

**Genetic Management and Selective  
Breeding in Farmed Populations of  
Gilthead Seabream (*Sparus aurata*)**

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Submitted for the Degree of  
Doctor of Philosophy

University of Stirling

2003

## **Abstract**

Gilthead seabream (*Sparus aurata*) is one of the most important species of intensively reared fish in the Mediterranean region. Its short history of domestication, along with the need to develop markets, new products and efficiency in the production process, has resulted in an increased interest in the potential genetic improvement of this species. Little work has so far been directed at establishing the procedures for selective breeding in gilthead seabream at a commercial level, although genetic parameters calculated in other studies have indicated that there is a large potential for improvement in certain traits. Selective breeding of commercial gilthead seabream populations is constrained by aspects of the biology that complicate the production of genetic groups and the maintenance of same-age offspring populations. The aim of this thesis was to develop a protocol for the selective breeding of gilthead seabream, specifically to serve a commercial hatchery and on-growing unit in Cyprus, where the fieldwork was carried out.

The hatchery broodstock was monitored over a three-year period to identify the rate of sex reversal in introduced fish and to quantify the sex ratio of the stock over time. The analysis of the egg production records was used to evaluate the success of photoperiod manipulation in each group. Size variation in the larval and juvenile stages is a common problem in the rearing of gilthead seabream, leading to cannibalism and labour-intensive sorting operations. Studies on larval populations, from first feeding through to metamorphosis, indicated the origin of size variation was the differences in early feeding ability. The size advantages could be maintained throughout the larval period. During the juvenile stage of the farm production system, a method to standardise the size sorting of populations by grading was developed in order to counter environmental effects of separating groups of fish. Using this method, grading would be suitable to form the first stage of a selection programme for growth rate. The potential gain of selection for growth rate during the on-growing stage was very high, using a simulated criterion and previous estimates of heritability. Other possible quality traits for selection were also examined and quantified in the hatchery populations. Existing and specifically developed microsatellite markers were used for the assignment of offspring to parents from mass spawning of the hatchery broodstock. The effective population size of single spawning events were found to be low and determined by a high variation in contribution to mass spawning. Contribution was found to be significantly linked to body size, which led to the formation of a replacement policy for the broodstock to maximise spawning performance. Survival of individual families through the larval period was also examined.

Based on the results of the experimental work, a two-stage selection programme was designed, along with the presentation of specific procedures for each stage of the production system. This project makes recommendations on various strategies that can increase the effective population size within a selection programme and these are discussed as part of the genetic management of hatchery populations. Significant progress has also been made in the use of genetic markers in monitoring the rate of inbreeding and contribution of individual broodfish, which are considered essential in this species.

## **Acknowledgements**

A number of people have been involved in the organisation and running of this doctoral project. Firstly, I would like to thank my supervisors Professor Brendan McAndrew and Professor John Woolliams, whose support, expert guidance and patience in steering me through the PhD process is greatly appreciated. I am also deeply indebted to the late Professor Niall Bromage, who, despite his illness, was able to make a substantial contribution to the format and content of this thesis.

I would also like to extend my thanks to the Alkioni Group plc for funding this project and providing me with the opportunity to conduct the field research at their hatchery in Cyprus. I am grateful to all the staff of the hatchery for their assistance in collecting the data presented here and for the advice and background information that they were able to provide in relation to my on-site work.

There are many staff and students at the Institute of Aquaculture, who have readily given me advice and assistance on the laboratory aspects of this project, most notably Tariq Ezaz, John Taggart and Tom Dixon. My thanks also go to Brian Howie for redesigning and manufacturing the grading sheets I used for my research work at the hatchery, which actually proved to be more accurate and more successful than I had anticipated!

During my extended visits to Cyprus, I was able to draw on the support of my wife's family, without which I couldn't have completed the fieldwork or even had the time to concentrate on writing this thesis. I am very grateful for all the help they have so willingly given to my wife and myself over the past three years and I hope they, too, feel it was worth the effort!

Last but not least, I wish to thank my wife for all her support and patience during the past three years, as we often seemed to travel in different directions for most of that time. She has given me invaluable assistance during the project by translating various documents and by single-handedly proof reading this thesis. I couldn't have done this without her.

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## **Glossary of Species Names**

<b>Common Name</b>	<b>Scientific Name</b>
Arctic charr	<i>Salvelinus alpinus</i>
Atlantic cod	<i>Gadus morhua</i>
Atlantic halibut	<i>Hippoglossus hippoglossus</i>
Atlantic herring	<i>Clupea harengus</i>
Atlantic salmon	<i>Salmo salar</i>
Brown trout	<i>Salmo trutta</i>
Chinook salmon	<i>Oncorhynchus tshawytscha</i>
Coho salmon	<i>Oncorhynchus kisutch</i>
Common goldfish	<i>Carassius auratus auratus</i>
European sea bass	<i>Dicentrarchus labrax</i>
Gilthead seabream	<i>Sparus aurata</i>
Matrinxã	<i>Brycon cephalus</i>
Mosquitofish	<i>Gambusia affinis</i>
Nile tilapia	<i>Oreochromis niloticus</i>
Plaice	<i>Pleuronectes platessa</i>
Rainbow Trout	<i>Oncorhynchus mykiss</i>
Red porgy	<i>Pagrus pagrus</i>
Red seabream	<i>Pagrus major</i>
Red snapper	<i>Pagrus aurata</i>
Rough dab	<i>Hippoglossoides platessoides</i>
Saddleback wrasse	<i>Thalassoma duperrey</i>
Turbot	<i>Scophthalmus maximus</i>
Winter flounder	<i>Pseudopleuronectes americanus</i>

## **Chapter 1: General Introduction**

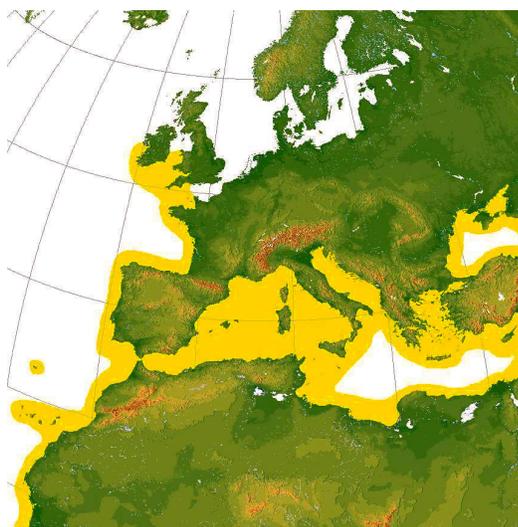
## **1.1 Biology and Aquaculture Production**

### **Of Gilthead Seabream**

#### **1.1.1 Biology of Gilthead Seabream**

Gilthead seabream is a member of the Sparidae family that contains a large number of species in several genera. Sparids are found throughout the tropical and temperate seas and are also found in cold and brackish waters. In the Mediterranean Sea eleven genera of the family are represented: *Dentex*, *Sparus*, *Diplodus*, *Pagellus*, *Pagrus*, *Lithognatus*, *Spondyliosoma*, *Oblada*, *Crenidens*, *Boops* and *Sarpa*. Many Sparid species are highly valued because of the quality of their meat and several species are already produced in intensive aquaculture systems.

The Gilthead seabream is found in coastal regions throughout the Mediterranean Sea and into the eastern Atlantic as far north as the British Isles (in summer), to the Canary Islands and the African coast to the south (Figure 1.1). Gilthead seabream generally occupy shallow waters up to 30m deep but adults may be found at depths of up to 150m (Bauchot and Hureau, 1990). They are normally associated with seagrass beds and sandy bottom habitats and are found within the surf zone. As with many other Sparids, gilthead seabream is mainly carnivorous, typically feeding on molluscs, decapods and annelids (Stergiou and Karpouzi, 2002). The maximum recorded size of the species is 2.5kg with a total length of 70cm (Frimodt, 1995).



**Figure 1.1: Distribution of gilthead seabream (indicated by ■). The species is found mainly in shallow coastal waters but ranges from the eastern Mediterranean to the Atlantic coast and as far north as Britain during the summer months.**

The family Sparidae has the most diversified expression of sexuality ranging from gonochorism through to protandrous and protogynous hermaphrodites (Buxton and Garratt, 1990; Yeung and Chan, 1987). Gilthead seabream is a protandrous hermaphrodite, beginning life as a male, while from the second to fourth year an increasing proportion become female (Zohar *et al.*, 1995). Ghislin (1969) proposed three models for the development of hermaphroditism: low-density, size-advantage and gene dispersal. The low-density model suggests that in cases where individuals are widely dispersed or sessile, it is advantageous to be able to mate with any individual to maximise reproductive success. The size-advantage model advocates that as the size of the animal increases, so the reproductive potential of each sex may change, for example larger individuals may have a greater capacity to produce gametes. Therefore, hermaphroditism is a way of maximising reproductive success by allowing sex change to suit the circumstances of the animal. The third model of gene dispersal suggests that sex change is an adaptation to maximise genetic diversity by reducing interbreeding between siblings (as they are the same sex). Due to marked size differences in males and females in some Sparid species, the size-advantage model has been proposed as the main process in the development of hermaphroditism in these species to maximise lifetime reproductive success (Buxton and Garratt, 1990). The low-density model is inapplicable because of the group spawning behaviour of many of these species, as is the gene dispersal model for the same reason – namely, that group spawning results in a high gene flow.

From studies of other protandrous Sparids, it can be deduced that both male and female gonadal tissues can be present in the fish, with male tissue active in young fish and female tissue appearing to mature at a particular stage of growth (Yeung & Chan, 1987). At the age of one year post-hatch, all fish in a natural photoperiod are typically male with 95% capable of producing sperm (Kissil *et al.*, 2001). At 24 months post-hatch under a natural photoperiod, the sex ratio approaches that of 1:1 ratio at the beginning of the spawning period (Kissil *et al.*, 2001). There is some suggestion that fish destined to be females are larger as juveniles, but also that this advantage is lost during maturation into females, which requires additional resources (Knibb *et al.*, 1997a). The actual proportion of fish that undergo sex reversal each year is variable and is likely to be under social control (Happe and Zohar, 1988). In other hermaphroditic Sparids, studies have shown that some fish remain the same sex throughout their lifetime suggesting that reversal is not obligatory (Yeung and Chan, 1987; Kokokiris *et al.*, 1999). Photoperiod (Kadmon *et al.*, 1985; Kissil *et al.*, 2001) and temperature (Villia and Canario, 2000) have also been found to have a large influence on the development of sex ratio in captive gilthead seabream stocks. Zohar *et al.* (1995) found that gilthead seabream enter an ambisexual (resting) stage outside of the spawning season at the end of which social and hormonal factors determine the rate of sex reversal before the beginning of the next spawning period.

Pheromones released into the water have been identified as a means of synchronising spawning in species such as the goldfish (Sorensson, 1992). This system could also be used as a mechanism to socially control sex reversal by stimulating the production of hormones. A hormonal mechanism of initiating sex reversal has been confirmed by Socorro *et al.* (2000), who were able to induce sex reversal in gilthead seabream using Estradiol-17 $\beta$  (E<sub>2</sub>) supplied within food. However, other sex reversing species such as saddleback wrasse have been found

to rely only on visual cues for the control of sex reversal in groups (Ross *et al.*, 1983). The complex mechanisms by which the sex ratio in gilthead seabream is determined are still not fully understood and, therefore, this is a limitation to the management of hatchery populations that depend on a captive stock of breeding individuals.

Gilthead seabream is a mass spawning species and spawning takes place between October to January, corresponding to natural water temperatures of 13 – 17°C (Pillay, 1990), but occurs later in the eastern Mediterranean where mean sea temperatures are higher. Females are daily sequential spawners producing 20,000 – 30,000 eggs per kg body weight per day over a 3 – 4 month period (Zohar *et al.*, 1995). Spawning in the wild takes place in large groups or schools and thus a minimum of 5 – 7 fish appears necessary for reduced stress and natural spawning in artificial environments (Gorshkov *et al.*, 1997). It is possible to induce spawning with human chorionic gonadotropin (hCG) or gonadotropin-releasing hormones (GnRH) followed by egg stripping or natural spawning over short periods (Zohar *et al.*, 1995). Gilthead seabream spawn readily in captivity when kept in large groups, but individual performance cannot be measured. Therefore, creating highly structured mating designs for the purposes of genetic improvement is impossible.

### **1.1.2 Intensive Production of Gilthead Seabream**

Early culturing of gilthead seabream was based on the capture of wild larvae and juveniles using valli and fish barriers at lagoon entrances. As the pressures of open water fishing and valli capture reduced the wild-seed resources, increased efforts were made to develop intensive production practices. Large-scale production of fry was achieved in the early 1980's in France and Italy and by the 1990's the intensive production of juveniles and methods to produce fish to market size in cages or ponds were accomplished (Moretti *et al.*, 1999). Continuing advancements in the nutritional and environmental requirements of larvae improved the survival and quality of fry production have allowed an expansion of the scale of production. Mediterranean hatcheries have often combined the culture of gilthead seabream and European seabass, because of the similarity in requirements and rearing techniques of the two species, both of which are now the most important commercial species in the Mediterranean region. The fieldwork for this project was carried out at a combined seabream/ sea bass hatchery and on-growing unit in Cyprus.

The production cycle of gilthead seabream in intensive aquaculture begins with the collection of eggs from the mass spawning activity of the broodstock (Figure 1.2). Viable, fertilised eggs float to the surface because of the presence of an oil droplet, whereas non-fertilised and damaged eggs sink due to the excessive absorption of water. The eggs are collected by a simple screen within the outflow system of the broodstock tank (Zohar *et al.*, 1995) and are normally collected in the morning following the spawning behaviour in the proceeding evening. Eggs are sorted by skimming the surface of the collecting tank to remove only the floating eggs. The eggs are then placed into conical incubating tanks with minimum water movement and no light for 36-48 hours.



at random from the cage populations. Broodstock groups are frequently held under photoperiod control, in order to manipulate the spawning time and provide eggs throughout the year. Spawning time coincides with the onset of winter, indicated by short days and low water temperatures. Therefore, manipulation is achieved by a progressively shorter light period and by reducing the water temperature by several degrees. Although manipulation can achieve spawning at any time of the year it has been found that rearing of larvae at higher ambient water temperatures can result in an increased susceptibility to diseases.

### **1.1.3 Industry Development**

Due to its robust nature, rapid growth, feeding habits and high quality meat, the gilthead seabream is particularly suitable for aquaculture and commands a high market value. Catches of gilthead seabream in open waters have steadily diminished and with high prices for unprocessed fresh fish, there has been considerable effort made towards intensive culture of the species in many countries. Annual production of this species has increased rapidly in recent years (Figure 1.3) as a result of technical developments in the production system. Greece maintains the highest production of gilthead seabream due to the number and size of on-growing units throughout the country. Production in Cyprus has followed the trend seen in other countries with the establishment of new fish farms in recent years and an improvement of culture techniques used. However, production in Cyprus has been mainly destined for the local market rather than export. This is mainly due to the increased taxes imposed within European Union countries on imports from non-EU countries (FEAP, 2003). With the accession of Cyprus into the EU in 2004, it is anticipated that production and exports will increase markedly.

The prices for market size adult gilthead seabream in Europe have declined over the last few years (Figure 1.4), mainly as a result of increased production (Bartley, 1998). In Cyprus the value of fish is generally higher, partly due to costs of importing from other countries, but the same downward trend can clearly be seen. As the value of the product falls, substantial efforts are needed to maintain profits by increasing the efficiency of the production system as well as developing new products and improving quality.

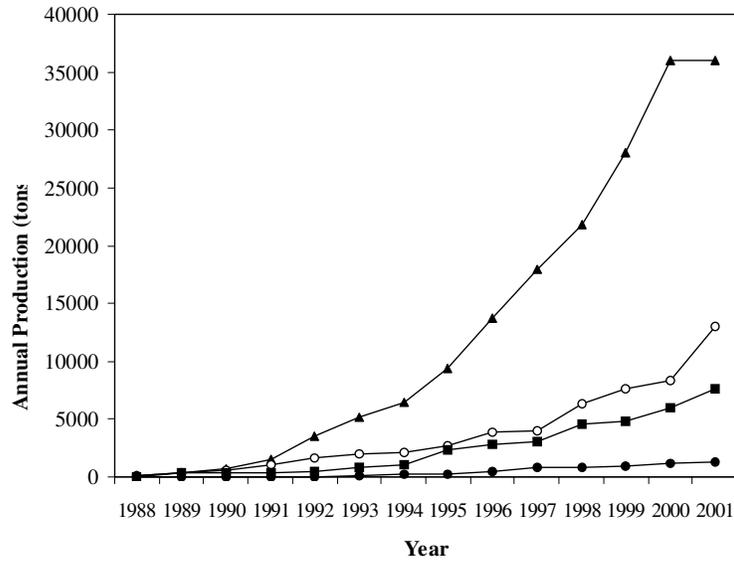


Figure 1.3: Trend of aquaculture production of gilthead seabream from 1988 to 2001. Production levels rose dramatically from 1994 onwards mainly in Greece (▲) which has the largest production of any country followed by Spain (◻) and Italy (■). Production in Cyprus (●) has risen to 1300 tons annually and this is expected to increase with new markets in Europe. Data from FEAP (2003).

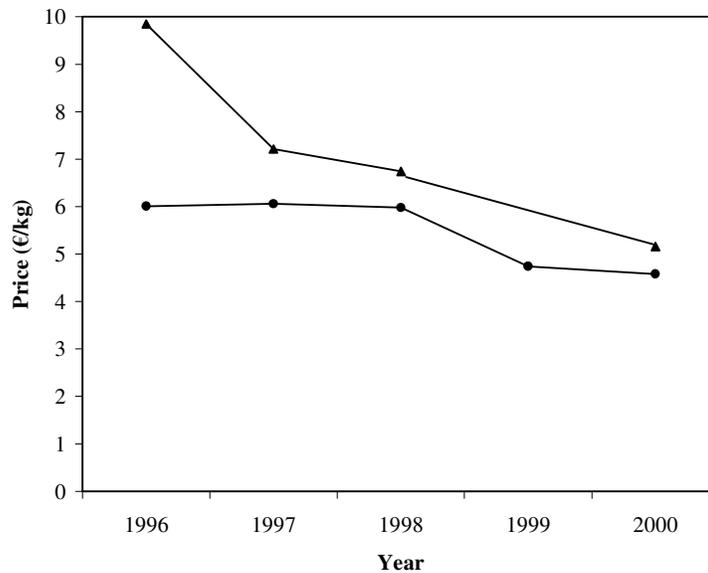


Figure 1.4: Market price of gilthead seabream in Cyprus (▲) and average within Europe (●) between 1996 and 2000. The decline in price is mainly due to increased production and year-round availability which has saturated markets. The declining price of the product will increase the need to improve efficiency in the farming system. Data from FEAP (2003).

The market for gilthead seabream is almost exclusively based on the sale of fresh and frozen whole fish. This is unusual considering the number of food products available for species such as salmon and tuna which include many types of steaks and fillets, preserved (tinned), frozen, processed and through to ready-prepared meals. Given the high quality of the meat from gilthead seabream and low fat content (Frimodt, 1995) the market for seabream products is likely to expand as its popularity increases. It is already commonly sold in supermarkets throughout northern European countries in addition to its natural Mediterranean country markets.

With the development of new products and markets the quality and size of the fish produced by aquaculture will undoubtedly change. Some of these improvements can be made by modifying the production system, but there is also a need to make genetic improvements with a species that has a very short history of domestication and is effectively still similar to the wild stock. The diminished price of fish will drive the industry to increase efficiency whilst the diversification of the markets will require improved quality of the products. Selective breeding can achieve both of these aims by improving traits such as growth rate, which will lead to a higher feed conversion ratio (FCR) and a reduced time to harvesting. Quality of the product, such as carcass fat and meat texture, can also be improved by selection as part of a multi-trait index, once standardised methods of quantifying the traits have been developed.

Selection programmes are only one approach to genetic improvement, as advances in biotechnology now allow a wide range of possibilities in determining genetic performance. Hybridization (crossings between strains and species), polyploidy (diploid and triploid gametes) and genetic manipulation (such as monosex populations, sterile offspring and inbred lines) have resulted in significant and rapid advancements in fish aquaculture systems (for examples, see Felip *et al.*, 1999; Hinitis & Moav, 1999; Pongthana *et al.*, 1999; Mair *et al.*, 1995). Techniques such as these are being used in countries across the world and are increasingly seen as a rapid way to improve yields (Bartley & Hallerman, 1995). However, not all of these techniques are suitable for all species of fish and may only offer short-term, non-cumulative gains in production (Gorshkov *et al.*, 1998; Bartley, 1998). Selective breeding offers long-term improvements to a farm population and can create fish specifically suited to the farm production system. Selection can also be practised on many different types of traits, either individually or as an index of several traits, with more reliability than many of the genetic manipulation techniques.

## **1.2 Selective Breeding Methods in Fish**

### **1.2.1 Choosing Traits for Selection**

Variation of a particular trait can be partitioned into several components reflecting the range of sources of genetic variation in a population. Of primary interest is the ratio of additive variation and phenotypic variation ( $V_A/V_P$ ) as this gives an indication of the degree to which phenotypes are determined by genes from the parents (heritability) and, consequently, the potential speed of genetic progress. Non-additive variation includes dominance variation ( $V_D$ ) and interaction or epistatic variation ( $V_I$ ), which can have important effects across the genome but are generally not heritable. Dominance results from the interaction between the pair of alleles at each locus, whereas epistasis is the interaction(s) of alleles between or among loci. Dominance variation can be exploited through methods such as hybridization and ploidy manipulation, which can increase the interaction between alleles (Tave, 1993). Variation due to environmental factors ( $V_E$ ) also plays a major role in the determination of all quantitative phenotypes throughout the life of the animal. This type of variation must be minimised in any selective breeding system, as changes in environment will mask any genetic gain or superiority within the population. For example, stocking density is known to affect growth rate (Gall and Bakar, 1999) and has been incorporated into experiments as a means of estimating this component of variation (Wohlfarth and Moav, 1985).

Heritability ( $h^2$ ) is the heritable element of the total variation and is used to calculate the breeding values of individuals. It can also be used to predict the response to selection and therefore it can provide an indicator of how successful selection for a particular trait will be. However, heritability must be estimated in the population to form the breeding stock as the value can vary between strains, populations and individuals (Tave, 1993). There are several methods of estimating heritability, which rely on performance data collected between relatives such as parents and offspring or between siblings. Ideally, estimates made from different methods will produce roughly equivalent results; however, variance from other sources can reduce the accuracy of each approach. The precision of heritability declines with the increasing distance of the relationship examined, so that with an estimate using half sib analysis the standard error is multiplied by a factor of four over a single parent regression (Falconer and Mackay, 1996). Bias can also enter into heritability estimation as a result of environmental variance and dominance in the case of full-sibs (Kinghorn, 1983). Another type of bias is the maternal effect, which can be quite marked when estimating the dam component of variance as opposed to the sire component (Gjerde and Shaeffer, 1989), although this can be hard to distinguish from other types of variance (Gjerde and Gjedrem, 1984). In many species, heritability has been found to increase with age as maternal effects become reduced (Kinghorn, 1983; McKay *et al.*, 1986).

Once an estimate of heritability has been obtained, the response to selection can be predicted and can even be used to determine which individuals to select to achieve a particular goal. Low heritability is often found for traits relating to

fitness or reproduction, as these are under strong selection pressures, resulting in a reduction of additive genetic variation (Kirpichnikov, 1981). If the estimate of heritability is low ( $<0.15$ ), the phenotype will not respond adequately to selection because of a lack of heritable variation (Tave, 1993). Genetic improvements can still be made through family selection, as this method is based on average family performance and not individual performance (Kirpichnikov, 1981). Heritability for traits such as body weight and length can be high, whereas, traits such as meat colour score are often much lower. Table 1.1 contains the heritability estimates for a range of characters in the Atlantic salmon and demonstrates how the estimates can vary at different ages as a result of changes in environmental conditions.

Realized heritability is based on the response of the population after selection has taken place. The reliability of this value is open to question, as selection will reduce a high heritability after the first generation and random drift or inbreeding can influence the response to selection (Hill, 1971). The method requires minimum environmental variance and trait information from at least two generations to improve the accuracy (Kirpichnikov, 1981). All estimates of heritability need to be produced with care, since error and experimental design can greatly affect the outcome. In order to avoid these problems, sample sizes should be as large as possible (Gill and Jensen, 1968; Cheverud, 1988), environmental conditions should be kept homogeneous within the hatchery (Kirpichnikov, 1981) or an accurate estimation of environmental variation should be used as a correcting factor (Wohlfarth and Moav, 1985).

Before a trait can be singled out for improvement by selection, the possibility of indirect selection of other traits as a result of pleiotropy (including linkage) must be investigated. Pleiotropy is a property of genes, which generates variation in two or more characters simultaneously during gene segregation (Falconer and Mackay, 1996). The correlation between traits is the value of all the segregating genes affecting the characters, which can be both positive and negative. Genes increasing both characters generate a positive correlation, while genes increasing one character and decreasing another will effect a negative correlation. There are several possible correlations that compare different factors of variance. These, however, can be summarised as the genetic correlation (all additive components) and the environmental correlation (all non-additive components). In combination, these two correlations produce the phenotypic correlation that is observable from the measurement of characters in the population. Genetic correlations are estimated in a manner similar to heritability by using an analysis of covariance on the components of variance of the two related characters (Falconer and Mackay, 1996). Estimates of genetic correlation are usually subject to high sampling error and, therefore, require larger sample sizes than those needed for heritability estimates. Environmental correlation values are estimated by separating the estimate of genetic correlation from the total phenotypic correlation. Once correlations between traits have been identified, they can expand the objectives of a breeding programme by increasing the number of traits that are being selected. For example, strong correlations between spawning body weight and egg quality (egg size, number and volume) in rainbow trout means that selection for one of these traits will result in improvement of both, even if heritability estimates are low for one of the traits (Su *et al.*, 1997). Correlations between traits may also be advantageous if one of the traits is not readily accessible to selection techniques (Fevoldan *et al.*, 1994).

**Table 1.1: Estimates of heritability in Atlantic salmon demonstrating how heritability can change over the lifetime of the animal. Heritability of morphometric traits (length and weight) are generally higher than quality traits such as meat colour score which can only be improved using family selection. Heritability of disease resistance is also high.**

Trait	$h^2 \pm \text{S.E.}$	Reference
12-week weight	$0.89 \pm 0.32$	Bailey and Loudenslager (1986)
6-month weight	$0.40 \pm 0.26$	
15-month weight	$0.67 \pm 0.32$	
12-week length	$0.79 \pm 0.31$	
6-month length	$0.57 \pm 0.28$	
15-month length	$0.73 \pm 0.32$	
3-year weight	$0.38 \pm 0.10$	Gjerde & Gjedrem (1984)
3-year length	$0.33 \pm 0.10$	
3-year gutted weight	$0.44 \pm 0.11$	
Dressing percentage	$0.03 \pm 0.02$	
Meat colour score	$0.01 \pm 0.03$	
Susceptibility to <i>Aeromonas spp.</i>	$0.48 \pm 0.17$	Gjedrem <i>et al.</i> (1991)

Environmental variation can markedly affect phenotypes and interaction between the genotype and environment can result in environment-specific performance of genotypes (Tave, 1993). The source of the variance is environmental, despite it being a property of the genotype, and is therefore included within the estimate of  $V_E$ . In selection programmes, genotype-environment interactions are important if the populations are raised in different environments. Each genotype has a mean value in each environment, but if there is no genotype-environment interaction then the best genotype will be better in all environments. Populations under selection in the best environment may produce genotypes that are poor performers in other environments after they have been sold or transferred. The difference in performance between the two environments can be predicted from the heritability of the performance in each environment and the genetic correlation between the two performances. This will indicate if selection should be carried out in the original or destination environment (Tave, 1993).

## **1.2.2 Methods of Selection**

A breeding programme is a long-term process aimed at producing incremental improvements to economically important traits. All programmes require substantial investment, labour input and record keeping to ensure their success. Before embarking on a programme, careful consideration should be given to the traits that should be improved, the selection methods, what the goals of the programme will be and how they can be measured (Shultz, 1986). These factors then need to be integrated into the farming system and often adapted to fit the biology of the species in question. Breeding programmes often focus on improving morphometric traits to improve yields; however, many other types of traits are suitable for selection that can result in increased production or value of

the product (Gjedrem, 1983). Table 1.2 contains a summary of several breeding programmes in different species. The type of selection method to use is dependent on the traits to be selected, the biology of the species and the resources available. Reliable estimates of heritability, phenotypic and genotypic correlations are also needed to decide on the optimal design of the programme (Gall & Huang, 1988).

**Table 1.2: Summary of breeding programmes undertaken in a range of fish species. Within family selection has frequently been used to increase the response to selection and to improve traits with low heritability. The selection method also has to reflect the biology of the species and the facilities available. To date, most selective breeding of fish has been carried out with salmonid species.**

Trait	Selection Method	Species	Author(s)
Growth Rate	Within-family	Tilapia	Abella <i>et al.</i> (1990)
	Mass	Tilapia	Huang & Liao (1990)
Weight	Within-family	Mosquitofish	Busack (1983)
Body Shape	Within-family	Salmon	Rye & Chavasuss (1994)
Stress Response	Within-family	Rainbow trout	Pottinger & Carrick (1999)
	Within-family	Rainbow trout	Fevolden <i>et al.</i> (1991)
Disease Resistance	Within-family	Atlantic salmon	Gjoen and Bentsen (1997)
Age at Maturation	Within-family	Tilapia	Horstgen-Schwark & Langholz (1998)
Flesh Quality	Within-family	Salmonids	Gjedrem (1997)

### 1.2.2.1 Mass (Individual) Selection

Mass selection is a relatively simple and inexpensive approach to selecting for the best individual fish, based exclusively on their phenotype. The phenotype is measured within the population as a whole and a cut-off value determined at a specific point so that all fish below this value are discarded, whilst those above are retained. Sexual dimorphism within the population may require two cut-off values so that fish of one sex are not overly-favoured and result in an imbalance of the male:female ratio in the population. Generally, mass selection is most effective at improving one trait at a time, as improving two or more traits risks severely reduced population numbers if too few fish meet the combined cut-off values (Tave, 1995). A more effective method in these cases is to use a selection index, combining measures of more than one trait to produce a 'score' for each individual. The index can be weighted to favour particular traits over others and allows simultaneous selection for several traits. For example, a single selection index has been used to improve traits at different life stages in salmon, including fry length and market size (Friars *et al.*, 1995; O'Flynn *et al.*, 1999). The effectiveness of mass selection combined with the simplicity of the technique means that it is the most frequently adopted method of selection in commercial fish farming (Bentsen, 1990). It is also the basis of informal selection, whereby farmers select the (phenotypically) superior fish for breeding. Mass selection can

even be applied in artisanal farming systems to increase production (Brzeski and Doyle, 1995).

Any form of selection will increase the risk of inbreeding through the loss of families, however, this can be a particular problem when using high intensity index selection. Some experiments have been unable to generate improvements in a population using mass selection, because of the lack of genetic variation within the starting population (Teichert-Coddington & Smitherman, 1988) or a large reduction in heritability in subsequent generations (Hulata *et al.*, 1986; Dunham & Brummett, 1999). Environmental variance can also have a significant impact on an individual's phenotype and, therefore, needs to be kept at a minimum to ensure that genetically superior individuals can be detected, based solely on the measurement of their phenotype (Kirpichnikov, 1981). Selection of phenotypes carries a risk because the genotypes of the discarded fish are unknown. The loss of genetic variation from the population is an inevitable consequence of discarding a significant proportion of the population based solely on phenotypic measurements. However, the loss of families under mass selection is not as severe as situations where family information is used to exclude whole family groups, such as classical (between-) family selection.

#### **1.2.2.2 Family Selection**

Using a measure of phenotypic value across a whole family is also a widely used technique, as it allows selection of traits with a lower heritable variation than would be possible with mass selection. Family selection allows traits such as disease resistance and carcass characteristics (which are only measurable after death) based on the family mean rather than the individual's own mean (Bentsen, 1990). Selecting from families also counteracts much of the variation due to environment and thus makes it easier to identify genetic differences in the population (Herbinger & Newkirk, 1990). Family selection can be carried out either as between-family selection, within-family selection or as a combination of these types.

Between-family selection ranks all the families in the population and the best families are retained to propagate the next generation. Either the whole family or a random sample from within the family can be kept. However, in the latter case, the number taken from each family must be equal to ensure a balanced contribution in future breeding. Within-family selection is simply individual selection applied within family groups, resulting in each family contributing the best individuals from that group. Combining both within- and between-family selection is a more powerful method, in that the best fish from the best families can be selected and, therefore, the greater selection intensity should result in a higher response in each generation (Gjerde & Rye, 1998). Best Linear Unbiased Prediction (BLUP) techniques combine the information from all family relationships, within and between generations, to accurately estimate the breeding values. However, by increasing the amount of family information used in selection, the greater the loss of families and consequently the greater the risk of inbreeding in subsequent generations.

Family selection is dependent on the ability to separate fish into family groups after fertilisation and is usually performed manually, by stripping eggs and mixing them with milt from specific fish. Families can be raised individually, which

requires extensive facilities. Alternatively, if marking is possible, the families can be communally reared (Wohlfarth and Moav, 1985). Often when using family selection, fish with an apparently poor phenotype are often selected, whereas better fish from other families are discarded (Tave, 1995). This is a consequence of basing selection on the family mean that can contain both good and poor individuals. Only combined selection avoids this apparent contradiction in selecting individuals and is estimated to be twice as effective as within-family selection (Gall & Huang, 1988). Even so, family selection has produced impressive improvements in characters such as growth – up to 12% per generation in tilapia (Bolivar, 1999) and also in tilapia, a 27% increase in body weight over two generations (Horstgen-Schwark, 1993). Family selection is often preferred over mass selection for higher selection intensities (Urawain & Doyle, 1986) and where carcass and disease resistance traits may need to be included into the breeding programme (Refstie, 1990).

### **1.2.3 Application of Genetic Markers to Selective Breeding in Fish**

#### **1.2.3.1 Types of Genetic Marker**

The use of genetic markers in fisheries and aquaculture has increased over the last 20 years as they have been applied to many aspects of production and management. Several types of marker exist, the most commonly used include protein products (allozymes), mitochondrial DNA (mtDNA), variable number tandem repeats (VNTR) and randomly amplified polymorphic DNA (RAPD). Each method has distinct properties and generates different types of data for a variety of applications. The discovery of new markers has not replaced previous techniques and by using a combination of markers many of the problems in aquaculture can be resolved.

Protein methods can be summarised as the analysis of electrophoretically distinguishable protein variants. Allozymes (product of different alleles at the same locus) are one of the earliest and therefore most widely used type of markers in population-level fisheries research. The techniques of electrophoretic separation and staining are easily adjustable to different species and any source of soluble protein can be used (Magoulas, 1998). Therefore, the use of allozymes has been widespread for population studies needing inexpensive, rapid stock identification on a large scale (for example, Rossi *et al.*, 1998). Allozymes can be particularly useful for analysing differences between species (Alarcon & Alvarez, 1999; Guiffra *et al.*, 1996). The method is, however, limited due to the sole focus on genes expressing proteins detectable with a stain, the restricted amount of polymorphism found in some species and the need for large tissue samples for analysis (Park & Moran, 1995).

Mitochondrial DNA (mtDNA) is characterised by high levels of sequence diversity at the species or infra-specific level. It is transmitted only through the mother, does not recombine and is present as multiple (and usually identical) copies within a cell. As a result of transmission through the maternal line, the effective population size of mtDNA is one-fourth that of nuclear DNA (Nei & Tajima, 1981). This allows population events such as bottlenecks and hybridizations to be detected more easily, but it cannot be used to detect male mediated genetic mixing of stocks (Magoulas, 1998). Different parts of the

mitochondrial genome have been looked at for particular applications: the cytochrome b, D-loop and ND genes in population studies (Carr & Marshall, 1991; Park *et al.*, 1993; Nesboe *et al.*, 1999) and the ribosomal genes in species or even family-level studies (Geller *et al.*, 1993).

The main advantage in using nuclear DNA (nDNA) markers is that they are widely dispersed throughout the genome in non-coding areas that are not normally under as great a selective pressure as that found in coding sequences. This allows a rapid evolution of markers that can be used for identification to the species and individual level. The first nDNA markers developed were randomly amplified polymorphic DNA (RAPD) in which short primers of arbitrary sequence were used to isolate anonymous regions of DNA (Williams *et al.*, 1991). The technique is quick and also cost effective, as there is no need to look for target sequences or to design specific primers. The interpretation of the markers, however, is often difficult and unreliable. The main application for RAPD's has been the comparisons between species and sub-species (Bardakci & Skibinski, 1994), for example stock identification (Gomes *et al.*, 1998; Elo *et al.*, 1997; Koh *et al.*, 1999) and biomarker experiments (Nadig *et al.*, 1998; Theodorakis *et al.*, 1998).

Repetitive DNA forms 10-30% of the genome and where this occurs as clusters, or blocks of tandemly repeated sequences, it is termed satellite DNA. The function of these blocks of repeat sequences is not fully understood, but the high frequency found in sub-telomeric areas and near centromeres suggests it may promote recombination or play a role in chromosome function (Twyman, 1998). Satellite DNA is highly polymorphic and markers can be divided into two types: i) minisatellite or variable-number tandem repeats (VNTR) – repeats of small length (10-64 base pairs); and ii) microsatellite – short tandem repeats (2-4 bp). Minisatellites are the basis of 'DNA fingerprinting' and, due to high degrees of mutation and heterozygosity in the sequences, they can be used to distinguish between individuals. This makes the markers ideal for pedigree analysis (Clifford *et al.*, 1998; Thomaz *et al.*, 1997) and stock identification (Beacham, 1996; Beacham *et al.*, 1996, 1999; Galvin *et al.*, 1996). However, minisatellites are not a good choice for population-level investigations as multiple loci are assayed simultaneously, allelic frequencies cannot be determined and the mutation rate is high (Magoulas *et al.*, 1998).

Microsatellite DNA markers are smaller and simpler than minisatellites, occurring as di-, tri- and tetra-nucleotide repeats. In addition, they are more numerous and widespread in the genome, with frequencies of  $10^4$  to  $10^5$  copies in fish (Park and Moran, 1995). Each microsatellite is usually flanked by unique sequences that can be used as primer binding sites for amplification of the microsatellite using the polymerase chain reaction (PCR) technique. An advantage of using PCR to assay the markers is that only small amounts of tissue are needed and these can be of poor quality, such as scales, fins and preserved or archival specimens (Palsbøll, 1999). The initial isolation of the microsatellites is the most time-consuming aspect of this method, but applying identical primers in related species has had some success (Smith *et al.*, 1998; Queller *et al.*, 1993). In addition to the properties of high polymorphism, microsatellites are also able to characterise alleles through differences in the sizes of PCR products. The number of alleles in each microsatellite is very variable and offers the opportunity to choose a marker with the suitable number of alleles at each locus for the particular application in

mind (Wright and Bentzen, 1995). Inheritance of microsatellites follows a codominant Mendelian fashion and is, therefore, suited to pedigree studies where information on conformation to Hardy-Weinberg expectations needs to be obtained. The versatility of microsatellite markers has made them suitable for application to many aspects of aquacultural genetics. These include stock identification (Noriss *et al.*, 1999; Carlsson *et al.*, 1999), parental assignment (Knight *et al.*, 1998; O'Reilly, Herbinger & Wright, 1998; Estoup *et al.*, 1998), assessing genetic manipulations and inbreeding (Pouyaud *et al.*, 1999; Chapman *et al.*, 1999) and genome mapping (Høyheim *et al.*, 1998; Kocher *et al.*, 1998). The use of microsatellites in selective breeding programmes is described below.

### 1.2.3.2 Microsatellite Markers in Selective Breeding

Genetic markers have a range of uses in selective breeding and broodstock management. The ability to monitor and evaluate populations by examination of the genotype is a great advantage in any programme aimed at genetic improvement. The use of PCR techniques in microsatellite analysis means that data can be obtained from all life stages including eggs and larvae, which would have previously been inaccessible to genetic testing. Large numbers of samples can also be rapidly analysed to produce data within the time-scales of the farming system.

Parental assignment in cultured stocks is one of the primary applications of microsatellites to a breeding programme. Microsatellites can identify the parents of high-performing progeny in communal rearing environments and, therefore, assist in the selection process (Garcia de Leon *et al.*, 1998). Family selection becomes possible by collecting genotype information on all the candidates and using family relationships as part of the selection criteria. Walkback-selection (Doyle and Herbinger, 1995) is an example of such a method, whereby fish are selected on size and then on family relationships following microsatellite analysis.

Parental assignment can also be used to reveal information on the contribution of individuals in mass spawning species (Waldbieser and Wolters, 1999; Magoulas, 1998 and references therein) which can subsequently aid broodstock management. The ability to identify families and individuals allows closer monitoring of stocks within a programme and also facilitates communal rearing methods to reduce environmental variance. Inbreeding can severely affect selection efforts and needs to be monitored, in all selection programmes. Rates of inbreeding can be estimated from pedigree information and changes in microsatellite allele frequencies over time (Tessier *et al.*, 1997; Galbusera, 2000). Inbreeding can then be minimised by the prior identification of the families and individuals that should be used for future breeding (Ryman and Laikre, 1991).

Discriminating between stocks and groups of fish can be important to a breeding programme where fish are released for restocking natural populations. Microsatellite tagging can indicate whether performance of cultured fish is different to that of wild fish in the same habitat (Perez-Enriquez *et al.*, 1999; Coughlan *et al.*, 1998). Genetic markers are also able to discriminate between farm escapees and wild fish (Beacham & Wood, 1999) which may be particularly important for farms undertaking genetic manipulations.

Microsatellites have a central role in genome mapping, as they serve as marker points distributed throughout the genome (Magoulas *et al.*, 1998). The production of a physical map of the genome allows the classification of genes affecting commercially important traits (Simm, 1998). In some cases the genes may control an observable phenotype, although regions that contribute a proportion of variation in a quantitative trait can also be determined (Simm, 1998). These regions are known as quantitative trait loci (QTL) and are particularly useful for genetic improvement programmes as complementary to breeding value estimates of genetic merit (Davis & Hertzler, 2000). Many of the traits of interest in a selective breeding programme are quantitative in nature and therefore controlled by many genes of small additive effects. Identifying markers linked to quantitative trait loci can greatly increase the rate of genetic improvement by permitting marker-assisted selection (MAS) (Poompuang & Hellerman, 1997).

Knowledge of quantitative trait loci has several applications for selective breeding in fish, as they allow selection of traits that may be difficult or expensive to measure and that can only be measured on one sex. In addition, traits that can only be measured after death or after selection has taken place, such as disease resistance (Davis and Hertzler, 2000) and lifetime reproductive performance (Simm, 1998), can also be selected. Detection of quantitative trait loci in fish species is still quite limited, but studies have been successful in locating QTL's in rainbow trout for spawning time (Sakamoto *et al.*, 1999) and temperature tolerance (Jackson *et al.*, 1998; Danzmann *et al.*, 1999). An increasing number of fish species are being mapped, including commercial species, such as Atlantic salmon (Høyheim, 1998), European seabass (<http://www.bassmap.org>) and gilthead seabream (<http://www.bridgemap.tuc.gr>), which raises the prospect of many more QTL sites being available in the future.

## **1.3 Selection of Gilthead Seabream**

### **1.3.1 Potential for Selection in Gilthead Seabream**

Gilthead seabream is a very important species in Mediterranean aquaculture, but has so far received little attention for selective genetic improvement. Between-strain hybridisation in Mediterranean stocks has found little heterosis and the appearance of a homozygous ebony form increased when using this technique (Knibb *et al.*, 1998). Hybridizations with closely related species such as *Diplodus puntazzo* and *D. vulgaris*, have been achieved (Jug Dujakovic & Glamuzina, 1990) and are continuing to generate animals better suited to aquaculture (Gorshkov *et al.*, 1998).

Very few heritability estimates are available for traits in this species, although Batargias (1998) calculated a heritability of  $0.55 \pm 0.18$  for weight at 22 months using an animal model. In addition, Knibb *et al.* (1997) found a realized heritability of  $0.34 \pm 0.02$  for weight at 710 days of age using regression coefficients from replicate selection lines. These calculations of heritability are very encouraging and are of the same magnitude with other intensively cultured species such as Atlantic salmon,  $0.38 \pm 0.15$  for 2-yr weight (Standal and Gjerde, 1987) and rainbow trout,  $0.38 \pm 0.22$  for 2.5-yr weight (Mackay *et al.*, 1987). This indicates there is a large potential for improvement of weight traits through selective breeding in this species. Selection in another Sparid species confirms the relatively large heritabilities reported in the studies mentioned above. Murata *et al.* (1996) calculated a mean realized heritability of  $0.33 \pm 0.28$  for 4<sup>th</sup>-year body weight in the red seabream. This study found the response to mass selection was positive each year but heritability of the trait increased after the third generation. Taniguchi *et al.* (1981) also found high heritability values for body weight and length in communally reared groups of red seabream.

Little published data exists for current breeding programmes of gilthead seabream, although larger hatchery companies are actively pursuing interests in this area (Selonda annual report 1999). Family selection has been used by Alfonso *et al.* (1998) to generate increased stress tolerance in gilthead seabream through lowering blood cortisol levels. However, there is no indication of how the family groups were created, given that manual stripping is ineffective (Gorshkov *et al.*, 1997). Mass selection is more suited to the constraints of this species and has been successfully employed to improve weight in gilthead seabream by an average of 7.3% in one generation (Knibb *et al.*, 1997). This constitutes evidence of the large and rapid results that can be gained using this method. A long-term selection procedure needs to be implemented, however, in order to continue this level of improvement in subsequent generations.

In order to generate family information from unknown parental crosses in a mass spawning, genetic markers are needed to assign parentage to offspring. Although a mass selection system can be conducted without genetic information, the long-term management of closed populations depends on the control of inbreeding.

Genetic markers are the most effective means by which inbreeding is monitored, especially when the contribution of individual parents is unknown and high selection intensities are used to select parents for the next generation. At present there are few microsatellite markers available for species of the Sparidae group, with only red seabream (Tagaki *et al.*, 1997) and gilthead seabream (Batargias *et al.*, 1998) being represented. Four of the latter markers were used to successfully assign parentage of over 900 offspring from a parental cross of 32 fish (Batargias *et al.*, 1998). Very high levels of heterozygosity and a large number of alleles in five of the loci emphasise their suitability for parental assignment. A D-loop mitochondrial DNA marker in gilthead seabream is also available (Magoulas *et al.*, 1995) but this type of marker is more suited to population level applications than parental assignment (Park and Moran, 1995).

### **1.3.2 Constraints of Biology on Selection Methods in Gilthead Seabream**

#### **1.3.2.1 Mass Spawning**

The group spawning behaviour in gilthead seabream presents a problem to a potential breeding programme, because independent family groups cannot easily be generated to evaluate genetic parameters. The use of family information offers greater selection accuracy, but requires the identification of pedigree structure to establish family means (Aleandri and Knibb, 1999). Egg stripping and paired-matings have proven to be ineffective in producing large numbers of eggs suitable for commercial production (Gorshkov *et al.*, 1997). In a typical group spawn, the number of contributing parents, pairings and family sizes are unknown and, therefore, family effects in phenotypic variation cannot be estimated. In circumstances where family data is not available, mass selection is the only option, as selection criteria can be based on the population as a whole (Kirpichnikov, 1981).

In mass selection genetic information, such as the breeding value of an individual can be estimated by comparisons to the population mean, but the accuracy of this method is poorer than when family information is used appropriately. In order to accurately calculate parameters such as breeding value and heritability, parental assignment of offspring is required (Magoulas *et al.*, 1998). Parentage of offspring from a mass spawning can be determined using genetic markers such as microsatellites (Batargias *et al.*, 1999). Parentage data also provides valuable information for the maintenance of genetic variation in the population by quantifying an individual's contribution to the next generation. Despite this, however, microsatellite DNA analysis is an expensive operation and requires DNA samples from all the potential parents in the spawning. The number of offspring needed to retrospectively construct family groups from a mass spawning may also be high, depending on the actual number of families generated in the crossings. The inclusion of parental information, nevertheless, may be essential in collecting genetic data and minimising the problem of inbreeding in the selection process.

### 1.3.2.2 Sex Reversal

The sex reversal of gilthead seabream from males to females could lead to problems with the selection and maintenance of broodstock in a breeding programme, as the structure of the broodstock population needs to be considered before any new fish can be introduced. Market size fish are exclusively male and thus selecting and introducing large numbers of new fish from this age group could affect the male:female ratio within the broodstock. However, there is an advantage in selecting fish at this age, as there will be no sexual dimorphism that favours the selection of one sex over the other. The timing of the introduction of new fish is important, as the sexual cycle of seabream includes a sensitive period (May – September) in which sex is determined (Zohar *et al.*, 1995). The addition of young males in the sensitive period has been found to increase the rate of change from males to females (Zohar *et al.*, 1995). The addition of selected broodstock will need to balance the ratio of males:females, so that future spawning will not result in a shortage of eggs or sperm. In this respect, the age structure of the broodstock is important to maintain, as older fish are likely to provide the majority of gametes, in particular eggs, which is a priority in a hatchery. The change of older males to females is detrimental to the broodstock because the quality of sperm from older males is usually better than that from young male fish, whereas larger females are likely to produce greater number of eggs than smaller females. These factors indicate that the replacement of broodstock needs to be monitored and conducted at a rate that minimises the disruption to the existing broodfish. The rate of replacement – and hence genetic improvement – could be limited by the need to maintain a broad age structure in the stock, so as to balance the sex ratio and maximise the production of gametes for commercial considerations.

### 1.3.2.3 Juvenile Variation

Growth differences between juveniles are often very marked in this species and can appear in early developmental stages (Goldan *et al.*, 1997). The origins of this early size variation are unknown and could result from random variables, such as feeding success, or from parental effects. At the juvenile stage, fish require frequent size sorting or grading in the hatchery, in order to reduce cannibalism and aggression (Moretti *et al.*, 1999). In other species, such as Arctic charr, social hierarchies are known to operate that maintain size disparities between dominant and subordinate fish (Koebele, 1985). Grading can disrupt such hierarchies, but needs to be carried out on a frequent basis to be effective (Jobling and Reines, 1987). Grading introduces an additional environmental variable into the juvenile stage, as larval batches that are communally reared up to the grading point become divided and reared separately. Fish density and other variables, such as feeding rate and type, may be different between groups which may then be mixed at a later stage. The lack of common environment means that comparisons between populations cannot be made. Moreover, determining the highest performing fish is difficult, due to the confounding factors of age, density and frequency of grading during this period. The effect of grading on growth rate is thought to be generally beneficial, especially in the smaller group where larger co-specifics have been removed. Grading, however, could also exert a selective pressure for particular growth patterns or body conformations that increase the likelihood of passing and being selected for on-growing.

#### **1.3.2.4 Selection in a Commercial Environment**

The development of a breeding programme within a commercial production system requires a balancing of two conflicting approaches. Selection methods involve measuring and quantifying variables in close-to-experimental conditions, whilst commercial operations depend on efficient management of time and resources. Therefore, a breeding programme that must be integrated into a farm situation needs to be straightforward and simple to operate, without disrupting other production activities. The risks associated with initiating a breeding programme from a commercial perspective centre on the additional resources required to measure and separate fish for selection, the maintenance of minimum environmental variance between groups of fish and the additional record keeping required. Commercial risk is also associated with changing the genetic structure of the farm population (decreasing variation), with the frequent replacement of broodstock and the increased handling of the fish, all of which may effect production in unpredictable ways. The benefits of the programme, however, will be an improvement of the stock, based on the trait being selected, a move towards the standardisation of management practices throughout the farming system and an increase in the biological information available on which management decisions are to be based. Record keeping is a central part of the programme and information needs to be collected throughout the farm – and not just the selected fish – in order to monitor correlated traits and to compare the performance between groups. Such information can be used to identify problems and deficiencies in the farming system and management, which can then be addressed. Efficiency within each stage of the farming system can also be evaluated and used to improve techniques and reduce costs.

#### **1.3.3 Development of a Selection Programme for Alkioni Fish Farms, Cyprus**

The primary consideration in the development of a breeding programme is to ensure a clear understanding of the biology of the species within the farming system, so that the constraints and possibilities of selecting for specific traits can be established. Allied to this is the evaluation of the farming system to identify potential conflicts between production and the requirements of a selection programme. The biology of the species and the limitations of the farming system may determine the methods of selection and, consequently, the objectives of the programme. Therefore, prior to the commencement of a selective breeding programme for gilthead seabream at Alkioni Fish Farms, studies were undertaken to examine the farming system, aiming to identify key factors that are likely to have an impact on the operation of a selection programme. This thesis contains the results of these studies and proceeds to discuss and propose procedures that will form the basis of a selective breeding programme at Alkioni Fish Farms.

Alkioni Fish Farms is a combined hatchery and on-growing unit located on the southern coast of Cyprus. Annual production exceeds 2 million fry from the hatchery, the majority of which are stocked into the associated on-growing unit, which produces over 300 tonnes of market-sized fish per year. The market for the

final product is mainly local, although juvenile fish are frequently sold to on-growers in Greece. A breeding programme will allow Alkioni to improve the fish's growth rate during the juvenile and seacage phases. This will reduce costs whilst also producing larger market fish of a higher quality that will be demanded by foreign markets, particularly in northern Europe, where the company wishes to increase its exports.

Given the constraints of the biology of the species outlined above, genetic markers were considered an essential tool to gather information on the farm population. Genetic markers such as microsatellites offer a convenient means of identifying family relationships and can provide information on the contribution of parents, on the effective population size and on the genetic differentiation between stocks. However, collecting genotype data is expensive and methods to reduce these costs, such as multiplex PCR reactions, are needed to apply these techniques on a large scale. The methodology of using microsatellite markers and multiplex reactions and the development of several new markers is described in Chapter 2.

One of the primary considerations in developing a selection programme is the rate of replacement of the breeding fish and, hence, the rate of genetic progress. The replacement of gilthead seabream broodstock is complicated by the sex reversal behaviour that can result in an unbalanced sex ratio if either too many fish are introduced to the stock or if these are introduced at the wrong time. It is also necessary to maintain an age structure, so that larger female fish are retained within the stock, in order to ensure the production of large numbers of eggs to support the farm's requirements. The broodstock at Alkioni are also maintained under three photoperiod regimes, which rules out the transfer of fish between groups and sets conditions on the timing of replacement for each stock. At present, the replacement policy is quite relaxed and new fish are introduced roughly every two years to replace the largest fish or those with injuries. New fish are collected at random from within the farm production system and, therefore, the farm population is completely closed.

Additional information is required on the rate of sex reversal and natural sex ratio, so that the broodstock can be properly managed for the more frequent replacement of broodfish demanded by a selection programme. Monitoring of the broodstock requires the PIT tagging of each fish, so that individual records can be maintained to provide a history of growth and sex ratio over time. This type of monitoring has been carried out in experimental seabream populations, but only for a short duration and thus, the long-term changes in sex ratio within broodstocks are unknown. Chapter 3 contains the results of observations to determine the optimum replacement rate of broodstock through a programme of detailed monitoring, including topics such as spawning performance, growth and the sex ratio of the broodstock groups. Individual fish were also monitored to follow the pattern of sex reversal within each of the groups.

Chapter 4 deals with one of the main problems in the culturing of gilthead seabream, which is the development of a substantial size variation in larval and juvenile stages. This variation has an uncertain origin in farm populations, but was hypothesised to be the result of mixing egg stocks, variable larval feeding success,

parental effects or behavioural interactions between larvae, such as dominance. Variation increases steadily throughout the larval period and into the juvenile stage, where frequent size sorting is required. Assuming that there is an additive genetic influence on growth during the larval stages, the grading could be used as an effective stage in the selection process. However, in order to estimate the effects of selection in these juvenile stages, an accurate assessment of the level of size variation within farm populations is required. In other species, the development of size variation often occurs at hatching and first feeding and this pattern needs to be confirmed in cultured stocks of gilthead seabream. Size and developmental differences at these very early stages are often maternal effects derived from differences in egg size or nutritional content. Maternal effects could have an impact on the selection of fish at grading if these developmental advantages persist through the larval stage. These issues will be addressed by using observational data on the farm production tanks, and on experimental larval populations, as well as by analysing genetic data obtained from the parental assignment of offspring.

Following on from the larval period, size variation continues into the juvenile stage and is controlled by frequent size grading. Grading is currently managed on an *ad hoc* basis, with the decision to grade being a subjective one and often based on the available facilities. The growth rates within the separated groups are often markedly different and this rules out the comparison of groups and populations, because the exact background of the fish is not known. Standardisation of the grading procedures is needed in order to reduce the environmental variance between groups and to separate genetically faster-growing individuals. To design a standard grading method, an experiment to control the variables between groups was conducted and is detailed in Chapter 5.

Little data is available to estimate the potential improvements selection could make to traits such as growth rate in gilthead seabream. Establishing a breeding programme requires substantial resources and, therefore, the potential gains from selective improvement of traits should be considered before making such an investment. Chapter 6 examines a number of possible traits for selection during the seage stage of the farming cycle and the potential genetic gain of selecting for growth rate in the Alkioni Fish Farm population.

Prior to beginning a selective breeding programme, an assessment of the suitability of the farm stock for forming the genetic base of the selected population is needed. As already noted, there is not a substantial heterosis effect when crossing between seabream populations, indicating that they are not genetically differentiated. Therefore, intra-strain testing is unnecessary and the current farm broodstock can be used on the assumption that performance will be effectively the same as any wild caught stock. This, however, does not take into account any domestication selection that may have been operating on the stock since its capture. The mass spawning behaviour may have led to inbreeding or genetic drift in the broodstock, as a result of the dominance of individual parents in the spawning process. As the replacement of broodstock occurs from within the farm populations, this could have led to the inadvertent selection of related individuals. Genetic parameters of the Alkioni broodstock including effective population size and genetic differentiation, are examined in chapter 7.

The value of a detailed assessment of the farm procedures is that each stage of the selection programme can be designed to overcome the limitations and constraints of both the species and the farming system. The most important aspect of the selection programme is the standardisation of some farm procedures so that environmental variance between populations in the farming system is minimised. Standardisation is needed in order to evaluate populations and determine the amount of progress made towards genetic improvement, whilst it is also beneficial to the management of the farm, by providing guidelines to develop formal procedures on methods such as grading. A detailed selection plan utilising the information generated on the farming system at Alkioni Fish Farms is presented in Chapter 8 as the conclusion of the work contained within this thesis.

## **Chapter 2: Materials and Methods**

## **2.1 Sample and Data Collection**

### **2.1.1 Tissue Sample Collection and Preservation**

Broodstock tissue sampling was carried out during the PIT (Passive Integrated Transponder) tagging of the stock. Fin-clips were taken from each fish immediately following the tagging stage and samples were subsequently designated by the PIT-tag code number. Offspring samples were collected at early or late larval stages to prevent mixing of stocks and the effects of high larval mortality prior to sampling. Moreover, the samples were small enough to allow convenient, individual storage of each of them. Offspring were collected and preserved as whole individuals. Table 2.1 contains a summary of the tissue types collected from different stages of the life cycle, as well as the corresponding DNA extraction method used. All samples were preserved in 95% ethanol at the time of collection. Most samples were stored individually to identify particular individuals and allow collection of other data, such as length measurements, gathered at the time of sampling. Fin clips and fry samples were stored in screw-top microcentrifuge tubes containing approximately 0.5ml ethanol. Larval samples were stored in microtitre plates and were air dried to remove excess ethanol so as to avoid interference with the adhesive plate seals. Bulk samples of 50 to 100 specimens – such as eggs and pre-feeding larval stages – were also collected, but without additional data, in order to serve as a backup for the dried samples.

**Table 2.1: Summary of sample types and extraction methods utilised for different tissues collected from all stages of the life cycle and farming system.**

<i>Fish Stage (approx. size)</i>	<i>Tissue type</i>	<i>Extraction method</i>
<b><i>Eggs (1mm)</i></b>	<b><i>Whole</i></b>	<b><i>Rapid Digestion</i></b>
<b><i>Pre-larvae (5mm)</i></b>	<b><i>Whole</i></b>	<b><i>Rapid Digestion</i></b>
<b><i>Larvae (15mm)</i></b>	<b><i>Muscle</i></b>	<b><i>Chelex</i></b>
<b><i>Fry (30mm)</i></b>	<b><i>Muscle / Fin</i></b>	<b><i>Chelex</i></b>
<b><i>Adult</i></b>	<b><i>Fin</i></b>	<b><i>Phenol – Chloroform</i></b>

## **2.1.2 Measurement Data Collection**

### **2.1.2.1 Broodstock**

On-site measurements were collected for the broodstock at the time of PIT tagging, to measure growth rate and determine an approximate age for each of the broodfish. All broodstock at Alkioni Fish Farms (n=240) were PIT tagged in May 2000. This included older broodstock and recently introduced fish that were restructured to form four broodstock groups (n=60) under three photoperiod regimes; advanced (2 groups), natural and delayed. Fish were anaesthetized with a Benzocaine solution until loss of equilibrium (approximately thirty seconds). The PIT tags used were 10mm glass-coated alphanumeric tags supplied by Trovan (<http://www.trovan.com>) and inserted into the abdominal cavity of the left flank of each fish by means of a scalpel incision and the tag numbers were recorded. A fin clip was collected and each fish was then measured using a measuring block ( $\pm 1\text{mm}$ ) and vernier calipers ( $\pm 0.1\text{mm}$  at the following points: fork length, body length, head height, body height, jaw length and body width). In addition, each fish was weighed ( $\pm 1\text{g}$ ).

The retention of PIT tags was very high, with only three lost tags over the two year period (>99% retention). No mortalities were associated with the tagging operation or subsequent measurement points. Although handling of the broodstock is prohibited immediately prior to, and during the spawning season, as it can lead to a cessation of spawning, it was noted that several fish in the delayed cycle continued to spawn after the capture and measurement process in 2002. Over the course of the study, one broodstock group was lost due to a disease outbreak. Also, a second tank could not be measured in 2002 due to technical problems.

### **2.1.2.2 Larval Stages**

All larval measurement collection involved lethal sampling because of the sensitivity of the fish to capture and measurement disturbance. Samples needed to be completely removed from the water in order to prevent movement and record an accurate measurement of body length. This technique frequently resulted in mortality and therefore, it was decided to cull all samples prior to measurement. Samples were chilled using iced seawater for a period of approximately 15 minutes and in common with the standard husbandry methods used within the farm. Chilling could not be used with first feeding samples however, as the cold water induced 'curling' of the body after death which prevented an accurate measurement and so these larvae were measured live.

### **2.1.2.3 Juvenile Stage**

Weighing of individual fish would require precision scales and can lead to inaccuracies because of the water on the surface of the skin and inside the gills, especially when measuring small fish (<0.5g). Therefore, given the strong correlation of length and weight in juvenile gilthead seabream (0.96), body length was used as a reliable and repeatable method of determining body size in live fish. A simple and rapid method of measuring the juvenile fish was developed which avoided the need for anaesthetic. Juvenile fish were captured and placed in groups of 50-100 fish in large (12-litre) buckets and allowed to calm for 15-20 minutes in

a dark room. Fish were then removed individually by hand nets and placed into a shallow plastic tray (10mm depth) with no excess water. Body length measurements were taken with a vernier caliper and each fish assessed for malformations before placing the fish into a second large bucket to recover.

## **2.1.3 Statistical Analysis**

### **2.1.3.1 Statistical Methods and Software**

Statistical methodologies were carried out as detailed in the material and methods sections within the individual chapters. All statistical data analysis such as linear regression, contingency tables log-linear modelling and t-test of means were carried out using GENSTAT ver5.0 software (Numerical Algorithms Group, Wilkinson House, Jordan Hill Road, Oxford). FSTAT software (Goudet, 1995) was used to calculate allelic richness scores and inbreeding statistics according to the methods of Nei (1987) and corrected for sample size. Heterozygosity and allele frequencies of microsatellite loci were calculated using the allele frequency module of the CERVUS software package (Marshall *et al.*, 1998). All parental assignment using microsatellite genotype data was performed using PAPA software (Duchesne *et al.*, 2002).

### **2.1.3.2 REML Analysis**

Restricted maximum likelihood (REML) was used to analyse the results of several experiments in this study because of its ability to attribute variance components to individual sources. This is particularly useful in situations where there are several confounding variables or the design of the experiment is unbalanced. REML, developed by Patterson and Thompson (1975), is a modification of ML that overcomes the bias in variance component estimates by incorporating the degrees of freedom of fixed effects. This increases the accuracy of the standard error for fixed effects that may otherwise be underestimated by standard maximum likelihood methods (Harville, 1977). REML is based on the general linear model with both fixed and random effects. The fixed effects relate to the treatment being tested and random effects to the sources of random error in the data. REML allows the experimental error to be divided into individual components to identify the sources of variation and develop a model of the data. Analysis of variance is able to partition the random error for each treatment term and a probability of significance is based on the F-distribution. In cases of unbalanced data, however, the F-distribution is inaccurate and instead a Wald statistic, based on the Chi-square distribution, is used to assess the fixed effects (Harville, 1977). A model of the variance components is chosen, based on the deviance value or 'fit' of the model to the data, tested using a log-likelihood ratio test (Sokal and Rohlf, 1995). The inclusion of marginal terms that do not improve the model is prohibited, because of the loss of error degrees of freedom. Therefore, the significance of each term is calculated independently, so that the model can be improved by removing or substituting individual terms. REML techniques are used in many situations where the experimental design or data is unbalanced. For example, combining data from different time periods or locations, or in the case of survey data and sire evaluation in breeding programmes (Robinson, 1987). REML analysis was used with the analysis of growth in first feeding larvae, growth data of larval groups in the farm and experimental conditions and variance component analysis of offspring body size and parentage.

## **2.2 Extraction of DNA from Gilthead**

### **Seabream Samples**

#### **2.2.1 DNA Extraction Methods**

Sampling of offspring at different stages of the farming system required DNA extraction from different types of specimens, for example eggs, pre-metamorphosed larvae and adults. Therefore, several extraction methods were employed to optimise the analysis of the different types of tissue collected. Details of DNA extraction from seabream tissues have previously only been reported using blood (Batargias, 1998), liver (Cavari *et al.*, 1992) and gill tissue (Rodriguez-Aziza *et al.*, 1999).

Amplification of DNA for microsatellite analysis requires only relatively low molecular weight DNA (<3kbp), because the sequences of interest are short (<500bp) and less likely to be disrupted by breaks in the DNA. This allows purification steps to be omitted, so that the quality of the DNA can be traded-off against the rapid processing of samples. Often low quality DNA is preferable for PCR amplification as shorter DNA fragments lead to more successful binding of primers with the target sequence. This project required a large number of samples to be extracted on a short time-scale, so the following methods were adopted mainly for their simplicity and speed rather than the production of high quality DNA. Two of the methods are single-tube reactions that can utilise a thermal cycler to carry out the incubation stage, which allows 96 samples to be extracted simultaneously and in a very short time period.

##### **2.2.1.1 Phenol-Chloroform Extraction**

Organic extraction is a standard method used for the extraction of DNA from most types of tissue and is the most widely used technique (Brown, 1995). After digestion of the tissue by a protease enzyme, the organic solvents phenol and chloroform are used to precipitate the excess proteins from the DNA in solution. The phenol-chloroform step can be repeated as necessary, in order to improve the quality or purity of the DNA by removing any remaining proteins. The DNA is subsequently precipitated by washing in isopropanol or ethanol.

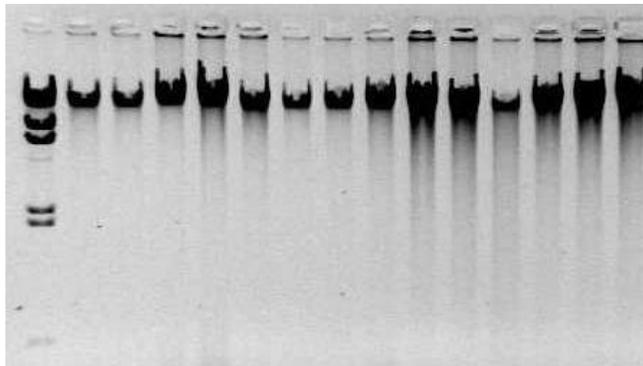
The protocol followed that of Taggart *et al.* (1992) for the rapid extraction of DNA from salmonid tissues. Approximately 2-3mm<sup>2</sup> of seabream fin tissue was used and air dried to remove excess ethanol. These pieces were placed in a microcentrifuge tube containing 375µl TEN buffer (100mM Tris, 10mM EDTA, 250mM NaCl; pH 8.0), 20µl 20% SDS (Sodium Dodecyl Sulfate) and 10µl Proteinase K (10mg/µl). The tube was incubated at 55°C in a rotating oven for 8-10 hours. After this period, 10µl RNaseA (10mg/µl) was added to the solution and incubated at 37°C for one hour. Following this, 400µl of phenol was added to the tube, mixed vigorously for 30 seconds, and placed on a rotator for 15 minutes. Then, 400µl of chloroform:isoamylalcohol (24:1) was added and vigorously mixed for 30 seconds and then placed on a rotator for 15 minutes, before centrifuging the tubes at 10,000rpm for five minutes. After centrifuging, the

solution separated into distinct layers and 300µl of the top aqueous (clear) layer was removed to a new tube, using a wide-bore tip. To this, 300µl of chilled isopropanol or 600µl absolute ethanol was added and then stored at  $-20^{\circ}\text{C}$  for 30 minutes. The solution was then gently mixed by over-end turning until the DNA precipitated and then concentrated into a pellet by centrifuging at 10,000rpm for 10 minutes. The isopropanol was removed and 1ml of 70% ethanol was added. The tube was placed on a rotator for 10 minutes, then centrifuged at 15,000rpm for five minutes. All the ethanol was removed and the DNA pellet allowed to air dry for 5-10 minutes. Finally, the pellet was dissolved in 100µl of TE buffer (10mM Tris, 0.1mM EDTA; pH8.0) and stored at  $4^{\circ}\text{C}$ .

Quantification of DNA using spectrophotometry indicated that DNA yields from this method ranged from 10 to 160µg. DNA was routinely checked on a 0.8% agarose gel to check the quality. The majority of fragment sizes were in excess of 2.3kbp (Figure 2.1). The large fragment sizes were found to inhibit PCR reactions so that very weak, or often no product, was obtained. Denaturing the DNA at  $95^{\circ}\text{C}$  for 15 minutes and storing at  $-20^{\circ}\text{C}$ , greatly improved product yields with all microsatellite loci.

### 2.2.1.2 Chelex Extraction

This technique is more rapid, convenient and safer than the phenol-chloroform method, due to the removal of substances, such as proteins, that inhibit PCR reactions by within-tube Chelex absorption. It is also a simpler protocol in that a thermal cycler can be used to carry out the incubation and denaturing stages and all solutions are added to a single PCR tube or plate well, before adding the tissue. It is effective with a range of tissue types (Estoup *et al.*, 1996) including scales and dried samples (Yue and Orban, 2001). The drawback of using the Chelex method is that the integrity of the samples is not guaranteed for long-term storage, even when stored at  $-20^{\circ}\text{C}$ . The usable lifetime of the sample may only be a matter of months after extraction (John Taggart, pers comm). Consequently, this method was employed to process large numbers of DNA samples from the progeny of the broodstock that did not require long term storage in the way that the broodstock samples needed to be.



**Figure 2.1: 0.8% Agarose gel of Ethidium bromide stained DNA extracted from broodstock fin clips using the phenol-chloroform method (lane 1 = Lambda *Hind*III size standard 2.3kbp - 564bp)**

A 10% Chelex solution was prepared (20g Chelex-100 beads (Bio Rad) in 20ml TE buffer (10mM Tris, 0.1mM EDTA; pH 8.0) and 0.1% SDS) and autoclaved. Before use, the solution was mixed and warmed to approximately 60°C, to aid the uptake of beads into the pipette tip. A wide-bore tip was used to aliquot 100µl of Chelex solution into the wells of a 96-well PCR plate to which 3µl of Proteinase K (10mg/µl) was added. Approximately 1mm<sup>2</sup> of tissue was added to each well and the plate sealed with an adhesive film before being centrifuged at 2000rpm for two minutes. The plate was then incubated in a PCR machine with the following programme: 55°C for three hours and a step of 95°C for 15 minutes, in order to denature the protease enzyme. The plate was removed and centrifuged at 2000rpm for two minutes then stored at -20°C. The solution was used directly in PCR reactions.

### **2.2.1.3 Rapid Digestion**

In cases where samples consisted of small amounts of tissue, such as eggs and pre-feeding larvae, a shortened incubation period was found to be sufficient for the digestion stage. Tissues of this type are also less likely to contain PCR inhibitors such as proteins, as the tissues are not fully developed. Therefore, a rapid technique of releasing the DNA, by simply digesting the tissues with a proteinase enzyme, was chosen. The method followed that of Neff *et al.* (2000), but with the length of the incubation stage reduced from 18 hours to three hours. The tissue was air dried briefly to remove excess ethanol and placed into the well of a PCR plate containing 50µl TE buffer (10mM Tris, 0.1mM EDTA; pH 8.0) and 2.5µl Proteinase K (10mg/µl). The plate was sealed with adhesive film and centrifuged at 2000rpm for two minutes. The incubation period followed the same programme as the Chelex method; i.e. 55°C for three hours followed by 95°C for 15 minutes. After incubation the plate was centrifuged at 2000rpm for two minutes and stored at -20°C. The extraction solution was used directly in PCR reactions.

## **2.2.2 Comparison of Extraction Techniques**

The extraction methods were employed to obtain DNA from different types of tissue, at various stages of the life cycle of gilthead seabream. All the techniques were successful in obtaining DNA suitable for use in PCR reactions. A consideration for this project was the length of time taken to process samples and, in this respect, the Chelex and rapid digestion methods had a significant advantage over the phenol-chloroform technique – the reactions being complete within a matter of hours. In terms of material costs, the rapid extraction method required the least resources, whereas the phenol-chloroform required more reagents and consumables. The Chelex method is similar to the rapid extraction method, but with the added costs of the Chelex beads. Testing of the Chelex method with different tissues indicates that it can be used with all the tissue types collected, as could the phenol-chloroform method. The rapid extraction method, however, could only be applied to certain tissues because there is no purification of the solution to remove the inhibitors found in the tissues from older fish stages. Therefore, it can be concluded, that in cases where the rapid extraction of a large number of samples is required, the Chelex method of DNA extraction from fin

clips is the most suitable. The drawback with the Chelex method, however, is the uncertainty over the long-term storage of samples. In most cases this is unlikely to be a significant problem, because only a small amount of tissue is required for the extraction, while the remaining portion of the tissue samples could be archived. Where long-term monitoring and analysis of populations is needed, an archive of samples could be useful in resolving, for example, genotyping errors or for the comparison of new analysis techniques.

## **2.3 PCR Techniques and Genotype Data Collection**

### **2.3.1 PCR Techniques**

#### **2.3.1.1 Available Microsatellite Loci for Gilthead Seabream**

At the beginning of this project only six polymorphic microsatellite loci had been identified for gilthead seabream (Batargias *et al.*, 1999). Four of these six loci were chosen based on their relative product sizes and high level of polymorphism, with the aim of developing a single multiplex PCR reaction. The remaining two loci were also used on some samples, on an individual basis, to supplement the genotypes produced by the multiplex. However, these two loci had a smaller number of alleles or a significant overlap in allele sizes with one or more of the first choice loci which made their inclusion in the multiplex problematic.

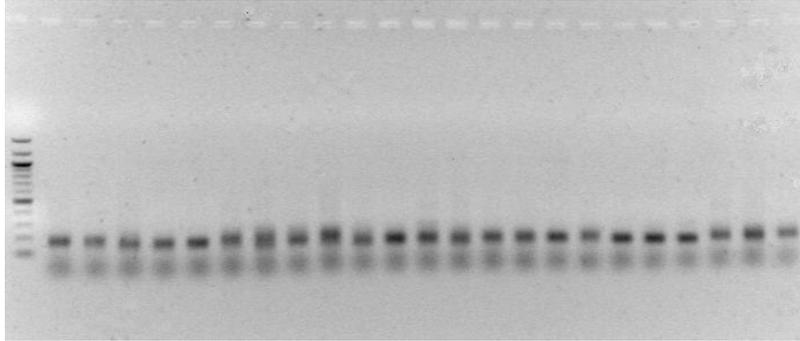
With the large numbers of parents contributing to a mass spawning in the farm production system, the resolution of the microsatellites needed to be very high in order to assign parentage at a high level of confidence. Therefore, six additional loci were developed, four of which were used for the genotyping of offspring and subsequent parental assignment. Section 2.4 will detail the development and operation of the new microsatellite loci, whereas this section focuses on the use of the existing microsatellites published by Batargias *et al.* (1999). All the microsatellite data was collected using semi-automated fluorescent genotyping on an ABI 377 sequencer, hereafter referred to as Genescan™ gels. Data collection and analysis was performed using Genescan™ and Genotyper™ software.

#### **2.3.1.2 Single Locus PCRs**

Single locus PCR conditions were based on the published conditions (Batargias *et al.*, 1999). For all PCRs the total reaction volume was 10µl, consisting of: 40ng genomic DNA template, 0.75µM forward and reverse primer, 130µM each of dGTP, dTTP, dATP and dCTP, 1.5mM MgCl<sub>2</sub>, 1x reaction buffer (75mM Tris-HCl, 20mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% (v/v) Tween) and 0.5 units Taq polymerase (AB Gene). The reactions were performed in a Biometra Gradient PCR machine with the following programme: initial denature step of 95°C for 3 minutes, followed by 30 cycles of 50 s at 95°C, 50 s at the corresponding primer annealing temperature (see below) and 1 min at 72°C and a final extension step of 30 minutes at 60°C. This final step was included to promote 3' adenylation (adenosine nucleotide additions to all the products).

Annealing temperatures were initially tested at 52°C, as suggested by Batargias *et al.* (1999). Large amounts of unspecific product were, however, noted when analysing the results of Genescan™ gels. Each primer pair were tested at a range of annealing temperatures from 52-62°C to find a temperature where non-specific stutter bands were reduced. It was found that the optimum annealing temperature to reduce the occurrence of stutter bands for all the loci fell between 57-60°C. Additional testing of MgCl<sub>2</sub> concentrations and increased Taq polymerase was not found to improve the appearance of the PCR products on Genescan™ gels, nor were any adjustments to the number of cycles, increases in the extension time of

each cycle and the use of touchdown PCR profiles. PCR products were visualised on agarose gels prior to loading on the ABI 377 in order to check the success of the reactions. 1.2% agarose gels in a 1X TAE buffer were run at 97volts, 104mAmps for 45 minutes. An example of a single locus PCR gel, stained with Ethidium Bromide, is shown in Figure 2.2.



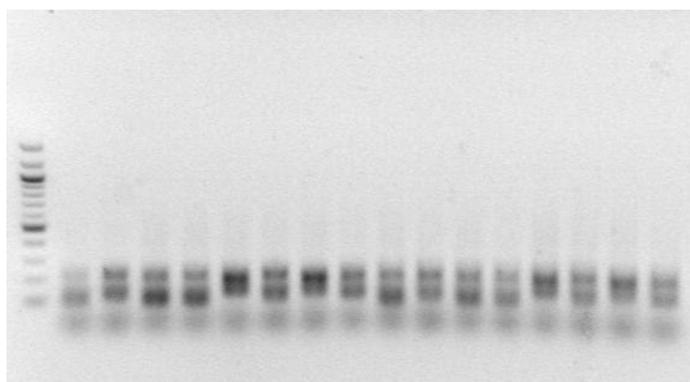
**Figure 2.2: 1.2% Agarose gel of a single locus PCR (SaGT32) indicating product sizes around 200bp. Lane 1= 100bp DNA ladder (1500-100bp)**

### 2.3.1.3 Multiplex PCR's

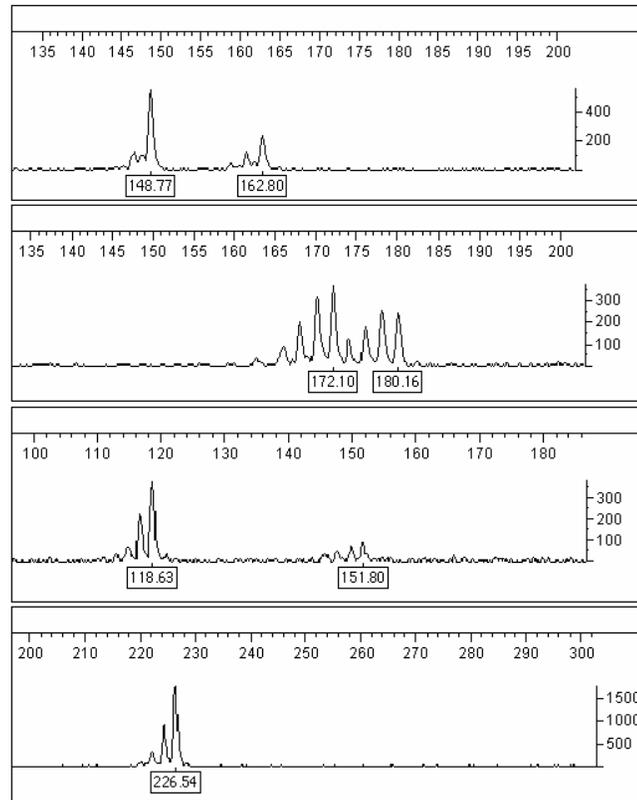
With the use of fluorescent markers and automated sequencing machines, the addition of two or more sets of primers to analyse different loci simultaneously has become possible. This technique is appealing because of the considerable saving of time and resources, offered by reducing the number of PCR reactions needed for analysis of several microsatellite loci. By using primers with different fluorescent markers, loci with overlapping product sizes can be differentiated in a single gel lane. However, developing a multiplex can be time consuming, as microsatellite loci are often designed in isolation and the PCR conditions can vary considerably between loci. Extensive optimisation may be necessary, involving all aspects of the reactions (see Henegariu *et al.*, 1997 for review). The specificity of PCR conditions among loci is the main limitation to the number of loci that can be combined, and as many as eight loci have successfully been combined (Fishback *et al.*, 1999). There is some evidence that allele sizes may be affected by the multiplex reactions (Deng *et al.*, 2000). Therefore, the use of single or multiplex reactions needs to be consistent, to avoid genotyping errors.

Four loci were chosen for inclusion in a multiplex reaction, based on their relative product sizes: SaGT1 (118 – 172bp), SaGT41b (148 – 192bp), SaGT26 (223 – 257bp) and SaGT32 (148 – 182bp). The reverse primer of each locus was labelled with a fluorescent marker of either HEX, FAM or TET (MWG Biotech). The annealing temperature for the multiplex reaction was set at 60°C, as all the loci were known to amplify sufficiently at this temperature. All other PCR conditions were identical to those used for single locus PCRs and equimolar amounts of each primer pair were used. Initially the loci were tested in pairs to determine how the primers would interact. This was also confirmed by testing the primer sequences in software such as Primer Select (DNASTAR Inc.). Significant amounts of primer-dimer were often observed in agarose gels of the multiplex PCR products (Figure 2.3). Where large interactions between primers were found, for example locus

SaGT32 and SaGT26, the primer concentrations were adjusted until all the loci were amplified equally. The multiplex reaction proved reliable when primer concentrations were used in the following quantities: SaGT1 – 0.75 $\mu$ M, SaGT41b – 0.75 $\mu$ M, SaGT26 – 1.05 $\mu$ M and SaGT32 – 0.3 $\mu$ M. Another feature of the multiplex PCR was the substantial reduction of stutter bands with some of the loci. Only locus SaGT32 showed any significant amount of stutter in the multiplex reaction, whereas the remaining loci all improved in clarity (Figure 2.4). This suggests that competition in the reactions for DNA template or Taq polymerase has led to the amplification only of the full target sequence, rather than incomplete products that were observed in the single PCR's as stutter bands.



**Figure 2.3: 1.2% Agarose gel of multiplex PCR (SaGT1, SaGT41b, SaGT26 and SaGT32) showing large amounts of primer-dimer and separation between the larger and smaller loci. Lane 1 = 100bp DNA ladder (1500 – 100bp)**



**Figure 2.4: Genotyper™ gel results of multiplex PCR. Loci (from top) SaGT41b, SaGT32, SaGT1 and SaGT26. Locus GT32 is the only one to still show significant stutter bands. Top scale = size in bp; Side scale = peak height. Each trace represents a different fluorescent marker colour.**

### **2.3.2 Results Analysis**

All PCR products were analysed using the Genescan™ and Genotyper™ software (PE Applied Biosystems). Polyacrylamide gels (0.2mm thickness) were made using 5.2ml 40% Acrylamde (Bio-Rad), 27.5ml dH<sub>2</sub>O, 18g Urea (Bio-Rad) and 0.5g mixed bead resin (Sigma). The mixture was de-ionised for 20 minutes on a magnetic stirrer, then sterilised through a 0.2µm filter before adding 5ml of 10X TBE solution. The mixture was then de-gassed for several minutes and 250µl APS (0.1g Ammonium Persulfate, 1000ml ddH<sub>2</sub>O) and 35µl TEMED were added to instigate polymerisation. The gel was injected between two glass plates (36cm) using the standard ABI plate cartridges, 0.2mm plastic spacers and injection clamp. The gel was allowed to polymerise at room temperature for 2.5 hours and a disposable well-comb inserted into the top of the gel once polymerisation was complete. After loading the plate cassette onto the ABI 377, the read region of the plates were checked for scratches on the glass and gel residues, using the plate check module. A 1X TBE solution was added to the buffer tanks and then the machine was set to pre-run for 10 minutes, in order to warm the gel to operating temperature. Prior to and after the pre-run period, the wells of the comb were flushed with the buffer solution using a 5ml pipette, in order to remove excess fluid, air bubbles and crystals of urea from the wells. After denaturing for 3 minutes, samples were loaded using a multi-channel pipette in two stages, with one minute of pre-run between loading points. The sample mixture consisted of

1.35µl de-ionised formamide, 0.5µl blue dextran dye, 0.3µl TAMRA 350 internal size standard (Genpak) and 1.0µl PCR product (diluted 1/10). From this mixture 1 – 1.2µl was loaded into each well. The gel was run for two hours at 3000volts, 50mA with a gel temperature of 51<sup>o</sup>C and 1200 laser scans per hour.

Gel images were analysed using the automated procedures of the Genescan™ collection software to track lanes and extract raw data. The size standard in each lane was aligned to standardise the size calling between lanes. Automated size calling (2 peaks per locus) proved unreliable in designating all alleles, because of the wide disparity in peak heights between samples and, therefore, allele designation was performed manually for all samples. Allele bin sizes were created to standardise the results from different gels and these were aligned using two reference samples on every gel. Bin sizes were determined using parental samples and corrected, where necessary, by comparison of the parental and offspring genotype data.

## **2.4 Development of New Microsatellite Loci**

### **2.4.1 Microsatellite Development**

#### **2.4.1.1 Production of Clones**

The sequences of positive clones were produced by the Institute of Marine Biology, Crete (IMBC) and provided by collaboration with Dr. Giorgos Kotoulas and Dr. Antonis Magoulas.

The clones were produced by the following procedure: a partial genomic library was constructed by size selecting fragments (300-800bp) of genomic *Sparus aurata* DNA digested with *Mbo*I. The fragments were ligated into pUC 18/*Bam*HI dephosphorylated vector. The ligation products were used to transform *Escherichia coli* competent cells (XL10-Gold Ultracompetent cells, Stratagene). Hybridization with a (GT)<sub>20</sub> oligonucleotide probe end labelled with [ $\gamma$ -<sup>32</sup>P] ATP produced 68 positive recombinants. These positive clones were sequenced by the Foundation for Research and Technology, Hellas (FORTH), Laboratory of Microchemistry. A total of 24 sequences were assembled using the Auto Assembler software (PE Applied Biosystems) and eight loci were selected for development, based on the quality of sequence, size of flanking sequences, size of the repeat sequence and the uninterrupted nature of the repeat. These sequences were then checked against existing, submitted sequences using the BLAST search engine (<http://www.ncbi.nlm.nih.gov/>), with no significant matches.

#### **2.4.1.2 Primer Design**

Primers were designed with an aim to multiplex at least four loci and, therefore, several primer sets were designed for each locus with a range of predicted annealing temperatures and product sizes. From this range, one primer pair was chosen for each locus, based mainly on similarity of annealing temperatures, primer length and GC content (stability). Primer sets were also chosen to obtain a wide range of non-overlapping product sizes for multiplexing of the loci or simultaneous loading onto Genescan™ gels. The primers were designed using the Primer Select software program (DNASTar Inc.). Table 2.2 contains a summary of the primer design. The nomenclature of the loci supports that suggested by G. Kotoulas (pers. comm.) to include the initials of the species (Sa) followed by the origin of the clones (Iraklio) and the identifying number code of the clone.

**Table 2.2: Characteristics of primer pairs chosen for the eight loci under development. The range of product sizes and similarity between annealing temperatures increased the likelihood of multiplexing the PCR reactions**

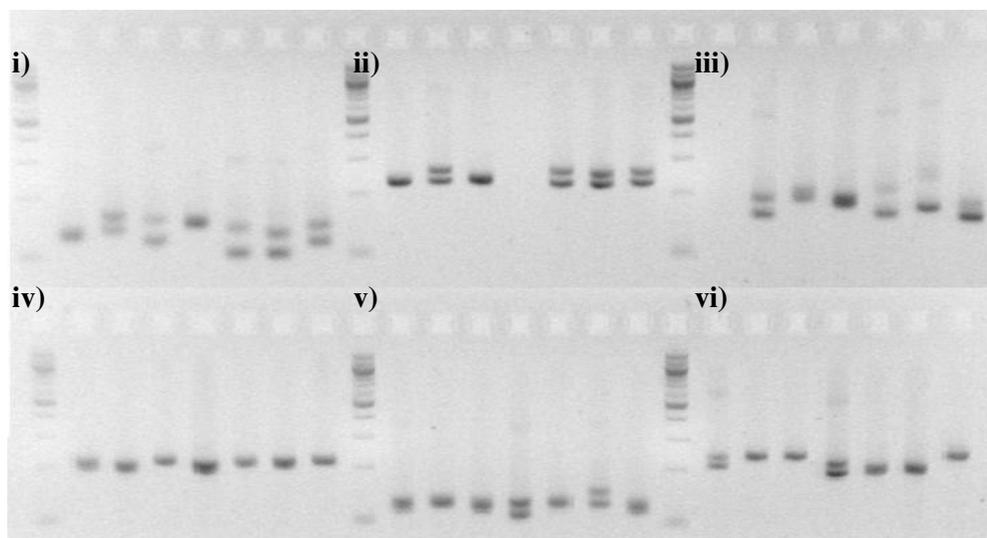
Primer Name	Primer Length (bp)	Annealing Temperature T <sub>a</sub> (°C)	Estimated Product Size (bp)
SaI10f	19	61.0	213
SaI10r	24	61.0	
SaI11f	23	57.1	242
SaI11r	19	58.8	
SaI12f	19	56.7	149
SaI12r	20	55.3	
SaI14f	24	61.0	219
SaI14r	24	59.3	
SaI15f	24	62.7	119
SaI15r	24	62.7	
SaI18f	20	49.1	157
SaI18r	18	51.4	
SaI19f	23	58.9	253
SaI19r	21	61.8	
SaI21f	19	58.8	196
SaI21r	24	59.3	

## **2.4.2 Testing of Microsatellite Loci**

### **2.4.2.1 Polymorphism and Selection of Loci for Genotyping**

Initially unlabelled primers were used in a testing phase to confirm polymorphism at locus before obtaining the more costly fluorescently labelled primers. The loci were tested using the PCR conditions and programmes outlined in section 2.4. Six of the eight loci produced product in these first tests and by using seven parental DNA samples these also appeared to be polymorphic. The two non-amplifying loci were re-tested at a higher annealing temperature and displayed a weak product. To accurately detect polymorphism, high resolution 3% Metaphor agarose (Sigma) gels of the PCR products were used as the results can be viewed rapidly using standard electrophoresis equipment, the DNA can be labelled with Ethidium bromide and individual alleles differing by several base pairs can be distinguished. The Metaphor gels were subsequently rejected in favour of using 3% (electrophoresis grade) agarose gels, which provided an adequate resolution at a reduced cost. Figure 2.5 is a 3% agarose gel showing the detected variability at six loci in the parental DNA samples. The remaining loci, SaI11 and SaI18 appeared to be monomorphic were not pursued further.

Further characterisation of the loci and testing of polymorphism focused on 32 individuals from the hatchery broodstock (natural photoperiod group). A summary of the results from these fish for each locus is given in Table 2.3 including the number of alleles, heterozygosity, PCR conditions and Genbank accession codes (Brown *et al.*, 2003).



**Figure 2.5: Preliminary indication of allelic polymorphism at six loci. PCR products from 7 parental DNA samples for each locus are resolved on a 3% agarose gel. Loci: i) SaI10, ii) SaI12, iii) SaI14, iv) SaI15, v) SaI19, vi) SaI21; First lane of each group is 100bp DNA ladder (1500 – 100bp)**

#### 2.4.2.2 Development of a Multiplex

Fluorescently labelled primers (reverse primer) were ordered for the six identified polymorphic loci with the intention of developing a multiplex of at least four loci. The loci were initially tested individually on Genescan™ gels to check the correct dilutions, product sizes and general appearance of the PCR products. SaI12 and SaI19 had almost no stutter bands, whereas, SaI14 and SaI21 had only moderate stutter. SaI10 and SaI15 had high levels of stutter bands, which indicated that further optimisation to the PCR conditions are needed. These results also indicated that the most likely combination of loci for multiplexing would be SaI 12, 14, 19 and 21.

Initial testing of the multiplex using equal amounts of each of the four primer sets resulted in amplification of all the loci. However, it also indicated that locus SaI21 would be problematic in the multiplex as it showed an inconsistent amplification and had a wide size range of alleles (142-266bp) that could overlap all of the other loci, leading to scoring errors. Therefore, SaI21 was dropped from the multiplex and only used as a single primer PCR in data collection. Adjustments to the concentration of primers in the multiplex was used to increase the amount of product, especially SaI19 which was the weakest of the loci. Final primer concentrations in the multiplex were as follows: SaI12 – 0.6µM; SaI14 – 0.75µM and SaI19 – 1.0µM. The three-loci multiplex (triplex) proved very reliable and was used for all offspring samples with very few repeat PCRs required. Size calling of the loci was also reliable because of the limited amount of stutter at each locus and good peak height obtained with the balance of primer concentrations.

**Table 2.3: Primer sequences of six microsatellite markers in *Sparus aurata* with Genbank accession number, repeat structure, annealing temperature, product size range, observed ( $H_0$ ) and expected ( $H_E$ ) heterozygosity and number of alleles. Values calculated from screening 32 individuals from the hatchery broodstock.**

Locus Name	Size Range	Repeat Structure	No. of Alleles	$H_0$	$H_E$	Primer Sequence (5' → 3')	Annealing Temp	Genbank Accession No.
SaI10	199-231	(GT) <sub>37</sub>	12	0.906	0.882	F: TCACGGGGGACCAAGACTG R: CTCACACTGCCTAATTAGCACAGA	62 °C	AY322107
SaI12	109-155	(GT) <sub>30</sub>	10	0.844	0.835	F: ACGGTATGGAGTCAACTGC R: CCCCTTTTGGTACATCATAG	60 °C	AY322108
SaI14	196-252	(GT) <sub>27</sub>	15	0.969	0.873	F: TGCCAGAATGAATACCAACTGGTG R: ATAAATCAAAAGTACCCCTGCATGTC	60 °C	AY322109
SaI15	101-161	(GT) <sub>26</sub>	18	0.969	0.917	F: ACACTGTCTTTCTGTCCCTCACAC R: GAGTAAACACAGCCTCAGTTGAAAGC	62 °C	AY322110
SaI19	233-282	(GT) <sub>25</sub>	15	0.875	0.910	F: ATTCTTCACAGGCCAACACAAA R: GAAAAACACCCGGCCAGTACGA	60 °C	AY322111
SaI21	142-266	(GT) <sub>41</sub>	25	0.750	0.969	F: GGACGCCACACCCATGTTCA R: AACCGAAGCTGATTGTTAGTGTGA	60 °C	AY322112

## **2.5 Parental Assignment Using Microsatellite Loci**

### **2.5.1 Assignment Methodology**

#### **2.5.1.1 Introduction**

The properties of microsatellites – such as high polymorphism, Mendelian co-dominant inheritance and the number of markers present in the genome – make them an ideal tool with which to determine parentage or ancestry. DNA profiling can now be carried out relatively quickly on a large number of samples and loci. Whereas initially, parental assignment was based on exclusionary power of a small number of loci, the statistical analysis of parentage can now use computer-based likelihood methods to determine parentage, based on the number of matches across all the loci being used (Marshall *et al.*, 1998). This increases the confidence level of the parental match by considering all the available information, rather than moving in a stepwise manner to exclude parents that fail to match at a particular locus. This is especially important when there are large numbers of similar genotypes, for example when small numbers of parents are used, and in cases where not all parents are sampled.

Microsatellite-based parental assignment has been used in fish populations for applications such as determining mating systems (Knight *et al.*, 1998; Mackiewicz *et al.*, 2002) and reproductive success (Taggart *et al.*, 2001; Fiumera *et al.*, 2002).

#### **2.5.1.2 Number of Loci Required**

Because of the limited number of loci available at the beginning of this project and the prospect of estimating parentage from a large number of broodstock, an investigation into the number of loci required for adequate parental assignment was conducted. Using the simulation module of CERVUS software (Marshall *et al.*, 1998) simulated genotypes were generated by randomising actual genotypes of loci SaGT32, SaGT26, SaGT41b and SaGT1 to represent the parents. This method was used so that the simulation would be based on the observed heterozygosity of the loci to be used, but without using actual genotypes. As indicated in Table 2.4, as the number of loci increases so does the resolving power. When four loci are used, the rate of assignment is only 47% at 80% confidence with 60 parents (one broodstock group). The table also indicates that eight loci would be necessary to achieve 94% assignment at the 95% confidence level when 60 parents are present. To assign offspring sampled at random in the farming system with no knowledge of parentage (240 possible parents) would require at least 11 loci to assign all samples at the 95% confidence level.

As the anticipated sampling regime was to sample progeny derived from single broodstock spawning events, it was concluded that eight loci would be sufficient to conduct the project. However, problems with incorporating locus SaI 21 into a multiplex meant that only seven loci were actually available for all progeny samples. Whilst all samples could be assigned at 80% confidence, the number assigned with 95% confidence would be reduced.

### 2.5.1.3 Assignment Process

Composite genotypes consisting of six to eight loci were collected for 670 offspring samples in 2001 and 2002. Offspring genotype data was collected from the Genotyper software and compared to the broodstock genotypes to detect errors and ensure bin sizes were matching, by using the allele frequency module of the CERVUS software. The PAPA software program (Duchesne *et al.*, 2002) was used for all parental assignment due to its ability to assign the most likely pair of parents. This was preferable to the CERVUS program, which is only able to assign the two most likely parents by using one as a known parent and, thus, restricting the alleles available to match the second parent. No sex data for the parents was included because of the unreliability of the method of sexing (see Chapter 3) and, therefore, all parental combinations were available including same-sex matches.

**Table 2.4: Simulation of the number of loci needed to achieve parental assignment at two confidence levels using different numbers of potential parents. Using one broodstock group, eight loci would be needed to assign an offspring, whereas this rises to 11 loci to assign an offspring derived from any of the farm broodstock.**

Number of Loci	Number of Parents	Unassigned	Assigned at 80% confidence	Assigned at 95% confidence
4	60	53%	47%	10%
5	60	13%	87%	36%
6	60	0	100%	59%
7	60	0	100%	82%
8	60	0	100%	94%
4	240	97%	3%	0%
10	240	0	100%	84%
11	240	0	100%	94%

In samples that were known to originate from more than one broodstock, each parental group was tested against the offspring separately and the results contrasted to determine the true parental matches. A 5% global error was allowed in the assignment calculations, equivalent to a 5% probability of a scoring error at each allele. Successful assignment was confirmed when at least 12 of the 14 alleles available were matching between assigned parents and offspring. Of the 670 offspring assigned, 23 were below the threshold of 12 matching alleles and nine were rejected because of same-sex matches in the parents, as determined using a parental matrix (see below). Therefore, the success rate of assignment using seven loci was 96%, which was very similar the predicted success of assignment at 80% confidence using this number of loci (Table 2.4).

The PAPA software was able to assign a pair of parents of unknown sex and this required checking to ensure that there were no same-sex parental matches and also to support the sex data collected on site. This was achieved by creating a matrix of the assigned pairs based on the shared offspring between multiple parental pairs. An example of a matrix is given in Figure 2.6. The sex of each side of the matrix was determined by a consensus with the observed sex data. The correlation of the observed sex data and parental matrices will be discussed in Chapter 3.

		Males																Total							
		DE44	3777	DD8D	3DBD	D79D	F210	2000	1C42	F28F	4063	2535	CA03	CA64	D23E	E6D8	BF7D	2C22	2181	239B	2B0D	455B	2C35	Total	
D88E	2							2			4														8
4280	5			1		2		2			2										1				14
1641		7		2		1					1			3							6		1		18
D157										5											1				6
EC42																					1				2
2C28																									0
E8D8	1	3								6															4
CFC4	5			5		1								1									1		12
275D																									7
1417																									0
EF1A										2															3
D65B																					1				0
3446	10	1		1																	7				19
D52D																									0
CC12								3													2				17
1EE4																									0
CF48	5											3													8
D265								2																	5
26C8	1																				1				2
2F73																									1
EA54				1																					0
28DD																									0
2E5B																									0
3091																									0
3BED																									0
2216																									0
1C4F	1												3												5
4271	4	1																							8
EF2D																							1		1
Total	0	34	13	0	10	0	6	4	4	0	26	5	3	6	0	1	0	0	0	0	28	0	3	1	140

Figure 2.6: Parental Matrix of Tank 5 offspring in 2002. Parental pairs were assigned using PAPA software without sex information. This was subsequently determined by separating parents on the basis of shared offspring with other fish. The sex of each side of the matrix was determined by comparison with the observed sex data to obtain a consensus of the representation of each sex on each side.

## **2.5.2 Sources of Error in Parental Assignment**

### **2.5.2.1 Number of Parents**

The genotypic data used in the assignment of offspring in this study had two major limitations. Firstly, information on parental contribution was unknown from the mass spawnings and, therefore, all fish in a broodstock group had to be considered as potential parents. Secondly, no sex data was available to confirm the viability of assigning parental pairs. However, in contrast to natural populations, the broodstock contained every parent and, provided that they were all sampled and correctly scored, it should be possible to assign every offspring with near certainty. Logically, when this does not happen, errors in the data collection must be responsible as, aside from mutations, inheritance of the microsatellite alleles follow Mendelian principles.

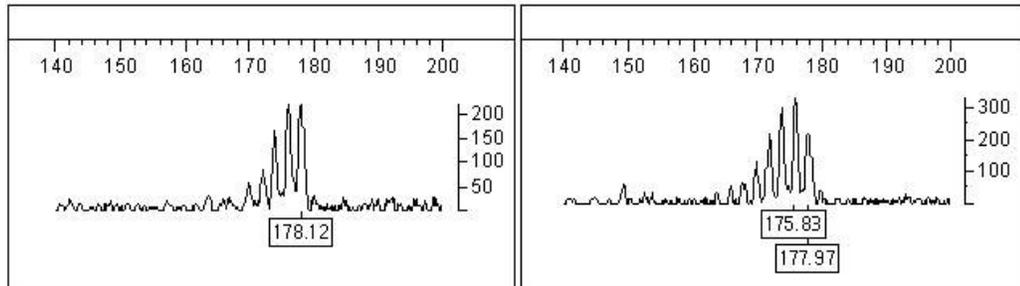
When the numbers of parents are high, the number of potential families can be very large and the resolving power of the loci can be severely reduced. The higher the proportion of similar genotypes, the more loci that will be needed to resolve parentage to an individual family. A measure of the resolving power of each locus is given by the exclusion probability, that is the probability of excluding an unrelated individual from the parentage (Marshall *et al.*, 1998), which can be calculated within the CERVUS package. The exclusion probabilities for the loci used ranged from 0.468 to 0.727 for the most likely parent. While these values are low for individual loci, when combined over all seven loci the exclusion probability was 0.997. This is a further indication of the number of loci needed to achieve reliable parental assignment and how exclusion based on a few loci is less reliable than considering matches over many loci.

### **2.5.2.2 Data Analysis and PCR Errors**

Size-calling errors are likely to be the greatest source of errors in the actual genotypes of both parents and offspring. PCR conditions are known to affect the size of products and the addition of erroneous base pairs can occur after PCR reactions are complete (Brownstein *et al.*, 1996). For this reason PCR conditions were kept as similar as possible by using the same brands of equipment, consumables and reagents. Data was gathered by using the same multiplex reactions and, where possible, combining parental and offspring genotypes in the same Genescan™ gel for direct comparison. Reference samples were also used in every gel to confirm base-pair additions and up or down bias in the size-calling between gels. There are several examples of mismatches in the data where one allele was repeatedly mismatched between parent and offspring. For example, the parent EF1A consistently mismatched known offspring at locus SaGT26 by two base pairs (258bp instead of 260bp), indicating that the parental genotype contained an error. This type of error can be overcome by repeating the PCRs and data collection process or by adjusting the parental genotype after the data has been checked and the consistent mismatches in the offspring identified.

Size calling is more prone to errors when the quality of the Genescan data is poor due to stutter bands on the gel. Stutter bands may mask a real allele or produce a false allele, if they are of sufficient strength (see Figure 2.7 for an example). This

problem becomes more acute if the alleles are separated by only a few base-pairs, as stutter will undoubtedly interfere with size calling. Taq polymerase is known to add an adenine nucleotide to the 3' end of PCR products which often results in the production of an additional band, one base-pair higher than the actual allele (Brownstein *et al.*, 1996).



**Figure 2.7: Occurrence of false alleles in locus SaGT32 by stutter bands. The same DNA sample produces either a homozygote or heterozygote depending on the strength of the additional band. This sample was classified as a homozygote at 178bp.**

In Figure 2.7 the genotype of this individual inconsistently appears as either a heterozygote (bands at 176 and 178bp) or homozygote (one band at 178bp) depending on the size of the peak at 178bp. As determined by repeated analysis of this individual, the correct genotype should be a homozygote at 178bp. This type of error can be avoided by changes to PCR cycles, type of Taq polymerase or the primer design (Brownstein *et al.*, 1996; Magnuson *et al.*, 1996).

Another aspect of mistyping is the occurrence of null alleles. These are alleles that consistently fail to amplify and this usually leads to the false typing of heterozygotes as homozygotes. In the current data set, locus Sai21 shows a particularly high rate of homozygotes and missing alleles in known parental matches, which indicates that null alleles may be present (Spruell *et al.*, 1999). Null alleles may be the result of sub-optimal PCR conditions or mutations in the primer binding sites, which prohibit amplification (Holm *et al.*, 2001). Null alleles can generally be treated in the same way as mismatches in that they may not effect assignment if other loci are available. At high frequencies, however, the locus may need to be dropped from the analysis or the primers redesigned, to avoid the mutation site (Jones *et al.*, 1998). Locus Sai21 was excluded from the analysis of parentage of most samples due to the problems of incorporating the locus into the multiplex PCR and the frequency of possible null alleles.

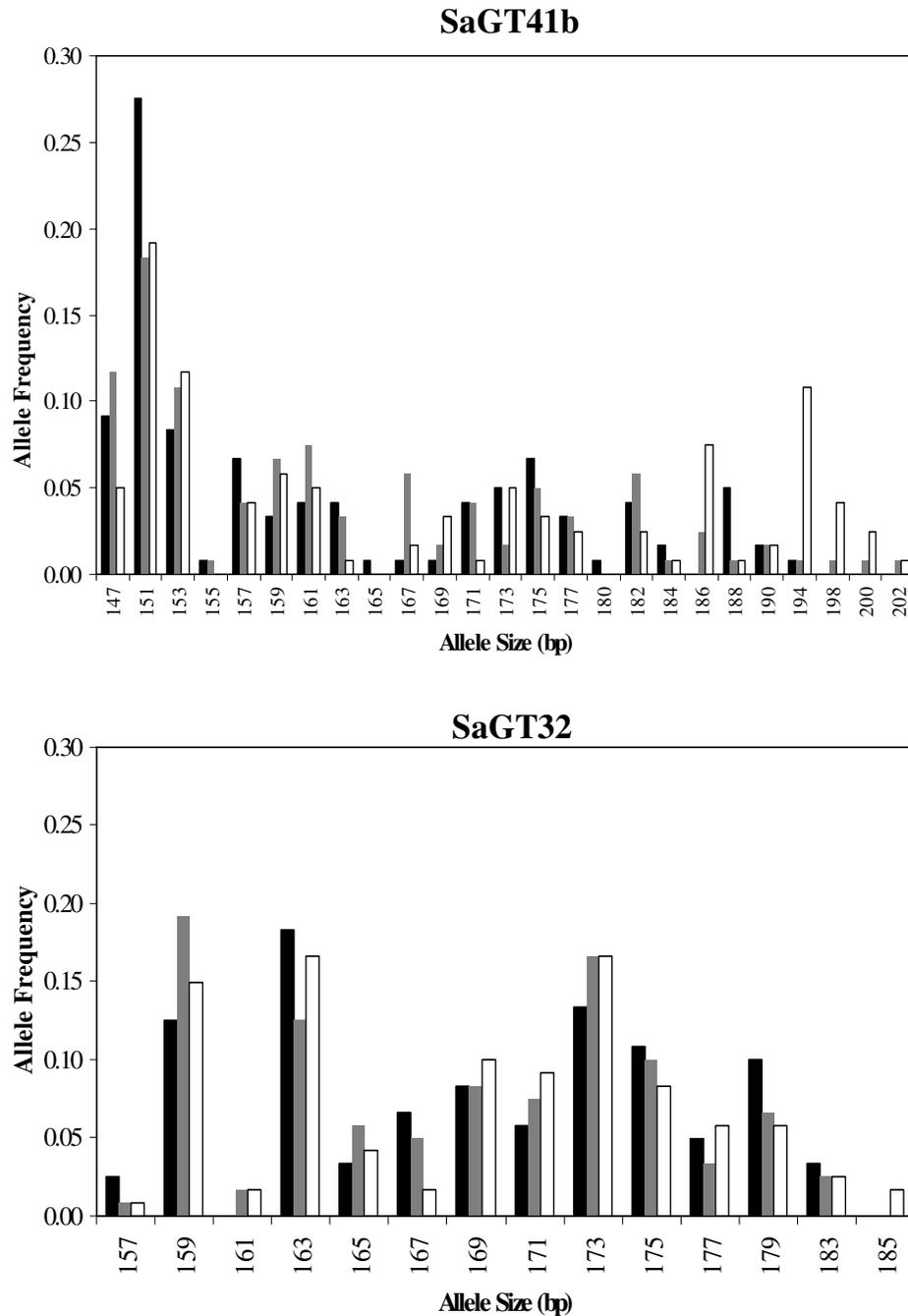
## **2.6 Characterisation of the Microsatellite**

### **Loci in the Hatchery Population**

#### **2.6.1 Characterisation of Microsatellite Loci in Hatchery Population**

Microsatellite loci are useful tools in the study of populations because of the large numbers of alleles and high levels of heterozygosity found at many loci. The structuring of populations can be revealed by genetic differentiation, heterozygosity and the number of alleles. Inbreeding and the size of the breeding population can also be determined from microsatellite loci and this aspect of their use will be dealt with in Chapter 7.

Figure 2.8 contains the allele frequencies of eight microsatellite loci in the Alkioni hatchery broodstock, plotted separately for each of the three groups of 60 fish. There is a high degree of similarity in the allele frequencies of each group that reflects the common origin of the stock and the recent separation of the group from the original population. Genetic drift can proceed rapidly in hatchery stocks because of the small number of breeding individuals and poor survival of offspring in culture conditions, which are then used to replace the breeding group. Figure 2.8 also indicates that many low frequency alleles are common to all the groups, which further suggests that the groups closely resemble the ancestral stock, as these low frequency alleles are more likely to be lost if inbreeding and genetic drift had been allowed to accumulate. The highest frequency allele was recorded in locus SaI14 (198bp) with a frequency of 0.30-0.45 in the three groups.



**Figure 2.8: Allele frequencies of microsatellite loci in Alkioni Fish Farm broodstock. Each broodstock group of 60 fish is plotted separately. The similarity of allele frequencies between groups indicates their recent division and departure from the ancestral population. Broodstock groups based on photoperiod control: (■) delayed group, (■) advanced group, (□) natural group.**

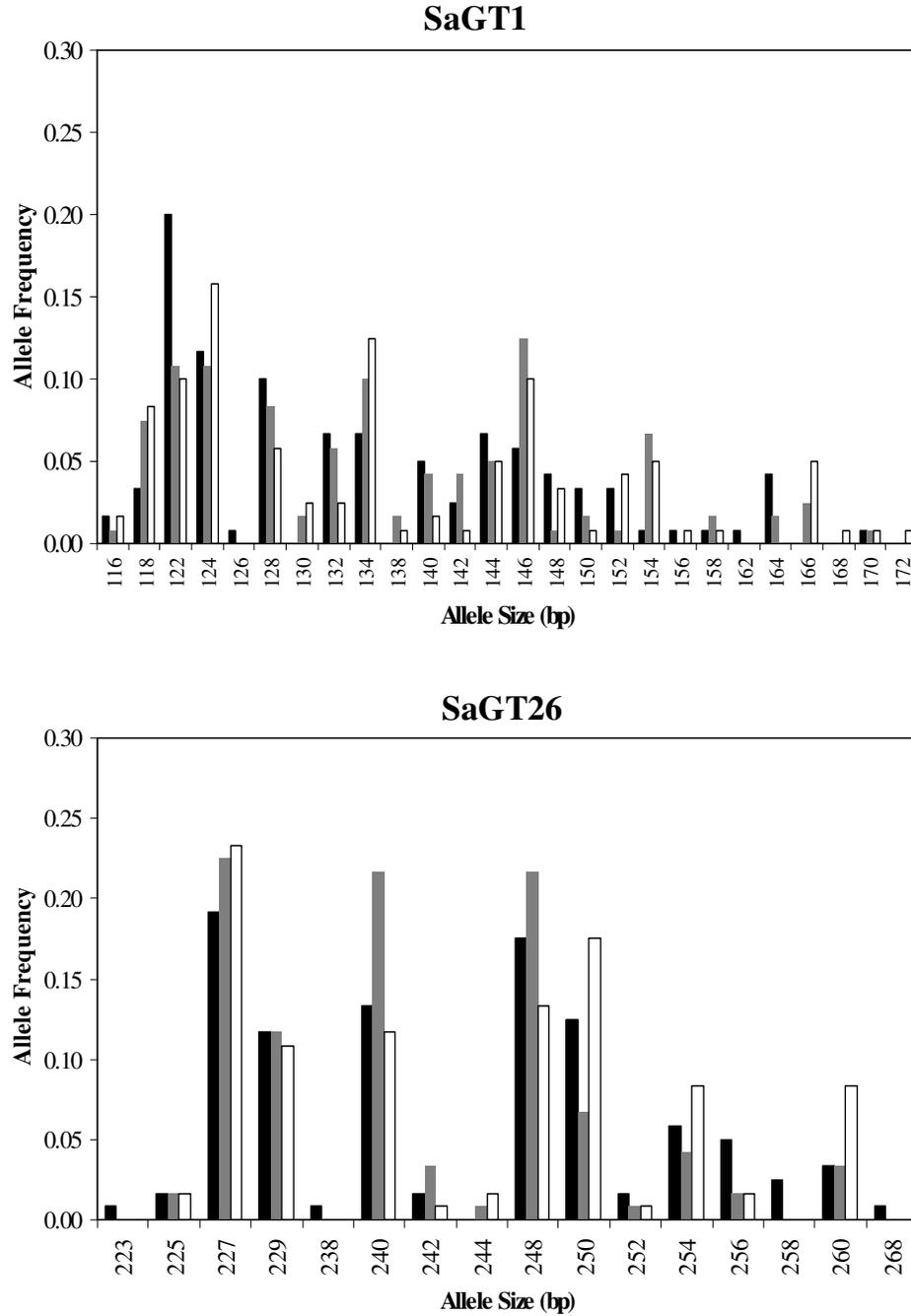


Figure 2.8 (cont.):

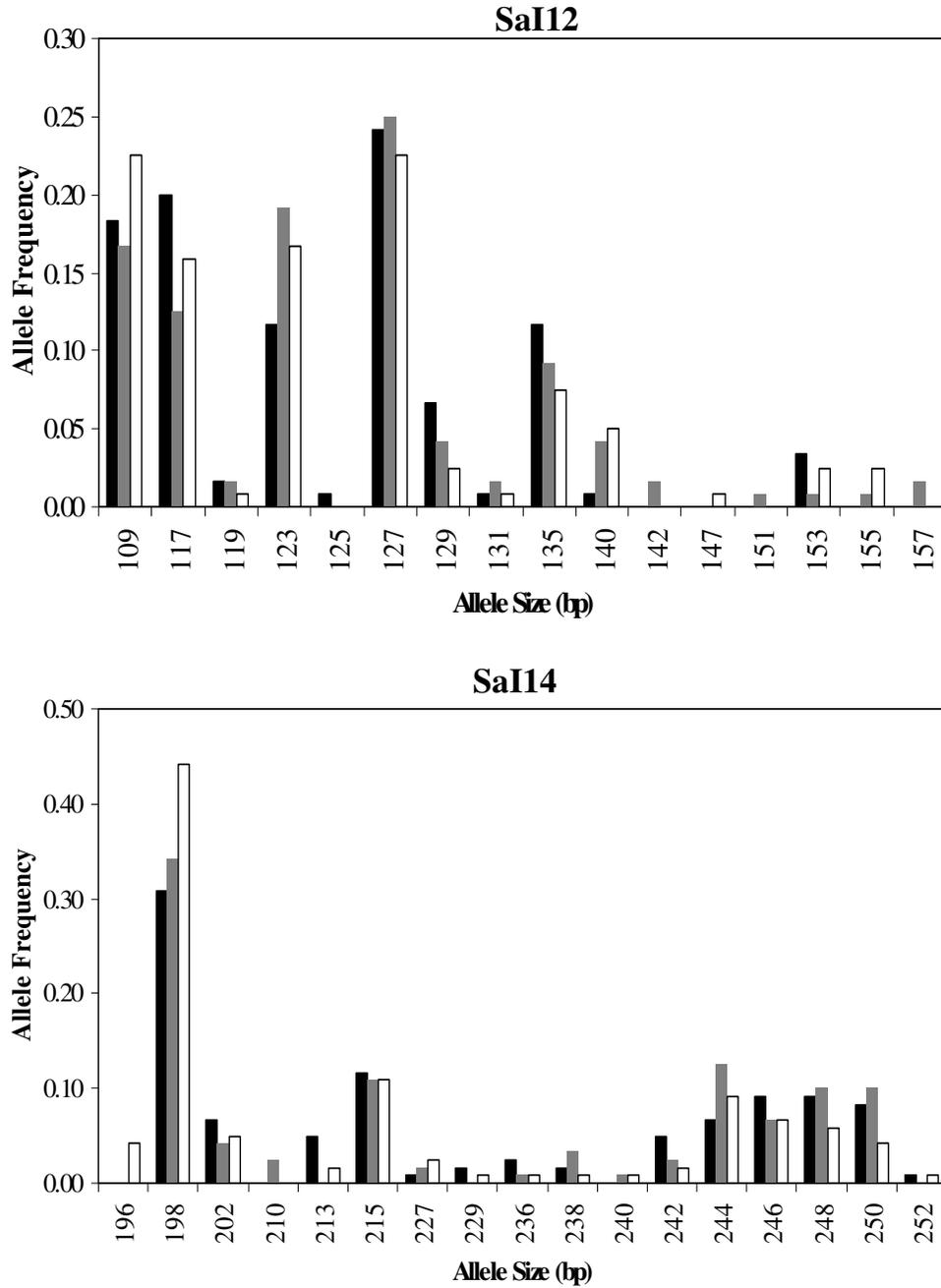


Figure 2.8 (cont.):

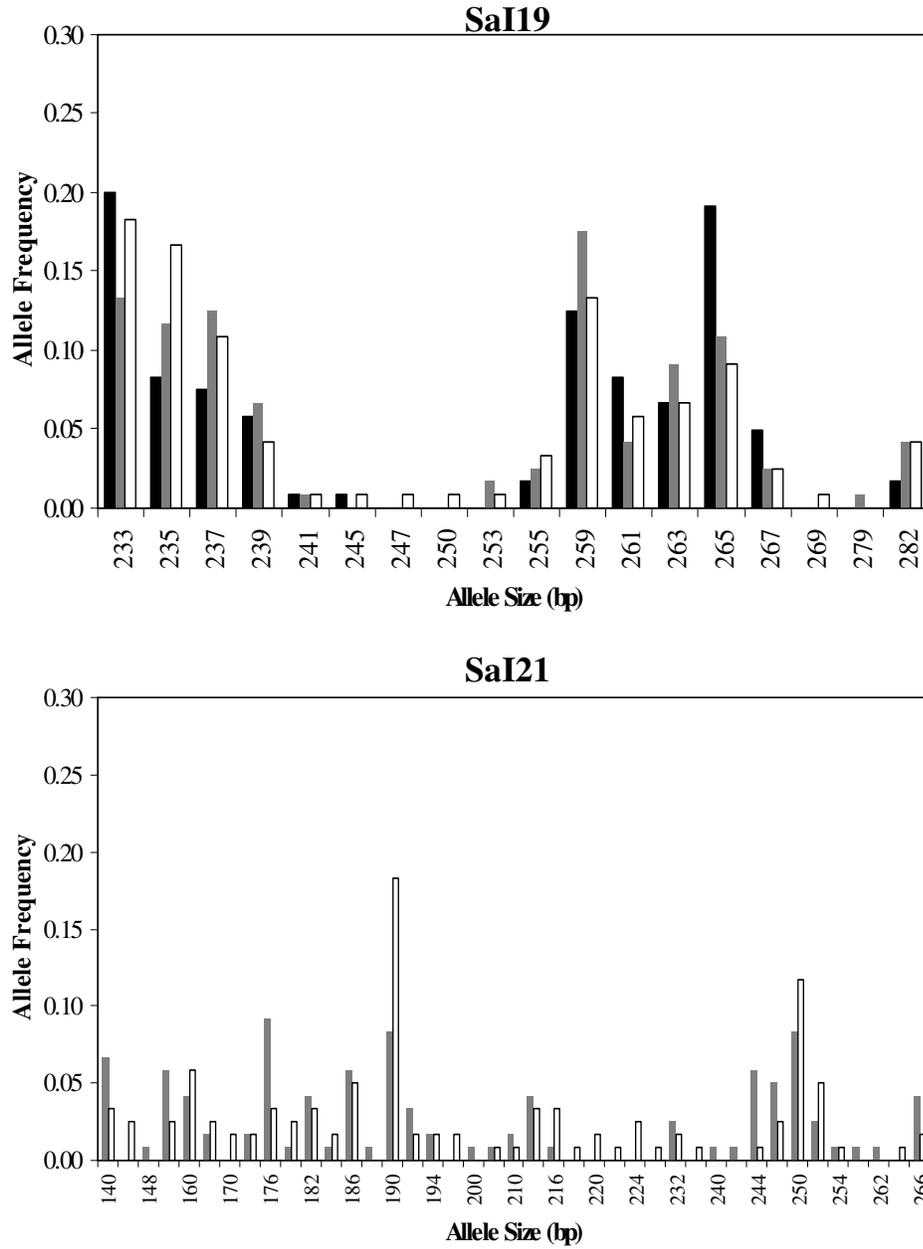


Figure 2.8 (cont.): Note – data not available for locus SaI21 in Delayed group.

## **2.6.2 Comparison of Hatchery and Wild Populations**

Due to the small number of microsatellite loci available for gilthead seabream, there is a paucity of genetic studies in wild populations of this species. In common with other species, there can be significant differences in heterozygosity and allele frequencies between wild and hatchery gilthead seabream populations (Palma *et al.*, 1998; Magoulas *et al.*, 1998). However, Magoulas *et al.* (1998) also note that the genetic differentiation between wild gilthead seabream populations are relatively minor. This was demonstrated in their study, by the small number of variants of a D-loop mitochondrial marker and similar allele frequencies at a number of microsatellite markers. This suggests that there is a high degree of mixing within wild gilthead seabream populations over large geographical ranges.

Batargias (1998) contains detailed allele frequency data for a small group of wild-caught gilthead seabream of a similar geographical origin (Crete), to the Alkioni hatchery broodstock (Cyprus). Table 2.5 shows the comparative data of the two stocks at four microsatellite loci, including number of alleles, size range and observed heterozygosity based on the genotypes of 32 fish. The observed heterozygosity was generally higher in the Alkioni stock, which may be attributed to the accuracy of the different methods of microsatellite analysis. Batargias's data was based on radioisotope labelled DNA fragments in contrast to the automated sequencer method used here. However, the number of alleles was greater in the wild population indicating that a small amount of variation may have been lost in the process of establishing the hatchery stock. A test of gene differentiation ( $G_{ST}$ ) (Nei, 1987) indicates that there are very minor differences between the two stocks suggesting that the ancestral population is undifferentiated at these loci and the source populations of both stocks are genetically similar.

**Table 2.5: Comparison of wild and hatchery broodstock at four microsatellite loci in 32 fish per stock. Observed heterozygosity ( $H_O$ ) was higher in the hatchery stock whereas, number of alleles was greater in the wild stock. Low gene differentiation values ( $G_{ST}$ ) indicate that the ancestral stocks are undifferentiated at these loci.**

Locus	Wild-Caught Broodstock			Alkioni Hatchery Broodstock			$G_{ST}$
	n	No. of Alleles	Size Range	n	No. of Alleles	Size Range	
SaGT1	32	22	118-172	32	20	116-172	0.061
SaGT26	32	13	223-257	32	11	225-260	-0.096
SaGT32	32	16	148-182	32	14	157-185	-0.021
SaGT41b	32	18	148-192	32	21	147-202	-0.042

**Chapter 3:**  
**Broodstock Management of**  
**Gilthead Seabream**

### **3.1 Introduction**

Intensive fish farming relies on the regular supply of fertilised eggs for the propagation of farm populations. Often the production system will require eggs outside of the natural spawning season and so management or manipulation of a captive broodstock is also necessary (Buss, 1980). Maintaining such a broodstock is often difficult because many species do not adapt well to captive conditions, which in turn leads to unpredictable or no spawning. Environmental requirements will be different for each species and likely to be determined by the ecology of the species. These may or may not be compatible with intensive aquaculture in land-based systems.

The management of broodstock aims to maximise the production and quality of eggs, whilst maintaining the performance of the stock and regularly introducing new fish to replace older ones. To manage the broodstock effectively, one should have a good knowledge of the biology of the species, meet the nutritional requirements and, take into account the performance of individual fish and the genetic makeup of the population. Nutrition of broodstock is an important determinant of the quality of gametes produced and will, therefore, be dealt with as a source of variation in the offspring in chapter 4.

This chapter will outline the major factors in the management of gilthead seabream broodstock, specifically spawning manipulation and the control of the sex ratio. Many aspects of the biology of seabream are able to influence the broodstock management strategies and these will be examined by utilising the results of long-term monitoring of a farm broodstock.

#### **3.1.1 Social Sex Determination in Fish**

A large number of fish species exhibit various forms of sexual plasticity mediated by both genetics (reviewed by Devlin and Nagahama, 2002) and environmental conditions, such as temperature (reviewed by Baroiller and D’Cotta, 2001). The social environment is also a key factor for many species in the determination of sex, especially in those that aggregate into groups for breeding (Warner *et al.*, 1975). In situations where the reproductive fitness of an individual is variable, the ability to change sex is more likely to develop (Godwin *et al.*, 2003). Ghiselin (1969) proposed a size-advantage model for hermaphroditism that states that if reproduction is more effective as one sex when smaller in size and as another sex when larger, changing sex becomes advantageous. The timing of this change should occur when the expected reproductive success of the secondary (reversed) sex exceeds the reproductive success of the primary (non-reversed) sex. However, the reproductive success of each sex is unstable in cases where sex change is under social control (Godwin *et al.*, 2003) and, consequently, the model may not apply to all species (Shapiro, 1989). Yet, when combined with a knowledge of the mating system, the size-advantage model is often a reasonable explanation of the decision to change sex in many sex changing fish species (Warner, 1988).

The mechanism by which the social environment influences sex change varies between species and, to a certain extent, reflects the ecology of each species. Open water, diurnally active and easily visible species, such as *Labroide* reef fish, tend to use direct visual stimuli or behaviour to influence sex change (Shapiro,

1983). The addition and removal of individuals (of the secondary sex) in bluehead wrasse has been found to have a direct effect on sex change (Warner and Swearer, 1991), suggesting that the encounter rate with other fish could be a primary mechanism for regulating sex change (Lutnesky, 1994). In species that are cryptic or isolated, for example benthic gobiid species, visual cues are less effective and pheromonal control of sex change has been found to operate (Cole and Shapiro, 1995). The role of pheromones in other sex-changing species is largely unknown, but has been ruled out in the case of saddleback wrasse, by Ross *et al.* (1983), where visual stimuli alone were able to induce sex reversal.

Gilthead seabream is a protandrous hermaphrodite, beginning life as a male and possessing the ability to undergo sex change from two years of age. Sex change is socially controlled (Happe and Zohar, 1988), although this may be restricted to a 'sensitive period' outside of the spawning season when all fish return to an ambisexual stage (Zohar *et al.*, 1995). The development of protandry is predicted by the size-advantage model to be the result of high reproductive costs of being a small female relative to being a small male (Warner, 1988). This, however, may only be applicable to the relative fecundity of the sexes at a given age rather than the total fecundity over the animal's lifetime. The high fecundity of gilthead seabream and the mass spawning mating system suggests that the size-advantage model is the most applicable to this species.

### 3.1.2 Manipulation of the Spawning Cycle

Many temperate fish have seasonal reproductive cycles that are influenced by environmental cues, of which photoperiod and temperature are the most important (Lam, 1983). Photoperiod control has long been recognised as a means to control the production of eggs from salmonid species (Bromage and Duston, 1985). In Atlantic salmon, photoperiod manipulation has been used to control growth rate (Kråkenes *et al.*, 1991), early maturation (Oppedal *et al.*, 1997) and spawning time (Taranger *et al.*, 1998) within commercial systems. Combined with temperature control of the water, any phase-shift of the natural environmental conditions can be achieved to produce eggs on a year round basis (Zohar *et al.*, 1995). Advancement and delay of the photoperiod can also be achieved by the use of short or long days at specific points of the year. For example in salmonids, advancement of spawning is performed by initially using long days (18 hours light: 6 dark) followed by short days (6 hours light: 18 dark) prior to the desired spawning time (Bromage *et al.*, 2001). Similar protocols are effective at advancing spawning in other species, such as European sea bass (Carrillo *et al.*, 1989). In contrast to changing day lengths, a constant photoperiod often results in a delay of the natural spawning period (Taranger *et al.*, 1998; Kissil *et al.*, 2001). The quality of gametes produced under photoperiod control is generally of the same quality as those under ambient conditions (Bromage *et al.*, 1992). However, there is also evidence of a trend towards smaller eggs from advanced groups and larger eggs from delayed groups in salmonids (Bromage and Cumaranatunga, 1988).

The seasonality of spawning is thought to be controlled by an endogenous rhythm, which may receive external environmental cues, but which can also operate in conditions of constant light and temperature (Bromage and Duston, 1986). The rhythm may be under genetic control, as suggested for other animals (Dunlap 1998), perhaps as the product of a hormonal cascade operating between the brain-

pituitary-gonad axis (Bromage *et al.*, 2001). Evidence of hormonal action on spawning time is very strong, given that administering gonadotropin releasing hormones can be used to stimulate spawning and to control spawning time (Zohar *et al.*, 1995). Photoperiod is thought to influence the endogenous rhythm through an increase in the duration of the secretion of melatonin from the pineal gland during the dark phase of the photoperiod (Ekström and Meissl, 1997). The duration of melatonin secretion is directly proportional to the length of this dark phase, thereby producing an accurate annual calendar based on day/night length and the direction of change (Randall *et al.*, 1995). However, direct evidence of the role of melatonin is lacking, due to the technical difficulties in administering and simulating melatonin levels in fish (Bromage *et al.*, 2001).

### 3.1.3 Broodstock Management of Gilthead Seabream

Gilthead seabream females are highly fecund and are able to produce 20,000 to 80,000 eggs per day over a 4-month period (Moretti *et al.*, 1999). Even considering a 90% fertilisation rate and a subsequent hatching rate of 80%, this results in a production potential of up to 50,000 hatched-larvae per female per day. Gilthead seabream readily spawn in captivity and little intervention is needed in the spawning process and handling of the fish will often lead to a cessation of spawning activity in the females. Gilthead seabream is a mass spawning species and prefer spawning in large groups of fish, with 5-7 fish judged to be the minimum number of fish required to induce natural spawning (Gorshkov *et al.*, 1997). These small groups of fish were, however, created after sex determination had taken place and the sex ratio was manipulated to favour females.

Alkioni Fish Farms currently hold 240 seabream broodfish to supply all the eggs used in the production system throughout the year. The broodstock are housed in four 5m<sup>3</sup> tanks, each containing 60 fish, located in internal and external facilities. The internal tanks permit photoperiod control of light and water temperature, whereas external tanks follow a natural cycle of light and water temperature. Two tanks are maintained on an advance cycle (three months ahead of the natural cycle), one tank on the natural cycle and one tank on a delayed cycle (three months behind the natural cycle). All broodstock are fed on the commercial compound diet Lansy Breed (INVE Hellas SA, 47 Fleming Street, Argiroupolis, Athens, Greece). Egg production begins in early November and continues through to June. No eggs are required after June, as the ambient sea temperature is at its peak (25°C) and larval survival in the hatchery falls rapidly at this point. Eggs are collected from the mass spawning by means of a screen (500µm) placed within outflow system (analogous to Zohar *et al.*, 1995). Fertilised eggs float, whereas non-fertilised eggs will sink to the bottom of the screen and can easily be separated. The screens are changed on a daily basis during the spawning season. Broodstock replacement is performed every two to three years by replacing 30% of each group with young male fish from the farm's on-growing units. Injured or diseased fish are the first to be replaced, followed by older fish regardless of sex. No routine monitoring of the sex ratio is performed.

The biology of gilthead seabream leads to two complications in the management of broodstock: firstly, the sex ratio of the broodstock changes over time, due to sex reversal in some fish, and secondly, in a mass spawning system, the actual number of fish spawning and an individual's contribution to the spawning is unknown. The sex ratio of the broodstock is determined primarily by social

factors and the age of the broodstock (Zohar *et al.*, 1995). The actual rate of reversal is unknown and may vary between broodstock groups, because of differences in the age structure, the effects of photoperiod manipulation and the behaviour of the fish. This has serious implications for the replacement of broodfish, especially in trying to achieve a balance in the sex ratio. Experiments have also shown that the timing of the introduction of new fish is also a determining factor in the subsequent sex ratio (Zohar *et al.*, 1995). The unknown contribution of broodstock in a mass spawning has consequences for the replacement of broodfish in that the relative performances of individual fish can not be established. Therefore, any replacement strategy is removing fish at random from the stock that could have either a positive or negative effect on spawning production or even the quality of eggs and offspring.

Most information on the process of sex ratio and rate of sex reversal in gilthead seabream has been based on the short-term experimental manipulation of small groups of fish. However, broodstock groups in commercial operations, involve large numbers of fish that must be managed on a long-term basis. The best way to gather information on the management of these stocks is to establish long-term monitoring procedures using individually tagged fish. Monitoring of the broodstock will allow the development of a suitable replacement policy for maximising egg production, and also increasing the reliability of egg production. The replacement rate of broodstock also determines the rate of genetic gain in a selection programme, so the frequency of replacement is an important aspect of the development of a breeding programme. This chapter details the monitoring of the Alkioni broodstock over a two-year period following formation of the broodstock groups and tagging of all the fish. The broodstock were also divided into groups under different photoperiod manipulation regimes and an assessment of the spawning characteristics in relation to the photoperiod was undertaken. Examination of the sex ratio and egg production over time should allow procedures for the replacement and management of the broodstock to be developed which maintain gamete production whilst maximising genetic gain.

## **3.2 Materials & Methods**

### **3.2.1 Broodstock Measurements**

All broodstock were measured in May 2000 at the time of PIT tagging. Subsequent annual measurements on the broodstock were collected for total length, body length and weight and also included the recording of the sex of each fish within a few days of the termination of spawning behaviour (dependent on the photoperiod regime).

### **3.2.2 Identification of Broodfish Sex – Observations**

This was achieved by applying pressure on the abdomen from the flanks and moving towards the anus. In male fish, this results in the release of sperm, whereas in females there will be no reaction. In this way male fish were positively identified and female fish identified by a negative result. Data was collected from two broodstock groups – natural and delayed photoperiods – over the three-year period (2000-2002). Both of these tanks contained 20 fish introduced in 2000 (new fish) and 40 existing fish of unknown age (old fish).

### 3.2.3 Identification of Broodstock Sex – Additional Evidence

Supporting evidence for the identification of sex was taken from the assignment of offspring to parental pairs within each broodstock group. Building up a matrix of these pairs (see section 2.5.1), sex could be allocated to fish on each side of the matrix by comparison to the observational data. This method, however, is limited to identifying only those fish that contributed offspring.

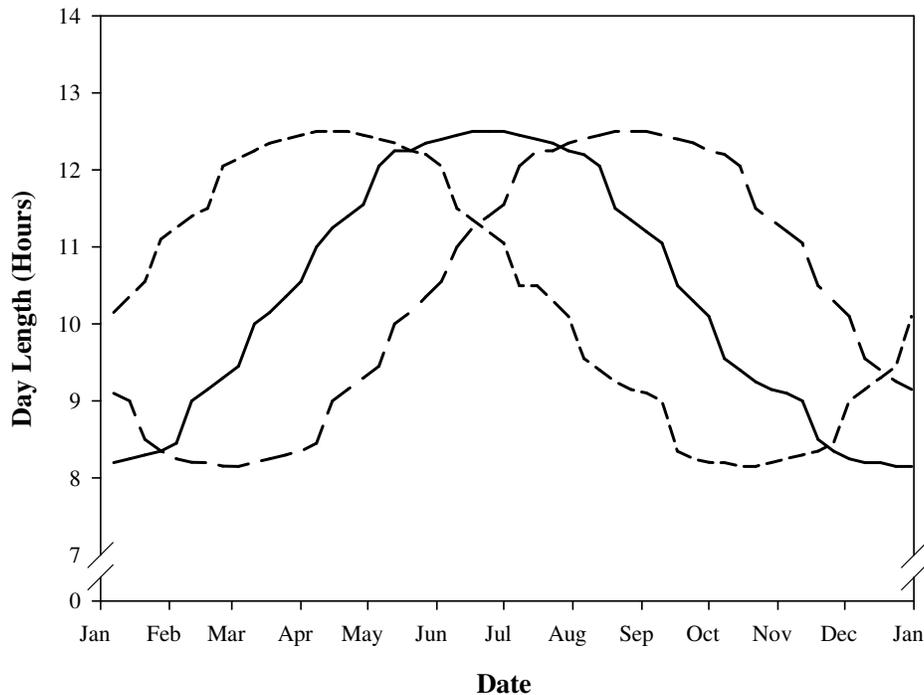
A further indicator of the sex ratio within each broodstock group was developed from the egg production data. Using an estimate of mean egg production of 40-80g per female per day (Morretti *et al.*, 1999), the number of females spawning at the point of maximum egg production was calculated. This value was then used to predict the ratio of males to females in the broodstock group. This method relies on a number of assumptions about the females, including that they all are the same weight, they all produce the same number of eggs and that this is constant over the spawning season.

### 3.2.4 Egg Production and Quality

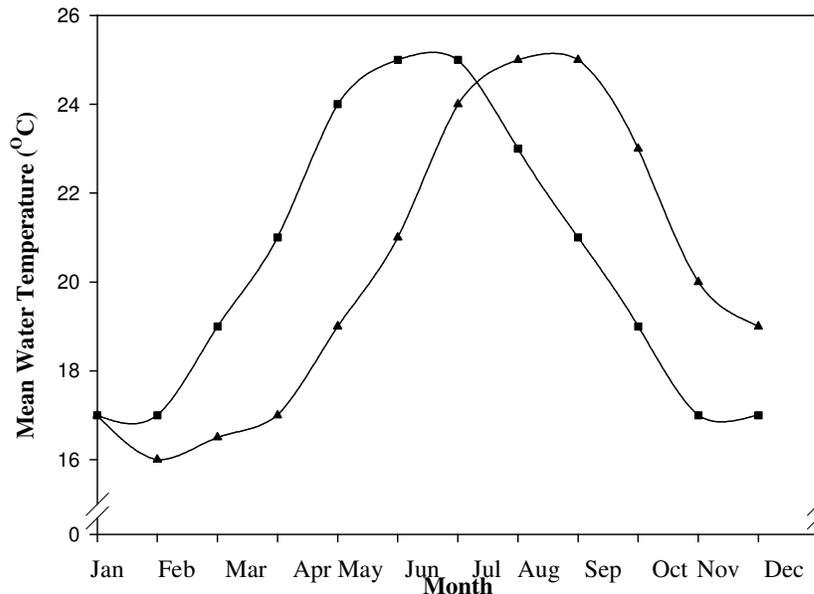
Records of egg production are collected by the farm staff on a daily basis and include weights ( $\pm 1$ g) of fertilised (floating) and non-fertilised (non-floating) eggs from each of the broodstock tanks. For this experiment, data was collected from each of the three photoperiod control groups: advanced cycle, natural cycle and delayed cycle. The light regimes and water temperatures used to manipulate the spawning time are presented in Figures 3.1 and 3.2. The natural photoperiod tanks receive no active environmental control, as this tank is located externally and is supplied by water directly from the borehole supply. The delayed tank receives photoperiod control, but water is also supplied directly from the borehole, as with the natural tank. The advanced photoperiod group is the only tank that is controlled through both photoperiod and water temperature.

Variations in light intensity between tanks were noted and are mainly due to the location of the tanks. The advanced and delayed groups have a constant 4-6 lux artificial light source, whereas the natural tank is external and receives direct sunlight for approximately one hour during the day. The peak light intensities in this tank were 48 lux during this hour but the mean outside of this time was 8.2 lux.

Measurements of egg quality (egg size) were collected in 2001 from three broodstock groups. Measurements were collected using a microscope graticule ( $\pm 0.05$ mm) in a stereo microscope (x20 magnification). Eggs were collected early in the morning from the collection unit in the broodstock tank outflow system. Several hundred floating eggs were collected in a Petri-dish along with approximately 5ml of water from the broodstock tank outflow. Measurements of the egg diameter were made from the outer edges of the egg case on 50 eggs per sample.



**Figure 3.1: Environmental manipulation regimes applied to the broodstock at Alkioni Fish Farms. Photoperiod control of day lengths: advanced photoperiod (---), natural photoperiod (—), delayed photoperiod (— —). Spawning corresponds to the period of short days (winter), beginning in November for the advanced group, January for the natural group and March for the delayed group.**



**Figure 3.2: Mean water temperatures of advanced (—■—) and natural (—▲—) photoperiod groups over season. Water temperature of the advanced group is controlled whereas the natural group receives ambient temperature water directly from a borehole supply. The delayed group also receives ambient water as the onset of spawning coincides with the natural low water temperature in March.**

## **3.3 Results**

### **3.3.1 Sex Ratio from Observational Data**

The ratio of males to females was generally close to a 1:1 ratio in both groups, as indicated in Figures 3.3 and 3.4. The natural group was found to be more stable in the ratio of males to females, as this was 1:1.04 in both years. The delayed group had an excess of males in 2002 resulting in a 1.66:1 ratio of males to females, in contrast to the previous year, which was 1:1.25 in favour of females. If the results are considered without the presence of the fish introduced in 2000 (new fish), the sex ratio of the existing fish in 2000 (old fish) is also generally balanced, with the exception of the delayed group in 2001. An equal division of sex in the older fish suggests that there is not a significant trend towards sex reversal from male to female with increasing age. The results of this experiment indicate that sex reversal was determined within the group as a whole although the numbers of each sex were unequal in two of the three groups. This statement is supported by the reversal of fish introduced in 2000 to the tanks as males. In both groups there is sex reversal of the new fish into females, involving six fish in the natural group in 2001. Although sex reversal is possible after 12 months of age, the frequency should be very low, due to the presence of older fish preferentially reversing into females. As the opportunity to undergo sex reversal appears to be equal for all fish in the group, it suggests that the social environment is the important factor in determining sex, rather than the physical status of the individual fish.

The rate of reversal in the two broodstock groups between 2001 and 2002 is summarised in Figure 3.5. In both groups, 60% of the broodstock do not display any sex reversal. The majority of reversing fish are older fish and a similar number of fish were found to reverse from male to female and female to male. The change from females to males has not been noted in this species before and, given the high frequency of the female to male reverses, raises doubts over the validity of the methodology of identifying the sex of fish in this study. Further analysis of the sex ratio of the broodstock can be inferred from egg production data and genotype data used in the assignment of offspring to parental pairs.

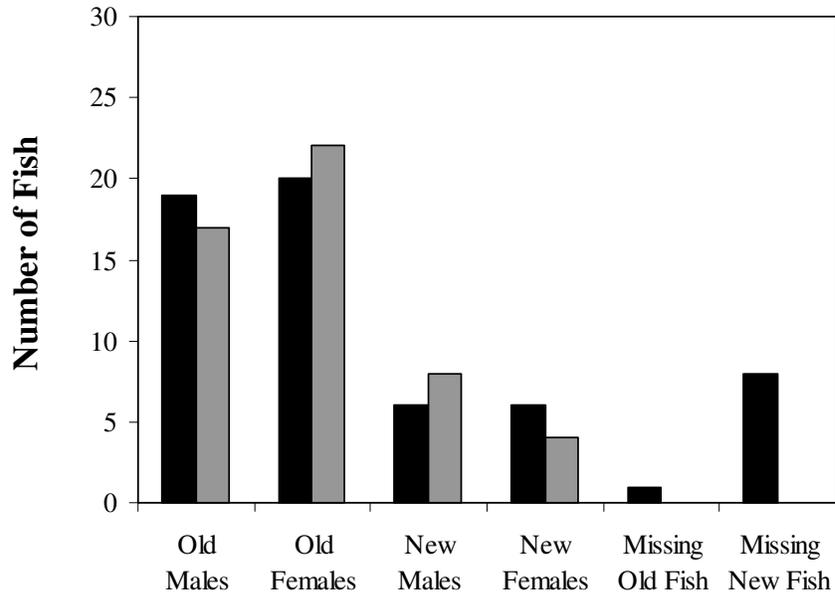


Figure 3.3: Number of males and females in natural group in 2001 (■) and 2002 (▒). New fish are those introduced to the broodstock in 2000, whereas old fish are those that were present in the broodstock prior to 2000. Missing fish are mortalities/ escapes incurred between sample points.

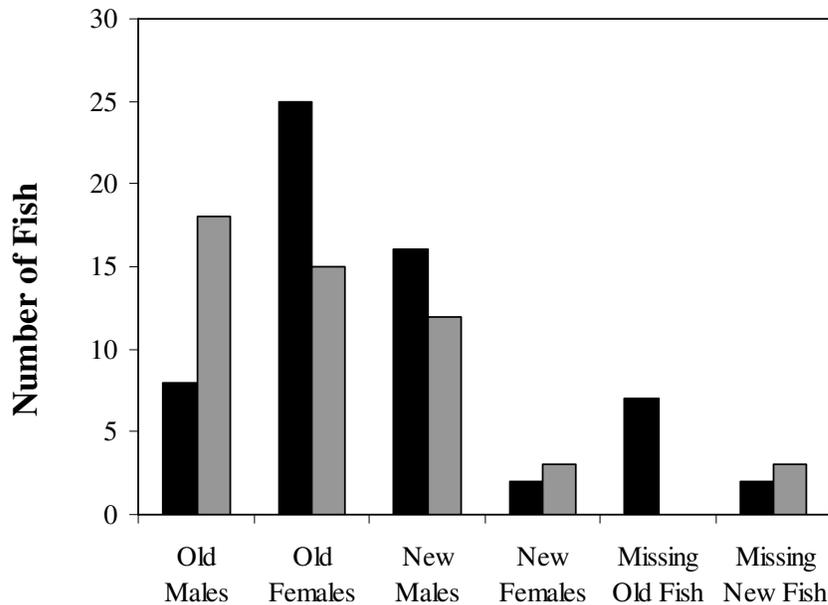
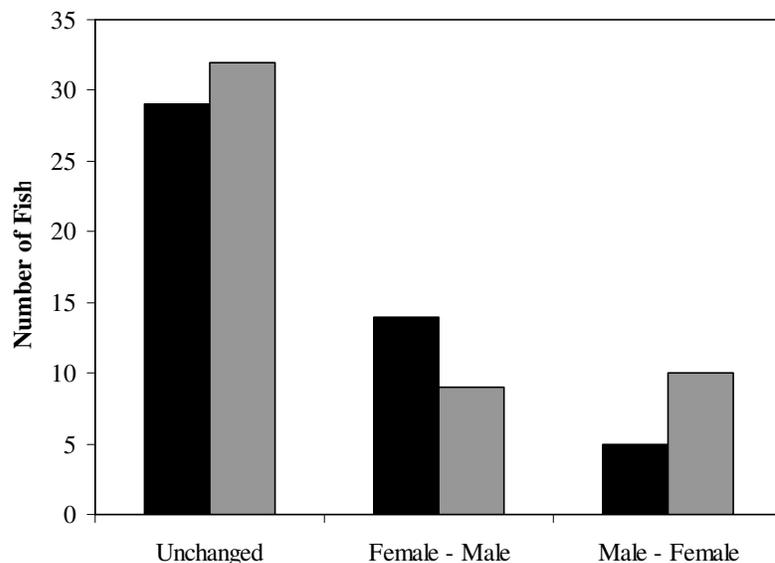


Figure 3.4: Number of males and females in delayed group in 2001 (■) and 2002 (▒). New fish are those introduced to the broodstock in 2000, whereas old fish are those that were present in the broodstock prior to 2000. Missing fish are mortalities/ escapes incurred between sample points.



**Figure 3.5: Summary of sex reversal in delayed group (■) and natural group (▒) between 2001 and 2002 as indicated by the observation method. Approximately 60% of the broodstock in both tanks did not undergo sex reversal during this period. Reversal from female to male was found to be as frequent as male to female reversals overall, but the former was more common in the delayed group.**

### 3.3.2 Additional Evidence for Sex Ratio

Table 3.1 contains the estimated number of females using the egg production method for the delayed and natural photoperiod groups. The results indicate that the low estimate of 40g eggs per kg body weight per day is a reasonable estimate in the 2001 season but overestimates the number of females in 2002 when egg production was generally much higher. In contrast, using the higher estimate (80g/kgBW/day) would seem to underestimate the number of females in 2001. As actual egg production levels of individual fish from this broodstock are unknown, this approach is only useful as a rough indication of the number of females contributing on a single day. However, using the low estimate in 2001 and the high estimate in 2002, the values are equal to a sex ratio of 3:1 (males: females) and 1:1 in the delayed group in 2001 and 2002, and 1:1 and 3:1 in the natural group. These results are subject to very large bias arising from the assumptions of egg production per female, but broadly support the view that the sex ratio in both groups was maintained at roughly equal proportions of males and females.

**Table 3.1: Estimation of number of females from maximum daily egg production. Low and high estimates of daily production taken from Moretti *et al.* (1999). All females are assumed to have a mean body weight of 1kg. Due to the difference in egg production levels between the 2001 and 2002 seasons the low estimate is more appropriate in 2001, whereas the high estimate is more applicable in 2002.**

Group/Year	Maximum daily egg production	Number of females (Ratio of M:F)	
		Low estimate (40g/kg/day)	High estimate (80g/kg/day)
Delayed 2001	600g	15 (3:1)	7 (8.5:1)
Delayed 2002	2400g	60 (0:1)	30 (1:1)
Natural 2001	1000g	24 (1.5:1)	12 (5:1)
Natural 2002	1450g	36 (1:1.6)	18 (3.3:1)

Using a parental matrix, it was possible to designate each parent as male or female based on the shared progeny with other broodfish. However, not all fish contributed to spawning and only the fish with offspring can be assigned sex using this method. The results of the assignment method are presented in Table 3.2 and are contrasted with the sex ratio data observed on the fish at capture. The assignment method indicates that the number of females was comparable between the two approaches, but the number of males was much lower. This may be due to differences in the success of males in contributing to spawning, but this will be discussed in chapter 7 in relation to the effective population size resulting from mass spawning. The ratio of males to females from the assignment data favoured females at a rate of 1.4-1.8:1 over the groups and two years. This again suggests that the sex ratio of the broodstock was close to a 1:1 ratio over the monitoring period.

Of more significance in the comparison of the two methods is the number of mismatches between observed sex and sex allocated from the parentage of offspring by genotyping, also shown in Table 3.2. The observed sex was used as the reference point because data was available for all fish. However, the genotype method was considered more reliable because of the high confidence in the assignment of offspring. The number of mismatches were similar between the groups and years, which suggests there was an intrinsic error rate in the observation method of approximately 30 – 40%. An inherent weakness of the observation method seemed to be the misidentification of females as they were frequently allocated as males by releasing fluids when pressure was placed on the abdomen. The fewer number of misidentified males indicates that the number of spent or non-spermiating males, was generally low even at the end of the season.

As many of the same fish contributed in both years, a large proportion of the stock could not be identified because of their non-contribution. Of the fish that could be assigned in both years in the natural photoperiod group, only two were found to have undergone sex reversal from male to female and none in the opposite direction as was suggested by the observational data.

**Table 3.2: Sex ratio of the broodstock determined by genotype data. Sex was allocated using a parental matrix following assignment of offspring to parental pairs. Mismatches between the assignment method and the observed sex of fish at capture indicate that the observed data had an error rate of 30-40%. More errors in the identification of females were noted. In the assigned sex data for the natural tank only two fish were found to have undergone sex reversal in 2002.**

Group/ Year	Observed		Assigned		Mismatch Observed-assigned
	Male	Female	Male	Female	
Delayed 2002	30	18	9	17	2 males, 9 females
Natural 2001	25	26	13	21	3 males, 7 females
Natural 2002	25	26	13	18	5 males, 9 females

### 3.3.3 Broodstock Growth

Tables 3.3 and 3.4 detail the growth of broodfish in the delayed and natural photoperiod groups, respectively. In general, the growth of fish in the delayed group was greater than that observed in the natural group for both the new and old fish. Weight differences between males and females were apparent in both of the groups studied, but they indicate conflicting results, possibly due to errors in sexing. In the delayed group, males are generally larger or similar sized, whereas in the natural group, females tend to be significantly larger (t-test,  $p < 0.008$ ,  $p = < 0.002$ ; Old fish in 2001 and 2002, respectively). If the mean body length is substituted for weight, then the pattern is similar in the natural group, but the mean lengths in tank two are almost identical between males and females. Sex of fish within Tables 3.3 and 3.4 was based on the observational sex data and errors in the sexing of fish, especially in the delayed group where the observed sex ratio was slightly uneven, could reduce the validity of comparisons between the sexes.

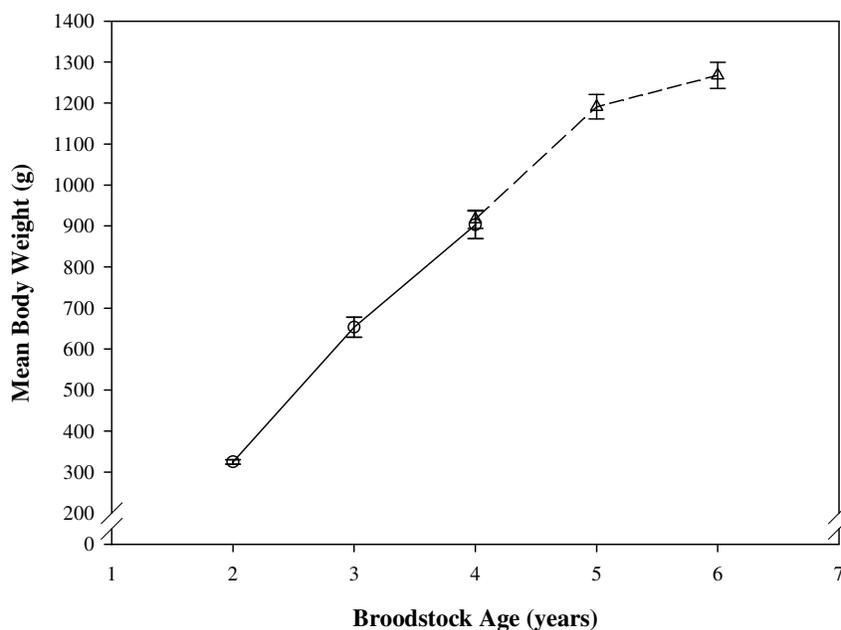
The growth of the new fish over the three-year period can be used as a predictor of the age of the old fish already present in the stock. New fish were introduced at two years of age and reached a mean weight of 903g by four years of age. Older fish in 2000 had an overall mean weight of 916g. This indicates that the old fish were, on average, four years old in 2000. As no farm records exist on these fish, the actual age structure is unknown, but the level of size variation measured in 2000 is similar to that seen in the new fish at four years of age. By combining data from the natural and delayed group, a plot of weight against age (Figure 3.6) for the new and old fish indicates a linear growth rate up to age five, before appearing to level-off by six years of age. However, this evidence needs to be treated with caution as the relationship between age and weight may not be linear over the lifespan of the fish, a fact emphasised by the slow growth of the oldest fish indicated in Figure 3.6. The maximum size of gilthead seabream is thought to be 70cm total length and 2.5kg body weight (Frimodt, 1995).

**Table 3.3: Summary of mean length, weight and growth (weight increase) measurements in delayed photoperiod group. All measurements  $\pm$ SEM. Calculations are divided between the new fish – the 20 fish introduced in 2000 – and the old fish – the existing 40 fish present in the stock in 2000. Mortalities in 2001 were 2 new fish and 7 old fish; in 2002, 3 new fish were lost. Measurements and observed sex data (not recorded in 2000) were collected at the end of each spawning season.**

Age Group	Sex	2000			2001			2002		
		Mean Weight (g)	Mean Length (mm)	No. of Fish	Mean Weight (g)	Mean Length (mm)	No. of Fish	Mean Weight (g)	Mean Length (mm)	No. of Fish
New Fish	Male	–	–	16	702 $\pm$ 29.73	280 $\pm$ 3.66	12	1005 $\pm$ 53.87	310 $\pm$ 8.20	12
	Female	–	–	2	700 $\pm$ 0.00	280 $\pm$ 10.00	3	917 $\pm$ 17.48	310 $\pm$ 2.89	3
	All	319 $\pm$ 8.41	212 $\pm$ 1.38	18	702 $\pm$ 26.33	280 $\pm$ 3.34	15	987 $\pm$ 43.82	310 $\pm$ 6.52	15
Old Fish	Male	–	–	8	1392 $\pm$ 64.57	350 $\pm$ 6.27	18	1399 $\pm$ 49.66	360 $\pm$ 4.29	18
	Female	–	–	25	1332 $\pm$ 42.22	349 $\pm$ 4.23	15	1400 $\pm$ 67.86	358 $\pm$ 5.43	15
	All	951 $\pm$ 31.01	324 $\pm$ 6.14	33	1347 $\pm$ 35.42	349 $\pm$ 3.50	33	1399 $\pm$ 40.38	359 $\pm$ 5.36	33

**Table 3.4: Summary of mean length, weight and growth (weight increase) measurements in natural photoperiod group. All measurements  $\pm$ SEM. Calculations are divided between the new fish – the 20 fish introduced in 2000 – and the old fish – the existing 40 fish present in the stock in 2000. Mortalities in 2001 were 8 new fish and 1 old fish. Measurements and observed sex data (not recorded in 2000) were collected at the end of each spawning season.**

Age Group	Sex	2000			2001			2002		
		Mean Weight (g)	Mean Length (mm)	No. of Fish	Mean Weight (g)	Mean Length (mm)	No. of Fish	Mean Weight (g)	Mean Length (mm)	No. of Fish
New Fish	Male	–	–	7	605 $\pm$ 37.44	264 $\pm$ 6.94	8	730 $\pm$ 30.95	290 $\pm$ 5.38	
	Female	–	–	5	543 $\pm$ 51.73	259 $\pm$ 8.30	4	932 $\pm$ 24.85	328 $\pm$ 5.15	
	All	330 $\pm$ 6.90	217 $\pm$ 2.02	12	579 $\pm$ 39.25	261 $\pm$ 5.19	12	797 $\pm$ 35.86	303 $\pm$ 6.64	
Old Fish	Male	–	–	19	976 $\pm$ 53.59	321 $\pm$ 7.22	17	1038 $\pm$ 35.03	338 $\pm$ 4.10	
	Female	–	–	20	1136 $\pm$ 35.49	339 $\pm$ 3.58	22	1246 $\pm$ 58.87	349 $\pm$ 4.93	
	All	882 $\pm$ 31.84	319 $\pm$ 3.46	39	1058 $\pm$ 33.98	330 $\pm$ 4.18	39	1155 $\pm$ 38.84	342 $\pm$ 3.50	



**Figure 3.6: Broodstock weight by age plot. Data combined from three consecutive years of measurements on new fish (—○—) (n=32) and old fish (—△—) (n=72) in the delayed and natural photoperiod groups  $\pm$  SEM. The mean weight of the four-year old new fish corresponds very closely to the mean weight of the old fish in 2000. The growth rate of the old fish appears to level-off after five years of age.**

### 3.3.4 Egg Production and Quality

Total egg production from each broodstock group was greater in 2002 than in 2001 (Table 3.5). The values of mean fertilised eggs per day were also significantly higher in 2002 ( $P < 0.001$ , all groups). This can be attributed to the increased mean weight of the stock between the two seasons and also to an increased acclimatisation to the photoperiod regimes set up in 2000. However, the duration of the spawning season was very similar between years for delayed and natural groups, indicating that these tanks were well adjusted to their respective light regimes. In contrast, the advanced group showed an extension of the spawning period by over four weeks in 2002, mainly in the latter part of the season, resulting in a spawning period over 30% longer than the natural group. Correspondingly, the total egg production from the advanced group was three times greater in 2002, compared to 2001, and was double the 2002 production of the delayed and natural groups. Manipulation of the photoperiod and water temperature were the most likely factor for the large differences observed in egg production between the tanks as the age structure, feeding regime and holding conditions of the three broodstock groups were identical. Genetic differences between broodfish may also be a factor but cannot be tested without individual records, which were unavailable in the current study.

**Table 3.5: Summary of spawning performance in the broodstock groups in 2001 and 2002 indicated by length of the spawning period, spawning dates, total egg production and mean fertilisation rate. The composition of the stock was identical between tanks suggesting the differences are due to the photoperiod manipulation.**

	<b>Parameter</b>	<b>Advanced Group</b>	<b>Natural Group</b>	<b>Delayed Group</b>
<b>2001</b>	Spawning Duration	158 days	147 days	123 days
	Season Dates	11/11/00 to 19/04/01	05/01/01 to 02/06/01	07/02/01 to 10/06/01
	Total Egg Production	43141g	35505g	10492g
	Mean Fertilisation Rate $\pm$ SEM	0.631 $\pm$ 0.03	0.474 $\pm$ 0.04	0.932 $\pm$ 0.02
	Mean Fertilised Eggs/ Day	172.3g	114.5g	79.5g
<b>2002</b>	Spawning Duration	193 days	143 days	131 days
	Season Dates	31/10/01 to 13/05/02	14/01/02 to 07/06/02	13/02/02 to 24/06/02
	Total Egg Production	127122g	65440g	65570g
	Mean Fertilisation Rate $\pm$ SEM	0.64 $\pm$ 0.01	0.59 $\pm$ 0.02	0.454 $\pm$ 0.03
	Mean Fertilised Eggs/ Day	421.5g	270.0g	227.2g

The number of floating eggs in the total daily egg production can be used as a measure of egg quality in gilthead seabream (Carnevali *et al.*, 2000). Floating eggs represent those that have been successfully fertilised, whereas eggs that sink have not been fertilised or may have been damaged or infected by pathogens. The mean fertilisation rate in the three photoperiod groups (Table 3.5), indicates that egg quality was consistent between years in the advanced group, whilst increasing in the natural group. The delayed group appears to have a greater fertilisation rate in 2001. However, this is concluded to be due to the failure to collect adequate data on the number of sinking eggs in the daily production during the early part of the season. Egg quality seems to be greater in the advanced group and corresponds to highest production levels and greater proportion of fertilised eggs per day observed within the three stocks. This suggests that the advancement of the photoperiod increases both egg production and quality in gilthead seabream, whereas, the delayed photoperiod reduces egg quality and production.

The pattern of egg production over the spawning season was consistent between all tanks and was characterised by a peak of production towards the middle of the spawning period (Figures 3.7-3.12). Gilthead seabream is a sequential spawner and the ovaries contain eggs at all maturation stages, allowing mature eggs to be released each day. The increase in production towards the middle of the spawning

season could be due to either an increased number of females spawning, or an increase in the egg production of each female.

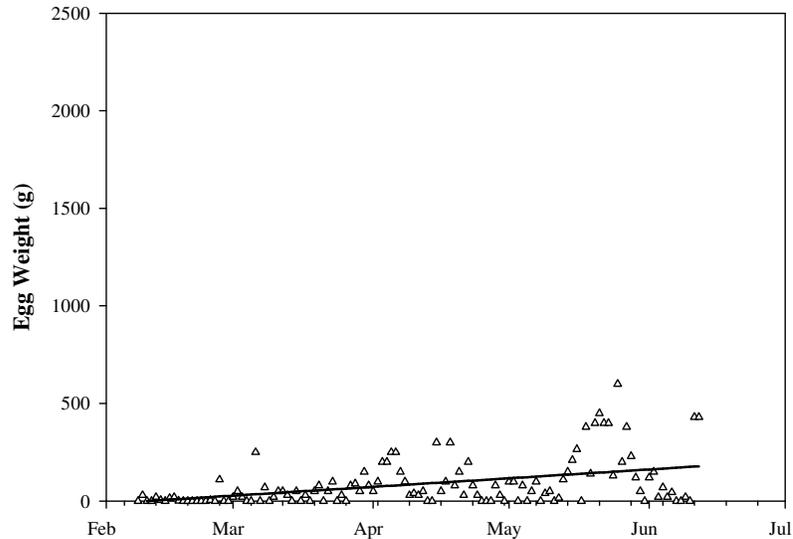


Figure 3.7: Daily egg production from delayed photoperiod group in 2001. Regression model indicates a linear relationship:  $\text{Egg weight} = -3.0 (\pm 19.9) + 1.461 (\pm 0.276) \times \text{Day}$ . Where day is number of days from first spawning.

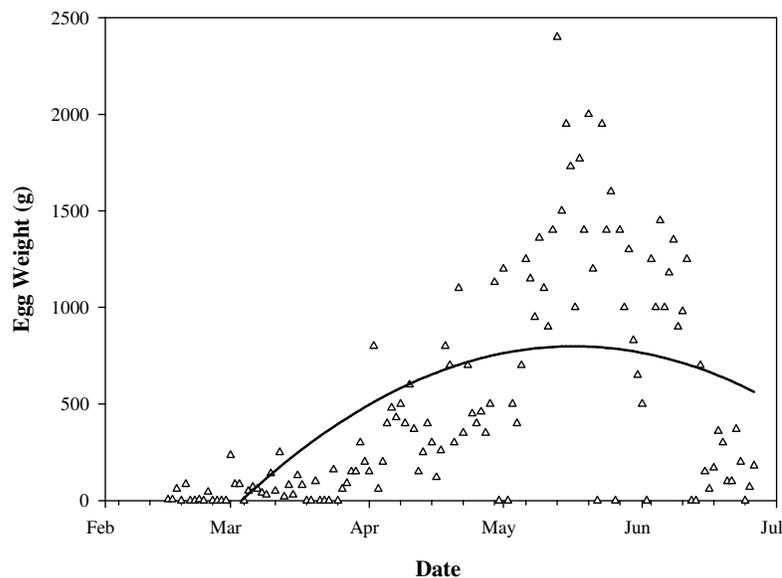
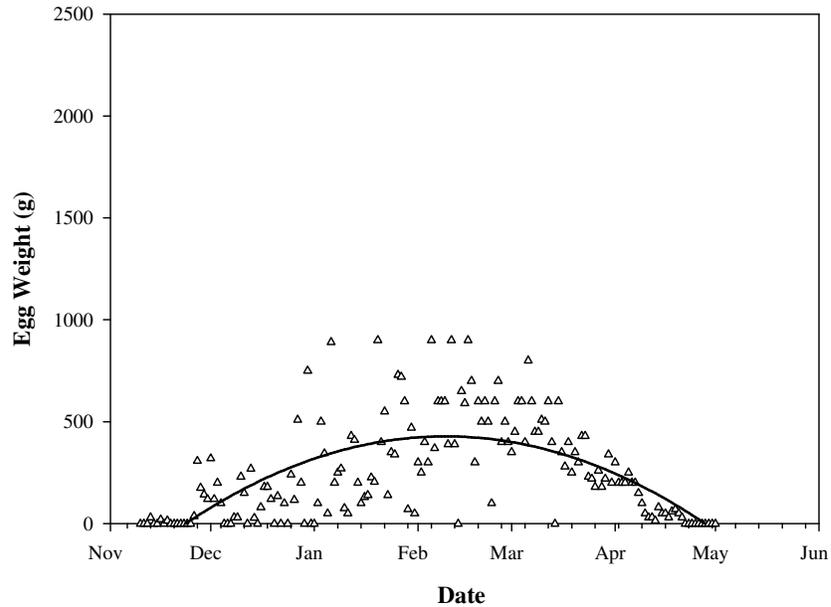
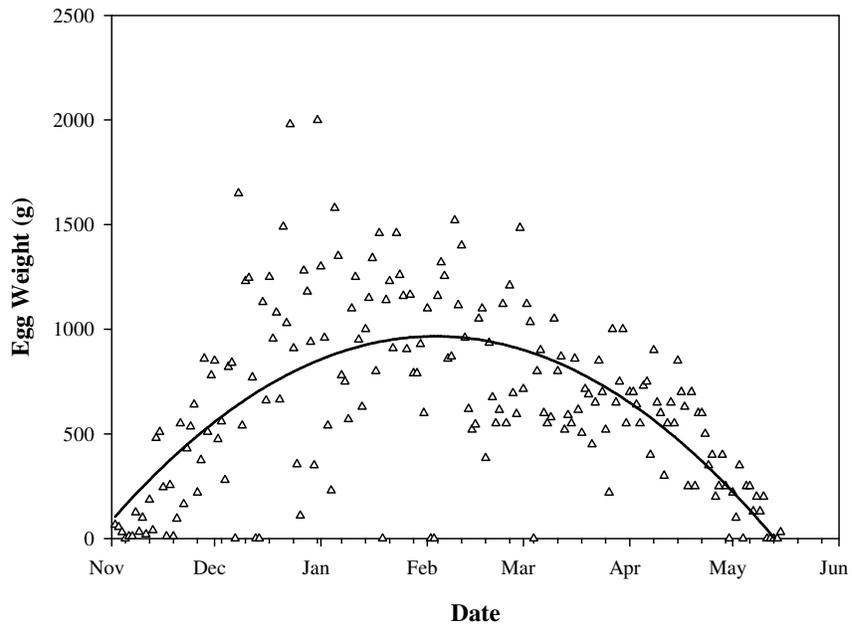


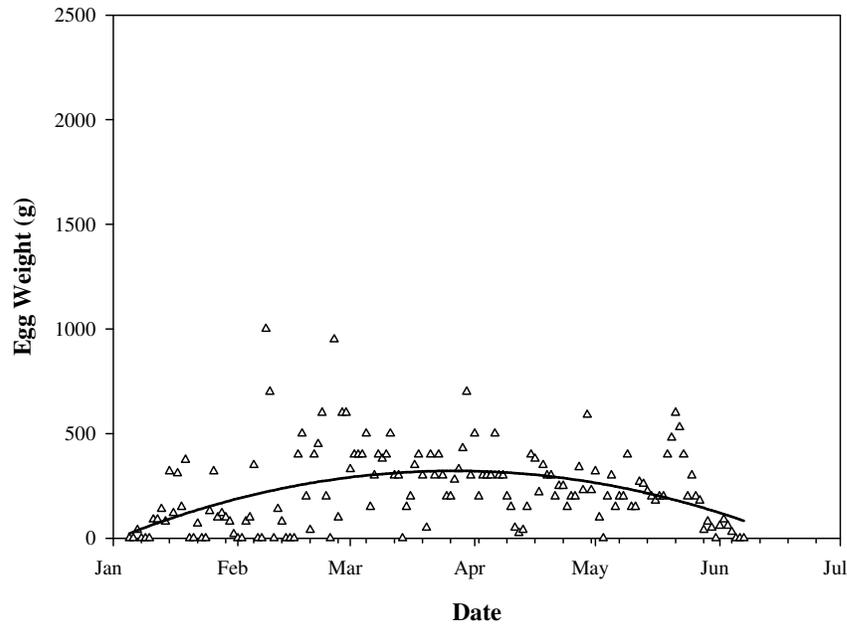
Figure 3.8: Daily egg production from delayed photoperiod group in 2002. Regression model indicates a quadratic relationship:  $\text{Egg weight} = -418 (\pm 121.0) + 26.54 (\pm 4.19) \times \text{Day} - 0.1447 (\pm 0.0305) \times \text{Day}^2$ . Where day is the number of days from first spawning.



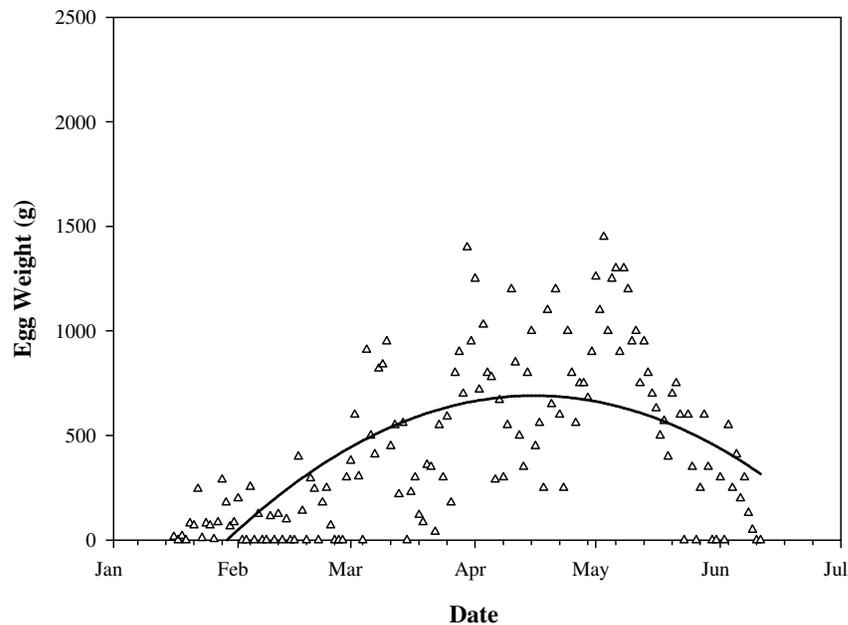
**Figure 3.9: Daily egg production from advanced photoperiod group in 2001. The regression model indicates a quadratic relationship: Egg weight =  $-160.9 (\pm 41.9) + 13.23 (\pm 1.213) \times \text{Day} - 0.07371 (\pm 0.006) \times \text{Day}^2$ . Where day is the number of days from first spawning.**



**Figure 3.10: Daily egg production from advanced photoperiod group in 2002. The regression model indicates a quadratic relationship: Egg weight =  $-86.7 (\pm 72.4) + 18.65 (\pm 1.71) \times \text{Day} - 0.09883 (\pm 0.008) \times \text{Day}^2$ . Where day is the number of days from first spawning.**



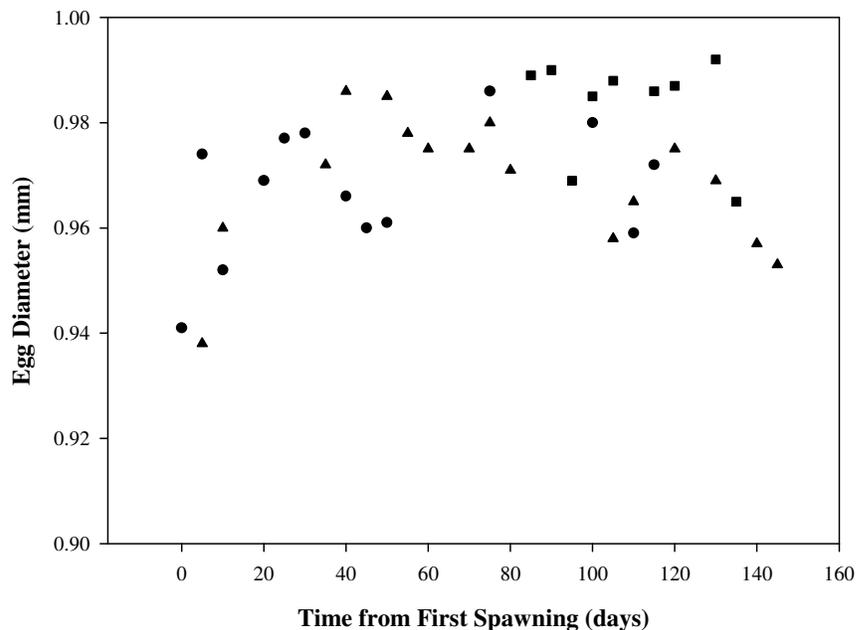
**Figure 3.11: Daily egg production from natural photoperiod group in 2001. Regression model indicates a quadratic relationship:  $\text{Egg weight} = -33.6 (\pm 43.1) + 7.22 (\pm 1.30) \times \text{Day} - 0.04532 \times \text{Day}^2$ . Where day is the number of days from first spawning.**



**Figure 3.12: Daily egg production from natural photoperiod group in 2002. Regression model indicates a quadratic relationship:  $\text{Egg weight} = -280.5 (\pm 75.9) + 21.42 (\pm 2.37) \times \text{Day} - 0.1181 (\pm 0.0155) \times \text{Day}^2$ . Where day is the number of days from first spawning.**

Modelling of the data by regression analysis is also plotted in Figures 3.7 – 3.12 and indicates a strong quadratic effect ( $p < 0.001$  for all tanks and years). The data concerning the delayed group in 2001 (Figure 3.7) reveals a linear relationship of egg production over time. However, the spawning period of this tank was interrupted in order to measure the broodfish and this resulted in the termination of spawning towards the middle of the season when production was at its greatest. Allied to the pattern of egg production, the daily fertilisation rate of eggs also increased towards the middle part of the season with a strong quadratic effect ( $p < 0.001$ ) (data not shown). The mean daily fertilisation rate was 45% in the delayed group, 64% in advanced group and 59% in natural group.

Measurements of the mean egg diameter over the 2001 spawning season indicate that within-broodstock group variation was generally very small, as shown in Figure 3.13. There was a general trend towards smaller eggs early on, and at the end of the spawning season. However, even these differences were slight, being less than a 0.1mm difference in mean egg diameter between the highest and lowest data points. The data is not modelled as each point represents a mean egg diameter measured from a sample of eggs from each broodstock group. The stability of egg size suggests that there are no negative effects of the photoperiod regimes on egg size and that environmental disturbances, such as temperature changes, are minimal. The stable egg size also indicates that there are no nutritional deficiencies within the females, which may be indicated by a fluctuating egg size.



**Figure 3.13: Mean egg diameter in the 2001 spawning season measured in advanced (■), natural (▲) and delayed (●) photoperiod groups. The lowest egg diameter was observed at the beginning and end of the respective seasons. There were no differences between photoperiod regimes.**

## **3.4 Discussion**

### **3.4.1 Sex Ratio in Gilthead Seabream Broodstock**

The observed sex ratio data produced unexpected results, in that fish were apparently reversing from female back to male fish. Examples were found in both the older and the new fish. It has been established that female is the terminal sex in gilthead seabream and, although fish return to an ambisexual phase out of the spawning season, they will remain female (Costandinos Mylonas, pers. comm.). However, during this ambisexual stage, the gonads consist of both ovary and testes and indeed, some fish are found to possess both functioning male and female gonads simultaneously (Kissil *et al.*, 2001; Happe and Zohar, 1988). This suggests that there are no physical barriers to becoming either sex in any particular season, while the very nature of social control of sex reversal points out that the reproductive success of each sex can change over the short term (Godwin *et al.*, 2003). In other species with social control of sex reversal, such as *Labriodes* spp., multiple reversals have been observed (Robertson, 1972) and can be very rapid in response to a change in social structure (Robertson, 1972; Warner and Swearer, 1991).

The genotype data, however, did not support the evidence for these secondary, female-male reversals and the sex of several fish was misidentified by the observational method. The identification of only two sex reversing fish in 2002 through the genotype data suggests that the rate of reversal may be lower than that suggested by observations, although only breeding individuals could be identified. Despite this limitation, the genotype data is the more reliable as it is based on offspring assignment at high confidence levels. The only firm conclusion that can be drawn from both the genotype and egg production evidence, is that the sex ratio is sufficient to produce large numbers of eggs with good rates of fertilisation in both years. The genotype data indicates a greater number of females but this could be due to the dynamics of the group spawning behaviour that allows particular males to dominate the fertilisation of eggs. The introduction of new fish did not have a negative impact on egg production, although few of the new fish contributed. The contribution of the new fish will be discussed in greater detail in Chapter 7.

The sex ratio in the natural group using the observation method was identical in 2001 and 2002, and the same as the normal 1:1 ratio observed by Zohar *et al.* (1995) in his experiments of sex reversal in gilthead seabream. This indicates that, despite the potential for errors in the methodology of sexing the fish, the results comply with those that would have been expected. The results in the delayed group show that, in 2002, the number of male fish far exceeds the numbers of females and leads to the conclusion that the method of sexing has two sources of error. Firstly, non-spermiating and spent males may be misidentified as females if no sperm is released, while secondly, females can be misidentified as males by the release of urine or fluids when pressure is applied to the abdomen. As the fish used in this experiment were part of a commercial broodstock, there was no opportunity for sacrificing fish in order to positively identify the sex by observing the gonads. In gilthead seabream, there are only minor external indicators of sex, such as colouration differences and a tendency for females to be bulkier in their stature. However, these are not reliable indicators, especially at the end of the

spawning season when fish are returning to the ambisexual stage. As indicated by the use of genotype data the errors of the observed data were high and included misidentification of males and females. One possible improvement to the methodology could be to collect a sample of any fluid produced by the fish and to examine it for the presence of sperm under a high power microscope. This should eliminate one source of misidentification by positively identifying all fish producing sperm. Alternatively, ultrasound can be used to examine fish for the presence of mature ovaries (Blythe *et al.*, 1994a; Martin-Robichaud and Rommens, 2001).

The introduction of new fish into the broodstock in 2000 gave an opportunity to follow these fish as they grew and became established with the broodstock groups. Initially these fish were all males, introduced at 24 months of age. The rate of sex reversal of these fish was higher in the natural group than in the delayed group in both years, suggesting that there were more old females in the delayed group and, therefore, the new fish remained as males. In the natural group, the balance between old males and females and new males and females was almost equal. These observations indicate that the sex ratio of the group is determined between all the fish, regardless of age or size and that young fish are capable of sex reversal to females at a rate determined by the social circumstances. The growth of the new fish was generally similar to the older fish in 2001, but greater in 2002. Differences in growth rate between male and female new fish were observed between 2000 and 2001, but growth between 2001 and 2002 was similar. Due to the small sample sizes, however, these values are possibly subject to large errors. One problem noted in the monitoring of the broodstock was that a large number of fish were lost, mainly from the new fish, in 2001. Mortalities are normally recorded, so these fish are presumed to have escaped the holding facilities by jumping out of the tanks. This represents a major loss to the stock (15% in the natural and delayed groups) and as these fish cannot be replaced until the end of the season, also a potential loss in egg production and fertilisation rate.

### **3.4.2 Photoperiod Manipulation and Egg Production**

The success of the manipulation of spawning time can be measured from the total egg production and the duration of the spawning period. The advanced spawning group produced the greatest amount of eggs and had a significantly longer spawning period than the other regimes. This group also showed the greatest increase in spawning duration in 2002, by an additional 35 days, mainly towards the end of the period. The natural and delayed groups showed no significant extension to the spawning period in 2002, which suggests that the manipulation of the advanced group may have been compromised by exposure to ambient light during the spawning season, thereby extending the spawning period. The light intensity measured in the internal broodstock facilities was 4-6 lux which is far below even the 20 lux minimum recommended by Bromage *et al.* (2001) for the successful manipulation of spawning. Improvements to the broodstock facilities are needed if the accurate control of photoperiod regime is to be achieved.

Mean egg diameter showed little variation over the season and was very similar within each broodstock group. Egg size has been found to decline over the season in other sparid species, such as red porgy, in response to increased water

temperature (Mihelakakis *et al.*, 2001). Egg volume may be a more indicative measure of egg size as small differences in diameter can have large effects on volume. The similarity in egg size between the photoperiod groups suggests that the manipulations of the spawning time have no effect on egg size, as other variables such as broodstock age and feeding regimes were identical between groups. Egg quality, as measured by the number of fertilised eggs, was found to be greater in the advanced group which is in contrast to studies in other species. Egg quality has been found to reduce under advanced photoperiods (Bon *et al.*, 1999; Hansen *et al.*, 2001) and increase with delayed cycles (Bromage *et al.*, 1984; Carrillo *et al.*, 1989). Similarly, advanced photoperiods have resulted in a reduced fecundity of the broodstock in pink salmon (Beacham *et al.*, 1994), although this has not been found in species such as rainbow trout (Bromage *et al.*, 1984) or striped bass (Blythe *et al.*, 1994b). The reason for the greater egg quality (as measured by fertilised eggs/day) in the advanced group is unknown but may reflect an optimal sex ratio or the presence of large males that are able to fertilise larger egg batches more successfully than the other broodstock groups. No sex data could be obtained from the advanced group in 2002 to confirm such a hypothesis.

In this experiment, egg production of the advanced group is almost double that of the natural and delayed groups and cannot be fully explained by the extended spawning period. The age structure of the group is also identical to the broodstock groups and there are no differences in the average size of fish. The pattern of egg production reflects that seen in other sequential spawners such as red porgy (Mihelakakis *et al.*, 2001) in that there is an initially slow production rising to a peak in the middle of the season, before progressively reducing again. Such a pattern could indicate that the number of spawning fish increases over the season to reach a maximum and then reduces again or that each individual's egg production can be increased towards the middle of the season.

Whether there is a periodicity to an individual's spawning pattern is unknown. However, it is unlikely that a single fish is able to spawn for the full 193 days, observed in the advanced group. Kadmon *et al.* (1985) observed that some fish terminate spawning sooner than others whilst some fish move between different stages of maturation throughout the season. Frequent gaps in egg production (i.e. days with no eggs) were noted and indicate that daily production may falter, although the exact reason is unknown. Periodicity in spawning has also been noted in gilthead seabream and other species, such as Atlantic halibut and Atlantic salmon, mainly as a result of the long maturational process of eggs (McEvoy and McEvoy, 1992).

### **3.4.3 Broodstock Replacement**

Observations on the sex ratio of broodstock groups can provide important information to determine the methods and frequency of broodstock replacement. The results presented here indicate that sex reversal in the broodstock will approach a 1:1 relationship, regardless of the number of fish replaced, because the sex ratio appeared to be determined in the group as a whole. This supports the view that the sex ratio is socially determined in this species, although the exact mechanism is unknown. If new fish are introduced at the appropriate time (end of the spawning season), the sex ratio should always approach a balanced ratio of

males and females. This should also be true in instances when the whole group is replaced, as beyond two years of age the fish are capable of sex reversal to become female (Zohar *et al.*, 1995). One possible drawback to replacing an entire group is that the egg production is likely to fall, due to the absence of large females, who are able to produce large quantities of eggs. Maintaining an age structure with older fish and new fish together is more reliable in commercial stocks where egg production is critical. This could be achieved by replacing a third of the stock each year at the end of the season. In this system the stock could be completely replaced in three years allowing a rapid genetic improvement whilst protecting egg production capacity.

The balance between replacement rate and egg production becomes more important when a selection programme is initiated, as genetic gain will be directly influenced by the replacement rate of the broodstock. A trade-off between replacement rate, generation interval and the intensity of selection also needs to be made.

### **3.5 Conclusions**

The sex ratio of broodstock, using direct observations at the end of the spawning season, was found to balance in the natural photoperiod group in both years, but favoured males in the delayed group in 2002. Observational data was found to be an unreliable method when compared to genotype evidence of sex especially in estimating the number of reversals from males to females. Re-reversals of females to males were observed but were not supported by genotype assignment of sex. The introduction of new fish did not have a negative effect on egg production. Growth of the broodstock was significantly higher in the natural group compared to the delayed group and there were only significant differences between males and females in the natural group. Egg production was higher in 2002 in all photoperiod groups and showed a similar pattern of production in all groups in both years. An extension to the spawning period in the advanced group in 2002 suggests a weakening of the photoperiod regime. Egg quality was greater in the advanced group in both years but egg size was similar in all groups.

**Chapter 4:**  
**Size Variation in the Larval**  
**Stages of Gilthead Seabream**

## **4.1 Introduction**

Substantial size variation in intensively reared populations of gilthead seabream can be evident even in the early stages of the larval rearing period (Goldan *et al.*, 1997). This type of variation occurs in many other fish species, both marine and freshwater, and within natural populations. The term ‘growth dispensation’ has been used to describe the differences in growth rate between individual fish that results in an increase in size variation over time (Magnuson, 1962; Umino *et al.*, 1997). Culturing species that display growth dispensation can lead to additional complications in their management, because a proportion of the population will grow more quickly and may have different requirements – such as environmental conditions and feed type – to the remainder of the population. Farmed juvenile and adult fish are often stocked at high densities. Under these conditions, size variation can lead to substantial losses through aggression and cannibalism. At older stages, fish can be divided into specific size-sorted groups by grading. However, prior to metamorphosis – approximately on day 47 post-hatch in gilthead seabream – size variation is difficult to control and may seriously affect the productivity of the farming system.

Size variation may potentially lead to difficulties in the operation of a breeding programme, because size differences – due to environmental variation between rearing tanks – would confound the selection for genetic merit at the juvenile stage. Therefore, an examination of the origins of size variation and its development through the larval rearing stage of the farming system, is essential in developing control strategies and assessing the impact on selection protocols.

### **4.1.1 Environmental Factors Affecting Growth**

The growth of fish, especially in the larval stages, is strongly influenced by environmental factors, which can modify the development, growth and survival of individuals. The effect of temperature on growth has been demonstrated in a range of marine species, including Atlantic cod (Bjornsson & Steinarsson, 2002), sea bass (Ayala *et al.*, 2001), halibut (Jonassen *et al.*, 1999) and plaice (Hovenkamp and Witte, 1991). Temperature increases the metabolic rate and sub-optimum temperatures can slow down growth and development sufficiently, to extend the time taken to reach metamorphosis (Benoit and Pepin, 1999). Photoperiod reduction has a limiting effect on growth in halibut (Jonassen *et al.*, 2000), but in salmon it can serve as a trigger for the development of disparate growth rates in juveniles as they prepare for over-wintering strategies (Skilbrei *et al.*, 1997).

The effect of fish density can act to limit growth, by increasing competition for food (Baskerville-Bridges and Kling, 2000; Ruzzante and Doyle, 1990) and the frequency of social interactions (Holm *et al.*, 1990). In farm conditions, many environmental factors can be manipulated, including temperature, fish density, feed type and availability. Yet, significant levels of variation are still found within individual rearing tanks and even within sibling groups (Metcalf *et al.*, 1992). Food availability in hatchery conditions is normally carefully managed and optimised for the population size. Nonetheless, variability in the size and nutritional content of food particles has been cited as a cause of differences in ingestion rates within larval farm populations (Goldan *et al.*, 1998). Feed density has also been found to have a direct influence on the success of first feeding in

gilthead seabream (Parra and Yúfera, 2000). Growth differences could originate from an external factor, such as localised temperature differences, feed availability and the nutritional content of particles. However, for the levels of variation to develop and increase to those observed in many cultured populations and on such a consistent basis, there must be additional physiological and behavioural processes involved.

#### **4.1.2 Parental Effects on Growth**

Size variation can appear very early in fish populations, which suggests that some individuals are able to gain an advantage prior to being able to facilitate their own growth by feeding. Parental effects may act on behavioural or developmental systems that allow some larvae to hatch earlier (Zhao *et al.*, 2001) or to begin exogenous feeding sooner than others (Valente *et al.*, 2001). Parental effects are often attributed to females, as they have a direct influence on the protein and energetic content of the egg and, therefore, on the energetic balance of the embryo (Nagler *et al.*, 2000). However, there are also instances where paternal effects contribute to improved offspring performance (Pakkasmaa *et al.*, 2001; Saillant *et al.*, 2001). Egg quality is strongly influenced by the nutritional state of the female parent. The enrichment of the broodstock diet, especially with highly unsaturated fatty acids (HUFA), results in improved growth and survival in the larval stages (Wantanebe *et al.*, 1984, 1985). Female body size has also frequently been linked to higher egg quality (Valling and Nissling, 2000), larval size (Gisbert *et al.*, 2000), and improved larval survival (Saillant *et al.*, 2001). In general, larger eggs give rise to larger larvae (Huang *et al.*, 1999; Imai and Tanaka, 1998; Pakkasmaa and Jones, 2002; Voellestad and Lillehammer, 2000). Therefore, the maternal influence on egg and early larval size can be considered a major source of the size variation that is observed within populations (Chambers and Leggett, 1996). Yet, once the transition to exogenous feeding has been made, the relationship between large larvae from larger eggs growing faster or surviving longer is much weaker (Bromage *et al.*, 1992) and, in some species, is absent altogether (Chambers *et al.*, 1989). Maternal effects are often only detectable in the early stages of development (Heath and Blouw, 1998) and not at older stages, as inter-individual differences in feeding success or environmental factors soon mask any parentally-derived advantage (Springate & Bromage, 1985). In contrast, there are circumstances where parental effects, in addition to heritable genetic variation, can have a far more extensive influence, such as the observed link between parental and offspring life histories (Thorpe, 1977).

#### **4.1.3 Life Histories in Fish Populations**

The life cycle of many marine fish species begins with a pelagic larval stage, mainly feeding on zooplankton. After metamorphosis, the juvenile fish disperse into their respective environments and appear much the same as adult fish. Metamorphosis is an important life history parameter, as this represents a significant developmental point and also the transition to new food sources and environments. It is also an opportunity for competitive advantage between individuals, which results in a diversification of life history approaches. Life history strategies have been described in marine species – for example rough dab (Stratoudakis *et al.*, 1997), winter flounder (Hunt von Herbing, 2001) and Atlantic herring (Jennings and Beverton, 1991) – but the most extensive research has been

carried out on Atlantic salmon. Salmon undergo a maturation phase (smoltification) prior to the seawater stage of the life cycle and have two distinct life history strategies, which become apparent at the juvenile stage as a bimodal size distribution (Metcalf *et al.*, 1990). Fast growing juveniles, the upper mode of the size distribution, grow throughout the winter to smolt early, whereas the lower mode, maintain a lower growth rate to undergo smoltification in the following year. Fast growth in the upper mode fish is associated with a higher metabolic rate (McCarthy, 2000), higher protein turnover (Morgan *et al.*, 2000) and lower rates of lipid deposition (Morgan *et al.*, 2002). There is also a behavioural shift between the two groups, with upper mode fish increasing their exposure to predators by feeding during daylight (Metcalf *et al.*, 1998; Valdirmarsson and Metcalf, 1999), whereas lower mode fish are able to minimise exposure by feeding less (Simpson *et al.*, 1996), and at lower light levels (Valdirmarsson and Metcalf, 1999). Modelling of salmon strategies suggest that the decision to smolt early or late is made during a sensitive period, when maturation can be triggered only if the individual's expected performance exceeds a threshold level (Metcalf, 1998). This threshold may be genetically determined (Thorpe *et al.*, 1992), but is also governed by environmental conditions and the individual's ability to obtain resources. Dominant (usually larger) fish will often maximise their feeding resources by the use of aggressive behaviour towards subordinate fish, thus promoting a social hierarchy in the population.

#### **4.1.4 Dominance and Social Hierarchies**

Once size variation has developed within a population, the most significant elements in the continuance of variation into post-metamorphosed stages are competition and social interactions (Imsland *et al.*, 1998). For the larger, dominant fish, a hierarchy will reduce competition for food by allowing access to superior feeding sites (Reinhart, 1999), a choice of higher quality food particles (Nakano, 1995) and by inhibiting the appetites of subordinates (Koebele, 1985). Fish grown in isolation often show a reduction in size variation (Koebele, 1985). The occurrence of size variation is also greatly reduced when food is not a limiting factor (Jobling and Keskelä, 1996). This indicates that competition can have a significant impact on the persistence of size variation and experiments have shown that feed type and availability can be used as a management tool to control variation in some larval populations (Goldan *et al.*, 1997, 1998). In the high-density environment of cultured populations, intra-specific aggression is common and suggests that hierarchies operate in such environments (Moutou *et al.*, 1998). Aggression often leads to mortalities through either an increased risk of disease infection or cannibalism, which can result in substantial losses of fish from a farm population. The economic consequences of this problem means that there is a high degree of interest in the control of size variation and the aggressive behaviour associated with dominance hierarchies (Moutou *et al.*, 1998).

#### **4.1.5 Size Variation in Farm Populations of Gilthead Seabream**

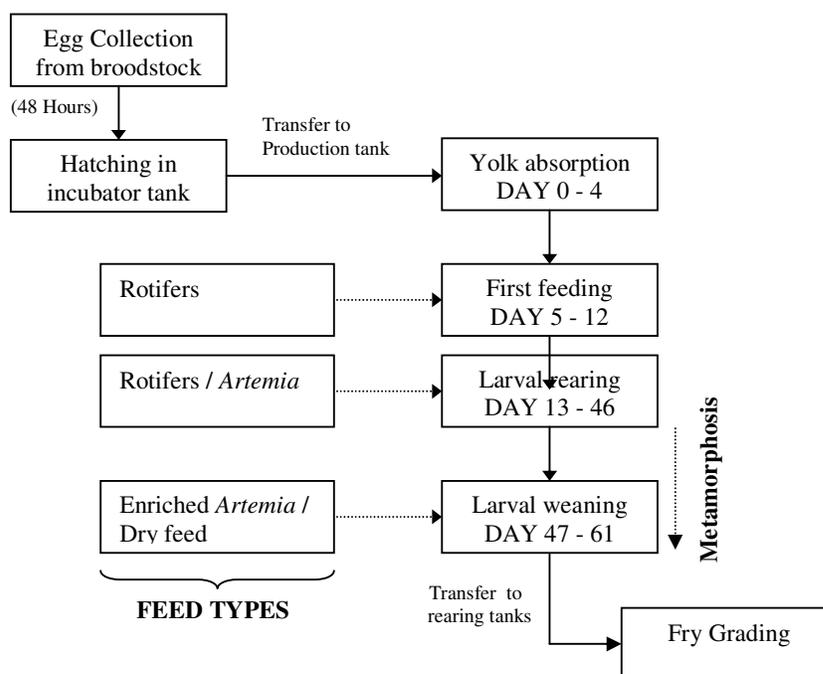
Growth dispensation is a well-known phenomenon in farm populations of gilthead seabream. However, very little work has so far been carried out to quantify size variation in the larval stages. Size variation becomes apparent early in the larval rearing stage, soon after the first feeding (Parra and Yùfera, 2000) and increases

markedly towards the onset of metamorphosis. Increased variation at metamorphosis has also been reported in other cultured sparid species, such as red porgy (Mihelakakis *et al.*, 2001). Metamorphosis occurs from day 47 post-hatch in gilthead seabream, and is characterised by the development of the skeleton, musculature and skin colour. The assumption of the definitive phenotype is often used as the classification of the beginning of the juvenile period (Balon, 1999).

Many sparids, including gilthead seabream, are sequential daily spawners and their egg quality can vary significantly over the season (Mihelakakis *et al.*, 2001). Improvements to egg quality – including egg size – have been achieved by the enrichment of the broodstock diet, in both gilthead seabream (Mourente and Odriozola, 1990; Zohar *et al.*, 1995) and red seabream (Wantanabe *et al.*, 1985). Dominance hierarchies are known to develop in the juvenile stages of gilthead seabream (Goldan *et al.*, 1997), but no work has quantified variation in the larval stages of development.

Growth differences in gilthead seabream larvae are of major importance for the successful rearing of larvae in large-scale facilities and the selection of fish at older stages. Figure 4.1 is a schematic diagram of the early life cycle of gilthead seabream in intensive farm production. Eggs collected from the broodstock are placed into conical incubator tanks, where hatching takes approximately two days at 20°C. After hatching, the larvae are transferred to indoor production tanks (3-metre diameter), where they remain until grading age. The larvae are kept in darkness with minimal water movement during the yolk absorption phase (days 0-4 post-hatch). By day 5 post-hatch the mouth, stomach and visual system is sufficiently developed in many fish to allow exogenous feeding and live feeds (rotifers) are introduced. Live feeds are supplied throughout the larval period, beginning with rotifers, to *Artemia* and then enriched *Artemia*, before weaning onto dry feeds after metamorphosis. Larval rearing extends for 60 days at 20°C, with metamorphosis occurring at approximately day 45 onwards.

First feeding is identified as a significant period for the development of size variation, as this is the point when somatic growth begins and when the first size differences between larvae can be observed. Experiment 1 focused on the growth of larvae during the first eight days of feeding and the differences between feeding and non-feeding larvae. Experiment 2 examined the practice of mixing batches of eggs from different broodstock and over two to three days of spawning, which is common in farm situations. It is not known whether this practice promotes size variation and, therefore, variation and growth throughout the larval period was evaluated in populations derived from both single and multiple egg batches. Parental effects on the growth of larvae could be significant, but have so far not been investigated in gilthead seabream. In experiment 3, genotype data collected on the larvae will allow this parameter to be estimated at the end of the larval stage and assist in determining the importance of size variation to the breeding programme.



**Figure 4.1: Early production stages of gilthead seabream in the hatchery environment. First feeding begins at 5 days post-hatch, after the absorption of the yolk sac. Live feeds are administered until metamorphosis begins and the larvae are weaned onto processed feeds. At the end of the larval phase, all fish are size-graded, to reduce size variation within groups.**

## **4.2 Materials and Methods**

### **4.2.1 Larval Rearing Conditions**

The farm tanks were all circular, of a 3-metre diameter and 1.5-metres in depth (4500 litre volume), whereas the lab tanks were circular, of 0.5-metre diameter and 40cm in depth (300 litre volume). All tanks were fed by the farm's borehole water supply (mean temperature  $19\pm 1^{\circ}\text{C}$ ). Fluorescent tube lights provided the only light source and followed the farm's standard 8-hr light/ 16-hr dark photoperiod. All tanks were cleaned daily.

All tanks utilised the 'green water' rearing method (Moretti *et al.*, 1999) of supplying algae and live feeds at high density for the first four to five weeks. After this point, algal density is reduced and weaning onto processed dry feeds takes place. Feeds were supplied by INVE (PROTON dry feeds (80–200 $\mu\text{m}$  and 150–300 $\mu\text{m}$ ), *Artemia* and Rotifer (*Branchionus sp.*) cysts – cultured on-site). Feeding regimes were comparable between tanks (scaled to tank size) and based on the manufacturer's recommended feeding plan (also reproduced in Moretti *et al.*, 1999). Dry feeds were presented on an hourly basis and distributed across the tank surface to ensure maximal feeding rates in all larvae. In addition, live feeds were distributed at lights on and again two hours later.

#### **4.2.2 Experiment 1: First Feeding Observations**

Observations on first feeding were conducted only on farm production populations at the hatchery of Alkioni Fish Farms, Cyprus. Four points during the egg-stocking period, over two successive seasons – one in 2001 and three in 2002 – were sampled, to minimise the effects of genetic background and spawning time. All populations were presented live feed (rotifers) on day 5 post-hatch. Feeding regimes were identical between tanks. First feeding larvae were sampled on the first day of food introduction (five days post-hatch) and on each of the subsequent seven days, five hours after the live feeds were introduced. The sampling method involved collecting approximately 250ml of water from three different locations at the tank's surface using a glass beaker. Larvae were individually measured by being placed on a Petri-dish and removing any excess water. Measurements were taken from the upper jaw to the termination of the notochord. The body length of one hundred larvae was measured each day using a stereo microscope and an ocular graticule (0.05mm graduations) and classified as either feeding or non-feeding. This classification was based on observations of the content of the stomach, in which ingested rotifers appear as a bright green mass within the gut, however, gut fullness (i.e. number of rotifers) was not recorded.

#### **4.2.3 Experiment 2: Variation in the Larval Stage**

Egg stocks were collected from the Alkioni Fish Farm broodstock, held under the advanced and natural photoperiods, in January 2001. Each farm production tank was stocked with eggs mixed from three broodstock groups (180 broodfish), collected over two days and pooled together at hatching. Three replicate farm tanks were each stocked with 600g of eggs, i.e. approximately 1,200,000 eggs (estimated at 2000 eggs/gram, using the method of Zohar *et al.*, (1995)). Three replicate laboratory tanks were also stocked with eggs produced from a single broodstock tank (60 fish) on two separate days, with two of the tanks stocked with the same egg batch. Stocking rate in the laboratory tanks was 100g of eggs (approximately 200,000 eggs). The hatching rate of eggs was estimated at 70% (based on farm records), giving an initial density of 186 larvae/litre for the farm tanks and 466 larvae/ litre for the laboratory tanks. Mortalities within the laboratory tanks were high over the experimental period and final densities were calculated to be 6-8 larvae/litre, compared to the farm tank estimated final density of 35 larvae/ litre.

Samples from the farm and the laboratory larval batches were taken at hatching (day 0), at first feeding (day 5) and then at seven-day intervals up to the end of the hatchery rearing stage (Day 61). Length measurements were taken using a stereo microscope and either an ocular graticule (0.05mm graduations) or a Vernier calliper (0.1mm graduations). Measurements were taken from the front of the upper jaw to the furthest extent of the spinal chord (body length). After metamorphosis, however, the spinal chord becomes ossified and the terminal portion differentiates to form the major spines of the tail. Therefore, all metamorphosed larvae were measured from the upper jaw to the base of the tail (final vertebrae).

#### **4.2.4 Experiment 3: Maternal Effects in the Larval Period**

In order to assess the magnitude of parental effects on growth during the larval stages, 100 offspring samples were collected in 2002 from each of two farm production tanks at day 60 post-hatch. Body length measurements were taken at the time of collection, using the methods described above. Each tank was stocked with eggs from the advanced photoperiod broodstock (60 fish) in December 2001. Using the genotyping methods described in Chapter 2, the parentage of these larvae was determined and a variance component analysis was carried out using REML with sires and dams as separate sources of variance.

#### **4.2.5 Data Analysis: Experiment 1**

For the first feeding observations, the results from all four tanks were pooled together for REML analysis. The fixed effects REML model consists of terms for feeding/non-feeding, day number and the interaction of feeding and day, because the number of feeding and non-feeding larvae fluctuated over the experiment. The random effects model consists of the tank and the interaction of tank and day. This model was used to assess the importance of feeding with growth and to produce predicted means for feeding and non-feeding larvae.

#### **4.2.6 Data Analysis: Experiment 2**

The REML model for the growth over the larval stages was used to partition the variation between the terms and to generate predicted means for growth in the two types of tanks. Before carrying out the analysis, the body length data was log transformed, to reduce variance in the measurement data and simplify the development of a model. The fixed effects model included terms for tank type and day effects because the data formed a time sequence of length measurements at fixed time-intervals. The interaction of tank type and day was also included, because observed growth rates were different between and within tank types over the time series. The random effects model includes the terms for differences between individual tanks and the interaction of tank and day, which represents the error variation of each tank over the series of measurements. The analysis was used to determine the growth differences between tank types and to produce predicted values for the growth in each tank after quantifying the variance components.

#### **4.2.7 Data Analysis: Experiment 3**

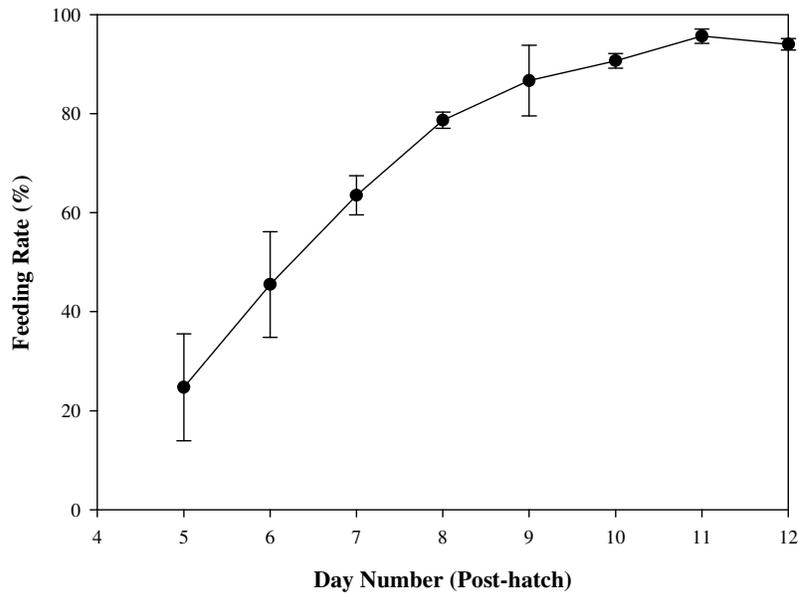
Parental effects were estimated using the male and female parents of each offspring as the random terms in the REML model. The fixed effects model comprised the tank from which the samples were drawn and the body lengths of individual larvae measured at day 60 post-hatch, as the variate. In this way the variance in offspring length could be partitioned between the male and female parents.

## **4.3 Results**

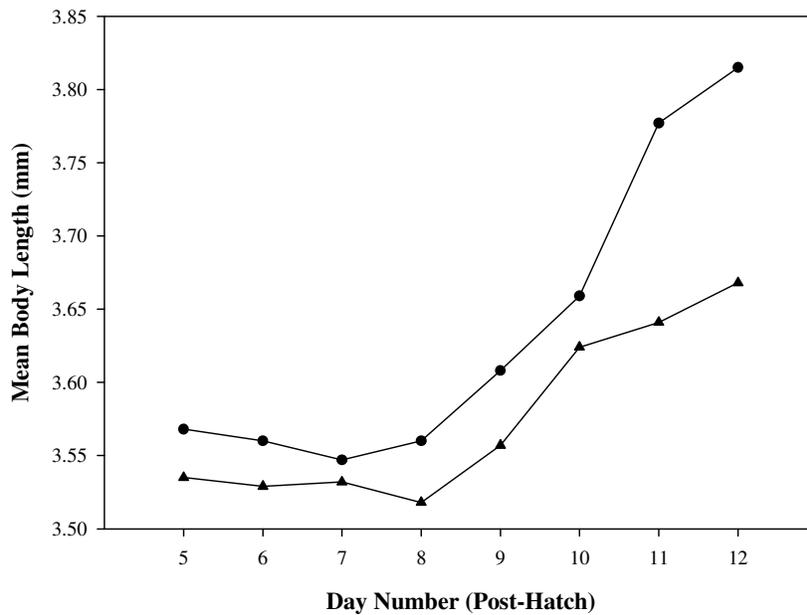
### **4.3.1 Experiment 1: First Feeding Observations**

First feeding in farmed gilthead seabream begins at five days post-hatch, when the feeding apparatus has developed and the yolk reserves have been fully assimilated. The timing of the changeover to exogenous feeding is not fixed, however, as the feeding rate over all tanks was observed to rise steadily from 15% on day 5 to 90% by day 10 (Figure 4.2). The maximum observed feeding rate reached 94% on days 11 and 12. This pattern suggests some larvae could feed before day 5 whilst others had not begun feeding even by day 12 post-hatch. Somatic growth is probably strongly linked to the ability to feed and could explain why the growth rates of feeding and non-feeding larvae were markedly different. Figure 4.3 presents the fitted values of growth of feeding and non-feeding larvae. Feeding larvae are consistently larger on all days ( $p < 0.001$ ), and as time progresses, the size disparity between feeding and non-feeding larvae increased.

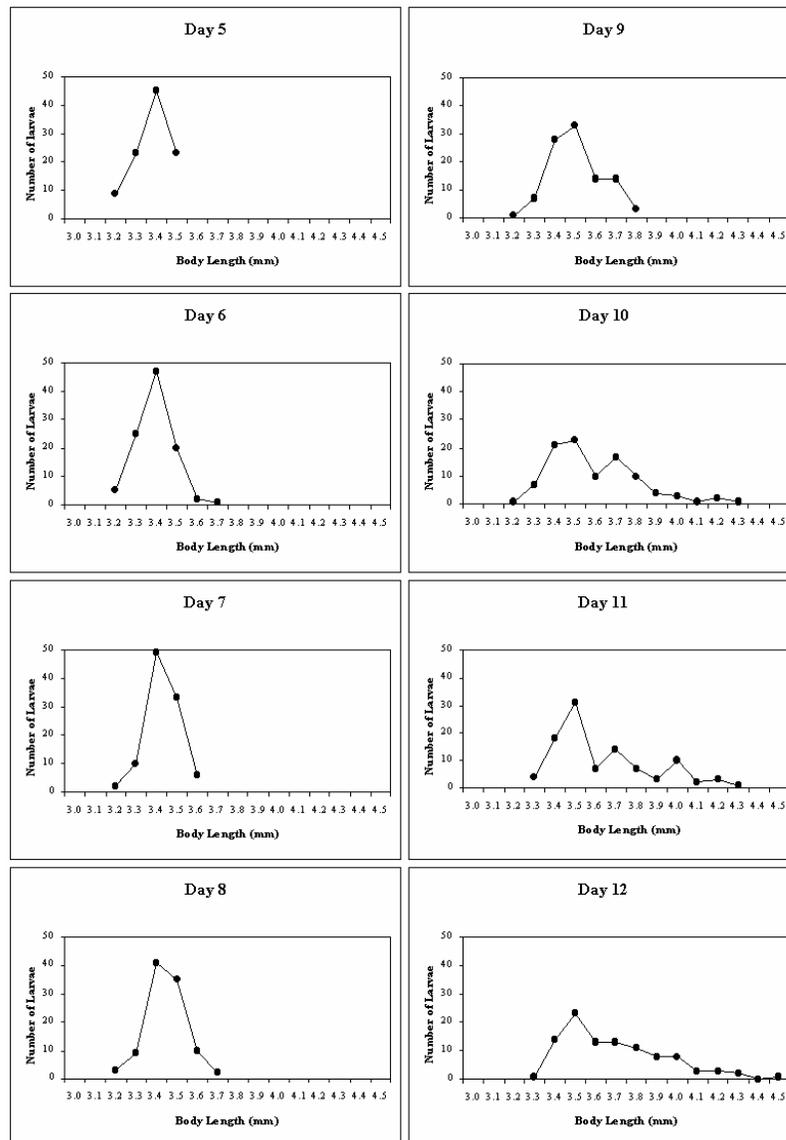
Both groups show a similar pattern of growth, which decreases from days 5 to 8, but then increases rapidly from day 9 onwards. This pattern may represent a period when the larvae are unable to use energetic resources on somatic growth, but instead these resources are required for the development of the digestive system, the visual system or the brain. Clearly, a delay in feeding at this point could lead to developmental problems, as well as mortalities. The day parameter of the model is also highly significant ( $p < 0.001$ ), possibly due to the changing numbers of larvae in each group over the experiment. Initially, the feeding group was small, but by day 8 the non-feeding group was only 6% of the population and, therefore, bias in such a small number of subjects is more likely. The interaction of day and feeding was significant ( $p < 0.05$ ) and represents the growth of the feeding and non-feeding groups over the experimental period. The growth of the larvae can also be visualised by the changes in the daily size distribution, seen in Figure 4.4. From a narrow size distribution on days 5 to 8 post-hatch, the growth of larvae on day 9 produced a distinct subset of the population at the larger end of the distribution. Through days 10, 11 and 12, this group expanded and the width of the size distribution enlarged as their size advantage increased. This supports the evidence that the size variation, seen throughout the larval stages, becomes apparent on day 9 post-hatch, as the feeding larvae begin to grow after the early transition to exogenous feeding.



**Figure 4.2: Daily feeding rate in farm production tanks after the introduction of food on day 5. Only 25% of the population is able to feed on day 5 and this is concluded to be the source of size variation in the larval stages. The feeding rate did not reach 100% in any of the tanks on any day.**



**Figure 4.3: Fitted values of feeding (●) and non-feeding larvae (▲) from REML analysis. Feeding larvae are consistently larger than non-feeding larvae and the difference increases towards the end of the first week of feeding. This confirms that larger larvae are the first to feed.**

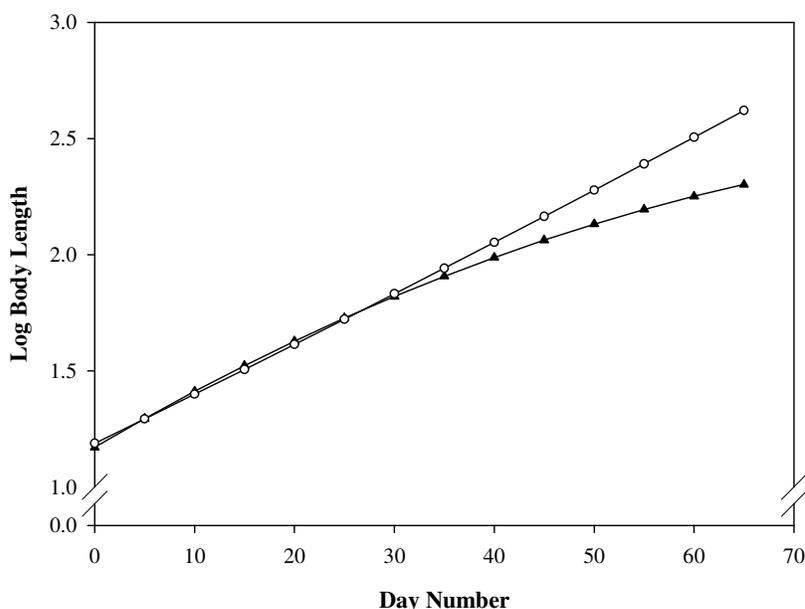


**Figure 4.4: Daily size distribution of larvae following first feeding (day 5). The size distribution is initially narrow on days 5-8. A larger group of larvae appear on day 9, and the size distribution progressively broadens as this group gain a size advantage. By day 12, the difference between the largest and smallest larvae is already 1mm.**

### 4.3.2 Experiment 2: Variation in the Larval Stages

In the laboratory tanks the analysis indicated that there was a linear increase in log length equivalent to a constant fractional growth rate. In the farm tanks there was a significant quadratic term ( $p < 0.01$ ) pointing to a decrease in fractional growth over the experiment, resulting in a greater average growth in the laboratory tanks ( $p < 0.01$ ). However, the interpretation was complicated by the presence of significant interactions between tank type and day number, both in the linear and quadratic terms ( $p < 0.001$  and  $p < 0.005$ , respectively).

Fitted values were constructed for each tank type from the REML analysis and are shown in Figure 4.5. In the initial period of the experiment, the growth in the two types of tank was almost identical. Beyond day 30 the fractional growth rate of the farm tanks gradually declines, whereas it remains constant in the laboratory tanks. This tailing off of the predicted mean length of the farm larval populations suggests that their growth was being limited. The likeliest cause for the decrease in growth was the higher rate of competition in the high-density environment of the farm production tanks. The laboratory tanks could not be maintained at comparable fish densities to the farm tanks, due to unexpectedly high mortalities during the experimental period. For this reason, the laboratory fish did not encounter the same degree of competition for resources and were able to grow at a higher rate, which may also have been promoted by the greater space available.



**Figure 4.5: Fitted values from REML analysis of larval growth in laboratory tanks (○) and farm tanks (▲). The laboratory tanks maintain a linear growth rate throughout the larval period. In contrast, growth rate in the farm tanks shows a significant quadratic effect. The cause of this decrease in growth is likely to be the formation of dominance hierarchies which limit the growth of sub-ordinate fish. Low fish density in the laboratory tanks is thought to have offset these effects.**

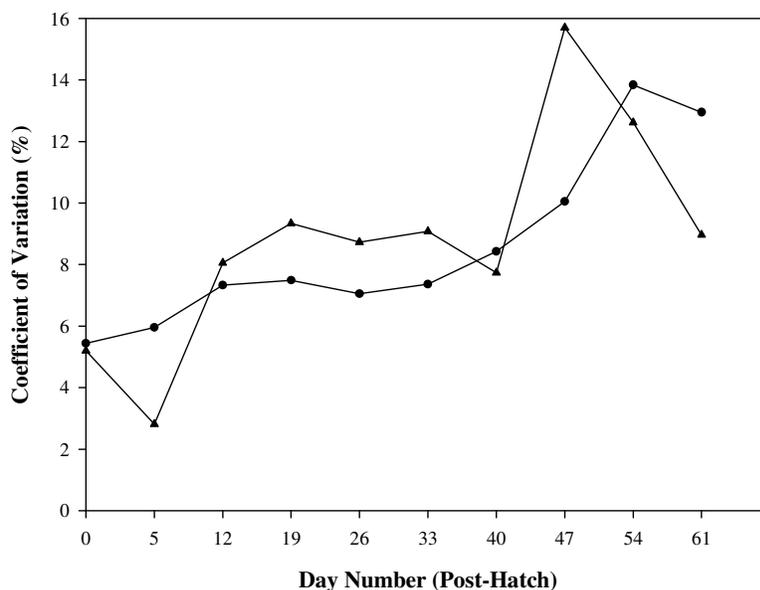
The pattern of variation in body length over the larval period was similar for the laboratory and farm groups. At day 0 (hatching), the coefficient of variation (CV) was the same in both groups, at just over 5% (Figure 4.6). By day 5 (first feeding), the variation has decreased in the laboratory group but then rose sharply by day 12. The farm tanks maintained a constant CV between days 0 and 5, but also showed an increase by day 12. Between days 12 and 40, the CV remained at a moderate 7-9% in both groups. On day 47, however, the laboratory group displayed a large increase in variation, to over 15%. The farm group had a correspondingly large increase on day 54, which remained at 13% on day 61. In

contrast, the CV in the laboratory group decreased to 8% by day 61. The REML estimated coefficient of variation over the sampling period for all tanks was 8.2%, which corresponds closely to the observed variance in the data collected.

This pattern of the development of variation may reflect the different developmental stages of the larvae over the rearing period. Initially, the CV is low as differences at hatching are small, possibly due to maternal effects such as egg size and hatching time. At first feeding, variation in the time of feeding leads to growth differences, which become apparent by day 12, causing an increase in the observed CV. Day 47 marks the onset of metamorphosis, the timing of which, is determined by size, rather than age. The first fish to undergo metamorphosis gain a distinct growth advantage and, consequently, the CV increases. On days 54 and 61, one would expect the CV to remain high as the larger, metamorphosed fish continue growing and an increasing proportion of the population reach metamorphosis. In the laboratory tanks, however, CV began to decrease at day 54, suggesting that the late metamorphosed larvae were able to narrow the size difference compared to the larger fish. This may be related to the lower stocking density in the laboratory tanks, which reduced competition and, therefore, maximised the growth of all fish.

### **4.3.3 Experiment 3: Parental Effects in the Larval Period**

Initially, the REML model included a term for the male parent, but this was found to be non-significant ( $0.00037 \pm 0.0238$ ) and was, therefore, dropped from the model. In contrast, the term for the female parent was significant ( $p < 0.005$ ). Residual variance was 0.869 and fraction of variance due to the female parent was  $0.0869 \pm 0.0616$ . Therefore, the proportion of variance in larval body length due to the female parent was approximately 10%. This represents a substantial maternal effect to be present at the end of the larval stage and could result in a weak selection for female parent at the first grading. The nature of the maternal effect was unknown, but as was confirmed in the first feeding observations, some larvae were able to feed earlier and so grow sooner than the majority of the population. Variation in egg diameter and hatching size was found to be minor which would indicate that the maternally-derived advantage occurs after hatching. This advantage appeared to persist throughout the larval rearing period until the first grading, which occurs from day 60 onwards.



**Figure 4.6: Increase in the coefficient of variation in the laboratory (▲) and farm tanks (●). Both groups show a similar pattern of increasing variation through the larval period. Variation increases after first feeding (day 5) and again at metamorphosis (day 47). The apparent decrease in variation towards the end of the larval period is attributed to the increasing number of larvae passing through metamorphosis and reducing the size variation in the population.**

## **4.4 Discussion**

### **4.4.1 First Feeding**

The observations of variation in larval seabream populations at first feeding indicate that size variation develops primarily after the onset of exogenous feeding. Growth differences between feeding and non-feeding larvae were highly significant and confirmed that feeding larvae are able to grow earlier than non-feeding larvae. The daily feeding rate varied little between tanks and displayed a characteristic pattern of increase. Only a small proportion of the population was ready to feed on day 5 (25%) but this increased steadily, until a plateau of 90-95% was reached on day 10. Delaying the timing of food introduction could be a means to reduce size variation by ensuring at least 50% of the population are ready to feed on the first day, which in this experiment would be day 6 or 7. However, this system may introduce mortalities and Parra and Yúfera (2000) concluded that the success of first feeding in gilthead seabream depended on a learning period in which larvae actively begin to prey on live food; the success of larval feeding increased with time, but was also highly dependent on prey density. This hypothesis could also mean that size variation will result from differences in the ability to feed soon after the introduction of food regardless of the timing.

The highest single feeding rate recorded was 97% and the reason for the presence of non-feeding larvae throughout the experiment is unknown. Gilthead seabream

larvae cannot survive beyond day 10 post-hatch without feeding (Yúfera *et al.*, 1993), so non-feeding larvae observed on day 12 must have fed at some point previous to that. This could be a result of the extended (16-hour) dark phase of the photoperiod used within the hatchery. First feeding in gilthead seabream is known to be a point of high mortality and recommended rearing techniques suggest that the dark phase should last a maximum of eight hours to prevent starvation (Moretti *et al.*, 1999). Some larvae may not be able to tolerate long periods without feeding or it may inhibit feeding when the light phase begins. Fielder *et al.* (2002) determined that the optimum photoperiod lengths for red snapper larvae was 12Light:12Dark at first feeding, and 18L:6D by the time of metamorphosis. In species such as Atlantic cod, a continuous light phase has also found to be effective in improving both growth and survival in larval stages (Puvanendran and Brown, 2002).

The period of yolk absorption is a critical period in the development of larvae, and also a point where environmental parameters can play a significant role (Polo *et al.*, 1991). Even relatively minor within-tank temperature differences could result in the faster development and adsorption of the yolk resources, which could place some individuals in a position to feed earlier than others, thus resulting in the development of size variation. However, the early appearance of size variation and the higher feeding rate in larger larvae, also provides the possibility that maternal effects may be responsible for feeding time and differential growth rates. Egg quality is normally mediated by the female, larger eggs have frequently been associated with larger larvae at hatching (Huang *et al.*, 1999; Pakkasmaa and Jones, 2002). Maternal effects could operate on developmental factors such as the speed of stomach development, and the stimulation of feeding behaviour that allows earlier feeding in some larvae (Valente *et al.*, 2001). This experiment was not designed to detect maternal effects in first feeding larvae, but these have been detected in other species, such as chinook salmon (Heath *et al.*, 1999) and rainbow trout (Wangila and Dick, 1996).

#### **4.4.2 Variation in Larval Stages**

The predicted means calculated from REML analysis indicate that the farm tanks exhibited a reduced growth rate in the later stages of the larval period, whereas the laboratory tanks maintained a linear growth rate. The reason for the significant disparity in growth rates between the two tank types can be attributed to the higher fish density in the farm tanks leading to increased competition and social interactions. The difference in density between the two groups was almost six-fold at the end of the experiment. Dominant larvae may be able to monopolise food resources by occupying preferred feeding areas, such as the tank surface, but are also likely to incur a metabolic cost in defending that location (Koebele, 1985). This could explain the overall drop in growth rates at the later larval stages, as larger larvae limit their somatic growth, in order to defend their access to the limited resources. Growth of subordinate individuals is also likely to fall in this situation, as food availability is reduced and more energy needs to be expended in the foraging process (Koebele, 1985). To compensate for low food availability, an increase in aggressive and cannibalistic behaviour is to be expected, as the formation of hierarchies has been found to be strongly linked to feed availability (Jobling, 1983; Soether and Jobling, 1999).

The data presented here suggests that feeding towards the end of the larval rearing period was insufficient to the needs of the farm tank populations. As a result of this, the growth of larvae in the farm production tanks was sub-optimal. The decrease in growth began at day 40, which may correspond to the onset of metamorphosis in the largest larvae of the population. It is likely that the first individuals to reach metamorphosis will become the dominant individuals in any social hierarchy. The period from day 47 to 61 was particularly critical, as the population contained larvae at different stages of development: pre- and post-metamorphosed larvae. Feeding regimes are developed to take this factor into account and provide a range of feeds with different particle sizes. However, this may serve to sustain the size differences by allowing dominant fish to monopolise the higher quality feeding areas, such as at the tank surface, whilst forcing subordinate fish to feed on poorer quality feed particles, lower in the tank. The reduced growth rate in farm tanks was evidence of a poor food availability that promoted the development of social hierarchies, which can increase the size variation in the populations. Larval populations in the farming system would benefit from a closer monitoring of feeding rates, growth rate and, where possible, an earlier separation of metamorphosed larvae to counter the formation of hierarchies.

Size variation in the two tank types was very similar at all sampling points and indicated that size variation in larval populations of gilthead seabream develops in a consistent way. The magnitude and pattern of size variation was the same irrespective of the origins and differences between egg batches. Egg batches used in the farm tanks were derived from almost three times the number of broodstock as the laboratory tanks, yet there were little differences in the coefficient of variation over the larval period. Therefore, it can be deduced that collecting eggs from multiple broodstock groups to stock production tanks has no effect on the early growth rate or the development of size variation in gilthead seabream populations.

Life history strategies have not yet been investigated in gilthead seabream, but, as a highly fecund, fast growing, shallow water pelagic species, survival of young fish is likely to be the greatest factor in the life history. Whether fast growth in the larval stages confers an ability to avoid predators is unknown. In farming situations, the most likely predator of larvae is generally other larvae and, therefore, large size does confer an advantage, especially when dominance hierarchies come into operation. The establishment of hierarchies soon after metamorphosis is a concern for the farming of this species, as size sorting is not possible until the larvae have all passed through metamorphosis and are more resistant to disturbance. Maternal effects are often masked by environmental variation in early developmental stages in other fish species (Heath *et al.*, 1999; Springate and Bromage, 1985). However, in this study the persistence of maternal effects through to day 60 indicates that hierarchies may play a significant role in sustaining these maternal effects.

These experiments relied on the measurement of body length as an indicator of growth throughout the larval stages, mainly due to the constraints of conducting the experiments on-site. In the early larval stages, such as at first feeding, changes in body mass may not be accurately estimated by length measurements. Therefore, a measure such as dry weight would be more suitable at these stages

when growth can be rapid and the accuracy of measuring body length is also problematic.

#### **4.4.3 Implications of Larval Size Variation for Selective Breeding of Gilthead Seabream**

Evidence from these experiments show that mixing egg stocks from a number of broodstock does not generate greater size variation in the larval farm populations. Therefore, selection procedures can be applied to a large base population by collecting eggs from several broodstock groups to stock into the production tanks and maximise the number of parents represented in the populations destined for selection. The similarity in rearing conditions between farm production tanks indicates that mixing larvae from several production tanks at first grading (day 60+) is possible because variation and growth is similar between production tanks. Age differences must be minimised, however, to avoid dominance by particular batches and large numbers of broodstock should contribute to reduce the prominence of maternal effects.

The development of size variation in larval populations causes additional management concerns, such as the correct feeding rates, particle size range, oxygen demands and water flow-rate. Evidence from this experiment and other studies indicates that size variation is probably the result of successful feeding by individual larvae in the days immediately after food introduction. Feeding success may be controlled by maternal effects – larger larvae were found to feed earlier – or could simply be a reflection of the encounter rate of food – as the feeding rate in each of the tanks was similar. Somatic growth after feeding meant that some larvae had gained a significant size advantage by the end of the first week of feeding. This advantage appeared to be carried throughout the remaining larval period, where dominance hierarchies were thought to maintain the size differences between larvae. Large larvae would continue to maintain an advantage in this environment, whilst suppressing the growth of others (Koebele, 1985). Significant maternal effects were found at the end of the larval rearing period (day 60), implicating them in the development of the initial size variation, and were sustained by the establishment of dominance hierarchies. Size variation was, therefore, mainly due to environmental influences on the growth of individual larvae, as maternal effects can be considered a form of environmental variance (Falconer and Mackay, 1996). In order to identify if the time to first feeding is a maternal or genetic effect, individual-based experiments observing feeding, or genotype data to verify the feeding performance of genetic groups at the end of the first week of feeding, would be required.

A selection point at the end of the larval rearing stage (first grading) would select juveniles for on-growing, based on body size. However, because growth during the larval phase is likely to be directed by environmental factors, it cannot be considered a reasonable measure of genetic performance alone. If maternal effects are responsible for larval size at the end of the rearing period, then grading would be biased to select related individuals and, therefore, reduce the genetic variance available to the selection programme. Growth in the early life stages is not always a reliable predictor of future growth, especially in a different environment (Falconer and Mackay, 1996). Compensatory growth is known to occur in other species once a dominance hierarchy has been disrupted (Jobling and Keskele,

1996). Therefore, selection at grading age needs to evaluate the potential growth of the population beyond the larval stage, so that only genetically superior individuals are selected. Selection for fast-growing larvae may have two important consequences: firstly, if maternal effects are inherited, selection of maternally derived traits may actually reduce size variation by increasing the proportion of larger larvae, which are able to feed earlier (de March, 1992) and, secondly, the criteria may indirectly select dominant and aggressive fish (Alarna, 1997). A method of standardising the grading process, to identify individuals for selection at the end of the larval stage, is the subject of the next chapter.

## **4.5 Conclusions**

Size variation in gilthead seabream larvae was found to increase after the onset of exogenous feeding in a majority of the population due to the spread of feeding ability. Initial size variation was low, but larger larvae were found to be able to feed earlier and begin somatic growth earlier than other members of the population. Size variation increased throughout the larval period, but no differences in variation between populations from mixed or single egg batches were found. Growth rates between the farm tanks and the laboratory tanks were significantly different, but this was attributed to a large disparity in larval densities between the groups. The lower growth rate in the farm tanks was also interpreted as evidence of dominance hierarchies operating under low feed availability. Maternal effects, which are implicated in the development of size variation, were detected at the end of larval period. These are presumed to promote the initial size differences, although the mechanisms were not tested fully, which are then maintained as the result of dominance hierarchies.

**Chapter 5:**  
**Control of Size Variation**  
**in Juvenile Stages**

## **5.1 Introduction**

The previous section summarised the mechanisms of the development of size variation in the larval stages of gilthead seabream. This section covers the subject of size variation as it develops in the juvenile stages of gilthead seabream and becomes a major economic consideration in the culturing of this species.

Dominance hierarchies are known to limit the growth of certain individuals, as aggressive behaviour is used by larger fish to block access to food or inhibit the appetite of subordinate fish. The fast growth rate of young fish means that the size difference between dominant and subordinate individuals will quickly increase, and with it the possibility of cannibalism. In the juvenile stages of gilthead seabream, aggressive behaviour becomes much more pronounced as size differences increase and when the fish are maintained at high densities. Even over the short term, losses of fish can be high and economically significant and, therefore, this behaviour needs to be limited as much as possible.

Size variation in juvenile fish also introduces additional complications for the farming system, such as providing the appropriate feeding type and rate, accurately calculating the population biomass and improving the quality of juvenile batches for sale or on-growing. For this reason, methods have been developed to disrupt the aggressive behaviour and reduce the size variation in juvenile farm populations. The principle method for controlling variation is size sorting – often termed grading – but other environmental factors, such as feeding type and light regimes, can also be manipulated to control size variation or aggressive behaviour.

### **5.1.1 Methods to Control Size Variation: Feeding**

Feeding plays a central role in the development and maintenance of size variation, as larger fish can dominate smaller individuals through competition for food, social stress and inhibition of appetite (Olsen and Ringø, 1999). Manipulation of feed type has been found to reduce size variation by restricting the competition between larger and smaller fish (Goldan *et al.*, 1998). In gilthead seabream, presenting food with a narrow range of particle sizes resulted in a reduced coefficient of variance in the population when compared to a wide particle size (Goldan *et al.*, 1997). Larger fish will preferentially feed on larger particles that have a higher nutritional content than smaller particles (Nakano, 1995). Larger fish are also able to physically consume more and this also leads to growth differences, particularly when live food is present, which acts as a continuous stimulus for the appetite (Goldan *et al.*, 1997). Therefore, minimising the range of particle sizes and early weaning from live foods can be used to reduce the development of size variation in juvenile populations.

There is conflicting evidence for the role of feeding frequency (feed availability) in size variation, as it has been found to both promote variation in some species, such as rainbow trout (Ruohonen *et al.*, 1998), and have no influence on others such as Arctic charr (Petursdottir, 2002). Cannibalism is often associated with the nutritional state of the fish – both in terms of quantity and quality – and when these are low, cannibalistic behaviour increases (Baras *et al.*, 1999; Folkvord and Otter, 1993). Gilthead seabream exhibits incomplete cannibalism (repeated biting

rather than swallowing whole) which is harder to control by mechanical means, such as grading, because it results from minor differences in size between predator and prey (Baras *et al.*, 2000). Increasing the supply and distribution of food can reduce the occurrence of cannibalism (Kubitza and Lovshin, 1999), as well as reducing stocking density and other environmental variables (Baras *et al.*, 2000).

### **5.1.2 Methods to Control Size Variation: Environmental Conditions**

Many environmental factors influence the growth of fish, and consequently, also the variation of growth rate between individual fish. Social interactions, such as aggression, are directly related to the stocking density of the environment and, therefore, also to size variation (Huntingford *et al.*, 1990). High stocking densities have been found to increase size variation in tilapia (Huang and Chiu, 1997), Arctic charr (Wallace and Kolveinshavn, 1988), matrinxã (Gomes *et al.*, 2000), turbot (Irwin *et al.*, 1999) and gilthead seabream (Canario *et al.*, 1998). However, high densities may also promote beneficial behaviours, such as shoaling, in which fish attempt to equalise their size so as to improve predator avoidance (Wallace *et al.*, 1988; Pitcher, 1993). Although a reduction of density can lead to a reduction of size variation, in hatchery conditions this is generally uneconomic.

Disruption of dominance hierarchies can be achieved by reducing light levels and by extending the length of the dark stage of the photoperiod. During the night, feeding hierarchies are thought to break down, in the absence of food and sufficient light to see competitors (Dou *et al.*, 2000). A reduction in light levels may allow subordinates to forage without fear of aggression from dominants and, consequently, growth differences between fish could be reduced. Low light levels also prevent cannibalism, and extending the dark phase of the photoperiod has been found to cut rates of cannibalism in certain species in the juvenile stages (Baras and Jobling, 2002; Dou *et al.*, 2000).

### **5.1.3 Methods to Control Size Variation: Grading**

Size sorting populations of fish is the most commonly used method of controlling size variation in hatchery conditions. Grading is a mechanical means of reducing variation and in young fish it can be achieved by passing large numbers through slots or gaps of a known size. Groups of fish are created on the basis of being either small enough, or too large to pass through the grader gaps. By separating the larger fish from the smaller fish, it is assumed that the growth of smaller fish will increase and, thereby, enhance the production. However, little gain in biomass has been observed in groups of smaller fish after grading (Baardvik and Jobling, 1990; Barki *et al.*, 2000) and, even when larger contemporaries are grouped together, there is often a decrease in growth due to competition (Brännäs *et al.*, 2002). Grading is able to disrupt the dominance hierarchies that promote size variation, but these are quickly replaced by new hierarchies in the post-graded groups (Jobling and Reinesnes, 1987). In order for it to be effective, a continuous and frequent grading system must be practised that may only be limited by the extent of the facilities available. Frequent grading may, however, lead to reduced growth and loss of fish through injury or stress, induced by the repeated handling (Tort *et al.*, 2001) and crowding of fish (Ortuno *et al.*, 2001).

To be effective, grading has to reduce the variation in the population by size sorting into homogenous groups. This is normally achieved by using bars spaced at equal distances through which fish can swim or pass through. The width of this spacing dictates the width of fish that can pass which is in turn, highly correlated with other physical traits, such as body length and weight. The accuracy of the grading sheets has a large impact on the variation of the population after grading, by restricting the size of fish that can pass through. Therefore, accuracy affects the speed at which variation develops and determines the length of time until the next grading is needed.

### **5.1.4 Standardisation of the Grading System**

Grading is one of the major tasks in the farm production system and requires a substantial input of labour. During the juvenile rearing stages of gilthead seabream at Alkioni Fish Farms, fish are graded almost weekly, but with only minor success in changing the degree of size variation of the populations. The grading sheets employed at the farm were found to have large differences in gap width, which decreases the accuracy and effectiveness of the grading. This suggests that the efficiency of the current grading system could be improved by increasing the accuracy of the grading sheets, thus reducing the frequency of grading. There is also no control on the density of fish groups after grading, which leads to different growth rates between groups of fish originating from the same batch. Subsequent grading then leads to the mixing of groups and batches, which further complicates the ability to separate faster growing individuals.

Managing the grading system to allow groups of fish with higher growth rates to be more easily separated is essential for the development of a selection programme and, generally, for the efficiency of the production system. A second reason for standardising the grading process is to utilise this aspect of farm management as a stage in the selection programme. This would select fish on the basis of larval growth rate as size at first grading. These fish would then form an 'elite line', held in isolation through the grading process and stocked into a seaage as an uncontaminated population. However, all processes in the selection programme need to be standardised, so that comparisons can be made between populations and over time. The current system introduces many sources of environmental variance, due to differences in density, number of grades and size of grading sheet. Therefore, at present, it is difficult to identify fish that are genetically faster growing and are suitable candidates to go through to further stages of selection. For the selection programme, a standardised methodology of grading is required, which may involve fixing the timing or size of graders used.

The aim of the experimental grading procedure detailed below was to determine how different groups of fish perform when environmental conditions, such as density and feeding, are kept constant. In particular, it was necessary to see how the performance of an 'elite line', created at the first grade, compared to the remainder of the population through the juvenile stages. The experimental grading could then be used as a basis for the grading methodology proposed for the selection programme. As part of this experiment, new grading sheets were designed that aimed to increase the effectiveness of grading, by increasing their accuracy in comparison to the grading sheets currently being used at the farm.

## **5.2 Materials & Methods**

### **5.2.1 Animals**

Approximately 5000 larval gilthead seabream were collected from the production system and communally reared to the age of 70 days post-hatch. Fish were then transferred to the experimental tanks and immediately graded with the 2.0mm sheet to create two categories (1 and 2). Measurements of body length were collected prior to the first grading, at each grading and three days after grading, on a random sample of 50 fish from each group. Measurements were taken using Vernier calipers (0.1mm scale) without anaesthetic.

### **5.2.2 Facilities**

All grading took place in circular experimental tanks (0.5-metre diameter, 40cm depth, 300-litre volume) with constant water exchange and aeration supplied from the main farm system. It was intended to keep stocking densities similar between groups by grading. However, no direct control of density was performed and so this varied from 2.0 fish/litre to 5.3 fish/litre within the four groups during the grading process. A 7-hour light photoperiod regime was followed, with feeding every hour during the light phase (8 feeds in total), replicating the conditions of the hatchery system. INVE dry feeds were used throughout the experiment consisting of: proton 3 (200-400 $\mu$ m) and NRD (300-500 $\mu$ m & 500-800 $\mu$ m). Additional *Artemia* was supplied only in the days following the transfer to the laboratory tanks to stimulate feeding and increase survival after the handling episode. Feeding type was identical within categories, but not between categories, due to the differences in size and developmental stage of the fish. Feeding rate was maintained at 1.5-2% of tank biomass per day for all groups over the course of the experiment.

### **5.2.3 Grading Sheets**

New grading sheets were designed and manufactured at the Institute of Aquaculture to an accuracy of  $\pm 0.1$ mm. Five sheets were produced with gap sizes of 1.5, 2.0, 2.5, 3.0 and 4.0mm (Figure 5.1). They were constructed using a plastic frame, with holes drilled at the relevant spacing along the side panels and 5mm steel circular bars slotting in to these holes to form the grading bars. A fibreglass box was constructed on-site at Alkioni Fish Farms, into which the new sheets could be fitted and exchanged as necessary. The box held the grading sheet approximately 10cm under the surface of the water and allowed large numbers of fish to be graded at a time. The fish were encouraged to swim through the bars by agitating the box up and down and from side to side for a period of one minute.

### **5.2.4 Experimental Design**

The first grading was used as a means to separate the population into two categories 1 and 2 that would remain isolated throughout the grading process, in order to observe their relative growth. Each of these categories was then further subdivided at the next grading into two groups termed 'large' and 'small'. These groups were not exclusive, because at each grading they were combined and graded to form the two groups again, on the basis of passing or not passing

through the grading sheet. A further three grades were carried out over the experiment using progressively larger grading gap sizes. The movement of fish between groups was recorded by counting the fish at grading and also by estimating the fish density from digital photographs of the tank.

### 5.2.5 Density Estimates

Digital photographs of the tanks were taken after grading, in order to estimate the number of fish present. The photographs were taken from directly above the tank to incorporate both the tank edge and the central outlet tube. A 'slice' of the tank corresponding to a known volume was then measured on the photograph and the number of fish within the slice counted. This value was then extrapolated to the tank volume to give the final density. This method was repeated three times to get an average of three photographs. The accuracy of the technique was tested where hand counts of fish were possible and showed that the estimates were within 20-100 fish of the actual number which is equal to approximately a 2-10 % error rate.



**Figure 5.1: Grading sheets designed for the experimental grading of gilthead seabream juveniles. The 5mm stainless steel bars are fitted into a plastic collar by holes drilled at fixed widths. The sheets are then slotted into a fibre-glass box which holds the sheets 10cm under the water's surface. Gap sizes of the sheets were 1.5, 2.0, 2.5, 3.0 and 4.0mm; accurate to 0.1mm.**

### 5.2.6 Results Analysis

Grader performance was evaluated by the probability of allowing larvae of certain body length to pass through the grading slots. The higher the accuracy of the grading sheet, the narrower the size-range of fish that will be able to pass through the grader, leading to increased homogeneity in the size of the groups after grading. Standard probability curves for each grader could then be used to predict the outcome of grading based on the mean length of the fish.

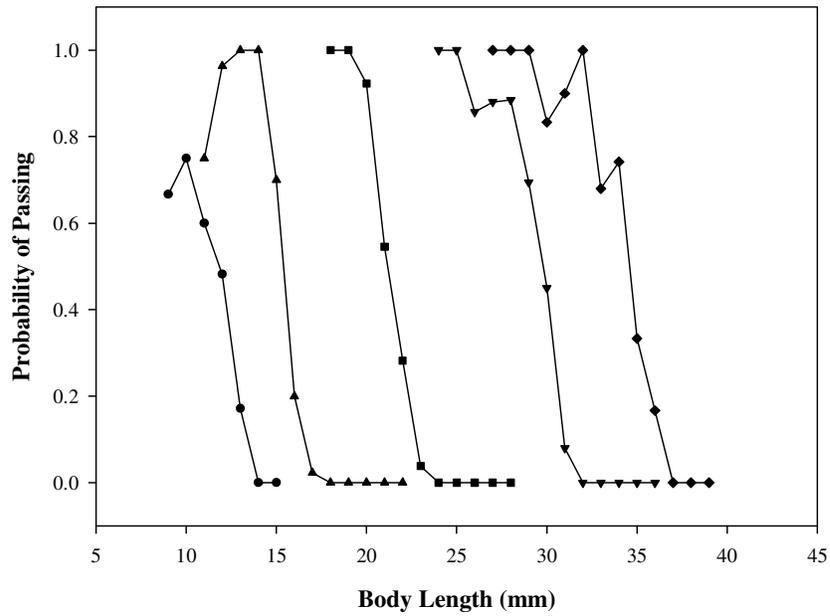
Growth rate in each group of the standardised grading method, was calculated using a linear regression of mean body length after grading. The fractional growth

rate and time to reach 2g mean weight were compared between groups. The probability of remaining with groups during grading was calculated using matrices of proportions moving between groups at each grade.

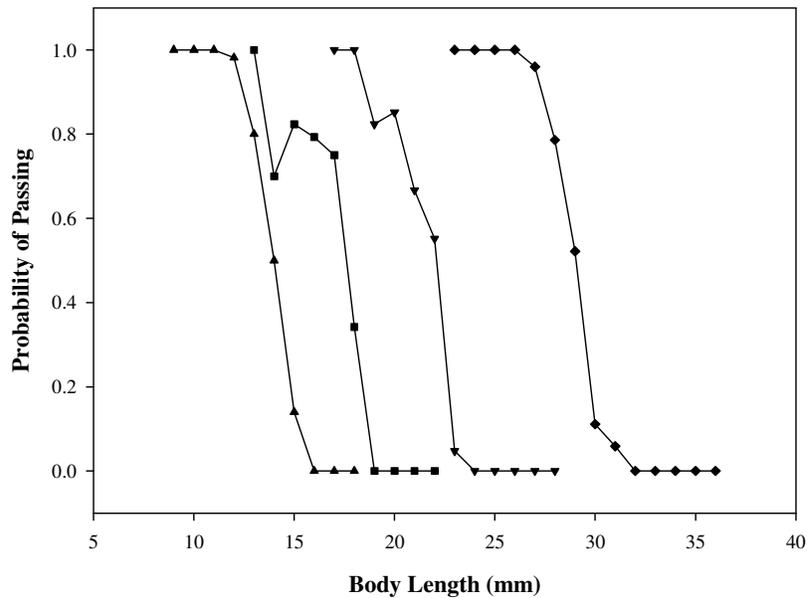
## **5.3 Results**

### **5.3.1 Grading Accuracy**

The accuracy of the grading sheets was tested by measuring 100 fish from post-graded groups for each size of grader. The new grading sheets produced a more consistent pattern of probability of passing for a given body length (Figure 5.2), whereas the farm graders were more unpredictable in their performance (Figure 5.3). It was found that the graders designated with a similar gap width operated at a much smaller size of fish – for example at the point of 50% passing for the 3.0mm grader, the values for the new and farm graders were 22.5mm and 30.0mm respectively. This proved so much of a problem that the new 1.5mm grader was found to be too small to grade the experimental population at the first grade. Variability in gap width in the farm graders is also evident from the distribution of probability curves in Figure 5.3. The spacing between each curve represents points where the probability of passing will be 1 for the previous grader and 0 for the next grader. Grading populations with a mean length at these points would be impractical with the existing graders and serve only to separate the extremes of the population. Both types of grader were effective at reducing the variance observed in the pre-graded groups and the difference in means of post-graded groups was highly significant for all graders ( $P > 0.001$ , Student's t-test, 198 d.f.). The lowest coefficient of variation was always achieved in the small group, compared to the large group, because grading was performed to remove the small section of the population and fish in the large groups never passed through the grader. Therefore, very large individuals were able to remain in the large group and increase the variation in that group. Variation in the large group didn't affect growth or survival, however, which were similar between all groups.



**Figure 5.2:** Performance of new grading sheets. Probability of a given body length passing through each grading sheet based on 100 measurements per sheet from post-graded groups. Grader sizes: 2.0mm (▲), 2.5mm (■), 3.0mm (▼) and 4.0mm (◆).



**Figure 5.3:** Performance of farm grading sheets. Probability of a given body length passing through each grading sheet based on 100 measurements per sheet from post-graded groups. Grader sizes: 1.5mm (●), 2.0mm (▲), 2.5mm (■), 3.0mm (▼) and 3.5mm (◆).

### 5.3.2 Growth and Variation under Controlled Grading

Category 1 was comprised of the large fish of the first grading (2.0mm) and category 2 of the small fish of this grade. The number of fish in each grade was not equal due to the low size range of the grading sheet, which passed fewer fish than anticipated. The categories were then subdivided at the second grading into large and small groups.

The difference in mean lengths between the groups within a category increases with each grade and by a similar rate in both categories. The difference in growth rates between categories is indicated by the time-scale of the grading process, where category 1 is graded with the 4.0mm grading sheet at day 29, whereas category 2 reaches the same point at day 38. Regression analysis of group lengths (grade 2 onwards) indicates that the large group of category 1 was the fastest growing with a fractional growth rate of 2.7% per day (Table 5.1). This group would be expected to reach the seacage stocking size (two grams or 45mm body length) in a total of 40 days, compared to 51 days for the small group of this category. The large group of category 2 also showed a marginally higher growth rate and would have overtaken the small group of category 1 to reach 45mm mean length at the slightly earlier time of day 49. The small group of category 2 showed the lowest fractional growth rate and would be the last to reach 45mm mean length, in a total of 58 days.

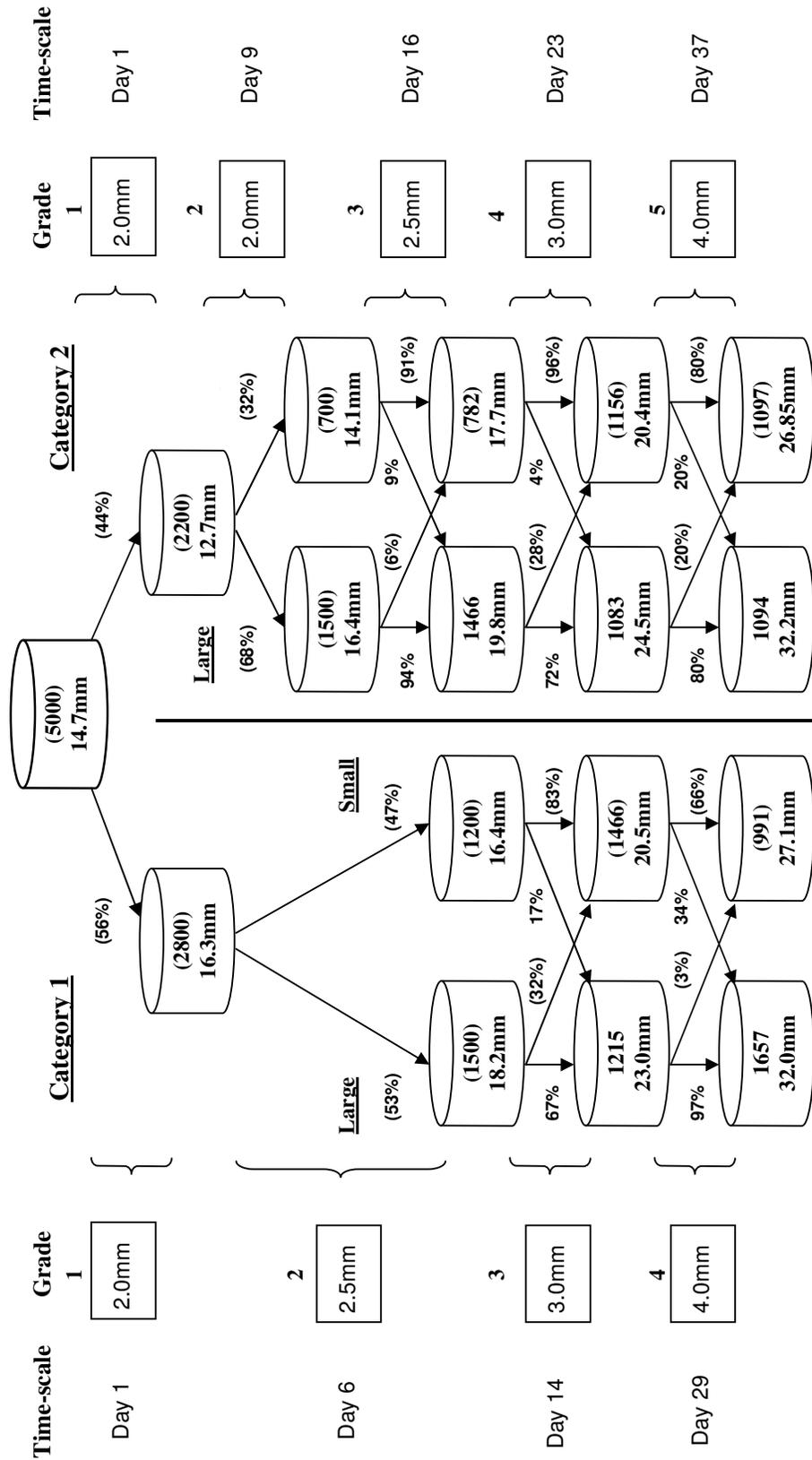
**Table 5.1: Growth of grading groups over the experiment including regression of time to reach seacage stocking size.  $\ln L = \log_e$  body length (mm);  $t =$  day from first grading.**

Growth Equation	Growth Rate / day <sup>-1</sup>	Days to reach 45mm (2g)
<b>Category 1: Large</b>		
$\ln L = 2.721(\pm 0.0307) + 0.0277(\pm 0.0018) \times t$	2.7%	40
<b>Category 1: Small</b>		
$\ln L = 2.673 (\pm 0.0147) + 0.0223 (\pm 0.00085) \times t$	2.2%	51
<b>Category 2: Large</b>		
$\ln L = 2.616 (\pm 0.0199) + 0.0233 (\pm 0.00087) \times t$	2.3%	49
<b>Category 2: Small</b>		
$\ln L = 2.547 (\pm 0.0437) + 0.0194 (\pm 0.0019) \times t$	1.9%	58

The number of fish in each group was quantified by both estimation of fish density in photographs and by hand counts of fish during grading. The movement of fish between groups indicates that there is some considerable movement both into and from each group. This is displayed in the schematic diagram of the grading experiment in Figure 5.4. This movement is strongly dependent on the timing of the grading, as the growth rates between groups were similar. In the experiment the timing of the grading was subjective, at least for category 1, because the performance of the grading sheets was unknown. The probability of fish remaining in individual groups is given in Table 5.2. The higher movement of fish in category 1 was reflected in the almost equal probability of moving from the small to large or staying within the small group at grade 4 (Day 29). However, fish in the category 1-large group, had a 76% chance of remaining in the large

group, which emphasises the size-advantage of fish within this group, above all the other groups. In category 2, the movement of fish was much lower, and the probability of remaining in the same group was over 90%, at both grades 4 (Day 23) and 5 (Day 37). The low probability of fish moving out of the category 2-small group, indicates that they were unable to grow at a sufficient rate to compensate for the size differences present at the beginning of the experiment.

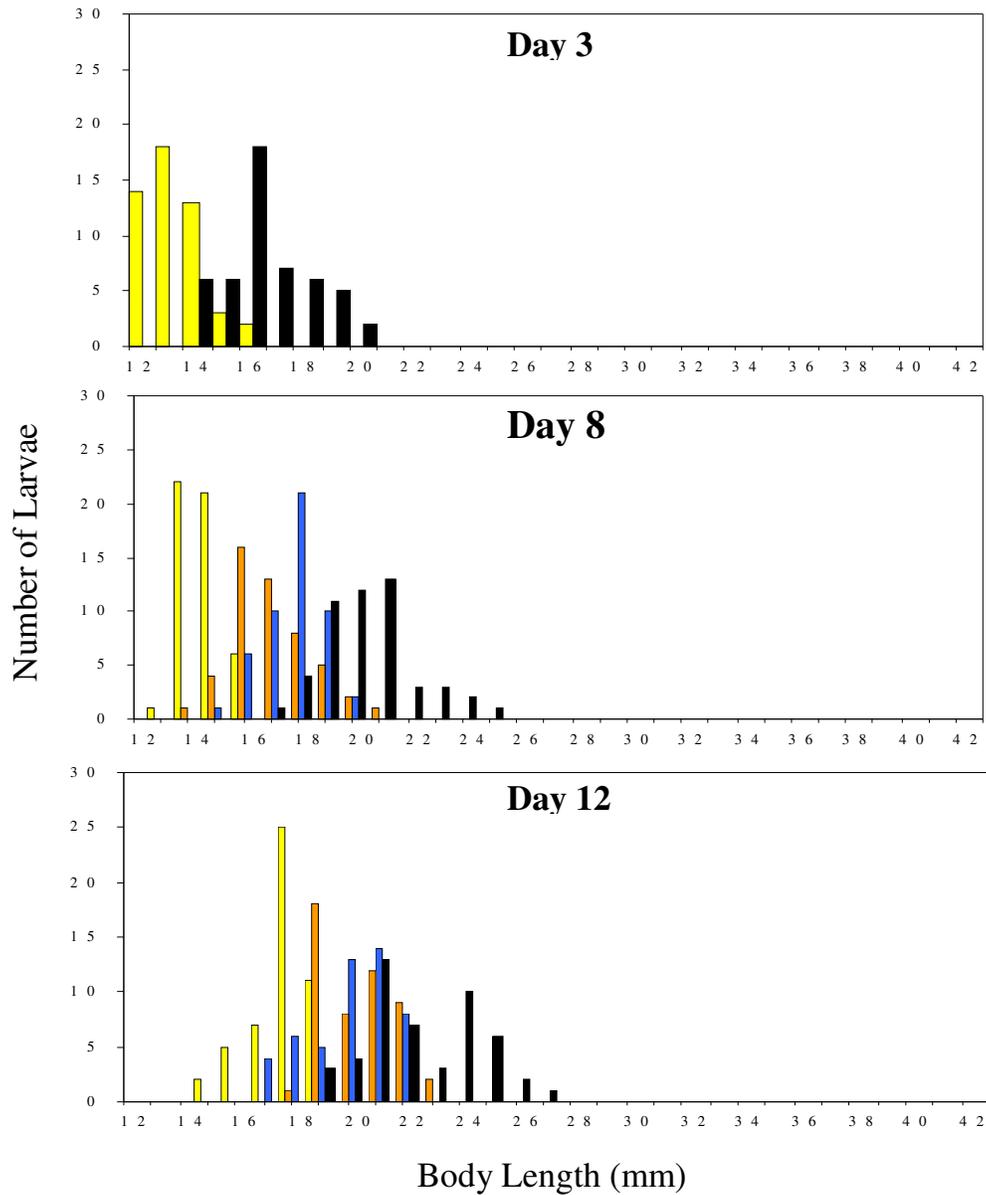
The movement of fish between groups is also indicated by the size distribution of fish lengths, shown in Figure 5.5. Early in the grading system the overlap between the groups is high in the centre of the distribution, although the largest and smallest fish are clearly distinguishable at the edges. As the fish grow, the overlap seems to increase (Figure 5.5 – Days 12, 16), indicating poor efficiency in the grading. However, by day 21 (Figure 5.5), the category 1-large group and category 2-small group fish are clearly moving further apart, whilst the remaining two groups overlap substantially. At the end of the experiment, there are three discernible groups with little overlap into the extreme groups (1-large and 2-small) by the central two groups (1-small and 2-large). These distributions demonstrate how few fish from the lower groups are able to catch up with the largest group, created at the second grade, and how the variance of the population as a whole increases during this period of rapid growth. The final histogram of series (Figure 5.5 – Day 26) marks the appearance of ‘shooters’, which, at over 40mm, are more than double the length of the smallest fish in the population.



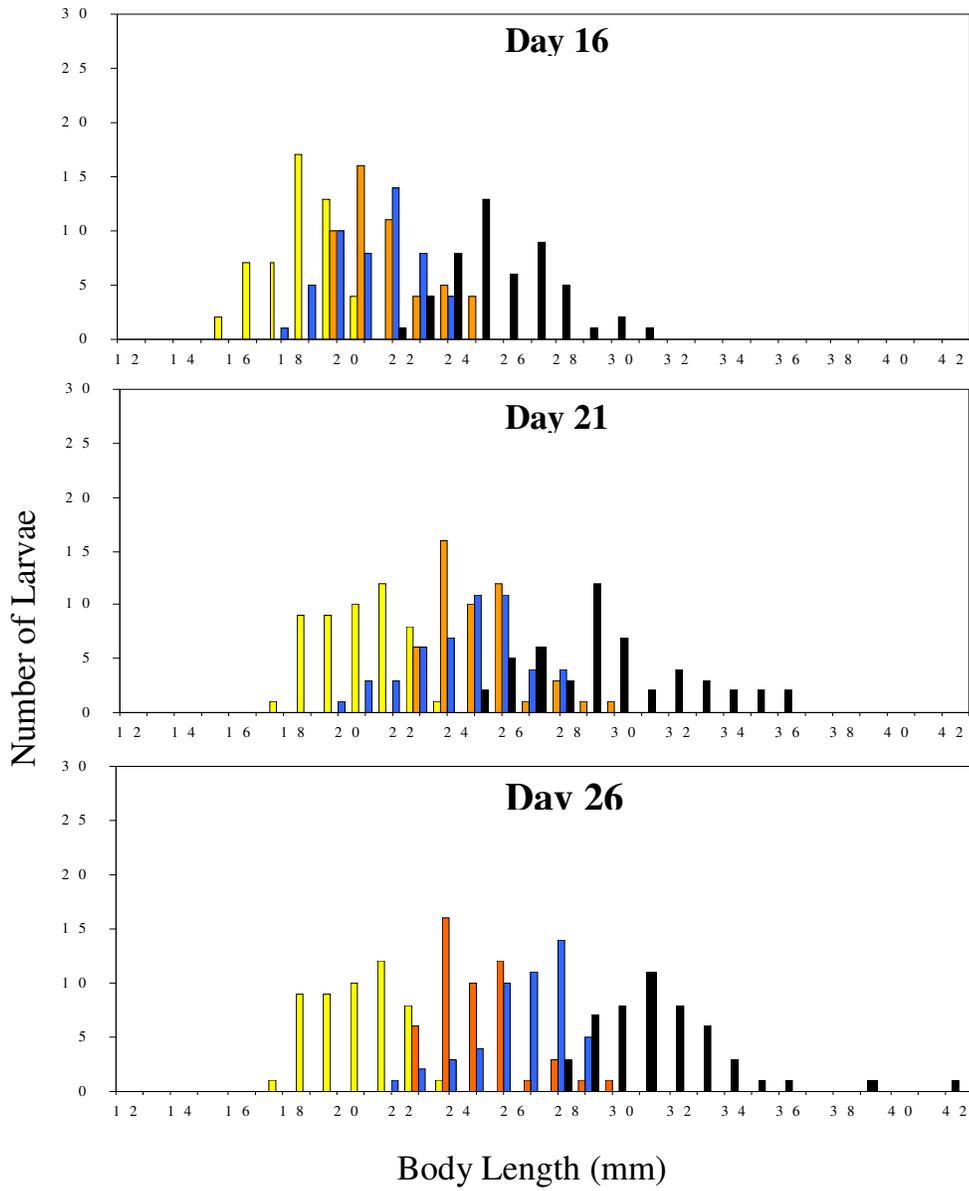
**Figure 5.4:** Schematic Diagram of the grading experiment. An initial population of 5000 larvae was graded into two categories, then graded again into four groups. Grading took place within the categories and the movement of fish between groups is recorded as a percentage of the tank population. The actual population size of each group and the mean length after grading are displayed within the tank symbols. Numbers in brackets are estimated values, whilst those without brackets are hand-counts of fish.

**Table 5.2: Probabilities of moving between grading groups in category 1 and 2. Movement out of the category 1-large group is low, as there is a 76% probability of staying within the group at grade 4. However, there is an almost equal probability of moving or staying in the category 1-small group. In category 2, the probability of staying within the same group is very high (>90%) for both groups at grades 4 and 5. This suggests that the largest fish and the smallest fish within category 2 retain their relative size differences throughout the grading process. But this result is not as clear in category 1.**

Destination Group	Starting Group	
	Large	Small
<i>Category 1:</i>		
<i>Day 14 to Day 29 (Grade 4)</i>		
<b>Large</b>	0.76	0.45
<b>Small</b>	0.24	0.55
<i>Category 2:</i>		
<i>Day 16 to Day 23 (Grade 4)</i>		
<b>Large</b>	0.94	0.09
<b>Small</b>	0.06	0.91
<i>Day 23 to Day 37 (Grade 5)</i>		
<b>Large</b>	0.93	0.08
<b>Small</b>	0.07	0.92



**Figure 5.5:** Size distribution of groups during the controlled grading experiment. Measurements collected at and between grading events. Group symbols: Category 1-large (■), -small (■); Category 2-large (■), -small (■).



**Figure 5.5(cont.):** Group symbols: Category 1 -large (■), -small (■); Category 2-large (■), -small (■).

## **5.4 Discussion**

### **5.4.1 Effectiveness of Grading in Controlling Size Variation**

The first aspect of this experiment aimed to determine if increasing the accuracy of the grading sheets would result in a reduced size variation of the post-graded groups. All the graders were effective at reducing size variation and producing groups with significantly different mean lengths, but no differences were found between the two types of grader and the degree of variation in the post-graded groups. The probability graphs (Figures 5.2 and 5.3) indicate that the new graders were much more predictable in their operation and, for a given body length, a good estimate of the probability of passing could be calculated. The farm graders, especially those of 1.5mm and 3.5mm sizes, were not as reliable and severely reduced the effectiveness of the operation, by allowing many smaller fish to remain in the grading box. The evidence suggests the farm graders should be replaced with the more accurate graders, however, this must be contrasted with the operation of the two types of grader, as the new graders were tested on a small experimental population, whereas the farm graders were tested on production populations of much greater size with greater time constraints. In this respect, residence time and number of fish in the grading box may be of significance for the accuracy of the grading sheets and not only the gap width of the sheet. Generating probability curves for the grading sheets may be a useful method of predicting the outcome of grading a particular population. If a sample of the population is measured, the mean length can be used to predict the proportion of the population that will pass through the grading sheet using the probability curves. In this way, population sizes could be controlled quite accurately in the post-graded groups and grading can be used as a management tool.

Grading is a labour intensive process, especially in the culture of gilthead seabream, because of the speed at which size variation re-establishes itself in juvenile populations. It is, however, effective at controlling variation (Panagiotakis *et al.*, 2001), and increases survival (Popper *et al.*, 1992) when performed frequently, as it can disrupt the dominance hierarchy that inhibits the growth of some individuals. Other methods to control variation, such as periodic feed frequency and utilising feeds with a narrow particle size range, may be effective over short time periods (Goldan *et al.*, 1997). Yet, these techniques would require additional management to monitor the level of feeding, and may be impractical in large populations and on a hatchery-wide scale. Therefore, grading is considered to be the best technique available for controlling size variation in gilthead seabream, and standardising the grading method is necessary to manage and improve the efficiency of the process.

### **5.4.2 Grading Strategy for the Selection Programme**

A standardised method of grading is required for the selection programme, in order to minimise environmental variation between populations and over time. This is especially important if grading is going to form the basis of the first stage of selection, as there needs to be a comparison of populations over time. Such data is useful to indicate how grading may influence the growth of fish, as it may itself exert a selective pressure on, for example, increased body width, deformations or the ability to remain in the grading box.

From the experimental data, it is perceptible that the category 1-large group fish grew the fastest over the experimental period and were projected to reach the stocking size approximately 10 days earlier than the nearest other group (category 2-large group). The growth rate was not significantly higher than in other groups, but the size advantage this group had at the first grading was maintained throughout the experiment. This result supports the rationale for developing an 'elite line' at the first grading, to be maintained separate from the remainder of the population and stocked for on-growing and, subsequently, broodstock selection. The movement of fish into the large group was high at grade 2 (502 fish), but an equivalent number of fish (477) were then removed at the next grade, suggesting that this movement was due principally to the timing of grading. The number of fish from other groups falling within the size range of the large group totalled 21, but all of these were in the lower end of the size distribution, and as such would not contribute significantly to the mean of this population. The growth rate in the remaining groups was high and, as the size distributions in Figure 5.5 indicate, the category 1-small and category 2-large groups were closely matched throughout the experiment. The size distribution of the whole experimental population on day 26 is close to the normal distribution, which is what would be expected of a typical population in the absence of grading. Therefore, controlling the density of graded groups, by means of probabilities, and grading within groups appears to be an effective way of separating the juvenile population into three distinct classes: an elite line, a large median group and a tail-end group. The use or destination of these classes of fish could vary according to production needs, but the value of fish – based on growth rate – is greatest with the elite line and lowest with the tail-end group.

The previous chapter indicated that environmental variation was an important influence on growth during the larval period. This experiment found that the largest larvae at the beginning of the experiment maintained their size differences and were also the largest larvae at the end of the grading period. Therefore, the maternal effects detected at the end of the larval period, would seem to also be present through the juvenile period. If the grading system is to be used as a selection point it must take these environmental effects into consideration, otherwise selection will be directed towards environmentally mediated traits such as hatching time, female parent size or aggressive larvae. Genetic improvements will not be made in this situation.

Standardisation of the grading process in this manner would be suitable for the operation of a selection programme and may also benefit general farm management. The ability to predict the number of fish moving between grades should allow better planning of facilities, such as the number of tanks required, and to monitor and predict the production levels of individual tanks and populations. As part of the selection programme, the grading period is important as a first stage of selection, as it increases the size of the base population on which the selection criteria will be applied.

## **5.5 Conclusions**

Size variation is a particular problem in the juvenile stages of gilthead seabream and grading is the most common means to control this variation. Improvements to

the grading system, such as increased accuracy and standardisation were proved to be effective. Grading could be used as the first stage of a selective breeding programme by standardising the grading process to minimise environmental variance in the post-graded populations. The growth rate between graded groups was very similar, however, a larger group of fish were distinct throughout the grading process. Environmental variance, carried over from the larval period, is considered to still have a large influence on size variation in the juvenile stages. This must be considered in any selection procedure utilising the grading system. A standardised methodology for grading has been developed from these results to separate the fastest growing fish into an elite line, which would remain isolated from the remainder of the production system and go forward to on-growing and, subsequently, broodstock selection.

**Chapter 6:**  
**Characteristics of Harvested**  
**Gilthead Seabream**

## **6.1 Introduction**

### **6.1.1 Seacages in Aquaculture**

Seacages are used for the long-term on-growing of many species of fish from the end of the weaning stage to the final market size of the product. The principle of seacages was developed from wooden, or bamboo, cages used in Asia more than a century ago (Beveridge, 1996). In the 1960's, floating cages began to be used for the on-growing of Atlantic salmon in Norway and Scotland, but it was not until the late 1970's when the use of seacages for the on-growing of gilthead seabream was tested with promising results (Pitt *et al.* 1977). The main advantage of using seacages over land-based ponds or open water ranching is the ability to monitor the growth and health, of the fish whilst reducing the facilities required for rearing in land-based facilities. In addition, the open water cages also resemble the natural environment of the fish more closely and can stock large numbers of fish into a single site. Recently, there has been an interest in open-water ranching and enhancement of natural stocks, which has been effective in species such as the red seabream (Kitada, 1999) though it is dependent largely on the rate of recapture of released fish (Svåsand *et al.*, 2000). A recent study suggests that gilthead seabream is suitable for such ranching operations in certain geographical locations (Sánchez-Lamadrid, 2002).

The cages used for the on-growing of seabream are generally very similar to those used for salmon. A large proportion of seabream fish farming is carried out in sheltered areas, such as those found in Greece (Basurco, 2000), where lightweight cage designs of plastic or fibreglass are sufficient. However, in the more exposed conditions found in Cyprus, round or square floating cages with a rigid floating collar of plastic (PolarCircle) or rubber coated steel (Dunlop) are generally in use (Stephanou, 2000). Recent developments in cage design have moved towards submerged designs that are even more robust and can hold larger numbers of fish (Scott and Muir, 2000) but require substantial investment to install.

### **6.1.2 Seacage Phase of Gilthead Seabream**

The seacage stage of the production of seabream is the longest of the farming cycle, lasting for up to 18 months. It is an important growth stage for the fish and, therefore, is a primary point for the development of potential selection traits for a breeding programme. Juveniles leave the hatchery rearing stage at a mean size of 2g (45mm body length) for stocking into seacages. Stocking density in the cages varies from 120,000 to 150,000 fish per 100m<sup>3</sup> cage, depending on the production cycle of the hatchery. During the cage period (547 days), the fish will reach a mean weight of 350g and are suitable for harvesting. Growth in the seacages is rapid, but the residence period inevitably involves a winter season in which the growth of fish decreases as a result of lower water temperatures (Pitt *et al.*, 1977). In Cyprus, mean sea temperatures are at their lowest during the period November to February, reaching down to approximately 16°C. Therefore, the ideal point to stock the cages is from March to May, so that the 18-month phase includes only a single winter period.

At Alkioni Fish Farms, harvesting is carried out by seine-netting the cage, to remove approximately 500 fish at a time for transport to the packing facility. The

fish are transported in large plastic containers, which are then emptied into a holding tank. From this tank, the fish are drained and drawn through to the packing line, where they are roughly size sorted into boxes of 5kg of fish and packed in ice. Harvesting is a rapid process and, in general the fish are packed within an hour of capture, which is important, as the market requires freshness. Chilling is an important feature of the harvesting process, as it can influence meat quality. Skjervold *et al.* (2001) found that crowding stress increases negative effects on quality, such as rapid *rigor mortis* and firm meat in salmon, whereas chilling reduces these effects. Depending on market demands, a cage will be harvested over a two-to four-week period and produce over 40 tonnes of fish.

### 6.1.3 The Seacage Phase in a Breeding Programme

The seacage phase represents an extended growth period and perhaps the most economically important stage of the farming cycle, due to the large amounts of feed used to maintain the high growth rate. The seacage environment is also relatively constant, in that all fish experience a winter period of reduced sea temperature and feeding rates are standardised to maintain similar growth between cages, so as to fix the harvesting order. This is also a stage where the measurement of specific traits can be carried out at the start and end of this distinct phase. A distribution of initial stocking size can be collected, as well as the final harvesting size of a large sample of fish. Measurement of traits is a fundamental part of the selection process and is required to measure the progress of any programme. The seacage stage is the point where improvements to traits such as growth and survival are likely to make the largest impact economically. For this reason, this is where the focus of a selection programme should be placed.

Selection can be performed on any measurable trait (if the heritability is  $>0$ ). However, it must have an economic value, if it is to form part of a breeding programme, to justify the expense of establishing the very programme (Refstie, 1990). In general, the most important trait in farming is that of growth rate, which is related to the feed conversion ratio (FCR), as this has the greatest potential to reduce the costs of on-growing. Other significant traits of adult stages are survival and disease resistance, which tend to have a lower heritability, but may also be weakly correlated to growth rate (Fjalestad *et al.*, 1993). Disease resistance to specific pathogens requires additional facilities to test groups and families, which will increase the costs of the programme. Quality characteristics are another factor that may influence the price of harvested fish and, therefore, increase the value of the stock. These traits can include body shape, colour and malformations. Characters such as body fat or flesh quality are harder to include in a selection programme, because fish need to be culled prior to measurement. This is a process that cannot readily be combined with mass selection techniques, as it requires pedigree data (Tave, 1993).

To estimate the phenotypic variation and hence, the potential to select for specific traits, a study of the harvested fish from Alkioni Fish Farms was conducted. From measurements of the fish at harvesting, it should be possible to propose various selection criteria and predict the response to selection in subsequent generations, if such fish were to be used as parents of the next generation. This should give an indication of the magnitude of the gains that could be achieved in a selection programme for characters such as body weight at harvest age. The frequency of

abnormalities and variations in body shape characteristics may be included in future selection efforts and baseline data for the population is presented here.

## **6.2 Materials & Methods**

### **6.2.1 Cages**

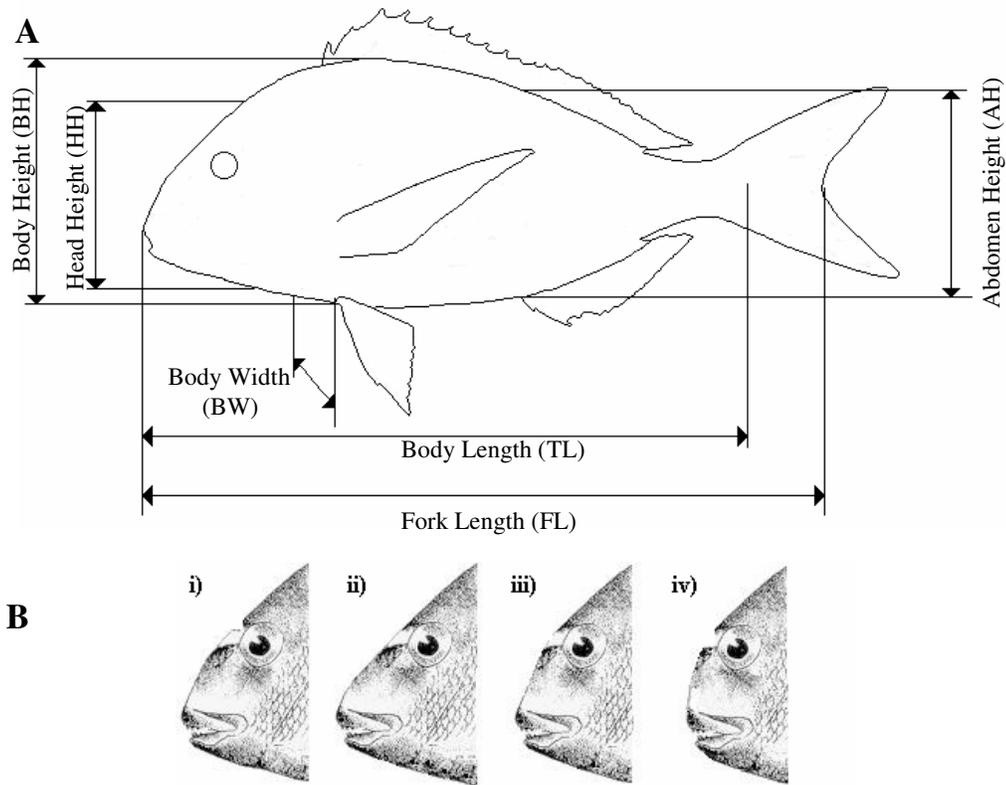
Measurements were collected on fish from two production cages harvested in May 2001. Both cages were stocked in October 1999 with approximately 120,000 fish from the Alkioni hatchery with a mean size of 2g. Total residence time, calculated as stocking date to the first harvesting date, ranged between 548 days (cage 14) and 586 days (cage 7). A total of 700 fish from cage 7 and 738 fish from cage 14 were measured over a one-month period as they were harvested.

### **6.2.2 Measurements**

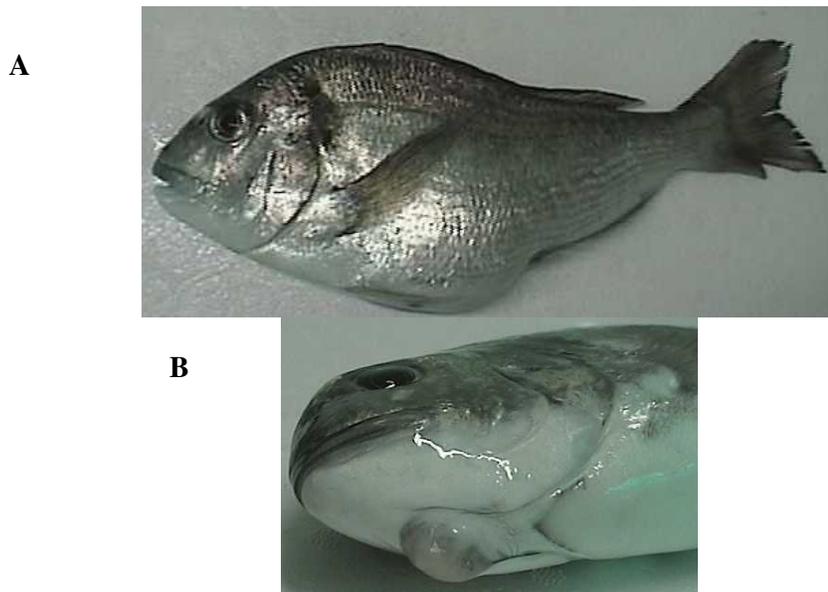
Fish were collected during the regular harvesting operations of the company at the point of entry to the packaging facility. This was chosen as a suitable sampling point because, it required no additional labour to catch and transfer the fish, it required no anaesthetic, due to the normal harvesting techniques, and had the ability to obtain a random sample of several thousand fish at each operation. Fish were removed, weighed and measured in batches of 20 fish, before being returned to the facility to be processed as normal, with no loss of quality. Fish were weighed on electronic scales ( $\pm 0.1$ g) and measured using a 30cm measuring block ( $\pm 1$ mm) and Vernier calipers ( $\pm 0.1$ mm). Measurements of body length (BL), fork length (FL), head height (HH), body height (BH) abdomen height (AH) and body width (BW) were collected, as indicated in Figure 6.1(A). As well as these physical characteristics, subjective estimates of the head shape were made on each fish, as well as recording the occurrence of deformities. Four head shape categories were identified: rounded, flat, normal and pointed (Figure 6.1(B)). Malformations were classified into four groups: spine (lordosis), gill (missing opercular cover), jaw (malformed jawbone) and throat (lesions resulting from extended pharyngeal bone). Other malformations, such as saddleback, were also be found in the stock, however, their frequency was very low ( $<0.5\%$ ), and so were excluded from this analysis. Examples of lordosis and throat lesion malformations are given in Figure 6.2.

### **6.2.3 Data Analysis**

The predicted response to mass selection is given by the equation:  $R = h^2 S$ , where  $h^2$  is the heritability of the trait and  $S$  is the selection differential (Falconer and MacKay, 1996). Heritability of the weight at harvest age was estimated as  $0.55 \pm 0.22$ , based on the findings by Batargias (1998) of heritability of weight in gilthead seabream at age 22 months. A selection criterion of two standard deviations above the mean was used to replicate a high intensity of selection. Therefore, the selection differential ( $S$ ) was equal to the difference between the population mean and the mean of the group of fish two standard deviations or more above the population mean. Using this method, the expected response in the offspring population of the selected individuals can be calculated as a mean weight increase. By calculating a daily weight increase of the parental generation, the response to selection can then be converted into a number of days taken to reach the mean harvest weight of 350g.



**Figure 6.1:** A) Morphological measurements collected on harvested fish: fork length, body length, head height, body height, abdomen height and body width. B) Head shape categories used to define harvested fish: i) normal, ii) pointed, iii) flattened, and iv) rounded.



**Figure 6.2:** Examples of malformations in the harvested fish. A) Lordosis is characterised by the fusing of vertebrae leading to curvature of the spine and an abnormal body shape. B) Lesions in the throat area may be the result of extended pharyngeal bones. In this study A) was classified as ‘spine’ and B) as ‘throat’.

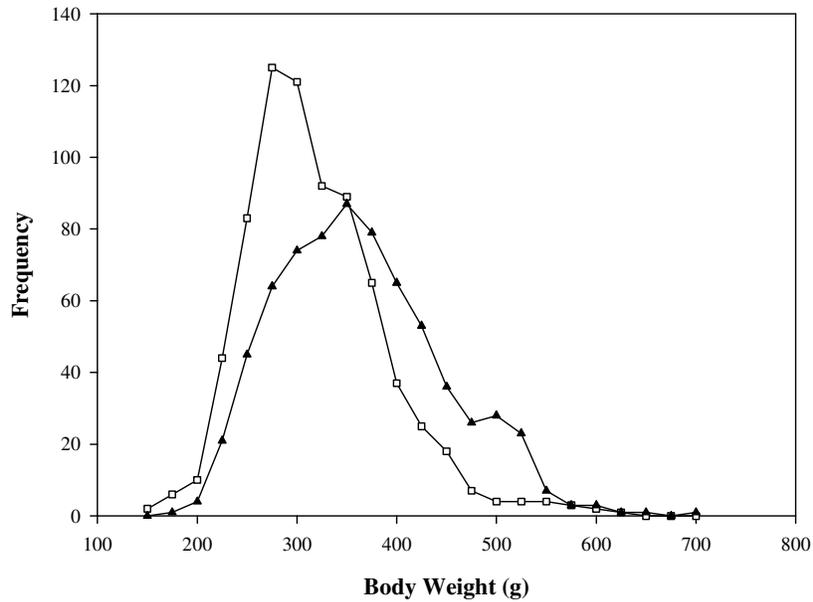
## **6.3 Results**

### **6.3.1 Morphological Characteristics**

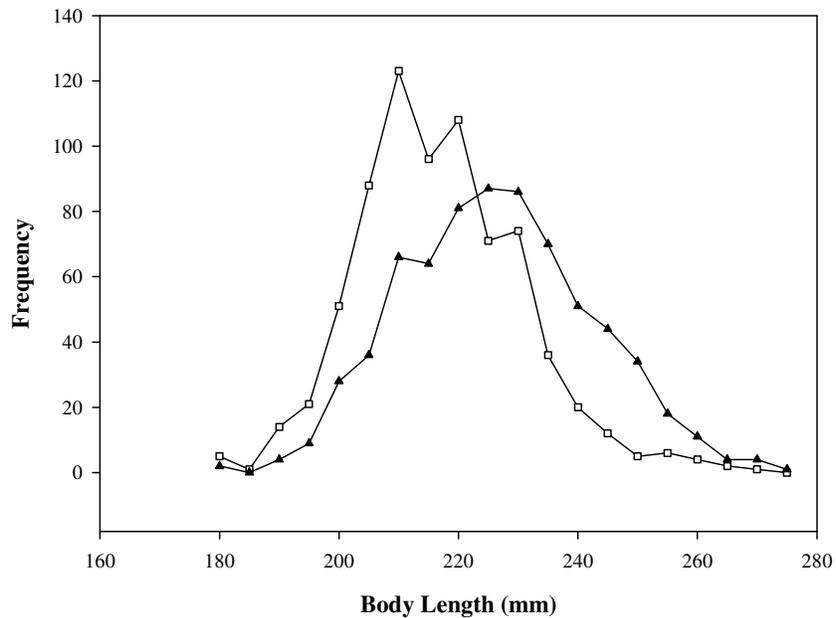
In both cages there was a wide distribution of weight and body length (Figures 6.3 & 6.4). The mean weight (standard deviation in brackets) in cage 7 was 376.78g (83.09g) and the mean body length 226.44mm (15.898mm). In cage 14, the mean weight was 329.92g (67.36g) and the mean body length was 217.22mm (13.787mm). The larger mean weight and body length, observed in cage 7, is mainly due to the extended residence time of an additional 38 days, in comparison to cage 14. The magnitude of the differences, however, also suggests that the mean size of juveniles at the time of stocking may also have been larger for cage 7, or that cage effects may have been responsible for the growth differences. Unfortunately, data on the mean stocking size of the fish in each of the cages was unavailable and this hypothesis cannot be confirmed. The distribution of weight and body lengths was close to a normal distribution in both cages. However, a small skewness exists in the weight data of cage 14, as indicated by a skewness value of 0.762 ( $\pm 0.09$ ). The absence of bimodal or strongly skewed distributions suggests that there are none of the strong hierarchy effects, seen in the early juvenile stages. There is, however, a distinct group of larger fish in the weight distribution of cage 7, at 500g. All measurement points were strongly correlated to body weight, ranging from 0.83 for abdomen height to 0.93 for body length and body length.

### **6.3.2 Quality Characteristics**

Little variation in body shape was observed and the subtle differences were difficult to classify, which meant that this characteristic could not be properly evaluated. Differences were noted towards a slightly elongated shape in some fish and also a rounding of the body, which was frequently associated with fish malformed by spinal curvature (lordosis). A group of fish ( $n=38$ ) in cage 7 were noted to have a distinct 'bulkiness' in the body, compared to the majority of the population. These 'bulky' fish had a significantly higher mean weight (430  $\pm 10.94$ g) than the cage mean (t-test;  $p < 0.001$ , 602d.f), but this character was not related to any other parameter measured. Differences in head shape were more obvious and the frequency of each type is presented in Table 6.1. Cage 7 showed a higher frequency of the normal type, whereas cage 14 displayed a more equal proportion of normal and pointed shapes. The number of fish with the flattened and rounded head shapes were similar in both groups. Fish with a malformation of the jaw, such as a lateral twisting of the lower jawbone, often displayed a round head shape. As shown in Table 6.1, the mean weights of each category of head shape were very similar and no significant differences were found between types. The overall rate of malformations in cage 7 was 15%, mainly consisting of jaw and throat defects. The mean weight of the malformed fish indicates that average weight was not significantly reduced in fish with jaw and throat malformations (t-test,  $p < 0.375$  &  $p < 0.175$ , 601 & 611 d.f., respectively). Sample sizes were too small in the cases of fish with gill and spinal deformations to be reliable, although the lower mean weight of these fish does suggest that these malformations reduce growth.



**Figure 6.3:** Distribution of body weight in Cages 7 (—▲—) and 14 (—□—). The body weight trait is close to the normal distribution in both cages. A small increase in the number of fish at 500grams is noted in cage 7. Differences in the mean weight between cages is due to the extra residence time of cage 7.



**Figure 6.4:** Distribution of body length in Cages 7 (—▲—) and 14 (—□—). Body length is also close to the normal distribution in both cages. Differences in the mean length between cages is due to the extra residence time of cage 7.

### 6.3.3 Predicted Response to Selection

Using a selection criterion of two standard deviations from the mean, the selection differential was 183.8g in cage 14 and 211.01g in cage 7. This gave a response of 101.13g and 116.05g for cages 14 and 7, respectively. Therefore, the expected gain in harvest weight of offspring produced by the selected fish would be over 100g in both cages. This additional weight at harvest can be interpreted as an increase in the total production of the cage, which would mean approximately an additional 10 tonnes of fish at the harvest point. Alternatively, the gain can be viewed as a reduction in the time taken to reach the harvest weight of 350g, which equates to over 60 days in both cages. In both cases, the expected gain represents a large increase in the production system of the cages.

**Table 6.1: Proportion of head shapes and malformations found in the two cages (n=700 & 738 for cages 7 and 14 respectively) with mean weight ( $\pm$  SEM). Malformations were recorded in cage 7 fish only.**

		Cage 7		Cage 14	
		Proportion	Mean weight	Proportion	Mean weight
<b>Head Shapes</b>	<b>Normal</b>	<b>61.15%</b>	<b>381.93 <math>\pm</math>4.50</b>	<b>44.85%</b>	<b>331.44 <math>\pm</math>3.17</b>
	<b>Point</b>	<b>25.85%</b>	<b>371.05 <math>\pm</math>4.91</b>	<b>41.87%</b>	<b>329.51 <math>\pm</math>4.69</b>
	<b>Flat</b>	<b>5.71%</b>	<b>365.62 <math>\pm</math>14.02</b>	<b>6.77%</b>	<b>333.52 <math>\pm</math>12.02</b>
	<b>Round</b>	<b>7.29%</b>	<b>385.66 <math>\pm</math>12.69</b>	<b>6.51%</b>	<b>315.69 <math>\pm</math>9.03</b>
<b>Malformation</b>	<b>Spine</b>	<b>0.85%</b>	<b>321.50 <math>\pm</math>25.12</b>		
	<b>Throat</b>	<b>6.86%</b>	<b>365.02 <math>\pm</math>13.30</b>	<b>Not</b>	<b>Recorded</b>
	<b>Gill</b>	<b>0.71%</b>	<b>342.60 <math>\pm</math>33.36</b>		
	<b>Jaw</b>	<b>7.00%</b>	<b>357.45 <math>\pm</math>12.68</b>		

## 6.4 Discussion

An analysis of the phenotypic variation in body weight in harvested fish indicates that there is a large potential for selection and that a significant increase in production could be achieved. The predicted response based on reported heritability estimates was a substantial 100g per generation, but this figure should be viewed with caution due to the standard error of the heritability of harvest weight. The heritability of body weight in fish is commonly around 0.20 – 0.40 (Tave, 1993), which suggests that the estimate of 0.55 by Batargias (1998) is a generous estimate, although the species is relatively new to captivity and figures of 0.67 have been reported for 15-month body weight in Atlantic salmon (Bailey

and Loudenslager, 1986). Batargias calculated a standard error of 0.22, meaning that the heritability could be as low as 0.33, but, even in this scenario, the predicted gains would be in the order of 60g or 35-40 days earlier to harvest weight. The response to selection is known to reduce once a selection process is in operation because of the reduction in the heritability of the selected trait (Falconer and Mackay, 1996). However, genetic gains have been shown to reach up to 10% per generation in fish populations (Knibb, 2000), which still represents a large incremental improvement in production. Hershberger *et al.* (2000) produced genetic improvements of 60% in seacage harvest weight over ten generations of family selection. The equivalent gains in gilthead seabream would result in a time to harvest of only 330 days.

The traits of body weight and fork length showed a normal distribution in both cages and the degree of variation is probably a reflection of the size distribution at the time of stocking. Both cages were stocked with juveniles from mixed grades (i.e. not equivalent to an elite line) which were graded immediately prior to stocking. The larger group identifiable in cage 7, may represent 'jumpers' that were first noted at the end of the grading experiment in the top grade, but which may be present in all grades and especially where many groups have been mixed. The regularity of the distribution of size characteristics indicates that the seacages represent an environment where all fish are able to grow to a high level. Even the malformed fish were able to feed, grow and survive in the cage environment. This suggests that either the dominance hierarchies found in the juvenile fish dissipate in the older stages, or that an increase in space and resources is enough to counter their negative effects. Gilthead seabream typically feed near the surface of the seacages (Andrew *et al.*, 2002), which may allow less competitive fish to remain lower in the water column where they are still able to forage effectively. The stability of growth during this communal stocking phase also emphasises the suitability for developing selection criteria that relate to traits expressed in this period, as they can be readily quantified without large environmental effects.

The initial focus of most selective breeding programmes is on growth rate (measured by various parameters). A breeding objective, however, may also include several other traits such as survival, body shape and other quality indicators, which are of importance to the production of the species. Quality traits are mainly related to the demands of the market and, at present, this is relatively undeveloped for seabream, in terms of range of products and the size of the market (Bartley, 1998). Seabream are mainly sold as unprocessed fresh fish and the market is still largely based in Mediterranean countries. With an increase in the amount of processing required to produce new products, body composition traits, such as fat content, meat quality and meat colour, become more significant. Therefore, traits such as these should be considered for future breeding objectives. In this experiment, body shape was not quantified, as a suitable method of classifying shapes could not be developed. There was, however, evidence of an elongated form and also a rounded body shape in the harvested fish, which could also be identified in the broodfish. Selection criteria can be developed once an economic benefit to particular body shapes can be established. In contrast to body shape, the head shapes of fish were variable, forming four distinct categories. Yet, no significance in terms of differences in mean weight and length or body shape could be detected. The different shapes may also relate to parental phenotypes, but their use as selection criteria are limited.

An interesting observation was the frequency of 'bulky' fish, as these had a significantly larger mean weight than the rest of the population. A possible explanation is that these are fish that are precociously maturing into females. It is generally assumed that seabream are all male up to the age of one year old and that from the second year onwards a proportion of males will reverse to become female (Zohar *et al.*, 1995). It has also been noted that faster-growing fish in the adult stages normally undergo sex reversal earlier than the slower-growing remainder of the population (Batargias, 1998; Knibb *et al.*, 1997). If this is indeed the case, then it has important implications for the selection of fish, as females may form the majority of selected fish, based on their size, and this could lead to large imbalances in the sex ratio of the selected broodstock. The number of bulky fish in the population was just over 1%, which is a very small proportion of the population, but it has the potential to impact on the recruitment of broodstock because of their potential frequency in the selected group. A second selection criterion may be necessary for the selection of males, set at a point below that of the females to ensure that the selected stock has a balanced sex ratio. This may not be necessary, however, if a broodstock group is able to balance the sex ratio in the next spawning season, regardless of the initial sex of the fish, which was hypothesised in Chapter 3. In order to resolve this issue, the 'bulky fish' at harvest age need to be unambiguously identified as male or female by examining the gonads. In addition, the sex ratio of same-age broodstock needs to be monitored to determine if fish are able to perform controlled sex reversal, in order to balance the sex ratio in the following spawning season.

Malformations are a quality indicator that could have economic weighting, as, in general, they command a lower market price than normal fish. Approximately 15% of the fish from cage 7 displayed some form of malformation, with varying degrees of severity. Spinal malformations (lordosis) appeared to be the most serious in terms of reduced growth, but occurred at a rate of less than 1%. This malformation is actively controlled during the hatchery stage by testing all fish in an anaesthetic bath that reduces the buoyancy of fish with a functional swimbladder, whilst those without a swimbladder sink (Chatain and Corrao, 1992). The latter are thus separated out and culled. Malformations of the opercular cover also reduce growth, but are harder to control, as affected fish must be removed by manually checking every fish (Koumoundouros *et al.*, 1997). This is often performed at the end of the juvenile stage, just prior to seacage stocking.

The low frequency of these two malformations, which are regularly controlled, indicates the effectiveness of the techniques used. This also suggests that the malformations occurring at a higher rate, 6.86% for throat and 7.00% for jaw, manifest themselves primarily during the seacage phase. If this is the case, then the origin of the malformations needs to be known before any reduction of their frequency can be considered. For example, jaw malformations could be the result of many factors of the larval rearing conditions, such as temperature (Boglione *et al.*, 2001; Koumoundouros *et al.*, 2001), light intensity and infection by pathogens (Cobcroft *et al.*, 2001) and nutritional deficiencies (Alexis *et al.*, 1997). However, many types of malformations have also been linked to particular family groups (Alfonso *et al.*, 2000) and, therefore, may be open to selective improvement.

Evaluation of a selective breeding programme relies on the comparison of populations over time. The end of the seacage stage is an ideal opportunity to

gather data on the performance of fish. Traits such as growth rate are straightforward to measure, because of the definitive beginning and end to this stage, as well as the ease of access to collect measurements on the fish. As the market for gilthead seabream develops, there may be additional quality traits that are expressed during this phase of the life cycle that will become increasingly important. This stage is the most economically significant in the farming of this species and, therefore, should form the focus of the selection effort. It is also a stage in which there are large environmental variables, but the evidence suggests that growth differences between cages are small. Therefore, comparisons between cage populations will allow a reliable evaluation of the gains made by selection.

## **6.5 Conclusions**

The seacage phase is an ideal point at which to develop selection criteria. The long growth period produced a broad distribution of phenotypes within the test population, which indicates that truncation selection is feasible and that the selection intensities could be very high. Environmental conditions are similar between all cages over the extended growth period, allowing comparisons between cages and populations. Many traits expressed during this stage, such as growth rate, malformations and survival, have an economic value and can form the basis of the selection programme. Given the high heritability of growth rate reported by other authors in gilthead seabream, the expected response to selection is large and will represent significant economic benefits to the farm.

**Chapter 7:**  
**The Effective Population Size in**  
**Gilthead Seabream Broodstock**

## **7.1 Introduction**

Within small populations, a restricted number of breeding individuals can lead to the non-random selection of gametes and changes in the gene frequencies between generations. The effective population size ( $N_e$ ) is an important measure of the number of individuals contributing to subsequent generations. Often  $N_e$  is lower than the observed number of parents, because of factors in the mating system, selection, the biology and the history of the species that reduce the actual contribution of parents over generations.

The effective population size is often used as a measure of the status and history of a population because of its close association with inbreeding and genetic drift. When  $N_e$  is low, there is an increased chance of the loss of alleles and a more rapid fixation of others. The rate of inbreeding will increase when  $N_e$  is low, because fewer individuals are contributing to the next generation or with a more unbalanced representation, which increases the chances of mating between related individuals in subsequent generations. There is a direct relationship between  $N_e$  and inbreeding, such that  $N_e$  is equal to  $1/(2\Delta F)$ , where  $\Delta F$  is the per-generation rate of inbreeding (Falconer and Mackay, 1996).

Many animal populations are subdivided to form discrete breeding groups through either geographical separation or behavioural differences. Fluctuations of  $N_e$  within these groups may be an important mechanism in genetic drift and molecular evolution (Kimura, 1983). In captive populations, managing  $N_e$  is of paramount importance in maintaining the maximum possible genetic diversity of the population and avoiding inbreeding as much as possible.

### **7.1.1 Calculation of Effective Population Size**

In the simplest of conditions, such as those in the idealized population (Fisher, 1930; Wright, 1931), all individuals ( $N$ ) have an equal chance of breeding and contributing to the next generation in an infinitely large population of constant size. In reality, most populations do not meet these conditions and the number of breeding individuals does not adequately describe the effects of inbreeding and gene frequency changes (Caballero, 1994). The concept of the effective population size ( $N_e$ ) was developed by Wright (1931, 1938) and defined as the size of an idealized population which would give rise to the change in gene frequency or rate of inbreeding observed in the population under consideration.

There are two principle methods by which information to calculate the effective population size can be obtained. Firstly, by pedigree data, which indicates the number of offspring per parent and the inbreeding coefficient derived between offspring. Secondly, through temporal changes in gene frequencies in the population, such as loss of alleles and increasing homozygosity. Inbreeding coefficients are more straightforward to calculate in captive populations, where all the parents can be sampled and the relatedness between offspring determined by the mating system or retrospective assignment. The temporal method relies on data from non-linked, selectively neutral loci, each with several alleles (Waples, 1989). In populations with overlapping generations, this method needs to be corrected to account for changes due to age-specific survival (Jorde and Ryman,

1995; Waples, 2002). However, the temporal method is widely used in studies of natural populations and can be used to assess long-term changes in  $N_e$  by using DNA analysis of archived samples (Hansen *et al.*, 2002; Hauser *et al.*, 2002).

A series of equations has been developed for the prediction of effective population size under a range of circumstances (reviewed by Wright, 1969; Caballero, 1994). Any deviation from the conditions of the idealized population will have an impact on the calculation of effective size. The most prominent difference between the idealized situation and natural populations is the number of individuals constituting the breeding group. Changes in survival and recruitment are strongly influenced by environmental variables and periods of reduced population size, such as a bottleneck, have a negative impact on the magnitude of  $N_e$  and a corresponding increase in the rate of inbreeding (Shaffer, 1987). In addition, later expansion of the population will not reverse the inbreeding caused by such bottlenecks, but only serve to counter the accumulation of new inbreeding (Falconer and Mackay, 1996). Differences in the sex ratio of parents will limit  $N_e$  through the number of the less numerous sex. This has implications for populations with mating systems such as polygyny, in which the contribution of males is greater than females (Nunney, 1993). Non-random mating within other mating systems also has similar implications. Differences in the contribution of parents, through variations in fertility or viability of offspring, also reduces  $N_e$ . Terms for the variance of family size need to be considered in the solution of  $N_e$  equations (Caballero, 1994). A common property of animal populations is that generations are not discrete, but overlap forming an age structure in which individuals will differ in reproductive capacity over time. Effective population size is a measure of reproductive contribution over time and can be measured per generation or per unit time. With a generation interval  $L$ , then  $\Delta F \approx 1/L$  ( $\Delta F/\text{year}$ ). Therefore, given this reciprocal relationship, a longer generation interval will act to decrease  $N_e$  in comparison to a shorter generation interval.

### 7.1.2 Effective Population Size in Captive Populations

In captive populations the census size of the population can be large but the  $N_e$  is often low because of deviations from the idealized population. This is a particular concern, given the importance of many captive populations in farming systems, supportive and captive breeding programmes. By maximising the effective population size through management of the populations, the risks of inbreeding and genetic drift can be reduced.

Inbreeding is the mating between related individuals, which results in an increase in homozygosity and the fixation of deleterious alleles. When the effective population size is low, there is an increased chance of relatives mating in subsequent generations, because the population would be derived from a restricted number of individuals. Inbreeding results in the loss of fitness, by acting on traits such as fecundity and survival. Inbreeding depression occurs with non-additive variation. In the absence of epistasis, inbreeding depression will increase linearly with  $\Delta F$ . Su *et al.* (1996) noted reductions in growth rate, egg size and hatching rate, with 5.2-7.8% levels of inbreeding in rainbow trout after five generations of selection. Inbreeding is a particular concern in selective breeding programmes, because high selection intensities may be used, which favour the selection of related individuals. Inbreeding depression rarely affects production traits, such as

growth rate, as these are not directly implicated in the 'fitness' of an individual (Falconer and Mackay, 1996). Therefore, inbreeding may often be ignored in favour of short-term gains. Natural selection acts to increase fitness through additive genetic variance and, if the  $N_e$  is sufficiently high, a balance between inbreeding and natural selection can be achieved (Gall, 1987; Meuwissen and Woolliams, 1994)

Management of the reproductive behaviour to maximise  $N_e$  can be achieved through equalising the sex ratio of breeding individuals and the number of offspring in each family. Effective population size has been noted to be reduced through the effects of age structure, but it is also a function of contribution over time. Therefore, any extension to the generation interval, such as multiple breeding seasons, would increase  $N_e$  when  $\Delta F$  is measured per year. By controlling the number and which individuals mate, the number of offspring they produce and how often, the ratio of  $N/N_e$  can be maximised (Rodriguez-Clarke, 1999). Models for controlled mating in small populations include circular breeding and maximum avoidance, which aim to restrict inbreeding over the long term (Wright, 1969).

Truncation selection is carried out in many farmed populations for particular traits important to production. The process of selecting restricted numbers individuals based on high genetic merit to propagate the next generation are in direct conflict with the methods of maximising  $N_e$ . In highly fecund animals, such as fish, high selection intensities can be used and frequently a limited number of individuals are used to produce all the progeny in each generation (Pante *et al.*, 2001a). Within a selection programme, the introduction of wild or external stock to counter inbreeding (Eknath and Doyle, 1990) is prohibited, because this would reduce the genetic gains being made. One solution is to subdivide the population into independent units with crossing between units whilst maintaining genetic gains within each unit (Gjedrem, 1993; Bentsen & Gjerde, 1994). Restricting the size of full-sib families during selection is also a means to increase  $N_e$  by increasing the number of parents with offspring selected for breeding (Gjerde *et al.*, 1996), but this method also leads to a corresponding decrease in genetic gain.

### 7.1.3 Effective Population Size of Gilthead Seabream

Gilthead seabream is a mass spawning species and the contribution of individual fish to a spawning event is unknown. Within such a mating system, it is likely that there will be a high degree of variation in contribution and mating success between individuals. The sex of individual fish is determined by social factors and, although evidence suggests that the sex ratio is balanced in broodstocks (Zohar *et al.*, 1995), this may not always be the case. A further factor that may affect the contribution of parents is the high mortality rate in the early larval stages of the life cycle, which may cause the selective mortality of families. These factors indicate that the effective population size may be significantly lower than the census size of the broodstock.

Batargias (1998) calculated the  $N_e$  of an experimental stock of gilthead seabream over two successive seasons, based on the variance of parental contribution. The census size of the population was 32 and the  $N_e$  calculated as 12.0 and 9.1 from mass spawning in two consecutive seasons. The low  $N_e$ , observed in the offspring

population, was attributed to the high variation in the contribution of individual fish, an unbalanced sex ratio (especially in the second season) and the non-participation of several fish. The results indicate that under this mating system the effective population size may be low and that within a small population, such as a farm broodstock, techniques to maintain  $N_e$  may be necessary. Broodstock replacement within farms also tends to use fish produced from within the production system, thereby increasing the chances of inbreeding, if these individuals are derived from a small number of parents.

The aim of this experiment was to determine the effective population size of a commercial broodstock of gilthead seabream by sampling offspring from a single day's spawning during the larval stages of development within the farm production system. Previous studies have also suggested a possible relationship between contribution and body size (Batargias, 1998). This will be examined in a larger number of fish in this experiment.

## **7.2 Materials and Methods**

### **7.2.1 Sample Collection**

Offspring samples were collected from three farm broodstock groups, each consisting of 60 fish of mixed age (4-6 years old). Samples were collected from production tanks that had been stocked with eggs produced from a single day of mass spawning (>500g eggs), from each broodstock group. All samples were collected during the hatchery larvae stage (hatching to 60 days post-hatch), but at different ages (see Table 7.1). The natural cycle broodstock group was sampled in both 2001 and 2002, whereas other groups were sampled only in 2002. All samples were preserved and stored individually in ethanol.

### **7.2.2 Genotype Data Collection**

Genotypes were generated for each sample at a minimum of six loci, published by Batargias *et al.* (1998) and Brown *et al.* (2003), using the methods described in section 2.5. An ABI 377 automated gene sequencer and Genescan™/Genotyper™ software (PE Applied Biosystems) was used for all raw data processing.

PAPA software (Duchesne *et al.*, 2002) was used for parental assignment of parental pairs with no sex information. A 5% probability of scoring errors was assumed at all alleles. Successful assignment was considered only with a minimum of five matching loci between the most likely parental pair and offspring. A parental matrix was constructed from the assignment of offspring to calculate family variance and to allocate sex to contributing parents.

**Table 7.1: Sampling regime and total sample numbers for each broodstock group. Samples taken at different points in the larval development were pooled for the analysis.**

Broodstock Source (& year)	Larval age				Total No. of Samples
	5 Days	12 Days	40 Days	60 Days	
Group 2	34	52	37	-	123
Group 3	-	-	-	176	176
Group 5 (2001)	196	-	-	-	196
Group 5 (2002)	52	38	50	-	140

### 7.2.3 Calculation of Effective Population Size

Due to the mass spawning characteristics of seabream, it was assumed that family sizes and individual contributions would be unequal and, therefore, the most applicable calculation of  $N_e$  would rely on the variation in contribution between individuals. This information can be obtained from the parental assignment of offspring to parental pairs. To obtain a preliminary estimate of  $N_e$ , the value of  $\Delta F$  was calculated assuming that the population was to be propagated in discrete generations with random selection from a single broodstock group:

$$\Delta F = \frac{1}{2} \sum_{parents} C_i^2 - \frac{1}{4} (\bar{C}_m)^2 - \frac{1}{4} (\bar{C}_f)^2$$

(John Woolliams, Pers. Comm.)

where,  $C_i$  is the fractional contribution of parents,  $\bar{C}_m$  is the average contribution of males ( $1/(2N_m)$ ), and  $\bar{C}_f$  is the average contribution of females ( $1/(2N_f)$ ). The effective population size ( $N_e$ ) was then calculated from the relationship  $N_e = 1/(2\Delta F)$ . Samples taken from different points were pooled for each broodstock group. Confidence intervals are not available for the calculation of  $N_e$  based on this method, as it is an estimate based on a single day of spawning for each tank.

### 7.2.4 Calculation of Heterozygosity

Evidence of genetic drift and inbreeding within the current broodstock was assessed using measures of heterozygosity and fixation statistics (F-statistics) (Wright, 1969).  $F_{IS}$  is defined as the inbreeding coefficient of an individual relative to whole population (heterozygote deficiency) and was calculated using the three broodstock groups as sub-populations. Nei (1987) defines all fixation indices by using the observed and expected heterozygosities of the population being studied. Therefore,  $F_{IS}$  and Nei's equivalent of Wright's  $F_{ST} - G_{ST}$  - was derived from the following equations:

$$F_{IS} = 1 - \hat{h}_o / \hat{h}_s \quad G_{ST}' = 1 - \hat{h}_s / \hat{h}_t$$

$$\hat{h}_o = 1 - \sum_{k=1}^s \sum_{i=1}^m \hat{x}_{kij} / s$$

$$\hat{h}_s = \frac{\tilde{n}}{\tilde{n} - 1} \left[ 1 - \sum_i \hat{x}_i^2 - \frac{\hat{h}_o}{2\tilde{n}} \right]$$

$$\hat{h}_t = 1 - \sum_{i=1}^m \bar{x}_i^2 + \frac{\hat{h}_s}{\tilde{n}s} - \frac{\hat{h}_o}{2\tilde{n}s}$$

Where  $\hat{x}_{ki}$  and  $\hat{x}_{kij}$  are the frequencies of the alleles  $A_i$  and genotypes  $A_iA_j$  in the sample from the  $k$ th subpopulation,  $m$  is the number of alleles,  $s$  is the number of subpopulations each in Hardy-Weinberg equilibrium and  $\tilde{n}$  is the harmonic mean of the number of individuals.

Offspring and parental genotype frequencies were compared using  $G_{ST}'$  which is the coefficient of gene differentiation (independent of sample size) (Nei, 1987). The calculation of unbiased heterozygosity was given by:

$$\hat{h} = \frac{2n(1 - \sum \hat{x}_i^2)}{(2n - 1)} \quad (\text{Nei, 1987})$$

where,  $\hat{x}_i$  is the population frequency of the  $i$ th allele at a locus and  $n$  is the sample number. The estimate of allelic richness was derived from:

$$R_s = \sum_{i=1}^{n_i} \left[ 1 - \frac{\binom{2N - N_i}{2n}}{\binom{2N}{2n}} \right]$$

(Petit *et al.*, 1998)

where  $N_i$  is the number of alleles of type  $i$  among the  $2N$  genes. All population statistics were calculated using FSTAT software (Goudet, 1995, 2001).

## 7.2.5 Parental Contribution to Spawning

Contingency tables of the proportion of offspring contributed by each parent were used to analyse changes in contribution at different offspring ages. Non-contributing fish were excluded from the analysis. The significance of table values were tested with both Chi-square distributions and maximum likelihood (ML) methods with a null hypothesis that the distribution of contribution among parents was constant over time. Rejection of the null hypothesis would suggest that differential selection between families may have occurred between sample points. The analysis was performed using Genstat software and only ML significance levels are presented in the text.

The relationship between parental contribution and parent weight was investigated by log-linear modelling of offspring counts (assuming Poisson distribution of counts). The regression model had terms for the deviation of weight from the mean weight of each sex within tanks (termed residual weight). The model also included terms for sex of the parent, the residual of weight from the tank mean, the residual weight-squared and interaction terms for sex and weight. In cases where these interactions were not significant, they were dropped from the model in a stepwise manner. It should be noted that offspring counts were found to deviate from the assumption of an independent Poisson distribution and the levels of significance were constructed from approximate F-tests using mean deviance ratios. Non-contributing fish were included in the analysis. The sex of fish was

determined by generating a parental matrix based on the assignment of parental pairs to the offspring using genotype data. Non-spawning fish were added to the matrix, based on the observed sex. In addition, parent weight in tank three broodstock was based on 2001 measurements, as these were not available in 2002.

## **7.3 Results**

### **7.3.1 Effective Population Size**

The effective population size calculated from a single event of mass spawning was very similar between broodstock groups and ranged from 14 to 18 (Table 7.2). The comparable values of  $N_e$  between tanks and over years suggest that there is a consistent relationship between the relative contributions and the number of broodstock. However, variation in family size was different between tanks, but was always higher for male parents compared to female parents (Table 7.2). Large variation in family size and a deficiency of males in the spawning groups were the main factors in reducing the magnitude of  $N_e$  and producing a ratio of  $N_e / N$  from 0.266 to 0.329. A small effective population size is also indicative of a higher rate of inbreeding and, across the tanks  $\Delta F$  ranged from 2.7 to 3.5% (Table 7.2). These values strongly suggest that methods to increase the effective population size are needed, in order to manage the genetic variation within the farm population, and allow the replacement of broodstock without recourse to wild stock.

Given the potentially high rate of per-generation inbreeding observed from a single mass spawning event, an assessment of the heterozygosity in the current broodstock was performed, to determine if genetic drift has already resulted from a limited number of parents contributing to the next generation. Comparisons between the parental and offspring populations, using indicators of gene diversity and differentiation ( $G_{ST}$ ), are shown in Tables 7.3-5. Offspring heterozygosity is lower at all loci and is expected to be reduced by the order of  $\Delta F$ , which was calculated as 2-3%. The coefficient of gene differentiation ( $G_{ST}'$ ) is low, less than 0.025 at all loci in all the offspring groups, indicating that there is no evidence of genetic drift over this single generation. In cases of random mating the values of  $F_{IS}$  are expected to be negative because of an excess of heterozygotes (Robertson, 1965). Most loci in the offspring group show  $F_{IS}$  values close or below zero.  $F_{IS}$  values in the parental group are generally higher, particularly at certain loci, such as SaGT41b and SaGT26, which could also indicate scoring errors. Comparisons of allelic richness indicate that several alleles present in the broodstock are not found in the offspring, a result mainly of the non-participation of some broodfish. Alleles found at low frequencies in the broodstock and will be the first alleles to be lost when there is a differential in parental contribution and the restricted sampling of gametes. Therefore, the high number of alleles in the broodstock indicates that it is not significantly inbred.

**Table 7.2: Effective population size calculated from a single day of mass spawning of each broodstock photoperiod group.  $\Delta F$  is the rate of inbreeding,  $N_e/N$  is the ratio of effective population size to number of parents present, while  $V_f$  and  $V_m$  is the observed variance of female and male parents, respectively.**

Broodstock	$N$	$\Delta F$	$N_e$	$V_f$	$V_m$	$N_e/N$
Advanced	58	0.0324	15.41	57.33	79.97	0.266
Natural (2001)	54	0.0272	18.34	73.54	162.54	0.294
Natural (2002)	51	0.0297	16.81	57.83	141.22	0.329
Delayed	48	0.0357	14.00	35.72	101.29	0.292

**Table 7.3: Comparison of heterozygosity, genetic differentiation and allelic richness between delayed photoperiod parents (P) and offspring (O).  $h'$  = unbiased heterozygosity;  $F_{IS}$  = heterozygote deficiency within groups;  $G_{ST}'$  = coefficient of gene differentiation (independent of sample size); Offspring n= 123, parents n= 60.**

Locus	Heterozygosity		Genetic Differentiation			Allelic Richness	
	$h'$ O	$h'$ P	$F_{IS}$ O	$F_{IS}$ P	$G_{ST}'$	O	P
SaGT41b	0.829	0.899	0.014	0.179	0.013	11.41	21.00
SaGT32	0.866	0.906	-0.076	-0.095	0.008	10.35	12.00
SaGT1	0.886	0.924	-0.052	0.061	0.013	13.88	22.00
SaGT26	0.857	0.892	-0.067	0.247	0.011	12.53	16.00
Sai12	0.805	0.849	0.014	0.110	0.024	8.91	11.00
Sai14	0.761	0.867	0.202	0.070	0.011	12.04	14.00
Sai19	0.818	0.892	0.159	-0.056	0.010	10.24	14.00
All Loci	0.832	0.890	0.024	0.073	0.013	-	-

**Table 7.4: Comparison of heterozygosity, genetic differentiation and allelic richness between advanced photoperiod parents (P) and offspring (O).  $h'$  = unbiased heterozygosity;  $F_{IS}$  = heterozygote deficiency within groups;  $G_{ST}'$  = coefficient of gene differentiation (independent of sample size); Offspring n= 200, parents n= 60**

Locus	Heterozygosity		Genetic Differentiation			Allelic Richness	
	$h'$ O	$h'$ P	$F_{IS}$ O	$F_{IS}$ P	$G_{ST}'$	O	P
SaGT41b	0.909	0.929	-0.059	0.188	0.005	18.06	23.00
SaGT32	0.858	0.899	-0.087	0.048	0.007	9.82	13.00
SaGT1	0.905	0.938	-0.035	-0.004	0.014	17.05	21.00
SaGT26	0.851	0.847	-0.025	0.188	-0.002	11.15	12.00
Sai12	0.828	0.858	-0.120	0.041	0.002	9.14	14.00
Sai14	0.822	0.841	-0.012	0.021	0.001	10.67	13.00
Sai21	0.916	0.964	0.275	0.252	0.005	20.778	32.00
All Loci	0.863	0.889	-0.025	0.071	0.008	-	-

**Table 7.5: Comparison of heterozygosity, genetic differentiation and allelic richness between natural photoperiod parents (P) and 2002 offspring (O).  $h'$  = unbiased heterozygosity;  $F_{IS}$  = heterozygote deficiency within groups;  $G_{ST}'$  = coefficient of gene differentiation (independent of sample size); Offspring n= 140, parents n= 60.**

Locus	Heterozygosity		Genetic Differentiation			Allelic Richness	
	$h'$ O	$h'$ P	$F_{IS}$ O	$F_{IS}$ P	$G_{ST}'$	O	P
SaGT41b	0.883	0.928	-0.026	0.096	0.006	16.19	22.00
SaGT32	0.888	0.902	-0.030	-0.101	0.002	11.41	14.00
SaGT1	0.914	0.932	0.016	-0.010	0.015	16.72	23.00
SaGT26	0.869	0.872	0.008	-0.005	0.010	11.11	12.00
Sai12	0.779	0.850	0.062	-0.049	0.008	9.45	12.00
Sai14	0.833	0.782	0.032	0.034	0.022	10.25	16.00
Sai19	0.881	0.902	0.018	0.050	0.011	10.79	16.00
All Loci	0.864	0.881	0.010	0.002	0.010	-	-

**Table 7.6: Comparison of genetic differentiation between broodstock groups (Del= delayed, Adv= advanced, Nat= natural photoperiod), indicated by inbreeding coefficients and allelic richness.  $F_{IS}$ = inbreeding coefficient of an individual relative to whole population (heterozygote deficiency);  $G_{ST}'$ = coefficient of gene differentiation (independent of sample size); n= 60 for all tanks.**

Locus	Wright's $F_{IS}$			$G_{ST}$	Allelic Richness		
	Del Tank	Adv Tank	Nat Tank		Del Tank	Adv Tank	Nat Tank
SaGT41b	0.179	0.188	0.096	0.001	21	23	22
SaGT32	-0.095	0.048	-0.101	-0.003	12	13	14
SaGT1	0.061	-0.004	-0.010	0.003	22	21	23
SaGT26	0.247	0.188	-0.005	0.003	16	12	12
Sai12	0.110	0.041	-0.049	-0.002	11	14	12
Sai14	0.070	0.021	0.034	0.003	14	13	16
Sai19	-0.056	0.019	0.050	0.002	14	15	16
All Loci	0.073	0.071	0.002	0.001	-	-	-

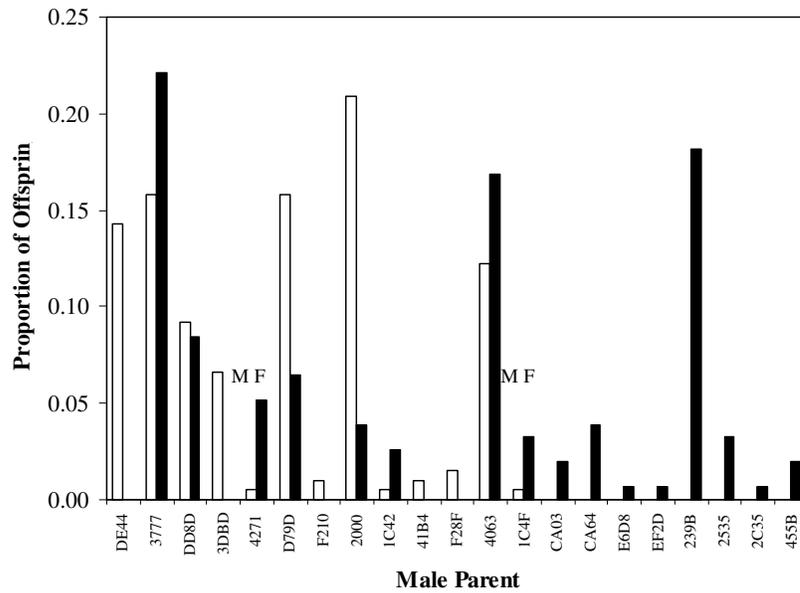
### 7.3.2 Parental Contribution Over Time

Using contribution data from the natural group in 2001 and 2002, parental performance in both years was investigated. Figures 7.1 and 7.2 contain plots of each male and female parent and their contribution in both years. As noted above, there was a large disparity in the contribution of individual broodfish, leading to a large variance in family size. In all broodstock groups, the number of fish spawning on a single day was similar, ranging from 25-35. This represents approximately 50-60% of the fish in each group actually spawning on any one day. In the natural group, all the females spawning in 2002 were also represented in 2001, whereas several new male fish are found in the 2002 season, compared to 2001. These additional male fish also consisted entirely of the fish introduced into

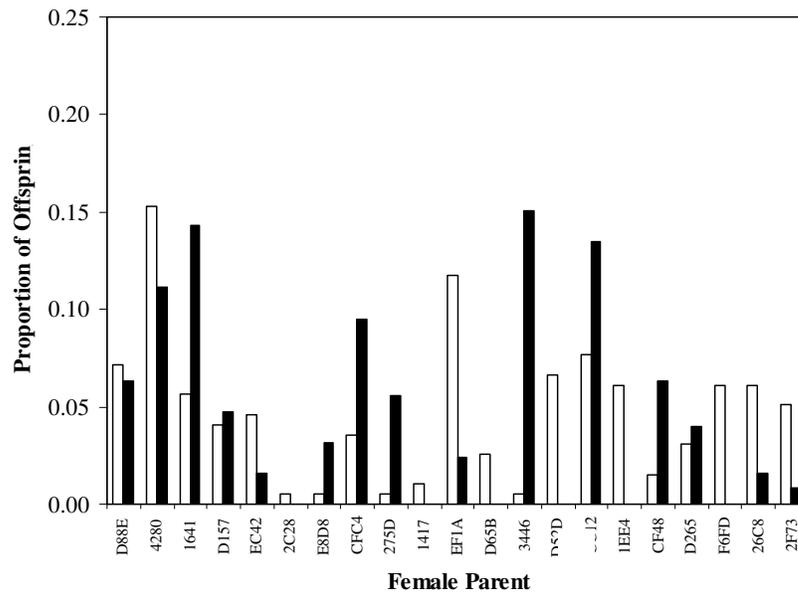
the broodstock in 2000, of which only one was represented in the 2001 season. In total, 16 females and eight males contributed in both seasons. Two fish were found to reverse sex (male-female) and contributed offspring in both years.

Contingency tables were used to test the hypothesis that relative numbers of offspring from individual fish followed a similar distribution in both years. However, contribution was significantly different between the two years for males ( $P < 0.001$ , 23 d.f.) and females ( $P < 0.001$ , 27 d.f.) and the null hypothesis was rejected. Parents with high contribution in 2001 were not necessarily the high contributors in 2002, examples of which can clearly be seen for the male fish (DE44, D79D, 2000) in Figure 7.1. Contribution of the two sex-reversed fish was higher in the second season as females than in the first season as males. This indicates that reversing sex increased reproductive success in these particular fish.

The contribution data in 2002 was further subdivided into the three sampling points: day 5 (first feeding), day 12 and day 40 post-hatch, in order to identify how mortalities in the larval stage may affect the contribution of parents. The data is also presented graphically in Figures 7.3 and 7.4. Again using contingency tables, there was a significant difference in the number of offspring at each sample point for both males ( $p < 0.001$ , 27d.f.) and females ( $P < 0.003$ , 34d.f.). This suggests that the survival of larvae is parentally determined. However, this analysis is based on a small sample size and contingency tables are less effective when using small numbers within categories. Hence, the strength of the relationship may be lower than that indicated by the significance level. The male parents (Figure 7.3) display good examples of how the contribution changes over the sampling period. For example, fish 239B shows a large reduction in offspring numbers between first feeding and day 12, possibly indicating mortalities due to the inability to feed. In contrast, fish 3777 and 4063 show a large increase in the proportion of offspring from the first two sample points to day 40.



**Figure 7.1: Contribution of male parents to mass spawning over successive years (□=2001; ■=2002) in the natural photoperiod group. Significant differences were found in individual contribution over the two seasons. Data excludes 8 non-spawning fish in both years. Two fish; 4271 and 1C4F, reversed sex between seasons and their contribution as each sex is highlighted as male (M) and female (F).**



**Figure 7.2: Contribution of female parents to mass spawning over successive years (□=2001; ■=2002) in the natural photoperiod group. Data excludes 10 non-spawning fish in both years.**

Proportion of Offspring

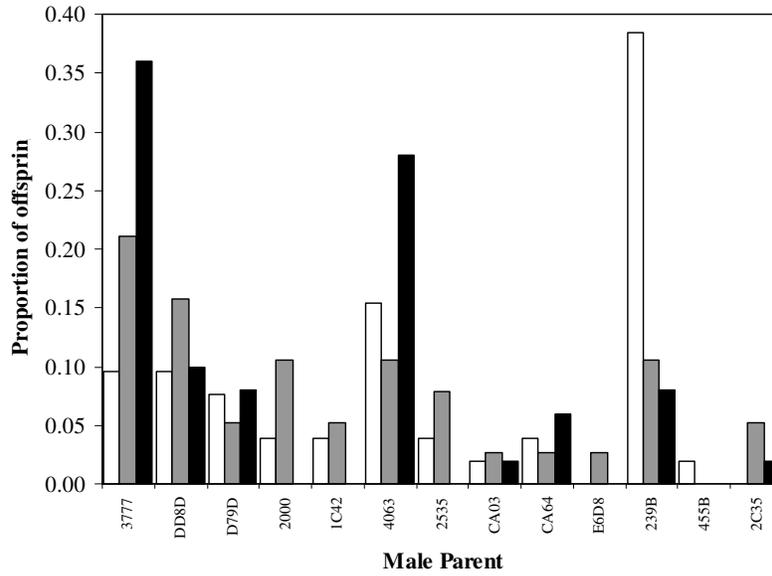


Figure 7.3: Proportion of offspring from each male parent (natural photoperiod) measured over the larval rearing period. Samples were taken in the time series: (□) day 5; (■) day 12 and (■) day 40 post-hatch. Data excludes nine non-spawning fish.

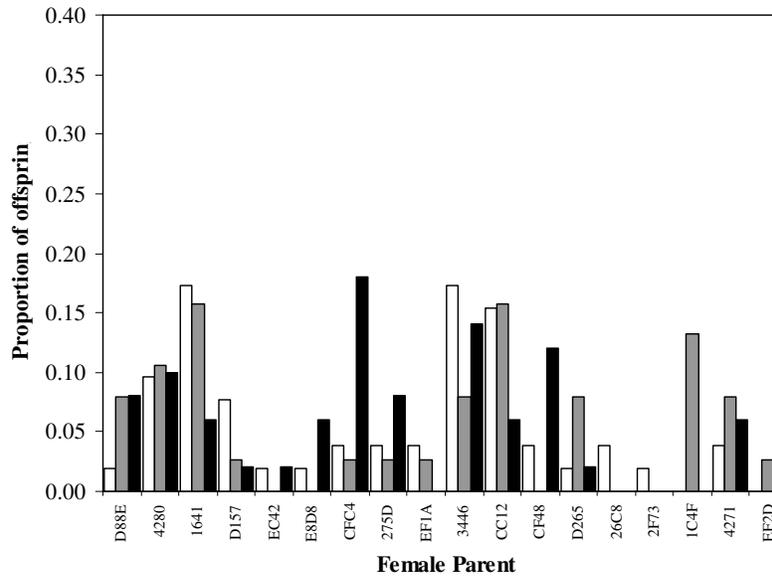


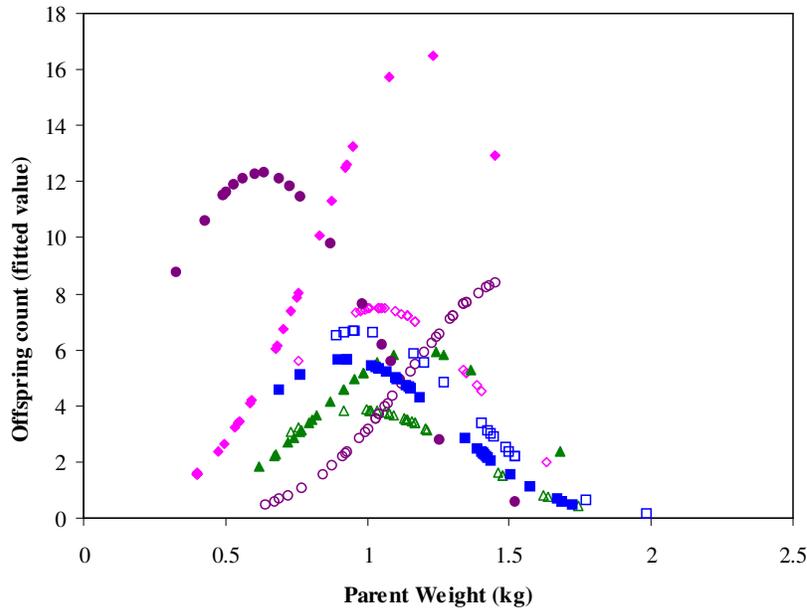
Figure 7.4: Proportion of offspring from each female parent (natural photoperiod group) measured over the larval rearing period. Samples were taken in the time series: (□) day 5; (■) day 12 and (■) day 40 post-hatch. Data excludes 11 non-spawning fish.

### 7.3.3 Contribution and Parental Weight

The distribution of parental contribution (offspring count) by weight of the parents indicated a possible quadratic effect with an intermediate optimum centred around the mean weight of each sex. This was investigated using a log-linear modelling of offspring counts and the deviation from the mean weight of each sex in each tank. A summary of the analysis results is given in Table 7.7. The terms residual weight, residual weight squared and the interactions with sex and tank were significant, but varied between tanks and individual terms were dropped from the model in some tanks. When all data was pooled, the terms for residual weight and residual weight squared were both significant ( $P < 0.01$ ), indicating a strong quadratic effect of contribution around the mean weight. Fitted values were derived from the model and are plotted in Figure 7.5. With the exception of advanced group females and natural group (2001) males, the pattern of contribution reaches a peak at approximately 1 – 1.25kg parental weight. The most striking feature of Figure 7.5 is the very low contribution of all the remaining groups beyond 1.5kg parental weight, which reaches almost zero at 1.75kg for most groups. This suggests that larger broodstock may have a reduced reproductive success, relative to fish at the mean size for the sex within the tank. Only the advanced group females do not fit the quadratic model but instead, show a more linear increase in contribution and weight. Advanced group parental weights were based on 2001 measurements as these were unavailable for 2002 when the offspring samples were collected. Therefore, the weight of both male and female parents was underestimated, and this may explain the poor fit of the data from the groups in this tank. Contribution of natural group males in 2001, was dominated by three fish and, therefore, the model indicates very high values between 1-1.5kg, corresponding to the weight of these dominating fish.

**Table 7.7: Summary of log-linear modelling of the contribution data and parental weight. The significant terms from each of the models are presented with the significance level and residual degrees of freedom. The residual weight is the deviance of weight from the mean of each sex within tanks.**

<b>Model:</b>			
Constant + Sex + Tank + Residual weight + Sex * Tank + Residual weight * Sex + Residual weight * Tank + Residual weight * Sex * Tank + (Residual weight) <sup>2</sup>			
<b>Group</b>			
<b>All Data</b>	Significant terms:	Residual weight	P=0.093 199 d.f.
		Residual weight * Tank (T3)	P=0.006 199 d.f.
		Residual weight * Sex * tank (T3)	P=0.093 199 d.f.
		(Residual weight) <sup>2</sup>	P=0.009 199 d.f.
<b>Natural (2001)</b>	Significant terms:	(Residual weight) <sup>2</sup>	P=0.012 55 d.f.
		Residual weight * Sex	P=0.014 55 d.f.
<b>Delayed</b>	Significant terms:	(Residual weight) <sup>2</sup>	P=0.110 46 d.f.
		Residual weight * Sex	P=0.113 46 d.f.
<b>Advanced</b>	Significant terms:	Sex	P=0.039 53 d.f.
		Residual weight * Sex	P=0.016 53 d.f.
<b>Natural (2002)</b>	Significant terms:	Residual weight	P=0.049 44 d.f.



**Figure 7.5: Fitted values from log-linear modelling of parental contribution against parent weight. Each broodstock photoperiod group and each sex are plotted individually: ( $\diamond$ ) natural group (2001) female, ( $\blacklozenge$ ) natural group (2001) male; ( $\triangleleft$ ) delayed group female, ( $\blacktriangleleft$ ) delayed group male; ( $\circ$ ) advanced group female, ( $\bullet$ ) advanced group male; ( $\square$ ) natural group (2002) female, ( $\blacksquare$ ) natural group (2002) male.**

## 7.4 Discussion

The effective population size, based on sampling from a single mass spawning event, was moderately low, given the number of fish actually contributing and the number of fish in the broodstock as a whole. This was due to a large variation in the contribution of individuals and an unbalanced sex ratio. The results were similar to those found by Batargias (1998) in that the  $N_e$  was equivalent to approximately 1/3 of the number of fish present at the time of spawning. The consistency of the results suggests that the dynamics of mass spawning in gilthead seabream may be an important consideration in the management of captive stocks. For example, doubling the number of potential parents does not result in a corresponding increase in  $N_e$ . However, these calculations are a single point measurement and do not take into account the effective population size over time. Each parent can potentially contribute on a daily basis over the spawning season and, therefore, contribution may vary between days. Under the current replacement regime, fish can remain in the broodstock for up to four years, which will significantly increase the contribution of individuals over time and, in turn,  $N_e$ . However, broodstock are replaced with fish taken from within the farming system and, as there is no information on the genetic background of candidates, the selection of related individuals (between candidates and existing broodstock) could inadvertently increase the rate of inbreeding.

The optimal  $N_e$  to maintain genetic diversity, may be in the order of several hundred, especially if the intention is to supplement wild populations (Lande and Barrowclough, 1987; Tave, 1993). In farmed animals, an  $N_e$  of 31 to 250 was suggested by Meuwissen and Woolliams (1994), in order to prevent a decline in fitness traits. Tave (1993) recommends an  $N_e$  of 45-250 for food fish farming and Gjerde (1993) and Jørstad & Nævdal (1996) conclude that an  $N_e$  of 100 per generation is sufficient to minimise inbreeding in salmonid breeding programmes. Over the medium term, a constant  $N_e$  of 100 is the most applicable to fish farming operations that are likely to be constrained in the size of facilities available for maintaining broodstock. An  $N_e$  of this magnitude should prevent substantial inbreeding and loss of alleles over a period of approximately ten generations (Tave, 1993).

These recommended estimates of  $N_e$  are substantially larger than the  $N_e$  observed in this experiment for gilthead seabream. This confirms the need to increase the  $N_e$  in the farm population, in order to avoid the risks of inbreeding in a selection programme. The most effective way to increase the  $N_e$  for a fixed number of parents would be to equalise the contribution of each parent so reducing the variance in family size. This is particularly problematic in this species, because of the difficulty in manually stripping the fish and the number of fish needed to stimulate mass spawning behaviour (Gorshkov *et al.*, 1998). However, there is evidence of an optimal size for contribution in males and females, which could indicate that minimising the size variance in the broodstock might increase the number of contributing fish. Another option is to maximise the number of fish spawning by synchronising the ovulation in females through the application of hormone treatments (Zohar and Mylonas, 2001). This method would ensure that a large number of females contribute to the spawning. Increasing the number of male parents is likely to be a more persistent problem than increasing females, as from the results of this experiment, reproductive success of males appeared more variable than that of females.

In selective breeding programmes, the risk of inbreeding is higher, due to the increased likelihood of recruiting related individuals into the broodstock through selection techniques and methods which place additional emphasis on family information, such as BLUP. However, there is a conflict between maximising the  $N_e$  and generating genetic gain in the stock, so that extension of the generation time must be balanced against replacing broodstock with animals of higher genetic merit and the need to select between families in a managed way. Yet, the advantage of most breeding programmes is that records should be available on the pedigrees of individual fish and, therefore, controlled selection and mating can be carried out to limit the rate of inbreeding (Pante *et al.*, 2001). Pedigree mating can effectively double the  $N_e$  by selecting equal numbers of offspring from each parental pair to use as parents in the next generation (Tave, 1993) but this severely limits the selection potential by ignoring between family selection. Another method is to subdivide the broodstock population so that gene flow – crossing of groups and the transfer of animals between groups – mitigates the genetic drift within each sub-population. This method is analogous to Wright's island model, in which a fraction of the population ( $m$ ) is randomly exchanged in each generation. Panmixia (equivalent to a single random-mating population) in this model can be achieved if  $m$  is greater than  $1/(4N_e)$  (Wright, 1969). Therefore, moving a small number of individuals at random between groups, and ensuring

that these individuals produce offspring, can result in a subdivided population being considered panmictic (Lande and Barrowclough, 1987). Selection could proceed in each of these sub-populations without the dilution of genetic gain because of the small number of individuals moving between groups (Bentsen and Gjerde, 1994). However, gains may be reduced if the transferred fish are selected out as poor performers and the number of fish transferred increased.

Differences in the heterozygosity between parents and offspring groups were very small and often a better indication of the genetic differentiation is the allelic richness (Wang *et al.*, 2002). This measure indicated that, over the single generation, there was a loss of low frequency alleles at almost all the loci. However, within the broodstock, the allelic richness was almost identical pointing to the fact that the stock had not experienced excessive genetic drift resulting from the subdivision of the population. The Alkioni broodstock has a short history, only being formed approximately 10 years ago and was only recently (in 2000) reduced to its current structure, of four groups of 50-60 fish per group. Prior to this, larger groups of around 80 fish were maintained without photoperiod control, which enabled mixing of fish. Replacement of the stock was done, as it still is, on an *ad hoc* basis with random replacement of older individuals (most likely to be females) with young males. Therefore, there is nothing to indicate that the stock may have suffered from any serious reduction in numbers or inbreeding and this is supported by the results presented here.

A selection programme benefits from a broodstock with an initially high level of genetic variation, since this will provide a more rapid genetic improvement. Using a small number of broodstock would reduce the genetic variation and encourage a high rate of inbreeding, which could only be countered by out-breeding the stock, resulting in a loss of any genetic gain already achieved. Based on the calculated  $F_{IS}$  and  $G_{ST}$  values, the Alkioni broodstock has very low levels of inbreeding across all of the loci examined. Higher values were noted for two loci in the delayed and the advanced groups but this could be the result of problems in the size calling of alleles, due to the types of error described in section 2.5.

As discussed above, the contribution of individual fish was highly variable in all of the broodstock tanks examined. In particular, there were fish in each group that contributed nothing to the spawning, with the result of decreasing  $N_e$  by reducing the number of parents, biasing the sex ratio and increasing the variance in contribution. Contribution variance could be an artefact from the mass spawning behaviour, in that females produce similar numbers of eggs, whereas the dispersal of milt from the males is a more random process (Peterson *et al.*, 1992; Shapiro *et al.*, 1994). Kiflawi *et al.* (1998) determined that fertilisation rates in group spawning were not improved by increasing the number of males and that fertilisation occurred very rapidly after the release of eggs. This suggests that fertilisation success may also be a product of sperm competition between males (Taborsky, 1998). Male success has also been found to be variable in other mass spawning species, such as cod (Bekkevold *et al.*, 2002), and this is proposed as a common limitation to  $N_e$  in other marine species (Hedgecock, 1994).

It was also noted that, in the consecutive season samples from the natural group, that there were many (18) non-spawning fish in both years. The reasons for non-spawning are unknown, as these particular fish were identified as both male and

female and covered a range of body sizes and ages. The number of non-spawners in each group ranged from 16 – 26. This indicates that this may be a common phenomenon in mass spawning situations. Examining the data in this experiment over consecutive seasons, it is apparent that several of the fish that spawned in 2001 did not contribute in 2002. The number of male parents in 2002 was increased by the presence of the new male fish and produced a corresponding fall in the contribution of older males. For larger males, who have a greater capacity for sperm production, the optimum strategy in high competition situations could be to defer spawning until a later point in the season when competition is reduced (Fuller, 1998).

Non-spawning could also be a failure of individual fish to adapt to the captive conditions (Zohar and Mylonas, 2001). The non-spawning fish in the current study had a generally larger mean weight – ignoring sex, delayed and natural groups combined; t-test,  $P=0.006$ , 96d.f. – indicating that their higher growth could be due to their not having expended resources on reproduction. Another possibility is a periodicity of spawning by individual fish over the spawning season, which has been noted in other sequential spawners (McEvoy and McEvoy, 1992), including gilthead seabream (Kadmon *et al.*, 1985). For example, a female fish may produce eggs during first six weeks of the season, whereas another female may produce eggs only during the final six weeks of the season. Applied throughout the broodstock, it would be expected that the majority of fish would spawn towards the middle of the season and fewer at the beginning and at the end of the season. Such a pattern was observed in the egg production of all the broodstock groups in 2002 (Chapter 3) although this could also be attributed to an increased egg production by each female towards the centre of the season. Individual records are needed to determine if egg production per female changes over the season and sampling of broodstock contribution at several points over the whole season would be required to observed if non-spawners are contributing at earlier or later times in the season.

In contrast to the consecutive non-spawners, the new fish introduced to the broodstock in 2000 provided a much greater contribution in 2002 than in the previous year. In 2001, only two of the twenty new fish were identified as parents. Although this data was based on a single tank (natural group) the number of new fish contributing in 2002 was close to 60% in all three of the broodstock investigated. The most likely explanation of the large increase in contribution of the new fish could be the increase in body size, achieved in the second year of residence. There was no evidence of a threshold body size at which spawning begins in this study, but these fish were added to an existing stock with a predicted age of four years old. There was, therefore, a disparity in body size that may have inhibited the spawning of the new fish in their first spawning year (2001). This finding has large implications for the replacement of the broodstock and the necessity for maintaining an age structure in each group. The frequency of replacement will have to account for the ‘missing year’ of new fish, when they are unlikely to spawn, by ensuring that there are enough older fish to sustain egg production. Again, this could point towards a preference for a single-age broodstock, which would be replaced as a whole group simultaneously and may not suffer from the same level of non-spawning observed in the age-structured groups. Such a system was not tested in this study and would require an

experimental approach to ensure that egg production is sufficient to sustain the farm production cycle using this method.

Analysis of parental contribution based on parent weight was found to be significant. Modelling of the data indicated that contribution was increased closer to the mean weight of each sex within the broodstock groups. Therefore, the larger the deviance of each fish from the mean weight, the lower the contribution in terms of offspring numbers. As discussed above, the new fish introduced to the broodstock contributed very little in the first spawning season, yet were high contributors in their second spawning season. The contribution analysis indicates that fish above 1.5kg in weight were unlikely to contribute as many offspring as the 1kg fish. These observations would suggest that the ideal weight – and by inference age – of broodfish lies in the range 0.5 to 1.5kg, or approximately 4 to 6 years of age. Therefore, a replacement policy aiming to maximise contribution would aim to replace each individual broodfish after three spawning seasons and thus maintain the majority of the broodstock within an optimum weight/age range.

Survival in the larval stages, as indicated by repeated sampling, was found to be different between parents but the evidence was mixed as to how strong this relationship was. The larval stages in gilthead seabream are characterised by high mortality, especially at hatching and first feeding. Family variability in survival has been noted in juvenile (Jonasson, 1993) and adult populations (Geiger *et al.*, 1997) and through the use of challenge tests (Schom, 1986; Kandl and Thompson, 1996). The data presented here indicates that the loss of families may be quite large, as almost 30% of the total number of parents at the beginning of the period end the sampling with none or a reduced number of offspring (at day 40 post-hatch). Such a large family effect on survival would be expected to have a bearing on the calculation of  $N_e$  using older larval stages, but this was not seen in the estimate of  $N_e$  in the advanced group, which was based on parental contribution to the larval population at day 60 post-hatch. The  $N_e$  in the advanced group was only marginally lower than the natural group (2002), suggesting that survival through the larval period is not family related and that mortality is mainly environmentally determined (Falconer and Mackay, 1996). The prominence of two males in the day 40 samples is a reflection of the number and size of families produced by these males. Contribution by female parents was more evenly distributed, but two females did show large changes in relative offspring proportions at day 40.

## **7.5 Conclusion**

The effective population size ( $N_e$ ) in a mass spawning event was determined using parental assignment to quantify individual contribution to the offspring generation.  $N_e$  was moderately low in all the broodstock groups sampled and was strongly influenced by a large variation in contribution of males and females and the non-spawning of a large number of fish. Analysis of the contribution by fish weight indicated that the greatest contribution came from fish close to the mean weight of both the sex and the broodstock group as a whole. This pattern was repeated for all the groups and the model indicated that the contribution falls significantly above a parental weight of 1.5kg for both males and females.

The genetic composition of the broodstock groups was also compared. It revealed that there is no evidence of genetic drift or inbreeding in the population and that

this would be a suitable stock to form the basis of a breeding programme. Due to the variation in parental contribution, many low frequency alleles were lost in the offspring generation. This supports the suggestion that the  $N_e$  needs to be increased, in order to avoid the detrimental effects of inbreeding in this population, when selection procedures are introduced as part of a breeding programme.

**Chapter 8:**  
**Outline of the Breeding Programme**

## **8.1 Procedures for Selection of Gilthead Seabream**

The aim of this study was to develop the procedures for conducting a genetic improvement programme in gilthead seabream, based on the investigation of the farming system in place at Alkioni Fish Farms, Cyprus. Key points in the production system were identified as uncertain variables or confounding factors to selection and demanded additional study, in order to identify the underlying biological processes. Having a greater understanding of these variables meant that they would become easier to manage in a more effective way and that the impact on the operation of a selection programme could be minimised. All stages in the production system were characterised by observations, so that procedures could be developed for a programme covering all aspects of the production system.

The development of a selection programme requires several important factors to be addressed concerning the objectives of the programme and its operation. The breeding objective is the ultimate aim of the selection programme and should consist of both the long and short-term goals. Other considerations on the operation of the programme should also be included, so that they are monitored and evaluated as part of the programme. The objectives must be able to be addressed by measurements and this may have an influence on the operation of the programme as the number and type of measures can have economic implications. Reducing environmental variables is a prime consideration for any selection programme, as they can confound any genetic improvement and reduce the efficiency of the selection methods. The selection methods and numbers of individuals to select are largely dependent on the species and traits under consideration, but need to be described so that the limitations of the system can be determined. Finally, disseminating the genetic improvement through the breeding stock and protecting the gains made will ensure that the expense of the breeding programme is justified and that the economic benefits are realised as quickly as possible. This chapter will cover each of these topics for the proposed breeding programme for gilthead seabream before discussing the future research needs to develop the programme further.

### **8.1.1 Breeding Objective**

The intensive production of gilthead seabream is still a relatively new industry, although it has expanded rapidly over the past 10 years. Many commercial stocks have only a short history of captivity and domestication selection has not been allowed to act on traits over a long time period, making them similar to wild fish in terms of performance. Growth rate (time to harvest size) in the sea cage stage is considered to be the primary trait for a breeding programme in gilthead seabream, as this will have the greatest economic importance to farm production. Examination of the harvested populations at Alkioni Fish Farms indicate that there is a large potential for genetic improvement in growth. The response to selection potentially achievable from only a single round of selection, using an estimated heritability obtained from other seabream populations, was a reduced time to harvest of at least 60 days. The main benefit of improvements to growth rate will be a reduced time to reach harvest weight and, consequently, a quicker rotation of cage stocking. Therefore, subsequent sections detailing the selection programme are based on the assumption that the selection programme has the objective of improving growth rate.

Other traits may become important as the industry develops, particularly quality traits such as body shape and fat content, as the market develops new products. In addition, more powerful means of selecting for traits, such as marker assisted selection (MAS) and QTL information, may become available in the future. These techniques would allow more intensive selection on a wider variety of traits. A secondary breeding objective would be to ensure that the selection on growth rate in the seacage and juvenile stages will not have an adverse effect upon these traits. The breeding programme will be designed around the commercial operations at Alkioni Fish Farms, a major consideration being the impact of the programme on production levels and in particular, the effect of frequent replacement of broodstock on fertilised egg production. Hence, the preservation of a viable breeding population is another objective that must be continuously observed in this programme.

### **8.1.2 Measurements to Address Breeding Objectives**

The breeding objectives include the maintenance of a viable population to continue egg production for the farming system and, therefore, monitoring of the broodstock population is an essential feature of the programme. Egg production and fertilisation rates are a straightforward measure of the performance of broodstock groups and can be collected by weighing fertilised and non-fertilised eggs each day during the spawning season. The sex ratio of the broodstock is an important factor in maintaining a viable breeding stock and should be assessed in both the established broodstock groups before the end of each spawning season and in the candidate fish prior to the introduction to the broodstock. The techniques to identify sex may be complicated and expensive, but the least invasive methods should be used, which could include the examination of milt and ultrasound imaging.

At the larval stages, measurements of body length in the pre-first grade larvae need to be carried out to evaluate the improvement from selection for fast larval growth. As the selection programme progresses, the mean larval size at first grading is expected to increase. In addition, the time to first feeding may also become more uniform and, consequently, reduce some of the size variation originating in this stage. Larval growth may also be correlated with aggression and, although this is difficult to monitor directly, it could introduce complications to the management of populations.

Growth rate during the seacage stage can be measured by the body size of fish at harvesting age, which is equal to 18 months (547 days) after stocking juveniles of a mean weight of 2g. Due to the inaccuracies of measuring body weight in live fish, length measurements can be used as a rapid and convenient method of calculating body size on-site, as there is a very strong correlation between body weight and body length in this species. The selection criteria can be established by measuring a sample of fish from the seacage prior to the selection of broodstock candidates. This data can then form the basis for comparisons between the selected populations over time, as the population mean should increase as the genetic gain is realised. During the initial stages of the programme, the genetic gain may be small due to the presence of unselected parents in the broodstock.

### **8.1.3 Managing Environmental Variance**

Reducing environmental variance is essential when making comparisons between populations and over time, as is required in evaluating genetic progress. The main sources of environmental variance in the breeding programme for gilthead seabream occur in the larval and juvenile stages, as during the adult stages the selected fish are more easily maintained as a single entity in a common environment.

#### **8.1.3.1 Number of Parents to Create the Base Population**

Due to the low effective population size observed from a single mass spawning in this species and the evidence of maternal effects at the juvenile stage, the base population for the selection programme must be created from a large number of parents. In addition to collecting eggs from all broodstock groups, the number of parents in each group needs to be increased by guaranteeing the contribution of all broodfish. As there is a limitation on the timing of egg collection for the programme, a means for synchronising female spawning, such as hormonal control (Zohar and Mylonas, 2001), can be used in order to ensure that the maximum number of fish are spawning at that time.

#### **8.1.3.2 Egg Collection**

Ensuring the contribution of broodstock groups within different photoperiod regimes relies on collecting eggs at a point when the respective spawning periods of each group overlap. Regression analysis of the egg production data at Alkioni indicates that a point of overlap occurs during a four-week period in March/April (Figure 8.1). Five larval cohorts are required to form the base population for the programme. Each cohort is created by stocking 600g of eggs, mixed in equal proportions from all broodstock groups, into a single hatchery tank for the duration of the larval period.

#### **8.1.3.3 Larval Cohorts**

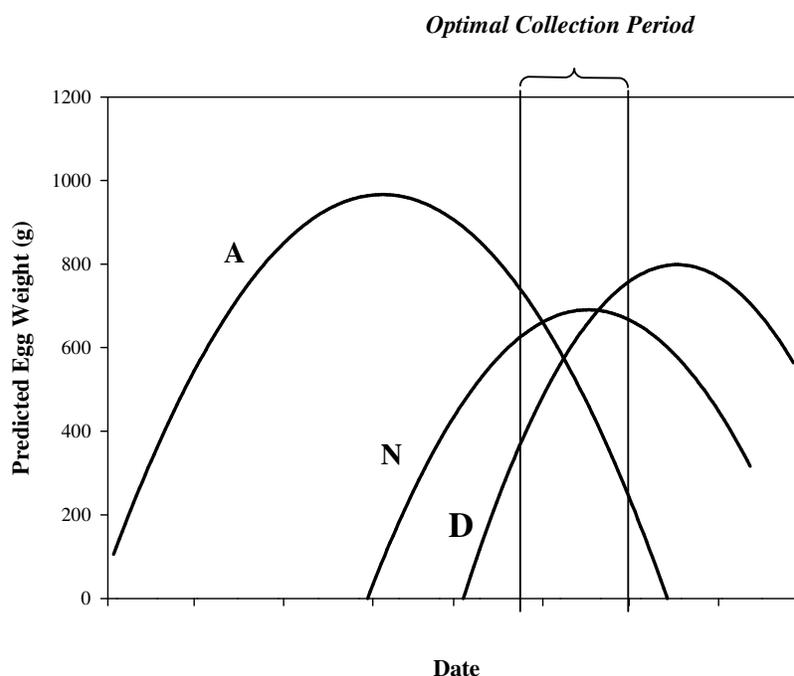
Larval cohorts forming the base population for the programme must be collected over as short a time period as possible, to reduce age effects between cohorts. Early development was indicated in this study to confer a competitive advantage, which may be maintained through to grading. This would disadvantage the offspring of parents who only contributed towards the end of the egg collection period. Ideally, the collection of eggs should occur on a single day. However, egg production was noted to be variable on a day-to-day basis. A limit on the period between stocking of the first and fifth larval cohorts is recommended to be 5 days.

#### **8.1.3.4 First Grading**

Each cohort will be maintained in separate hatchery tanks up until the first grading (day 60 post-hatch). Cohorts will be graded individually using the same criterion (grader size) to divide the population equally. The group of large larvae (not passing through the grader) will subsequently be pooled to form the elite line, whereas the small larvae (passing through the grader) will be incorporated into the farm production. This system will select the top 50% of the initial base population as an elite line, which can then be maintained as a single population through the programme.

### 8.1.3.5 Grading

Grading is an essential management tool in the production of this species. In this study, there was no evidence for more rapid later fractional growth amongst fish that were initially slow growers, compared to those that were initially rapid growers. Therefore, there was no evidence for an adverse relationship between fast larval growth and fast growth in the juvenile stages. Consequently, an elite line of fish – created from the largest fish at the first grading – would appear to contain those fish that will also have the genetic potential for more rapid growth from first grading to marketing. Additional selection within the elite line will be achieved at each grading by selecting the top 75% of the population (Figure 8.2). Three such grades, in addition to the first grading, are required to reduce the elite line population to the numbers required to stock an individual seacage.



**Figure 8.1:** The optimum egg collection point for maximising the number of broodstock is at a point where the photoperiods regimes converge and egg production from each tank is at its maximum. Based on the 2002 egg production a four-week window of simultaneous spawning of all photoperiod groups occurs between late March and late April. Key: A= advanced photoperiod group, N= natural photoperiod group, D= delayed photoperiod group.

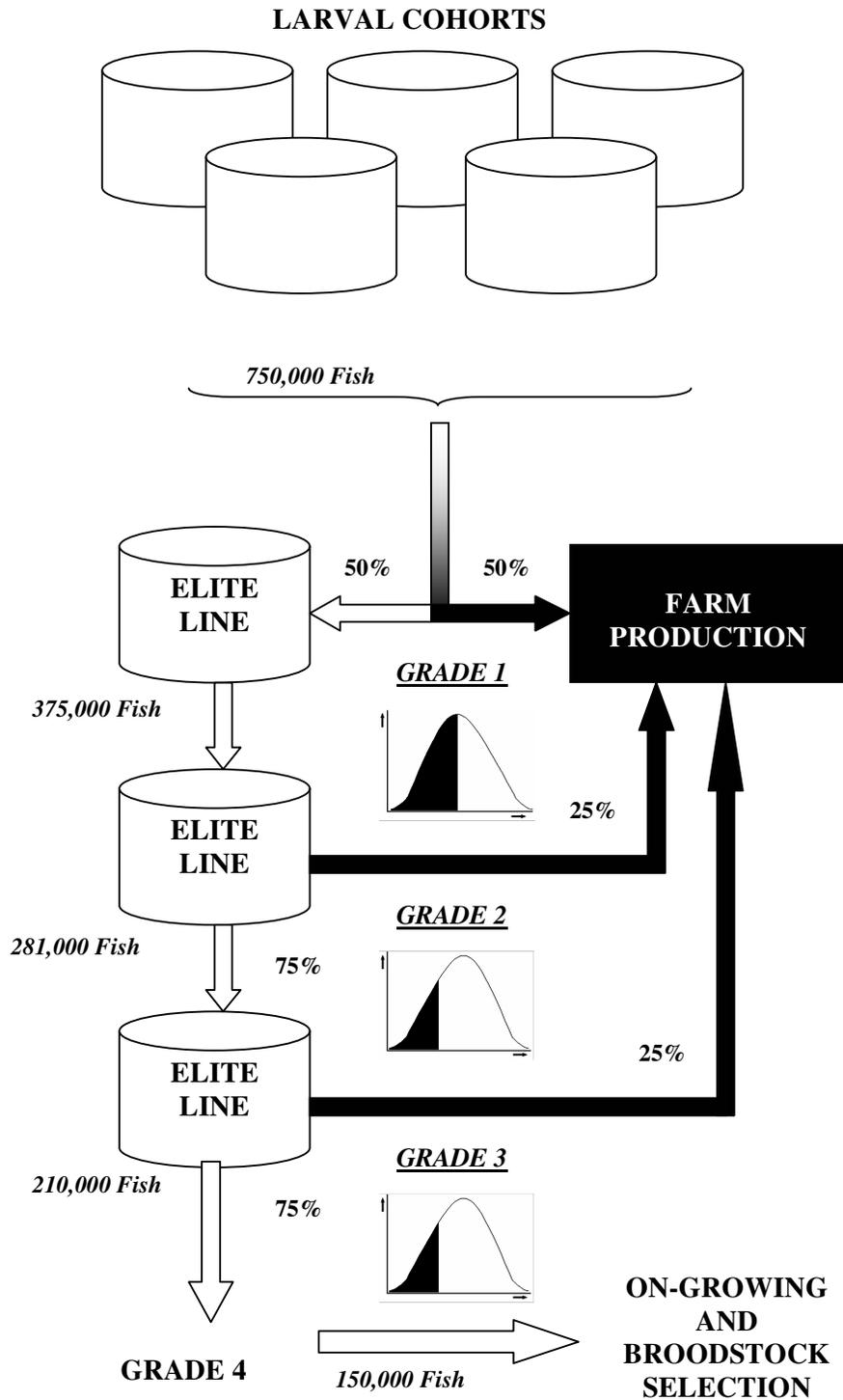


Figure 8.2: Diagram of elite line grading system. Each cohort is divided into two equal groups at the first grading, with the lower size group going into the farm production system. At each subsequent grading, a proportion of the elite line group (25%) is removed from the lower end of the size distribution. This is repeated until the elite line group reaches the density suitable for seagee stocking (approx. 150,000 fish).

### **8.1.4 Size of the Selected Populations**

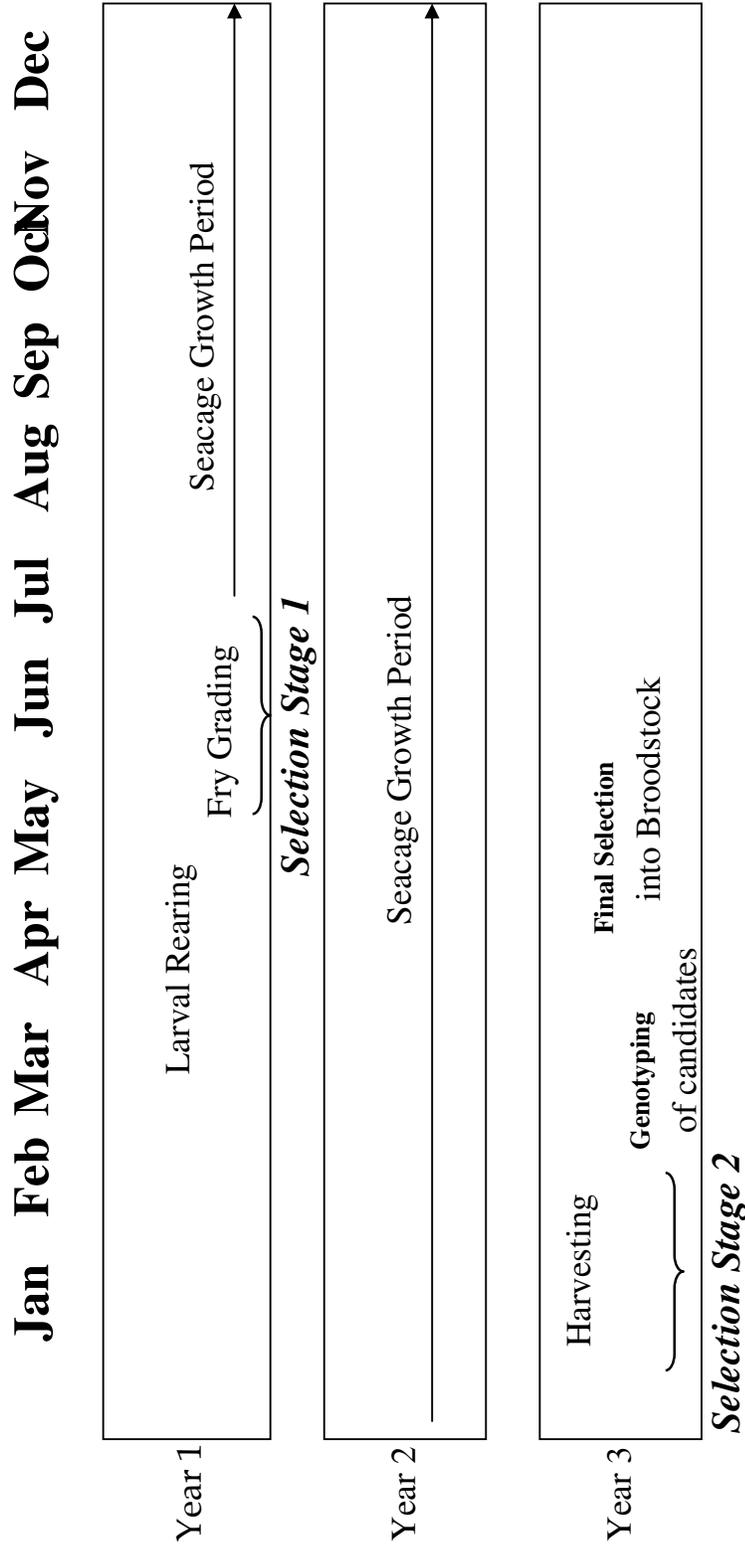
A total of five larval cohorts are required for the creation of the base population for the selection programme, as each cohort is anticipated to consist of 150,000 larvae at the age of first grading. The first grading selection criteria will be applied to a population of 750,000 larvae and, initially, the elite line will contain 50% of this population. Each subsequent grading of the elite line will reduce the size of the group by removing the smallest 25% of the population until the seacage stocking size is reached (Figure 8.2). The number of fish required to stock into a single seacage is between 100-120,000. This would allow for three gradings of the elite line in the manner outlined with a total mortality rate of 20% over the grading period. Controlling the number of fish passing through the grading sheets was achieved in this study by using accurate grading sheets and predicting the outcome of grading, based on the population mean prior to grading.

The seacage population will remain unchanged until the second selection criteria is applied at harvest age and a total of 1000 broodstock candidates are selected. The number of candidates needs to be high, to ensure a sufficient number of families are selected to facilitate the genetic management of the broodstock. These candidates will then be transferred to dedicated facilities where they will be PIT-tagged, measured and genotyped. A total of 60-80 fish will be introduced across the broodstock groups at the end of the spawning season each year in order to ensure a rapid genetic gain in all the photoperiod groups simultaneously.

### **8.1.5 Selection Protocols**

The programme will consist of two stages of mass selection aimed at selecting for growth in the larval and juvenile stages and at the end of the seacage stage. The criteria for selection at these points will be developed based on measurements of body length, collected from a sample of the population immediately prior to the selection point. At first grading, this sample will comprise of 200 fish from each larval cohort that will be measured for body length. A selection criterion based on the mean length within each cohort will be applied by using a grading sheet that will allow half the larval population to pass through the sheet whilst retaining the other half to form the elite line. At subsequent grades, measurements of 200 fish will be used to determine the appropriate grader sheet size that will retain 75% of the elite line population.

To accurately develop selection criteria for selection at harvest age, measurements of 500 fish are recommended to obtain a good representation of the seacage population. This stage of data collection could be carried out during the process of first harvesting of the cage, so as to minimise the costs and simplify the process of measuring large numbers of fish. The size distribution of this sample will be used to determine the selection criterion, which may typically be set at two standard deviations above the cage mean. Given a Normal distribution of body length in a population of 120,000 fish, such a criterion would be exceeded by 3000 individuals. Fish will then be captured from the cage and those exceeding the criterion will be retained as broodstock candidates, whilst those failing to meet the criterion can be returned to the production system. The time scale on which the two selection stages will take place is outlined in Figure 8.3.



**Figure 8.3:** Time-scale of the selection programme. Eggs are collected in March/ April, followed by 60 days of the larval rearing period. The first grading represents the first selection stage at which point the elite line will be created. After approximately 50 days, the elite line will then be stocked into a seacage for the 18-month period of on-growing. Harvesting of the seacage is the second selection stage after which only the broodstock candidates remain. These candidates are then genotyped prior to the final selection into the broodstock. This system will be repeated annually to generate replacement fish each year.

Procedures for controlling inbreeding under a mass selection scheme will be used as described in Grundy *et al.* (1998) and Bijma *et al.* (2000) and applied to the selection of broodstock candidates. These selection tools will allow the rate of inbreeding to be constrained below 1% per generation. The procedures will need to be modified to account for (i) the single sex nature of the selection, (ii) the uncertainty in parental contributions within the elite line and, (iii) initially the use of phenotypic measurements, rather than BLUP breeding value estimates. These modifications are technically feasible (J. Woolliams, pers. comm.), but require further development.

### 8.1.6 Replacement Policy for Broodstock

The rate of renewal of the broodstock is one of the important criteria (the other being selection intensity) for setting the pace at which genetic improvement can advance within the population and, therefore, this needs to be as rapid as possible. The impact of introducing new fish on the existing broodstock can only be inferred from the sex ratio and the egg production. As the level of egg production is an important commercial consideration, this would need to be monitored in the future, in order to establish if there is a negative correlation with the replacement rate. This study found a decrease in egg production in the season following the introduction of new broodstock, which indicates that maintaining an age structure to the broodstock is an important consideration for sustaining egg production.

The optimum rate of replacement is concluded to be one third of the broodfish in each group per year (Figure 8.4). This equates to 20 fish per group of 60 broodstock. Total renewal of each group would take three years, giving any one fish three consecutive spawning seasons, but not retaining any over five years old.

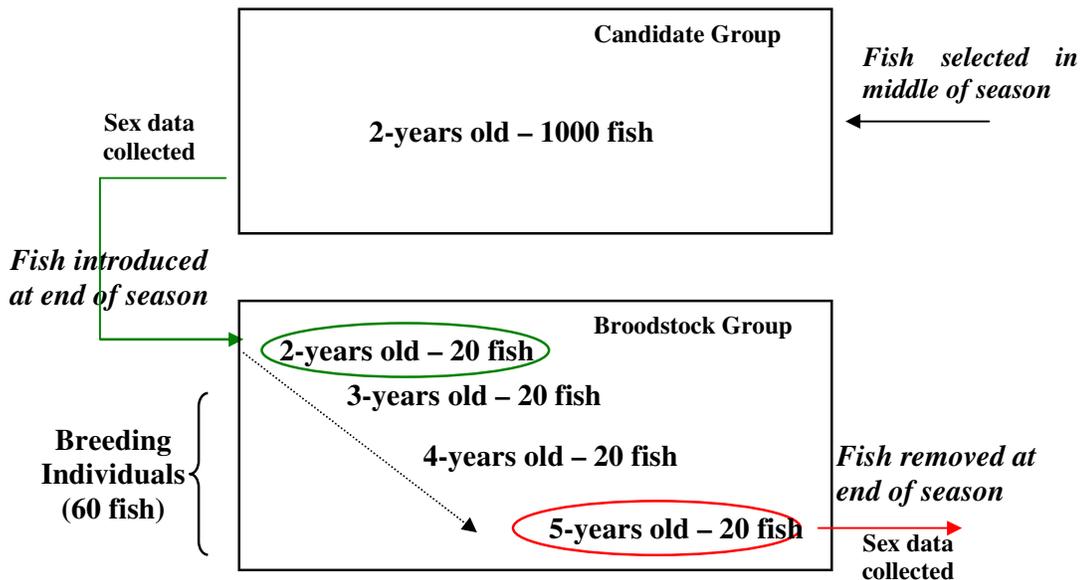


Figure 8.4: Proposed replacement rate within each broodstock group (60 fish) to maximise genetic gain. The age structure of each group is preserved by replacing one third of the stock annually, at the end of the spawning season. The sex ratio would be monitored in the existing broodstock at the end of the season and in the candidate group prior to introduction.

The age at which to replace broodstock was determined from the analysis and modelling of offspring contribution with broodstock weight. Above a body weight of approximately 1500g (6 years of age), the contribution of both males and females reduces to zero, which indicates that, above this body size, spawning contribution either decreases or the viability of offspring is reduced. By reducing the average age of the broodstock, it is also hoped to reduce the deviation from the mean weight of the broodfish, in order to increase the contribution to spawning by all fish in all seasons. As the selection programme progresses, the mean weight of the new fish should increase and size differences between existing and new fish would be reduced. This frequency of replacement would still permit an age structure within the broodstock to maintain egg production, but older fish would be replaced prior to the observed decrease in contribution, at greater body size deviation from the group mean. The monitoring of the sex ratio within both the broodstocks and the candidate group would need to be maintained in order to highlight any preferential selection for one of the sexes, using the stringent selection criteria at the harvest point. The management of the broodstock will remain the same as the current practices, with three photoperiod regimes supplying eggs over the year. Monitoring of all the broodstock will take place annually to collect measurements and sex ratio data at the end of the respective spawning periods.

Replacement fish will be introduced at the end of the spawning season. The result of this would be to have fish breeding at 3, 4 and 5 years of age and a generation interval of approximately 4 years. This may be an underestimate if newly introduced fish were found to be largely non-contributors, as was found in the current study. With pedigrees established for all the candidates, it may be desirable to target individual replacements to particular tanks, in order to avoid potentially close matings.

### **8.1.7 Use of Pedigree Information**

The mass spawning behaviour of this species prevents using a structured mating design and, therefore, pedigree information is needed to monitor family representation and to calculate genetic parameters. Microsatellite analysis will be carried out on all broodstock candidates by PIT-tagging and collecting fin clips at the time of transfer to the holding facilities. Rapid DNA extraction using the Chelex method and genotyping using multiplexed microsatellite loci, as developed in this study, will allow the rapid analysis of samples, so that the candidates can be identified prior to their introduction to the broodstock.

Genetic management of the broodstock will rely on genotype information to (i) minimise the group co-ancestry among those selected as replacements through family representation and (ii) limit the potential for consanguineous mating within each broodstock group by targeting individual replacements to broodstock tanks. Therefore, selection into the broodstock will be based on the number and nature of relatives within the existing stock, to minimise the potential for close relative mating within each group.

### **8.1.8 Protection of Genetic Gains**

An important development to the selection programme, once it is established, is to establish a means to protect the genetic gains that have been made. Alkioni Fish

Farms is a supplier of fingerlings to other on-growing farms and the gains made within the broodstock could be transferred by exporting offspring that could then be used for broodstock at other farms, after on-growing. A commercial imperative, therefore, is to safeguard the genetic gains by ensuring that the juveniles exported from the farm are not suitable for use as broodstock.

A common method of achieving this is to use crossbreeding with other strains or species, so that only the  $F_1$  hybrids are sold and, as the quality of the  $F_2$  generation from these fish would be unpredictable, these fish are unsuitable for use as broodstock. However, these fish must also retain the qualities of the selected line to be of market value. Another option is to ensure that the juvenile population is comprised of a restricted number of families by using a small number of parents. The low  $N_e$  of the offspring population would result in rapid inbreeding if the stock were used as broodstock and, if mixed with other broodfish, the genetic gains would be largely diluted.

### **8.1.9 Economic Potential of Selection in Gilthead Seabream**

#### **8.1.9.1 Costs**

The proposed selection scheme is relatively straightforward in that mass selection can be carried out on communally reared stocks and no family information is required prior to selection. However, the lack of information on family representation and the need to genetically manage the broodstock means that significant costs will be incurred through the need to genotype large numbers of fish. The results of this study indicate that eight loci are needed to assign parentage at a high confidence from a 30x30 parental cross (one broodstock group). This may need to be increased with the mixing of offspring from a number of groups. Using rapid DNA extraction methods and multiplex PCR techniques brings the costs of generating genotypes to approximately £5 per sample.

Routine data collection such as fish measurements and selection criteria, will need to be incorporated into the management system of the farm and may require a substantial labour input at harvest time. A consultant geneticist will be required to analyse the farm and genotype data, to calculate genetic parameters, manage selection of candidates into the broodstock and monitor the progress of the programme. Another fixed cost of the programme will be the holding facilities needed for the candidates prior to their selection into broodstock. Given the large number of fish (1000), hatchery based facilities may be too expensive and, therefore, seacage facilities will be needed. Each candidate will also require a PIT tag, although the fish not selected for the broodstock will be culled and the PIT tag retrieved.

#### **8.1.9.2 Benefits**

The heritability of growth rate in gilthead seabream has been shown by other authors to be similar to other fish species, such as salmon and trout. Breeding programmes in these species have been very successful in improving a variety of traits in addition to growth. The economic returns of programmes such as the national Norwegian breeding programme for salmon have been high, resulting in a cost / benefit ratio of 1:15 (Gjedrem, 1998). In many respects, the proposed programme for gilthead seabream makes substantial savings, in comparison to the

procedures used in salmon, as a result of using genotype data to monitor factors such as inbreeding.

A simulated response to selection using the data from a typical production seacage in the current study, indicated that time to harvest could be reduced by 60 days (10% of the seacage period) from a single round of selection. This is a substantial improvement and benefits will be realised by both a reduction in the time to harvest fish and an increase in the number of fish that can be produced in the cage per unit time. Such improvements are necessary if companies such as Alkioni wish to stay competitive in demanding markets such as the European Union.

## **8.2 Further Research Needs**

A more accurate assessment of  $N_e$ , taking into account the effects of selection and maternal effects, is needed in order to ensure that the number of candidates selected at harvest age is comprised of a sufficient number of families. This can be achieved using the analysis of lifetime contribution outlined in Rönnegård & Woolliams (2003) and Bijma & Woolliams (2000). With this information, the number of fish selected as candidates can be refined whilst ensuring enough families are represented to genetically manage the broodstock effectively. As maternal effects were noted to persist through to grading age, these may also continue through the grading period and beyond. The establishment of an elite line is intended to apply a series of criteria early in the life history and narrow the proportion of the population proceeding to the later selection point. However, it may serve to sustain the maternal effects by maintaining the largest fish within the elite group and at the top of the hierarchy, where they can extend their growth advantage. The effect of an elite line on the representation of families needs to be investigated, as this may influence the methodology of this stage. The use of two stringent selection criteria – 20% of the population at grading and 0.003% at harvest age – would inevitably lead to the loss of a large proportion of families. However, the larger the number of families present at the candidate stage, the higher the potential  $N_e$  of the population, as the rate inbreeding can be reduced by increasing parental representation.

As the programme progresses, additional statistics can be calculated as the information becomes available. For example, heritability of body length at harvest age is essential to calculate the rate of genetic gain. In addition, genetic correlations of grading age and harvest age would confirm the suitability of using two stages of selection and the influence of maternal effects in the later farm stages. This data can be obtained from the broodstock candidates that will be routinely measured and genotyped as part of the selection programme. Measurements of other traits on these fish, such as body shape, could also be introduced to establish the potential for selection on these traits at a future point.

An important aspect of the broodstock management, which could not be resolved here, was the sex ratio in the broodstock over time. In the experiment, the addition of new fish did not result in an unbalanced sex ratio in the following season and a small number of new fish actually underwent sex reversal in both the monitored groups. This suggested that the social control of sex reversal always results in a balanced sex ratio. However, the limitations of the sexing methodology meant that the 1:1 balance could not be confirmed and some fish were identified as females in one season and male in the next, which is in contrast to the prevailing view that

the maturation to female is final. Long-term monitoring of the broodstock by tagging at introduction and annual sexing by genotyping and analysis should consolidate the data on the sex ratio over time and confirm the frequency of sex reversal in fish of different ages. The sex of candidate fish also needs to be monitored, because of the evidence that larger fish at harvest age tend to be females.

Allied to the monitoring of the broodstock, the establishment of single age broodstock groups needs to be investigated, so that the optimal replacement rate of the broodstock can be determined. Whole group replacement could increase the rate of genetic gain by shortening the generation interval. Results from this study also indicated that a single-age broodstock may be preferable in order to increase offspring contribution by reducing weight differences between broodfish. However, as commercial production needs to be maintained, the effects on gamete production need to be investigated before the technique can be established in farm situations. Small-scale experimental groups could be an alternative means to study this question and could provide additional insights into the social control of sex in this species. For example, experiments could be performed to test if sex reversal is stimulated by the removal of dominant fish, as has been noted in other sex reversing species (Robertson, 1972).

Additional traits for future selection in the programme include body shape and carcass quality traits. These need to be monitored, as discussed previously, but work is also needed to establish standardised methods of measuring these characters, so that appropriate selection criteria can be developed. An analysis of body shape can be performed using digital photographs and imaging software (Bates and Tiersch, 1997; Loy *et al.*, 1999), to create distinct shape categories. Preliminary work on the body shapes of the market fish indicated that there were two or three forms, but these were not as distinct as the head shapes. The head shapes could be identified within images of the broodstock, which suggests that this is an effective method of analysis. One head shape (round) was strongly associated with malformations of the jaw. This could form the basis for identifying malformed fish at the juvenile stage, when most batches of fish are manually checked for quality, including malformations and swim-bladder inflation. Malformations have been linked to environmental effects (Alexis *et al.*, 1997; Boglione *et al.*, 2001) but may also have a genetic component (Alfonso *et al.*, 2000), which needs to be investigated further, as they can comprise significant losses at the juvenile stage and have a reduced value at harvest age.

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