Genetic Management of the Atlantic halibut (*Hippoglossus hippoglossus*)

A thesis submitted for the Degree of Doctor of Philosophy

By

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August 2005

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.

Signature of Candidate: _____

Signature of supervisor: _____

Date _____

Abstract

The Atlantic halibut (*Hippoglossus hippoglossus*) was selected as a new aquaculture candidate towards diversification from salmonid culture. The species was chosen because of its high market value and perceived good growth in the cold waters of the target farming regions. Extensive and collaborative research efforts formed the basis for the culture of this benthic marine species. Broodstock populations were established from a limited number of wild individuals due to the high costs involved in their capture. First generation hatchery reared offspring are now being selected as replacements but with no knowledge of a broodstock replacement strategy to manage this valuable genetic resource and to maximize the potential of this species the industry runs the risk of genetic degradation with the associated problems of inbreeding depression. This thesis investigates the processes involved with developing a genetic management strategy, using genetic profile technology, based on a population in Scotland at Otter Ferry Seafish Ltd.

The level of genetic variability of 70 individuals in the parental population and 802 of their offspring (F_1) in two year classes (1995 and 1998) was assessed at 7 microsatellite loci. The parental population which was comprised of three different stocks was genetically diverse, however, when compared with their offspring, substantial reductions in genetic variation, as judged by allelic diversity, were observed. The parentage of these F_1 individuals was determined by exclusion principles and 91% of all the offspring genotyped were unambiguously assigned to a single parental pair. The assignment revealed that only half of the parents succeeded in contributing to the F_1 generation. This problem was compounded by the fact that the family sizes were highly skewed such that the entire population consisted of a small number of large families resulting in an unacceptably low effective population size of 8.11. The inbreeding coefficient in the F_1 generation was 6.16% however this differed markedly between the 1995 (7.74%) and 1998 (10.64%) year classes.

The repeatability of reproductive performance defined by five performance traits reflecting quantity and quality of eggs, frequency of stripping events and viability was assessed by REML using data collected over three spawning seasons from 239 F_1 females. The phenotypic correlations obtained between quality and quantity traits were low in magnitude and the study showed that with the exception of seasonal activity, all traits studied improved with age. There was also an effect of photoperiod in that fish kept under a 4 month-delayed regime did not perform as well as the fish under a one-month extended regime. Of all five traits, only volume of eggs collected was repeatable ($r=0.37\pm0.07$). This suggests that it is the only reproductive character influenced by the fish and all other traits, especially those pertaining to egg quality are determined by management practices.

The heritability of body weight at four stages in the growout phase of production was estimated by REML using data from 486 F_1 individuals. These animals were reared in two different sites, a land based tank and a sea cage. Significant effects of site, sex and grade were observed and by the end of the trial the average weight of fish in land based system was higher by 2 kg. Females were over 3 kg heavier than males and this difference was attributed to precocious maturation while fish in the smaller grade were consistently though decreasingly lighter than their larger counterparts. Heritabilities, derived from sire, dam and combined estimates ranged from 0.09 to 0.53 with wide confidence intervals. The poor precision of the estimates was due to the highly imbalanced family structure and the small number of offspring studied. Values varied depending on the age of fish and the source of variance used to estimate them. Heritabilities increased with age and although sire estimates were higher than dam estimates these differences were tested and the results obtained consistent with the conclusion that they represent nothing more than chance. The results suggested that selection for increased body weight in the halibut was likely to be successful.

Based on the findings of the study a genetic management strategy for the Atlantic halibut was proposed structured on controlling the rate of inbreeding and a theory of domestication. The need to introduce additional individuals into the population was strongly recommended.

Acknowledgements

I would like to start by thanking my primary supervisors, Professors Brendan McAndrew and John Woolliams, for their advice, support, guidance and tenacious belief that this thing will get done in spite of all the challenges. It has been a great privilege to learn from you, oh and John I am grateful, *very* grateful to you for being O. I would also like to thank Dr. Dave Penman, my secondary supervisor for all his advice and for proof reading the thesis. In thanking my supervisors I cannot but express my heartfelt gratitude to Dr. John Taggart without whom the word "Gislon" would have been a squash racket bought on the cheap. John, I owe you. Olorun ni kan lo ma ba mi dupe.

I must thank the management at Otter Ferry Seafish Ltd and Knowledge Transfer Partnerships (K.T.P) for funding this project. My thanks goes to Mr Alastair Barge for being an understanding boss and an excellent neighbour while I was in Otter Ferry, to Mr Dave Patterson for his much appreciated help with halibut husbandry and his patience as well as to all the staff on the farm for putting up with me. I am grateful to my K.T.P consultant, Mr Tony Mitchell for all his help with the K.T.P process especially during the "atmospheric" L.M.C meetings. Thank you for your friendship, guidance and help in salvaging the project. I would, at this point, also like to thank the trustees and management of Russell and Aitken, Falkirk, for their financial support during the writing up period and Mrs Melanie Cruickshank for helping me with the finance problems.

I wish to thank the people at the institute in Stirling who helped me find my way around a laboratory and the world of molecular biology: Dr. Morris Agaba, Dr Margaret Cairney, Mrs Ann Gilmore, Mr Steve Powell and Dr. Cameron Brown. I

must also thank Dr. Andrew Shinn (Andy Pandy) and Dr Antonio Campos (Toño, me machismo amigo) first being wonderful friends and second for all the help they gave me with the project. Special thanks to Mrs Alison Giles for all her help with the word processing and Dr Nigel Jordan for all his work with the heritability data.

My PhD experience has been a rich one and I must thank the people who supported me, listened to my Olympic-style grumbling, prayed with me and added colour to my four years, four months, three weeks and six days (almost anyway!), my friends: Bro. A, The Adetayos, Arinola, Andy-I'm better than you at squash-Forrest, Bolaji Brian Tee, Barry&Marcia, Ben&Ruth, Cameron, Judy, Karen, Paula and The Thomases

I must acknowledge the fact that without the support of my family I would not be here typing these words. Words cannot express the gratitude I have for my mother and grandparents. Their emotional and financial support over the past twelve years and the last two in particular is the biggest act of selflessness I have experienced. Thank you. I am grateful for all your love and your belief in me. I thank my wife, the real "Dr Frank-Lawale" for being the better side of me, for all her love, kind words of encouragement, support and for helping make the completion of this project a reality. Ife, Olorun ti ba awa se. I thank my sister, Layo, for being the big sister and cheering me on when I wanted to quit. I thank my mother-in law and the Abereojes for their constant prayer and pep talks. I especially thank Dr. Joshua, my father-in law, for being the man who gave me the desire to study genetics.

Finally I thank God for this PhD experience. The sweat, blood, smiles and tears that cumulated in the completion of this phase of my "spiritual domestication" where I am becoming adapted to Him and to the environment He provides.

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Chapter 1

General introduction

1.1 Introduction

Fishing remains the only significant practice of hunter gathering still used today in man's quest to feed an ever-expanding population. It is estimated that fishing communities thrived throughout the African continent as far back as 4000 BC (Shillington, 1995). Instead of evolving towards cultivation, as in agriculture and animal husbandry, methods of processing protein from the aquatic environment developed along a different path, towards more efficient methods of tracking and hunting (Pillay, 1990; Beveridge and Little, 2002). However, reduction in fish stocks of desirable species, increase in fuel costs, changes in laws governing the oceans and in particular increase in knowledge of aquatic biology and technology led to the science of aquaculture.

Aquaculture, the large-scale husbandry or rearing of aquatic organisms for commercial purposes (Landau, 1992), has increased at an average compound rate of 9.2% per year since 1920 (FAO, 2003). FAO statistics show that aquaculture accounted for 41% of the total world aquatic production in 2001. In the countries of Northern Europe and Chile salmonid production has dominated aquaculture over the past 30 years. Today over one million tonnes of farmed salmon is produced worldwide (www. Salmonchile.cl). This has resulted in intensive competition among producers forcing reduced prices to the point where salmon is no longer considered a luxury item and profit margins are slim. Consequently, there has been a growing interest in diversifying the range of species for cold water, and in particular marine, aquaculture (Tilseth 1990; Alvial and Manríquez, 1999). The Atlantic halibut (*Hippoglossus hippoglossus* L.), turbot (*Scophthalmus maximus*), cod (*Gadus morhua* L.), ocean wolfish (*Anarhichas lupus*) and spotted wolfish (*Anarhichas minor*) were

considered to be the most promising marine species candidates for the aquaculture industry in the North Atlantic (Tilseth 1990; Olsen *et al*., 1999). However, the main focus on domestication and cultivation was placed on the Atlantic halibut and in the 1980s it was selected as the most suitable marine fish species for farming in the UK (Olsen *et al*., 1999; Shields *et al*., 1999).

The Atlantic halibut was identified as an ideal candidate based on its high market value (£10/Kg compared to £2/Kg for salmon) and good growth in relatively cold waters. Farmed stocks can reach the desired market weight of 3-5 kg within 3 years post weaning (Bromage *et al*., 2000; Adoff *et al*., 1993). Progressive decreases in the wild capture of this species, especially in the past 30 years, as shown in Figure 1.1 below, prove that wild stocks have been overexploited (Haug, 1990) and strengthens the market value of this fish. Comprehensive research programmes, particularly in Norway, formed the basis for the commercial culture of the halibut. Today the Atlantic halibut is farmed in Norway, Scotland, Iceland, Ireland, Canada, USA and Chile (Brown, 2002; Alvial and Manríquez, 1999).

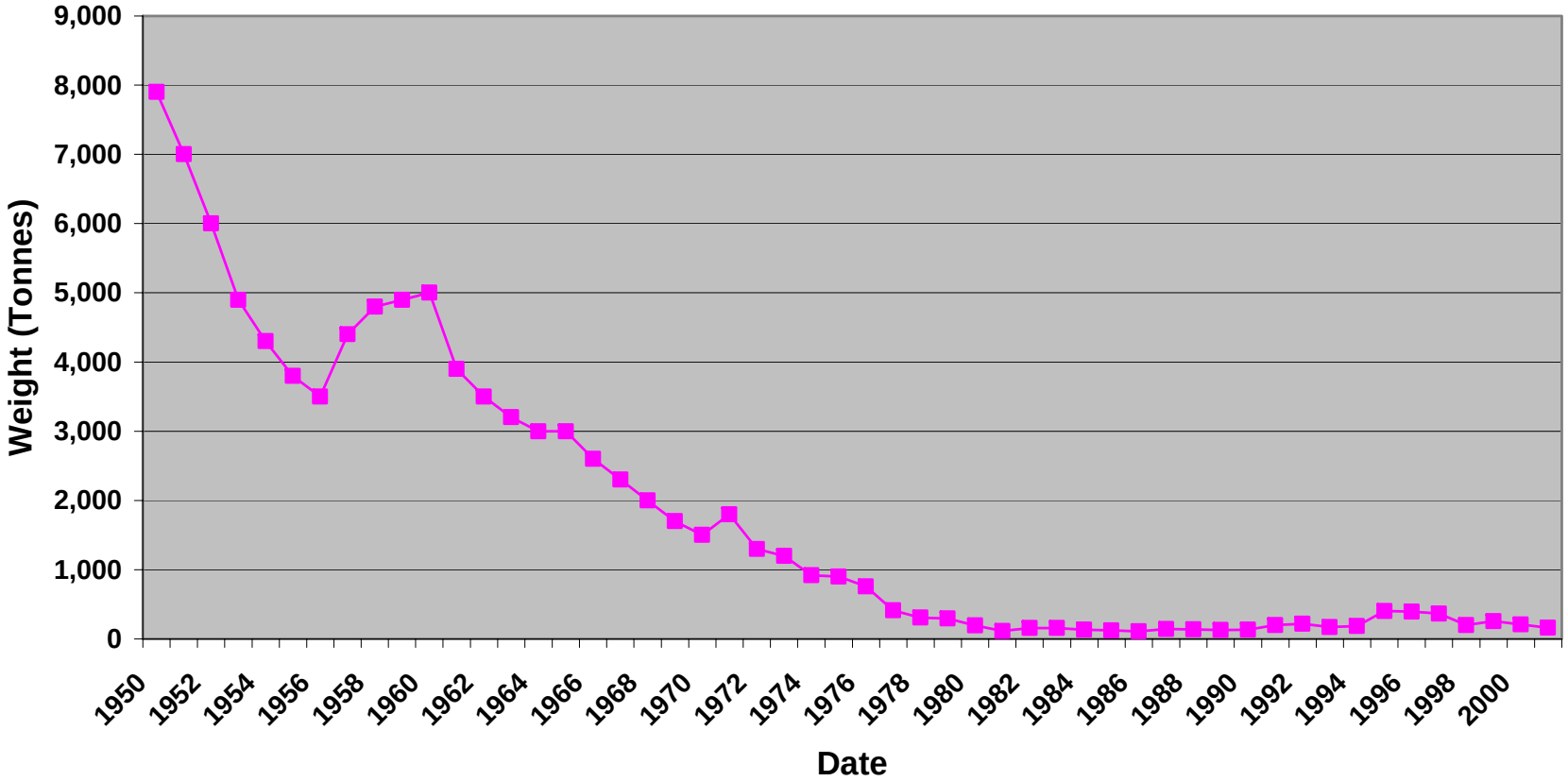

Figure 1.1 Graph showing the total capture of Atlantic halibut in the northeast Atlantic of the UK between 1950 and 2001 (FAO, 2003).

1.2 The Atlantic halibut

The Atlantic halibut, *Hippoglossus hippoglossus* (L.), is a right-eye flat fish and it is the largest member of the Pleuronectid family. The fish was first described by Linnaeus in 1758 as *Pleuronectes hippoglossus* but following closer observations of fin structure Cuvier reclassified the genus and thus the name was changed to *Hippoglossus hippoglossus* in 1885 (Bromage *et al*., 2000). The halibut is widely distributed in the North Atlantic Ocean and in parts of the Arctic Ocean, along the coasts of Norway, Iceland, southern Greenland and the East Coast of Canada, on occasion to as far south as the Bay of Biscay and New York (Andriyashev, 1954: as cited by Haug, 1990).

Although the first description of the successful hatching of halibut eggs was reported by Rollefsen in 1934 (Mangor-Jensen *et al*., 1998b) the first attempts to rear Atlantic halibut larvae were carried out in Norway between 1974-1980 when ripe adult specimens were net caught and stripped for eggs and milt. Researchers have since then pieced together the early, complex, life history and culture requirements of this deep-water species. In the late 1980s and early 1990s the main focus of research was on the biology and rearing techniques for eggs and yolk-sac larvae. Below is a brief summary of some of their main findings.

1.2.1 Life Cycle

In the wild, mature halibut congregate for spawning in well defined spawning grounds at depths between 100-700 meters. The spawning season extends over several months and there are clear inter-area differences in the halibut spawning times throughout the North Atlantic. Generally halibut will spawn between the months of November and

April at which times the bottom water temperatures are in the range of 5-7^{o}C and salinity 34.5-34.9% (Haug, 1990; Neilson, *et al*., 1993; Arthur, 1999).

Male halibut reach sexual maturity at a younger age and smaller size than females. Haug (1990) observed that after the onset of maturity (between 6 –7 years) the total weight of males seldom reached 50kg whereas females as heavy as 330 kg had been recorded. There also appears to be a relationship between location and maturity and he postulated that maturity was more a function of growth rate rather than age. This agrees with Roff (1982) who suggested that in some flatfish species maturity is governed by size and not age. In wild Atlantic halibut from Faroese waters, which exhibit the best known growth rates (Kjørsvik and Holmefjord, 1995), Jákupsstovu and Haug (1988) observed that on average males matured at 4.5 years old, weighing only 1.7kg and 55cm long. The average female matured at 7 years old, weighing about 18kg and between 110-115 cm in length.

Atlantic halibut are batch spawners. Females release discrete successive batches of eggs over several (4-6) weeks during a single spawning season. Sexually mature halibut have immense reproductive potential and under favourable conditions the amount of eggs released can equate to approximately 40% of body mass (Mangor-Jensen *et al*., 1998a). Their total fecundity is about half a million eggs in a single spawning season (Norberg and Kjesbu, 1991) giving up to 200 000 eggs per batch with mean inter-ovulation intervals of 70-90 hours (Norberg *et al*., 1991). Each female can be expected to produce 6-16 batches of eggs in one season (Kjørsvik and Holmefjord, 1995). The pelagic halibut egg is large, with a diameter in the range of 3.06-3.49 mm (Haug *et al*., 1984), when compared with eggs of other planktonic

fishes such as the cod for example, which has eggs in the range of 1.13-1.50 mm (Kjesbu, 1989).

After fertilisation the eggs gradually move upwards in the water column and most eggs in the wild are found in areas where temperature and salinity range between 4.5 and 7.0 ^{o}C and 33.8% and 35.0% respectively. This vertical distribution of the eggs is thought to be determined by their specific density and is closely related to seawater salinity (Haug *et al*., 1984). Little is known of the pelagic larval development stage of the halibut in the wild. Records of the larval stage in the wild are scarce, and no more than 70 planktonic halibut larvae have been reported (Haug *et al*., 1989). This is because they are scattered at low density and distributed unevenly over very large areas (Haug, 1990). Therefore most of our knowledge on hatching, development, behaviour, nutritional and environmental requirements of yolk sac larvae is based on observations made during rearing experiments with artificially fertilised eggs (Haug, 1990).

Eggs from halibut hatch when the larvae are still at an early, undeveloped, stage and so they are dependant on the environment for survival through this phase as well as transport to the photic zone where first feeding will occur (Rabben *et al*., 1986). Approximately 82 degree-days after fertilisation the halibut larva emerges from the egg when the ring of hatching enzymes has broken down the zona radiata in a circle around the region of the larval head (Pittman *et al*., 1990b; Mangor-Jensen *et al*., 1998a). This newly hatched larva is about 7mm long. It is not pigmented and has a non-functional mouth and eyes. The yolk sac stage in the halibut is long and it lasts for about 265 degree-days (50 days at 5.3^{o}C) (Blaxter *et al*., 1983). Following this stage the halibut metamorphose from a round shape to a horizontal flatfish shape.

This is a very complex stage of development during which the body twists and the left eye migrates to the right side of the fish while the upper body becomes gradually pigmented. This brown pigment gives the halibut some camouflage and a degree of UV protection (Arthur, 1999). Schmidt (1904) described the presence of minute orange-red chromatophores scattered over the head and abdominal region of the juveniles (Haug, 1990). The transition from pelagic to bottom life probably takes place at a length of 34-40mm and in the wild young halibut are localised in well-defined nursery grounds in coastal areas 20-60m deep with a sandy bottom (Haug, 1990). They remain in these grounds for 4-6 years after which a period of migration occurs and the cycle begins again when the fish mature (Arthur, 1999).

1.2.2 Domestication and Culture of the Atlantic Halibut

The first two metamorphosed fry were produced in 1980 at the Flødevigen Research station in Norway by Øiestad and Haugen from gametes acquired by stripping net-caught mature adults (Mangor-Jensen *et al*., 1998a). This breakthrough sparked a large-scale research programme into halibut production in Norway joined by smaller programmes in the UK.

In 1983 cultivation work began in the UK, comprising a small demonstration project based at Sea Fish Industry Authority's Marine Farming Unit, Ardtoe. Initial fish populations were established from the wild capture of individuals from Shetland, Faroese and Icelandic waters. Commercial hatchery development began six years later in 1989 accompanied by a larger applied research programme (Shields *et al*., 1999). The commercial hatcheries were established by small independent companies making use of sites with existing seawater facilities and staff experienced in rearing turbot and salmon provided the initial nucleus of the UK workforce. The basic husbandry

procedures for halibut rearing were developed during the initial collection programmes of the 1980s, however refinements to handling methods and holding conditions were constantly being made from the results of research on reproductive physiology and broodstock environmental requirements (Mangor-Jensen *et al*., 1998a). An overview of halibut production cycle is shown in Figure 1.2. Rearing techniques and production in the UK, based on observations at the largest halibut hatchery in the UK, Otter Ferry Seafish, are outlined below.

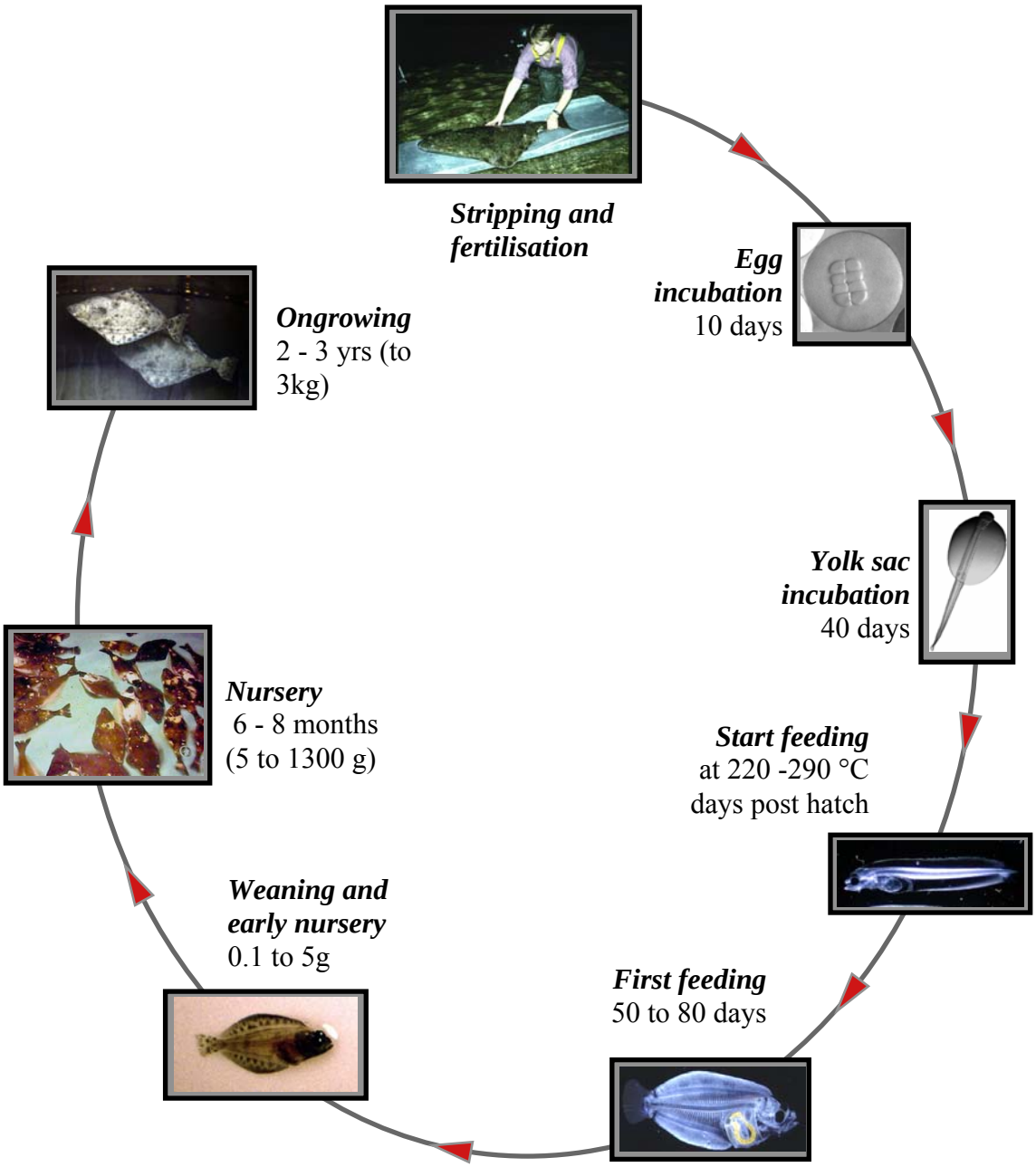

Figure 1.2 The production cycle of the Atlantic halibut with photographs of each life stage. Approximate times and/or average sizes for each stage are given. Pictures not to scale (Presented with permission from Brown, 1998).

1.2.2.1 Broodstock maintenance

Broodstock are held in large covered circular tanks ranging in diameter from 3.5-10m and depths of 1-2m. Sex ratios are usually maintained at 1-2 females per male. Good stable water quality is maintained by pumping ashore water from a deep-sea water supply. Salinity values of 33-34% are regarded as optimal; flow rates and water exchange rates should be as high as possible, however they are limited by the capacity of water chilling systems. Two exchanges per day appears to be sufficient in some systems (Shields *et al*., 1999).

Light appears to be important for growth and maturation in the Atlantic halibut (Simensen *et al*., 2000; Smith *et al*., 1991) and low intensity artificial lights are used in broodstock tanks. Results from experiments conducted by Smith *et al*. (1991) showed that manipulation of photoperiod resulted in changes of spawning time. Thus lighting is artificially controlled with 12-month out-of-phase photoperiods applied to different broodstock populations on the farm facilitating an all year round supply of eggs. Broodstock diets are prepared by wetting a formulated meal (TROUW, UK) with fish oil and water which is then extruded into a sausage. This sausage meal is presented to the fish three times a week. Feeding behaviour varies throughout the year with a minimum feeding response during the spawning season.

1.2.2.2 Stripping and fertilisation

Spawning performance has been shown to vary according to water temperature. Brown *et al*. (1995) showed that fish kept at 6^{o}C during the spawning season had improved fecundity and egg viability compared with those held at ambient water temperature. Fertilisation and hatch rate fell dramatically once water temperature exceeded 8^{o}C. Chilling broodstock water supplies is now standard practice in halibut

hatcheries. Two months before stripping the water temperature in the tank is lowered to 8°C and one month prior to stripping the water temperature is lowered to 6°C. Out of the spawning season stocks are maintained at ambient in order to retain a good feeding response. The requirement to provide chilling adds significantly to hatchery installation and running costs, particularly where broodstock are manipulated to spawn beyond the natural winter season.

Impending spawning time can be estimated from the visible abdominal swelling of the females, an indication that their ovaries are full of eggs. Although domesticated halibut are able to release eggs naturally in holding tanks and fertilisation rates of up to 90% have been reported (Holmefjord and Lein, 1990), the general method of gamete collection is by stripping. Stripping is a process where fish are guided onto a table and pressure is applied to the abdomen to release eggs/milt. Eggs and milt have to be stripped without coming into contact with water as salt water activates sperm motility and hardens the eggs.

Stripping is the preferred method of gamete collection because it is difficult to obtain fertilised eggs from natural spawning in tanks. However, the timing of stripping is very important because it affects the quantity and quality of eggs as well as their fertilisation rates (Norberg *et al*., 1991; Basavaraja, 1991; Holmefjord, 1991). Egg viability is time restricted and eggs must be stripped and fertilised within six hours of ovulation at 6-7°C (Bromage *et al*., 1991). Therefore the ovulatory cycle of each female has to be accurately estimated before stripping is attempted. Ovulatory rhythms seem to be more or less constant for individual females over the whole spawning season (Kjørsvik and Holmefjord, 1995), however this can be disturbed by changes in water temperature and handling stress (Norberg *et al*., 1991). Egg retention

within the ovarian lumen has also been seen to be a problem, causing blockages when this material begins to degenerate inside the fish. Blocked females can be injected intramuscularly with antibiotics which induces the release of egg debris within three days (Bromage *et al*., 2000).

After collection gametes are transferred in the dark, because light affects the osmolarity of the eggs (Mangor-Jensen and Waiwood 1995) to be incubated. 1ml of milt is stirred into 3ml of sea water (chilled and U.V treated) to activate sperm motility. Once the milt is checked for motility the solution is immediately added to the volume of stripped eggs in a bucket. The eggs are left to water harden for thirty minutes. After the water-hardening period all excess fluid is poured off and the fertilised eggs are poured into an incubator (egg conical). A sample of the fertilised eggs from each bucket is collected in a 250ml beaker; the volume is topped up with chilled water and kept overnight. After 24 hours each sample is checked for fertilisation rate. This is done by counting the number of eggs showing symmetric division at the 4 or 16 cell stage under a microscope, as described by Shields *et al*. (1997). It is not unusual, in fact it is common practice, to keep the eggs in their buckets overnight until the fertilisation rate is checked before incubating them. Samples that have less than 60% fertilisation will be discarded. These are always recorded. The volume of eggs that go into a conical (incubator) is called a "BATCH".

Towards the end of the spawning season male and female gamete production becomes unsynchronised, in that milt becomes viscous and difficult to express with poor fertilisation properties while females continue to produce good quality eggs. Vermeirssen *et al*. (2004) describe a method of implanting Gonadotrophin-releasing

hormone antagonist (GnRHa) into male muscle tissue, resulting in increased fluidity of the milt.

1.2.2.3 Egg room

Traditional down-welling incubators used for turbot eggs are unsuitable for halibut eggs because of their high density and so egg incubation is carried out in up-welling cylindro-conical tanks with volumes of 450 litres. Mangor-Jensen *et al*. (1998b) described the design and operation of halibut egg incubators. The architecture of these incubators is such that they mimic the conditions found in the wild. Strict temperature control is important, salinity must be in a narrow range and maintenance of good water quality is required as sub-optimal conditions can cause developmental abnormalities and / or mortalities (Pittman *et al*., 1990a). Light also has a direct effect on egg buoyancy as it causes an increase in osmotic water loss thereby increasing overall density. Total darkness is maintained in hatcheries because of the increased ease of handling low-density buoyant eggs (Mangor-Jensen and Waiwood, 1995; Mangor-Jensen *et al*., 1998a)

The incubation water is filtered to 5 μm and UV-sterilised. Temperature is maintained between 5-6^{o}C. Due to the high cost of ensuring a supply of seawater at this temperature it is recycled without detriment to egg survival rates. Eggs are maintained in the water column using an up-welling flow of 1-4 litres per min with stocking densities of 200-1000 eggs per litre. Dead eggs are removed daily in order to prevent bacterial activity. This is done by means of a hypersaline (40 ppm) salt plug. This procedure was described by Jelmert and Rabben (1987: as cited by Bromage *et al*., 2000; Shields *et al*.,1999 ; Mangor-Jensen *et al*., 1998a). It relies on the fact that dead eggs are non-buoyant and will sink rapidly through a salt plug whereas live eggs do not pass into this layer and rest on the pychnocline. In order to reduce bacterial

infection, every three days each incubator is disinfected using peroxyacetic acid. The eggs are kept in the egg room for 10 days or up to 65 degree-days. At 65 degree-days eggs are gently siphoned from their incubators. They can be successfully transported without causing any damage at this stage (Holmefjord and Bolla, 1988). The eggs are then disinfected in peroxyacetic acid at 250:1 concentration for one minute to prevent carry over of bacterial contamination, after Harboe *et al*. (1994). They are then transferred to the yolk sac room. The volume of eggs transferred is measured and recorded. Generally this reflects the fertilisation rate (usually 5% lower than the fertilisation rate). At this stage to justify the larger incubators yolk sac batches, may be mixed.

1.2.2.4 Yolksac

Hatching occurs 16-19 days post-fertilisation at 5^{o}C (Kjørsvik and Holmefjord, 1995), however it is influenced by external factors such as oxygen, turbulence and light (Helvik and Walther, 1993). Light arrests the hatching of halibut eggs and hatching can be synchronised by applying light regimes (Bromage *et al*., 2000; Kjørsvik and Holmefjord, 1995). The yolk sac stage in the halibut lasts for 265 degree-days (Opstad *et al*., 1998). It is very long compared to other cultured species and this developmental phase has represented a major bottleneck in the establishment of a reliable production system. Intensive culture systems routinely exhibit elevated and highly variable mortality, up to 50% (Opstad *et al*., 1998; Shields, *et al*., 1999), and a large fraction of larvae develop deformities. There is a high frequency of jaw deformity which is thought to be related to abrasion of the head by contact with the rearing container followed by the penetration of eroded tissue by bacterial and fungal organisms present in the water (Morrison and MacDonald, 1995). The fragile yolk sac larvae are very sensitive to both physical and microbial conditions and therefore

must be handled with care (Opstad *et al*., 1998; Rabben *et al*., 1986). During this period larvae are held in conditions similar to those in the egg room. Large conical (silo) shaped flow through systems are used with volumes of 1750 litres. Water temperature is maintained at 5-6^{o}C for reasons similar to those in the egg room (Lein *et al*., 1997); temperature also appears to affect muscle growth in yolk sac larvae (Galloway *et al*., 1999). Stocking densities of 10–20 larvae per litre are generally used. Water flow rates are relatively low, in the order of 1-2 litres per min, as this has been shown to be negatively correlated with larval survival (Opstad *et al*., 1998). After hatch the dead material is removed daily by opening a valve at the bottom of the conicals and allowing 4 litres of water to pour out. An estimate of the dead material components is recorded daily (eggs, shells, larvae).

Soon after 150 degree-days post hatch substantial losses have been known to occur. In response to this phenomenon UK operators have adopted an early transfer procedure that involves stocking the yolk sac larvae into feeding tanks before 200 degree days post hatch (Shields *et al*., 1999).

1.2.2.5 First feeding

At 150 degree-days the larvae are attracted to the surface using a torchlight and transferred to tanks containing similar temperatures and treatment to the conicals. First feeding tank design was described by Harboe *et al*. (1998). 4 metre tanks are used. They are covered and the water is chilled until the fish are 215 degree-days old. Unlike the other developmental stages the first-feeding yolk sac fry require light to feed. The photoperiod is 24 hr continuous light regime (Bromage *et al*., 2000). At 215 degree-days the covers are taken off and live algae added to the water to make it turbid. The presence of algae has been shown to improve growth and survival as well as having a positive effect on feeding frequency (Gulbrandsen *et al*., 1996; Nass *et al*.,

1991). Algae seem to help with the perception of light by the larvae and the development of normal patterns of feeding behaviour. Ambient water is added to the tanks at 1^{o}C per day until ambient temperature is reached. At this stage the fish are still absorbing their yolk sac.

First feeding starts at 240-250 degree-days. A major constraint in the commercial production of the halibut has been the onset of exogenous feeding. The rate of initial feed uptake is low with typically only 30% of the larval population accepting live diet. Like several marine fish species the halibut requires live foods at this stage. This low uptake represents the main bottleneck in the hatchery process for UK operators (Shields *et al*., 1999). *Artemia* and copepods form the basis of larval diets in most cases.

Initial rearing attempts used the rotifer/*Artemia* systems developed for the turbot but they resulted in high mortality of larvae (Shields *et al*., 1999). Presenting *Artemia* only at the onset of exogenous feeding gave positive results with initial rapid feed intake and growth rate (Næss *et al*., 1995; Næss and Lie, 1998). However, over the course of the 60-day live feed period the fish developed problems with malpigmentation, failure of eye migration and poor growth. The reason for this is thought to be due to the absence or shortage of certain essential nutrients, especially the polyunsaturated acids (Næss *et al*., 1995). Halibut larvae have a high requirement for decosahexaenoic acid, 22:6 n-3, (DHA), and the supply of DHA through *Artemia* is a major problem because of the high DHA catabolism of Artemia (Evjemo, 2001; Olsen *et al*., 1999). *Artemia* has been shown to rapidly metabolise DHA for energy production (Evjemo *et al*., 1997). In order to prevent the problems associated with nutritional deficiencies of *Artemia*, most halibut producers use large amounts of

copepods supplemented with *Artemia*. Copepods are known to be nutritionally superior to *Artemia* (Evjemo, 2001), however it is difficult to secure a reliable supply of the organisms and there are concerns about pathogen and parasite transmission because they are grown in open systems.

The juveniles are fed *Artemia* and copepods daily while keeping high levels of algae at the same time. The age of the juveniles determines the type of live feed they are presented with in Otter Ferry. At 230-350 degree-days they are fed copepod nauplii; at 350-450 degree-days they are fed juvenile copepods and post 450 degree-days they are fed copepod adults. All the time they are supplemented with *Artemia*. However, there is a lot of variation in the quality of live feed (copepods) produced and this affects the performance of the juveniles. Consequently there has been a significant research effort into manipulating and enriching the nutritional composition of *Artemia* particularly with respect to essential fatty acids. Gara *et al*. (1998) demonstrated a method of achieving normally metamorphosed fry using enriched *Artemia* as the exclusive food source. In their study up to 60% of fry exhibited excellent metamorphosis characteristics. Næss *et al*. (1995) and Næss and Lie (1998) found that normally metamorphosed fry can be produced using a straightforward enriched *Artemia* diet provided that copepods are supplied during part of the larval phase where pigmentation is thought to be determined. This larval phase, called a "copepod window", was found to be after 19 days of feeding or after the larvae are 16 mm long.

The period of development up to weaning from first feeding is often problematic. The energy demand of the larvae is very high in order to achieve good growth. In this phase of the production cycle heavy demands are placed on the hatchery in terms of labour, facilities, live feed and enriched media (Brown, 1998). Most mortalities occur

during the initial phases of first feeding and after 350 degree-days losses are minimal, however morphological abnormalities are often encountered, even when they are fed copepods. Failure of eye migration, elongated body shape, malpigmentation, abnormal jaws, fins and lateral line canals, are examples (Pittman *et al*., 1998). By 600-700 degree-days the fish are fully metamorphosed and they start to settle on the bottom of the tanks (Bromage *et al*., 2000). The quality of the juveniles is assessed in this stage by the behaviour of the fish. If they settle at the bottom when they are 650 degree-days old they are considered "good". At this stage a report is made on the behaviour and appearance of the batch this is recorded.

1.2.2.6 Weaning

When the fish are 650 degree-days old, which is about 3 months, they are transferred to weaning systems. Poor batches will be held back and may be mixed. Approximately 15,000-25,000 fish are put into a weaning tank. The tanks are brushed daily. Initially the juveniles are fed *Artemia* in the evening and artificial diets are presented ad libitum all day long. The tanks are treated with formalin every three weeks and mortalities are recorded daily. Average weight in each tank is measured and recorded every 2 weeks from 1g. At 2g the juveniles are well established and they are graded to prevent problems of cannibalism. Growth rate at this stage is significantly influenced by temperature and photoperiod (Jonassen *et al*., 1999; Jonassen *et al*., 2000a). Optimum conditions were found to be water temperature of between 12 ^{o}C and 15^{o}C under continuous light.

1.2.2.7 Grading & Nursery

Fish are graded at 1500-2000 degree days post hatch. The fish in all tanks are standardised according to four grades: Large, Medium, Small, Runt. Pigmentation and metamorphosis are recorded. At 5g, approximately 5 months old, the fish are

graded once again and at 100-150 g they are ready to be dispatched to growout systems.

1.2.2.8 Growout

The on growing stage of production is usually done by salmon producers who wish to diversify their production. Once weaned, the halibut is a robust animal and the growout phase is done in either land-based tanks (Adoff *et al*., 1993) or in sea cages (Martinez Cordero *et al*., 1994). Difficulties with cage design are often encountered because of the bottom dwelling habit of the fish and mortalities resulting from excessive current speeds, high water temperatures and sunburn are common (Bromage *et al*., 2000; Brown, 2002). Surface cages are the most commonly used and they are constructed from a variety of materials. Cages are generally 3-7 m deep and net bases are fabricated from 6–15mm netting. Predator netting is essential and problems associated with fat cell necrosis following sunburn are avoided by the use of shade netting (Brown, 2002). In both land-based and sea cage ongrowing systems shelving is used to increase the available substrate area to enhance the carrying capacity of facilities.

In the ongrowing facilities halibut exhibit good growth in temperate waters and mean growth rates of 3.2 Kg/yr and 1.4 Kg/yr have been reported for females and males respectively (Björnsson, 1995). As observed in the wild by Jákupsstovu and Haug (1988) and Haug and Tjemsland (1986), males also tend to grow slower than females in captivity. They mature at a smaller size and earlier age. Björnsson (1995) observed that males matured at an average weight on 3.2 Kg while females matured at 12.7 Kg. However, there tended to be variation in the age at which males matured. Growth rate was seasonally dependent and the fish grew slower between the months

of December and March (Haug *et al*., 1989). This was thought to be as a result of photostimulation.

From the industry perspective, eliminating the effect of early sexual maturity in males by the production of all female stocks would undoubtedly have significant economic benefits. Triploidy has been successfully induced in halibut using temperature cold shocks (Holmefjord and Refstie, 1997) and pressure shocks (Brown, 1998). Hendry *et al*. (2003) reported the successful feminisation of males using sex steroids. Though it is questionable whether such fish would be suitable for commercial culture in the European Union due to legislation, it allows the investigation into the possibilities of creating "neomales" (hormonally masculinised females) for the production of all-female offspring for commercial ongrowing. The use of photoperiod in order to control sexual maturation in male halibut is well documented (Björnsson *et al*., 1998; Norberg *et al*., 2001). However the effects of photoperiod in delaying maturity were not conclusive.

1.3 Closing the production cycle and broodstock replacement (selective breeding)

Despite the strong interest and significant investment in research the growth of the Atlantic halibut industry has been slow. Survival through the egg and yolk-sac incubation periods and the times to first feeding and metamorphosis up to weaning has been poor, unpredictable and disappointing. Historically survival rates have been 0.5-2% from egg incubation to weaning (D. Patterson pers comm). Consequently there has been only a limited growout of the fish and therefore small quantities of farmed product have reached the market. Commercially the high costs of running a hatchery that requires strict environmental controls and intensive labour increases the investment risk and the initial enthusiasm and sharing of experience has been tempered by the need to recoup investments. Notwithstanding, there is an urgent need within the industry to close the production cycle (Figure 1.2) and identify individuals from within the first generation farmed population to replace the ageing wild caught broodstock.

1.3.1 Selective Breeding and Aquaculture

A breeding program is often considered after other problems associated with production have been solved. On the contrary, Shultz (1986) argues that "Genetics begins at the beginning". This is because success in any aquaculture enterprise depends on organisms genetically suited for the production systems. Moreover in any production system some genotypes will make success come easy while others will make the job difficult if not impossible.

In agricultural and livestock production systems the infrastructure exists, and is utilized, for the farmer to replace his parental generation. This is done frequently and efficiently to maintain and often improve average yields. This replacement with

better performing parents was made possible by the application of principles of animal breeding. The application of animal breeding theory to the genetic improvement of breeds has been the primary method for increasing production in farmed populations, consequently agriculture productivity of mammals and birds is often at least 3-5 times higher than that of their wild progenitors (Bentsen and Gjerde, 1994; Knibb, 2000).

Until recently selective breeding has rarely been practiced in aquaculture and Gjedrem (1997) estimated that only about 2% of the world fish and shellfish stocks are genetically improved. This contrasts immensely to animal production where the world supply of animal protein is mostly dependent on the genetically enhanced farmed species. Most aquaculture production relies completely on wild broodstock, and the majority of the genetic material used in fish farming has not been definitely separated from the wild populations to form specialised domesticated breeds, e.g. several marine populations are refreshed by introducing wild spawners (Bentsen, 1990; Bentsen and Gjerde, 1994; Gjedrem, 2000).

This slow application of selective breeding mirrors the general trend in the development of aquaculture as a science in that it was or rather has been limited by technology and an understanding of the biology of the species of interest. The genotype-phenotype relationships in fish are different from those of other vertebrates and the fact that fish live in water makes it difficult to observe them and to obtain estimates of the basic genetic parameters needed for planning breeding schemes (Allendorf *et al.*, 1987). In order to develop breeding programmes the reproductive biology of the species must be understood and their production cycles closed. The inability to control mating and reproduction were identified as significant hindrances

to the development of breeding programmes in aquaculture (Bentsen and Gjerde, 1994; Bentsen and Olesen, 2002). Furthermore breeders were faced with the problem of choice. Compared with other vertebrates breeders were presented with a very wide range of inter and intra specific variation from which to select. This contrasts to livestock production where many of the progenitors of chickens, cattle and pigs no longer existed at the onset of selective breeding as a significant science within the field of agriculture. Finally early breeding experiments showed a lack of, or variable, selection response and formed a school of thought that there was little or no additive genetic variance in fish (Gjedrem, 1998).

1.3.2 Genetic Improvement

Genetic improvement is the process of selecting animals of higher genetic merit than average, to be the parents of the next generation, such that the average genetic merit of their progeny will be higher than the average of the parental generation (Cameron, 1997). The process of genetic improvement begins with a breeding goal. This reflects the ultimate aim of the breeding programme and concerns the decision of which trait(s) to select for or improve the selection objective. Once the selection objectives have been defined the second step is to evaluate the available breeding candidates for genetic merit.

The best measurement(s) on the animal that reflects the trait(s) of interest, the selection criteria, are determined. In animal production one of the most important selection objectives is improved growth rate. The selection criterion is often live weight at a certain age. In beef production for example birth weight and 200-day weight are measured and the growth rate is then calculated. Often it is not possible to measure desired traits directly on the selection candidates, for example reproductive

performance in a young animal. Selection must therefore be made on the basis of either information from relatives or from correlated measurements that can be made on the live animal. The next step is to develop a system of testing the candidates in order to determine which individuals from within the population perform significantly better than average. The selection regimes, the set of conditions under which animal are tested and how they are tested in a particular programme are then fashioned to suit the selection criteria. Having obtained measurements on the animals this information is used to predict their genetic merit or their breeding values. The breeding value of an animal is a measure of its genetic superiority or inferiority relative to the population to which it belongs (Gall, 1990). It cannot be measured directly but it is predicted from the measurements made on the animal and or that of its relatives.

The whole focus of a breeding programme is to identify animals with high genetic merit that can be used to produce the next generation; the processes outlined above are usually conducted within the nucleus of a breeding company. The final stage of a breeding programme is to disseminate the genetic material of superior animals to the farmer, because improvement is only worthwhile if it is of benefit to the producer and the consumer. Depending on the species this is usually done through 2-4 tiers of a pyramid of multipliers, where the genetic material is amplified to meet the numbers required by the producers.

Selective breeding in aquaculture is a relatively new practice; most breeders within the field were trained in agriculture and resisting the temptation to reinvent the wheel based their programmes on traditional poultry and pig breeding schemes. The first breeding programmes were established in Norway for salmon and trout in 1971

(Refstie, 1990) and they formed the template on which subsequent programmes were fashioned.

Fish and shellfish present geneticists with several advantages over other livestock species (chickens, pigs and cattle), mainly due to their reproductive biology. Firstly compared to terrestrial animals they are highly fecund. Even tilapias (*Oreochromis spp.*) that are infamous in aquaculture for having low fecundity (Little *et al*., 1993) can produce up to 2,400 eggs in a single spawning (Campos-Mendoza *et al*., 2004). Secondly, fertilisation is external and in many species it presents the possibility to obtain a wide variety of family group designs. A large number of maternal and/or paternal half sib groups may be produced in hierarchical or factorial designs by stripping and collecting eggs and milt (Bentsen and Gjerde, 1994; Gjerde and Rye 1998). To the animal breeder, the advantage that aquatic organisms possess over farmed terrestrial animals is demonstrated in the calculation of response to selection, R.

$R = i\, h^2\, \sigma_p$

Where:
i= selection intensity
h^2= heritability
σ_p = phenotypic standard deviation

The response to selection, R, is the difference between the mean performance of the progeny and parental generations (Cameron, 1997). The high fecundity of fish allows higher genetic gains to be obtained through high selection intensities (within and between families). Also fish typically exhibit higher levels of variation both within and between populations than other vertebrates (Allendorf *et al*., 1987; Gjedrem, 1998; Kinghorn, 1983; Friars and Bailey, 1990). However, Allendorf *et al*. (1987)

suggest that the larger phenotypic variation observed in fish species is not necessarily associated with greater genetic variability but highlights the greater susceptibility of fish to environmental factors, a fact that seems obvious because they are poikilothermic. This contrasts with conclusions by Bentsen and Gjerde (1994) who argue that the differences are indeed genetic. Nonetheless, from the equation above, provided that the heritability is moderate, higher levels of variation will result in a greater response to selection. It should be noted though that because aquatic animals are highly fecund only a small number of animals might be used as parents for subsequent generations. This could result in a high rate of inbreeding and have deleterious effects on the breeding programme, a point that will be discussed later.

The extremely high fecundity in fish species ensures that the genetic gains achieved in the nucleus have an extensive and immediate impact on the industry with minimum time and money spent in the dissemination process. In developed aquaculture breeding systems the genetic lag, the number of years of selection that a production animal is behind an elite animal in the nucleus of a breeding programme, is often comparable with the generation interval. Large family sizes negate the need to amplify the genetic material for the industry and so genetic improvement reaches the producers with a minimum delay through zero, or one level of multipliers (Gjerde *et al*., 2002).

Finally fish species have been proven to be more tolerant than higher animals to manipulations of the reproductive and early developmental processes. Viable and often fertile hybrids between related species are well known; this increases the range of combinations that may be tested for commercial hybrid production (Bakos, 1994). Induced triploidy may be applied to prevent sexual maturation. Gynogenesis (all

maternal inheritance) and androgenesis (all paternal inheritance) may be used for rapid development of inbred lines. Phenotypic sex reversal may be induced in both sexes to produce monosex offspring, as these are highly relevant in the culture of several species that show large sexual differences in productivity (Mair *et al*., 1995; Beardmore *et al*., 2001).

1.3.3 Selective Breeding in Aquaculture

The breeding goals in aquaculture must be defined for each species and each population due to the diverse range that exists. However, historically most fish breeding programmes start with improved growth rate, defined by weight at harvest as the selection criterion (Refstie, 1990; Knibb, 2000) and then other traits are included in subsequent generations. In general all economically important traits should be included in the breeding goal and Gjedrem (2000) outlined the traits most commonly selected for. They include feed conversion efficiency, disease resistance, survival, flesh quality, fecundity and age at sexual maturation.

In order for selection to be successful genetic variation must exist within the population for the trait(s) of interest. If there is no genetic variation between animals for the traits of interest, there can be no improvement as there will be no genetically superior animals to select.

The phenotypic (physical) differences (variation) observed between individuals are due to genetic and environmental influences:

$V_P = V_G + V_E + V_{GE}$

Where:

V_G= Variation due to the action of genes

V_E= Variation due to environmental factors

V_{GE}= Variation due to the interaction of genetic and environmental factors

V_P= Total phenotypic variation

Genetic variation, differences due to the action of genes, is caused by segregation of genes and differences in allele frequency. It can be further divided into three components, each due to different types of gene action. These are additive, dominance and epistasis (interaction)

$V_G = V_A + V_D + V_I$

Where

V_G= Variation due to the action of genes

V_A= Variation due to the additive effect of genes

V_D= Variation due to the action of dominance

V_I= Variation due to the interaction of genes (epistasis)

In selective breeding probably the most important component of genotypic variation is that due to the additive effect of genes. This is because most traits of economic value, breeding objectives, are inherited quantitatively due to the action of additive effects of genes (Falconer and Mackay, 1996). Consequently a first step for many fish selection projects is to estimate heritabilities for economic traits (Knibb, 2000).

The heritability (h^2) of a trait is the proportion of the total phenotypic variation of that trait among individuals in a population attributable to the additive effect of genes. It provides an estimate of the degree to which differences between animals are repeated in their progeny and it tells us to what extent the differences we observe in animal performance are due to genetics (Falconer and Mackay, 1996). Some estimates of heritabilities of economically important traits are given in Table 1.1 below.

In their reviews of the literature, Toro and López-Fanjul (1998) and Gjedrem (2000) concluded that most heritability estimates for body weight were intermediate (0.2-0.3). These estimates are similar to those established in traditional farmed terrestrial

species and therefore the prospects for improving productivity in aquaculture by selective improvement are very good. Disease resistance when measured by challenge tests also showed high heritability, however, estimates for survival were low. Feed conversion efficiency is a difficult and expensive trait to measure, thus with the present technology it is recommended that the trait should not be measured in a breeding programme (Gjerde *et al*., 2002).

Table 1.1 Heritability estimates of some economically important traits in aquaculture.

Species	Trait	Heritability Estimate	Reference
Rainbow trout	Body weight	0.35±0.30	Henryon et al. (2002)
Rainbow trout	Body length	0.53±0.27	Henryon et al. (2002)
Rainbow trout	Disease resistance	0.13	Henryon et al. (2002)
Rainbow trout	Body weight	0.41	Gjerde and Schaeffer (1989)
Rainbow trout	Flesh colour	0.29	Gjerde and Schaeffer (1989)
Rainbow trout	Spawning time	0.66	Quinton et al. (2002)
Rainbow trout	Carcass yield	0.50	Chevassus et al. (2002)
Atlantic salmon	Body weight	0.22-0.34	Rye and Mao (1998)
Atlantic salmon	Body weight	0.24	Gjerde et al. (1994)
Atlantic salmon	Growth rate	0.04-0.26	Gjerde et al. (1994)
Atlantic salmon	Sexual maturity	0.34	Gjerde et al. (1994)
Coho salmon	Alevin weight	0.52-0.80	Martínez et al. (1999)
Marron	Growth rate	0.35	Henryon et al. (1999)
Marron	Survival (eggs)	0.02	Henryon at al. (1999)
White shrimp	Harvest weight	0.70	Argue et al. (2002)
Tilapia	Body weight	0.55	Bolivar and Newkirk (2002)
Catfish	Body weight	0.27-0.62	Bondari (1986)
Turbot	Body weight	0.70	Gjerde et al. (1997)

Simm (1998) outlined the three main strategies that have been used for the genetic improvement of livestock. These are:

a) Selection between strains: substituting one strain for another that is recognised to have a better performance under local conditions.

b) Selection within strains: choosing better parents within a particular strain to improve performance.

c) Crossbreeding: mating parents of two or more complementary strains or species together that will give a lift in performance through hybrid vigour.

In aquaculture it is common practice to use a combination of at least two methods for genetic improvement. Usually selection between strains precedes either selection within strains or crossbreeding. Due to the amount of variation that exists between

captive and wild strains for many species, all breeding programmes should start with the collection, comparison and selection of the best genetic material available (Refstie, 1990; Dunham and Liu, 2003). Choosing the best strains at the start of a breeding programme could equal the genetic gains made by years of within-strain selection using inferior strains (Kinghorn, 1983; Knibb, 2000). The Norwegian national salmon breeding programme began with the testing of 40 different strains (Gjøen and Bentsen, 1997). The GIFT (genetically improved farmed tilapia) project started with eight different strains of tilapia and comparison tests showed that some wild stocks performed better than farmed stocks in culture (Eknath *et al*., 1993). The choice of multiple lines at the start of a breeding programme not only allows the testing of various strains but also develops the platform for a founding population with a broad genetic base, the base population. If this base population is made up of different strains as seen in the establishment of the GIFT tilapia it is referred to as a synthetic population.

1.3.3.1 Selection within strains

When selecting within strains the aim is to increase the frequencies of the favourable genes controlling the desired trait(s). The choice of animals to breed from may be based solely on their appearance or on a subjective assessment of their own, or their relatives' performance (Simm, 1998). Methods used for selection depend on several factors based on the nature of the trait(s). The heritability of the trait(s), whether the trait is normally distributed or binary and if records can be obtained on live individuals are considered to be some of the most important factors. Due to the high fecundity in aquaculture species the methods used are individual selection (mass selection), family selection or a combination of the two (combined selection) (Gjerde and Rye, 1998). When the heritability of the trait is approximately 0.5 both family

and individual selection are equally efficient. When the heritability is lower, family selection is more efficient and when it is higher individual selection is a more efficient method of selection. For binary traits such as age at sexual maturity and survival, family selection should be chosen (Gjedrem, 2000).

The most common system in aquaculture is mass selection, where breeding stocks are chosen on the basis of individual performance (Toro and López-Fanjul, 1998; Lymbery, 2000). This method is widely used, probably because it is simple to apply and has the advantage that individuals reared from different families can be mixed in the same environment just after fertilisation. It is however limited in that it can only be applied for traits that can be recorded on live breeding candidates. This method is thus difficult to practice for carcass quality traits and will also be inefficient for binary traits like age at sexual maturity and survival. It is of paramount importance when applying mass selection that the environmental influences are kept the same for all individuals that are being compared at any stage of the life cycle. To obtain the most equal environmental conditions possible and prevent bias, all individuals should be hatched on the same day or within a few days of each other and thereafter reared under identical conditions. Individual selection requires substantial precautions in order to avoid inbreeding, which are often not taken or have been uncontrollable until recently.

When traits such as carcass quality that cannot be measured on a live animal are included in the breeding objective, family selection may be successfully applied using information from relatives. Selection is then made either within-families, between-families or a combination of the two. Family identification must, however, be maintained either by tagging or stocking in separate units. The emphasis on

developing family selection also reflects concerns over inbreeding that may arise through mass selection (Lymbery, 2000).

Within-family selection is simply individual selection applied within family groups, resulting in each family contributing the best individual(s) from that group. Between-family selection on the other hand is the method in which family groups are ranked according to the mean performance of each family and whole families are rejected or selected as units.

Between-family selection is efficient because random environmental effects that affect individuals will tend to cancel each other out when the family mean is calculated. It is especially important when environmental deviations constitute a large part of the phenotypic variance i.e. when the heritability is low (Gjerde, 1993). Because the selection is among whole families, the selection differential is a function of the differences among families, not differences among individuals. Therefore to obtain an acceptable rate of genetic gain and to keep inbreeding low the number of family groups tested must be high (>100) (Gjerde and Rye, 1998). It should be noted however that environmental variation common to members of the same family can mask the true genetic potential and impair the efficiency of family selection. If common environmental effects are large they can conceal the true genetic differences between families and prevent the accurate ranking of the families according to their genetic values. As such the environment for all family groups should be standardised as much as possible in order to reduce the common environmental component to a minimum.

A combination of between- and within-family selection will almost always provide the most efficient method of genetic improvement, however it requires individual

identification and pedigree determination (Gjedrem, 1983; Gall, 1990). By combining between- family and within-family selection, the additive genetic variance both between and within families will be utilised in an optimal way. Families may be ranked on a continuous scale even for discontinuous traits like mortality and sex-limited traits like fecundity (Bentsen and Gjerde, 1994).

1.3.3.2 Crossbreeding

The objective in crossbreeding is to exploit non-additive genetic variance (V_D). The resulting hybrid vigour is produced by the interaction of alleles at each locus and therefore varies with genotype and is often unpredictable (Clayton and Price, 1994). It requires the maintenance of pure species lines and the degree of differentiation between lines to produce measurable hybrid vigour appears to be important (Beaumont, 1994). Very closely related strains may produce none while more distantly related groups may suffer from outbreeding depression, probably brought about by the break-up of co-adapted gene complexes or other changes in gene architecture (Gharrett *et al*., 1999). Crossbreeding should be considered if non-additive genetic variance exists for traits included in the breeding goal (Refstie, 1990). The hybrid performance should not only exceed the best parent strain, but also all other available purebred stocks. It is however, considered to be a short-term measure as it does not produce any additive genetic improvement over time and there are conflicting data on the importance of heterosis effects in aquaculture species e.g. the common carp (Dunham *et al*., 1990; Eknath *et al*., 1998; Knibb, 2000). It has been suggested that it should be seen as a supplement to a programme for additive genetic improvement and be used at the start of a within strain scheme in order to make new synthetic populations (Gjedrem, 1983; Gjerde, 1993).

1.3.3.3 Breeding programmes and selection experiments in aquaculture

The quintessential example of a selective breeding programme in the literature will probably be the Norwegian Atlantic salmon breeding scheme. A summary of this programme described by Gjøen and Bentsen (1997) is summarised below.

The Norwegian Atlantic salmon breeding programme utilises all the methods of selection outlined above and has been running now of about 30 years. A base population was established by testing strains from 40 different rivers (between strain selection). Due to the fact that little heterosis was found between the strains, pure breeding was chosen as the breeding method for the scheme. However, synthetic lines were made by crossing some of the strains (crossbreeding) in order to avoid inbreeding and secure a broad genetic base. Family selection is used for all traits in the breeding goal, while individual selection is used within families for growth rate (combined selection). The breeding goal started with growth rate and expanded to include other traits as stated above. Their research facility allows the testing of 200 families each year. A hierarchical mating system is used (one male is mated to two or three females) and full sib families are kept separate until they are tagged at about 20g. Electronic PIT (Passive Integrated Transponder) tags are used to identify each fish and the 120 best ranked full sib groups are mixed together to avoid common environmental effects influencing selection. One hundred fish from each full sib group are evenly distributed among three or four private farms to allow testing under ordinary conditions. Growth rate is recorded as body weight at slaughter. Family averages of frequency of early sexual maturation after one year in sea cages and survival rates after challenge tests to infectious salmon anaemia (ISA) and Furunculosis are also recorded. Flesh quality is measured by computerised tomography for fat content and distribution. A Minolta Chroma Meter CR-300 is

used to measure flesh colour. When all records have been collected, a selection index is used to calculate breeding values and the families are ranked according to their breeding value. In the index, each trait is weighted by its variance, heritability and economic value. Males are selected from the 10-15 highest ranking families and females from the top 15-20 families. When the fourth generation of selected fish were compared with their wild progenitors they grew 77% faster, giving a mean genetic gain per generation of greater than 15%. For reduced frequency of early maturation the improvement was estimated at 22% per generation (Gjedrem, 2000). Estimates from 1994 indicated that flesh pigmentation was improved at approximately 8-10% per generation (Gjøen and Bentsen, 1997). In the trout enterprise of the Norwegian National Breeding programme, Gjerde (1986) reported a 26% improvement in growth rate after only 2 generations of selection.

A similar breeding programme was established in Canada for Atlantic salmon in 1984 using a synthetic base population from crosses between fish from seven different rivers (Friars *et al*., 1990). O'Flynn *et al*. (1999) reported significant gains for survival after a BKD challenge test and growth rate in that population. The mean harvest weight of the selected fish was 0.88 Kg higher than the control line after 2 generations of selection, a gain of 0.83 standard deviations. Other salmonid breeding programmes include the coho salmon selective breeding program developed in 1977 by the University of Washington (Hershberger *et al*., 1990). Hershberger at al. (1990) and Myers *et al*. (2001) described how they achieved a 60% improvement in weight after 4 generations of selection using a combined selection technique. The "PROSPER" (a French acronym for Optimised Individual Selection Procedure with Repeated Challenges) breeding programme was developed in France to improve growth rate in Brown trout (Vandeputte *et al*., 2002; Chevassus *et al*., 2003). Using

mass selection with a large population they were able to obtain a mean response to weight of 6.3% per generation in one of their selected lines.

Selective breeding has been successfully practiced in the catfish industry in the USA. The catfish genetic improvement program at Auburn University in Alabama uses an integrated method of genomics, hybridisation and selective breeding to develop superior lines. Mass selection for body weight has resulted in up to a 55% increase after four generations (Dunham and Liu 2003). The interspecific hybrid between the female channel catfish and the male blue catfish is known to show improved growth rate, increased tolerance to low oxygen levels and survival. However, there appears to be a genotype-environmental interaction with rearing conditions (Dunham *et al*., 1990).

Despite early reports that mass selection is ineffective for the improvement of growth in the tilapia (Hulata *et al*., 1986; Huang and Liao, 1990) and evidence of stock genetic deterioration (Eknath *et al*., 1993) the GIFT project was established in 1988. Eknath *et al*. (1998) described the structure of the breeding programme, modelled on the Norwegian salmon programme. Eight different strains from different countries were used to establish a base population. 64 hybrid and pure strain combinations were made and the top performing 25 groups were selected. Using a combined selection technique Bentsen *et al*. (2003) reported an estimated 88% accumulated improvement relative to the base population, about 12-17% per year (Eknath *et al*., 1998). The response to selection in the GIFT project were similar to those obtained by Bolivar and Newkirk (2002) who used a within-family selection method while selecting for growth rate in Nile tilapia. The results of some other selection

experiments and selection responses in breeding programmes are shown in Table1.2 below.

Table 1.2 Results of some selection experiments and selection responses in aquaculture breeding programmes.

Species	Trait	Gain (%)	No. Generations	Selection method	Reference
White Shrimp	Growth rate	21	one	Combined	Argue et al. (2002)
White Shrimp	Disease resistance	18.4	one	Combined	Argue et al. (2002)
Penaid Shrimp	Growth rate	21	five	Mass	Goyard et al. (2002)
Red Sea Bream	Body weight	66.46	eight	Mass	Murata et al. (1996)
Rainbow trout	Body weight	27	two	Combined	Hörstgen-Schwark (1993)
Channel catfish	Body weight	29	three	Mass	Rezk et al. (2003)
Common carp	Growth rate	25	one	Crossbreeding	Bakos (1994)

1.3.3.4 Inbreeding in aquaculture

The long term selection goal for any breeding programme should be sustainable genetic gain for the traits in the breeding goal (Gjøen and Gjerde, 1998). In order for this to be achieved programmes will have to be managed so that the additive genetic variation is maintained in every generation. Avoidance of inbreeding and the use of breeding schemes to avoid inbreeding are critical for the maintenance of genetic variance.

Inbreeding means the mating of individuals that are related to each other by ancestry (Falconer and Mackay, 1996). It results in a reduction in the amount of genetic variation and therefore reduces the response to selection. Even when steps are taken to avoid it some inbreeding in closed populations is inevitable (Kincaid, 1983). In closed populations, such as those in most aquaculture production systems, the

probability that inbreeding will occur depends on the size of the population and when selection is practiced it is increased. This is because related animals have genes in common and their performance or breeding values will be more alike (Simm, 1998).

Any fish production system that results in a limited number of fish being available to produce progeny for broodstock in the next generation can lead to a constriction in the gene pool and thus inbreeding. Kincaid (1983) identified several reasons why this should occur in fish production systems. They include selection of superior performing individuals, use of fish from a small segment of the spawning season and the use of a small number of broodstock because they provide adequate numbers of eggs to meet management requirements.

Aquaculture species are highly fecund and so extremely high selection intensities may be applied in breeding programmes, however this will result in only a few animals being selected as parents for the next generation. For production and commercial purposes this does not pose the problem of few progeny that other livestock production systems have, as only a few paired matings are sufficient to reproduce a population. But this will inevitably result in high rates of inbreeding. The rate of inbreeding will not only be determined by the number of spawners but also by the relationships between them.

Inbreeding is measured by the inbreeding coefficient, F. The inbreeding coefficient is the probability that two alleles at any locus are identical by descent and values range from 0-1. Inbreeding coefficients express the amount of inbreeding that has accumulated from a specific point in the ancestry of a population. Within a specific population the rate of inbreeding is a function of the base population and the population sizes of subsequent generations (Falconer and Mackay, 1996).

Inbreeding not only results in a loss of genetic variation but generally also in a reduction in performance. This decline in performance is known as inbreeding depression. Inbreeding depression is measured as the average performance difference between an inbred population and the base population (Kincaid, 1983). Traits that frequently exhibit inbreeding depression are quantitative traits associated with fitness (reproductive capacity, disease resistance and survival) and physiological efficiency (fry deformities and growth rate). The effects can be explained by the phenotypic expression of increased numbers of masked recessive alleles and reduced frequency of heterozygous loci expressing dominance. Reproductive traits such as egg size and hatchability are doubly sensitive to inbreeding depression. This is because of the negative influences, not only of the inbred genotype of the individual, but also that of its mother as this affects the embryonic developmental stages.

Estimates of the deleterious effects of inbreeding in aquaculture species in the literature are mostly limited to salmonids and often based on a small number of families (Toro and López-Fanjul, 1998; Pante *et al*., 2001). Inbreeding is a particular consideration in rainbow trout due to the length of time they have been domesticated (Lutz, 2002). Gjerde *et al*. (1983) estimated reduced performance of up to 9% on survival of fry with an inbreeding coefficient of 0.25 equivalent to a full-sib mating. Over three successive generations this accumulated to a reduced survival of over 18%. Su *et al*. (1996a) found that for a 10% increase in inbreeding coefficient, spawning age of females was delayed by 0.53% and egg number decreased by 6.10%. Pante *et al*. (2001) studied the effect of inbreeding on harvest weight in rainbow trout and estimated that for a 10% increase in inbreeding coefficient a reduction of between 1% and 5% resulted. In Atlantic salmon, Rye and Mao (1998) reported a 0.6%-2.3% reduction in growth per 10% increase in inbreeding coefficients. Inbreeding

depression has also been documented for growth and reproduction traits in the coho salmon (Myers *et al*., 2001; Gallardo *et al*., 2004)

Inbreeding depression can reduce production considerably and the lack of response to selection in experiments with tilapia by Hulata *et al*. (1986) has been explained by the depletion of genetic variation due to inbreeding (Bentsen, 1990; Gjedrem, 1998). Eknath and Doyle (1990) estimated the rate of inbreeding in 18 polyculture systems in Indian carp and found that inbreeding increased at rates of between 2-17% per year. It should be stressed that steps must be taken to control inbreeding because without formal genetic programmes local industries attempting 'in house' selection with few broodstock inevitably result in severe inbreeding (Knibb, 2000). Kincaid (1983) outlined three approaches to avoid inbreeding: the use of large random mating populations, the use of systemic line crossing schemes to eliminate the mating of close relatives and strain crossing to produce hybrid populations.

When starting a breeding programme establishing a wide genetic base cannot be overemphasised. Synthetic populations have been used to secure genetic variability in several breeding schemes as outlined above but maintaining this variation has been the focus of fish breeders in recent years. Their work has been aimed at either reducing the rates of inbreeding while keeping genetic gains at the same level, or by increasing selection response under a restriction on inbreeding. Simulation studies by Bentsen and Olesen (2002) and Gjerde *et al*. (1996) examined optimum mating designs in mass selection schemes. Bentsen and Olesen (2002) found that to keep inbreeding rates low, at about 1% per generation, a minimum of 50 pairs of breeders should be selected. The number of progeny tested should also be restricted and should be standardised to not less than 30-50 progeny per pair. Testing hierarchical

designs, where each male was mated to more than one female, Gjerde *et al*. (1996) found that in order to keep the rate of inbreeding at 1% per generation the number of matings were 50-100 at a ratio of 1 male: 2 females and when each male was mated to 10 females the numbers rose to 150-250 matings. Results by Bentsen and Olesen (2002) are in agreement with recommendations by Kincaid (1983), who stated that the minimum number of breeding adults for maintaining a random mating broodstock should be at least 50 pairs. Generally, if the rate of inbreeding is to be kept constant, the number of individuals that may be selected per family will be reduced as the number of families in the test decreases. Consequently the selection intensity between families will be reduced but the intensity within families will increase (Bentsen and Gjerde, 1994).

In order to prevent accumulation of inbreeding the number of selected individuals per sib family should be restricted and pooling a restricted number of individuals from each family shortly after or at fertilisation can resolve this. The main requirement of this strategy is that all breeding candidates are individually tagged or branded. In fish external tags or brands may only be applied after a certain growth period because of the small size of fry at hatching. Up until recently one of the biggest problems with applying efficient selective breeding programmes for fish is that newly born individuals are too small to be tagged physically and so family groups need to be reared separately until individuals are large enough to be tagged (Gjerde and Rye, 1998; Villanueva *et al*., 2002). This method of genetic improvement is not only costly; it can induce environmental effects common to members of the same family (Doyle and Herbinger, 1994).

1.3.3.5 Identification in aquaculture breeding schemes

The ability to uniquely identify individuals or families is key to any selective breeding programme. In aquaculture this appears to be more important due to problems associated with inbreeding caused by high fecundity. Emulating livestock breeding schemes where all individuals purposed for selection are tagged at, or shortly after, birth using a variety of external tags probably caused the limitations imposed on the aquaculture breeding schemes. Aquaculture breeding programmes presents a distinctive opportunity to merge various disciplines within the field of genetics: molecular genetics, population genetics and quantitative genetics.

Molecular genetics approaches were first applied to fisheries, and the techniques developed over the years were then applied to aquaculture. In breeding programme applications the interest is in their use as markers or tags. The problem of individual identification of fish can be solved by using nuclear DNA (nDNA) polymorphic markers.

DNA profiling has been used as a tool for reconstructing the pedigree of communally reared aquaculture populations (Herbinger *et al*., 1995). This not only allows the rearing of different families in the same tank from hatching but also provides the necessary pedigree information required to avoid inbreeding. A wide range of genetic markers is available for studying genetic variation, mitochondrial DNA and nuclear DNA, which can either be studied directly or indirectly through the use of proteins (allozymes). At present it can be argued that the most useful markers to assess genetic parentage are microsatellite DNA loci (O'Connell and Wright, 1997). Microsatellites are a class of co-dominant DNA markers, which are inherited in a Mendelian fashion. They are widely dispersed throughout the genome and each locus is characterised by a known sequence (DeWoody and Avise, 2000). These sequences

consist of both unique DNA and repetitive DNA motifs. The repetitive elements consist of tandem repeats of two $(AT)_n$ or four $(GATTA)_n$ nucleotides where n varies between individuals ranging from 5 to 50 (DeWoody and Avis, 2000).The variation in the length of the repetitive section (polymorphisms) can be amplified by Polymerase Chain Reaction (PCR), using primers in the unique flanking sequences, and the individual sizes (alleles) scored exactly on high resolution sequencing gels.

Molecular markers provide the tools to measure variation directly at the DNA level. This allows the impact of selection on the genetic diversity of a farmed population to be monitored and thus enables better tools for managing the breeding population.

1.3.3.6 Selective breeding and the Atlantic halibut

A selective breeding programme is structured around the existing production system of the animals. The production system of the halibut outlined in section 1.3 above brings to light the fact that individual rearing of families with this species is very difficult.

Egg volumes are variable and the cost of maintaining 250 litre egg incubators for a single batch of eggs of less that 1 litre cannot be justified. Therefore several batches (families) are mixed repeatedly as they progress through the system and by the time a batch of fertilised eggs advance to the weaning stage of production they would have been combined with at least seven other batches. Thus the halibut production system as it stands resembles a mass spawning structure of the cod or sea bream, as far as breeding is concerned, especially when the repeat egg batches of females are considered. Due to the fact that the cost of producing halibut juveniles is so high it leaves very little margin for error so the costs of building new hatchery facilities with

smaller egg incubators that facilitate separate family tanks cannot be justified, at least until problems with juvenile production are solved.

In agreement with the statement by Shultz (1986) that genetics begins at the beginning, the application of genetics to the culture of the halibut is imminent. This is because the establishment of breeding stocks was difficult due to the expense involved in capturing individuals, the low abundance of mature individuals in the wild and the time taken for acclimation. Therefore only a small number of wild individuals formed the base population within the U.K industry. Wild fish do not thrive in captivity as they are not accustomed to artificial food and high densities in narrow enclosures (Gjedrem, 1990), therefore only a proportion of the existing broodstock will succeed in breeding and be represented in the next generation. This domestication selection effect has been demonstrated in salmon culture (Cross and King, 1983; Norris *et al*., 1999; Koljonen *et al*., 2002). Also the generation interval in the halibut is long, 5 years, therefore early problems of inbreeding, if they exist, need to be identified and dealt with as soon as possible.

1.3.4 Aims of the Study

The overall purpose of this PhD study is to develop ways to manage this rare and valuable genetic resource. In order to prevent the deleterious effects of inbreeding that comes with the mating of closely related individuals within the F_1 generation broodstock population, the parentage of individuals identified as potential broodstock needs to be known. This study focuses on the use of microsatellite markers for pedigree analysis as a tool to identify selected individuals within a population in the establishment of a sustainable selective breeding programme for the Atlantic halibut industry in the UK.

The structure of the base population on the farm will be characterised and the level of genetic variation after one generation of hatchery rearing will be determined (in Chapter 2).

The parentage assignment of the previously selected F_1 potential replacement broodstock from the 1995 and 1998 year class will be the focus of Chapter 3.

Some of the problems associated with deformities in the juvenile stage of production have been attributed to poor egg quality (Pittman *et al*., 1998). Thus the repeatability of reproduction traits will be examined in Chapter 4.

Growth rate has been identified as the most desirable trait for improvement by members of the British Marine Finfish Association (BMFA). A significant amount of variation in growth has been reported by various authors (N Jordan pers comm.) however the heritability has yet to be estimated in this species. The heritability of body weight will be investigated in Chapter 5.

Based on the outcomes of work conducted in Chapters 2 to 5 a breeding programme or broodstock management and replacement strategy will be designed.

Chapter 2

Genetic characterisation of the founder population and the effect of hatchery practises on genetic variability after one generation

2.1 Introduction

The primary resource for the success of any animal breeding programme is genetic variation. Consequently the number of individuals and the level of variability present in the base population is crucial when establishing a hatchery population. This is because, without the introduction of new individuals, the alleles present therein, and any subsequent mutations, represent the upper limit of allelic variation available for selection in subsequent generations (Allendorf and Ryman, 1987). A genetic improvement strategy aimed at selecting "better" performing individuals will inevitably change the genetic composition of the population by reducing its genetic variability as "poor" alleles are replaced with "good" ones. Thus the conservation, i.e. preservative management of genetic variation, becomes an essential component of broodstock management and regular monitoring of hatchery stocks is necessary to check that breeding programmes are not leading to inappropriately rapid erosion of genetic variability (Ward and Grewe, 1995; O'Connell and Wright, 1997). The high fecundity of many aquaculture species and small sizes of offspring at hatching makes genetic identification of individuals/families a fundamental requirement for effective genetic management in any aquaculture programme. An important route to achieving this objective, particularly where pedigree is difficult, is the analysis of distinct alleles at defined loci (Ferguson, 1995). The technology and its effectiveness of accomplishing this goal has evolved over the past 50 years.

2.1.1 Molecular Markers

Molecular genetics approaches to the study and management of fish populations were first applied to fisheries in the 1950s (Utter, 1991). For the first time it was

possible to look beyond the phenotype in order to discriminate between fish stocks. Early studies involved the use of blood group polymorphisms to discriminate between different fish populations. However, fish erythrocytes were more fragile than those of birds and mammals and difficulties arose in producing and preserving discriminating antisera (Utter, 1991). The procedures were labour intensive, expensive and results were unreliable because patterns were difficult to interpret and the variation identified could not be attributed to any single locus (Utter *et al*., 1987; O'Connell and Wright, 1997). Electrophoretic procedures were then developed which permitted the rapid and reliable identification of protein variations reflecting simple genetic differences (Utter *et al*., 1987; Ward and Grewe, 1995; Ferguson *et al*., 1995).

2.1.1.1 Allozymes

Allozymes were the first generation of widely used genetic markers used in aquaculture. They are the products of genetic variation at enzyme-encoding loci. They arise from heritable, electrophoretically detectable differences in the amino acid composition of enzymes that share a common substrate. In order to identify and score allozymes, a piece of tissue is obtained from an organism and is ground up with buffer solution to release proteins from the cells. After centrifugation, these proteins present in the supernatant are subject to gel electrophoresis that separates the proteins on the basis of charge and size. This technique is relatively inexpensive to perform because reagents are cheap and there are few requirements for specialised equipment, making it possible to assay large numbers of samples quickly and easily. Their codominant nature is well suited to population studies allowing the estimation of gene frequencies and heterozygosity thus they provide

estimators of genetic differentiation and population structure in relation to the Hardy-Weinberg concept (Carvalho and Hauser, 1995).

The use of allozyme markers for describing population structure often assumes that they are selectively neutral and that genetic drift is responsible for population differentiation. However, there is evidence that some allozymic differentiation is determined by locus-specific selection, arising from forces that may be independent of mating patterns and gene flow (Utter, 1991; Carvalho and Hauser, 1995). The selection-neutrality controversy remains unsolved but is has become clear that if selection pressures operate they are generally small in magnitude (Ward and Grewe, 1995)

Allozyme electrophoresis has certain limitations. The resolution of this method is not always adequate for detecting differences between populations or individuals. Many genetic variants are not detected by protein electrophoresis and only proteins detectable with histochemical stains can be examined thereby limiting the proportion of the genome to only enzymatically active proteins (Park and Moran, 1995). In addition only fresh or freshly frozen tissue can be used. Furthermore, a relatively large amount of tissue is sometimes required and many important loci are assayed from organs such as the heart or liver and thus may require killing the animal (Ferguson *et al*., 1995; Park and Moran, 1995).

In aquaculture allozyme electrophoresis has been used to assess the level of genetic variation in hatchery stocks (see section 2.2 below) but in recent times its use has been limited. One of the main limitations of allozymes as genetic markers in aquaculture is the low level of observed polymorphism. Only a small fraction of enzyme loci appear to be polymorphic in many species (Magoulas, 1998),

prompting a continued search for markers with greater genetic resolution. Allozyme studies provided limited information on how the loci related to each other and thus no indication of genomic organisation or metabolic pathways. In the late 1970s to 1980s as molecular techniques developed, workers turned to a direct investigation of DNA sequence (Ferguson *et al*., 1995; O'Connell and Wright, 1997).

2.1.1.2 DNA markers

Rather than the indirect method of examining gene products, direct assessment of DNA variability came about with the isolation of restriction endonucleases that are capable of cutting DNA at specific nucleotide sequences (Ward and Grewe, 1995). The fragments produced are of variable size and can be separated on electrophoretic gels allowing the direct study of DNA sequence variation. Genetic variation identified in this way is called restriction fragment length polymorphism (RFLP). With this technology the number of markers available for the genetic study of populations increased dramatically. RFLP markers were very popular because they are codominant and easy to score. The major disadvantages of this technique were that it is time consuming and observed polymorphisms were low.

2.1.1.2.1 Mitochondrial DNA

When the target molecule is the mitochondrial DNA (mtDNA) rather than nuclear genomic DNA the markers are known as "mitochondrial DNA markers". The mitochondria is a discrete organelle that could be isolated and mtDNA analysis became popular for genetic studies, particularly in studies designed to answer questions about phylogeny and population structure (Ward and Grewe, 1995; Magoulas, 1998; Liu and Cordes, 2004). MtDNA markers were seen to be more powerful for such studies for various reasons not least because mtDNA has a mutation rate about an order of magnitude higher than that of nDNA (Dunham,

2004). MtDNA is haploid and has a near exclusive maternal mode of inheritance. It therefore has an effective population size approximately only one quarter that of nDNA, resulting in greater genetic differentiation due to drift, making it more likely to provide population specific markers. MtDNA also exhibits a lack of recombination therefore using mtDNA markers individuals can be organised into matriarchal lineages even after inbreeding has taken place (Ferguson *et al*., 1995; Ward and Grewe, 1995). MtDNA analysis was hailed as a more powerful tool than allozyme electrophoresis, often revealing genetic differences among populations of fish that were homogeneous for isozyme variability (Dunham, 2004; Reilly *et al*., 1999), and from a practical perspective analysis could be carried out on fresh, frozen or alcohol preserved samples.

MtDNA marker analyses have made a significant contribution to the management of cultured fishes. The technique has been used extensively to investigate stock structure and is quite popular among aquaculture geneticists due to their use in the identification of broodstocks (Liu and Cordes, 2004). However due to its non-Mendelian mode of inheritance, mtDNA is usually treated as a single character and does not permit the examination of independent loci. Furthermore, because mtDNA is maternally inherited, the phylogenies and population structure derived from mtDNA data may not reflect those of the nuclear genome due to gender-based migration (Birky *et al*., 1989: as cited by Liu and Cordes, 2004).

2.1.1.2.2 Random amplified polymorphic DNA

In 1990 random amplified polymorphic DNA (RAPD) markers were first developed (Welsh and McClelland, 1990: as cited by Liu and Cordes, 2004). Analysis using these markers involves the use of random oligonucleotide primers (8-12 base pairs

(bp) long) in a polymerase chain reaction (PCR) to amplify anonymous regions of genomic DNA. Amplified products are then separated by gel electrophoresis and scored for the presence or absence of each product indicating changes in the DNA sequence of target loci. RAPD polymorphisms occur as a result of base substitutions at the primer binding sites or insertions in the regions between sites (Liu and Cordes, 2004). RAPD markers are easy to develop, economical, quick to analyse and are highly polymorphic but these markers have significant drawbacks. Even though they are inherited in a Mendelian fashion, they are genetically dominant and a single band is produced for homozygotes and heterozygotes alike, making scoring difficult. Also they are subject to low reproducibility due to the low annealing temperatures used in the PCR amplification (Liu and Cordes, 2004; Dunham, 2004). Because they are cheap and easy to analyse, RAPDs expanded the scope of DNA studies in aquaculture; whilst they are useful in gene mapping studies the fact that they are not codominant has severely restricted their use in population analyses.

2.1.1.2.3 Variable number of tandem repeats markers

Since 1985 attention turned from the analysis of changes in the DNA sequence as a result of point mutations to differences in the number of repeated copies of a segment of DNA. Spread throughout the genomes of most eukaryotic organisms are regions that contain tandem repeats of specific DNA motifs. These sequences may contain very long (100 to 5000 bp) repeated units termed satellites, short repeat units (5 to 100 bp) termed mini satellites or very short repeat units (2 to 4 bp) called microsatellites or STR (Beaumont and Hoare, 2003). Individual alleles at a locus differ in the number of tandem repeats of the unit sequence and can be easily differentiated by gel electrophoresis according to size; the observed variation in the

number of repeats at these loci can be very extensive in populations and provide a valuable tool for investigation of population genetic changes. These markers, collectively called VNTRs (variable number of tandem repeats), were initially discovered in 1980 (O'Connell and Wright, 1997) however it was not until 1990 that they were first used as molecular markers in aquaculture. Following work by Jeffreys *et al*. (1985) on multilocus DNA fingerprinting using minisatellite loci for paternity tests in humans, Fields *et al.* (1989) and Taggart and Ferguson (1990) pioneered this technology in aquatic organisms- applied to three salmonid fish species. Although multilocus DNA fingerprinting gave complex banding patterns unique to each individual difficulties in the interpretation of results instigated a move to single locus VNTR markers (reviews by O'Reilly and Wright, 1995).

Over the past decade it has became apparent however, that the most popular DNA Mendelian markers are microsatellites because they are, arguably, the most powerful marker type (Jarne and Lagoda, 1996; Liu and Cordes, 2004). The power of a molecular marker for detecting polymorphism in a population and thus their usefulness can be measured based on their polymorphic information content (PIC). Microsatellites have the highest PIC values of any DNA marker (Liu and Cordes, 2004). The PIC reflects the fraction of heterozygotes and is dependent on the number and frequency of alleles. Microsatellites differ from minisatellites in size and contents of repeat; microsatellite repeat units are simpler and the lengths of the loci are much shorter. They are also much more abundant in the genome, particularly in vertebrates (Magoulas, 1998). In fishes microsatellites have been estimated to occur as often as once every 10 kb (Wright, 1993).

2.1.1.2.4 Microsatellites

Microsatellites consist of multiple copies of tandemly arranged simple sequence repeats that range in size from 2 to 4 base pairs. However the most common are dinucleotide repeats (Jarne and Lagoda, 1996; Schlötterer, 1998). Each microsatellite locus is flanked by a sequence that is assumed to be unique. If the sequences flanking the microsatellite are known, primers can be synthesized complementary to these flanking sequences such that the tandem array of the microsatellite locus can be amplified by the polymerase chain reaction (PCR) (Wright and Bentzen, 1995). Microsatellite markers are ideal molecular markers because they are highly polymorphic, codominantly inherited, abundant and evenly distributed in genomes (Dunham, 2004). In addition microsatellite loci are relatively short in size ranging from a few to a few hundred repeats; this characteristic facilitates rapid genotyping by PCR from small or even degraded quantities of DNA, negating the need to sacrifice or biopsy animals. Furthermore specimen samples need not be fresh and can be stored in alcohol, dried or frozen which makes the transport of samples much easier.

Microsatellite polymorphism is based on size differences due to varying numbers of repeat units contained by alleles at a given locus. The length of each allele can be accurately determined by electrophoresis. Slippage during DNA replication is believed to be the main mutational process accounting for this change in the length of repeat units (Wright, 1993; Schlötterer, 1998). The high rate of mutation at many of these loci leads to extensive allelic variation and high levels of heterozygosity making them useful for population genetic analysis, strain and individual identification. The drawbacks with using microsatellites are that a large amount of investment and effort is required in designing primers. Each microsatellite locus

has to be identified and its flanking region sequenced before a microsatellite locus can be amplified. This involves constructing genomic libraries, isolating loci by screening DNA libraries with repeat motif probes, and sequencing clones that hybridise to the probes. Finally effective PCR primers need to be designed. For the most part microsatellite scoring is generally reproducible and accurate however, it is important to note that additional bands, called stutter bands, may be present and some alleles may not amplify. These alleles, termed null alleles, occur if a deletion or point mutation in the primer binding site of a specific allele interferes with priming. The presence of null alleles is suspected if a surplus of homozygous individuals in observed (Schlötterer, 1998).

2.1.1.2.5 Single Nucleotide polymorphisms (SNPs)

Despite the dominance of microsatellites over the past decade, another marker type, Single nucleotide polymorphism (SNP), is beginning to gain popularity. The interest in these markers developed from the need for a higher density of genetic markers for gene mapping as they are the most abundant polymorphism in any organism, occurring approximately 1 every 1000 bases (Dunham, 2004; Hayes and Andersen, 2005). Although sequence differences resulting from base substitutions have been characterised for over 25 years, the ability to identify, isolate and genotype SNPs rapidly in large quantities has only recently been made possible (Liu and Cordes, 2004).

SNP polymorphisms are caused by point mutations that give rise to different alleles containing alternative bases at a given nucleotide position within a locus (Liu and Cordes, 2004). In order for a base position with sequence alternatives in genomic DNA to be considered as an SNP, the least frequent allele should have a frequency

of 1% or greater (Vignal *et al.,* 2002). In principle a SNP within a locus can produce as many as four alleles, each containing one of the four bases: A, T, G and C, however in practice they are usually bi-allelic, comprised of either the two purines (A&G) or the two pyrimidines (C&T) (Vignal *et al.,* 2002; Liu and Cordes, 2004). SNPs are inherited as co-dominant markers and they can either be in non-coding regions or in specific genes. This single base variation can be determined by DNA sequencing, primer extension typing, the designing of allele-specific oligo and gene-chip technology (Dunham, 2004).

Due to the fact that a maximum of only two alleles are present with these markers, they are not as polymorphic as microsatellites but this drawback is balanced by their high abundance. Furthermore, unlike with microsatellites, allele definition is much simpler with SNPs so automation is expected to be less problematic and comparisons between laboratories more likely (Vignal *et al.,* 2002). The cost of genotyping SNPs can also be comparatively lower than microsatellites, using high throughput technology such as mass arrays for genotyping large numbers of animals for large numbers of markers (Vignal *et al.,* 2002). SNP analysis does however have several disadvantages including the need for sequence information, the necessity of probes and hybridization, high expense involved with specialised equipment and difficult genotyping (Liu and Cordes, 2004; Dunham, 2003).

2.1.2 Molecular Markers in Fisheries and Aquaculture

The ability to detect, visualise and quantify DNA level polymorphisms has revolutionised the field of aquaculture genetics. By detecting genetic variations at the DNA level aquaculture geneticists are able to provide information on and address various important issues that would otherwise have been impossible.

Studies in several species have been conducted in areas related to population structure in various species including salmonids (e.g. McConnell *et al*., 1995; Wenburg *et al*., 1996; Colihueque *et al*., 2003; Tessier *et al*., 1997; Nelson *et al*., 1998; Heath *et al*., 2002) carps, (Gross *et al*., 2002), cyprinids (Salgueiro *et al*., 2003), gadoids (Ruzzante *et al*., 1999) and tunas (Appleyard *et al*., 2002), phylogenetic relationships (Hansen *et al*., 1999), linkage mapping and identification of quantitative trait loci for facilitating marker assisted selection (Danzmann *et al*., 1999; Sakamoto *et al*., 2000; Palti *et al*., 2002; Sakamto *et al*., 1999) as well as parentage assignment (Norris *et al*., 2000; Taggart *et al*., 2001; Jerry *et al*., 2004). Whilst various molecular markers have been applied to these areas with varying degrees of success the current study is focused on their use in monitoring gene flow within and between cultured and wild populations in order to detect any changes in genetic variation that might occur as a consequence of artificial or selective breeding strategies in the farming environment. Table 2.1 below gives a summary of the markers discussed and their uses in aquaculture.

The origin of these techniques lie in fisheries science where the most common objective is often to determine if samples from culture facilities or natural populations are genetically differentiated from each other. The resolution of these studies usually involves an analysis of allele frequencies and levels of heterozygosity.

Farming production practices or the effect of the hatchery environment often cause losses of genetic variability in cultured populations (Allendorf and Ryman, 1987). Reduced variability can result from genetic drift promoted by the use of small numbers of breeders used as replacements, particularly in species with high

fecundities, resulting in inbreeding and inbreeding depression. The effect of inbreeding depression and reduced genetic variation is expressed as poor phenotypic performance and limited potential for future responses to selection (Kincaid, 1983).

Genetic variability within populations is usually considered in terms of average heterozygosity. Since genetic drift will lead to a loss of rare alleles and a reduction in heterozygosity, molecular markers are used to quantify how much degradation, if any, has occurred. Levels of inbreeding can be calculated by examining decreases in heterozygosities in and between populations using Wight's F statistics (Hartl and Clark, 1989).

Studies examining the levels of genetic variation in hatchery populations started using allozymes to compare levels of heterozygosity between farmed and wild strains. A number of these studies demonstrated significant differences in allele frequencies and reductions in mean heterozygosity between wild and farmed population in various species: Atlantic salmon (Cross and King, 1983; Verspoor, 1988; Cross and Challanain, 1991), Turbot (Bouza *et al*., 1997), Pacific oyster (Hedgecock and Sly, 1990) and tilapia (Macaranas *et al*., 1995) amongst others. Allozyme electrophoresis has also been used to determine levels of genetic variability in successive years of hatchery stocks (Butler and Cross, 1996) and to establish links between lower levels of heterozygosity with reduced fitness (Danzmann *et al*., 1989).

Table 2.1 A comparison of molecular markers, their characteristics and potential applications.

Characteristics	Allozymes	RFLPs	MtDNA	RAPDS	Minisatellites	Microsatellites	SNPs
Abundance in genome	Low	High		High	Medium	High	High
Level of Polymorphism	Low	Low	Low	Medium	High	High	Low
Likely allele numbers	2-6	2	Multiple	2	Multiple	Multiple	2
Codominance	Yes	Yes	No	No	No/Yes	Yes	Yes
Reproducibility	High	High	High	Low	High	High	High
Labour intensity	Low	High	Medium	Low	High	Low	Medium
Set-up Cost	Low	Medium-High	Medium-High	Low	Medium-High	High	High
Running costs	Low	High	Low-Medium	Low	High	Low-Medium	Medium-High
Technical demands	Low	High	High	Low	High	Low-Medium	High
Neutrality	Questionable	Yes	Yes	Yes	Yes	Questionable	Yes/No
Major applications	Population studies	linkage mapping	Maternal lineage Phylogeography	Population studies Hybrid identification Linkage mapping	Parentage analysis	Linkage mapping Population studies QTL studies Parentage analysis	Linkage mapping QTL studies Parentage analysis

Although mitochondrial DNA and RFLP markers have been used to study the genetic variation between wild and cultured species (e.g. Hilsdorf *et al*., 2002; Reilly *et al*., 1999; Romana-Eguia *et al*., 2004) the marker of choice for these studies is usually microsatellites. This is because their very high polymorphism enables the detection of genetic variation between populations where differences may be limited.

Studies monitoring the levels of genetic variability by comparing levels of heterozygosity, allelic frequencies, allelic diversity and the number of alleles per locus at microsatellite loci between hatchery and wild stocks are becoming common in the literature, particularly in the Atlantic salmon. Reilly *et al*. (1999), Norris *et al*. (1999), Koljonen *et al*. (2002) and Spidle *et al*. (2004) all observed differences in genetic variability between cultured and wild populations of Atlantic salmon based on one or more of the criteria outlined above. Was and Wenne (2002) compared polymorphisms in 5 microsatellite loci between hatchery reared and wild sea trout. They found that while heterozygosity levels were similar between both populations allelic diversity was significantly lower in the hatchery population. Such reductions in genetic variability have also been reported in invertebrates such as shrimp (Xu *et al*., 2001) and abalone (Evans *et al*., 2004). On the contrary Romana-Eguia *et al*. (2004) compared the levels of heterozygosity between hybrid strains of tilapia developed for aquaculture production and their wild progenitors. They found that the levels of genetic variation were higher in the hybrid populations indicating good genetic management practises and the success of the introgression programmes used in establishing the improved lines.

In flatfish both Coughlan *et al*. (1998) and Stefánsson *et al*. (2001) observed reduced levels of variability between hatchery and wild populations of turbot. However, whilst the study by Stefánsson *et al*. (2001) revealed reductions in both heterozygosity and allelic diversity, Coughlan *et al*. (1998) noted only changes in allelic diversity. Sekino *et al*. (2002) reported similar result to Coughlan *et al*. (1998) in the Japanese flounder.

2.1.2.1 Molecular markers and the Atlantic halibut

Research involving the use of molecular markers in the Atlantic halibut began in the 1980s when the studies involved using both morphological characters and allozymes in population studies. The emphasis was placed on testing the hypothesis that the species consisted of several reproductively isolated populations. Studies by Haug and Fevolden (1986) and Fevolden and Haug (1988) comparing fish from Greenland and Norway did not reveal significant genetic differences between fish from both areas. However, a more extensive study by Foss *et al*. (1998) examined fish from Norway, Faroe, Iceland and Greenland and genetic variation at four enzyme loci revealed deviations from Hardy-Weinberg equilibrium, suggesting that the populations were not from a single panmictic population.

A series of studies involving fish from different geographical sites revealed performance differences in a range of commercial traits. Jonassen *et al*. (2000b) studied the growth patterns of juvenile halibut from Canada, Iceland and Norway at four different temperatures (8, 12, 15 and 18^{o}C). Their results intimated that Norwegian and Icelandic fish demonstrated superior growth capacities than the Canadian fish and also that the Norwegian animals had better feed conversion efficiencies across all temperatures. Their results agree with a hypothesis of countergradient growth where more northerly populations need to be able to grow

quickly during a relatively short growing season. The Norwegian fish had a decreased temperature interval that shifts the growth curve to the left enabling them to grow quicker at all temperatures.

Hoare *et al*. (2002) examined the susceptibility, immune response, of halibut from off the coast of the three countries to *Vibrio anguillarum* at optimal (12^{o}C) and super optimal (18^{o}C) growth temperatures. The results of their experiment showed that Canadian and Icelandic fish were significantly more susceptible to infection at 18^{o}C than Norwegian fish. In addition, the total mortality at 18^{o}C for the Canadian and Icelandic fish was almost double that at 12^{o}C whereas there were no significant differences in Norwegian fish at both temperatures. Specific antibody levels increased with rising temperatures and differences between strains were observed. The main antibody was specific for Lipopolysaccharide (LPS) and the immunoblot reactions were stronger in the Norwegian fish suggesting that this may account for the difference in strain resistance.

Imsland *et al*. (2002) also analysed the performance of juvenile halibut from the three countries. They assessed the fish for growth performance and disease resistance to *Vibrio anguillarum*. Again differences in performance were observed among the strains. Norwegian fish showed the best growth rates followed by the Icelandic fish and the Canadian fish ranked lowest. The Icelandic fish were the most susceptible to the bacterial infection while again the Norwegian fish showed the best survival rates of all three. In general they observed that the fish which survived the disease challenge were those that had grown fastest in the growth trial, however, this varied across strains. The Canadian fish showed no correlation between either size or growth and survival while size was correlated with survival

in the Icelandic group. In the Norwegian fish both size and growth were correlated with survival.

2.1.2.1.1 Microsatellites and the Atlantic halibut

The Atlantic halibut karyotype consists of 24 pairs of subtelocentric chromosomes (Brown *et al*., 1997). The first microsatellite loci identified in the Atlantic halibut were isolated by McGowan and Reith (1999). They developed five loci; *Hhi*C17, *Hhi*I29, *Hhi*D34, *Hhi*J42 and *Hhi*A44, which were tested on 55 individuals from the Bay of Fundy in Canada. A total of 76 alleles were observed over all five loci and apart from one locus, *Hhi*J42, were found to be in apparent Hardy-Weinberg equilibrium. *Hhi*J42 had a significant excess of homozygotes suggesting the presence of a null allele at this locus within the population. The loci were tested successfully for cross-amplification on three other species of flatfish; the winter flounder (*Pleuronectes americanus*), the yellowtail flounder (*Pleuronectes ferrugineus*) and Canadian plaice (*Hippoglossoides platessoides*).

Coughlan *et al*. (2000) developed 11 more microsatellites for the species and tested them on 20 Icelandic individuals. All loci were in Hardy-Weinberg equilibrium and a total of 95 alleles were observed.

To date, two studies on the application of microsatellites to the monitoring of genetic variability in the halibut have been published. Using six microsatellites (4 from Coughlan *et al*. (2000), one from McGowan and Reith (1999) and one unpublished locus developed for the Pacific halibut (*Hippoglossus stenolepis), Hst*-16), Stefánsson *et al*. (2001) investigated the effect of artificial rearing on genetic variability in the halibut. They did this by comparing levels of heterozygosity, number of alleles and allelic variation between wild-caught broodstock from

Canada, Iceland and Norway, some of which were used as parents, and their F_1 hatchery reared offspring. They found significant reductions between parental and offspring populations for the mean number of alleles in the Canadian and Icelandic populations. Only allelic diversity was significantly lower in the Canadian group while only observed heterozygosity was significantly lower in the Icelandic group. It appears that there were no significant reductions in any of the parameters used to estimate genetic variability in the Norwegian population.

Jackson *et al*. (2003) conducted a similar study within the Canadian halibut industry. Using the five microsatellites isolated by McGowan and Reith (1999), they examined the same three parameters between 149 F_1 individuals from 27 potential parents. They found that although there were no significant differences in heterozygosity there were significant reductions in the number of observed alleles and allelic diversity.

Results from studies such as these emphasise the need to assess and monitor the amount of genetic variation present within captive populations in aquaculture broodstock replacement or selective breeding programs. The aim of the current chapter is therefore to assess the level of genetic variability within the parental population using microsatellite loci and evaluate the success of the hatchery practices, within the UK industry, at maintaining the genetic variation by comparing this with the observed levels in their offspring.

2.2 Materials and Methods

2.2.1 Broodstock Origin and Husbandry

The study was carried on in a converted salmon farm on the West Coast of Scotland, 130 km west of Glasgow on the East coast of Loch Fyne, Otter Ferry Seafish Limited. The parental population on the farm was started in 1987 and established over a period of five years based on wild caught fish acquired from Icelandic and Shetland waters and first generation farmed stock from the United Kingdom Sea Fish Industry Authority research facility in Ardtoe, Scotland. At the start of the study 70 individuals were left on the farm, 28 males and 42 females. 33 individuals (47%), 12 males and 21 females, were wild caught from Icelandic waters. 11 individuals (16%), 6 males and 5 females, were wild caught from Shetland. The remaining 26 individuals (37%), 10 males and 16 females, were first generation farmed stock acquired from Sea Fish Authority Ardtoe. Each fish was identified using a Passive Integrated Transponder (PIT) tag number.

The fish were held in covered 5m diameter raceway tanks. Water depth was kept at 80cm. Oxygen saturation of the water was maintained at above 90% with a flow rate of 2.5 litres per minute. Artificial lighting was applied using 8.5 lux under a simulated natural photoperiod. Fish were fed an industry composite diet (Trouw) of 64% protein, 9% fat, 13% ash, 7% N.F.E (Nitrogen Free Extract) and 7% moisture. The feed was presented to the animals three times a week and stocking density kept at 26 fish per tank. Although the fish matured in 1994 a potential replacement F_1 broodstock population was not established until 1995.

The F_1 population used in this study comprised fish from the 1995 and 1998 year classes. About 350 individuals were selected by farm managers as potential

broodstock replacements from the 1995 spawning season. The animals were selected based on size at three years old (see Chapter 4). The fish from the 1998 year class were a random sample of 532 individuals taken from a group of fish chosen at the nursery stage for a PhD study by Nigel Jordan, University of St Andrews, aimed at comparing the growth performance between fish reared in sea cages and fish reared in land based tanks through the grow-out period (see Chapter 5). Some of the fish reared in the land based tanks would later be selected as replacement broodstock on the farm. Throughout the text all the offspring in both the 1995 and 1998 year classes will be referred to as "F_1" fish as they were the first generation hatchery reared stock on the farm.

2.2.2 Sample Collection and Storage

In 1999 blood samples were collected from seventy of the parental broodstock using heparin treated syringes. The samples were put in 1ml microcentrifuge tubes (Alpha) and spun down to separate the serum from the red blood cells. The serum was discarded and the cells were stored –20^{o}C.

In 2001 before the onset of the breeding season fin clips were taken from all the F_1 individuals within the 1995 year class (350). The samples were taken from the pectoral fins and each sample was placed in an Eppendorf (Thermo Life Sciences) tube containing approximately 1ml 95% ethanol (Sigma). At the same time that the fin clips were collected the fish were tagged using Trovan® ID-100 protocol PIT tags (Identify UK limited, Hessle, East Yorkshire, HU13 0RD, UK). Each sample was subsequently identified by the PIT tag number. The fin samples were stored at 4^{o}C.

At the last stripping event of each group in the 2001/2002 spawning season, following anaesthesia with phenoxyethanol, blood samples were taken from a random sample of 351 fish from the 1995 year class. However, only tissue samples from a random sample of 270 individuals were analysed. Approximately 300µl of blood was extracted from the caudal vein and immediately fixed in 700µl 100% ethanol (Sigma) in an Eppendorf tube (Thermo Life Sciences). Again each sample was placed in an individual tube and the tubes were identified using the PIT tag numbers of the fish. They were stored at 4^{o}C. Blood samples were also collected by Nigel Jordan from 532 individuals from the 1998 F_1 year class using a similar protocol. All these samples were processed and analysed.

2.2.3 DNA Extraction

As the fish were the initial base for the UK halibut industry and because of the use of molecular genetic approaches in the future, such as QTL analyses, it was important to acquire good quality DNA for archiving. Therefore DNA was extracted from samples using a phenol-chloroform protocol modified from Taggart *et al*. (1992). All attempts to extract good quality DNA from fin clips using this technique were unsuccessful (Figure 2.1).

Using the blood samples fixed in ethanol, 70µl of each solution was aliquoted into sterile Eppendorf tubes (Thermo Life Sciences). Each tube was placed in a centrifuge (MSE Micro Centuar) and pulse spun at maximum speed for 30 seconds. Excess ethanol was taken off the top of the solution and the tubes were left open to air dry on the bench for 5-10 minutes.

After the ethanol had evaporated, 10µl proteinase K (ABgene, 20 mg/ml) and 340µl of TEN (50mN Tris HCl pH 8.0; 100mM EDTA pH 8.0; 100mM NaCl; 1% SDS

(Sodium Dodecyl Sulfate)) buffer was added to each tube. The SDS causes the cells to rupture and initiates protein denaturation while the proteinase K reduces proteins to their component amino acids. The tubes were vortexed for 30 seconds and incubated at 55^{o}C in a rotating oven (Techne Hybridiser HB-1) for 4-6 hours. After incubation 10µl RNAase (DNAse Free, Abgene, 2mg/ml) was added to each tube and incubated at 37^{o}C, also in a rotating oven, for one hour in order to digest and remove traces of RNA.

Following the incubation period 370µl of phenol was added to each tube to extract the denatured protein from the DNA solution. The solutions were mixed vigorously for 10 seconds and shaken gently every 5 minutes for 20 minutes. After the 20 minute period an equal volume of chloroform was added to the tubes to absorb and eradicate traces of phenol. The solutions were again mixed vigorously for 10 seconds and then shaken gently every 5 minutes for another 20 minutes. Each tube was then spun for 5 minutes at 10,000g. This causes the solutions to separate into two distinct layers. 270µl of the top aqueous layer, which contains the DNA, was removed and pipetted into a new tube, using a wide-bore tip. 870µl of 92% ethanol (Sigma) was added to the aqueous solutions and each tube was mixed by vigorous inversions, causing the DNA to precipitate out of the solution. After 3 minutes the tubes were pulse spun (10s at 10,000g) to form a DNA pellet at the bottom of the tube. The ethanol was decanted off and 1ml of 70% ethanol was added to each tube to wash the pellet.

Tubes were placed in a rotator overnight and the alcohol was removed using a micropipette, after which they were then left open to air dry for 10-15 minutes. The

pellets were then resuspended in 100µl TE pH 8.0 (10mM Tris, 1mM EDTA) buffer.

The same protocol was followed for the frozen blood samples collected from the parents in 1999, however because the samples were not diluted in alcohol only 35µl of the each sample was used.

2.2.3.1 Quantifying extracted DNA

The extracted DNA was allowed to dissolve for 24-48 hours. Half the volume (50µl) of the resuspended DNA in TE pH8.0 buffer was taken out of each tube and put in a new tube. This formed a "working solution" on which subsequent analysis was performed. The remaining 50µl in the original tube contained the "stock solution" and was archived at –20^{o}C.

The amount of DNA in all the "working solutions" was quantified using spectrophotometry (GENEQUANT, Pharmacia Biotech). The quantities obtained were highly variable between samples and ranged from 29-1940µg/ml. The concentration of each sample was then made up to 100µg/ml with the addition of TE pH 8.0 buffer. The quality of each sample was checked on a 0.8% agarose gel against a ØX74 RF DNA Hae III Ladder (ABgene) and revealed that, as desired, the majority of the DNA samples extracted were of high molecular weight (Figure 2.2).

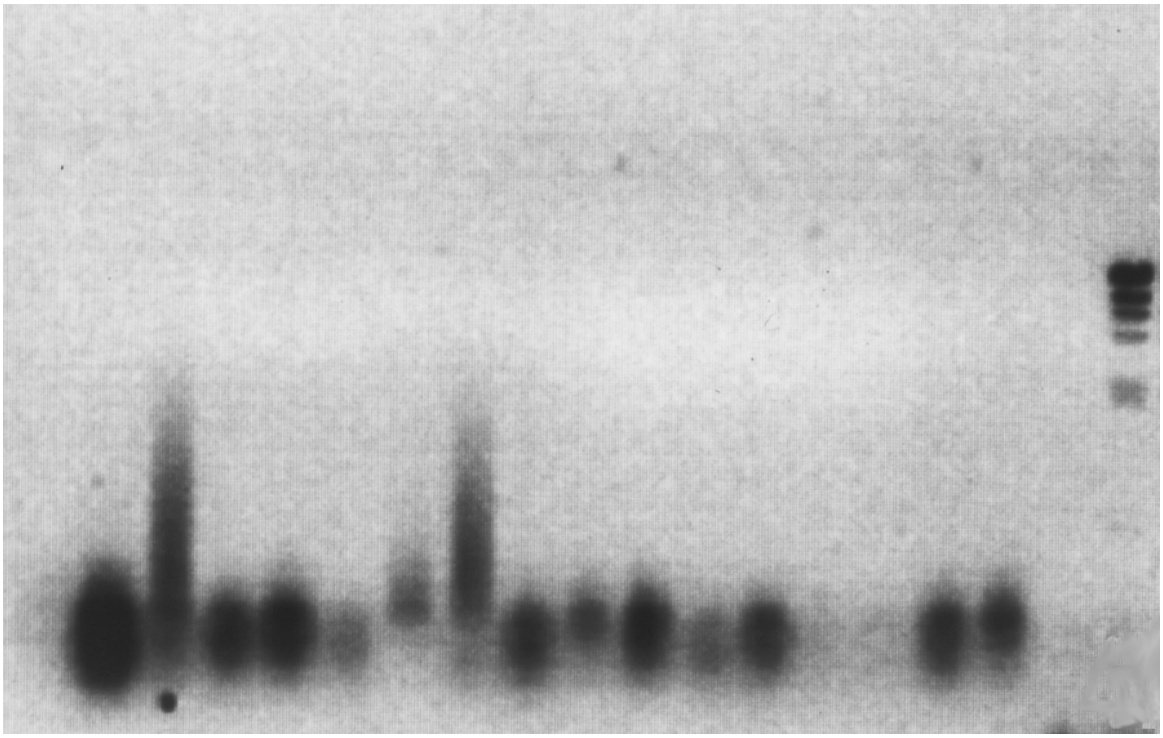

Figure 2.1 0.8% Agarose gel of Ethidium bromide stained DNA extracted from fin clips fixed in ethanol. Lane 16 contains the DNA ladder, ØX74 RF DNA HaeIII

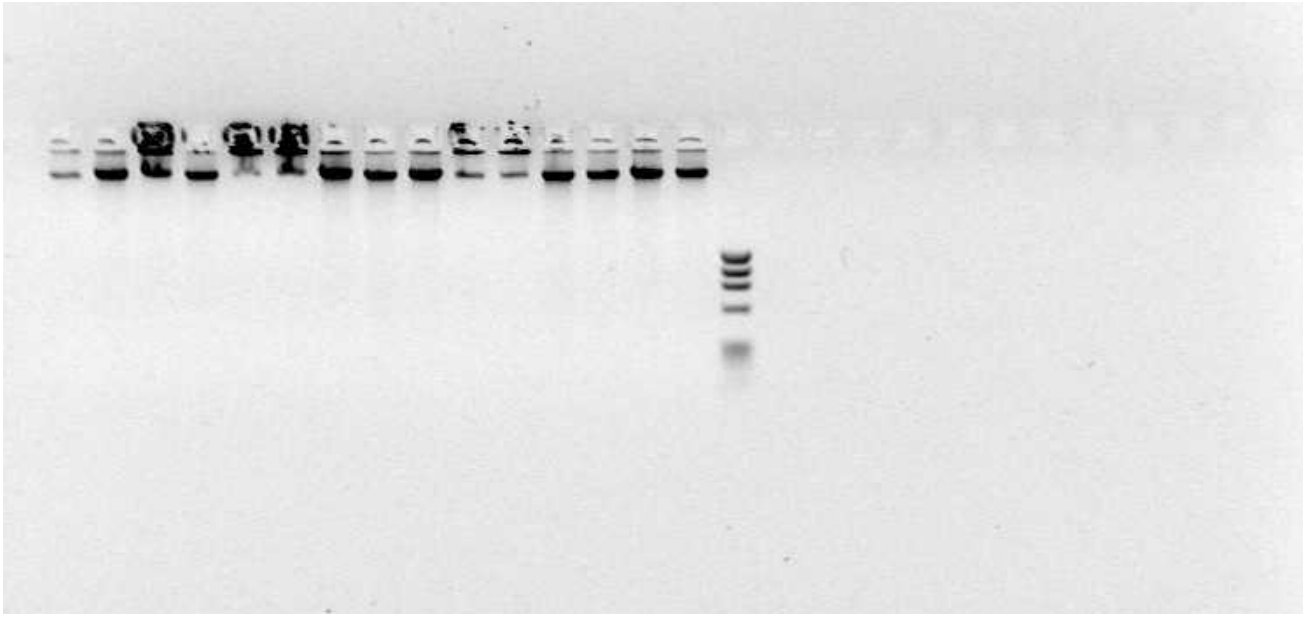

Figure 2.2 0.8% Agarose gel of Ethidium bromide stained DNA extracted from from blood fixed in ethanol. Lane 16 contains the DNA ladder, ØX74 RF DNA HaeIII

2.2.4 Polymerase Chain Reaction (PCR)

Large fragment sizes and high concentrations of DNA, of the sort obtained from blood in the current study, have been found to inhibit PCR reactions. It was therefore necessary to reduce the concentration of each sample to 20µg/ml. This was done by diluting 10µl of 100µg/ml DNA stock solution with 40µl PCR grade water (dH_20) in 100µl non-skirted PCR plates (Abgene). The DNA was then denatured by heating to 95^oC for 15 minutes, and the plates were subsequently stored at –20^oC until they were used in PCR reactions.

Five microsatellite loci previously characterised by McGowan and Reith (1999), *Hhi*A44, *Hhi*J42, *Hhi*D34, *Hhi*I29 and *Hhi*C17, were chosen for the present study (details are presented in Table 2.2). Forward primers for all five microsatellite loci were fluorescently labelled with one of three fluorescent dyes: 6-FAM, HEX and TET (MWG Biotech).

The decision was taken to follow the McGowan and Reith (1999) protocol of single locus PCR. This approach was followed because initial multiplex reactions produced inconsistent results and optimising the multiplex reaction for all five loci would have added to the analysis time, which would have resulted in a long delay in applying parentage data to farm management procedures.

Each PCR reaction consisted of 1.5mM $MgCl_2$ (Abgene), 150µM of each dNTP (Abgene), 1.0 µM of each forward and reverse primer,1x reaction buffer (Buffer II, Abgene: 75mM Tris-HCl, 20mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween® 20), 0.2 units of Taq DNA polymerase (Abgene) and 50ng genomic DNA template. The total volume of every reaction was 10µl. Reactions were conducted in a Biometra Gradient PCR machine under the following thermal cycling protocol:

Initial denature step of 3 minutes at 96^{o}C

30 cycles of

30 seconds at 94^{o}C (denaturing)

40 seconds at 55^{o}C (primer annealing)

60 seconds at 72^{o}C (extention)

A final extension step at 72^{o}C for 10 minutes to promote 3' adenylation.

PCR products were stored at –20^{o}C immediately after the reactions were completed. Products were visualised on agarose gels in order to check the success of the reactions (1.2% agarose gels in a 1X TAE buffer were run at 2.5 volts/cm for 45 minutes).

Following preliminary analyses two more loci were employed in the analysis. *Hhi*-3 and *Hhi*-53 isolated by Coughlan *et al*. (2000) were selected because they were highly polymorphic and robust (Jamie Coughlan pers comm.). Forward primers were fluorescently labelled using TET (*Hhi*-3) and FAM (*Hhi*-53) (see Table 2.2). All parents and 121 F_1 offspring were genotyped at these loci. PCR conditions were the same as those used for the first five loci.

Table 2.2 Nucleotide sequences, repeat motifs, fluorescent dye labels and published allele size ranges of microsatellites used in the current study.

Locus	Primer sequence (5'-3')	Repeat motif	Fluorescent Dye	Allele size range	Source
Hhi-3	F:GGAATAAAAGAAGGGGGTGCG R:TGTGGTGGGTGGCAGTGGCTA	$(CA)_{32}$	TET	175-217 bp	Coughlan *et al*.(2000)
Hhi-53	F:ACCAACAGTGACACATAGCTCCT R:ATGCTAATGGGCTCTAAAATC	$(CA)_{29}$	FAM	226-270 bp	Coughlan *et al*.(2000)
*Hhi*C17	F:TTAGGTCTGATCACCGCTATG R:GTTTACAAAGGTTTCTGATGGC	$(AC)_{24}$	FAM	114-168.bp	McGowan and Reith (1999)
*Hhi*I29	F:GCTTCGGTTACSCCTTTGC R:AGGACAGTGAGGATGTCCG	$(GT)_{25}$ (GTGTG)	HEX	98-134 bp	McGowan and Reith (1999)
*Hhi*D34	F:GCCTGGTCTCATTGTGTTCC R:AGGTTAAATGATTTCCTGAAGCTG	$(CA)_{12}$	FAM	184-226 bp	McGowan and Reith (1999)
*Hhi*J42	F:CACAAACTCAAGATGTTGCG R:AAGCTCACTGGAAAATAATACCC	(ACACACACAG) $(AC)_{20}$ (ACACA)	TET	112-144 bp	McGowan and Reith (1999)
*Hhi*A44	F:CAACTGTGGGTATGTGCCTG R:GTGTCAGCACTGTGCTTAAACC	(GTGTGCGTCT) $(GT)_{27}$	TET	136-234 bp	McGowan and Reith (1999)

2.2.5 Microsatellite Screening: Genescans

A 50ml polyacrylamide gel solution was prepared using 18g of Urea (Bio-Rad), 5.2mls Long Ranger® solution (Cambex) and 27.5mls MilliQ dH_2O. 0.5g mixed bed resin beads (Sigma) was then added to deionise the solution. The gel mix was stirred using a magnetic stirrer for about 25 minutes after which 5mls 10x TBE buffer was added to it and then it was filtered through a 0.2µm Whatman filter to remove the beads. The filtered gel mixture was de-gassed for 4 minutes to remove air bubbles, then 35µl TEMED and 250µl APS (Ammonium Persulphate solution; 0.1g in 1ml dH_20) were added to initiate polymerisation. The gel mix was injected between two 36cm glass plates using a 50ml syringe through an injection clamp. The plates were already assembled on a standard ABI cassette separated by 0.2mm plastic spacers. Once the gel was poured it was left for 2-3 hours at room temperature to polymerise fully.

Polymerisation was checked by expressing the excess gel from the syringe. After the gel had polymerised the injection clamp was removed and the plates were wiped clean. A disposable cardboard sharks tooth well comb (Web Scientific) was inserted into the top of the cleaned gel slot and the cassette was placed inside the ABI 377 DNA sequencer and clamped into place. A plate check module was run to check that the read region had no gel residues and there were no scratches on the plates, as these would influence the results of the run. A 1X TBE solution was added to the buffer tanks and the wells of the comb were flushed with the buffer solution using a 50ml syringe and 23mm gauge needle, in order to remove excess fluid, air bubbles and crystals of urea from the wells. The pre-run module was then run for 10 minutes in order to warm the gel to about 42^{o}C, the machine was then paused and the wells flushed again.

A loading buffer was prepared using 1.40µl deionised formamide, 0.30µl 50 mg/ml blue dextran dye, 25mM EDTA buffer and 0.3µl TAMRA 350 internal size standard (Genpak). 0.5µl of PCR product was added to this 2µl buffer, the mixture was denatured for 4 minutes at 95^{o}C and kept on ice until loading was complete. Samples were loaded manually using a single pipette. Odd numbers were loaded first followed by a 1-minute pre-run period. The wells were flushed again using a dripping pipette and then the even numbers were loaded. The volumes loaded depended on the size and number of the wells in the combs used. When a 48 well comb was used 1.20µl was loaded in the wells and when a 64 well comb was used 0.85µl was loaded. Gels were run for 2.2 hours at 3000volts, 50mA, with a gel temperature of 51^{o}C and 1200 laser scans per hour using filter set C.

2.2.5.1 Multiplex screens

PCR products from each individual were mixed prior to loading on the gene sequencer. The use of fluorescent markers made the simultaneous analysis of two or more different loci possible. This "multi-loading" technique of PCR products is appealing because of the considerable saving of time and resources, offered by reducing the number of gene scan runs needed for the analysis of several microsatellite loci.

In the first round of the analysis two runs were performed for each individual. The runs were a triplex of *Hhi*C17, *Hhi*I29 and *Hhi*A44 and a duplex of *Hhi*J42 and *Hhi*D34. Samples were diluted using the following protocol: for the triplex 2µl each of *Hhi*C17 and *Hhi*A44, 4µl *Hhi*I29 and 1µl dH_20. For the duplex 2µl each of *Hhi*J42 and *Hhi*D34 were diluted in 6µl dH_20. Two control samples were used on every PCR plate and genotyping run. In the second round of the analysis only one run was performed because both primers were labelled with different dyes. Data

collection and analysis was performed using Genescan™ and Genotyper™ softwares.

2.2.6 Genotype Analysis

Gel data were analysed using GenescanTM Analysis Software v3.2.1 (Applied Biosystems) and fragments were sized using the Local Southern method. Following the installation of a gel matrix, the matrix compensates for some fluorescent emission in the detection ranges of other dyes being detected in the wavelengths of each specific dye. The lanes on the gel images were tracked, cross checked and extracted using automated procedures of the Genescan collection software. The size standard in each lane was aligned to standardise the size calling between lanes. Extracted lanes containing fragment size data for each sample were exported into Genotyper™ Analysis software V3.21 (Applied Biosystems).

Genotyper is a software application that enables the analysis and interpretation of nucleic acid fragment size and quantifies data by converting it into user defined results. Allele sizes were determined manually and data for each allele at each locus from all individuals in the wild broodstock population was used to define a bin size for each allele. Bin sizes were created in order to account for the slight changes that occur in fragment sizes between gels and thus ensure a measure of consistency between results. Fragment sizes of the two controls were compared across all five loci after every Genescan run with the results from the first run and the bin sizes were adjusted accordingly if there was a need to do so.

2.2.7 Statistical Analysis

Allele frequencies and observed and expected heterozygosities for all loci were calculated using the programme GENEPOP 3.3 (Raymond and Rousset 1995).

Assumptions for Hardy-Weinberg equilibrium (HWE), genic and genotypic differentiation between all parental and offspring populations were also tested using GENEPOP 3.3 (Raymond and Rousset 1995). Exact P values were determined through a Markov chain process using the default settings in the programme (1000 dememorisations, 100 batches and 1000 iterations per batch) for all tests. Polymorphic Information Content (PIC) was estimated using the allele frequency analysis option in Cervus (Marshall *et al.*, 1998).

F-statistics (F_{ST} and F_{IS} values) were calculated for each locus, across generations and populations according to Weir and Cockerham (1984) using the programme Fstat (Goudet, 1995). Significance values for each locus and test were determined by bootstrapping over samples.

Effective Number of Alleles (a_e) allows the comparison of two or more different populations where the numbers and frequencies of alleles differ. It was calculated using the formula from Ferguson (1980):

$$a_e = \frac{1}{\Sigma q_i^2}$$

Where:

q_i= Frequency of the i^{th} allele at a locus.

Allelic Diversity (A_d) is a measure of the proportion of genetic variation remaining in reared strains from founding populations based on the number of alleles (n') retained at a polymorphic locus (Allendorf and Ryman, 1987). This value was calculated using the formula:

$$A_d = \frac{n'-1}{n-1}$$

Where n is the initial number of alleles present in the base population. Allelic diversity ranges from 1, where all alleles are retained, to 0 where all alleles but one are lost (Allendorf and Ryman, 1987).

2.3 Results

Genotypic data was successfully collected for all but one of the individuals in the parental population. Data for 69 individuals was collected over all seven loci through singleplex PCR and gel electrophoresis. No data was generated for one female from the wild Icelandic population: this was because the frozen tissue sample was compromised and several attempts to extract DNA from it were unsuccessful. However, the genotype of a missing female parent was derived based on observations of offspring genotypes. This female was assumed to be of wild Icelandic origin based on data acquired from the farm. The reconstruction of the unknown female's genotype is discussed in Chapter 3. Genotypic data was collected from a total of 802 F_1 animals.

2.3.1 Levels of Genetic Variability in the Parental Population

In order to conduct population genetic analysis successfully the loci employed should be robust and polymorphic. Both of these conditions were found to be true for all loci used in this study. Observed heterozygosities at all loci were greater that 70% and the number of alleles detected ranged from a minimum of ten in *Hhi*D34 to a maximum of twenty-six in *Hhi*C17. These as well as other indicators of the levels of polymorphism are shown for each locus in Table 2.3 below.

Table 2.3 Polymorphic indicators for each locus in the parental population; Number of allele(A), effective number of alleles (a_e), observed heterozygosity (H_o), expected heterozygosity (H_e) and Polymorphic Information Content (PIC).

Locus	***Hhi*-3**	***Hhi*-53**	***Hhi*C17**	***Hhi*I29**	***Hhi*D34**	***Hhi*J42**	***Hhi*A44**
A	24	20	26	17	10	15	23
a_e	13.55	11.85	13.53	9.95	3.73	4.21	5.05
H_o	0.971	0.857	0.871	0.957	0.743	0.786	0.857
H_e	0.933	0.922	0.933	0.906	0.737	0.768	0.808
PIC	0.924	0.908	0.921	0.891	0.692	0.745	0.789

The most polymorphic loci in this study averaged across all criteria were *Hhi*-3, *Hhi*C17 and *Hhi*-53. Even though the number of alleles present at *Hhi*A44 was high the effective number of alleles was, by comparison, very low. This was due to the high incidence of a common allele, 143. The disproportionate frequencies of alleles in the population and consequent low effective number of alleles was also observed at *Hhi*D34 and *Hhi*J42 as shown in Figures 2.3 and 2.4 below.

The ratio of the actual number of alleles to the effective number of alleles for each locus shows that *Hhi*A44, *Hhi*D34 and *Hhi*J42 (4.55, 2.68 & 3.56) are likely to be less informative in a pedigree analysis. This is also reflected in their relatively low PIC values.

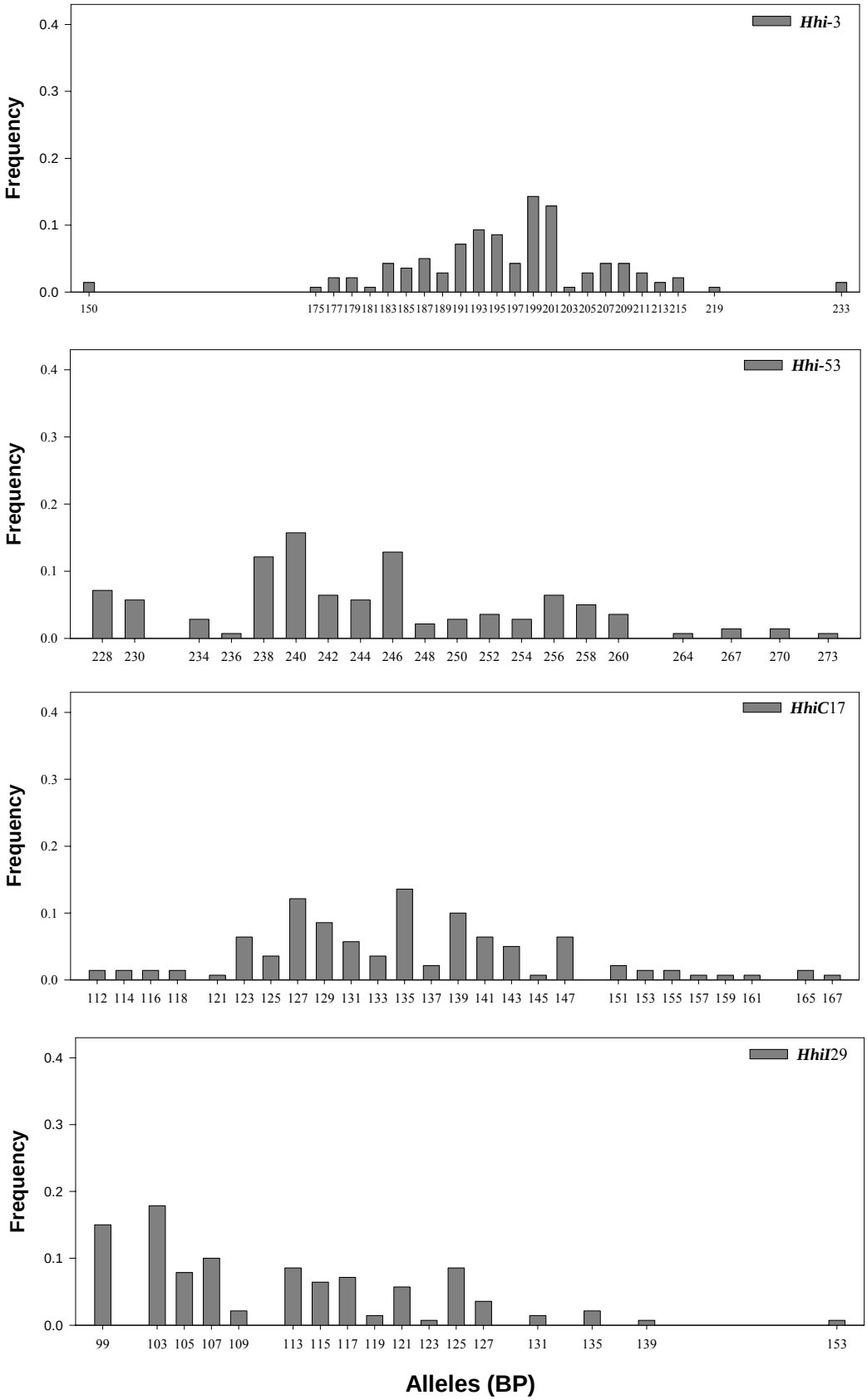

Figure 2.3 Frequency distribution of alleles in parental population for loci :H*hi*-3, H*hi*-53, H*hi*C17 and H*hi*I29.

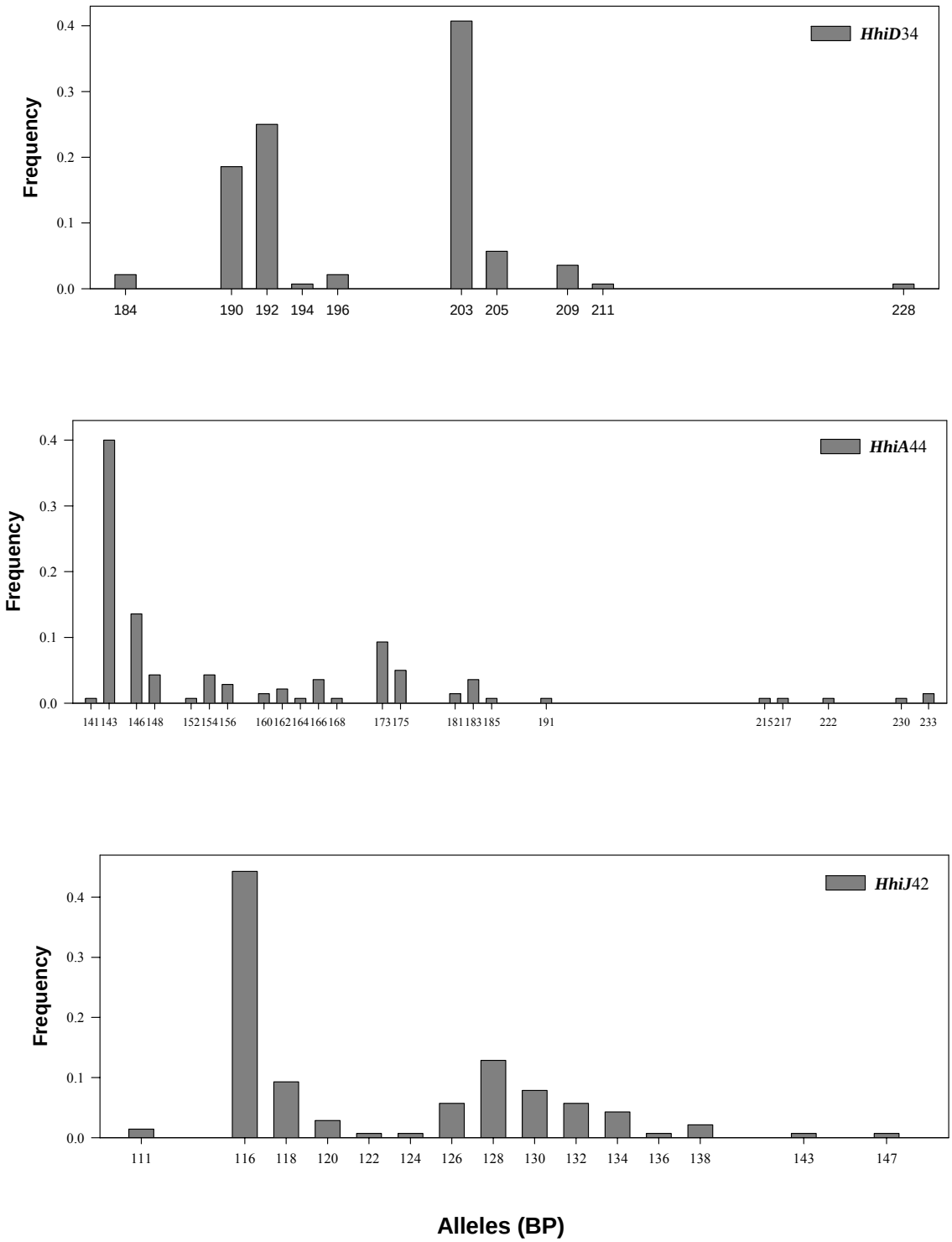

Figure 2.4 Frequency distribution of Alleles in parental population for loci: H*hi*D34, H*hi*A44 and H*hi*J42.

2.3.2 Hardy-Weinberg Equilibrium in the Parental Generation

In order to determine if the bands identified as alleles in the programme Genotyper were acting in a Mendelian fashion, Hardy–Weinberg equilibrium (HWE) tests were conducted. Global tests across all loci did not reveal significant departures from HWE (P=0.700) i.e. the parental population as a single entity was in HWE. Tests for individual loci, however, showed that one locus, *Hhi*C17, was not seen to be in HWE. Exact P values and their standard errors for each of the loci are shown in Table 2.4 below.

The Departure from HWE at *Hhi*C17 was due to an excess of homozygotes for six alleles; 123, 127, 139, 147, 151 and 153. Observations of this nature can imply large allele drop out but this was not evident in the parentage assignment (see Chapter 3). It could also indicate, amongst other things, that the sample is a mixture of genetically distinct populations or point to the influence of selection on a locus (Ferguson *et al*., 1995).

Table 2.4 Summary of Hardy-Weinberg analysis in the parental generation.

Locus	Probability Value	Standard Error
Hhi-3	0.418	0.037
Hhi-53	0.089	0.019
*Hhi*C17	<0.0005	<0.0005
*Hhi*I29	0.051	0.013
*Hhi*D34	0.334	0.024
*Hhi*J42	0.459	0.036
*Hhi*A44	0.944	0.019

The parental population in Otter Ferry was made up of three separate groups; 33 individuals from Icelandic waters, 11 individuals from Shetland and 26 F_1 (Icelandic fish) from Ardtoe. Due to the fact that three separate populations made up the parental population each population was examined separately. The levels of

polymorphism within each population and the exact test for HWE are shown in Table 2.5 below.

Table 2.5 Comparison between groups in the parental population.

Origin	*Hhi*-3	*Hhi*-53	*Hhi*C17	*Hhi*I29	*Hhi*D34	*Hhi*J42	*Hhi*A44
Iceland	*n*=33						
A	22	17	24	16	10	12	20
a_e	13.79	10.37	15.90	10.23	3.90	5.93	5.78
H_o	1.00	0.906	0.940	0.970	0.788	0.879	0.849
(S.E)	(0.00)	(0.052)	(0.042)	(0.030)	(0.071)	(0.057)	(0.062)
H_e	0.945	0.916	0.951	0.916	0.755	0.844	0.840
P	0.100	0.550	0.018	0.754	0.264	0.684	0.766
Shetland	*n*=11						
A	17	14	11	9	5	8	8
a_e	15.13	10.52	6.72	6.54	3.36	4.94	4.65
H_o	1.00	0.818	0.818	0.818	1.00	0.909	1.00
(S.E)	(0.00)	(0.12)	(0.12)	(0.12)	(0.00)	(0.09)	(0.00)
H_e	0.978	0.948	0.918	0.887	0.736	0.836	0.823
P	1.000	0.181	0.720	0.167	0.513	0.510	0.714
Ardtoe(F_1)	*n*=26						
A	15	16	13	11	6	9	10
a_e	9.20	7.60	6.60	8.40	3.23	2.36	3.69
H_o	0.923	0.808	0.808	1.00	0.577	0.615	0.808
(S.E)	(0.052)	(0.077)	(0.077)	(0.00)	(0.097)	(0.095)	(0.077)
H_e	0.909	0.885	0.865	0.898	0.704	0.587	0.743
P	0.011	0.081	0.714	0.417	0.408	0.484	0.931

N: Number of individuals in sample, A: Number of alleles, a_e: Effective number of alleles, H_o: Observed heterozygosity, H_e: Expected heterozygosity, P: Probability of exact test for departure from HWE (Considered significant at $P<0.05$).

Having separated the parental population into its three composite fractions it would seem that the significant deviation from HWE was caused by an excess of homozygotes at alleles at the *Hhi*C17 locus in the Icelandic group. It is important to note though that the sample sizes are relatively small thus inferences made should be treated with caution. Significant deviations from HWE were also observed at both *Hhi*-3 and *Hhi*-53 in the group from Ardtoe. There was an excess of heterozygotes with *Hhi*-3 and a deficit in *Hhi*-53. These deviations from Hardy-Weinberg equilibrium were not unexpected as these animals were first generation

farmed stock from parents that had been subject to some level of artificial selection and thus non-random mating.

The table also shows that, on average, both wild parental populations, Iceland and Shetland, are more polymorphic than the individuals from the Ardtoe group. All the polymorphic indicators were markedly different between the Icelandic group and the Ardtoe group, however the differences between the Shetland and F_1 Ardtoe group were not as large.

The Icelandic parental group had a higher number of effective alleles than the Shetland but this was not significantly different. The number of alleles (A) was notably different between the Icelandic and Shetland group but this could be due to the fact that there were more representatives in the Icelandic group (more than double the number). It should however be noted that the number of alleles across all loci in the Shetland group were very high considering the small number of fish within the group. Also the apparent reduction in levels of polymorphism between the first generation farmed fish from Ardtoe and wild groups suggest that a level of selection might have occurred in the parental population at Ardtoe.

2.3.3 Allelic Frequencies in the Parental Generation

Comparisons of allele frequency distributions between each of the parental populations are presented in Figures 2.6 and 2.7 below. The histograms for a few of the loci suggested differences between the parental stocks. Thus tests to assess both genic and genotypic differentiation between each pair of populations were conducted using GENEPOP version 3.3 (Raymond and Rousset 1995). Across all loci the only significant differences detected were between the Ardtoe group and

both wild groups ($P<0.001$). None of the differences between the wild groups, Shetland and Iceland, were significant.

Although these values are significant the sample sizes are too small to make confident conclusions about differences between wild populations but they do suggest that there is no indication of population sub-structuring and noteworthy effects of hatchery practices between farmed and wild stock.

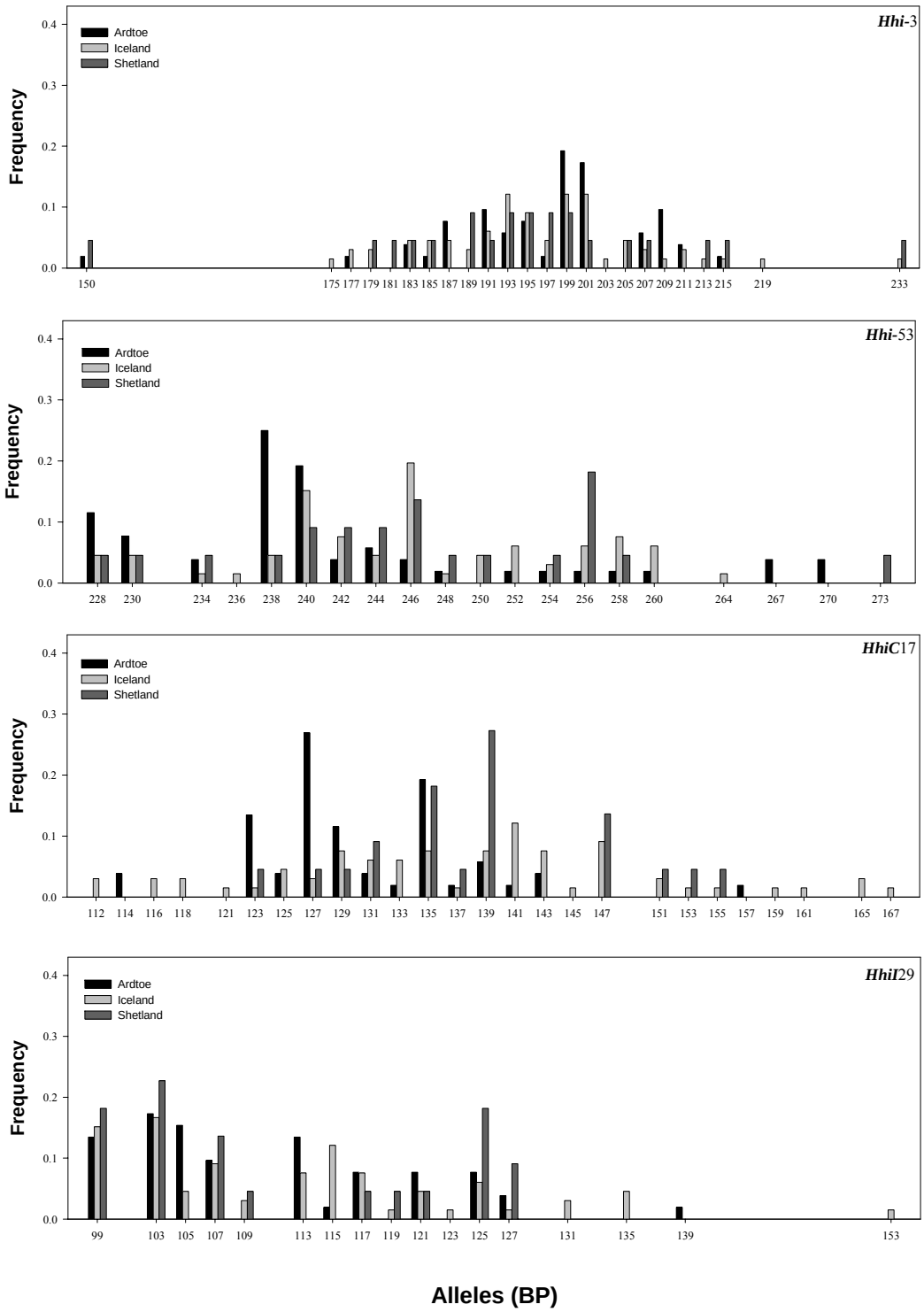

Figure 2.5 Frequency distribution of alleles within the three different parental populations (Ardtoe, Iceland and Shetland) at H*hi*-3, H*hi*-53, H*hi*C17 and H*hi*I29.

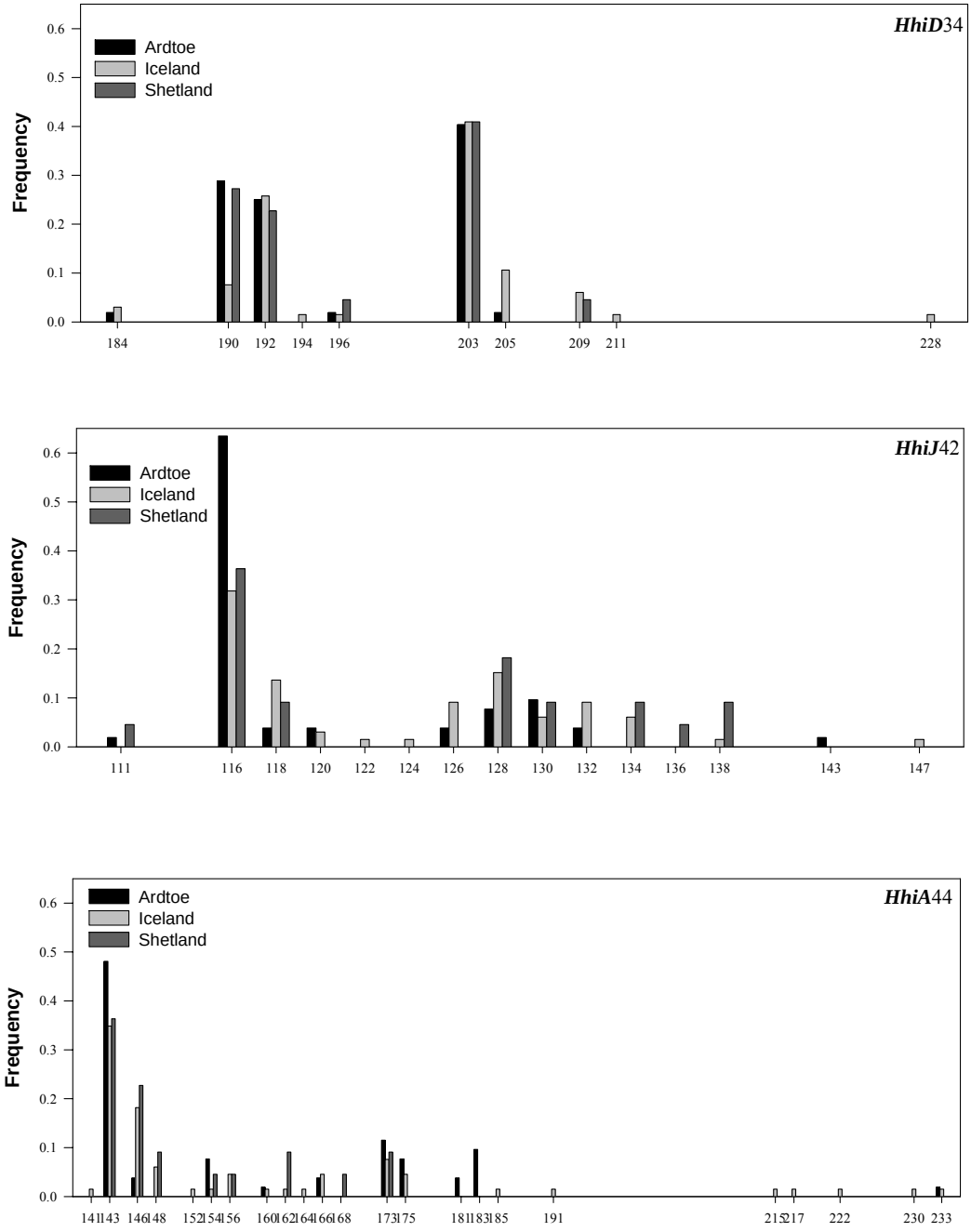

Figure 2.6 Frequency distribution of alleles within the three different parental populations (Ardtoe, Iceland and Shetland) at H*hi*D34, H*hi*A44 and H*hi*J42.

2.3.4 Genetic Variation in the F_1 Generation

The offspring group was made up of 270 individuals from the 1995 year class and 532 from the 1998 year class. As with the parental generation high levels of polymorphism were observed in the F_1 generation. Observed heterozygosities ranged from 0.675 to 0.940. The number of alleles per locus ranged from eight to twenty three.

2.3.5 Comparison Between Parental and F_1 Generations

When the genetic variation in the F_1 generation was compared with that of their parents, a general trend towards the loss of diversity was observed. Table 2.6 shows a comparison in polymorphic indicators between the parental and offspring generations. The mean heterozygosity estimates over all loci were lower in the F_1 generation (both observed and expected). Five out of the seven loci showed lower values for both H_o and H_e in the first generation of farmed individuals but none of these differences were significant.

In *Hhi*C17 the observed heterozygosity was higher in the F_1 population and in HhiA44 both the observed and expected heterozygosities were higher in the F_1 population. Although heterozygosity is widely used as a measure of genetic variation it is very insensitive for multiallelic loci. For this reason Allendorf and Ryman (1987) defined the parameter Allelic diversity (A_d) as a measure of the proportion of genetic variation remaining based on the number of alleles retained at a polymorphic locus (see materials and methods). Alleleic diversity ranged from 0.73 to 0.88.

The actual number of alleles per locus decreased substantially across all seven loci. In some cases up to 6 alleles were lost including alleles of medium frequency

(≥0.03); the average number of alleles lost per locus was 4.14. It is however important to recognise that not all the offspring produced on the farm in both the 1995 and 1998 year classes were sampled and some of the alleles that were not detected may still be present in the F_1 population on the farm but in low frequencies. There were also marked reductions in the effective number of alleles, but at the *Hhi*A44 locus it was higher in the F_1 population. Interestingly this was also the locus with the lowest value for A_d indicating that more alleles were lost at this locus.

Table 2.6 Comparison of polymorphic indicators between parental and offspring generations.

Locus	Population	A (A_d)	a_e	H_o	H_e
Hhi-3	Offspring (F_1)*	18 (0.74)	10.26	0.940	0.907
	Parental	24	13.88	0.971	0.935
Hhi-53	Offspring (F_1)*	17 (0.84)	10.88	0.914	0.912
	Parental	20	11.70	0.855	0.921
*Hhi*C17	Offspring (F_1)	23 (0.88)	8.27	0.907	0.880
	Parental	26	13.53	0.871	0.933
*Hhi*I29	Offspring (F_1)	14 (0.81)	6.05	0.920	0.872
	Parental	17	9.95	0.957	0.906
*Hhi*D34	Offspring (F_1)	8 (0.78)	2.91	0.675	0.656
	Parental	10	3.73	0.743	0.737
*Hhi*J42	Offspring (F_1)	13 (0.86)	3.63	0.725	0.725
	Parental	15	4.21	0.786	0.768
*Hhi*A44	Offspring (F_1)	17 (0.73)	6.05	0.797	0.835
	Parental	23	5.05	0.857	0.912

A: Number of alleles, A_d: Allelic diversity, a_e: Effective number of alleles, H_O: Observed heterozygosity, H_E: Expected heterozygosity, *Used in second round of analysis, only 121 offspring sampled.

The allele frequency distributions between the parental and offspring generations are shown below in Figures 2.7 and 2.8. Most loci showed quite different distributions. In *Hhi*D34 for example the second most common allele in the offspring generation was 209 (frequency =0.19), however this allele was present in a comparably low frequency in the parental generation (0.04). This same pattern was repeated to varying degrees in *Hhi*C17 and *Hhi*I29. Generally across all loci in

the F_1 generation there was a trend towards a higher frequency of fewer numbers of alleles.

Allelic and genotypic frequencies over all loci were tested for differentiation between the parental and offspring generations using GENEPOP version 3.3 (Raymond and Rousset 1995). The results were highly significant ($P<0.001$) over all loci used in this study suggesting a considerable effect of hatchery practices on gene and genotypic frequencies. In natural populations a change in allele frequency, genetic drift, of the sort observed in this study will normally be accompanied by a loss of heterozygosity. However, if a small number of parents were involved in the establishment of a captive population it is possible that the heterozygosity at a single locus could be higher in the new population than in the founder population (Allendorf and Ryman, 1987)

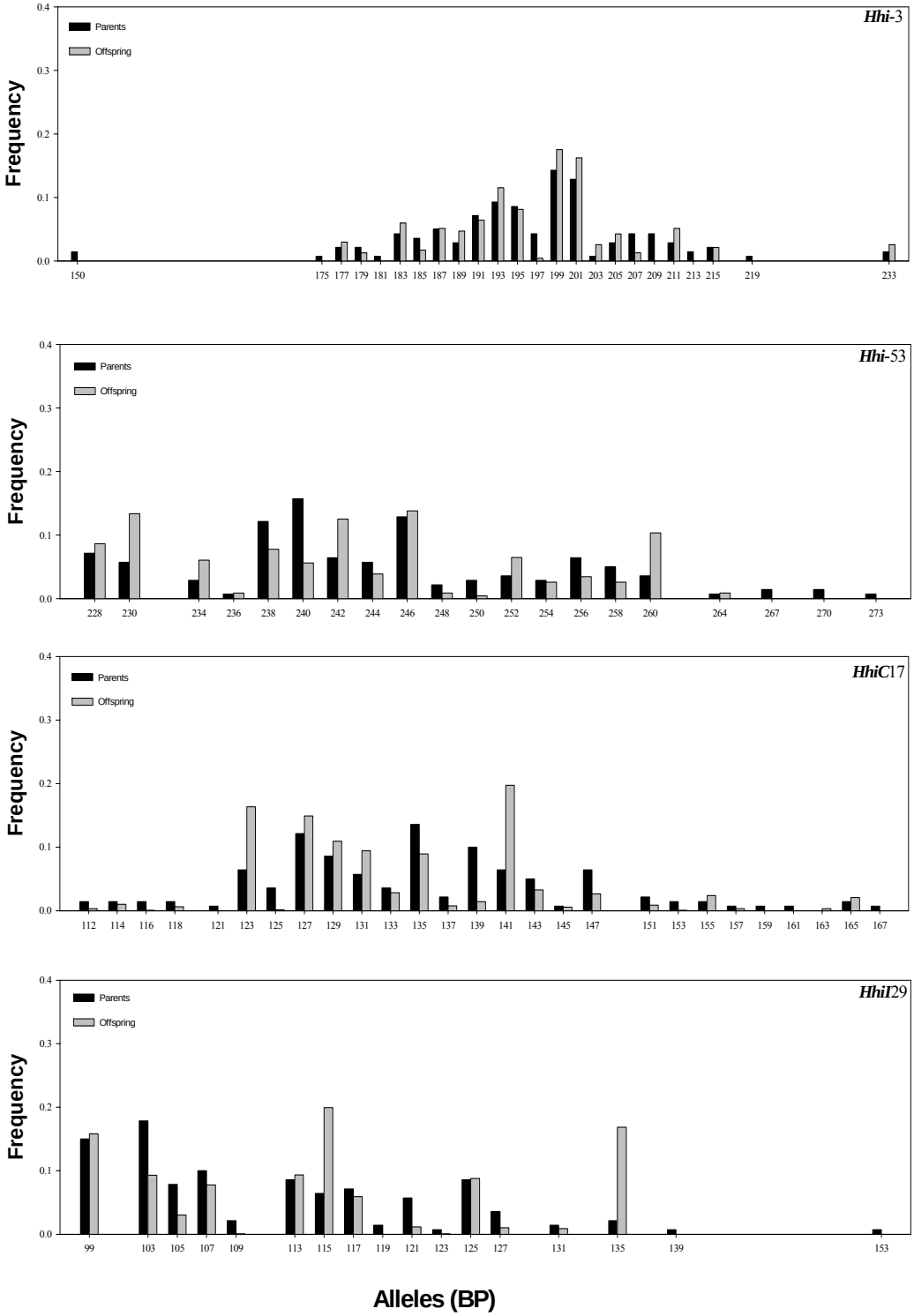

Figure 2.7 A comparison of the frequency distribution of alleles within the parental and offspring populations for H*hi*-3, H*hi*-53, H*hi*C17 and H*hi*I29.

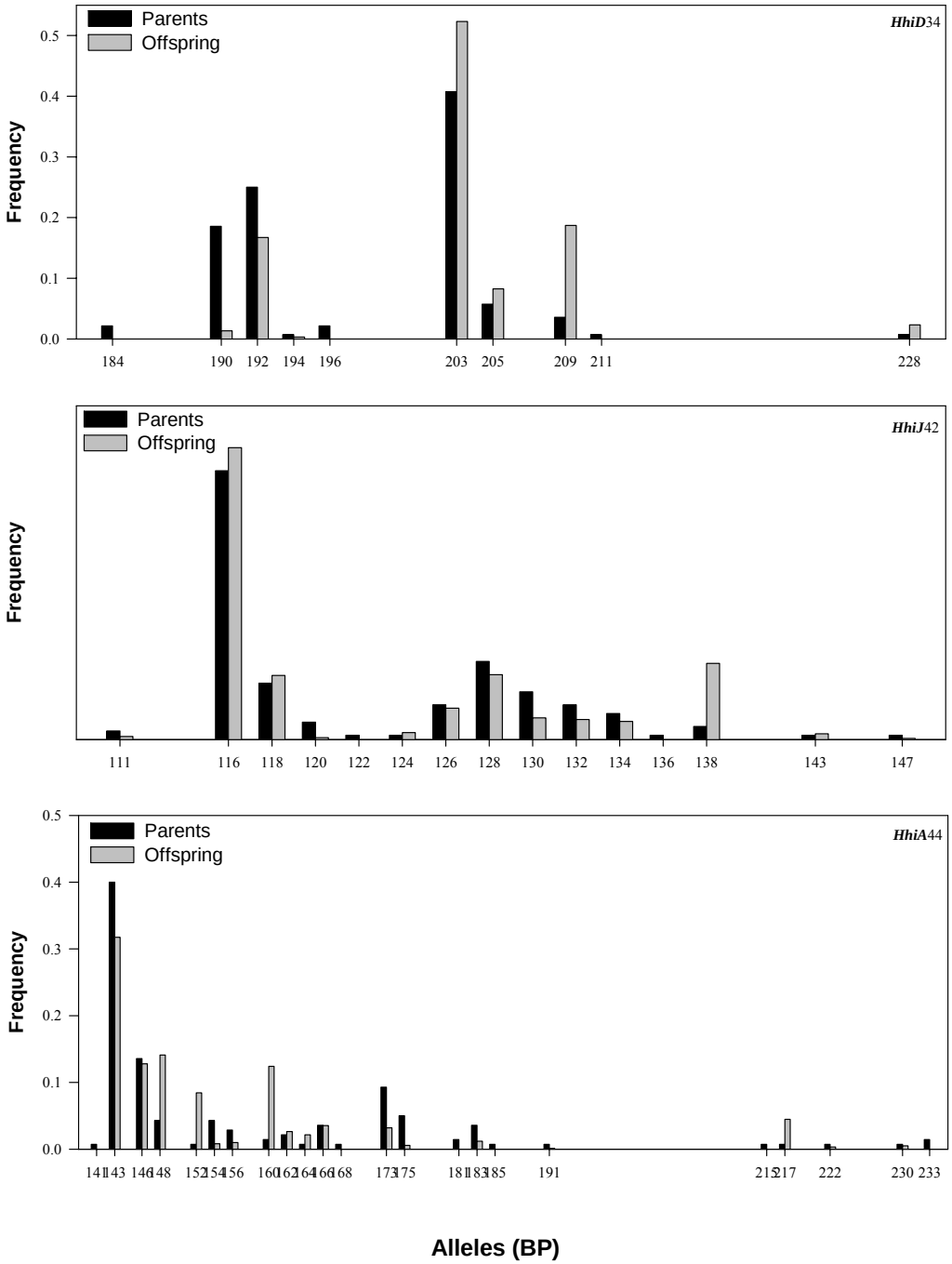

Figure 2.8 A comparison of the frequency distribution of alleles the parental and offspring populations for H*hi*D34, H*hi*J42 and H*hi*A44.

2.3.6 Hardy-Weinberg Equilibrium in the F_1 Generation

As expected all the loci in the F_1 generation showed highly significant deviations from Hardy –Weinberg equilibrium ($P<0.001$). In all but one case (*Hhi*A44) an excess of heterozygotes was observed. Deviations from Hardy-Weinberg equilibrium in an F_1 population following selection that results in reduced heterozygosity and allelic diversity will be expected to be towards an excess of homozygotes however this was not the case. The overall inbreeding coefficient, Weir and Cockerhams' (1984) estimation of the $F_{IS,}$ for all loci in the F_1 generation is shown in Table 2.7 below.

Table 2.7 Probability value of Hardy-Weinberg deviation test and estimate of F_{IS} for all loci in the F_1 generation.

Locus	Probability value (SE)	F_{IS}
Hhi-3	<0.0005 (<0.005)	-0.037
Hhi-53	<0.0005 (<0.005)	-0.002
*Hhi*C17	<0.0005 (<0.005)	-0.031
*Hhi*I29	<0.0005 (<0.005)	-0.056
*Hhi*D34	<0.0005 (<0.005)	-0.028
*Hhi*J42	<0.0005 (<0.005)	-0.001
*Hhi*A44	<0.0005 (<0.005)	0.046
Total	<0.0005 (<0.005)	-0.016

2.4 Discussion

The level of allelic variability in the parental (base) population and their F_1 offspring were assessed and compared at 7 microsatellite loci. The study revealed that the parental generation which was comprised of three different stocks was genetically diverse. After one generation of artificial rearing practises substantial reductions in genetic variation were observed. This was evident as a loss of allelic diversity but not as a decline in overall heterozygosity.

2.4.1 Polymorphic Parameters and Hardy-Weinberg Equilibrium

Table 2.8 below is a summary of the comparisons between values obtained in this study for polymorphism in the parental generation and those published by other authors using the same loci in wild captured populations. Whilst it is difficult to make fair comparisons, because laboratory and analytical procedures were not standardised across research groups, these results show that the loci used are at least as polymorphic in the population studied as reported by other authors.

The values obtained from the Ardtoe group were excluded because they were not wild fish but first generation hatchery stock. It worth noting that on average, the number of alleles per locus observed in the current study, across populations, was higher than those published in other studies (Table 2.3). These differences could be due, in part, to the fact that the number of individuals observed in this study was higher and the population was made up of individuals from different genetic origins.

All loci used in the analysis of the parental population apart from *Hhi*C17 were seen to be in HWE. The departure from equilibrium was due to an excess of homozygotes at certain alleles in the Icelandic population. This study was,

however, limited by small sample sizes between populations and results such as these could have occurred by chance alone.

There was no evidence to suggest that any population substructuring between the parental groups but the F_1 Ardtoe group appeared to be less polymorphic. Studies looking at genetic differences in geographically distinct Atlantic halibut populations have given variable results.

Table 2.8 Comparisons of the levels of polymorphism observed in captured wild halibut between fish in the current study and published estimates.

Locus	Study	*n*	A	H_o	H_e	Origin
Hhi-3	Stefánsson *et al*. (2001)	54	24	0.93	0.93	Norway
	Coughlan *et al*. (2000)	20	16	0.95	0.88	Iceland
	This study	33	22	1.00	0.95	Iceland
	This study	11	17	1.00	0.98	Shetland
Hhi-53	Stefánsson *et al*. (2000)	47	16	0.85	0.87	Norway
	Coughlan *et al*. (2000)	20	14	0.90	0.94	Iceland
	This study	33	17	0.91	0.92	Iceland
	This study	11	14	0.82	0.95	Shetland
*Hhi*C17	Stefánsson *et al*. (2001)	51	21	0.86	0.94	Norway
	McGowan and Reith (1999)	55	22	0.89	0.95	Canada
	Jackson *et al*. (2003)	52	23	0.90	0.95	Canada
	This study	33	24	0.94	0.95	Iceland
	This study	11	11	0.82	0.92	Shetland
*Hhi*I29	McGowan and Reith (1999)	55	14	0.85	0.86	Canada
	Jackson *et al*. (2003)	52	14	0.89	0.87	Canada
	This study	33	16	0.97	0.92	Iceland
	This study	11	9	0.82	0.89	Shetland
*Hhi*D34	McGowan and Reith (1999)	55	9	0.80	0.73	Canada
	Jackson *et al*. (2003)	52	10	0.81	0.74	Canada
	This study	33	10	0.79	0.76	Iceland
	This study	11	5	1.00	0.74	Shetland
*Hhi*J42	McGowan and Reith (1999)	55	13	0.67	0.79	Canada
	Jackson *et al*. (2003)	52	15	0.71	0.79	Canada
	This study	33	12	0.88	0.84	Iceland
	This study	11	8	0.91	0.84	Shetland
*Hhi*A44	McGowan and Reith (1999)	55	18	0.87	0.86	Canada
	Jackson *et al*. (2003)	52	18	0.89	0.86	Canada
	This study	33	20	0.85	0.84	Iceland
	This study	11	8	1.00	0.82	Shetland

n: Number of individuals sampled, A:Number of alleles, H_O: Observed heterozygosity, H_E: Expected heterozygosity, Origin; geographical origin of stock

Stefánsson *et al*. (2001) reported significant pair wise strain differences between Icelandic, Norwegian and Canadian fish stocks, however no significant differences were found in a separate study between three Canadian populations and an Icelandic one (M. Reith pers comm.). Differences in growth performance and disease resistance between strains have been reported between fish from these three regions (Imsland *et al*., 2002; Jonassen *et al*., 2000b). If similar results are obtained for these performance characters under commercial (farm) conditions and sufficient genetic data is available to support the findings, they present an excellent opportunity for between strain selection.

2.4.2 Genetic Variation in the F_1 Generation

One objective of this chapter was to quantify the allelic variation between the parental and F_1 broodstock on the farm and detect the impact of hatchery practice on the breeding population by comparing this with the parental generation. After one generation of selection it would seem that there was a considerable reduction in genetic variation. Genetic variation defined by heterozygosity showed no significant difference between the parental and F_1 generations whereas allelic diversity did. It also appears from differences in allelic distributions that the two groups are becoming genetically distinct.

The contradictions between heterozygosity and allelic diversity are because the parameters utilized measure different things (Butler and Cross, 1996). Artificial breeding practices result in a decrease in genetic variability in terms of allelic diversity, which is not necessarily detected by measures of heterozygosity (Norris *et al*., 1999). This is because fewer numbers of breeders are selected as broodstock than would normally happen in nature, thereby reducing the number and frequency

of alleles present in the offspring generation with less effect on the number of heterozygotes present at these loci (heterozygosity). Thus heterozygosity is insensitive and therefore not an appropriate indicator of polymorphism in studies examining differences in genetic variation between wild and farmed populations (Butler and Cross, 1996; Norris *et al*. 1999).

Reductions in the levels of genetic variation between farmed fish and their wild counterparts have been reported in various studies involving a wide variety of species. It would seem that in aquaculture systems there is a steady decline in genetic variation from the first generation onwards after the establishment of the base population. In Atlantic salmon Koljonen *et al*. (2002) estimated that in a short term breeding programme (2 generations) in Finland the observed average rate of allele loss was 4.7% per generation. Similar results were obtained with other salmonids, the Sea trout (Was and Wenne, 2002) and rainbow trout (Butler and Cross, 1996) to name two. Evans *et al*. (2004) estimated a 62% reduction in the number of alleles when they compared an F_1 population of abalone with wild stock. Xu *et al*. (2001) compared the genetic diversity between wild and cultured tiger shrimp, and also found significant differences in the number of alleles. In flatfish, Coughlan *et al*. (1996) reported a loss of rare alleles in a farmed strain of turbot but and two separate studies in the past three years have examined the impact of captive rearing on the genetic variation in the halibut.

Stefansson *et al*. (2001) conducted a study to determine the effects of artificial rearing on genetic variability of both turbot and halibut from 3 different geographical locations using microsatellites. They found all the F_1 individuals from the three locations were out of HWE. In all cases an excess of heterozygotes was

observed. The halibut from Canadian and Icelandic hatcheries in their study all exhibited losses of genetic variation between the wild parents and cultured stocks. There were no significant differences in heterozygosity but there were significant differences in the number of alleles. Mean Allelic diversity (A_d) in each group was 0.60±0.12 (Canada), 0.83±0.08 (Iceland) and 0.88±0.09 (Norway).

Jackson *et al*. (2003) looked at the genetic variation between wild and cultured stocks in the Canadian industry. Following a prior selection of 27 (13 males and 14 females) broodstock parents from a wild capture of 52 individuals, they found no significant losses in either heterozygosity or number of alleles between the parental and F_1 generation. However significant differences were found in allelic diversity between the 52 original wild caught individuals and the F_1 group, in that 26% of alleles were lost. They noticed a highly significant difference in genotype frequencies between the parental and three separate F_1 groups. In both studies it was suggested that the populations showing losses in allelic diversity had been through a bottleneck.

The results obtained in the present study are very similar to those of previous authors looking at the effect of artificial rearing in genetic variation in the Atlantic halibut and indeed other farmed aquatic species. There was an insignificant reduction in observed heterozygosity (1.5%) and a highly significant reduction (18.56%) in the number of alleles. Mean allelic diversity (A_d) was estimated at 0.82±0.05. Deviations from HWE were expected as certain conditions required for a population to be in HWE were not met i.e., the number of breeders was not "large" and mating was certainly not "random" as a level of selection was practiced. The direction in which the departure occurred though, towards an excess of

heterozygotes, was unpredicted, especially when the number of available parents is considered.

Heterozygosity excess or deficit can occur after a recent change of the effective population size and populations exhibiting significant excess would be considered as having experienced a recent genetic bottleneck (Cournuet and Luikart, 1996). This is because when a population goes through a bottleneck many low frequency alleles are eliminated. These rare alleles contribute little to heterozygosity and in this situation the heterozygosity measured at a locus will exceed the expected heterozygosity computed from the number of alleles sampled assuming mutation and drift equilibrium (Cornuet and Luikart, 1996; Norris *et al*. 1999). If this information is considered with the significant differences in the distributions of alleles between the parental and F_1 generations, the deviations from HWE may be indicative of small founding population size. The heterozygote excess due to a bottleneck event may be calculated using the program Bottleneck (Cornuet and Luikart, 1996) however data from a larger number of polymorphic loci (>20) is required. Using microsatellites the parentage of each individual F_1 offspring can be determined and the effective population size estimated with precision. When the pedigree structure of the population on the farm is determined an exact picture of whether or not a bottleneck has occurred within the population will be known.

Several authors have reported the loss in genetic variation between wild and cultured halibut populations using microsatellites. Reduction in genetic variability was demonstrated using allelic diversity because it is more sensitive than heterozygosity to population bottlenecks.

These differences in genetic variation observed between the parental and offspring generation may be a result of selection, using a limited number of broodstock as parents. In aquaculture systems the loss of genetic variation has been attributed to these causes. Whatever the case it is important to avoid losses of genetic variation because in many cases there is a positive relationship between variability and viability (Allendorf and Ryman 1987). Nonetheless the ultimate goal in aquaculture breeding programmes is to select fish that perform maximally under aquaculture systems and this process, if not properly managed, will inevitably lead to reduced variability and a loss of genetic diversity. Here lies the problem for many geneticists, particularly aquaculturalists, who work with highly fecund organisms; finding the balance between genetic gains by high selection intensities and maintaining genetic variation.

It is obvious from these findings that some element of domestication or husbandry practice selection may have led to changes in the genetic composition of the F_1 stock. The source of this is yet unknown, but could be due to genetics, biological limitations of the fish to adapt to their new captive environment, the environment, poor husbandry practices, or all. The contribution of each fish to the offspring generation will be the subject of the next chapter. It is possible that insufficient numbers of parents were used as broodstock or the breeding regime may be inappropriate to minimize genetic drift. Artificial selection can reduce the effective population size by using only individuals with certain attributes as broodstock. This was demonstrated in the Canadian hatchery where the number of broodstock was effectively halved prior to the establishment of the F_1 population.

Chapter 3

Parental Assignment

3.1 Introduction

In 1976 a group of scientists from the Hebrew University in Jerusalem published a paper in the journal *Aquaculture* (Moav *et al*., 1976) and in it claimed that "the need for parental identification in fish breeding by genetic markers was so acute that in its absence no serious breeding work beyond basic mass selection could be effective". They also proposed that due to their high fecundity, fish should become models for the applications of genetic markers to animal breeding. While their claim might have seemed immoderate at the time, and consequently proven wrong, today the application of microsatellite markers for parentage determination in aquaculture is gaining both acceptance and popularity. It would however take almost 20 years for their vision to be realised. Herbinger *et al*. (1995) were the first to assess the feasibility of establishing pedigrees in mixed aquaculture populations under commercial conditions. They successfully assigned the parentage of a group of rainbow trout from complete factorial crosses between ten sires and ten dams after one year of communal rearing, using microsatellite markers.

3.1.1 The Need for Genetic Profiling In Aquaculture

Unique individual and pedigree identification is central to the framework and success of all selection programmes. Without the ability to routinely identify better performing individuals correctly the exercise of selective breeding is futile. Secondly the performance of these individuals needs to be related to their relatives' performance otherwise the process can be compromised. This is because in order to evaluate the genetic merit (breeding value) of selection candidates accurately techniques are employed that rely on pedigree information. In the last 15 years BLUP (Best linear unbiased prediction) techniques, using an animal model have become

standard methodology for large scale selective breeding programmes. The animal model uses information on all animals within a pedigree, tracing genetic links via a relationship matrix, to provide solutions for the genetic merit for each individual within the pedigree.

The greatest problem in implementing a selective breeding programme in aquaculture is that newly hatched aquatic animals, unlike farmed terrestrial species, are too small to be tagged, uniquely identified, and subsequently pedigreed, by physical methods. In order to circumvent this problem and apply more efficient and sustainable selection techniques, progeny from family groups are reared for long periods in separate facilities until the animals are large enough to be tagged and thus identified at the family or individual level. This approach limits the number of families available for selection and is cost, space and labour intensive.

Whilst the construction of large hatchery family unit facilities was possible for the genetic evaluation of many separate families in some government funded research institutes, they are limited in commercial production farms and proved to be a deterrent for small scale producers wanting to start broodstock selection or replacement programmes. Furthermore, this approach introduces environmental effects common to full-sibs which are confounded with the genetic effects under study, therefore statistically adequate experiments tend also to be large and expensive (Doyle and Herbinger, 1994; Herbinger *et al*., 1999). In addition separate family units cannot be used in species with mass spawning and complex mating systems such as the cod and sea bream. The alternative, as pointed out by Moav *et al*. (1976), was mass selection which is simple and relatively cheap to perform. However, the estimation of genetic merit is much less accurate with this system and it impossible to

assess the genetic variability present in the chosen broodstock population or the relationships between the selected breeding candidates. Thus pedigree information is not only necessary for the estimation of genetic merit of breeding candidates but also for the genetic management of broodstock populations, to minimise the deleterious effects of inbreeding associated with mating related individuals, and to prevent the unnecessary loss of genetic variation. This risk of inbreeding is particularly high in aquaculture due to the high fecundity of most of the cultured species. In its most extreme form it is possible that the offspring of a single mating could account for an entire generation (McDonald *et al*., 2004).

An alternative approach to mass selection, as suggested by Harris *et al*. (1991) and Doyle and Herbinger (1994) amongst others, involves the application of genetic profiling, using molecular markers, to offer solutions to many of these problems. This presents the opportunity to allow communal rearing, high selection intensities, the avoidance of inbreeding and the use of complex genetic merit evaluation techniques. They suggested the pooling of progeny from multiple families at hatching and rearing them communally. This maximises the number of families available for testing and increases the potential for higher selection intensities while allowing a combination of individual and family selection. Individuals could then be retrospectively assigned to family groups using molecular markers and pedigree structures could then be constructed. A lot of work has been focused on the practice of this theory in aquaculture and successful reports have been reported for a number of commercially important species including the rainbow trout (Herbinger *et al*., 1995), Atlantic salmon (O'Reilly *et al*., 1998), turbot (Estoup *et al*., 1998) and shrimp (Jerry *et al*., 2004). While these studies centred on parental assignment, other authors considered the potential for using pairwise estimates of relatedness to avoid the risk of inbreeding

depression (Norris *et al*. 2000; McDonald *et al*., 2004). The results of these studies and others like these will be discussed later but it is important first to consider the statistical and analytical methods employed in pedigree analysis using molecular markers.

3.1.2 Statistical Methods of Genetic Profiling Used in Aquaculture

Molecular pedigree analysis is the use of molecular data to infer pedigree; this includes all methods that test hypothesised relationships between individuals (e.g. putative sibships or parent-offspring relationships) and the estimation of the level of relatedness between them (Wilson and Ferguson, 2002). The current review will deal primarily, but not exclusively, with the former.

Molecular pedigree analyses began with the advent of DNA fingerprinting where individual animals could be identified from genetic data by the composite genotype at polymorphic VNTR loci (Jeffreys *et al*., 1985). Due to the fact that no two individuals, with the exception of identical twins and clones, are genetically identical, these differences, reflected in the genes, are identified as unique DNA profiles and compared between individuals and families (Hånstein *et al*., 2001). The critical parameter for the feasibility of pedigree analysis is the degree of polymorphism within the population (Plasbøll, 1999). Although parentage assignment can be achieved by any type of genetic marker, provided it is sufficiently polymorphic, dominant markers are less efficient than codominant markers (Geber *et al*., 2000) and microsatellites provide the best results, since genetic variation among individuals can be extremely high with these markers. The large numbers of alleles segregating at many loci make obtaining unique genotypes for every individual in a study feasible (Wright and Bentzen, 1995; Liu and Cordes, 2004).

The earliest and simplest assignment technique used in parentage analysis is exclusion. This method is based on Mendelian rules of inheritance and uses incompatibilities between parents and offspring to reject particular parent-offspring pairs. Given a codominant marker, two individuals related as parent and offspring will share at minimum of one allele at each locus. In order to assign parentage, multi locus genotypes of offspring are compared with all possible parent-pair genotypes. Parents that could not have produced the offspring's genotypes are excluded leaving one or more pairs of individuals assigned as possible parents (Villanueva *et al*., 2002; Wilson and Ferguson, 2002; Jones and Arden, 2003). With sufficent genotypic data and sampling of all parents, it is possible to assign any given offspring to a single parental pair. This is an appealing approach because exclusion of all but one parental pair from a complete sample of all possible parents for each offspring can be considered perfect. It is most practical when there are a few candidate parents and highly polymorphic loci available. One of the potential weaknesses of this system is that genotyping errors (e.g. null alleles and mutations) will contribute to false exclusions of true parents. These problems become more acute as more data are included in the analysis because the likelihood of erroneous genotyping and mutations increases (Jones and Arden, 2003). Also difficulties arise when the available genotypic data (number of alleles and or loci) are insufficient to exclude more than one parent or parental pair. This can occur if the number of candidate parents becomes too large so that the number of loci needed becomes impractical. In these instances, likelihood methods can be employed.

Progeny can also be assigned to non-excluded parents based on likelihood scores derived from their genotypes. Likelihood approaches to parentage assignment occurs in two ways, either categorically or fractionally. Although the basic principles are the

same, the former assigns the entire offspring to a particular parent while the latter divides an offspring among putative parents.

Likelihood methods involve calculating Log-likelihood ratios (LOD score) for each offspring. This is done by determining the likelihood of an individual or pair of individuals being the parent(s) of a given offspring divided by the likelihood of these individuals being unrelated. After an iterative process of evaluating all genetically possible parents, offspring are assigned to the parent(s) with the highest LOD score. Unlike with exclusion methods, likelihood-based allocation methods usually allow for some degree of transmission errors due to genotyping or mutation and incomplete sampling of parental candidates (Marshall *et al*., 1998). Categorical allocation selects the most likely parent(s) from a pool of non-excluded parents while the fractional approach assigns some fraction, between 0 and 1, of each offspring to all non-excluded candidate parents. The portion of an offspring allocated to a particular candidate parent is proportional to its likelihood of parenting the offspring compared to all other candidate parents (Jones and Arden, 2003).

There are several software packages available for performing pedigree analyses using both exclusion, e.g. FAP (J. Taggart, unpublished), PROBMAX (Danzmann, 1997) and likelihood, e.g. PAPA (Duschesne *et al*., 2002), CERVUS (Marshall *et al*., 1998) for parentage analysis and other packages for relatedness, e.g. KINSHIP (Goodnight and Queller, 1999). These packages differ with respect to the types of problems that they are designed to answer and most have been reviewed by Wilson and Ferguson (2002) and Jones and Arden (2003). Whatever the package used and method it employs, it appears that the accuracy of assignment is dependent on the amount of information available. Therefore the more complete the data set the higher the

degrees of success. Bernatchez and Duchesne (2000) demonstrated in a simulation study that the probability of assigning offspring to a parental pair is dependent on the number of loci used, the average number of alleles per locus and the number of candidate parents. A simulation study by Villanueva *et al*. (2002) showed that not only is the number of parents involved important but also the mating structure. They found that predictions based on exclusion probabilities are accurate provided that the number of parents involved in the crosses was large. The power of discrimination increased with the number of parents, for a given number of crosses, and it was substantially reduced when the number of crosses increased from 100 to 400. For a given number of crosses the best predictions were with the highest number of parents involved in the crosses as the sampling of parents is less important when more parents are used. The most informative 4 microsatellites developed for Atlantic salmon were sufficient to assign at least 99% of offspring to the correct pair when 100 males and 100 females were used to produce 100 crosses. An additional locus was required to correctly assign 99% when only 10 males and 10 females were used for the same amount of crosses. The differences among different mating schemes in the power to discriminate among crosses were because of the differences in the number of breeders. With a small number of breeders there will be a higher chance of losing alleles i.e. the frequency of heterozygotes will decrease, as a consequence of random drift, making the levels of polymorphisms lower, leading to a requirement for more loci.

3.1.3 Studies Using Genetic Profiling in Aquaculture.

In rainbow trout Herbinger *et al*. (1995) reported that 91% of 873 offspring resulting from a complete factorial cross of 10 sires and 10 dams were assigned, using exclusion methods, to one or two parental pairs, out of a possible 100, with four or

five microsatellites. Using 14 microsatellites Fishback *et al*. (2002) were able to assign 93.3% of offspring reared under commercial hatchery conditions from the mating of 2 sires and 48 dams. They found highly significant differences in family structure, i.e. number of offspring assigned to each dam and each parental pair, with 12 females not having any offspring assigned to them at all. In the Atlantic salmon, O'Reilly *et al*. (1998), also using exclusion techniques were able to assign 99.5% of 792 offspring from 12 full sib families communally reared after fertilization (each of 12 males was crossed with one of 12 females) with four microsatellite markers. In the same population, using the same analytical techniques, Herbinger *et al*. (1999) obtained 100% assignment from 12 full sib families. In addition, they observed statistically significant differences in rates of survival at various stages of juvenile production, through first feeding to ongrowing. In sea bass Garcia de Leon *et al*. (1998) reported that 781 offspring from a complete factorial design of 3 sires and 3 dams were unambiguously assigned using only two microsatellites. In their experiment they noted differential representation between individuals and significant sire and dam effects on survival. However due to the small size of their study a larger sample size was needed to support their findings. Also using exclusion techniques Perez-Enriquez *et al*. (1999) were able to assign 73.5% of 200 offspring using 5 microsatellites from a population of 250 potential parents in two mass spawning tanks of red sea bream. Although no significant parental effects were observed in family structures, only 91 breeders succeeded in contributing to the offspring generation in the sampled population. Using eight microsatellites Vandeputte *et al*. (2004) successfully assigned 95.3% of 550 offspring to a single parental pair from a complete factorial cross of 24 sires and 10 dams in the common carp. They observed unequal contributions of both sires and dams to the offspring generation despite a determined

effort to standardise egg volumes at the start of the experiment. Sekino *et al*. (2003) achieved 100% assignment from mesocosm spawned Japanese flounder offspring using exclusion methodologies. They were able to work out the parentage of 736 fish because unique alleles were found for each of the 18 potential parents for the 4 microsatellites used in the study. An interesting finding in their study was that more than 99% of the offspring were sired by a single male.

Using likelihood methods, Norris *et al*. (2000) obtained 98% assignment of 200 offspring from an Atlantic salmon group of 10 full-sib families (2 sires and 10 dams). Borrell *et al*. (2004) were able to assign the parentage of turbot broodstock populations in two different Spanish hatcheries. In the first hatchery 50 offspring were sampled from 5 full sib families (5 sires and 5 dams). In the second hatchery a multifactorial cross using 6 sires and 6 dams was used to make 11 full and half sib families. 110 offspring were sampled from this group. Eight microsatellites were employed in the analysis and in the first hatchery 81.2% of the offspring were assigned successfully while in the second hatchery only 38.7% of offspring were assigned. The reasons for the poor results obtained in the second hatchery were associated with the presence of null alleles and low levels of polymorphism in the hatchery population. A similarly poor result was obtained by Jerry *et al*. (2004) with Kuruma shrimp where only 47% of 98 offspring were assigned from the mating of 168 sires with 22 dams, using six microsatellites. They also used a likelihood approach and attributed the low level of success to the presence of null alleles. In both studies there were significant differences between expected levels of assignment obtained in simulations and those obtained with the real data set.

In situations where no genetic information is available on potential parents, the coefficient of relatedness (r) among pairs of potential breeders can be estimated. This is the fraction of alleles in the genome that two related individuals share by descent. If this information is obtained for potential breeding candidates unrelated pairs of individuals can be identified based on their lower levels of estimated relatedness and inbreeding can, theoretically, be minimised. The relatedness between individuals in aquaculture populations of Atlantic salmon (Norris *et al*., 2000), white sturgeon (Rodzen *et al*., 2004) and trout (McDonald *et al*., 2004) have been determined, however the effectiveness and precision of this system is heavily dependent on number of loci employed and levels of polymorphism at each locus. Thus its use may be limited in populations which have gone through bottlenecks such as those often seen in aquaculture.

At present only one study has involved the use of genetic profiling to determine parentage in the Atlantic halibut. Jackson *et al*. (2003) reported the use of 5 microsatellites to successfully assign 98% of 145 hatchery reared F_1 individuals from the pooled gametes of 27 (13 sires and 14 dams) wild caught parents. They used an exclusion programme, PROBMAX, and found that the family structure in the offspring population was heavily skewed, consisting of a small number of large full and half sib families. A few individuals did not contribute at all to the F_1 generation.

Although studies involving the use of genetic markers for parentage assignment have reported high and promising levels of success, the results have been varied. Most of the published experiments have involved relatively few numbers of families and in commercial breeding programmes they are likely to incur the need for a greater number of loci in order to achieve high rates of assignment because the families

involved will be up to and over 10 times those reported. Furthermore because family identification is retrospectively assigned the geneticist has no control of family size. This could lead to high variances in family sizes and high genotyping costs associated with trying to even out the number of individuals per family. Also threats of substantial biases leading to high frequencies of false parentage exclusions due to null alleles, genotyping errors and mutations are significant (Dakin and Avise, 2004; O'Reilly *et al*., 1998). However, whilst not perfect, the use of this technology is undoubtedly a cost-effective, realistic and more informative alternative to separate rearing facilities

3.1.4 Effective Population Size and Pedigree Analysis

Once the pedigrees of the candidate offspring have been determined this information can be used to estimate genetic parameters such as heritabilities, evaluate genetic merit of breeding candidates and, as mentioned above, make management decisions particularly those involving the avoidance of inbreeding. Effective management of inbreeding, the mating of related individuals, cannot be overemphasised in aquaculture enterprises simply because the success of broodstock management programmes depends on it (Myers *et al*., 2001). The level of inbreeding, usually expressed as the percentage rate of inbreeding per generation, (ΔF), can be estimated through the direct measurement of changes in genetic variability (as seen in the previous chapter) or through pedigree analysis. In aquaculture an alternative measure of determining rate of inbreeding is adopted, the notion of effective population size (N_e).

The effective population size is a concept used to estimate the rate of inbreeding expected from small populations, when there are deviations from the idealised

population, including unequal numbers of males and females, non-random distribution of family sizes and unequal numbers of breeders in successive generations (Gall, 1987; Falconer and McKay, 1996). These conditions, as seen from the results of pedigree studies, ring true in aquaculture populations. The effective population size is directly related to the rate of inbreeding per generation, ΔF, such that:

$$N_e = \frac{1}{2\Delta F}$$ (Falconer and McKay, 1996)

In aquaculture populations, ΔF is sometimes estimated from the observed value of N_e based on results from the pedigree analyses using molecular data or from the number of sires (Ns) and dams (Nd) and additional information about variance in family sizes. The N_e is often lower than the total number of individuals in the parental generation because not every member in the parental generation successfully contributes to subsequent generations due to the biology of the species and the effect of the hatchery environment on individual reproductive performance and offspring survival. The magnitude of the N_e is increased or decreased depending on the size of founder members of populations, unequal sex ratios in breeding populations and variation in family sizes (Gall, 1987). The actual number of parents and the estimated effective population sizes of some aquaculture populations are shown in Table 3.1 below.

Table 3.1 A comparison between the actual number of parents (N), the number of sires (Ns), number of dams (Nd) and the estimated effective population sizes (N_e) in different farmed populations.

Species	N	Ns	Nd	N_e	Source
Japanese Flounder	18	6	12	3.3	Sekino *et al*. (2003)
Atlantic halibut	27	13	14	13	Jackson *et al*. (2003)
Gilthead seabream	48			14	Brown (2003)
Red seabream	91			63.7	Perez-Enriquez *et al*. (1999)
Common carp	34	10	24	22.3	Vandeputte *et al*. (2004)

The halibut hatchery population under study here is the largest broodstock group of wild caught and hatchery reared Atlantic halibut in the United Kingdom. Given the difficulties involved with the capture of wild broodstock this population represent an extremely valuable resource to the future of the British halibut industry. The results obtained in Chapter 2 show that a significant amount of genetic variation has been lost in only one generation of hatchery practices and in the absence of pedigree information the risk of mating related individuals may lead to inbreeding in the F_1 population of potential broodstock. The number of wild parental fish that succeeded in contributing to the F_1 population and how evenly the potential crosses were represented in the retained 1995 and 1998 F_1 groups is of major concern. This is because it could, potentially, set the upper limits to the amount of genetic variation available for a generation, which in the halibut is a considerable amount of time. The purpose of this chapter is to address these issues by using microsatellite profiling to identify the parentage of all the potential broodstock in the 1995 F_1 population and a select group of the 1998 F_1 group The rate of inbreeding will also be estimated using the effective population size to inform of potential problems.

3.2 Materials and Methods

All the information on the animals and laboratory procedures followed are described and outlined in Section 2.2 of the previous chapter.

3.2.1 Parentage Assignment

Parentage assignment was carried out using a programme called Family Assignment Program (FAP) version 3.0 (J. Taggart unpublished). FAP performs two tasks using exclusion principles. It predicts the resolving power of specific parental genotypic data sets for unambiguously discriminating among families and it assigns family of origin to progeny genotypic data. Both analyses performed in the programme assume a closed population- i.e. all individuals are the progeny of known parental combinations for which full genotypic data is available. It also assumes that the nuclear loci employed in the analyses are independently inherited in simple Mendelian fashion.

Due to the fact that no accurate breeding (fertilisation) records were made of the crosses used in the production of the populations examined and batches were mixed repeatedly throughout the production cycle the assumption was made that any single F_1 individual could be the offspring of any sire or dam in the parental population. Thus all 1176 potential crosses between the 28 sires and 42 dams needed to be examined. A utility program within the package, MERGE1.EXE, was used to generate all possible offspring genotypes using a factorial crossing design such that each female is mated to each male and vice versa. Parentage was then assigned to a pair of individuals by matching progeny composite genotypes to those of known parental crosses generated by the programme.

In order to account for genotyping errors two features are included in the programme, allele size tolerance (minor errors) and allele mismatch tolerance (major errors). Allele size tolerance compensates for allele measurement error. If allele size tolerance is set at zero the family and progeny alleles must be identical for a match to be recorded. However, for example, if it is set at 2 then alleles within a two base pair size tolerance or less would be considered a match. Allele mismatch tolerance allows imperfect matches to be identified, where no perfect matches have been found. This can be useful for detecting scoring errors, mutations or null alleles. Assuming allele mismatch tolerance is set to one when parentage is being assigned for each progeny, an attempt is made to assign family of origin from the full number of loci, if successful the program records match(es). If however a match is not found the progeny genotype data are reanalysed looking for a match for N-1 alleles where N is the total number of alleles employed in the analysis.

3.2.2 Estimation of Effective Population Size (Ne) and Inbreeding Coefficient (F).

The inbreeding coefficient can be estimated through direct experimental measures of changes in genetic diversity or through pedigree analyses (Myers *et al*., 2001). In aquaculture the effective breeding number or the effective population size (N_e) has been adopted as the measure by which inbreeding is estimated. N_e is the size of the ideal population that would undergo the same amount of random genetic drift, measured by the rate of loss of selectively neutral heterozygosity, as the actual population (Lande and Barrowclough, 1987). Estimating Ne is central to assessing inbreeding because it is inversely related to the rate of loss of genetic diversity and the rate of increase in inbreeding in a finite population (Falconer and Mackay, 1996; Arden and Kapuscinski, 2003);

N_e and subsequently ΔF was estimated using the method developed by Woolliams and Bijma (2000). The inbreeding coefficient was estimated using the effective population size, which was calculated from the fractional contributions of the male and female parents after Woolliams and Bijma (2000) and Brown (2003). This is because the precision of the N_e estimate is highly dependent on the variance in family size.

$$\Delta F = \frac{1}{2}\sum c_i^2 - \frac{1}{4}(\bar{c}_m)^2 - \frac{1}{4}(\bar{c}_f)^2$$

Where:

c_i = the fractional contribution of each parent

$\bar{c}_f$ = the average contribution of females

$\bar{c}_m$ =the average contribution of the males

3.3 Results

3.3.1 Missing Parent 64A

When all the offspring had been genotyped, an allele (217) was observed at the *Hhi*A44 locus that was not present in the parental population. Archive data of female spawning performance revealed that samples had not been collected from all the females present on the farm between 1994 and 1998 for genotyping. Furthermore because no genotypic data was generated for one potential female parent from the wild Icelandic population (see Chapter 2) and the new allele was present in 71 of the 802 sampled F_1 offspring, it was deemed authentic.

The complete set of alleles for the new parent was reconstructed from the genotypes of the individuals in which the new allele, at the *Hhi*A44 locus, was present. Inspection of the genotypes at each locus showed one or two alleles common to each of the 71 individuals at all loci. This was consistent with a single parental genotype and therefore with there being only one unknown parental individual.

When the reconstruction of the missing parent was complete the allelotype of the other parents were deduced. Two male individuals were found that repeatedly complemented the newly derived genotype, including one with a unique allele at the same locus, 164 at *Hhi*A44. This corresponds to the information on the farm that the missing individual was indeed a female.

Table 3.2 Genotype of missing female reconstructed from genotypes of offspring with new allele-217.

Locus	***Hhi*-3**	***Hhi-53***	***Hhi*C17**	***Hhi*I29**	***Hhi*D34**	***Hhi*J42**	***Hhi*A44**
Genotype	199-201	242-260	141-133	107-113	192-203	116-118	143-217

3.3.2 FAP Simulations

Using the five loci characterised by McGowan and Reith (1999) the programme predicted that, assuming equal family representation, 83.1% of the offspring would be unambiguously assigned to a family. The proportion of progeny, though, with distinctively identifiable genotypes for each family ranged from 0.05 to 1.00. However, 77.90% of all the families were expected to produce offspring in which ≥0.75 of their progeny had uniquely identifiable genotypes and 15.30% where all offspring had uniquely identifiable genotypes.

Increasing the number of loci to seven by including two, *Hhi*3 and *Hhi*53, of the 11 loci characterised by Coughlan *et al.* (2000) provided more resolution and the prediction improved by 14.30%. 97.40% of all offspring were expected to be unambiguously assigned to a family and the proportion of all progeny for each family with uniquely identifiable genotypes ranged from 0.44 to 1.00. With seven loci 99.90% of all the families were expected to produce offspring in which ≥ 0.90 of their progeny had uniquely identifiable genotypes.

3.3.3 FAP Results

The total number of offspring (F_1) genotyped was 802: 270 individuals were from the 1995 year class and 532 individuals from the 1998 year class. Based on the output from FAP each individual may be assigned to "no parental pair" (no match), "one parental pair" (single match), with zero or more than one mismatches (error tolerance), and "multiple matches" (more than one parental pair).

The initial scoring of alleles was likely have errors, therefore allele mismatches were used to highlight potential problems with either parental or offspring genotypes. The exercise revealed that 114 different alleles over all loci screened in the offspring were

spurious. The data set was then refined and the assignment was run with zero error tolerances and zero mismatches. Only fish in the "one parental pair" category were considered a positive match. All fish in either the "multiple match" or "no match" groups were classed as "unassigned"

3.3.3.1 Parentage assignment using five microsatellite loci

Due to experimental errors in the PCR procedures not every individual was screened at all 5 loci. Failure to amplify in PCR reactions resulted in 109 of the 802 offspring (13.59%) being screened at 4 out of the 5 loci. This was, in some cases, enough to assign parentage. Using the 5 original loci the application of genotyping results was successful at assigning 666 individuals (83.04%) unambiguously to a single parental pair.

Table 3.3The total number of offspring analysed using the five original loci and the partitioning of results into the various output categories from FAP.

Result	**Genotypes from:**					
	All 5 loci	4 loci No *Hhi*C17	4 loci No *Hhi*I29	4 Loci No *Hhi*D34	4 Loci No *Hhi*J42	4 Loci No *Hhi*A44
Single matches	598	6	42	2	14	4
Multiple matches	47	1	27	1	2	4
No matches	48	0	6	0	0	0
Total number analysed	693	7	75	3	16	8

Multiple matches in FAP mean that the individuals' genotypes matched more than one possible parental pair. There were 82 individuals in this group, approximately 10% of the sampled F_1 population, and reflects the resolving power of the loci used in the analysis within the parental population. As the original 5 loci were insufficiently informative two additional microsatellite markers *Hhi*-3 and *Hhi*-53 isolated by Coughlan *et al*. (2000) were screened for these individuals. They were also used in

cases where fish were typed for 4 out of the 5 loci and were not assigned to a single parental pair i.e. for individuals with incomplete genotypes.

3.3.3.2 Parentage assignment using seven microsatellite loci

A total of 121 F_1 animals were typed using the two extra loci. Included in the number were 8 previously screened, and assigned, at the original five loci to act as positive controls and 36 individuals in the "no match" category to act as negative controls. Unfortunately 5 fish in the "multiple matches" category were not included in this analysis due to personal errors. All the parents were typed at the two new loci and parentage was determined using FAP. Table 3.4 below shows a summary of the results.

Table 3.4 The total number of offspring analysed using seven microsatellite loci and the partitioning of results into the various output categories from FAP.

Result	Genotypes from:					
	All 7 loci	6 loci *Hhi*C17	6 loci *Hhi*I29	6 loci *Hhi*D34	6 Loci *Hhi*J42	6 Loci *Hhi*A44
Single matches	46	1	16	0	3	4
Multiple matches	2	0	4	0	0	0
No matches	34	0	10	1	0	0
Total number analysed	82	1	30	1	3	4

All eight positive controls used in the second round of the analysis assigned as they should have and gave the same results, parental pair, as when only five loci were used. 62 new individuals were assigned to a single parental pair using the two new loci. This, excluding the positive controls, accounted for 51.23% of the sampled population. They all came from the original "multiple matches" category within the "unassigned" group in the first round of the analysis shown in Table 3.3.

3.3.3.3 Unassigned fish

After both rounds of analysis 75 individuals were unassigned. Twenty one fish were assigned to more than one parental pair (multiple families) and fifty four were not assigned at all (no match) because their genotypes did not match any of the 1176 family combinations possible from the available parental genotypes.

A "no match" can result from erroneously typed parental or offspring genotypes, marker mutation or null alleles. 36 of the 54 individuals in the "no match" category were scored at 7 loci and required allowing at least 2 mismatches at 2 different loci to assign parentage. It was therefore highly unlikely that the mismatches were as a result of mutations, mistyping and or false parental exclusion. The remaining 18 fish were typed at 5 loci and required a single mismatch to assign parentage. The chromatographs of each mismatch locus was scrutinised thoroughly and it was concluded that mistyping could confidently be excluded. If however mistyping and or mutations are discounted as sources of error it can be hypothesised that other parents were involved in the mating regimes, creating extra parental contributions, for which no genotype information was available. This corresponds to the fact that when the two extra loci were screened for the 36 fish the number of mismatches required to assign parentage increased and affirms the information from farm records that not all spawning females on the farm were sampled. All 75 individuals were excluded from further analysis.

Prior to the routine use of PIT tags for broodstock identification on the farm in 1999 fish were identified by appearance and physical features only. Each fish was given a name and even though external tags were fitted farm records indicate that all fish were identified by name using physical appearances only. In 1995, when the replacement population was being established, 22 fish spawned on the farm but only 15 fish

produced eggs that were good enough to be incubated. Farm records indicate that eggs from just 6 fish were kept as replacements in the 1995 year class. Table 3.5 below is a summary of the stripping sheet showing the eggs incubated in 1995 and tissues collected in 1999.

Table 3.5 Summary of 1995 stripping sheet showing whether eggs collected from females were incubated and genotype ID if tissue samples were collected in 1999 for genotyping.

Fish Name	Last 4 digits of PIT tag number	Genotype I.D	Eggs Incubated
Tiny	4803	167	YES
Hope	138F	4A/171	YES
Small Blind	OA28	6A	YES
LYF	8295	3A	YES
Left	B11A	11A	YES
Large 1 Tag	AEC8	165	YES
Olive	D5D8	12A	NO
Mrs T	41D7	18A	YES
3 Fingers	3AD4	19A	YES
Rag Tag	0821	10A	YES
Pale fish	A4A7	NO GENOTYPE	YES
Lesley	AC57	NO GENOTYPE	NO
2 Tags	3D31	NO GENOTYPE	YES
Moonbeam	CULLED '96	-	YES
Largest fish	CULLED '96	-	NO
Speckless	CULLED '96	-	YES
Beauty	CULLED '96	-	YES
Small fish	CULLED '96	-	YES
Speckled Blinky	CULLED '96	-	NO
RBO	CULLED '97	-	NO
Nicky	CULLED '97	-	NO
Nutter	CULLED '99	-	YES

() Farm records indicate that eggs were kept for replacement broodstock

Tissue samples were collected for genotyping in 1999 from only 10 of the 21 individuals within the 1995 spawning group because they were the only ones available at the time. As such there are potentially seven female parents that contributed to the 1995 year class of which no genotype data is available. Due to the management procedures i.e. the mixing of batches at the egg, yolk sac and weaning phases of production as well as the mode of identifying spawning females, the

possibility that errors were made in recording female contributions to broodstock replacement batches cannot be ignored.

In 1998 more fish spawned on the farm, including some F_1 fish acquired by the farm from the captive population in Ardtoe, Table 3.6 contains a list of the extra fish that spawned in 1998. Based on farm records, the information in Tables 3.5 and 3.6, there were 10 females that were potential parents in the 1995 and 1998 year classes for which no genotype information is available. Also because no DNA was extracted from a female fish (see Section 2.3) the total number of missing female genotypes becomes 11. No information is available for male mortalities thus a missing male parent, even though unlikely, cannot be excluded. If these individuals had unique alleles it would be easy to deduce their composite genotypes based on the offspring genotypes. This was demonstrated with the female 64A. However if their genotypes are common this becomes a difficult task.

Table 3.6 Summary of 1998 stripping sheet showing whether eggs collected from females were incubated and genotype ID if tissue samples were collected in 1999 for genotyping.

Fish Name	Last 4 digits of PIT tag number	Genotype I.D	Eggs Incubated
Blacky	F067	8A	YES
Smiley			NO
Grey	3461	25A	YES
Spot	227D	48A	YES
Twisted head dark			NO
Narrow tail	A3E3	24A	YES
Poorly fish	80A4	22A	NO
Sandy			YES
Nice dark	7E1D	27A	YES
Twisted tail	CD81		YES
Grey thick	01CD	21A	YES
Tiny	4803	167	YES
Hope	138F	4A	YES
Small Blind	OA28	6A	YES
LYF	8295	3A	YES
Left	B11A	11A	YES
Large 1 Tag	AEC8	165	YES
Olive	D5D8	12A	YES
Mrs T	41D7	18A	YES
3 Fingers	3AD4	19A	YES
Rag Tag	0821	10A	YES
Pale fish	A4A7	NO GENOTYPE	NO
Lesley	AC57	NO GENOTYPE	YES
2 Tags	3D31	NO GENOTYPE	YES
Nutter	CULLED '99	-	YES

() Farm records indicate that eggs were kept for replacement broodstock

In summary, using strict exclusion principles employed by FAP, 90.77% of the sampled F_1 population were assigned unambiguously to a single parental pair (see Table 3.7). In the 1995 year class 229 individuals were assigned out of 270 (85%) and in 1998 year class 499 were assigned out of 532 (94%).

Table 3.7 Final summary of the parentage assignment after the first and second rounds of analyses.

Category	Number
Assigned to a single family	728
Assigned with one or more mismatches	54
Assigned to multiple families	21
Total	802

3.3.4 Family Structure

Table 3.8 gives a glossary of identification numbers (identifiers used for) of the sires and dams used at different stages of the study and the pedigree structure of the F_1 population on the farm (both 1995 and 1998 year classes) in Table 3.9. The results of the parent assignment show that, in the offspring population studied, there was a significant reduction in the levels of polymorphism, as described in Chapter 2, because very few parents succeeded in contributing to the F_1 generation. 17 males and 18 females contributed to the F_1 generation, exactly half of the numbers in the parental generation. However this fraction was not even across the sexes, 60% of the sires were successful and 42% of the dams.

The percentage contribution of each individual was also heavily skewed. Certain individuals contributed significantly more than others and one family in particular (17A-sire- and 10A –dam-) made up 30% (215 offspring) of the whole population. It appears that the entire F_1 population was composed of few large half sib families. Out of all the males that were represented only four had more than thirty offspring and one male, 17A, sired 393 individuals i.e. 55% of the F_1 population. Nine males had less than ten offspring each (Figure 2.1). A similar picture is seen with the females (Figure 2.2). Five dams had more than thirty offspring and seven had less than ten. It should be noted that the distribution, though badly skewed, was less extreme in the dams than the sires. Therefore even though 35 individuals were successful in contributing

offspring to the F_1 generation, a lot of them did not make significant contributions and thirteen parents (6 males, 7 females) had five or less offspring.

Table 3.8 Glossary of identifiers used for individuals in the parental generation and their places of origin.

Sire I.D	Tag	Name	Origin		Dam I.D	Tag	Name	Origin
1A	106B	NICK	Iceland		3A	8295	LYF	Iceland
2A	30F0	WHITE TAIL	Iceland		4A	138F	HOPE	Iceland
5A	3BB8	-	Iceland		8A	F067	BLACKIE	Iceland
7A	F29D	-	Iceland		10A	0821	RAG TAG	Iceland
9A	0DA5	-	Iceland		11A	B11A	LEFT	Iceland
13A	43CC	-	Iceland		19A	3AD4		Iceland
14A	4A59	-	Iceland		20A	0BA1		Iceland
15A	545F	-	Iceland		23A	F96C	GREEN	Ardtoe
17A	D821	PEE PEE	Iceland		24A	A3E3	NARROW TAIL	Ardtoe
32A	A594	-	Ardtoe		26A	CD81	TWISTED TAIL	Ardtoe
33A	F219	-	Ardtoe		27A	7E1D	NICE DARK	Ardtoe
34A	Ae3e	-	Ardtoe		30A	0B3F	TWISTED HEADI	Ardtoe
35A	5ED2	LARGE TESTS	Ardtoe		42A	0E4A	-	Ardtoe
37A	3629	-	Ardtoe		50A	AEBF	-	Shetland
44A	85DF	-	Ardtoe		64A	****	?	Iceland
54A	391D	-	Shetland		165	AE38	LARGE 1 TAG	Iceland
170	A7EA	-	Iceland		166	4FDB	-	Iceland
					167	4803	TINY	Iceland

Tag= Last 4 digits of the PIT tag numbers, I.D= Genotype Identity numbers

Table 3.9 Total number of offspring assigned to males and females in the sampled offspring population.

	3A	*4A*	*8A*	*10A*	*11A*	*19A*	*20A*	*23A*	*24A*	*26A*	*27A*	*30A*	*42A*	*50A*	*64A*	*165*	*166*	*167*	**Male total**
1A		8													56			9	**73**
2A		28													3			49	**80**
5A	1										1				2				**4**
7A	2														53			1	**56**
9A			4	12			1												**17**
13A							1											1	**2**
14A																		1	**1**
15A			5	1	8														**14**
17A	1		58	215	82	17	16	1						2			1		**393**
32A						1													**1**
33A	2														1				**3**
34A											7				2				**9**
35A									5	14		3			6				**28**
37A	18									1					13				**32**
44A	1									4	2		1						**8**
54A			1																**1**
170						1										5			**6**
Female total	***25***	***36***	***68***	***228***	***90***	***19***	***18***	***1***	***5***	***19***	***10***	***3***	***1***	***2***	***136***	***5***	***1***	***61***	***728***

Dams

Sires

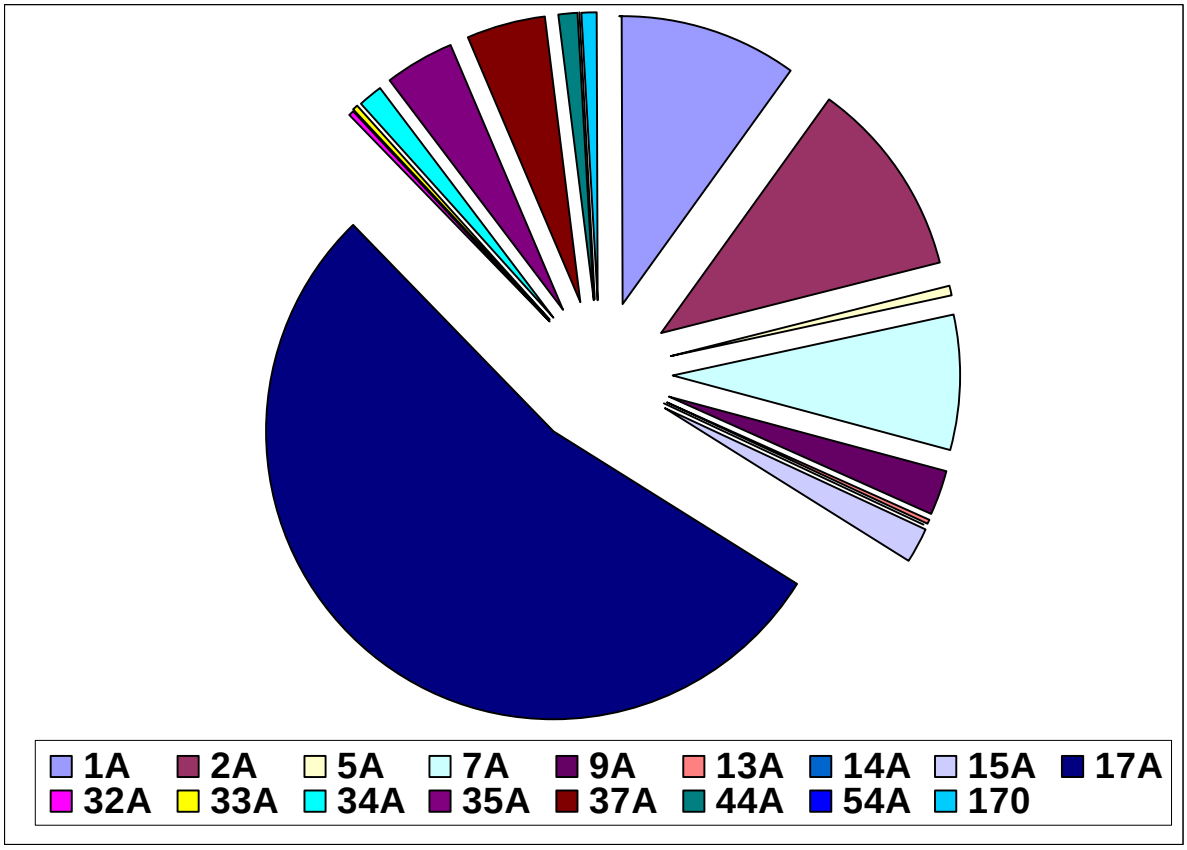

Figure 3.1 Percentage contributions of sires to the sampled F_1 gene pool

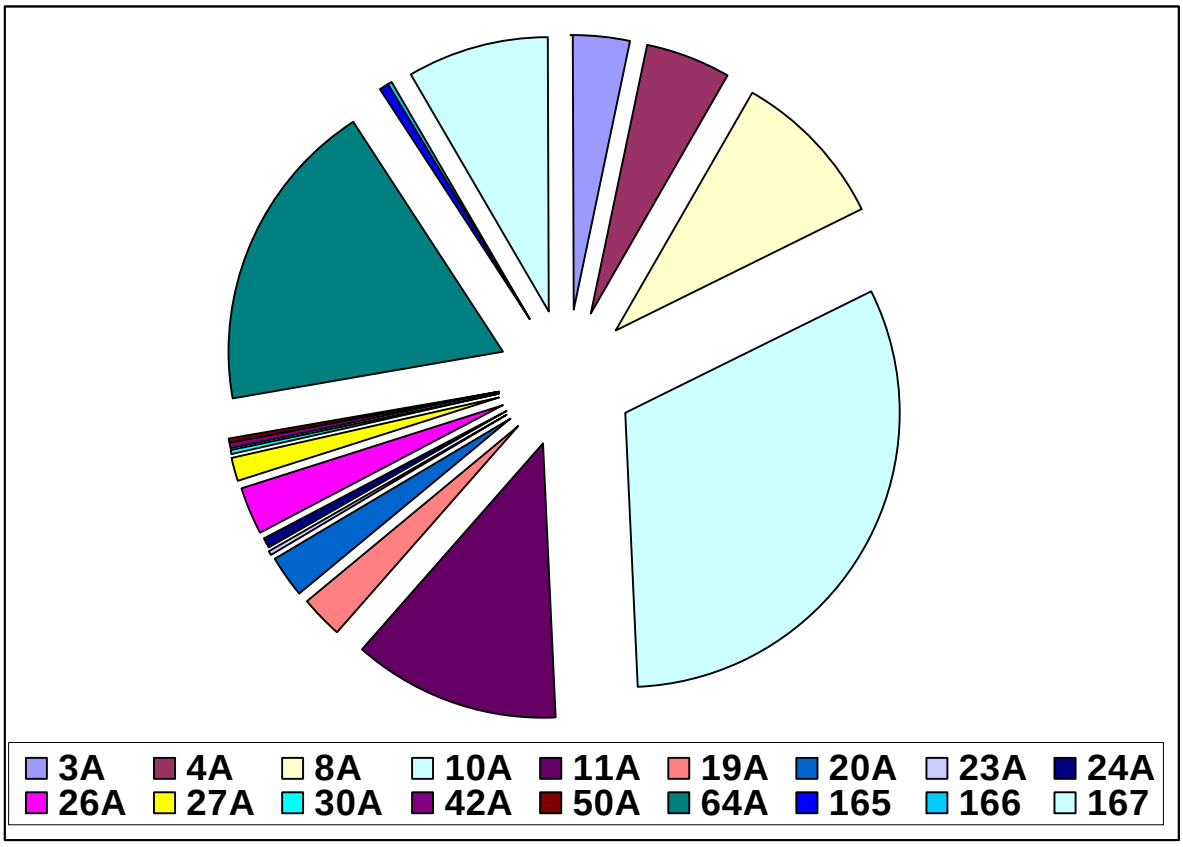

Figure 3.2 Percentage contribution of dams to the sampled F_1 gene pool

3.3.4.1 Contributions per origin

60% (21 individuals) of the parents that contributed to the F_1 generation were from Iceland. Only 2 individuals, one from each sex, were from Shetland and 12 (34.2%) were from the Ardtoe group. Most of the offspring were the result of crosses between Icelandic parents and as such were pure bred Icelandic individuals. There were no pure bred Shetland individuals in the F_1 population, the Shetland fish gave only 3 offspring and they were half Icelandic. 42 individuals (6%) were crosses between the Icelandic and Ardtoe fish and 5.2% were from crosses between two Ardtoe parents.

3.3.4.2 Contributions per year class

When the parental contributions to the 1995 and 1998 year classes are examined separately it becomes clear that although they both consist of large sib families, their compositions are different. Figures 3.3 and 3.4 below show the pedigree structure of the F_1 populations in both year groups. In 1995 only eight sires and nine dams were detected in the offspring sampled. Offspring from three sires, 1A, 2A and 7A made up most of the population. A single dam, 64A, produced 41% of the pedigreed population. She was mated to the three sires mentioned above.

In 1998 more parental contributions were detected, 12 males and 15 females had offspring in this year class. However a single parental cross made up 215 out of the 494 pedigreed offspring. Also just as in the 1995 year class a single individual, this time a male, 17A, was found to dominate the entire population. He was mated to eight females, and 77% of the pedigreed individuals in 1998 were sired by him. There was an overlap in the representation in that some fish gave offspring in both year classes.

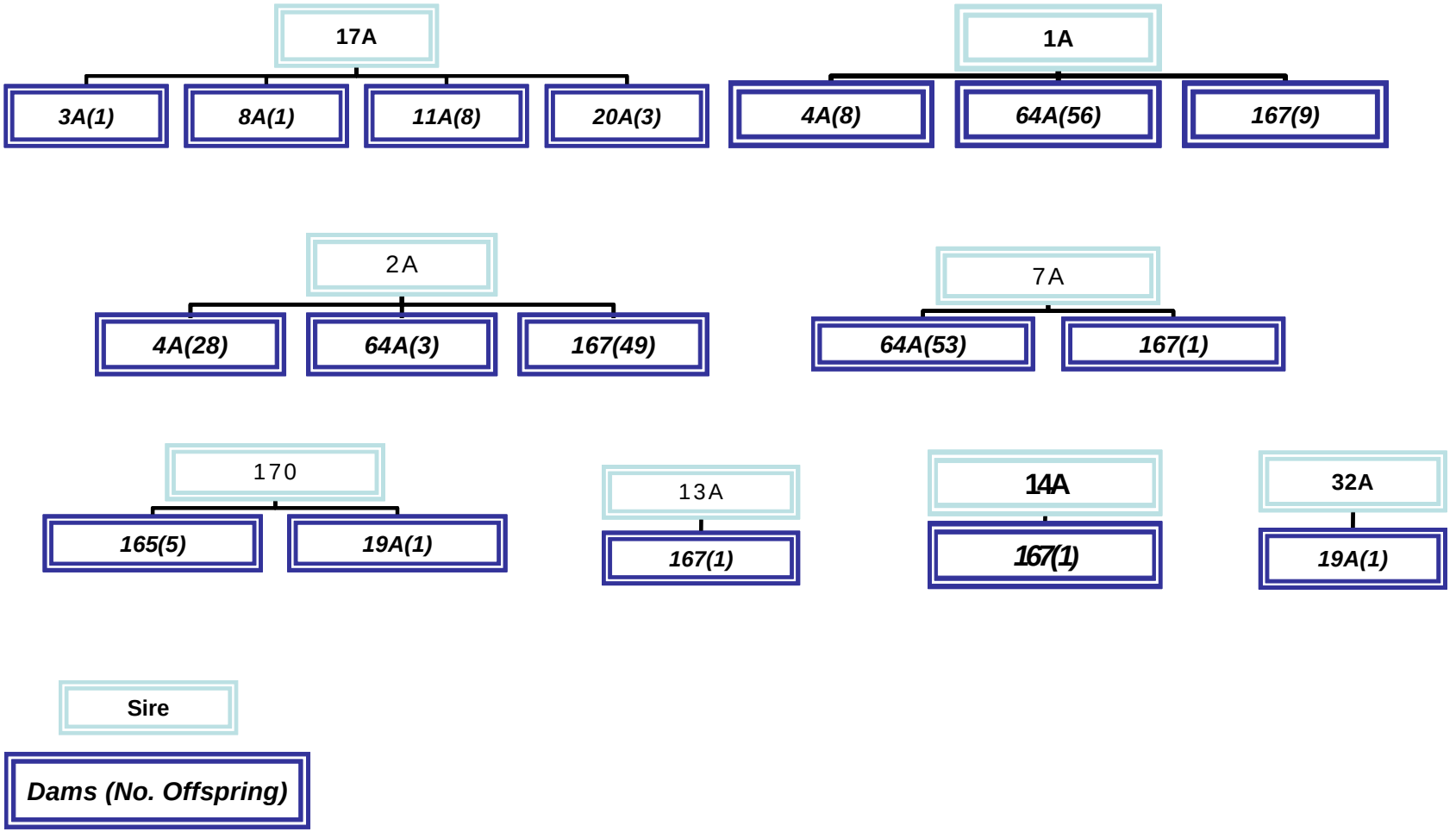

Figure 3.3 Pedigree structure of the 1995 year class

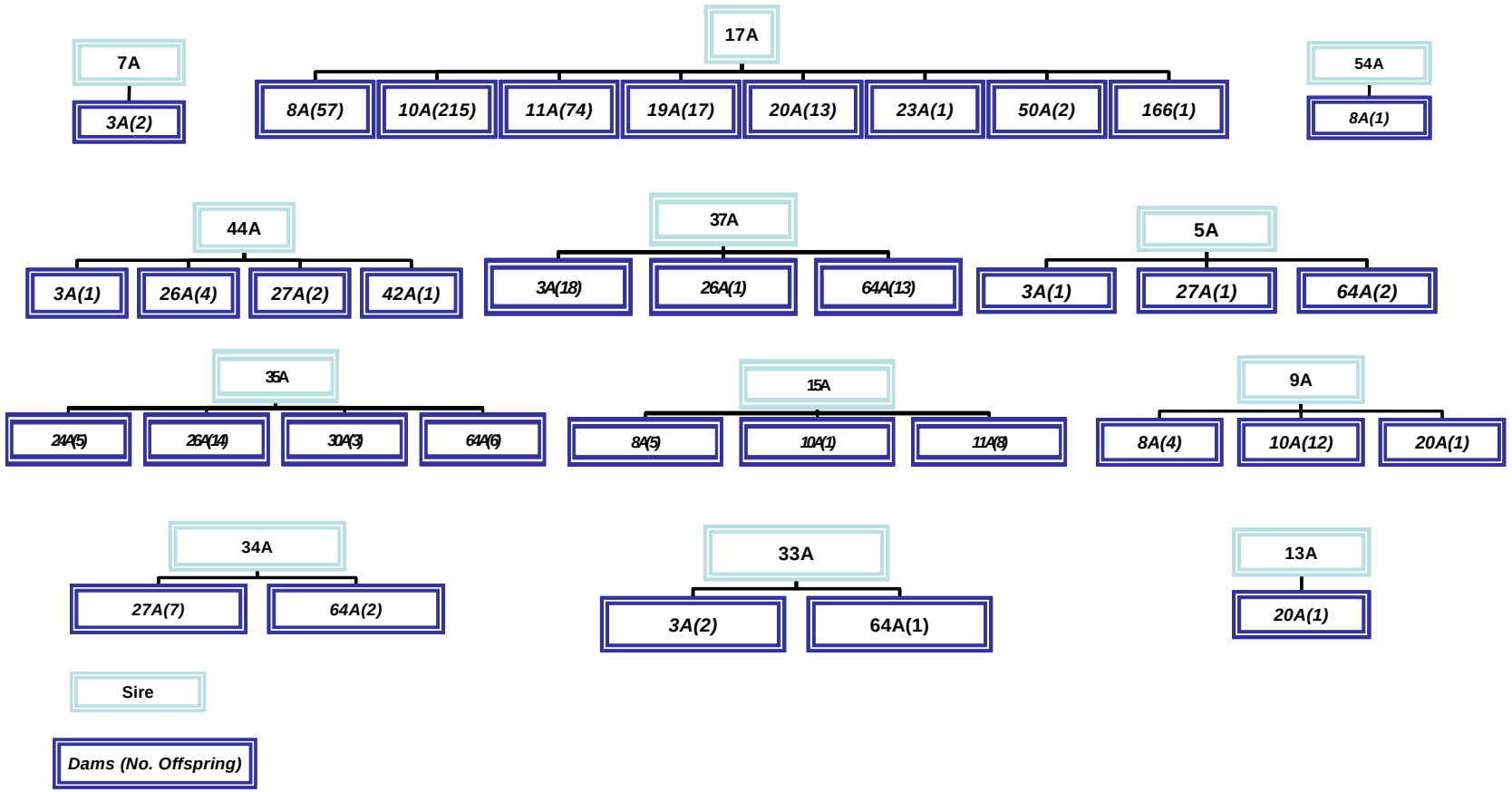

Figure 3.4 Pedigree structure of the 1998 year class

3.3.5 Effective Population Size and Inbreeding

The inbreeding coefficient in the F_1 generation as a whole was 6.16%. This is almost as high as and to a half-sib mating at 6.25%. However, when the year classes are examined independently it becomes apparent that the 1998 year class is at an even more alarming level of inbreeding (10.64%) and most breeding programmes run on the assumption that the effects of inbreeding through sib matings predict significant declines in reproduction and growth traits with inbreeding coefficients of 10% or more (Myers *et al.*, 2001). It is important to note though that these values are the potential inbreeding levels that could occur if related F_1 individuals were mated together.

Table 3.10 Effective population size and inbreeding coefficient in the F_1 group.

Population	N_e	ΔF
All F_1 individuals	8.11	0.0616
1995 Year class F_1	6.46	0.0774
1998 Year class F_1	4.69	0.1064

Even though no loss of heterozygosity was observed between the parental and offspring generations as seen in Chapter 2, the pedigree structure has revealed how deceptive this measure is at quantifying inbreeding in hatchery populations. Variation of family size or parental contribution is one of the most important causes of N_e being less than the actual number of breeding individuals (Falconer and Mackay, 1996). The variance in sire and dam contributions to the F_1 generation was large (8790 and 3565 respectively) especially the sire contribution in 1998 (11465). This was because so many of the offspring in that year class were sired by a single individual. The present offspring population as it stands is unsustainable and without controlled mating (using pedigree information to avoid sib matings) the effect of inbreeding depression will very shortly be expressed.

3.4 Discussion

One of the difficulties in implementing a selective breeding programme in aquaculture is establishing and maintaining pedigree information. In this study approximately 91% of the offspring genotyped were unambiguously assigned to a single parental pair using exclusion principles after an extensive period, up to six years, of communal rearing. The parentage assignment revealed that the F_1 population on the farm consists of a small number of large full- and half-sibling families which was reflected in a low effective breeding number that varied between year classes. The 1998 year class had a particularly poor N_e (4.69) and the inbreeding coefficient estimated was unacceptably high.

3.4.1 Parentage Assignment

Considering the number of loci used in the analysis the results obtained are comparable to those published by several authors over various species. In the Atlantic salmon Norris *et al*. (2000) and O'Reilly *et al*. (1998) both reported over 98% assignment using four and eight micosatellites respectively. Using four microsatellites Herbinger *et al*. (1995) were able to assign 65.4% of offspring to a single parental pair in rainbow trout while Ferguson and Danzmann (1998) assigned over 90% with six microsatellites in the same species. Brown (2003) assigned 96% of offspring using fourteen microsatellite loci in the gilthead seabream while Perez-Enriquez *et al*. (1999) assigned 73.5% using four loci in the red seabream. Jerry *et al*. (2004) were only able to assign 47% of offspring using six loci but this was due to the presence of null alleles.

Studies in parentage assignment of flatfish have had high success rates, within excess of 90% assignment. In a project involving the Japanese flounder Sekino *et*

al. (2003) were able to achieve 100% assignment while using the same five microsatellite loci characterised by McGowan and Reith (1999), Jackson *et al*. (2003) reported 98% assigned using an exclusion method of assignment (PROBMAX).

In the first round of the analysis of the present study 83% of the F_1 individuals typed were assigned using five loci. When the two extra loci were included in the analysis this value went up to 91%. The study by Jackson *et al*. (2003) had to discriminate among 182 potential families (13 males and 14 females) whereas in the present study there was six times the number of potential families (1176) and the number of individuals typed was five times higher. In all the studies cited above the actual number of families were limited to less than 200 with the exception of Jerry *et al*. (2004), Brown (2003) and Perez Enriquez *et al*. (1999). Furthermore except for where mass spawning was unavoidable (Sekino *et al*. (2003); Brown (2003) and Perez Enriquez *et al*. (1999)), the mating structure was known. Due to the fact that the mating structure of the fish in the current study was unknown, all potential crosses had to be tested thus decreasing the overall power of the analysis. When the mating structure is not known and the number of potential parents is high many more progeny/parent matches may result, necessitating the use of additional microsatellite for complete discrimination (Ferguson and Danzmann 1998). Norris *et al*. (2000), O'Reilly *et al.* (1998) and Rodzen *et al*. (2004) all observed that increasing the number of potential parents in the analysis resulted in a reduction of the number of offspring unambiguously assigned to a parental pair. This increases the likelihood that individuals and families will share common alleles and thus the "multiple families" group will tend to be higher, explaining why the number of

assigned individuals went up by 7% when more loci were employed in the analysis. In all the studies cited the genotypes of all the parents were known.

In this study approximately 7% of the fish sampled were not assigned to a single family, 36 individuals from the 1998 year class and 18 from the 1995 year classes. Proportionally more fish were unassigned in the 1995 year class (16%) than in the 1998 year class (3%). Due to the fact that not all the parental fish were sampled the reason for the unassigned fish could, discounting genotyping errors, be attributed to missing parental genotypes or mutations. This then suggests that more contributions were made from un-typed parent(s) in 1995 than in 1998. From an understanding of the biology and management system of the halibut it is unlikely that the missing parent (s) is or was a male. In the stripping season each female is handled at least once every three days. The females are guided on a stripping table and the process of stripping can be stressful on the animal. It sometimes results in lesions on the underside of the fish which get progressively worse as the stripping season develops. Spawning females tend to have depressed appetites, the water temperature is also maintained at lower temperatures, consequently damaged animals tend not to recover and if they are not isolated and returned to tanks containing higher temperatures they die. Egg retention within the ovarian lumen is also a problem; this causes blockages when this material begins to degenerate inside the fish and if not treated this condition can be fatal. Unlike females, males are handled less often in the stripping season and the loss of appetite is less pronounced, therefore the probability of mortality is greatly reduced. Farm records also show that 20% of the female broodstock group (nine females) died or were culled between 1995 and 1998 (Table 3.8). Unfortunately no data on male mortalities was available.

As mentioned earlier in section 3.3 the stripping records for 1998 and 1995 were acquired from the farm. The volume of fertilized eggs for all the fish that spawned in both years was calculated. This was done by multiplying the fertilization rate by the volume of eggs produced at each stripping event and adding the value over the whole stripping season. In 1995 two fish gave eggs with zero fertilization rates and in 1998 the number of fish that gave no fertilized eggs was five. Lists of the volume of eggs produced, the volume of fertilized eggs incubated and the number of offspring detected by genetic profiling for females spawning in 1995 and 1998 are shown in Tables 3.11 and 3.12. The lists include only fish that gave eggs with fertilisation rates higher than zero.

Table 3.11 Volumes of eggs produced and volumes fertilised by broodstock in 1995.

Name*	I.D	Volume of eggs produced (ml)	Volume of fertilized eggs(ml)	Number of offspring detected by profiling
Large 1 Tag	165	9000	4991.35	5
Hope	4A	8900	4689.35	36
Pale fish*		7700	4675.05	
Left	11A	6400	2949.45	8
LYF	3A	2700	1136.35	1
Small Blind	6A	2870	2028.95	
Beauty*		4800	1367.4	
3 Fingers	10A	3500	1217	
Small Fish		1700	945.4	
Tiny	167	3200	887.5	61
Lesley*		5900	660	
Speckless*		900	384.3	
Olive	12A	2600	280	
Moonbeam*		600	231	
RBO		2800	195.6	
Largest fish*		2400	168	
Nutter*		2400	166.2	
Rag Tag	10A	200	115	
Mrs. T	18A	900	108	
2 Tags*		1300	102.5	

*No genotype available because fish died before the start of the study

Table 3.12 Volumes of eggs produced and fertilised by broodstock in 1998.

Name*	I.D	Volume of eggs produced (ml)	Volume of fertilized eggs(ml)	Number of offspring detected by profiling
Large 1 Tag	165	14200	10859	
Rag Tag	10A	8250	6410	228
Mrs. T	18A	6800	4968	
Hope	4A	5850	3744.5	
Olive	12A	5500	3535	
LYF	3A	5500	3399	24
Small Blind	6A	3500	2705.2	
Twisted Head	30A	4600	2507	3
Pale Fish*		6100	2474	
Lesley*		4100	2133	
Blacky	8A	2750	1656	67
3 Fingers	10A	3550	1647.50	
2 Tags*		7900	1505	
Left	11A	2800	1088	82
Sandy Twisted	29A	2300	1084	
Dark twisted head	28A	1600	1048	
Grey	25A	3300	889.2	
Nutter*		3300	872	
Narrow Tail	24A	2050	797	5
Nice Dark	27A	1250	543	10
Grey Thicky	21A	1200	166	
Poorly Fish	22A	600	87.6	
Spot	48A	1200	50	

*No genotype available because fish died before the start of the study

In 1995 nine fish produced eggs that were incubated for which no genotype information was available. The total volume of fertilized eggs incubated from un-typed females was 8895.45 ml, or 32.6% of the total volume. In 1998 7164 ml were incubated from un-typed females, which made up 12.9% of the total volume

From Tables 3.11 and 3.12 there appears to be a very poor correlation between the volume of fertile eggs produced per female and the number of offspring detected by the genetic profiling exercise. However, because not every fish in the F_1 generation was genotyped this inference is, at least, indicative of a genuine trend within the wider population. The number of fertile eggs going into the system is not necessarily a good indicator of batch survival or dam representation as would have

otherwise been expected. Alternatively because physical descriptions and names were used to identify the fish, prior to the use of PIT tags on the farm, mistakes could have been made in the identification process and the wrong volume of eggs credited to the same fish. This was shown by the fact that in 1995 the parentage assignment revealed one fish (19A) had offspring represented and farm records indicated that it did not spawn until 1996. In 1998 this problem was repeated with nine other different females.

This study clearly shows the importance of genetic profiling in halibut culture. It has become clear that farm records were not accurate in predicting the outcome of family survival i.e. the parental contribution to the batches that survived until weaning. The farm predicted that the 1995 year class were comprised of offspring from batches spawned by 5 females, but the genetic profiling results showed that the group of fish were offspring from 9 females, of which only 3 were identified correctly by the farm. In 1998 only 4 of the 11 fish proposed by the farm as potential female parents were accurate.

Due to the fact that the population on the farm is not a strictly closed system, i.e. where all the parents are known, a likelihood method of analysis could have been employed using a programme such as CERVUS (Marshall *et al*., 1998). The program however relies heavily on the sample population being in Hardy-Weinberg equilibrium and since this population departs significantly from that equilibrium predictions are that CERVUS will not perform as well as it is expected to (Villenueva *et al*., 2002). The limitations of using exclusion principles are that individuals could be wrongly excluded from further analysis, however, when the

population structure in this study is considered, i.e. poorly proportioned families (see Section 3.3.4), a strict method is desirable.

3.4.2 Family Structure

Genetic profiles from the microsatellite markers used have allowed the assignment of individuals into family groups. The result of this assignment has shown that a significant bottleneck has occurred within one generation of the population on the farm. Only half of the total number of individuals in the parental generation contributed to the F_1 generation in the sampled population. The proportional representation of the individuals was extremely skewed such that the offspring generation is essentially a small number of large family groups (full and half sibs). When the offspring group is separated into the two year classes it appears that even though more individuals were represented in 1998, the 1995 year class is better proportioned. This is because in 1998 a single full-sib family made up 43% of the whole group, and furthermore the same male sired 76% of the year class. It is important to reiterate that not all the offspring produced on the farm, in both years, were sampled and financial constraints meant that 80 potential broodstock candidates were not genotyped. Nonetheless the candidates tested were randomly sampled and the probability of finding other large, hitherto undetected families is very low.

In most hatchery strains, an unequal contribution of broodstock to the next generation seems to be typical, particularly when mass spawning method is used to establish hatchery strains (Sekino *et al*., 2003; Perez-Enriquez *et al*., 1999). Sekino *et al*. (2003) suggested that the most effective hatchery option to obtain a more homogenous contribution by broodstock would be to employ the stripping method

with one-to-one crossing using a large number of broodstock. Jackson *et al*. (2003) reported findings very similar to those in the current study with first generation cultured halibut. In both studies a stripping method was used to obtain and fertilise gametes. In their study Jackson *et al*. (2003) used milt from 3 males to fertilise each batch of eggs. More males were represented than females, 3 males out of 13 and 4 females out of 14 dominated the offspring generation. They also noticed that quite a lot of crossings performed achieved no representation in the F_1 group and attributed the causes to lack of established techniques for the spawning of broodstock and the culture of larvae. In their experiment with rainbow trout Herbinger *et al*. (1995) observed an unexpectedly higher number of offspring from certain females i.e. uneven representation of dams in the offspring population. They concluded that the differences from the expected number of offspring were due to the initial difference in female fertility, with some females produced poorly surviving offspring, possibly due to maternal effects. In a small scale study of parentage assignment in the sea bass, Garcia de Leon *et al* (1998) observed a significant family effect in survival rate resulting from both male and female effects.

Interviews with managers in Otter Ferry can explain why the distribution of family size was so poor in 1998. A single male 17A (D821) was used because he was the largest male in the tank (male halibut are prone to early maturation and they stop growing at the onset of sexual maturity), thus the farm managers hypothesized that if he hadn't matured early his offspring would not either, therefore they used him with most of the females. Other males were "favourites" and were used more often than others so not every male got a chance to contribute to the next generation. The observed unevenness in the family structures cannot be explained by management alone; the phenotype of an animal is made up by its genotype, the environment (all

things non-genetic) and the interaction between the two. All the females were kept under similar environmental conditions; tanks, lighting regimes, feed water conditions etc, and yet half of them failed to produce viable offspring while others seemed to dominate the subsequent generation. Dunham and Lui (2002) argue that when wild fish are moved from their natural environment to the aquaculture environment, a new set of selective pressures is exerted on them resulting in changes in performance. These domestication effects are dramatic and can be observed in fish in as little as one generation. Doyle (1983) defines domestication selection as natural selection on traits which affect survival and reproduction in a human-controlled environment. Could it be that in the halibut the variability in reproductive performance observed in this study and in the study by Jackson *et al*. (2003) are partly due to the effects of the artificial environment on the halibut and a failure to adapt to new environments? Again it is unlikely that there are single independent reasons for this observation and a combination of genetic and management factors probably caused the shift. It is obvious though that the effect of the "domestication selection" can be estimated better in the females than the males. Each female was given an equal chance to breed. She was stripped every three days and the family groups represent an equal chance whereas only certain males were selected as broodstock.

The selection, or in this case exclusion, of certain individuals masks the true genetic effect of hatchery management on the halibut. This also brings to light the conflict between production and breeding. At the time when the replacement broodstock was being established the focus was on meeting production targets and so only the "best" males were used, inadvertently narrowing down the gene pool. Had every male been given a chance to breed this may not have been the case. The production

cycle of the farm also plays a part in masking the outcome because the fry might have been picked as a batch was coming through the system. In 1995-1998 the females spawned in batches over a period of 6 months and so the poor representation might have been caused by a manager picking only fish from a few parents, a snap shot of the production at the time. Rodzen *et al*. (2004) reported consistent family structure and conserved genetic diversity between 4 year classes of cultured white sturgeon. This is probably due to the mating system used as they employed a factorial mating design.

3.4.3 Effective Population Size and Inbreeding

A small fraction of the wild parental population was used for broodstock replacement. This therefore increased the total variance of family size, a parameter of critical importance to the effective population size and consequently the inbreeding coefficient. It compares the degree of relationships between the individuals now, with that between the individuals in the base population (Falconer and Mackay, 1996). The figures obtained for F were not surprising considering the population structure nonetheless they strongly suggest that immediate measures must be taken to avoid further degradation of the genetic resource in the offspring generation on the farm.

As a strategy, the avoidance of matings between close relatives in a closed population delays the increment of inbreeding but very little reduction of the subsequent rate of inbreeding is achieved (Falconer and Mackay, 1996). Only by increasing the number of breeding animals and ensuring an equal number of contributions from each family can the rate of inbreeding be reduced. Thus the introduction of new genetic material into the gene pool is strongly recommended.

Chapter 4

Repeatability of reproduction traits

4.1 Introduction

Aquaculture has gained international acclaim and attention as a means of producing large quantities of high quality protein quickly. In this context therefore reproductive efficiency is an important component of profitability in the industry (Su *et al*., 1997; Su *et al*., 2002); it could even be considered the basis for the success of any fish farming enterprise. The high fecundity of cultured aquatic species is a major difference which makes their culture so lucrative when compared with other domesticated species. Aquaculture is therefore centred on the production of large volumes of eggs from hatcheries, where the life cycle of species are closed with broodstock replacements bred from closed populations, or their collection from wild broodstock to be sold as "seed" or fry for ongrowing.

Selective breeding schemes are being developed for a number of fish species, mainly salmonids, in order for the industry to live up to its full potential. Historically the primary selection objective of aquaculture breeding programmes has been to increase growth rate whilst relying on the inherent fecundity of fish to allow higher genetic gains, compared to farm animals, through increased selection intensities. This could result in a very small number of individuals contributing to the genetic make up of successive generations and coupled with differential fecundity could severely erode the genetic variation in captive populations. Therefore greater care must be taken with the monitoring of reproductive traits in broodstock management and selective breeding programmes for aquatic species, perhaps even including them in the selection objectives.

Yet, with few exceptions, the management of reproductive traits are rarely regarded as essential components of breed improvement programmes because of the high

fecundity of the species involved. Nonetheless, an understanding of the sources of variation for reproductive performance traits should also be assessed so the breeder can identify any changes in reproductive productivity that may occur during selection and breed improvement (Gall and Neira, 2004). These processes have yet to be conducted in the halibut, probably due to the fact that it is still a newly developing industry. In contrast, cultured salmonid species such as the trout and salmon provide an exception to the trend in aquaculture in that reproductive traits have been defined and estimates of genetic parameters have been published.

4.1.1 Genetics of Reproduction Traits in Aquaculture

The reproductive traits defined in aquaculture are focused on the quantity of eggs, time of spawning and weight of spawning individuals. The latter is assessed because in general fish size is positively correlated with fecundity and egg diameter (Bromage, 1995) with larger fish producing more eggs, although a gradually diminishing increase in egg size with increasing body size is observed in the rainbow trout (Bromage *et al*., 1992). Other traits include egg size, egg number, egg volume and hatchability.

The first published heritability estimates for reproductive traits were from Gall (1975) who studied spawning body weight and egg traits in the rainbow trout. These estimates came from full-sib analysis of female progeny from crosses between two domestic rainbow trout populations. Curiously the heritabilities estimated were very similar, approximately 0.2 (±0.05) for all traits studied: post-spawning body weight, egg volume, egg size, egg number and egg number per 100g body weight. Kanis *et al*. (1976) estimated the heritability for mortality in eggs, alevins and fry for three salmonid species; salmon, sea trout and rainbow trout. In

general pooled heritabilities, estimated from sire components, over all three species for all traits were very low, 0.08±0.04 for eggs at the eyed stage and 0.05±0.01 for alevins while the estimates for fry mortality were not significantly different from zero. Gall and Gross (1978) evaluated the inheritance of reproductive performance in three different strains of rainbow trout. They showed that there were significant genetic effects and differences between the strains in reproductive performance. Pooled heritability estimates over the three strains for egg volume, egg size and egg number ranged from 0.32±0.11 to 0.52±0.13. Fertility, defined by the proportion of eggs alive at the eyed stage, was also studied and a value of 0.23±0.12 obtained for its heritability. Also in the rainbow trout, Gall and Haung (1988b) estimated heritabilities of 0.15 (±0.14) for post spawning weight, 0.30 (±0.15) for egg volume, 0.32 (±0.14) for egg number and 0.28 (±0.16) for egg size.

Advances in statistical techniques such as mixed model methodologies have allowed more precise estimates of genetic parameters to be made and also the use of data from animals within the same population for estimating them. Sue *et al*. (1997) reported heritability values for the rainbow trout of 0.65 for spawning date, 0.60 for egg size, 0.55 for egg number, 0.52 for egg volume and 0.13 for hatchability. Gall and Neira (2004) estimated genetic parameters of reproduction in a coho salmon population, obtaining heritability values of 0.42 (±0.08) for number of eggs, 0.39 (±0.08) for weight of eggs spawned, 0.32 (±0.07) for egg size and 0.33 (±0.08) for number of eyed eggs (fertilisation rate). Both studies were conducted using maximum likelihood procedures.

The relationships between the traits of interest are of great importance in a breeding programme. Selecting for one trait might be detrimental to the expression of

another. In the dairy industry for example there appears to be a negative correlation between fertility and milk yield, thus high yielding cows are likely to be less fertile (Weigel Rekaya, 2000; Pryce *et al*., 2004). Alternatively the correlations might be positive and thus selecting for one of the traits will result in a gain in another trait removing the need to select for both traits independently. However the genetic and phenotypic correlations between traits can be different with the phenotypic correlation being the sum of the environmental and genetic components. In breeding programmes the genetic correlations are of the most interest.

As previously stated there appears to be a positive phenotypic correlation between size and fecundity. The genetic correlations between reproductive traits that define quantity and body weight have been estimated by a handful of authors, again in salmonid species (Gall 1975; Gall and Gross 1978; Su *et al*., 1997, 2002; Gall and Neira, 2004). They all reported positive genetic correlations between body weight and egg volume, egg number and egg size, ranging from 0.18-0.69 with the highest correlations found between egg volume and body weight. Consequently, selecting for bigger fish will simultaneously result in fish that are highly fecund; therefore both objectives will be achieved without the need to consciously select for reproduction traits, had they been included in the selection objectives. Genetic correlations between reproductive traits (egg size, egg volume and egg number) were also estimated and found to be positive (moderate to high).

4.1.2 Reproductive Performance in the Atlantic halibut

Reproductive performance in the Atlantic halibut has yet to be studied quantitatively. Although some traits similar to those used in the salmonid industry have been defined, the relationships between traits have not been analysed.

Furthermore, since reproduction parameters are species-specific due to the significant biological differences and stages of domestication between cultured salmonids and cultured flatfish, the traits are likely to be different.

The halibut is a large animal and of all cultured aquaculture species it is more like the dairy cow in some aspects of its biology and husbandry than the layer chicken. This is because it matures at a comparatively older age and adult individuals are maintained for production over several years. Female reproductive success (the ability to produce eggs) is extremely important to halibut broodstock genetic management because of the difficulties encountered in recruiting wild individuals for domestication. Captive populations in the UK were established from a few, no more than 60, wild progenitors. Like other marine species they are also more highly fecund than salmonids and an adult female can give up to half a million eggs in a season (Norberg and Kjesbu, 1991). This characteristic of the species could possibility hide the reality that not all the fish are productive or even fertile, especially in a mass spawning environment or in a farm where eggs are pooled in large conical incubators. For example results from Chapters 2 and 3 have clearly shown that not all females contribute offspring to the next generation, therefore there is a need to identify fertility or reproductive activity as a selection objective and study the trait. This is to inform on and suggest solutions for the observed poor family representation.

Halibut are multiple spawners ovulating at intervals of 70-90 hours over the spawning season and adult females are capable of producing between 6 and 16 batches in a season (Norberg *et al*., 1991; Kjørsvik and Holmefjord, 1995).

Therefore the frequency of spawns will be of interest as well as the volumes observed.

A dependable supply of halibut juveniles for ongrowing remains the major significant limitation to the halibut industry in the UK and indeed the world. The ability to produce more fry from the same number of broodstock facilities would dramatically reduce the production cost of fingerlings. Although juvenile production has proven to be the bottleneck for successful commercial halibut culture the problem is multifactoral and little understood. However, varying egg quality has been identified as one of the limiting factors for mass production of fish fry.

Egg quality is defined as the egg's potential to produce viable fry (Kjørsvik *et al*., 1990). This has been shown to be highly variable and dependant on environmental conditions such as water temperature, microbial colonisation, timing of stripping and diet of broodstock (Holmefjord, 1991; Norberg *et al*., 1991; Bromage *et al*., 1994; Brown *et al*., 1995; Mazorra *et al*., 2003).

Indicators that predict egg quality reliably and accurately in marine fish species have been the subject of a lot of debate. Bromage *et al*. (1994) recommended that they should be simple to perform and be conducted as soon as possible after stripping and or ovulation. Bromage *et al*. (1994) developed the definition of egg quality by Kjørsvik *et al*. (1990) into different components thus allowing for further study of the trait. They define egg quality as the ability to show low levels of mortality at fertilisation, hatch and first-feeding, with the underlying assumption that good quality eggs would also be expected to produce the healthiest and fastest growing fry.

Post-fertilisation checks to predict the survival potential of eggs are important for the assessment of eggs entering the rearing system in order to guide management decisions on resource allocation, particularly for those species with prolonged incubation periods such as the halibut. Assessments can be done adequately by fertilisation rate in salmonids but while this has been shown to be a good indicator of larval survival in these species it does not appear to be the same in marine fish (Kjørsvik *et al*., 1990; Bromage *et al*., 1994). Shields *et al*. (1997) and Brown (1998) defined five parameters of blastomere morphology; symmetry, cell size, adhesion, margins and inclusions (blastomeres are cells resulting from mitotic cytoplasmic cell divisions after fertilisation) and showed a high correlation between blastomere morphology at the 8-cell stage and survival. The time-consuming procedures required to fully assess the egg batches were not appropriate for routine commercial checking but since all five parameters correlated strongly with each other they recommended cell margins or cell size, the uniformity of cell size between all eight blastomeres, as a quick alternative in combination with fertilisation rate.

Even though there are undoubtedly genetic factors involved in the control of egg quality, as with most reproduction traits, the environment plays a major role. Many authors (e.g. Kjørsvik *et al*., 1990; Norberg *et al*., 1991; Bromage *et al*., 1994) have reported that over ripening of eggs, an aging process that results in reduced viability after ovulation beyond a 6 hour window, is a particular problem with the halibut. Thus the timing of stripping is of central importance to the quality of eggs as they must be fertilized within a 6 hour window after ovulation (Bromage *et al*., 1994).

Norberg *et al*. (1991) showed that when stripping times are synchronised with the ovulatory rhythms of females not only is egg quality improved but total egg yield increases. Therefore optimal results can only be obtained when individual ovulatory cycles of females are known before stripping is practiced. This presents a problem when large numbers of broodstock are maintained for egg production, as is the case in most commercial halibut hatcheries where first generation replacement broodstock are now being established. Nonetheless, because of the high fecundity of the halibut, if the major determinants of quality are identified and addressed the need to maintain a large number of broodstock could be negated.

Halibut broodstock managers observe differences in reproductive performance within and between individuals over years but underlying biological parameters have yet to be quantified, including the relationships between egg quality and egg production. How much variation in these highly economically important traits is due to the animal and how much is due to the environment or management? When multiple measurements of a trait are made on individuals, as is the case with the multiple spawnings over years in the halibut, the repeatability of the trait can be estimated.

4.1.3 Repeatability

Repeatability is the correlation between repeated measurements of the same individual and is thus useful in predicting future performance from past records. It expresses the proportion of the variance of single measurements that is due to the permanent differences between individuals, both genetic and environmental (Falconer and Mackay, 1996). Repeatability, r, is defined as the ratio of the between-individual to the total phenotypic variance and thus:

$$r = \frac{v_G + v_{Eg}}{v_P}$$

Where:

v_G =the genetic variance

v_{Eg} =the *permanent* environmental variance between individuals

v_P = the phenotypic variance

The question is often asked "is a good fish always a good fish"? Perhaps the preceding question "what is a good fish" may be a more pertinent one. If these questions are answered it might be possible to select better performing animals as replacement broodstock and not only improve the average performance of the group but also reduce the running costs of the hatchery.

Before any form of selection can take place the selection objectives have to be defined and genetic parameters need to be estimated. Selection in this instance is not based on genetic merit but on the individual's phenotypic performance. Despite the established importance of reproductive performance and the value of quantifying the observed variation between individuals for management and selection, only limited work has been done in the halibut. The purpose of this chapter therefore is to define reproductive traits that may be used to describe a "good" fish in commercial terms and provide information on their interrelationships and the sources of variation within them. These traits should not only include egg quality traits but also egg quantity and frequency of collection. With this information it may be possible to establish procedures for keeping replacement females and apply them to the development a broodstock replacement policy for the Atlantic halibut.

4.2 Materials and Methods

4.2.1 The Animals.

The broodstock used in this study were first generation (F_1) farmed individuals established from the 1995 and 1996 spawning season of wild parents on the farm. In 1995 following the normal hatchery procedures outlined in Chapter One, about 1000 individuals were selected from the best performing batches throughout the season. They were selected based on superior physical appearance and good growth.

Selection occurred in the nursery at the 10-20g stage and these fish were grown in tanks, 5m wide and 1m deep, until they were between 50 and 100g. At this stage they were all transferred to large tanks 12 meters wide and water depth was kept at 1.2m. These large tanks were in polytunnels and illuminated by artificial lighting using two 400 watt sodium bulbs augmented by natural light in the day time. Sea water was supplied to both tanks and oxygen concentration was maintained at above 90% by water exchange.

At three years old the all the individuals heavier than 6 kg were kept as potential broodstock and eggs obtained were intended for the production of juveniles to be sold for ongrowing. The fish were separated into eight tank groups; however, data from only two of the groups, SP2 and SP3, was used and analysed in this study.

At four years old two different photoperiod regimes were applied to the groups. Fish in SP2 were maintained under a one month-advanced photoperiod cycle and spawned from March to May while the fish in SP3 spawned between July and September in a four-month delayed regime. The number and sex ratios in both tanks changed every year because some individuals died and more fish were added to the

tanks from the 1996 year class F_1 population within the farm in order to augment egg production.

Table 4.1 Population and sex ratios in fish tanks used for repeatability study.

Year	SP2			SP3		
	Females	Males	Total	Females	Males	Total
2001	81	6	95	68	5	74
2002	100	6	106	87	8	95
2003	92	30	122	115	17	134

Temperatures were at ambient for most of the year but approximately two months prior to the stripping season the water temperature was lowered to 8^{o}C and one month before the stripping season the water temperature was lowered to 6^{o}C and maintained at this temperature until the end of the stripping season. Fish were fed to satiation three times a week on an industry sausage diet mixed by TROUW.

4.2.2 Data

The data used in this study was obtained from farm records. Information from a total of 239 females were analysed in this study. Most of the fish, with the exception of 11 individuals, were from the 1995 year class group and began spawning activity in the year 2000 at 5 years old. The 11 females were 1996 year class fish and matured in 2001, then were put into SP3 in 2003. The data analysed was collected over three years between 2001 and 2003 when the fish were 6 – 8 years old.

Fish were checked for swelling, an indication of impending spawning time, from the end of February and the end of June in SP2 and SP3 respectively. The date of the first strip and the number of subsequent strips of each female were recorded. From the onset of the spawning season the fish were checked for readiness every three days and attempts were made to strip eggs from each individual, however these

were not always successful. Due to the large number of fish involved within each group individual spawning rhythms were not determined and fish were only examined and stripped on three day intervals throughout the spawning season. The volume of eggs collected at each stripping event was measured and recorded immediately after the stripping was over. The eggs were then stored in covered cold boxes to protect them from ultraviolet light damage and inflated temperatures until they were fertilised.

Eggs were fertilised and incubated following the protocols outlined in Chapter 1. Samples from every batch of fertilised eggs were collected and fertilisation rates determined. These estimated values were recorded for every individual. Whenever lesions or other ailments caused by handling or otherwise were noticed on the fish they were recorded by the manager.

4.2.3 Traits

Five traits pertaining to reproductive performance were defined. These were seasonal reproductive activity (ACT), Number of successful stripping events (NSS), Volume of eggs collected (VEC), Percentage of fertile eggs (PFE) and Volume of fertile eggs (VFE).

Seasonal activity (ACT) defines whether a fish gave any eggs at any of the stripping opportunities presented within a spawning season or not. A value of 1 is given if any eggs were collected and a value of 0 is given if no eggs were collected.

Number of successful stripping events (NSS) is the total number of strippings per female where eggs were obtained, regardless of quality as assessed by appearance, within a single spawning season.

Volume of eggs collected (VEC) is the total volume of eggs collected in millilitres from a female within a single spawning season. Egg volume was measured to the nearest ml by allowing the eggs to settle in a volumetric cylinder after stripping.

Percentage of fertile eggs (PFE) is the mean fertilisation rate estimated from all the fertilised batches produced by a female within a single stripping season. Fertilisation rate is the total number of developing eggs divided by the total number of eggs (expressed as a percentage) assessed at 20hrs post-fertilisation in triplicate samples of eggs. This trait is linked with the blastomere morphology trait of symmetry (see introduction). It should be noted that the assessment of symmetry is subjective and estimates were made by different members of staff on the farm over the three years and therefore this was not standardised.

Volume of fertile eggs (VFE) is the volume of eggs collected at each stripping event multiplied by the fertilisation rate observed for that batch of eggs summed up over all the batches collected from a female within a single stripping season.

4.2.4 Statistical Analysis

A total of 538 individual reproduction data records were obtained from the 239 females over the 3 years. The data was a catalogue of repeated seasonal measurements from the individuals in the two separate groups of fish. Once all five reproduction traits described above were computed the following restrictions were applied prior to the onset of the analysis. Information on egg volume collected was not recorded on 15 individuals in 2002, these data points were removed from the analysis in order to avoid the introduction of bias.

Descriptive statistics were estimated using the programme GenStat for Windows version 7.0. The analysis was done in two stages. Stage I contained all the available data including non fertile females and all the zeros. Stage II was data from only fertile females.

4.2.4.1 REML Analysis

The data was analysed with REML to fit mixed linear models using GenStat software. Fixed effects included in the model were age, tank and physical damage to broodstock (such as lesions or abrasions) as well as an interaction between age and photoperiod.

The random effect fitted was the individual.

Model:

$Y_{ijkm} = \mu + A_i + P_j + D_k + (AP)_{ij} + f_{ijkl} + e_{ijklm}$

Y= The ijklmth observation of a fish for any of the production traits defined with mean-μ at age-i in photoperiod-j and physical condition-k

Fixed effects:

A_i =Age of fish in years.

P_j =Photoperiod regime. Confounded in this was the tank effect. This was coded 2 for SP2 and 3 for SP3.

D_k = if the fish were recorded as having stripping associated wounds within a season. This was coded 1 for damaged and 0 for no damage.

Random effects:

f_{ijkl}= random effect of individual fish.

e_{ijklmn}= residual error.

4.2.4.2 Transformations

Maximum likelihood analyses require that the data is normally distributed. This was not the case with the volume of eggs collected (VEC). A standard procedure in most quantitative genetic investigations is to transform the data to resemble

normality as closely as possible. The Box-Cox transformation provides a general method for achieving this (Lynch and Walsh, 1998). The Box-Cox transformation is defined by:

$$T(y) = \left(y^{\lambda} - 1 \right) \Big/ \lambda$$

Where y is the response variable, λ is the transformation parameter and T(y) is the transformed value of y. The objective of this method is to find the value of λ that gives the best fit to normality.

In the current study transformation of the data was required. This was conducted using a Box-Cox type of the family of power transformations adapted by Darwash *et al*. (1996). The method considers the family of power transformations indexed by λ, thus, $(VEC)_{\lambda}$.

The transformations for a dependent variable y is given by:

$$y_{\lambda} = \left(y^{\lambda} - 1 \right) \dot{y}^{1-\lambda} \lambda^{-1}$$

Where $\dot{y}$ is the geometric mean of y.

The linear model was fitted to all values of λ in steps of 0.1 in the range of -1 to 1.0 inclusive. When λ = 0 the dependent variable, y, is estimated as the natural logarithm multiplied by the geometric mean. When λ = 1 the additive model was fitted to the observed values of VEC and when λ = -1 an additive model was fitted to the reciprocal of VEC.

The optimum, most likely, transformation for VEC was determined by obtaining the minimum deviance (-2xlog likelihood) value after fitting all the defined values of λ to the model. This was done by plotting a deviance profile of the various values for λ.

4.3 Results

4.3.1 Descriptive Statistics

Descriptive statistics; phenotypic means, standard deviations, standard errors and coefficients of variation are presented in Table 4.2. Results show that the most variable traits appear to be those that assess egg quality, PFE and VFE.

Table 4.2 Descriptive statistics of defined reproductive traits for all fish.

TRAIT	μ	s.e. (μ)	σ	max	c.v (%)
ACT. (%)	0.71	0.02	0.45	1.00	NA
NSS	4.80	0.20	4.60	21.00	95.20
VEC (ml)	2810	121	2768	11400	98
PFE (%)	13.00	1.00	15.00	88.00	123.46
VFE. (ml)	664	42	960	7070	145

ACT= seasonal activity, NSS= Number of successful stripping events, VEC= Volume of eggs collected, PFE= Percentage of fertile eggs, VFE= Volume of fertile eggs, μ= mean, s.e= standard error, σ= standard deviation, max= maximum recorded value, c.v= coefficient of variation. NA=Not applicable

Records show that 71% of the individual stripping records summed up over each season resulted in the collection of eggs, therefore 29% of the fish population in any one season over the three years were not reproductively active i.e. gave no eggs. The "inactive" group includes fish that did not produce any eggs over the three year period of data collection as well as fish that gave eggs in either one or two years. The number of active and inactive females in each of the three years of the study is shown in Table 4.3 below.

Table 4.3 Number of reproductively active and inactive females in the three years of the study.

Year	Number of active females	Number of inactive females
2001	114	35
2002	144	49
2003	113	83

The average number of successful stripping events was approximately 5 however one fish, F519, gave eggs 21 times within a single season. The average volume of

eggs collected was 2.79 litres; in contrast the mean volume of fertilised eggs was much less at 641ml. Perhaps the most interesting result is the average percentage of fertilised eggs. The average fertilisation rate was only 13% even though the maximum-recorded value was 88%. The farm discards all egg batches of fertilisation rate less than 40%; this therefore indicates that most of the batches collected from this group of fish were not incubated. It is worth noting that the range of values obtained and means estimated were spread and reduced respectively because of the data from the non-fertile females.

4.3.1.1 Reproductively active fish

Data from only active females within each season were extracted from the data set and summarised. The results obtained are shown in table 4.4 below. Not surprisingly means were increased. However, as previously observed, traits associated with quality were still the most variable. The variance in both PFE and VFE appeared to increase while the coefficient of variation decreased because the means were higher. Even though only reproductively active females were examined the mean percentage of fertilised eggs was still only 18%.

Table 4.4 Descriptive statistics of defined reproductive traits for "active females".

TRAIT	μ	s.e. (μ)	σ	max	c.v (%)
NSS	6.80	0.20	4.00	21.00	59.40
VEC (ml)	3962	130	2497	11400	63
PFE (%)	18.00	1.00	16.00	88.00	88.92
VFE. (ml)	935	53	1022	7070	109

NSS= Number of successful stripping events, VEC= Volume of eggs collected, PFE= Percentage of fertile eggs, VFE= Volume of fertile eggs μ= mean, s.e= standard error, σ= standard deviation, max= maximum recorded value, c.v= coefficient of variation.

4.3.2 Phenotypic Correlations

Phenotypic correlations between all the traits were positive and moderate to high (Table 4.5). The correlations with the highest magnitude were between traits that

describe the quantity and frequency of strips. The lowest correlations were between the number of successful strips and the percentage of fertilised eggs. Again the presence of zero values from the non-active females in the data set was likely to exaggerate the correlations, so they were re-estimated using data from fertile females only.

After excluding the inactive females from the analysis all correlations, on average, although still positive, were lower in magnitude. The correlation between NSS and PFE reduced from 0.38 to 0.05. This results suggest that the number of times a fish is stripped may not have an effect or may have very little influence on egg quality, as defined by the percentage of fertile eggs and also that the volume of eggs collected or produced by a fish is poorly associated with egg quality. As expected, the correlations between VFE and PFE were strong due to the partial auto-correlations originating from commonality among biological components and methods of measuring these traits.

Table 4.5 Phenotypic correlations between the defined reproductive traits.

TRAIT	ACT	NSS	VEC	PFE	VFE
ACT (%)	1				
NSS	NA	1	0.70	0.05	0.42
VEC (ml)	NA	0.83	1	0.22	0.71
PFE (%)	NA	0.38	0.48	1	0.63
VFE (ml)	NA	0.58	0.77	0.72	1

Figures above the diagonal are the correlations estimated for active females only and figures below the diagonal are those of all females. ACT= seasonal activity, NSS= Number of successful stripping events, VEC= Volume of eggs collected, PFE= Percentage of fertile eggs, VFE= Volume of fertile eggs, NA=Not applicable

4.3.3 Predicted Means Of Fixed Effects.

Predicted means of all fixed effects fitted in the model were estimated from the REML analysis in the "predict" option from GenStat version 7.0. The overall tests for age effects were consistently significant ($P<0.001$) for all the traits studied. In

general the results suggest that the older a fish was the better she performs however, reproductive activity within the population declined in fish from 7 to 8 years old.

The predicted means of seasonal activity over the three years in both groups are shown in Figure 4.1. The fish in SP2 under the one-month extended photoperiod were more active ($P<0.001$) than the fish in SP3. In the 3 years of data collection there was an average difference of 43% (±4.5%) in the levels of seasonal activity between both groups i.e. more fish gave eggs in SP2. There were no significant interactions between photoperiod and age and whether a fish was damaged or not did not appear to influence activity.

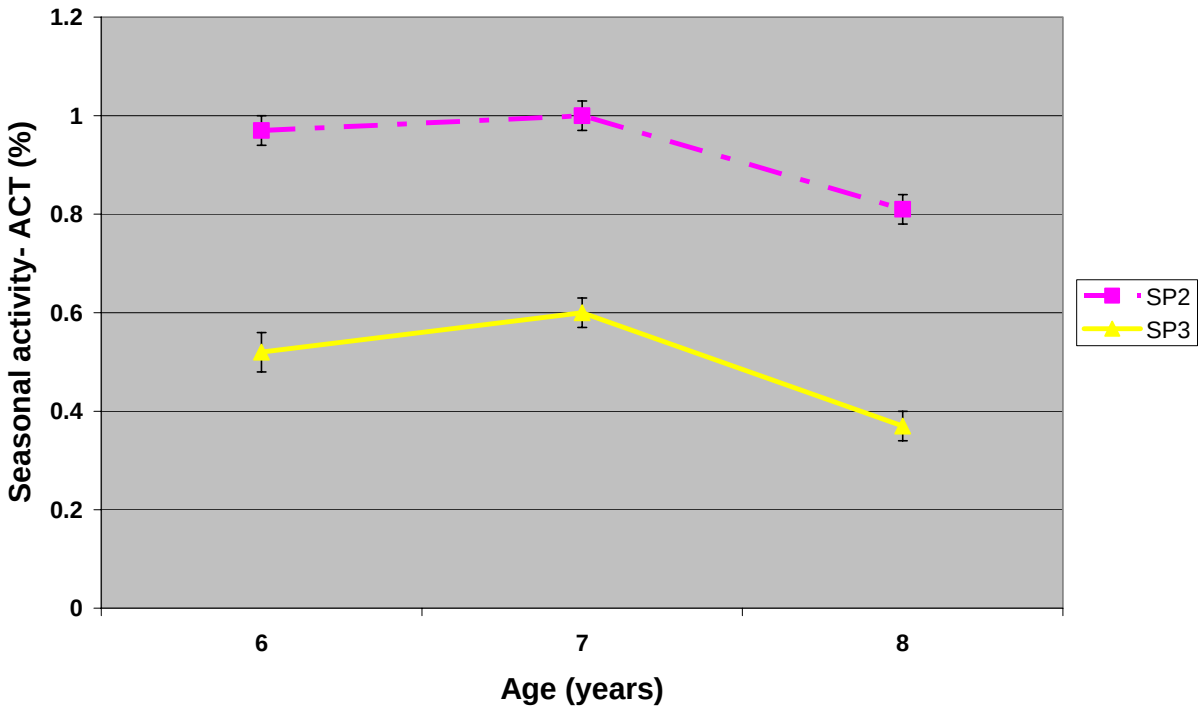

Figure 4.1 Graph showing the predicted means of age for seasonal activity over the three years of the study in both tank groups (SP2 and SP3).

The older a fish was the more times she could be stripped. The average numbers of successful strips were 1.63 (±0.68), 4.97 (±0.62) and 6.95 (±0.63) for fish aged 6, 7 and 8 years respectively. Due to the fact that the stripping conditions and protocols were kept constant, i.e. the fish were stripped every three days, this indicates that

the stripping season is extended as fish mature. There were no significant differences in the number of strips between photoperiod regimes but there appeared to be a significant interaction effect between age and photoperiod. As expected ailments or lesions had negative effects on stripping ($P<0.001$).

The strong correlation (0.70) between volume of eggs collected (VEC) and NSS was not surprising and implies a similar response to age for the trait. Older fish did produce more eggs as shown in Figure 4.2. However there was a significant difference between photoperiods in that an estimated volume of 780 (±279.20) ml ($P<0.05$) more eggs were collected from fish in SP2.

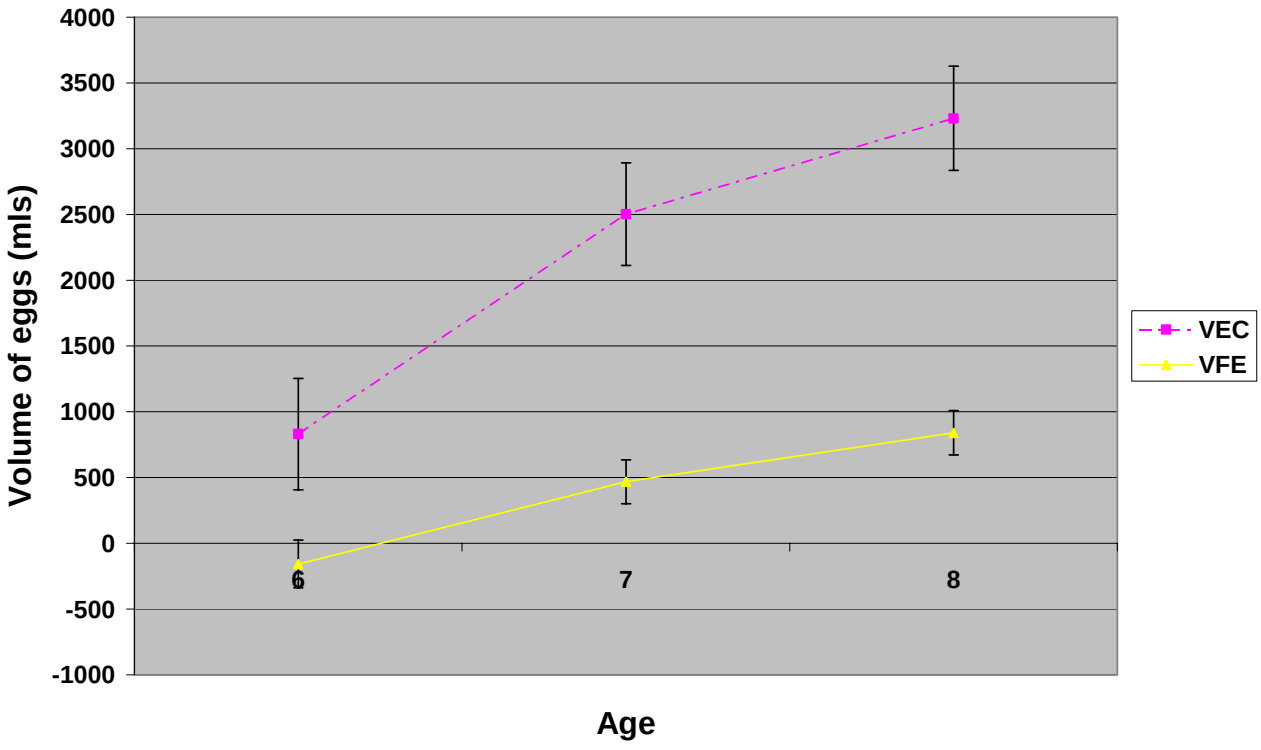

Figure 4.2 Graph showing the predicted means of VEC and VFE averaged over both tank groups (SP2 and SP3) in the three years of the study.

Egg quality, defined by PFE, improved with age. Predicted means are shown in Figure 4.3. Fish in SP2 performed better by 13% (±1.5%). This difference was highly significant (P<0.001). There was also a significant interaction between age and photoperiod (P=0.002) and fish with ailments gave poorer quality eggs (P<0.05). VFE showed the same pattern however, there was no significant interaction between age and photoperiod.

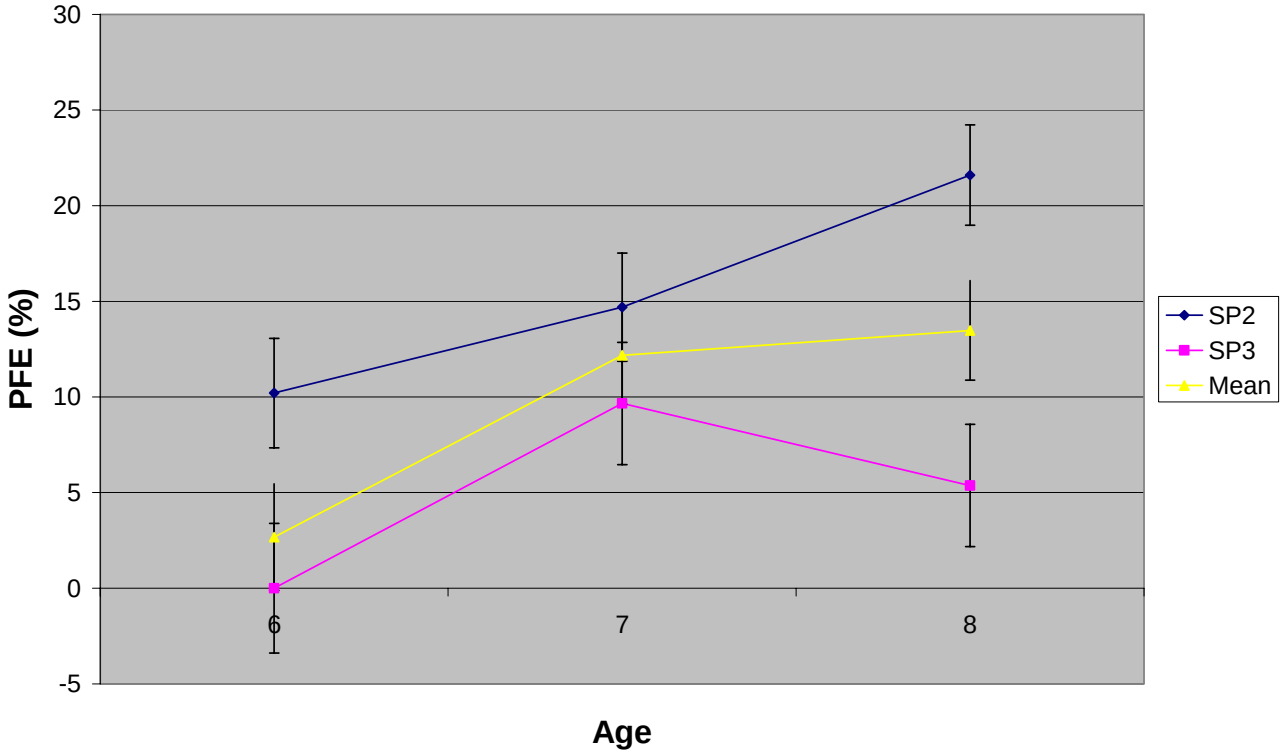

Figure 4.3 Graph showing the predicted means for the Percentage of fertile eggs (PFE) of fish in SP2, SP3 and the mean over both tanks.

4.3.4 Repeatability

Estimates of repeatability for the five traits studied are given in Table 4.6. The figures estimated ranged from 0.05 for egg quality traits to 0.37 for quantity traits however, most values were low. It is perhaps not surprising that repeatability estimates of reproductive measures were low because of the complex nature of the traits involved.

Fertility was very poorly repeatable (0.08); this therefore means that the same individuals were not productive every year. The volume of eggs per fish though was moderately heritable and points to the fact that good producers are likely to be consistently good performers. No significant evidence was found however that egg quality defined by fertilisation rate was repeatable.

Table 4.6 Repeatability of reproduction traits with standard errors and likelihood ratio tests statistics (L.R.T.S) with Chi squared significance values.

Trait	Repeatability	Standard Error	L.R.T.S	χ^2_1
ACT	0.08	0.06	1.83	0.5
NSS	0.12	0.08	2.25	0.1
VEC	0.37	0.07	30.86	0.001
PFE	0.05	0.07	0.63	0.9
VFE	0.15	0.07	4.18	0.05

χ^2_1 Significance with 1 degree of freedom

4.3.4.1 Transformations

The likelihood profile obtained from fitting the model to different power transformations applied to VEC is shown in Figure 4.4 below. The estimate of repeatability was sensitive to the transformation across the range from 0 (λ=-1) to 0.37 (λ=1). The effects of power transformations on estimates of repeatability of VEC are presented in Table 4.7. The optimum transformation was λ=0.5 for which the deviance value was 5907.56 and repeatability was 0.35±0.07. This result presents the best estimate of the character for the trait. Further work looking at models with appropriate error distributions may uncover evidence of repeatable variation for the other traits but this was not pursued.

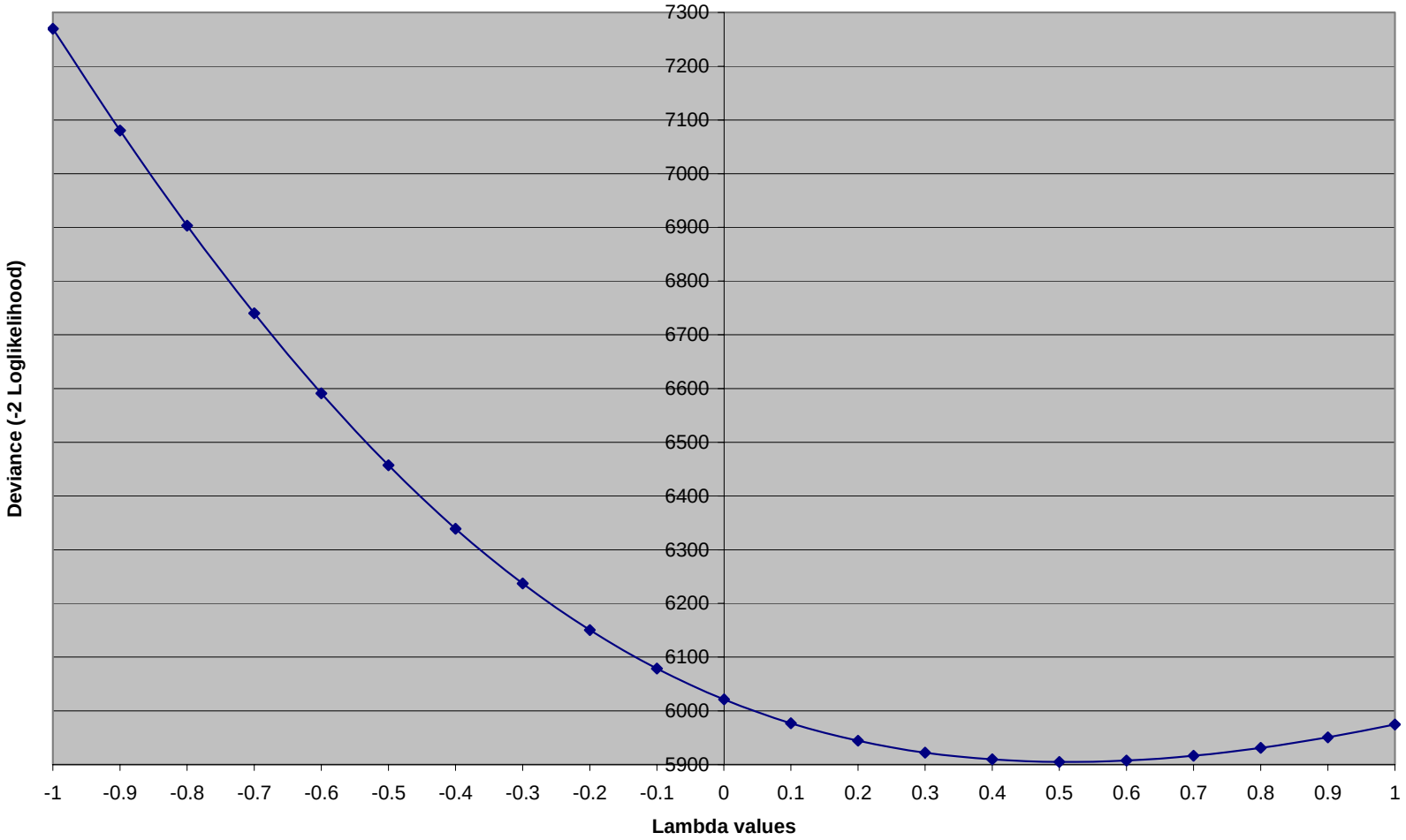

Figure 4.4 Deviance profile of fitting the model to power transformation of λ values in the range of -1 to 1 for VEC.

Table 4.7 The effect of power transformations (λ) on estimates of repeatability values estimated for volumes of eggs collected (VEC).

λ	Repeatability (r)	s.e	Deviance
-1.0 (reciprocal)	0.00	0.00	7269.74
-0.6	0.09	0.08	6591.02
-0.5	0.13	0.08	6457.19
0 (logarithm)	0.28	0.07	6021.47
0.5	0.35	0.07	5904.96
0.6	0.36	0.07	5907.56
1.0 (observed)	0.37	0.07	5974.62

4.4 Discussion

Hitherto reproductive performance has not been defined or quantified in the Atlantic halibut under commercial conditions. This study has defined five performance traits reflecting quantity and quality of eggs, frequency of stripping events and viability. Analysis of these traits has shown that phenotypic correlations between "quality" and "quantity" traits are low in magnitude and that whilst egg volume was repeatable, providing the fish were reproductively active, for individual fish across seasons i.e. indicative of intrinsically good producers, the quality of eggs showed little repeatability. The study also quantified the effects of age and photoperiod on reproductive performance.

4.4.1 Effect of photoperiod on reproductive traits

On average the fish in SP3 kept under a 4 month- delayed photoperiod did not perform as well as the fish under the one-month extended regime. Whilst there were no significant differences in the number of successful strips between fish under the two different photoperiod regimes there was a 56.25% reduction in the total volume of eggs collected, an 18.21% drop in the volume of eggs collected per fish and a 20.9% difference in mean fertilization rate over the three years of the study. Therefore there were approximately 182 litres more fertile eggs produced by the group SP2 than SP3. Furthermore the fish in SP2 were more reproductively active by 43%. In the first year this could be explained by the fact that in 2001 the fish in SP3 were moved and fin clipped 3 months prior to the start of the stripping season and the stress associated with the move may have affected the group's performance in that they stopped eating. Female halibut with suppressed feed intake are known to reabsorb their gonads and miss a spawning season (Haug, 1990). This

however does not explain the observed differences over the next two years of the study where although the level of activity improved they still performed poorer than their counterparts. Björnsson *et al*. (1998) also reported that over three spawning seasons when a four month delayed photoperiod regime was applied to females spawning activity was negatively affected and 20% less fish spawned compared with the control group under a natural cycle.

A further reason for this could be due to the fact that in both studies the annual temperature profile of the tank was not synchronized with the photoperiod profile. In their pioneering work on photoperiod manipulation of spawning in the halibut Smith *et al*. (1991) experienced problems with the timing of spawning in females and recommended that water temperature be altered as well as photoperiod. Although the water temperature was lowered prior to the start of spawning season in accordance with the recommendations of Smith *et al*. (1991), this reduction in temperature was not integrated with an annual rhythm applied to a full annual cycle. Future work should therefore attempt to explore the potential of synchronizing annual rhythms of both water temperature and photoperiod.

4.4.2 Effect of age on reproductive performance

With the exception of reproductive activity (Figure 4.1), all the traits studied improved with age. Older fish were more fertile, gave more eggs and were stripped more often. Kjesbu *et al*. (1996) conducted a similar study with cod. They monitored the reproductive performance of 10 females over three spawning seasons and observed that as fish grew older and larger they spawned more often, gave more eggs and the fertilisation rate of batches increased. It was concluded that maternal weight was positively and significantly correlated with the number of batches,

length of the spawning season, total number of eggs shed and the weight of eggs. In their analysis however the positive effects of increased size over the three seasons on reproductive performance, were confounded with maturity and advancing age.

Haug and Gulliksen (1988) examined the potential for egg production (fecundity) in wild caught Atlantic halibut females off the Northern Norwegian coast between 1981 and 1986. They observed a positive, significant, relationship between total number of eggs produced and body size. Fitting a power – curve regression to the data they derived an equation to predict the future performance of females based on body length and concluded that fecundity was dependent on body size. In their study however the ages of all fish sampled were not known. It appears that, as previously stated, maternal size is the main determinant of reproductive performance in fish and body weight usually explains a great proportion of the variance in fish fecundity (Wootton, 1998) nonetheless in a repeat spawner the effects of maturity, advancing age, cannot be excluded.

Evans *et al*. (1996) conducted a study on the composition of eggs from repeat and first-time spawning in captive Atlantic halibut. They found that though there was a large difference in weight between first spawners (up to 8.7 Kg), there was no difference in the mean dry weight of eggs between batches. There was however a highly significant difference between first and second time spawners. Female halibut are expected to keep growing until they are about 20 years old (Haug, 1990). Reproductive performance will therefore be expected to improve with age but in the framework of a selective breeding programme this is potentially conflicting. The rate of genetic progress is inversely proportional to the generation interval, therefore maintaining a broodstock population for 15 years or more will be unfavourable for

any programme unless their performance is offset by the need to recruit replacements. It is also irrational to believe that performance will improve progressively with time without eventually diminishing. There is thus a need to understand the profile of reproductive performance over time in order to determine the optimum period for which a fish is kept active in the broodstock population.

4.4.3 Repeatability of reproductive performance traits

In the context of the lifetime of the female halibut, repeatability estimates of reproductive traits are of particular value because this represents that portion of the variance that is due to some intrinsic property of the fish. Although environmental conditions may change from year to year, the repeatability represents the strength of the tendency for a fish to either be a superior or inferior performer and will thus allow the manager to predict future performance. The repeatability also sets an upper limit of the heritability of a trait because it includes all genetic variance, not just additive variance and permanent environmental variance. Neither reproductive activity nor egg quality appear to be an intrinsic property of fish, however the volume of eggs produced given that the fish was active did appear to be substantially repeatable.

The estimates obtained for repeatability of all reproduction traits were low compared to other published heritability values for the same traits in trout and coho salmon, except perhaps the volume of eggs (Sue *et al*., 1997; Gall and Neira 2004). Unlike with other domesticated species published works suggest that reproduction traits are highly heritable in fish. This then means that there is considerable additive genetic variance and might explain why certain families dominate captive populations. This however does not appear to be the case with the halibut, in which

it would seem that factors apart from genetics and permanent environment determine reproductive performance.

Reproductive activity over seasons did not appear to be repeatable or predictable (r=0.08±0.06). All the fish were checked every three days for over 2 months and so it will be highly unlikely, though not impossible, for a fish to have spawned undetected. Studies by Kjesbu *et al*. (1996) in the cod showed that high fecundity in one season negatively affected both the next season's performance and the fish body weight. This might provide a reason for the apparent low repeatability but it also shows why the family representation in any one year group can be poor. It also has significant implications for broodstock management. If all the fish do not spawn every year then the numbers kept for production of eggs will need to be higher.

Volume of eggs collected was the only repeatable trait (r=0.35±0.07). This then means that a "good" fish will, on average, always be a good producer. Alternatively a poor performer will consistently under perform and can be culled from the group. Number of successful strips was poorly repeatable but because of its high correlation with volume of eggs collected this is an unexpected result.

Percentage of fertile eggs was not repeatable and therefore draws attention to the fact that this trait is not a function of the fish but a function of its environment. Falconer and Mackay (1996) define the variance between different measurements on the same individual as "the special environmental variance, Ves" because this is entirely non-genetic, calculated as one minus the repeatability. In this case the estimate is almost unity. Therefore egg quality is mostly dependent on good management practice not on "good" fish.

Previous authors (Holmefjord, 1991; Norberg *et al*., 1991; Bromage *et al*., 1994; Brown *et al*., 1995; Mazorra *et al*., 2003) identified four factors that influence egg quality in the halibut; water temperature, disease, timing of stripping and diet. All fish on the commercial farm were held in similar tanks, fed on the same diet and water temperature was maintained at 6^{o}C throughout the stripping season. Of these four factors only timing of stripping was uncontrolled. Nonetheless a large amount of variation was observed between animals and fertilisation rates from 0-88% were observed. Norberg *et al*. (1991) reported fertilisation rates of > 80% when ovulatory rhythms were synchronised with stripping times in an experiment using only 4 females. Under commercial conditions checking the fish to determine individual rhythms when 70 females are spawning is a highly labour intensive and seemingly impossible task. This routine places an upper limit on the number of fish that can be properly managed over a season. Furthermore checking fish daily to see if they are ready to be stripped is perceived as being stressful on the farmers and fish, so fish are stripped every three days. There appears to be a trade off between a small volume of good quality eggs obtained from a small number of females and a large quantity of poor quality eggs obtained by random chance from a large spawning group.

It appears therefore that in a halibut hatchery the only trait that is "controlled" by the fish is volume of eggs and that in turn is limited by size and maturity. All the other traits are influenced by management practice. Therefore, the concept of a "good fish" is limited to volume alone. As far as the perceived limitation to the halibut industry is concerned, egg quality, the quest is for a "good manager" or rather "good management practice". The results of this study have shown that a good fish is one that gives a large quantity of eggs and yes, a good fish is always a

good fish. The quality of eggs is not a function of the fish but of its environment. Nutrition has been shown to influence egg quality significantly and a study by Mazorra *et al*. (2003) has shown that egg quality traits, fertilisation rate and blastomere scores, are significantly improved with the addition of arachidonic (1.8%) acid to the composite diet.

The findings of this study permit the proposal of a management strategy that might allow more fertile eggs to be produced from fewer fish. Due to the fact that egg volume is repeatable, provided the fish are reproductively active, performance can be predicted (volumes of eggs collected) therefore only the best performing individuals should be maintained as broodstock. With fewer females in the broodstock population efforts could be concentrated on paying more attention to spawning rhythms. A better approach to the management of broodstock for improved egg quality might be to reduce the number of broodstock and focus efforts on synchronising stripping times with the individual rhythms. However, due to the fact that reproductive activity between seasons is not repeatable the number of females in a broodstock group will need to be higher i.e. reduce the selection differential. The design and implementation of a broodstock replacement strategy will be discussed in Chapter 6.

Chapter 5

Heritability of body weight

5.1 Introduction

The Atlantic halibut is famed for its large size and ongrowers are paid differentially based on the weight of individual fish. Consequently the primary breeding objective, as defined by members of the British Marine Finfish Association (BMFA), for commercial production of the Atlantic halibut in the UK is to increase growth rate, more specifically body weight at harvest. Despite the fact that breeding objectives are considered to be species-specific (Gjedrem, 2000), growth is universally accepted as one of the first, and most important, traits chosen for improvement in aquaculture (Gjedrem, 1983; Kinghorn, 1983; Gjerde, 1986; Hershberger *et al*., 1990). This is because rapid growth speeds up the turnover of production and larger animals attract a higher price per unit weight compared to smaller ones. Furthermore because it is an easy trait to measure it is likely to dominate selection criteria and indices.

In breeding programmes the objective is to select the individuals with the best genetic performance. This is usually done, in its simplest form, by selecting individuals with observed superior phenotypic features; body weight at a specific age is most commonly used in improving growth rate (Crandell and Gall, 1993). However, unless additive genetic variation exists within the population for the trait under selection efforts can be wasted. Additive genetic variation between individuals for the trait(s) of interest is the most important prerequisite for genetic improvement through selection, because it is this component of the observed phenotypic differences between individuals that permits genetic change in the mean of the population, and so makes genetic improvement by selection possible.

Moreover the amount of this variance component determines the rate of response to selection (Gjedrem, 1983; Gall and Haung, 1988a; Beaumont, 1994).

Significant research efforts have been focused on estimating both genetic and phenotypic parameters for growth traits in several aquaculture species in order to predict the success of selection, choose the most appropriate method from various types of breeding techniques, estimate economic returns and predict breeding values of selection candidates (Gjerde and Schaeffer, 1989). The results of these studies have shown that, in general, a sufficient amount of additive genetic variation exists for most growth traits to justify a selective breeding approach to improve growth (reviews by Gjedrem, 1983; Kinghorn, 1983; Gjerde, 1986; Gjedrem, 2000). Furthermore selection experiments have resulted in significant improvements in growth performance (see general introduction). However such studies have been limited in marine species and have not been conducted in the halibut.

5.1.1 Heritability

Additive genetic variance (V_A) is normally expressed as a proportion of the total phenotypic variance (V_P), termed the heritability (h^2). The predictive role of this character is its most important feature in selective breeding in that it expresses the reliability of the phenotypic value as a guide to the breeding value. For this reason, heritability enters into almost every formula connected with breeding methods and many practical decisions about procedures depend on its magnitude (Falconer and McKay, 1996).

5.1.1.1 Estimating heritability

Heritabilities are estimated from the degree of resemblance between relatives. This is usually done by creating a pedigree structure through controlled matings in order

to compare the variance in performance of traits(s) among different relatives (full and half- sibs, parent/offspring). Alternatively following actual selection, the realised heritability can be calculated from the deviation of the mean performance between the parental and offspring generations. It is however difficult to estimate values with great precision and most estimates have relatively high standard errors (Beaumont, 1994; Falconer and McKay, 1996). The precision of heritability estimates are generally weak because the scale of experiments are often constrained by physical limitations such as the number of family crosses that can be made and subsequently accommodated, and the number of individuals per family that can be measured. In addition, if estimates are derived from full-sib families they are based on maternal variance components which tend to include supplementary variance components other than additive genetic variance such as non-additive genetic variance e.g. dominance, and maternal effects which can lead to over estimation of the heritability values. Estimates based on half-sibs tend to give a more robust estimate because they are generally based on the sire components of variance, which in general do not include, or tend to include to a smaller degree, these non-additive genetic and common environmental effects (Falconer and McKay, 1996).

5.1.1.2 Heritability estimates in aquaculture

When compared with other organisms, aquaculture species present both advantages and disadvantages with regard to the genetic study of quantitative characters. The high fecundity and oviparousness of many species provide the opportunity to simultaneously fertilise numerous eggs and create full-and half-sib families using various experimental designs, including powerful factorial breeding plans. However the need to keep families separate until the offspring are large enough to be tagged results in the introduction of common environmental effects shared by

family members which have proven difficult to remove (Winkelman and Peterson, 1994a). Even if genetic profiling methods are used to minimise the environmental correlation between family members, fish geneticists are limited by the numbers of fish that can be genotyped under mixed rearing conditions (Blanc, 2003).

The first estimates of heritability for body weight in fish based on half-sib data were published in 1972 for the rainbow trout and the common carp (Aulstad *et al*. : cited by Gjedrem, 2000). Since then, many estimates for body weight at specific ages have been estimated from sire, dam and family variance components, particularly for cold-water species. In general, however, many of them were based on a few families and as such have large standard errors or due to common environmental, maternal and non-additive effects were deemed biased. A more recent list of published estimates is shown in Table 5.1 below.

Table 5.1 Heritability estimates for body weight in various aquaculture species.

Species	Age /size	Design (No. of Sires, No. of Dams)	Numbers tested	h^2(±S.E)	Method	Reference
Rainbow trout	years 1.5 years 2.0 years 2.5 years	Nested (32S, 76D)	1781	0.28(0.07) 0.40(0.07) 0.48(0.08) 0.51(0.04)	AM	Elvingson and Johansson (1993)
Rainbow trout	4.16(±1.55)Kg	Nested (278S, 682D)	67,280	0.44 (0.02)	AM	Pante *et al*. (2002)
Peneaid shrimp	20g	Nested (16S; 8-16D)	400	0.11(?)	4 generations	Goyard *et al*. (2002)
Rainbow trout	2.5 years 4 years	Nested (2S, 2D)	747 699	0.37(0.22) 0.27(0.20)	S	McKay *et al*. (1986)
Atlantic salmon	1 winter at sea 2 winters at sea	Nested (194S, 512 D)	4198 840	0.36(0.11) 0.00(0.15)	S	Jónasson *et al*. (1997)
Common carp	8 weeks	Factorial (24S, 10D)	516	0.33(0.07)	AM	Vandeputte *et al*.(2004)
Chinook salmon	3-6g 9 months in Sea 22 months in Sea	Factorial (16S, 32D)	7649 5107	0.24(0.09) 0.39(0.08) 0.25(0.10)	AM	Winkelman and Peterson (1994a&b)
Rainbow trout	2.58(±1.43)g 153.8(±35.44)g 1.45(±0.39)Kg 1.95(±0.49)Kg	Nested (49S, 192D)	1920 2140 892 1363	0.52(0.15) 0.20(0.11) 0.18(0.12) 0.20(0.10)	S	Gall and Haung (1988a)
Rainbow trout	52 days post 0.15 g 215 days post 0.15g	Factorial (30S, 30D)	3290	0.00(0.32) 0.53(0.27)	AM	Henryon *et al*. (2002)

S=sire componenet. D=dam component, AM= additive component from animal model, x gen, number of generations for realised heritability

In marine finfish species only two estimates of heritability have been presented in the literature, Gjerde *et al*. (1997) in turbot and Gjerde *et al*. (2004) in cod. Gjerde *et al*. (1997) analysed the body weights of 6400 fish recorded at 15-16 months of age. The fish were offspring from a nested design of 35 sires and 19 dams and full-sibs were kept in separate tanks for about one-third of the studied growth period. The estimated values of heritability for body weight were 0.45(±0.28) and 0.70(±0.19) based on dam and sire variance components respectively. Gjerde *et al*. (2004) reported heritability estimates for body weight in the Atlantic cod. 8236 individuals were tested from a nested design using 34 sires and 50 dams. Full-sib families were kept separate but estimates were derived using an animal model. They reported heritability estimates of 0.29(±0.27) and 0.52(±0.26) depending on the model fitted.

Differences in growth performance between halibut from different areas of the North Atlantic (Canada, Iceland and Norway), reared in the same environment under similar conditions have been reported (Jonassen *et al*., 2000; Imsland *et al*., 2002). These suggest significant genetic differences for the trait. However, the heritability of growth traits within domesticated populations has yet to be estimated. Therefore the aim of this chapter was to estimate the heritability of body weight at different stages in the growout phase of the production cycle for hatchery-reared juveniles under current commercial conditions.

5.2 Materials and Methods

5.2.1 The Animals.

The fish used in this study were part of a wider experiment, forming the basis of a PhD project by Nigel Jordan, University of St Andrews, aimed at comparing the growth performance between fish reared in sea cages (Teacius, owned by Marine Harvest McConnell) and fish reared in land based tanks (Otter Ferry Seafish) through the grow-out period. The study was also designed to examine the growth patterns between male and female halibut in order to ascertain the point at which divergence in growth occurred between the two sexes.

All the fish used were first generation (F_1) offspring established from the 1998-spawning season of wild parents on the farm. 1516 fish were randomly selected at the nursery stage of the production cycle for the experiment. Prior to this point normal protocols for hatchery procedures were followed, as described in Chapter 1. The animals used were taken from batches that were of similar ages in order to limit bias in the experimental design. Therefore the fish studied were likely to be offspring from a small group of fish that spawned within a one-month period.

The fish were graded into four categories based on size; two groups of "large" fish and two groups of "small" fish. They were grown in external trial tanks, 5m wide and 1m deep until every individual was fitted with a PIT tag and weighed. The average weights of the fish in the two large groups were (a) 486.07±89g and (b) 505.66±87g and the average weights of the fish in the small groups were (c) 326.79±65g and (d) 343.41±65g. At this stage a random sample of 832 fish, sampled approximately equally from one large

group (b) and one small group (c), were transferred to two separate tanks 12 meters wide with a water depth of 1.2m in Otter Ferry (OFS). The remaining 693 fish in the other two tanks (b&d), which were the larger i.e. had higher mean weights in both categories were moved to two separate sea cages in Teacius owned by Marine Harvest McConnell (MH).

Each fish was weighed at approximately 3-month intervals and males were checked for sexual maturity until harvest in November 2002. Sex was assigned by ultrasound scanning and sexual maturity in males was determined by gentle palpation of the abdomen at weighing times to check for running milt. The age of sexual maturity for males was classified into three categories; first, second and third winter males. First winter males were those that "ran" (produced milt) in February 2001, second winter males were those that ran in February 2002 and third winter males were those that did not show signs of sexual maturity at the end of the trial period.

In May 2002 blood samples were collected from 544 randomly selected individuals from within both groups (OFS and MH) for genotyping to determine their parentage and subsequently to analyse the quantitative inheritance of body weight within the population. The procedures followed for the molecular genetics analysis and the results obtained were described and reported in Chapters 2 and 3. The number of fish sampled from both groups and their initial grade is shown in Table 5.2.1

Table 5.2 Number of fish used in Nigel Jordan's trial and numbers sampled for heritability study.

Fish	Otter Ferry			Teacius		
	Large	Small	Total	Large	Small	Total
No. In trial	405	418	823	396	297	693
No. Sampled	191	168	359	104	69	173
No. Assigned			339			160

5.2.2 Traits and Data

The performance data used in this study was acquired from the trial records compiled by Nigel Jordan in his PhD study. Information from all the 1516 fish tested in the trial (dates of sampling, weights, lengths, condition factor, point of sexual maturity and sex) were obtained. However, only data from fish with assigned pedigrees were used for genetic analysis. The fish were weighed 12 times in the course of the study in both sites. The dates of sampling are shown below; harvest weight was recorded for fish in Teacius only because different management procedures pertaining to harvest are followed in Otter Ferry and some fish were retained as potential broodstock

Four traits were analysed in this study. They were: body weight after three months acclimatising to new test environments (acclimatised weight- Acc wt-), body weight at the 6th and 9th sampling events that corresponded approximately to the 1st and 2nd years on test and the final weight (end weight). There were differences in weighing times between both sites with no consistent patterns (see Table 5.3).

Table 5.3 Dates when fish were weighed in the growth trial.

Measurements*	Dates of weighing in Otter Ferry	Dates of weighing in Teacius
Start weight	25/10/99	25/10/99
Acclimatised weight*	24/01/00	18/01/00
2	21/03/00	13/03/00
3	12/06/00	21/06/00
4	12/0900	28/08/00
5	07/1200	13/12/00
First year on test*	26/02/01	13/03/01
7	12/06/01	12/06/01
8	10/10/01	25/09/01
Second year on test*	16/01/02	27/02/02
10	28/05/02	28/05/02
End weight*	18/09/02	12/09/02
Harvest weight		04/11/02

*Measurements for which heritability estimates were derived

5.2.3 Statistical Analysis

Of the 532 fish sampled, 33 were excluded because no pedigree information was derived (see Chapters 2 and 3), and a further 13 were removed because of insufficient growth information. Therefore data from 486 individuals were analysed with a sex ratio of approximately 55:45 (271 females and 215 males). Descriptive statistics and phenotypic correlations were estimated using the programme GenStat for Windows version 7.0.

5.2.3.1 REML Analysis

The data was analysed using REML to fit mixed linear models using GenStat software. The model used for analysis was:

$Y_{ijklmn} = \mu + S_i + G_j + I_k + (SG)_{ij} + (SI)_{ik} + \Sigma_{ijkl} + \Delta_{ijkm} + e_{ijklmn}$

Where:

Y= weight measurement of the n^{th} fish of i^{th} sex, initially graded in the j^{th} category reared in the k^{th} site, by l^{th} sire and m^{th} dam.

μ= Population mean.

S_i = fixed effect of sex i.

G_j = fixed effect of initial grade j of the fish coming out of the nursery.

I_k = fixed effect of site for rearing while on test.

$(SG)_{ij}$= fixed interaction between the sex i and initial grade j.

$(SI)_{ik}$= fixed interaction between the sex i and site of rearing k.

Σ_{ijkl}= random effect of l^{th} sire.

Δ_{ijkm}= random effect of m^{th} dam.

e_{ijklmn}= residual error.

Σ_{ijkl} , Δ_{ijkm} and e_{ijklmn} were considered to be independent random normal variables with mean 0 and variances σ_S^2,σ_D^2 and σ_e^2 respectively. The phenotypic variance, σ_T^2 was estimated by:

$$\sigma_T^2 = \sigma_S^2 + \sigma_D^2 + \sigma_e^2$$

Estimates of heritability and standard errors were obtained from the variance components using the VFunction procedure in GenStat based on formulae from Falconer and McKay (1996).

$$h_S^2 = 4\left(\sigma_S^2\right) \Big/ \sigma_T^2$$

$$h_D^2 = 4\left(\sigma_D^2\right) \Big/ \sigma_T^2$$

$$h_C^2 = \frac{2(\sigma_S^2 + \sigma_D^2)}{\sigma_T^2}$$

Where:

h_S^2=Heritability from sire variance component.

h_D^2=Heritability from dam variance component.

h_C^2=Heritability from combined sire and dam variance components.

The combined estimates are expected to give the most precise values of heritability because they use more of the available information. However they are thus based on the assumption that the additive genetic component (V_A) from the sire and the dam are equal. From the initial scrutiny of the results the sire components were observed to be higher for most traits. In order to test the hypothesis that the sire and the dam variance components were equal, the combined estimate of heritability (h^2_{cf}) was re-estimated for each of the four traits.

The sire and dam variance components were fixed relative to the residual variance (σ_e^2) such that:

$$\gamma_S = \sigma_S^2 / \sigma_e^2$$

$$\gamma_D = \sigma_D^2 / \sigma_e^2$$

The heritability was then estimated using the formula outlined for h^2_{cf} below:

$$h_C^2 = \frac{2(\sigma_S^2) + 2(\sigma_D^2)}{\sigma_S^2 + \sigma_D^2 + \sigma_e^2}$$

$$h_C^2 = \frac{2\left(\sigma_S^2 / \sigma_e^2\right) + 2\left(\sigma_D^2 / \sigma_e^2\right)}{\sigma_S^2 / \sigma_e^2 + \sigma_D^2 / \sigma_e^2 + 1}$$

$$h_C^2 = \frac{2\gamma_S + 2\gamma_D}{\gamma_S + \gamma_D + 1}$$

If $\sigma_S^2 = \sigma_D^2$

then $\gamma_S = \gamma_D = \gamma$

$$h_{cf}^2 = \frac{4\gamma}{2\gamma + 1}$$

From the equations above, h^2_{cf} was calculated through a manual iterative process (26 iterations) for various γ values ranging from 0.001 to 0.5, corresponding to heritability values from 0 to 1.0 respectively. The maximum likelihood for the h^2_{cf} was obtained by finding the value of γ that minimised the deviance value (-2 x log-likelihood).

95% confidence intervals for h^2_{cf} were defined by the minimum deviance values required to reject the null hypothesis ($h^2_{cf} = h^2$), where h^2 is the true estimate of the heritability. This threshold value was taken as "Dmin" plus 3.84 where, Dmin is the deviance for the maximum likelihood estimate and 3.84 is the critical value of χ^2 for 95% significance.

In order to test the hypothesis that the sire and dam variance components are equal, i.e. $\gamma_S = \gamma_D$, the hypothesis ($h^2_{cf} \neq h^2$c), was tested. A likelihood ratio test statistic was calculated by subtracting the deviance obtained in the model described earlier, allowing σ_S^2 and σ_D^2 to vary freely from Dmin. The significance of the statistic was tested by comparing with critical values of χ^2 at the 95% significance level.

Additional models were used for analysis of male weights including the season of maturity for the males as a factor with 3 classes.

5.3 Results

5.3.1 Descriptive Statistics

The observed means and their standard errors, standard deviations, maximum values, minimum values and coefficients of variation for body weight taken at the four defined stages for all the fish used in the study are given in Table 5.4. After acclimatising to their new environments, over the duration of the test period, the fish grew from an average weight of 0.65kg to 5.90kg. The variation in body weight increased with advancing age and at the end of the experiment the minimum weight of fish was 1.04kg while the heaviest fish was 14.56kg. The coefficient of variation also increased as the experiment progressed and this level of phenotypic variation suggests a high potential for genetic improvement, assuming moderate to high heritability.

Table 5.4 Descriptive statistics of defined weight traits for all fish used in the heritability study.

TRAIT	μ	s.e. (μ)	σ	Min	Max	c.v (%)
Acc wt (kg)	0.65	0.01	0.18	0.27	1.17	27.97
1st Year wt (kg)	2.12	0.04	0.79	0.68	4.61	37.16
2nd Year wt (kg)	4.37	0.09	1.91	1.09	10.95	43.71
End wt (kg)	5.90	0.13	2.84	1.04	14.56	48.18

μ= mean, s.e= standard error, σ= standard deviation, Min=minimum recorded value, Max= maximum recorded value, c.v= coefficient of variation.

Figure 5.1 Graph showing the unadjusted mean weights of fish-averaged over sex, site and grade- plotted against the times at which the four defined stages of the trial; Acc weight, 1st year weight, 2nd year weight and end weight were measured

5.3.2 Unadjusted Phenotypic Correlations

Phenotypic correlations between body weights at the points defined in the study are presented in Table 5.5. All correlations were positive and moderate to high in magnitude. The correlations of the lowest magnitudes were those between acclimatised weight and other body weight traits, particularly with end weight (0.36).

Table 5.5 Unadjusted phenotypic correlations between acclimatised weight, 1st year weight, 2nd year weight and end weight for all fish.

TRAIT	Acc Wt	1st Year Wt	2nd Year Wt	End Wt
Acc Wt	1			
1st Year Wt	0.55	1		
2nd Year Wt	0.40	0.76	1	
End Wt	0.36	0.68	0.92	1

It appears that the strongest correlations, those of highest magnitude, were those between measurements made within close time periods particularly late in the production cycle. The correlation between 2nd year weight and end weight was of the highest magnitude, almost unity (0.92), indicating that fish did not change their ranking position between these dates.

5.3.3 Predicted Means of Fixed Effects

Assessments of the phenotypic means analysed separately for each group showed that all fixed effects fitted (sex, site and initial grade) had notable influences on body weight defined at the four stages in the study.

5.3.3.1 Effect of sex

The differences in body weight between both sexes were not significant until after the acclimatising period. A graphical illustration of the predicted effect of sex on body weight of fish in the experiment is shown in Figure 5.2 below. From the first year onwards females were significantly ($P<0.001$) larger than males and this difference increased progressively throughout the experiment. The observed mean weights of males were 85.3%, 52.8% and 44.3% of the corresponding mean female weights in the first year, second year and final weights respectively. Unlike in the females, the average weight of males did not change significantly after the second year on test indicating a

plateau in the growth curve. By the end of the experiment the heaviest female was 1.6 times larger than the heaviest male; the average "end weight" of the females was 6.07±0.35kg while the corresponding value for males was only 2.69±0.36kg, approximately 300g less than the desired minimum harvest weight of 3kg

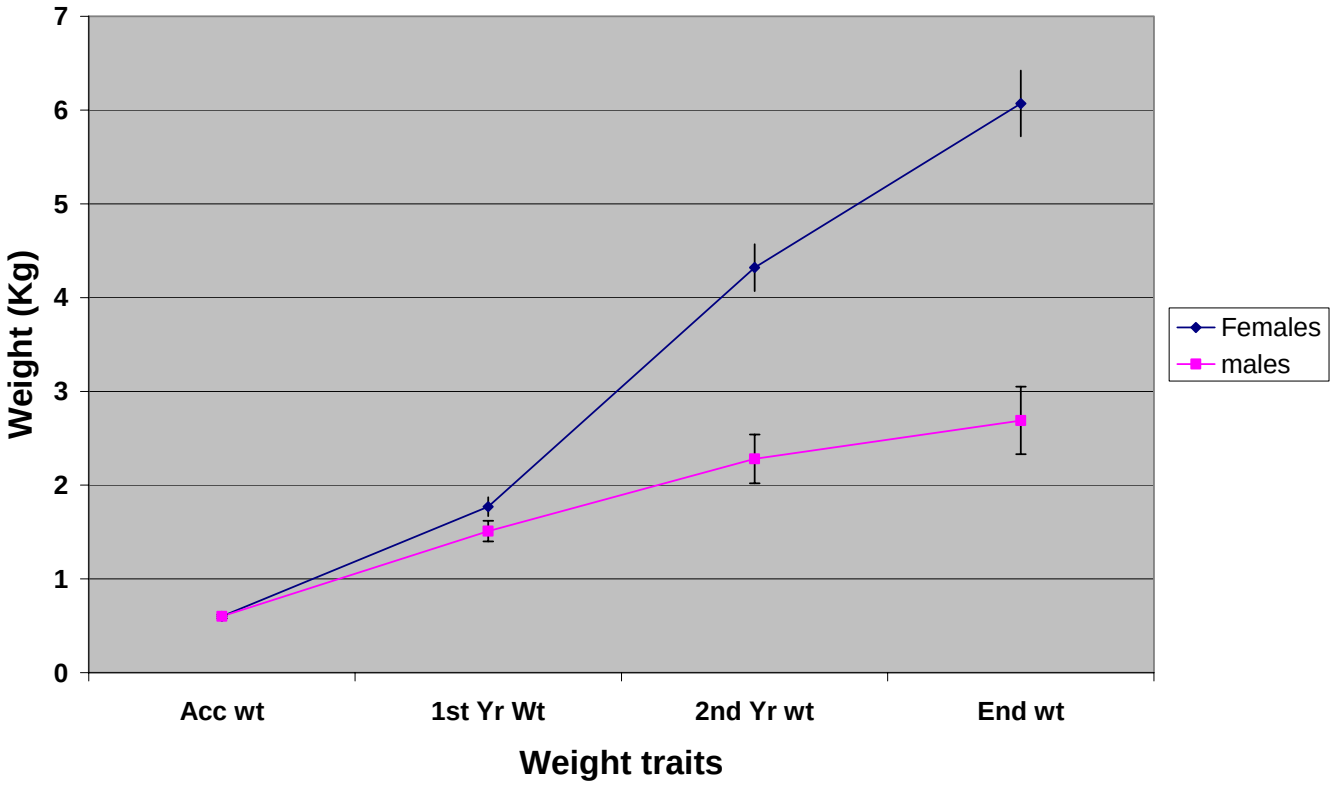

Figure 5.2 Graph showing the predicted means of the effect of sex (females and males), averaged over site, on body weight measured at the four defined stages of the experiment; Acc weight, 1st year weight, 2nd year weight and end weight.

Significant interactions were observed between sex and site. However, they were not apparent until the second year on test. The average weights of females and males in Otter Ferry and Marine Harvest are presented in Table 5.6 below. The result of significant interactions between sex and site for 2nd year weight (P=0.036) and end weight (P=0.004) imply that the effect of sex was dependent on which site the fish were reared in. This

interaction was one of scale rather than rank in that although females were larger overall, they grew better in land based tanks, Otter Ferry, than in sea cages, Marine Harvest, such that the difference between the sexes was larger in the more conducive environment.

Table 5.6 The average weights of females and males in Otter Ferry and Marine harvest showing the interactions between sex and site of rearing.

	2nd Year weight		End weight	
Site	Otter Ferry	Marine Harvest	Otter Ferry	Marine Harvest
Females	5.19(±0.25) kg	3.46(±0.27) kg	7.33(±0.36) kg	4.80(±0.38) kg
Males	2.87(±0.26) kg	1.69(±0.29) kg	3.42(±0.36) kg	1.97(±0.41) kg
Differences	2.32(±0.17) kg	1.77(±0.20) kg	3.91(±0.21) kg	2.83(±0.31) kg

5.3.3.2 Effect of site

The environmental effect of site on weight between fish grown on land based tanks (OFS) and fish grown in sea cages (MHM) was highly significant ($P<0.001$) throughout the course of the experiment. The fish in Otter Ferry (OFS) grown in the land based tanks were consistently larger than those in sea cages at the Marine Harvest McConnell (MH) site in Teacius. Figure 5.3 shows the predicted means of weight of fish reared on both sites. Although the patterns of the growth curves were similar there was a substantial difference in scale. At the end of the experiment the fish in the land based tanks were, on average, 2kg heavier than the fish in sea cages. There were also significant interactions, with grade ($P<0.001$) from the first year of the experiment and with sex (see previous section 5.3.3.1), which rule out the possibility of discussing this effect independently.

It appears that, as with sex, the effect of site was dependent on which group the fish were graded in at the start of the experiment. The interaction is again one of scale rather than rank. The fish in the land based site (OFS) grew better than the fish in the sea site (MH), however with the exception of acclimatised weight, the difference between the "large"

grade and the "small" grade was greater in OFS as shown in Table 5.7. These differences were progressive in OFS and remained relatively constant in MH. The nature of these interactions suggest that animals predisposed to perform better did so given the opportunity, in this case a superior or more conducive environment.

Table 5.7 The average weights of fish within and between initial grade (Small and Large) reared in the land based site (OFS) and the sea site (MH) showing the interaction between the two factors.

	OFS			MH		
Trait	Large	Small	Difference	Large	Small	Difference
Acc weight (kg)	0.74±0.02	0.54±0.02	0.20± 0.01	0.68±0.02	0.45±0.02	0.23± 0.01
1st Year weight (kg)	2.30±0.11	1.77± 0.11	0.53± 0.07	1.35±0.11	1.16±0.12	0.19± 0.10
2nd Year weight (kg)	4.45±0.26	3.61±0.26	0.84±0.17	2.71±0.27	2.43±0.29	0.28±0.20
End weight (kg)	6.00±0.36	4.76±0.36	1.24±0.24	3.55±0.39	3.21±0.41	0.34±0.29

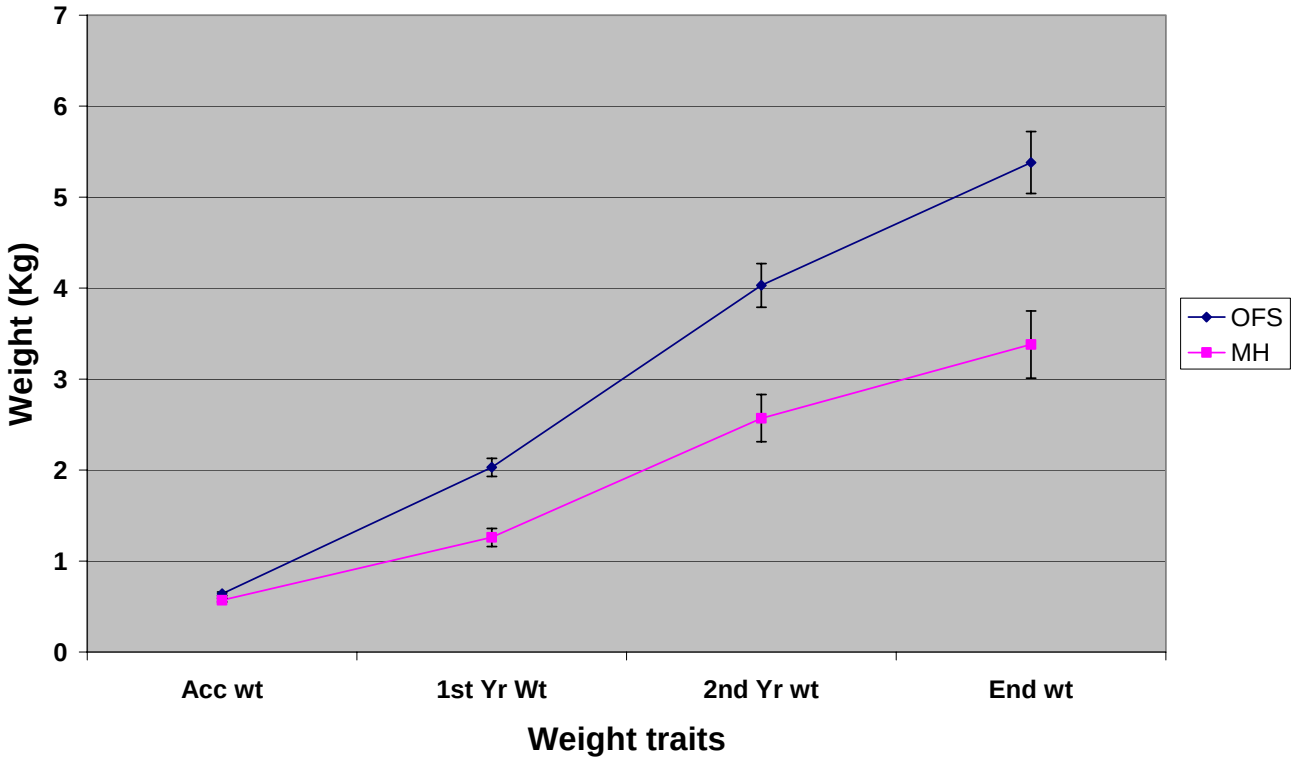

Figure 5.3 Graph showing the predicted means of the effect of site (OFS and MH), averaged over sex and grade, on body weight measured at the four defined stages of the experiment; Acc weight, 1st year weight, 2nd year weight and end weight.

5.3.3.3 Effect of grade

Due to the fact that there were significant interactions between grade and tank (see previous section 5.3.3.2), a general overview of this effect is reported. The overall effect of initial grade on body weight was significant ($P<0.001$) throughout the experiment and, as expected, larger fish maintained their initial weight advantage, however, the impact of initial grade/size on fish performance diminished as the fish grew older. It seems that as time passed the difference in relative body weight between initially small fish and their larger counterparts gradually decreased. At the start of the experiment the smaller grade fish were 70% of the weight of the larger fish. In the first and second year they were 80% and 84% of the weight of their contemporaries respectively. However, by the end of

the experiment they were 83% of the larger group. These results are presented graphically in Figure 5.4

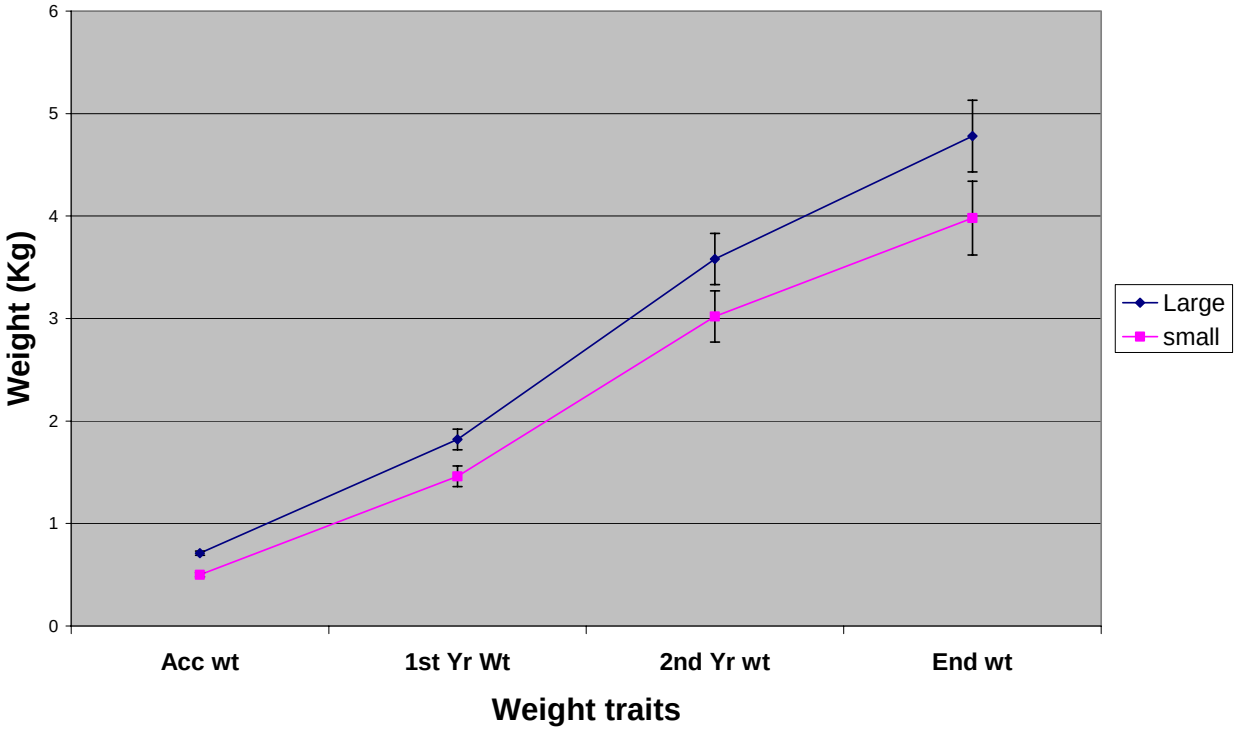

Figure 5.4 Graph showing the predicted means of the effect of initial grade, averaged over site, on the body weight measured at the four defined stages of the experiment; Acc weight, 1st year weight, 2nd year weight and end weight.

5.3.4 Parentage Assignment

The results obtained from the parentage assignment are presented and discussed in Chapter 3. The animals used in this study were identified as the "1998 year class". A summary of the contributions of sires and dams to this group are presented in Table 5.9 below.

Table 5.8 The actual number (N) and effective number (n) of sires and dams together with their individual percentage contributions to the 486 F_1 fish studied in the heritability trial. Effective number of sires is defined by $(\sum p_i^2)^{-1}$ where p_i is the contribution of a sire i. Effective number of dams is defined analogously.

Sires: N=12 & n=1.66	Contribution (%)	Dams: N=15 & n=3.78	Contribution (%)
5A	0.80	3A	4.73
7A	0.40	8A	13.79
9A	3.41	10A	45.88
13A	0.20	11A	16.46
15A	2.81	19A	3.50
17A	76.15	20A	3.09
33A	0.60	23A	0.21
34A	1.81	24A	1.03
35A	5.61	26A	3.91
37A	6.41	27A	2.06
44A	1.60	30A	0.62
54A	0.20	42A	0.20
		50A	0.41
		64A	3.91
		166	0.21

5.3.5 Heritability

Heritability estimates for body weight at the four stages of the experiment derived from sire, dam and combined components of variance are listed in Table 5.9. Values ranged from 0.09 to 0.53 with high standard errors that resulted from the small number of parents and numbers of offspring per family used in the study.

Table 5.9 Heritability of body weight traits estimated from (i) sire components h_S^2, (ii) dam components h_D^2 and (iii) combined sire and dam components of variance h_C^2, together with the associated likelihood ratio test statistic (L.R.T.S).

TRAIT	h_S^2± s.e	L.R.T S	χ^2_1	h_D^2± s.e	L.R.T.S	χ^2_1	h_C^2± s.e	L.R.T.S	χ^2_2
Acc Wt.	0.09±0.13	1.60	P>0.05	0.11±0.12	2.55	P>0.05	0.10±0.08	5.98	P<0.05
1st Year Wt	0.48±0.32	20.08	P<0.001	0.11±0.10	7.57	P<0.001	0.29±0.16	50.38	P<0.001
2nd Year wt	0.53±0.34	23.18	P<0.001	0.25±0.18	15.62	P<0.001	0.39±0.17	60.75	P<0.001
End Wt	0.49±0.32	19.20	P<0.001	0.28±0.19	25.37	P<0.001	0.39±0.17	63.67	P<0.001

s.e= standard error,χ^2_1 =Chi squared significance value with one degree of freedom, χ^2_2 =Chi squared significance value with two degrees of freedom

The estimates for body weight heritability were not constant and increased in magnitude with age, particularly h_D^2 which could be taken as evidence of declining maternal effects with age. Depending on the variance component used to estimate the value, the highest estimate was either 2nd year weight (sire component) or end weight (dam component or combined). The presence of dominance was tested and found to be absent.

In order to test if the differences between sire and dam components were due to genuine differences in additive genetic variance (V_A), a likelihood test was conducted. The deviance, -2loglikelihood ratio, profiles of the combined heritability values using the formula described in Section 5.3.2.1 for each of the traits are shown in Figures 5.5-5.8 below. The newly estimated heritability values obtained from the minimum deviance values with their corresponding 95% confidence intervals are presented in Table 5.10.

The likelihood ratio test statistics calculated for each trait show that there is no evidence to reject the null hypothesis of $\sigma_S^2=\sigma_D^2$. It can therefore be concluded that although the heritability estimated from sire variance components were higher for most traits there is no evidence to suggest that they represent legitimate additive genetic influences.

Since the deviance profiles are markedly asymmetric the confidence intervals will provide the best estimate of precision for the heritabilities because the standard errors assume quadratic profiles obtained with large data samples.

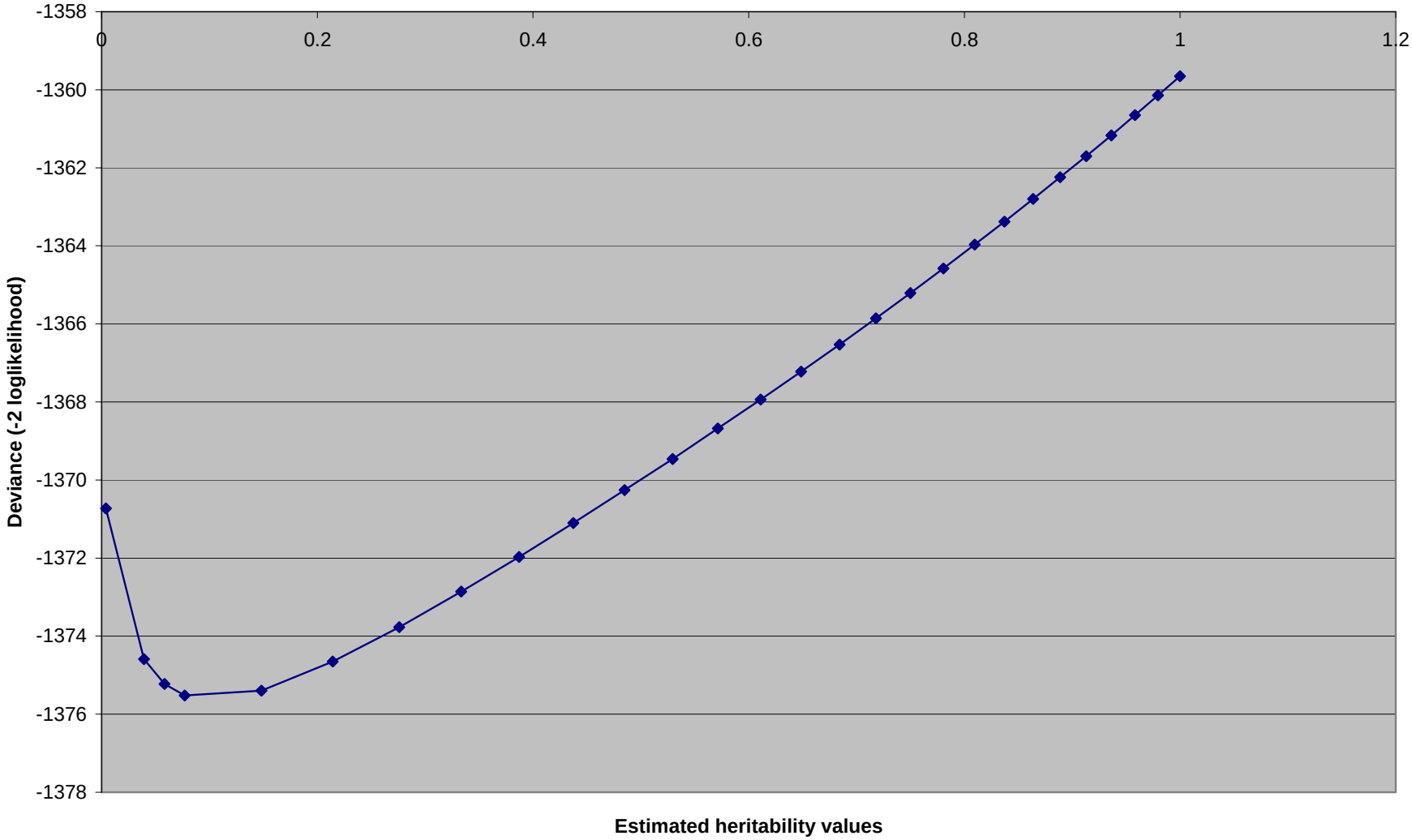

Figure 5.5 Deviance profile of the various estimates of heritability for acclimatised weight.

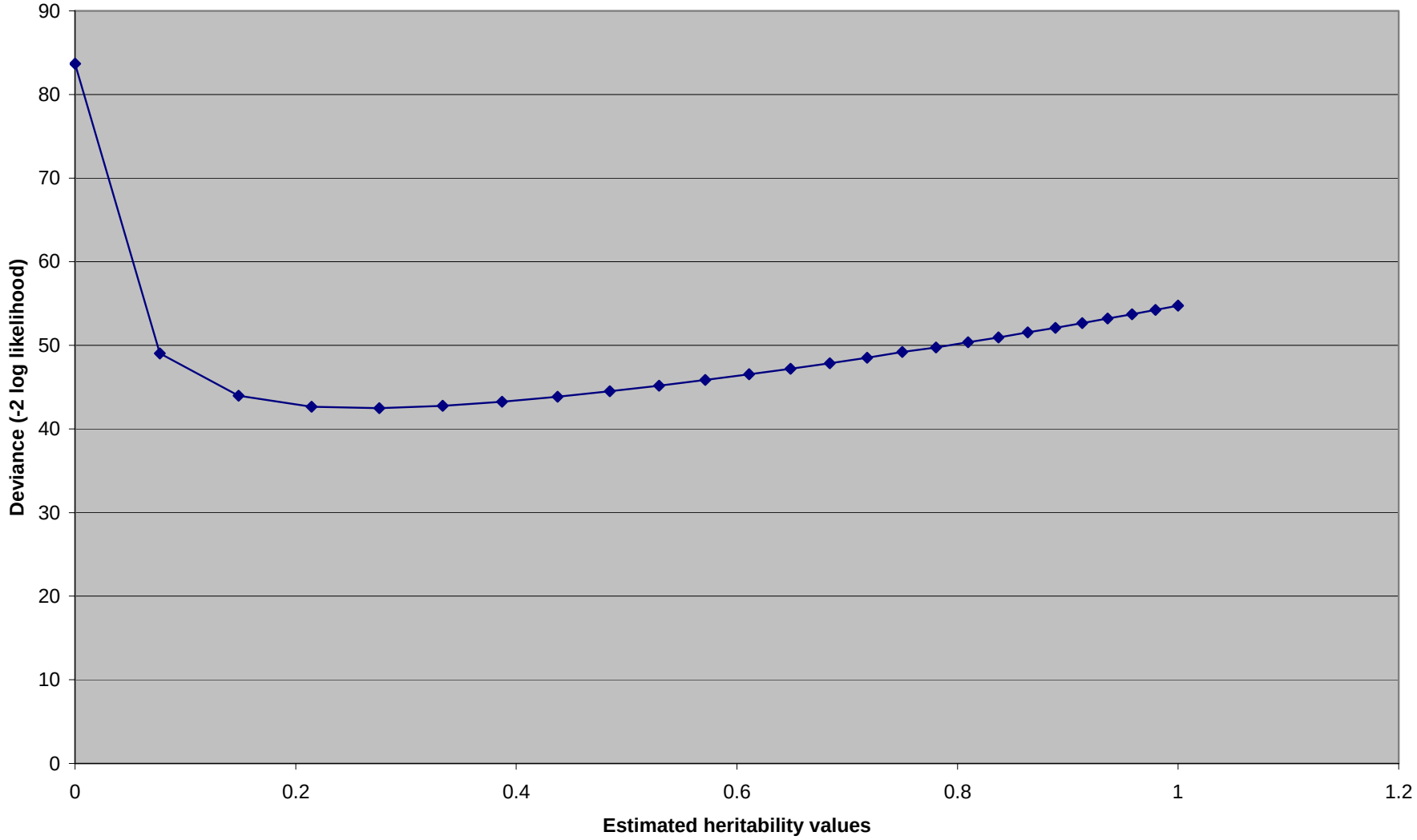

Figure 5.6 Deviance profile of various estimates of heritability for 1st year weight.

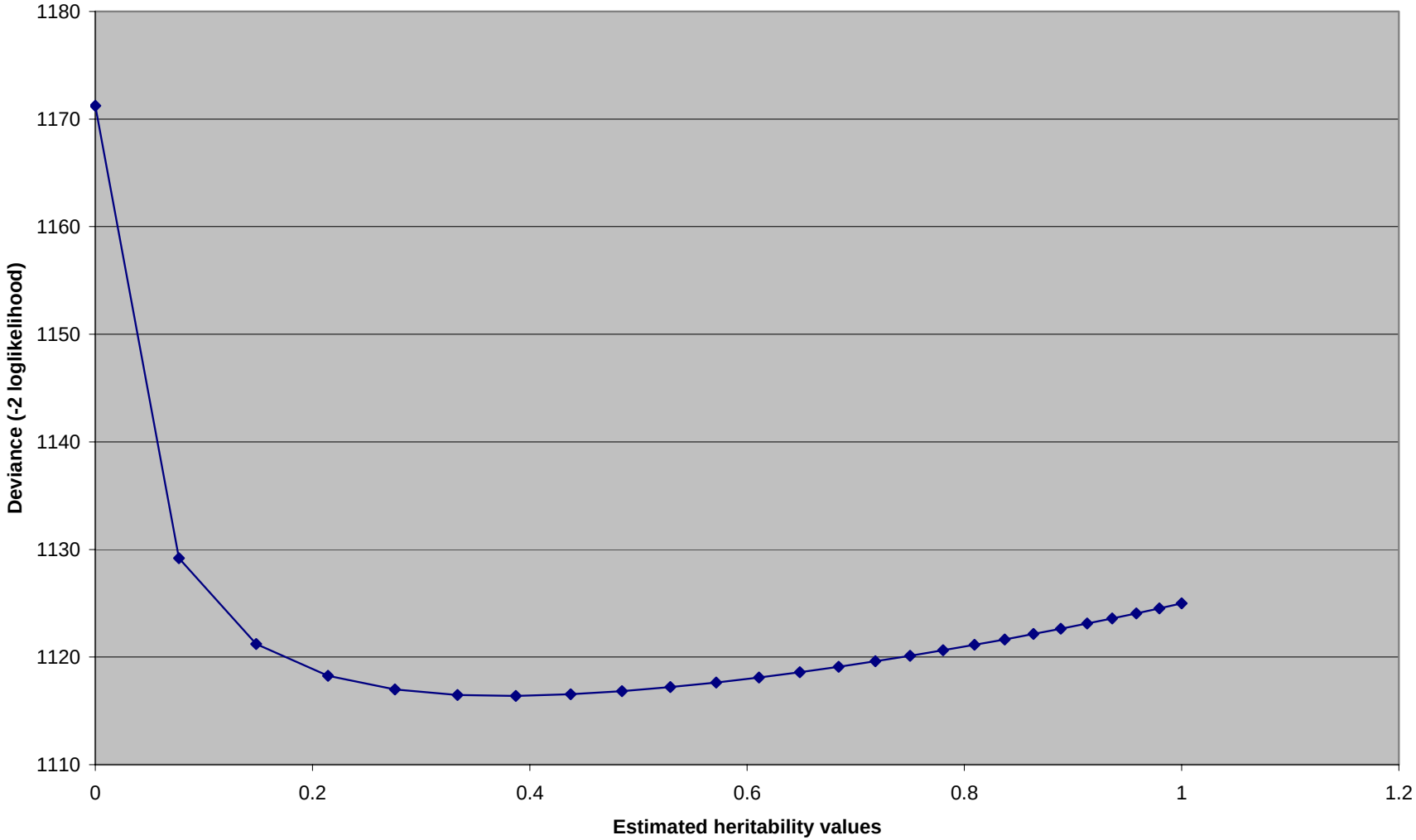

Figure 5.7 Deviance profile of various estimates of heritability for 2nd year weight.

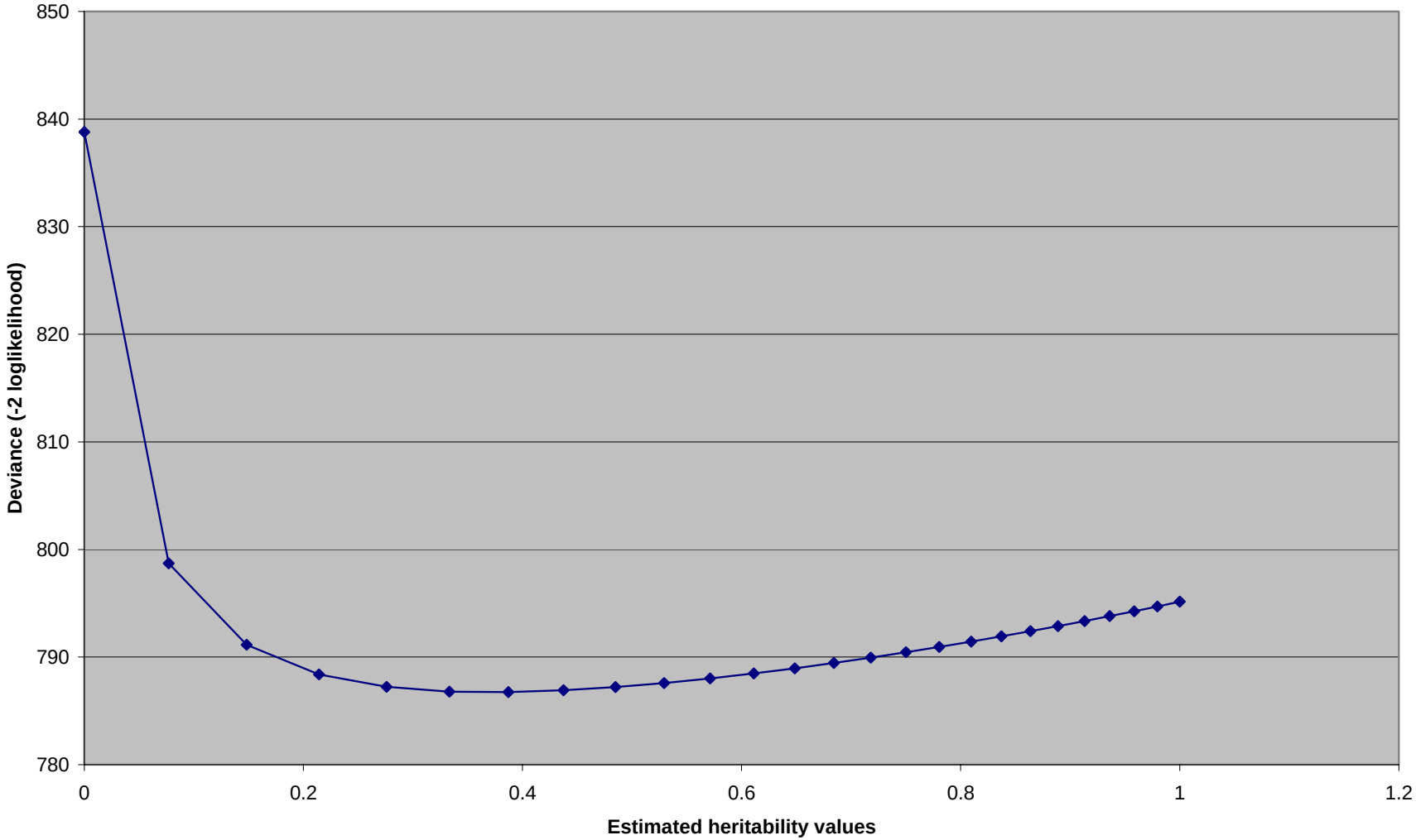

Figure 5.8 Deviance profile of various estimates of heritability for end weight.

Table 5.10 Heritability of body weight traits estimated from combined sire and dam variance components using γ, fixed –equal- sire and dam variance relative to the residual variance (h_{Cf}^2) with the calculated likelihood ratio test statistic (L.R.T.S) obtained from comparing these estimates with those previously obtained from the REML analysis.

TRAIT	h_{Cf}^2	95% Confidence interval	L.R.T.S	χ^2_1
Acc wt	0.08	0.00-0.44	0.13	N.S (P>0.05)
1st Year wt	0.28	0.12-0.53	1.72	N.S (P>0.05)
2nd Year wt	0.39	0.17-0.72	0.62	N.S (P>0.05)
End wt	0.39	0.17-0.72	0.33	N.S (P>0.05)

χ^2_1 =Chi squared significance value with one degree of freedom, N.S= Not significant

5.4 Discussion

This is the first study to present heritability estimates for body weight in the Atlantic halibut. Estimated heritabilities for body weight varied depending on the age of the fish and the source of variance used to estimate the value. In general values obtained were of the same magnitude found for body weight in other marine and cold-water species (0.08-0.53). In the present study although the confidence intervals and/or the standard errors of the early values obtained were large and thus not significantly different from zero, the magnitude and precision of the later estimates indicated that there is a substantial amount of additive genetic variation for growth in the Atlantic halibut and suggest that selection for increased body weight is likely to be successful. The phenotypic correlations between body weights at different stages obtained show that 2nd year weight is a good predictor of future performance.

5.4.1 Fixed effects

End weight, defined as body weight after 3 years, is dependent on sex. At harvest females are 3.37±0.19 kg heavier than males; this dimorphism in growth performance is consistent with observations by other authors (Björnsson, 1995). From the differences observed in body weight of males it appears that growth in the Atlantic halibut is affected by the onset of sexual maturity in males. Accounting for this in the statistical model used has improved the estimates of heritability for body weight (McKay *et al*., 1986) however, in the current study it was excluded from the statistical model because age of maturity was not recorded in females.

The observed difference in body weight between both sexes can be explained by the onset of sexual maturity in males. Maturity significantly affected the performance,

mean weights, of males for all traits ($P<0.05$) particularly 2nd year weight ($P<0.001$).The predicted mean weights of the males within each group are presented in Table 5.11. The numbers of first, second and third winter males are 49, 149 and 17 respectively. On average males did not reach the preferred market weight of 5kg. The divergence in mean body weight between the sexes occurred when most of the males matured in the second year of the experiment. The differences in mean body weight between males seemed to depend on whether they matured in the first year or not. The differences between second and third winter males were not significant but this could be due to the small sample size of the latter in the current study.

Table 5.11 Comparison of mean weights, and their standard errors, between males maturing in the first, second and third winters of the experiment.

Trait	1st winter males	2nd winter males	3rd winter males
Acc weight (kg)	0.64 (±0.02)	0.59 (±0.21)	0.57 (±0.04)
1st year weight (kg)	1.22 (±0.15)	1.59 (±0.14)	1.61 (±0.20)
2nd year weight (kg)	1.95 (±0.24)	2.55 (±0.22)	2.24 (±0.32)
End weight (kg)	2.73 (±0.29)	3.29 (±0.26)	3.11 (±0.40)

In order to improve the mean harvest weight of a population the issue of early maturation in males needs to be addressed. Due to the limited number of males within the three classes of maturity (1st, 2nd and 3rd winter males) in the study, analysis of heritability of maturity was impossible. Heritability of sexual maturity has however been estimated in other aquaculture species and is seen to be of low magnitude. Gjerde *et al*. (1994) obtained values of 0.09±0.04 and 0.15±0.08 in a population of Atlantic salmon.

The fish grown in sea cages were on average 2.00±0.19 kg lighter at the end of the growout period than fish reared in land based tanks. Due to the significant interactions between all the fixed effects in the study it is not prudent to discuss them independently. All the phenotypic correlations observed in the current study

were scaling interactions showing that animals predisposed to better performance (females and larger fish at the start of the experiment) grew better in the more favourable environment i.e. the sheltered, controlled, tank in Otter Ferry. Scaling interactions such as these could be removed by logarithmic transformations. Nonetheless they suggest that there might be genotype by environmental interactions introduced as broodstock (replacement candidates) are selected in land based systems and commercial producers rear their offspring in sea cages. Thus improved performance in land tanks may not be captured by ongrowers who grow juveniles in different and often more hostile environments. In order to quantify this effect, if it exists, a further study looking at the ranking of families reared in both systems should be conducted. However, the possibility of carrying out a genotype by environmental analysis in the current study was prevented by the uneven family structure of the test population, the limited number of parents detected and the small sample size.

5.4.2 Heritability estimates

The wide range of the confidence intervals, and thus poor precision, obtained for heritability values in the current study is consistent with the nature of the experimental design, the number of offspring studied and more importantly the immensely imbalanced family structure. Heritability estimates reported for body weight in aquaculture species are notorious for having large standard errors; even in recent times when utilising complex statistical methods to analyse data from large numbers of individuals (≥ 1500) several authors still report values of low accuracy (Gjerde *et al*., 1997; Henryon *et al*., 2002; Gjerde *et al*., 2004).

One reason for this problem is unequal family representation. It is of particular concern when experiments are designed with mixed spawning or hatching groups (Fishback *et al*., 2002). Most heritability studies however, are designed from carefully structured nested or factorial mating designs and equal family numbers are reared separately, at least initially, (e.g. McKay *et al*., 1986; Winkelman and Peterson 1994a; Su *et al*., 1996b; Gjerde *et al*., 2004) in order to address and alleviate this problem. With the advent of genetic profiling it is now possible to rear families in communal tanks so as to reduce the common environment effects shared by sibs. In these cases steps are taken to ensure that the numbers of offspring per parental pair are standardised. These include the repeated incubation of equal volumes of eggs from different females (Vandeputte *et al*., 2004), or the pooling together of equal numbers of hatched fry following separate incubation in order to limit problems of unequal fertilization success (Fishback *et al*., 2002).

In the current study, however, neither of these measures was taken and financial constraints meant data from relatively few individuals (486) was analysed. In addition, although the test population was comprised of offspring from 12 sires and 15 dams, the mating strategy was not controlled, resulting in a highly unbalanced family sizes highlighted by the very small effective number of parents (1.66 and 2.48). This was because the experiment was conducted within a commercial hatchery and results from the parentage analysis revealed managers' preferences for particular males over others. One possible benefit of standard commercial practice in halibut hatcheries is the mixing of batches several times in the production cycle which could reduce the possibilities of introducing common environmental effects between siblings.

The differences in heritability estimates between sire and dam components of variance for body weight were tested and the results obtained were consistent with the conclusion that they represent nothing more than chance. For this reason there are no grounds to invoke the notion of non-additive and or other genetic effects. Nevertheless the differences observed were marked and several other authors have noted similar disparities, including substantial effects, particularly at the early stages of growth (McKay *et al*., 1986; Gall and Haung, 1988a; Gjerde and Scheaffer, 1989; Silverstein and Hershberger, 1994 and Gjerde *et al*. 1997). It is therefore conceivable that the differences observed have some basis in reality.

Unlike many studies, estimates from sire components of variance were observed to be much higher than those derived from dam components. It is, however, more common for the reverse to occur (Beaumont, 1994). In his review of eight heritabilities estimates in the rainbow trout, Gjedrem (1992) found that those derived from dam variance components were, on average, 0.3 higher than those estimated from sire components of variance. Differences in heritability estimates between maternal and paternal components are usually attributed to common environmental, maternal and or non-additive genetic effects caused by dam-nested-within sire mating designs.

In the unusual cases where the sire heritabilities were higher than the dam heritabilities (Gall and Haung, 1988a; Silverstein and Hershberger, 1994; Gjerde *et al*., 1997) the experimental designs were structured such that sires were nested with the dams and offspring were reared separately. Therefore sire components of variance also contained common environmental variance and dominance which is usually the case when the dams are nested within sires (Vandeputte *et al*., 2003).

A more plausible explanation for these differences observed in the current study is that they are due to the variance in sire contributions to the offspring generation compared with the variance in dam contributions. The sire variance estimates were exaggerated as a result of a single male accounting for a high percentage (76.15%) of the parentage in the test population (Table 5.8).

Although tests revealed there was insufficient evidence to suggest that sire and dam variances were not significantly different from each other, the opposite is also true in that there is insufficient evidence to exclude the presence of a small amount of maternal effects

In the present study, heritability estimates for body weight increased with age. Changes in heritability estimates for body weight with advancing age of the fish are consistent with some findings in the literature but reports are conflicting. Studies by Gall and Haung (1988a), Crandell and Gall (1993), Gjerde *et al*. (1994) and Winkelman and Peterson (1994b) all found that heritability estimates for body weight in trout and salmon decreased with age. Yet in his review, Gjerde (1986) concluded that heritability of body weight in juvenile trout and salmon was generally low (0.1) but increased up to 0.4 in adults. McKay *et al*. (1986), Elvingston and Johansson (1993) and Su *et al*. (1996b) also observed that heritabilities increased with age. Kinghorn (1983) suggested that in cases where heritability of body weight increases with age it might be due to diminishing maternal effects.

Maternal effects in fish species are likely to be caused through differences in egg sizes or by egg quality however, they are not expected to be large because offspring are not actively nurtured (Gjedrem, 1992). This is consistent with the findings of

several authors concluding that differential growth in salmonid fry, associated with variation in egg sizes disappears soon after first feeding (Crandell and Gall, 1993). In this study the presence of maternal effects in the form of varying egg sizes might have had an effect on early growth performance and thus the heritability estimates as suggested by Crandell and Gall (1993). This is because the halibut egg is large (3.06-3.49 mm) and it is expected to contain enough energy to sustain a developing larva through a very long yolk sac period up until first feeding, which sometimes lasts up to 80 days (Haug *et al*., 1984). Thus the maternal environment, i.e. the egg, received by a developing halibut is comparatively more important for early growth than that received by a developing salmonid which start first feeding at 50 days because it is required for a longer period of time. Secondly, first feeding in the halibut is also different as fry are fed on live feed. This requires a greater amount of activity by the larvae to feed successfully and so the maternal effects may be expected to last longer because if a halibut "inherits" a good quality and/or large egg from its mother it is more likely to feed better and thus grow quicker in the early stages than one that "inherits" a poor quality and/or smaller egg.

Previous results from Chapter 4 suggest that egg quality in the F_1 population was not repeatable. If this is the case with the parental population used to produce the fish used in this study then maternal effects (differences in egg quality between females) will occur not only between females but also between different spawnings of the same female. Also because batches were mixed frequently, environmental effects common to maternal sibs could be discounted.

The experimental design could have led to lower estimates of heritability obtained for acclimatised weight due to a division of the population distribution as a result of

grading. By incorporating differences between fish in the small and large grade into the statistical model the extent of the genetic differences between the offspring of different sires and dams could have been masked, i.e. the effect of grade could be confounded with genuine additive genetic differences and the resulting heritabilities biased downwards. The statistical analysis was repeated and the trait heritabilities for all traits were re-estimated without including grade in the model in order to explain the potential magnitude of this bias. The results are presented in Table 5.12 below.

Table 5.12 Heritability of body weight traits estimated from (i) sire components h_S^2, (ii) dam components h_D^2 and (iii) combined sire and dam components of variance $h_C^2$when grade was excluded from the statistical model compared with when grade was included in the model $_{(G)}$.

TRAIT	h_S^2± s.e	χ^2_1	$h_S^2{}_{(G)}$± s.e	h_D^2± s.e	χ^2_1	$h_D^2{}_{(G)}$± s.e	h_C^2± s.e	χ^2_2	$h_C^2{}_{(G)}$± s.e
Acc Wt.	0.21±0.21	P<0.05	0.09±0.13	0.36±0.23	P<0.001	0.11±0.12	0.29±0.14	P<0.001	0.10±0.08
1st Year Wt	0.52±0.34	P<0.001	0.48±0.32	0.17±0.14	P<0.001	0.11±0.10	0.34±0.17	P<0.001	0.29±0.16
2nd Year wt	0.64±0.38	P<0.001	0.53±0.34	0.28±0.19	P<0.001	0.25±0.18	0.46±0.19	P<0.001	0.39±0.17
End Wt	0.55±0.34	P<0.001	0.49±0.32	0.33±0.21	P<0.001	0.28±0.19	0.44±0.17	P<0.001	0.39±0.17

s.e= standard error,χ^2_1 =Chi squared significance value with one degree of freedom, χ^2_2 =Chi squared significance value with two degrees of freedom

In general the results of the analysis are not surprising in that the trend in the estimates is similar but the magnitudes of the estimates are greater. They do however suggest that for acclimatised weight, a significant amount of the genetic variation could have been masked by the grading process. Not only have all the estimates increased but they are all now highly significant. This second analysis is nevertheless also biased but in this case upwards.

The purpose of this study was to test whether additive genetic variance exists for body weight in the Atlantic halibut. This was the case. The farmed population of halibut appears to be consistent with other farmed aquaculture species where additive genetic variation for growth traits has been found. Undoubtedly values obtained from sire variance estimates were inflated and thus the combined estimates from both sire and dam variance components will be used. In addition because of the skewed family sizes and the over-representation of a single sire the best estimates were those calculated from the manual iterations using fixed sire and dam variance components. The confidence interval is also a better measure of precision because standard errors are calculated based on the assumption of normality which, from the deviance plots, is not the case in the current study. Based on these estimates breeding values for candidate replacement broodstock can be calculated and the predicted response to selection evaluated.

Chapter 6

General Discussion and Genetic management Strategy

6.1 Introduction

The Atlantic halibut industry in the UK is now 22 years old but contrary to predictions made has not grown at the desired rate. The impetus to culture the halibut developed from a need for diversification within the European and North American aquaculture industries which were, and still are, dominated by the production of salmonid species, in particular the Atlantic salmon. However, interest in the species is beginning to wane due to the numerous technical challenges associated with the hatchery phase and disease that has resulted in disappointing and variable levels of fry production. It has also become apparent that in contrast to the Atlantic salmon, the Atlantic halibut is likely to remain a high value product in a niche market, due to the high cost of juvenile production caused by the fixed costs associated with running a hatchery and the level of expertise and labour involved.

The industry in the UK started from a small demonstration project based at the Sea Fish Industry Authority's Marine Farming Unit in Ardtoe, Scotland and was developed by Otter Ferry Seafish as a commercial enterprise. The species was originally selected as the most suitable marine fish species for farming in the UK based on its high value, good market demand and perceived growth characters in the prevailing water conditions (Shields *et al.,* 1999). Market demand was high because of consumers' image of a premium fish and demand for the product out weighed supply as natural stocks had declined causing high prices.

The farming of the Atlantic halibut was only possible due to comprehensive research efforts and collaborations between commercial growers and research scientists but the progressive decline in salmon prices is having a knock on, negative, effect on the progress of the culture of this species. This is because the

aquaculture industry, like many others, is presently following the universal trend for consolidation and globalisation. It is increasingly one dominated by a small group of multinational companies such that the financial input needed to fund research towards finding the solutions to the problems associated with poor juvenile production observed in the halibut is being eroded by low profits from the salmon enterprises. Thus research grants are being withheld or, in the certain instances in the UK, withdrawn.

Whilst the objective of the current study was to establish a strategy for the effective genetic management of captive populations and the establishment of selective breeding programmes for the halibut it was also, by default, about furthering and contributing to the collective knowledge for and towards the successful routine culture of this newly selected aquaculture candidate.

The results obtained in Chapters 2 and 3, describing the application of molecular makers to assess the level of genetic variation and contribution of wild parents to the first generation of hatchery reared fish, were very similar to those previously reported by other authors working in Canada, Norway, Ireland and Iceland (Stefánsson *et al.,* 2000; Jackson *et al.,* 2003; Cross *et al.,* 2005). Marked reductions in the levels of genetic variation, as judged by allelic diversity, were observed between the parental and offspring generations with only half of the parents succeeding in contributing any offspring to the F_1 populations. These problems were compounded by the fact that family sizes were highly skewed and certain families were greatly over represented, compared to what would have been expected from random mating. The F_1 population was in effect a small number of large full and half sib families.

Despite the fact the heritabilities obtained in Chapter 5 point to the fact that selecting for improved growth is likely to be successful; the original idea of designing a selective breeding programme has been compromised by the poor family structure within the sampled population which, from the small number of published studies appears to be typical for this species. The poor family representation and losses of genetic variability can be explained by a combination of forces: (1) management practices, (2) the nature of the reproductive biology of the halibut and (3) an interaction between the fish and its environment- which given the stage of development of the industry, can be viewed as the process of domestication.

These are large issues and they can only be resolved by understanding domestication in relation to the halibut. Thus, while the issues related to genetic diversity need to be addressed immediately, they do however raise the interesting question of why these reductions occurred. Therefore domestication and its implications will be reviewed and discussed, because in my opinion, it gives insight into the genetic management of this species.

6.1.1 Domestication

The issue of domestication in aquaculture is often discussed in terms of the impact of farmed stock on the population structure of their wild counterparts when they escape from cultured systems (e.g. Gross, 1998; Waples, 1999; Cross, 2000; Hansen, 2002; Glover, 2002). The focus in the current context, however, is the *process* of domestication itself, i.e. the reverse flow of wild fish into captivity. Several definitions of domestication can be found in the literature among them the definition by Price (1984) bests suits the present purpose. He defines domestication

as "that process by which a population of animals becomes adapted to man and to the captive environment which he provides by some combination of genetic changes occurring over generations and environmentally induced developmental events reoccurring during each generation". In this sense domestication can be viewed as a bi-factorial phenomenon, firstly an evolutionary process of the animal; and secondly an ongoing experience provided for and controlled by man. By comparison Hale (1969) defines domestication as "that condition wherein the breeding, care and feeding of animals are more or less controlled by man" which excludes the notion of evolution; a time dependent, cumulative change by the animal.

6.1.1.1 Domestication and aquaculture

Of the vast number of animal species, very few have been successfully domesticated (Simm, 1998; Diamond, 1998; Mignon-Grasteau *et al*., 2005). The reasons for this were discussed by Francis Galton (1865) in his essay "The first steps towards the domestication of animals". He proposed that the process of domestication occurred by trial and error and argued that "*the animal creation has been pretty thoroughly, though half consciously, explored and there is no animal worthy of domestication that has not frequently been captured, and might ages ago have established itself as a domestic breed, had it not been deficient in certain necessary particulars*". Galton identified six criteria (particulars) for successful domestication of animals which have formed the basis of all subsequent theories (e.g. Hale 1969; Price 1999; Mignon-Grasteau *et al.,* 2005). In brief they are:

1. They should be hardy

2. They should have a fondness for man

3. They should have a desire for comfort

4. They should be useful to man

5. They should breed freely

6. They must be easy to tend

Whilst it is clear that man is well placed to experiment and work towards items 2, 3, 5, & 6 on land animals, these objectives are much more challenging with aquatic species as man was dealing with organisms and an environment that were far removed from his experience.

Modern aquaculture however, represents a shift in Galton's criteria for domestication. This shift, from the definition by Price (1984) given above, is a change in focus from "the animals and their inherent ability to adapt to man and the environment he provides" to "man and his ability to provide a suitable environment and experience for the animals". As such the success of domestication today draws heavily upon advances in technology and human understanding of animal biology. Man now has the potential to create an environment that meets many of the needs of animals allowing the process of inherent adaptation to happen without or with very little incidence. The process of fish domestication, for example, is underpinned by complex industrial technology informed by an extensive body of scientific knowledge relating to many aspects of fish biology including nutrition, health and physiology (Huntingford, 2004). Consequently it can be said that of Galton's criteria for domestication item 4 ("usefulness to man") is the only one relevant to aquaculture today.

6.1.1.2 Domestication and the Atlantic halibut

The halibut is a case in point. The justification for domesticating the halibut were based on the desire to mass produce a high value product at a profit, i.e. usefulness to man, irrespective of the other criteria outlined by Galton (1865) such as "easiness to tend" and "the ability to breed freely". It was assumed that technology would be developed to overcome the apparent challenges and any biological criteria that were not fulfilled. In this case the technology needed for successful culture includes a steady flow of salt water, maintaining precise temperature control that is vital for egg quality in broodstock and pre-weaned juvenile survival as well as the production and culture of live feed for newly hatched fry to name a few.

Although observed losses in genetic variation have been attributed to the lack of established techniques for the culture of this animal (Jackson *et al*., 2003) it is my view that they also represent an inability of some of the fish to adapt to the farmed environment, a natural phenomenon called "natural selection in the captive environment" or "domestication selection".

6.1.1.3 Domestication selection

The domestication process concerns adaptation to the captive environment which is characterised by genetic changes in behaviour, morphology and physiology achieved over generations (Doyle, 1983; Price, 1984). The genetic forces with the greatest impact on the process of domestication are inbreeding, genetic drift and selection. Whereas inbreeding and genetic drift produce random changes in gene frequencies, the changes resulting from selection are non-random and often directional (Price, 1984; Price 1999; Vandeputte and Launey, 2004).

Selection is the final stage of the domestication process and it was by continued selection of the tamest individuals that our domestic breeds became established (Galton, 1865). Selection in the context of domestication is a continuum of unconscious "natural" selection and methodical "artificial" selection (Price, 1984; Diamond 1998). In contrast to "natural" selection the so called "artificial" selection involves an effort by man who selects those individuals which he believes will produce a desired phenotype (Price 1984; Price 1999). It has been defined as "any non-deliberate change in selection, resulting from natural interactions between fish and the domestic environment in which they are reared, relative to that experience by the natural population" (Glover, 2002) and as "natural selection on traits which affect survival and reproduction in human controlled environments" (Doyle, 1983).

In the absence of artificial selection, domestication selection provides the basic mechanism for genetic change in captive populations and this process eliminates animals unable to reproduce in captivity (Price, 1984; Price 1999; Mignon-Grateau *et al.,* 2005). The influence of domestication selection on the gene pool depends on (1) the extent to which the captive environment allows the development and expression of species-typical biological characteristics and (2) the number of generations in captivity (Price, 1984). The intensity of this selection is expected to be inversely related to the degree of preadaptation for the specific captive environment provided, such that the adaptive capabilities of animals are severely tested when new environments do not match the physical environment to which the population is inherently adapted. Thus, species that possess few preadaptations for their respective environments will experience rather intense selection and show relatively poor and highly variable survival or reproductive success in the early stages of domestication (Price, 1984; Price 1999). In general, domestication

selection is most intense during the first few generations following the transition from wild to captive environments and the degree of adaptation is expected to increase as the frequencies of "favourable genes" accumulate in response to selection (Price, 1984; Price 1999).

In halibut culture the environment in which the animals are reared in is very different from, and almost alien to, their natural one. The halibut is a benthic dwelling creature: coastal areas at depths of 20-60 m are thought to serve at nursery grounds and mature halibut migrate to spawning grounds in deep water at 300-1000 meters, with eggs floating upwards to depths of 100- 200 m (Haung 1990; Arthur, 1999). In contrast captive broodstock, larvae and juveniles are maintained in tanks that are comparatively shallow and confined compared to their natural environment; in most cases in the UK they are kept in converted salmon facilities. Although conditions of low light intensities and water flow are mimicked in the hatchery incubators other conditions such as pressure are not. Such differences in environment could increase, to an unknown degree, the intensity of domestication selection.

Our ability to minimise the intensity to selection is limited by the difficulty of observing normal behaviour of the halibut in its normal habitat. In fact all knowledge of yolk sac larvae stem from observations made during rearing experiments with artificially fertilised eggs because of a scarcity of records of pelagic stages (Blaxter *et al*., 1983). In the wild, larvae are scattered randomly at low density over a very large expanse of water (Haung, 1990). Thus the factors that make modern domestication successful i.e. an in depth understanding of biology

and the creation of the "experience and environment" that ease preadaptation is absent.

It can therefore be proposed that the severe losses in genetic variation and poor family representation observed in halibut culture are due, for the most part, to a very high selection pressure imposed on the species by the captive environment and man's inability to determine those factors that maximise reproductive success in the wild. An example of limiting reproductive success through current management practices is the routine practice of stripping every three days regardless of the stage of females' reproductive cycle which has an effect on egg quality as argued in Chapter 4.

Doyle (1983) concluded that variation in survival and fecundity was inescapable in the domestication phase of aquaculture stocks. The lower levels of reproductive success between cultured and wild stocks of Atlantic salmon reported by various authors (e.g. Gross, 1998; Fleming *et al.,* 2000) have been attributed to the negative effects of domestication selection (Vandeputte and Launey, 2004). Low reproductive success between families was also observed at the initial stages of the Norwegian Atlantic salmon selective breeding programme (Gjedrem *et al*., 1991). These effects however, are not as drastic as those observed in the halibut probably because the hatchery environment is much closer to their natural environment.

A complementary explanation for these reductions could be that because the animal is very highly fecund these losses could be normal for this species in their natural habitat. Also organisms which are highly fecund such as the Pacific oyster tend to carry a large genetic load (Launey and Hedgecock, 2001). Genetic load is defined as the decline in fitness caused by deleterious alleles relative to fitness of an optimal

phenotype (Rowe and Beebee, 2003); it describes the relative chance that an average individual will die before reproducing because of the disadvantageous genes that it possesses (Ridley, 2004). Following a severe population bottleneck, of the sort that occurred when captive halibut populations were established, genetic load caused by recessive mutations always increases (Kirkpatrick and Jarne, 2000). This happens because loci at which deleterious alleles increase in frequency causing those mutations to be exposed as homozygotes. Furthermore because small populations tend to suffer larger genetic loads (Lynch *et al.,* 1995; Batallion and Kirkpatrick, 2000) it could be that the high mortality rate of juveniles was due to the increased genetic load in the parental population resulting from the small number of individuals in the founding populations.

6.1.1.3.1 Domestication selection and the Atlantic halibut

How important is domestication selection and how does this influence the establishment of a genetic management and genetic improvement system for the Atlantic halibut? Genetic management involves the assessment and control of forces that exert selection pressures on the population resulting in the loss of genetic variation of which, as proposed in the halibut, domestication selection in the halibut is a significant component. Knowledge of these forces is essential for the design of efficient genetic improvement schemes.

While significant amounts of progress have been made in several aquaculture breeding schemes involving a wide range of species such as the trout, Pacific and Atlantic salmon and catfish, (see Chapter 1) it is my opinion that selective breeding in the halibut is, at this stage premature. To illustrate this point I will compare the halibut with the species it was domesticated to compete with, the Atlantic salmon.

After just 25 years of production the output from salmon and trout farming was greater than the sum of meat production from pig, cattle and poultry (Gjøen and Bentsen, 1997). At the present rate of growth, the world wide halibut industry is unlikely to equal this feat. A potential problem with domestication and selective breeding in the halibut is the long generation interval. The process of domestication occurs over generations and the generation interval in the halibut is at least twice as long as in the Atlantic salmon. Consequently in the 18 years of halibut commercial culture in the UK, only one generation of mature fish have been produced whereas at this stage in the salmon process 5 generations had been produced. Therefore the accumulation of "domesticated" individuals is as least five times as rapid. Going by the criteria given by Price (1984) it can even be argued that compared to the halibut, salmon and the trout are already domesticated. He proposes and I quote that "*given a stable captive environment and animal management system, the domestication process is complete only when the fitness of the population has reached some reasonable maximum*". The successes reported in salmonid breeding suggest that this "maximum" is being approached within the aquaculture environment for the Atlantic salmon and the trout but the same cannot be said of the present situation of halibut culture and points to one of the objectives for the future of the industry. Until the production of juveniles is routinely successful a selective breeding strategy, of the sort employed in domesticated livestock and established aquaculture species, will exacerbate the depletion of genetic diversity already occurring through domestication.

The selection pressures imposed on the halibut population by the hatchery environment and management practices have resulted in half of the candidate parents failing to breed successfully. Put into an animal breeding context, the

selection intensity for the trait of survival alone was approximately 0.50. Following natural selection for survival if added pressures are imposed on the population for traits such as growth, coupled with the limited population size, it is conceivable that the survival of the Otter Ferry halibut population may be under serious threat. This is because compared to mass selection modern methods of breeding value estimation, such as BLUP assign the highest predicted breeding values to animals that will tend to be more related than expected in comparison to mass selection. When these animals are crossed their offspring will be less able to breed due to inbreeding depression, which consequently will limit their ability to adapt further retarding the domestication process. So how can the principles of animal breeding theory best serve this species and what is the future perspective of the world wide captive halibut industry?

Animal breeding theory and techniques should be applied to ensure that the domestication period of the species progresses with the minimal losses of genetic diversity. The goal of the geneticists is to bring about a steady improvement in the profitability of the stock (Doyle 1983) to this end; based on findings of the current study the present breeding goal for the halibut industry should be redefined. It is my view that the goal should be to acquire a domesticated strain of the Atlantic halibut.

Fitness, reproductive success, of newly domesticated animals in captivity is likely to increase with time i.e. subsequent generations are expected to out perform their parents. In a study with the fruit fly (*Drosophila melanogaster*) Gillingan and Frankham (2003) found that the rate of improvement in fitness reached 25% of its maximum after 6 generations. Whether the same will happen in the halibut is as yet

unknown however what is clear at this stage is that unless the captive populations are “domesticated” i.e. adapted to their new environment, added selection pressures other than those imposed by the environment will be detrimental. Thus animal breeding theory should be applied to ensure that the domestication period progresses with the minimal losses of genetic diversity.

6.2 Genetic management strategy

The overall purpose of this strategy is to ensure a wide genetic base by employing methods used in conservation genetics to minimise the rate of inbreeding over subsequent generations. These methods are discussed briefly and their application to a general programme for the genetic management of the halibut is proposed, structured around the routine husbandry practices outlined in Chapter 1. Following this the specific case of Otter Ferry is discussed.

6.2.1 Minimising the Rate of Inbreeding

In order to minimise the rate of inbreeding within a population three factors need to be considered: 1) the number of selected parents (census of population), 2) the number of sires and dams (sex ratio) and 3) the numbers selected per family (variance in family size). The concerns with the halibut broodstock replacement populations are that none of the three factors were considered or due to biological and technical limitations were not resolved. Few of the wild parental individuals are being represented in the F_1 populations, i.e. families are being lost, and the variance in family sizes are very large resulting in the effective population sizes being much less than the actual number of parents. Furthermore, as revealed by the genetic profiling, milt from a single male was used to fertilise most females' eggs resulting in a highly skewed sex ratio.

Inbreeding is kept lowest when families contribute equally to the next generation (Sonesson *et al*., 2005). If an equal number of parents are taken from each family, the variance in family size will be zero, and the resulting effective population size will be twice the actual number (Falconer and Mackay, 1996). Also if the number

of each sex is the same, using single pair matings as is commonly done in the halibut, the effective population size is maximised.

While the common selection methods employed in fish breeding like mass selection and family selection will result in the reduction of the amount of genetic variation through limiting the number of families that are selected and increasing the variance in family sizes as previously observed on the farm (see Chapter 3), using within-family selection, on the other hand will allow the control of these highlighted factors.

In within-family selection, each family can contribute a fixed number of parents of each sex and because this method does not select between families, the level of genetic variation is maintained, however only half of all the available genetic variation is utilised resulting in reduced gain (Sonesson *et al*., 2005). Nonetheless using this method it will also be possible to improve growth rate while the animals adapt over generations with minimal effects on the increase of inbreeding and obvious benefit to the industry. Individuals are selected because they have the largest deviation from their family mean, in this case body weight, so the processes of artificial and domestication selection can occur side by side. The largest animals in each family can be chosen as broodstock and although the primary objective of this strategy is to conserve the genetic diversity, under the seemingly intense domestication selection and/or through the developmental bottleneck of successful rearing protocols, it will also be possible to simultaneously improve growth rate.

6.2.2 Broodstock Replacement

6.2.2.1 Overview

The proposed strategy is, in effect, a broodstock replacement policy using within-family selection. This method reduces the selection intensity imposed by the breeder thereby allowing the process of domestication to "select" the best members within rather than between family groups maximising the level of genetic variation in the hatchery population by maintaining a constant effective population size. The animals selected as broodstock replacements will therefore go on to produce the eggs and offspring for commercial culture with zero genetic lag, a crucial factor considering the generation interval of this species. There will also, as a corollary, be no distinctions between the "breeding nucleus" and "production fish" thereby conserving space.

6.2.2.2 Broodstock population, sex ratio and family sizes

The objective of this programme is to maintain a low rate of inbreeding therefore as previously stated in Section 6.2.1; the base population should be large, ideally comprised of at least 250 unrelated individuals, with an equal sex ratio, 125 males and 125 females. To establish a broodstock replacement population using within-family selection, one male should be mated to one female only and visa-versa, resulting in the creation of 125 full-sib families. Two offspring per family, one of each sex, should be retained as replacements and because the variance in family size will be zero the effective population size becomes 500. Using the formula derived from Falconer and Mackay (1996) for estimating the rate of inbreeding with zero variance in family sizes:

$$\Delta F = \frac{1}{16N_m} + \frac{1}{16N_f}$$

Where:

N_m= Number of males

N_f = Number of females

This gives a desired rate of inbreeding of 0.1% per generation.

6.2.2.3 Rate of replacements

As previously stated the major draw back with this species is the long generation interval. Although females start producing eggs at 5 years old they produce their best eggs later on in life, however a quick turn over of generation time is required to speed the rate of adaptation therefore as a compromise replacements will be retained from females at 7 years old. Males maturing in their first or second winters will not be desirable as replacements therefore third winters or males maturing at three years old will be used, bringing the generation interval to five years. In order to maintain a consistent production of eggs and to ease the work load on the hatchery only 25 families will be replaced in the broodstock population each year.

6.2.3 Selection Regime

The current study has shown that without the application of genetic profiling technology for the identification of selection candidates, successful broodstock genetic management will have to incur the expenditure of building and designing new and smaller egg incubators and yolk sac incubators which, considering the current state of the industry is highly improbable. Therefore the alternative "walk back" strategy, proposed by Doyle and Herbinger (1994) and developed in this study, is recommended whereby pedigree is retrospectively assigned after communal rearing of offspring.

To cause the minimum amount of inconvenience to hatchery and management staff the genetic management strategy will be structured around normal production procedures. This will involve actions at all phases in the production cycle, however, the replacement procedures will run concurrently to the production practices. Throughout the production cycle though, the batches that contain potential replacement broodstock (replacement batches) will be kept separate from production fish in order to ensure minimal age effects within the batches and to facilitate a greater accuracy of parental assignment as the parents that comprise each batch will be known before hand. A flow diagram of the replacement strategy is presented in Figure 6.1 below.

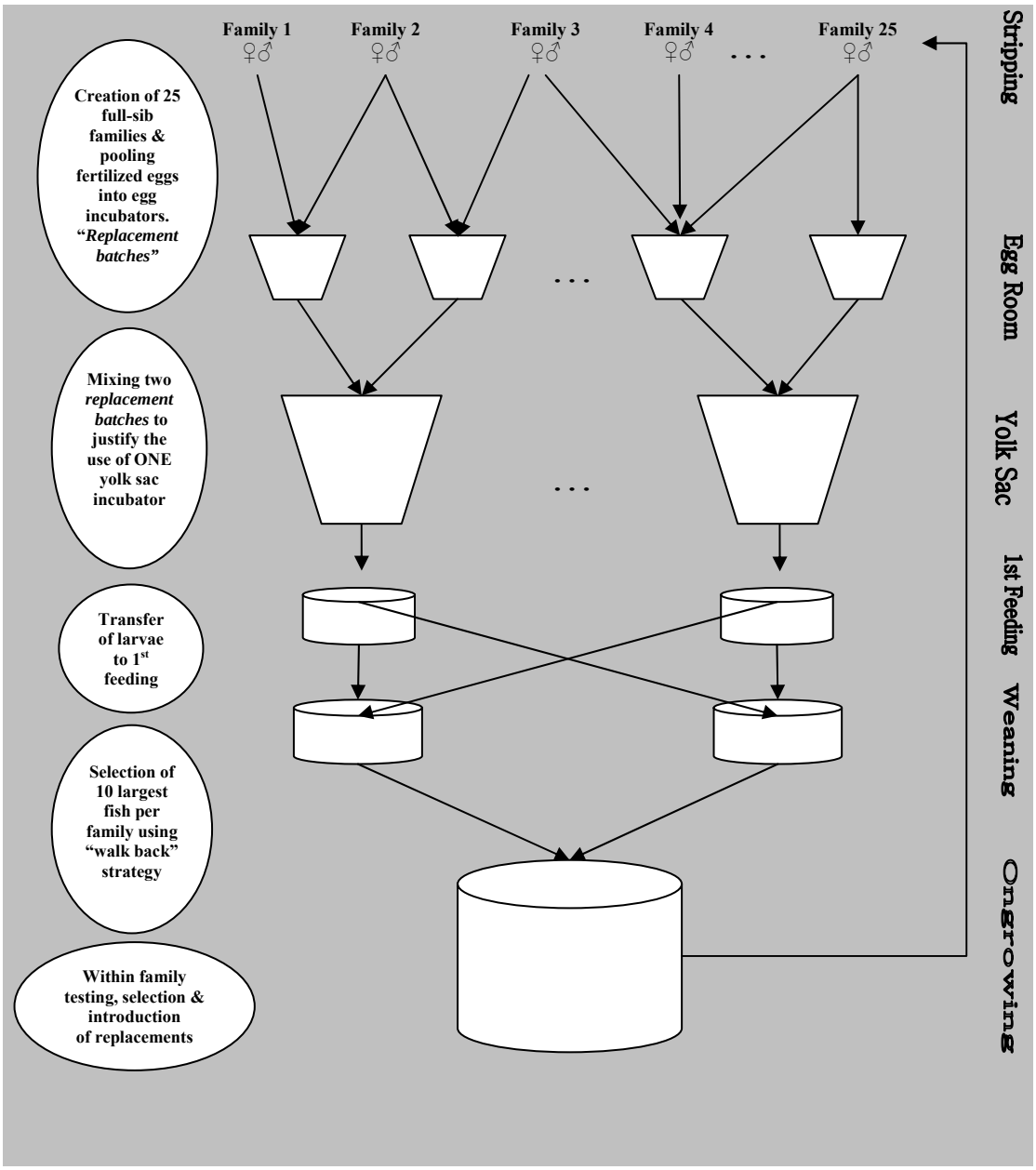

Figure 6.1 Flow diagram of broodstock replacement procedures through the production cycle

6.2.3.1 Stripping and fertilisation

The external fertilisation, high fecundity and batch spawning characteristic of the halibut allows the production of a wide variety of maternal and paternal full-and half-sib families several times in a single spawning season. For the purpose of the broodstock replacement strategy only 25 (one fifth) of the 125 full-sib families will be replaced every year. So in five years the entire broodstock population would have been replaced with more "domesticated" fish. The families will be previously determined by the geneticists and fixed for at least 2 generations. Every one of the 25 families will be set up by routine practices (milt from one male used to fertilise eggs from his designated mate as described in Section 1.2.2.2) and the process will be repeated at least 3 times for each family in a single spawning season to ensure that offspring from each family is represented.

6.2.3.2 Egg room and yolk sac

In order to keep the replacement programme separate from normal production a "replacements batch" is defined. A "replacements batch" will be comprised of as many fertilised eggs from the 25 full-sib families that will be required to justify the running of a single egg incubator (approximately 1500 ml). Because the contents of two egg incubators are required to fill a yolk sac incubator two "replacement batches" will be used to make up one yolk sac incubator.

6.2.3.3 First feeding, weaning and nursery

It is usual practice to put the contents of two yolk sac incubators into one large first feeding tank. Therefore for the purpose of the replacement strategy at least two smaller first feeding tanks will be required to hold larvae from one yolk sac "replacements batch", at very little cost to the hatchery. The potential broodstock

will be placed in these tanks until they are transferred to weaning and nursery facilities. At weaning another two or more tanks will be set apart for the replacements and the grading will be "lessened" into only two of the regular four grades for fish within a "replacement batch" cohort: Large and small to minimise environmental variation, age effects, cost and space.

6.2.3.4 Grow out and selection

At about 500g the largest 250 fish from all "replacement batches" will be PIT tagged and genotyped at the cost of approximately £6 per fish. The largest ten from each family will be retained as potential broodstock. If however no representatives from a family are found, the "walk back" strategy will be employed until 10 members of each family are found. All selected fish will be fitted with PIT tags.

Testing protocols will be almost identical to those conducted in the trial by Nigel Jordan as described in Chapter 5. These fish will be weighed twice every year at six monthly intervals. Sex will be determined by ultra sound and by gentle palpitation of the abdomen to check for running milt. Males that mature within two years will be removed from the group, unless every male within a single family is a precocious maturer in which case the largest males form that family will be retained. Every year the smallest two individuals from each family will be culled from the group and the best four fish, two from each sex will be kept as potential replacements. Although only two are required as replacements doubling the number of fish per family reduces the risk of loosing families. At the time of introduction into the broodstock population only two will be selected.

6.2.3.5 Replacement policy and broodstock population

The selected replacements will be introduced into the broodstock populations depending on their sex. The replacement broodstock have two functions: their first purpose is to produce offspring for production and secondly to produce future broodstock. Males will be introduced into the broodstock population for commercial production at three years old and the females at five years old. In order to reduce the generation interval males will be used to produce "replacement batches" at three years old whereas females will be used once at seven years old. If however males from a certain family are later maturing, they can be used again at four years old. This causes a mixing of the gene pool between successive year classes.

6.2.3.5.1 The use of photoperiods

Photoperiod control of reproduction is successfully used to delay and advance spawning to manage egg production, however it can also be used to manage the gene pool and to prevent consanguineous matings. Under the proposed replacement strategy, if each sex from one family is placed under different photoperiod regimes they will effectively be in two different populations. Subsequently they will never, unless by a deliberate action of linking both populations (photoperiods) e.g. by cyropreservation, be mated together. Dividing the families into two photoperiods also spreads the risk.

The results from the repeatability study in Chapter 4 showed that egg quality was more likely to be a management issue (i.e. timing of stripping) than any factors controlled by the fish. It was therefore recommended that fewer fish be kept and efforts concentrated on getting the ovulatory rhythms of females correct. If however 125 females are required for genetic management it appears that these two

recommendations are conflicting. To overcome this problem the females can be divided into two or three photoperiods: ambient, delayed and advanced. This will mean that there will either be 42 or 62 females per group. In order to improve the detection of individual ovulatory rhythms, the fish within each photoperiod group can be further divided into two or more groups of manageable numbers. Because the fish will spawn several times in a spawning season each group can be allotted a period of two weeks where hatchery staff can concentrate on each fish. After the two week period the next group of fish is stripped and so on (Lesley McElvoy pers comm.)

6.2.4 SWOT Analysis of Each Phase of the Genetic Management Strategy

The above strategy is a simple one because of the present state of the halibut industry. The programme was structured to suit the inability and /or unwillingness of halibut producers to complicate their production systems and spend meagre or non existent profits on building new facilities. The strategy demonstrates that their aforementioned perceived notions about effective selective breeding programmes and their reasons for divorcing animal breeding from broodstock replacement, a strategy that has proved detrimental, need not always be true. If this strategy is to be adopted there are, however, some practical considerations that need to be discussed and these are presented in the SWOT analysis below.

6.2.4.1 Stripping and Fertilisation

Strengths

- External fertilisation so families can be created with ease.
- High fecundity so despite high mortality rate a sizable number of offspring from each family may be represented.
- Batch spawning therefore 1) each family "batch" can be repeated several times within a spawning season and 2) presents the adoption of an

opportunist strategy whereby farm managers can decide the best times to allocate resources towards the replacement batches when interests of production and replacements are not conflicting.

- Pooling families reduces 1) the introduction of common environmental and age effects and 2) Cost on hatchery for building and running new facilities.
- Photoperiod manipulation will allow the spread of replacement batches between families over the course of a year and ease the load on the hatchery staff.
- The protocols of fertilisation are already established and because the mating system is simple, i.e. only full sib families, the margins for error are much smaller when compared with large scale factorial mating.

Weaknesses

- Limited number of unrelated broodstock within captive halibut populations
- Poor repeatability of reproductive traits such as seasonal reproductive activity and egg fertility mean that all designated females may not spawn and of the females that spawn their egg quality might be too poor to be incubated.
- Asynchrony between female spawning and stripping procedures may result in not enough fertile eggs from every designated female to justify the composition of a separate "replacement batch".
- Robust methods of identification and fertilisation procedures are required to prevent mistakes with the setting up of families.
- The full-sib family structure prevents the detection of individual sire and dam effects.

Opportunities

- Due to the universal problem of restricted broodstock population sizes a pre-competitive collaboration between halibut producers can be proposed, a halibut breeder's consortium. The process will involve a mutual exchange of eggs, milt or fry from F_1 offspring for a period of 2-3 years in order to reduce the impact of domestication selection.
- Following the exchange of fish, inter-specific differences in performance between fish from different origins can be compared under commercial conditions and the existence of heterosis can be tested.

- After the first two generations more complex mating designs can be explored.

Threats

- An inability to acquire the desired number of broodstock, i.e. the reluctance of halibut members to form a consortium.
- Disease transfer between producers.

6.2.4.2 Egg room and yolk sac

Strengths

- Pooling of families reduce the financial burden on the hatchery.
- All fish in the same "replacements batch" will be of similar ages.

Weaknesses

- The cost of running separate incubators for non-commercial purposes might appear high if production batches are particularly poor.
- Requires that incubators containing replacement batches be identified to prevent mixing with production batches.

Opportunities

- Pedigree analysis of dead eggs and larvae will indicate if there are family effects on mortality at this stage in the production cycle.

Threats

- High mortalities or large "drop out" volumes at these stages of production will lead to the "dumping" of the replacement batches.

6.2.4.3 First feeding and weaning

Strengths

- Grading only within batches keeps age effects minimal.
- Separate facilities for replacement strategy at this stage are relatively inexpensive to build and maintain.

Weakness

- High mortalities of fry at this stage is expected.

Opportunities

- Pedigree analysis of dead fry will determine of there are family effects associated with what has been described as the major bottleneck within the halibut industry.

Threats

- Disease.
- Mixing with non-replacement batches.

6.2.4.4 Selection procedure

Strengths

- With genetic profiling technology the parentage of every individual can be accurately determined.
- As parents of replacement batches are determined before hand the power of parentage assignment analysis is increased.
- Selection is done at an early age so costs of maintaining a large number of potential replacements is reduced.
- A sufficient number of offspring per family is kept to allow for the desired number of fish from each sex.
- A standard number of representatives per family is retained.
- As all fish communally reared there are few common environmental effects introduced.
- Age effects limited as selection will be done within batches.

Weaknesses

- High genotyping costs associated with the walk back strategy, particularly if the mortality rates in certain families are large.
- A robust method of data and sample collection is critical.
- Poor correlations between the measurements made of body weight early in the growth curve and those later on in life mean that selection might be made at too early an age.
- Errors made in the pedigree analysis can be immensely detrimental therefore protocols need to be highly rigorous.

Opportunities

- Using photoperiod manipulation the replacement family batches can be set up at different times within a year. Therefore the number of different families within one batch is reduced and the chances of finding different families within a batch are increased thus lowering the cost of the walk back strategy.

- The genotyping costs can be shared by all members within the proposed halibut breeder's consortium. Alternatively the genotyping can be centralised and all members within the consortium can send samples to a central laboratory, because the number of samples will be large the cost per sample will be reduced.
- Data generated from regular measurements between and within families can be used to obtain better estimates of genetic parameters.

Threats

- By selecting too early traits such as precocious maturation in males will not be determined until it is too late.

6.2.4.5 Ongrowing and Replacement

Strengths

- Post weaning mortality in the halibut is low
- Estimates of heritability for body weight indicate that selection will be successful
- Test procedure requires relatively little effort as fish are examined and measured only twice a year.
- Methods of sex determination are already established.
- After replacements are kept from females they can either be culled from the broodstock population or kept on for the production of commercial fry, i.e. replacement and production procedures can co-exists with only the best performing females retained for production.

Weaknesses

- The potential broodstock are kept in land based systems and because their offspring will be reared in sea cages genotype by environmental interactions might be introduced.
- The replacement procedure will incur the additional fixed costs of maintaining a constant number of fish every year.
- Sex and precocious maturity is assigned post selection so the selection intensity between sexes and within families will be variable. Also some families will be made up of only precocious maturing males.

Opportunities

- Reproductive records from females can be used to estimate genetic parameters.
- Data acquired while animals are on tests can be used to estimate genetic and phenotypic parameters for precocious maturity in males.

Threats

- Disease
- Mortality of selected broodstock families due to stripping associated injuries in the spawning season.

6.2.5 Alternative selection strategy

An alternative to the within family strategy proposed is to use the optimum contribution methods (OCM), originally described by Meuwissen (1997) to manage the rate of inbreeding over time. This method maximises genetic response with a constraint on the rate of inbreeding. This restriction is set at the level of the relationship among all of the parents instead of inbreeding at the level of individual offspring (Sonesson *et al*., 2005) such that the maximum number of offspring that each selected candidate can contribute to the next generation to achieve the desired, pre-set, level of inbreeding. A quadratic index is used to assess the potential contribution accounting for the magnitude of the estimated breeding value of a candidate and its relationship with other individuals in a population. Effectively the best selection candidates will be those with high estimated breeding values that have few relatives in the test population. The appeal of this system is that it will be possible to achieve significant genetic gains while keeping a certain level on inbreeding. In regard to the specific case of the halibut this method allows selection both within and between families for a restricted level of inbreeding set by the geneticist. Sonesson (2005) explored the application of the optimum contribution to the walk-back strategy for pedigree identification using stochastic simulations and

concluded that compared with genotyping all selection candidates this system would save costs while simultaneously maintaining high genetic gains.

The application of mass selection using optimum designs to the initial stage of halibut breeding, however, presents various challenges. Gjrede *et al* (1996) concluded that an optimum breeding design may easily turn into a suboptimal design due to different survival rates and/ or different proportions of sexual maturing, two problems central to halibut culture. Due to the highly skewed family sizes observed the amount of genotyping required for the OCM will not be different to that in a family-based breeding programme. Imposing the severe restrictions on the rate of inbreeding proposed will not only imply lower selection intensities but will require a greater number of offspring per families to be tested and a greater number of families to be generated. Also because of the poor survival rates the number of individuals needed to maintain the desired rate of inbreeding (0.001) would far exceed the 250 fish proposed. Ultimately this system will result in the selection of certain individuals over others, further degrading the already depleted genetic resource compounding the effect of domestication selection already in progress. It is conceivable, though, that following the next stage of the domestication process when families are reproducing routinely the OCM will be of more benefit.

6.2.6 Otter Ferry Seafish Broodstock Population

The aim of this project was to design a selective breeding programme for the company. However, in the second year of the project all the wild caught broodstock died in an incident on the farm. Therefore the population on the farm is comprised of only F_1 fish from the 1995, 1996 and 1998 year classes. Although not all the fish

in the 1995 year class were genotyped the coefficient of inbreeding estimated from all the fish sampled was 6.16%. This level of inbreeding is highly unacceptable and requires immediate attention.

Pedigree information should be used to prevent consanguineous matings it is, however, only a short term measure. The program outlined above requires that the population is made up of 125 families. Although this might appear exaggerated it contrasts to the F_1 population on the farm which is made up of only 17 full-sib families. It is my recommendation that new fish should be introduced into the population. Based on the findings of this study these new individuals should be F_1 hatchery reared individuals which will be better adapted and much cheaper to acquire than wild fish. These fish can be obtained from commercial hatcheries in Iceland, Shetland, Norway, Canada and the USA. If issues related to disease are a concern eggs and milt can be delivered to the hatchery and tested for viruses before they are used.

6.3 Future Research and Concluding Remarks

The process of culturing the Atlantic halibut has and continues to be a challenging one. It is worth noting that considering the generation interval of the species and the number of individuals involved in commercial culture, the industry is still in its infancy. The future of the industry will, however, depend on how the domestication process is managed. This process of how the halibut adapts to the aquaculture environment and/or more importantly the process of how the aquaculture environment is adapted to allow maximum reproductive success is crucial to the success of this industry.

The findings of the current study suggests a significant bottleneck is occurring somewhere in the production system that affects the survival of certain families however, this process is not yet understood. Identifying the source(s) of this problem and addressing the issues related to them will allow the routine establishment of a viable, consistent and genetically diverse broodstock population, a factor central to the survival of the industry. The reasons for the loss of so many families are undoubtedly multifactorial and multidisciplinary, covering a wide range of issues related to broodstock and larval nutrition, husbandry practices and disease. Nonetheless from the geneticists' perspective certain families appear to do well under these conditions so the question is why isn't this level of success repeated across all families?

In this study survival was only determined at the end of the production cycle and although most authors attribute the mortalities to the first feeding stage it will be important to determine whether there is a genetic effect on mortality at this point in the production cycle or if families were lost earlier on. The stage at which different

families “drop out” of the system is crucial in obtaining solutions and improvements in the culture of this species.

This can be determined by setting up a factorial mating design and pooling a standard volume of fertile eggs from all the families into discrete egg and yolk sac incubator(s) to emulate normal hatchery conditions, as described earlier for “replacement batches”. This batch of eggs should be kept discrete following the protocols for broodstock replacements already described. By determining the parentage of animals that “drop out” of the system i.e. analysing mortalities using DNA profiling technology throughout the production cycle i.e. (DNA from dead eggs, larvae and fry), or those that fail to enter the system due to poor egg or milt quality, it will be possible to discern where the families are lost and where research efforts need to be centred in order to create a more suitable environment for the adaptation of this species to aquaculture. Environment in this sense will include not only the physical compartments where the fish are held but all factors that are not genetic such as nutrition and broodstock or larval management

Growth rate was identified as the primary breeding objective of the industry, this trait, however, defined as body weight at various points from weaning to harvest, is determined to a great extent by sex. Results from Chapter 5 showed that the onset of sexual maturity in males significantly retarded their growth and the genetic influences of this needs to be investigated further. The heritability of age of maturity and the phenotypic and genetic correlations with body weight at various points in the growth curve should be estimated to determine a long term strategy for dealing with the problem. In the short term, the production of all female stocks by the indirect route of sex reversal can be explored.

As the global population increases so does the need to culture rather than capture food from the aquatic environment. This fact coupled with the low prices of the hitherto domesticated species that dominate world markets, such as the Atlantic salmon, necessitates the culture of new species to provide multinational producers a competitive financial advantage as they meet the ever increasing demands made by consumers for variety. Despite the apparent challenges with its culture the halibut is an excellent fish that gives a highly desirable and profitable product, a fact that has lead to a concreted effort by fish farmers in Iceland, Norway, Canada, the USA, Ireland and Scotland to continue pursuing the culture of this fish.

After 22 years of commercial experience valuable lessons have been learnt and the present study shows that there are still a few steps to secure the future of the halibut industry, at least in Scotland. The establishment and maintenance of a broodstock population comprised of a large number of genetically diverse individuals, as described in this thesis, through the application of molecular and quantitative genetics techniques and a collaboration between halibut producers will contribute to this goal.

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