *Tch*11 only).

Assignment outcome	Predicted assignment		Actual assignment	
	PAPA	FAP	PAPA [1] 0.02 uniform error	FAP 1 allele mismatch
Single-match	91.9%	85.0%	257 (92.5%)	186 (66.9%)
Multiple-match	8.1%	15.0%	21 (7.5%)	72 (25.9%)
No-match	--	--	0	20 (7.2%)
Contributing families	--	--	59	25

[1] 'Correctness' computed as 0.97 (allocation and production error model values = 0.02)

3.3.1.6. Parentage allocations of simulated offspring datasets

Differences between FAP and PAPA analysing methods were further investigated by comparing the allocation outcomes from two simulated offspring datasets (Table 3.10). As expected, the allocation results from the "real error" and the "5 loci raw" datasets concurred. Overall, PAPA allocated 97% of the simulated offspring to a single family while FAP only managed to allocate 59%. However, due to the extensive genotyping errors present in the offspring file, 18% of the families matched by PAPA were incorrect for either one or both parents (vs. only 5% for FAP). The allocation outcomes from the "10% typing error" offspring dataset were also as expected. By reducing the occurrence of genotyping errors in the simulated offspring file, the percentage of single-matches increased significantly, from 59% to 86.5%, with FAP (Table 3.10) but remained almost identical with PAPA (96%). The reduction in typing errors however influenced greatly the correctness of the results given by PAPA since only 5% of the allocated families were incorrect this time around (vs. 1% for FAP).

Table 3.10. Assignment results for two simulated offspring datasets given by PAPA and FAP.

Assignment outcome	"real error" dataset		"10% typing error" dataset	
	PAPA [1] 0.02 uniform error	FAP 1 allele mismatch	PAPA [1] 0.02 uniform error	FAP 1 allele mismatch
Single-match	287 (97%)	174 (59%)	283 (96%)	256 (86.5%)
Multiple-match	9 (3%)	70 (24%)	13 (4%)	34 (11.5%)
No-match	0	52 (17%)	0	6 (2%)
Incorrect family allocation	54 (18%)	14(5%)	14 (5%)	2 (1%)

[1] 'Correctness' computed as 0.99 (allocation and production error model values = 0.02)

3.3.2. Comparison between FAP and CERVUS parentage allocations

Predicted assignments were computed, using CERVUS, for the three genotyping datasets ("5 loci raw", "5 loci corrected" and "4 loci corrected"). The predicted assignment power for CERVUS derived first parent allocations was high assuming the data was error free – but much lower if even a modest 1% error rate model was invoked (Table 3.11). For the 'five loci corrected' dataset only 70% first parent assignments were predicted at 'relaxed' confidence level, and 29% at strict level. Predicted assignment dropped even more dramatically for the four loci dataset (24%, "relaxed"; 4% "strict"). Predicted performance allowing for 3% error rate, still a relatively low value, was extremely poor (0-7% of sample likely to be assigned, even with relaxed confidence; Table 3.11).

In actual assignments, performance was poorer. For clarity only data for assignments allowing for a 1% error rate are presented (Table 3.12). In all cases when a first parent was identified CERVUS also managed to assign a second parent, at the same confidence level. Reassuringly, the "five loci corrected" dataset generated the highest number of allocations, i.e. 168 (60%) offspring were assigned to a family with

80% confidence. This dropped to 23% of offspring assigned parentage at 95% confidence level. In both these cases the vast majority of assignments concurred with those produced by FAP (Table 3.12). Assignments based on the four loci dataset were very low (18%, “relaxed”; 6% “strict”) and therefore concurred with the predicted results.

Table 3.11. CERVUS predictions for assignment power of first parent for each of the three datasets (based on simulations generated from 1000 iterations, all parents known).

	0% typing error		1% typing error		3% typing error	
[1] Confidence: Dataset	relaxed	strict	relaxed	strict	relaxed	strict
'5 loci raw'	99%	87%	44%	18%	7%	2%
'5 loci corrected'	100%	83%	70%	29%	6%	0%
'4 loci corrected'	90%	54%	24%	4%	0%	0%

[1] Relaxed = 80% confidence ; strict = 95% confidence

Table 3.12. Summary of parental-pair assignments identified by CERVUS for 278 cod offspring (assuming a 1% error rate), and comparison with FAP allocations.

	Assignment outcome - 80% confidence level			Assignment outcome - 95% confidence level		
Dataset	Family allocations Number & (%)	Number of contributing families.	Concurs with FAP [1]	Family allocations Number & (%)	Number of contributing families.	Concurs with FAP [1]
'5 loci raw'	134 (48%)	61	45 / 60	62 (22%)	32	23 / 31
'5 loci corrected'	168 (60%)	66	110 / 117	64 (23%)	32	43 / 47
'4 loci corrected'	50 (18%)	29	16 / 28	18 (6%)	14	5 / 8

[1] Number of family assignments that agreed / number of offspring assigned a parental-pair by both CERVUS and FAP

3.3.3. Comparison between FAP and VITASSIGN parentage allocations

As expected, the allocation results given by FAP and VITASSIGN for the "5 loci raw", "5 loci corrected" and "4 loci corrected" datasets were identical. However, in predictive mode, the answers given by the two programs differed slightly due to the different algorithms they each used (see Chapter 2). Choosing between FAP and VITASSIGN to conduct exclusion-based parental assignment exercises mainly comes down to personal choice. In this research project, FAP was chosen to run exclusion-based parental analyses simply because VITASSIGN was not made available until late 2006.

3.3.4. Summary

To conclude this section, the principal results of this Chapter are recapitulated in Table 3.13.

Table 3.13. Summary of parental pair assignments by FAP, VITASSIGN, PAPA and CERVUS for '5 loci raw', '5 loci corrected' and '4 loci corrected' offspring datasets.

Dataset	FAP and VITASSIGN 1 allele mismatch			PAPA 0.02 uniform error			CERVUS 80% confidence level		
	Prediction Single-match	Single-match	Contributing families	Prediction Single-match	Single-match	Contributing families	Prediction Single-match 1% typing error	Single-match	Contributing families
'5 loci raw'	90.0%	41.0%	29	94.0%	98.2%	74	44.0%	48.0%	61
'5 loci corrected'	97.0%	77.0%	24	98.4%	96.4%	54	70.0%	60.0%	66
'4 loci corrected'	85.0%	66.9%	25	91.9%	92.5%	59	24.0%	18.0%	29

3.4. Discussion

It is clear that there were potentially serious flaws among the initial genotypes (i.e. '5 loci raw' dataset) generated for this cod parentage assignment study. Indeed the extent of errors in the initial dataset (mean 20%) was much higher than that usually reported in the literature (from 0.25% to 2.0%; see Blouin, 2003). These errors were uncovered after the assignment results obtained with PAPA were challenged with FAP. The assignment program PAPA was used, in the first instance, following the results published by Wesmajervi and colleagues (2006), where 91.2% of assignments were solved by PAPA, based on the genotyping information from the same 5 loci analysed in this study. The discrepancies observed between the allocation results from FAP and PAPA prompted a detailed study of the performance of different assignment approaches under 'extreme' conditions. The success of parentage allocation in a closed system, such as that under investigation here, is dependent on both the number of genetic markers employed and their reliability / robustness during data collection. In the case of species recently introduced to aquaculture like Atlantic cod, the number of available markers can be a limiting factor. This serious constraint is clearly illustrated by the results of the present study. Initially 37% of the fry analysed with five markers could not be reconciled with any expected parental-pair by exclusion (even allowing for one allele mismatch). Two of the five markers (*Gmo*19 and *Gmo*8) on later inspection, informed by assignment outcomes, were found to be prone to large allele dropout, a major recognised source of genotyping error (e.g. Blouin, 2003; Pompanon et al., 2005). This large allele dropout was mainly technical in nature. Minor allele peaks were evident, but not registered by the scoring software. Other sources of scoring errors were also detected, including size marker bands being

wrongly identified, evidence for null alleles and binning issues for similar sized alleles. However these were comparatively minor in frequency.

It should be noted that even after extensive correction of the dataset (400 allelic designations changed), more than 14% of offspring (n = 40) could still not be allocated to any expected parent-pair by exclusion, even allowing for one allele mismatch. Significant errors evidently remained in the corrected dataset. It required a tolerance of up to three mismatched alleles to 'force' an allocation for all these un-matched progeny (data not shown). From inspection of genotypes it is likely that large allele dropout could account for most of these non-assignments. However, as detailed in Chapter 4, other causes such as misidentification of the sex of parents (i.e. due to the misreading of ultrasound scans), presence of extraneous fish in the spawning tank (i.e. due to unrecorded fish movements) and later sample contamination during farm rearing, cannot be ruled out as contributory factors.

The performance of the four types of parentage analysis varied greatly both within and between the three datasets that were explored. Compared to the parental-pair likelihood approach computed using PAPA, the exclusion-based analysis (computed either by FAP or VITASSIGN) was much more conservative in both the number of offspring for which an assignment could be made and for the overall number of parental-pairs involved during spawning over this single day. Assignment success for the error prone 'five loci raw' dataset was low, but nearly doubled (to 77%) after major correction to the dataset. Crucially, while the overall number of assignments increased, only c. 1% of them were allocated to different parental-pairs. This was to be expected from exclusion principles. Genotyping errors are much more likely to result in false exclusions than false inclusions. The four loci dataset again

behaved as predicted, having reduced power, but where assignments were made these concurred with the five loci dataset.

Likelihood based parental-pair assignments appeared, on first look, to provide a more powerful means of family allocation for cod fry from this mass spawning tank. Based on the 'five loci raw' genotypic dataset, parentage was assigned to the vast majority of fry (> 95%) with apparently high precision and accuracy. However similarly high assignment levels were attained from the five loci and four loci corrected datasets, with simulations again suggesting that these too were highly reliable allocations. Yet a fairly large number of assignments (up to 18%) changed of identified parental-pairs - for individual offspring - among the three analysed datasets. Since this later observation agreed with the analysis results obtained from a simulated offspring file reflecting a similar typing error level, the reliability of the outcomes generated by PAPA, as implemented in this study, must therefore be questioned. We opted to use the uniform error rate (UER) model and at the default 0.02 level as 1) most errors detected involved large allele drop out, and not mistyping of closely spaced alleles; and 2) this is the most widely used parameter and value quoted in published studies using PAPA for aquaculture / fisheries related work (Fessehaye et al., 2006; Johnson et al., 2007). In order to increase the reliability of assignments, Morrissey and Wilson (2005) have recently argued that likelihood equations should be applied with error rates set to values considerably lower than the rates at which genotype errors are believed to occur. We reanalysed the datasets (not shown) with a much reduced error value (UER = 0.0001) and obtained exactly the same allocations in each case as for a UER value of 0.02. This underlines the difficulty in defining what this parameter represents in error terms and how it is likely to modulate parental-pair assignments. Single-family assignments identified by exclusion always concurred

with PAPA. Likewise in multiple-match cases identified by FAP (or VITASSIGN), the family assigned by PAPA was always represented. A few of these cases were explored by comparing the expected relative frequency of the offspring composite genotype in each of the potentially matched families (this frequency is calculated by FAP and stored in an ancillary file for inspection). In most cases the PAPA selected parental-pair was only twice as likely to be the parent *c.f.* another parental pair (assuming all families were equally represented in the sample). As PAPA reports only the most likely family, the output gives no indication as to the extent of close ties among different families. Confidence in the outcomes relies on the simulated 'correctness' value, which was not found to be robust in this particular study. The PAPA 2.0 user guide recommends that the first allocation check should be run under the 'no-error' model, with more than 10% resultant no-match ('null') assignments being indicative of potential problems with the input data. This was the case for all three datasets used in this study ('5 loci raw' – 76% nulls; '5 loci corrected' – 26% nulls; '4 loci corrected' – 25% nulls). The major concern with the performance of this package was that allowing for apparently low levels of error (UER = 0.0001 – 0.02) appeared to solve these 'issues' with a predicted high level of confidence. Generally, the results of 'no error' model assignments are not detailed in published papers.

In this study, CERVUS resolved parentage for the fewest number of offspring. The low numbers of confident allocations produced by CERVUS is not unexpected as the software was not designed specifically for parental-pair assignments, but rather to assign a single parent, where one is already known. The large numbers of potential parents and remaining genotype errors within the dataset were likely to be significant factors in the relatively poor performance of this software. CERVUS has been used to assign family to offspring in aquacultural contexts (e.g. Castro et al., 2006; Dong et

al., 2006; Porta et al., 2006). In these three publications alone, a number of points are worth mentioning. The number of potential families involved in those parentage studies was notably lower (100 to 400 families) than in our case study. In each case, the methodology followed to allocate parents using CERVUS was poorly described. Although the allocation outcomes were not transparent it was suggested by the authors that virtually all the offspring analysed were successfully and reliably allocated to a single family. Without any detailed numerical data on the assignment outcomes, it is difficult to assess whether the results of those studies were robust or not.

Although considered far from ideal for routine parentage analysis due to high error levels (Jones and Ardren, 2003; Morrissey and Wilson, 2005), the five loci selected for DNA profiling were the best available for use at the start of this project. The papers published by Delghandi et al. (2003) and Wesmajervi et al. (2006) suggested that these markers were perfectly suitable to solve the parentage of a complex dataset (using the assignment program PAPA). Looking back now, developing new microsatellite markers would have most probably been a better approach to undertake rather than using this set. Unfortunately, the project was far too advanced when this issue emerged.

A panel of new DNA microsatellites for cod have been recently isolated (Jakobsdóttir et al., 2006; Wesmajervi et al., 2007) and others are currently being tested / optimised for parentage assignment (M. Delghandi, personal communication; see also Chapter 5). These should allow improvements in the overall success of parentage studies in Atlantic cod, particularly in complex situations such as mass spawning tanks (see Chapter 5).

As parentage assignments studies become ever more complex and ambitious (i.e. involving more families, and hence more loci and samples) it is inevitable that greater reliance will be placed on automated or semi-automated screening procedures. This study (along with many others e.g. Hoffman and Amos, 2005; Pompanon et al., 2005) has demonstrated that relying solely on automated allele detection can introduce substantial mistakes into genotyping data. Combined with the inappropriate use of, or reliance on, likelihood-based assignment, the potential for serious misinterpretation of datasets is likely to be significant. For example the question regarding the appropriate number of markers to include in an analysis is often discussed. With this particular issue, it is important to bare in mind that two factors must be considered: the number of markers and the level of polymorphism (which can greatly vary from one population to other). A number of recent publications for various species, have recommended the use of as few as 4 to 6 markers to successfully solve 99% of the allocations (Jackson et al., 2003; Borrell et al., 2004; Fessehaye et al., 2006; Porta et al., 2006). Authors of these studies advocated likelihood approaches for solving parentage. However, the results of the present study outline the substantial potential risks of an approach combining few markers with a likelihood analysis method that has not been sufficiently characterised / tested for the task being undertaken.

The application of likelihood approaches to determine mating outcomes in mass spawning tanks is itself open to scrutiny. These methods invariably rely on a number of assumptions which may include: mating assumed to be random; equal productivity of parents; equal survival / performance of offspring; parental sex determination is error free; a closed system. Often, some of these factors are unknown and constitute the reason why the study is being carried out. Thus, likelihood-based

assignments may actually bias the outcomes in favour of the model assumptions. In the current study, although all 99 parents were present, it was not expected (from physiological grounds alone) that many females would actually spawn in any twenty four hour period. Also the reproductive output / fertilisation rate / survival rate among parents was likely to be highly skewed. For these reasons, power predictions (whether likelihood or exclusion based) should be interpreted with caution. Similarly such factors may explain, in part, the erratic performance of the likelihood based parental-pair assignment observed for this mass spawning event.

3.5. Conclusions

Few parental assignment exercises are likely to be conducted without error, especially when large numbers of samples are involved. However, the extent to which false assignments may be problematic will depend on the aims of the investigation (e.g. whether for detecting the main contributing families or identifying all offspring from a particular family / parent). Both the reliability of the data and the analytical approach taken must be carefully weighed up in context. Perceived error (or lack of) may have implications beyond the particular dataset being investigated. Apparent (but erroneous) assignment could lead to undue confidence being place on other data (e.g. population allele frequencies) derived using the same screening panel and protocols. In light of the conflicting assignments observed in this study we would recommend for future mass spawning assignment projects that 1) likelihood-based assignments be performed in conjunction with an exclusion-based method (see Jones and Ardren, 2003 for suitable packages); 2) genotype errors are rigorously investigated, quantified and reported (as urged by Pompanon et al. 2005) and 3) assignment protocols employed are fully described and detailed in publications.

Chapter 4. Spawning dynamics of cod broodstock in tank systems

4.1. Introduction

4.1.1. Physiology of reproduction

4.1.1.1. Spawning behaviour in the wild environment

Cod are long-lived iteropars which reproduce annually during several consecutive years. Males and females aggregate on spawning grounds and females spawn repeatedly over a period of 4 to 8 weeks (Hutchings et al., 1999; Bekkevold et al., 2002). Ovarian development starts on average 8-9 months prior to spawning (Pavlov et al., 2004). Cod females release from 15 to 20 batches of eggs throughout the spawning season (Walden, 2000) at intervals of 2 to 6 days (Hutchings et al., 1999). Atlantic cod in Norwegian and Scottish waters spawn naturally between February and May, with a peak of egg production around April (Engen and Folstad, 1999; Walden, 2000; Hansen et al., 2001).

Cod mating behaviours are complex (see Figure 4.1) and still not fully understood (Hutchings et al., 1999). The few studies published suggested that cod display lekking behaviour (Hutchings et al., 1999; Nordeide and Folstad, 2000). The behaviour of males and females differs in terms of residence time and activity in spawning grounds (Robichaud and Rose, 2003). Males aggregate at the spawning ground and establish territories while females remain free to move in and out of the ground periodically (Robichaud and Rose, 2003). Cod do perform extensive courtship, including fin display, flaunting, prodding and sound production. Both fin display and sound production are believed to constitute criteria of selection for females (Nordeide and Folstad, 2000). Reproductive competition among cod males has frequently been observed both in the wild and in captivity and seems to be based upon aggressive interaction and body size (Hutchings et al., 1999; Hansen et al.,

2004). Release of eggs has been suggested to mainly occur during the night (Engen and Folstad, 1999; Hutchings et al., 1999).

Sex ratio within a given spawning ground is equal to 1:1 overall but a vertical segregation exists (females are found in the water column whereas males are nearer to the sea bed; Hutchings et al., 1999). Times of arrival and departure from the spawning grounds depend on both the sex and the size of the fish (Lawson and Rose, 2000). Males tend to arrive and leave the spawning ground earlier.

4.1.1.2. Spawning behaviour in captivity

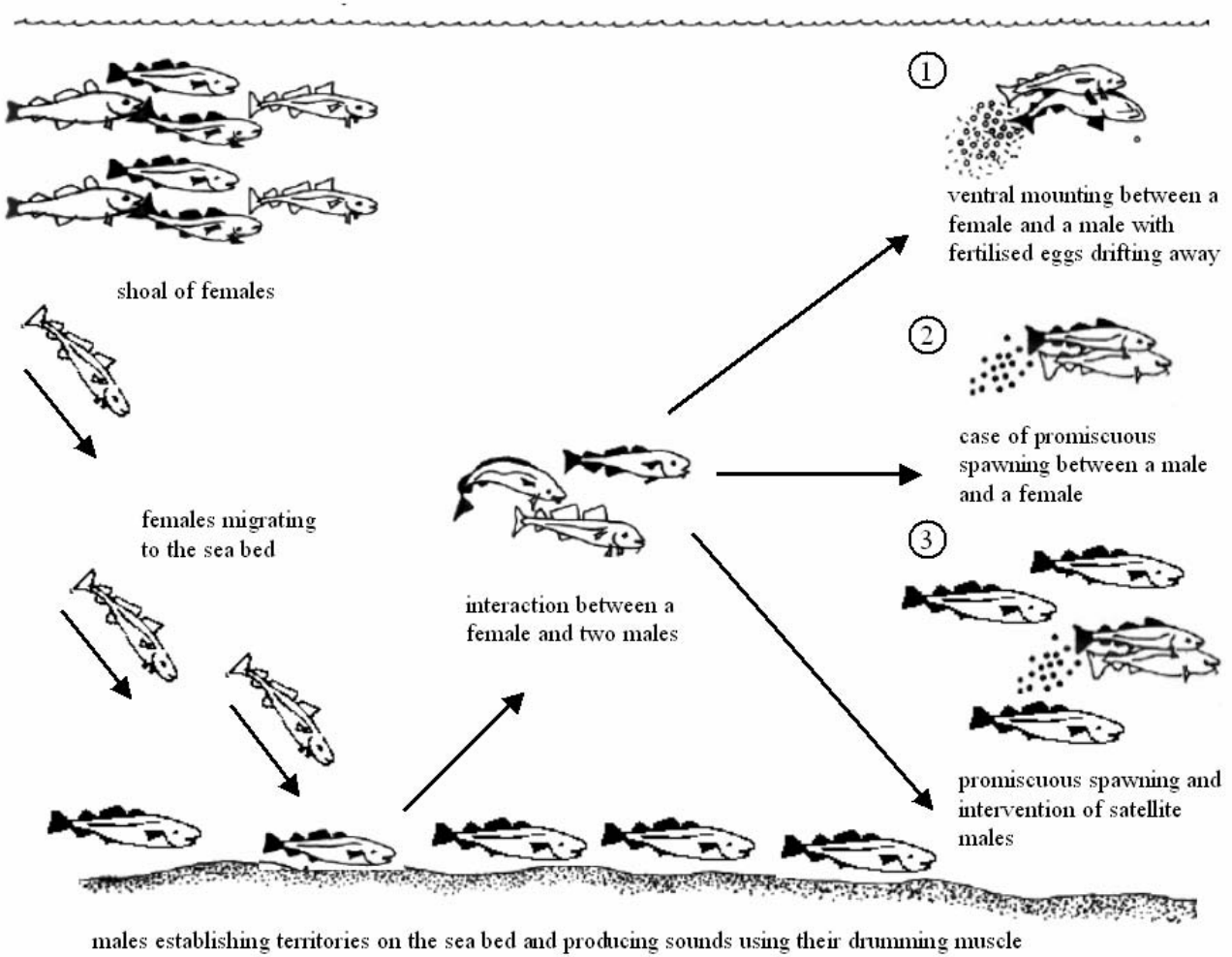
Most captive cod, once adapted to enclosed tanks and aquaculture rearing conditions, display courtship behaviour and spawn naturally (Kjesbu et al., 1996; Pavlov et al., 2004) during both day and night (Kjesbu, 1989).

Egg size, fecundity and duration of the spawning period generally increase with maternal size (Kjesbu et al., 1996). New recruit females usually have a short spawning duration. Their fecundity is generally inferior to repeat spawners and eggs/larvae produced are also smaller (Saborido-Rey and Junquera, 1999). Survival of cod larvae usually decreases as the spawning season reaches an end (Choa, 2004). It was also shown that egg size decreases with the number of batches shed (Kjesbu et al., 1996; Larsen, 2002; Choa, 2004).

Male size rank, in both wild and captive cod populations, is believed to have a significant effect on paternity success of individual males (Engen and Folstad, 1999; Bekkevold et al., 2002). Nonetheless, sperm competition is often observed in tanks with satellite males releasing milt among female eggs without courting (Nordeide and Folstad, 2000; Hansen et al., 2004). Multiple paternity is common in any given egg

batch. This is rendered possible by the fact that unfertilised eggs remain viable in seawater for about one hour (Bekkevold et al., 2002).

A recent study on captive broodstock behaviour suggested that not all individuals contribute equally to the spawn. One can expect that around 50% of females produce 90% of eggs (Hansen et al., 2004). Similarly some males may only display courtship behaviours without actually producing milt (Hansen et al., 2004).



Note: Figure inspired by Trippel (2003)

Figure 4.1. Illustration of Atlantic cod spawning behaviours in the wild environment.

4.1.2. Marine hatchery practices regarding cod broodstock management

Cod spawning strategy is typical of a so-called r-strategy where large numbers of small offspring are produced with little maternal investment in care and nutrients per egg (Choa, 2004). As a result, in the wild environment, the survival rate of cod eggs is extremely poor (about one egg out of a million succeeds in completing the life cycle). For a commercial cod hatchery, the foremost economical requirement, which consists in guaranteeing a year round supply of good quality eggs to meet the production target of juveniles, may therefore prove particularly challenging. Most commercial cod farms rely solely on their own broodstock to supply eggs. However, in Norway and Canada, cod hatcheries have now the opportunity to purchase cod eggs from private companies and / or governmental research institutions (i.e. MarineBreed AS in Norway; the Ocean Sciences Centre from the University of Newfoundland in Canada).

Both the quality and the quantity of fertilised cod eggs produced under commercial conditions are largely affected by the rearing conditions of the captive broodstock populations (Pavlov et al., 2004; Salze, 2004).

4.1.2.1. Nutrition

Under natural photoperiod, gonadal growth starts during late autumn for cod. Broodstock feeding regime and quality are critical at that stage as they will both influence greatly the egg quality (i.e. lipid/energy content) (Bromage and Roberts, 1994; Salze, 2004). Feeding during vitellogenesis is also believed to promote an increase in the total number of eggs produced by a captive female (Kjesbu, 1989).

4.1.2.2. Environment

Fertilisation rates obtained by natural spawning are often high if the fish are spawning regularly (Bromage and Roberts, 1994). However, egg over-ripening in captivity may occur if the broodstock are subjected to stress (Morgan et al., 1999; Pavlov et al. 2004). Environmental factors such as water temperature, water salinity, mechanical and light stress or pathogen exposure can potentially affect both egg and larval survival (Bromage and Roberts, 1994; Eveillard, 2004). The use of artificial photoperiod, coupled with water temperature control, is commonly used in cod hatcheries to spread the production of eggs across the year (by advancing or delaying the natural spawning cycle).

4.1.2.3. Mass spawning vs. hand-stripping

Most commercial cod hatcheries rely on mass spawning in tanks coupled with automated egg collection. Generally no control over matings is realised, since stripping and artificial fertilisation are likely to result in casualties among the broodstock due to excessive handling stress (Richard Prickett, personal communication). Therefore, parameters such as the effective breeding population size, the spawning dynamics and the individual spawning performances are unknown.

4.1.2.4. Hatchery broodstock populations: origin and characteristics

The opinion is currently largely divided among cod breeders over the issue raised by using wild vs. farmed fish as broodstock. Several commercial hatcheries have reported poor reproductive performances of farm-bred stocks. Females may be mostly to blame as they are reported to frequently develop ovarian blockages and tumours (Richard Prickett, personal communication). As a result, cod hatcheries are

still widely relying on wild caught broodstock, even though this may not be sustainable in the long term.

Commercial cod hatcheries tend to skew the sex ratio in broodstock tanks towards females: 3 ♀:1 ♂ is considered to be optimal for obtaining high yields of fertilised eggs (Pavlov et al., 2004; Richard Prickett, personal communication). The stocking densities of cod broodstock in commercial breeding tanks from the Marine Farms AS group are maintained between 10 and 15 kg/m^3 (Richard Prickett, personal communication).

4.1.3. Aims of the study

The lack of information surrounding the management of Atlantic cod broodstock populations in commercial hatcheries motivated this study. The principal aims of this research work were: 1/ to study and compare the genetic diversity of three distinct cod broodstock populations 2/ to study the spawning dynamics in force in a mass spawning cod tank.

4.2. Materials and methods

4.2.1. Collection of samples for DNA profiling

4.2.1.1. Norwegian case study (wild broodstock): fry parentage assignment from a single spawning day

The "Norwegian" broodstock population consisted of 99 fish of wild origin (see Chapter 2, Figure 2.1). Farm records showed that the population was made up of 38 males, 54 females and 7 fish where the sex remained uncertain (these fish were considered as both males and females when performing parentage analyses, but eliminated from the size analysis of males and females). The fish were PIT-tagged as a routine farm procedure and a fin tissue sample was taken for DNA analysis.

Three hundred cod fry originating from a single day of spawning (19/11/03) were sampled in 95% ethanol. At the time of sampling the fry were 83 days post hatch. They had already undergone size grading at 15 mm and belonged to the smallest group (average standard length = 1.3 cm; average wet weight = 97 mg). This particular group was chosen for being the only fry tank of unmixed origin in the hatchery.

4.2.1.2. Scottish case study (wild broodstock): fry parentage assignment from four single spawning days

The "wild Scottish" broodstock population consisted of 141 fish of wild origin (see Chapter 2, Figure 2.1). The population was made up of 55 males, 73 females and 13 fish where the sex remained uncertain (these fish were considered as both males and females when performing parentage analyses, but eliminated from the weight analysis of males and females). The fish were PIT-tagged as a routine farm procedure and a fin tissue sample was taken for DNA analysis.

Four samples of three hundred cod fry, originating from four days of spawning (04/02/05, 18/02/05, 21/02/05 and 26/02/05), were sampled in 95% ethanol. These spawning dates correspond to the four unmixed egg batches (i.e. unique spawning date and broodstock tank origin) which were stocked in individual hatchery tanks during this particular season. At the time of sampling the fry were 50 days post hatch and had not undergone size grading. The sampling was done by the hatchery staff and the fry (preserved in 95% ethanol) were directly sent to the Institute of Aquaculture. There are no available records on their average size or their average wet weight.

4.2.1.3. Farmed broodstock genotyping

The "farmed Scottish" broodstock population consisted of 249 fish which were held in two broodstock tanks at MMF (see Chapter 2, Figure 2.1). Fin clips of the fish were sampled in 95% ethanol for subsequent DNA analysis. No fry were sampled from that particular stock.

4.2.2. DNA profiling

DNA was extracted from fin samples (all adults) and fry heads using the Dynabeads® genomic universal DNA kit (see Chapter 2).

Five loci (*Gmo*8, *Gmo*19, *Gmo*35, *Gmo*37 and *Tch*11) were used to both assess and compare the genetic diversity from the three broodstock populations. The loci were coamplified as the pentaplex described by Wesmajervi and colleagues (2006).

A total of eight loci (*Gmo*8, *Gmo*19, *Gmo*35, *Gmo*37, *Tch*11, *Gmo*3, *Gmo*34 and *Gmo*36) were used for analysing the parentage of the offspring samples. The loci were coamplified as three separate multiplex PCR reactions (a tetraplex and two

duplexes) as described in Chapter 2. Part of the genotyping analyses was realised at Fiskeriforskning, Norway (i.e. the pentaplex amplification).

The amplified DNA fragments were processed on two different laser-based capillary electrophoresis instruments: the ABI 310 Avant Genetic analyser (ABI) (pentaplex) and/or the CEQ 8800 Genetic Analysis System (Beckman Coulter) (tetraplex, two duplexes). The broodstock samples were run on three separate occasions (i.e. three PCR reactions per multiplex and per sample) to obtain high quality scores. Fry samples were screened only once.

4.2.3. Additional information gathered from hatchery records

Records from the commercial hatcheries were used to complete the information provided by the genotyping analyses. The data gathered concerned: 1) the age, gender, individual weights and total lengths of the "wild Norwegian" stock; 2) the age, gender, individual weights, daily collected egg quantities and fertilisation rates (2005 spawning season) of the "wild Scottish" broodstock. For the "farmed Scottish" stock, only the information related to the average age was disclosed (see Chapter 2, figure 2.1).

4.2.4. Parentage analysis

The genotyping data were analysed using the exclusion-based program FAP (see Chapter 3). An error tolerance of one allele mismatch was included in the parentage analyses.

4.2.5. Video recording of cod mating behaviour

Two CCD Night-Vision cameras 802C (Shenzhen Lianyida Science Co., Ltd.) were installed outside a cod breeding tank at MERL, during the spring season 2006.

The studied breeding tank was somewhat smaller than the commercial breeding tanks from both the Scottish and the Norwegian hatcheries (4 m in diameter, 1 m deep) and contained 16 cod males and 9 females of wild origin. Fish behaviours were recorded every other night, during April 2006, from 11pm to 3am (240 min video tape) on a VCR linked to one infrared camera, the other one only providing additional lighting. The recordings were made over 2 consecutive weeks.

4.3. Results

4.3.1. Comparison of the genotyping profiles of three hatchery broodstock populations

The degree of allelic polymorphism encountered at each of the five loci genotyped varied greatly amongst the three hatchery broodstock populations studied (Table 4.1). Both hatchery stocks of wild origin showed very similar levels of genetic diversity for four out of five loci. However, fewer alleles were accounted for, at all loci, for the "farmed Scottish" stock. For the locus *Gmo*19, eight alleles were found in the farm bred population versus 20 and 23 in the Norwegian and Scottish wild stocks (see Table 4.1). The degree of allelic polymorphism (adjusted to the population size), of the "farmed Scottish" stock was significantly different from the "wild Scottish" stock ($T = -3.31$, $P = 0.03$). Overall, the genetic diversity of the "wild Scottish" broodstock population was four times greater than the diversity of the farm bred stock for the five markers tested.

Table 4.1. Number of observed alleles, at five different loci, among three hatchery broodstock populations (semi automated allele detection followed by systematic manual correction of allele sizes).

Number of observed alleles	Farmed Scottish stock N = 249	Wild Scottish stock N = 141	Wild Norwegian stock N = 99
*Gmo*8	**11**/4.4	**40**/28.4	**22**/22.2
*Gmo*19	**8**/3.2	**23**/16.3	**20**/20.2
*Gmo*35	**7**/2.8	**9**/6.4	**7**/7.1
*Gmo*37	**8**/3.2	**14**/9.9	**13**/13.1
*Tch*11	**9**/3.6	**20**/14.2	**19**/19.2
Overall	**43**/17.2	**106**/75.2	**82**/82.8

Note: format **11**/4.4 where the first number refers to the number of alleles observed in the population and the second refers to the adjusted number of alleles for 100 fish (adjusted number of alleles = (number of alleles observed x 100) / N).

The level of heterozygosity was somewhat more homogenous across the three broodstock populations (Table 4.2) except, perhaps, for the locus *Gmo*19 where a significant reduction of heterozygote genotypes was observed for the farmed population (29%) compared to the two wild stocks (94% and 76%). Overall, the level of heterozygosity, at all five loci, of the "farmed Scottish" stock was not significantly different from the "wild Scottish" stock (T = -1.90, P = 0.13).

Table 4.2. Percentage of observed heterozygote genotypes, at five different loci, among three hatchery broodstock populations(semi automated allele detection followed by systematic manual correction of allele sizes).

Observed Heterozygosity	Farmed Scottish stock N = 249	Wild Scottish stock N = 141	Wild Norwegian stock N = 99
*Gmo*8	78.7	86.5	83.8
*Gmo*19	29.3	93.6	75.8
*Gmo*35	61.0	83.7	71.7
*Gmo*37	59.0	77.3	79.8
*Tch*11	93.6	90.1	87.9
Overall	64.3	86.2	79.8

Figure 4.2 shows the distribution of allelic frequencies, at the locus *Tch*11, for the three hatchery broodstock populations. Both populations of wild origin showed very similar profiles. This was also the case for the four other markers genotyped

(data not shown). As expected, the allelic frequency profiles of the farmed bred stock differed from the two wild populations for four loci (*Gmo*8, *Gmo*19, *Gmo*37 and *Tch*11) out of five. For example, at the locus *Tch*11, three alleles were highly represented in the farmed bred population (i.e. Freq.>15%) while no allele dominated in both populations of wild origin (see Figure 4.2).

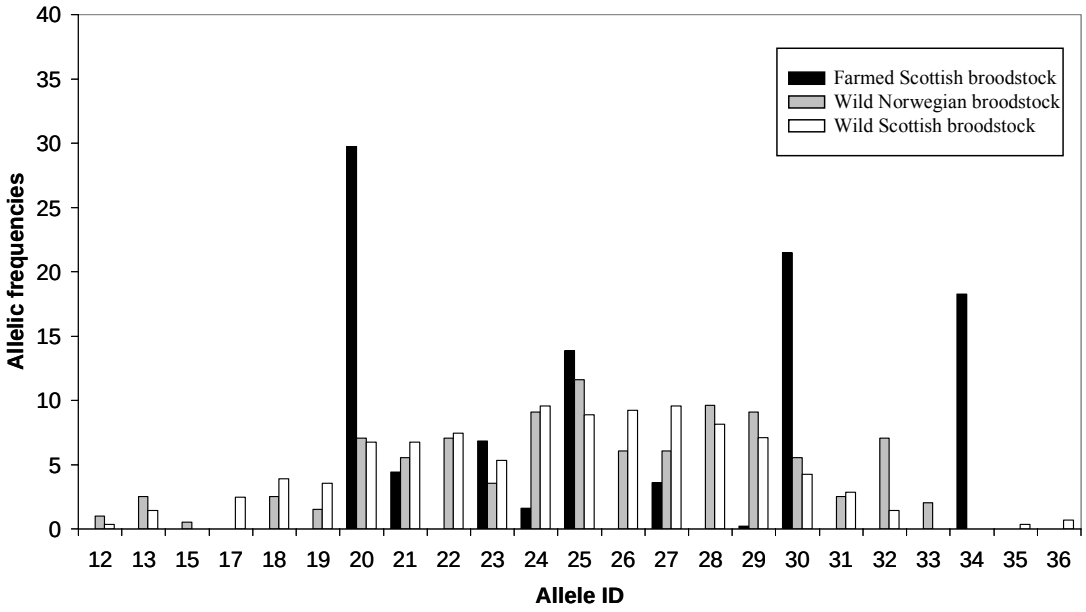


Figure 4.2. Comparison of allelic frequencies between three hatchery broodstock populations for the locus *Tch*11.

Out of the three hatchery populations, only the "wild Scottish" broodstock appeared to be in Hardy-Weinberg equilibrium (P = 0.22). The GENEPOP test on population differentiation revealed that all populations were different.

4.3.2. "Wild Norwegian" broodstock: parentage assignment study of fry from a single day spawning

4.3.2.1. Broodstock genotyping data

The genotyping data gathered from the "wild Norwegian" broodstock population showed striking differences in the degree of polymorphism amongst the markers. Four markers (*Gmo*8, *Gmo*19, *Gmo*37 and *Tch*11) displayed a large number of alleles (n≥12) while the others were much less polymorphic, thus limiting their informative value in the parentage analysis (see Table 4.3). Both from the parental genotype data and initial assignment trials it was apparent that scoring of three loci (*Gmo*8, *Gmo*19, and *Gmo*35) was compromised due to obvious large allele dropout and / or presence of null alleles (see Chapter 3). Efforts were made to minimise screening errors by optimising PCR conditions and careful manual checking of pertinent chromatograms. Nevertheless, scoring errors were expected to be present in the final dataset.

Table 4.3. Description of the polymorphic microsatellite markers used to genotype the "Wild Norwegian" broodstock.

Microsatellite Name	Locus assignment set [1]	Number of alleles identified in the "wild Norwegian" broodstock population
*Gmo*8	5	22
*Gmo*19	5	20
*Gmo*35	5	7
*Gmo*37	5	13
*Gmo*3	5+3	6
*Gmo*34	5+3	8
*Gmo*36	5+3	3
*Tch*11	5	19

[1] 5 – used in original 5 loci multiplex (Wesmajervi et al., 2006).
5+3 – used for 8 loci assignment

4.3.2.2. Parentage assignment

A very large number of families (n = 2745) could potentially be generated within the experimental tank holding 54 females, 38 males and 7 unsexed fish; i.e. (54x38) + (7x54) + (7x38) + (7x7). FAP predicted unambiguous allocation of offspring to a single pair of parents in 88.8% and 92.1% of cases, based on the parental genotype data for five and eight markers respectively. This gives only an approximate indication as to the resolution that would be achieved in the actual assignment as the figure was predicated on two assumptions (i.e. equal representation of all families in the sample and absence of genotyping errors / mutations), both unlikely to be met in this study. However, the prediction did confirm that the full set of eight polymorphic loci would not be sufficiently informative to fully resolve all parentage issues.

Of 300 offspring that were screened initially for five loci, complete genetic profiles were obtained for 278 individuals. Only 64% of these were assigned to a single family without error tolerance (Table 4.4). Assignment was increased to 77% by allowing one allele mismatch. Re-examination of a subset of these chromatogram traces identified the expected genotyping errors (mostly large allele dropouts). Even allowing for one allele mismatch 40 individuals could not be assigned to any expected parental pair, suggesting significant genotyping problems remained.

Table 4.4. Outcome of first assignment round for 278 fully genotyped offspring from the "Wild Norwegian"broodstock, at five loci (a further 22 individuals were not included due to incomplete data).

Assignment outcome	Predicted assignment	Actual assignment with allele mismatch tolerance #	
		0	1
Single-match	88.8%	179 (64%)	214 (77%)
Multiple-match	11.2%	11 (4%)	24 (9%)
No-match	--	88 (32%)	40 (14%)

To explore this further, the entire parental data set was re-screened for 8 loci (5 original + 3 additional) together with the 86 offspring not yet assigned to a single family when allowing for one mismatch allele (i.e. 24 multiple-matches + 40 no-matches + 22 incomplete data). Fourteen offspring samples were not scored for all loci and were omitted from further analysis. Of the remaining 72 fry, 19 were assigned to a single family under the most stringent condition (no errors permitted; Table 4.5). Allowing one allele mismatch per assignment increased single family resolution to 28 individuals, though further relaxation of stringency had little effect on single-match numbers (Table 4.5). Again, re-examination of chromatogram traces from these additional nine single-mismatch family assignments did identify predicted genotyping errors. Sixteen offspring were assigned to multiple families (Table 4.5). In 12 of these cases at least one identified single-match family was a candidate. Even with one allele mismatch allowed, 28 fry could not be reconciled to any potential family. Three mismatches were required to "force" assignment to all offspring (Table 4.5). Not surprisingly, given the reduced power of the analysis, all but one of these assignments were to multiple-match families.

Table 4.5. Outcome of second assignment round for 72 fully genotyped offspring from the "Wild Norwegian" broodstock, at eight loci (a further 14 individuals were not included due to incomplete data).

Assignment outcome	Predicted assignment	Actual assignment with allele mismatch tolerance #			
		0	1	2	3
Single-match	92.1%	19 (26%)	28 (39%)	28 (39%)	29 (40%)
Multiple-match	7.9%	7 (10%)	16 (22%)	36 (50%)	43 (60%)
No-match	--	46 (64%)	28 (39%)	8 (11%)	0 (0%)

4.3.2.3. Parental contributions

Analysis of parentage (based on 242 offspring assigned to single-match families, allowing up to one allele mismatch) indicated that at least 27% of the males and 23% of the females present in the broodstock tank actually contributed to the fry analysed (Table 4.6). The range and extent of spawning contributions were comparable between the two sexes. Thus, one male (M42) predominated, siring 50% (n = 121) of assigned fry. A further six males made substantial contributions (siring 5-41 offspring) while low levels of contribution (1-2 fry) were detected for another four males (Table 4.6). Similarly, a single female (F51) was responsible for 45% (n = 108) of offspring, five dams were assigned to 9-46 fry, while a further eight females had low level contributions (1-3 fry). This pattern of spawning has resulted in a markedly skewed contribution, with four males and five females (i.e. 9% of the fish in the tank) being responsible for 90% of successfully assigned fry. The most numerous family was M42 x F51 with 70 progeny, 29% of the total assigned (Table 4.6).

Both sexes were involved in multiple fertilizations (7 of 11 sires; 5 of 14 dams). Though more numerous in males, this likely reflects sampling bias, as there were more low frequency female assignments (8) *c.f.* males (4). For both sexes four of the top five most successful contributors were detected as multiple spawners. The

most successful male (M42) fertilised eggs from six different females, while the most successful female (F51) had eggs fertilised by three different males.

Table 4.6. Parental contribution to the offspring sample from the "Wild Norwegian" broodstock as determined by exclusion-based parentage, based on the genotyping of 5-8 DNA microsatellites.

Females ID	Males ID												No. of offspring	%	Cum. %
	M42	**M14**	**M72**	**M65**	**M83**	**M10**	**M30**	**M15**	**M38**	**M39**	**M54**	**M90**			
F51	**70**/66	--	**27**/20	**11**/5	--	--	--	--	--	--	--	--	108	44.6	44.6
F40	--	**35**/33	--	**2**/0	--	**4**/4	**2**/1	--	**1**/1	**1**/0	--	**1**/1	46	19.0	63.6
F29	**35**/34	--	--	**6**/2	--	--	**1**/1	--	--	--	--	--	42	17.4	81.0
F49	**12**/11	--	--	--	--	**1**/0	--	--	--	--	--	--	13	5.4	86.4
F51C	--	--	--	--	**11**/3	--	--	--	--	--	--	--	11	4.5	90.9
F19	**2**/2	**1**/1	**6**/6	--	--	--	--	--	--	--	--	--	9	3.7	94.6
F22	--	--	--	**1**/1	--	--	**2**/1	--	--	--	--	--	3	1.2	95.9
F25	--	**3**/3	--	--	--	--	--	--	--	--	--	--	3	1.2	97.1
F63	--	**2**/0	--	--	--	--	--	--	--	--	--	--	2	0.8	97.9
F44	--	--	--	--	--	--	--	--	--	--	**1**/0	--	1	0.4	98.3
F58	--	--	--	--	--	--	--	**1**/0	--	--	--	--	1	0.4	98.8
F59A	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	0.4	99.2
F68	--	--	--	--	--	--	--	**1**/0	--	--	--	--	1	0.4	99.6
F82	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	0.4	100.0
No. of offspring	121	41	33	20	11	5	5	2	1	1	1	1			
%	50.0	16.9	13.6	8.3	4.5	2.1	2.1	0.8	0.4	0.4	0.4	0.4			
Cum. %	50.0	66.9	80.6	88.8	93.4	95.5	97.5	98.3	98.8	99.2	99.6	100.0			

Notes: format **70**/60 where the first number refers to the number of offspring allocated allowing up to one allelic mismatch and the second refers to the number of offspring allocated allowing no error. Results are based on FAP allocations, one allele mismatch allowed.

4.3.2.4. Influence of male and female sizes on reproductive success

The average sizes (total length, mouth to tail) for the male and female broodstock were very similar (respectively 77 cm SD±7 cm and 76 cm SD±5 cm) and were not significantly different ($T = -0.03$, $P = 0.98$). The size distributions of the contributing males and females were not significantly different from the overall broodstock population (Figure 4.3; $T = 0.06$, $P = 0.96$ for contributing males vs. the rest of the male population; $T < 0.005$, $P > 0.99$ for contributing females vs. the rest of the female population). Figure 4.4 shows the male and female reproductive successes (both as percentage of offspring produced and as number of successful matings) against the male-female size difference. It shows that a small, positive male-female size difference (0 to 5 cm) resulted in both the highest number of successful matings and the highest percentage of offspring sired. The hypothesis of size-assortative matings was tested further by comparing actual spawning data with simulated matings. The mean male-females size difference computed from 1000 sets of random matings (whereby each of the 14 known female spawners was paired with a randomly selected male, with replacement, from the spawning tank) was ranked (see Figure 4.5). The observed male-female size difference (+ 2.7 cm) was mid ranking (629 of 1000 simulations), suggesting no obvious size bias in the mating pattern.

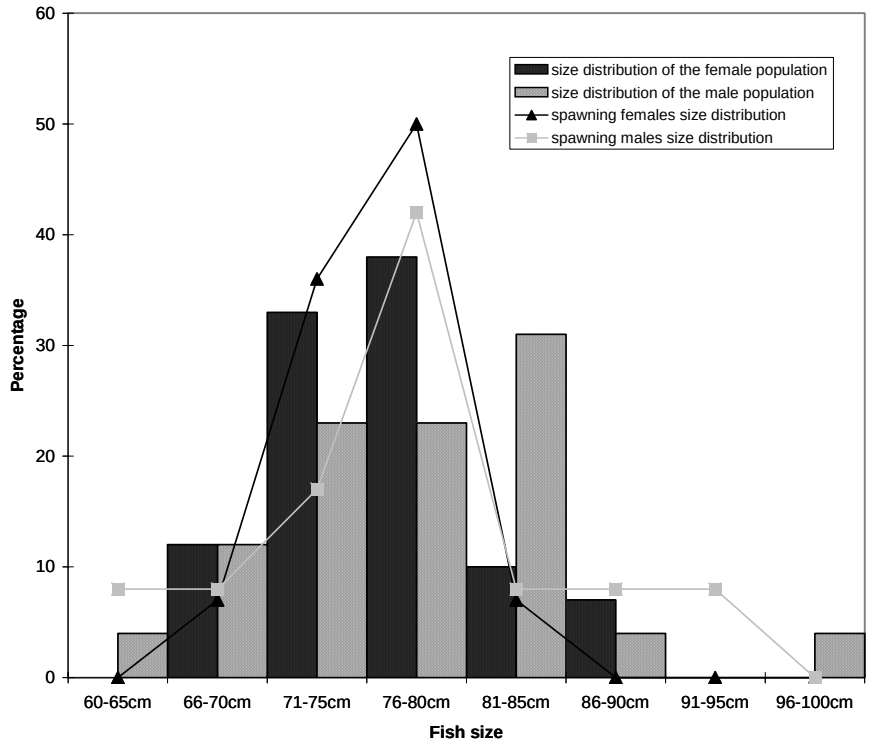


Figure 4.3. Comparison of the size distribution of the "Wild Norwegian" broodstock male and female populations with the size distribution of the spawning males and females.

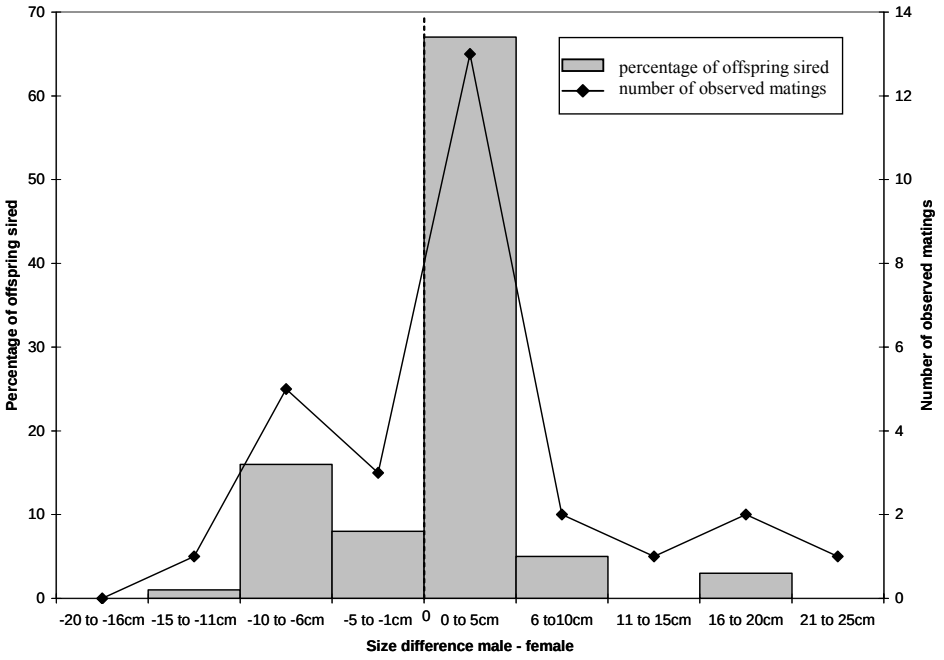


Figure 4.4. Overall reproductive success and number of successful matings plotted against parental size difference (sire length - dam length; values are grouped into 5 cm intervals) for the "Wild Norwegian" broodstock.

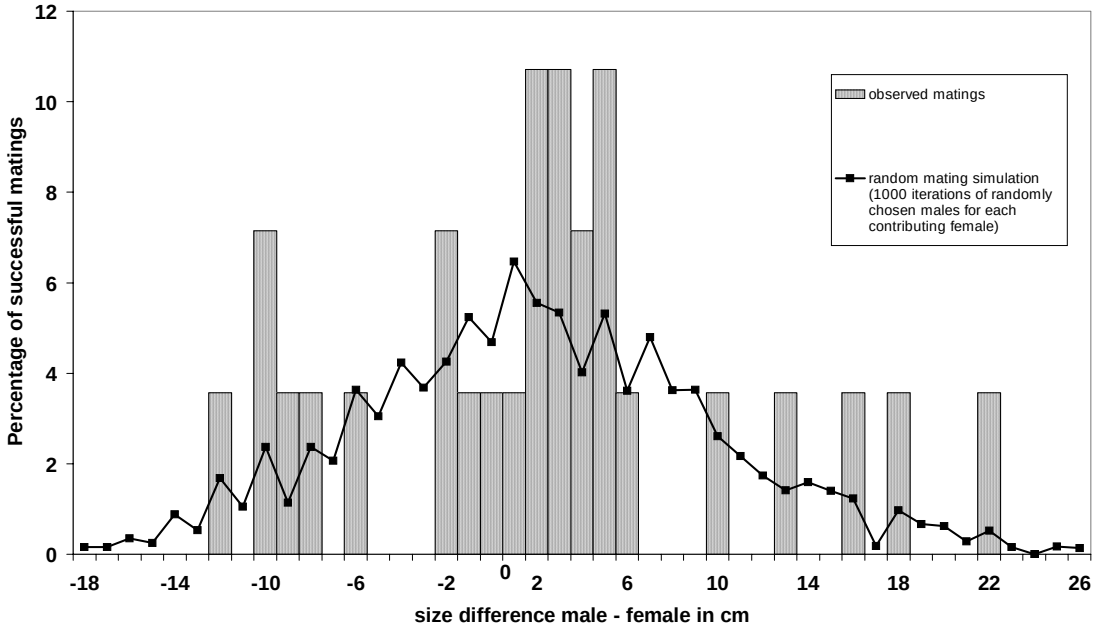
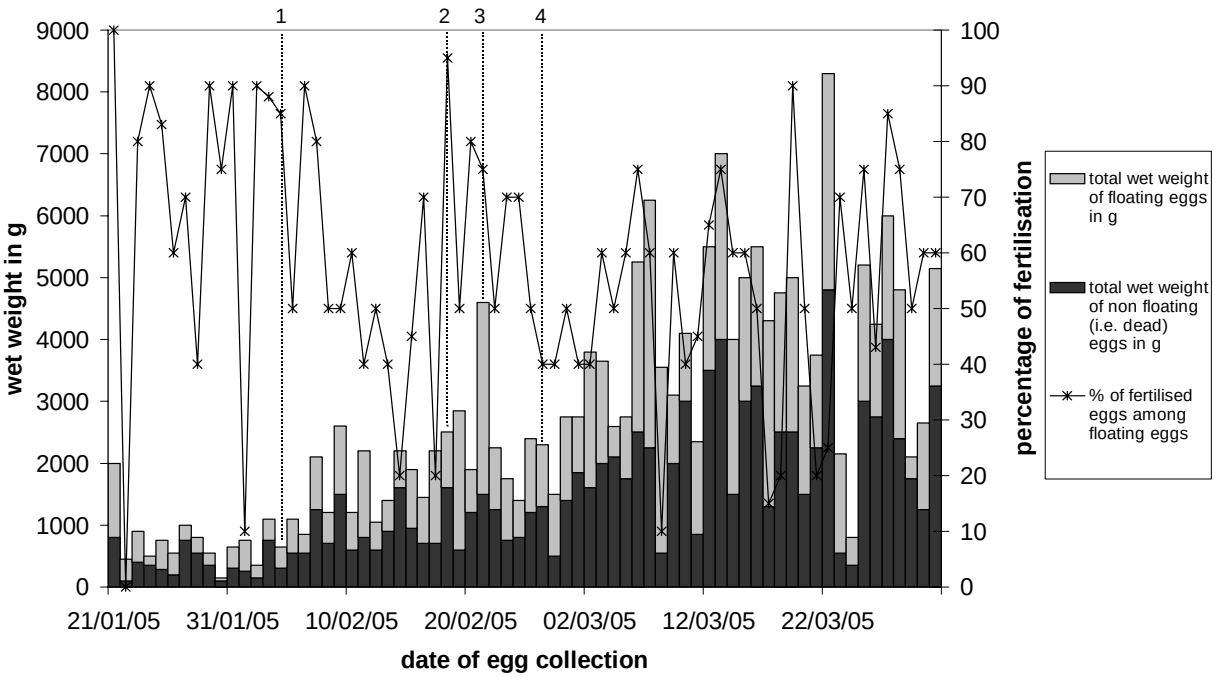


Figure 4.5. Comparison of the distribution of parental size differences (sire length - dam length) between the "real" matings and 1000 sets of randomly generated pairings created from the "Wild Norwegian" tank population.

4.3.3. "Wild Scottish" broodstock: comparison of parentage assignments of fry sampled from four days of spawning

4.3.3.1. Spawning season

The 2005 spawning season of the "wild Scottish" broodstock lasted for 70 days (i.e. from the 21st of January to the 31st of March). During this period, 99 kg of floating eggs were collected with an average fertilisation rate of 57%. A total of 19 egg batches (each from a single day of collection) were stocked in hatchery incubators and, subsequently, 13 fry batches were transferred to larval tanks. The four fry batches sampled in this study originated from the first five weeks of egg production (Figure 4.6). Records provided by MMF indicated that at least 75% of the eggs from the first three batches sampled were fertilised vs. only 40% for the last batch (Figure 4.8).



Note: 1 to 4 indicates the sampling dates. Weights were recorded at collection.

Figure 4.6. Records of daily egg collections for the "wild Scottish" broodstock during the 2005 spawning season (data provided by MMF).

4.3.3.2. Parentage assignment

In theory, a maximum of 5848 families could have been generated within the "wild Scottish" broodstock tank holding 73 females, 55 males and 13 unsexed fish (i.e. (55x73) + (13 x 55) + (13 x 73) + (13 x 13)). Based on the genotyping data from eight loci, FAP predicted unambiguous allocation of offspring to a single pair of parents in 95.9% of cases (assuming both the equal representation of all families and the absence of genotyping errors / mutations). It also identified 156 families with a predicted allocation success of below 9%. Such low predictions -although they only concerned 2% of the possible families- could potentially affect the outcome of the allocation exercise.

From the 1200 fry originally sampled, 915 were successfully typed for at least six loci (i.e. 76%). Individuals typed for less than six loci were not included in the parentage analysis. The allocation results of this set of data were very poor. Indeed, only 43% of the fry successfully typed for at least 6 loci were assigned to a single family without error tolerance (Table 4.7). The assignment rate increased to 56% when allowing for up to one allele mismatch. As expected, and despite re-examination of chromatogram traces, a large number of offspring (i.e. 285) could not be reconciled to any parental pair, suggesting that a significant amount of errors remained in the dataset. It took up to three allele mismatches to assign virtually all the offspring to at least one family (Table 4.7).

Table 4.7. FAP assignment outcome for 915 cod fry successfully genotyped for at least 6 markers.

Assignment outcome	Predicted assignment	Actual assignment with allele mismatch tolerance #		
		0	1	3
Single-match	95.9%	390 (42.7%)	511 (55.9%)	549 (60.0%)
Multiple-match	4.1%	48 (5.2%)	119 (13.0%)	359 (39.2%)
No-match	--	477 (52.1%)	285 (31.1%)	7 (0.8%)

4.3.3.3. Parentage contributions

Analysis of the parentage was carried out, for each of the four spawning dates, based on 511 offspring assigned to single-match families (see Tables 4.8, 4.9, 4.10 and 4.11). Overall, 156 full-sib families were found to contribute to the fry analysed, with an average of 44 families participating on a daily basis. 78% of the breeding population was involved in at least one spawning event (i.e. 64 females and 47 males). On average, 28 females and 18 males (i.e. 26% ± 10% of the males and 32% ± 5% of the females) contributed to the daily production of eggs. The extent of spawning contributions was highly unbalanced between the sexes (Figures 4.7 and 4.8). Three spawning dates were largely dominated by the contribution of one male (tag ID: 455F). This fish sired a total of 271 offspring (i.e. 53% of the fry analysed). The contribution of females appeared more balanced in comparison with on average 3 fish responsible for 50% of the fry produced on a daily basis (Figure 4.7). Overall three females dominated the four spawning dates analysed (tag IDs: E462, 7459 and F0CD). Together they contributed to 162 offspring (i.e. 32% of the fry analysed). On the other hand, 47 families (i.e. 26% of the total number of full-sib families) were only represented by a single offspring.

As previously found in "wild Norwegian" broodstock case study, in each of the four samples analysed, both sexes were involved in multiple fertilisations (see

Tables 4.8, 4.9, 4.10 and 4.11). The most successful male (tag ID: 455F) fertilised eggs from 40 different females, while the most successful female (tag ID: 7459) had eggs fertilised by only two different males.

Out of the 13 unsexed fish, 12 were found to contribute to at least one offspring sampled. 5 unsexed fish were identified as females, 3 as males and the remaining four were identified as both male and female (tag IDs: BE0F, CE2E, CF7D and 6043). This last result confirmed the existence of false assignments in this parental allocation exercise. These four "problematic fish" were found to contribute to 25 offspring (i.e. 4.9%).

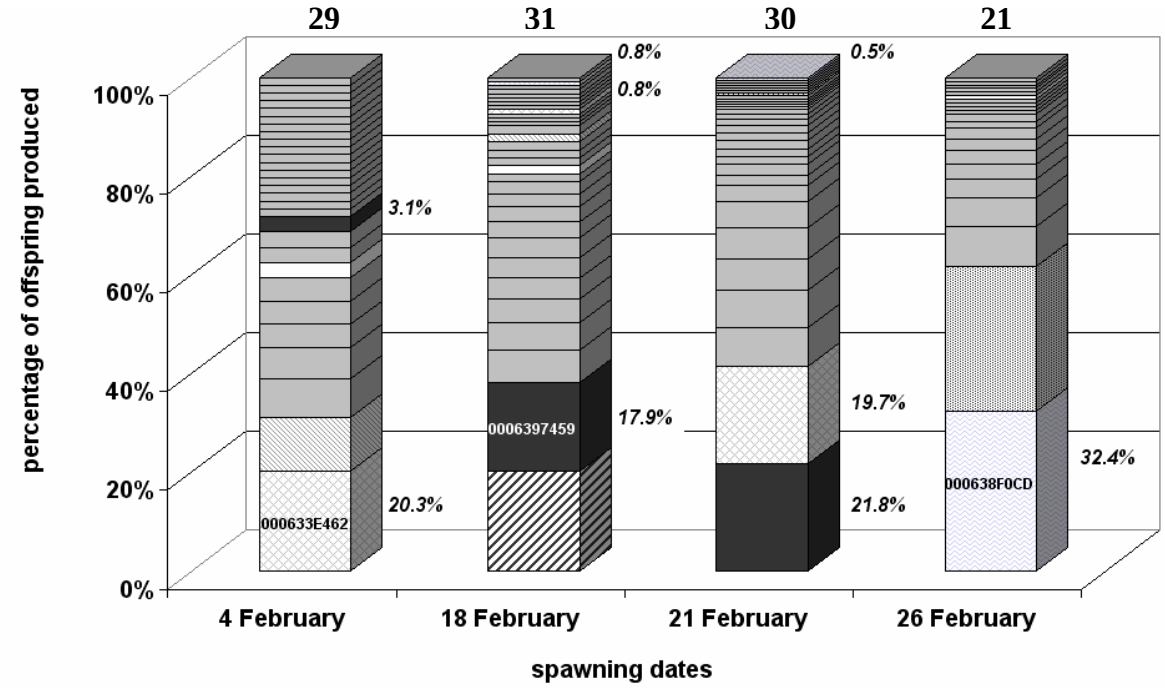


Figure 4.7. Female contributions among the "wild Scottish" broodstock to the four fry samples, as determined by exclusion-based parentage, based on the genotyping of 8 DNA microsatellites.

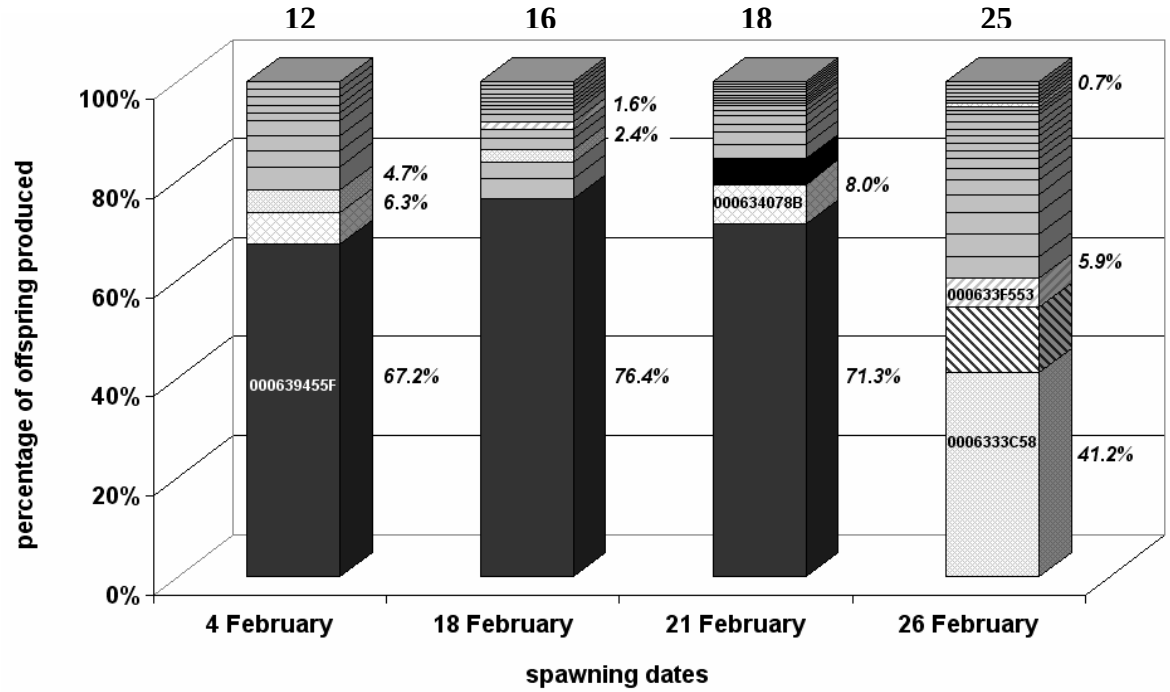


Figure 4.8. Male contributions among the "wild Scottish" broodstock to the four fry samples, as determined by exclusion-based parentage, based on the genotyping of 8 DNA microsatellites.

Table 4.8. Parental contribution of the "wild Scottish" broodstock to the fry sample from the 4th of February, as determined by exclusion-based parentage, based on the genotyping of 8 DNA microsatellites.

Females ID	Males ID												No. of offspring	%	Cum. %
	455F	078B	3C58	DD7A	2AC0	CE2E	DDE3	4CF2	B4B4	CBC8	EF82	4EE2			
E462	**12**/11	--	--	--	--	--	--	**1**/0	--	--	--	--	13	20.3	20.3
E384	**3**/1	--	**1**/0	**1**/1	**2**/2	--	--	--	--	--	--	--	7	10.9	31.3
4DF3	**2**/1	**1**/0	**1**/1	--	--	--	--	--	--	--	--	**1**/1	5	7.8	39.1
3F77	**4**/4	--	--	--	--	--	--	--	--	--	--	--	4	6.3	45.3
2E9C	--	**1**/1	**1**/1	--	--	**1**/1	--	--	--	--	--	--	3	4.7	50.0
DDEF	**3**/3	--	--	--	--	--	--	--	--	--	--	--	3	4.7	54.7
BE0F	**3**/3	--	--	--	--	--	--	--	--	--	--	--	3	4.7	59.4
2361	**1**/1	--	--	--	--	--	--	--	--	**1**/1	--	--	2	3.1	62.5
FD7B	**2**/0	--	--	--	--	--	--	--	--	--	--	--	2	3.1	65.6
E8BE	**2**/0	--	--	--	--	--	--	--	--	--	--	--	2	3.1	68.8
7459	--	--	--	--	--	--	**2**/2	--	--	--	--	--	2	3.1	71.9
21D1	**1**/0	--	--	--	--	--	--	--	--	--	--	--	1	1.6	73.4
2405	--	--	--	--	--	--	--	--	**1**/0	--	--	--	1	1.6	75.0
28EB	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	1.6	76.6
28F0	**1**/0	--	--	--	--	--	--	--	--	--	--	--	1	1.6	78.1
3931	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	1.6	79.7
409A	--	--	--	--	--	**1**/1	--	--	--	--	--	--	1	1.6	81.3
48CC	--	**1**/0	--	--	--	--	--	--	--	--	--	--	1	1.6	82.8
5686	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	1.6	84.4
D861	--	--	--	--	--	--	--	--	--	--	**1**/0	--	1	1.6	85.9
D8B6	**1**/0	--	--	--	--	--	--	--	--	--	--	--	1	1.6	87.5
ED54	--	**1**/1	--	--	--	--	--	--	--	--	--	--	1	1.6	89.1
F185	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	1.6	90.6
C252	--	--	--	**1**/1	--	--	--	--	--	--	--	--	1	1.6	92.2
CF7D	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	1.6	93.8
F63F	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	1.6	95.3
FEB1	**1**/0	--	--	--	--	--	--	--	--	--	--	--	1	1.6	96.9
5272	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	1.6	98.4
6D9F	--	--	--	**1**/0	--	--	--	--	--	--	--	--	1	1.6	100.0
No. of offspring	43	4	3	3	2	2	2	1	1	1	1	1			
%	67.2	6.3	4.7	4.7	3.1	3.1	3.1	1.6	1.6	1.6	1.6	1.6			
Cum. %	67.2	73.4	78.1	82.8	85.9	89.1	92.2	93.8	95.3	96.9	98.4	100.0			

Notes: format **12**/11 where the first number refers to the number of offspring allocated allowing up to one allelic mismatch and the second refers to the number of offspring allocated allowing no error. Results are based on FAP allocations, one allele mismatch allowed.

Table 4.9. Parental contribution of the "wild Scottish" broodstock to the fry sample from the 18th of February, as determined by exclusion-based parentage, based on the genotyping of 8 DNA microsatellites.

Females ID	Males ID																No. of offspring	%	Cum. %
	455F	**59D1**	**BE0F**	**2F10**	**3C58**	**4F52**	**F553**	**B8ED**	**31B3**	**3EFD**	**5330**	**E477**	**0626**	**CE2E**	**EF82**	**4EE2**			
45FA	**25**/25	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	25	20.3	20.3
7459	**22**/18	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	22	17.9	38.2
DDEF	**8**/8	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	8	6.5	44.7
E0E5	**7**/5	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	7	5.7	50.4
6043	**6**/4	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	6	4.9	55.3
2E9C	**3**/3	--	--	**1**/0	--	--	--	--	--	--	--	**1**/1	--	--	--	--	5	4.1	59.3
DDF6	**3**/2	--	--	--	--	**2**/1	--	--	--	--	--	--	--	--	--	--	5	4.1	63.4
DCA2	--	--	**3**/0	--	**2**/1	--	--	--	--	--	--	--	--	--	--	--	5	4.1	67.5
BE0F	**3**/3	--	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	4	3.3	70.7
D506	**4**/4	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	4	3.3	74.0
2405	**1**/1	**2**/2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	3	2.4	76.4
F63F	**1**/1	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	**1**/0	--	3	2.4	78.9
2361	**1**/1	--	--	--	--	--	--	--	--	**1**/0	--	--	--	--	--	--	2	1.6	80.5
44DE	**2**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2	1.6	82.1
042A	--	--	--	--	--	--	--	--	**1**/0	--	--	--	--	**1**/1	--	--	2	1.6	83.7
0BE8	--	--	--	--	--	--	--	**2**/1	--	--	--	--	--	--	--	--	2	1.6	85.4
E384	--	**2**/2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2	1.6	87.0
FEB1	--	--	--	**1**/1	--	--	**1**/0	--	--	--	--	--	--	--	--	--	2	1.6	88.6
39B2	--	--	--	--	--	--	--	--	--	--	**1**/1	--	**1**/0	--	--	--	2	1.6	90.2
28EB	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.8	91.1
2E11	--	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.8	91.9
32DE	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.8	92.7
409A	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.8	93.5
48CC	--	--	--	--	--	--	**1**/1	--	--	--	--	--	--	--	--	--	1	0.8	94.3
5298	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.8	95.1
E462	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	**1**/0	1	0.8	95.9
CE2E	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.8	96.7
DB2F	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.8	97.6
E9E4	--	--	--	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	0.8	98.4
F0CD	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.8	99.2
5272	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.8	100.0
No. of offspring	94	5	4	3	3	2	2	2	1	1	1	1	1	1	1	1			
%	76.4	4.1	3.3	2.4	2.4	1.6	1.6	1.6	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8			
Cum. %	76.4	80.5	83.7	86.2	88.6	90.2	91.9	93.5	94.3	95.1	95.9	96.7	97.6	98.4	99.2	100.0			

Note : format **25**/25, see Table 4.8.

Table 4.10. Parental contribution of the "wild Scottish" broodstock to the fry sample from the 21st of February, as determined by exclusion-based parentage, based on the genotyping of 8 DNA microsatellites.

Females ID	Males ID																		No. of offspring	%	Cum. %
	455F	**078B**	**0625**	**41B4**	**B8ED**	**295C**	**5634**	**FA3D**	**CE2E**	**2F10**	**39B8**	**3D0E**	**3EFD**	**F6FB**	**02A4**	**BC8C**	**CF7D**	**06A8**			
7459	**41**/41	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	41	21.8	21.8
E462	**36**/28	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	37	19.7	41.5
E57B	--	--	**7**/7	**2**/1	--	--	--	**2**/2	**2**/2	--	**1**/0	--	--	--	--	--	--	**1**/0	15	8.0	49.5
0BE8	**12**/9	--	**2**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	14	7.4	56.9
39B2	--	**11**/11	--	--	--	--	--	--	--	--	--	--	--	**1**/1	--	--	--	--	12	6.4	63.3
D506	**12**/4	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	12	6.4	69.7
41A8	**10**/10	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	10	5.3	75.0
E0E5	**6**/6	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	6	3.2	78.2
3F42	**3**/3	--	--	--	--	--	--	--	--	--	--	--	**1**/1	--	--	--	--	--	4	2.1	80.3
01B1	--	--	--	--	**1**/0	**1**/0	**2**/2	--	--	--	--	--	--	--	--	--	--	--	4	2.1	82.4
389E	**1**/1	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	**1**/1	--	--	3	1.6	84.0
54E8	--	--	--	--	**3**/2	--	--	--	--	--	--	--	--	--	--	--	--	--	3	1.6	85.6
CF7D	**3**/3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	3	1.6	87.2
E328	--	--	--	**3**/3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	3	1.6	88.8
E8BE	**2**/2	--	--	--	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	3	1.6	90.4
3931	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	**1**/0	--	2	1.1	91.5
D8B6	**1**/1	--	--	--	--	--	--	--	--	**1**/0	--	--	--	--	--	--	--	--	2	1.1	92.6
DDF6	**2**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2	1.1	93.6
2361	--	--	--	--	--	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	1	0.5	94.1
28EB	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.5	94.7
2E9C	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.5	85.2
32DE	--	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.5	95.7
3EFB	--	--	--	--	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	1	0.5	96.3
3FC6	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.5	96.8
3FE6	--	--	--	--	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	1	0.5	97.3
48CC	--	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.5	97.9
4DF3	--	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.5	98.4
5298	--	--	--	--	--	--	--	--	--	--	--	--	--	--	**1**/0	--	--	--	1	0.5	98.9
DB2F	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.5	99.5
F0CD	--	--	--	--	--	--	--	--	--	--	--	**1**/1	--	--	--	--	--	--	1	0.5	100.0
No. of offspring	134	15	10	5	5	3	3	2	2	1	1	1	1	1	1	1	1	1			
%	71.3	8.0	5.3	2.7	2.7	1.6	1.6	1.1	1.1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5			
Cum. %	71.3	79.3	84.6	87.2	89.9	91.5	93.1	94.1	95.2	95.7	96.3	96.8	97.3	97.9	98.4	98.9	99.5	100.0			

Note : format **41**/41, see Table 4.8.

Table 4.11. Parental contribution of the "wild Scottish" broodstock to the fry sample from the 26th of February, as determined by exclusion-based parentage, based on the genotyping of 8 DNA microsatellites.

Females ID	Males ID																									No.	%	Cum.
	3C58	DD79	F553	6043	7C60	F6FB	D986	CF7D	48C1	4F52	2AC0	2C26	2F10	2FAF	3572	F818	295C	3D0E	59D1	FA3D	078B	B5B1	B8ED	D649	D7C4	offsp.		%
F0CD	**44**/44	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	44	32.4	32.4
3FE6	--	**15**/14	**2**/2	**5**/3	**1**/0	**1**/1	**4**/1	**2**/2	**3**/1	--	**2**/2	--	--	**2**/0	--	**1**/0	--	**1**/0	**1**/0	--	--	--	--	--	--	40	29.4	61.8
3EFB	--	**2**/1	**1**/1	--	--	--	--	**1**/0	--	**3**/1	--	**1**/0	--	--	--	--	**1**/0	--	--	--	**1**/0	--	--	--	**1**/0	11	8.1	69.9
4D71	--	--	--	**1**/1	**1**/0	**4**/0	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	**1**/0	--	--	--	8	5.9	75.7
58E3	**3**/3	--	**2**/2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	5	3.7	79.4
5686	--	--	--	--	**3**/2	--	--	--	--	--	--	--	--	--	--	**1**/0	--	--	--	--	--	--	--	--	--	4	2.9	82.4
5272	**4**/3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	4	2.9	85.3
F25C	--	--	--	--	--	--	--	--	--	--	--	**1**/1	--	--	**1**/1	--	--	--	--	--	--	--	--	**1**/1	--	3	2.2	87.5
E328	**3**/3	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	3	2.2	89.7
303C	--	--	**1**/0	--	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	2	1.5	91.2
F083	--	--	--	--	--	--	--	--	--	--	--	--	**1**/0	--	--	--	--	--	--	--	--	--	**1**/1	--	--	2	1.5	92.6
2361	--	--	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.7	93.4
2E11	--	--	--	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.7	94.1
2E9C	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.7	94.9
409A	--	--	--	--	--	--	--	--	--	--	--	--	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	1	0.7	95.6
41A8	--	**1**/1	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.7	96.3
483D	--	--	--	--	--	--	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.7	97.1
5462	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	**1**/1	--	--	--	--	--	1	0.7	97.8
D861	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.7	98.5
DDEF	--	--	**1**/0	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	1	0.7	99.3
E922	--	--	--	--	--	--	--	--	--	--	--	--	--	--	**1**/1	--	--	--	--	--	--	--	--	--	--	1	0.7	100.0
No. offsp.	56	18	8	6	6	6	5	4	3	3	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1			
%	41.2	13.2	5.9	4.4	4.4	4.4	3.7	2.9	2.2	2.2	1.5	1.5	1.5	1.5	1.5	1.5	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7			
Cum. %	41.2	54.4	60.3	64.7	69.1	73.5	77.2	80.1	82.4	84.6	86.0	87.5	89.0	90.4	91.9	93.4	94.1	94.9	95.6	96.3	97.1	97.8	98.5	99.3	100.0			

Note : format **44**/44, see Table 4.8.

4.3.3.4. Effective breeding populations

The average effective breeding population size, for the fish successfully allocated on the four spawning dates sampled, was 7 fish (representing 5% of the total breeding population; see Figure 4.9). This result was largely explained by the markedly skewed contributions to the spawning events by very few fish (especially amongst the males). The effective breeding population size appeared not to be correlated with the quantity of eggs collected (Figure 4.9).

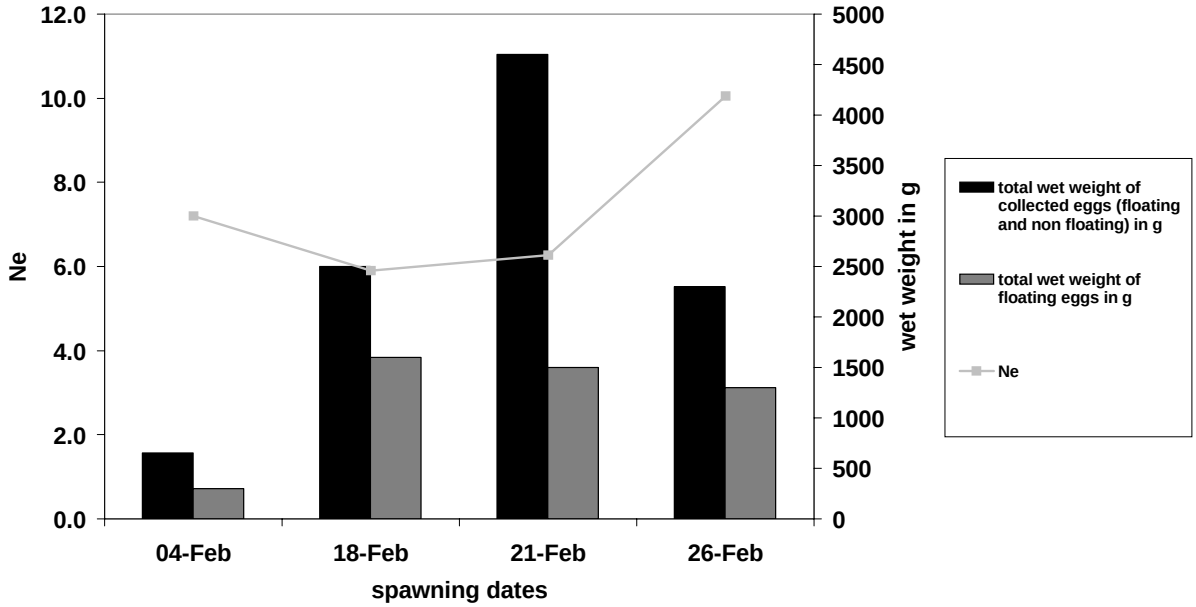


Figure 4.9. Effective breeding population sizes and egg production of the "wild Scottish" breeding tank.

4.3.3.5. Influence of male and female weights on reproductive success

The average weight of the "wild Scottish" broodstock population was 4.6 kg, the females being slightly heavier than the males (respectively 5.0 kg SD±1.5 kg and 4.1 kg SD±1.1 kg). Figure 4.10 shows the male and female reproductive successes (both as percentage of offspring produced and as number of successful matings)

against the male-female weight difference. Both the percentage of offspring produced and the number of successful matings followed a normal distribution centred around -1 to -0.1 kg which corresponds to the average weight difference between the males and females present in the tank. This later result indicates that there are no obvious weight biases in the mating pattern in the studied breeding tank. Finally, both the dominant male (455F weight = 4.7 kg) and the dominant females (E462 weight = 3.1 kg; 7459 weight = 4.6 kg; F0CD weight = 4.9 kg) were of average weight.

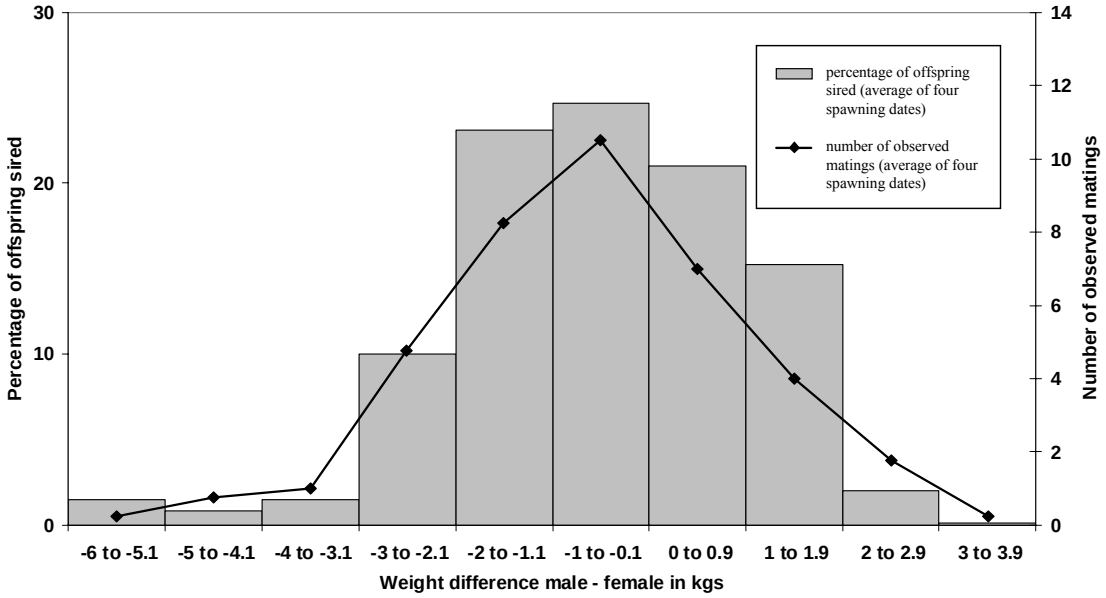


Figure 4.10. Overall reproductive success and number of successful matings plotted against parental weight difference (sire weight – dam weight; values are grouped into 1 kg intervals) for the "wild Scottish" breeding tank.

4.3.4. Mating behaviour of wild cod broodstock in tank systems

Both courtship behaviour and apparent mating behaviour were observed when analysing the video recordings gathered from the spawning activity of the breeding tank held at MERL. Over a recording session of four hours, on average, seven peaks of activity occurred. Those peaks of activity were relatively brief (3 minutes at most) and could involve up to 8 fish at a time. Behaviour associated with ventral mounting was clearly observed on one occasion (Figure 4.11). The video recordings also suggested the possible fertilisation of a single egg batch by several males. However, the distance of the camera from the water surface (approx. 1m) did not allow direct observation of release of gametes into the water.

4.3.5. Summary

The principal results of this Chapter are summarised in Table 4.12.

Table 4.12. Summarised results of Chapter 4.

	Wild Scottish stock N = 141	Wild Norwegian stock N = 99	Farmed Scottish stock N = 249
N of observed alleles (5 markers)	106	82	43
Observed heterozygosity (5 markers)	86.2%	79.8%	64.3%
Number of offspring analysed	915	286	0
N offspring assigned unambiguously (1 mismatch)	511 4 spawning dates	242 one spawning date	--
N contributing families	156 overall 44 daily	28	--
N contributing parents	111/78% overall 46/33% daily	26/26%	--
N contributing females	64/74% overall 28/33% daily	14/23%	--
N contributing males	47/69% overall 18/26% daily	12/27%	--
Ne	7/5% daily	--	--

Notes: format **111**/78% where the first number refers to the number of contributing individuals and the second refers to the percentage it represents within the broodstock (female/male) population.

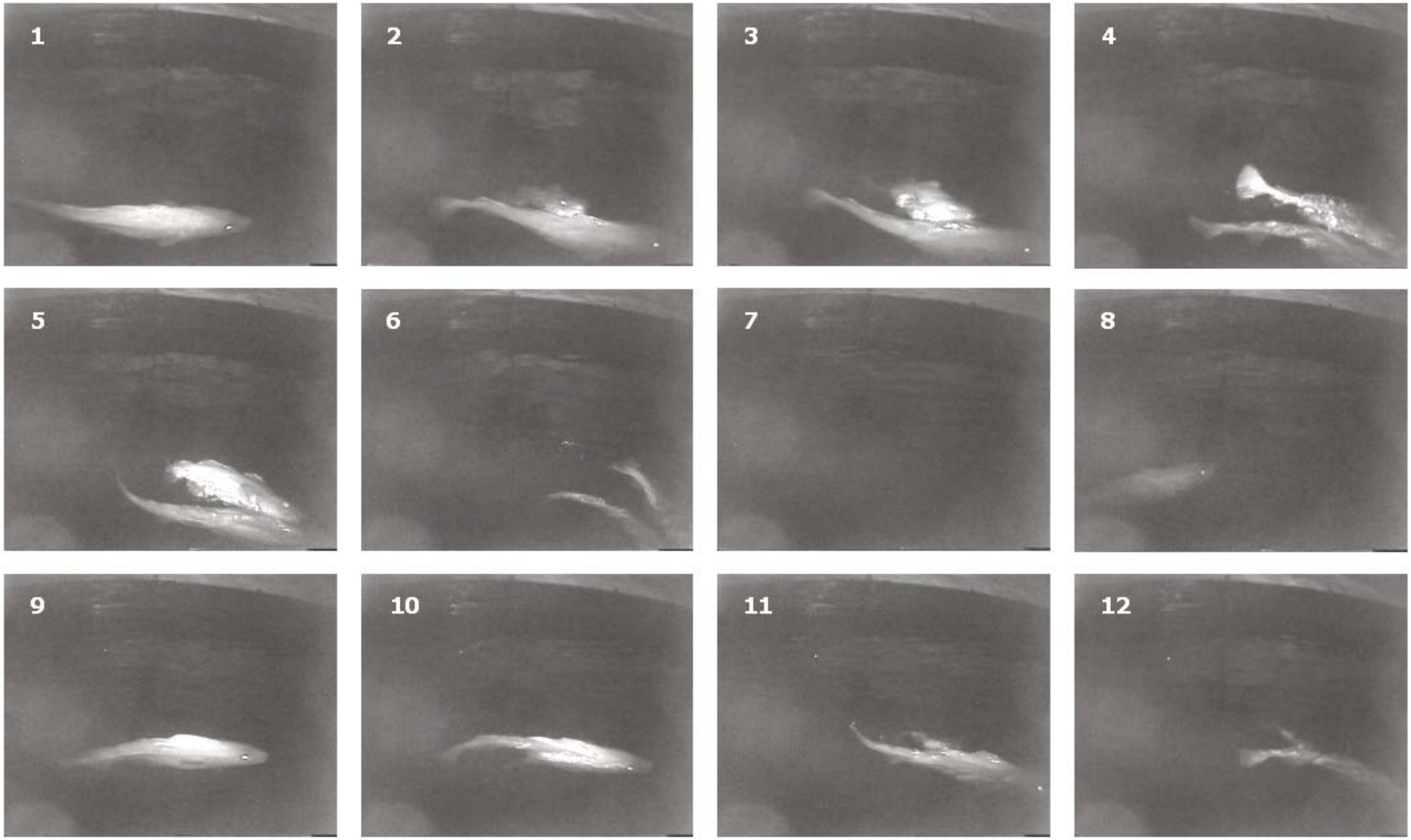


Figure 4.11. Sequence snap shots of the video recordings of MERL broodstock showing pair-mating and ventral mounting between a male and a female cod (real time length of the sequence: 165 seconds – time: 12.20am).

4.4. Discussion

Assigning parentage to offspring produced under commercial hatchery conditions proved to be a challenging exercise mainly because of the very large number of potential parental pairs which needed to be resolved (2800 in the Norwegian case study and as many as 5800 in the Scottish case study). The limited choice of published microsatellites for Atlantic cod, at the start of this research project, also constituted an important limiting factor in these studies. Of the eight loci used, two loci (*Gmo*8 and *Gmo*19) showed evidence of frequent large allele dropout. This was not fully resolved by PCR optimisation / genotype calling programs. Despite manual re-inspection of problematic chromatograms, the rate of allocation success in the study of spawning dynamics was extremely poor (i.e. 55.9%). The presence of unsexed fish amongst both the "wild Scottish" and the "wild Norwegian" broodstock populations further complicated the exercise. Not only did these fish create additional artificial families, they were also responsible for false assignments in the study of spawning dynamics. Despite allowing for up to one allele mismatch to allocate a given offspring, 28 fry (c. 10% of the total number screened), in the Norwegian case study, and 285 fry (c. 31%), in the Scottish case study, could not be reconciled to any expected parental pair. This may be due to further unresolved genotyping errors in the parental and / or offspring genotype data. However, more basic problems with the sample set may also explain some of the non-assignments. Parents were sexed by ultrasound and this may not have been 100% accurate. Although no novel alleles were observed in the offspring dataset, the presence of extraneous parents in the spawning tank or offspring in rearing tanks (i.e. due to unrecorded fish movements) also cannot be completely ruled out. The FAP predictive analysis indicated that the eight loci used would not be completely discriminatory in both parental studies. Thus, the presence of

multiple-matches was to be expected. The observed multiple-matches in the Norwegian case study, 16 out of 258 parental pair matched offspring (c. 6%), was similar to that predicted (8%) by FAP. However, in the Scottish case study, the observed multiple-matches were three times greater than expected (13% observed vs. 4% expected). This suggested that, in the Scottish case study, the loci used were not discriminatory enough. Due to the presence of these multiple matches and the fact that not all low level spawning participation is likely to have been represented in the samples taken, the data presented here represent the minimum numbers of parents involved in spawning events occurring on a daily basis.

Although exclusion-based assignment is known to be particularly sensitive to genotyping errors and / or allele mutations (Jones and Ardren, 2003; Vandeputte et al., 2006), this method was still considered preferable to a likelihood-based approach. The offspring dataset from the "wild Norwegian" broodstock was assigned using the likelihood method (see Chapter 3). The conclusions from this exercise were that: 1) it was difficult to interpret likelihood assignments in light of the possibly extensive errors within the genotype dataset; 2) it forced a detailed documentation / assessment of the errors to be undertaken (see Chapter 3).

For technical reasons (in terms of analysing costs and processing time), the number of offspring sampled per group was limited to 300 individuals. This means that none of the fry samples was statistically representative (i.e. hatchery tank populations ranged from 150 000 to 300 000 cod fry at the time of the sampling).

The parentage results from the exclusion based analysis indicate that, on a single day, at least 25 to 30% of the total breeding population contributed to fertilised

eggs that resulted in viable offspring among the analysed groups (at 50 and 83 days post-hatching). Family representation was, however, highly skewed in the five samples analysed. One family comprised between 20% to 30% of the surviving progeny while over 90% of the allocated offspring were the progeny of on average only 10% of the broodstock population. The results of the parentage analyses were similar between the "wild Scottish" and the "wild Norwegian" broodstock populations (in terms of ranges of family / individual contributions), although the fry sampled from the Norwegian hatchery had already undergone size grading. It seems to indicate that the first hatchery size grading did not have a marked effect on family representations. However, the possible selective loss of families linked with poor egg or fry survival prior to 50 days post-hatch was however impossible to quantify.

Cod females are batch spawners, shedding eggs on average once every 53 hours (Kjesbu et al., 1996) over a period of 1-2 months (Bekkevold et al., 2002). It is known that, in cod mass spawning tanks, not all females are perfectly synchronised and they will start spawning at different points in time (Kjørsvik et al., 2004). The maximum daily egg production (by volume) of a commercial broodstock tank is typically reached six to eight weeks after the first egg collection occurred (Richard Prickett, personal communication; Figure 4.6). None of the five fry samples originated from the peak of egg production. In the Norwegian case study the fry sampled was spawned three weeks after the peak of production and, in the Scottish case study, all four samples were spawned in the first five weeks of egg collection. Therefore, it is perhaps not surprising that, on average, only 35% of the females were found to contribute to the five spawning dates studied. The low percentage of males (31%) involved in the progeny of all five fry batches sampled is perhaps more surprising. It is known that cod males are capable of continuously producing milt

during the spawning season (Rakitin et al., 1999; Trippel, 2003). Therefore, they are, in theory, capable of fertilising eggs during the entire spawning season. The study of spawning dynamics brought to light the occurrence of male dominances within a population of captive cod but did not show any clear effect of the timing of the spawning season. Indeed, in the Scottish case study, for three spawning dates (out of the four studied), the same male fertilised on average 71% of the fry. This dominance had an extremely negative impact on the effective breeding population sizes. This observation is backed up by several publications on cod which have all showed the existence of male competition in spawning aggregations, whether in the wild or in small captive groups (Hutchings et al., 1999; Nordeide and Folstad, 2000; Bekkevold et al., 2002). In a broader context, evidence of low effective breeding populations in mass spawning tanks has also been reported in both gilthead and the red seabream commercial hatcheries (Perez-Enriquez et al., 1999; Brown et al, 2003; Nugrohoa and Taniguchi, 2004). In gilthead seabream, consistent low effective breeding populations, over a given spawning season, were attributed to a large number of non-contributing fish, particularly amongst males (Brown et al., 2003).

The data gathered in this study was not best suited for studying the possible occurrence of size-assortive mating in cod as described by Rakitin et al. (2001). Cod mating behaviour has been documented in both its natural wild environment and experimental tank-based rearing systems (Engen and Folstad, 1999; Hutchings et al., 1999; Nordeide and Kjellsby, 1999; Bekkevold et al., 2002). It is associated with complex sequences of events including courtship dance performance, sound production by the males, fin display and both dorsal and ventral mounts. The observations made using the infrared cameras showed that pair matings involving ventral mountings also occurred in large commercial breeding tanks. In this situation,

males in the same size range as the spawning females may benefit from an increased probability of successfully mating compared to bigger or small males. The data presented in this chapter did not allow to draw any firm conclusion on size-based mating choices. However it appears to rule out the assumption of male dominance based on larger body size (Hutchings et al., 1999) as the dominant males were never the largest. Furthermore, the close match between the number of successful matings and the percentage of offspring sired (Figures 4.4 and 4.10) suggests very little success of "sneaky" males - if we assume that sneaky males are less well matched in size than a male fertilising the bulk of the eggs in a ventral mount position. In that case we can expect the number of successful matings to be more broadly spread than the percentage of offspring sired (Figure 4.4). This is somewhat surprising as this result does not echo the strong evidences (provided by the analyses of parentage and video recordings) in favour of the existence of frequent matings between single females and multiple males. Overall, the spawning population matched the size distribution of the entire broodstock population, suggesting that reproductive success in cod is not skewed towards larger or smaller fish for either sex.

The genetic make-up of commercial cod breeding tanks is very heterogeneous not only between hatcheries but also between tanks within a same hatchery. For captive populations of wild origin, genetic differences might be attributed to the geographical origin of the stocks. The hypothesis of significant structuring of wild Atlantic cod populations was indeed reported in the literature on cod. It might be linked to the complex migratory patterns and fidelity to spawning grounds of wild cohorts (Nordeite and Folstad, 2000; Sarvas et al., 2004). The significant reduction of genetic diversity observed in the farm-bred stock illustrated the risks associated with "non-informed" decisions in managing hatchery breeding populations. Two

generations of inbreeding were sufficient to induce a significant genetic drift. Finally, out of the three breeding tanks studied, only the "wild Scottish" population was in Hardy-Weinberg equilibrium. It does suggest that even captive populations of wild origin might be subjected to genetic drift.

Chapter 5. Influence of hatchery practices (i.e. repeating size gradings and mixing of fish batches) on the genetic diversity of the juvenile production from a commercial mass spawning cod broodstock tank

5.1. Introduction

5.1.1. Stocking of larval tanks in Marine Farms ASA cod hatcheries

In cod hatcheries belonging to Marine Farms ASA (i.e. Grieg Marine Farms AS and Machrihanish Marine farm Ltd), initial stocking densities range from 50 to 100 larvae per litre (Richard Prickett, personal communication). A larval tank (8 m^3 average capacity) is typically stocked with 400,000 to 800,000 newly hatched larvae. In an ideal case scenario, a larval tank is to be stocked with fry originating from a single day of spawning from a broodstock population / tank. However, insufficient numbers of larvae may lead to the mixing of batches from several breeding populations and / or several days of spawning. Mixing of ages and / or origins is also likely to occur later on during hatchery rearing, as a direct consequence of size grading / pooling of similar size graded fish batches.

5.1.2. Growth dispensation

Under commercial rearing conditions, fish growth within the same age group and / or the same rearing unit can be very variable. Heterogeneous growth rates are likely to arise at a very early stage (Brown, 2003) and may progressively increase the variance of size distribution within a fish batch if no measures of control are taken. This phenomenon, referred to as growth dispensation, has been reported in various marine species including seabream, haddock and Atlantic cod (Goldan et al., 1997; Hamlin et al., 2000; Watson et al., 2006).

The reasons behind growth dispensation are not clearly understood and may be related to genetic variation, parental effects, environmental conditions and / or fish behaviour (Björklund et al., 2003).

5.1.2.1. Genetic variation for growth in farmed fish

The existence of an additive genetic variation for growth rate has been extensively documented in several fish species including Atlantic salmon (Thodesen et al., 2001), rainbow trout (Henryon et al., 2002), channel catfish (Silverstein et al., 2001), turbot (Gjerde et al, 1997) and, most recently, cod (Gjerde et al., 2004). In a paper published in 2004, Gjerde and colleagues studied the heritability for body weight and survival of 200 days post hatch Atlantic cod juveniles issued from two geographically distinct wild broodstock populations. Two mathematical models were created to calculate the heritability for body weight, including or not a fixed "region effect". The first model which included the "region effect" gave an heritability estimate for body weight of 0.29, SE± 0.27. The second model which did not include the "region effect" gave an heritability estimate of 0.52, SE± 0.26. As a result, the practice which consists in rearing together mixed families and / or mixed stock types of cod fry, is very likely to induce some range in fish sizes of genetic origin.

5.1.2.2. Parental effects

Panagiotaki and Geffen (1992) reported the existence of important size variation in newly hatched herring larvae which they attributed to parental effects. These effects are broadly described as the non-genetic influences derived from both maternal and paternal phenotypes (Bang et al., 2006). Since Panagiotaki and Geffen' s work, parental effects have been described in several other species including rainbow trout and haddock (Henryon et al., 2002; Probst et al., 2006). Parental effects are often exclusively attributed to females as they are responsible for the energetic content of eggs (Brown, 2003). The existence of a maternal effect in cod is still argued by scientists. Larsen (2002) stated that the spawning history of cod females did not have an influence on egg size or larval survival while previous studies suggested the

opposite (Kjesbu et al., 1996; Saborido-Rey and Junquera, 1999). However, even if parental effects are apparent at hatching, they are often progressively lost or masked by overriding environmental and / or behavioural effects (Brown, 2003).

5.1.2.3. Environmental parameters affecting fish growth

Environmental factors play a major role in the productivity of intensive hatchery rearing systems. Growth performances and survival of cod juveniles are known to be profoundly affected by water quality and temperature (Anderson and Dalley, 2000; Foss et al, 2004). Poor water quality and sub-optimal water temperature will both slow down cod juvenile growth (Rosenlund and Halldórsson, 2007). For example, Kling et al. (2007) showed that 80 days post hatch cod fry had a higher feed efficiency when reared at 10^{o}C compared to 16^{o}C. High stocking densities may also be detrimental to fish growth (Ashley, 2007), although this does not seem to be the case for cod juveniles providing feed is not a limiting factor (Puvanendran and Brown, 1999; Baskerville-Bridges and Kling, 2000). Intensive cod hatchery operations widely rely on artificial photoperiod to increase feed intake and promote growth in juveniles. Applying continuous light regime during first feeding was shown to have a positive effect on both cod juvenile growth and survival (Puvanendran and Brown, 2002; Rosenlund and Halldórsson, 2007). However, Monk et al. showed that better feed efficiency was achieved if the light regime was reduced from 2200 lux to 600 lux after day 28 post hatch.

Environment conditions can interfere with the expression of genetic variation for growth in fish. Saillant et al. (2006) recently found that heritability estimates for growth in European sea bass juveniles varied depending on both stocking densities and water temperature. Genotype x environment interactions have also been described

when studying heritability for growth in other fish species including common carp (Wang and Li, 2007).

5.1.2.4. Fish behaviour

Atlantic cod juveniles are aggressive feeders. In case of noticeable variances in growth and / or feed shortage, cannibalism is likely occur in hatchery larval tanks (Baskerville-Bridges and Kling, 2000). Cannibalism can lead to important mortalities, especially within the first four months of rearing (Brown et al, 2005; Höglund et al, 2005).

5.1.3. Size grading as a mean to control growth dispensation

In commercial farming, grading fish according to size is a well established procedure to control growth dispensation and simplify the feeding of tanks (i.e. by using the same type / particle sizes of feed) (Goldan et al., 1997). The effect of size grading on the growth rate of biomass remains unclear, although some studies suggest that size grading does not promote faster growth (Martins et al., 2005).

In Marine Farms cod hatcheries, cod fry are first size graded when reaching 50 days post hatch (Richard Prickett, personal communication). Grading boxes (supplied by Catvis, Netherlands) with interchangeable grids are used for sorting small sizes. The grading grids consist of parallel bars separated by spaces of standard width (to let small fish go through). The frequency of size grading is adapted to the growth performances of the batches. Limiting the number of gradings during larval rearing remains however in the hatchery's best interest since grading is a labour demanding task.

Size grading was recently reported to have a detrimental effect on the genetic diversity of commercially farmed barramundi (Frost et al, 2006). Results from this study suggested that family representation within the various size grades could significantly differ (caused by the existence of genetic variation for growth in this species). Although no such results have been yet reported for cod, the possible negative impact of repeated size gradings on the genetic diversity of commercially produced cod juveniles can not be ruled out.

In commercial hatcheries, grading of fish is often followed by the mixing of different batches of fry (i.e. different ages and / or different origins). To date, no published data describes the combined effect of size grading and mixing on the genetic diversity of commercially produced fish juvenile batches.

5.1.4. Aim of the study

The aims of this experimental study were first to analyse the genetic diversity of a cod juvenile batch produced by a commercial hatchery and, second, to test new DNA microsatellite markers for parentage analysis. To do so, the parentage of 960 cod juveniles produced by MMF (mean weight of 20g) was analysed using a "new" set of eleven loci. The allocation outcomes were compared with the results previously obtained using the set of eight loci (see Chapter 4). Parental contributions to the juvenile sample were analysed and compared with the contributions to the four fry batches analysed in Chapter 4. On this occasion, the effects of hatchery rearing procedures (i.e. size grading and mixing of batches) were investigated. Finally, the genetic diversity of the juvenile batch was assessed to determine whether or not this population was a suitable candidate for broodstock replacement.

5.2. Materials and methods

5.2.1. Collection of samples for DNA profiling

A parentage study was carried out on a batch of commercially produced 20g cod juveniles from MMF. Fin clips from 960 fish (i.e. corresponding to ten 96 well plates for DNA extraction and PCR amplifications) were sampled in 95% ethanol for subsequent DNA analysis. The batch sampled originated from the "wild Scottish" broodstock 2005 winter spawn (see Chapters 2 and 4) and regrouped juveniles from up to six different spawning dates (including the four dates previously sampled and analysed for parentage in Chapter 4). These fish were fin clipped towards the end of July 2005 (at an average age of 5 months). Other information concerning the batch sampled (for example the overall number of fish constituting the batch or the grading group) were not disclosed by the hatchery.

5.2.2. DNA profiling

DNA was extracted from fin samples using the Dynabeads® genomic universal DNA kit (see Chapter 2).

Eleven loci (*Gmo*C18, *Gmo*C20, *Gmo*C42, *Gmo*C52, *Gmo*35, *Gmo*37, *Tch*11, *Gmo*C71, *Gmo*C80, *Gmo*C90 and *Gmo*C88) were used for analysing the parentage of the cod juvenile samples. The loci were coamplified as 3 separate multiplex PCR reactions. *Gmo*C18, *Gmo*C20, *Gmo*C42, *Gmo*C52, *Gmo*C71, *Gmo*C80, *Gmo*C90 and *Gmo*C88 were coamplified as two tetraplexes as described in Chapter 2. *Gmo*35, *Gmo*37 and *Tch*11 were coamplified as a triplex derived from the pentaplex assay according to Wesmajervi et al (2006).

The amplified DNA fragments were processed on the ABI 310 Avant Genetic analyser (ABI). The broodstock samples were run on three separate occasions to obtain high quality scores. Juvenile samples were screened only once unless PCR reactions had failed.

5.2.3. Parentage analysis

The genotyping data were analysed using the exclusion-based program FAP (see Chapter 3). An error tolerance of two allele mismatches was included in the parentage analysis after the presence of null alleles among several broodstock DNA profiles was discovered (see section 5.3.1.1.).

5.3. Results

5.3.1. Analysis of the parental contribution to a batch of commercially produced cod juveniles

The parentage exercise involved a larger set of loci than in previous studies (eleven vs. eight; *cf.* Chapter 4). Overall, this set of eleven loci showed a greater level of allelic polymorphism among the "wild Scottish" broodstock population (Table 5.1). However, although eight markers (*Gmo*C18, *Gmo*C20, *Gmo*37, *Tch*11, *Gmo*C71, *Gmo*C80, *Gmo*C90 and *Gmo*C88) displayed at least 12 alleles (vs. four markers in the previous set of eight loci), the effective number of alleles was notably low for four markers (*Gmo*C42, *Gmo*C52, *Gmo*C90 and *Gmo*C88; see Table 5.1).

Table 5.1. Description of the eleven polymorphic microsatellite markers used to solve the parentage of commercially produced cod juveniles.

Microsatellite Name	Number of alleles identified in the "wild Scottish" broodstock population	Effective number of alleles (AE)
*Gmo*C18	15	9
*Gmo*C20	20	9
*Gmo*C42	6	3
*Gmo*C52	8	3
*Gmo*35	9	5
*Gmo*37	14	5
*Tch*11	20	15
*Gmo*C71	12	7
*Gmo*C80	18	10
*Gmo*C90	25	3
*Gmo*C88	12	4

5.3.1.1. Parental allocation

Based on the genotyping information provided by the "new" set of eleven loci, FAP predicted unambiguous allocation of offspring to a single pair of parents in 99.9% of cases.

A total of 951 cod juveniles - out of the 960 screened - were genotyped for at least 6 loci and were subsequently included in the parentage analysis. Of these 951 juveniles, 591 (i.e. 62%) were allocated by FAP to a single pair of parents with two allele mismatches allowed (Table 5.2). Allocation success varied according to the number of markers genotyped per offspring. The less markers were typed, the less single-matches were attributed; this became even more evident as the number of allelic incompatibilities between parents and offspring increased (see Table 5.2).

Table 5.2. FAP assignment success according to the number of markers typed per offspring and the number of allelic mismatches allowed.

Number of loci typed	Actual assignment with allele mismatch tolerance #			Total
	0	1	2	
11	**256**/256	**89**/89	**56**/56	**401**/401
10	**32**/32	**17**/17	**5**/5	**54**/54
9	**14**/14	**6**/7	**3**/6	**23**/27
8	**10**/10	**5**/6	**0**/6	**15**/22
7	**62**/62	**28**/35	**0**/29	**90**/126
6	**6**/9	**2**/3	**0**/1	**8**/13
Total	**380**/383	**147**/157	**64**/103	**591**/643

Note: format **256**/256 where the first number refers to the number of offspring allocated to a single family and the second refers to the number of offspring allocated to one or more families.

380 offspring (i.e. 40%) were allocated by FAP to a single pair of parents with no allele mismatch (Table 5.3).The manual inspection of chromatograms, which took place after the first round of FAP allocations, revealed the existence of at least seven null alleles among the broodstock population. A null allele (Ø) was exposed when a

parent appeared homozygous at a given locus but this allele was apparently not transferred to the offspring (for example: Male genotype: 119/Ø, Female genotype: 137/140 and Offspring genotype: 137/Ø). These null alleles were exposed at five different loci: *Gmo*C20 (2), *Gmo*C52 (2), *Gmo*37 (1), *Tch*11 (1) and *Gmo*C90 (1). More importantly, null alleles were identified in the genotype profiles of two main contributing parents (i.e. males 455F and DD79). Consequently, an error tolerance of two allele mismatches was allowed in the final FAP analysis to account for the errors possibly generated by the presence of null alleles. When allowing for up to two mismatches, assignment was further increased to 62% (Table 5.3). However, a large number of offspring (32%) still remained unassigned. Up to six allele mismatches were necessary to "force" assignment to all juveniles.

Table 5.3. FAP assignment outcome for 951 cod juveniles successfully genotyped for at least 6 markers (out of 11).

Assignment outcome	Predicted assignment	Actual assignment with allele mismatch tolerance #				
		0	1	2	3	6
Single-match	99.9%	380 (39.9%)	527 (55.4%)	591 (62.1%)	693 (72.8%)	791 (83.2%)
Multiple-match	0.1%	4 (0.4%)	14 (1.5%)	53 (5.6%)	61 (6.4%)	160 (16.8%)
No-match	--	567 (59.7%)	410 (43.1%)	307 (32.3%)	197 (20.8%)	0 (0%)

Overall, the outcome of this allocation exercise was very similar to the previous case study which only employed eight loci (section 4.3.5). Introducing new polymorphic loci did not improve the percentage of offspring successfully assigned to a unique parental pair when up to one allele mismatch was allowed (Table 5.4). It did however significantly reduce the number of multiple matches (13% with eight loci vs. only 2% with eleven loci in case of one allele mismatch). The percentage of no-matches was always greater in the case of 11 loci, which immediately suggested that additional typing errors had been introduced as more loci were genotyped (Table 5.4).

Table 5.4. Comparison of FAP assignment outcomes between the two parentage exercises realised on offspring produced by the "wild Scottish" broodstock.

Assignment outcome	Assignment with 0 mismatch		Assignment with 1 mismatch		Assignment with 3 mismatches	
	8 loci*	11 loci*	8 loci	11 loci	8 loci	11 loci
Single-match	43%	40%	56%	55%	60%	73%
Multiple-match	5%	0%	13%	2%	39%	6%
No-match	52%	60%	31%	43%	1%	21%

* refers to the set of markers used in the parentage analysis.

5.3.1.2. Parental contributions

157 families were found to contribute to the juvenile sample analysed. 108 fish (77% of the total breeding population) were identified as parents (i.e. 50 males and 58 females). As expected, individual contributions were extremely uneven for both sexes (Table 5.5). The dominant male 455F - identified previously through the study of spawning dynamics (Chapter 4) - sired 228 of the juveniles analysed (i.e. 39%). Once again, in comparison, the range of female contributions was more balanced with 7 fish responsible for 52% of the juveniles produced (Table 5.5). However, as many as ten females and thirteen males (16% of the breeding population) were found to be only represented by a single offspring. Not surprisingly, family contributions were also highly skewed (Table 5.6). The most represented family accounted for 11.5% of the juveniles analysed. On the other hand, 90 families (i.e. 57% of the total number of contributing families) were only represented by a single juvenile in the sample analysed.

Table 5.5. Parental contributions to the juvenile sample (produced by the "wild Scottish" broodstock), as determined by FAP, based on the genotyping of 11 DNA microsatellites.

Contributing Females	No. of offspring	%age	Cumulative %age	Contributing Males	No. of offspring	%age	Cumulative %age
38A6	92	15.6	15.6	**455F**	228	38.6	38.6
7459	68	11.5	27.1	**DD79**	68	11.5	50.1
3FE6	36	6.1	33.2	**078B**	28	4.7	54.8
FEB1	34	5.8	38.9	**F553**	21	3.6	58.4
41A8	31	5.2	44.2	**9619**	20	3.4	61.8
39B2	24	4.1	48.2	**7C60**	20	3.4	65.1
E0E5	24	4.1	52.3	**0626**	19	3.2	68.4
0BE8	22	3.7	56.0	**CE2E**	19	3.2	71.6
F0CD	19	3.2	59.2	**3C58**	18	3.0	74.6
E462	17	2.9	62.1	**2AC0**	11	1.9	76.5
E57B	14	2.4	64.5	**2C26**	10	1.7	78.2
D506	14	2.4	66.8	**B8ED**	10	1.7	79.9
58E3	13	2.2	69.0	**2FAF**	8	1.4	81.2
6043	13	2.2	71.2	**46A8**	8	1.4	82.6
45FA	12	2.0	73.3	**F818**	7	1.2	83.8
DCA2	12	2.0	75.3	**295C**	6	1.0	84.8
5462	10	1.7	77.0	**51E0**	6	1.0	85.8
DDEF	10	1.7	78.7	**2F10**	5	0.8	86.6
409A	9	1.5	80.2	**41B4**	55	0.8	87.5
2E9C	8	1.4	81.6	**57B1**	5	0.8	88.3
5686	8	1.4	82.9	**BE0F**	5	0.8	89.2
CF7D	8	1.4	84.3	**CF7D**	5	0.8	90.0
4D71	6	1.0	85.3	**FB92**	5	0.8	90.9
3EFB	5	0.8	86.1	**3EFD**	4	0.7	91.5
3F77	5	0.8	87.0	**4F52**	4	0.7	92.2
F25C	5	0.8	87.8	**CBC8**	4	0.7	92.9
01B1	5	0.8	88.7	**D7C4**	4	0.7	93.6
21D1	4	0.7	89.3	**E9E4**	4	0.7	94.2
28EB	4	0.7	90.0	**48C1**	3	0.5	94.8
3F42	4	0.7	90.7	**5634**	3	0.5	95.3
F083	4	0.7	91.4	**06A8**	3	0.5	95.8
E328	4	0.7	92.0	**211A**	2	0.3	96.1
48C1	3	0.5	92.6	**5330**	2	0.3	96.4
F185	3	0.5	93.1	**E475**	2	0.3	96.8
042A	3	0.5	93.6	**FA3D**	2	0.3	97.1
CBC8	3	0.5	94.1	**D986**	2	0.3	97.5
0082	3	0.5	94.6	**6043**	2	0.3	97.8
2668	2	0.3	94.9	**3458**	1	0.2	98.0
3916	2	0.3	95.3	**3776**	1	0.2	98.1
44DE	2	0.3	95.6	**39B8**	1	0.2	98.3
483D	2	0.3	95.9	**3D0E**	1	0.2	98.5
48C9	2	0.3	96.3	**4CF2**	1	0.2	98.6
48CC	2	0.3	96.6	**59D1**	1	0.2	98.8
DDF6	2	0.3	97.0	**DD7A**	1	0.2	99.0
BE0F	2	0.3	97.3	**E477**	1	0.2	99.2
E8BE	2	0.3	97.6	**B5B1**	1	0.2	99.3
F1F6	2	0.3	98.0	**C483**	1	0.2	99.5
516A	2	0.3	98.3	**EF82**	1	0.2	99.7
2361	1	0.2	98.5	**45B5**	1	0.2	99.8
247B	1	0.2	98.6	**4EE2**	1	0.2	100.0
26D4	1	0.2	98.8				
323B	1	0.2	99.0				
5298	1	0.2	99.2				
D861	1	0.2	99.3				
E922	1	0.2	99.5				
02A4	1	0.2	99.7				
E9E4	1	0.2	99.8				
F63F	1	0.2	100.0				

Table 5.6. Contributing families to the juvenile sample (produced by the "wild Scottish" broodstock), as determined by FAP, based on the genotyping data provided by 11 DNA microsatellites.

Family (female x male)	No. of offspring	Percentage	Cumulative %age
7459 x 455F	68	11.5	11.5
38A6 x DD79	45	7.6	19.1
E0E5 x 455F	23	3.9	23.0
3FE6 x DD79	21	3.6	26.6
39B2 x 078B	20	3.4	29.9
41A8 x 455F	18	3.0	33.0
0BE8 x 455F	17	2.9	35.9
E462 x 455F	16	2.7	38.6
F0CD x 3C58	16	2.7	41.3
D506 x 455F	14	2.4	43.7
38A6 x 7C60	13	2.2	45.9
6043 x 455F	13	2.2	48.1
38A6 x CE2E	12	2.0	50.1
45FA x 455F	12	2.0	52.1
FEB1 x F553	12	2.0	54.1
DDEF x 455F	10	1.7	55.8
E57B x 0625	10	1.7	57.5

5.3.1.3. Evolution of the genetic diversity of a commercially produced cod juvenile batch throughout hatchery rearing

Table 5.7 compares the number of contributing parents / families as well as the effective breeding population sizes between the four fry samples previously analysed (see Chapter 4) and the present juvenile sample. Overall, the genetic diversity of the juvenile sample was greater than the diversity of any of the four fry samples. The number of contributing families identified in the juvenile sample was, on average, four times greater than the number of families represented in any of the four fry samples (see Table 5.7). The effective breeding population size of the juvenile sample represented 14% of the breeding population (i.e. 20 fish) which was two to three times greater than the effective population size of a single day of spawning (i.e. 7 to 10; see Table 5.7).

Table 5.7. Contributing parents / families and effective breeding population sizes for four samples of cod fry (representing 4 days of spawning) and a batch of 591 juveniles, issued from the production of the "wild Scottish" broodstock.

	Fry spawn on the 04/02/05	Fry spawn on the 18/02/05	Fry spawn on the 21/02/05	Fry spawn on the 26/02/05	Juveniles sampled on the 22/07/05
No. of offspring assigned	64	123	188	136	591
No. of contributing families	39	43	46	50	157
No. of contributing males	12	16	18	25	50
No. of contributing females	29	31	30	21	58
Ne	7.20	5.90	6.27	10.05	20.03

The number of contributing families identified in the juvenile sample was almost identical to the number of families found to have contributed to the combined four fry samples previously analysed, if we remove all duplicate families which appear to contribute in more than one spawning date (i.e. respectively 157 vs. 156 families; see Chapter 4 section 4.3.3.3). However, only 47 families were shared

between the fry and the juvenile samples analysed (i.e. approx. 30%). Each of the "original" 109 families found in the fry samples, and subsequently "lost" in the juvenile batch analysed, were only marginal contributors (contributing at most to 1% of the fry analysed). Amongst the additional 110 families identified in the juvenile sample, only four contributed to more than 1% of the juveniles analysed. The second most contributing family found in the juvenile sample (i.e. 38A6 x DD79) was one of them (with 7.6%).

Table 5.8 compares the family representations in the fry and juvenile samples (with the four fry samples combined together), for the sixteen most contributing families identified in the fry samples. Overall, these families accounted for 58% of the fry and 44% of the juveniles analysed. Only one of these sixteen "original" families (i.e. 3FE6 x 6043) was no longer detected amongst the juveniles. The most represented family in the fry batches analysed (i.e. 7459 x 455F) maintained its level of contribution throughout hatchery rearing (i.e. 12.3% amongst the fry vs. 11.5% amongst the juveniles). However, both the families E462 x 455F and F0CD x 3C58 saw their contributions significantly decrease (from 9% to 3%; see Table 5.8). None of the marginally represented families in the fry samples saw a significant increase of its contribution in the juvenile batch analysed (data not shown).

Table 5.8. Family contributions to both the fry and juvenile samples -issued from the "wild Scottish" broodstock production- as determined by exclusion-based parentage, based on the genotyping of 8-11 DNA microsatellites.

Family (female x male)	Four fry samples combined			Juvenile sample		
	No. of offspring	Percentage	Cumulative %age	No. of offspring	Percentage	Cumulative %age
7459 x 455F	63	12.3	12.3	68	11.5	11.5
E462 x 455F	48	9.4	21.7	16	2.7	14.2
F0CD x 3C58	44	8.6	30.3	16	2.7	16.9
45FA x 455F	25	4.9	35.2	12	2.0	19.0
D506 x 455F	16	3.1	38.4	14	2.4	21.3
3FE6 x DD79	15	2.9	41.3	21	3.6	24.9
E0E5 x 455F	13	2.5	43.8	23	3.9	28.8
0BE8 x 455F	12	2.3	46.2	17	2.9	31.6
39B2 x 078B	11	2.2	48.3	20	3.4	35.0
DDEF x 455F	11	2.2	50.5	10	1.7	36.7
41A8 x 455F	10	2.0	52.4	18	3.0	39.8
E57B x 0625	7	1.4	53.8	10	1.7	41.5
BE0F x 455F	6	1.2	55.0	1	0.2	41.6
6043 x 455F	6	1.2	56.2	13	2.2	43.8
3FE6 x 6043	5	1.0	57.1	0	0.0	43.8
DDF6 x 455F	5	1.0	58.1	1	0.2	44.0

Note: only the 16 most contributing families figure in this Table.

5.3.1.4. Genetic makeup of the juvenile batch

Table 5.9 compares the number of alleles observed, at 11 loci, between the juvenile batch sampled and the "wild Scottish" broodstock. Overall, there was no significant losses of alleles in the first generation of farmed juveniles analysed. Four markers (*Gmo*C18, *Gmo*C42, *Gmo*C52 and *Gmo*C71) maintained the same level of polymorphism in the F1 batch analysed. The reduction of allelic polymorphism was the most important in the case of two markers (*Gmo*37 and *Gmo*C80), with 3 alleles less being observed in the F1 (Table 5.9). Allelic frequencies - at each of the 11 loci typed - for the juvenile batch and the "wild Scottish" broodstock were not significantly different (Table 5.10). These results suggested that there was no major loss of genetic diversity between the broodstock of wild origin and the F1 population sampled.

Table 5.9. Comparison of allelic diversities, at 11 loci, between the "wild Scottish" broodstock and the batch of commercially produced F1 juveniles.

Locus	"wild Scottish" broodstock	Juvenile sample
*Gmo*C18	15	15
*Gmo*C20	20	19
*Gmo*C42	6	6
*Gmo*C52	8	8
*Gmo*35	9	7
*Gmo*37	14	11
*Tch*11	20	18
*Gmo*C71	12	12
*Gmo*C80	18	15
*Gmo*C90	25	24
*Gmo*C88	12	10

Table 5.10. Results of Paired T-tests realised on the allelic frequencies, at 11 loci, between the "wild Scottish" broodstock and the batch of commercially produced F1 juveniles.

Locus	T-Value	P-Value
*Gmo*C18	0.00	1.00
*Gmo*C20	0.00	1.00
*Gmo*C42	0.00	0.99
*Gmo*C52	0.00	1.00
*Gmo*35	0.00	1.00
*Gmo*37	0.00	0.99
*Tch*11	0.00	0.99
*Gmo*C71	0.00	0.99
*Gmo*C80	0.00	1.00
*Gmo*C90	0.01	0.99
*Gmo*C88	0.00	0.99

However, both the calculation of the per-generation rate of inbreeding and the outcome of the Hardy-Weinberg test suggested that the juvenile population studied was subjected to a certain degree of inbreeding. Indeed, the Hardy-Weinberg test revealed that the juvenile batch analysed was not at equilibrium (data not shown) and the rate of inbreeding, ΔF, reached 2.5%.

5.4. Discussion

The assignment exercise proved to be, once again, a challenging task despite introducing a new set of loci with a greater number of polymorphic alleles. Although the FAP predictive analysis seemed to indicate that the set of eleven loci was discriminatory enough for the dataset considered (i.e. 99.9% of predicted assignments), the rate of assigned offspring remained very low (i.e. 62% with two mismatches allowed). Moreover, quite surprisingly, the addition of new polymorphic loci made little difference to the percentage of offspring successfully assigned to a single pair of parents, when compared to the previous assignment exercise employing just eight loci (section 4.3.5). Both the high number of potential families (i.e. 5800) and the presence of 13 unsexed fish among the broodstock population remained essential problems. In addition, the manual inspection of chromatograms revealed the presence of null alleles among parental genotypes. Null alleles were uncovered at four different loci and, more worryingly, affected the genotypes of two main contributing parents (males 455F and DD79). The presence of null alleles further complicated the allocation exercise by adding substantial errors to the dataset. As a result, it was decided to allow for up to two mismatches for assigning offspring. However, despite raising the error level to two allelic mismatches, 307 juveniles (c. 32%) could not be reconciled to an expected parental pair, suggesting that unresolved genotyping errors remained in the parental and / or offspring genotype data. It is also worth mentioning at this point that other reasons including the presence of extraneous parents / offspring in the sampled populations may also explain some of the non-assignments.

It is more than likely that not all family contributions were detected in the sample of juveniles analysed. Therefore, the data presented here represent the minimum numbers of parents involved in the production of the studied cod juvenile

batch. The results from the exclusion-based parentage analysis showed that at least 77% of the "wild Scottish" broodstock population contributed to the juvenile batch analysed. However, family representations were highly skewed with 57% of the contributing families only represented by a single offspring. Not surprisingly (given the outcomes of previous analyses presented in Chapter 4), 39% of the juveniles analysed were sharing a common parent. This parent was the previously identified dominant male 455F (see Chapter 4).

The number of families identified in the juvenile sample was equivalent to the number of families found in the combined four spawning dates analysed (respectively 157 vs. 156 families). Only 30% of the families initially present in the four fry batches analysed were represented in the "ongrown" juvenile population sampled. These however included the ten most contributing families initially identified amongst the fry. Repetitive size grading were previously reported to have marked effect on the genetic diversity of commercially produced barramundi juvenile: Frost et al. (2006) showed that repetitive size grading alone (without mixing of batches) were responsible for significant losses of families within a single fish cohort (i.e. slow growing families were progressively separated from the main cohort). Although the family losses observed in this case study might well be directly related to size grading, it is impossible to rule out other possible causes including sampling bias, selective mortalities and cannibalism.

Frost et al. (2006) showed that size grading modified the relative family / parental contributions over time in a population of barramundi juveniles. The data presented in this Chapter was unfortunately not best suited to compare to these findings since it was impossible to separate the likely effect of size grading from the

effect of mixing fry batches (since part of the information regarding the history of the juvenile batch was not disclosed by MMF). However, it is interesting to note that the dominant family initially identified among the four fry batches analysed appeared to have maintained its level of contribution throughout hatchery rearing.

The juvenile batch analysed in this study was noticeably more genetically diverse than any of the four individual fry samples previously analysed. The effective breeding population size of the juvenile batch was two to three times greater than the effective size observed on a single day of mass spawning (this will be further discussed in Chapter 7). The most immediate conclusion is that the mixing of fry batches from different days of spawning did override the possible adverse effect of size grading.

The effective breeding population size of the juvenile batch remained relatively low (i.e. represented approx. 14% of the breeding population), despite the diversity brought in by mixing fry batches from several spawning dates. This result was similar to the findings of Brown (2003) for gilthead seabream. According to Meuwissen and Woolliams (1994), the effective population size which contributed to the juvenile sample analysed was, however, not sufficient to prevent detrimental effects caused by inbreeding (i.e. decline in fitness traits). The per-generation rate of inbreeding ($\Delta F = 2.5\%$) was six times greater than the rate of inbreeding which would be expected in an "idealized" population subjected to random matings and where no selection occurred (Falconer, 1986; with $\Delta F = 1/8Nm + 1/8Nf = 0.4\%$).

The analysis of allelic frequencies at eleven loci showed no significant difference between the "wild Scottish" broodstock and the juvenile batch analysed.

Despite the relatively low Ne and the high ΔF, the juvenile population studied could still be considered for broodstock replacement providing the variance in family size was altered. By removing fish from the most represented families, the individual parental contributions would become more homogeneous (Brown, 2003). Indeed, by bringing the contributions of the three most represented females and the two most represented males down to 30 offspring each, the effective breeding population size would increased to 101 fish while the rate of inbreeding would be brought down to 0.5% (data not shown). This simple measure would minimise inbreeding and make the juvenile population a good candidate for broodstock replacement (Falconer, 1986; Jørstad and Naevdal, 1996).

5.5. Conclusions

This study highlighted once again the difficulties associated with solving the parentage of extremely large and complex genotyping datasets (i.e. a set of offspring issued from the mass spawning of more than a hundred broodstock). Adding new microsatellites, quite surprisingly, did not improve the overall success of this particular allocation exercise. The most plausible explanation for this was that additional errors were introduced to the dataset as more markers were genotyped (Borrell et al., 2004). Despite the problems associated with this parentage exercise, interesting results were obtained from analysing family contributions. Repeated size grading and mixing of fry batches had an overall positive effect on the genetic diversity of the juvenile batch produced (compared to the genetic diversity from a single day of mass spawning). However, family contributions remained highly skewed. This made the juvenile population a rather unsuitable candidate for broodstock replacement (as 39% of the offspring shared a common parent) unless fish from excessively represented families were removed.

Chapter 6. Preliminary testing of gynogenesis induction in Atlantic cod

6.1. Introduction

The presence of two to three years old sexually mature males and females, in sea cages, is a growing concern for cod ongrowers. Although different approaches to control maturation are being investigated (including the use of photoperiod, selective breeding and the production of triploids), no clear-cut method has yet been identified. Uncovering the sex determination mechanism(s) which operates in cod would most probably help inform on the best suited method of maturation control to implement for this species.

6.1.1. Sex determination and identification of sex chromosomes / genes

6.1.1.1. Sex determination mechanisms in fish

Sex determination refers to the sum of genetic elements responsible for the existence of gonads (Piferrer, 2001). The so called "sex determining" genes can either be spread throughout the genome or mostly concentrated in a pair of chromosomes (i.e. sex chromosomes). Contrary to mammals, there is no evidence of the existence of a unique sex gene in fish. This said, several fish species (including rainbow trout and common carp) present sex chromosomes in a early stage of differentiation (Nada et al., 1992; Ezaz, 2002). It is commonly believed that morphologically differentiated chromosomes - which constitute a characteristic of higher vertebrates (such as the sex chromosomes X and Y in humans) - have evolved from originally identical homologues. So far, eight chromosomal systems have been identified in fish, some involving more than one pair of sex chromosomes or different numbers of chromosomes depending on the sex (Nanda et al., 1992; Piferrer, 2001; Devlin and Nagahama, 2002). Studies of sex ratios among offspring can give clues about the likely system in place. In species where individual crosses yield 1:1 sex ratios,

monofactorial determination of sex, with sex determining genes located on a single chromosome, can be suspected. However, many fish species like tilapia or seabass display, under certain conditions, unbalanced sex ratios suggesting the existence of a more complex polyfactorial system (Campos Ramos, 2002; Devlin and Nagahama, 2002), possibly involving environmental factors. In cod, due to the poor survival of larvae at the age of sex differentiation, it might not be appropriate to solely rely on the study of sex ratios, obtained from single crossings, in order to elucidate sex determination mechanisms.

6.1.1.2. Tools to elucidate sex determination

6.1.1.2.1. Cytogenetic

Cytogenetical studies have been performed on more than 1700 fish species so far. Only 10% were found to possess distinct sex chromosomes (Devlin and Nagahama, 2002). However, many commercially cultured fish, such as carps and salmonids, exhibit the commonly encountered XX/XY system (Dunham et al., 2000). Extended research has been carried out in Mozambique and Nile tilapia to isolate sex determining genes. As these species do not possess apparent differentiated mitotic sex chromosomes, examination of chromosomes pairing in synaptonemal complexes, were carried out. They revealed the existence of sex-chromosome regions on the largest chromosome pair but no specific sex genes have been so far identified and sequenced (Campos Ramos, 2002; Ezaz, 2002; Campos Ramos et al., 2003; Ezaz et al., 2004). However, such techniques are still rarely performed (Devlin and Nagahama, 2002). In cod, chromosome spreads revealed the existence of 46 chromosomes (2n) (Klinkhardt et al., 1995) but no published information is yet available on its sex determination system.

6.1.1.2.2. Molecular markers

Molecular approaches, focusing exclusively on DNA, can also be used to elucidate sex determination mechanisms. Indeed, DNA markers have been extensively used and proved useful for examining sex linkage in fish (Nanda et al., 1992; Dunham et al., 2000; Hulata, 2001; Devlin and Nagahama, 2002). Among these DNA-based tools, the AFLP technique presents numerous advantages. It is based on the selective PCR co-amplification of high numbers of restriction fragments from genomic DNA (Vos et al., 1995). This technique is able to generate DNA fingerprints regardless of its origin or its complexity and does not require prior knowledge of its sequence (Mickett et al., 2003). AFLP techniques have already been used with success on plant and animal species (Singh et al., 2002) and more recently on fish such as channel catfish (Mickett et al., 2003) and tilapia (Agresti et al., 2000; Ezaz et al., 2004). Such a technique could be also applied to cod in order to localize potential sex-linked or sex-specific sequences.

6.1.1.2.3. Gynogenesis

Gynogenesis refers to the process whereby offspring which exclusively possess genetic information of maternal origin are produced. Gynogenesis can either occur naturally by exclusion of the paternal genetic input from the zygote, or be induced experimentally by the destruction of spermatozoa's DNA with UV light or ionizing radiation (Devlin and Nagahama, 2002). In all cases, spermatozoa remain able to fertilise the egg and activate embryonic development but do not provide any genetic material to the zygote. Two types of gynogenesis can occur (see Figure 6.1). In mitotic gynogenesis, the fertilised egg goes through the first mitotic cell division and the application of a physical shock (i.e. pressure or temperature) prevents the cell from physically dividing, thus re-establishing the diploid state. Alternatively, meiotic

gynogenesis is achieved by applying a shock treatment (i.e. pressure or temperature) to the fertilised egg - at the stage of the second meiotic division - which results in the retention of the second polar body (Figure 6.1). Mitotic gynogenetics are homozygous for all loci while meiotic gynogenetics are not. Gynogenesis can be used to elucidate sex determination in fish by studying the sex ratio of gynogenetic offspring. Gynogenetic experiments have been carried out extensively, with success, in numerous fish species belonging to both fresh and marine water environments (e.g. Chourrout, 1984; Hollebecq et al., 1986; Pongthana et al., 1995). Extensive gynogenetic works in marine fish have been already done on European seabass (Peruzzi and Chatain, 2000; Peruzzi et al., 2004; Francescon et al., 2004) and Atlantic halibut (Tvedt et al., 2006).

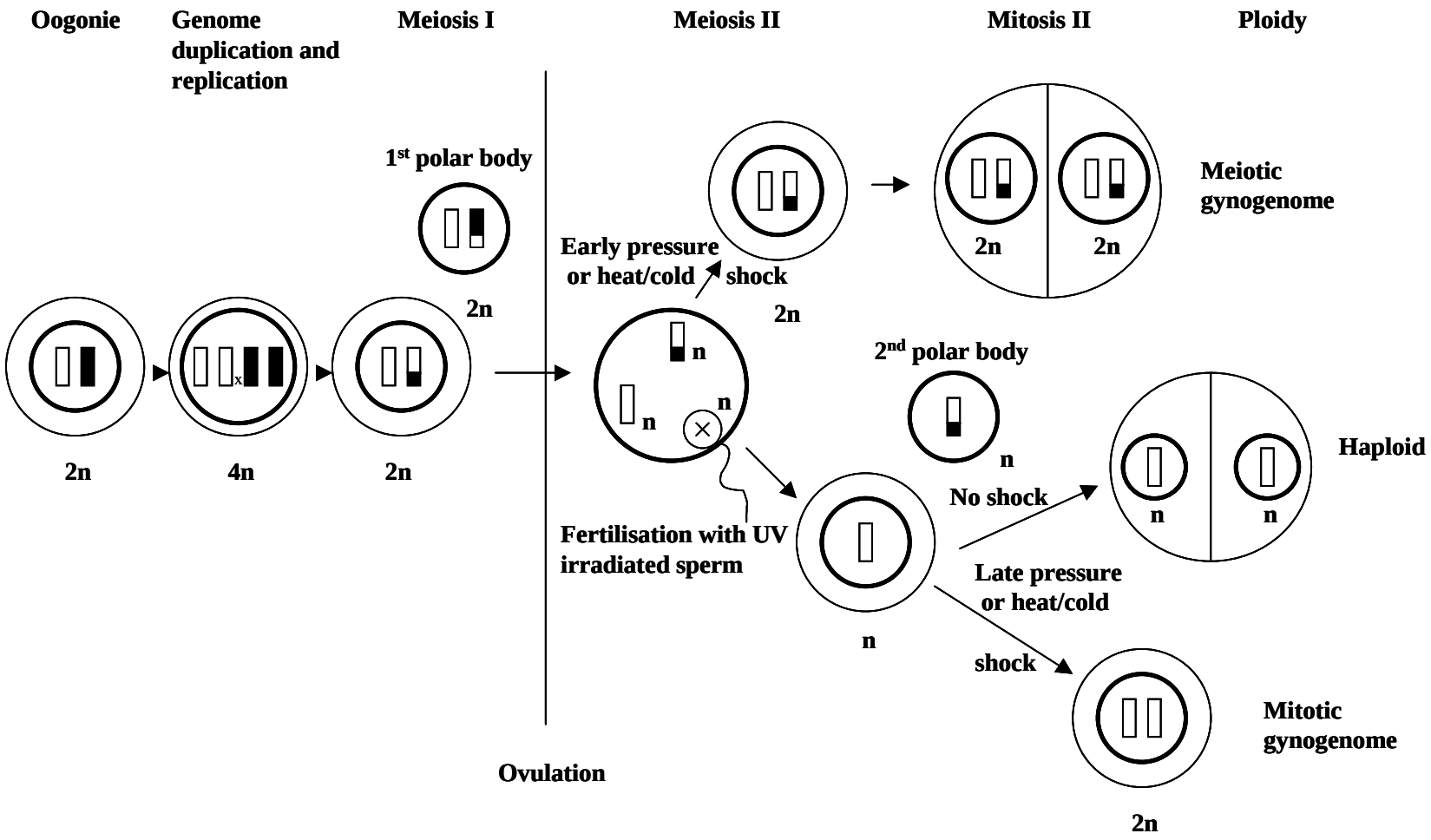


Figure 6.1. Induction of gynogenesis in fish. Figure adapted from Hussain (1992).

6.1.2. Aims of the study

The aims of this Chapter were: 1) to develop a successful protocol to induce gynogenesis in cod using temperature shocks, 2) to induce gynogenesis on a large batch of cod eggs and ongrow the gynogenetic cod fry until sexing was possible, 3) to study the sex ratio of ongrown gynogenetics and possibly conclude on the sex determination mechanism(s) operating in cod. First, a protocol to artificially induce gynogenesis was developed at a relatively small experimental scale using a combination of UV irradiation treatment (milt) and cold temperature shock (fertilised eggs). Gynogenesis was then induced on a large batch of cod eggs (approx. 200 000) in order to both test the efficacy of the technique and to study the sex ratio of ongrown gynogenetics.

6.2. Materials and methods

The experiments described in this Chapter took place over three consecutive spring seasons (from 2004 to 2006) at the Marine laboratory facilities in Machrihanish (MERL). Milt and eggs were collected from captive broodstock held at the laboratory. The fish (approx. 80 males and 60 females) were of both wild (West Scottish coast) and farmed origin (issued from MMF juvenile production) and kept in six breeding tanks (4m in diameter, 1m deep). The farmed origin cod were first year spawners in 2004 (i.e. approx. two years old). The wild origin cod first spawn in captivity during the spring season 2004 (age unknown but estimated to be 4+ years old). Both farmed and wild origin broodstock were kept under natural photoperiod.

6.2.1. Milt collection and treatments

6.2.1.1. Fish handling and stripping

Cod milt was collected from mature cod males, held at MERL, during the course of the spring seasons 2004 to 2006. The fish were anaesthetised (benzocaine, 100 ppm) and subsequently stripped for milt into 25 ml universal tubes. Any sample contaminated with either water or urine was discarded. Up to five different males were stripped on a given occasion (depending on the nature of the experiment). For the fertilisation trials, the milt from a single male was used but up to three males were stripped, at times, to obtain both the quantity and the quality of milt desired.

6.2.1.2. Mounib's extender

Milt samples were diluted to a fixed concentration of gametes in a formulated extender developed by Mounib (1978) and later modified by Degraaf and Berlinsky (2004). The "modified" Mounib's extender (10 g/L $KHCO_3$, 2 g/L glutathione reduced, 42.7 g/L sucrose, pH 7.8) was chosen for this experiment since it had

previously been reported to suit both the dilution and the storage of milt from several marine fish species including seabass, turbot and cod (Suquet et al., 2000).

The extender was prepared at the IoA and stored at 5°C, in aliquots of 50 ml, until being used. Its shelf life lasted for several months.

6.2.1.3. Estimation of the percentage of spermatozoa activated by seawater

Freshly collected milt samples were checked for activation using seawater. 4 µl of milt and 5 µl of seawater were thoroughly mixed on a microscope slide and the percentage of activated spermatozoa was estimated (using a compound microscope, x400 in magnification) after approx. 10 seconds. The entire slide was checked rapidly at a lower magnification (i.e. x100 or x200) to find an adequate zone to study (i.e. a zone representative of the global level of spermatozoa activity with a concentration in cells low enough for counting). In the selected zone, at x400 magnification, the number of motile spermatozoa, within a randomly chosen group of 100 cells, was counted (using a hand counter). This number was used as an estimate, for the milt sample, of the percentage of spermatozoa activated by seawater.

6.2.1.4. Estimation /adjustment of the concentration in spermatozoa

Concentrations of milt samples were estimated based on the average result of three spermatozoa counts. Semen samples were first diluted 500-fold in Mounib's extender (i.e. 1 µl of milt in 499 µl of extender). About 15 µl of diluted milt were then loaded on a hemacytometer. Samples were allowed to settle for approx. three minutes before beginning to count (so that the cells had stopped drifting and were in

the same focal plan). Spermatozoa were hand-counted in five 0.04 mm^2 squares across the principal chamber of the hemacytometer (using a compound microscope, x400 in magnification). For each milt sample, three spermatozoa counts were made (from three different hemacytometer preparations) and the average was used to calculate the concentration as follow:

Spermatozoa concentration / ml = Average of the total cell count in five 0.04 mm^2 squares x 50 000 x dilution factor

Based on the previous result, samples were adjusted to working concentrations using Mounib's extender.

6.2.1.5. UV treatments

The collected milt was irradiated using a 254 nm ultraviolet lamp (UVP Inc., USA). The intensity of the radiation delivered to the sperm was adjusted to 240 $\mu W/cm^2$ with a UV radiometer (UVP Inc., USA). The intensity used in this project was voluntarily set in the range of the radiation intensities used by Ezaz (2002) to produce gynogens in Nile Tilapia. Two millilitres aliquots of diluted cod milt were exposed to the UV light (from 10s up to 25min) while being stirred in a 5 cm Petri dish (see Figure 6.2). Constant stirring during irradiation ensured that the samples were homogeneously treated (i.e. all the spermatozoa received the same dose of UV).

The treated milt was kept for the shortest time possible at 5°C until being used for fertilising eggs as the damaged spermatozoa suffered from a short lifespan (less than 3 hours in this trial).

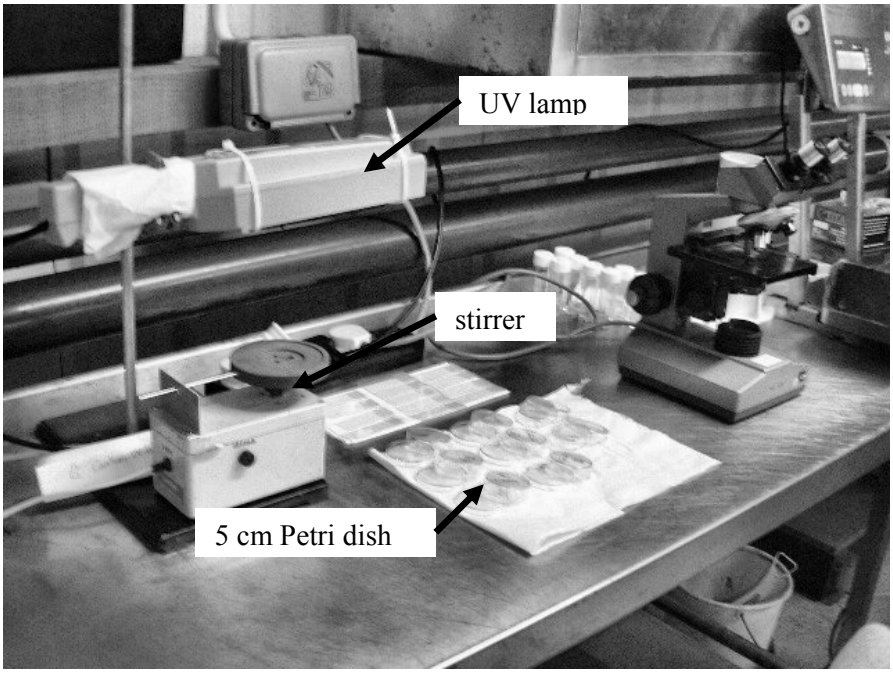


Figure 6.2. Picture showing the experimental set up used to perform UV irradiation of cod milt samples.

6.2.2. Collection of eggs, fertilisation and temperature shock treatments

6.2.2.1. Fish handling and stripping

Ripe cod females, held at MERL, were stripped for eggs during the course of the spring seasons 2004 to 2006. They were anaesthetised (benzocaine, 100 ppm) and subsequently stripped for eggs in either 25 ml universal tubes or 500 ml glass beakers. Ripe females were identified in the tanks by their swollen abdomen. Two to four females were fished and anaesthetised each time eggs were needed. On occasions, cod females started release eggs while being anaesthetised. This was a sign the females were ripe and the quality of the eggs collected from these "running" females was most of the time very good. Generally speaking, it was more difficult to obtain good quality eggs towards the end of the spawning season (i.e. end of May). "Egg binding" was observed on several occasions in females of farmed origin. Eggs stripped from these fish were always overiped and of very poor quality (most of the time they were dead). A fertilisation trial was realised with the eggs from only one female (to avoid any "female effect" on egg survival). Therefore, the quantity of eggs collected constituted another critical parameter. On several occasions, an experiment was abandoned because either the quantity or the quality of the eggs was insufficient. A piece of fin, from the stripped females whose eggs were used for the fertilisation trials, was sampled in 95% ethanol for further DNA analysis. The eggs were kept in a cool box (temperature maintained at approx. 6^{o}C) until being fertilised.

6.2.2.2. Artificial fertilisation

Eggs were fertilised in 25 ml universal tubes. Kjørsvik et al. (2004) recommended a ratio of 200 000 spermatozoa per ovule for achieving an optimum fertilisation rate of cod eggs. Based on this theoretical ratio and the assumption that

there are 500 000 eggs per litre (Moksness et al., 2004), a fertilisation protocol was decided. In a 25 ml vial containing 3 ml of seawater, 2 ml of eggs (approx. 1000 eggs) were fertilised by 2 ml of diluted milt (adjusted concentration of $9x10^8$ spermatozoa/ml). The contact time between the gametes was standardised to three minutes while the temperature was maintained at 7°C throughout. The fertilised eggs were then directly transferred, without being rinsed or disinfected, to 500 ml glass beakers which played the role of incubators (see section 6.2.3).

6.2.2.3. Temperature shock treatment

Cold temperature shock treatments were used in this study, based on the promising results published by Kettunen et al. (2004) on cod triploidy induction. Cold shocks were applied to fertilised eggs in order to block one cell division (either the second meiotic division of the ovule or the first mitotic cell division of the fertilised egg). Three temperatures were tested: -1°C, -3°C and –6°C. Figure 6.3 presents the profiles of the three cold shocks tested in this study. The shocks were carried out in 25 ml universal vials, each containing approx. 1000 fertilised eggs and 15 ml of seawater. The tubes were stacked horizontally in a ventilated incubation chamber. A control panel located on the door of the chamber was used to adjust its temperature. The cooling rate of the eggs was monitored using a waterproof thermometer probe inserted in a test vial containing approx. 15 ml of seawater. Different shock durations (i.e. 30 min, 60 min, 90 min and 120 min) and starting times (i.e. 10 min, 15 min, 20 min, 30 min, 40 min, 50 min and 60 min post-fertilisation) were tested.

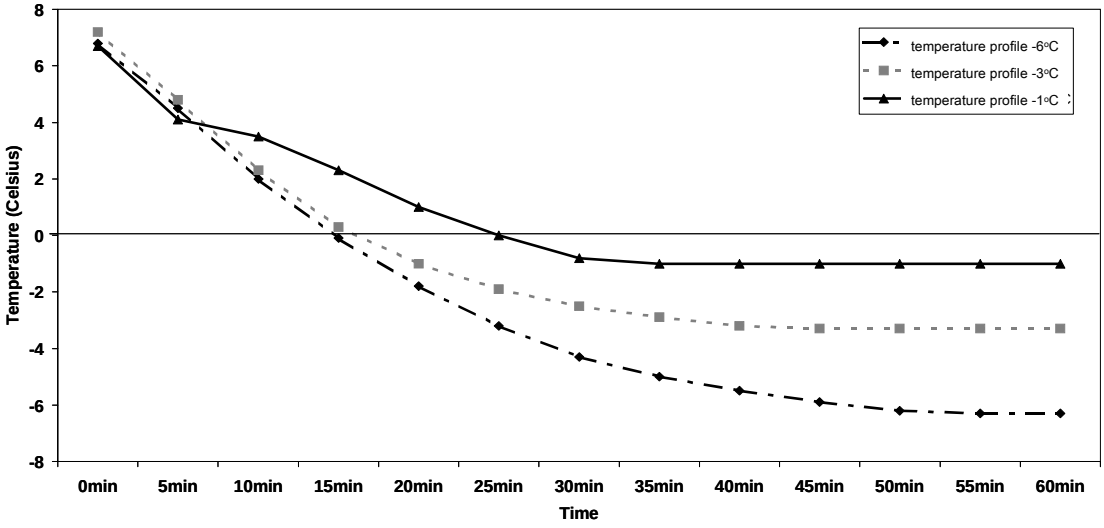


Figure 6.3. Temperature profiles of three cold shocks designed to induce gynogenesis in cod eggs fertilised with UV irradiated milt.

6.2.2.4. Diploid and haploid controls

One or two control treatments were used for each fertilisation trial performed. A diploid control - consisting of eggs fertilised with non irradiated milt - was included in each trial which aimed to produce haploid cod eggs. Both a diploid and an haploid control (i.e. eggs fertilised with UV irradiated milt (240 μW/cm^2 intensity, 4 min duration)) were included in the trials which aimed to produce gynogenetic cod fry.

6.2.3. Egg incubation and larval ongrowing

6.2.3.1. Egg incubator design and management

Eggs were incubated in 500 ml glass beakers. An air stone provided the water current necessary to maintain the eggs in motion in the water column (see Figure 6.4). The beakers were kept in a shallow water bath with a water recirculation system (including a water pump and a water cooler). By doing so, the water temperature of the beakers was maintained between 7^{o}C and 11^{o}C (corresponding to the optimum

temperature range for incubating cod eggs). The temperature varied at most ± 1°C in 24 hours. The daily maintenance of the incubation system consisted in renewing the water of the beakers and removing the dead eggs (using a 1.5ml Pasteur pipette). The eggs were exposed to artificial light (natural photoperiod) during the entire incubation time.

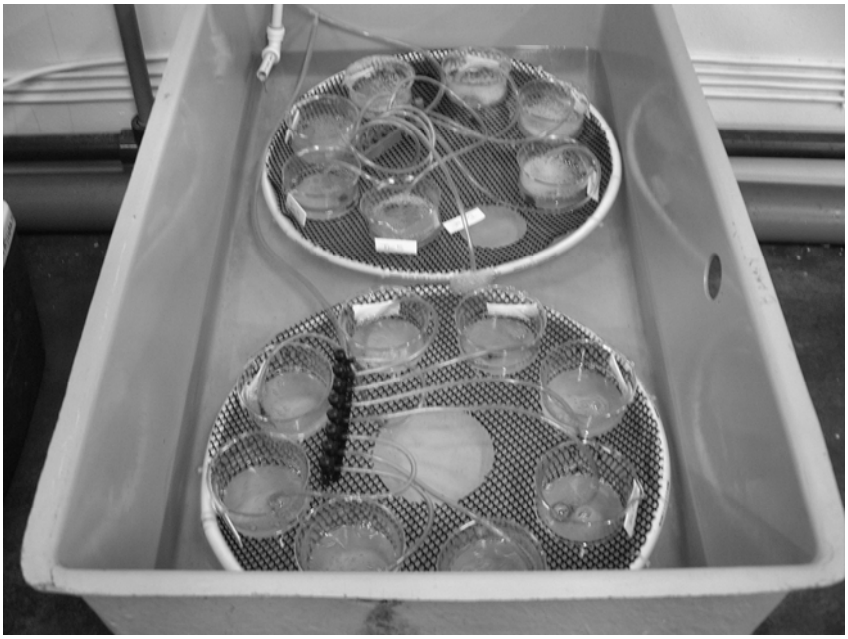


Figure 6.4. Picture showing the experimental set up for incubating cod eggs in 500ml beakers.

6.2.3.2. Calculation of fertilisation and hatching rates

Both fertilisation and hatching rates per incubator / treatment were back calculated from both the number of surviving hatched fry and the egg mortality (i.e. sum of the daily egg mortality counts).

6.2.3.3. Larval rearing protocol

The eggs were transferred into two 1 m^3 circular larval tanks before hatching (i.e. a control treatment and a gynogenetic treatment tank). The larvae were then reared following a commercial rearing protocol developed by MERL / MMF and described by Fletcher et al. (2007).

The newly hatched larvae were fed live prey (rotifers and / or *Artemia*), three times a day, for the first 55 days. During this period, the tanks were kept under continuous light and the water temperature was progressively increased from 8 to 12^{o}C. From day 56 onwards, the juveniles were exclusively fed on commercially formulated diets ("Gemma micro" and "Europa" diet ranges from Skretting, UK). Because of low stocking densities (due to logistic limitations in the number of eggs treated and poor larvae survival(i.e. <1%)), the fish were kept for the entire duration of the experiment (i.e. seven months) in the same experimental tanks.

6.2.4. DNA profiling

DNA profiling was used for two different purposes: 1) to verify the ploidy status of eggs fertilised by UV treated milt and 2) to confirm the presence of diploid gynogenetic fish among the fry ongrown.

6.2.4.1. Identification of haploids

Thirty eggs - issued from a trial experiment looking at the survival of cod eggs fertilised with UV irradiated milt- were sampled, at 65 degree days (^{o}C days) and stored in 95% ethanol for subsequent DNA analysis. Fin clips from both parents were also similarly sampled while stripping for gametes.

DNA was extracted from fin samples and each individual egg using the Dynabeads® genomic universal DNA kit (see Chapter 2).

Four loci (*Gmo*8, *Gmo*19, *Gmo*35 and *Gmo*37) were used to check the ploidy status of each egg. The loci were coamplified as a tetraplex assay for fin clip samples (see Chapter 2) and as separate singleplex reactions for eggs (due to the law amount of DNA extracted from eggs). Singleplex amplifications were carried out in 10 µl reactions. Each contained 2-5 ng of DNA template, 2x PCR-buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 300 µM of each DNTP, 2 mM $MgCl_2$,1 µM of each forward and reverse primer and 0.5 U of *Taq* polymerase (ABgene, UK). The PCR amplification program was: initial denaturation at 95^{o}C for 5 minutes, 40 cycles of 95^{o}C for 35 seconds, 57^{o}C for 35 seconds, 72^{o}C for 1 minute, and a final extension step at 72^{o}C for 10 minutes.

Amplified DNA fragments were analysed on the CEQ 8800 Genetic Analysis System (Beckman Coulter). Samples were run three times to verify repeatability.

6.2.4.2. Detection of gynogenetics

Eggs and tissue samples were collected from an ongrowing trial of gynogenetic fish. Samples included: 25 eggs (60^{o}C days) from the haploid control treatment (i.e. eggs fertilised with UV irradiated milt (240 µW/cm^2 intensity, 4 min

duration)) 10 fin clips from fish belonging to the diploid control treatment (i.e. eggs fertilised with non treated milt) and 7 tissue samples (fin or head) from fish belonging to the diploid gynogenetic treatment (i.e. eggs fertilised with UV irradiated milt + cold shock). The number of collected fin clips samples for both the diploid control and gynogenetic treatments corresponded to all the surviving fish in these two groups at 120 days.

DNA was extracted from tissue samples and individual eggs using the Dynabeads® genomic universal DNA kit (see Chapter 2).

Seven loci (*Gmo*C18, *Gmo*C20, *Gmo*C42, *Gmo*C52, *Gmo*35, *Gmo*37 and *Tch*11) were used to genotype the samples. The loci were coamplified as two separate PCR reactions: a tetraplex (*Gmo*C18, *Gmo*C20, *Gmo*C42 and *Gmo*C52) assay and a triplex (*Gmo*35, *Gmo*37 and *Tch*11) assay (see Chapter 2). 1x QIAGEN Multiplex PCR master mix was used in the reaction mix, for both PCR assays, as it made it possible to coamplify loci from cod egg DNA.

Each 10 µl PCR reaction contained 5-30 ng of DNA template, 1x QIAGEN Multiplex PCR Master MIX (Qiagen, UK) and 0.1 µM each of *Gmo*C18, *Gmo*C20, *Gmo*C42 and *Gmo*C52 primers (or *Gmo*35, *Gmo*37 and *Tch*11 primers). Both PCR amplification conditions were: initial denaturation at 95^{o}C for 15 minutes followed by 35 cycles of 94^{o}C for 30 seconds, 56^{o}C for 90 seconds, 72^{o}C for 1 minute, and a final extension step at 72^{o}C for 30 minutes.

Amplified DNA fragments were analysed on the ABI Avant Genetic analyser (Applied Biosystems). Samples were run three times to verify repeatability.

6.2.5. Sexing cod fry

6.2.5.1. Histology preparation

Six dead fry recovered from the "gynogenetic" larval tank were sampled in 10% neutral buffered formalin (10% formaldehyde in Phosphate buffered saline). Samples were sent to the IoA laboratory of histology for processing. Histological sections were stained with hematoxylin / eosin.

6.2.5.2. Gonad squash

The surviving fish from the gynogenesis ongrowing trial at 264 days post-hatch (i.e. 10 fish from the control treatment and one fish from the diploid gynogenesis treatment) were dissected for gonads. Fresh dissected tissues were "squashed" between a microscope slide and a cover glass. Observations were made, using a compound microscope, at x400 magnification.

6.2.6. Summary of the experiments carried out in this study

To facilitate the reading of this Chapter, a summary of all the experiments carried out in this study is given in Table 6.1.

Table 6.1. Summary of the experiments carried out for the gynogenesis induction study.

Experiment	N replicate experiments	N broodstock stripped	Treatments	N offspring sampled for DNA profiling	Other parameters studied
Evaluation of sperm concentration	16	16 males	NA	NA	sperm concentration
Activation of spermatozoa motility in seawater / extender	5	5 males	fresh, stored 24h non diluted, stored 24h diluted in extender	NA	motility, dilution in extender, storage at 5°C
Determination of an optimum milt concentration to perform UV treatments	3	3 males	four standard concentrations for each milt sample and exposition time varies from 10s to 25min	NA	sperm motility
Egg fertilisation using UV treated milt, short irradiations	4	4 males 4 females	diploid control, 10s, 30s, 1min, 1.5min, 2min, 2.5min and 3min, 4min irradiation	NA	survival at blastula, eye pigmentation and hatching
Egg fertilisation using UV treated milt, long irradiations	3	3 males 3 females	diploid control, 10s, 30s, 1min, 1.5min, 2min, 2.5min, 3min, 3.5min, 4min, 5min, 6min, 7min, 9min, 11min, 13min and 15min irradiation	30 eggs from the third replicate experiment (i.e. 10 eggs from the diploid control, ten eggs from the 1.5min treatment and 10 eggs from the 4min treatment)	daily survival during incubation and at hatching. morphology study of haploid eggs
Effects of the cold shock timing on larvae survival	2 (shock at -3°C for 60 minutes)	2 males 2 females	diploid control, haploid control 10min, 15min, 20min, 30min, 40min, 50min and 60min PF	NA	daily survival during incubation and at hatching
Effects of the cold shock duration on larvae survival	1 (shock at -3°C, applied 10min PF)	1 male 1 female	diploid control, haploid control, 30min, 60min, 90min and 120min duration	NA	daily survival during incubation and at hatching
Effects of the shock intensity on larvae survival	1 (shock of 60min)	1 male 1 female	diploid control, haploid control, 10min PF/-6°C, 20min PF/-6°C, 30min PF/-6°C, 10min PF/-1°C, 20min PF/-1°C, 30min PF/-1°C	NA	daily survival during incubation and at hatching
Induction of gynogenesis on a "large" scale	3 but 2 unsuccessful attempts	3 males 3 females	diploid control (approx. 50 000 eggs), haploid control (approx. 1 000 eggs), gynogenesis treatment (approx. 140 000 eggs)	25 eggs from the haploid control, 10 fin clips from the diploid control and 7 fin clips from the gynogenesis treatment	sexing of the control and gynogenetic fry (gonad squash and histology)

PF: post-fertilisation

6.3. Results

6.3.1. Cod milt properties

Cod milt was readily available throughout the spawning seasons during which the experiments described in this chapter took place. Large quantities of milt - 5 to 20 ml - were stripped from ripe males. Direct observation of spermatozoa required the use of a compound microscope (x400 magnification). The length of sperm cells was in the range of 10 to 20 μm (see Figure 6.5).

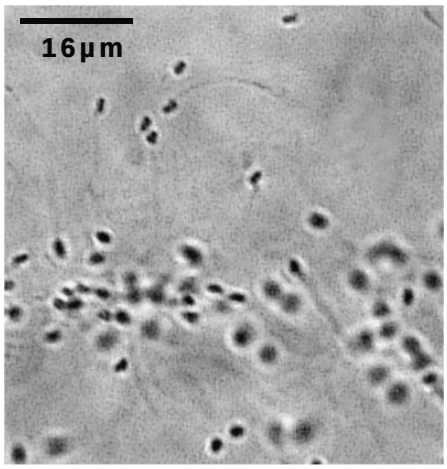
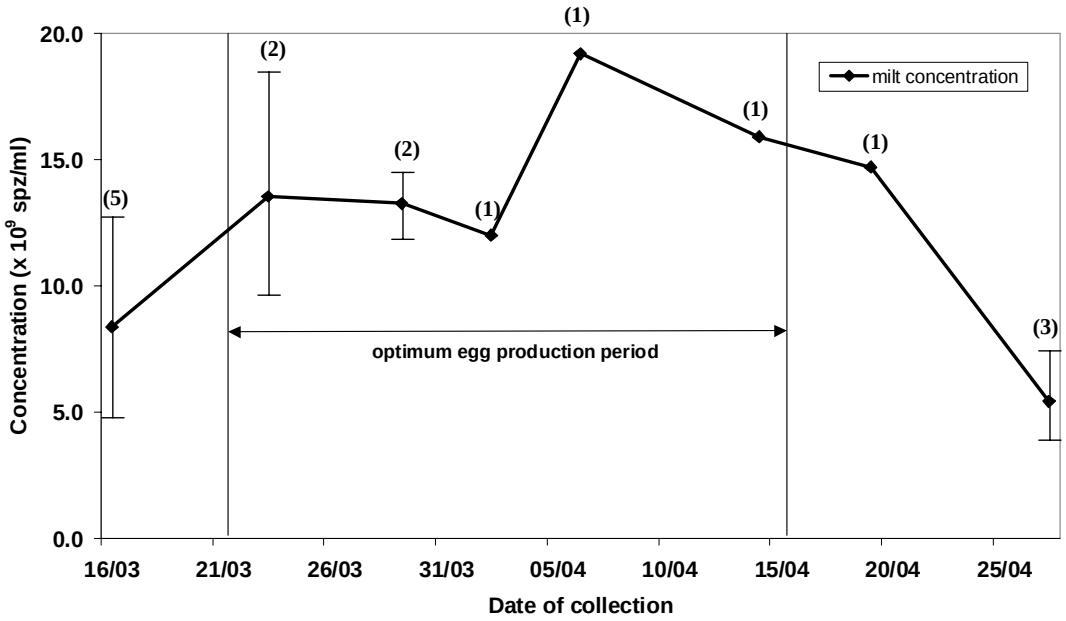


Figure 6.5. Atlantic cod spermatozoa as seen through a compound microscope (magnified x400).

6.3.1.1. Sperm concentration

The concentration of semen samples varied from 4.5 to 19.2x10^9 spz/ml (data not shown). Variation in sperm concentration was observed among 1) individual males belonging to the same breeding population and 2) across the spawning season (see Figure 6.6). The concentration in semen increased as the spawning season progressed (from 8.4x10^9 spz/ml on the 16th of March 2004 to 19.2x10^9 spz/ml on the 6th of April 2004). The production of gametes from a given broodstock tank was synchronised between both sexes, the optimum of egg production coinciding with the peak in semen concentration (Figure 6.6).



Notes: **(5)** indicates the number of milt samples (from different males). The number of males stripped on a given date varies from 1 to 5 according to the trials realised. Where the data from more than one males is available, the standard error is indicated.

Figure 6.6. Variation in sperm concentration among milt samples collected during the 2004 spring spawning season.

6.3.1.2. Activation of spermatozoa motility in seawater

The activation rate of fresh milt samples was extremely variable, ranging from 30% to 80%. On average, cod spermatozoa remained motile in sea water for a little less than two minutes (Table 6.2). However, motility was only efficient during the first 70 seconds (i.e. with spermatozoa moving actively in one direction). The activation rate of milt samples significantly decreased as storage time increased. Within 24 hours of storage at 5°C, the rate of activation dropped, on average, by 60% while the duration of motility was shortened by 40 seconds (see Table 6.2). The maximum shelf life of undiluted milt samples, stored at 5°C, was approximately 72 hours.

6.3.1.3. Milt extender

Mounib's extender was successful in diluting cod milt samples without activating gametes. It also greatly improved the storage conditions of cod semen. By diluting one part of fresh milt in 5 parts of extender, the overall rate of sperm activation only decreased by 16% after 24hrs of storage, compared to 60% for untreated milt (Table 6.2).

Table 6.2. Motility of cod semen after activation with sea water and effects of storage conditions.

Sample	Proportion of activated spz 10s after addition of seawater (%age ± SE)	Extinction of efficient motility (seconds ± SE)	Total extinction of motility (seconds ± SE)
Fresh milt	58 ± 22	73 ± 18	116± 10
Stored milt at 5°C 24hrs / no dilution	24 ± 16	53 ± 22	77 ± 47
Stored milt at 5°C 24hrs / diluted in Mounib's extender (1/5 dilution)	49 ± 27	53 ± 11	79 ± 32

Note: the average values are based on data from five replicate milt samples (from five different cod males).

6.3.2. Ultra-violet treatments of cod milt

6.3.2.1. Determination of an optimum working concentration to perform UV treatments

A motility index was designed to quantify the effects of UV irradiation on milt samples (see Table 6.3). A score of 4 was given to samples which showed no effects related to UV irradiation. As spermatozoa were exposed to greater UV doses, the score attributed to milt samples decreased until reaching 0, when virtually no motile spermatozoa were observed (see Table 6.3).

Table 6.3. Details of the motility index used to quantify the impact of UV irradiation on cod milt samples.

Score	Signification
0	no cell movement
1	most cells not moving
2	cells active and moving in circles
3	most cells moving in circles or moving in one direction
4	cells active and moving in one direction

Figure 6.7 shows the effects of UV irradiation on cod milt. Three samples, originating from different males, were diluted down to four standard concentrations (i.e. $1x10^9$ spz/ml, $9x10^8$ spz/ml, $1x10^8$ spz/ml, $1x10^7$ spz/ml) and exposed to increasing doses of UV irradiations.

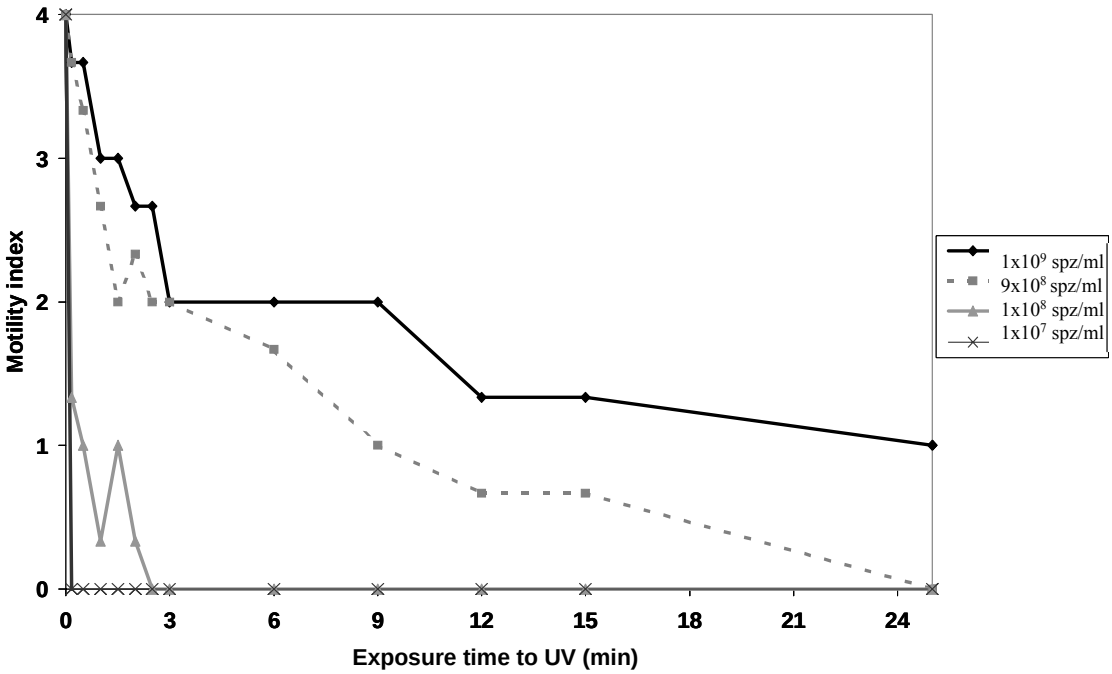


Figure 6.7. Effects of UV radiations (240 µW/cm^2) on cod milt samples. The results plotted are average values for three milt samples from different males; milt samples were diluted in Mounib's extender.

Cod spermatozoa were extremely sensitive to the damaging effects of UV irradiation. The three milt samples tested behaved the same way. Sperm motility

rapidly declined during the first three minutes of treatment for all four concentrations tested (Figure 6.7). However, the damage caused by UV radiations was more accentuated as the dilution factor increased. A lethal dose of UV was delivered in only 10s for milt samples diluted down to $1x10^7$ spz/ml while 25 minutes were necessary to deliver a similar dose to samples diluted down to $9x10^8$ spz/ml (see Figure 6.7). Samples diluted to $1x10^9$ spz/ml were non-homogeneously irradiated by UV (i.e. not all the cells of the preparation were scoring equally using the motility index), suggesting they were too concentrated to receive an efficient treatment. On the other hand, both the dilutions $1x10^8$ and $1x10^7$ were too penetrant to UV radiations, with extensive damage to the motility functions of gametes occurring within the first 60 seconds of treatment.

Of the four concentrations tested, $9x10^8$ spz/ml was selected as the most suitable to conduct UV treatments which aimed at destroying the genetic material of cod gametes without altering their motile functions and fertilisation power.

6.3.2.2. Egg fertilisation using UV treated milt

Overall, egg batches fertilised with irradiated sperm had lower hatching rates than diploid control batches fertilised with non-irradiated sperm. Figure 6.8 illustrates the large discrepancies in survival rates which arose, at hatching, between treated and diploid control batches. In this particular trial, the survival rate for the control treatment was 52% and 0% for eggs fertilised with milt irradiated for at least 1.5 min. Incubation of eggs was punctuated by two episodes of mortality: from 0 to 30°C days and from 63 to 81°C days. These mortality episodes were much more pronounced when eggs were fertilised with irradiated sperm (Figure 6.8).

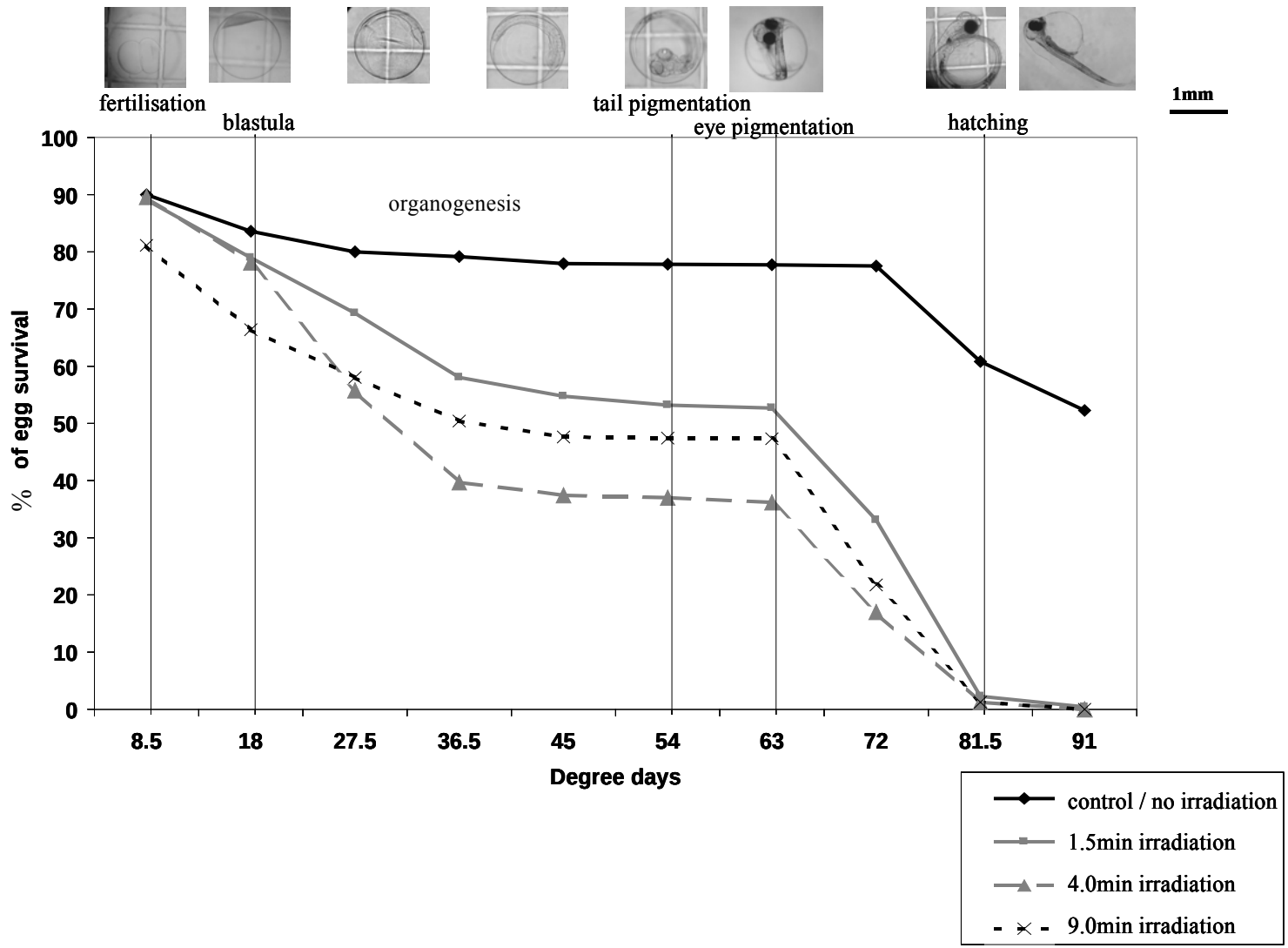


Figure 6.8. Variation in daily egg survival, during incubation, for a batch of cod eggs fertilised with UV irradiated milt (240 μW/cm^2). Milt concentration was adjusted to 9x10^8 spz/ml.

Table 6.4 shows average survival rates of eggs - based on the results from four replicate experiments - at three different stages of embryogenesis. In this trial, three shorter UV irradiation durations than in the previous experiment were tested: 1 min, 2 min and 3 min. Like in the previous experiment (see Figure 6.8), differences in survival were apparent at early stages of embryogenesis (although in this trial the survival rates were, on average, a lot lower in both the diploid control and the UV treatments, thus most probably reflecting an egg quality issue). At 20°C days (blastula stage) an average rate of 19% survival was observed for UV treated batches vs. 30% for control batches (see Table 6.4). These differences in survival increased further, until they became highly significant, at hatching. On average, 22% of eggs hatched from control treatments vs. 0.9% from UV treated batches. As the exposure time of milt samples to UV increased, survival rates at hatching decreased (from 1.5% for 1 min treatment to 0.6% for 3 min treatment). For two replicate experiments out of four, the survival rate at hatching was zero for egg batches fertilised with milt samples exposed to 3 minutes of UV. By applying longer UV treatments (i.e. at least 4 minutes), hatching rates eventually became zero.

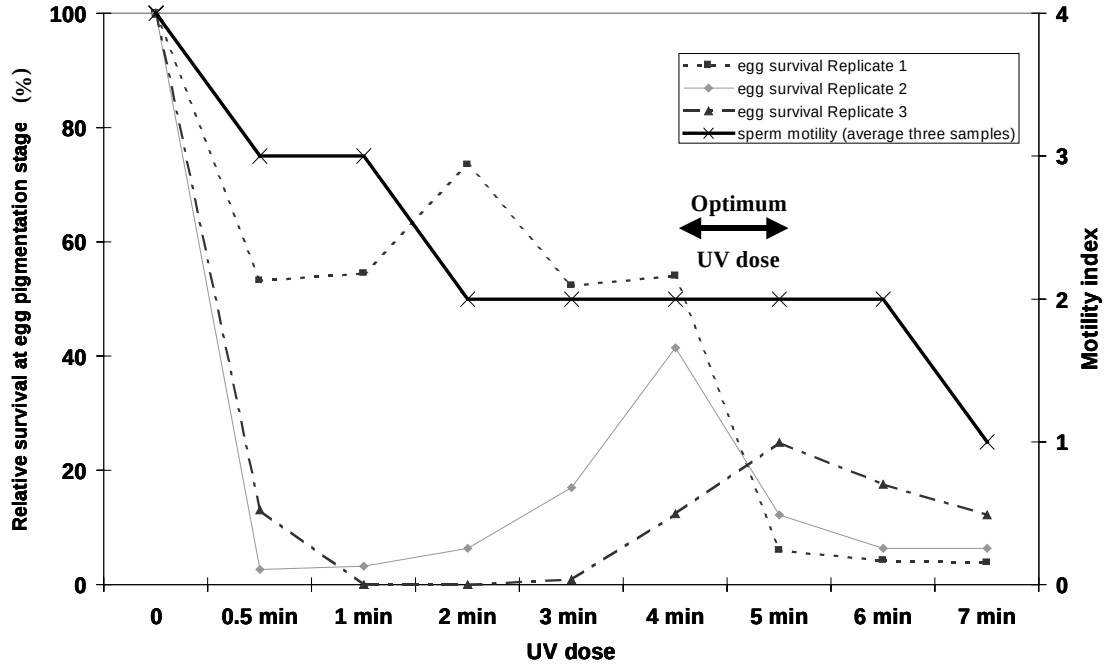
Table 6.4. Average survival of cod eggs fertilised with UV irradiated milt (240 μW/cm2) - based on four replicate experiments - at three stages of embryogenesis. Milt concentration were adjusted to $9x10^8$ spz/ml.

Treatment	Survival (%age ± SE)		
	Blastula (20°C days)	Eye pigmentation (63°C days)	Hatching (90°C days)
Diploid control	30.7 ± 18.9^a	22.8 ± 17.2^a	21.8 ± 16.6^a
1 min UV	18.0 ± 22.0^b	1.9 ± 2.7^b	1.5 ± 2.3^b
2 min UV	20.0 ± 13.3^b	3.8 ± 3.5^b	0.7 ± 0.8^b
3 min UV	19.5 ± 14.7^b	3.9 ± 3.9^b	0.6 ± 1.1^b

Note: common superscripts in the same column identifies means which are not significantly different ($P<0.05$), based on the results from one-way ANOVA statistical analyses.

Figure 6.9 presents the survival data of three egg batches, at eye pigmentation stage (approx. 60°C days), when fertilised with milt exposed to increasing UV doses. The pattern of the survival curves (especially for the replicates 2 and 3) is characteristic of the "pseudo Hertwig" effect (Porter, 1998). When subjecting cod milt to low doses of UV (time exposure below 3 min), survival of embryos at 60°C days was extremely low. With exposition time to UV further increasing to 4 - 5 min, survival of embryos increased and reached a peak (see Figure 6.9). Survival then decreased with UV doses exceeding six minutes.

Based on the results presented in Figure 6.9, UV doses of 4 to 5 mins maximised the percentage of viable eggs at 60°C days.



Note: Relative survival = (survival of the treatment x 100)/survival of diploid control.

Figure 6.9. Effects of increased UV exposure times on the sperm motility and the survival of cod eggs at eye pigmentation stage (approx. 60°C days). Milt concentrations were adjusted to $9x10^8$ spz/ml.

6.3.2.3. Haploidy in cod eggs

Three egg batches fertilised with milt irradiated for four minutes were sampled at 61°C days. The time of collection coincided with the peak of mortality, previously described in section 6.3.2.2, which occurred at the eye pigmentation stage. Treated embryos were observed under a dissecting microscope and compared with eggs sampled from the control treatment (Figure 6.10). Eggs fertilised with treated milt possessed several distinctive morphological features which normally characterise the haploid syndrome in fish: an enlarged yolk sac, a short deformed body and a small deformed head (see Figure 6.10). Most of these suspected haploid eggs died before hatching.

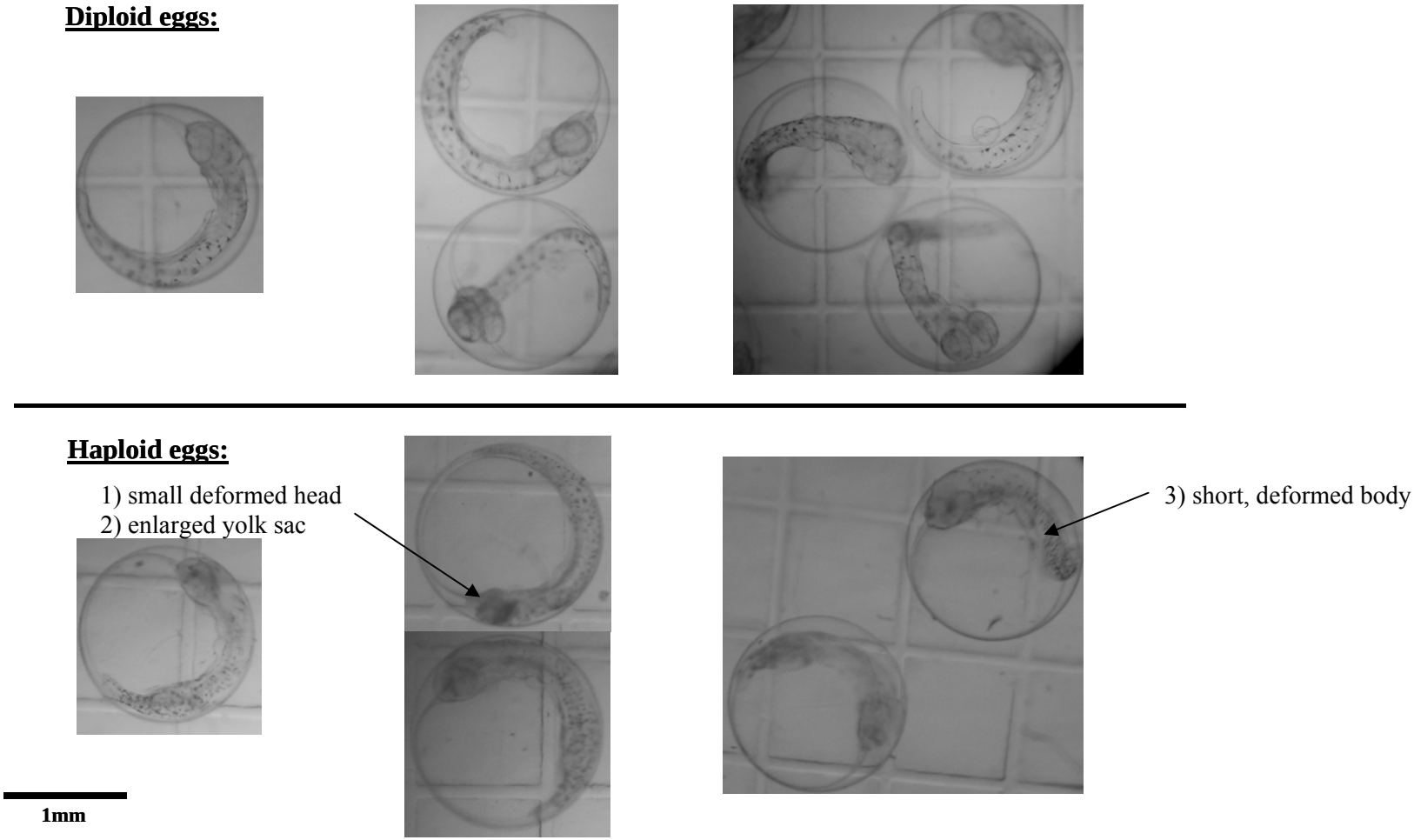


Figure 6.10. Comparative morphology of diploid vs. haploid cod embryos at 61°C days. Eggs were observed under a dissecting microscope (x20 in magnification).

From the 30 eggs originally sampled to study ploidy, 24 were successfully genotyped for at least two loci. The analysis of chromatograms revealed that the eggs genotyped from the 4 min irradiation treatment were all haploids (Table 6.5), the only allele expressed being of maternal origin (detailed data not shown). However, quite surprisingly - given the previous results from the survival and morphology studies - all the eggs genotyped from the 1.5 min treatment were diploids.

Table 6.5. Ploidy status of 65°C days cod eggs -fertilised with UV irradiated milt (240 μW/cm^2)- based on the genotyping data from four DNA microsatellites.

	Diploid control	1.5 min UV	4.0 min UV
Number of eggs genotyped	8	10	6
Number of haploids	0	0	6

6.3.3. Egg shocks

6.3.3.1. Effects of the cold shock timing on larvae survival

The highest survival rate at hatching was observed among egg batches which were exposed to a cold shock 10 minutes after being fertilised (Table 6.6). The timing of cold shocks influenced greatly the survival of cod larvae at hatching. A shock initiated ten minutes after an egg batch was fertilised resulted in an average hatching survival rate of 3.1% (equivalent to 44% of the survival rate observed in the diploid control treatment). By beginning the shock only 5 minutes later (i.e. 15 minutes post-fertilisation), the average survival rate fell below 0.7% (i.e. 10% of the survival rate observed in the diploid control treatment).

Table 6.6. Effects of cold shock starting times on the survival of cod eggs, at three stages of embryogenesis.

Treatment	Survival (%)			Relative survival of the treatments to the survival of the diploid control at hatching (%)
	Blastula (20^{o}C days)	Eye pigmentation (63^{o}C days)	Hatching (90^{o}C days)	
Diploid control	30.3	22.2	7.1	100.0
Haploid control	33.7	13.5	0.0	0.0
10 min PF	37.2	10.6	3.1	43.7
15 min PF	28.4	7.0	0.5	7.0
20 min PF	26.1	9.7	0.7	9.8
30 min PF	20.9	4.6	0.4	5.6
40 min PF	26.5	2.5	0.1	1.4
50 min PF	20.4	0.6	0.1	2.4
60 min PF	16.1	3.2	0.4	5.6

PF: post-fertilisation

Notes: the results presented are based on the data provided by two replicate experiments; treated eggs were exposed to a cold shock at -3^{o}C for 60 minutes. Relative survival = (survival of the treatment x 100)/survival of diploid control.

6.3.3.2. Effect of the cold shock duration on larvae survival

A shock duration of 30 or 60 minutes led to the highest survival rate at hatching (i.e. equivalent to 27% of the survival rate observed in the diploid control treatment). Virtually no difference existed, in terms of survival, between conducting a shock of 30 min or a shock of one hour (based on the data provided by the results of a single experiment). The tolerance of cod eggs to longer exposition times was however extremely limited: applying a cold shock of two hours was lethal to the entire batch (Table 6.7).

Table 6.7. Effects of the duration of cold shocks (at –3°C, initiated 10min post-fertilisation) on the survival of cod eggs, at three stages of embryogenesis.

Treatment	Survival (%)			Relative survival of the treatments to the survival of the diploid control at hatching (%)
	Blastula (20°C days)	Eye pigmentation (63°C days)	Hatching (90°C days)	
Diploid control	15.2	9.0	8.3	100.0
Haploid control	12.2	8.0	0.0	0.0
-3°C / 30 min	14.2	9.1	2.2	26.5
-3°C / 60 min	19.1	6.7	2.3	27.7
-3°C / 90 min	10.7	6.3	0.7	8.4
-3°C / 120 min	9.5	0.0	0.0	0.0

Note: the results presented are based on the data from a single experiment.

6.3.3.3. Effects of the cold shock intensity on larvae survival

Important discrepancies in hatching survival rates arose as both the intensity and the starting time of cold shocks varied. The highest rate of survival, at hatching, was achieved after exposing cod eggs to a shock at -6°C, for one hour, 10 minutes post-fertilisation (see Table 6.8). The coldest temperature tested in this experiment (-6°C) was by far the most efficient (7.3% hatching survival for -6°C vs. 1.2% for -1°C when treatments were initiated 10 min post-fertilisation).

Table 6.8. Effects of the intensity and the starting time of cold shocks on the survival of cod eggs, at three stages of embryogenesis.

Treatment	Survival (%)			Relative survival of the treatments to the survival of the diploid control at hatching (%)
	Blastula (20^{o}C days)	Eye pigmentation (63^{o}C days)	Hatching (90^{o}C days)	
Diploid control	27.9	22.8	8.5	100.0
Haploid control	24.1	10.1	0.0	0.0
10 min PF / -6^{o}C	14.5	9.2	7.3	85.9
20 min PF / -6^{o}C	8.3	3.0	2.1	24.7
30 min PF / -6^{o}C	21.8	6.4	0.9	10.6
10 min PF / -1^{o}C	18.6	8.3	1.2	14.1
20 min PF / -1^{o}C	22.0	12.2	1.1	12.9
30 min PF / -1^{o}C	22.2	9.0	0.0	0.0

PF: post-fertilisation

Notes: the results presented are based on the data from a single experiment; cold shocks lasted for 60 minutes. Only two temperatures could be compared for technical reasons.

6.3.4. Induction of gynogenesis on a large scale

Three attempts were made to induce gynogenesis on a large batch of eggs. The firsts two attempts were unsuccessful due to the extremely low survival of eggs at hatching. Therefore, the data presented in this section is based on the results from a single experiment.

6.3.4.1. Induction of gynogenesis

Gynogenesis was artificially induced in a batch of approx. 140 000 cod eggs, following the conditions previously established. The experiment also included both a diploid (approx. 50 000 eggs) and a haploid (approx. 1 000 eggs) control treatment.

6.3.4.2. Survival

Survival at hatching was poor in both diploid control and diploid gynogenetic treatments. Less than a thousand hatched larvae were stocked in each of the two experimental tanks (i.e. diploid gynogenetic and diploid control treatments). The

course of the experiment was further affected by high mortalities of fry during early stages of rearing (i.e. live prey / dry feed weaning transitions). The trial was terminated after 264 days with only ten fish in the diploid control and one fish in the diploid gynogenetic treatment having survived.

6.3.4.3. DNA analysis

Genetic profiles for the seven loci screened were obtained for all the 17 fry analysed (i.e. 10 "control" and 7 "gynogenetic" fish). 23 out of the 25 eggs sampled from the haploid control were also successfully genotyped for at least three loci.

The genotyping data gathered from these samples informed on the partial success of the experiment. Based on the typing information from the seven loci screened, only three out of the seven fish sampled from the gynogenesis treatment were true diploid gynogenetics (Table 6.9). The four remaining fish possessed at least an allele of paternal origin. The partial success of the experiment was further confirmed by the results of the genotyping analysis carried out on eggs collected from the haploid control. Only 10 eggs out of the 23 successfully genotyped for at least 3 markers (i.e. 43%) were haploids (the only allele expressed being of maternal origin).

Table 6.9. DNA profiles of seven cod fry sampled from the gynogenesis treatment.

	DNA profiles (allele sizes in bp)						
	*Gmo*C18	*Gmo*C20	*Gmo*C42	*Gmo*C52	*Gmo*35	*Gmo*37	*Tch*11
Female	149/161	119/119	164/173	292/292	116/122	268/292	162/194
Male	**158/161**	**113/119**	**167/173**	**295/298**	**119/128**	**264/292**	**166/170**
G1	149/**158**	**113**/119	164/173	292/**298**	116/**119**	268/292	162/**170**
G2	149/**158**	119/119	**167**/173	292/292	116/122	268/268	162/194
G3	149/161	119/119	173/173	292/**295**	122/**128**	**264**/292	**170**/194
G4	**158**/161	**113**/119	**167**/173	292/**298**	116/**119**	292/292	**166**/194
G5	161/161	119/119	164/173	292/292	116/122	268/292	162/194
G6	161/161	119/119	173/173	292/292	122/122	268/292	162/194
G7	149/149	119/119	164/173	292/292	116/122	292/292	162/194

G: gynogenetic

Note: format **158**, in the offspring profiles, for alleles of paternal origin.

6.3.4.4. Observed sex ratio

The sex of fish sampled at 264 days post hatch was easily assessed by gonad squash. As expected, the sex ratio of the diploid control was balanced (i.e. 6 females and 4 males out of 10 fish sampled). The sex of the surviving gynogenetic fish (confirmed via DNA profiling at seven loci) was female. The histology study of the recovered mortality from the gynogenesis treatment (i.e. six fry aged between 120 and 203 days) remained however inconclusive (see Figure 6.11). The gonads observed were all undifferentiated.

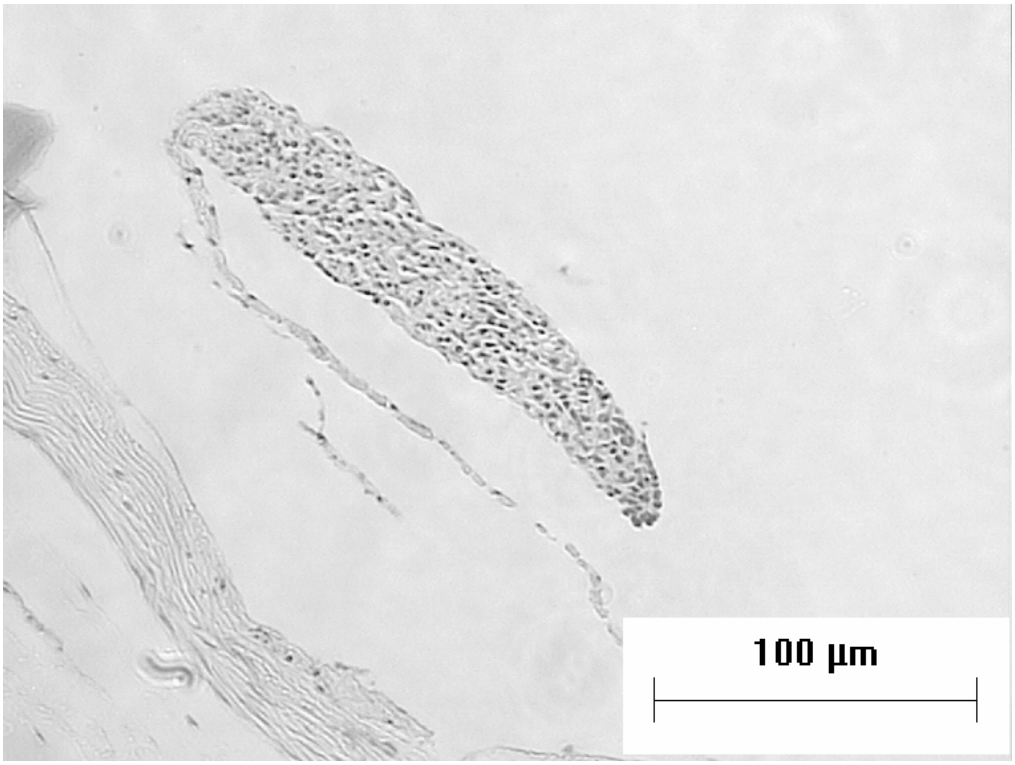


Figure 6.11. Undifferentiated Atlantic cod gonad at 203 days post hatch (compound microscope x400).

6.4. Discussion

This study suffered from several major drawbacks and rescheduling due to the lack and / or the poor quality of cod eggs. Although gametes could be collected from up to six different breeding tanks at times (each containing approx. 10 females), experiments had to be cancelled, on several occasions, due to the lack of available eggs (especially towards the end of the spawning season). The important variability in egg survival proved equally problematic. Hatching rates of diploid control batches varied from 0 to 52%, which means that part of the collected data had no real scientific value and, therefore, was not included in subsequent analyses. The incubating system is unlikely to be the sole factor responsible for the variability in hatching rates observed. Inconsistencies in both egg sizes and percentages of floating eggs suggested that the egg quality greatly fluctuated between collected batches. This might be related to the timing of stripping and / or the time of the season. As a result, not all experiments were carried out in triplicates.

Through a succession of experiments, a protocol to artificially induce gynogenesis in Atlantic cod was established. This protocol consisted of three main steps: 1) diluting cod milt to a working concentration of $9x10^8$ spz/ml in Mounib's extender; 2) delivering to the diluted milt a UV dose equivalent to 240 $\mu W/cm^2$ for four minutes; 3) conducting a temperature shock sustained for one hour, at -6°C, ten minutes after fertilisation.

Cod sperm was extremely sensitive to UV irradiation. When increasing the exposure time to UV and / or the diluting factor of the semen, the percentage of activated spermatozoa decreased dramatically. Similar results were previously

reported in several other marine species including turbot and Atlantic halibut (Piferrer et al., 2004; Tvedt et al., 2006).

A "pseudo Hertwig" effect was observed when cod eggs were fertilised with milt which had been irradiated with low doses of UV (see Figure 6.9). Low UV doses - in this case below three minutes - did not totally destroy the sperm DNA. The partially potent paternal DNA was then passed on to the eggs (during fertilisation) and the created aneuploid embryos died prior to the eye pigmentation stage. Higher UV doses, comprised between 4 and 5 minutes, resulted in the complete destruction of the sperm DNA and allowed for the development of haploid embryos, viable at 60°C days. With UV doses exceeding 6 minutes, further damage was inflicted to the sperm (i.e. progressive destruction of proteins). These damages progressively decreased the capacity of the milt to fertilise eggs, which in turn explained the decrease in egg survival observed with prolonged UV exposure (> to 6 min).

Cold shocks (ranging from -2 to 0°C) were successfully used on several cold water marine species to artificially induce both gynogenesis and triploidy (Piferrer et al., 2004; Rani, 2005). For Atlantic cod, promising results were first obtained by Kettunen et al. (2004) when using cold shocks on fertilised eggs to induce triploidy: up to 14% triploids were produced by applying a cold shock of two hours at -1.7 ± 0.1°C. The choice to use cold shocks for inducing gynognesis in this study was primarily motivated by the results of this early publication. However, later work by Peruzzi et al. (2007) failed to achieve the same level of success, in using cold shocks to induce triploidy in cod, and proved that 20°C heat shocks were far more efficient. In the present study, cold shocks of greater intensity than previously reported in the literature on cod were tested. The best result, in terms of egg survival at hatching, was

achieved with the coldest temperature applied (i.e. -6°C). However, the tolerance of cod eggs to such extreme temperatures was limited: survival rates, at - 3°C, decreased with exposure times exceeding 60 minutes. The protocol used to shock eggs at these temperatures was somewhat unusual: the eggs were not directly immersed in seawater at the shock temperature but rather progressively cooled (see section 6.2.2.3). This method was adopted after a trial which consisted in immersing fertilised eggs directly in seawater at -3°C proved unsuccessful (i.e. lethal to all eggs including eggs from the control treatment; data not shown).

Multilocus DNA microsatellite profiles were successfully used to assess the ploidy status of both cod eggs and fry. DNA profiling was chosen over other available techniques (including chromosome preparation and flow cytometry) because of the possibility it offered to detect alleles of paternal origin in the genomes of offspring (Tvedt et al., 2006). Although quantities of DNA extracted from 60°C days cod eggs were extremely low (i.e. on average 0.05 μg ± 0.03 μg), PCR amplifications of loci were successfully conducted. The best results, in terms of both quality and quantity of PCR products, were obtained when using the QIAGEN Multiplex PCR master mix in the PCR reaction mix (see section 6.2.4.2). Being able to obtain genotyping information from cod eggs, at such an early stage of development, made it possible to confirm the haploid status of "suspected haploid" embryos. DNA profiling was also used to detect the presence of diploid gynogenetics among fish sampled from the "large scale" gynogenesis experiment. On this occasion, as many as seven loci were screened so the chance of having at least one "decisive" locus (with no shared alleles between the parents) was high. In this particular case study, the two parents shared no common alleles at three loci (*Gmo*C52, *Gmo*35 and *Tch*11) which made the detection of diploid gynogenetics highly reliable.

Inducing gynogenesis at a relatively large scale proved extremely difficult. Three attempts were necessary before enough hatched larvae were produced to stock two 1 m^3 larval experimental tanks (i.e. approx. 1000 hatched larvae per tank / equivalent to a total survival rate of just 1.4%). Such a result was not totally unexpected as poor hatching survival rates of gynogenetics were reported in several other freshwater and marine fish species (Tvedt et al., 2006; Komen and Thorgaard, 2007).

In addition, both the control and the gynogenesis treatments suffered from high mortalities during the first 100 days of rearing. Among the factors likely to have affected the survival of fry, egg quality and rearing conditions largely prevailed: 1) the eggs used in this large scale trial were collected at the very end of the spawning season for technical reasons (i.e. when egg quality is known to be at its lowest); 2) larval feeding proved extremely challenging, two weeks into the rearing trial, due to a sudden shortage in live rotifers.

The DNA analyses undertaken on the surviving fish from the experimental groups revealed that the gynogenesis induction was only partially successful. The UV treatment was clearly to blame as only 43% of the eggs from the "UV / no cold shock" group genotyped were found to be gynogenetic haploids. The other 57% were mosaics, with at least one allele of paternal origin detected, implying that the intensity of the UV treatment was not sufficient to entirely incapacitate the DNA material contained in the male gametes. This result was not totally unexpected given the difficulties encountered, in this experiment, when calibrating the intensity of radiations, delivered by the UV lamp (the radiometer used in the previous experiments had to be replaced, following several inaccurate readings).

Lastly, since only one diploid gynogenetic was sexed, no conclusion on the sex determination mechanism(s) operating in cod could be drawn.

6.5. Conclusion

In this study, a protocol to induce gynogenesis in Atlantic cod was tested using extreme cold shocks (-6^{o}C). Although promising results were obtained from small scale experimental trials (approx. 1000 eggs treated), scaling up the experiment proved extremely difficult.

In early 2007, further work on the induction of gynogenesis in cod was undertaken at the University of Bodø (Norway) - using the results from this study - by Professor Igor Babiak and colleagues. A second protocol, using a combination of UV radiations (on sperm) and pressure shocks (on fertilised eggs), was developed (Igor Babiak, personal communication).This work will be resumed next year in an attempt to study the sex ratio of at least one cohort of cod gynogenetics and possibly uncover the mechanisms behind sex determination in Atlantic cod.

To conclude, if I could rewind time, I would 1) try to better plan the experiments so they would all coincide with the optimum peak of the spawning season, 2) work on both warm and cold temperature egg shocks and 3) dedicate more time to this study (I think I truly underestimated the amount of work that was required to carry out all the experiments I planned at the start of this project).

Chapter 7. General Discussion

7.1. Summary of the findings

In this project, a substantial effort was put into the development of a robust parentage assignment system which could support the analysis of large and complex genotyping datasets. A review of recently published work exposed a lack of consensus / coherence in the analytical methodologies adopted to analyse parentage in case of complex aquaculture mating systems (Borrell et al., 2004; Fessehaye et al., 2006; Porta et al., 2006). In light of this observation, a detailed comparison of two assignment principles (i.e. strict exclusion and probabilities) and four parentage software programmes was conducted, using a dataset representing the output of a mass spawning cod tank. This study highlighted the discrepancies which could arise between assignments based on strict exclusion and assignments based on probabilities, when typing errors were present in the dataset. Based on these results, a "three steps" method to analyse large genotyping datasets was developed. This method consisted in: 1/ performing an exclusion-based analysis - using the software programme FAP - in order to assess the quality of the genotyping dataset, 2/ reviewing and correcting the typing errors according to the results of the previous analysis and 3/ running a second and definite exclusion-based analysis. This study was also an occasion to challenge and eventually disagree with the recommendation recently made by several authors to screen less loci for parentage analyses in order to limit costs (e.g. Fessehaye et al., 2006; Porta et al., 2006).

A total of five samples, issued from five single days of mass-spawning of two captive broodstock populations, were analysed for parentage (following the method previously described). Information on the ranges of individual contributions, the spawning behaviour and dynamics of captive cod populations were gathered (see Chapter 4). The main findings are summarised in the four following points: 1/ on a

single day, at least 25 to 30% of the total breeding population contributed to fertilised eggs that resulted in viable offspring, 2/ individual contributions were highly skewed, mostly because of male dominance, 3/ male dominance was not based on size or weight and 4/ effective breeding populations were consistently low (approx. 5% of the breeding population). These results mostly agree with the information already published from smaller scale studies on cod (i.e. Hutchings et al., 1999; Nordeide and Kjellsby, 1999; Nordeide and Folstad, 2000; Bekkevold et al., 2002) and also concur with suggestions made by commercial breeders.

The analysis of parental contributions to a batch of commercially produced cod fingerlings was the occasion to assess the impacts of hatchery practices (i.e. repetitive size gradings and mixing of batches) on the genetic diversity. The main findings of this analysis were that: 1/ the effective breeding population size of the juvenile batch was two to three times greater than the effective size observed on a single day of mass spawning, 2/ the effective breeding population size of the juvenile batch represented approx. 14% of the breeding population and 3/ the per-generation rate of inbreeding (ΔF), based on broodstock replacement using this single batch of fingerlings, equalled to 2.5%. The effective breeding population size of the batch analysed was comparable to the findings of Brown (2003) for gilthead seabream.

Finally, in an attempt to study the sex determination mechanism(s) operating in cod, a protocol to induce gynogenesis was developed. This protocol consists of three main steps: 1) diluting cod milt to a working concentration of $9x10^8$ spz/ml in Mounib's extender, 2) delivering to the diluted milt a UV dose equivalent to 240 $\mu W/cm^2$ for four minutes, 3) giving a temperature shock sustained for one hour, at -6^oC, starting ten minutes after fertilisation. Although the expectations from the

ongrowing trial were not entirely met (i.e. in terms of the number of surviving gynogenetic fish which could be sexed), gynogenetic fish were successfully produced (verified by molecular analyses). The protocol used to shock eggs (using cold temperature shocks) was inspired by early work on triploidy induction in cod, by Kettunen et al. (2004). However, it was later on suggested that heat shocks (at 20^{o}C) were more efficient than cold shocks in inducing triploidy in cod (Peruzzi et al., 2007). Due to time constraints, the induction of gynogenesis via heat or pressure shocks could not be investigated further and only one ongrowing trial of gynogenetic fish could be carried out.

7.2. Recommendations for improving genetic management and implementing selective breeding in cod hatcheries

Based on the findings of this PhD research project, recommendations can be made regarding both the genetic management and the implementation of selective breeding in intensive commercial cod hatcheries.

7.2.1. Short-term broodstock management

In mass spawning systems, the fry production is likely to be largely dominated by the spawning activity of very few males. Since spawning dominance in cod is not based on size, the common practice which consists in culling small males after the spawning season (to reduce stocking densities or to partially replace the breeding stock), might be detrimental to the production. In that sense, being able to identify dominant fish (based on the results of behaviour and / or parentage studies) might prove extremely useful for optimising the management of commercial mass spawning cod broodstock tanks.

Another question which arose from the results of this research project concerns the "optimum" broodstock population size necessary to maintain in a commercial cod hatchery to: 1) meet its needs in terms of daily volume of fertilised eggs produced, 2) ensure a sufficient genetic diversity level among the juveniles selected for broodstock replacement. The results of the parentage exercise realised on offspring sampled from the "wild Scottish" broodstock seem to indicate that a large number of males per breeding tank (i.e. N=55) encourages the establishment of spawning dominances (see Chapter 4).In addition, the parentage analyses realised on offspring sampled from both the "wild Scottish" and the "wild Norwegian" broodstock populations also suggest that, on a daily basis, only 25 to 30% of the fish contribute to viable 50 days post hatch fry. Given these two results, it might be preferable for a hatchery to stock two breeding tanks with 50 cod each (i.e. approx 30 females and 20 males) rather than one tank with a hundred fish.

7.2.2. Long-term broodstock management: implementing genetic selection for improving growth of Atlantic cod produced in an intensive commercial hatchery

The following "enhanced" mass selection model is based on the results from the study cases presented in this thesis and takes MMF hatchery as a model. This model presents a set of measures which do not take into account both the space availability (i.e. number of tanks) and the financial constraints of the farm.

Providing the broodstock population held in the hatchery is suitable to select from (i.e. genetically diverse enough), the programme begins with the establishment of a F1 population which reflects the genetic pool of the farm. This can be achieved by retaining a fraction of several mixed and graded fry groups, at the end of hatchery

rearing, from the overlap production of all four hatchery broodstock tanks held at MMF (see Figure 7.1). In each cohort, age and environmental effects would be minimised during hatchery rearing.

A fast growing fraction of the base population (approx. 20 000 fish) would then be retained following the first nursery size grading (see Figure 7.1). Parentage contribution to this elite line would be assessed by sampling fin clips from approx. 1000 fry and realising DNA profiles (using an adequate set of DNA microsatellite markers and an exclusion-based parentage analysis). A fraction of the slow growers (i.e. small grade) would be also retained for comparison of growth performances over time. Environmental variances between the fast and the slow growing groups would be kept minimal during nursery rearing, as well as the variances between selected groups and the rest of the commercial production (i.e. in terms of fish density, feeding regime, water temperature, health status, etc.).

The selected fast and slow growing lines would then be sent to sea cages for ongrowing and a representative sample of this line would be kept in the hatchery for broodstock replacement (Figure 7.1). Information on the harvest time, average weight of the fish and possibly other quality parameters gathered on the filleting / processing lines would be collected. If possible, fin samples from the best performing fish would also be taken post mortem, on the processing lines, to identify the best performing families. Heritability for growth would be estimated based on these data.

In the meanwhile, the fish retained in the hatchery for broodstock replacement would be tagged and genotyped. Family contributions would be evened out by removing fish from over-represented families.

Based on the performance results of the elite line in sea cages, fast growing families would progressively be introduced in the hatchery broodstock tanks (see Figure 7.1).

All the fish used in this selection programme (except the fish ultimately retained for broodstock replacement) can eventually rejoin the commercial production and be sold (in order to minimise costs).

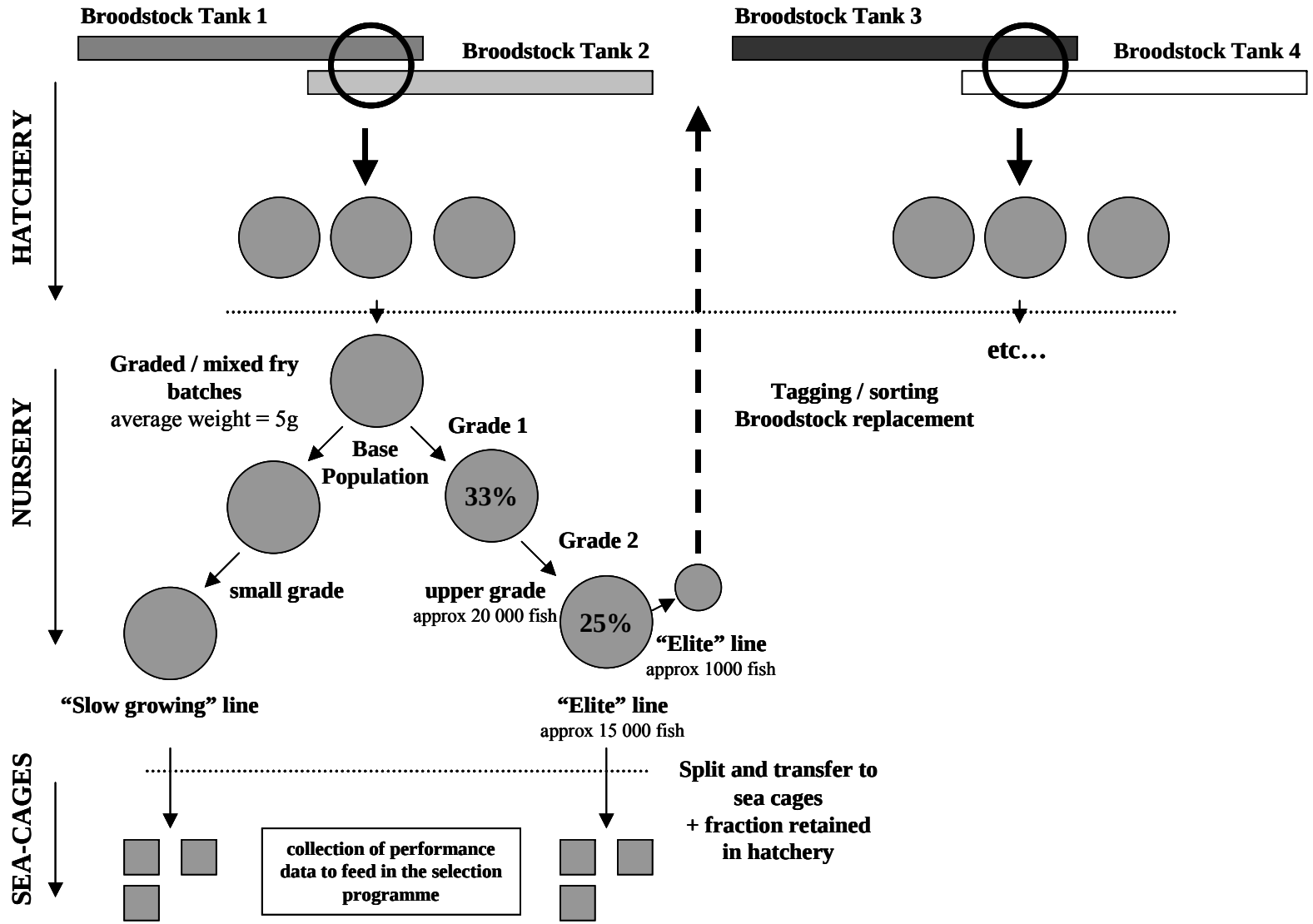


Figure 7.1. Diagram of an "enhanced" mass selection programme for the genetic improvement of growth in Atlantic cod.

7.3. Scope for future work

The following paragraph lists some research aspects which could be further explored in a follow-up to this project:

1/ refinement of the parentage analysis (i.e. by working on primer designs / PCR conditions to remove null alleles) to find a better suited set of DNA microsatellite markers to analyse the parentage of fry batches issued from the mass spawning of commercial cod breeding tanks.

2/ establishment of a fry population, in MMF hatchery, to study the genetic variation for traits of commercial interest in cod (i.e. improved growth, increased disease resistance, late sexual maturation).

3/ refinement of the protocol to induce gynogenesis on a large scale to further study the sex determination mechanism(s) operating in cod.

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Appendix

Communications to conferences and workshops

3rd November 2004	British Marine Finfish Association workshop, Oban, Scotland.
	Oral presentation: "Introduction of genetic management in new marine farm hatcheries: the case of Atlantic cod".
17th June 2005	Marine Farms Technical meeting, Machrihanish, Scotland.
	Oral presentation: "Introduction of genetic management in new marine farm hatcheries: the case of Atlantic cod".
27th October 2005	British Marine Finfish Association workshop, Oban, Scotland.
	Oral presentation: "Introduction of genetic management in new marine farm hatcheries: the case of Atlantic cod".
19th May 2006-10-13	Aquaculture International Exhibition 2006, Glasgow, Scotland.
	Oral presentation: "Genetic management of Atlantic cod broodstock".
25th-30th June 2006-10-13	International Symposium for Genetics in Aquaculture IX, Montpellier, France.
	Oral presentation: "Analysis of parental contribution and spawning dynamics in commercial Atlantic cod (*Gadus morhua*) breeding tanks".
12th-13th September 2006	Sustainable Animal Breeding Conference, Edinburgh, Scotland.
	Poster (1st prize in the Genesis Faraday Associates poster competition): "Genetic management of Atlantic cod broodstock".
14th-15th November 2007	British Marine Finfish Association workshop, Inveraray, Scotland.
	Oral presentation: "Genetic management of Atlantic cod hatchery populations".

Publications

Herlin, M., Delghandi, M., Wesmajervi, M., Taggart, J.B., McAndrew, B.J., Penman, D.J., 2007. Analysis of the parental contribution to a group of fry from a single day of spawning from a commercial Atlantic cod (*Gadus morhua*) breeding tank. Aquaculture 272, 195-203.

Herlin, M., Taggart, J.B., McAndrew, B.J., Penman, D.J., 2008. Parentage allocation in a complex situation: a large commercial Atlantic cod (*Gadus morhua*) mass spawning tank. Aquaculture 274, 218-224.