

Thesis
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Egg Quality, Triploidy Induction and
Weaning of the Atlantic Halibut
Hippoglossus hippoglossus

*Thesis presented for the degree of
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by

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NUMEROUS ORIGINALS IN COLOUR



Table of Contents

List of Figures	vi
List of Tables	ix
Acknowledgements	x
Abstract	xi
1. General Introduction	1
2. General Materials and Methods	8
2.1. <i>Introduction</i>	8
2.2. <i>Facilities</i>	8
2.2.1. History	8
2.2.2. Site location	9
2.2.3. Seawater supply	10
2.2.4. Hatchery	10
2.3. <i>General Rearing Techniques</i>	12
2.3.1. Broodstock management and gamete production	12
2.3.2. Egg incubation	14
2.3.3. Yolk sac incubation	15
2.3.4. Larval rearing	16
2.3.5. Weaning	17
2.3.6. Live food production	17
2.3.7. Weaning diet	19
2.3.8. Hygiene	19
2.4. <i>General experimental methods</i>	20
2.4.1. Handling of gametes	20
2.4.2. Handling of fish	20
2.4.3. Weaning tank facilities	20
2.4.4. Sampling and measurement of fish	21
2.4.5. Photography	23
2.4.6. Measurement of physical parameters	23
2.5. <i>Data handling and statistical analysis</i>	24
3. Egg Quality Assessment	26
3.1. <i>Introduction</i>	26
3.2. <i>Material and Methods</i>	35
3.2.1. Egg handling	35
3.2.2. Egg assessment	35
3.2.3. Statistical analysis	42
3.3. <i>Results</i>	44
3.4. <i>Discussion</i>	52
3.5. <i>Conclusions</i>	67
4. Broodstock Temperature Requirements	69
4.1. <i>Introduction</i>	69
4.2. <i>Materials and Methods</i>	73

4.3. Results	79
4.3.1. Spawning pattern and egg production	79
4.3.2. Egg size	82
4.3.3. Temperature profiles	82
4.3.4. Fertilisation and hatching	87
4.4. Discussion	90
4.5. Conclusions	102
5. Induction of Triploidy Using Hydrostatic Pressure Shock	103
5.1. Introduction	103
5.2. Materials and Methods	109
5.2.1. Fertilisation and pressure shocking	109
5.2.2. Post fertilisation checks and incubation	111
5.2.3. Ploidy determination	111
5.2.4. Batch production of triploid Atlantic halibut	112
5.2.5. Statistical analysis	114
5.3. Results	115
5.3.1. Survival to hatching	115
5.3.2. Ploidy	118
5.3.3. Appearance of larvae	122
5.3.4. Batch production trial	122
5.4. Discussion	125
5.5. Conclusions	134
6. Weaning and Co-feeding	135
6.1. General Introduction	135
6.1.1. Weaning in the hatchery production cycle	135
6.1.2. Inert diets versus live prey	136
6.1.3. Total replacement of live prey with inert diets	138
6.1.4. Early transfer to inert diets	139
6.1.5. Partial replacement of live feeds	140
6.1.6. Experiments and aims	144
6.2. Experiment 1: The effect of live feed history on weaning	147
6.2.1. Introduction and aims	147
6.2.2. Materials and methods	149
6.2.2.1. Live material and facilities	149
6.2.2.2. Feeding regime and husbandry	151
6.2.2.3. Sampling	152
6.2.2.4. Vitality tests	152
6.2.2.5. Statistical analysis	152
6.2.3. Results	153
6.2.3.1. Survival	153
6.2.3.2. Feed response	153
6.2.3.3. Growth	156
6.2.3.4. Vitality	159
6.2.4. Conclusions for Experiment 1	162
6.3. Experiment 2: The effect of age and size at weaning	164
6.3.1. Introduction and aims	164
6.3.2. Materials and methods	165
6.3.2.1. Live material and facilities	165
6.3.2.2. Feeding regime	165
6.3.2.3. Sampling	167

6.3.2.4. Vitality tests	167
6.3.2.5. Statistical analysis	168
6.3.3. Results	168
6.3.3.1. Survival	168
6.3.3.2. Feed response	170
6.3.3.3. Growth	170
6.3.3.4. Vitality	173
6.3.3.5. <i>Artemia</i> consumption	177
6.3.4. Conclusions for Experiment 2	180
6.4. <i>Experiment 3: The effect of size variation during weaning</i>	182
6.4.1. Introduction and aims	182
6.4.2. Materials and methods	183
6.4.2.1. Live material and facilities	183
6.4.2.2. Grading procedure	183
6.4.2.3. Feeding regime	185
6.4.2.4. Sampling	185
6.4.3. Results	186
6.4.3.1. Survival	186
6.4.3.2. Feed response	186
6.4.3.3. Growth	188
6.4.3.4. Vitality	194
6.4.3.5. Metamorphosis and pigmentation	194
6.4.4. Conclusions for Experiment 3	198
6.5. <i>Experiment 4: Co-feeding and early weaning pilot experiment</i>	200
6.5.1. Introduction and aims	200
6.5.2. Materials and methods	201
6.5.3. Results	204
6.5.3.1. Survival	204
6.5.3.2. Feed response	204
6.5.3.3. Growth	206
6.5.4. Conclusions for Experiment 4	211
6.6. <i>Experiment 5: Co-feeding and early weaning I</i>	213
6.6.1. Introduction	213
6.6.2. Materials and Methods	213
6.6.2.1. Live material and facilities	213
6.6.2.2. Feeding regime	214
6.6.2.3. Sampling	215
6.6.2.4. Statistical analysis	217
6.6.3. Results	218
6.6.3.1. Survival	218
6.6.3.2. Feed response	218
6.6.3.3. Growth	220
6.6.3.4. Metamorphosis and pigmentation	220
6.6.3.5. Carcass lipid content	223
6.6.3.6. Water quality	224
6.6.4. Conclusions for Experiment 5	225
6.7. <i>Experiment 6: Co-feeding and early weaning II</i>	227
6.7.1. Introduction and aims	227
6.7.2. Materials and methods	228
6.7.2.1. Live material and facilities	228
6.7.2.2. Feeding regime	228
6.7.2.3. Sampling	229
6.7.2.4. Statistical analysis	230
6.7.3. Results	231
6.7.3.1. Survival	231

6.7.3.2. Feed response, settlement and stomach development	231
6.7.3.3. Growth	234
6.7.3.4. Metamorphosis and pigmentation	234
6.7.3.5. Water quality	239
6.7.4. Conclusions for Experiment 6	240
6.8. Discussion	243
6.9. Conclusions	265
7. General Discussion	268
8. References	273
9. Appendices	322
9.1. Appendix I. Proximate composition, gross energy values and fatty acid profiles of dry diet (Nutra Marine) and Artemia.	322
9.2. Appendix II. Classification of eye migration and pigmentation characteristics. (After B. Gara).	324
9.3. Appendix III. Common and scientific names of fish species mentioned in thesis text	325

List of Figures

General Introduction

- 1.1 Production cycle of the Atlantic halibut. 2

General Materials and Methods

- 2.1 Site map. 11
2.2 Hatchery floor plan. 13
2.3 Diagram of experimental weaning tank and automatic feeder. 22

Egg Quality Assessment

- 3.1 Photomicrographs of halibut egg at 8 cell stage showing normal divisions. 36
3.2. Photomicrographs of halibut eggs illustrating blastomere characteristics. 37-41
3.3 Photomicrographs of halibut eggs showing abnormalities during incubation, normal larvae hatched in microtitre plate wells, dead eggs, normal and abnormal hatched larvae. 45 - 46
3.4 Plot of hatch rate against fertilisation rate and egg buoyancy. 47
3.5 Plots of arcsine hatch rate against each of the blastomere scores and total score. 49
3.6 Data from other authors on egg viability in relation to blastomere morphology. 57

Broodstock Temperature

- 4.1 Diagram of experimental broodstock groups. 74
4.2 Diagram of broodstock tank facilities. 75
4.3 Spawning period for two broodstock groups in years 1 and 2. 80
4.4 Cumulative egg production for two broodstock groups in years 1 and 2. 81
4.5 Average spawning period for two broodstock groups in years 1 and 2. 81
4.6 Mean egg production per female for two broodstock groups in years 1 and 2 and average relative fecundity in year 2. 83
4.7 Mean egg diameter for two broodstock groups in years 1 and 2. 83
4.8 Change in egg diameter for each female in two broodstock groups in years 1 and 2. 84
4.9 Temperature profiles, fertilisation rates and hatch rates for two broodstock groups in year 1 and 2. 85-86
4.10 Scatter plot comparing total egg production against length for two broodstock groups in year 2 with fecundity relationship from Haug and Gulliksen (1988). 93

Induction of Triploidy

5.1	Diagram of pressure shocker.	110
5.2	Flow diagram of experimental procedures.	113
5.3	Relative hatch rate and triploidy yield for control and pressure treated eggs.	117
5.4	Percentage frequency distribution of chromosome counts from control and pressure treated groups.	119
5.5	Percentage of different ploidy types from control and pressure treated groups.	120
5.6	Photomicrographs of diploid and triploid metaphase chromosome spreads.	121
5.7	Photomicrographs of diploid and triploid at hatching and at the end of the yolk sac phase.	123
5.8	Percentage survival rates of diploid and triploid halibut at each stage of the production cycle.	124

Weaning and Co-feeding Experiment 1

6.2.1	Cumulative survival and feed response.	154
6.2.2	Mean survival rate.	155
6.2.3	Time taken to reach 50% feed response.	155
6.2.4	Final wet weight at day 107 PFF.	157
6.2.5	Final dry weight at day 107 PFF.	157
6.2.6	Change in wet weight during experimental period.	158
6.2.7	Specific growth rate from day 84-107 PFF.	158
6.2.8	Cumulative mortality during high salinity challenge.	161

Weaning and Co-feeding Experiment 2.

6.3.1	Cumulative survival and feed response .	169
6.3.2	Mean survival rate.	169
6.3.3	Mean percentage of fish taking inert diet at the end of weaning period.	171
6.3.4	Change in wet weight during experimental period.	171
6.3.5	Mean final wet and dry weight at day 109 PFF.	172
6.3.6	Coefficient of variation of wet weights before weaning and at day 109 PFF.	172
6.3.7	Specific growth rates from day 58-109 PFF.	174
6.3.8	Change in specific growth rate from day 58-109 PFF.	174
6.3.9	Cumulative mortality during high salinity challenge.	175
6.3.10	Regression of wet weight vs. time of mortality in salinity challenge	176
6.3.11	Daily <i>Artemia</i> ration and cumulative total number of <i>Artemia</i> fed per fish from first feeding until weaning.	178

<i>Weaning and Co-feeding Experiment 3</i>		
6.4.1	Diagram illustrating grading procedure.	184
6.4.2	Feed response and cumulative survival rate .	187
6.4.3	Feed response at the end of weaning period.	187
6.4.4	Initial and final length at day 68 PFF and at day 101 PFF.	189
6.4.5	Initial and final wet weight at day 68 PFF and at day 101 PFF.	190
6.4.6	Change in wet weight during experiment.	191
6.4.7	Specific growth rates during experiment	191
6.4.8	Initial and final dry weight at day 68 PFF and at day 101 PFF and specific growth rate over whole period.	193
6.4.9	Cumulative mortality during high salinity challenge and stress index.	195
6.4.10	Metamorphosis and pigmentation characteristics	196
6.4.11	Photographs of representative fish from each group.	197
<i>Weaning and Co-feeding Experiment 4</i>		
6.5.1	Diagram of experimental procedures.	202
6.5.2	Final wet weight, survival rate and feed response for each stage.	205
6.5.3	Final length, dry weight and specific growth rate for each stage.	207
6.5.4	Photomicrographs of representative larvae from treatment groups.	208 - 210
<i>Weaning and Co-feeding Experiment 5</i>		
6.6.1	Diagram of experimental feeding regime.	216
6.6.2	Survival rates at the end of the experiment.	219
6.6.3	Feed response during experimental period.	219
6.6.4	Initial and final wet weights and length on day 69 PFF and 86 PFF	221
6.6.5	Pigmentation and eye migration characteristics.	222
<i>Weaning and Co-feeding Experiment 6</i>		
6.7.1	Final survival rates, change in feed response and feed response at the end of the weaning period.	232
6.7.2	Frequency of demersal fish on day 41 and 81 PFF and proportion of fish with true stomachs on day 50, 56 and 80 PFF.	233
6.7.3	Change in wet weight during experiment.	235
6.7.4	Final dry weight on day 81 PFF and specific growth rate from day 58 to 81 PFF.	236
6.7.5	Final length and condition factor.	237
6.7.6	Pigmentation and eye migration characteristics.	238
6.7.7	Daily <i>Artemia</i> ration and cumulative total number of <i>Artemia</i> fed per fish from first feeding until weaning.	242
<i>Weaning and Co-feeding Discussion</i>		
6.8.1	Summary of growth of all experimental groups.	244

List of Tables

2	General Materials and Methods	
2.1	Products used for <i>Artemia</i> enrichment.	19
2.2	Details of meters used for measurement of physical environmental parameters.	24
3	Egg Quality Assessment	
3.1	Description of blastomere characteristics assessed for halibut eggs.	42
3.2	Correlation coefficients for blastomere characteristics and hatch rate.	44
3.3	Best subset models of multiple regression for blastomere scores on hatch rate.	51
4	Broodstock Temperature Requirements	
4.1	Formulation of broodstock moist feed sausage.	76
4.2	Data for Year 1 and 2 egg production in ambient and chilled broodstocks; number of batches, mean fertilisation rate and hatch rate.	88 – 89
5	Induction of Triploidy Using Hydrostatic Pressure Shock	
5.1	Mean fertilisation rate and hatch rates for control and pressure treated eggs.	116
6	Weaning and Co-feeding	
6.1.1	Review of results from other early weaning and co-feeding studies.	141- 143
6.2.1	Live feed history, initial wet and dry weights of larvae for Experiment 1.	150
6.2.2	Cumulative mortality during 10 h salinity challenge.	160
6.3.1	Age, initial wet and dry weight and length of fish for Experiment 2.	166
6.3.2	Standard 9 day weaning regime.	167
6.3.3	Stress indices for fish before and after weaning from salinity challenge.	177
6.3.4	Savings in <i>Artemia</i> according to weaning age.	179
6.6.1	Feeding regimes applied to 4 groups of larvae in Experiment 5.	214
6.6.2	Fatty acid composition of 69 day PFF larvae at the end of Experiment 5.	223
6.6.3	Lipid class composition of 69 day PFF larvae at the end of Experiment 5.	224
6.7.1	Treatment groups for Experiment 6.	229

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Abstract

The supply of juvenile Atlantic halibut, *Hippoglossus hippoglossus*, has been sporadic and until recently, has fallen short of expectations, due to difficulties associated with the hatchery phase. This thesis focuses on some specific aspects of intensive hatchery production which needed to be addressed in the areas of egg production and quality, triploidy induction and weaning.

A quality assessment technique for halibut eggs, based on observations of morphological anomalies occurring during early blastomere divisions was devised. The degree of abnormality in the appearance of five features was quantified and a strong relationship between these characteristics and hatch rate of eggs incubated in microtitre plates was revealed. It was concluded that this method is of great potential use as an early predictive indicator of egg viability.

The effect of temperature on egg production was studied in two broodstock groups held either at ambient or low stable temperature during spawning. High temperatures caused a delay and shortening of the spawning period as well as a reduction in egg quantity and quality. The results indicated that temperature control is a necessary feature of broodstock management at sites where ambient temperature profiles are unsuitable.

The efficacy of hydrostatic pressure shocking for the induction of triploidy was tested on newly fertilised eggs. A 5 min pressure shock of 8500 psi, administered around 15 min after fertilisation resulted in high triploidy yields. This treatment had little effect on survival to hatching. However, a preliminary experiment indicated that triploid halibut may be prone to higher mortality through the hatchery cycle.

Six weaning experiments were conducted to determine the influence of size, age, and developmental stage of halibut larvae on diet uptake, survival, growth and fry quality. Gradual replacement of *Artemia* with dry diet, co-feeding, and the use of intermediate diet types, were evaluated. In the absence of live prey, pre-metamorphic larvae (< 100 mg) would accept non-living feed particles but total replacement with a conventional dry diet was unsuccessful. However, following good growth prior to weaning, 700 °day old larvae could be successfully weaned over a nine day period. Improved growth and substantial savings in live feed were among the positive benefits resulting from early weaning.

1. General Introduction

Supported by a considerable research effort and investment from the public and private sectors, commercial culture of the Atlantic halibut is now in its initial phases in Norway, Scotland, Iceland and Canada. The potential profitability of halibut farming, owing to its high market value and a demand that exceeds the declining wild catch, has provided the impetus for this development. World landings fell from nearly 8000 tonnes in 1984 to less than 4000 tonnes in 1996 (FAO, 1998) and the unit value and volume of imports to the U.K., a major consumer, have more than doubled in the last ten years.

Aside from the earliest trials (e.g. Rollefson, 1934), research into the techniques for the culture of halibut began in the 1980's and a few juveniles were reared past metamorphosis in the first attempts (Blaxter *et al.*, 1983; Anon 1988). There are a growing number of fry producing operations world-wide (Waiwood and Brown, 1997; Engelsen, 1998, Shields *et al.*, in press). Recent years have seen encouraging results in terms of fry output (an estimated total of 350,000 were produced in 1997) and this has translated to significant volumes of farmed halibut entering the market (Pittman, 1996; Olsen, 1993; Berg, 1997).

Due to the lack of a reliable and sufficient number of juveniles to supply the ongrowers, the development of the industry has been slow compared to other fish species. Initial difficulties with hatchery production were attributable to a poor understanding of the biological requirements of the halibut. As the techniques for mass production have evolved, it has become apparent that investment in technology and labour costs are high. Early stages require precise environmental control and the hatchery cycle is relatively long (see Fig 1.1). Progress for research groups and industry

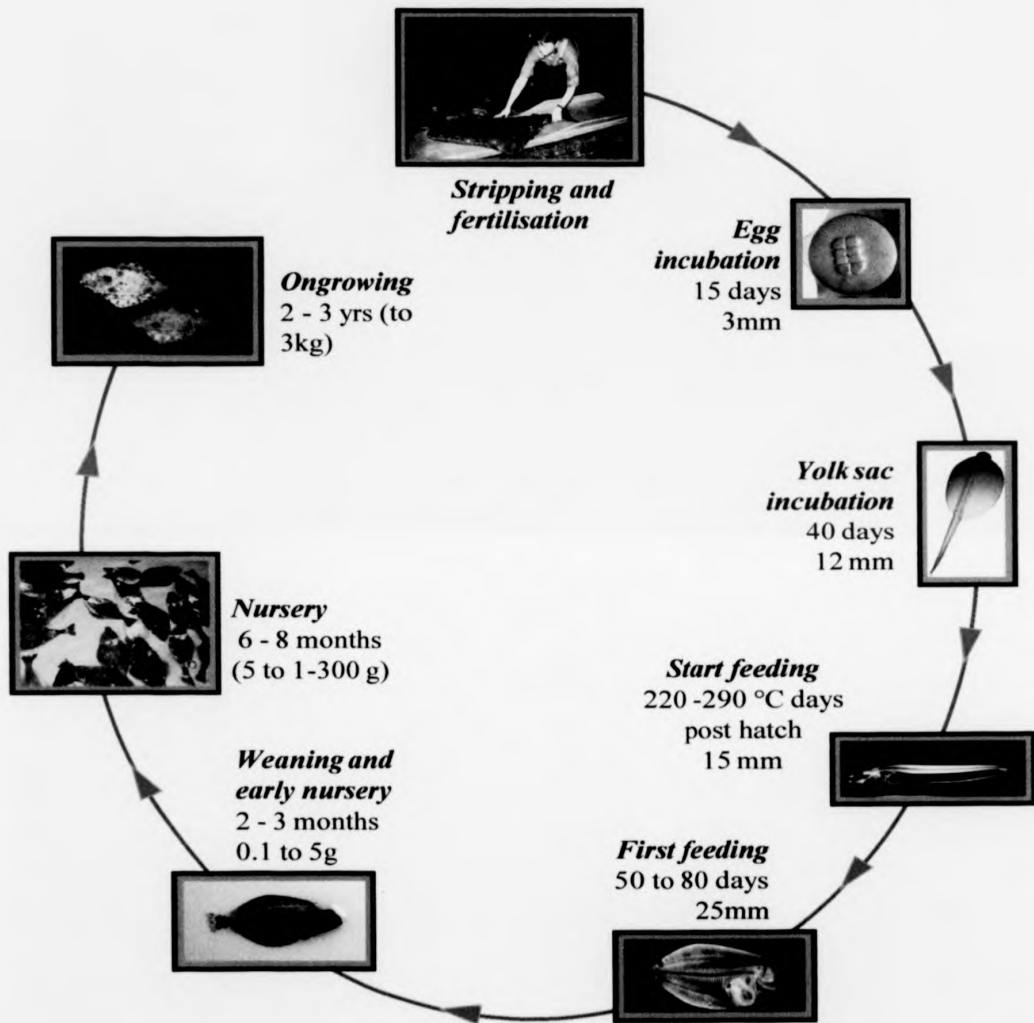


Figure 1.1. The production cycle of the Atlantic halibut with photographs of each life stage. Approximate times and typical sizes for each stage are given. Pictures not to scale.

has been hampered by a scarcity of material caused by low survivals to metamorphosis and by a shortage of eggs, particularly in Scotland.

Owing to the expense involved in their capture, the low abundance of mature individuals in the wild and the time taken for acclimation to holding facilities, the establishment of spawning stocks has been difficult. Captive broodstock populations were first set up in Scotland and Norway in the early 1980s (Blaxter *et al.*, 1983; Rabben *et al.*, 1986; Smith, 1987). The Atlantic halibut is a batch spawner, producing several batches of eggs in relatively regular intervals of 3-4 days (Smith 1987; Haug, 1990; Holmefjord and Lein, 1990; Norberg *et al.*, 1991). The spawning season occurs between December and April under natural photoperiod (Kjorsvik *et al.*, 1987; Haug, 1990). However, year-round egg production is possible using altered photoperiod (Smith *et al.*, 1991; Holmefjord *et al.*, 1993; Naess *et al.*, 1996).

The incubation period is relatively long and egg quality can be highly variable (Kjorsvik *et al.*, 1990; Norberg *et al.*, 1991; Bromage *et al.*, 1994). Estimates of viability are commonly based on fertilisation rate, gross morphology or buoyancy. These techniques are neither accurate nor reliable (Kjorsvik *et al.*, 1990; Bromage *et al.*, 1994) and a more effective technique would be of great benefit. During early divisions, the large cells (or blastomeres) can display abnormalities. The morphological symptoms of these abnormalities were documented and quantified in a study described in Chapter 3. To assess the exploitative value of blastomere morphology as an egg quality indicator, these characteristics were related to hatch rate in small scale incubation experiments

Atlantic halibut are known to spawn at great depths where temperatures are generally stable and between 5 to 7°C (Haug, 1990). Ambient temperature profiles found in many

broodstock holding facilities, such as those at Ardtoe, Scotland, are subject to seasonal fluctuations of a greater magnitude than those found at the natural spawning grounds. Temperature is one of the major environmental cues for spawning in a number of fish species (Lam, 1983; Bye, 1984) and may also affect gamete quality and quantity. For the management of halibut broodstock, which is a limited and valuable resource, it is important to understand the effect of temperature on egg production, since this may have implications for the strategies and costs involved. The influence of temperature on egg production characteristics, in terms of volume and quality was investigated in Chapter 4. Spawning performance of broodstock groups held under either controlled or ambient temperatures was monitored for a two year period.

The period from hatching to first feeding, when the endogenous reserves stored in the yolk sac are absorbed, can last up to 50 days depending on temperature. During this period larvae are held in upwelling cylindroconical incubators. These range in volume from 450 l, such as those used at Ardtoe, to large silos of 3000 to 13000 l, favoured by Norwegian operators (Harboe *et al.*, 1994; Berg, 1997). Strict temperature control is necessary during this phase since suboptimal temperatures can cause developmental abnormalities or high mortality (Lein *et al.*, 1997; Bolla and Holmefjord, 1988; Ottesen and Bolla, 1998). Salinity must also be within a narrow range (Lein *et al.*, 1997; Ottesen and Bolla, 1998; Bolla and Ottesen, 1998) and maintenance of good water quality is required. Light, which has been shown to affect egg buoyancy during incubation (Mangor-Jensen and Waiwood, 1995) and the timing of hatching (Helvik and Walther, 1993), is also important during the yolk sac stage. The animals are generally kept in near or complete darkness due to the detrimental effects that light can cause (Bolla and Holmefjord, 1988). They are also strongly positively phototactic towards the end of the

yolk sac period (Naas and Mangor-Jensen, 1990). Halibut larvae hatch in a very primitive developmental state and organogenesis proceeds at a slow pace (Lonning *et al.*, 1982; Blaxter *et al.*, 1983; Pittman *et al.*, 1990a). At around 150°Cdays, the eyes, mouth and intestine become functional (Blaxter *et al.*, 1983; Pittman *et al.*, 1990b; Kvenseth *et al.*, 1996). The transition to exogenous feeding can occur between 200 and 290°Cdays and the duration of the live feed stage is typically 50 to 70 days (Harboe *et al.*, 1990; Lein and Holmefjord, 1992; Harboe *et al.*, 1997). Larvae are fed live prey: rotifers, *Artemia*, cultured copepods, wild zooplankton or a mixture of these (Holmefjord *et al.*, 1993; Naess *et al.*, 1995; Shields and Bell, 1995). "Green water" is generally used in intensive systems since it has been found to be beneficial for first feeding success (Naas *et al.*, 1992; Holmefjord *et al.*, 1993; Gulbrandsen *et al.*, 1996).

The energetic requirement of the larvae is very high and to achieve good growth, the provision of up to 5000 *Artemia*/fish/day is necessary (van der Meer, 1996). During this stage of the cycle, heavy demands are placed on a hatchery in terms of labour, facilities, live feeds and enrichment media. One of the primary goals in marine fish rearing is to reduce the length of the live feeding period, by bringing forward the time at which the switch to inert diets can be made. This, together with the mortalities that can often occur during this transition, led to the study of different aspects of the weaning stage, detailed in Chapter 6. Several long term experiments were conducted with the aim of identifying some of the attributes of the fish, as well as exogenous influences and inputs that affect the success and efficiency of weaning.

Once weaned, the Atlantic halibut is a comparatively robust and disease resistant fish, which demonstrates good growth rates in temperate waters (Adoff *et al.*, 1993;

Bjornsson *et al.*, 1992; Bjornsson, 1994, 1995; Blanquet and Lyngren, 1997). There are two basic approaches to ongrowing; using land-based tanks or raceways (Adoff *et al.*, 1993; Blanquet and Lyngren, 1997) or at sea in cages (Martinez Cordero *et al.*, 1994). As with many species, the halibut exhibits sexual dimorphism in terms of growth rates. Males tend to grow more slowly than females and mature at a smaller size and younger age (Jakupsstovu and Haug, 1988; Haug, 1990; Bjornsson, 1994, 1995). There are economic benefits to be gained from the exclusion of males from the population (i.e. produce all female stocks), or by limiting the impact of sexual maturation by sterilisation. The latter has been achieved for a number of species through the induction of triploidy, which can be done using various physiological shocks soon after fertilisation. Triploidy has already been successfully induced in halibut using cold shocks (Holmefjord and Refstie, 1997). An alternative method employing hydrostatic pressure shock, widely practised with other species, was tested during this study and is reported in Chapter 5.

Mass production of halibut was initially achieved in Norway using semi-intensive techniques and these have been described by Berg and Oiestad (1986), Rabben *et al.* (1986) and Berg, (1997). Larvae reared in indoor incubators are moved to outdoor bag enclosures prior to first feeding and fed harvested wild zooplankton and *Artemia*. Though this technique can potentially generate large numbers of fry and was the mainstay of production in 1994, output from these systems fell drastically in 1995 (Olsen, 1997; van der Meer and Naas, 1997) and it is now accepted that the method has drawbacks. Seasonal variations in wild zooplankton harvests can result in shortages of live prey. There is also a greater risk of exposure to pathogens e.g. nodavirus (VNN) or infectious pancreatic necrosis (IPN), which can cause serious mortalities in halibut (Grotmol *et al.*, 1997; Rodgers and Frerichs, 1997). Large size variations are also a

characteristic of fry reared in these systems and this can cause problems at weaning (Berg, 1997). The development of methods for hatchery production in the U.K. has focused on intensive techniques using *Artemia* or cultured copepods as the primary live food source. The experimental work described in this thesis was carried out in an intensive hatchery. However, since the studies were conducted on those stages either side of the live feeding stage, where the main area of divergence between the two techniques lies, the findings can be considered relevant to both rearing methods.

2. General Materials and Methods

2.1. Introduction

All the experiments described in this thesis were conducted at the Marine Farming Unit of the Seafish Industry Authority at Ardtoe. The following chapter describes the characteristics and development of the site and its facilities. All the material used for the experimental work was derived at some stage of the production cycle, from the Ardtoe stock and therefore it is necessary to describe briefly the origin of this material and the rearing techniques employed. Also, some of the general experimental methods are detailed in this section.

2.2. Facilities

2.2.1. History

The site at Ardtoe was selected by the White Fish Authority in 1965 as a suitable location for setting up an experimental hatchery at which to continue the development of rearing techniques for the plaice, originally established by MAFF scientists at Port Erin, Isle of Man. Unfavourable economics for plaice culture meant that attention was turned to more valuable species. The Dover sole, was selected as the next candidate species based on early success achieved at Port Erin and the Whitefish Authority's second hatchery opened at Hunterston. Sole were reared semi-extensively using naturally occurring zooplankton, but it became apparent that ambient temperatures were below optimum. Turbot, was chosen next but larval rearing proved difficult, so initially wild caught fry were ongrown. Larval rearing techniques for this species were later successfully developed at the Hunterston hatchery (formerly White Fish Authority and

later Golden Sea Produce) and Mannin Seafarms (Isle of Man). The Sea Fish Industry Authority took over from the White Fish Authority in 1981 with essentially the same remit, that is to assist the UK fishing industry with all aspects of operations from catching to marketing.

As early as the beginning of the 1970s, interest was directed towards the Atlantic halibut but initial problems including a lack of broodstock males, low survival of yolk sac larvae and disease in the broodstock led to the abandonment of halibut rearing for ten years. Meanwhile further progress was made with turbot rearing and also small scale sea bass and cod rearing trials.

A new broodstock was collected from the wild and established in 1983. Fertilised eggs were collected in 1985 and the first fish survived to metamorphosis in 1987. After an extensive fire in 1988, the facilities were reconstructed and tailored specifically for halibut rearing. Research has gathered pace since the 1980s fuelled by commercial interest particularly from salmon farming companies looking to diversify. A collaborative approach involving the S.F.I.A., industry partners coordinated by the British Halibut Association and University research groups particularly from the Institute of Aquaculture at Stirling University has resulted in significant improvements in culture techniques.

2.2.2. Site location

The Marine Farming Unit is located in Ardtoe (Lat.; 56°46'N, Long.; 5°52'W), a small crofting township 4 miles from Acharacle and 45 miles by road from Fort William in north Argyll on Scotland's west coast. The site is northwest facing onto Ardtoe bay at the mouth of Kentra bay into which a number of streams feed providing some freshwater

input. It is relatively sheltered from most wind directions and virtually free from pollution of any kind. Tidal range is between 1 m at neaps and 4 m at springs.

The main building houses the offices and the halibut hatchery and laboratories. There are other separate buildings containing the halibut broodstock, the halibut nursery, the shellfish facility, the lobster unit, and a workshop. A site map is shown in Figure 2.1.

2.2.3. Seawater supply

The sea water inlet is just covered at low water springs and the salinity of the supply may fluctuate when surface water is diluted during high rainfall and a brackish water wedge forms with onshore winds. Sea water for the broodstock and nursery areas are pumped by 15 cm submersible pumps (Flygt, Sweden) capable of delivering 2000 l/min from an intake 80 m offshore via welded polyethylene pipes. Water for the hatchery is delivered by 7.5 cm submersible pumps delivering 1000 l/min via a separate line to two 5 m³ onshore header tanks. All water is filtered through sand filters to 25 µm. Water is further filtered (using cartridge filters) to 5 µm for the larval rearing tanks, 5 µm for egg and yolk sac incubation and *Artemia* culture and down to 0.2 µm for algal culture. Further treatment in the hatchery includes U.V sterilisation, foam fractionation (protein stripping) and biofiltration for recirculating systems. Automatic brining equipment is installed to provide water of constant salinity where required.

2.2.4. Hatchery

The hatchery is divided into six main areas; algal production, *Artemia* culture, egg incubation, yolk sac incubation, production larval rearing and production weaning. There are also two separate rooms for experimental larval rearing, one for experimental

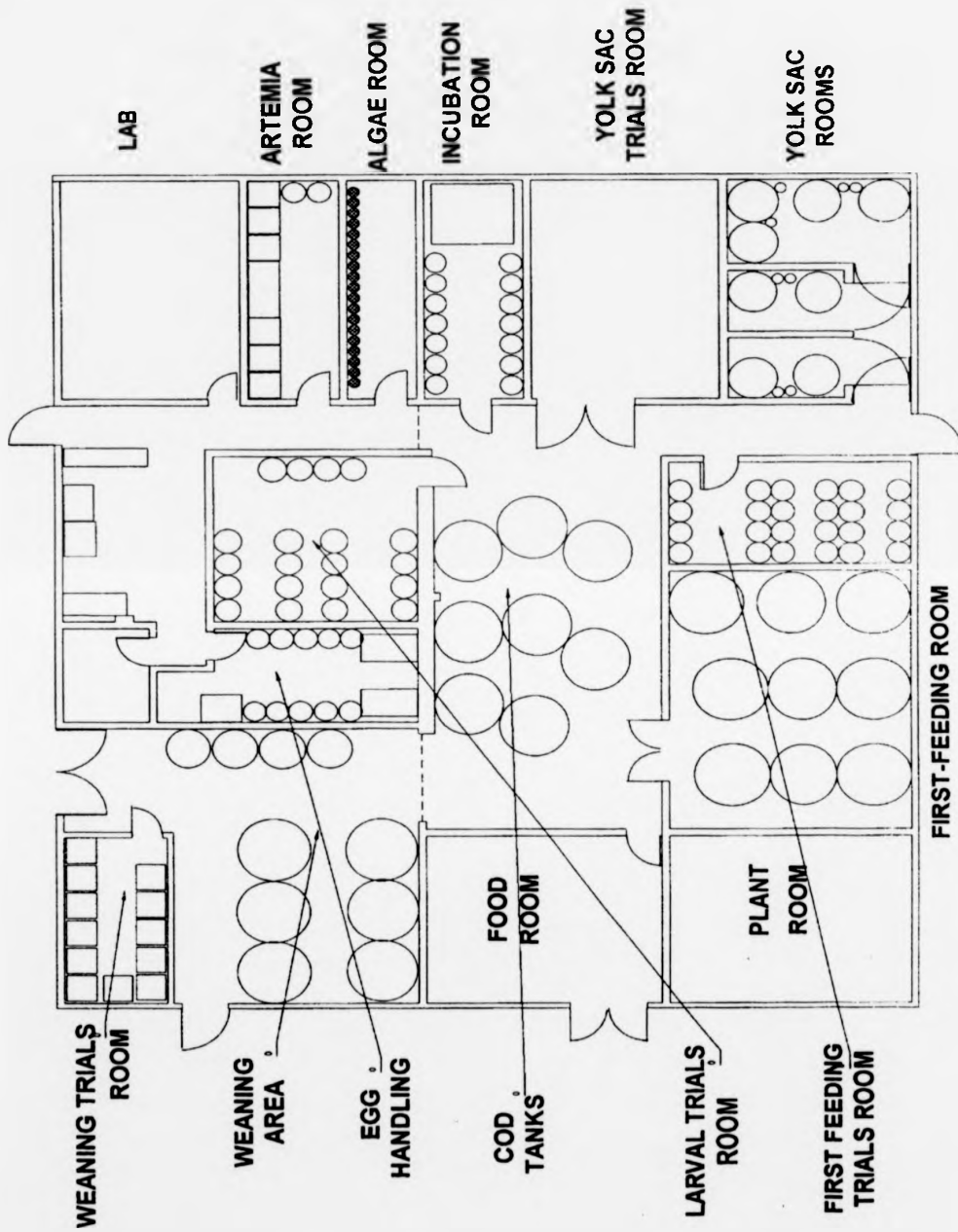


Figure 2.2. Floor plan showing facilities at halibut hatchery site, Ardtoe.

weaning and areas for testing yolk sac tank designs and recirculation systems. The holding facilities are continually updated as rearing technology is improved and fine tuned. The current floor plan is shown schematically in Figure 2.2.

2.3. General Rearing Techniques

2.3.1. Broodstock management and gamete production

The original broodstock at Ardtoe were caught from the wild in the early 1980's from waters around Shetland and the Faroes (Smith, 1987). Many of these fish are still spawning and have reached in excess of 30 kg body weight. Since then, a number of the first hatchery reared fish have reached maturity and have subsequently been recruited to the broodstock population.

Fish are held in covered 5-10 m diameter, vitreous enamelled steel tanks with concrete bottoms. Stocking densities are low (<5 kg/m²) and a typical spawning group may consist of three females and three males per 5 metre tank. Some groups are held under ambient photoperiod, salinity and temperature whilst some or all of these environmental variables are controlled for other groups. The fish are fed once per day on a semi-moist sausage comprising minced wet fish, fishmeal, marine body oil, vitamins, minerals and binder.

Ovulating females are observed closely and the first spawning is usually signalled by a release into the tank water of ripe eggs, which are collected at the outlet. The spawning rhythm is subsequently established by regular checks. When a female is to be stripped, the tank is drained to a convenient depth and a stripping table is lowered onto the tank floor. The female is ushered onto the table, which is then lifted out of the water, and if the eggs are released with gentle hand pressure to the abdomen, they are collected into a

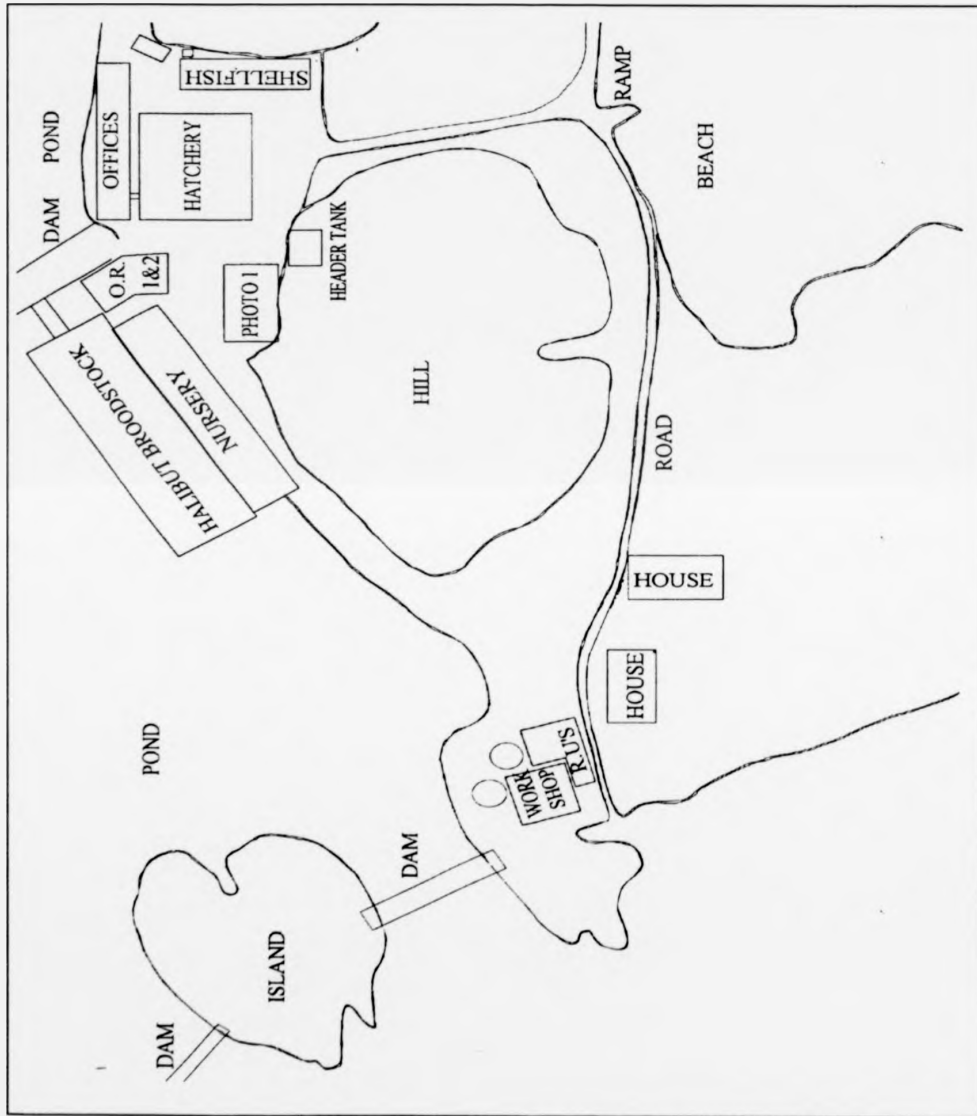


Figure 2.1. Map of Seafish Aquaculture site at Ardtoe, Acharacle, Argyll, Scotland.

dry plastic jug and the total volume is noted. Males are stripped in a similar fashion after the spermatopore is dried with tissue to prevent sperm activation in the presence of seawater.

The gametes are taken to the hatchery in a light proof insulated box to a temperature controlled room held at a steady 6°C. One ml of milt is diluted in 1 l of seawater and added immediately to the eggs. Fertilisation occurs within 1 min and sperm activity ceases within 3 min. After 20 min when the eggs have water hardened, the eggs are rinsed in a soft hand net with two changes of fresh seawater. They are then placed in static seawater of 34-35ppt at 6°C held in an 80 litre cylindroconical black polyethylene tank in a dark room where the air temperature is maintained at 6°C. Dropout is removed after 30 minutes and noted. Batch size is estimated from total and sample volumes.

2.3.2. Egg incubation

After 16 h in static seawater at 6°C, fertilised eggs will normally have reached the 8 cell stage. At this point the batch is checked for fertilisation rate and appearance. Any dropout occurring between fertilisation and this stage is collected and noted. On the basis of the performance of the batch up to this point, a decision is made as to whether to discard the batch or stock it into the egg incubation system. The number of eggs transferred to egg incubation is estimated from the total weight and counts of replicate 1g samples.

The egg incubation system is now a well established part of the hatchery system and its design was arrived at following some of the work detailed in this thesis. It consists of 14 black polyethylene cylindroconical 80 L tanks. The seawater is ~50% recirculated and the fresh seawater at 32 - 33 ppt enters the 600 l header tank. Water is circulated from

the header tank through a chiller to bring it to 6°C. Water passes from the header tank through a U.V steriliser before flowing into the incubation tanks through a bottom inlet. The inlet consists of a narrow funnel and flow rates are adjusted to <1 l/min to give a gentle upwelling flow keeping the slightly negatively buoyant eggs in suspension. The outlet is a spherical 1mm mesh positioned at the surface. Water returns to the header tank via a sump tank, a 5 µm cartridge filter, a U.V. steriliser and a foam tower. The air temperature is maintained at 6°C with an air chiller and the room and is light proof, all procedures being carried out using a torch.

Once per day, dead eggs are removed from the tanks using the "salt plug technique" developed in Norway. The flow is turned off and about 6 l of high salinity (40 ppt) seawater is injected into the bottom of the tank. Live eggs generally float on the resulting halocline and non-viable eggs drop to the bottom where they can be tapped off with the salt plug. The flow is then restored and the volume of dead eggs is recorded. Up to about 1 l of eggs (~40,000) can be stocked per incubator.

Hatching takes place in the incubators after approximately 75-80°Cdays post fertilisation. Hatched larvae will usually float in the surface layer and can be removed using 2 l jugs. Larvae are transferred in jugs to yolk sac incubators in light proof, insulated boxes.

2.3.3. Yolk sac incubation

Several designs of yolk sac incubator have been tested at Ardtoe and since this stage has proved one of the most problematic, much of the research effort at Ardtoe has been directed towards improving system design. Tanks of volume ranging from the now redundant 4 m³ silos down to 200 l (also redundant) have been tested. The system now

comprises 450 or 950 l conical tanks with short vertical sections. Flows are upwelling and inlet diameters and flow rates vary. Environmental conditions are strictly controlled as for egg incubation. Tanks are housed in dark rooms at 6°C and water is filtered to 5 µm, U.V. sterilised, and maintained at a constant 6°C and stable salinity.

The duration of the yolk sac stage is long for Atlantic halibut and typically lasts for 220 - 290°Cdays. When the larvae are almost ready to start exogenous feeding the temperature of the yolk sac tank is allowed to rise to approximately 10°C gradually over a few days. The larvae are then counted out and transferred in water to first feeding tanks again using plastic jugs.

2.3.4. Larval rearing

Larval rearing tanks used for production purposes are black GRP, flat bottomed and circular (top diameter: 150 cm; bottom diameter: 125 cm; height: 89 cm), with a central double standpipe giving a depth of 85 cm and a volume of 1.2 m³. The inlet is at the surface and the outlet is positioned at the bottom of the outer standpipe and covered with fine mesh. Flow rates are increased gradually through the first feeding phase from 0 to 1l/min (5 µm filtered, U.V. sterilised seawater, ambient salinity). The temperature is maintained at 10-12°C with immersion heaters and an air chiller. Illumination is provided by overhead tungsten lights to achieve light levels starting at around 30 lux and increasing gradually to 600 lux at the water's surface as the fish feed more actively. Aeration is provided to each tank by a single airstone.

Algae (*Nannochloris* sp.) is added to the tanks to green the water to achieve cell densities of 1-3000 cells/µl during first feeding. This density is gradually reduced as the larvae grow. At the start of exogenous feeding, newly hatched *Artemia* nauplii are

presented for the first 10-12 days. From day 5 PFF "24 hour ongrown" enriched *Artemia* are fed until weaning. On occasion, "48 hour ongrown" enriched *Artemia* was also presented to larvae during the latter stages of larval rearing.

An estimate of first feeding success is obtained at day 14 PFF when larvae are counted into a clean tank.

When larvae have begun to settle on the bottom and are large enough to consume inert diet, they are captured with soft nylon hand nets and moved out to weaning tanks.

2.3.5. Weaning

The tanks generally used for production weaning are black GRP, flat bottomed, and circular (top diameter: 150 cm; bottom diameter: 140 cm; height: 60 cm) with a central double standpipe giving a depth of 40 cm and a volume of 660 l. Flow rates of 5-10 l/min are supplied via a sub-surface 15 mm diameter spreader bar angled tangentially to induce a circular flow and improve self-cleaning characteristics. Airstones provide aeration and each tank is illuminated from above by a single tungsten light producing around 600 lux at the surface.

This phase of the production cycle is dealt with in considerable detail in this thesis and weaning techniques will not be described in this section.

2.3.6. Live food production

Algae are grown *en masse* in batch cultures in well-aerated 0.2 µm filtered, U.V sterilised seawater of 33-34 ppt in closed, vertical, 100 l food grade plastic bags lit from behind with fluorescent tubes (~700 lux). Essential nutrients are provided by addition of Walnes media and trace metal solution. Air temperature is maintained at around 20°C by

waste heat from the light source. A batch is harvested typically 10 days after inoculation when the cell density has reached approximately 40,000 cells/ μ l. Axenic stock cultures are held static in 500 ml flasks. The species generally used for "greening" of halibut first feeding tanks is *Nannochloris atomus* which is fast growing and reasonably easy to maintain.

Artemia nauplii are hatched from decapsulated cysts (EG, *Artemia* Systems) suspended in strongly aerated 5 μ m filtered, U.V sterilised seawater of 33-34 ppt preheated to 28°C in 80 l cylindroconical polyethylene tanks lit from above with fluorescent tubes (~400 lux at water's surface). Nauplii hatch (typically 100,000/l) within 16-18 h and once the air supply is turned off and they are allowed to settle, the harvest is siphoned off and rinsed in fresh seawater. Nauplii are fed to first feeding halibut larvae or set up for on-growing at a density of approximately 150,000/l into similar tanks and water. Enrichment media (see Table 2.1.) are blended with seawater and added to the culture tank at specified concentrations (typically 0.6 g/l). Newly hatched nauplii are generally harvested in the morning, receive the first enrichment dose in the afternoon and are harvested and rinsed the following morning after an 18 h enrichment as "24 hour on-grown". These are either fed to larvae or set up as before for a further 24 h period of on-growing with enrichments to be harvested the following day as "48 hour on-grown". In some cases enrichment periods of 1-2 h may be employed just prior to feeding.

Artemia for feeding are stored during the day at a concentration of 250/ml. Feeding rates were generally on a number of prey per individual basis and a correction factor was applied to allow for reduction of prey density due to flow rate according to Beck and Bengtson (1979).

Table 2.1. Products used for *Artemia* enrichment in this study.

Product	Supplier	Notes
Super Selco	Inve, Gent, Belgium	Liquid blended lipid formulation.
DC Selco	As above	As above
SSF Microfeed	Norwegian Herringmeal and Oil Industry, Norway.	Fish meal based powder.
Algamac 2000	Biomarine Aquafauna, USA	Powdered enrichment derived from spray dried <i>Schizotrychium</i> sp.(heterotrophic algae).
Tuna orbital oil	Roche Pharmaceuticals	High purity oil extracted from eyes of tuna. Emulsified using soy lecithin.
Herring Oil	Norwegian Herringmeal and Oil Industry, Norway	Purified oil extracted from herring. Emulsified using soy lecithin

2.3.7. Weaning diet

The weaning diet used in all experiments was Nutra Marine (T. Skretting AS, Stavanger Norway). Details of the proximate and fatty acid composition and particle sizes of this diet are given in Appendix I and II.

2.3.8. Hygiene

All equipment are washed with hot water and surfactants (Kick-Off, RS Hygiene Ltd, Suffolk England) and sterilised with peroxyacetic acid, 1:250 (Kick Start, RS Hygiene Ltd). Equipment used for live feeds, larval rearing and weaning are kept separate.

2.4. General experimental methods

2.4.1. Handling of gametes

All experiments with unfertilised and fertilised eggs were conducted in an adapted walk-in insulated cold store (3 m x 3 m x 3 m) which was fitted with an air chiller to maintain the air temperature at $6^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. The cold room served as a mini laboratory and housed a binocular stereo zoom microscope (SZH-ILLD Olympus, Tokyo, Japan), an incubator (LMS Ltd, Kent, England) and a hydrostatic pressure shocker (SOAFD Marine Laboratory, Aberdeen, Scotland). A ceiling mounted light controlled by a dimmer switch provided low illumination.

Stripped gametes were transferred via plastic jugs to this laboratory in insulated picnic boxes so that temperature and light shocks were minimal

2.4.2. Handling of fish

Larvae for weaning experiments were carefully collected from first feeding tanks using 2 l jugs and transferred to experimental weaning tanks directly. It was ensured that the temperature of the two systems did not differ by more than 1°C to avoid physiological disturbance.

2.4.3. Weaning tank facilities

All weaning experiments except Experiments 1 and 4 were conducted in the designated experimental weaning facility containing 12 tanks. These were black, moulded GRP rounded square tanks 70 cm x 70 cm x 40 cm with flat bottoms (C & H Aquaculture, Edinburgh, Scotland). An integral conical sump was covered by a disc perforated with 2 mm holes and a central double standpipe took water to waste. Each

tank was fitted with a single airstone providing aeration and the 15 mm inlets were either surface (reduced to 1 mm) or subsurface (15 mm 90° elbows with 2 pinholes) oriented to direct the flow in a circular direction to aid self cleaning. A schematic diagram of a tank is shown in Figure 2.3. The seawater supply was filtered to 5 µm through a cartridge filter. Depending on the season, water was at ambient temperature or controlled with chilling units and/or an immersion heater fitted to a 600 l header tank situated above the room. Seven overhead tungsten lights controlled by a dimmer switch provided variable illumination. Feeding of fish in the first weaning experiments (1-4) was by hand. In subsequent experiments, automatic feeders, designed for the project, were installed. A diagram of one such feeder is shown in Figure 2.3. The feed was contained in a steel hopper and delivered via a motor driven rotating nylon screw onto a spinning disc, driven by another motor, which spread the feed evenly over the tank. These feeders were attached to the ceiling and suspended over the tanks, one feeder per tank. The feed delivery rate and feed interval was adjusted by individual timers which controlled the two motors.

Experiments 1 and 4 were conducted in the larval rearing trials room and the early nursery area respectively (see floorplan shown in Figure 2.2.). These tank facilities are described in detail in the relevant sections.

2.4.4. Sampling and measurement of fish

Samples for measurement were collected randomly from throughout the tanks using soft nylon 3" hand nets.

Weights of fish larvae or fry were measured unblotted, blotted or dry using a micro-

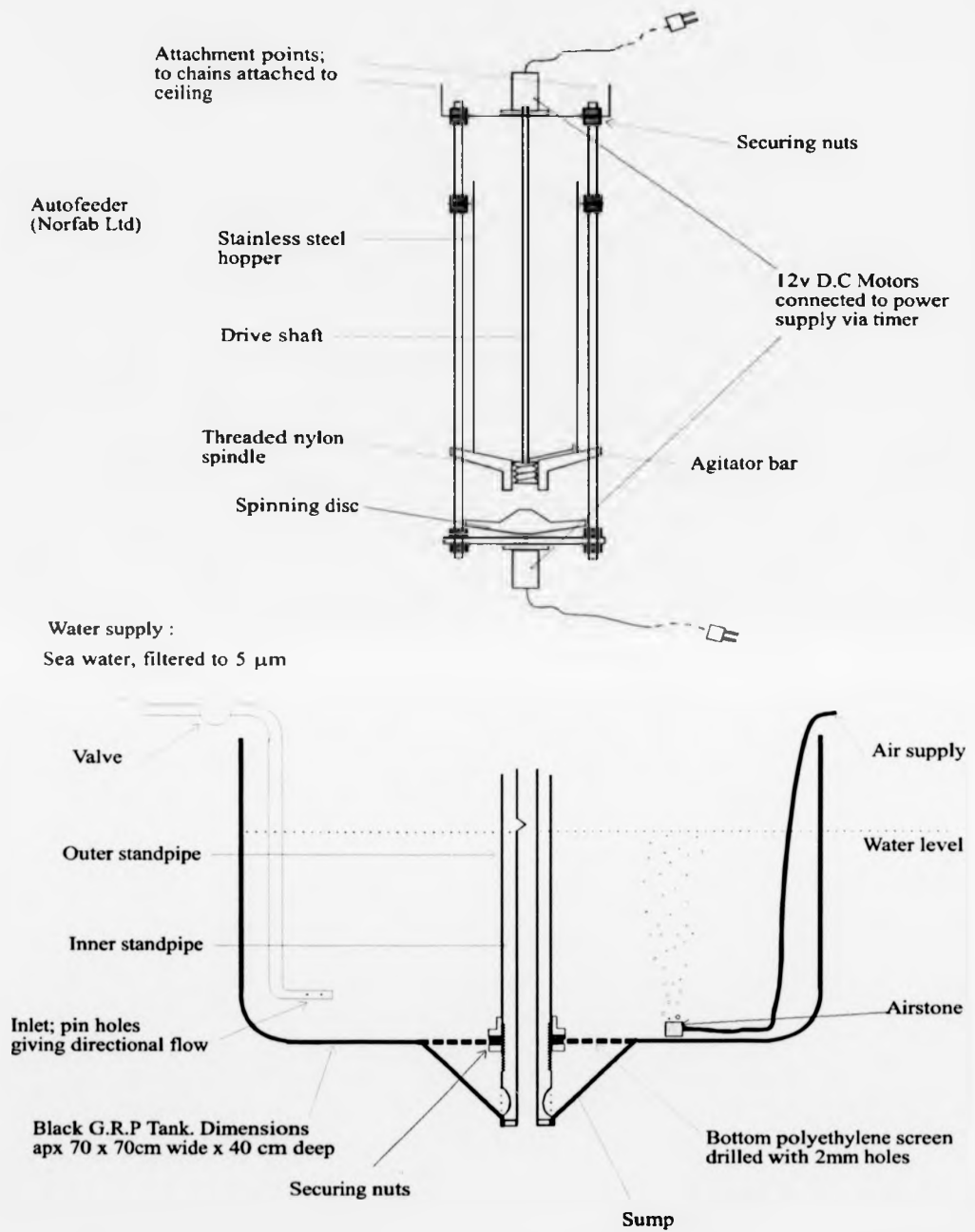


Figure 2.3. Diagram of automatic feeder and experimental weaning tank.

balance (Sartorius H110, Göttingen, Germany). Live wet 'unblotted' weights were taken by catching a fish on a flattened nylon tea strainer, shaking off excess water, blotting the underside of the strainer and then tapping the fish off into a 100 ml beaker of seawater on a tared balance. Fish of over 50 mg weighed in this way survived without damage.

Fish sampled for purposes other than live wet weight were killed by a lethal dose of anaesthetic (MS 222, Sigma Chemicals Ltd, Poole, England). Fish for blotted wet weights were blotted for 3 s on both sides on absorbent tissue paper. Fish for dry weight measurement were rinsed in freshwater to remove excess salt, blotted and stored in individual pre-weighed cryovials at -20°C until freeze drying. Samples were freeze dried in an Edwards High Vacuum freeze drier (B.O.C. Ltd, Sussex, England) until all moisture was removed (after 24 - 48 h).

Total length was measure from the tip of the snout to the end of the caudal tail fin. Small larvae of less than 20 mm were measured under the binocular microscope with a calibrated eyepiece graticule and fry greater than 20 mm were measured with a ruler.

2.4.5. Photography

Photographs of eggs and small larvae were taken on Kodachrome 200 ASA slide film using an SLR 35 mm camera (Nikon F-301, Tokyo, Japan or Olympus OM-2, Tokyo, Japan) attached to a binocular stereo zoom microscope (SZH-ILLD Olympus, Tokyo, Japan × 0.5 objective).

2.4.6. Measurement of physical parameters

Most physical parameters were measured using hand held meters, details of which are shown in Table 2.2. Ammonia readings were taken from water samples using the salicylate method with Hach reagents and a Hach DR/2000 spectrophotometer (Hach,

Colo, U.S.A). Values are expressed in [NH₃-N] (mg/l). To convert to NH₃ or NH₄⁺ conversion factors of x 1.22 or x 1.29 must be applied respectively.

Table 2.2. Details of meters used to measure physical environmental factors.

Parameter	Units	Device	Manufacturer
Temperature	°C	WTW LF196	WTW GmbH, Weilheim, Germany
Salinity	ppt	WTW LF196	WTW GmbH, Weilheim, Germany
pH	pH	WTW pH320	WTW GmbH, Weilheim, Germany
Oxygen concentration	mg/l	Dryden Oxyguard O ₂ meter	Dryden Aquaculture, Edinburgh, Scotland
Light level	lux	RS 180-7133	RS Components.

2.5. Data handling and statistical analysis

Data were processed and manipulated using Excel 5.0 (Microsoft). All statistical analyses were carried out either by hand or using Minitab for Windows, release 9.0 (Minitab Inc., USA).

For the weaning trials, treatments were applied in triplicate where possible and animals and treatments were allocated randomly to replicates.

Possible treatment effects were generally analysed using parametric analysis of variance (ANOVA) or generalised linear model ANOVA for unbalanced designs (Sokal and Rohlf, 1981) after checking for normality and homogeneity of variances (Bartlett's test, Zar, 1980). Pairwise comparisons between means are made by means of Tukey tests (Zar, 1980).

Where percentage data were analysed, arcsine transformations were applied (Sokal and Rohlf, 1981).

Data are normally expressed as means \pm standard deviation (s.d.) and error bars on graphs or bar charts normally represent standard error of means, S.E.M. (s.d./ \sqrt{N}). Coefficient of variation, C.V. is an expression of relative variability and given by (s.d / X) $\times 100$. Specific growth rate (SGR) is derived from the formula:

$$\text{SGR} = [(\log_n W_t - \log_n W_i) \times 100] / \Delta t$$

where W_t = final weight

W_i = initial weight

Δt = time between sampling

Growth Factor, GF3 (Finlay, 1997) is calculated according to the formula:

$$\text{GF3} = (\text{Final weight}^{1/3} - \text{Initial weight}^{1/3}) \times 1000 / \text{Period (in } ^\circ\text{Cdays)}.$$

3. Egg Quality Assessment

3.1. Introduction

Established broodstocks for the supply of eggs to Atlantic halibut hatcheries are growing in numbers (Rabben, 1987; Smith, 1987; Holmefjord and Lein, 1990; Shields *et al.*, 1993) and knowledge of the requirements of captive broodstock is constantly improving (Holmefjord, 1996). Despite this, the supply of good quality eggs is still somewhat unreliable and represents a primary bottleneck for cultivation, particularly in the U.K. Acquisition of wild broodstock remains a priority among hatchery operators since the number of mature hatchery reared animals is still relatively low. The cost of obtaining wild adult fish can be prohibitively expensive given the complexity of the techniques required to capture these fish live and the logistics of transporting them from the fishing grounds to the broodstock facilities in good condition.

Captive halibut are generally stripped by hand although natural spawning can occur (Holmefjord and Lein, 1990). The natural spawning period differs between stocks from each geographical location in the north Atlantic and occurs between late December until late March (Kjorsvik *et al.*, 1987; Jakupsstovu and Haug, 1988; Haug, 1990). During oocyte development, rapid growth of the ovaries occurs, largely due to the incorporation of yolk. In response to an elevation in the level of steroid hormone 17β -oestradiol, the phosphoprotein precursor to yolk, vitellogenin (VTG) is synthesised in the liver and transported to the ovary via the blood (Nagahama, 1983). In immature halibut females, levels of plasma VTG are low (c. 0.017 mg/l) and increase at maturity to around 4.1 mg/l (Norberg and Kjesbu 1991). During the autumn, plasma VTG rises slowly and reaches a peak close to the first ovulation. Plasma 17β -oestradiol levels increase in a

similar fashion and fall slightly just prior to ovulation. Both VTG and 17β -oestradiol show multiple peaks during the subsequent ovulatory cycles through the spawning season (Norberg and Kjesbu, 1991). Halibut are determinate batch spawners ovulating at intervals of 70 to 90 hours over the spawning season (Holmefjord, 1991; Norberg *et al.*, 1991). During the maturation process batches of oocytes are sequentially hydrated. This involves the uptake of up to several litres of water following an increase in osmotic potential caused by hydrolysis of yolk proteins to amino acids (Craik and Harvey, 1984; 1987; Holmefjord, 1996). Adult female halibut have large gonads and are highly fecund. Adult females of between 20 and 60kg fish are capable of producing between 6 and 16 batches of 10 to 200×10^3 eggs in a spawning season (Haug and Gulliksen, 1988; Kjorsvik and Holmefjord, 1995). A single production 'run' in an average hatchery can therefore be accounted for by just a few egg batches which could be derived from a small number of individual females. The egg incubation phase is lengthy (75 -85 °C days) and the proceeding yolk sac phase lasts from 220 to 290 °C days. Once facilities are committed to a batch of eggs or yolk sac larvae, they are thus occupied for a long period of time. From a broodstock spawning during the natural season (under normal photoperiod), a hatchery manager is unlikely to achieve more than two production runs. It is critical therefore that only the best possible quality eggs are utilised in order that the production is optimised.

Egg quality is highly variable from captive Atlantic halibut broodstock (Norberg *et al.*, 1991; Bromage *et al.*, 1994). For the hatchery operator, a means of assessing egg quality early on in embryonic development would be of great benefit both for the purpose of resource allocation and to monitor broodstock performance. The need for a procedure for assessment of egg quality was highlighted by the I.C.E.S. working group

(Anon, 1991).

Egg quality has been defined as "the egg's potential to produce viable fry" (Kjorsvik *et al.*, 1990). Several criteria based on physical or physiological properties have been proposed for measuring fish egg quality. Rapid egg assessment procedures, such as buoyancy and fertilisation checks, are routinely employed to screen out dead or non-fertilised egg batches (Bromage *et al.*, 1994).

In salmonids, fertilisation rate has been shown to provide a good predictive indicator of egg viability (Springate *et al.*, 1984; Bromage and Cumaranatanga, 1988). In marine fish, the correlation between fertilisation rate and viability of fertilised eggs is not always strong, e.g. sole (Baynes *et al.*, 1993) and the relationship is not generally applicable (Kjorsvik *et al.*, 1990; Bromage *et al.*, 1994).

Buoyancy has been correlated with larval survival in sole (Dinis, 1982), sea bass (Carrillo *et al.*, 1989), red sea bream (Sakai *et al.*, 1985), cod (Kjorsvik and Lonning, 1983; Kjorsvik *et al.*, 1990), turbot (McEvoy, 1984) and Pacific halibut (Liu *et al.*, 1993), where high neutral buoyancy was thought to correlate with higher mortality. Buoyancy can be measured using a calibrated density column such as that described by Mangor-Jensen (1987) for use on cod eggs and later for halibut eggs (Holmefjord, 1996). For halibut, whose eggs float at depth (Vedal-Taning, 1936), about 87% of the buoyancy is accounted for by the water content (Craik and Harvey, 1987) accumulated during meiotic maturation. In general, the eggs of the Atlantic halibut have a relatively high specific gravity owing to their high inorganic content (Riis-Vestergaard, 1982), and so often sink at ambient salinities found in most coastal marine hatcheries. There is considerable variation in buoyancy between and within broodstocks and this may be due to stock differences, diet or environmental factors. Solemdal (1967) showed that salinity

at spawning influenced the relative buoyancy of flounder eggs and hence this may play a role in halibut broodstock management. Kjesbu *et al.* (1992) found that large cod in good condition tended to produce larger eggs and successive batches decreased in specific gravity through the spawning season. Conversely smaller eggs from smaller or poorly conditioned broodstock tended to have a consistently high specific gravity throughout the spawning season. They speculated that the resulting wide horizontal distribution of the former is of adaptive advantage. There is no consensus on the importance of buoyancy of eggs of Atlantic halibut (Bromage *et al.*, 1994) and sinking eggs can be effectively reared to hatching in upwelling incubators, producing viable larvae.

Following fertilisation, the permeability of the egg membrane reduces drastically and in marine fish eggs the low osmotic potential relative to the external medium is maintained. Osmolarity itself has been linked to egg quality in lumpsucker (Kjorsvik *et al.*, 1984a) and herring (Alderdice *et al.*, 1979). In their study on cod Kjorsvik *et al.* (1984b) showed that poor quality eggs maintained a relatively higher osmotic potential through the incubation period.

In halibut eggs the perivitelline space is relatively narrow (Rollefsen, 1934) and as in all teleosts, is formed following the cortical reaction. This occurs during fertilisation and activation and involves a breakdown of the cortical alveoli releasing colloids from the cortical layer. A disfunction of this process has been observed in poor quality eggs of cod (Kjorsvik and Lonning, 1983; Kjorsvik *et al.*, 1984b) and turbot (McEvoy, 1984).

Hardening of the chorion occurs during activation through an enzymatic reaction requiring the presence of calcium ions. The relative hardness of the chorion can be measured mechanically (Davenport *et al.*, 1986; Mangor-Jensen *et al.*, 1994) and it has

been demonstrated that good quality eggs are more resistant to mechanical stress (Kjorsvik and Lonning, 1983; Kjorsvik *et al.*, 1984b; Kjorsvik *et al.*, 1990).

Bacterial contamination of halibut eggs, which may lead to a reduction in viability, has given some cause for concern (Hansen and Olafsen, 1989) and it is common practice to use surface disinfectants e.g. glutaraldehyde, particularly in Norway (Harboe *et al.*, 1994). Increased survival rates during first feeding have been attributed to such treatments however this practice is not universally adopted. An alternative and less toxic egg disinfectant, peracetic acid has been tested in the U.K. with promising results (Kristjansson, 1995).

Interest in egg quality determinants and nutritional requirements during embryonic and larval development has stimulated numerous investigations into the biochemical composition of eggs. The endogenous reserves laid down during oocyte development and vitellogenesis are of maternal origin and likely to reflect amongst other factors, the diet of the broodstock, the age of the fish and environmental conditions. Of the essential nutrients most commonly studied, lipid profiles of have been investigated in a number of marine fish including cod, herring, haddock and saithe (Tocher and Sargent, 1984) and halibut (Falk-Petersen *et al.*, 1986; Daniel *et al.*, 1993; Bruce *et al.*, 1993). Total lipid levels in halibut eggs account for between 12 to 16% of the total dry weight. There is contradictory evidence as to the relationship between lipid levels and egg viability (Kjorsvik *et al.*, 1990). Kamler *et al.* (1982) demonstrated that high lipid levels in eggs of the freshwater vendace were associated with high viability whereas the opposite was found in the eggs of turbot, sole and sea bass (Devauchelle *et al.*, 1982) and high lipid levels are often associated with overripening (Devauchelle *et al.*, 1988; Rainuzzo 1993). Total lipid levels in eggs vary from species to species and comparisons between wild and

cultured broodstocks have been made in an attempt to determine optimal levels (Komis *et al.*, 1991). Daniel *et al.* (1993) reported that egg lipid levels were similar for the wild and captive broodstock halibut they sampled. Rainuzzo (1993) proposed an EFA index as an indicator of quality in plaice eggs and in a recent study on cod (Pickova *et al.*, in press), a positive correlation between hatching rate and EPA/DHA ratio was demonstrated. Bruce *et al.* (1993) classified halibut eggs as non-viable or viable according to their symmetry, fertilisation rate and hatching success. Analysis of lipid class and fatty acid composition of the two classes revealed no differences with the exception of cholesterol which was higher in non-viable eggs.

The importance of amino acids to egg quality has received considerably less attention though it has been revealed that they are a major energy substrate for halibut eggs (Fhyn, 1989; Finn *et al.*, 1991). Egg and larval viability have been linked to amounts of free amino acids in sturgeon (Federova, 1976) and common bream (Golovanenko, 1975).

Studies on the vitamin content of eggs have concentrated on vitamins C and E. For example it is known that a deficiency of vitamin C is detrimental to hatchability in tilapia (Soliman *et al.*, 1986) and rainbow trout (Sandnes *et al.*, 1984). In sea bream, a high level of incorporation of vitamin E had positive effects on egg quality (Watanabe *et al.*, 1991).

Pigments are thought to be important constituents of some fish eggs, particularly salmonids and it is thought that carotenoids play an important role as antioxidants and as protection against U.V. radiation (Craik, 1985). A recent study on Atlantic salmon has concluded that dietary astaxanthin, an important pigment added to improve flesh colour, has no importance for egg viability (Christiansen and Torrisen, 1997). The role of pigments in marine fish however has not received attention and may not be of

importance to the survival of transparent pelagic marine eggs such as those of the halibut.

Levels of maternal hormones, important for regulation of growth, development and physiological functions may also be a determinant of egg quality. Those hormones that have been studied include thyroid (T3 & 4), corticosteroids, sex steroids, growth hormone, and prolactin (see Lam, 1994 for review). Evidence exists that levels of T3 and T4 influence survival of striped bass larvae (Brown *et al.*, 1988). Cortisol is present in newly-fertilised eggs and it has been demonstrated that the level of this hormone influences the survival of sea bass larvae (Sampath-Kamur *et al.*, 1993).

Egg quality criteria assessed by means of complicated assays or involving sophisticated laboratory procedures are of little use to a hatchery operator who needs a rapid technique with which to establish the potential of an egg batch early on in embryonic development.

A number of morphological characteristics may be of use as indicators of viability in marine fish eggs. Egg size has generally been rejected as a useful criterion following work on salmonids which showed that under favourable conditions, larvae from small eggs were as viable as those from larger ones (Springate and Bromage, 1985; Thorpe *et al.*, 1984) although the point-of-no-return may be earlier in the absence of food (Blaxter and Hempel, 1963). In general, larger eggs give rise to larger larvae and as shown for cod larvae, these larvae will have a bigger jaw gape and better swimming abilities (Knutsen and Tilseth, 1985). There is no evidence that egg diameter is of importance as a quality criterion in marine eggs (Devauchelle *et al.*, 1988; Kjorsvik *et al.*, 1990). In wolffish a high variation in egg diameter was thought to be a better indication of poor quality than average diameter *per se* (Pavlov and Moksness, 1996). In many species, egg

size fluctuates with fish age and also through the spawning season. The average diameter of eggs tends to increase with female age in salmonids (Christiansen and Torrisen, 1997). A decrease in egg diameter occurs as the spawning season progresses for halibut, cod and sole broodstock (Kjorsvik *et al.*, 1987; Baynes *et al.*, 1993; Mangor-Jensen *et al.*, 1994).

The gross appearance of halibut eggs prior to fertilisation may give an indication of ripeness and overripe batches often contain a large proportion of opaque eggs. Consistency of size and spherical shape were shown to be indicative of good egg batches for halibut (Basavaraja, 1991) and chorion appearance is believed to be of use in gauging ripeness and is routinely observed by some broodstock managers (P. Smith pers. comm.). Sakai *et al.* (1985) described a wrinkling of the chorion of overripe red sea bream eggs.

In all animal eggs, fertilisation is followed by the process of cleavage, a rapid series of mitotic cell divisions that partition the cytoplasm into numerous cells called blastomeres. In contrast to most mitotic divisions, there is no cytoplasmic growth between cycles, consequently the blastomeres decrease in size with each successive division. In fish eggs, the large yolk volume restricts cleavage to a small area of cytoplasm at the animal pole. This is referred to as a discoidal meroblastic cleavage pattern (Gilbert, 1994). Owing to their peripheral displacement, the large early blastomeres are easily visible in non-pigmented eggs. For most fish species "normal" early blastomeres are regular in size and shape. Routine observations of halibut eggs have revealed that the normal morphology is often disrupted. Aberrations generally take the form of irregular spatial interactions (asymmetry) or cleavage anomalies; unequal sizes of sister cells, incomplete cell margins, vesicles or blisters within, between or on the surface of blastomeres or incorrect cell numbers. The morphology of the blastomeres has been studied in a number of species

and gross abnormalities have been linked to poor viability (Kjorsvik *et al.*, 1990; Bromage *et al.*, 1994).

Abnormal fish blastomeres have been recorded in a range of studies, mostly concerning the effects of pollutants on egg development. Toxicology studies have demonstrated that very abnormal eggs, including blastula-stage embryos, do not complete embryogenesis (Westernhagen, 1988) and it has been suggested that abnormal blastomeres may generally correspond to low egg viability and therefore provide a useful predictive tool for egg assessment.

Limited data, generally of a qualitative nature, has been published linking the survival rate of fish eggs to embryo morphology and the embryonic abnormalities under investigation are usually not closely defined. At Ardtoe, blastomere observations are routinely made for Atlantic halibut eggs, but it has not been possible using production incubators, to adequately test the relationship between morphology and egg survival. Earlier work had demonstrated the feasibility of incubating halibut eggs to hatching in separate wells of microtitre plates (Brown, 1994). This enabled close observation and classification of individual eggs whose performance to hatching could be assessed. This study showed that there was a relationship between blastomere symmetry and egg viability. It was felt that this technique could be refined, and that a range of abnormalities which are observed in early halibut embryos could be further defined. A study was therefore undertaken to measure the survival of individual halibut eggs in relation to specific blastomere characteristics. This chapter describes a set of experiments designed to test this relationship. Blastomere morphology is discussed as a tool for egg quality assessment and the possible causes of these abnormalities are considered.

3.2. Material and Methods

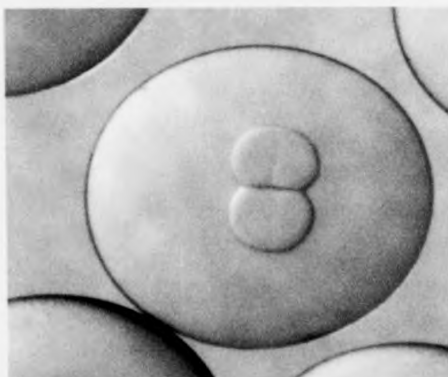
3.2.1. Egg handling

Samples from single batches of stripped, wet fertilised halibut eggs were collected from incubators (described in Section 2.3.1) at approximately 96 hours post-fertilisation, once they had reached the 8-cell stage (Stage IIc according to Ahlstrom, 1943). The samples were taken from the surface of the incubation water so excluding any eggs that might have sunk to the bottom of the conical since fertilisation. Each egg sample was viewed in a petri dish using a binocular microscope (SZ60, Olympus, Tokyo, Japan) located in a temperature controlled room at 6°C. Fertilised eggs were randomly stocked by pipetting into the wells of a 96 well microtitre plate (Greiner, No. 655180), one egg per well containing 200 µl of 5 µm filtered, u.v. sterilised seawater.

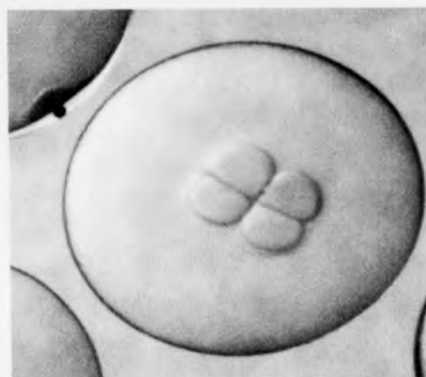
3.2.2. Egg assessment

Each egg was observed at high magnification and low light intensity and given a score from 1 ("very abnormal") to 4 ("normal") for each of the parameters listed in Table 3.1. Photomicrographs illustrating the normal appearance of halibut eggs during the first 3 divisions are shown in Figure 3.1. Examples of eggs exhibiting the range of abnormal characteristics are presented in Figure 3.2.A-E. The photomicrographs are arranged in order of severity of abnormality to give an indication of how the scoring system relates to morphology. They are shown at high magnification to enhance some of the features, particularly cell adhesion and inclusions.

2 cell
7 hours



4 cell
11 hours



8 cell
16 hours

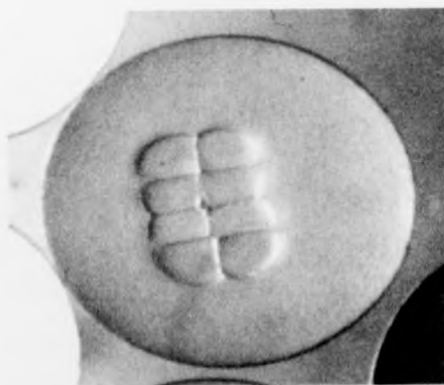


Figure 3.1. Photomicrographs of Atlantic halibut eggs at 2, 4 and 8 cell stages showing normal cleavage pattern. Approximate time from fertilisation at 6 °C to reach each stage is given. (Magnification x17).

A) Symmetry

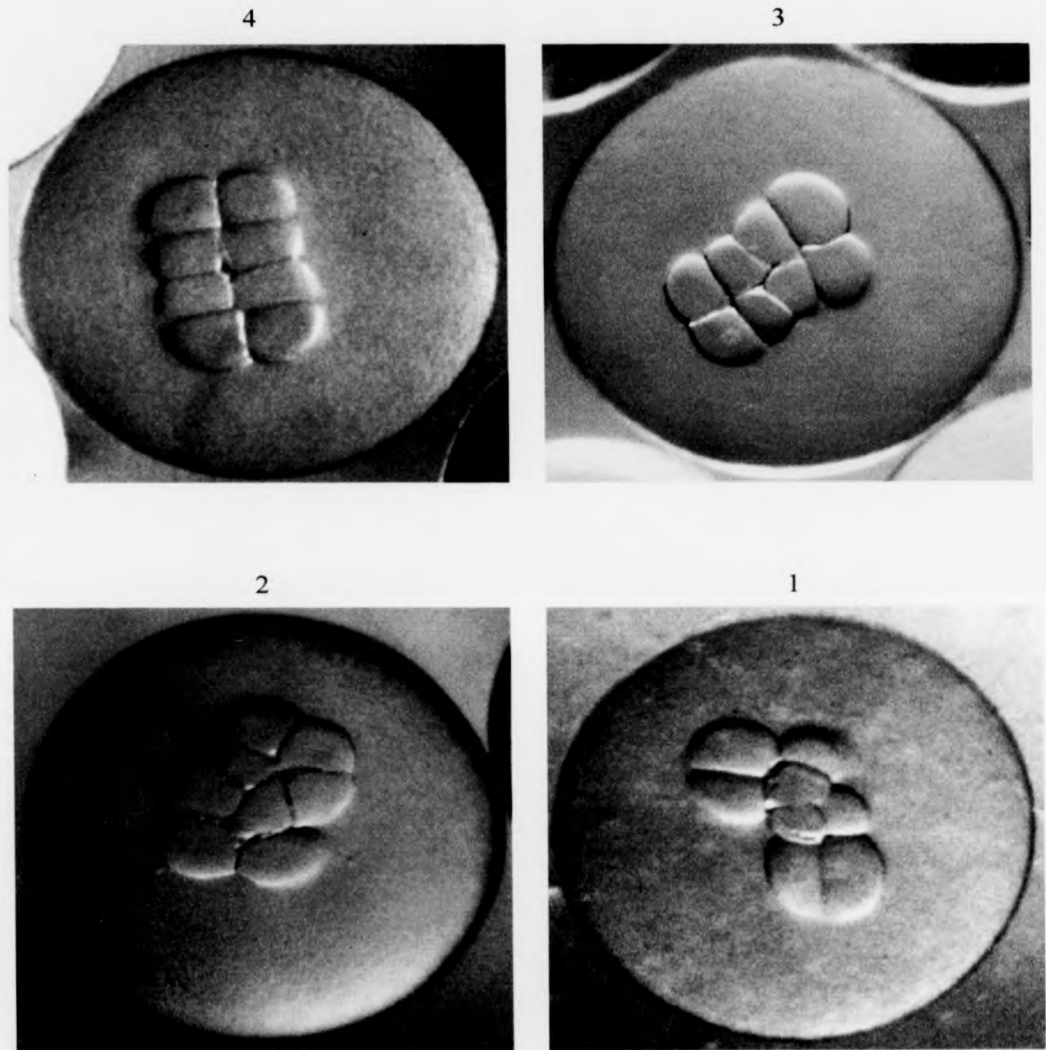


Figure 3.2.A. Photomicrographs of Atlantic halibut eggs at 8-cell stage illustrating examples of blastomere abnormalities; Category: Symmetry. Images are arranged in order of increasing severity from 4 (normal) to 1 (severely abnormal) to illustrate the scoring system. Magnification x23.

B) Cell size

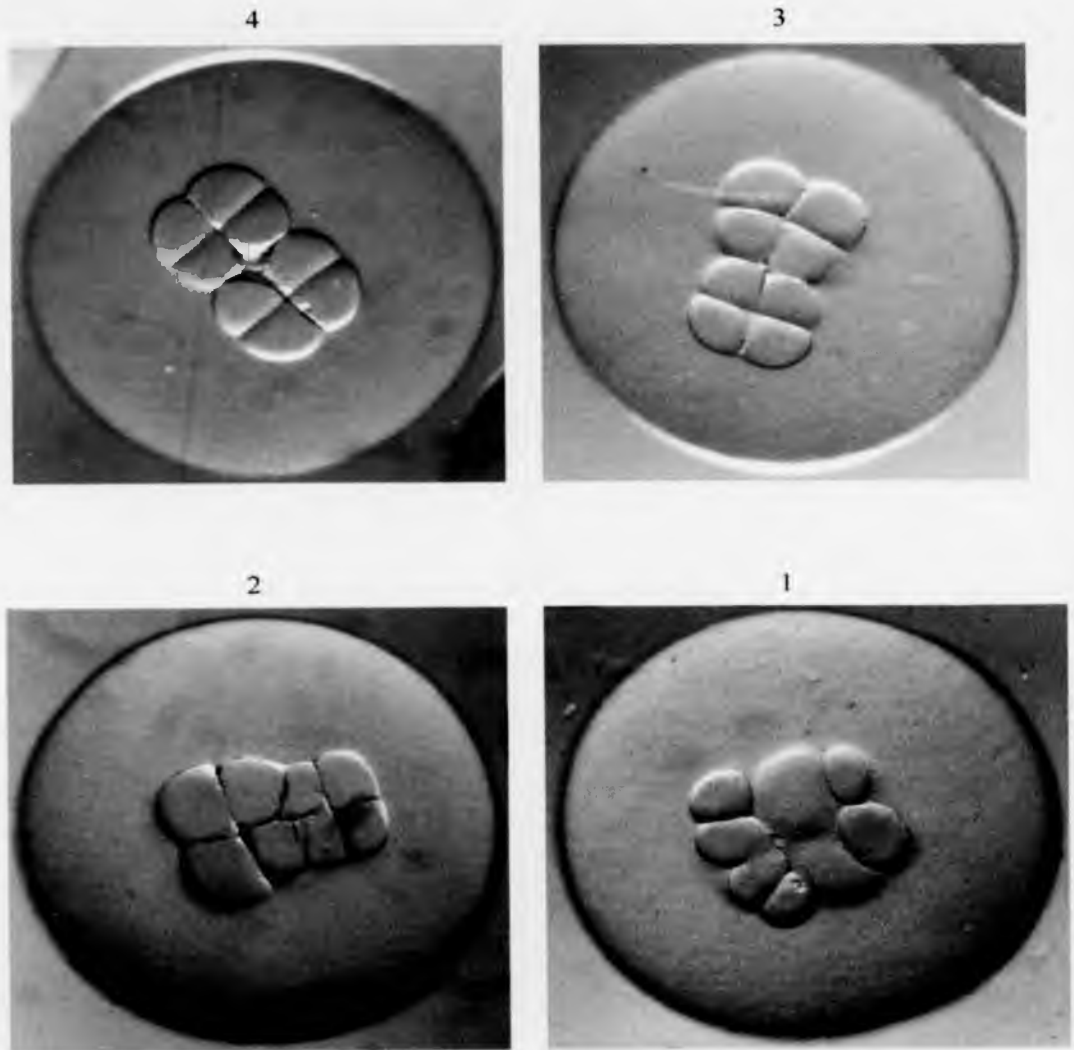


Figure 3.2.B. Photomicrographs of Atlantic halibut eggs at 8-cell stage illustrating examples of blastomere abnormalities; Category: Cell size. Images are arranged in order of increasing severity from 4 (normal) to 1 (severely abnormal) to illustrate the scoring system. Magnification x23.

C) Adhesion

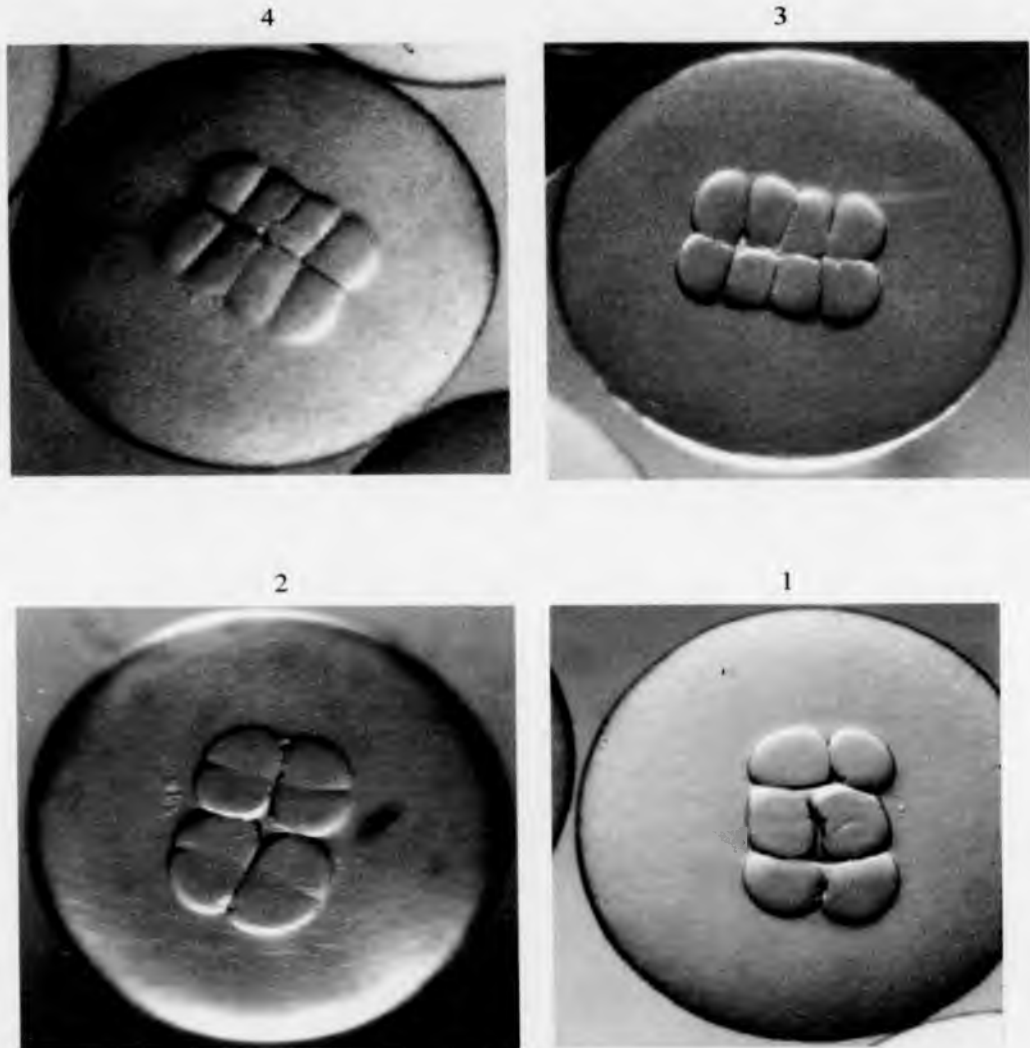


Figure 3.2.C. Photomicrographs of Atlantic halibut eggs at 8-cell stage illustrating examples of blastomere abnormalities; Category: Adhesion. Images are arranged in order of increasing severity from 4 (normal) to 1 (severely abnormal) to illustrate the scoring system. Magnification x23.

D) Margins

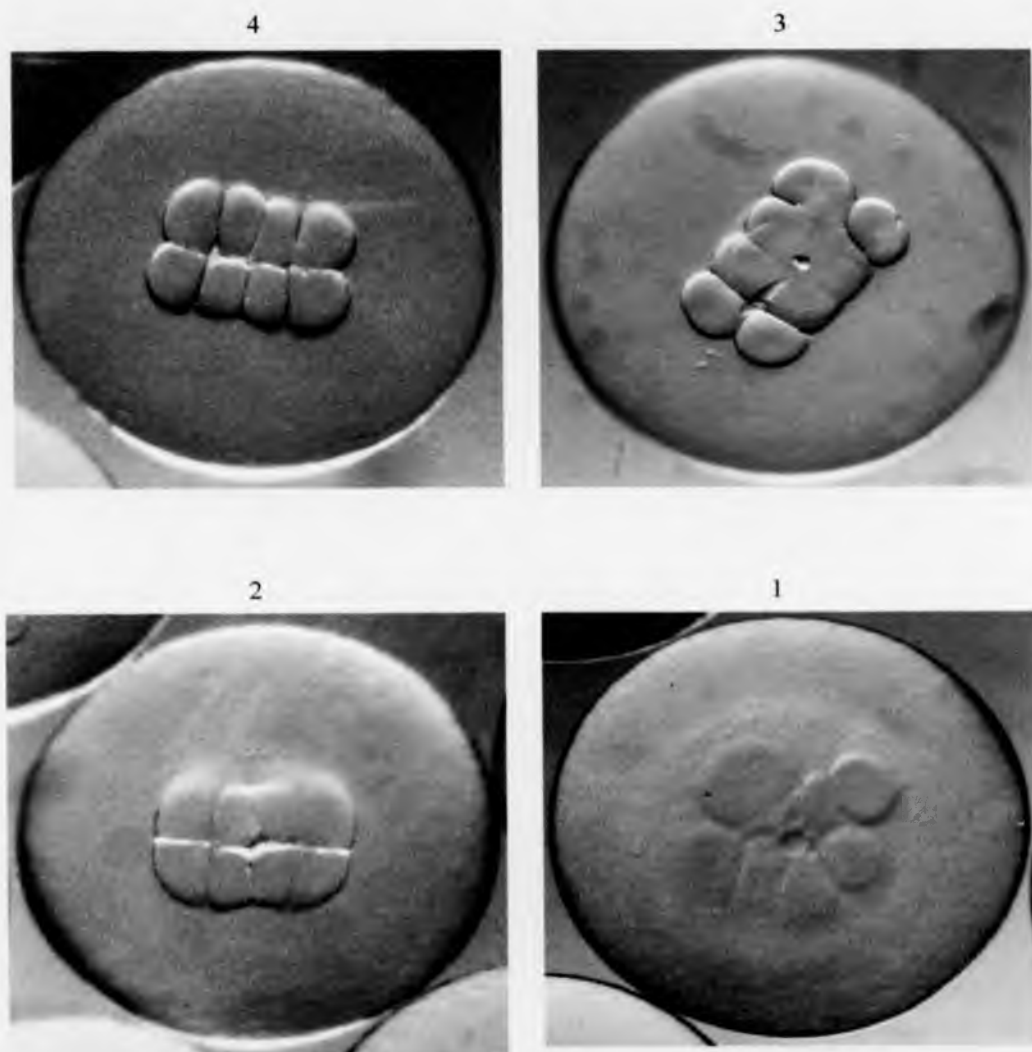


Figure 3.2.D. Photomicrographs of Atlantic halibut eggs at 8-cell stage illustrating examples of blastomere abnormalities; Category: Margins. Images are arranged in order of increasing severity from 4 (normal) to 1 (severely abnormal) to illustrate the scoring system. Magnification x23.

E) Inclusions

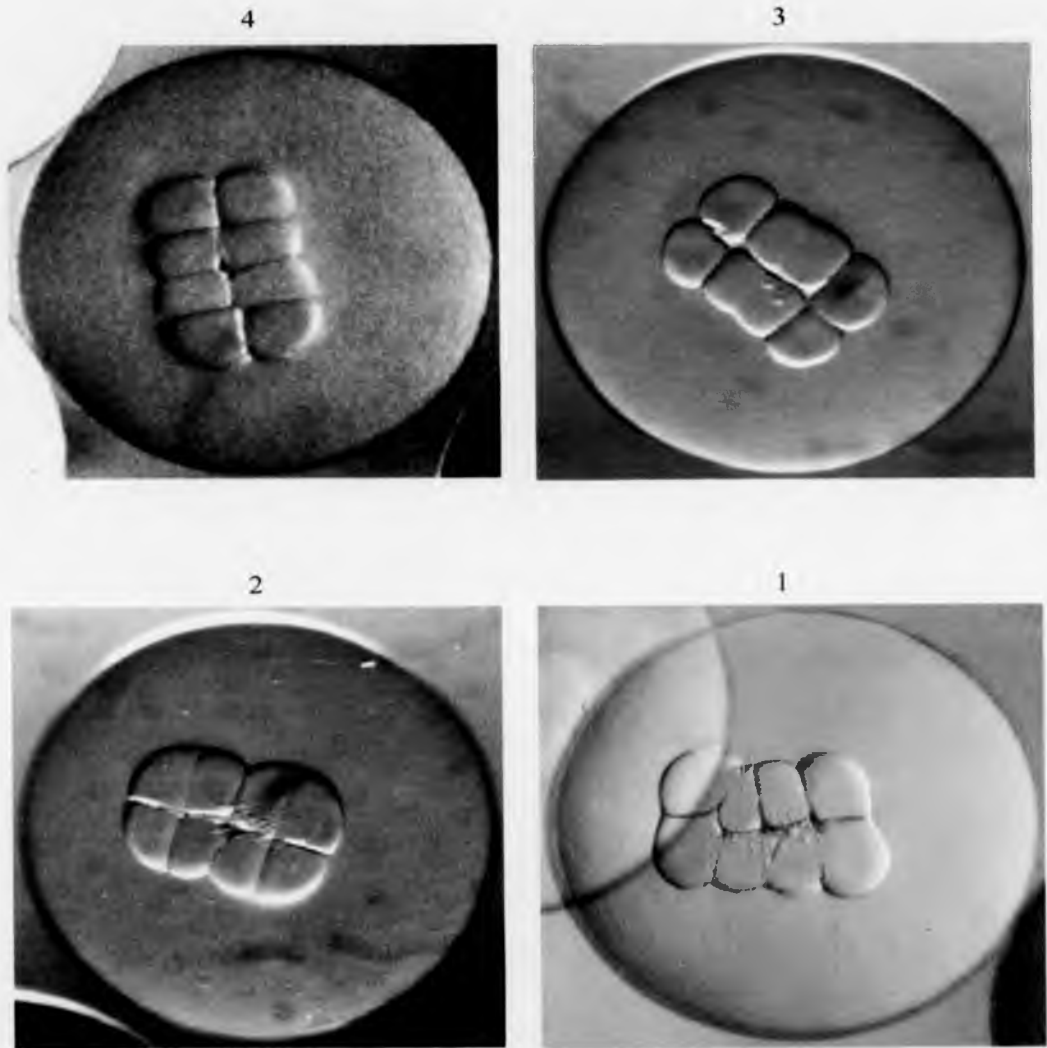


Figure 3.2.E. Photomicrographs of Atlantic halibut eggs at 8-cell stage illustrating examples of blastomere abnormalities; Category: Inclusions. Images are arranged in order of increasing severity from 4 (normal) to 1 (severely abnormal) to illustrate the scoring system. Magnification x23.

Table 3.1. List and description of the blastomere characteristics assessed for halibut eggs at the 8 cell stage.

<i>Term</i>	<i>Description</i>
Symmetry	Bilateral symmetry about the axes of the 8 blastomeres
Cell size	Uniformity of cell size
Adhesion	Intimacy of contact between adjacent blastomeres
Margins	Distinctiveness or completeness of cell margins
Inclusions	Vacuoles or vesicles within or between blastomeres

Once each egg batch had been assessed the microtitre plate was sealed with tape and incubated in darkness at 6°C. At 75 - 85 °Cdays post-fertilisation when hatching was complete, each egg was examined and recorded as hatched or dead. Twenty-one egg batches from 14 females were treated in this way.

The following data were collated from each egg batch: fertilisation rate as a proportion of eggs floating at 16 hours post-fertilisation, assessed from 3 samples of at least 100 eggs; hatch rate as a proportion of fertilised eggs; mean score for each blastomere characteristic for fertilised eggs. For 27 batches also incubated in microtitre plates, the proportion of sinking eggs was assessed from a 2.5ml sample just post fertilisation placed in a beaker containing seawater at a salinity adjusted to separate viable and non-viable eggs.

3.2.3. Statistical analysis

Linear regression of percentage hatch on fertilisation and buoyancy rates (all arcsine transformed) was performed for data pooled from all batches. Linear regression was also

used to test the relationship between percentage hatch and mean score for each of the blastomere parameters (independent variables). Using multiple regression a number of best subsets of these independent variables were tested in combination in order to find the optimum model. All analyses were carried out using the MINITAB statistical computer package.

3.3. Results

There was considerable variation between batches of eggs in terms of fertilisation rate, hatch rate and morphological characteristics. Eggs appeared to hatch normally in the microtitre plates and a photograph of such a larvae hatched *in situ*, is shown in Figure 3.3. Hatching rates of fertilised eggs correlated well with the proportion of buoyant eggs measured post fertilisation and with the fertilisation rate. There were significant positive correlations ($p < 0.005$) for both relationships (see Figure 3.4.).

The relationships between the severity of blastomere abnormalities and hatch rates were even stronger. The matrix of correlation coefficients is given in Table 3.2.

Table 3.2. Correlation coefficients (r) between hatch rate (arcsine %) and mean scores for each of the blastomere characteristics.

	Hatch Rate (sine -1)	Mean Symmetry Score	Mean Cell Size Score	Mean Cell Adhesion Score
Mean Symmetry Score	0.815			
Mean Cell Size Score	0.830	0.946		
Mean Cell Adhesion Score	0.786	0.649	0.719	
Mean Margins Score	0.835	0.813	0.861	0.755

Figure 3.3. Photomicrographs of Atlantic halibut embryos and larvae incubated in microtitre plates;

A) Live egg in microtitre well at morular stage showing disintegration of blastodisc. Magnification $\times 17$.

B and C) Live embryos in microtitre wells in developmental arrest, post gastrulation, showing poor differentiation. Magnification $\times 15$.

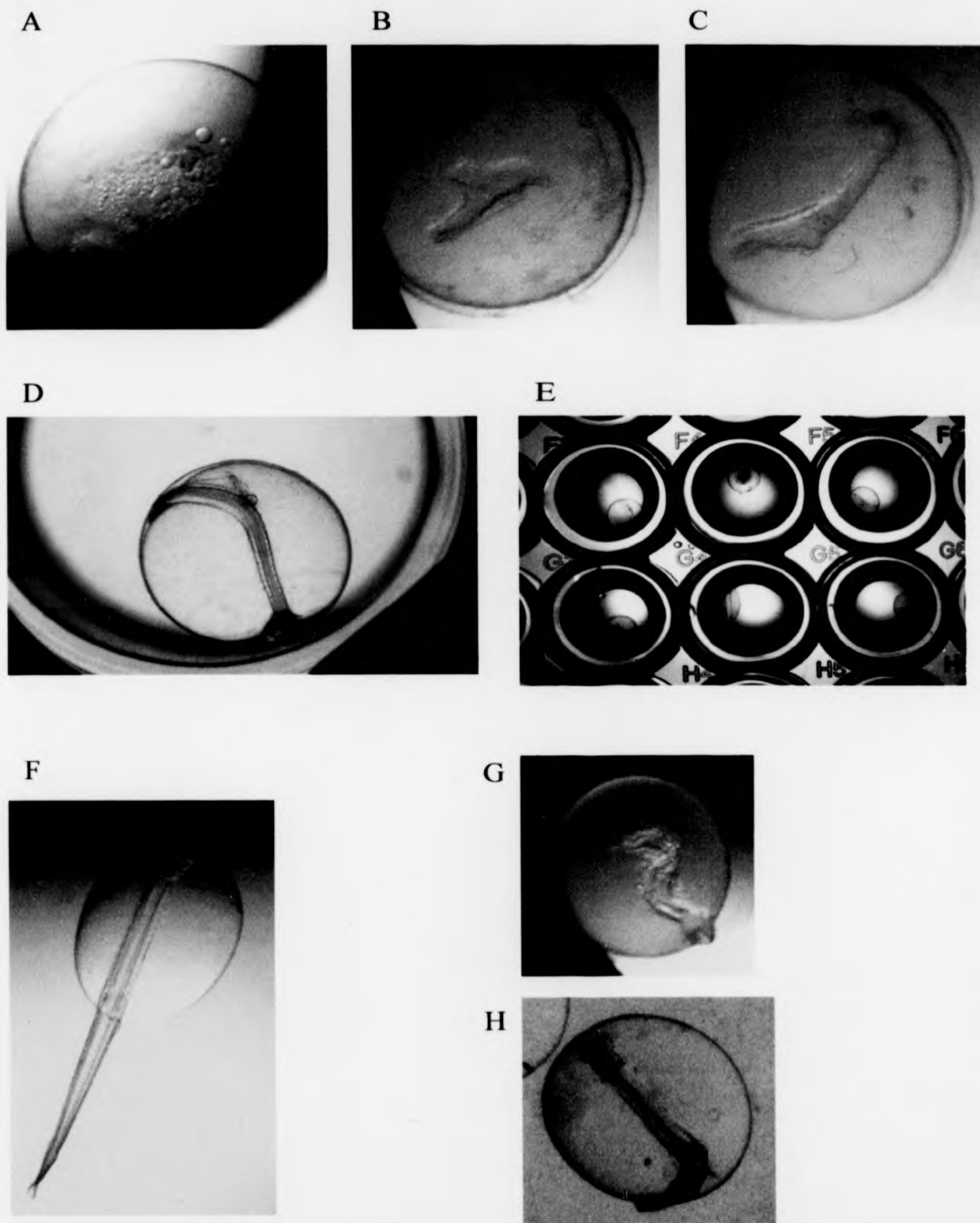
D) Normal live embryo in microtitre well just pre-hatch. Hatching ring can be seen. Magnification $\times 11$.

E) Dead eggs and hatched larvae in microtitre wells. Magnification $\times 1.7$.

F) Live normal hatched larvae removed from microtitre well. Magnification $\times 10$.

G) Live hatched larvae removed from microtitre well showing serious deformity of abdomen and tail region. Magnification $\times 10$.

H) Live hatched larvae removed from microtitre well showing bent, foreshortened tail. Magnification $\times 11$.



A)

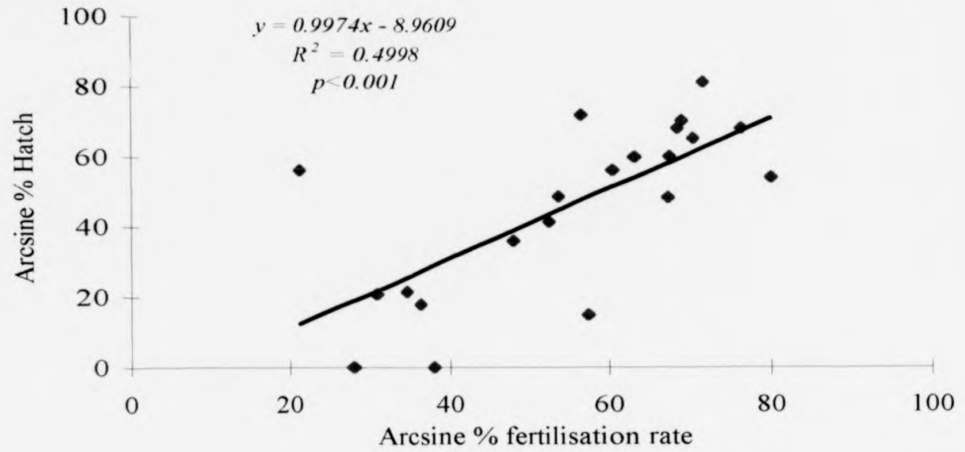


Figure 3.4.A. Hatch rate (as % of fertilised eggs) in microtitre plates plotted against fertilisation rate (as % of floating eggs at 16 hrs post fertilisation). Values are arcsine transformed. Regression equation and R^2 value are given.

B)

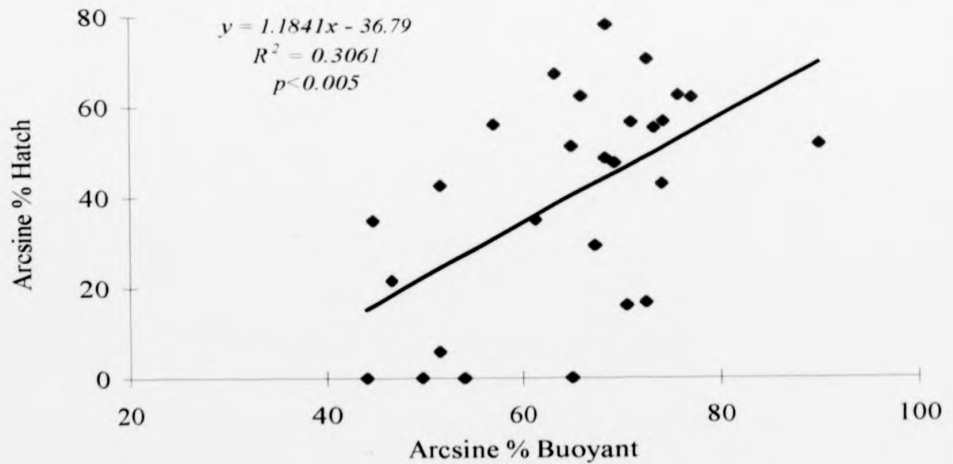


Figure 3.4.B. Hatch rate (as % of fertilised eggs) plotted against buoyancy rate (as % of stripped eggs). Values are arcsine transformed. Regression equation and R^2 value are given.

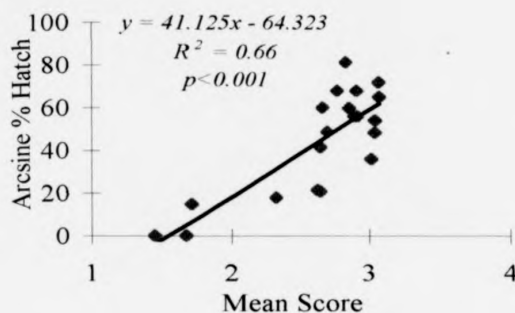
Figure 3.5.A-F. illustrates scatter plots with fitted regression lines for percentage of eggs hatched (arcsine transformed) versus each of the 5 indices of blastomere characteristics and on total mean score. Each blastomere characteristic and total score exhibited a significant positive correlation with hatch rate ($p < 0.001$).

The vast majority of hatched larvae appeared normal. There were however some abnormalities noted including bent or foreshortened tails (see Figure 3.3.). The incidence of these deformities was generally higher in batches with poorer hatch rates. Abnormal eggs often continued to divide until developmental arrest. Whilst still alive, these embryos exhibited a variety of abnormalities such as poor differentiation, loose cell aggregates and structural deformities. Some examples are shown in Figure 3.3.

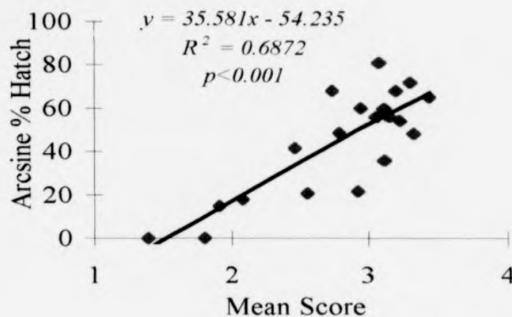
Table 3.3. presents the best subset models of the multiple regression using the maximum r^2 criterion (Sokal and Rohlf, 1981). Though all five models showed significant F values, t tests on each of the partial regression coefficients revealed that when more than 2 variables are considered, their partial regression coefficients were not significant (i.e. $H_0: \beta_i = 0$ is not rejected). There was a degree of correlation between the independent variables as indicated by the matrix of correlation coefficients (Table 3.2.) but these were not high enough to affect the accuracy of the models as indicated by the acceptably low variance inflation factors (VIFs).

A test was performed to assess the significance of the increased increment of determination of hatch rate due to the addition of additional variables over and above the single best variable (margins score). This showed that adding more than one variable to the regression equation does not add significant further explanation of the variation in hatch rate. Thus, although models II to V account for successively greater proportions

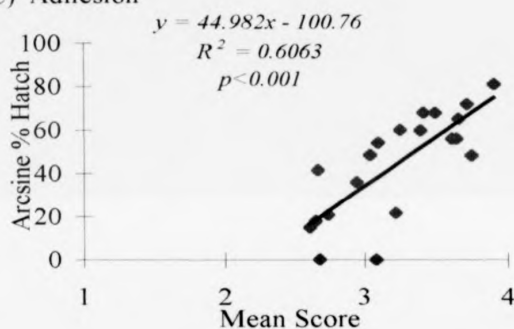
A) Symmetry



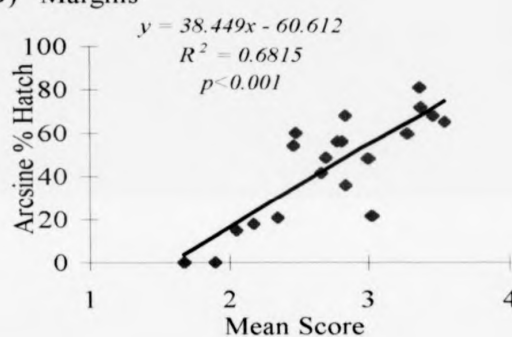
B) Uniformity



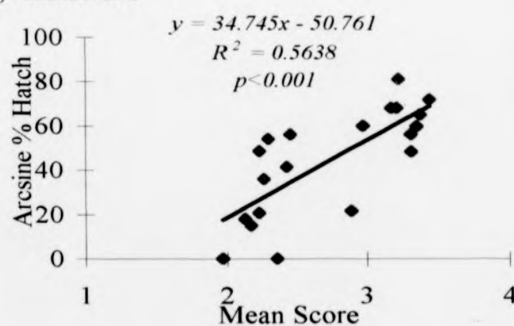
C) Adhesion



D) Margins



E) Inclusions



F) Total Score

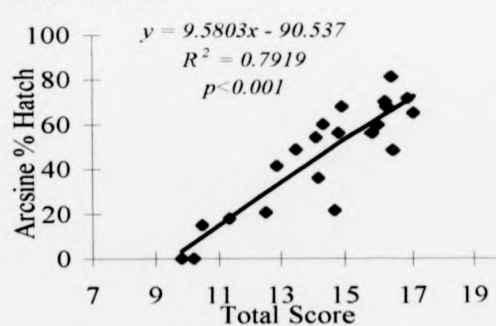


Figure 3.5. A-E: Hatch rate (as % of fertilised eggs) plotted against mean score for each of the blastomere indices for all batches. F: Hatch rate against total mean score (sum of all indices). Values are arcsine transformed and based on 96 eggs per batch. Regression equation and R^2 values are given.

of the variance in hatch, the fits are not significantly better than for model I, hatch on margins score. From this it can be concluded that although Model II is the most effective model for predicting hatch rate but Model I is sufficient. No more than two variables are needed for the regression model.

Table 3.3. Best subset models of multiple regression analysis: percentage of eggs hatched (arcsine transformed) on mean scores of 1 to 5 blastomere characteristics, using the maximum r^2 criterion. Statistics include F value and multiple regression coefficient (r^2), partial regression coefficient β , t values, variance inflation factor (VIF) and probability values for each partial regression coefficient.

<i>Model</i>	Independent variable	β	<i>t</i>	VIF	<i>P</i>
Model I Hatch on best single variable.					
Intercept = - 59 <i>F</i> = 43.74 <i>P</i> < 0.001 r^2 = 69.7%					
	Margins score	37.79	6.61		<0.001
Model II Hatch on best two variables.					
Intercept = - 111 <i>F</i> = 31.65 <i>P</i> = <0.001 r^2 = 75.4%					
	Adhesion score	26.03	3.05	1.7	0.007
	Symmetry score	27.03	3.62	1.7	0.002
Model III Hatch on best three variables.					
Intercept = - 93.8 <i>F</i> = 17.31 <i>P</i> = <0.001 r^2 = 77.9%					
	Inclusions score	-0.52	-0.04	4.6	0.967
	Margins score	25.58	2.81	2.8	0.012
	Adhesion score	21.59	1.51	4.1	0.149
Model IV Hatch on best four variables.					
Intercept = -102.0 <i>F</i> = 16.02 <i>P</i> = <0.001 r^2 = 80.0%					
	Inclusions score	3.93	0.34	4.8	0.738
	Margins score	11.24	1.00	4.9	0.332
	Adhesion score	16.40	1.21	4.3	0.243
	Symmetry score	19.56	1.94	3.1	0.071
Model V Hatch on all five variables.					
Intercept = - 102.0 <i>F</i> = 12.02 <i>P</i> = < 0.001 r^2 = 80.0%					
	Inclusions score	3.93	0.33	4.8	0.746
	Margins score	11.47	0.94	5.5	0.362
	Adhesion score	16.55	1.17	4.4	0.261
	Symmetry score	20.5	1.11	9.8	0.286
	Cell size score	-1.12	-0.06	13.1	0.952

3.4. Discussion

This study has characterised some of the abnormalities which can be observed in the morphology of blastomeres of eggs derived from captive halibut broodstock. The results of the incubation experiments have confirmed that the degree of severity of these abnormalities can be used as an estimator of egg viability to hatching. It is not suggested that the entire procedure is used by the hatchery operator as it is extremely time consuming. There are however, certain morphological features which have been documented and illustrated, which are symptomatic of poor egg quality and which can be readily and rapidly observed at the same time as the measurement of fertilisation rates.

Multiple regression analysis revealed that although including more than two of the variables resulted in higher r^2 values, the t values of the partial regression coefficients were not significant. On this basis, the extra variables must be discarded and it must be concluded that model II is the best one. On analysing all the subsets, the difference in r^2 values between each combination of 2 variables was marginal. In practice, since all characteristics are indicative of egg viability, it is not recommended that one should only use observations of cell adhesion and symmetry (as in model II) in isolation to assess egg quality.

The data show that both egg buoyancy and fertilisation rate are correlated with viability although the former is a relatively weak relationship. Neither of these factors are as strongly correlated with survival to hatching as any of the blastomere indices but could be used in combination with observations on morphology. A batch with low fertilisation rate, say less than 30%, would probably be discarded, especially if those fertilised eggs were highly negatively buoyant. Using blastomere observations, combined

with batch size and fertilisation rate, potential numbers of viable larvae might be predicted with a degree of confidence. Decisions as to whether to stock or discard borderline egg batches with moderate fertilisation rates would certainly be better informed in the light of blastomere morphology assessment.

Assessment of cleavage patterns is best done at the 8-cell stage. Before or after the third cleavage, spatial changes and deviations from the normal cleavage pattern are more difficult to discern. In general, it was found easier to assign scores for certain characteristics, especially the cell size and cell margins categories. The five blastomere characteristics analysed: symmetry, cell size, adhesion, margins and inclusions, were sometimes strongly associated with each other. Thus, egg batches exhibiting poor survival tended to have low scores for more than one of the characteristics and *vice versa*. For certain morphological characteristics, an association was expected, for example disparity in blastomere size can disrupt the symmetry of cleavage. It is possible that indistinct margins may indicate weak or incomplete cell membranes and leakage of cytoplasm might result. This may manifest itself as the vacuoles or vesicles associated with the blastodisc.

The use of microtitre plates for small scale, repeatable incubation experiments provided a useful way of following egg batches through till hatching without employing tank facilities and ancillaries. The hatch rates achieved in these plates were remarkably high for some batches and indeed, often out-performed the hatchery egg incubation system when batch for batch comparisons were made. This suggests that normal respiration was not impaired and that levels of metabolic wastes in the culture water were not critical. According to Finn *et al.* (1991), the respiration rate of a halibut egg increases during incubation to a maximum rate of oxygen consumption of 220 nl/egg/hr

and ammonia excretion of 1.5 nmoles/egg/hr.

Grossly abnormal eggs were often observed to continue surviving and dividing during incubation. The resulting embryos usually showed signs of poor differentiation and tissue organisation and persisted until developmental arrest and eventually death. Many died at some point during gastrulation and few survived beyond blastopore closure. Abnormalities at hatch were relatively rare but were more likely to occur in poor batches. Such larvae, exhibiting malformations such as bent or foreshortened tails and deformities of the head region, were certainly not viable. The fate of halibut larvae hatched from eggs incubated in microtitre plates has never been followed owing to the difficulties associated with maintaining good water quality in microtitre wells for individual larvae.

In most species studied, division of the blastomeres occurs symmetrically and uniformly and this pattern is thus considered normal. However, this is not the case for all species, for example eggs of the wolffish, have unequal blastomere sizes during early divisions (Pavlov *et al.*, 1992).

Previous studies investigating the relationship between fish egg viability and embryo morphology have recorded survival rate in relation to the estimated proportion of abnormal embryos in an egg batch (Lincoln, 1976; Westernhagen *et al.*, 1988; Pavlov and Moksness, 1994; Kjørsvik, 1994). Generally, a positive relationship is observed between percent survival and the proportion of 'normal' embryos, but statistical analysis has not always been applied. The present work sought to overcome these limitations by scoring individual Atlantic halibut eggs according to blastomere morphology and recording the hatch success of the same individual eggs.

Literature referring to abnormal fish blastomeres broadly concerns either egg

sensitivity to environmental variables (physical and chemical) or "egg quality" studies, where gamete viability is measured from known broodstock. Most of the work falling in the first category concerns the effects of pollutants, applied either directly to laboratory-incubated eggs, or measured at sea in association with abnormal embryos from plankton collections. For plankton-caught eggs the embryonic stage at which abnormalities have been recorded is variable and has not always been reported. Thus, Yannopoulos and Yannopoulos (1981) reported unspecified embryo malformations in the sardine and the European anchovy from the Mediterranean, while abnormalities in Baltic cod and sprat have been recorded during late embryonic development (Grauman and Sukhorukova, 1982). More comprehensive studies have partitioned egg samples according to their developmental stage (Westernhagen *et al.*, 1988; Cameron and Berg, 1992; Cameron *et al.*, 1992; Longwell *et al.*, 1992). Marked geographic and species-specific differences have been found in the incidence of abnormalities. In some cases embryonic deformities have been statistically associated with measured pollutant concentrations (Longwell *et al.*, 1992), but more often abnormal eggs are seen to predominate in locations of known high pollutant loading (for example Westernhagen, 1988).

In their studies on developmental defects in planktonic flatfish eggs, Cameron *et al.* (1992) and Cameron and Berg (1992) observed a variety of abnormalities in early embryos. These included "incomplete and irregular cleavage, abnormal shaped blastodiscs, wrinkles and blisters on the inside and surface of cells and loose cell aggregates". At more advanced stages of development, "blister like outcrops from the body axis, bent notochords, twisted tails, head and eye deformities and abnormal pigmentation" were also noted. The prevalence of these defective embryos reached high levels, for example up to 50% deformities among flounder eggs in the North Sea

(Cameron and Berg, 1992). The clustered distribution was associated with high pollution loads of heavy metals and chlorinated hydrocarbons along the coast, particularly near estuaries of major rivers. They speculated that these toxins may be either accumulated in the ovary or might directly affect the free embryos.

A common feature observed in field studies is a decrease in the incidence of abnormal eggs with increasing developmental stage. This is thought to indicate selectively high mortality among abnormal early embryonic stages (Westernhagen, 1988). Apparently, only Westernhagen *et al.* (1988) have reported direct measurements of survival in relation to morphological abnormalities in wild caught eggs. In their study, the deformities observed were similar to those reported by Cameron *et al.* (1992) and prevalence of abnormalities of up to 28, 32 and 44% were recorded for plaice, cod and flounder respectively. The proportion of normal embryos of cod and plaice obtained from different plankton hauls and incubated in the laboratory showed a significant positive correlation with hatch (Figure 3.6.A.).

Other studies on cytological defects in early fish embryos include observations of abnormal chromosome behaviour (Longwell, 1977; Longwell and Hughes, 1980; Longwell, 1982; Kjorsvik *et al.*, 1984b; Dethlefsen *et al.*, 1987; Longwell, 1988; Longwell *et al.*, 1992) such as delayed anaphases, anaphase bridges, failure of chromosomes to orient on the spindle and mitotic arrest at anaphase. In most cases this method has not been deliberately applied to evaluating egg viability in relation to deformities, however statistical associations have been found between pollutant concentrations, abnormality rates and egg mortality for Atlantic mackerel, *Scomber scombrus* (Longwell, 1988; Longwell *et al.*, 1992). Experimental exposure of eggs to aromatic hydrocarbons such as benzene and xylene is effective in inducing irregularities

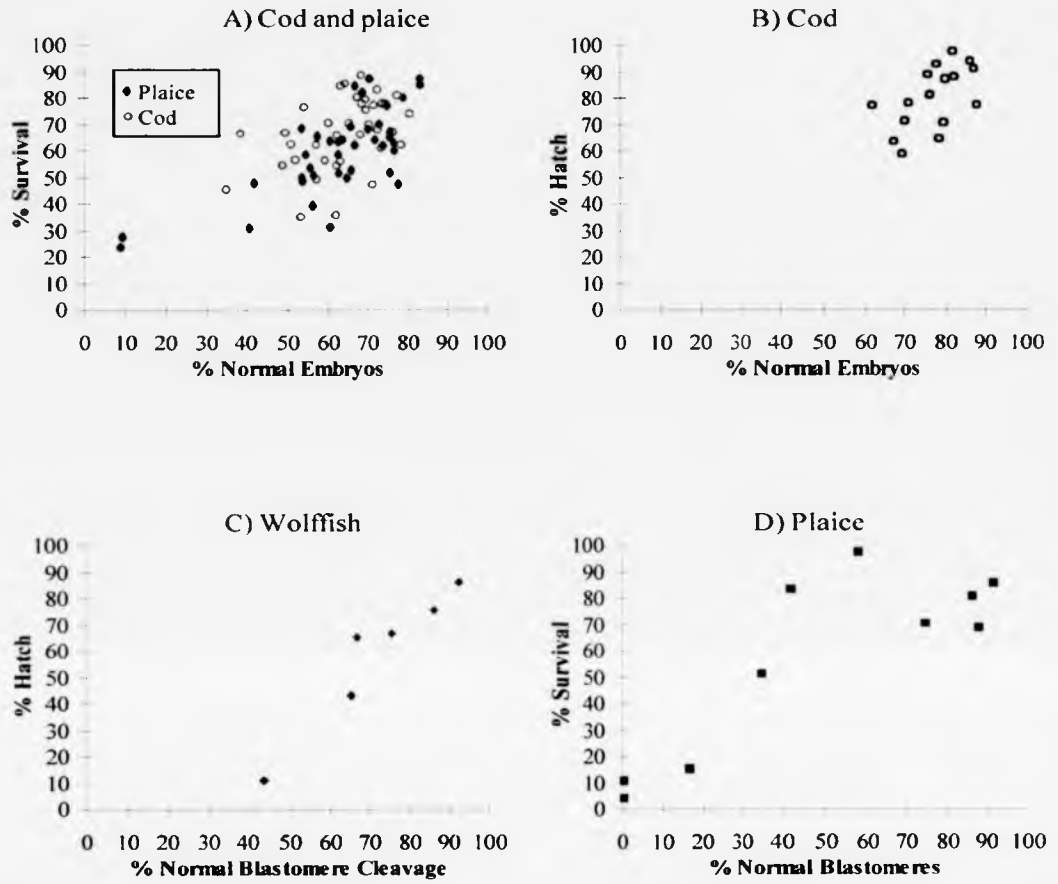


Figure 3.6. Survival rate of eggs in relation to proportion of normal embryos for A) Wild caught cod and plaice eggs incubated in the laboratory (n=38 batches), redrawn from Westernhagen *et al.* (1988); B) Broodstock derived cod eggs (n=16 batches), redrawn from Kjorsvik (1994); C) Broodstock derived wolffish eggs (n=6 batches), redrawn from Pavlov and Moksness (1994) and D) Broodstock derived plaice eggs (n=10 batches), redrawn from Lincoln (1976).

in early cleavage of plaice (Lønning, 1977) and cod (Kjørsvik *et al.*, 1982). Similar effects on cod eggs were achieved with treatments of DDT and DDE (Dethlefsen, 1977). Relatively few publications have discussed the blastomere characteristics of fish eggs obtained from broodstocks. The work includes studies of post-ovulatory ageing effects, fertilisation practices and direct measures of egg viability from wild-caught adults. In recent studies on wild cod broodstocks from Baltic and Skagerrak origin (Vallin and Nissling, 1994; Pickova *et al.*, in press) fish were spawned in captivity and observations made of blastomeres of the resulting eggs. Both studies revealed a relationship between the rate of symmetrical cleavage and hatch with the symmetrical eggs showing around 30% greater hatch rates than the asymmetrical eggs in the former study. The latter study showed that eggs from the Skagerrak stock were generally of better quality than the Baltic stock. In work on developing the techniques for cultivation of wolffish, much attention has been focused on the morphology of the very large eggs, since the egg incubation phase dominates the hatchery cycle (Pavlov *et al.*, 1992). The eggs are fertilised internally and if they are not released before the first cleavage, subsequent divisions are typically abnormal and viability low (Pavlov, 1994). Features which can be observed and indicate potential viability are; the completeness of the cortical reaction, incomplete or irregular cleavage, indistinct cell borders, spaces within the blastodisc, yolk constriction and damaged yolk membranes (Pavlov and Moksness, 1994). In an investigation on gynogenesis and triploidy of plaice×flounder hybrids using cold shock to inhibit meiosis II, Lincoln (1976) found that the success of these treatments depended on egg quality. Poor egg viability was evidenced by high rates of abnormal cleavage patterns during the first divisions.

Figures 3.6. A-D illustrate some of the aforementioned data where a positive

relationship between percentage normal embryos and egg survival rate were recorded for plaice, wolffish and cod. Wolffish and cod eggs were incubated to hatch (Pavlov & Moksness, 1994; Kjærsvik, 1994), whereas Lincoln (1976) recorded survival to post-gastrulation in plaice eggs.

A deviation in cell numbers is a relatively rare abnormality in early halibut embryos and hence this characteristic was not included in the analysis. The causes of this phenomenon can only be speculated on. Possible causes include fertilisation anomalies (e.g. failure of polar body extrusion or polyspermy) or unusual chromosome numbers. Work on *in vitro* fertilisation has revealed similar defects in human embryos. For example, Kola *et al.* (1987) observed a division of the initial cell into 3 cells mediated by a tripolar spindle. Uneven cleavage patterns, cytoplasmic fragmentation and degenerate cells are common features of pre-implantation human embryos. Hardy *et al.* (1993) found a relationship between defects in cytokinesis occurring at the third cleavage, including cleavage arrest and uneven cell size, and abnormalities in cell nuclei distribution e.g. incidence of anucleate or binucleate blastomeres or those with fragmented nuclei.

When compared to other criteria commonly employed as indicators of viability, such as fertilisation rate and buoyancy, blastomere assessment provides more information on the inherent viability of a batch. The abnormalities are probably manifestations of disruptions at a cellular level and closer investigation into the fundamental causes was beyond the scope of this study. It is nevertheless worth considering what might be the cause of these aberrations, how far back in the life cycle these causes may lie and what factors might be better optimised to improve egg quality and supply.

The fertilised egg derives all the material necessary for biosynthesis and metabolism

from reserves acquired during vitellogenesis and oocyte development. The inherent quality of the egg is therefore likely to reflect the biochemical make-up, which in turn will almost certainly be affected by the nutritional and physiological status of the broodstock.

The nutritional requirements of mature fish maintained for seed production have proved more difficult to fulfil using artificial diets than those necessary to ensure rapid somatic growth of juvenile fish grown to market size. As a result, broodstock managers have historically been reluctant to switch from wet fish diets to dry formulations. Due to the desire to use dry diets for reasons of convenience, reliability of supply and quality and the perceived risk of introducing disease via wet fish diets, broodstock nutrition is receiving considerable attention. Several broodstock and egg quality studies have demonstrated that manipulation of various dietary components can have a direct effect on egg viability (for reviews of earlier work see Watanabe, 1985; Kjorsvik *et al.*, 1990). As for other stages in the life cycles of fish, nutritional studies concerning broodstock and egg quality are dominated by lipid requirements. Polyunsaturated fatty acids are important for the formation and function of membranes and as precursors in the synthesis of certain molecules involved in a variety of biochemical pathways (Bell *et al.*, 1986). EPA, eicosapentaenoic acid, and DHA, docosahexaenoic acid are incorporated into the phospholipid fraction of marine fish eggs (Rainuzzo, 1993) and are an important source of energy during embryonic development (Tocher *et al.*, 1985; Rainuzzo *et al.*, 1992). Describing the energy metabolism in halibut eggs, Finn *et al.* (1991) also established that lipid and carbohydrate are the dominant sources for energetic requirements during early embryonic development, the emphasis switching to free amino acids as hatching approaches. Tandler *et al.* (1995) demonstrated an improvement in egg

quality in broodstock sea bream fed an n-3 HUFA enriched diet. The resulting larvae grew faster and were less likely to develop problems associated with swim bladder inflation. Harel *et al.* (1994) showed that incorporation of essential fatty acids in the reproductive organs is very rapid in gilthead sea bream, reaching an equilibrium with the diet within 15 days. A decrease in egg viability reflected the switch to a diet deficient in n-3 HUFA in 10 days. Similar results using essential fatty acid deficient diets have been obtained for other fish species (Watanabe, 1985). Enhancement of levels and ratios of certain essential fatty acids; AA (arachidonic acid), EPA and DHA, translated to improvements in egg hatching and larval survival in sea bass (Navas *et al.*, 1997). Recent developments in halibut broodstock diets have shown promising results and diets capable of replacing wet fish have been formulated (Mazorra, *et al.*, 1997; John Williamson pers. comm.).

It is important to ensure that broodstock feeds are formulated with the highest quality ingredients. Watanabe *et al.* (1991) showed the benefits in terms of egg quality in red sea bream, of using cuttlefish meal as a protein source and krill as a lipid source in place of white fish meal. In a study on sea bass, low dietary protein : lipid ratios fed to broodstock resulted in low fecundity and poor egg quality. The authors (Cerdá *et al.*, 1994) proposed that survival rates of larvae were related to the availability of high energy components in broodstock diet.

It appears that there is a definable window during which incorporation of the correct balance of essential fatty acids into the diet is critical to egg quality. In sea bass this period has been shown to be during vitellogenesis (Navas *et al.*, 1997). Halibut tend to suspend feeding during the spawning season so it is important to ensure that in the period leading up to spawning, the correct ration is fed. In cod, a high ration and fast

growth during the autumn vitellogenic period ensures high fecundity at spawning, whereas a high ration all year tends to bring the spawning season forward (Kjesbu and Holm, 1994).

During early cleavage the embryo relies heavily upon endogenous reserves of metabolic energy which are accumulated during oocyte development (Boulekbache, 1981). In the course of fertilisation and cell cleavage, high demands are placed on storage energy. Assembly of the cytoskeleton and mitotic spindle contractile proteins utilises high energy phosphate groups and this is evidenced by aggregation of mitochondria about the spindle at cell division, as shown by studies on mice (Van Blerkom and Runner, 1984).

It has been demonstrated for trout (Boulekbache, 1981) and red drum (Vetter, 1983) that levels of ATP and energy charge reflected by relative levels of adenylc nucleotides (AMP, ADP and ATP), are highest in the unfertilised oocyte and generally fall during the period between fertilisation and hatching. A chemically-induced blockage of ATP synthesis can cause embryonic defects resulting from arrested differentiation or dedifferentiation, reminiscent of those caused by other environmental stressors (Rosenthal and Alderdice, 1976; Westernhagen, 1988). It is possible that a reduction in the energy budget caused by such a blockage or by increased metabolic activity in response to such stressors may result in an impairment of cleavage processes (Westernhagen, 1988).

Energy charge decreases during the ageing process that occurs following ovulation. This phenomenon was demonstrated by Boulekbache *et al.* (1989) who associated energy charge with viability in carp embryos and suggested that the ageing process resulted in an energy shortage which compromised early cell cleavage.

Direct evidence of fertilisation and cleavage aberrations caused by over ageing of hamster oocytes was given by Juetten and Bavister (1983). Incidence of polyspermy, unequal blastomere sizes and abnormal shapes was higher in eggs whose fertilisation had been delayed. Similarly, Eichenlaub-Ritter *et al.* (1986) and Webb *et al.* (1986) reported that ageing in mouse oocytes was linked to disruptions in cytoskeletal organisation causing abnormal cleavage and chromosome behaviour. Similar effects were reported in oocytes of mice approaching the end of their reproductive life (Eichenlaub-Ritter, *et al.*, 1988). Abnormalities in the form of poor cleavage and ruptured blastodiscs were recorded in eggs of wild caught White Sea herring, aged in sea water before fertilisation (Dushkina, 1975). The incidence of deformed larvae resulting increased with ageing. Similarly, overripening in eggs of the Japanese flounder was associated with discoloration and shrinking of the cytoplasm and resulting larvae showed deformities including lordosis (Hirose *et al.*, 1979). McEvoy (1984) observed abnormal cleavages, discoloration, dimpled chorions and negative buoyancy in overripe turbot eggs and Sakai *et al.* (1985) reported wrinkled chorions, spotted egg cortexes and plasma bulges in the cytoplasm of aged red sea bream eggs.

Overripening has been linked to poor viability for a number of fish species (see review by Kjorsvik *et al.*, 1990). In halibut, a marked reduction in viability can occur if fertilisation is delayed longer than 4-6 hours after ovulation (Basavaraja, 1991; Bromage *et al.*, 1991; Bromage *et al.*, 1994). It has been shown that close observation of individual female ovulation cycles can help to pinpoint the timing of stripping and improve viability and fertilisation rates for halibut (Norberg *et al.*, 1991; Holmeffjord 1996) and turbot (Howell and Scott, 1989; McEvoy, 1984; McEvoy and McEvoy, 1992). Post-ovulatory ageing is relatively rapid in marine batch spawners and occurs

over a similar time scale in cod, 9 hours (Kjorsvik and Lonning, 1983) and turbot, 10 hours (McEvoy, 1984). The process is considerably slower in one-off spawners such as rainbow trout where oocytes can remain viable between 4-10 days after ovulation (Nomura *et al.*, 1974; Springate *et al.*, 1984; Bromage *et al.*, 1992). In turbot, the pH of the ovarian fluid falls during the ageing process from around 8.1 at ovulation to 7.1 and thus pH is a potential tool to test ripeness and egg quality (Fauvel *et al.*, 1993). The timing of spawning for a particular female can be predicted from analysis of oocyte diameter and developmental stage by biopsy (Kjesbu, 1994) or by ultrasonography (Shields *et al.*, 1993). From a practical standpoint, females are generally tested every few days as the spawning season approaches, evident from the amount of swelling apparent in the abdomen. The first ovulation is often signalled by a release of eggs into the water. Close observation from this point allows a female's ovulatory rhythm to be determined.

Reproductive processes are well known to be influenced by environmental factors, both as cues dictating the timing of events and as physiological stressors when conditions are sub-optimal. Effects on reproductive physiology may be mediated by hormones intimately involved both with reproductive processes and also with the stress response (Billard *et al.*, 1981). Handling, confinement and extremes of temperature, salinity and oxygen can all induce a stress response, leading to an elevation of corticosteroids and catecholamines. High levels of these hormones could have direct and detrimental effects on reproductive processes. Experiments with rainbow trout have shown that repeated acute stresses or confinement can result in delayed spawning and a reduction in egg viability and survival of larvae (Campbell *et al.*, 1992; 1994). The stripping of a large female halibut which may weigh in excess of 30 kg, necessitates a lot of handling. Females can become accustomed to routine handling and many seem

unaffected. Norberg *et al.* (1991) commented that handling was thought to have a negligible effect on ovulation in halibut.

Environmental factors are also likely to play a key role both during oocyte development and in the period between ovulation and early embryo development when the abnormalities become apparent. Egg handling and incubation procedures should be designed to ensure environmental stressors including low oxygen, temperature fluctuation, extremes of salinity and light exposure are kept to a minimum. Low oxygen and salinity are known to cause developmental defects in fish embryos (Westernhagen, 1970; Alderdice and Forrester, 1971; Rosenthal and Alderdice, 1976). Fertilising and incubating *Bairdiella icista* eggs outwith optimum salinities or temperatures increased the likelihood of irregular cleavage during development in a study by May (1975). Embryos exhibiting these abnormalities, including "clover leaf" patterns, a characteristic occasionally seen in the current study, "usually died before gastrulation and never hatched". Investigating tolerance to low temperature shocks during embryonic development in some marine fish eggs, Sasaki *et al.* (1988) found that such a stressor applied during cleavage stages could induce irregular cleavage patterns. In the aforementioned and indeed many other studies, abnormal morphology was often accompanied by chromosome abnormalities. In this case aneuploidy was the reported defect.

To understand the causes of cleavage anomalies, knowledge of the mechanics of cell division is required. In animal cells, which usually divide symmetrically, it is the mitotic spindle which is the architect of cytokinesis, determining when and where division occurs (Alberts *et al.*, 1994). Since the spindle is also responsible for the organisation of chromosomes during cell division, it is not surprising that chromosome and cleavage

aberrations are often documented simultaneously.

Cleavage is accomplished by contraction of an actomyosin band, beginning with the formation of a furrow at the junction between the parent cells. This progresses out toward the edge of the blastodisc and culminates in cell partition. This is accompanied by two 'calcium waves', first as the furrow lengthens and then after cleavage, a second 'zipping wave' as the cells close together to obscure the groove (Fluck *et al.*, 1991).

Defects in cell surface components have been proposed as one of the possible causes of abnormal cleavage in early embryos (Hardy *et al.*, 1993). Certain types of protein molecules contained within the cell membrane are responsible for maintaining inter-cell connections and communication between adjacent cells (Gilbert, 1994). Cell adhesion molecules (CAMs) protrude from the surface of the cell membrane and bond with similar molecules attached to neighbouring cells. One class of these molecules, calcium dependant glycoproteins known as cadherins, are believed to be crucial for maintaining cell adhesion and spatial segregation and eventual tissue differentiation. Both the CAMs and the mitotic spindle are closely associated with the cell's 'scaffold', the cytoskeleton.

The extent to which aberrations occurring in cleavage stage fish embryos affects subsequent embryogenesis is likely to depend on the role of individual blastomeres in tissue differentiation. Early work on *Lymnaea stagnalis* embryos (Verdonk and de Groot, 1969) showed that heat shocks interfering with the third cleavage stage lead to specific abnormalities associated with the head region since these structures could be mapped to the first quartet of micromeres arising from this division. This subject has more recently been investigated in detail for zebrafish, embryos. Early zebrafish blastomeres (8 and 16 cell stages) have been shown to map to specific subsequent embryonic tissues according to their cleavage planes (Strehlow *et al.*, 1994). The

specificity with which this occurs is debated (Helde *et al.*, 1994), nonetheless the agreed mechanism of cell differentiation, whereby spatial inter-relations of blastomeres are important, supports the view that irregular blastomere positioning or damaged blastomeres may be deleterious.

The fertilised egg is the starting material for the hatchery production cycle and it is essential that egg viability is optimal. In order that high quality eggs are produced, broodstocks must be carefully selected and held under optimum conditions. Mature halibut broodstock females are valuable animals and it is critical that a healthy nutritional and physiological status is maintained to ensure that reproductive capacity of the broodstock is not compromised. Careful attention must therefore be paid to both the dietary requirements of the broodstock and the environmental factors which determine spawning success. The next chapter deals specifically with one of the most important considerations for broodstock management i.e. the effect of temperature on egg production and quality.

3.5. Conclusions

- A method has been developed and tested for small scale experimental incubation of halibut eggs using microtitre plate wells. This technique provides a reliable and repeatable way of incubating eggs through to hatching with potentially high survival rates.
- A range of blastomere abnormalities, easily visualised at the 8 cell stage, have been identified and quantified using a simple scoring system.
- Good correlations between viability, evident in hatch rates, and the severity of each of the blastomere characteristics have been demonstrated. It is proposed therefore

that blastomere observations may be used alone or in conjunction with other parameters such as fertilisation rate, for the assessment of halibut egg quality.

- Higher incidence of deformity at hatching was observed in batches exhibiting blastomere abnormalities at the 8 cell stage.
- Normal cell morphology at the third division can be considered symmetrical, with equal cell sizes, clear unbroken cell margins and few or no vesicular inclusions.
- The relationship between egg viability and blastomere morphology is stronger than with fertilisation rate. It is hypothesized that these characteristics are more indicative of inherent viability. Abnormalities apparent during early cell divisions are likely to be manifestations of physiological disruption at a cellular level.

4. Broodstock Temperature Requirements

4.1. Introduction

The relationship between physical environmental variables, the control of fish reproduction and egg quality, places great emphasis on the importance of understanding the requirements of captive broodstocks. The majority of fish species in temperate zones spawn on an annual basis and reproductive strategies are geared such that the timing of spawning best ensures that environmental conditions are optimum as the resulting progeny emerge. Mechanisms for synchronising the cascade of events culminating in the ovulation and spawning of ripe eggs are governed by the pattern of change in ambient environmental conditions.

Environmental signals reach the brain and act on the hypothalamus. This organ secretes gonadotropin releasing hormones (GnRH) which control the release of gonadotropin hormone (GtH) from the pituitary. The control of GtH synthesis is under dual control in some teleosts; stimulated by GnRH (a decapeptide) and inhibited by GnRIH. The inhibitory dopaminergic mechanism is not present in many of the marine species investigated thus far (Lin and Peter, 1996). The ovary responds to GtH by secreting steroid hormones which control vitellogenesis and final maturation (Goetz, 1983; Bhattacharya, 1992). These central processes appear common to many fish species and recent studies suggest that the Atlantic halibut is no exception (Methven *et al.*, 1992; Norberg, 1995).

The change in day-length provides the best natural chronometer and it is well known that photoperiod is strongly associated with the reproductive cycles of most temperate teleosts (Breton and Billard, 1977; Lam, 1983; Bye, 1984). This fact has been exploited

to great effect by many fish culturists. Manipulation of photoperiod is routinely used to influence natural spawning cycles enabling the production of out-of-season eggs and, when multiple broodstocks are used, all year round production. These techniques are best documented and most extensively used for salmonids (Bromage *et al.*, 1993) but have also been applied successfully to marine fish, for example, sea bass (Carrillo *et al.*, 1989; 1995), cod (Norberg *et al.*, 1991), turbot (Devauchelle *et al.*, 1988; Stoss and Røer, 1993), sole (Baynes *et al.*, 1993) and halibut (Smith *et al.*, 1991, Holmefjord *et al.*, 1993; Naess *et al.*, 1996).

A possible mechanism by which these stimuli are transmitted into physiological events is through the diel fluctuation in circulating melatonin. As shown in salmonids, daily and annual fluctuations in light level are reflected by melatonin levels which rise to a maximum during darkness (Bromage *et al.*, 1995). It has been proposed that environmental cues entrain an endogenous rhythm which has been demonstrated for a number of species, e.g. salmonids (Bromage *et al.*, 1993) and sea bass (Zanuy *et al.*, 1991).

Water temperature also exerts a strong influence on spawning rhythm and although its influence is thought to be secondary to photoperiod for most temperate species (Bye, 1984; Bromage *et al.*, 1993) there is an important interaction between the two (Devauchelle *et al.*, 1988). In many studies on photoperiod, the effects of temperature have often not been considered (Lam, 1983; Bye, 1984) and where photoperiod regimes are overlaid on normal temperature cycles, the synchronising effects of the latter may be masked (Scott *et al.*, 1984).

Apart from the role of temperature in synchronising gonadal activity, direct effects on gamete quantity and quality have been reported in both captive and wild broodstocks.

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Apart from the role of temperature in synchronising gonadal activity, direct effects on gamete quantity and quality have been reported in both captive and wild broodstocks.

Recorded adverse effects resulting from sub-optimal temperature regimes during gametogenesis and spawning include reduced fecundity (Devauchelle, 1987), oocyte retention and delays in spawning (Gillet 1991; 1994) and atresia (Kuo, 1995; Gillet, 1991; 1994). High temperatures during gametogenesis have been implicated in the reduction of gamete viability in many species e.g. Atlantic salmon (Taranger and Hansen, 1993), rainbow trout (Pankhurst *et al.*, 1996), Arctic charr (Gillet, 1991), sole (Baynes *et al.*, 1993), turbot (Devauchelle *et al.*, 1988), sea bass (Carrillo *et al.*, 1995) and carp (Statova *et al.*, 1982). Temperature is known to play a role in the rate of ripening of eggs following ovulation (Billard and Gillet, 1981) and can also influence egg size (Mihelakakis and Kitajima, 1995; Kawaguchi *et al.*, 1990) or nutritional composition (Jobling *et al.*, 1995).

Under normal environmental conditions, gonadal growth in the halibut starts in early autumn (Haug and Gulliksen, 1988). In the wild, the Atlantic halibut spawns in very deep water where the ambient temperature is stable and typically between 5 and 7°C (Kjorsvik *et al.*, 1987; Haug, 1990). Since most marine fish hatcheries are located on land, their water supply is derived from surface waters which are generally subject to larger annual fluctuations in temperature. Furthermore, halibut reared in Scotland are at the southerly extreme of their distribution and ambient thermal profiles are outwith those experienced by wild stocks. Broodstocks held at ambient temperature in Scotland, spawning at the normal time of year during the spring, and particularly those stocks which have been induced to spawn out-of-season using photoperiod manipulation, will be subject to abnormal temperature profiles.

The capital and running costs of controlling water temperature at the throughputs required for broodstock facilities are considerable. It is important therefore to assess the

effects of ambient temperature on broodstock performance and subsequent quality of gametes to determine whether this is a necessary feature of Atlantic halibut broodstock management in Scotland.

4.2. Materials and Methods

The broodstock used for this study were wild fish originally caught off Iceland. They began spawning in captivity at Ardtoe 3 years after capture. At the start of this experiment, 6 years after capture, the fish had already been established for 2 years in their respective groups, under the conditions described. A total of 28 fish were used in the experiments with a sex ratio of 1:1. The fish were separated into 2 groups (see Figure 4.1.), each consisting of 7 pairs of adult male and female fish held in 3 vitreous enamelled steel tanks, 1 of 5.3 m diameter and 2 of 4.6 m diameter. The larger tanks held 3 pairs of fish and the smaller tanks held 2 pairs of fish each. The water depth in each tank was 1.2 m. All tanks received 25 l/min (flow to waste) sea water maintained automatically above a minimum salinity of 33 ppt. The tanks were illuminated by diffuse natural light and thus were under ambient photoperiod. The sea water supplied to the first group ('ambient group') was at ambient temperature all year round. For the second group ('chilled group'), the sea water temperature was controlled using a chiller to maintain the temperature below a maximum of 10°C during the summer months and at approximately 6 °C prior to and during the spawning period (from December until June). Waste water from the chilled tanks was routed through a heat exchanger to pre-cool incoming water. A diagram of the system is shown in Figure 4.2. Each female was allocated a code signifying the group, tank and individual. All ambient group females were given the prefix 'B' and the chilled group the prefix 'RU'. This was followed by tank number and female number. Thus, RU11 represents female 1, tank 1, chilled group.

The fish were fed 3 times per week to appetite on a moist 'sausage' diet (formulation shown in Table 4.1.).

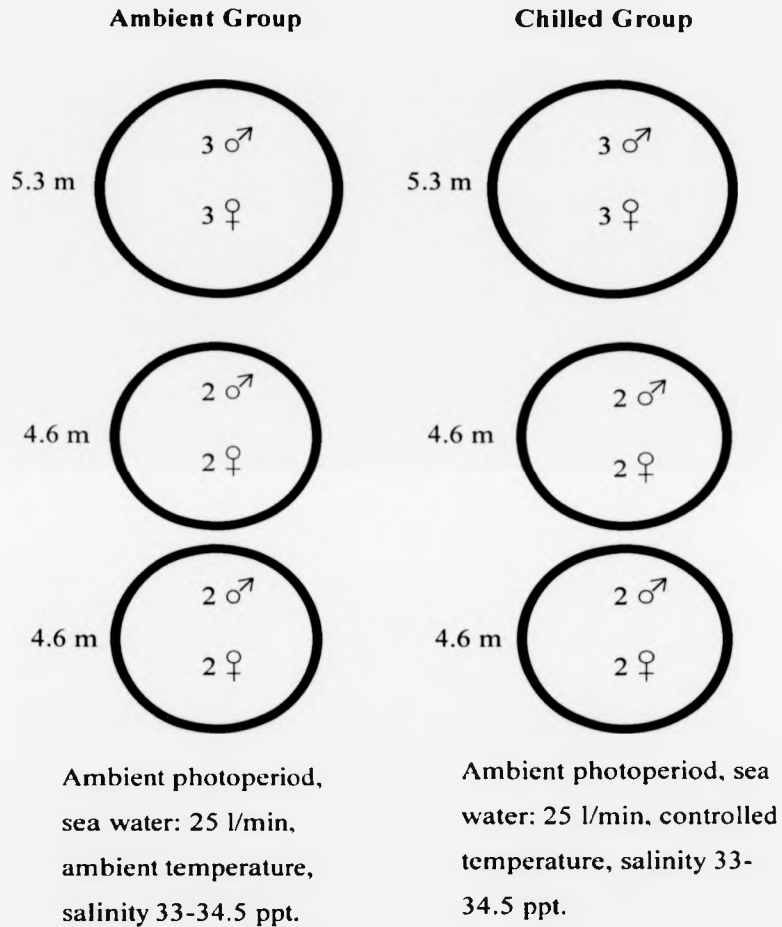


Figure 4.1. Schematic representation of broodstock groups giving details of tank sizes, stocking regimes and environmental conditions for ambient and chilled groups.

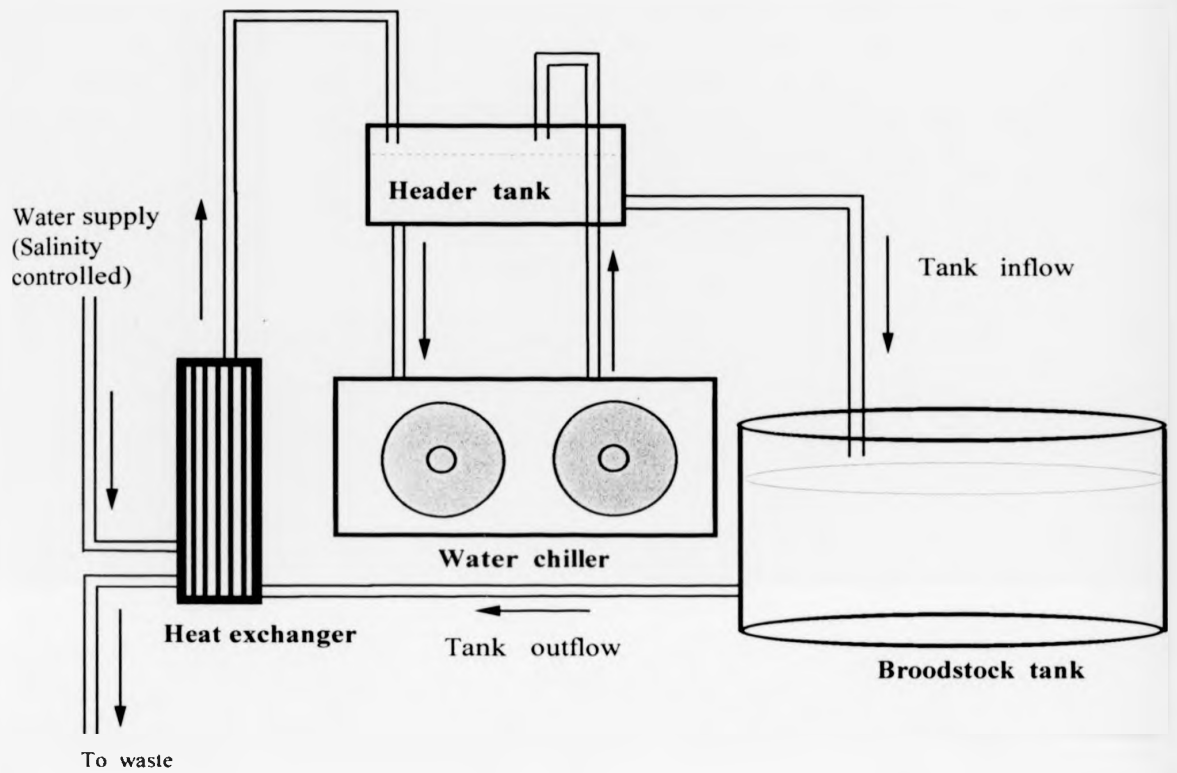


Figure 4.2. Schematic representation of system used for chilled halibut broodstock. Each chilling unit serves three broodstock tanks. Water temperature is controlled by an inbuilt chiller thermostat. Facilities for ambient group do not include chilling equipment.

Table 4.1. Formulation for preparation of 17.7 kg of broodstock moist "sausage" diet fed to fish during the experiment. The mixed ingredients were extruded into a 28 mm sausage skin and cut into sections (approximately 10-12 cm long) for feeding.

<i>Ingredient</i>	<i>Quantity</i>	<i>Supplier</i>
Dry components		
Norse LT 94 fishmeal	5 kg	Trouw Aquaculture, Northwich, U.K.
Vitamin mix	200 g	Trouw Aquaculture, Northwich, U.K.
Vitamin booster	100 g	Trouw Aquaculture, Northwich, U.K.
Binder (Guar gum Emulcol "U")	100 g	Trouw Aquaculture, Northwich, U.K.
Wet components		
Herring	6 kg	Local supplier
Squid	3 kg	Local supplier
Cod roe	3 kg	Local supplier
Oil (30% PUFA fish oil)	300 g	Isaac Spencer & Co Ltd, Fleetwood, U.K.

Spawning rhythms were closely followed for all fish involved in the experiment and eggs were stripped from ovulating females by hand and wet fertilised as described in Section 2.3.1. Eggs released naturally by females were collected in net baskets at the tank outlets. These were checked daily and any eggs were removed and weighed.

Fertilised egg batches were placed in static incubators (described in Section 2.3.1) and left for approximately 16 hours at 6°C until the 8-cell stage. Non-buoyant eggs (dropout) were removed and weighed just after fertilisation, again at 16 hours and again just prior to moving to the upwelling incubation system (described in Section 2.3.2). Fertilisation rate was assessed at 16 hour post fertilisation from 3 samples of at least 100 eggs taken from the floating fraction in the static incubator. Fertilisation rate was taken

as the number of developing (actively dividing) eggs at the 8-cell stage expressed as a percentage of the total number of eggs floating 16 hours post-fertilisation. Egg diameter was also measured at this stage. A sample of 10 eggs were lined up in adjacent fashion in a channelled microscope slide and viewed under low magnification with a compound microscope. The slide carriage was moved to position the eye-piece cross hairs at one end of the line of eggs. The reading was then taken on the micrometer of the carriage scale, the carriage was then moved till the eye-piece cross hairs were positioned at the other end of the line of eggs and the micrometer read once more. The two readings were then subtracted and the result divided by 10 to give an average egg diameter.

Samples of floating eggs were also removed at this stage and stocked into microtitre plates as described in Section 3.2.1. These eggs were incubated to hatching in these plates held in an incubator at 6°C. At hatch, the total number of hatched larvae were counted to give a hatch rate. The hatch rate was taken as the number of eggs hatched into larvae expressed as percentage of the original number of floating eggs at the 8-cell stage. This value equated to that routinely used in the hatchery as opposed to the alternative method of expressing hatch rate as a percentage of fertilised eggs. The experiment monitored egg production over 2 spawning seasons. In the first season, a sample of 192 fertilised eggs from each batch were incubated in 4 microtitre plates, 48 eggs per plate and in the second season, a sample of 288 randomly selected eggs (fertilised and unfertilised) per batch were incubated in 3 plates, 96 eggs per plate.

To obtain data on relative fecundity, all females were weighed and measured at the end of the second season.

Differences between ambient and chilled broodstock groups in terms of spawning period, total egg production per female and fecundity (year 2 only) were analysed

separately for each of the two seasons using one way ANOVA. Fertilisation rates and hatch rates were arcsine transformed and also analysed using one way ANOVA for each season's data.

4.3. Results

4.3.1. Spawning pattern and egg production

In the first year, spawning commenced in early February in the chilled stock and by late March most fish in this group had started ovulating (see Figure 4.3.). In the ambient group, spawning started an average of 19 days later with 3 females starting spawning in late March and 4 females starting in mid-late April. Only one female failed to spawn in year 1. This fish, in the chilled group, had damaged eyes and did not feed well during the months preceding the spawning season. In year 2, it was the chilled group females which again started spawning earliest and it was the same females as in year 1 which were first to spawn in year 2. Most females were spawning in this group by late March. As in year 1, the ambient females were delayed and spawning did not get under way until mid April, an average of 21 days later, the last fish coming into season well into May. In the second year, 3 females failed to produce eggs, 2 from the chilled group and 1 from the ambient group. Figure 4.4. shows the cumulative egg production of the two groups and illustrates the spawning patterns of the groups as a whole for the 2 years and indicates the delay of the ambient group compared to the chilled group.

The average spawning period (see Figure 4.5) appeared longer for the chilled females in both years than for the ambient fish in years 1 and 2 respectively although this difference was not significant (ANOVA, $p > 0.05$). A large proportion of the total eggs produced from each female were released naturally. An average of 49% and 34.6% of total egg production came from natural releases in years 1 and 2 respectively. Some of these releases occurred just prior to or just following stripping. Occasionally, when an ovulation was not anticipated, females released a complete batch. Certain females were

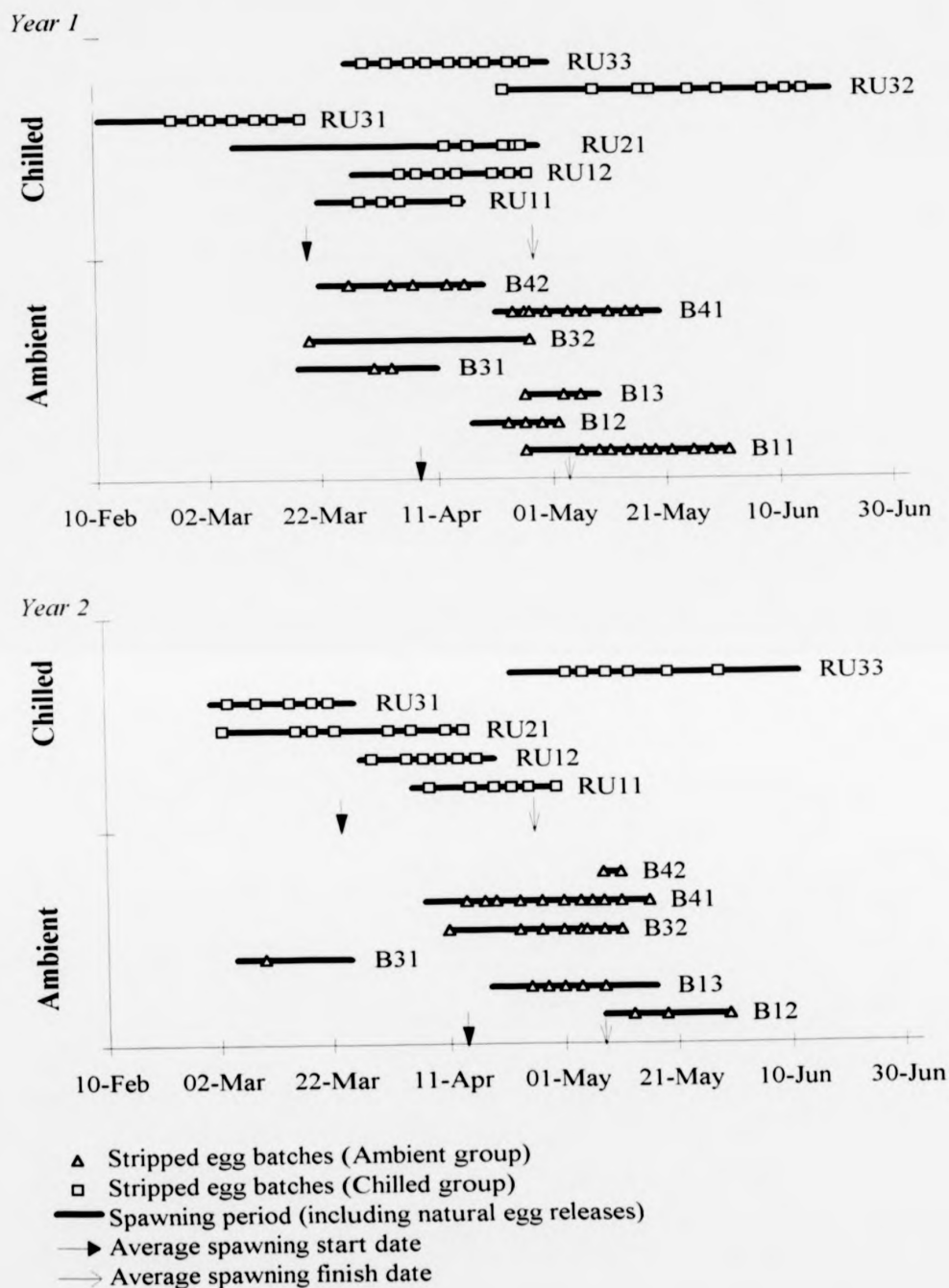


Figure 4.3. Spawning period of each female in ambient and chilled groups in years 1 and 2. See Section 4.2. for explanation of female codes.

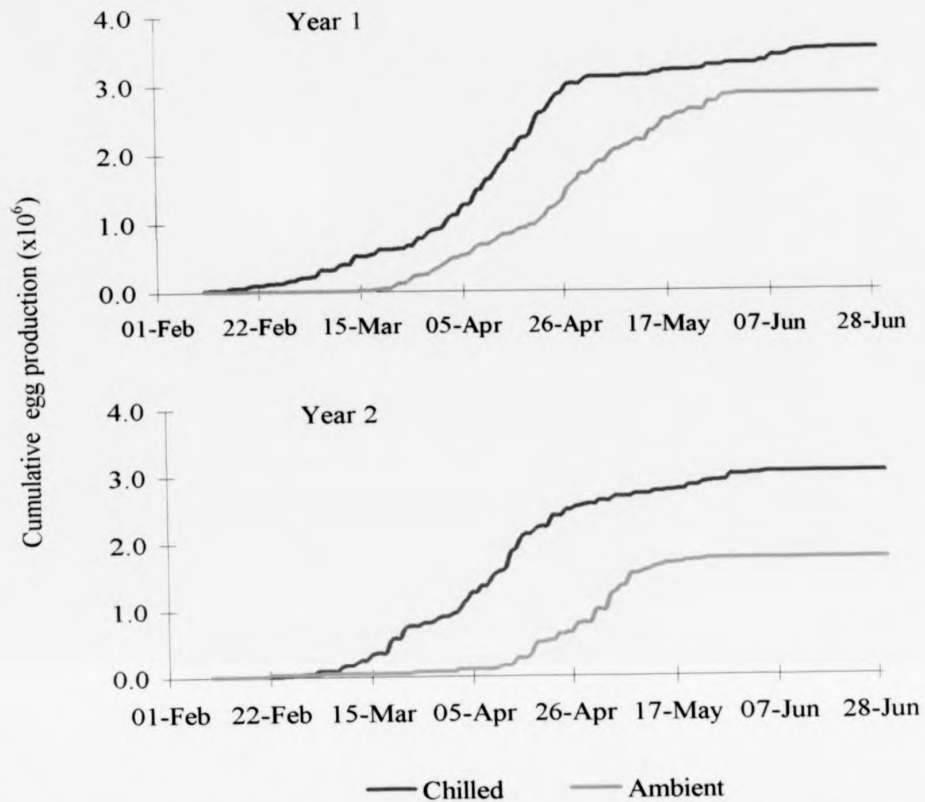


Figure 4.4. Cumulative egg production of ambient and chilled groups in years 1 and 2. Values are the sum of all egg batches including natural releases from all females in each group.

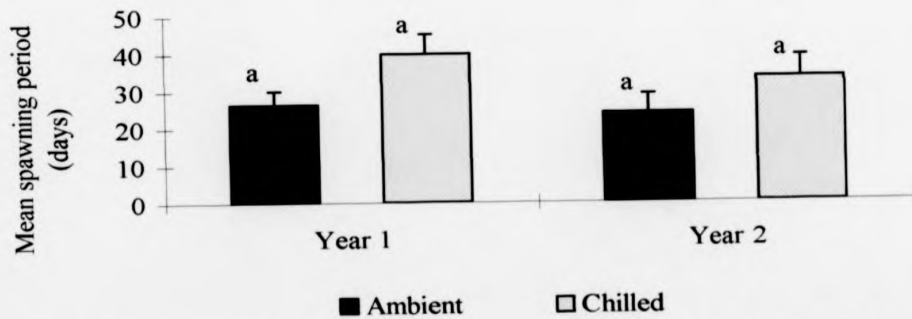


Figure 4.5. Average spawning period from first to last ovulation (including natural releases for ambient and chilled groups in both years. Error bars represent standard error of means. a,b denote significant difference between means (ANOVA, $p < 0.05$).

also known to 'dump' their eggs as the tank was being drained ready for stripping. The phenomenon of 'queuing' was noted in some tanks where females ready to spawn did not commence spawning until another individual in the same tank already ovulating had completed the spawning cycle.

Total egg production in terms of eggs produced per female, shown in Figure 4.6.A., was significantly higher in the chilled group during both seasons (ANOVA $p < 0.05$). This difference was not detected when fecundity was expressed in terms of eggs/kg (Figure 4.6.B.), calculated from the weights of broodstock measured at the end of year 2.

4.3.2. Egg size

Mean egg diameters for each group in years 1 and 2 are shown in Figure 4.7. Egg diameter was similar for both groups in year 1 but was significantly lower in the ambient group in year 2 (ANOVA, $p < 0.001$). There was a tendency for egg diameter to decrease during the course of the spawning season for most females and the relationship between egg diameter and time was significant in many cases (see Figure 4.8.).

4.3.3. Temperature profiles

The temperature profiles for each broodstock group for the two seasons are shown in Figure 4.9. The temperature remained below 6.5°C for most of the spawning period in the chilled stock for both seasons. In the ambient group however, the temperature fluctuated. In year 1, the water temperature started to increase in February and had already risen above 6°C by early March. A rapid increase during April took the temperature above 8°C by mid-April. A maximum of 11°C was reached during the spawning season of the ambient females in year 1. In year 2, a warmer winter meant that the ambient groups' pre-spawning temperature was already fluctuating around 6°C

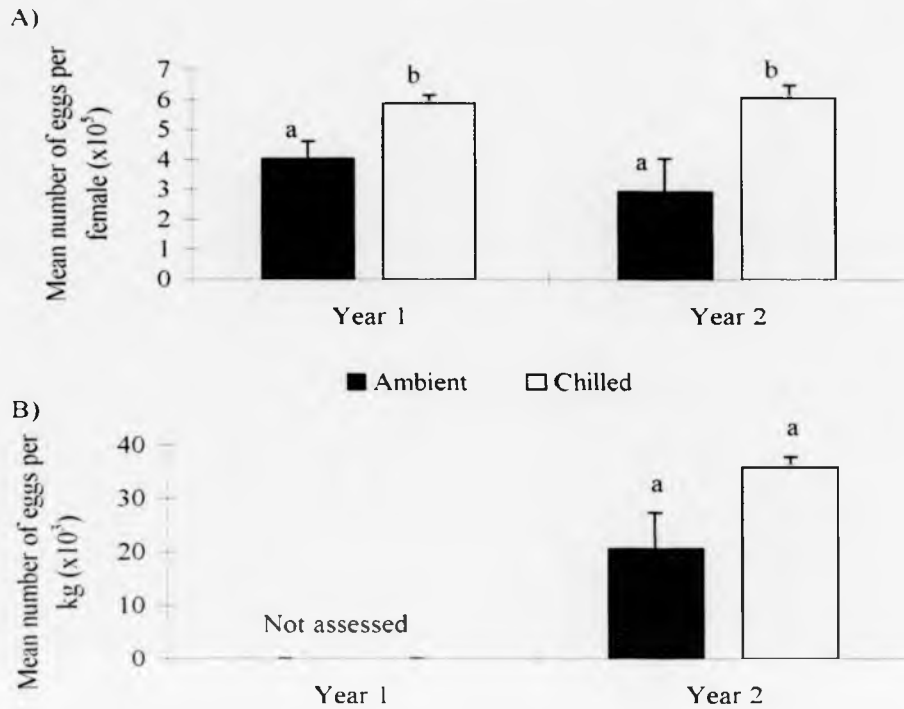


Figure 4.6. A) Mean egg production per female for ambient and chilled groups in years 1 and 2. B) Relative fecundity (eggs/kg) for females from ambient and chilled groups in year 2. Females were not weighed in year 1 so this data was not available. Error bars represent standard error of means. a,b denote significant differences between means (ANOVA, $p < 0.05$).



Figure 4.7. Mean egg diameter for ambient and chilled groups in years 1 and 2. Error bars represent standard error of means. a,b denote significant differences between means (ANOVA, $p < 0.05$).

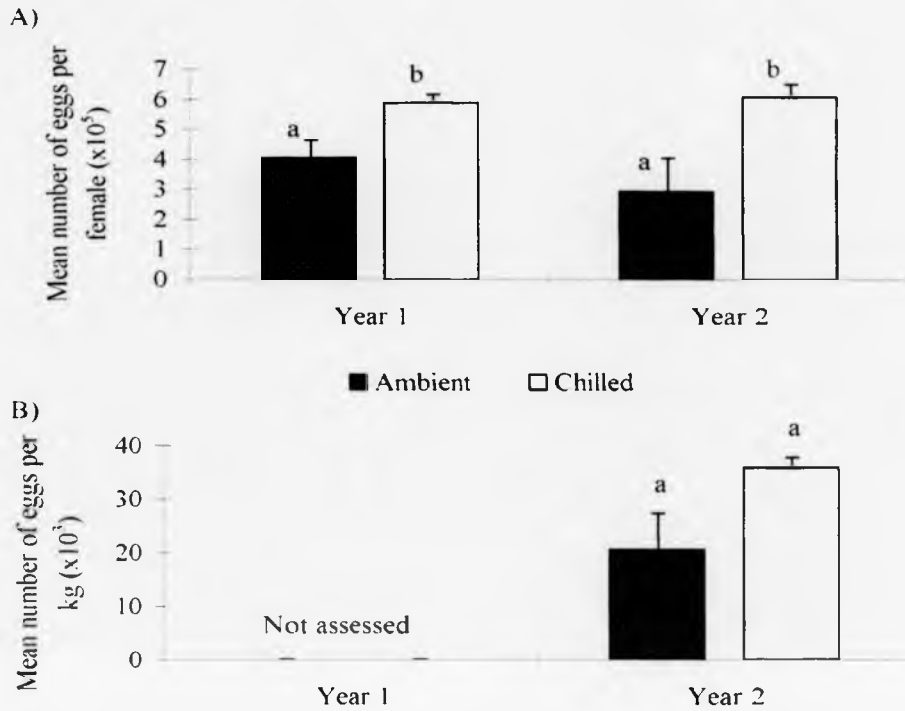


Figure 4.6. A) Mean egg production per female for ambient and chilled groups in years 1 and 2. B) Relative fecundity (eggs/kg) for females from ambient and chilled groups in year 2. Females were not weighed in year 1 so this data was not available. Error bars represent standard error of means. a,b denote significant differences between means (ANOVA, $p < 0.05$).



Figure 4.7. Mean egg diameter for ambient and chilled groups in years 1 and 2. Error bars represent standard error of means. a,b denote significant differences between means (ANOVA, $p < 0.05$).

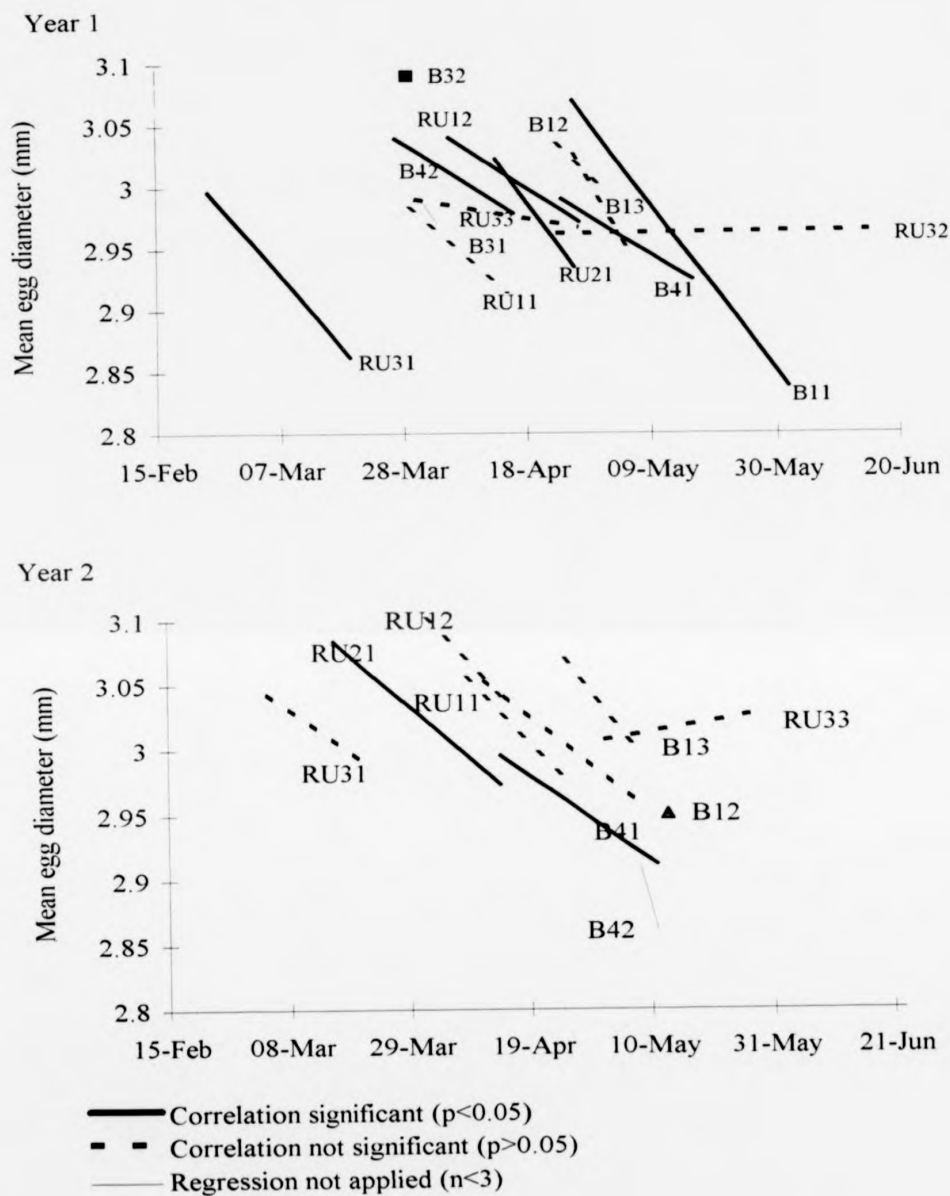
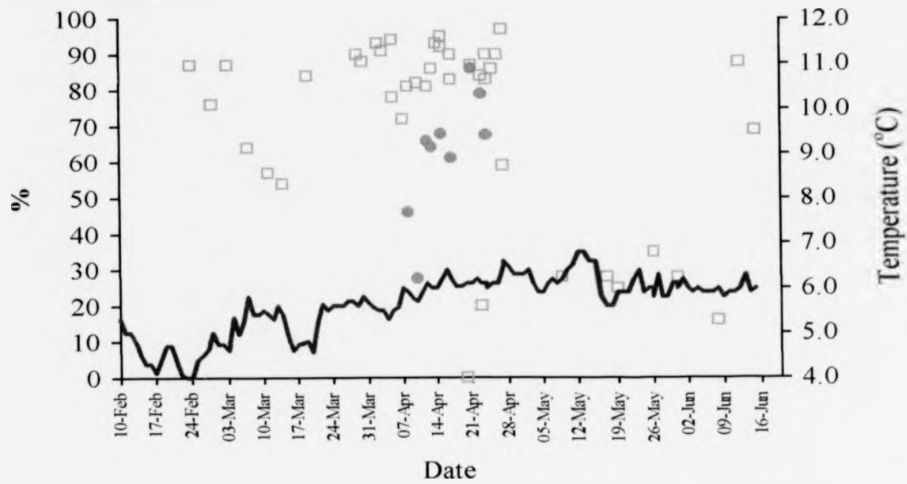
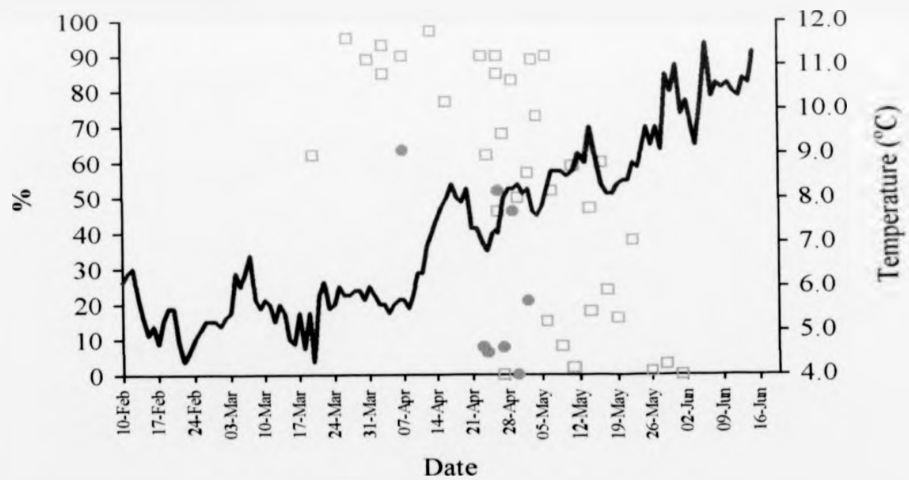


Figure 4.8. Change in mean egg diameter for each female during their respective spawning seasons in year 1 and 2. Regressions were applied to each data set, see legend for indications of significance of correlations.

Chilled group; Year 1



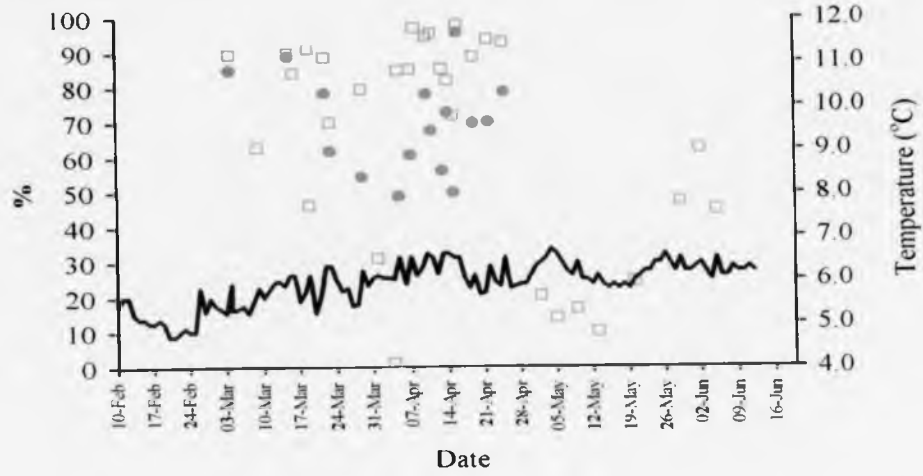
Ambient group; Year 1



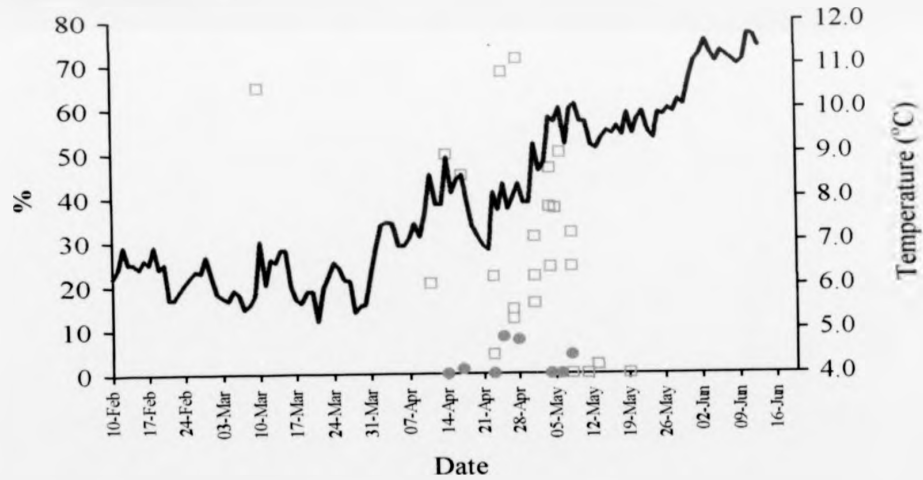
- Fertilisation rate
- Hatch rate
- Temperature

Figure 4.9.A. Temperature profiles, fertilisation rate of stripped batches and hatch rate of batches incubated in microtitre plates for chilled and ambient groups in year 1.

Chilled group; Year 2



Ambient group; Year 2



- Fertilisation rate
- Hatch rate
- Temperature

Figure 4.9 B. Temperature profiles, fertilisation rate of stripped batches and hatch rate of batches incubated in microtitre plates for chilled and ambient groups in year 2.

during February to March and had not reached the low extremes of 4-5°C as in year 1. Similar to the spring rise of year 1, the temperature rose to 8°C by mid-April.

4.3.4. Fertilisation and hatching

The fertilisation rate and hatch rates of all egg batches fertilised and incubated in microtitre plates are shown in Figure 4.9A-B. The number of batches fertilised and incubated in microtitre plates and the mean fertilisation and hatch rates of these egg batches are shown in Table 4.2. and are related to corresponding females therein. The performance of egg batches from the chilled group was significantly better than that of batches from the ambient group both in terms of fertilisation rate and hatch rate (ANOVA, $p < 0.05$) for both seasons. In the main, egg batches from the chilled groups exhibited fertilisation and hatch rates above 50% in both seasons. Lower rates of fertilisation were obtained from eggs ovulated by a late spawning female (RU33) in both seasons. None of these batches were incubated in microtitre plates. In the ambient group, batches spawned earlier in the season in year 1 showed reasonable fertilisation rates. The fertilisation rates of egg batches declined later in the season, coincident with the ambient temperature rising above 8°C (see Figure 4.9.A). With the exception of 3 batches from 2 females, hatch rates were all below 30% in the ambient group. From year 1 to 2, the overall fertilisation and hatch rates for the chilled group were similar. They were much lower however, for the ambient group in year 2 than in year 1 (see Figure 4.9.B.).

Table 4.2. Data for year 1. Performance of egg batches from females of ambient and chilled groups; number of batches fertilised, mean % fertilisation rate for each female and for each group \pm (s.d.), number of batches incubated in microtitre plates and mean hatch rate ([number hatched \div number of buoyant eggs at 16 hours post fertilisation] \times 100) \pm (s.d.) for each female used and for each group. Different superscripts indicate a significant difference (ANOVA, $p < 0.05$).

Tank	Female	Number of batches fertilised	Mean % fertilisation rate \pm (s.d.)	No of batches incubated	Mean % hatch rate \pm (s.d.)
Ambient group					
B1	1	11	15.5 (15.4)	0	31.8 (20.8)
	2	4	78.8 (14.8)	4	
	3	3	89.7 (0.6)	0	
B3	1	2	87.0 (2.8)	0	
	2	2	31.0 (43.8)	0	
B4	1	8	58.9 (9.0)	3	4.7 (4.1)
	2	5	90.4 (7.9)	1	63.5
n	7	35		8	
Mean \pm (s.d.)			54.7 (33.6) ^a		25.6 (24.6) ^a
Chilled group					
RU1	1	4	92.3 (1.7)	1	67.9
	2	7	87.0 (7.4)	3	66.0 (20.0)
RU2	1	5	85.8 (2.7)	3	68.1 (9.6)
	2	Not spawned			
RU3	1	7	72.7 (14.3)	0	47.6 (28.3)
	2	9	37.4 (24.4)	0	
	3	9	73.1 (29.5)	2	
n	7	41		9	
Mean \pm (s.d.)			71.0 (30.7) ^b		62.8 (17.3) ^b

Table 4.2. (continued). Data for year 2. Weight and length of broodstock measured at the end of year 2 and performance of egg batches from females of ambient and chilled groups; number of batches fertilised, mean % fertilisation rate for each female and for each group \pm (s.d.), number of batches incubated in microtitre plates and mean hatch rate for each female used and for each group \pm (s.d.). Different superscripts indicate a significant difference (ANOVA, $p < 0.05$).

Tank	Female	Weight (kg)	Length (cm)	Number of batches fertilised	Mean % fertilisation rate \pm (s.d.)	No of batches incubated	Mean % hatch rate \pm (s.d.)
Year 2 Ambient group							
B1	1	20.2	130	Not spawned			
	2	13.1	92	2	1.0 (1.4)	0	
	3	8.9	90	5	43.7 (23.9)	2	8.0 (0.5)
B3	1	15.4	130	1	64.8	0	
	2	18.6	110	7	21.1 (13.3)	2	0.0 (0.0)
B4	1	14.5	106	9	29.1 (20.0)	4	1.3 (2.0)
	2	9.7	95	2	0.0 (0.0)	0	
n	7			26		8	
Mean \pm (s.d.)		14.3	107.6		26.7 (20.0) ^a		2.7 (1.9) ^a
Chilled group							
RU1	1	23	117	5	69.7 (39.4)	4	67.2 (12.2)
	2	14.8	100	6	89.9 (7.6)	5	69.1 (17.6)
RU2	1	19.2	108	7	70.5 (23.4)	4	62.9 (10.3)
	2	12.6	92	Not spawned			
RU3	1	12.9	99	5	84.4 (12.2)	3	84.1 (5.3)
	2	19.8	99	Not spawned			
	3	16.2	105	8	30.1 (19.1)	0	
n	7			31		16	
Mean \pm (s.d.)		16.9	102.9		65.9 (30.9) ^b		69.9 (13.9) ^b

4.4. Discussion

Under conditions of ambient photoperiod, the spawning performance of the two groups of broodstock halibut was clearly different under the two temperature regimes used in this study.

The temperature profile experienced by the ambient group delayed spawning in both years, demonstrating that temperature exerts a strong influence on the timing of ovulation and spawning in the Atlantic halibut. This had the effect of exposing the broodstock to even higher temperatures during spawning than would have been the case had they ovulated at the same time as the chilled stock. The average start date for spawning was slightly later for both groups in year 2, being on average of 4 and 6 days later for the chilled and ambient groups respectively.

Though photoperiod is the proximal cue for spawning in most teleosts of temperate latitudes, temperature usually works in concert with day-length to synchronise reproduction (Bye, 1984). In some fish, temperature is considered to be the primary cue and controlling temperature can be an effective method for the manipulation of spawning season. For example, research into the spawning of sole has demonstrated that out-of-phase spawning may be achieved with temperature cycling alone (Lenzi and Salvatori, 1989). Some species lend themselves well to temperature manipulation particularly e.g. the red drum can be induced to spawn continuously if maintained at late summer spawning temperature (Thomas *et al.*, 1995). If spawning does not commence, a sharp reduction of temperature by 3 to 4°C followed by a reversion to the spawning temperature is usually effective in inducing spawning. Kelley *et al.* (1991) found that for striped mullet, photoperiod cues initiated reproductive processes but beyond the onset of

the cortical vesicle stage, events were under the influence of water temperature. Kuo (1995) reported that while short photoperiod triggers the onset of vitellogenesis in the grey mullet, temperature plays a dominant role in regulating vitellogenesis to functional maturity when ovulation and spawning can be induced hormonally. For this species, a temperature of around 21°C is optimal for promoting the maturation process.

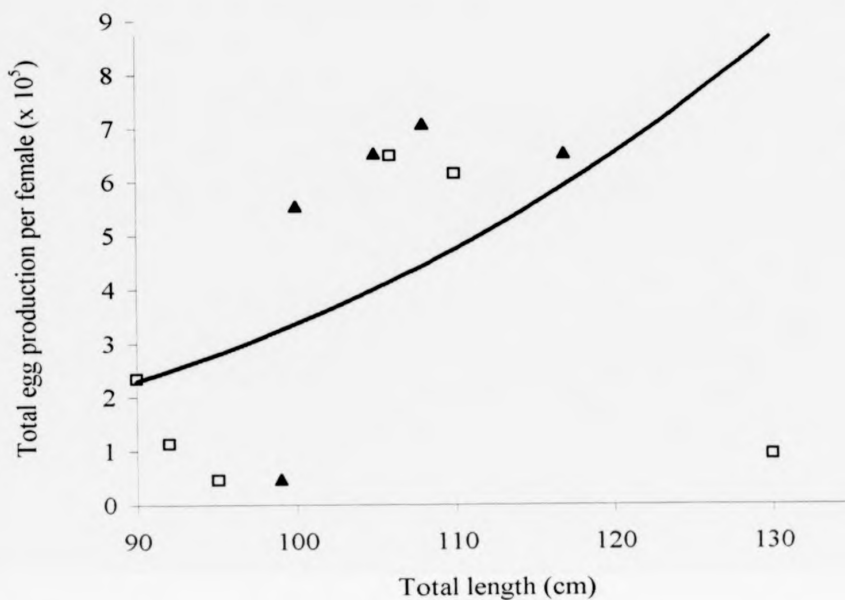
Manipulation of spawning time is generally most effective when both photoperiod and temperature are shifted in parallel. For example the spawning rhythm for gilthead sea bream can be most effectively controlled using compressed cycles of photoperiod and temperature together (Devauchelle, 1984; Zohar *et al.*, 1995). The response in terms of spawning time to elevated temperature is species specific. In sobaity *Sparidentex hasta* and Arctic cod for example, an early rise in temperature can advance spawning (Al-Marzouk *et al.*, 1994; Graham and Hop, 1995).

It is possible that the delay in spawning time of the ambient stock was caused by thermal environment during the winter months, although the delay relative to the chilled stock was similar in years 1 and 2, despite rather different seasonal ranges of temperature. A close relationship between minimum winter temperature and the time of spawning exists for sole (Baynes *et al.*, 1993) and rising temperature in the spring is thought to be an important cue for the initiation of spawning. Spawning ceases by mid-May when the temperature rises above 12.5°C and spawning of captive French stocks tends to be later than their wild counterparts indicating that sole has a narrow thermal preference with respect to reproductive processes (Devauchelle *et al.*, 1987).

It is evident that the Atlantic halibut is stenothermal with respect to reproductive developmental processes. This is not surprising in view of the habitat and environmental characteristics of the natural spawning grounds. A somewhat similar situation to that

found for the halibut in this study applies to the Arctic charr of lake Geneva, described by Gillet (1991; 1994). These fish normally spawn at depth where the temperature is stable around 6°C. Under natural photoperiod, various experimental regimes have been tested in order to elucidate the thermal requirements of the reproducing females. Fish held at 11°C failed to spawn whilst fish held at 8°C were delayed relative to those at 5°C. Transfer from 8 to 5°C stimulated spawning almost immediately. Fish held at low temperature prior to gametogenesis did not spawn early and it was concluded that low temperature was only required during gametogenesis and spawning.

In the current work, the number of eggs produced by the chilled stock was higher in both years despite the fact that there was one less female spawning in the chilled stock in both years. This suggests that the temperature regime for the ambient group was not conducive to optimal egg production. Due to the limited data (number of females), the difference in relative fecundity was not significant (ANOVA, $p=0.079$). For the purpose of comparison with published figures on fecundity for the Atlantic halibut, Figure 4.10 shows the relationship between egg production and total length for wild halibut derived by Haug and Gulliksen (1988). This is superimposed on the egg production figures from year 2 where length measurements allowed similar treatment of the data. This exercise shows that in general, while the ambient group females compare poorly with the wild fish sampled by these authors, the chilled group did rather better in terms of egg production. Lower rates of fecundity in sub-optimum temperatures have been reported for other species. For example, studies on the requirements of broodstock sole in France demonstrated that egg output was lower following warm winters (Devauchelle *et al.*, 1987).



□ Ambient egg production

▲ Chilled egg production

— Fecundity/Length relationship ($F=0.019 * L^{3.624}$). After Haug and Gulliksen (1988)

Figure 4.10. Scatter plot showing relationship between total egg production per female and total length in year 2, for both the ambient and chilled stocks. The fecundity equation derived by Haug and Gulliksen (1988) is drawn on the chart to indicate the comparison between the two studies.

The quality of eggs from the ambient group was also reduced both in terms of fertilisation rate and hatch rate. It is possible that egg quality is compromised by high temperature through an increase in the rate of overripening. Temperature exerts a direct influence on the rate of post-ovulatory ageing which is accelerated at high temperature (Billard and Gillet, 1981). This would effectively narrow the window from ovulation to stripping during which good quality eggs could be stripped and one would expect at least some egg batches to be viable. However, egg batches obtained at high temperature from the ambient stock were universally poor.

Thermal preferences for reproduction have been investigated in a wide range of species and beyond limits of tolerance, negative effects on spawning performance range from a reduction in egg quality to total inhibition of spawning. For example, in the sea bass, temperatures exceeding 17°C can impair spawning but above 20°C, gonadal regression occurs (Zanuy *et al.*, 1986; Carrillo *et al.*, 1991, 1995). As with halibut in this study, high temperatures are detrimental to the quality of sole gametes and when gametogenesis takes place at temperatures higher than 13°C, egg quality is poor (Devauchelle *et al.*, 1987). In order to improve egg quality and egg production, it is evident that chilling is required. This requirement is not unique to halibut, and the value of water chilling was also demonstrated in terms of egg quality for Atlantic salmon spawning under natural photoperiod in Norway (Taranger and Hansen, 1993) and for sea bass (Carrillo *et al.*, 1995).

The observations on egg diameter revealed a tendency for egg size to decrease during the course of the spawning season. This might be explained by a gradual depletion of metabolic reserves during spawning resulting in decreasing amounts of yolk nutrients sequestered into successive batches of oocytes. A similar conclusion was reached by

Kjesbu (1993) following studies on cod. For the Atlantic halibut, the investment in egg production is considerable, with up to 30% of the body weight given over to egg production; furthermore, feeding is generally suspended during spawning.

A comparison between the egg sizes from the females from the two groups showed that in year 2, following a warm winter, egg size was significantly smaller in the ambient stock. This suggests that the amount of material incorporated into the oocytes of these females was affected by temperature, indicating that the thermal regime has an impact on vitellogenesis. Accounts of a relationship between egg size and temperature and/or time in the spawning season have been given in similar studies on other species. For example egg size tends to decrease during the spawning season of sole (Devauchelle *et al.*, 1987), turbot (Howell and Scott, 1989) and silver sea bream (Mihelakakis and Kitajama, 1995). The trend in the latter study was also coincident with increasing spawning temperature whilst a study by Kawaguchi *et al.*, (1990) directly demonstrated an inverse relationship between temperature and egg diameter for the Japanese anchovy. For the grey mullet lower temperatures result in insufficient yolk deposition, whereas higher temperatures can cause incomplete vitellogenesis and atresia (Kuo, 1995). If water temperature can exert an influence on the processes of vitellogenesis, then the metabolic reserves available during fertilisation and to the developing embryo following fertilisation might be affected. A recent study on the Arctic charr provided evidence that temperature regime during oogenesis and vitellogenesis has an important role to play in determining the amounts of lipids deposited into oocytes. The authors (Jobling *et al.*, 1995) found that Arctic charr exposed to high temperatures for a few weeks during the summer produced eggs with higher levels of triacylglycerols and lower levels of phospholipids. Furthermore, the phospholipid fraction contained lower proportions of certain essential

fatty acids including DHA and EPA.

Fisheries investigations on annual fluctuations in abundance of wild stocks have shown that seasonal temperature profiles can affect stock recruitment e.g. South African pilchard (Le Clus, 1992). Studying the effects of El Niño, Tanasichuk and Ware (1987) reported a relationship between fecundity in the Atlantic herring and water temperature. They suggested that warm temperature during a decisive period of vitellogenesis, occurring 60 to 90 days prior to spawning, determined the number of oocytes undergoing pre-ovulatory atresia possibly as a consequence of elevated GtH levels.

An interesting hypothesis was put forward by Kjesbu (1994) who suggested that localised water temperatures may be important in governing the synchronisation of spawning in wild cod stocks. Larger, more fecund females tend to spawn later than smaller individuals under the same thermal regime. He suggested that this effect is counteracted by a temperature differential experienced by different size classes. Higher temperature tends to advance spawning in cod and larger individuals may inhabit warmer water thus bringing ovulations forward. In this species, a decrease of 1°C can delay spawning by 8-10 days.

A number of hypotheses have been put forward to explain the mechanisms by which temperature can influence gametogenesis (Lam, 1983). Temperature may have a direct effect on gametogenesis, an influence on pituitary GtH secretion, or modify the rate of clearance of hormones. Changes in the responsiveness of the liver to steroid hormones or the gonad to pituitary hormones might also be induced affecting production of vitellogenins or steroids. There is evidence for all of these mechanisms in the literature. Pankhurst *et al.* (1996) held rainbow trout broodstocks at 9, 12, 15 and 21°C and recorded spawning characteristics and egg viability as well as levels of circulating GtH

and gonadal steroids. They found that between 9 and 15°C, spawning proceeded normally and egg quality was good. The number of ovulating females was reduced at 18°C and almost nil at 21°C, and none of the resulting eggs survived to the eyed stage. Though *in vivo* levels of GtH and plasma steroids were unaffected, they showed that production of gonadal steroids 17- β oestradiol (E₂) and testosterone (T) was depressed at high temperatures *in vitro* and the responsiveness of isolated ovarian follicles to steroid precursors or GtH was not retained as long at high temperatures. They concluded that temperature effects were most critical during final maturation and ovulation. Further evidence for a relationship between gonadal steroid secretion and temperature was given by Chang *et al.* (1993) for ayu. Lower levels of circulating T and E₂ were found in female fish exposed to high temperature resulting in an inhibition of vitellogenesis and the hepatosomatic index.

There is conflicting evidence regarding the effects of temperature on levels of pituitary GtH which may to a certain extent be due to species specific responses. Early work on rainbow trout suggested that high temperature depressed GtH production (Billard and Breton, 1977) whereas Pankhurst *et al.* (1996) did not report such a response. In cyprinids, the response to temperature elevation is generally an increase in plasma GtH (Gillet and Billard, 1977; Gillet *et al.*, 1978). In the latter study, high temperature inhibited gonadal development in the goldfish despite elevated GtH. More recent studies on another cyprinid, the honmoroko, *Gnathopogon caeruleus*, showed similar results in that high temperature stimulated GtH synthesis but inhibited oocyte development. The authors (Okuzawa *et al.*, 1989) were of the opinion that photoperiod effects on gonadal development were mediated by GtH but temperature effects were more likely to act through gonadal steroidogenesis. Later work on the same species led

the authors to postulate that high temperature might increase binding of GnRH to pituitary receptors leading to increase synthesis of GtH (Okuzawa *et al.*, 1994).

Evidence of temperature affecting the response of the liver to gonadal steroids was demonstrated in salmon when post-smolts were injected with E₂. At low temperature (3°C), the vitellogenic response was insignificant compared to that of fish held at 10 and 15°C (Korsgaard *et al.*, 1986). It is likely that temperature influences a range of processes associated with gonadal development right through to post-ovulatory follicle degeneration (Fitzhugh and Hettler, 1995).

For the Arctic charr, high temperature can cause retention of oocytes, atresia and accelerated maturation (Gillet, 1991; 1994) and for the hatchery broodstock, water chilling was needed to achieve a spawning performance comparable to that of the wild stock. Alternatively, it was proposed that photoperiod could be shifted to advance the spawning season into ambient temperature regimes which are more conducive to gamete quality.

Further work is required to establish the critical period over which temperature control is needed for Atlantic halibut. The ability to shift the spawning using photoperiod manipulation (Smith *et al.*, 1991; Holmefjord *et al.*, 1993; Naess *et al.*, 1996) enables the production of eggs and milt all year round. However, if shifted photoperiods are simply overlaid on ambient temperature, then spawning performance will be poor if fish are programmed to spawn at a time of year when the ambient temperature is high. If the requirement for low temperature is only during the spawning season itself however, then an advanced photoperiod regime, bringing the spawning season forward by say one month, will reduce the requirement for water chilling.

Whether the temperature effects described in this study impact during vitellogenesis or final maturation is unknown. In year 2, the spawning performance was drastically reduced in the ambient stock. Though the temperature during spawning was similar to that in year 1, at around 7 to 8°C, the preceding winter months were unusually warm in year 2. This fact, and observations on egg size, point to the need for chilling during gametogenesis and vitellogenesis as well as oocyte maturation and ovulation. Further work is required to investigate the precise timing of events involved in the reproductive cycle of the Atlantic halibut. In a study on turbot (Devauchelle *et al.*, 1988), spawning took place at a wide range of temperatures but though conditions during maturation did not seem to be critical, high temperature (>16°C) 1.5 months prior to spawning led to a significant decrease in egg viability. The authors concluded that temperature during gametogenesis was an important factor determining the success of fertilisation. Work on wolffish has indicated that temperatures below 10°C are required during the 4 months preceding final maturation (Pavlov and Moksness, 1996). Statova *et al.* (1982) showed that if the temperature rose to the level normally occurring during spawning for carp before completion of vitellogenesis, the maturation process was initiated and gamete quality was reduced.

The number of eggs released naturally by the broodstock in this study demonstrate that this species can spawn adequately in captivity without manual stripping. Though this method of egg production has been employed in Norway, it tends to be unreliable (Holmefjord and Lein, 1990). These egg releases occurred despite close observation of spawning rhythm which is crucial for optimising egg output and quality in view of the short window determined by the relatively rapid ripening processes (Bromage *et al.*, 1991; Norberg *et al.*, 1991).

On several occasions, it appeared that social interactions within a spawning group had a bearing on spawning time for individuals in the tank. The result was that a female apparently ready to begin ovulating would postpone final maturation until another member of the group had finished ovulating. It would be conjecture to explain this phenomenon but the role of pheromones cannot be ruled out. Research is currently under way to identify possible hormonal interactions between broodstock halibut (E. Vermiersen pers.comm.). Measures to combat such interactions include separating spawning females from non-spawners though this has yet to be properly tested.

All egg batches were incubated in the hatchery at 6°C. Eggs from the ambient group were stripped at temperatures of up to 10°C. Fertilisation was carried out at the same temperature as that of the broodstock tanks and following fertilisation, egg were stocked into static incubators at this temperature in a room with an air temperature of 6°C. This meant that eggs from the ambient group experienced a drop in temperature from the time of stripping to stocking both in production incubators and in the microtitre plates. A temperature change of up to 4°C over several hours was therefore inevitable and it is possible that this might have affected survival in these egg batches.

The supply of high quality eggs is an essential prerequisite for successful hatchery operation. To ensure this, optimum conditions must be provided for existing broodstock. Since a stable temperature of around 6°C is necessary for the adequate production of viable eggs from halibut broodstock, temperature control is recommended. For captive stocks located in U.K. facilities supplied with surface sea water, this will mean the provision of chilled water. This is true for stocks which spawn at the normal time of year under ambient photoperiod and for retarded or advanced stocks programmed to spawn in the summer or autumn months. A larger temperature differential at these times of year

will mean that the energy inputs required to chill supplies for the latter will obviously be greater. Research is currently under way to investigate the possibility of using recirculated water in order to help reduce the costs incurred by chilling.

Looking to the long term, new recruits for breeding stock will be required, either from the wild or from selected, high performing, hatchery reared populations. The first groups of fish reared in the U.K. have now started to mature and some of these fish have already been selected as potential broodstock. For the ongrower, whose priority is the production of meat, sexual maturation is to be avoided. The next chapter presents work on a method for the induction of sterility as a possible means for the prevention of gonadosomatic occurring at the expense of somatic growth.

4.5. Conclusions

- Spawning time and duration were affected by temperature profiles for both years. The average start spawning time was delayed by approximately 3 weeks in the stock held at ambient temperature compared to the group held at 6°C. The duration of the spawning season was also shorter for the ambient stock.
- Total egg production per female and for the whole group was lower for the ambient stock. However, the difference in relative fecundity, measured in the second year, was not statistically significant.
- Egg quality in terms of fertilisation rate and survival to hatching was adversely affected by high temperatures. Egg quality was lower in the second year in the ambient group following a warm winter. A decline in egg viability late in the season during both years was noticeable following a rise in temperature above 8°C.
- Egg diameter was significantly lower in batches spawned from the ambient stock in the second year compared to the chilled group. It is hypothesized that this may indicate that vitellogenesis was affected by the warmer ambient temperature experienced during the winter months preceding the second season.
- Ambient temperature profiles at Ardtoe are considerably different to those found on the natural spawning grounds and relatively high temperatures are likely in the months preceding and during spawning. For captive halibut broodstocks held at such locations, temperature control is necessary to ensure good egg production.

5. Induction of Triploidy Using Hydrostatic Pressure Shock

5.1. Introduction

An increasing production of juvenile Atlantic halibut in Northern Europe and Canada is resulting in an expansion of the ongrowing sector. One of the most important factors affecting the economics of ongrowing is growth rate and feed conversion efficiency of the stock. Analysis of wild populations (Jákupsstovu and Haug, 1988; Haug, 1990) and recent growth trials (Björnsson, 1994, 1995) have demonstrated sex dependant growth patterns for this species. Growth and maturation in the Atlantic halibut exhibits sexual dimorphism whereby males tend to grow at a slower rate than females and become sexually mature at an earlier age and smaller size. This characteristic is common to other flatfish; turbot (Imstrand *et al.*, 1997), sole (De Vecn, 1976), Indian halibut (Druzhinin and Petrova, 1980), flounder (Berner and Sager, 1985, cited in Imstrand *et al.*, 1997), dab, plaice and sand soles (Déniel, 1990) and is well known in other families e.g. salmonids (Bye and Lincoln, 1981; Lou and Purdom, 1984; Johnstone, 1993).

While early estimates from wild caught fish suggested that male halibut will mature at an average size of around 90-100cm (McCracken, 1958; Rae, 1959; Kohler 1967), the more recent study conducted by Jákupsstovu and Haug (1988) indicated that 50% maturation would occur at around 55-60cm corresponding to a weight of about 1.7kg. They found that no females were mature at less than 15kg, and 50% maturity occurred at a weight of 18kg. Males were smaller than females at all ages sampled and yearly growth rates for males and females were approximately 2 and 8 kg/yr respectively. The fact that maturity seemed to occur at a smaller size in this study than in earlier investigations led to the hypothesis that depletion of halibut populations through heavy

fishing pressure had caused a shift in size at maturity. The Faroese stock which was the subject of this study was, however, a relatively fast growing population compared to the captive stock studied by Björnsson (1995) who reported a roughly linear growth from 2 initial average weights of 1.8 and 3.2kg. Over 3 years, average growth was markedly different between males and females averaging 1.4 kg/yr and 3.2 kg/yr respectively with final weights of 7 and 12 kg being attained. Average weight at maturation was 3.2 and 12.7 kg for males and females respectively and fluctuations in growth were more pronounced in males. Indeed, males tended to lose weight during a period between December and March, coinciding with the spawning period. In a study on the same stock involved in a stocking density trial, Björnsson (1994) reported a two fold difference in the growth rate of male and female halibut. Despite a constant food supply and steady water temperature of 7°C, males showed a seasonal depression in growth rate compared to females, possibly related to annual fluctuation in photoperiod.

Concomitant with reduced growth there is also the possibility that carcass quality might be diminished due to maturation, from diversion of somatic metabolic reserves to gonadal growth and secondary sex characteristics e.g darkening of the skin. There are difficulties associated with sexing young juveniles, and a scarcity of published data on the growth rates of halibut below 1.5 kg. To establish growth rates realistic long term growth trials conducted in commercial scale facilities with optimum diets are required before it is known whether early maturation will pose a problem to the ongrower. Recent commercial growth trials in the U.K. have revealed a significantly slower growth rate in male halibut prior to market weight (D.Mitchell pers. comm.). Current thinking and consumer preferences presently suggest that farmed Atlantic halibut will be harvested undressed at a weight of between 2-6 kg. It is therefore likely that there will be a

disadvantage for the ongrower in having male fish amongst the stock.

Imsland *et al.* (1997) found that fast growing turbot generally matured earliest suggesting that maturity was related to size rather than age. Differences in growth between sexes may be due to a number of reasons. Lozán (1992) found that female dab had longer digestive tracts than males and hypothesized that females had a more efficient digestion than males. Roff (1983) put forward the theory that while large size might be an advantage in terms of fecundity for females, smaller size in males might be due to a survival strategy stemming from reduced foraging in order to reduce exposure to predation.

In order to combat the potential problems associated with sexual dimorphism and early maturation, hatchery managers have a number of tools at their disposal. These include hybridisation, the production of all-female stocks and sterilisation.

Hybridisation has been investigated in certain flatfish and beneficial effects stemming from hybrid vigour have been reported in plaice \times flounder and turbot \times brill and in some cases the resulting progeny can be sterile (Purdom, 1976; Purdom and Thacker, 1980). Though there are no reports of hybridisation with Atlantic halibut, experiments using halibut sperm to fertilise plaice or flounder eggs showed that activation occurs without fusion of the sperm and egg nuclei. Without further treatment this lead to a typical haploid syndrome but cold shocks restored diploidy to produce gynogenetic progeny (Purdom and Lincoln, 1974; Purdom, 1976). This technique was later used by Howell *et al.* (1995) with sole eggs and halibut sperm to produce gynogenetic sole.

The production of all-female stocks without the direct use of hormones requires knowledge of the sex determination mechanism of halibut and this is currently under

investigation. There is a strong possibility that the XY heterogametic male /XX homogametic female mechanism that exists in salmonids and some tilapia does not operate in halibut. The early work on the production of gynogenetic plaice (another Pleuronectid) using irradiated sperm (Purdom, 1969) or using halibut sperm (Purdom and Lincoln, 1974) and more recently on the production of gynogenetic sole (Soleidae) using halibut sperm (Howell *et al.*, 1995) resulted in the appearance of both sexes at maturation. This strongly suggests that in these two flatfish, the female is heterogametic (WZ) and the male is the homogametic sex (ZZ). The sex determining mechanism in the Japanese flounder (Bothidae) is reported to be of the XX female, XY male type but spontaneous sex reversal of gynogenetic females during early development can occur at extreme temperatures (Yamamoto, 1999). If the sex determining mechanism in the Atlantic halibut is similar to plaice or sole then the production of all-female stocks would require more complicated indirect techniques or direct sex reversal using hormone treatment.

Direct techniques to induce sterility in fish include the use of exogenous hormones (Hunter and Donaldson, 1983), surgical castration (Brown, 1982), gamma irradiation (Konno and Tashiro, 1982; Thorpe *et al.*, 1987) and auto-immune sterilisation (Secombes, 1987). All of these techniques have their drawbacks in terms of legality, consumer acceptance, risk to operators and cost.

The most common technique employed to induce sterility in teleosts involves the induction of triploidy (Thorgaard, 1983). Triploid fish are generally considered to be functionally sterile due to the failure of reduction division during gametogenesis (Ihsen *et al.*, 1990) which seems to be due to a disruption in the chromosome pairing mechanisms occurring during meiosis (Gui *et al.*, 1992). A number of methods have

evolved for the production of the triploid condition. Triploids may be produced from the crossing of tetraploids and diploids (Chourrout *et al.*, 1986). However, it is generally the case that the viability of tetraploids is low (Myers, 1986; Cassani *et al.*, 1990; Yamazaki and Goodier, 1993).

Most techniques commonly used to induce triploidy involve a traumatic interference to the normal cell cycle during the first divisions occurring shortly after fertilisation. In fish, the second meiotic division occurs shortly after fertilisation culminating in extrusion of the second polar body as the extra set of maternal chromosomes is removed from the genome. This process can be interrupted to induce triploidy (Thorgaard, 1983; Ihssen *et al.*, 1990) and various physiological shocks have been shown to be effective in preventing the extrusion of the second polar body. These include temperature shocks alone (Thorgaard *et al.*, 1981; Wolters *et al.*, 1981; Solar *et al.*, 1984; Cassani and Caton, 1985) or in conjunction with electrical shock (Teskeredzic *et al.*, 1993) or the use of chemical inhibitors such as colchicine (Smith and Lemoine, 1979), cytochlasin B (Refstie *et al.*, 1977) or nitrous oxide (Shelton *et al.*, 1986).

Hydrostatic pressure shocks have also been applied for these purposes and were first used to induce polyploidy in Amphibia (Dasgupta, 1962; Gillespie and Armstrong, 1979; Reinschmidt *et al.*, 1979) through interruption of second polar body extrusion or by blocking of the first mitotic division. Pressure shocks were first used in fish for the retention of the second polar body for the purpose of producing gynogens of zebra fish (Streisinger *et al.*, 1981). Numerous studies have since demonstrated the value of this method for the induction of polyploidy (Lou and Purdom, 1984; Benfey and Sutterlin, 1984; Chourrout, 1984; Johnstone 1985; Cassani and Caton, 1986; Benfey *et al.*, 1988). For some species, the use of hydrostatic pressure shock has proved more consistently

effective than thermal shocks for the induction of triploidy e.g. salmon (Johnstone, 1989) and Nile tilapia (Hussain *et al.*, 1991).

Apart from the benefits to the ongrower in producing sterile fish there are other considerations. As environmental concerns grow over the expansion of the aquaculture industry, there is increasing pressure for the physical as well as the genetic segregation of cultured stocks from native populations. The production of sterile fish is seen as one way that reproductive isolation can be achieved (Hindar *et al.*, 1991).

Despite the growing interest in the culture of the Atlantic halibut, there has been only one study on the production of triploids in this species (Holmefjord and Refstie, 1997). The authors employed both heat and cold shocks and concluded that cold shocks were effective in producing triploids. The resulting individuals were only reared until some point during yolk sac absorption.

In this study, the first preliminary data on the effective use of hydrostatic pressure for the induction of triploidy in the Atlantic halibut is presented. In an attempt to establish the feasibility of mass production of triploid halibut, half a complete egg batch was treated with hydrostatic pressure. To compare the viability of triploids versus their diploid counterparts, this treated batch was reared alongside their sibling diploid controls through the entire hatchery cycle.

5.2. Materials and Methods

5.2.1. Fertilisation and pressure shocking

Eggs were collected from spawning females as described in Section 2.3.1. Four egg batches, each from a different female were used in separate experiments and a sample of approximately 400 ml was used from each batch. Shortly after stripping, the eggs were transferred in a light proof insulated box to the cold room described in Section 2.4.1. where all procedures were carried out at 6°C. The eggs were mixed and then divided equally into lots according to the number of treatments; 8 for batches I-III and 4 for batch IV. Each sample of eggs was poured into a 5 cm diameter, 10 cm high sieve constructed from 5cm PVC pipe covered at one end with 1mm mesh. All sieves containing the eggs were placed in a 10 l plastic aquarium. Fertilisation occurred when 2 ml of fresh milt mixed with 2 l of sea water was poured into the aquarium and thoroughly mixed through the eggs. Two min after fertilisation (a.f.), the eggs were rinsed in clean sea water and then the first sample was divided into 3 lots, each lot poured into a 200 ml screw top jar with sea water. The lids were screwed down and tightened under water once any air bubbles had been expelled. The jars were then placed under the fresh water in the pressure vessel (see Figure 5.1), the lids were then loosened slightly to allow pressure equalisation and then the cap of the pressure vessel was screwed on to seal the pressure chamber. The pressure was then increased using the manual pump over about 30 sec until it reached 8500 psi above atmospheric pressure at 5 min a.f. The pressure was left at 8500 psi for 5 min after which time the pressure was released over a period of about 20 sec. The pressure vessel lid was unscrewed, the jars taken out and the eggs poured into clean sea water in 500 ml plastic beakers which were

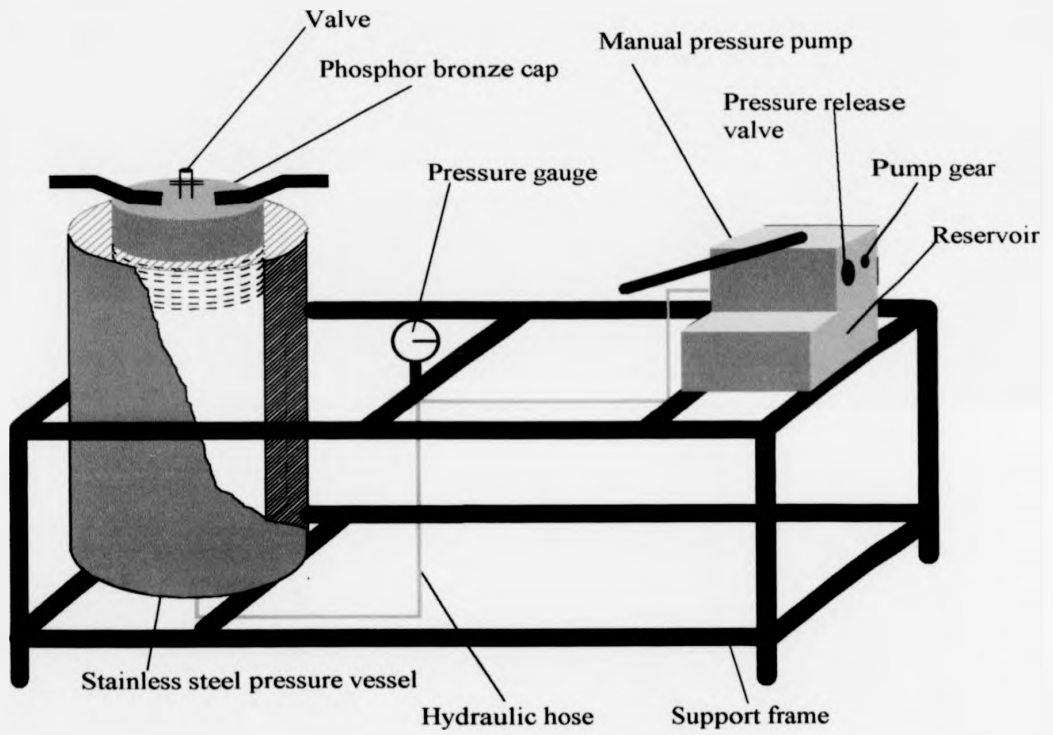


Figure 5.1. Diagram of pressure shocker.

placed in an incubator at 6°C. In experiments I-III this was repeated at 10 min intervals until 65 min a.f. and in experiment 4, intermediate times of 10, 20 and 30 min a.f. were used. In each experiment, a fertilised unshocked control batch was treated in an identical manner but the pressure treatment was not applied.

5.2.2. Post fertilisation checks and incubation

At 16 h post fertilisation at least 100 eggs from each replicate were examined under a dissection microscope to assess fertilisation rate as a proportion of floating eggs. For each replicate, one 96 well microtitre plates was prepared as described in Section 3.2.1. Fertilised eggs were stocked into each plate, one per well and the microtitre plates were incubated at 6°C until hatching when hatch rate was determined.

5.2.3. Ploidy determination

The protocol for chromosome preparations from halibut larvae was modified from the methods described by Kligerman and Bloom (1977), Chourrout and Itzkovich (1983) and Chourrout (1986). Newly-hatched larvae were carefully removed from the microtitre plates using a plastic 3 ml pipette and placed in petri dishes. Once the larvae had been removed from all the microtitre plates they were incubated at 6°C in 0.05% colchicine for 5 - 8 h. The tail and trunk of each larva was then dissected under the microscope using surgical needles, immersed in 0.4% potassium chloride hypotonic solution for 20-30 min and then fixed in Carnoys fixative (methanol : acetic acid, 4:1). This material was stored at 4°C for up to 3 months. The tissue samples were blotted and macerated in a few drops of 50% glacial acetic acid using a glass rod. After 10 min, the resulting cell suspensions were dropped onto slides placed on a slide warmer at 45°C using a capillary tube. The slides were then air-dried, stained in 10% Giemsa stain (BDH: diluted in

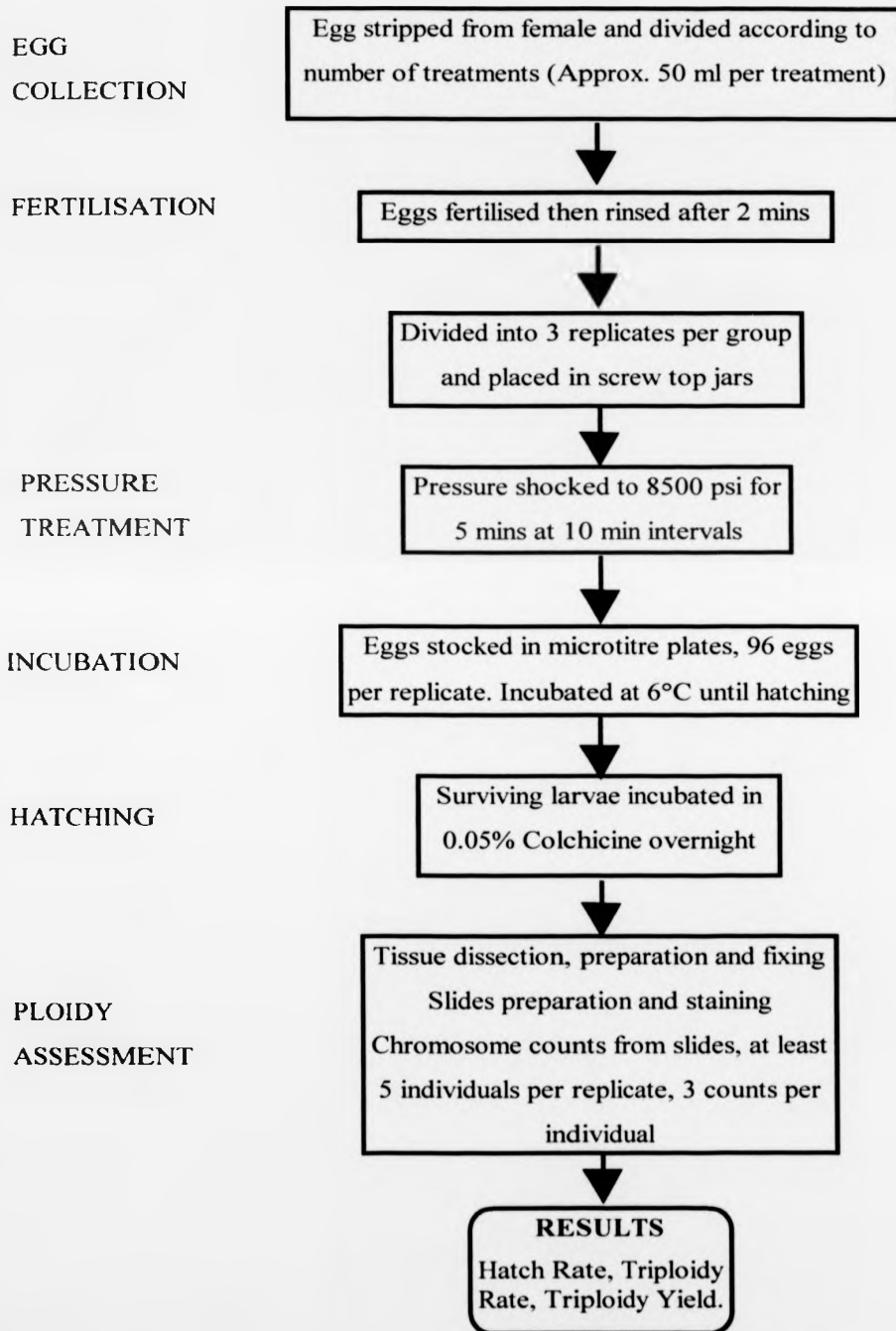
0.01M phosphate buffer) for 20 min, rinsed, dried, washed in xylene and mounted with cover slips using DPX mountant.

For each replicate where there were 5 or more survivors, 5 individuals were karyotyped and 3 counts were made from each individual. The diploid number of chromosomes has been determined as $2n=48$ (Brown *et al.*, 1997). The mean number from the 3 counts was calculated and chromosome counts within 5 of the triploid karyotype ($3n=72$) were judged to be triploid. Individuals with a chromosome number within 5 of the diploid number ($2n = 48$), were counted as diploid. Individuals falling outside these values were deemed to be either aneuploid where the standard deviation of the 3 counts was less than 10 or mosaic where the standard deviation was greater than 10. The rate of triploidy was expressed as a percentage of the total number of hatched larvae. Relative hatch rate was calculated as a percentage relative to the control batch for each experiment. The triploidy yield was expressed as % relative hatch \times % triploidy. A general summary of experimental procedures is shown in Figure 5.2.

5.2.4. Batch production of triploid Atlantic halibut

Using the optimum protocol developed in the initial experiments an attempt was made to produce larger numbers of triploid progeny. One entire batch of eggs, of a total volume of 825 ml was divided into two. One half was pressure shocked and the other half was treated in the normal manner. Both groups of eggs were then incubated separately in egg incubators, transferred to yolk sac conicals at hatching and to separate larval rearing tanks at first feeding and reared according to the methods described in Section 2.3. The survival rates of the diploid controls and the triploids were compared at each stage of the rearing schedule; fertilisation rate, survival to hatching, survival to

Figure 5.2. Flow diagram summarising experimental procedures.



completion of yolk sac absorption and survival from first feeding until weaning.

Both the controls and triploids were karyotyped as described above at hatching.

5.2.5. Statistical analysis

For replicated groups (control group and groups shocked 5, 15, 25, 35, 45, 55, 65 mins a.f.), comparisons of mean relative hatch rates and mean frequencies of each ploidy type resulting from different shock times were made using one-way ANOVA after arcsine transformation of percentage data. Where replication of ploidy analysis was not possible due to zero hatch rates, certain groups could not be included in the ANOVA.

5.3. Results

5.3.1. Survival to hatching

Table 5.1 shows the fertilisation rates and hatch rates for the control and pressure shocked groups from each egg batch. The fertilisation rates for all batches varied between 28.6% and 96.1%. Though there was not a great deal of variation between the fertilisation rates in the control groups for the different females, there was large variation between pressure shocked egg batches. For female I, all fertilisation rates were above 70% irrespective of treatment. Eggs from female II and particularly from female III seemed to be more affected by pressure shock and exhibited lower fertilisation rates when given pressure shocks at certain times after fertilisation.

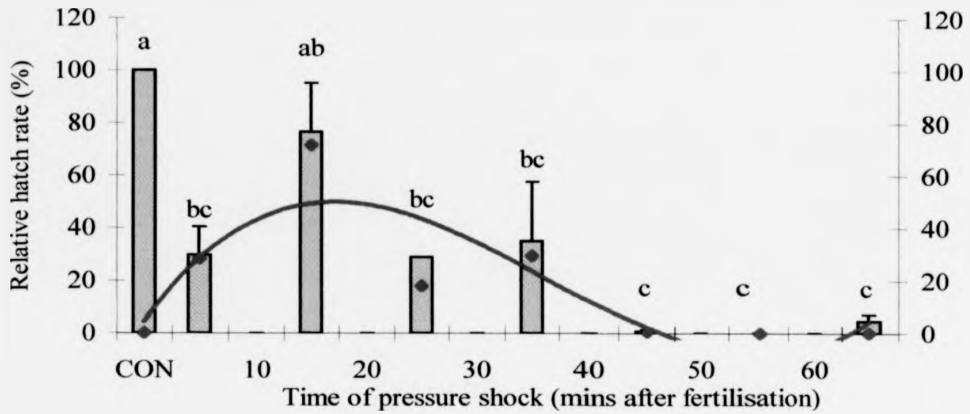
Hatch rates were highest in the control groups with the exception of those eggs from female I shocked 15 min a.f. and those eggs from female IV shocked 20 min a.f. Hatch rates were low in all groups, including the control group from female III and high in all groups from female IV. The latest shock given to eggs from female IV was 30 min a.f. All eggs shocked later than 35 min a.f. gave negligible or zero hatch rates. Although the hatch rates were very poor in egg batches from females I-III shocked 25 min a.f., eggs shocked 30 min a.f. from female IV gave a high hatch rate.

To compensate for the inter-batch variability and to allow comparisons between treatment times, the relative hatch rates were used. Figure 5.3. shows the relative hatch rates for each batch and treatment. Analysis of variance carried out on relative hatch rates for eggs from females I-III (where replication allowed) revealed that survival to hatch was significantly higher in control groups than in all treated groups except those

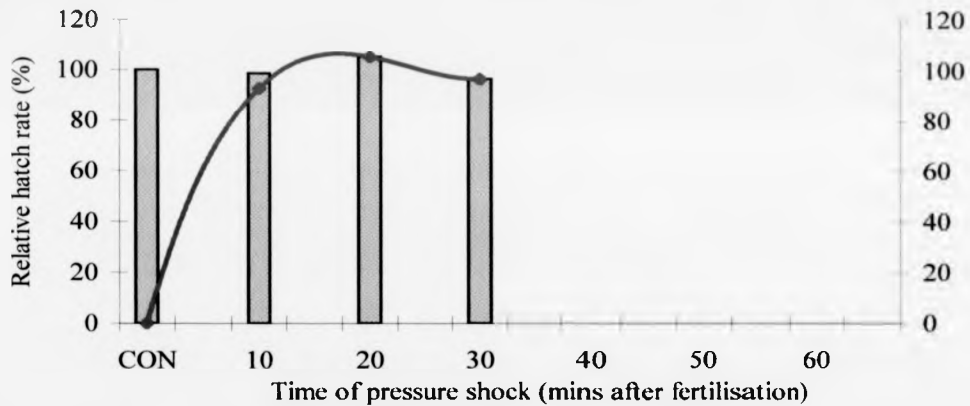
Table 5.1. Mean fertilisation rate and hatch rate of 3 replicates per female (\pm standard deviation) and for control and pressure treated batches from 3 females I-III shocked at 5,15,25,35,45,55 and 65 min a.f. (\pm s.d) and 1 female (IV) for 10, 20 and 30 min a.f.

Treatment time (min a.f)	Mean fertilisation rate (\pm s.d.)					Mean hatch rate (\pm s.d.)				
	Female					Female				
	I	II	III	IV	Mean	I	II	III	IV	Mean
CONTROL	84.1 (5.87)	89.0 (2.74)	86.3 (4.81)	96.1 (0.51)	88.8 (5.22)	49.7 (5.24)	80.6 (5.83)	36.5 (3.76)	75.4 (11.04)	60.5 (20.96)
5	71.7 (4.32)	79.5 (5.78)	59.2 (1.48)	* (0.51)	70.1 (10.22)	18.1 (3.18)	35.4 (14.69)	3.1 (2.76)	* (0.51)	18.9 (16.16)
10	* (0.51)	* (0.51)	* (0.51)	93.9 (2.97)	93.9 (2.97)	* (0.51)	* (0.51)	* (0.51)	74.3 (10.64)	74.3 (10.64)
15	88.8 (11.65)	84.5 (11.66)	57.9 (15.06)		77.0 (16.73)	55.2 (6.25)	57.3 (7.20)	17.4 (3.94)	* (0.51)	43.3 (22.48)
20	* (0.51)	* (0.51)	* (0.51)	93.8 (1.68)	93.8 (1.68)	* (0.51)	* (0.51)	* (0.51)	79.2 (2.08)	79.2 (2.08)
25	71.5 (6.21)	28.6 (8.54)	29.2 (3.18)	* (0.51)	43.1 (24.62)	28.8 (0.60)	0.0	0.0	* (0.51)	9.6 (16.64)
30	* (0.51)	* (0.51)	* (0.51)	94.3 (0.36)	94.32 (0.36)	* (0.51)	* (0.51)	* (0.51)	72.6 (3.18)	72.6 (3.18)
35	87.7 (15.48)	73.3 (14.21)	48.6 (16.38)	* (0.51)	69.9 (19.78)	39.9 (3.94)	10.8 (9.53)	4.2 (2.08)	* (0.51)	18.3 (19.03)
45	82.7 (1.45)	86.8 (3.45)	56.0 (14.25)	* (0.51)	75.15 (16.74)	0.0	1.7 (2.69)	0.0	* (0.51)	0.58 (1.00)
55	92.0 (6.9)	92.5 (1.77)	44.8 (21.32)	* (0.51)	76.44 (27.43)	0.0	0.0	0.0	* (0.51)	0.0
65	78.8 (3.21)	83.5 (3.55)	63.5 (12.06)	* (0.51)	75.27 (10.45)	4.2 (2.76)	4.5 (2.69)	0.0	* (0.51)	2.9 (2.51)

A: Females I-III



B: Female IV



Relative hatch rate ● Triploidy Yield
 Triploidy yield

Figure 5.3.(A) Results for females I-III and (B) results for female IV. Relative hatch rate (%) and triploidy yield (%) for larvae hatched from untreated (CON) and pressure shocked eggs shocked at various times after fertilisation. Where treatments were carried out on more than one batch i.e. females I-II, standard error bars represent standard errors of mean relative hatch rates. a,b,c denote significant differences between relative hatch rates (ANOVA $p < 0.05$).

shocked 15 min a.f. ($p < 0.05$). Hatch rates were also significantly higher in controls and those shocked at 15 min a.f. than all batches shocked more than 45 min a.f. ($p < 0.05$). Batch IV shocked at 10, 20 and 30 min a.f. resulted in hatch rates close to or even higher than 100% of controls.

5.3.2. Ploidy

Figure 5.4. shows the distribution of chromosome counts from each treatment. This shows that the modal count for the control groups was 48 confirming the diploid condition in the untreated fish. In all pressure shocked groups with the exception of those shocked 65 min a.f., the modal count was 72 indicating the triploid condition. The percentage of individuals amongst the hatched larvae showing the different levels of ploidy is given in Figure 5.5. The controls exhibited 100% diploidy and this was significantly higher than in all other treatments (ANOVA, $p < 0.05$). The mean frequency of diploids was significantly higher in the groups shocked 65 mins a.f. than in all the other shocked groups (ANOVA, $p < 0.05$). The triploidy rate among those groups pressure shocked between 5 and 45 min a.f. ranged from 93.3 to 100% and was significantly higher than in the controls and the batch shocked 65 mins a.f. Individuals exhibiting aneuploidy or mosaic type patterns were rare in all treatments and there were no significant differences between treatment groups. Figure 5.6 shows examples of triploid and diploid karyotypes.

The triploidy yield, a product of relative hatch rate and triploidy rate, is given in Figure 5.3. The best fit line fitted to this result indicates that at this pressure and temperature, high triploidy yields (above 90%) can be achieved when the pressure shock is administered between 10 and 20 min a.f.

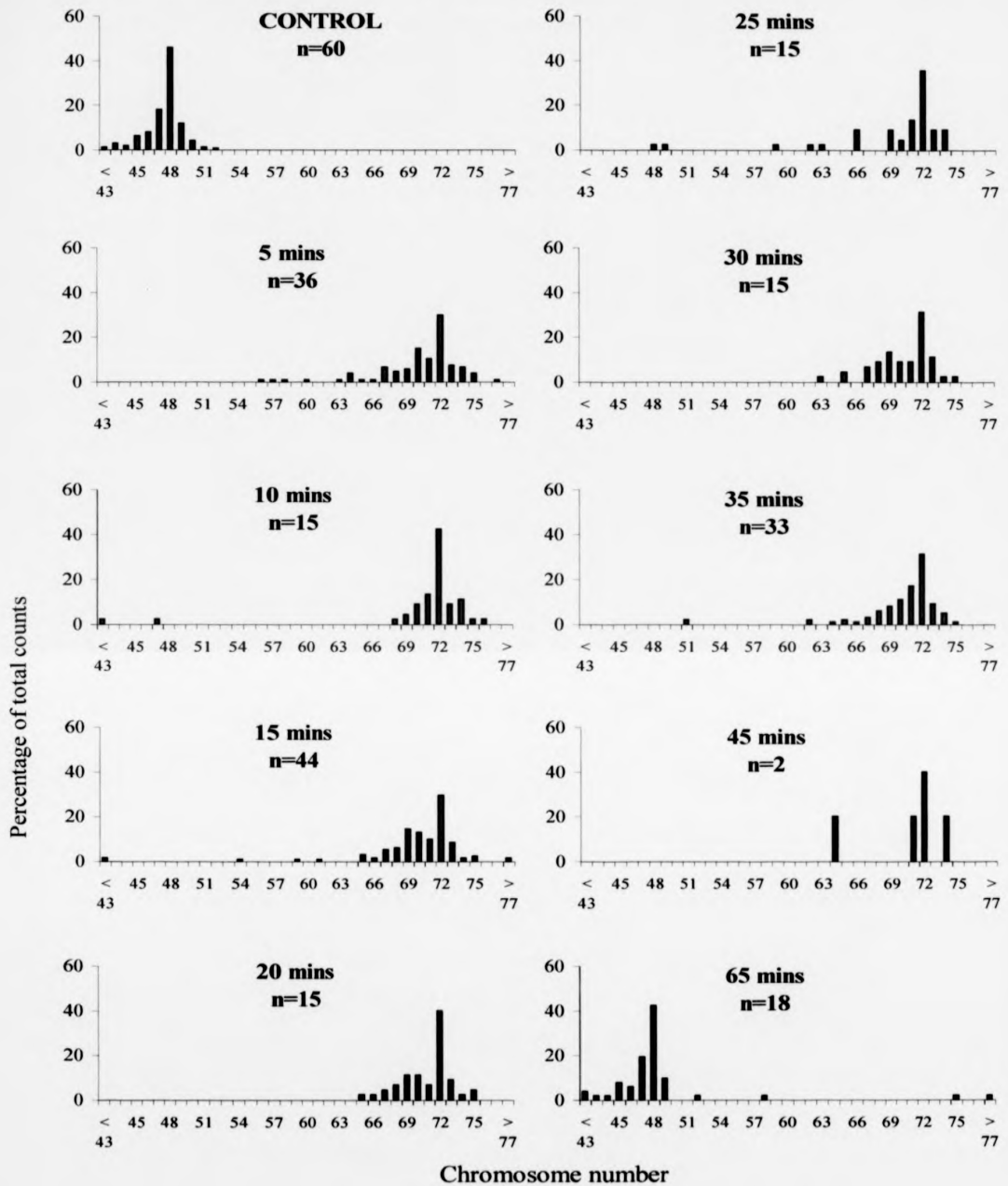


Figure 5.4. Percentage frequency distribution of chromosome counts for each treatment (time in mins after fertilisation of pressure shock and controls). Data are pooled from all experiments. The number of individuals karyotyped from each treatment (n) is given. There were 3 counts per individual.

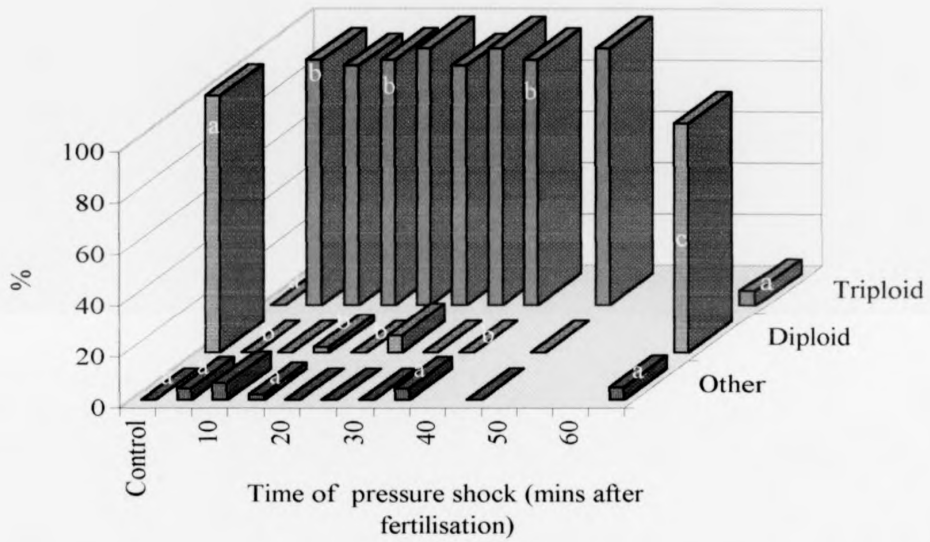


Figure 5.5. Percentage of diploid, triploid or other (aneuploid or mosaic) karyotypes found in hatched larvae from control and pressure treated groups shocked at various times after fertilisation. a,b,c denote significant differences between treatment means (ANOVA, $p < 0.05$).

A:



B:



Figure 5.6. Photomicrographs of examples of metaphase chromosome spreads from A: an untreated control (diploid) and B: a pressure shocked (triploid) group. Magnification x 3200.

5.3.3. Appearance of larvae

Observations of hatched larvae from treated and untreated eggs suggested that though the majority of larvae hatched normally, the incidence of deformities in hatched larvae or incomplete embryonic development was higher in treated groups. The rate of abnormality was not recorded but these features are indicated in the photographs in Figure 5.7.

5.3.4. Batch production trial

The pressure shocked group were administered an hydrostatic pressure shock of 8500 psi for 5 min starting 15 min a.f. The comparison between the control (diploid) and pressure shocked (triploid) groups taken through the whole rearing cycle is shown in Figure 5.8. The results indicate that for the batch used, triploids were much less viable than diploids at every stage of the cycle. Though fertilisation rates were similar, survival rates at each stage were lower for the triploid batch. Deformity rates, assessed at day 20 of the yolk sac stage were higher in the triploids and some examples are shown in the photographs in Figure 5.7. The cumulative survival rate for the control group was 1.9% compared to 0.1% for the pressure shocked group. Karyotypical analysis confirmed that the pressure shock group was 100% triploid as for the other groups.

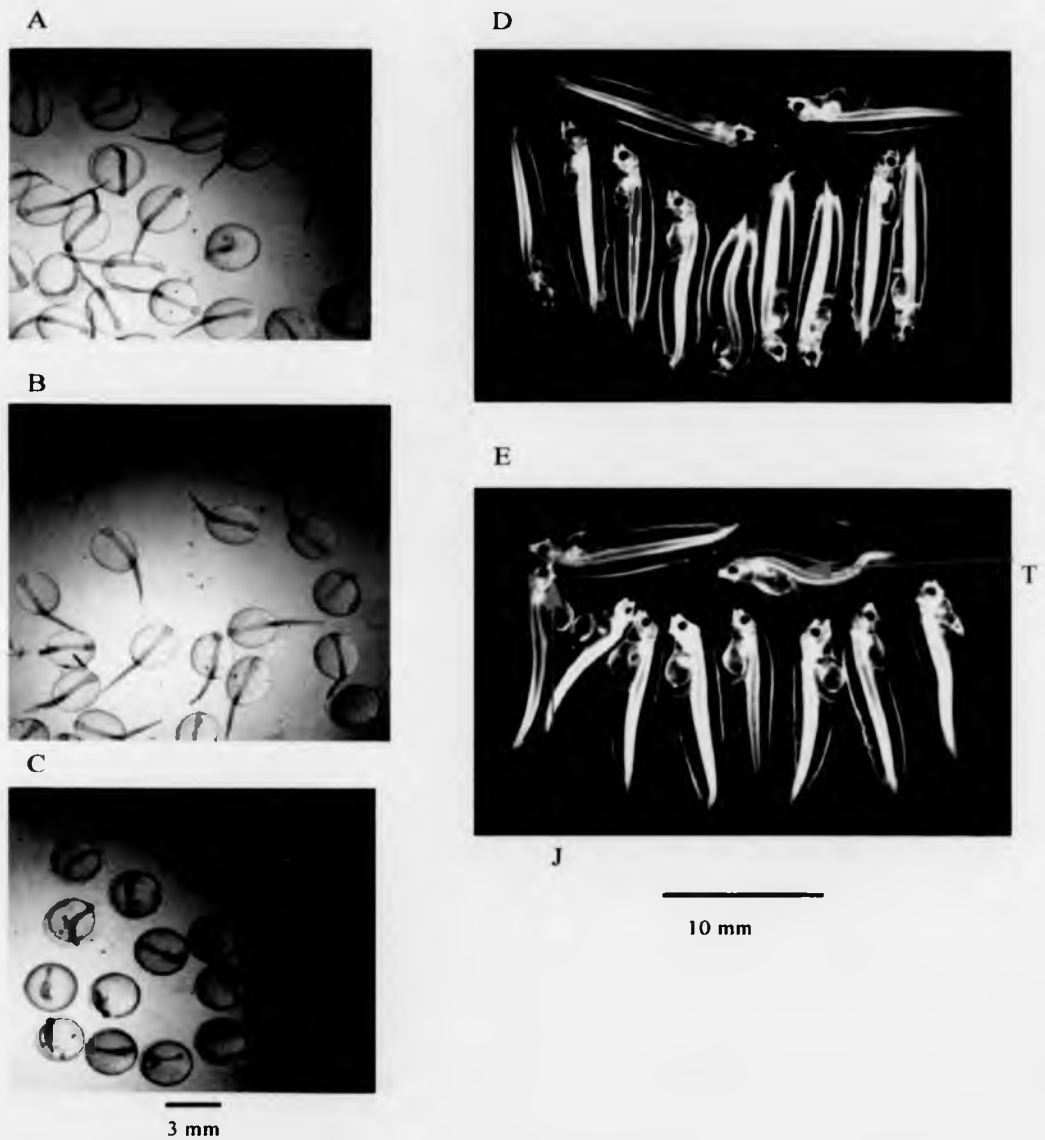


Figure 5.7. *A-C. Examples of larvae at hatching; A) Control (untreated), B) Pressure shocked (normal appearance) and C) Pressure shocked (abnormal appearance). Note many prehatched embryos with incomplete development. D-E. Larvae at end of yolk sac phase; D) Control and E) Pressure treated. Examples of jaw (J) and tail (T) deformities can be seen.*

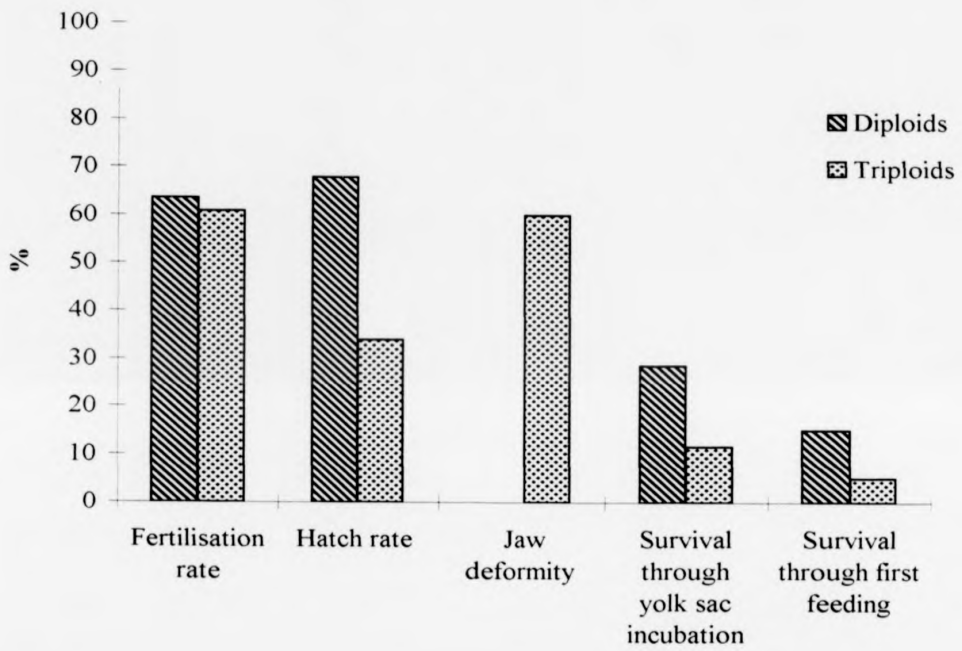


Figure 5.8. Percentage survival rates of triploid and diploid halibut through each stage of the hatchery process. The incidence of jaw deformity at day 20 of yolk sac stage is also shown.

5.4. Discussion

This work has produced preliminary results indicating the feasibility of using hydrostatic pressure shocks to induce triploidy in the Atlantic halibut. High rates of triploidy were obtained in larvae hatching from eggs shocked at 8500 psi for 5 min starting between 10 and 45 min a.f. Survival rates were generally lower than unshocked controls though relative hatching rates and triploid yields of close to 100% or even above were achieved when shocks were given between 10 and 20 min a.f. The high triploidy yields achieved in this study were of similar magnitude to those reported by Holmefjord and Refstie (1997) using cold shocks. Shocks applied later than 30 min a.f. resulted in poor survival and shocks applied later than 55 min a.f. produced low numbers of larvae which were mostly diploid. It appeared that the suppression of second polar body extrusion was best achieved at about 15 min a.f. and that the window of opportunity for doing so closed at about 50 min a.f. Though there was a high degree of variability between batches from different females in terms of survival of shocked eggs, there was consistency in terms of optimum timing of shocks for triploidy induction. The results show that the production of triploid Atlantic halibut using hydrostatic pressure is a relatively reproducible and reliable procedure which is neither complicated or time consuming.

Compared to the vast number of studies on triploidy induction in freshwater fish species, triploidy induction among marine fish has been reported relatively rarely. Successful attempts at blocking second polar body extrusion to induce triploidy or gynogenesis have been reported for plaice (Purdom, 1972), red and black sea breams (Arakawa *et al.*, 1987; Kitamura *et al.*, 1991; Sugama *et al.*, 1992), sea bass (Carrillo *et*

al., 1993; Gorshkova *et al.*, 1995; Colombo *et al.*, 1995; Felip *et al.*, 1997), gilthead sea bream (Gorshkova *et al.*, 1995; Garrido-Ramos *et al.*, 1996), California flounder and longsnout flounder (Yano and Sakai, 1988), sole (Howell *et al.*, 1995), hiramé (Tabata *et al.*, 1986), turbot (Piferrer *et al.*, 1997) and halibut (Holmefjord and Refstie, 1997). In the vast majority of these studies cold shock was the method employed.

In some species, pressure shock has proved more effective than temperature shock. This is especially true for warm water species where the window for interruption of second polar body extrusion is narrow due to the relatively rapid progress of cell divisions. For example, in a study on triploidy induction in Nile tilapia (Hussain *et al.*, 1991) both heat and pressure shocks were tested. Though heat shocks induced triploidy, they resulted in higher variability in the rates of survival and triploidy compared to pressure shocks. In a later study on the same species concerning the suppression of first cleavage for the production of gynogens, Hussain *et al.* (1993) found a wider window was available for pressure shocks (9000 psi, 40-50 min a.f.) than for heat shocks (28°C, 27.5-30 min a.f.). Similar findings were reported for triploidy induction of salmonids (Benfey and Sutterlin, 1984; Johnstone, 1985; Johnstone, 1989) and carp (Cassani and Caton, 1986). The sensitivity of the egg to interference with extrusion of the polar body changes rapidly following fertilisation (Cherfas *et al.*, 1990) and since the cell metabolic processes are extremely heat labile, a change in temperature will affect the speed of processes involved with cell division. An increase in temperature will effectively accelerate these processes and reduce the time during which the spindle apparatus, which controls cell division, can be affected and polar body extrusion or first mitosis can be suppressed. This makes it more difficult to accurately pinpoint the timing of a shock. It has also been suggested that pressure shocks affect a batch of eggs more uniformly than

temperature since pressure is transmitted uniformly through a medium (Malison *et al.*, 1993a) and eggs of different sizes may be differently affected by temperature shock (Teskeredzic *et al.*, 1993). For some species however, the use of temperature shock is more effective e.g. common carp (Linhart *et al.*, 1991). Though the use of temperature shocks provides a relatively inexpensive method of producing large numbers of triploids, industrial sized pressure vessels can cope with high volumes of eggs, as shown by Benfey *et al.* (1988). The pressure vessel used in this study could easily be used to shock up to 2 l of eggs at a time which would equate to approximately 80,000 eggs.

The results of these experiments demonstrate that the period of time during which polar body extrusion can be interrupted is relatively long with halibut. It is likely that this is due to the low incubation temperatures used which mean that early developmental processes progress at a slow pace. To relate the timing of physiological shock to embryonic age, a useful unit of measurement devised by Detlaff and Detlaff (1961) representing the duration of one mitotic cycle during synchronous cell divisions in early embryogenesis (τ_0), can be used. This can be calculated from the mean of the time periods between the first and second divisions and the second and third divisions (Shelton and Rothbard, 1993). This unit has been used in measuring and predicting the optimum timing for interruption of second polar body extrusion or blocking of first mitosis in carp (Gomelsky *et al.*, 1989; Cherfas *et al.*, 1990; Linhart *et al.*, 1991; Shelton and Rothbard, 1993). These authors found that the optimum time for interrupting second meiosis occurred between 0.03 and 0.26 τ_0 . Shelton and Rothbard (1993) determined that first mitosis can be blocked at between 1.2 and 1.9 τ_0 . Similar timings have been reported for tench (Flajshans *et al.*, 1993) where hydrostatic pressure applied at 0.07 - 0.17 τ_0 produced triploidy, while at 1.7-1.9 τ_0 tetraploidy resulted. To compare with

halibut in this study, τ_0 derived from a mean of the interval between the 2 cell stage and initiation of the 4 cell stage and between the 4 cell and initiation of the 8 cell stage (2 h and 5 h at 6°C respectively from Pittman *et al.*, 1990a; Brown, 1994) is 3.5. Relating this to the optimum shock time for triploidy induction found in this study of between 10 and 30 min at 6°C gives a comparable value of 0.05-0.14 τ_0 .

The triploid Atlantic halibut produced recently by Holmefjord and Refstie (1997) were reared through to some point during early yolk sac absorption. In the present study, constraints in both egg supplies and time meant that only one batch of eggs was used to test the viability of triploid halibut larvae through the entire production cycle. These are the first data comparing the viability of triploid and diploid fish in this species. It was apparent from this study that triploid individuals are less likely to survive through each stage of the production cycle. This result should be interpreted with caution given the fact that this was an unreplicated comparison dealing with a single batch of eggs. At all stages, from fertilisation to post metamorphosis, the triploid batch performed poorly compared to the untreated diploid control. However, in view of the batch variability in terms of fertilisation and survival to hatching demonstrated in the previous experiments, it is clear that good quality batches can be relatively unaffected by the trauma of pressure shock following fertilisation.

Variability in susceptibility to physical shocks has been found in other studies (Scheerer and Thorgaard, 1983; Lou and Purdom, 1984; Johnstone, 1985; Shelton *et al.*, 1986; Taniguchi *et al.*, 1986; Levanduski *et al.*, 1990) and much of this variability could be attributed to egg quality, possibly overripening (Lincoln *et al.*, 1974; Refstie *et al.*, 1982; Cassani and Caton, 1985; Diaz *et al.*, 1993). Where possible, egg batches chosen for triploidy induction should be of the highest quality. Whether viability, apparent in

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high survival to hatching seen in many of the experimental batches, can be extrapolated beyond hatching is unknown, but it is likely that batches with higher inherent viability would show higher survivals at all stages in the production cycle. To clarify this, more experiments designed to directly compare triploids against diploids from the same egg batch are needed. Given the low survival rates currently achieved in the rearing of halibut, it is unlikely that induction of triploidy will be adopted as a production procedure in the near future.

The performance characteristics of triploids and diploids have often been compared in other species and lower viability during rearing among triploids has been reported in red sea bream (Sugama *et al.*, 1992), rainbow trout (Lincoln and Scott, 1983), Atlantic salmon (Galbreath *et al.*, 1994), coho salmon (Johnson *et al.*, 1986) and tench (Flajshans *et al.*, 1993). It is not always the case however, as demonstrated by Joyce *et al.* (1994) who showed no difference between triploid and diploid hybrids of chinook salmon, chum salmon, and pink salmon in terms of yolk sac stage survival or salinity challenge at 90 days. In earlier studies no problems were reported in the rearing of triploid plaice (Purdom, 1972) or stickleback (Swarup, 1959). Comparisons between triploids and diploids reared separately or communally have indicated that diploids might out-compete triploids when maintained in mixed groups and there are also indications that triploids may be less robust under stressful conditions (Galbreath *et al.*, 1994). It has been postulated that lower viability in triploids may be due to higher levels of inbreeding or shock trauma (Solar *et al.*, 1984).

A higher incidence of deformity was observed in pressure treated larvae than in diploid controls both at hatching and at day 20 of the yolk sac stage. The cause may lie either with the shock treatment or with the triploid condition itself. Similar findings have

been reported in triploid pejerrey (Strüssman *et al.*, 1993), red sea bream (Sugama *et al.*, 1992) and Atlantic salmon (Sutterlin *et al.*, 1987). In the last study, the authors were able to isolate the cause of the deformities in salmon to ploidy status rather than shock treatment.

There was no indication of differences in the growth characteristics of triploids and diploids during early rearing in this study. Pioneers of triploidy induction in fish originally postulated that because triploids have larger cells than diploids, their body size would be larger. This however, is often not the case (Ihssen *et al.*, 1990) and there are conflicting results with respect to juvenile growth patterns from experiments with triploid fish. Valenti (1975) found that growth of triploid tilapia was faster during the first 14 weeks compared to diploids. Juvenile triploid Atlantic salmon were also found to grow faster in two studies by Benfey and Sutterlin (1984) and Galbreath *et al.* (1994). For juveniles of plaice x flounder triploid hybrids (Purdom, 1972), common carp triploids (Gervai *et al.*, 1980), triploid mud loach (Kim *et al.*, 1994) and triploid channel catfish (Wolters *et al.*, 1982) growth was not different from diploids. Thorgaard *et al.* (1982) and Solar *et al.* (1984) found that growth of triploid rainbow trout was in fact slower than diploids as was the growth of triploid common carp studied by Cherfas *et al.* (1994). The situation is further complicated by the possibility that the actual trauma used to induce triploidy might override the effect of triploidy itself as was shown in perch (Malison *et al.*, 1993b). These authors demonstrated that unshocked diploids grew faster than triploids but shocked diploids grew more slowly.

In view of the lack of corroborated positive evidence for the improvement of growth in juveniles of most species studied, it is likely that any benefits to the ongrower of halibut in terms of flesh production will come from preventing sexual maturation.

Growth improvements following sexual maturation resulting from triploidy have been reported for several species including channel catfish, *Ictalurus punctatus*, (Wolters *et al.*, 1982) and rainbow trout (Thorgaard, 1986; Chourrout *et al.*, 1986).

Long term trials are required to assess the effect of triploidy on growth and sexual maturation in the Atlantic halibut. In Atlantic salmon it has been demonstrated that though triploid males are functionally incompetent producing aneuploid sperm, they are endocrinologically competent (Benfey *et al.*, 1986). Conversely, the female triploids of this species are both functionally and endocrinologically incompetent (Lincoln and Scott, 1983). In general, the gonadosomatic index (GSI) is more reduced in female than male triploids. For example, in coho salmon, triploid males possess testes of a similar size to normal diploids (Pifferer *et al.*, 1994), whereas female triploids have reduced ovaries containing no oocytes. Similarly, triploid female hybrid plaice x flounder were found to be sterile, shown cytologically by the absence of normal oocytes in 6 month old fish (Purdom, 1972), though the testes of the diploids and triploids had a similar histological appearance. The sex ratio in Purdom's study was normal but in some studies it has been shown that the sex ratio can be affected by ploidy. For example Lincoln (1981a) who produced triploid plaice using cold shocks and reared them to 3 yrs found that males predominated more than in diploids. Furthermore, triploid males developed larger than normal testes which although macroscopically normal, produced abnormal spermatocytes and spermatids. Lower fertilisation rates were obtained from triploid sperm and steroid production was lower. However, it appeared that steroid production was sufficient to allow sexual development. It was concluded that the production of male triploids offered no benefit over diploids for aquaculture purposes. Conversely, the resulting females from triploidy induction developed gonads of less than half the size of

their diploid counterparts containing mostly undifferentiated cells and small numbers of abnormal oocytes (Lincoln, 1981b). Degenerate oocytes in reduced ovaries were also found in 3 year old triploid plaice, whilst the normal condition was apparently reverting by year 5. Lack of ovulation was hypothesized to be due to problems with expulsion of eggs due to reduced hormone levels. Following induction of triploidy in zebra fish (Kavumparath and Pandian, 1990) and red sea bream (Kitamura *et al.*, 1991), it was discovered that all, or almost all of the fish respectively, were male. Kitamura *et al.* (1991) concluded that either triploid females were less viable or that the sex determination mechanism of this species was affected by the treatment. Evidence from a recent study on triploid rainbow trout (Carrasco *et al.* in press) suggests that triploidy affects ovarian development at an early stage and causes gonadal hermaphroditism later on in females. In males, it was observed that development during the early proliferative and meiotic stages of spermatogenesis progressed normally. It appeared that triploidy only caused disruption of spermiogenesis resulting in a low occurrence of normal free spermatozoa and high numbers of spermatocytes or abnormal spermatids.

Apart from the relative viability, growth rates and sexual development of triploids, it is also important to establish how triploids perform under normal farm conditions. Other aspects such as feed conversion, stress tolerance and disease resistance need to be considered. The latter was investigated in diploid and triploid ayu exposed to a *Vibrio* challenge (Inada *et al.*, 1990) and no difference in resistance was found between the two ploidy states.

There are various techniques available for the assessment of ploidy in fish (see review by Thorgaard, 1983). These include measurements of blood cell volumes/diameters (e.g. Wolters *et al.*, 1982; Lou and Purdom, 1984), nuclear density (Johnstone and Lincoln,

1986), measurement of DNA content by fluorescence (e.g. Dubé *et al.*, 1991) or by flow cytometry (e.g. Utter *et al.*, 1983), by observation of phenotype e.g. melanophore counts (Purdom, 1972), by counting nucleolar organising regions or nucleoli (e.g. Phillips *et al.*, 1986; Felip *et al.*, 1997) or by using gel electrophoresis to examine allozyme markers (e.g. Sugama *et al.*, 1992). In many studies the results of such techniques are confirmed by karyotypic analysis. In this study chromosome counts alone were used. Though extremely laborious, this technique is both reproducible and reliable. None of the techniques involving preparations of blood cells are applicable to yolk sac larvae of the halibut since true blood cells are not developed until well into first feeding. Attempts to stain nucleoli with silver chloride were unsuccessful and more complex techniques involving measurement of DNA were not available on site.

This work was certainly not exhaustive in terms of defining the optimum conditions for triploidy production using pressure shock. Further work would be necessary to determine the optimum pressure and duration of the shock and whether there is any interaction between the various optima.

The first halibut ongrown in the U.K. to reach the market were harvested in 1997. At slaughter, undertaken at around 3kg average weight, males were smaller on average than females in this stock (D. Mitchell pers. comm.). The investigation of methods to reduce the proportion of precociously maturing males in a stock, either by sterilisation or through the production of monosex stocks will continue and it is hoped that this work will contribute towards this aim.

5.5. Conclusions

- Hydrostatic pressure shock is effective in inducing triploidy in Atlantic halibut.
- High survival rates to hatching and triploidy rates of up to 100% resulted in excellent triploidy yields when a shock of 8500 psi of 5 mins duration was administered between 10 and 20 mins after fertilisation (a.f.) at 6°C.
- Shocks applied later than 30 mins a.f. resulted in poor survival rates and when applied later than 55 mins a.f., the low numbers of survivors were mostly diploid. It was concluded that polar body extrusion had been completed by about 50 mins a.f. at 6°C.
- Ploidy status was verified by karyotypic analysis of colchicine treated hatched larvae.
- The incidence of deformed larvae at hatching seemed to be higher in pressure shocked batches.
- Preliminary investigation has indicated that pressure shocked triploid larvae are less viable than unshocked diploids through all stages of the hatchery cycle.

6. Weaning and Co-feeding

6.1. General Introduction

6.1.1. Weaning in the hatchery production cycle

The final phase of the hatchery production cycle begins with weaning and is perhaps one of the most critical. This stage marks the transition from live feed to the final diet type, ideally an inert formulated feed, which will probably continue until harvest. During weaning the larvae or fry must be established on an artificial diet which sustains them completely. The diet must therefore be ingested, digested, assimilated and fulfill all the nutritional requirements of the fish at that developmental stage, in order that the health status is maintained and good growth is promoted.

Prior to this, the animals have passed through two distinct nutritional phases. During embryonic development and subsequent yolk sac stage, nutritional sustenance is derived from endogenous reserves of maternal origin laid down during vitellogenesis and oocyte development. During the second phase, 'first-feeding', nutritional requirements for maintenance and growth are provided exogenously from live prey. During the endogenous feeding phase, rearing success is determined by inherent quality (maternal and paternal contributions), environmental factors (biotic and abiotic) and husbandry. Once exogenous feeding begins, additional factors come into play, notably, diet quality and its presentation.

The switch from endogenous to exogenous feeding is pre-programmed, and occurs at a specific developmental stage heralding the imminent exhaustion of yolk sac reserves and is basically beyond external control. For most fish larvae, exogenous feeding starts

before complete absorption of the yolk sac (Heming and Buddington, 1988). First-feeding for halibut larvae can start between 200°Cdays (Lein and Holmefjord, 1992) and 290°Cdays (Harboe *et al.*, 1997). The feeding of live prey, whether wild zooplankton as with semi-extensive operations or with cultured *Artemia* as in intensive operations, approximates to the natural diet of halibut larvae. The appropriate time for transition to inert diets, an essentially unnatural diet for a species which is carnivorous throughout its life cycle, is not outwardly so apparent or predetermined. It is likely that halibut juveniles would continue to feed on zooplankton indefinitely if it were the only food presented.

Most research to date on the rearing technologies for Atlantic halibut has focused on the primary areas of difficulty with production or "bottlenecks": egg production, yolk sac larvae incubation and first feeding. Whilst weaning is not considered a bottleneck to successful juvenile production, losses have been incurred at this stage in both Norwegian and U.K. hatcheries. The optimisation of this transfer from live to inert diets is crucial to the efficiency of the hatchery production cycle and deserves considerable attention. The investment in terms of labour and materials represented by animals surviving to this stage is substantial and losses occurring during weaning are particularly important and expensive.

6.1.2. *Inert diets versus live prey*

The Norwegian approach to the live feeding stage has until recently been essentially semi-extensive and based on the use of a mixture of wild or mesocosm-reared zooplankton, predominantly copepods, and hatchery-produced *Artemia* (Berg and Oiestad, 1986; Rabben *et al.*, 1986; Harboe *et al.*, 1990; Boxaspen *et al.*, 1990). In contrast, most U.K. commercial hatcheries have adopted an intensive approach relying solely on the use of *Artemia* which is initially presented as newly-hatched nauplii and

subsequently ongrown enriched metanauplii.

The provision of live prey has a number of drawbacks. It is costly both in terms of material and labour. For example, for the culture of sea bass, the provision of live prey represents up to 79% of the production cost of a 45 day juvenile (Coves *et al.*, 1991) and a considerable proportion of the total cost of a halibut juvenile (Sutherland, 1997). It requires dedicated facilities; tanks, lighting, heating, clean sterile water supplies, complex and high quality enrichment media and trained personnel. The culture of live prey can be problematic with variations in performance between batches in terms of survival and nutrition. Cultures are prone to sudden collapse (Watanabe *et al.*, 1980, 1983; Fukusho *et al.*, 1980; Tandler, 1985), particularly those involving dense enrichment media. Bioencapsulation is an inaccurate and quite unreliable technique for providing nutrition to young fish larvae and subject to the vagaries of *Artemia* nutritional physiology. *Artemia* are nutritionally suboptimal when compared to the natural diet (Witt *et al.*, 1984), consisting chiefly of copepods, which have a higher content of some essential fatty acids such as DHA, required by marine fish larvae (Watanabe, 1991; Sargent *et al.*, 1993). A high bacterial loading is commonly associated with *Artemia* cultures, despite best efforts to control this with the use of rinsing and physical or chemical disinfection (Verner-Jeffreys, 1996). The effect this has on the fish to which the *Artemia* are fed is often unknown but is likely to have a profound influence on the bacterial flora associated both with the fish and the culture tank. Supplies of *Artemia* cysts have historically been subject to fluctuation since the habitat from which they are harvested is part of a delicate ecosystem and the demand on this resource is heavy and likely to grow.

It is clearly desirable to reduce the use of live feeds as much as possible and replace them with inert formulated diets. The advantages accompanying the employment of inert

diets as opposed to live prey are numerous. Once prepared in bulk at the point of manufacture, they can be conveniently packaged, dispatched and stored ready for use. They require no preparation at the hatchery but simply need suitable storage conditions and are easily presented to the fish by hand or automatically with mechanised feeders. In theory, the diet formulation can be tailored to the nutritional needs of the culture species.

6.1.3. Total replacement of live prey with inert diets

Total replacement of living prey with inert diets from the onset of exogenous feeding has been accomplished for relatively few fish species. Adaptation to dry feeds is generally less of a problem with freshwater fish owing to their relatively large size and advanced stage of development at hatching. Salmonids for example, which hatch at a size of 12 to 25 mm and possess a functional stomach at first feeding can be fed exclusively on artificial diets from the onset of feeding (Dabrowski, 1984a; Jones *et al.*, 1993). Other freshwater species which can be reared successfully on artificial diets alone include whitefish, (Rosch and Appelbaum, 1985; Champigneulle, 1988), common carp (Charlon and Bergot, 1984; Charlon *et al.*, 1986), silver carp (Dabrowski, 1984b), ayu (Kanazawa *et al.*, 1985) and smallmouth bass (Ehrlich *et al.*, 1989). However, not all freshwater species can be successfully reared exclusively on dry diets as demonstrated for goldfish (Abi-Ayad and Kestemont, 1994), striped bass and hybrid striped bass (Baragi and Lovell, 1986; Tuncer *et al.* 1990), African catfish (Verreth and van Tongeren, 1989; van Damme *et al.*, 1990) and European minnow (Kestemont and Stalmans, 1992).

Conversely, marine fish larvae tend to hatch at smaller sizes (typically 2 - 3 mm) and an earlier stage of development. Kissil (1984) suggested that larvae of less than 7mm needed live feed at first feeding. In relation to most marine fish, the larva of the Atlantic

halibut is large at first feeding (12 mm) but is similarly primitive in terms of developmental stage (Blaxter *et al.*, 1983; Pittman *et al.*, 1990b).

Total replacement of live feeds has rarely been achieved for most marine species (Jones *et al.*, 1993) and the limited successes have always been at the expense of reduced growth and survival when compared to the use of live prey. Rearing on artificial diets exclusively has been attempted for plaice (Adron *et al.*, 1974; Garatun-Tjeldsto *et al.*, 1989), sole (Appelbaum, 1985; Gatesoupe *et al.*, 1977), sea bass (Gatesoupe *et al.*, 1977), gilthead sea bream (Kolkovski *et al.*, 1990), barramundi (Walford and Lam, 1991), cod (Garutun-Tjeldsto *et al.*, 1989), herring (Fox, 1990), red drum (Holt, 1993), sea bass (Saroglia and Ingle, 1989) and red sea bream (Kanazawa *et al.*, 1982).

6.1.4. Early transfer to inert diets

Much of the work on weaning of fish has focused on the timing of transition in order to define the earliest possible stage that live feed can be replaced by artificial diet without heavy losses. The early transfer to artificial diet is often difficult and may be associated with retarded growth, reduced survival and juvenile quality (Person le Ruyet, 1989a). In weaning trials with sea bass described by Person le Ruyet *et al.* (1993), conventional weaning at day 40-45 (20 mg) produces consistently good results (85% survival and 1.25 g at day 90) but earlier weaning at day 5 resulted in much poorer survival (15%) and less than 10% of the growth potential. However, substantial savings in live food (80%) are achievable when weaning is attempted at day 20 (3-4 mg) and acceptable growth and survival is attainable, although the incidence of skeletal abnormalities causing scoliosis and lordosis was higher. Early weaning of sea bass and a number of other marine species has been the subject of considerable attention by a number of

researchers and the results of some of these studies are summarised in Table 6.1.1. It is apparent that when weaning is attempted too early, high mortality and reduced growth are generally the result. In some instances a delay in development has also been observed. For example, Appelbaum (1985) showed that early weaning of sole resulted not only in poor growth but that metamorphosis was delayed. Similar results were obtained with milkfish by Duray and Bagarinao (1984).

6.1.5. Partial replacement of live feeds

Another possible strategy for the reduction of live feed consumption is to substitute part of the ration with inert feeds. This technique, often known as co-feeding, may involve partial substitution of live prey or supplementation over and above the usual live prey ration. Conventional weaning practices often involve a gradual transition between live and inert feed, hence there is often a period when the two overlap, so the distinction between weaning and co-feeding may become blurred. Co-feeding might be defined as a prolonged simultaneous presentation of live and inert diets without a reduction in either.

Co-feeding has been utilised successfully for some marine species where it has proved beneficial in terms of survival and/or growth compared to live prey alone or resulted in savings of live feed without detriment to larval performance. Examples of such experiments include those described by Person le Ruyet *et al.* (1989a) for 6 to 13 day old sea bass larvae, by Rosenlund *et al.* (1997) for 140 mg Atlantic halibut fry and by Comeillie *et al.* (1989) for 20 day old gilthead sea bream larvae. Improvements in

Table 6.1.1. Review of results from literature on early weaning and cofeeding experiments on marine fish larvae.

Species	Age	Initial Size	Regime	Results	Authors
Atlantic halibut		80-270mg	Different size groups fed microparticulate feed alone or in combination with <i>Artemia</i> for 2 weeks.	Larger larvae survived direct transfer to dry diet (~80% survival). Smaller size groups did not. Survival and growth always enhanced by cofeeding for 2 week period but no comparison to live feed control.	Opstad, 1995.
Atlantic halibut		140mg	Microparticulate diet fed in combination with 100% <i>Artemia</i> for 10 days, then with reduced <i>Artemia</i> for 10 days, followed by inert diet alone for 20 days (compared to <i>Artemia</i> control.)	Higher growth and survival in cofed group than control.	Rosenlund <i>et al.</i> , 1997
Turbot	d15-25 & 30-40	5mg-150mg	Review of weaning practices.	Weaning at d15, 25 or 30-40 results in average survival rates of 10-20%, 50-70% and 60-90% resp.	Person le Ruyet, 1989b.
Turbot	d10-30	6-17mm	Weaned abruptly or gradually to salmon diet at different sizes.	Survival 10-70%. Smaller larvae suffered high mortality with abrupt weaning but larger larvae could adapt. Feed response quicker for larger larvae.	Bromley, 1978.
Sole	d10-15	3.1mg	Weaned @ d10 from <i>Artemia</i> to compound diet.	Survival 28-60%.	Gatesoupe, 1983.
Sole	d14-20	18-190mg	Weaned on a variety of diets and at different sizes.	Best survival rate for smaller group (initial wt 18-92mg); ~65% and for larger group (initial wt 190mg); 76%. Growth rate 0.12 and 0.3mm/day resp.	Bromley, 1977.
Sole	d0-10		Transferred directly to dry diet on d0, 5 or 10 compared to <i>Artemia</i> only control.	Total replacement resulted in poor growth and survival (20% vs 60% for control). Transfer at d5 and d10 gave 20-50% and 50-80% survival resp. compared to 84-90% for control. Similar growth to control when weaned at d10.	Appelbaum, 1985.
Sole	d31	25mg	Weaned at d31 onto rehydratable pellets.	30-43% survival at day 131.	Dinis, 1992.
Cod		13,49 & 465 mg	Weaned 3 size groups from zooplankton onto different diets.	Moist feed gave the best results. Survival increasing with initial size was 39, 64 and 90% resp.	Ottera and Lie, 1991.
Sea bass	1st feeding	4mm	Compared 100% <i>Artemia</i> control to 100% inert diet or mixture.	Similar growth in all groups but lower survival on inert only (3.4% vs 16.5%).	Saroglia and Ingle, 1989.

Table 6.1.1. (Continued).

Species	Age	Initial Size	Regime	Results	Authors
Sea bass	d20 / d40	1.43-3.7mg vs 10 - 27mg	Direct weaning @ ~d20 compared to control @ d40. Assessed @ d60.	Similar or better survival than control with equivalent growth.	Person le Ruyet <i>et al.</i> , 1989a.
Sea bass	d6 / 13		50% substitution of live feed with microdiet from d6 or d13.	Better survival in cofed than control. Similar growth. Lower survival than weaning @ d22.	
Sea bass	d20 till 39	1.2mg dry wt	At d22, 27, 32 and 39, fed 14C labelled microdiet (MD) with or without enzyme supplementation alone or in combination with live prey.	Addition of <i>Artemia</i> enhanced assimilation of MD and improved growth. Addition of enzymes had no effect on growth or assimilation.	Kolkovski <i>et al.</i> , 1997.
Sea bass	d15-25		Weaned onto MD at d15 and 25 compared to live prey control.	Final weights proportional to time on live prey; 40, 21 and 7mg for control, weaned d15 and 25 resp.	Cahu and Zambanino Infante, 1994 & 1997
Sea bass	d23 and 30	4.4 & 10.8 mg	Control fed <i>Artemia</i> till day 50 compared to weaning @ d23 and d30.	Similar growth and survival for all groups but skeletal abnormalities in weaned groups.	Person le Ruyet <i>et al.</i> , 1989b.
Sea bass	1st feeding	5mm	Fed immobilised rotifers or <i>Artemia</i> and inert diet, <i>Artemia</i> (enriched or newly hatched), or rotifers and <i>Artemia</i> till d30.	Cofeeding immobilised rotifers and inert diet resulted in survival comparable to live feeds (>15%) and reasonable growth.	Barnabe and Guissi, 1994.
Sea bass	1st feeding	<2mg-4mg	Fed MD from 1st feeding or weaned onto MD @ d5 or d20 vs control @ d37.	Control survival and growth; ~50% and 35mg @ d40. Total replacement of live feed or weaning at d5 gave v. poor survival (~15%) and growth (10% of control). Weaning @ d20 resulted in similar survival and growth to control.	Person le Ruyet <i>et al.</i> , 1993.
Sea bass	d10-37		Weaned @ d10, 15, 20, 25 compared to <i>Artemia</i> control till d37.	Survival 0% in group weaned d10 but similar in other groups (~20-27%). Growth proportional to time on <i>Artemia</i> . <5, 7, 13 and 34mg @ d37 for weaning d15, 20, 25 and control resp.	Cahu and Zambanino Infante, 1994.
Milkfish	d2 till 20	3mm	Fed microbound diet (MBD) only compared to live prey alone or in combination with MBD.	Growth highest in cofed group and lowest in group fed only MBD. Survival in cofed group 50% of live prey control.	Marie and Duray, 1991.
Barramundi	1st feeding		Fed all-protein-membrane microcapsules alone or in combination with rotifers. Examined digestion.	Fed alone, microcapsules were ingested but not digested and 100% mortality resulted. Digestion of microcapsules occurred when fed in combination with rotifers; 2.4% survival.	Walford <i>et al.</i> , 1991.

Table 6.1.1. (Continued).

Species	Age	Initial Size	Regime	Results	Authors
Barramundi	d20 or d30	15-40mg	Weaned with 10% <i>Artemia</i> (live or frozen) + MBD (different formulations) for 10 days from d20 and d30 vs <i>Artemia</i> control.	Weaning at d20 generally resulted in lower survival (20-60%) but similar growth to control. Weaning at d31 resulted in high survival rate (90%).	Fuchs and Nedelec, 1989.
Gilthead sea bream	d20 to 46		<i>Artemia</i> vs dry diet + 50% <i>Artemia</i> .	Cofeeding resulted in similar growth and survival to control.	Cornellie <i>et al.</i> , 1989
Gilthead sea bream	d20-25	3.5-6.0mm	Feeding behaviour and gut contents of larvae fed mixtures of rotifers or <i>Artemia</i> with microcapsules observed.	Positive selection for live prey even when larger than optimum prey/mouth width ratio.	Fernandez-Diaz <i>et al.</i> , 1994
Gilthead sea bream	d8-22	0.2mg dry wt	Substituted 100, 80, 50 or 0% (control) of rotifer ration with MD.	Total replacement gave poor survival (6.3%). Replacement of up to 80% rotifers with MD gave similar survival to control.	Tandler and Kolkovski, 1991.
Red sea bream	d10	4.2mm	Tested 2 diets in combination with reduced live prey ration vs live prey control.	d40 better survival but lower growth compared to live feed control.	Kanazawa <i>et al.</i> , 1989.
Red sea bream	d3	3.2mm	Fed Agar polyacrylate MBD or Nylon protein MED only or rotifers only.	Poor growth and survival on artificial diets alone.	Kanazawa <i>et al.</i> , 1982.
	d10	4mm	Fed 50%MBD + 50% rotifers; 50% MED + 50% rotifers; MED only or rotifers only.	Growth of cofed groups inferior to rotifer fed groups.	
Red sea bream	d10	4mm	4 microbound diets substituted 67 - 88% of live feed vs live feed control.	Cofed groups all had better survival after d20 than live feed control. Growth similar or better than control and vitality dramatically improved.	Hayashi, 1995.
Japanese flounder	d10	4.5mm	As above.	Cofed groups showed similar survival and growth to controls. Vitality was improved.	" "
Red drum	d1-5	2mm	Cofed MD with rotifers for 1,2,3,4 or 5 days then on MD alone + control on rotifers alone.	Cofeeding until d5 produced similar growth and survival to controls weaned after 3 weeks. Shorter periods on live feed reduced survival and growth.	Holt, 1993.
Yellow-finned black porgy	d10	4 mm	100% live prey; vs 33%, 67% or 100% substitution with MD. Assessed at d30.	Best growth and survival from 67% substitution with MCD. Lowest growth and survival with MCD only.	Leu <i>et al.</i> , 1991.
Atlantic silverside		84mg	Artificial diet (prawn or salmonid flake) substituted with <i>Artemia</i> nauplii every 2nd, 4th or 8th day compared to <i>Artemia</i> control.	Growth proportional to <i>Artemia</i> ration but diets supported reasonable growth and similar survival to control.	Beck and Bengtson, 1979.

growth, survival and vitality were reported by Hayashi (1995) when substituting up to 88% of live feeds for Japanese flounder and red sea bream. Similarly, replacement of 80% of live prey with microdiet was possible without compromising growth or survival of young gilthead sea bream larvae according to Tandler and Kolkovski (1991). These and other co-feeding experiments with marine fish larvae are summarised in Table 6.1.1.

6.1.6. Experiments and aims

This chapter describes six experiments conducted on weaning which address weaning techniques for halibut larvae and on the effect of age and/or size, the developmental stage and the status or vitality of the larvae as they enter the weaning period.

A different batch of fish was used for each experiment and whilst there is little doubt that there are variations in the inherent quality between batches, the rearing practices preceding weaning are likely to have a strong influence on subsequent performance through weaning. The first experiment assessed the effects of different live feed regimes prior to weaning on the success of transfer to inert diet. The response to weaning was observed in a group of larvae which had been divided early in the first feeding stage and presented with differently enriched *Artemia*.

Possibly the most important criteria in relation to weaning is the timing of transition to formulated diets. The second experiment addressed this question directly by testing a standard weaning regime implemented on a batch of larvae at intervals of increasing age.

A population of hatchery reared larva is typically well dispersed in terms of individual size and this variation can be accentuated at weaning due to a differing response to inert diet. Experiment 3 was designed to evaluate the effect of size dispersion on weaning performance by comparing graded and ungraded groups of larvae from a single

population.

The potential of presenting inert and live diets together known as "co-feeding" was explored in the last three experiments. The first of these, a time series of short duration unreplicated tests of combinations of *Artemia* and formulated feed was designed to define the earliest possible stage that halibut larvae would ingest inert diet. This experiment served as a pilot trial for the subsequent experiment which pursued the investigation of co-feeding along with the use of frozen *Artemia* as a potential tool for conditioning halibut larvae to inert diets.

A final experiment was conducted to clarify the effect of co-feeding compared to an early weaning strategy and combined observations on behaviour and development with growth, survival and morphology.

The results from these experiments are discussed in terms of the response to weaning, evident in survival, growth and morphology, the implications to the efficiency of hatchery operations and in reference to knowledge of physiological and behavioural development of fish larvae.

The experiments are presented in chronological order and the techniques gradually evolved as experience was gained and the characteristics of halibut larvae were revealed. This is true not only of weaning techniques but also of the knowledge that has accrued from first-feeding experiments from season to season. In particular, growth rates through the first feeding phase increased dramatically over the 3 year period that this experimental work was conducted owing to an improved understanding of enrichment techniques and appropriate rates for feeding of live prey. This had a significant bearing on both the results obtained in the weaning experiments and on the final objectives.

The experiments are described in the following order:

Experiment 1. The effect of live feed history on weaning

Experiment 2. The effect of size and age at weaning

Experiment 3. The effect of size variation during weaning

Experiment 4. Co-feeding and early weaning pilot experiment

Experiment 5. Co-feeding and early weaning I

Experiment 6. Co-feeding and early weaning II

6.2. Experiment 1: The effect of live feed history on weaning

6.2.1. Introduction and aims

There are a host of potential criteria which may affect the ability of larvae to adapt to formulated feeds during weaning. Those pertaining to the characteristics of the larvae themselves, e.g. size, vitality, nutritional status and physiological development, are likely to be influenced strongly by the nutritional history and husbandry during the first feeding stage. Live feed history has been implicated in weaning success in a number of studies. Bromley and Howell (1983) and Gatesoupe (1982) showed that weaning performance in turbot was influenced by *Artemia* enrichment protocols during first feeding. Ablett and Richards (1980) demonstrated that feeding 24 h unenriched *Artemia* to sole larvae produced superior growth to 48h unenriched *Artemia* and that these differences persisted through weaning. Mourente and Tocher (1992) suggested that low levels of brain DHA in turbot larvae due to deficiencies in *Artemia* feeds could account for poor performance through weaning.

Intensive rearing techniques for marine fish involve the culture of live organisms to replace the wild zooplankton of the natural diet. The two organisms predominantly utilised are the rotifer *Brachionus plicatilis* (100 - 200 μm) and the brine shrimp *Artemia salina* (400 - 500 μm). The Atlantic halibut is large at first-feeding in comparison to most marine species and is able to consume *Artemia* nauplii directly from the initiation of exogenous feeding. The nutritional content of *Artemia* alone is sub-optimal for feeding to halibut larvae and once the larvae are large enough to consume ongrown *Artemia*, their nutritional profile can be enhanced using a technique known as "bioencapsulation". *Artemia* are filter feeders and will indiscriminantly ingest particulate

nutrients suspended in their surrounding medium. There has been considerable effort directed towards the development of enrichment media to boost the nutritional value of *Artemia*, particularly essential fatty acids and a number of proprietary commercial compounds are available. Much of the work at S.F.I.A. Ardtoe on the live feeding stage has been concerned with experiments evaluating the potential of various commercial and experimental enrichment media for halibut larvae (e.g. Shields and Bell, 1995; Gara *et al.*, in press). The fish from one such experiment were the subject of the following study.

This experiment was designed to assess whether possible differences in nutritional status brought about by different *Artemia* enrichment strategies during first feeding emerge through weaning and affect the adaptation to dry diets.

6.2.2. Materials and methods

6.2.2.1. Live material and facilities

Five groups of fish from a single batch of larvae were used for this experiment. Each group had been fed differently throughout the first feeding period until the start of this experiment. The fish were aged 83 days post first-feeding (PFF) or 1453°Cdays post hatch. The mean blotted wet weights varied between 114.18 - 229.41 mg and dry weights varied between 23.80 - 51.86 mg. The live feed history of each group and their respective initial wet and dry weights are given in Table 6.2.1.

During first feeding the first 4 groups were reared in the same experimental facility used for this experiment and the fifth was reared in a production tank (1 m³).

The fish were allocated randomly to 11 tanks. For the 'Production', 'Tuna' and 'Selco' groups, 3 replicate tanks were stocked with between 25 - 29 fish per tank. Due to poor survival through the live feed stage, there were sufficient fish to stock only one tank of 31 and 27 fish for the 'Herring' and 'SSF' groups respectively.

Artemia fed during the experiment were enriched with either SSF alone or SSF and Selco according to the protocols described in Table 6.2.1.

The experiment was conducted in a light-proof room and the air temperature was maintained with an air-chiller. The tanks were black polyethylene, flat-bottomed and cylindrical, (height: 70 cm, internal diameter: 50 cm). The water supply was filtered (5 µm), U.V. sterilised seawater entering the tank via a surface inlet reduced to 1mm and oriented tangentially to give directional, circular flow (300 ml/min).

Table 6.2.1. Live feed history, initial blotted wet and dry weights of the 6 groups of halibut larvae to be used in Experiment 1. Values are the mean of 10 individuals for wet weight and 10 individuals for dry weight \pm s.d.

Group	Enrichment protocols for <i>Artemia</i> fed to halibut during first feeding period. Enrichments carried out at 27°C.	Initial wet wt in mg (\pm s.d.)	Initial dry wt in mg (\pm s.d.)
SSF	Ongrown for 24 h with SSF microdiet (0.6 g/l) then a further 24 h with the same product.	120.95 (41.87)	23.80 (8.15)
SELCO	Ongrown for 24 h with SSF microdiet (0.6 g/l) then a further 24 h with Super Selco (0.6 g/l).	189.43 (58.62)	37.90 (20.07)
TUNA	Ongrown for 24 hs with SSF microdiet (0.6 g/l) then a further 24 h with tuna orbital oil.	229.41 (95.37)	51.86 (17.36)
HERRING	Ongrown for 24 h with SSF microdiet (0.6 g/l) then a further 24 h with herring oil.	114.18 (66.27)	32.69 (10.89)
PRODUCTION	Reared in the "production system" but fed on <i>Artemia</i> enriched with the same products as the Selco group.	119.79 (21.63)	20.44 (4.52)

The central bottom outlet (3 cm diameter, covered with 1 mm square mesh) was connected to an external 15 mm standpipe rising to an elbow giving a water depth of 40 cm, then a down-pipe taking water to waste.

Aeration was supplied to each tank with a single airstone. Overhead tungsten lights were fitted above each tank giving illumination of approximately 10 lux at the water surface. Water temperature was 16.4 ± 2.5 °C, salinity 33.9 ± 0.7 ppt and dissolved oxygen 8.1 ± 0.85 mg/l.

6.2.2.2. Feeding regime and husbandry

Weaning began on day 84 PFF. For the first 5 days of the experiment, *Artemia* was fed twice per day, at a rate of 500 prey/fish/feed, first at 14:00 hrs and again at 18:00 hrs. The first feed was SSF /Super Selco enriched *Artemia* and the second feed was SSF/SSF enriched *Artemia* (see Table 6.2.1.). After 5 days, the first feed was withdrawn and only one feed of SSF/Super Selco *Artemia* was given at 18:00 hrs. The proportion of fish ingesting inert diet was observed by eye from a sample of 10 fish per tank. Once the feed response in a tank had reached 50%, the *Artemia* feeds to that tank were reduced to one feed on alternate days for 4 days, then ceased altogether.

Inert diet (Nutra Marine, T.Skretting AS, Stavanger, Norway) was presented to all tanks from day 84 PFF several times per day by hand. Crumb size increased gradually from 0.3-0.5 mm, 0.5-0.8 mm and 0.8-1.4 mm as the fish grew.

Tank bottoms were cleaned once a day with a siphon to remove waste feed and faeces. Water quality parameters (dissolved oxygen, salinity and temperature) were measured on a daily basis.

6.2.2.3. Sampling

Fish were sampled for unblotted and blotted wet weight and dry weight at the start of the trial (day 84 PFF). To obtain intermediate sample weights for a growth curve, a sample of 10 fish per tank were removed once per week, weighed live unblotted and replaced. Final wet and dry weights were obtained from a sample of 10 fish per tank at the end of the experiment on day 107 PFF, 1830°Cdays post hatch.

6.2.2.4. Vitality tests

At the end of the trial (day 107 PFF) 10 fish per tank were subjected to a salinity challenge. These fish were placed in 3 litre rectangular plastic boxes containing aerated sea water of 65 ppt salinity at the same temperature as the weaning tanks. Every hour, mortalities were counted, removed and weighed (wet and dry weight).

Cumulative mortality was recorded and an index of vitality obtained from the sum of hourly cumulative proportional mortality values in a fashion similar to that proposed by Dhert *et al.* (1990).

6.2.2.5. Statistical analysis

Final weights were analysed with two factor ANOVA (General linearised model for unbalanced design) with live feed history and tank as factors and tank as a random factor nested within treatment. Survival rates, stress indices, feed response and SGR were compared using one way ANOVA on replicated treatments.

6.2.3. Results

6.2.3.1. Survival

Figure 6.2.1. shows the cumulative survival over the experimental period. There was a steady mortality in the 'SSF' group starting at day 91 PFF and a later mortality in the 'Tuna', 'Selco' and 'Herring' groups starting at day 104 PFF. Final survival rates shown in Figure 6.2.2. range from between 51.6% for the 'Herring' group to 88% for the 'Production' group. There were no differences between the replicated groups (ANOVA, $p > 0.05$). The unreplicated groups could not be included in the analysis.

6.2.3.2. Feed response

The feed response of the five groups is shown in Figure 6.2.1. The 'Tuna' and 'Selco' groups adapted to inert diet most rapidly while the SSF and Herring groups were slowest to respond. No group reached 100% response (as % of total number of remaining fish). In the 'Tuna', 'Selco' and 'Herring' groups, some fish that had actually begun ingesting dry diet did not survive. The mean number of days for each group to reach 50% response to inert diet were 7.7, 9.0, 10.7, 11.0, and 11.0 days for 'Tuna', 'Selco', 'Production', 'Herring' and 'SSF' groups respectively. Differences between groups in this respect were not significant (ANOVA, $p > 0.05$). A plot of mean weight for each tank at day 91 against the time taken to reach a 50% feed response (Figure 6.2.3) shows a significant negative correlation ($r = -0.827$, $p < 0.001$).

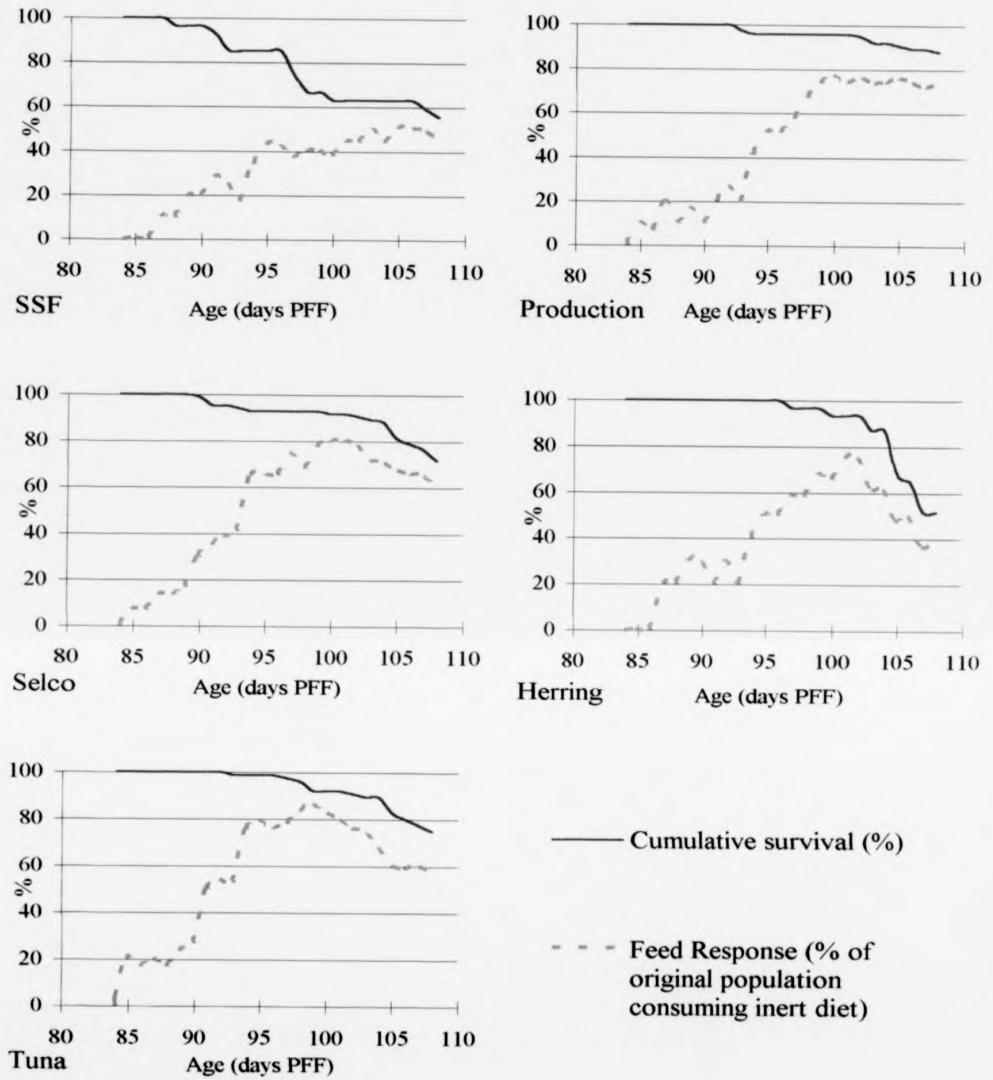


Figure 6.2.1. Cumulative survival (%) and feed response (% of original population taking inert diet) during the course of the experiment. Values are based on the mean of 3 replicates for each treatment except 'SSF' and 'Herring'.

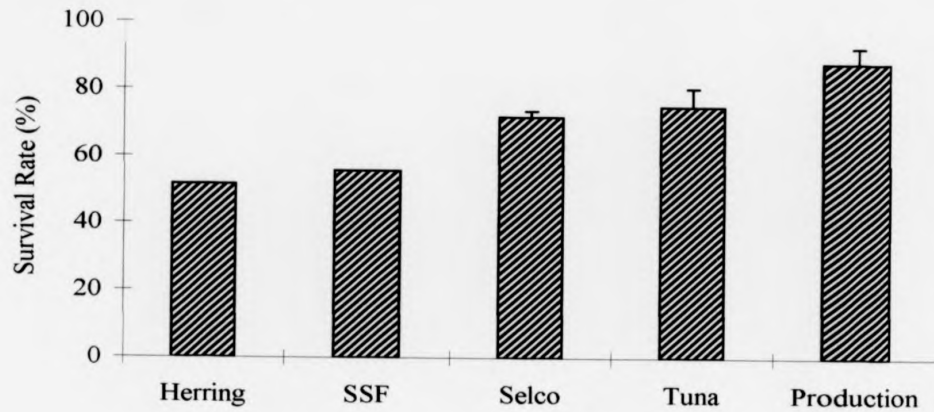


Figure 6.2.2. Survival rate (%) at day 107 PFF for each group. Error bars represent standard error of mean of 3 replicates in replicated groups. Herring and SSF groups were not replicated.

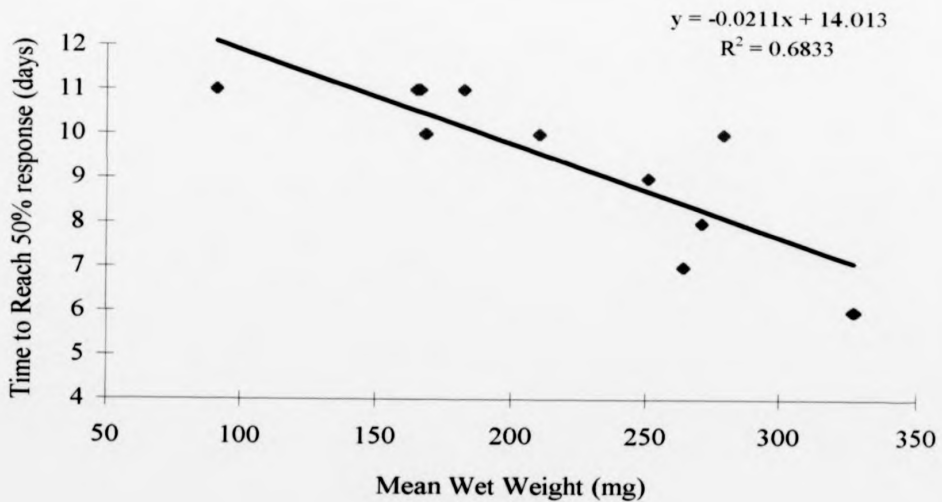


Figure 6.2.3. Regression of time taken for a tank of fry to reach a 50% feed response (days) against mean wet weight (mg). Regression equation and R^2 value are given.

6.2.3.3. Growth

Final wet weights were highest in the 'Tuna' and 'Selco' groups and lowest in the 'SSF' and 'Herring' groups (see Figure 6.2.4). There was considerable variation in all groups. The largest individuals were found in the 'Tuna' group and the smallest in the 'Herring' group. The maximum and minimum wet weights, in mg for each treatment were 46.3 and 241.7 (Herring), 64.1 and 317.3 (SSF), 73.4 and 691.2 (Selco), 112.5 and 1087.5 (Tuna), 80.1 and 544.2 (Production).

A preliminary test with two way ANOVA revealed no significant difference in mean wet weights between replicates among treatments for replicated groups 'Selco', 'Tuna' and 'Production'. It was therefore assumed that a tank effect could be discounted and replicates were pooled to allow comparison with the unreplicated treatments. Although analysis with oneway ANOVA shows a significant difference between final wet weights of different groups, the Tukey test did not detect pairwise differences. It was concluded that no significant differences exist between treatments for wet weight (ANOVA, $p > 0.05$). Analysis of dry weights (see Figure 6.2.5.) revealed a significant difference between 'Herring' and 'Tuna' groups with the lowest and highest mean dry weights respectively ($p < 0.05$).

Figure 6.2.6. shows the growth curves for the five groups based on the unblotted wet weights. Initial differences in wet weights were maintained throughout the experiment for the 'Production', 'Tuna' and 'Selco' groups. The 'SSF' and 'Herring' groups which started the experiment with mean weights similar to the 'Production' group grew more slowly. Specific growth rates shown in Figure 6.2.7. reveal that the 'Tuna' group had the lowest specific growth during the experiment while the 'Production' group

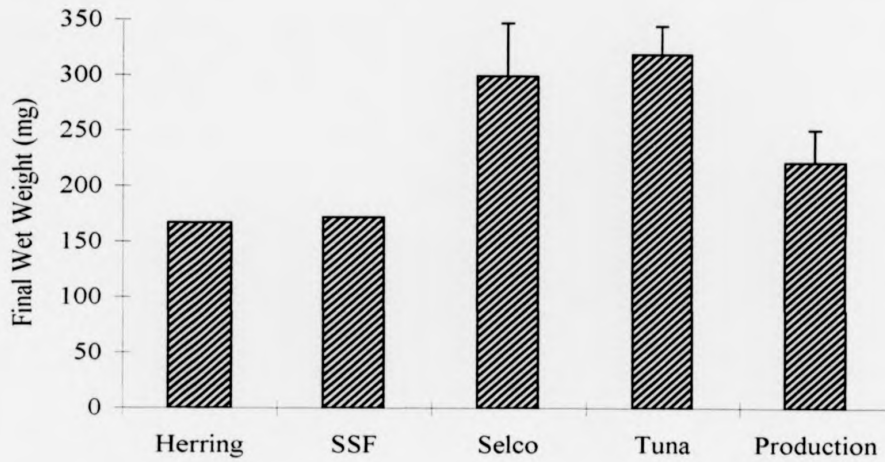


Figure 6.2.4. Final wet weight (mg) at day 107 PFF for each group. Error bars represent standard error of mean of 3 replicates in replicated groups. Herring and SSF groups were not replicated.

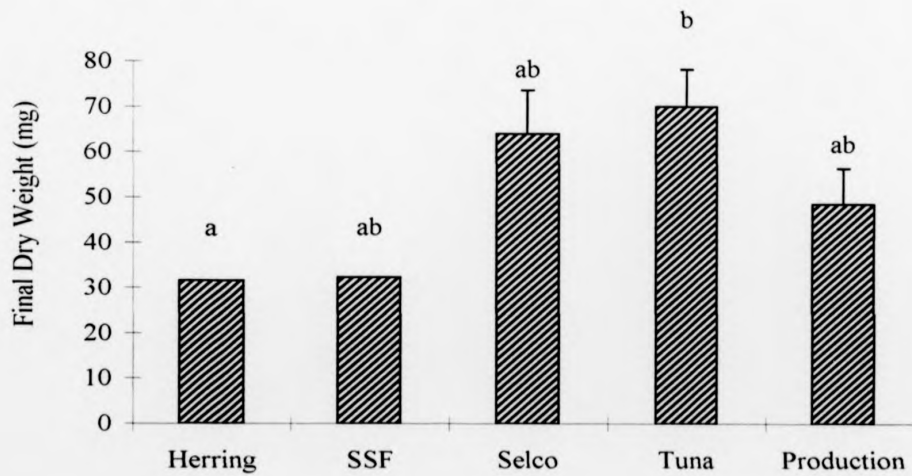


Figure 6.2.5. Final dry weight (mg) at day 107 PFF for each group. Error bars represent standard error of mean of 3 replicates in replicated groups. Herring and SSF groups were not replicated. a,b denote significant differences (ANOVA $p < 0.05$)

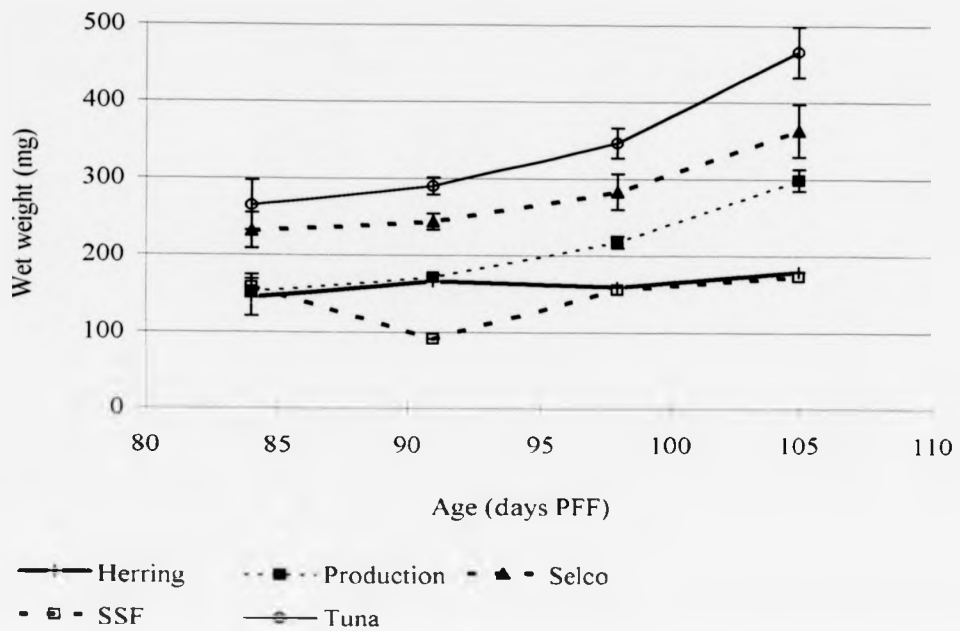


Figure 6.2.6. Growth during experimental period of 5 groups of fish. Error bars indicate standard error of mean of 10 individuals (initial sample) or 3 replicate groups for subsequent samples except for Herring and SSF which were not replicated.

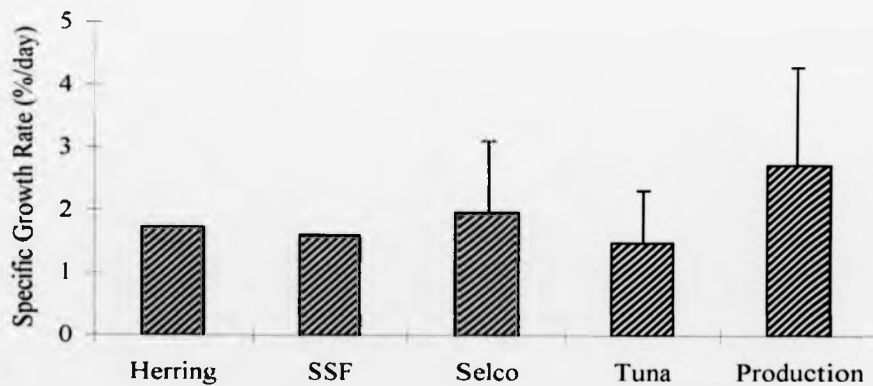


Figure 6.2.7. Specific growth rate (%/day) during the experimental period day 84-107 PFF for each group. Error bars represent standard error of mean in replicated groups. Herring and SSF groups were not replicated.

had the highest. There were no significant differences between SGRs for the three replicated groups (ANOVA, $p > 0.05$). Those groups with lowest mean weights and survival rates, 'Herring' and 'SSF', had the lowest coefficients of variation (CV) of 40.1 and 52.7% respectively, while the Selco, Tuna and Production groups had CVs of 60.0, 58.8 and 59.0% respectively.

6.2.3.4. Vitality

Table 6.2.2 shows the cumulative proportional mortality for the five groups and the calculation of stress index (SI). During the high salinity challenge there was little mortality in any group for the first three hours and by 10 hours, all fish had succumbed. This data is represented in Figure 6.2.8. A rapid mortality after 5-6 hours for the 'Tuna' group resulted in the highest SI and the 'Production' group had the lowest SI. There were no significant differences between the 'Tuna', 'Production' and 'Selco' groups (ANOVA, $p > 0.05$). The 'SSF' and 'Herring' groups could not be included in the analysis since there was only one replicate for each.

Table 6.2.2. Cumulative proportional mortality during a 10 hour 65 ppt salinity challenge and the resulting stress indices (SI). There were 10 fish per replicate.

TIME (hrs)	Group										
	SSF	Herr'	Tuna			Production			Selco		
Rep'	A	A	A	B	C	A	B	C	A	B	C
0	0	0	0	0	0	0	0	0	0	0	0
1	0	0.1	0	0	0	0	0.1	0	0	0	0
2	0	0.1	0	0	0	0	0.1	0.1	0.1	0	0
3	0.1	0.1	0.2	0.2	0	0	0.2	0.1	0.1	0	0.1
4	0.2	0.1	0.4	0.4	0.1	0	0.2	0.1	0.1	0	0.3
5	0.3	0.4	0.6	0.7	0.4	0.2	0.2	0.3	0.2	0.1	0.4
6	0.9	0.7	0.7	0.9	0.7	0.3	0.4	0.5	0.6	0.4	0.6
7	1.0	0.9	0.7	1.0	0.7	0.7	0.4	0.6	0.7	0.6	0.8
8	1.0	1.0	0.8	1.0	1.0	0.9	0.8	0.7	1.0	0.8	1.0
9	1.0	1.0	1.0	1.0	1.0	0.9	0.9	0.9	1.0	0.9	1.0
10	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SI	5.5	5.4	5.4	6.2	4.9	4.0	4.3	4.3	4.8	3.8	5.2
Mean SI	5.5	5.4	5.5			4.2			4.6		

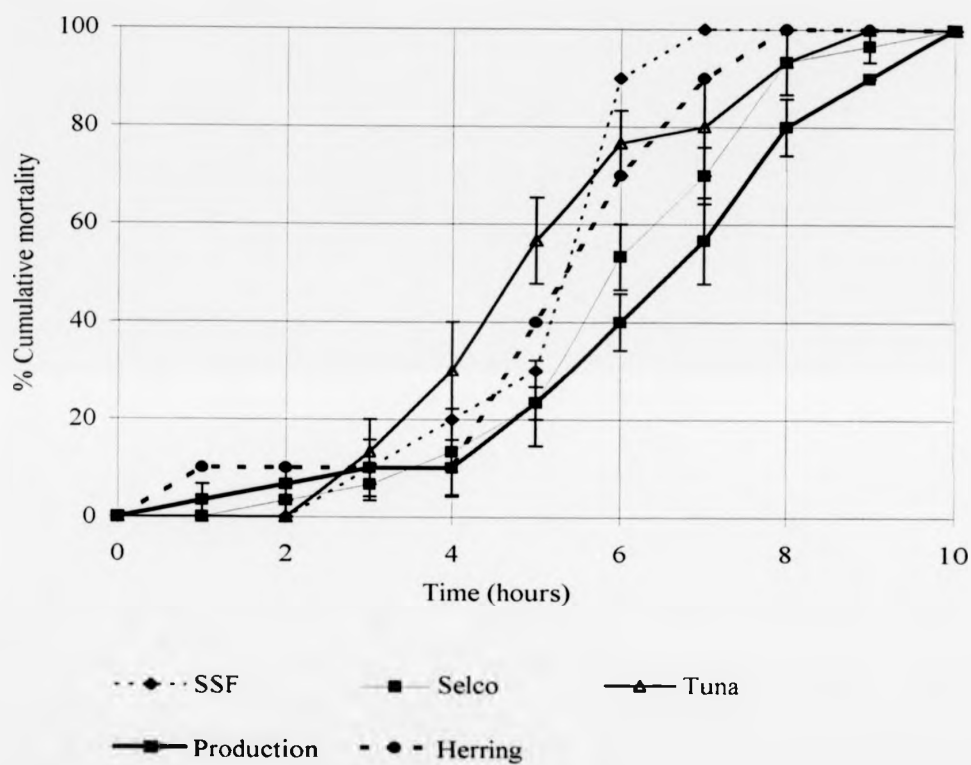


Figure 6.2.8. Cumulative mortality curves of 5 groups of fry in response to exposure to high salinity (65ppt). Values are means of 3 replicates for Tuna, Selco and Production groups. SSF and Herring were not replicated. Error bars represent standard error of means. There were 10 fish per replicate.

6.2.4. Conclusions for Experiment 1

This was the first of a series of experiments concerning weaning and served to develop an introduction to weaning methods for halibut and provide indications of how subsequent experiments might be designed.

This experiment demonstrated how the performance of fish from the same batch can be affected by differing treatments during the first feeding phase. Although there were perceived differences in the performance of the different groups through transfer to inert diet these were not always revealed statistically due to the lack of replicates in the poorer performing groups. This was the case for the comparison of survival rates which were much lower in the 'SSF' and 'Herring' groups than the other three groups. These results are similar to those obtained for these treatments during the preceding first feeding phase (F.Luizi pers. comm.), indicating that performance characteristics persist.

The 'Production' group performed best in terms of survival, resistance to salinity challenge and SGR. These statistics are perhaps the most important and may give the best indication of relative fitness of the population. Though the 'Production' group had received *Artemia* enriched with the same products as the 'Selco' group they entered the experiment at a considerably lower mean weight (119.8 and 189.4 mg wet weight respectively). Differences between these groups must be attributed to environmental conditions during early rearing and/or feeding rate.

A good correlation between wet weight and the time taken to reach a 50% feed response, as expected, suggests that larger fish are more likely to accept inert diet. However an examination of other criteria indicates that there are other factors which influence the relative performance through the weaning phase. The 'Production',

'Herring' and 'SSF' groups were all of similar mean wet weight at the start of the experiment. The 'Production' fish showed a similar response to inert diet in terms of uptake but survival, SGR and resistance to salinity challenge were all higher for this group than the 'Herring' and 'SSF' groups. The larger 'Tuna' and 'Selco' groups showed a relatively rapid uptake of inert diet during weaning and finished the experiment with the largest mean weights and contained the largest individuals, evidently growing rapidly on inert diet. However the 'Tuna' group had the lowest SGR overall during the experimental period. This may be due to the fact that during weaning this group received less *Artemia* on a N^o prey/larval weight basis than the other groups since rations were given on a number of prey/larvae basis. Alternatively these fish may have already entered a slower growth phase characteristic of late larval/early juvenile halibut.

In the larger groups some aggressive behaviour was noticed and the overall feed response started to decline towards the end of the experiment. Cannibalism was also observed in the 'Tuna' and 'Selco' groups. This reflects the wide size distribution evident in the coefficient of variation and that the largest individuals were up to 10× larger than the smallest. This was an indication that social interactions might influence feeding behaviour in halibut larvae during weaning.

6.3. Experiment 2: The effect of age and size at weaning

6.3.1. Introduction and aims

For the hatchery operator, the most important criteria in relation to weaning is when the transfer from live prey to an artificial diet can safely take place. In terms of live feed usage and labour, the earlier this transition can take place, the more efficient the juvenile production will be. As discussed in the introduction (Section 6.1.), a considerable effort has been focused on determining the age and size at which larvae of different culture species will adapt to formulated diets. In comparison to the earlier life stages, this aspect of halibut hatchery production cycle has received little attention. At present, due to the difficulties associated with earlier stages of the life cycle, particularly yolk sac absorption and first feeding, most hatchery operators err on the side of caution when it comes to weaning. The tendency is to wean larvae at a size of around 200 mg rather than attempt to bring this phase forward and risk losing valuable and all too scarce fry. This results in a very lengthy period on live feeds (typically >70 days) and this places a heavy demand on *Artemia* and hatchery resources in general.

The aim of this experiment was to assess the importance of age or size of halibut larvae at the start of weaning and to try and gauge at what time it is possible to remove live prey without inducing serious mortality or growth depression.

6.3.2. Materials and methods

6.3.2.1. Live material and facilities

A single batch of 984 larvae were taken from a first-feeding tank aged 55 days PFF (980°Cdays post hatch) and distributed evenly and randomly into 12 tanks. They were allowed to acclimate for 3 days.

The experiment was conducted in the purpose built facility consisting of twelve tanks described in Section 2.4.3. Filtered sea water was supplied through surface inlets at ambient temperature (mean 15.6°C; range: 13.6 - 18.5°C) and salinity (33 - 34 ppt). The mean dissolved oxygen concentration was 8.3 mg/l; range: 7.8-8.6 mg/l. The flow rate was initially set at 300 ml/min and the water depth at 40 cm (volume: 125 l). To facilitate removal of waste feed and faeces, the flow was increased for each group first to 800 ml/min at the start of weaning and then gradually up to 1200 ml/min by the end of the weaning period. The depth was also reduced to 30 cm (volume: 85 litres) on the first day of feeding inert diet to increase water exchange. Illumination was set at approximately 200 lux at the surface. Tanks receiving dry diet were siphoned once a day to remove waste feed and faeces until day 61 PFF and then swirled once per day once the fish were more robust.

6.3.2.2. Feeding regime

The fish were fed three times per day at 09.00, 14.00 and 18.00 hrs with enriched *Artemia* at a rate of 500 prey/fish/feed. The first and third *Artemia* feeds were enriched at 27°C for 18 hours with SSF microfeed and then for 18 hours with Super Selco. The second *Artemia* feed was enriched at 27°C for 18 h with SSF microfeed

only.

The 12 tanks were allocated randomly to 4 groups, 3 replicates per group. Algae (*Nannochloris atomus*) was added (2 l/tank/day) for the first three day acclimation period until the fish were observed to be settled and feeding well. Weaning started at 58 days PFF in one group (A) of 3 replicates, at 68 days PFF in group B, 78 days PFF for group C and 88 days PFF for group D. The weaning age, initial weights and lengths are shown in Table 6.3.1.

Table 6.3.1. Age, initial wet and dry weight and lengths of each group of fish at the start of weaning. Values are based on the mean of 3 replicates \pm s.d.

Group	Age at start of weaning (days PFF)	Initial wet weight (mg) \pm (s.d.) <i>n=21</i>	Initial dry weight (mg) \pm (s.d.) <i>n=15</i>	Initial length (mm) \pm (s.d.) <i>n=21</i>
A	58	106.1 (41.90)	21.2 (8.20)	25 (5.7)
B	68	161.1 (56.71)	38.4 (11.14)	28 (6.8)
C	78	194.3 (84.50)	43.9 (22.89)	30 (7.7)
D	88	285.7 (111.70)	65.2 (27.29)	34 (9.3)

Larvae were weaned gradually over a 9 day period according to the weaning regime shown in Table 6.3.2. From the first day of weaning inert diet (Nutra Marine, T.Skretting AS, Stavanger, Norway) was fed *ad libitum* by hand several times per day. Crumb sizes of 0.5-0.8 mm were used to start weaning and then this was mixed with 0.8-1.4 mm crumb to provide an appropriate range of particle sizes for the wide distribution of size classes of fish.

Table 6.3.2. Standard weaning regime applied to all groups of fish

	Dry diet all day		
Feeding Period	1st <i>Artemia</i> feed	2nd <i>Artemia</i> feed	3rd <i>Artemia</i> feed
Day 1 - 3	Half ration*	Full ration	Full ration
Day 4 - 6	None	Half ration	Full ration
Day 7 - 9	None	None	Full ration
Day 10 onwards	None	None	None

*Full ration was 500 *Artemia*/fish

6.3.2.3. Sampling

Fish were sampled from each group one day prior to the start of weaning; 7 fish per replicate for live wet weight and blotted wet weight and 5 per replicate for dry weight. Samples of 10 fish per replicate were also weighed live at 10 day intervals from all groups to obtain intermediate weights for a growth curve. At the end of the trial (day 109 PFF), survivors were counted and weighed live. 14 fish per replicate were wet weighed (blotted) and 10 dry weighed.

6.3.2.4. Vitality tests

Prior to weaning, 5 fish per replicate were subjected to a 65 ppt salinity vitality test as

described in Section 6.2.2.4. At the end of the weaning period 5 fish per replicate were again subjected to the same test. Stress indices were calculated according to the method described in section 6.2.3.4.

6.3.2.5. Statistical analysis

Two factor nested analysis of variance were carried out on final wet and dry weights with time of weaning as the treatment factor and tank as a random factor nested within treatment. Where treatment effect was found to be significant and tank effect was found to be non-significant, pairwise comparisons were performed using Tukey tests following one-way ANOVA on pooled data. All other analyses of variance were carried out using one way ANOVAs.

6.3.3. Results

6.3.3.1. Survival

Cumulative survival rate curves shown in Figure 6.3.1. show that the mortality had ceased in groups A and B and in both cases this plateau was reached approximately 25 days after the end of the weaning period. The later weaned groups C and D however had not reached a plateau. The experiment had stopped 20 days after weaning had finished in group C and 10 days after group D had finished weaning. Survival rates, shown in Figure 6.3.2. ranged between 54.4 and 80.5% and were higher the longer larvae remained feeding on *Artemia*. A number of fish escaped from one replicate in group C so only two replicates are included in the survival rate calculation for this group. There was a significant difference between the survival rates of group A and D (ANOVA, $p < 0.05$). A linear regression was carried out on initial age and initial size on survival rate. Both

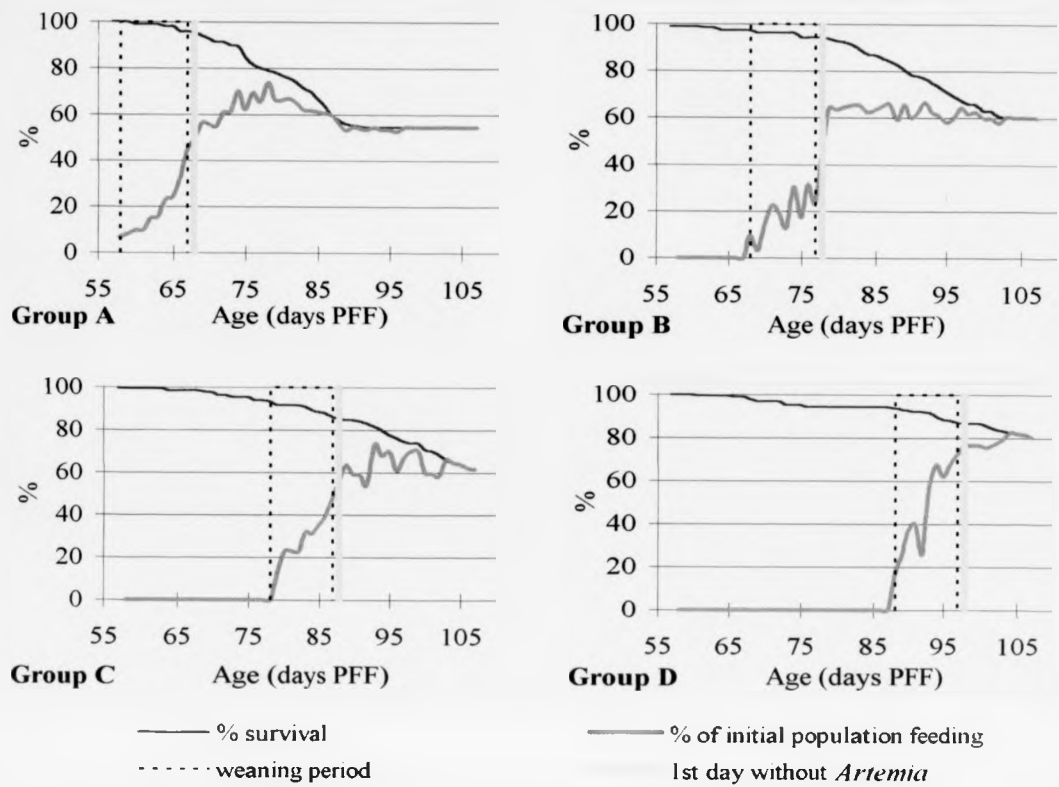


Figure 6.3.1. Cumulative survival (%) and feed response (% initial population feeding on inert diet) for each group.

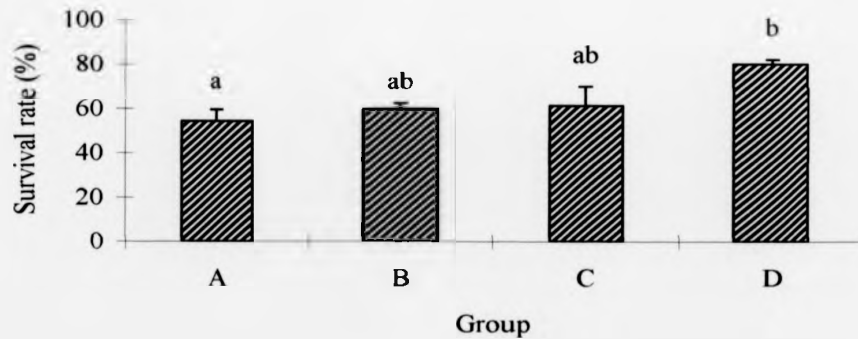


Figure 6.3.2. Mean survival rates at day 109 PFF. Values are the means of 3 replicates. Error bars represent standard error of means. a,b denote significant differences between means (ANOVA $p < 0.05$).

were significantly positively correlated with survival rate and gave R^2 values of 0.907 and 0.971 ($p < 0.05$ and $p < 0.025$) respectively. Survival rate was therefore better correlated to initial size than age.

6.3.3.2. Feed response

The change in feed response during the course of the experiment is shown in Figure 6.3.1. By the end of the experiment all fish were feeding on inert diet. The feed response curves show that the percentage of fish eating inert diet on the first day without *Artemia* tallied well with the final survival rate indicating that fish not feeding by this time would not survive. The feed response at the end of the weaning phase, shown in Figure 6.3.3. was significantly higher for the latest weaned group, D than in the other groups (ANOVA, $p < 0.05$). When assessing feed response it was noted that in the early weaned groups the majority of fish taking inert diet were demersal, but there were a significant number of small pelagic larvae taking crumb.

6.3.3.3. Growth

Figure 6.3.4. shows the growth curves for the four groups of fish. The general pattern was that growth tended to accelerate once the fish were established onto dry diet and thus a growth advantage was conferred upon those groups weaned earliest. Final wet weights (shown in Figure 6.3.5.A.) were highest and similar for the two earlier weaned groups A and B, and lowest for group D, weaned latest. Final dry weights (see Figure 6.3.5.B.) also increased with a decrease in weaning age. Coefficients of variation shown in Figure 6.3.6. remained at about 40% whilst larvae were feeding on *Artemia* and increased following weaning to 60 - 70%. Significant differences in final wet weight

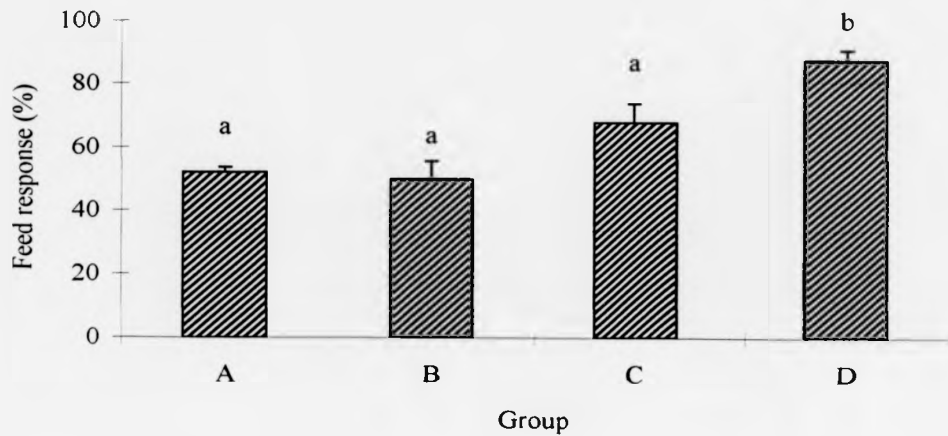


Figure 6.3.3. Mean percentage of fish taking inert diet (feed response) on first day without *Artemia*. Values are the means of 3 replicates. Error bars represent standard error of means. a,b denote significant differences between means (ANOVA $p < 0.05$)

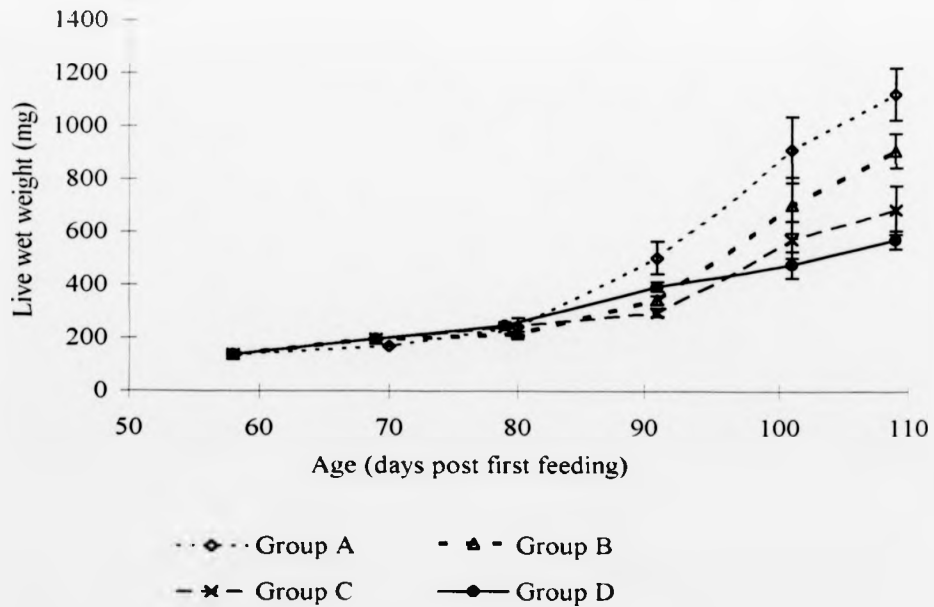
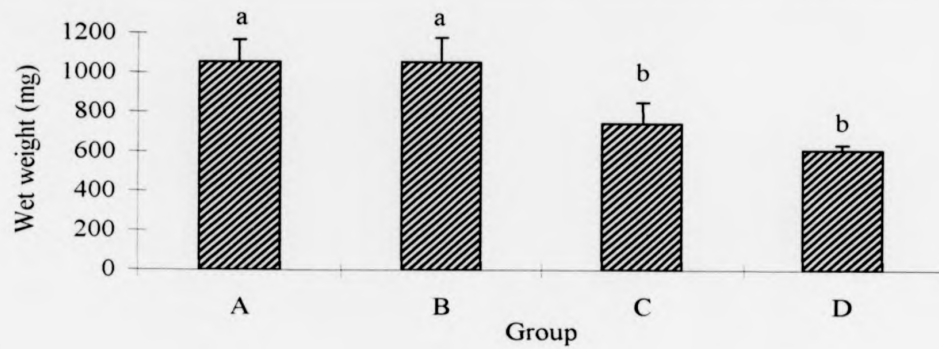


Figure 6.3.4. Change in live wet weight during experimental period. Values are means of 3 replicates. Error bars represent standard error of means.

A:



B:

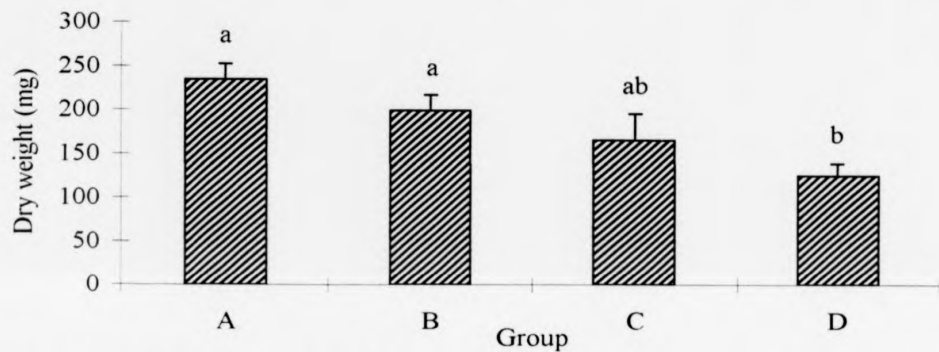


Figure 6.3.5. A: Mean final wet weights at day 109 PFF and B: Mean final dry weights at day 109 PFF. Values are the means of 3 replicates. Error bars represent standard error of means. a,b denote significant differences between means (ANOVA $p < 0.05$).

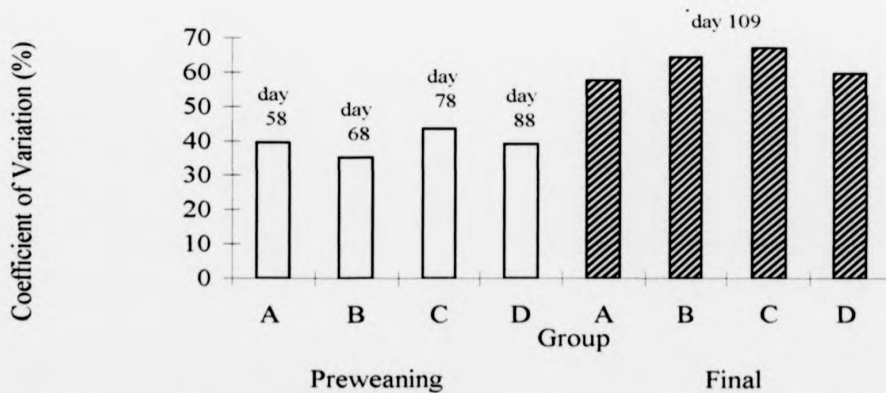


Figure 6.3.6. Coefficient of variation for wet weights of each group of fish before weaning (at the age indicated above the bars) and at the end of the experiment (day 109 PFF).

were revealed between the two later weaned groups and the two earlier weaned groups and in final dry weight between group D and groups A and B (ANOVA, $p < 0.05$).

Specific growth rates for the whole period based on dry weights are shown in Figure 6.3.7. and tended to decrease with later weaning age. Incremental SGRs calculated from live wet weights are presented in Figure 6.3.8. This graph shows the change in SGR for each group during the experimental period. There is a general trend for the SGR to decrease to a minimum during weaning and then increase following weaning to a maximum. This pattern is most obvious for groups A-C. By the end of the experiment the SGR for all groups had fallen to a similar level.

The maximum and minimum final live wet weights were; by group (A: 3395.7 & 128.7 mg); (B: 3183.3 & 132.9 mg); (C: 2223.8 & 110.0 mg); (D: 1954.3 & 124.7 mg) for groups A-D respectively.

6.3.3.4. Vitality

Figure 6.3.9. shows the cumulative mortality patterns of the four groups of fry during the stress test. It can be seen that as the fry increase in age the curves shift towards the right as the resistance increases. The rate of mortality tended to be higher prior to weaning in groups A-C but in group D, the pattern of mortality was very similar in pre and post weaned fry. The stress indices given in Table 6.3.3. also indicate that halibut larvae become more resistant to salinity challenge as they grow. This was also evident from the regressions of wet weight on time of death during the test shown in Figure 6.3.10. which reveal significant positive correlations between wet weight of mortalities and time of death for pre-weaned and post-weaned fish ($R^2 = 0.31$ and 0.59 respectively, $p < 0.005$ in both cases). A comparison of stress indices of weaned and pre-weaned fish of

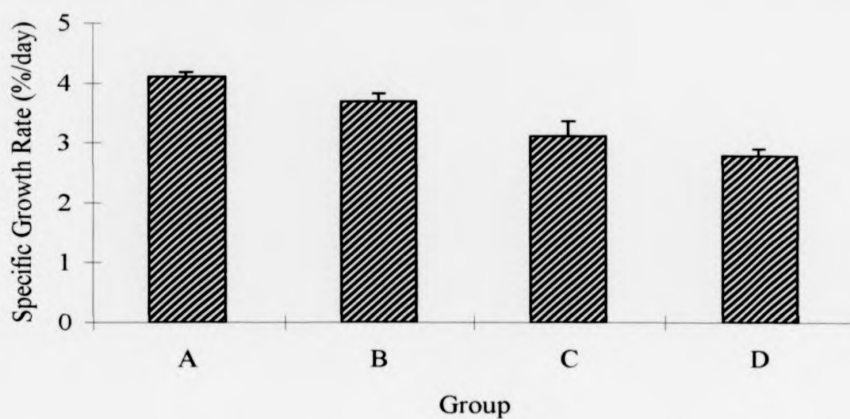


Figure 6.3.7. Mean specific growth rates during the experiment from day 58-109 PFF based on live wet weights. Values are the means of 3 replicates. Error bars represent standard error of means.

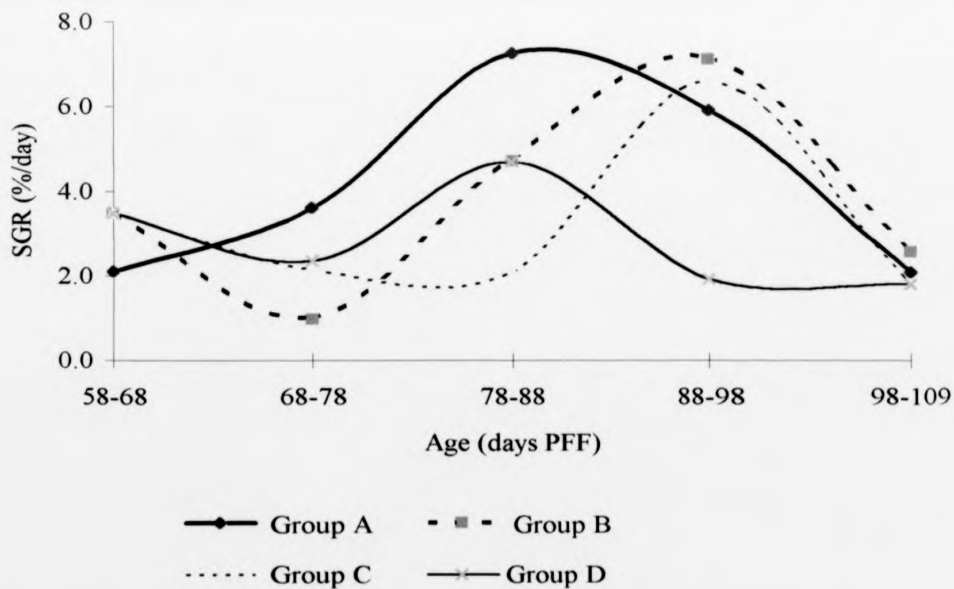


Figure 6.3.8. Change in incremental specific growth rate for each group of larvae during the experiment (day 58 - 109 PFF). Values are mean of 3 replicates.

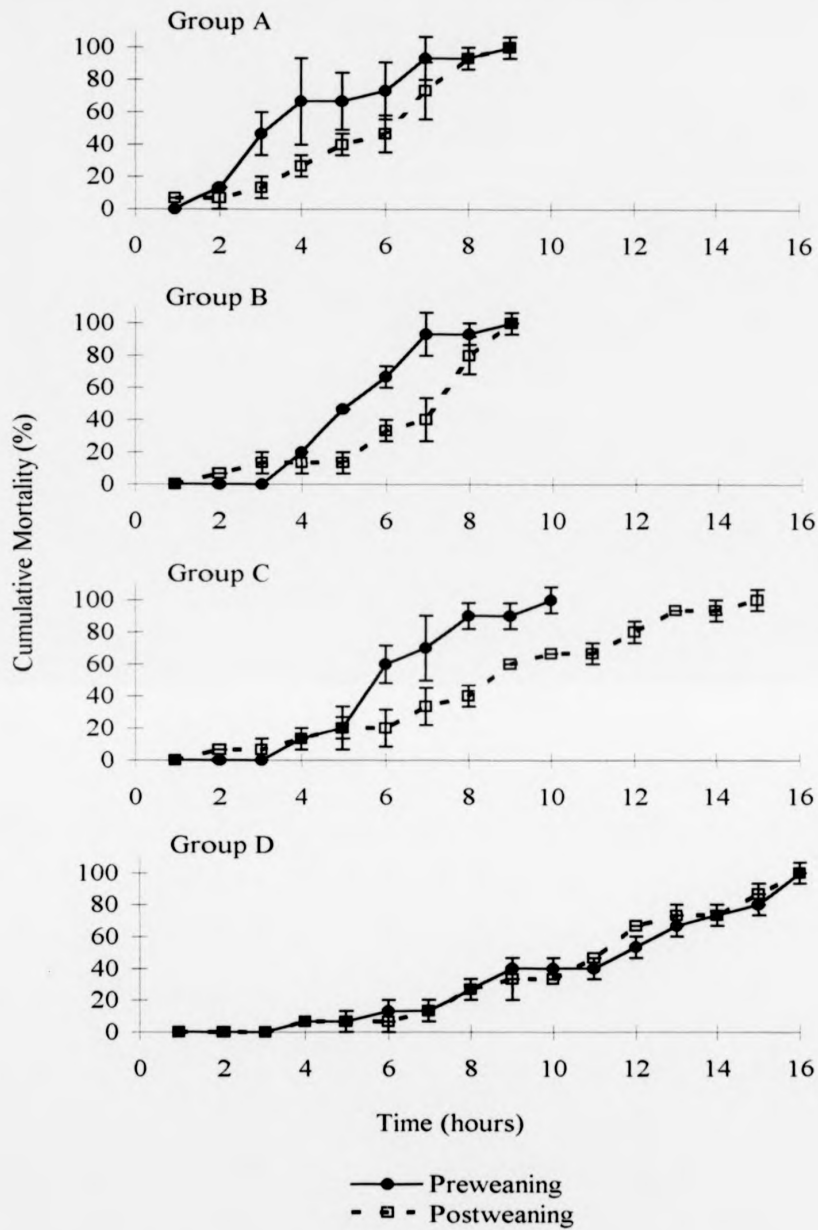
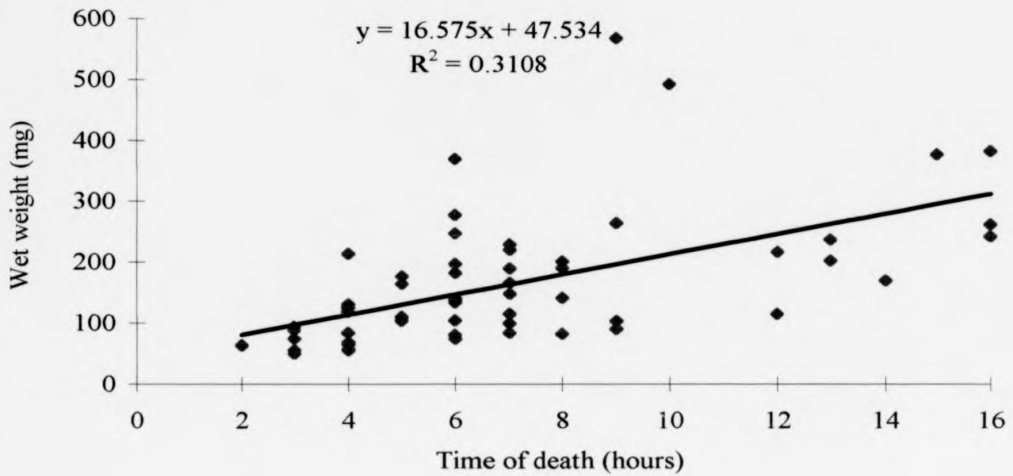


Figure 6.3.9. Cumulative mortality during high salinity (65ppt) challenge for each group before and after weaning. Values are means of 3 replicates, 5 fish per replicate in each test. Error bars represent standard error of means.

A: Pre-weaned fish



B: Weaned fish

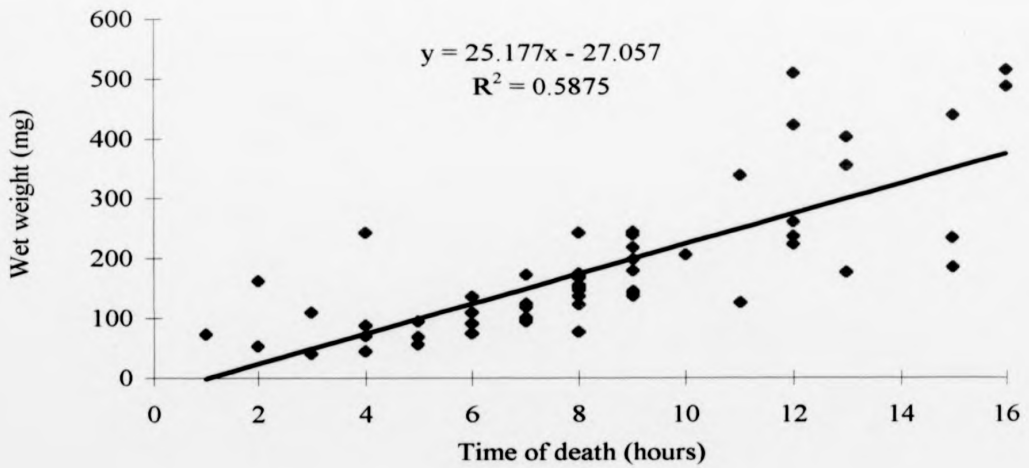


Figure 6.3.10. Regressions of wet weight of mortalities against time of death during high salinity (65ppt) challenge for A; preweaned fish and B; weaned fish.

a similar age revealed that for two out of the three groups where such a comparison was possible (age 68 and 78 days PFF), weaned animals were more resistant. This is not the case for the larvae at 88 days PFF.

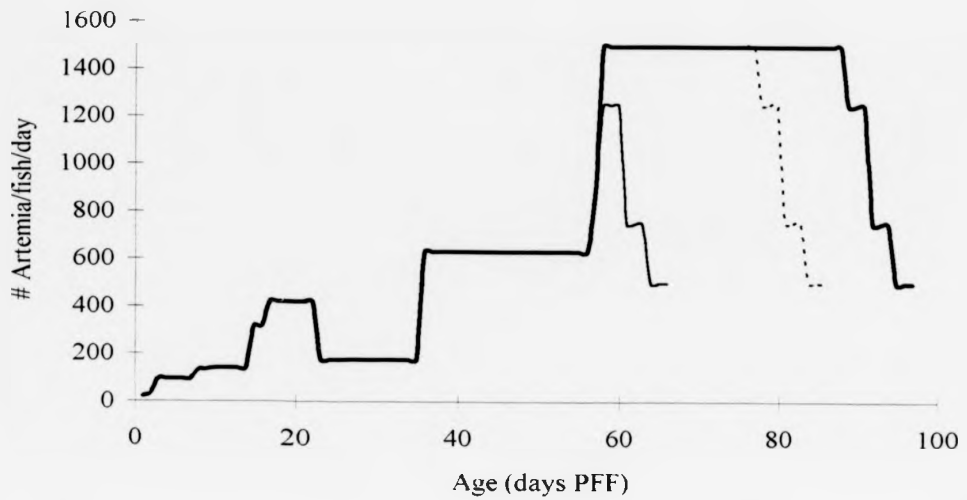
Table 6.3.3. Ten hours stress indices for each group of fish resulting from 65 ppt sea water vitality tests conducted before and after weaning. Values are mean cumulative proportional mortality for 3 replicates calculated as in Section 6.2.3.4.

Age (days PFF)	PRE-WEANING						POST-WEANING					
	Group	Stress Index			Mean	s.d.	Group	Stress Index			Mean	s.d.
58	A	5.6	5.6	8.4	6.5	1.62						
68	B	5.6	5.4	4.6	5.2	0.53	A	5.6	5.2	4.4	5.1	0.61
78	C	4.2	5.8	3.8	4.6	1.06	B	4.2	3.2	4.6	4.0	0.72
88	D	1.6	0.8	2	1.5	0.61	C	2.8	3	2.2	2.7	0.42
98							D	2	0.8	1	1.3	0.64

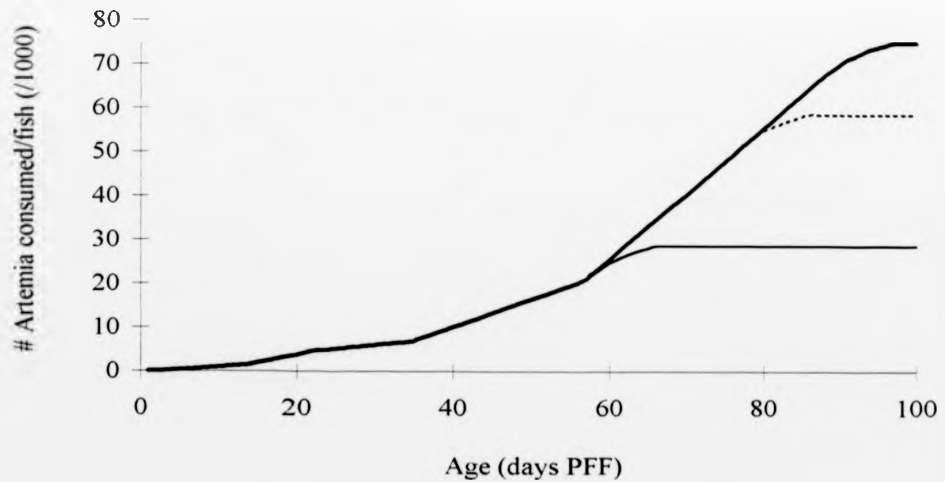
6.3.3.5. *Artemia* consumption

Figure 6.3.11. shows the daily *Artemia* consumption and cumulative *Artemia* consumption from the start of first feeding to the end of weaning for each group of fish. From this, the savings in *Artemia* have been calculated and are shown in Table 6.3.4.

A: Daily *Artemia* ration



B: Cumulative *Artemia* consumption



— Group A Group B
 Group C — Group D

Figure 6.3.11. A: Daily ration of *Artemia* from first feeding until weaning for each group and B: Cumulative total number of *Artemia* fed per fish over the same period.

Table 6.3.4. Savings made in *Artemia* usage based on cumulative consumption (#*Artemia* /fish) from day 1 PFF according to weaning age.

Group	Weaning period (age, days PFF)	Total # <i>Artemia</i> consumed per fish	% saving of <i>Artemia</i> relative to weaning from day 88-97 PFF
A	58-67	28600	62
B	68-77	43600	42
C	78-87	58600	22
D	88-97	75100	0

6.3.4. Conclusions for Experiment 2

A first glance at the final survival rates indicate that early weaning caused significantly higher mortality in this batch. The losses in the groups weaned at day 58, 68 and 78 PFF were however similar and the mortalities had ceased by the end of the experiment in the first two groups. The mortality had seemingly not finished in the later weaned groups. If the mortality patterns from groups A and B were carried over to groups C and D, it might be argued that had the experiment continued longer, the survival rates might have been more similar.

On this basis it could be that delaying weaning for this batch beyond 100 mg merely delayed mortality using the techniques applied to the whole batch. The mortality rates were certainly more rapid in the earlier weaned groups and this probably reflects a more rapid exhaustion of storage energy in the smaller fish. Mortalities collected during the course of the experiment were usually from the smaller size classes. It is the smaller fish of the cohort that are likely to fail during weaning and it seems that size at weaning is more important than age.

A clear growth advantage was conferred upon the larvae by early weaning. Though size selective mortality may cause some bias in growth estimates, comparisons between groups A to C are entirely valid since the survival rates were very similar. The largest individuals were found in group A, the next in group B and so on. This demonstrates that for halibut of this age/size, the growth potential is better realised when fed inert diet than *Artemia*.

Growth rates, evident from the specific growth rates calculated at 10 day intervals, decrease to a minimum during weaning as *Artemia* ration is reduced to zero and initial

uptake of dry diet is slow. Once ingesting inert diet, growth accelerates and wide variations in size distribution result as smaller fish which are slower to adapt to inert diet are left behind. This causes an increase in the coefficient of variation following weaning. Feed response observations showed that uptake of diet was slowest in the groups weaned earliest reaching only 50% by the end of the weaning period. Older, larger fish adapt to the artificial diet more rapidly.

Though the sensitivity of the stress tests might be called into question being based on five fish per replicate, they consistently showed that vitality increased with age and size. Comparison of weaned and unweaned fish was more difficult. In two out of three comparisons for weaned and unweaned fish of identical age, it was apparent that fish were more robust after weaning. These tests might be better performed some while after weaning once the majority of fish have been established on dry diet for a period. Immediately following removal of *Artemia*, it is likely that some fry will be weak from reduced feed intake or even starvation whereas others which adapted to dry diet immediately will be more robust than their counterparts.

6.4. Experiment 3: The effect of size variation during weaning

6.4.1. Introduction and aims

During the weaning phase, size variation has a tendency to increase. Those fish which adapt to the diet rapidly soon increase their food consumption above levels which were previously limited by a maximal intake and assimilation of *Artemia*. An increase in growth rate accompanies this as shown in the previous experiment. However some fish, generally in the smaller size classes which are slower to adapt to artificial diet, are left behind and their growth rate will reduce during the weaning period as *Artemia* feeding and thus intake is lowered. These factors conspire to widen the size distribution in the population and this can lead to aggressive behaviour and cannibalism in many cultured fish species (Hecht and Pienaar, 1993). In the first experiment described in this chapter, some aggressive behaviour was observed in some of the largest individuals and this was directed towards the smaller conspecifics. This extended to fin nipping and in a few extreme instances, cannibalism was also noted. To study more closely the importance of size variation at weaning an experiment was designed in which groups were graded to reduce the size range and weaned alongside ungraded groups. A comparison of the performance of different size classes from a single batch of fish also served to demonstrate the importance of size at weaning and the influence of size selective mortality.

6.4.2. Materials and methods

6.4.2.1. Live material and facilities

A total of 636 larvae were moved from a first feeding tank at 55 days PFF (740 °C days post hatch) and stocked randomly to 12 experimental tanks (described in Section 2.4.3) 53 per tank. The tanks were supplied with 5 µm filtered sea water via surface inlets at an initial flow rate of 100 ml/min. Water depth was set initially at 40cm and later dropped to 30 cm on day 90 PFF. Illumination was initially set at 20 lux at the water's surface. Algae (*Nannochloris atomus*) was added, 2l/day, for the first 3 days till day 58 PFF. The flow rate was increased to 200 ml/min on day 60 PFF, to 350 ml/min on day 74 PFF and finally to 500 ml/min on day 77 PFF. Light was increased gradually to 35 lux on day 58, 50 lux on day 60, 70 lux on day 70 and finally to 100 lux on day 84 PFF.

6.4.2.2. Grading procedure

After an acclimation period of 12 days and once it was deemed appropriate to commence weaning, 4 treatment regimes were allocated to the tanks, 3 replicates per treatment. The grading procedure, shown in Figure 6.4.1., was carried out as follows. All fish from each treatment group were pooled into a plastic aquarium and then redistributed evenly between the 3 tanks using a soft nylon net. The controls were left ungraded and redistributed equally and randomly between the 3 tanks, whilst the other 9 tanks were each divided into 3 graded groups of equal number. From the pool, the largest individuals, selected by eye were allocated to one tank and the smallest to another tank and the remaining fish were allocated to the third tank. This resulted in 3 tanks containing the smallest fish in their respective groups, 3 containing the largest, 3

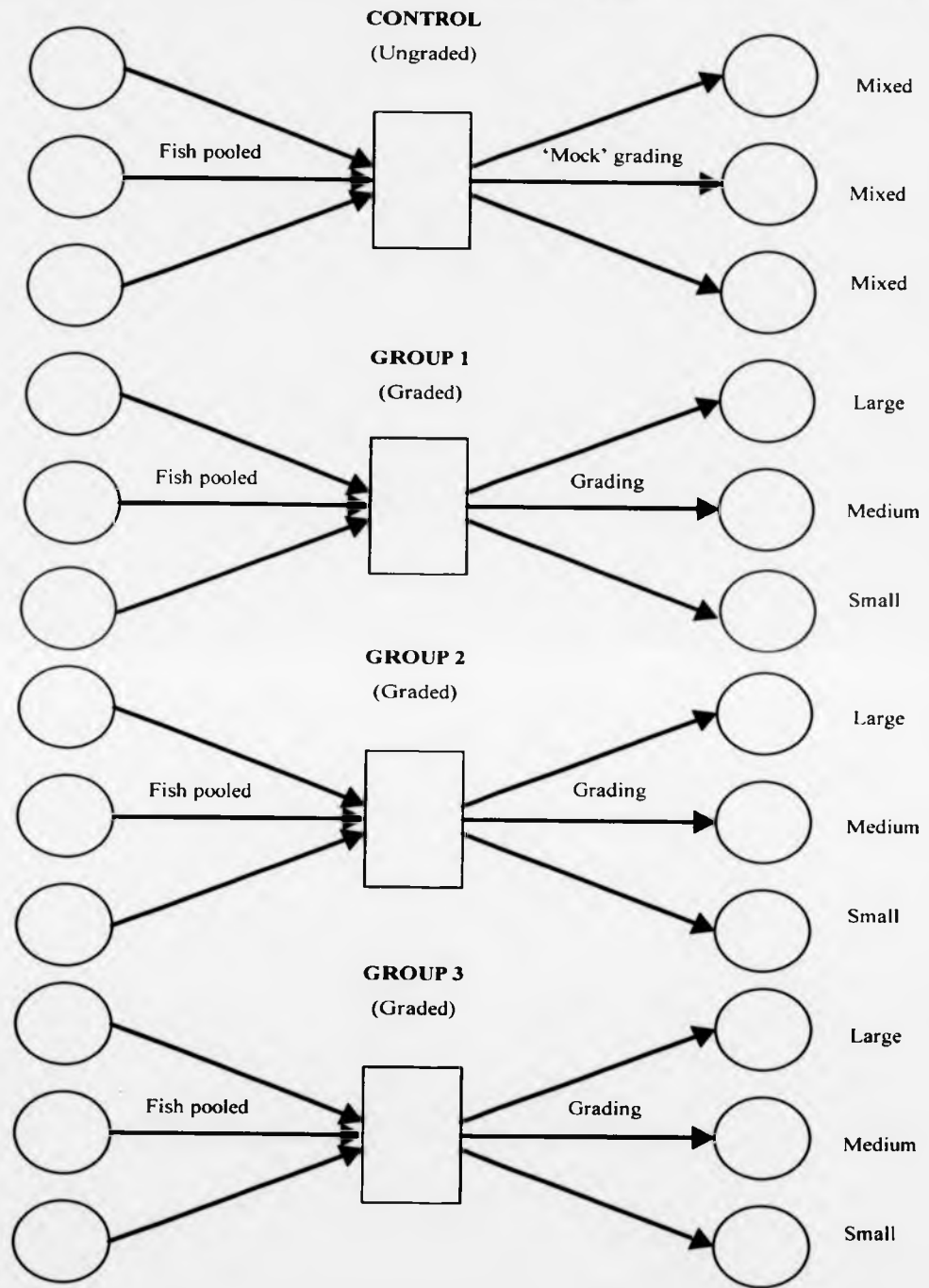


Figure 6.4.1. Schematic representation of initial grading procedure.

containing fish of medium size and three tanks containing ungraded populations.

Further minor grading was carried out on days 78, 88 and 98 PFF. This was done by moving equal numbers between tanks within groups, up to 5 fish per tank up or down a size class. In this way, numbers were not altered in each tank so that survival rates within size classes could be assessed. Similar numbers of randomly selected fish were moved between Control tanks to ensure handling was identical between treatments.

6.4.2.3. Feeding regime

During the acclimation period fish were fed 3 times per day at 09.00, 14.00 and 18.00 hrs with enriched *Artemia* at a rate of 500 prey per fish per feed. The first *Artemia* feed was enriched at 28°C for 24 h with tuna orbital oil. The second and third *Artemia* feeds were enriched at 28°C for 48 h with Algamac 2000. Weaning commenced for all groups on day 68 PFF at a mean wet weight of 118.3 mg \pm 30.52, dry weight 20.6 mg \pm 6.38 and length 25.0 mm \pm 2.01 according the 9 day regime described in Section 6.3.2.2.

6.4.2.4. Sampling

The day immediately following grading (day 68 PFF), a sample of fish were taken for live wet weight, blotted wet weight, dry weight and length. Intermediate live wet weights were measured on days 80 and 90 PFF to obtain information for a growth curve. At the end of the trial on day 101 PFF, all survivors were counted and 10 fish per replicate were subjected to a salinity challenge as described in Section 6.2.2.4. Blotted wet weight, dry weight and length were measured for 10 fish per replicate and all survivors were weighed for live wet weight. Surviving fry were also assessed for metamorphosis and pigmentation characteristics according to the indices proposed by B. Gara (pers.comm) described in Appendix II.

6.4.3. Results

6.4.3.1. Survival

The cumulative mortality graph shown in Figure 6.4.2. illustrates that the pattern of mortality in all groups was very similar in that most mortality came shortly after the weaning period. The survival rates for the Control, Medium and Large groups were all over 80% but lower for the Small group. Analysis of variance revealed no significant differences in survival rates between groups.

6.4.3.2. Feed response

The change in feed response to artificial diet during the experiment for each group is shown in Figure 6.4.2. All groups began to take crumb during the weaning period. The Small group were slowest on the uptake of diet and a significantly higher proportion of fish were taking crumb on the first day without *Artemia* (ANOVA, $p < 0.05$) in the Large group which had the best feed response (see Figure 6.4.3.). The other groups showed intermediate responses to diet. Interestingly there was a decrease in feed response to dry diet in all groups on the first day without *Artemia*. The average time taken for 50% of the larvae for each group to accept weaning diet was 76 days (Control), 73 days (Large), 74 days (Medium) and 76 days (Small).

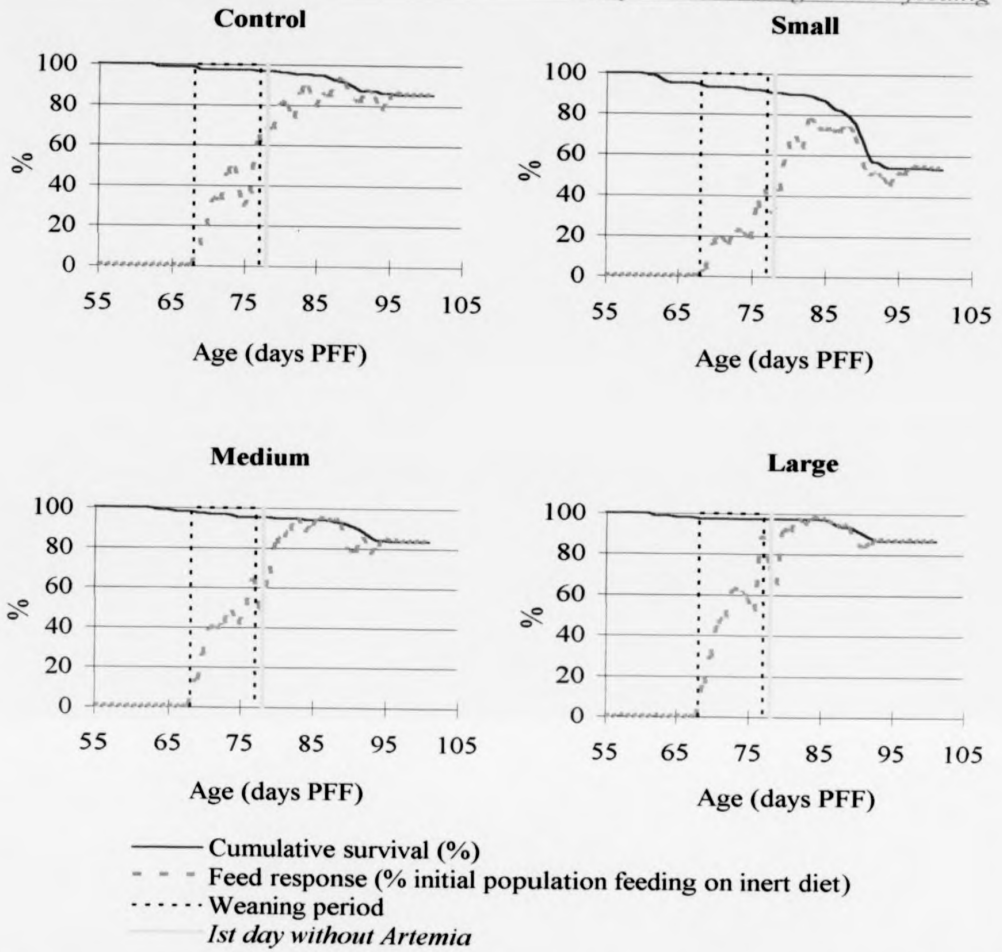


Figure 6.4.2. Change in feed response (% fish of initial population taking inert diet) and cumulative survival rate (%) vs age (days PFF). The weaning period and 1st day without *Artemia* are indicated. Values are means of 3 replicates.

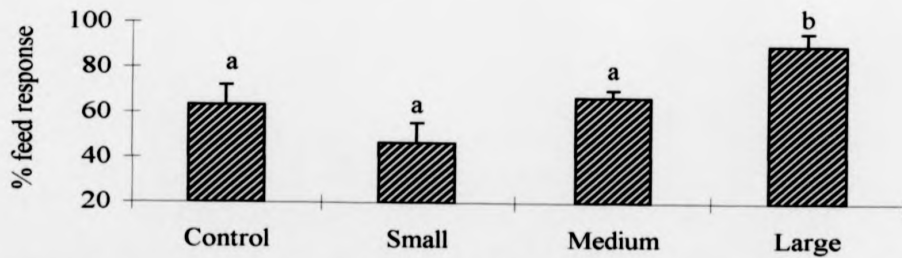


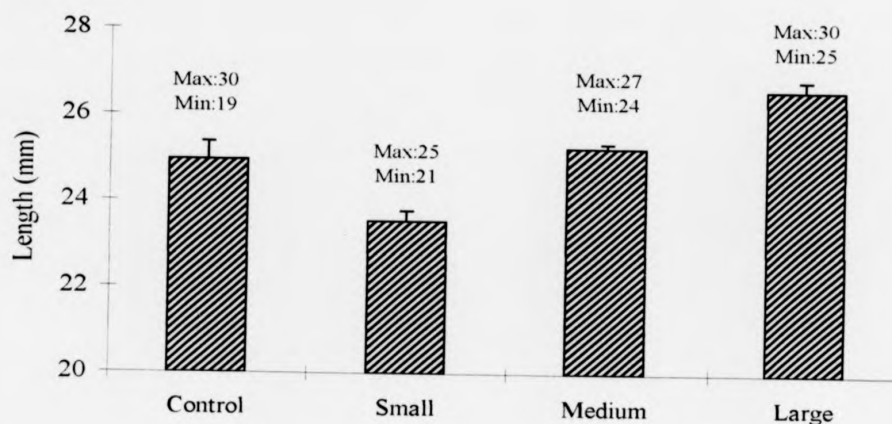
Figure 6.4.3. Feed response (% taking inert diet) on first day without *Artemia* for each group. Values are means of 3 replicates. Error bars represent standard error of means. a, b denote significant differences between means (ANOVA $p < 0.05$)

6.4.3.3. Growth

The mean lengths following grading at the start of the experiment and at the end of the experiment are shown in Figure 6.4.4.A. and 6.4.4.B. while the corresponding wet weights are shown in Figure 6.4.5.A and 6.4.5.B. Mean wet weights for the graded groups ranged from 136.5 mg for the Small group to 344.4 mg for the Large group. The Control and Medium group means were very similar at around 260 mg. There was some overlap in size between size classes indicated by the maximum and minimum values for the size parameters shown in these figures. The reduction in size variations following grading were confirmed by a decrease in the coefficients of variation for wet weights from 25.8% in the Control, to 16.5%, 9.7% and 18.7% in the Large, Medium and Small groups respectively. Maximum and minimum values for live weight at the end of the experiment indicate that there was considerable overlap between the size classes due to possible differences between subgroups, condition factors (grading was judged by length, not weight) or size dispersion following the previous grading.

The growth curves for each group based on live weights taken at regular intervals during the experiment and the corresponding specific growth rates are shown in Figures 6.4.6. and 6.4.7. During weaning between days 68 and 77 PFF, growth rates were low or even slightly negative in the case of the Control and Large groups. The growth rates for all except the Small group then increased in the two subsequent periods. Maximum SGR was around 2.2 %/day for the Control and Large groups for the second 11 day period and close to 5 %/day for the Large group in the final 11 day interval.

A)



B)

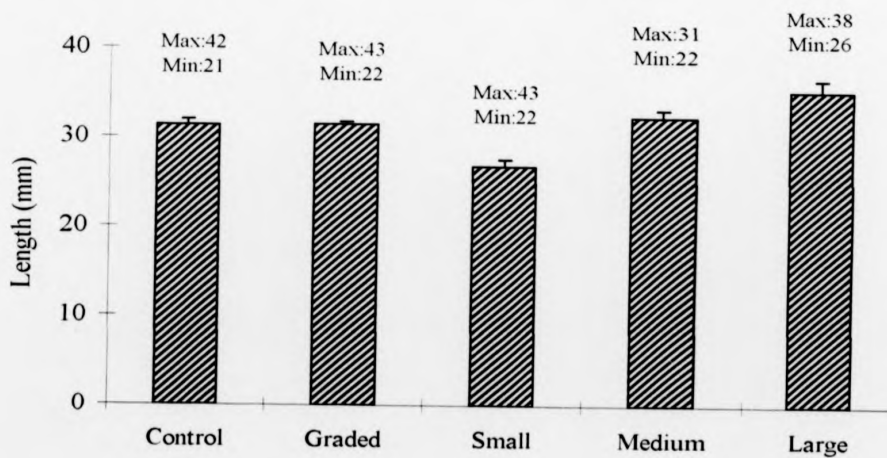
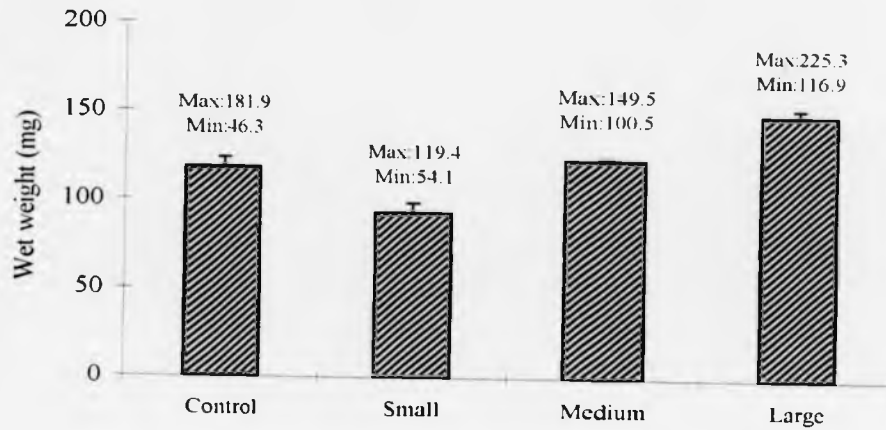


Figure 6.4.4. A) Initial length (mm) at start of experiment (day 68 PFF) and B) Final length at end of experiment (day 101 PFF) for control, graded (pooled size classes) and each size group. Values are based on the means of 3 replicates, 10 individuals per replicate. Maximum and minimum values are shown. Error bars represent standard error of means.

A)



B)

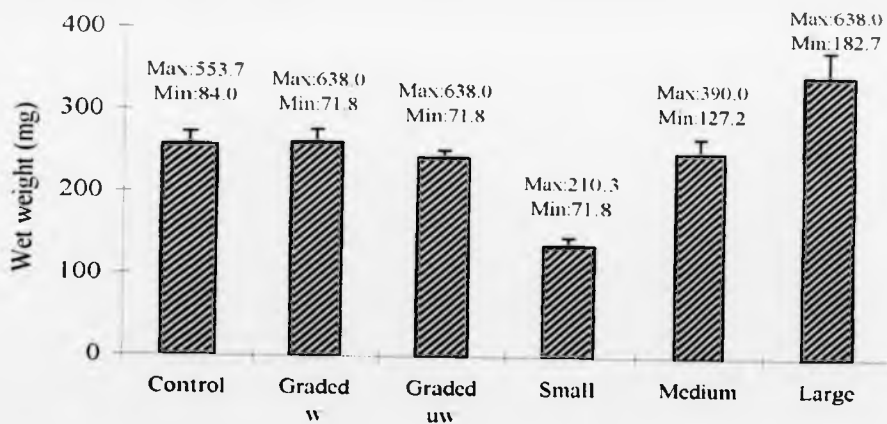


Figure 6.4.5. A) Initial wet weight (mg) at start of experiment (day 68 PFF) and B) Final wet weight at end of experiment (day 101 PFF) for control, graded (pooled size classes) and each size group. Values are based on the means of 3 replicates, 10 individuals per replicate. Maximum and minimum values are shown. Graded _w indicates weighted mean and Graded _{uw} indicates unweighted mean values (see Section 6.4.3.3). Error bars represent standard error of means.

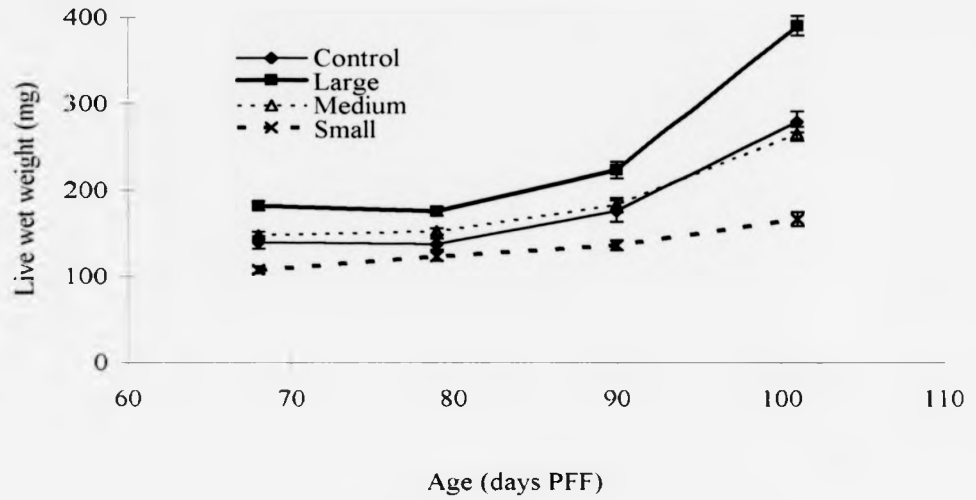


Figure 6.4.6. Growth curves for each size class and control group during experimental period. Values are means of 3 replicates (10 individuals per replicate for first three samples and all survivors for the final weights). Error bars represent standard error.

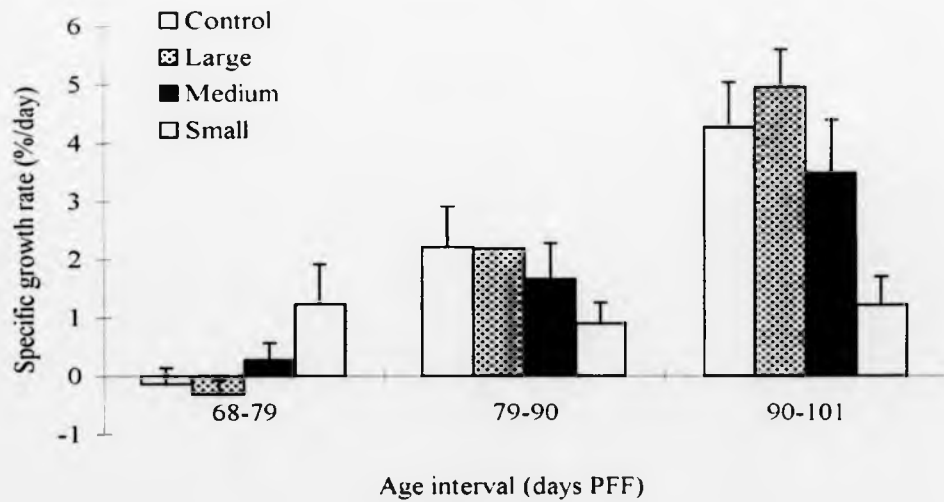


Figure 6.4.7. Incremental specific growth rates for 3 age intervals based on live wet weights for each size class and control group. Values are means of 3 replicates. Error bars represent standard error of means.

The dry weights at the beginning and end of the experiment are shown in Figure 6.4.8.A and 6.4.8.B. respectively. There was no significant difference ($p > 0.05$) between graded and Control wet or dry weights when analysed using two factor nested ANOVA (General Linearised Model), nesting replicate within treatment.

The average condition factor, calculated from the wet weight/length³ was lowest in the Small group (0.68) indicating that these were the “thinnest” fish. The Medium, Large and Control groups had condition factors of 0.72, 0.76 and 0.77 respectively.

Specific growth rates calculated on the change in dry weight over the experimental period are shown in Figure 6.4.8.C. When comparing the graded and ungraded (Control) groups, samples for the former are pooled from all size classes. Since there were equal numbers (10/tank) taken for each replicate, giving equal weight to each size class will result in bias towards the smaller size class where mortality was highest. A weighted mean is also given to reflect more accurately the mean weight of the pooled size classes for each group and is calculated thus:

$$(\text{Mean Wt}_{S_m} \times \#_{S_m}) + (\text{Mean Wt}_{M_d} \times \#_{M_d}) + (\text{Mean Wt}_{L_g} \times \#_{L_g})$$

$$\text{Group mean} = \frac{\text{Mean Wt}_{S_m} \times \#_{S_m} + \text{Mean Wt}_{M_d} \times \#_{M_d} + \text{Mean Wt}_{L_g} \times \#_{L_g}}{\#_{S_m} + \#_{M_d} + \#_{L_g}}$$

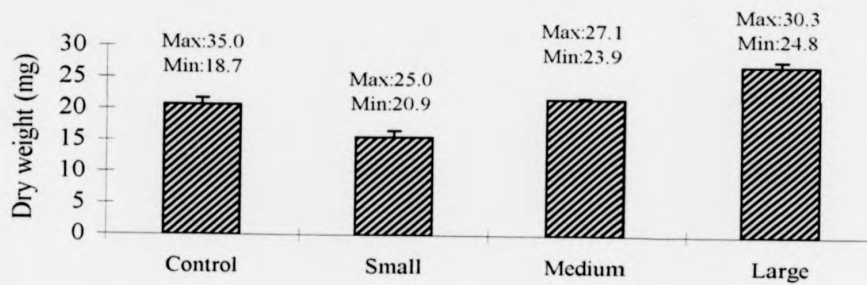
Where;

Mean Wt = Mean wet weight of sample.

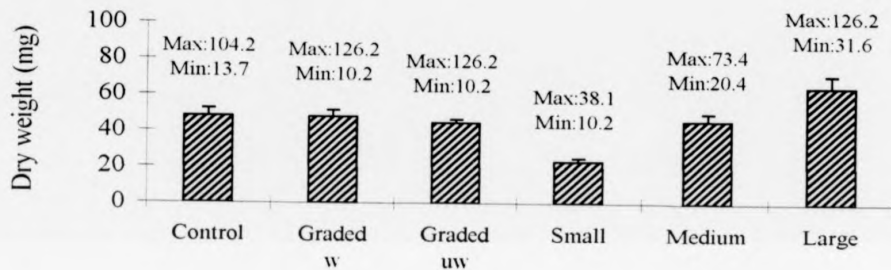
= Total number of fish remaining in tank.

Sm, Md and Lg = Small, Medium and Large subgroups.

A)



B)



C)

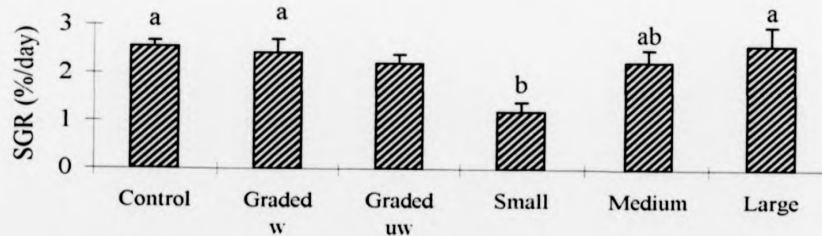


Figure 6.4.8. A) Dry weight (mg) at start of experiment (day 68 PFF), B) Dry weight at end of experiment (day 101 PFF) and C) Specific growth rate based on change in dry weight from day 69-101 PFF for control, graded (pooled size classes) and each size group. Values are based on the means of 3 replicates, 10 individuals per replicate. Maximum and minimum values are shown. Graded _w indicates weighted mean and Graded _{uw} indicates unweighted mean values (see Section 6.4.3.3.). Error bars represent standard error of means. Different letters denote significant differences (ANOVA, $p < 0.05$)

Although the unweighted mean does not reflect the actual mean weight of the remaining fish in a graded group, it actually contributes towards correcting for size specific mortality which tends to bias growth estimates which arise from calculating specific growth rates from mean weights. SGR calculated on the basis of unweighted mean weights gave a lower value than that based on the weighted mean. Analysis of specific growth rates revealed a difference between that of the lowest SGR (Small group) and the Control, Large and Graded groups (ANOVA, $p < 0.05$). The latter is included in the analysis to demonstrate the lack of difference in overall growth performance of the graded and ungraded populations.

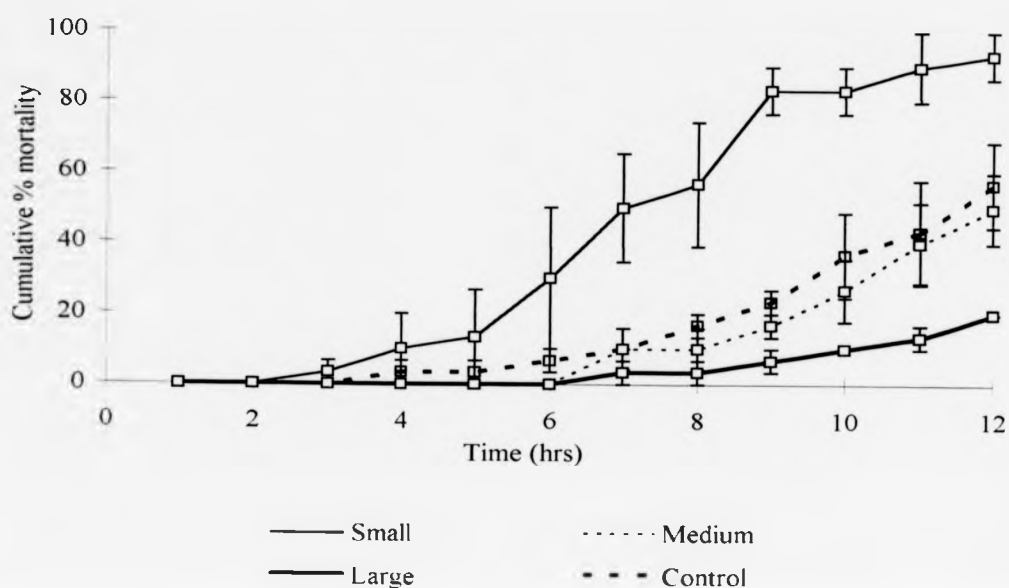
6.4.3.4. Vitality

Figure 6.4.9.A shows the pattern of mortality during the salinity challenge vitality test and the stress indices are given in Figure 6.4.9.B. These indices decrease with increase in size showing that larger fish are more resistant to the challenge. A significant difference was only detected between the Small group and the rest (ANOVA, $p < 0.05$).

6.4.3.5. Metamorphosis and pigmentation

In all groups, the majority of fish were abnormal with respect to metamorphosis and pigmentation characteristics. Eye migration was poor in all groups (mean ≤ 1.6) and a large proportion were pigmented on both dorsal and ventral surfaces (see Figure 6.4.10). No normal fish were present in any of the groups. There were no significant differences between any group with respect to these traits (ANOVA, $p > 0.05$). The appearance of the fish can be seen in the photographs in Figure 6.4.11. which show the metamorphosis characteristics and size range of the fish at the end of the experiment.

A)



B)

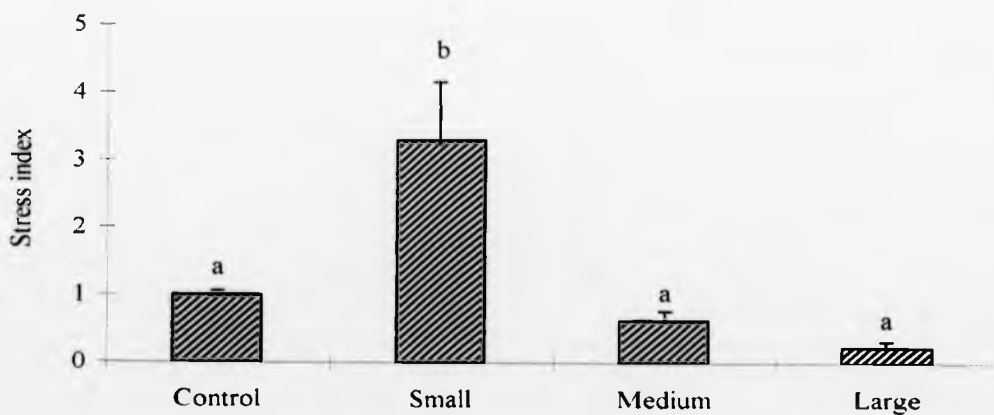


Figure 6.4.9. A) Cumulative mortality for Control and 3 graded groups of fry during 65 ppt salinity challenge test. B) Stress index (calculated as in Section 6.3.3.4.) Values are means of 3 replicates. Error bars represent standard error of means, a,b denote significant differences (ANOVA, $p < 0.05$).

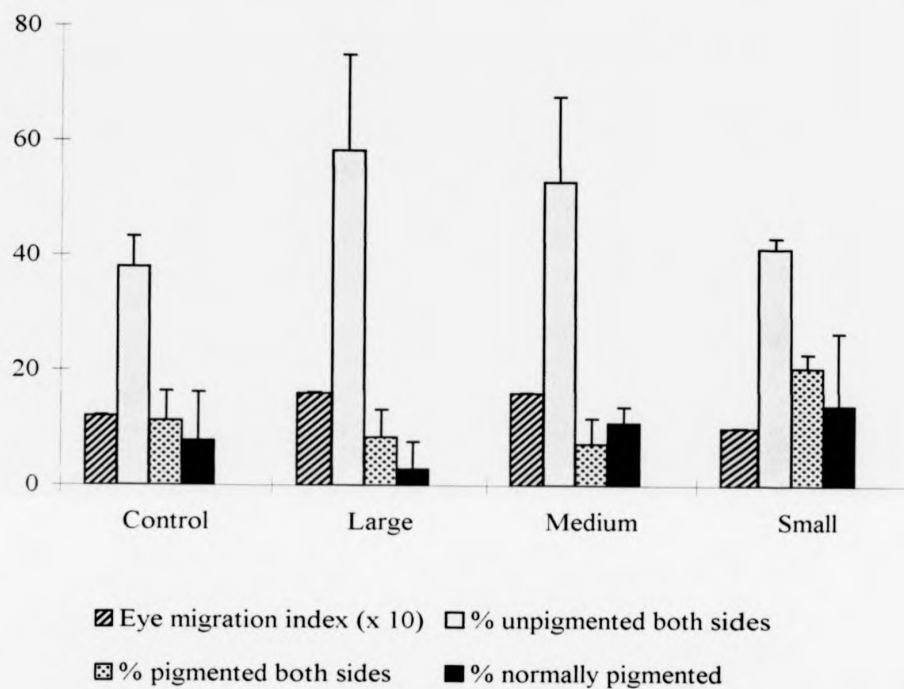


Figure 6.4.10. Metamorphosis and pigmentation characteristics for each group of fish. Mean eye migration index, % unpigmented on both dorsal and ventral sides, % pigmented on both dorsal and ventral sides, % normally pigmented (dorsal side only). Values are means of 3 replicates. Error bars represent standard error of means.

Control



Large



Medium



Small

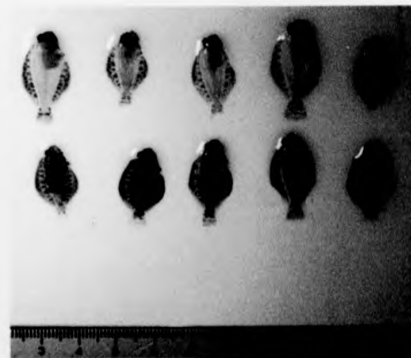


Figure 6.4.11. Photographs of representatives from each group of fish sampled at the end of the experiment. Control group (ungraded), Large group, Medium group and Small group. Photographs are to scale indicated by the cm graduations on ruler.

6.4.4. Conclusions for Experiment 3

Since the graded and ungraded populations were similar in terms of their survival and growth characteristics (CV, CF, SGRs, stress index and final biometric statistics) it can be assumed that they are similar populations. It must be concluded that grading in itself and the stratification of a population of halibut larvae will not improve overall survival or growth rates. However, by dividing the population, the slow growers were isolated and although this did not reduce their mortality through a putative reduction in aggression or competition, it did to some extent localise the population mortality.

It is common commercial practice to remove slower growing fish at this point to delay weaning or extend the overlap period between live and artificial diet to minimise losses. Meanwhile weaning can proceed relatively rapidly for the faster growing fish and their growth potential can be realised once established onto inert diet.

In the Control and Large groups there was a slight decrease in average wet weight during the weaning period whilst in the Medium and Small groups, specific growth rates were slightly positive. The fish were fed *Artemia* at a rate/fish not per weight. The larger fish would have a higher requirement for *Artemia* which is reduced through the transition period and possibly the smaller individuals would have received a higher proportion of their requirement during this time. Once weaned, the larger fish exhibit higher specific growth rates on average than the smaller fish. The fact that minor grading was necessary to maintain size classes demonstrates that individual rates of growth change resulting in promotion or demotion of certain individuals.

From a theoretical point of view, the division of the population into discrete size classes enabled the demonstration and partial correction of size specific mortality which

is common to most growth studies on fish larvae. The only way to completely avoid the over estimation of average growth rates in populations is to tag individual animals, a solution which is clearly impractical with larvae.

Metamorphosis characteristics were poor in this batch of fish and this is a common trait with hatchery-reared halibut and is likely to result from nutritional deficiencies during the live feeding stage.

Cannibalism was not witnessed in any of these groups of larvae and since survival rates were similar in both graded and ungraded groups, it has to be assumed that this was not a factor influencing survival. The ratio between maximum and minimum weights in the ungraded group was approximately 7.5:1 but in spite this large size distribution no aggressive behaviour was exhibited by the larvae. This is not the only factor influencing cannibalistic behaviour in fish larvae and other criteria such as feeding rate and density also play a role (Hecht and Pienaar, 1993).

Despite regular handling during the experiment for the purposes of grading or sample weighing, survival rates were high and fish were not unduly affected. It seems that halibut of this size can be quite robust and not highly susceptible to handling stress.

6.5. Experiment 4: Co-feeding and early weaning pilot experiment

6.5.1. Introduction and aims

The previous experiments concentrated on weaning larvae of 100 mg mean wet weight and above. Observations of feeding behaviour in these experiments had indicated that in some instances, larvae smaller than the population average, i.e. considerably less than 100 mg in size, consumed artificial diet. Since these larvae were the slower growing individuals, it was hypothesised that the faster growing individuals may accept artificial diet at a much earlier stage. To determine at what age/size halibut will accept inert feed, it was necessary to assess the uptake of dry diet starting soon after first feeding.

The perception that the energetic requirements of first feeding halibut larvae are not entirely met through the provision of *Artemia* based nutrition, and the possibility that supplementation with formulated artificial diets may boost the nutritional intake of halibut larvae led to a research priority which was highlighted by the British Halibut Association. It was proposed that an investigation be conducted to examine whether the presentation of artificial dry diet alongside live *Artemia* during the first feeding phase could improve growth, survival and metamorphosis characteristics of halibut fry.

The first step in this investigation was to assess the response of very young halibut larvae to the presence of an inert particulate diet in the water column and to ascertain whether any uptake occurred. Thus, a pilot trial was conceived with the purpose of comparing the uptake of inert diet by halibut larvae fed different combinations of *Artemia* and inert diet presented exclusively or simultaneously. This was done with halibut larvae of different ages, starting soon after first feeding.

6.5.2. Materials and methods

This experiment was conducted on a single batch of larvae at three different ages (stages 1-3, see Figure 6.5.1.). At stage 1, larvae from a group of established feeders were taken at day 23 post first-feeding (459°Cdays post hatch) at a mean wet of 18.8 ± 5.64 mg, dry weight 2.83 ± 0.96 mg and length 17.5 ± 1.66 mm and allocated randomly, 30 per tank to 4 tanks (circular GRP black, volume 245 l, bottom/ top diameter, 42/45 cm resp., depth 33 cm). Lighting was supplied by over head tungsten lights, giving illumination of approximately 150 lux at the surface. Filtered (5 μ m) seawater was supplied at an ambient temperature of 11 - 15.5°C and at a flow rate of 120 - 160 ml/min. These larvae were acclimated for 3 days whereupon 4 treatments were applied, one tank per treatment group. In the first tank, group A was fed three *Artemia* rations per day to satiation at 10.00 hrs, 14.00 hrs and 18.00 hrs. In the second tank, group IA was fed the same amount of *Artemia* as group A as well as inert diet (Nutra Marine) by hand several times per day. Group IA/3 (tank 3) was fed the third ration of *Artemia* at 18.00 hrs only and inert diet as for group IA. Group I (tank 4) was fed inert diet exclusively. *Artemia* enrichments were as described in Experiment 3.

The fish were held under these conditions for 10 days until day 33 PFF.

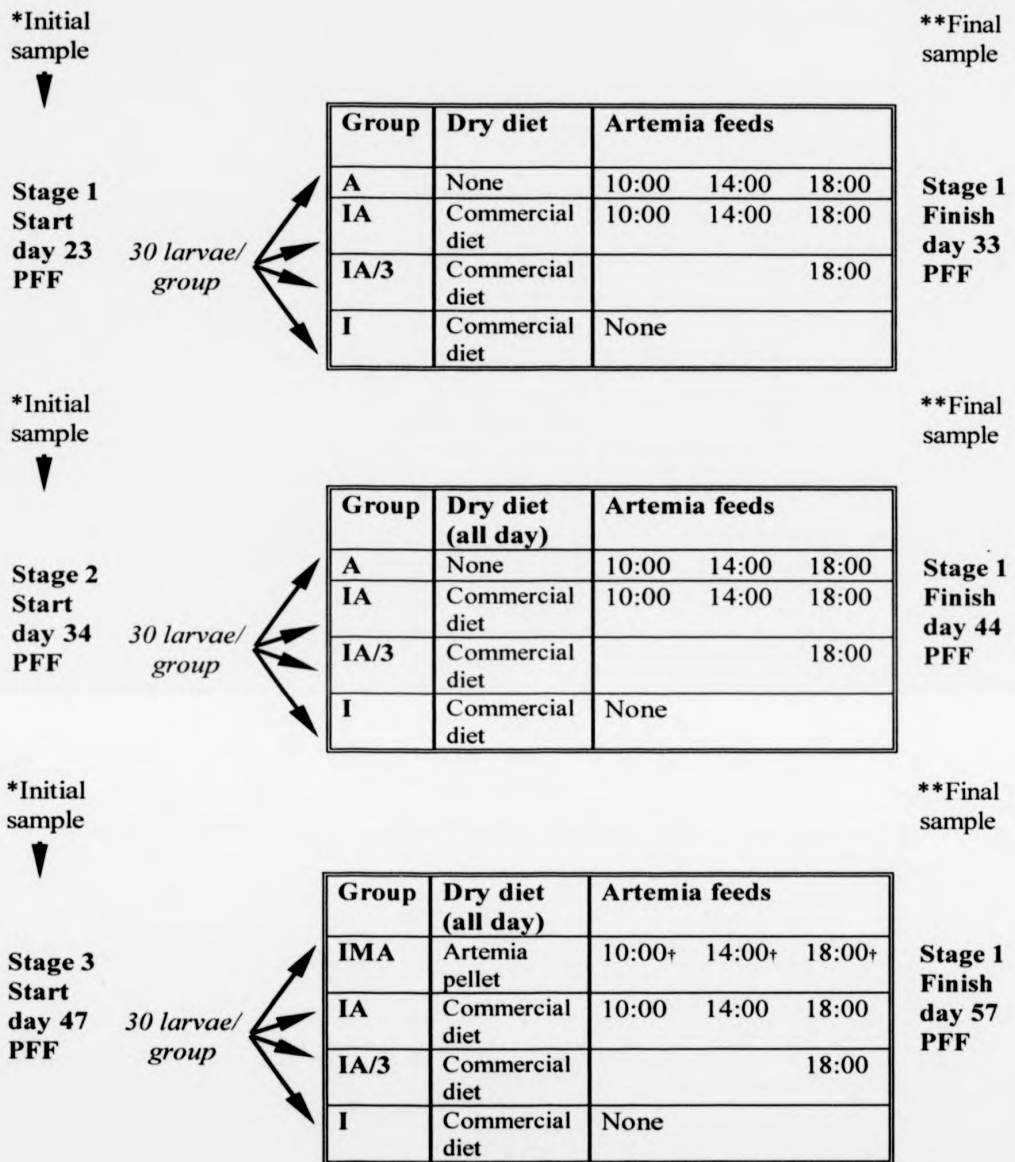


Figure 6.5.1. Diagram of experimental procedures indicating age of larvae at each stage, feeding regimes and sampling programme. *Initial sample for wet weight & length (n=14) & dry weight (n=9) **End sample for survival, wet weight, length and feed reponse (all survivors) and dry weight (n=9). †immobilised *Artemia*(see text).

This was repeated with larvae from the same initial batch starting at day 34 PFF (589°Cdays post hatch); mean wet weight 23.3 ± 5.74 mg, dry weight 2.87 ± 2.27 mg and length 17.2 ± 0.88 mm (Stage 2) and day 47 PFF (754°Cdays post hatch); mean wet weight 43.4 ± 13.59 mg, dry weight 5.82 ± 5.65 mg and length 19.5 ± 0.83 mm (Stage 3). The treatments were identical apart from the oldest larvae where group A was replaced by a treatment in which immobilised *Artemia* (frozen and thawed the same day) were fed in place of live *Artemia* and a crumbed pellet containing fishmeal, marine body oil, vitamins and minerals, binder and freeze dried *Artemia* was presented rather than the commercial pellet (group IMA).

Larvae were sampled at the beginning of each time period for wet weight and length (14 larvae) and dry weight (9 larvae, in groups of 3). At the end of each period, all survivors were counted wet weighed and measured for length and up to 9 larvae per treatment were dry weighed. The gut contents were observed under a dissection microscope (Meiji Labrax, Tokyo, Japan, 0.5× objective) and the number consuming inert diet was recorded for each group. This was done prior to the final *Artemia* feed so that group IA/3 had been fed only inert diet that day.

Tank bottoms were siphoned clean every other day and water quality parameters (temperature, O₂, salinity and pH) were measured daily. Algae (*Nannochloris atomus*.) was added to every tank each day to 'green' the water.

6.5.3. Results

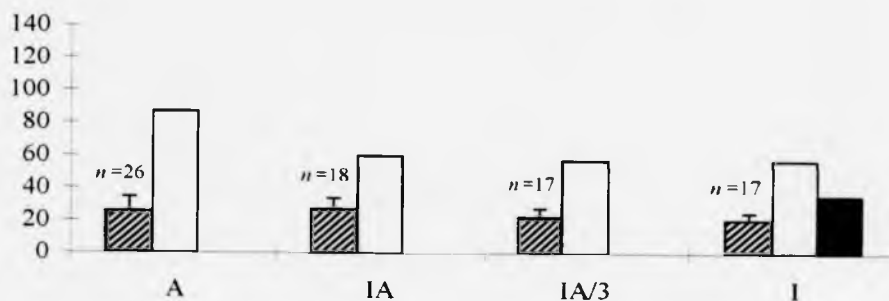
6.5.3.1. Survival

Survival rates, shown in Figure 6.5.2. were lowest in those groups transferred directly to dry diet (group I) and this mortality was most severe in the oldest group. In those groups receiving *Artemia*, survival was higher in those groups fed 3× per day than those fed 1× per day at all stages. In the group that received immobilised *Artemia* and *Artemia* pellet, survival was relatively high (77%). No statistical analysis was performed on survival rates since treatments were unreplicated.

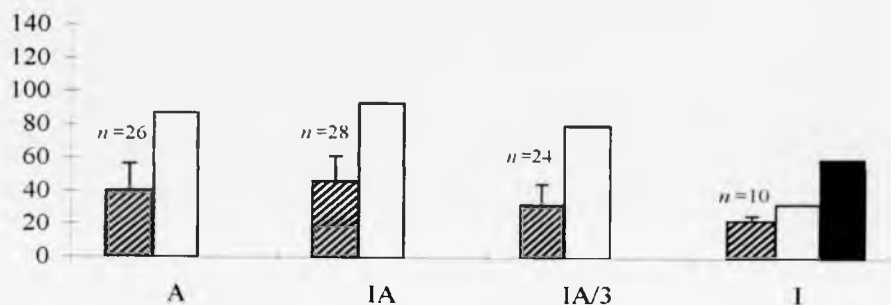
6.5.3.2. Feed response

Examination of gut contents at the end of each 10 day period revealed that during the first 2 stages, larvae fed *Artemia* and dry diet simultaneously rejected the latter even when only one ration of *Artemia* was given at the end of the day (see Figure 6.5.2.). When dry diet and a reduced ration of *Artemia* was presented to group IA/3 at the final stage, some uptake of diet was apparent. At each stage some larvae ingested dry diet when this was presented exclusively (group I) and the percentage of survivors accepting dry diet in this group increased with age. However, given the high mortality in these groups, the actual number of fish taking dry diet was small. The best feed response to inert diet occurred in the group fed immobilised *Artemia* followed by *Artemia* pellet at stage 3 (group IMA). Larvae in this group immediately accepted the immobilised *Artemia* and within 3 days most of the surviving fish were feeding on the *Artemia* pellet.

Stage 1. Day 23-33 PFF



Stage 2. Day 34-44 PFF



Stage 3. Day 47-57 PFF

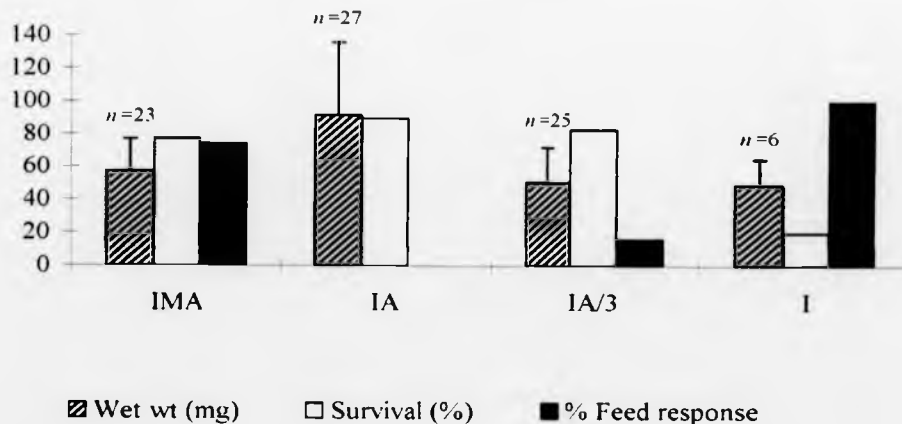


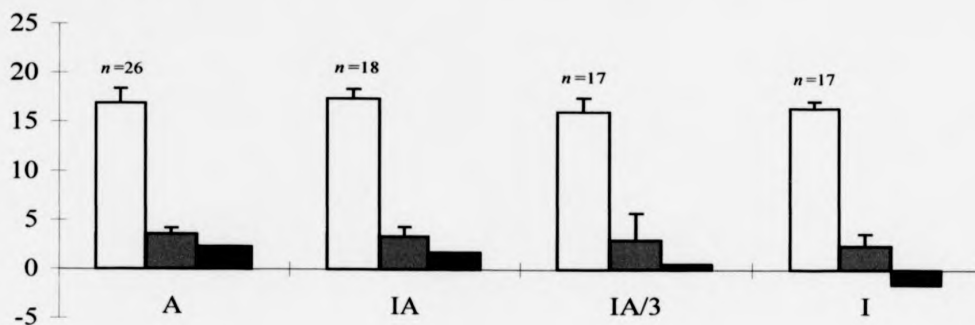
Figure 6.5.2. Final mean wet weight (mg), survival during experimental period (%) and feed response (% of survivors taking inert diet at the end of each period) at each stage of the experiment. Values are based on samples of n individuals from the single replicate tanks. Error bars indicate standard deviation of sample.

6.5.3.3. Growth

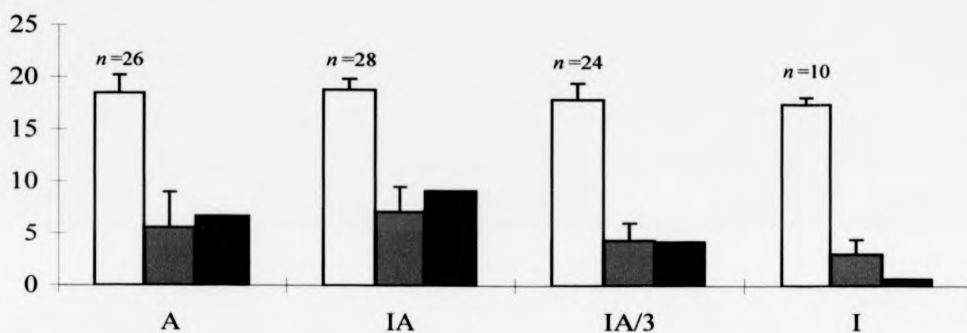
At each stage, final wet weights and, shown in Figure 6.5.2. were highest in the group fed both a full ration of *Artemia* and dry diet simultaneously, followed by those fed *Artemia* alone in stage 1 and 2 and by group IMA at stage 3. The final lengths (shown in Figure 6.5.3.) did not differ greatly but followed a similar pattern to the wet weights. Final dry weights at each stage were also highest in groups fed a full *Artemia* ration (A and IA) and lowest in those fed inert diet only (I). This was reflected in the specific growth rates which, in group I, were apparently negative (see Figure 6.5.3.). In stage 3, some growth was apparent in the group fed immobilised *Artemia* and *Artemia* pellet (IMA).

Figures 6.5.4. to 6.5.6 shows photomicrographs of representative larvae from different groups at each stage of the experiment indicating the size and developmental stage of the larvae at each age and the gut contents.

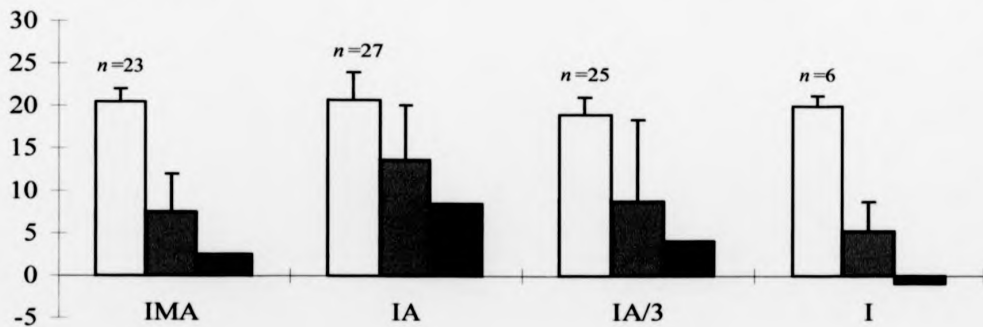
Stage 1. Day 23-33 PFF



Stage 2. Day 34-44 PFF



Stage 3. Day 47-57 PFF



□ Length (mm) ■ Dry Wt (mg) ■ SGR (%/day)

Figure 6.5.3. Final mean length (mm), dry weight (mg) and specific growth rate (SGR, %/day) based on dry weight for each group at each stage of the experiment. Values are based on samples of n individuals from the single replicate tanks. Error bars indicate standard deviation of sample.

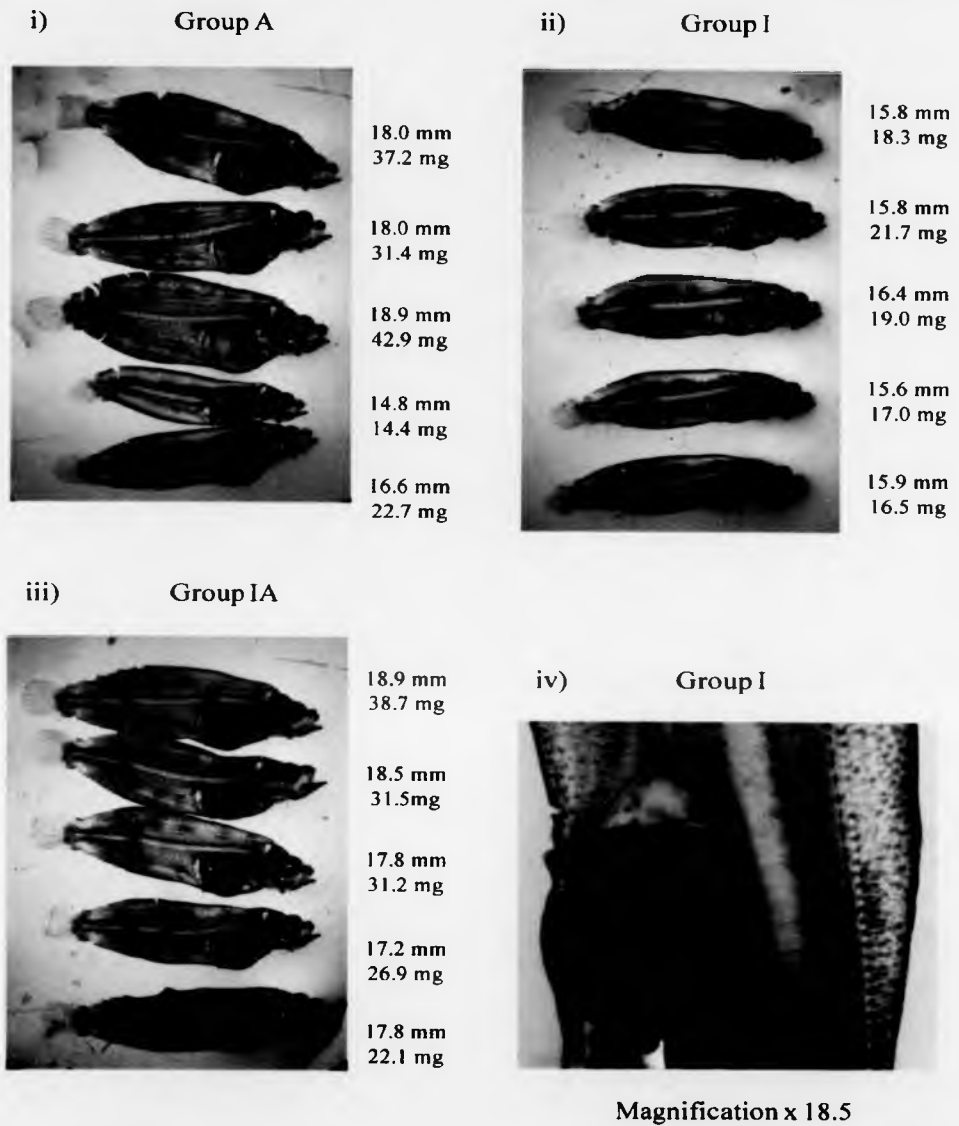


Figure 6.5.4. Photomicrographs of representative halibut larvae from some of the treatment groups for Stage 1, aged 33 days PFF. i) 5 larvae from group A, fed *Artemia* only. Guts contents is *Artemia*. ii) 5 larvae from group I, fed inert diet only. These larvae have ingested inert diet. iii) 5 larvae from group IA, fed both *Artemia* and inert diet. These larvae have only *Artemia* in the gut. iv) Close up of gut contents of larva from group I, fed inert diet only. Wet weights and total lengths are given for each fish.

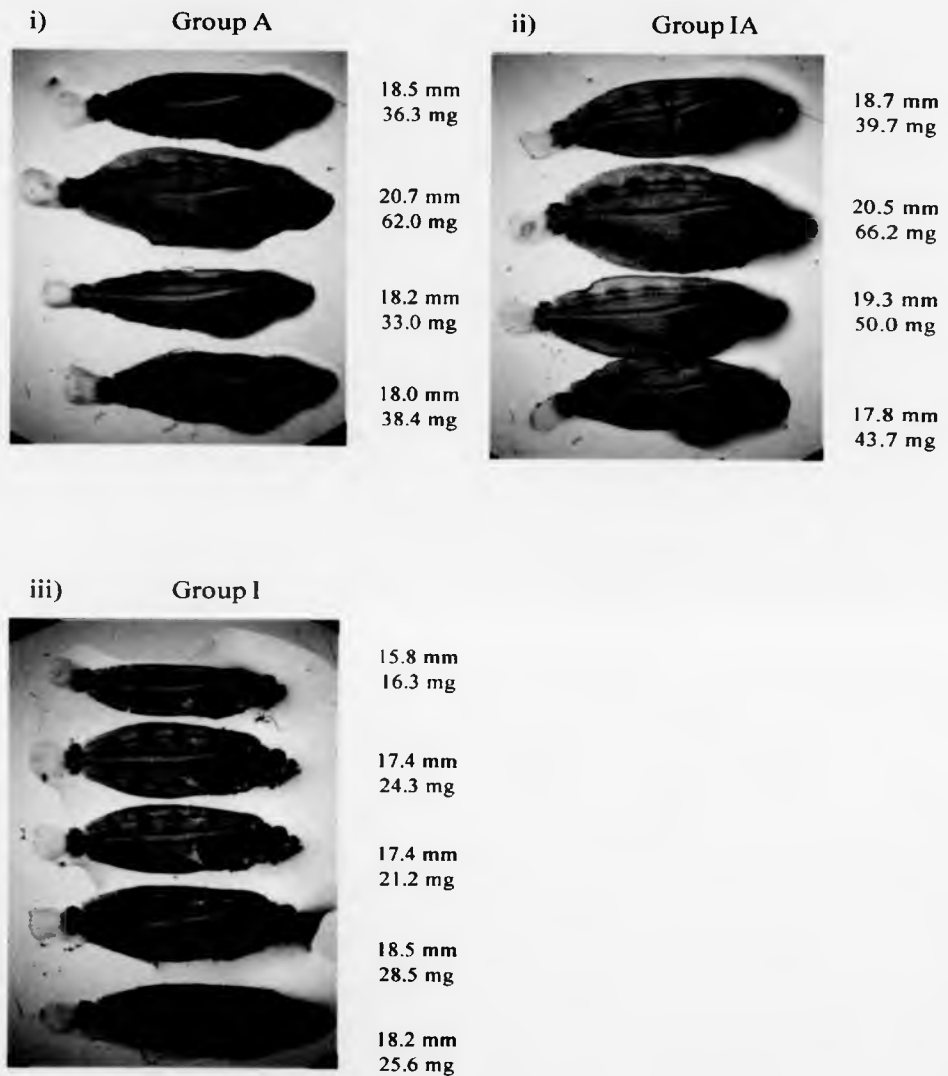


Figure 6.5.5. Photomicrographs of representative halibut larvae from some of the treatment groups for Stage 2, aged 44 days PFF. i) 4 larvae from group A, fed *Artemia* only. Guts contents is *Artemia*. ii) 4 larvae from group IA, fed both *Artemia* and inert diet. These larvae have only *Artemia* in the gut. iii) 4 larvae from group I, fed inert diet only. These larvae have ingested inert diet. Wet weights and total lengths are given for each fish.

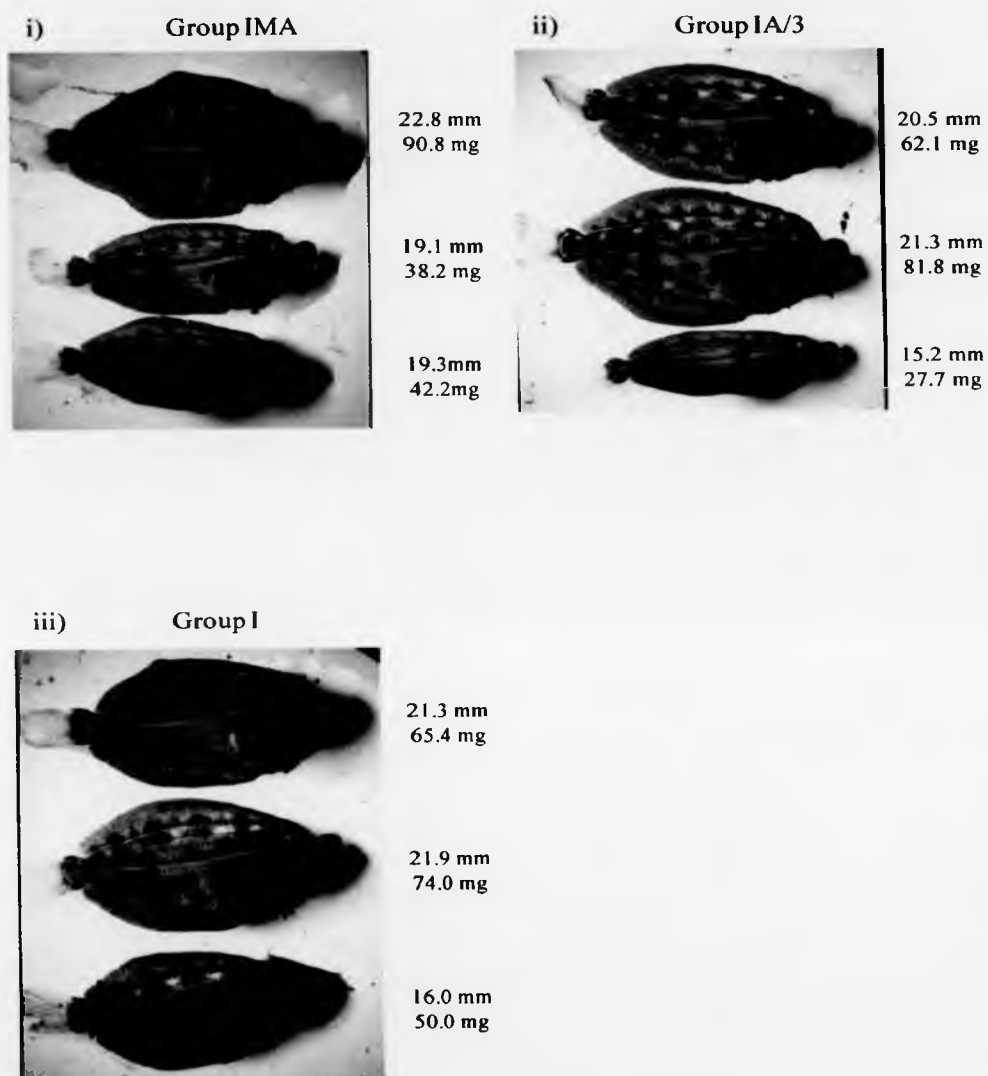


Figure 6.5.6. Photomicrographs of representative halibut larvae from some of the treatment groups for Stage 3, aged 57 days PFF. i) 3 larvae from group IMA, fed immobilised *Artemia* then *Artemia* pellet. Guts contents is *Artemia* pellet. ii) 3 larvae from group IA/3, fed inert diet and reduced *Artemia*. These larvae have both diet types in the gut. iii) 3 larvae from group I, fed inert diet only. These larvae have ingested inert diet. Wet weights and total lengths are given for each fish.

6.5.4. Conclusions for Experiment 4

Immediate transfer to dry diet caused high mortality and growth depression at all stages. However, it was only in the group (I) where larvae were completely deprived of *Artemia*, that any uptake of dry diet was observed in the first two stages. Larvae fed *Artemia* and dry diet simultaneously, preferentially selected *Artemia* and showed no uptake of dry diet between day 23 and 44 PFF and limited uptake from day 47 - 57 PFF in the group fed a reduced *Artemia* ration (group IA/3). In the group fed *Artemia* 3× per day, no ingestion of dry diet was observed at any stage but growth was highest at stages 2 and 3 in this group.

These results show that direct transfer to inert diet is severely detrimental for halibut larvae of less than 40 mg wet weight resulting in heavy losses and poor growth. The limited uptake of dry diet in very young larvae in the complete absence of live prey indicates that it is possible to induce to some extent the ingestion of inert particles. The fact that the oldest group fed a reduced *Artemia* ration showed some interest in dry diet, whilst the younger larvae completely rejected it, demonstrates the gradual development in capacity to adapt to inert diets with increasing age/size. This points to the possibility of weaning at least part of a population of halibut larvae at under 50 mg wet weight. Though some larvae were feeding on the inert diet at this age/size range, it is unknown whether the diet would fulfill all nutritional requirements of halibut larvae and sustain growth for extended periods since each of the experiments was only of 10 days duration.

The immediate acceptance of immobilised *Artemia* used in the final age group demonstrates that halibut larvae of this size will feed on prey which is not motile but presumably looks, smells and tastes similar to living *Artemia*. The feed response was

triggered without movement. This group also readily accepted the pellet containing freeze dried *Artemia* and there are a number of possible explanations for this including:

- a) Immobilised *Artemia* helped to condition the larvae to an inert particle.
- b) The *Artemia* pellet contains substances derived from *Artemia* that act as feed stimulants.
- c) The *Artemia* pellet is more attractive as an artificial diet due to other characteristics e.g. texture, colour or sinking rate.

If explanation a) were true, then immobilised *Artemia* may be a useful intermediate for weaning to a conventional weaning diet such as Nutra Marine. If explanation b) were true then conventional weaning diets may be improved by the addition of specific feed attractants and if c) were the case then they may be improved through manipulating their physical characteristics. The *Artemia* pellet was a much softer, lower density feed than Nutra marine with a 'fluffy' texture and coloured orange/red. The response both to immobilised *Artemia* and *Artemia* pellet reveals much about the adaptability of feeding behaviour in the halibut larvae and lead to further investigation into possible improvements in weaning methods in the following experiment.

The apparent growth advantage conferred upon the cofed group IA suggests possible advantages in the simultaneous presentation of inert diets and *Artemia*. Consequently, this question was further investigated in Experiment 5.

6.6. Experiment 5: Co-feeding and early weaning I

6.6.1. Introduction

Based on the observations from Experiment 4, a replicated experiment set up was then designed. The aims of this experiment were to test more rigorously the performance of the *Artemia*/commercial dry diet co-feeding regime over an extended period and compare this with a conventional, phased weaning strategy. The promising results from the pilot study using immobilised *Artemia* and *Artemia* pellet led to the speculation that halibut larvae could be conditioned to commercial weaning diets using these feeds. This required closer scrutiny and so treatments contrived to assess the use of these as intermediate feeds for facilitating transfer to commercial dry diet were also included in this experiment. The effects of presenting inert diets during the early feeding phase on water quality were also monitored.

6.6.2. Materials and Methods

6.6.2.1. Live material and facilities

Twelve tanks (described in Section 2.4.3.) were stocked with 65 larvae per tank from a single batch aged 24 days PFF (520 °Cdays post hatch) at a mean wet weight of 19.0 ± 5.7 mg, length 1.63 ± 0.12 cm, dry weight 2.8 ± 0.50 mg.

The tanks received chilled, filtered (5 μ m) sea water, at a mean ambient salinity of 33.8 ppt (range: 33.1 - 34.4 ppt). Temperature was maintained at between 11.3 - 15.3°C (mean: 13.2°C). Mean oxygen concentration was 8.6 mg/l (range: 7.0-10.0 mg/l) and the mean pH was 8.09 (range: 7.97-8.17). The tank inlets were sub-surface horizontal spreader bars angled to induce a directional water flow and the depth was set at 30 cm

(tank volume of 125 l). Flow rates were set at 150 ml/min until day 36 PFF and then increased to 200 ml/min. Algae (*Nannochloris atomus*) was added at a rate of 2 l per day until day 69 PFF. Illumination was set at approximately 150 - 200 lux at the water surface.

The tank bottoms were siphoned once every 2 days to remove waste feed and faeces. Water quality measurements included daily O₂, temperature and salinity and weekly ammonia readings to monitor the effect of waste feed.

6.6.2.2. Feeding regime

After 5 days acclimation period during which all tanks received 3 *Artemia* feeds per day, 4 treatments were applied (3 replicate tanks per treatment) as follows:

Table 6.6.1. Feeding regimes applied to 4 groups of larvae.

Group	Treatment
Control	Fed 3x per day with live <i>Artemia</i> to satiation until normal weaning age.
CF	Fed 3x per day with live <i>Artemia</i> to satiation and commercial dry diet (Nutra Marine, T.Skretting AS, Norway) until normal weaning age.
SK	Weaned from live to immobilised <i>Artemia</i> and then to dry diet; timing of transition determined by feed response.
AP	Weaned from live to immobilised <i>Artemia</i> and then to a crumb including dried <i>Artemia</i> then to commercial dry diet; timing of transition determined by feed response.

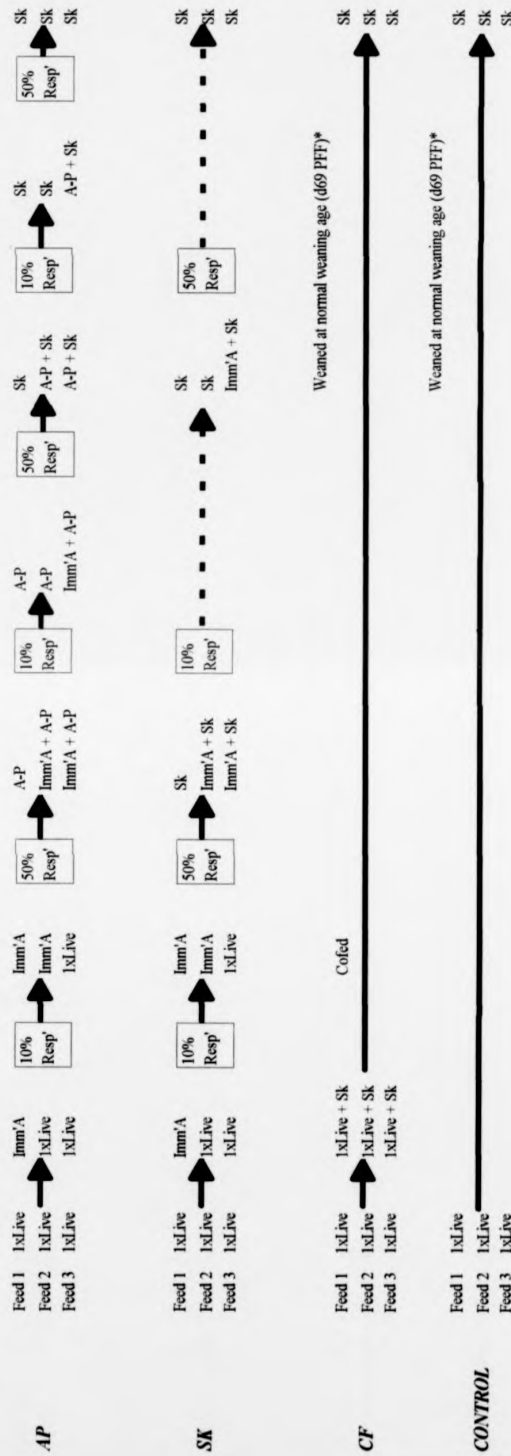
The transition to new feed types was determined by the feed response of the larvae which was assessed visually from the gut contents of 10 larvae per tank. A detailed feeding protocol is shown schematically in Figure 6.6.1.

The Control and CF groups received 3 equal *Artemia* rations per day until weaning according to the 'standard' 9 day weaning regime (see Experiment 2) starting at day 69 PFF. Larvae were fed to satiation and *Artemia* rations were increased from approximately 600 prey/larva/day to approximately 4000 prey/larva/day just prior to weaning (day 69 PFF). The *Artemia* were enriched separately using Super Selco and Algamac 2000.

The commercial dry diet (Nutra Marine) was delivered by hand at the start of the experiment approximately 5 times per day until the installation of automatic feeders (Norfab Ltd, Fort William, U.K.) which were commissioned on day 44 PFF. The feeders were controlled by time switches and calibrated to deliver approximately 0.2g per feed once every hour between 08.00 and 18.00 hrs. The feed was spread evenly over the surface of the tank via a spinning disc-type distributor. The feeders are described in Section 2.4.3. Crumb size range increased from 0.3 - 0.5 mm to 0.5 - 0.8 mm and finally to 0.8 - 1.4 mm as the fish grew.

6.6.2.3. Sampling

Larvae were sampled at the start of the experiment on day 30 PFF (22 larvae) for wet weight, dry weight and length, again when the Control and CF groups began weaning at day 69 PFF (4 fish per tank) for wet weight and lipid analysis and at the end of the experiment at day 86 PFF for wet weight (all survivors), dry weight (up to 10 fish per tank) and length (all survivors). The lipid analysis was carried out by the NERC



Key: Live = Live *Artemia*
 Imm'A = Immobilised *Artemia*
 #% Resp' = Percentage feed response (as criteria for progressing to next stage)
 AP = *Artemia* pellet
 SK = Skretting Nutra marine commercial dry feed
 * weaned through 9 day weaning regime as described experiment 2

Figure 6.6.1. Diagram showing experimental feeding regime for the 4 groups of fish

laboratory, Department of Aquatic Biochemistry, Stirling University. All surviving fish were also assessed for metamorphosis and pigmentation characteristics at the end of the experiment using the methods described in Appendix II.

6.6.2.4. Statistical analysis

Results were analysed statistically using ANOVA and Tukey pairwise comparisons. Where percentage data were analysed, values were transformed using arcsine transformation before statistical analysis.

6.6.3. Results

6.6.3.1. Survival

Survival rates were initially calculated on the basis of daily removal of mortalities, until a recount at day 42 PFF revealed that a number of fish were missing due to swimming or falling through the perforations in the bottom plates. The fish were therefore recounted and a fine mesh was placed over the plates. Survival rates displayed for days 26 - 42 PFF account for these missing fish, while rates for days 43 - 86 PFF are based on the recount and numbers of fish remaining at the end of the experiment (see Figure 6.6.2.). Survival rates were generally poor and for the SK group, significantly lower (ANOVA, $p < 0.05$) than the other groups.

6.6.3.2. Feed response

The response to immobilised *Artemia* presented to the AP and SK groups was immediate and exceeded 10% on the first day (day 30 PFF), and 50% on the second day in all tanks. All tanks in these groups were therefore presented with dry diets by day 33 PFF. Uptake of dry diet was very slow (see Figure 6.6.3) and in the SK group eventually reached 50% by day 61 PFF. This level was not reached in the AP group and so this group was weaned according to the 'standard' 9 day regime along with the Control and CF groups starting at day 69 PFF. The response of these latter groups increased rapidly once the *Artemia* feeds were almost completely removed. During the co-feeding period there was no uptake of diet in the CF group.

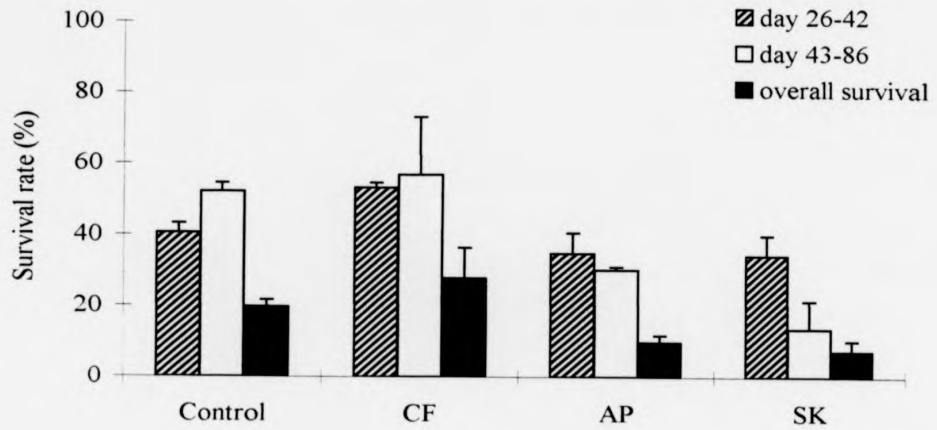


Figure 6.6.2. Survival rates for each group at day 26-42 PFF, day 43-86 PFF and overall survival. Values are means of 3 replicates. Error bars represent standard error of means.

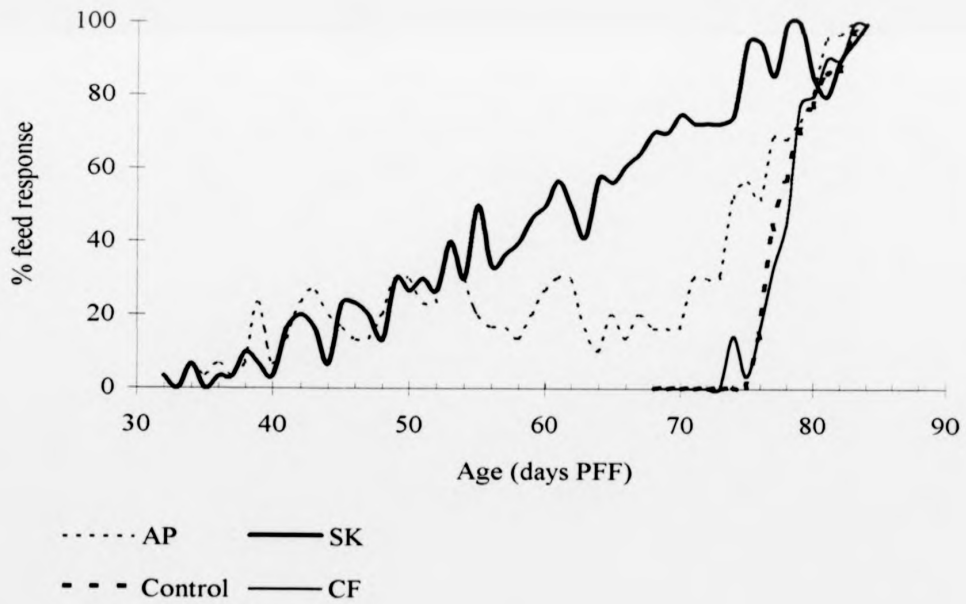


Figure 6.6.3. Feed response (% taking inert diet) of each group during the experiment, from the start of feeding inert diet. Values are the means of 3 replicates.

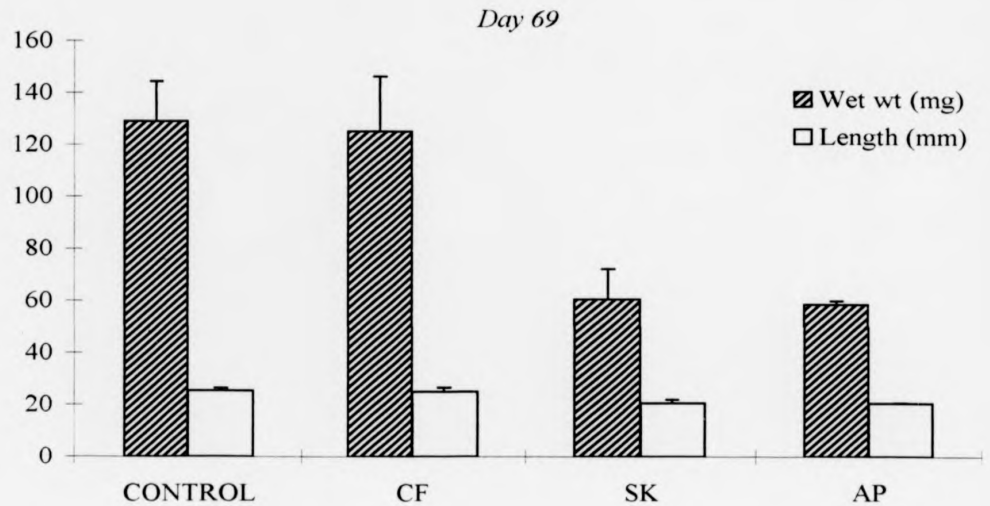
6.6.3.3. Growth

Wet weight and length at day 69 and final wet weight, dry weight and length for each group are shown in Figure 6.6.4. The intermediate sample (day 69 PFF) revealed lower mean weights in the AP and SK groups. The final wet and dry weights were lower again in the AP and SK groups than in the CF and Control. Wet weights of the latter 2 groups were significantly higher (ANOVA, $p < 0.05$). There was an apparent loss of wet weight between day 69 and 86 PFF in the Control and SK groups.

6.6.3.4. Metamorphosis and pigmentation

Pigmentation was generally poor in all groups with a maximum of 20.2% perfectly pigmented fish in the Control group (see Figure 6.6.5.). The majority of fish (100% in groups SK and AP and >60% in groups CF and Control) were pigmented on both dorsal and ventral sides and the difference between these pairs of groups was found to be significant (ANOVA, $p < 0.05$). There were no other significant differences between treatments with respect to metamorphosis characteristics. Eye migration was higher in the Control and CF groups than in the SK and AP groups, although migration indices were low in all treatments.

A)



B)

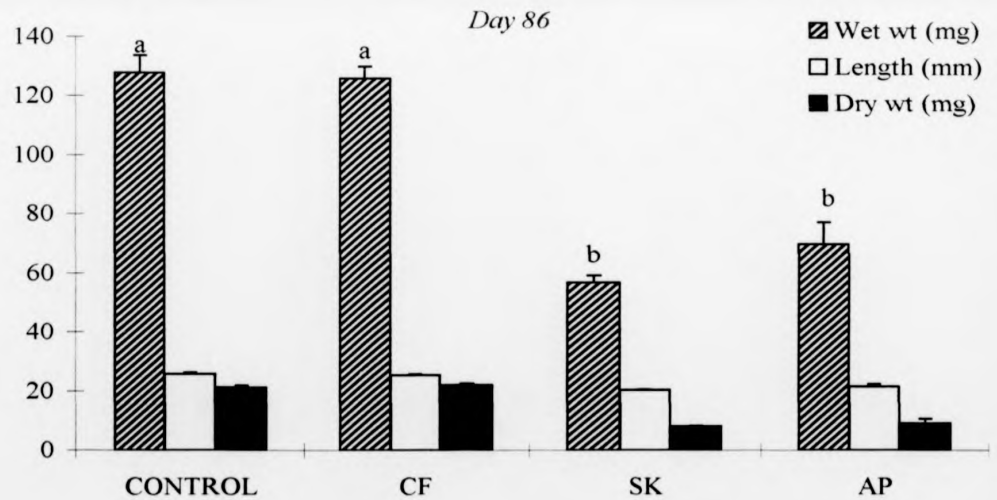


Figure 6.6.4. A) Wet weight (mg) and length (mm) on day 69 PFF. B) Wet weight (mg), dry weight (mg) and length (mm) at the end of the experiment on day 86 PFF for each group. Values are the means of 3 replicates. Error bars represent standard error of means. a,b denote significant differences (ANOVA, $p < 0.05$).

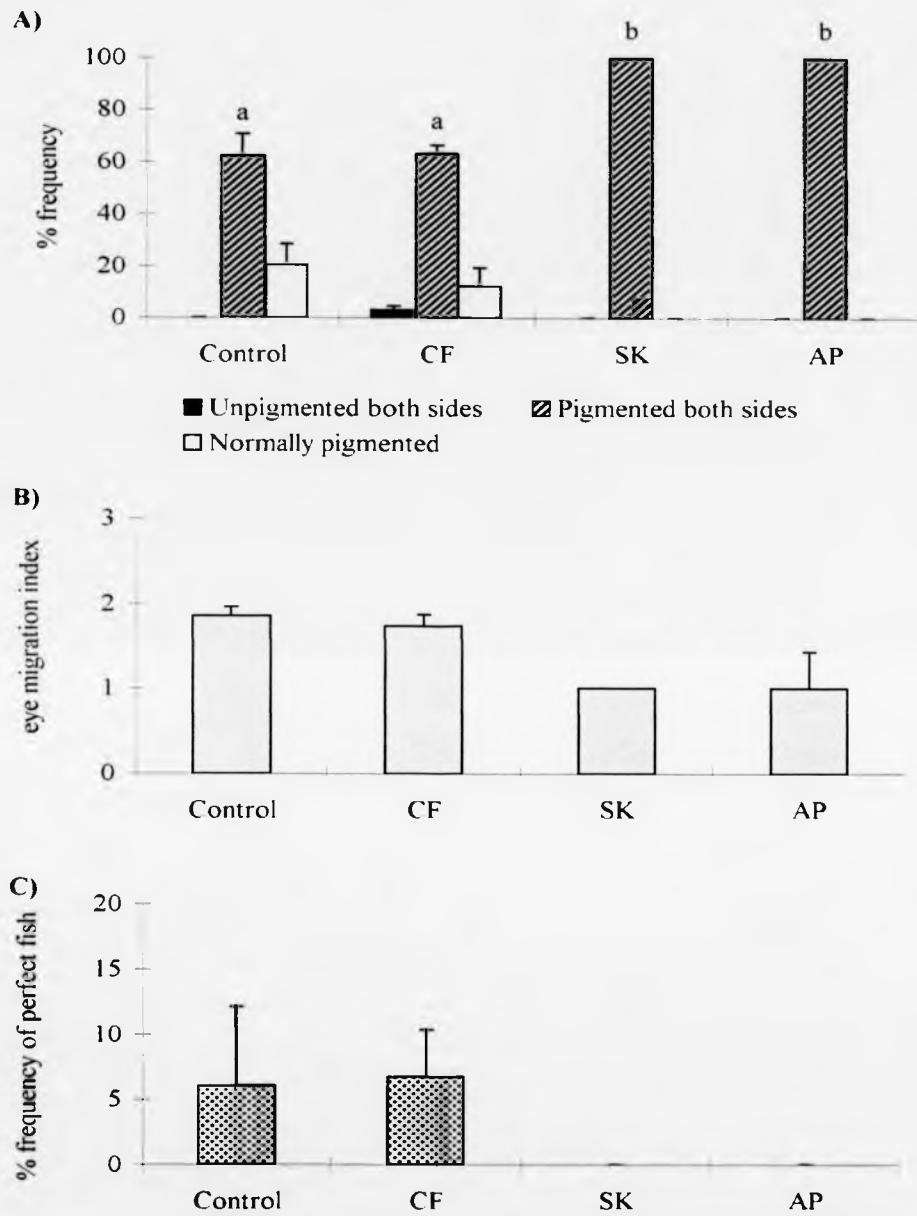


Figure 6.6.5. A) Percentage frequency of pigmentation categories, B) Mean eye migration index and C) Percentage frequency of perfect fish for each group of fish. Values are the means of three replicates. Error bars represent standard error of means. a,b denotes significant differences between means (ANOVA, $p < 0.05$).

6.6.3.5. Carcass lipid content

Table 6.6.2. Fatty acid composition of 69 day old larval carcasses in Control and CF groups. Values are the means (area %) of two samples (4 larvae per sample) \pm s.d. a,b denotes significant difference between groups (ANOVA $p < 0.05$). Data provided by NERC lab, Aquatic Biochemistry, Stirling University.

	CONTROL area% (\pm s.d.)	CF area% (\pm s.d.)
Total saturates	21.0 (0.3)	20.6 (0.5)
Total monoenes	34.2 (2.0)	31.7 (0.3)
18:2n-6	4.1 (0.0)	3.9 (0.0)
18:3n-3	8.7 (0.0)	8.7 (0.5)
20:4n-6	3.9 (0.3)	3.7 (0.1)
20:5n-3	15.2 (0.7)	16.4 (1.0)
22:5n-6	1.1 (0.1)	1.2 (0.1)
22:6n-3	6.9 (0.4) a	9.1 (0.1) b
Total n-6 HUFA	9.5 (0.4)	9.1 (0.1)
Total n-3 HUFA	34.3 (1.2)	37.9 (1.0)
DHA/EPA	0.5 (0.0)	0.6 (0.0)
EPA/AA	3.9 (0.1)	4.5 (0.1)

The fatty acid composition of carcasses from the CONTROL and CF treatments were very similar, except for the level of DHA, which was significantly higher in CF carcasses.

Table 6.6.3. Lipid class composition of 69 day old larval carcasses in Control and CF groups. Values are the means (area %) of two samples (4 larvae per sample) \pm s.d. Data provided by NERC lab, Aquatic Biochemistry, Stirling University.

	Control area% (\pm s.d.)	CF area% (\pm s.d.)
LPC Lysophosphatidylcholine	trace	0.1 (0.1)
SM Sphingomyelin	1.6 (0.2)	1.7 (0.4)
PC Phosphatidylcholine	21.4 (0.1)	19.0 (1.9)
PS Phosphatidylserine	3.4 (0.1)	3.2 (0.7)
PI Phosphatidylinisitol	3.1 (0.1)	3.0 (0.7)
CL/PG Cardiolipin/Phosphatidylglycerol	1.6 (0.1)	1.6 (0.5)
PE Phosphatidylethanolamine	13.0 (0.1)	11.8 (1.6)
Pigment	1.3 (0.1)	1.5 (0.2)
Cholesterol	15.8 (0.2)	14.7 (0.2)
FFA Free fatty acids	1.8 (0.1)	1.9 (0.5)
TAG Triacylglycerol	31.2 (0.3)	35.4 (5.9)
Sterol esters	5.7 (0.8)	6.1 (0.5)

The lipid class composition of the two groups did not differ significantly in any respect.

6.6.3.6. Water quality

Ammonia levels reached excessive values during the dry diet feeding phase (up to 0.6

mg/l) at flow rates of 150 ml/min. These values were reduced only slightly after flow rates were increased to 200 ml/min. Fouling of the tank floors was caused by accumulation of uneaten dry feed and in the AP group, immobilised *Artemia*.

6.6.4. Conclusions for Experiment 5

The relatively low growth and survival rates obtained under the Control weaning regime indicate that the batch of larvae used for this experiment was of poor quality. In particular, the Control larvae did not achieve any weight increase between days 69 and 86 PFF, and this performance compared poorly with batches of larvae used in the other weaning experiments described in this chapter. Despite these limitations, clear treatment effects were discernible.

There was no benefit to be gained from co-feeding from day 30 PFF, evident in similar growth and survival rates, and responses to dry diet through weaning, in both the Control and CF groups. No significant uptake of dry diet was observed during the co-feeding period and this is supported by the near identical lipid compositions of the carcasses in Control and CF groups. The significantly elevated level of DHA in CF carcasses may point to some uptake of inert diet, since the DHA level in the Nutra Marine diet was higher than that generally found in the enriched *Artemia* (Gara and Shields, 1997).

Early weaning using the inert *Artemia* feeding regimes (SK and AP treatments) caused high mortalities and severe growth retardation in these groups. Immobilised *Artemia* were obviously attractive to the halibut larvae, even without movement, due to their appearance and/or taste. Although the uptake of immobilised *Artemia* was rapid,

the fish in these groups would not accept dry diet readily. Thus, since much of the immobilised *Artemia* sank before consumption, there was a prolonged period of low feed consumption with minimal intake of dry diet. The better response to the Nutra Marine dry diet, compared to *Artemia* pellet, may have been due in part to the method of presentation, i.e. continuously by auto-feeder rather than intermittently by hand.

Water quality deteriorated rapidly once feeding with inert diets commenced, due to the requirement to maintain green water conditions and therefore low tank exchange rates. Tank husbandry (removal of waste feed) was very labour intensive under these conditions and would not be practical in a commercial rearing system.

Following these findings a further experiment was devised to further test the simultaneous presentation of live and inert diets against a normal weaning protocol on another batch of larvae. Considering the problems encountered with water quality due to feeding of dry diet during the "green water" period it was decided that co-feeding might best be done soon after the time when algae could safely be phased out. The next experiment was therefore conducted with these aims in mind.

6.7. Experiment 6: Co-feeding and early weaning II

6.7.1. Introduction and aims

In view of the low larval survival and growth rates obtained in Experiment 5, a revised experimental design was applied to a batch of halibut larvae to further test the *Artemia*/Nutra Marine co-feeding regime. The batch used in this experiment was a fast growing population which had performed well through the first feeding stage. The inert *Artemia* regimes that had been tested in Experiment 5 were replaced by a "standard" early weaning treatment, involving the standard phased transition to Nutra Marine diet over 9 days. On this occasion the experimental feeding regimes were not started until day 43 PFF to increase the likelihood of successful uptake and assimilation of the inert diet.

Weaning was initiated in the Control treatment from day 58 PFF in order to compare performance, age for age, with larvae from Experiment 2. In this previous experiment, halibut larvae at day 58 PFF had a mean wet weight of 106 mg, whereas larvae for this experiment had already reached this weight by day 41 PFF and weighed 218 mg (mean wet weight) by day 58 PFF. Comparison of these two batches would therefore provide an indication of the relative significance of age versus size on halibut weaning success.

6.7.2. Materials and methods

6.7.2.1. Live material and facilities

A single batch of 495 larvae, aged 40 days PFF (687°Cdays post hatch), were allocated randomly (55 per tank) to 9 experimental weaning tanks (described in Section 2.4.3.). Filtered sea water supplied to the tanks was maintained at $12^{\circ}\text{C} \pm 1^{\circ}\text{C}$, at ambient salinity ($34 \text{ ppt} \pm 0.5 \text{ ppt}$). Flow rates were set at 200 ml/min initially and increased to 400 ml/min at day 58 PFF. The fish were acclimated to the new tank conditions for two days prior to the start of the experiment. The tanks were initially siphoned and then later swirled once every day to remove waste feed and faeces.

Initial wet weight (\pm s.d.) of the larvae was $105 \text{ mg} \pm 9.72$; dry wt (\pm s.d.) $18.34 \text{ mg} \pm 2.16$; length (\pm s.d.) $24.7 \text{ mm} \pm 0.7$.

Tanks were 'greened' with *Nannochloris atomus* (2 l per day) only for the first 3 days of acclimation, until day 43 PFF. Daily water measurements were made of dissolved oxygen, temperature and pH and ammonia was also monitored intermittently.

6.7.2.2. Feeding regime

Three treatments were applied in triplicate, as shown in Table 6.7.1. Until weaning, the fish received 3 equal *Artemia* feeds per day, e.g. if total ration was 2100 *Artemia*/ fish/ day, each feed was 700 *Artemia*/ fish/ day.

In the Control and Cofed groups, *Artemia* was fed to satiation and the total daily *Artemia* ration increased from 1500 prey/fish per day at the start of the

Table 6.7.1. The three treatment groups for Experiment 6.

Group	Treatment
Control	Fed <i>Artemia</i> 3x / day until day 58 PFF. Started weaning on day 58 PFF (219 mg wet wt), feeding inert diet 10x / day and gradually reducing <i>Artemia</i> over 9 day period.
Cofed	Fed <i>Artemia</i> 3x / day until day 58 PFF. Also fed inert diet 10x / day from day 43 PFF onwards. Reduced <i>Artemia</i> over 9 day period as with control, starting on day 58 PFF(195 mg wet wt).
Early	Inert diet 10x / day, from day 43 PFF (105 mg wet wt), reducing <i>Artemia</i> over 9 days as above.

experimental period to 2700 prey/fish/day by weaning time. As during the first feeding period, the *Artemia* were enriched separately using DC Selco and Algamac 2000. Fish received equal amounts of each enrichment type every day.

The 9 day weaning regime was as described in Section 6.3.2.2. The Nutra Marine dry diet was presented in progressively increasing crumb sizes (0.3 - 0.5 mm, 0.5 - 0.8 mm then 0.8 - 1.4 mm) according to fish size. The diet was distributed to the tanks using the automatic feeders (Norfab Ltd) described in Section 2.4.3. The feeders were calibrated to deliver approximately 0.2 g per feed once every hour between 08:00 hrs and 18:00 hrs.

6.7.2.3. Sampling

Fish were sampled for length and wet and dry weight and assessed for metamorphosis and pigmentation at the beginning of the experiment (day 41 PFF) at day 58 PFF prior to

weaning the Control and Cofed groups and at the end of the experiment on day 81 PFF (1200°Cdays post hatch). Mortalities were recorded and removed daily.

Feeding behaviour in terms of response to dry diet (based on presence of diet in the gut) and the position of fish in the tank (demersal / pelagic) was noted daily for all fish. The appearance of the digestive tract was also observed daily and the presence of an obvious stomach was recorded when an enlargement was visible at the anterior of the intestine.

6.7.2.4. Statistical analysis

Results were analysed statistically using ANOVA (one-way or two level nested) and Tukey pairwise comparisons with MINITAB statistical package. Where percentage data were analysed, values were transformed using arcsine transformation before statistical analysis.

6.7.3. Results

6.7.3.1. Survival

Survival rates, shown in Figure 6.7.1.A. were very high in all treatments with no significant differences between groups.

6.7.3.2. Feed response, settlement and stomach development

Figure 6.7.1.B. shows the increase in feed response with age of the 3 groups. The Early group exhibited a relatively slow response to dry diet during the weaning phase in comparison to the Control and Cofed groups which were weaned later. This increased more rapidly after day 52 PFF, once the *Artemia* was removed completely. The low feed response (<10%) in the Cofed group during the co-feeding period (day 43 PFF to day 58 PFF) indicates that a small number of fish were consistently taking diet. The response to diet through the weaning phase was similar in both Cofed and Control groups and increased rapidly before the total removal of *Artemia*.

When feed response is compared at the end of each 9 day weaning period, the Early group had a significantly lower response ($p < 0.001$) at day 52 PFF than the Control and Cofed groups at day 67 PFF. The latter two groups did not differ significantly in feed response (see Figure 6.7.1.C).

At the start of the experiment, around 50% of fish were settled on the bottom and this had increased to around 90% in all treatments by day 81 PFF (see Figure 6.7.2.A.). Response to inert diet was generally greater amongst the demersal fish than the pelagic fish during the weaning phase in all treatments.

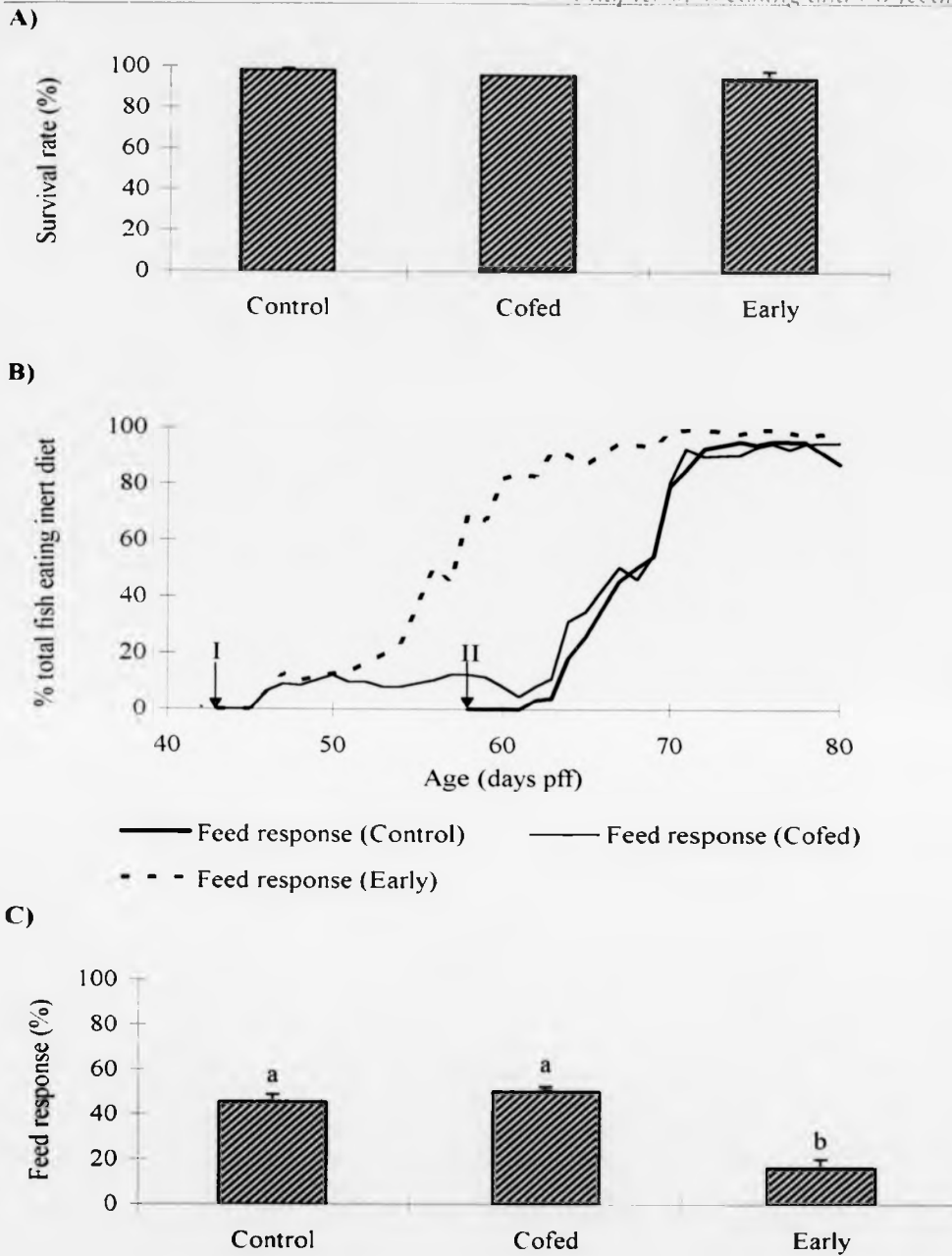
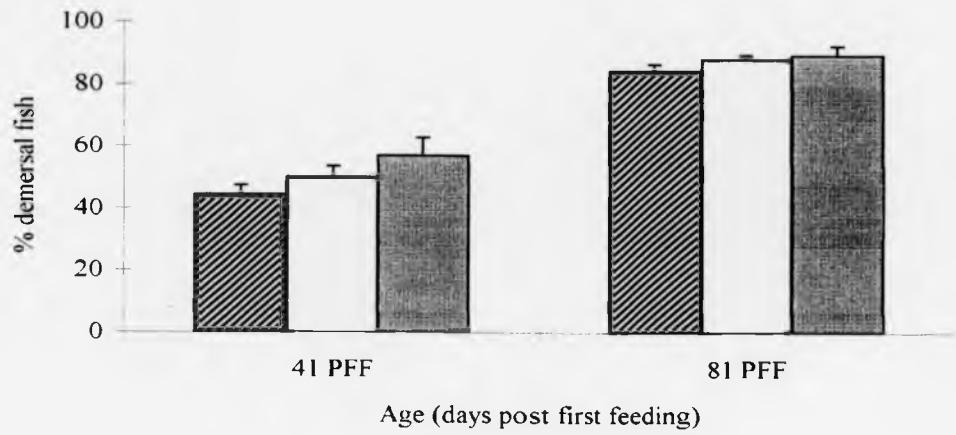


Figure 6.7.1. A) Final survival rates B) Change in feed response (mean % of initial population feeding on inert diet) during experimental period for each group. (I) started weaning Early and Cofed groups, (II) started weaning Control group. C) Feed response at the end of the weaning period (first day without *Artemia*) for each group. Values are the means of 3 replicates. Error bars represent standard error of means. a,b denote significant differences between means (ANOVA, $p < 0.05$).

A)



B)

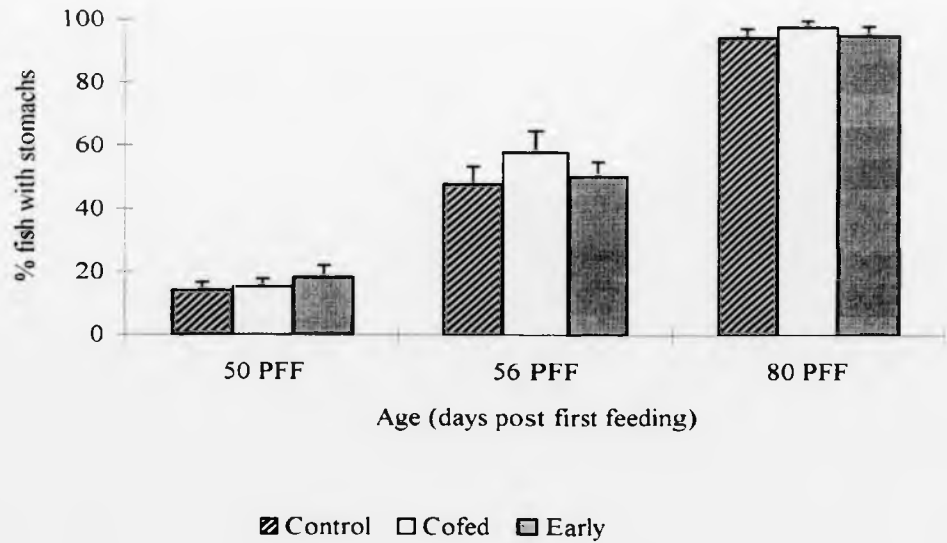


Figure 6.7.2. A) Frequency (%) of demersal fish at day 41 and 81 PFF. B) Frequency (%) of fish with obvious stomachs at day 50, 56 and 80 PFF. for each group of fish. Values are the means of 3 replicates. Error bars represent standard error of means.

The proportion of fish with obvious stomachs increased from around 15% at day 50 PFF to around 50% by day 56 PFF and to 95% by day 81 PFF (shown in Figure 6.7.2.B.).

6.7.3.3. Growth

Figure 6.7.3. shows the growth of the three groups of fish from day 41 PFF until day 81 PFF. The intermediate sample at day 58 PFF revealed a significantly lower weight (ANOVA, $p < 0.05$) in the Early weaned group than in the Cofed and Control groups. The Control group had the highest wet weight. The Early group had the highest final weight which was significantly different from the other two groups (ANOVA, $p < 0.05$). Final dry weight was significantly lower (ANOVA, $p < 0.05$) in the Cofed group than either the Control or Early group (Figure 6.7.4.A). Specific growth rate, shown in Figure 6.7.4.B., calculated from the change in dry weight between day 58 and 81 PFF was significantly higher in the Early group than the other two groups (ANOVA, $p < 0.05$).

The Early group had the highest and the Cofed group the lowest mean final length and condition factor respectively (see Figure 6.7.5.). The Control and Cofed groups had the highest and lowest coefficients of variation 59.7% and 52.3% respectively and the Early group and intermediate value (52.3%).

6.7.3.4. Metamorphosis and pigmentation

The mean eye migration index was very low in all groups and no fish showed complete eye migration (3 on a scale of 0 - 3) . Eye migration was best in the Early group as shown in Figure 6.7.6.A., but there were no significant differences between

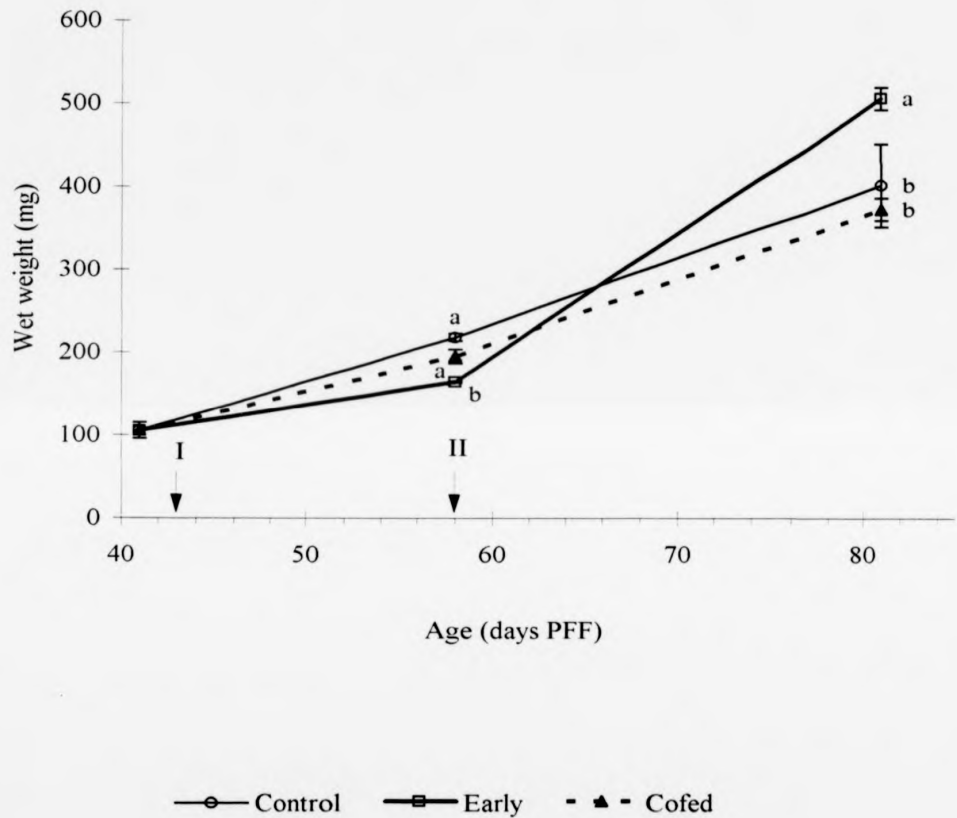


Figure 6.7.3. Change in wet weight for each group of fish during experiment (I; Started weaning Early group, II; Started weaning Control and Cofed groups). Values are means of 3 replicates. Error bars represent standard error of means. a,b denote significant differences between means (ANOVA, $p < 0.05$).

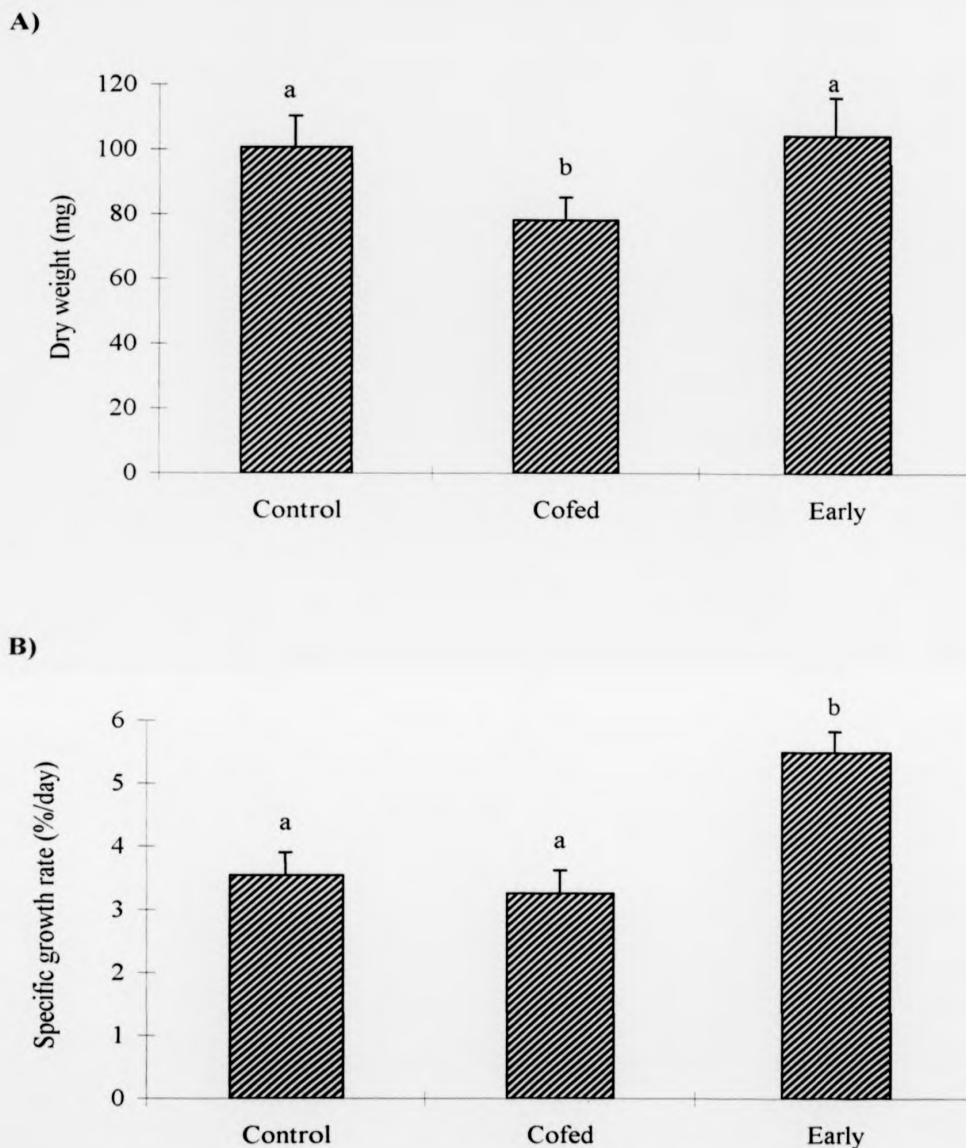


Figure 6.7.4. A) Final dry weight B) Specific growth rate based on change in dry weight between day 58 and 81 PFF for each group of fish. Values are means of 3 replicates. Error bars represent standard error of means. a, b denote significant differences between means (ANOVA, $p < 0.05$).

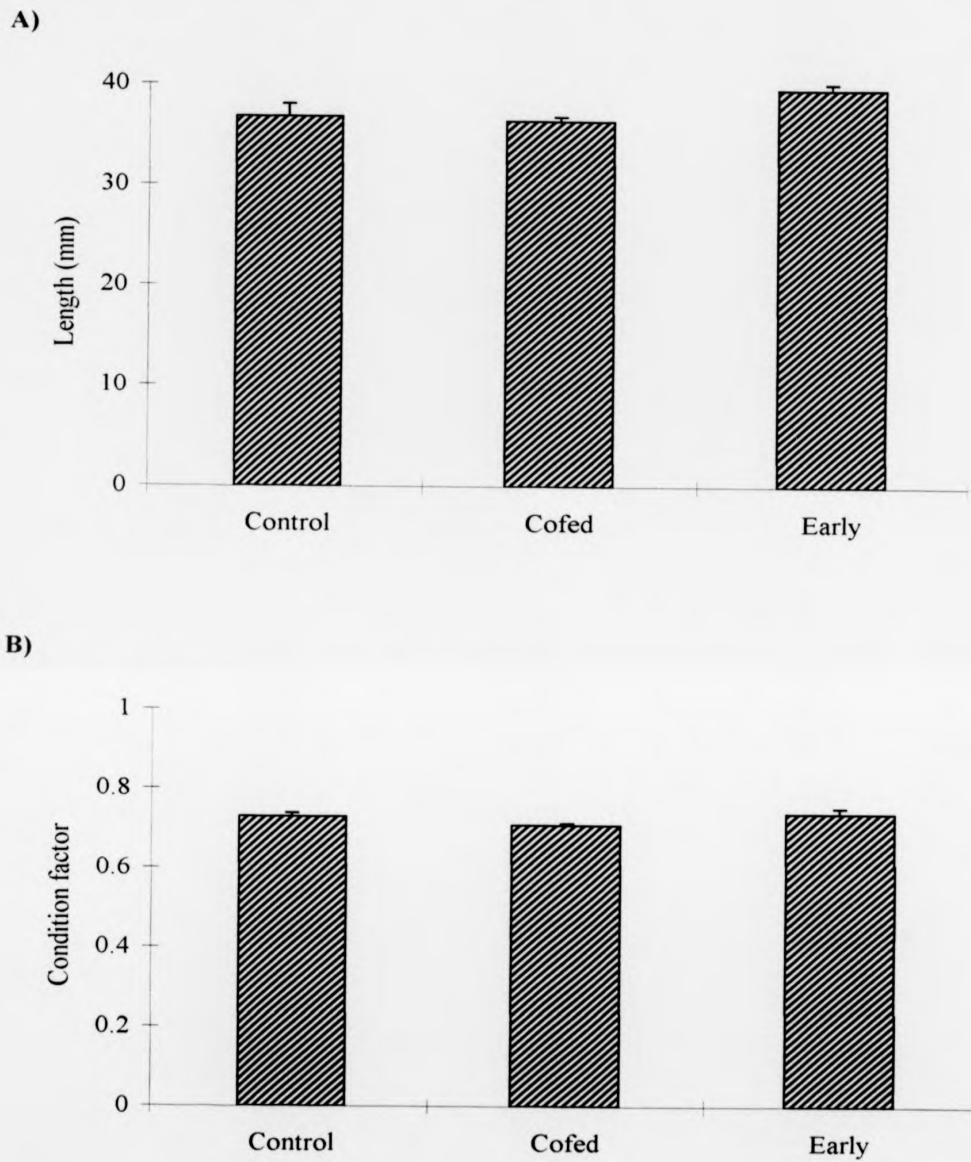


Figure 6.7.5. A) Final length B) Condition factor (W/L^3) for each group of fish. Values are means of 3 replicates. Error bars represent standard error of means.

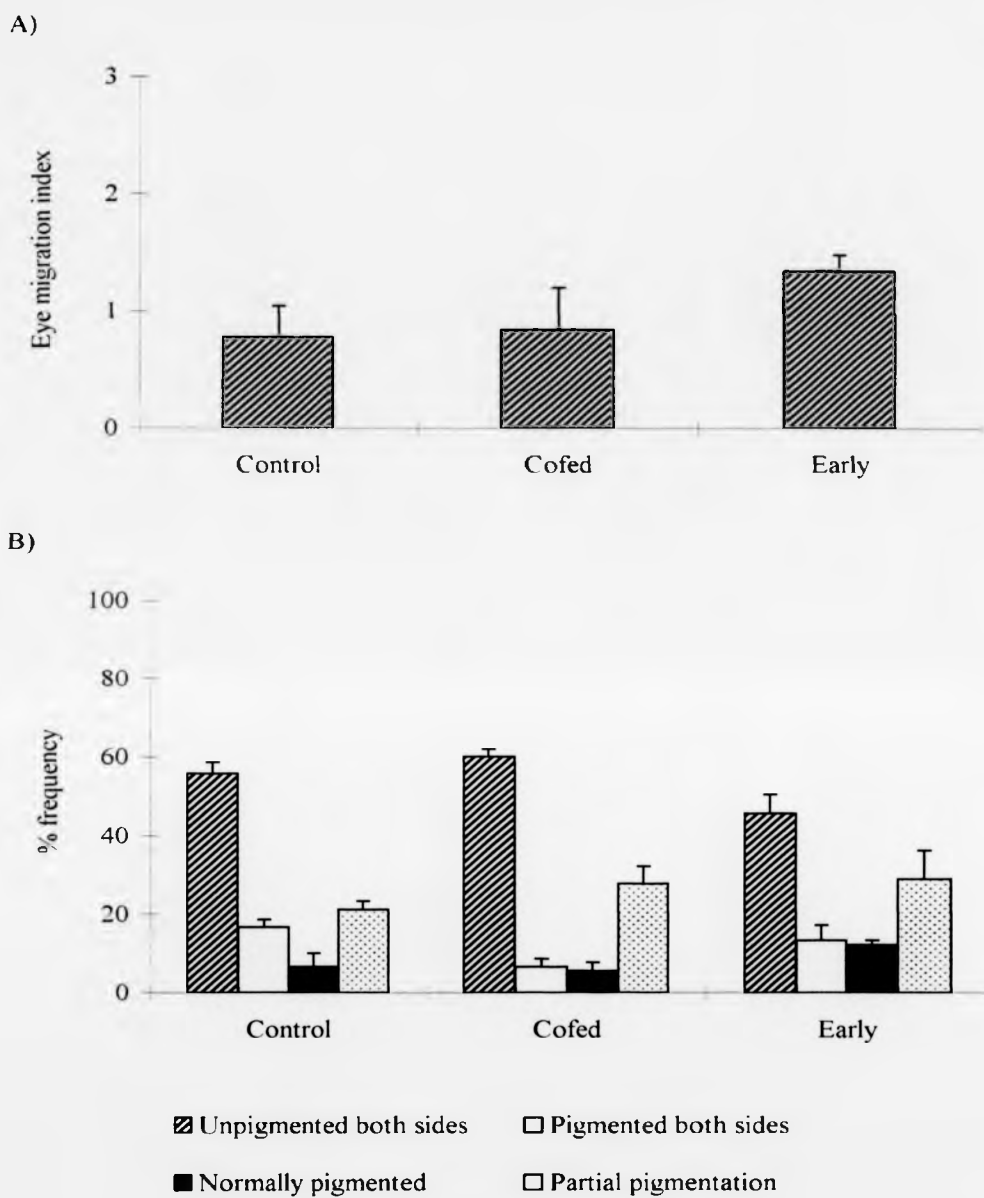


Figure 6.7.6. A) Mean eye migration index B) Percentage frequency of pigmentation categories assessed at day 81 PFF at the end of the experiment for each group of fish. Values are the means of 3 replicates. Error bars represent standard error of means.

groups (ANOVA, $p > 0.05$).

Pigmentation was also poor in all treatments, with very few fish perfectly pigmented (complete dorsal pigmentation and absent from the ventral side). A large percentage of the fish in all groups were unpigmented on both sides (see Figure 6.7.6.B.). Pigmentation characteristics were slightly better in the Early weaned group but there were no significant differences between groups (ANOVA, $p > 0.05$).

There were no significant differences between groups for any of the metamorphosis characteristics investigated. The number of perfectly metamorphosed fish was zero in all groups.

6.7.3.5. Water quality

Water quality remained high in all treatments during the experiment with ammonia concentrations remaining below a maximum of 0.16 mg/l, pH 8.0 ± 0.06 , dissolved O_2 8.85 ± 0.80 mg/l.

6.7.4. Conclusions for Experiment 6

Survival rates were very high in all treatments in this experiment. This is due in part to the good growth performance of this batch of larvae during the live feeding phase, achieved through improvements in *Artemia* feeding regimes. The fact that mean larval wet weight had reached 105 mg by the start of the experiment (day 43 PFF) was exceptional in view of the typical growth rates being achieved at the site at the time of the experiment. The resilience of the larvae was evident in their tolerance of clear water rearing from day 43 PFF, which greatly facilitated tank management in the Cofed and Early treatments.

As in Experiment 5, co-fed larvae again failed to show any significant uptake of inert diet during the period of joint diet presentation, despite the greater size and age of the larvae compared to Experiments 4 and 5. Feed response during the 9 day weaning period was only slightly better than the Control (naive) group, suggesting that any 'conditioning' effect of co-feeding was negligible. Also, larval size in terms of length and wet weight at the end of the weaning period was not significantly different from larvae weaned under the Control regime.

The Early weaning of halibut larvae using a phased 9 day regime was straightforward for this batch of larvae. Larvae in the Early treatment did not suffer any mortality associated with weaning. Furthermore, their mean wet weight at the end of the experiment was significantly greater than the Control group, demonstrating an energetic advantage for formulated crumb over *Artemia* for larvae in this size range. The Early weaned larvae did not exhibit increased size variability relative to the Control group, nor was there any cannibalism following transfer to dry diet.

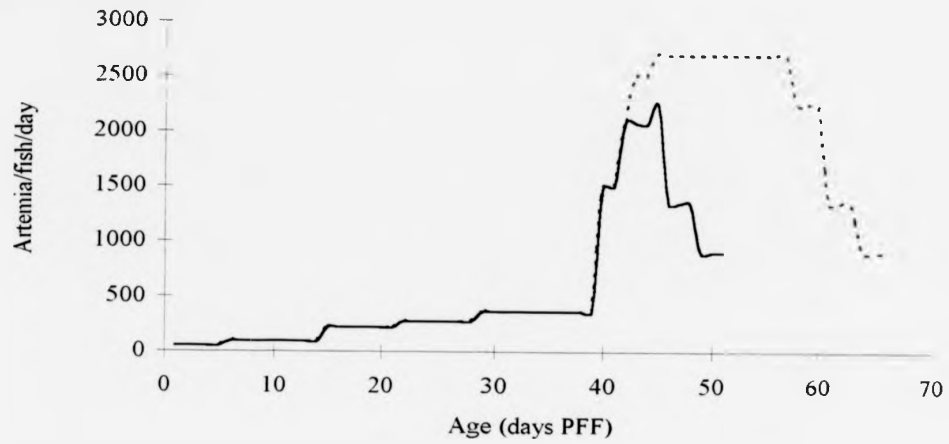
The accelerated growth rate in the Early treatment was achieved despite a lower initial feed response at the end of the 9 day weaning period. It was not until *Artemia* had been completely withdrawn that the Early larvae started to ingest dry diet well and a significant proportion of the fish endured a starvation period of several days. Their ability to cope with this starvation period indicated that this group had sufficient reserves of energy on entering the weaning period.

Aside from the growth advantage, early weaning also enabled major savings in *Artemia* consumption. Figure 6.7.7. illustrates the daily and cumulative consumption of *Artemia* per fish for this larval batch, from first-feeding to day 80 PFF. From these data it was calculated that weaning from day 43 PFF reduced *Artemia* usage by 60%, in comparison to the Control treatment that was weaned 15 days later.

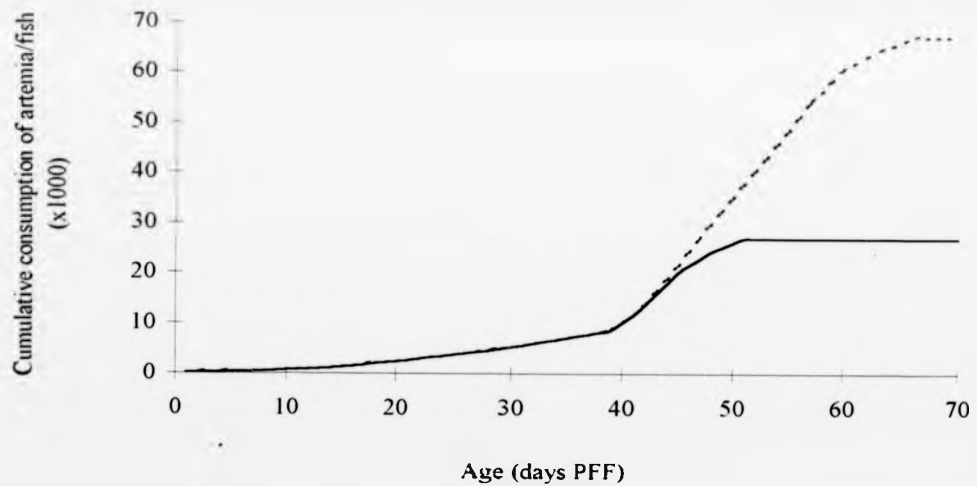
By comparing the results from this experiment with Experiment 2, it was concluded that halibut weaning performance is primarily determined by larval size and growth parameters, rather than chronological age. Larvae aged 58 days PFF (mean wet weight 218 mg) attained a 98% survival rate through weaning in this experiment, compared to 54% in Experiment 2 (larval wet weight 106 mg). Of greater significance, larvae weaned from the Early group weaned from day 43 PFF also achieved greater than 90% survival, although their mean wet weight (105 mg) was virtually the same as the earliest weaned group in Experiment 2 (106 mg).

As in Experiment 5, there were no significant differences between treatments with regard to metamorphosis characteristics. It is likely that eye migration and pigmentation characteristics were already determined before the start of the experiment, as a result of low ration levels and the *Artemia* enrichments used (Gara and Shields, 1997).

A: *Artemia* ration



B; Cumulative total use of *Artemia*



..... Control — Early

Figure 6.7.7. A; Graph showing daily consumption of *Artemia* (prey/fish/day) for Early weaned and Control groups from first feeding until weaning and B, cumulative total use of *Artemia* for the same period.

6.8. Discussion

The weaning period is undoubtedly a critical phase in the halibut production cycle when poor adaptation to inert diet can result in low feed consumption or even starvation causing heavy mortality. Husbandry demands also change as the introduction of inert diets inevitably leads to a build up of wastes which influence environmental conditions in the culture tanks.

The performance of the different batches of larvae through weaning varied considerably. Excluding Experiment 4, all the rest included a group of fish which started weaning at wet weights of between 100 -150 mg. The age at which this weight range was reached for each batch differed significantly. Figure 6.8.1 shows the initial and final weights of representative groups from all batches used in the experiments at the corresponding age in degree days. This comparison reveals a steady improvement from season to season, with the exception of Experiment 5, resulting in a shortening of the time taken to reach a given size, and this reflects the advances made in rearing procedures at the live feeding stage. The batch used in Experiment 6 showed the best performance through weaning. Compared to the other batches, the growth of these larvae was rapid during the first-feeding stage and had reached 100 mg by day 43 or 723 °Cdays. The larvae of this fast growing batch were evidently well equipped to adapt to dry diet and even the smaller larvae of this batch were able to cope with the transition. This probably reflects a healthy nutritional status, efficient digestive function and adequate stored energy. This enabled those larvae which did not show an immediate uptake of dry feed to survive long enough until the switch was eventually made.

A comparison of all batches used for the experiments indicates that the absolute age

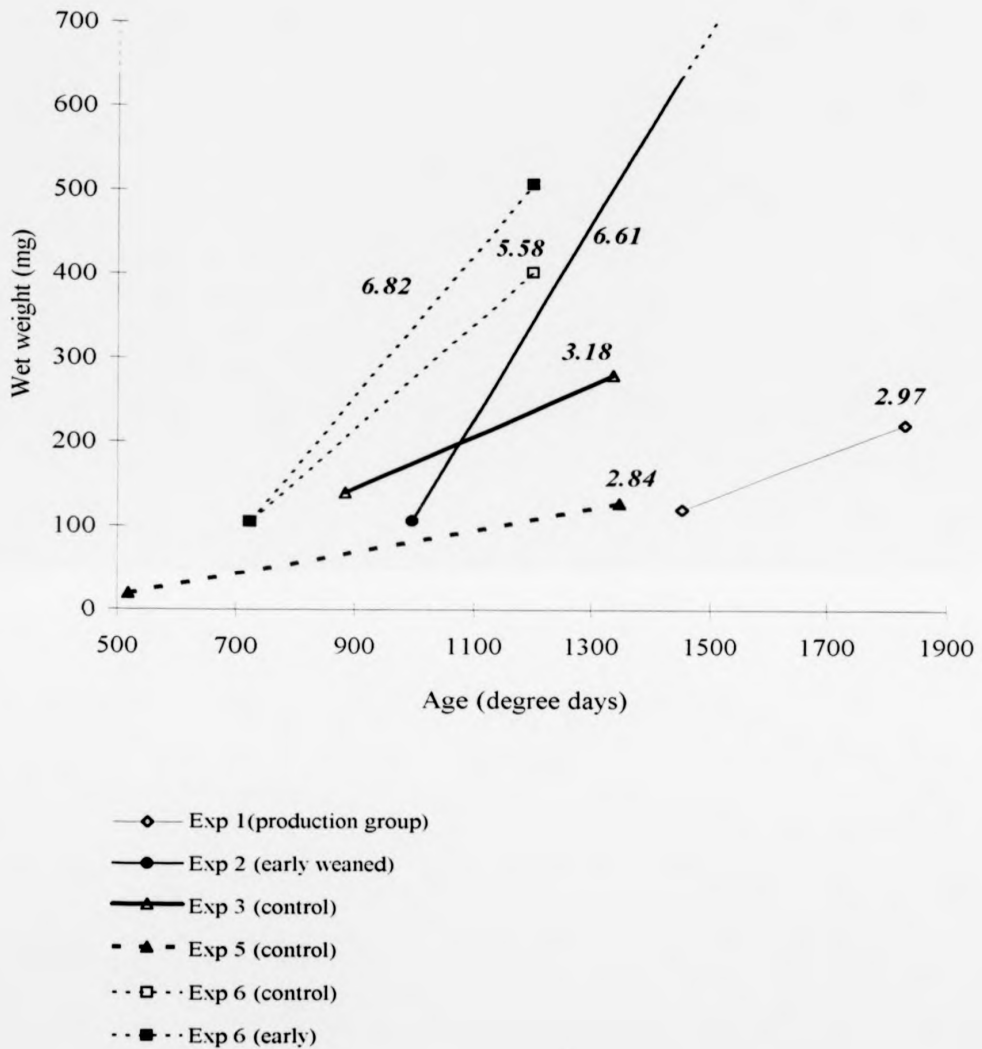


Figure 6.8.1. Graph showing initial and final wet weights against age (day degrees) of representative groups from all long term weaning experiments (i.e excluding experiment 4). Growth factor (GF3) is shown for each group in bold italics.

of larvae as they enter the weaning phase is of little importance and that size, developmental stage and condition are of greater significance.

In order to compare the performance of all batches used in the experiments, the growth factor (GF3; see Section 2.5.) was calculated taking into account the different rearing temperatures used. The highest growth coefficients were found in Experiment 6 and the highest of these was the early weaned batch. The values of GF3 for the batch used in Experiment 2 are of a similar order. The figures indicate the poor quality of larvae used for Experiments 1 and 5 where values for GF3 of under 3 were calculated for the Production and Control batches.

The performance of larvae during weaning is highly dependant upon their inherent quality and inter-batch variations are often observed. Such variations were clearly shown by the different batches used in this study. The conditions experienced prior to weaning are of major importance and Experiment 1 illustrated the significance of nutrition during the first-feeding stage. The need to adapt feeding behaviour and the reduced feed intake or even temporary starvation that can occur during weaning, places extra demands on fish larvae. A healthy nutritional status, well developed sensory and digestive apparatus and sufficient stored energy are therefore prerequisites for larvae entering this transitional phase. The importance of the nutritional status of fish larvae as they enter the weaning phase is well established and the quality of live feed and their enrichments fed to larvae prior to weaning has often been implicated in the success or failure of larvae through weaning (Bromley and Howell, 1983; Bromley and Sykes, 1985; Person le Ruyet *et al.*, 1993). Person le Ruyet *et al.* (1993) demonstrated that poor quality larvae resulting from feeding a DHA-deficient *Artemia* during the first feeding phase performed badly through weaning. Mourente and Tocher (1992) found that pre-weaning

turbot larvae were deficient in DHA in the brain tissue and suggested that this may jeopardise the performance through weaning.

The successful transition to artificial diets is determined by several factors. The first of these is the attractiveness and acceptability of the inert particles and this will govern initial acceptance and ingestion rate by the larvae. This is dependant on the feeding behaviour of the larvae and their ability to recognise and adapt to a new diet type. For many marine species, where weaning has been attempted on very young larvae, difficulties have been encountered in relation to initial acceptance and ingestion of artificial feeds. This was also the case for halibut larvae in this study. Where co-feeding was attempted, young halibut larvae repeatedly showed a reluctance to consume dry diet, selecting *Artemia* preferentially. Where both *Artemia* and dry diet were presented simultaneously, little or no uptake of dry diet was observed. It seemed that the larvae needed to be almost deprived of live feed before dry diet was accepted indicating that they were conditioned to *Artemia*. The preference for live prey shown by fish presented with both live and inert diets has been demonstrated in gilthead sea bream (Fernandez-Diaz *et al.*, 1994). Weinhart and Rosch (1991) compared feed intake of *Artemia* and dry diet and found that whitefish larvae ingested more *Artemia* than artificial feed. Using ¹⁴C labelled microdiet, Tandler and Kolkovski (1991), showed that the ingestion of inert diet was only one tenth compared to *Artemia* for gilthead sea bream larvae and suggested that attractiveness was the main reason for low ingestion.

Halibut larvae, like most marine fish, are carnivorous visual feeders from the onset of first-feeding (Hunter, 1981), and feeding commences in the surface water layers amongst the rich zooplankton populations. The natural diet comprises a host of living prey organisms made up of numerous species at different developmental stages. The choice

of type, behaviour and size of prey will be wide and typically the fish larvae actively seek a certain size and species of prey depending on its age, body size, gape size and stage of development (Dutton, 1992; Detwyler and Houde, 1970). Feeding efficiency improves as larvae develop and varies from species to species (Houde and Schekter, 1980). Prey recognition is thought to be an important factor and the feeding behaviour of fish larvae probably involves an element of learning (Dutton, 1992). Barnabe and Guissi (1994) made an attempt with some success to reverse this by pre-conditioning sea bass larvae to non-motile particles using immobilised rotifers. The use of frozen *Artemia* in Experiment 5 demonstrated that halibut larvae will readily accept non-motile *Artemia* suggesting that movement is not the most important criteria eliciting a feed response. The use of frozen *Artemia* is common for both marine and freshwater fish (Kirk, 1972; Metailler *et al.*, 1981; Dabrowski, 1984a; Dinis, 1992; Kestemont and Stalmans, 1992). Fuchs and Nedelec (1989) found that frozen *Artemia* was as effective as live for the weaning of barramundi. Kentouri (1981) demonstrated species-specific differences in performance on frozen plankton. Larvae of sea bass, sea bream, and mullet all accepted and grew well on frozen zooplankton whereas gudgeon and gurnard rejected it. The two flatfish studied, turbot and sole both accepted frozen zooplankton but failed to thrive. This may be due to the rapid leaching of amino acids that follows thawing of frozen zooplankton and suspension in sea water reported by Grabner *et al.* (1981). Frozen *Artemia* were observed to sink rapidly and were only available to the larvae for a short period, a problem which was also encountered by Barnabe and Guissi (1994). The extended use of frozen *Artemia* in Experiment 5 indicated that halibut larvae will not grow well on such a diet but the effectiveness of immobilised *Artemia* cannot be ruled out as an intermediate diet for weaning.

Fish larvae generally identify prey by sight and perhaps to a lesser extent through detection of vibration via the ear or lateral line. At first-feeding they are generally inefficient feeders and improvement in visual acuity develops during ontogenesis (Blaxter, 1980). In turbot, visual acuity increases from 6-7° to 11° between first feeding and early metamorphosis (Neave, 1984). The light levels/ wavelengths used in culture conditions, colour of diets and backgrounds i.e. tank walls and turbidity are all likely to affect the larva's perception of prey/diets. The colour of an artificial diet can be altered with the use of dyes or pigments such as the carotenoids canthaxanthin or astaxanthin. In a study on the feeding success of Dover sole larvae Dendrinis *et al.* (1984) highlighted the importance of contrast perception, by demonstrating differences in response to *Artemia* dyed different colours.

Kolkovski *et al.* (1995) showed that both movement and olfactory stimuli were important in eliciting a feed response for gilthead sea bream feeding on *Artemia*. They illustrated this by showing that ingestion of microdiet could be stimulated through the addition of *Artemia* culture water to the feeding tank or by holding live *Artemia* in a separate container within view of the feeding larvae.

To induce ingestion of artificial diet by fish larvae, the particles must first and foremost be attractive. This attractiveness may be determined by size, texture, colour, movement, sinking rate, and taste or smell. All these criteria can to a certain degree be manipulated when producing and/or presenting an artificial diet. For olfactory feeders such as sole, the taste and smell of a diet is of obvious importance.

Chemical feeding stimulants can enhance acceptance of inert diets and various substances (including amino acids, quaternary amines and nucleosides), particularly glycine betaine and inosine, have been identified as effective feeding stimulants for larval

Dover sole (Mackie *et al.*, 1980; Metailler *et al.*, 1983) and turbot (Mackie and Adron, 1978; Person le Ruyet *et al.*, 1983). These attractants are present in the natural diet and extracts of invertebrate prey added to dry diets can improve attractiveness (Metailler *et al.*, 1981). In Experiment 4, there was an indication that the inclusion of freeze-dried *Artemia* in the weaning diet might have improved acceptance. Fluchter (1982) presented evidence of an essential substance present in *Artemia* which, when extracted with acetone, added to inert diet and fed to whitefish larvae, improved survival rate and metamorphosis. Kolkovski and Tandler (1995) discussed the importance of free amino acids and the hormone bombesin present in *Artemia* which when added to microdiets stimulated peristaltic movement of the intestine and improved assimilation.

The size of a pellet or microparticle is also important and can be tailored to the preferences of a particular species and size or age of larvae. Active particle size selection has been observed in sea bass larvae fed micocapsules (Walford *et al.*, 1991). These authors observed that if larvae were fed particles that were too large, they could become lodged undigested in the digestive tract. Both turbot and halibut larvae show selective feeding behaviour, tending to optimise energetic intake by increasing prey size as they grow (van der Meer, 1991; Olsen, 1997). During the course of this study, experience showed that halibut larvae of around 100 mg would readily accept particles in the range of 300-500 μm .

The water stability of an artificial diet has important consequences for both nutritional value and water quality. One potential problem with inert diets is leaching of essential nutrients, particularly water soluble vitamins and amino acids (Meyers, 1979; Yamamoto, 1979; Lopez-Alvarado *et al.*, 1994) and this problem may be overcome with the use of microencapsulation (Leibovitz, *et al.*, 1991; Lopez-Alvarado *et al.*, 1994).

There are a variety of microdiets available which have been tested at laboratory and commercial scales (Person le Ruyet, 1989a; Melotti *et al.*, 1992) and are produced using a range of processes (Langdon *et al.*, 1985). Flaked or expanded feeds produced by pressure cooking techniques require special equipment for their manufacture. Digestibility of certain components is high due to the use of high temperatures and it is relatively straightforward to supplement the basic diet with feed stimulants or dyes to enhance attractiveness due to their absorbant nature. Microbound diets are simple to manufacture without special equipment and consist of dry or raw ingredients bound by substances such as agar, caragheen, alginate, gelatin or sodium polyacrylate. The temperature required depends upon the type of binder used and is of importance in influencing the rate of loss of thermolabile substances. Microcoated or microencapsulated diets are manufactured using coacervation, interfacial polymerisation or lipid wall capsules (Person le Ruyet, 1989a). These particles may have a water proof wall which should be easily broken down by biting or in the gut by enzyme, bacterial or pH action (Jones and Gabbott, 1976). The production of microencapsulated particles by coacervation involves precipitating a polymer (e.g. gelatin, zein nylon or polystyrene) around the particle in liquid phase. Microcoated diets are produced by evaporating a coating solution (e.g. zein or a cholesterol-lecithin mixture) from the particle. The structure of the capsule can also be made slightly porous to allow some leaching of amino acids which may enhance the attractiveness of the particle (Person le Ruyet 1989a). Further development of microdiets for marine fish larvae is an important area of research which could prove extremely rewarding in terms of improvements in efficiency.

The motion of an inert particle can to a limited degree be manipulated by the manufacturing process. The sinking rate will be determined by density which will depend

upon composition. The buoyancy of microdiets, which should be neutral or slightly negative, can be adjusted with the balance of lipid, carbohydrate and protein components (Jones and Gabbott, 1976). At the point of presentation, the movement of the diet can be enhanced with use of water currents and air bubbles. However, excessive aeration or water flow may have detrimental effects for the larvae. For instance, it was noted in some of the weaning experiments that feeding was inhibited by high flow rates, particularly for recently metamorphosed larvae which had settled on the tank bottom. The hydrodynamics of the culture tank are of particular importance during weaning since water movement determines the motion of suspended inert particles. During the course of the weaning experiments described, much attention was paid to flow dynamics. For example, it was found that surface flows tended to draw feed particles rapidly through the surface water film causing them to sink rapidly. Sub-surface inlets on the other hand, caused no disturbance to the surface tension and particles remained for longer at the surface before sinking.

For a pelagic fish larvae, the longer a particle remains in the water column, the greater the chance of encounter. The residence time of a particle in the water column will depend on the depth and sinking rate and the presence of water and air currents. Care was taken with inlet and airstone position in order to maximise the length of time feed remained in suspension. When weaning onto artificial diet, it is important to present feed particles as often as possible so that encounter rates are maximised. Ideally, feed should always be available throughout the water column. Automatic feeders assist considerably in this respect and the design used for Experiments 5 and 6 effectively dosed small amounts of food at frequent intervals through the day and feed was spread evenly through the water column. The use of automatic feeders reduces the labour demand

during weaning and was considered to be highly beneficial to the weaning process and an important factor in the high performance of the last batch of larvae weaned in Experiment 6.

Once ingested the suitability of a diet in terms of satisfying nutritional requirements will depend firstly on the inclusion of the correct balance of nutrients and secondly the availability of these nutrients to the larvae. The latter will be determined by the form in which the nutrients are present and the digestive capability of the larvae. Most fish are carnivorous at first-feeding but there is considerable diversity in the nature and rate of development of digestive capability amongst species. Those that develop a stomach prior to the onset of first-feeding such as salmonids are able to survive well on artificial diets from first feeding (Dabrowski, 1984a). Cyprinids, although not possessing a stomach throughout life, rapidly acquire tryptic enzyme activity and are able to adapt to an inert diet at an early stage. The digestive organs and enzyme activity in marine fish generally evolve during larval life. The poor performance of many species on inert diets is thought to be due to the incomplete development of the digestive tract (Dabrowski, 1984a; Lauff and Hofer, 1984).

At the start of exogenous feeding the digestive system is quite well developed in most fish. The intestine is generally differentiated into three specialised regions (Stroband and Dabrowski, 1981; Govoni *et al.*, 1986). In a description of the histology of the enterocytes of the anterior intestine of first feeding turbot, Segner, *et al.*, (1993) showed that it was already fully differentiated to the adult form. Lipid absorption in turbot, whitefish and African catfish (Segner *et al.*, 1993) and in sea bass (Deplano *et al.*, 1991) is well developed at this stage. The presence of the brush border enzymes alkaline phosphatase, maltase and amino peptidase has been demonstrated histochemically in the

African catfish, whitefish (Segner *et al.*, 1993) and turbot (Cousin *et al.*, 1987). The functionality of both the exocrine and endocrine pancreas has been demonstrated in larval fish for a number of species (Segner *et al.*, 1993; Govoni, 1980; Cahu *et al.*, 1995). The liver is also well developed as a site for nutrient metabolism at first feeding (Segner *et al.*, 1993). The apparent inability to digest formulated feeds has been widely linked to the lack of a stomach in fish larvae. Indeed the appearance of the stomach is often considered to be the end of the larval phase and is associated with transformation or metamorphosis.

The ontogeny of the digestive system in halibut has not yet been described in detail. The gross observations of the digestive tract in Experiment 6 indicated that halibut larvae were able to assimilate inert diet before the formation of an obvious stomach. These observations need to be supported by histological and histochemical examination techniques. A recent histological study carried out by F.Luizi (Phd thesis) showed that a true stomach and functional gastric glands appear at around day 45-50 PFF, coinciding with metamorphosis and demersal settlement.

In some fish species the appearance of the stomach is a discrete and rapid event and corresponds to an increase in pepsin-like activity within an acidic lumen. This is true for catfish (Verreth *et al.*, 1992) and turbot (Segner *et al.*, 1993). In whitefish, the process is more prolonged but digestion of inert diets becomes efficient before complete formation of the stomach. In turbot (Segner *et al.*, 1993) and catfish (Verreth and van Tongeren, 1989; Verreth *et al.*, 1992), growth rates on inert diet are lower than on live prey until the stomach is fully functional. In sole, the gastric glands start to develop around day 22 post-hatch (Boulhic and Gabaudan, 1992), and the stomach does not become fully functional until day 200 (Clark *et al.*, 1986) but sole may be weaned well before these

events at around day 10 (Gatesoupe, 1983; Appelbaum, 1985). Conversely, Zambanino Infante and Cahu (1994) showed that growth was poor in sea bass weaned at day 25 despite the appearance of pepsin activity from day 24. In fish larvae that lack a stomach until transformation, pepsin activity is not present or at a very low basal level until the gastric glands become functional (Tanaka *et al.*, 1972; Kawai and Ikeda, 1973; Vu, 1983; Walford and Lam, 1993). Until this point protein digestion in larval fish is effected through the activity of pancreatic enzymes: trypsin, chymotrypsin and aminopeptidase. The activity of these enzymes may be similar for both larvae and adults as reported for turbot (Munilla-Moran and Stark, 1989) or may decrease coincident with the formation of the stomach as with sea bass (Walford and Lam, 1993) and ayu (Tanaka *et al.*, 1972). This decrease may be due either to a lower secretion or a denaturing of exogenous trypsin in the acid lumen of the stomach. Extracellular digestion is incomplete in the absence of pepsin and HCl and may be compensated for by the increased pinocytotic activity and intracellular digestion in the hindgut, a process which has been observed in a number of species (Govoni, 1980; Watanabe, 1984; Govoni *et al.*, 1986; Kjorsvik *et al.*, 1991; Walford and Lam, 1993).

As well as changes in digestive capacity, there are changes in larval nutritional physiology and energy metabolism during development, especially at transformation or metamorphosis, which alter the nutrient requirements (Segner *et al.*, 1993).

The response of the larval digestive system to ingestion of dry diets depends largely upon the developmental state of the larvae and consequently whether or not the digestive system is ready to cope with the diet. The formulation of the diet is also important and Zambanino Infante and Cahu (1994) showed that incorporation of an amino acid mixture to a basal diet significantly enhanced trypsin activity. Feeding exclusively on commercial

dry feed from the start of exogenous feeding in goldfish stimulated low trypsin activity compared to a live prey control (Abi-Ayad and Kestemont, 1994) and a state of malnutrition was evident from an ultrastructural examination of the liver and pancreas. Conversely, an increase in trypsin activity was occasioned by transfer to dry diet in sea bass larvae (Person le Ruyet, 1989b) and roach larvae (Hofer, 1985). Cahu and Zambanino Infante (1997) showed that the activity of the pancreatic enzymes, amylase and trypsin, in sea bass larvae fed dry diet was at least equal to that of larvae fed live prey. They argued that this was not a limiting factor for weaning to inert diet and that deficiencies were due to inadequate nutrition with respect to the digestive specificities of larvae.

There is much evidence of compensatory responses in larval digestion and metabolism brought about by changes in diet. Segner *et al.* (1993) used four indices to measure nutritional status in whitefish, turbot and catfish: activity of alkaline proteolytic enzymes, height of midgut epithelium enterocytes, RNA/DNA ratio and hepatocyte nuclear diameter. These four indices all exhibited a rapid decline compared to levels on live food under conditions of starvation in all three species. When whitefish and catfish were fed dry diets there was also a decline for all parameters and an eventual recovery at a more advanced stage of digestive ontogeny. An increase in amylase activity was occasioned by weaning in sea bass (Cahu and Zambanino Infante, 1994). In these animals compensatory increases of activity of intestinal peptidases were accompanied by a decrease in brush border enzymes relative to live prey fed larvae indicating malnourishment in weaned larvae. Deplano *et al.*, (1991) demonstrated a diminished absorption of protein in the enterocytes of the postvalvular intestine of sea bass larvae fed dry diet. Changes in the morphology of the second intestinal segment were also

observed in response to dry diets and may be a response to differences in pinocytotic activity, since this is the suggested site of protein absorption in fish larvae (Govoni *et al.*, 1986). Zambanino Infante and Cahu (1994) showed an increase in specific pepsin activity following weaning of sea bass. The ingestion of dry diets also promotes an increase in incidence and size of lipid droplets in the enterocytes of the first intestinal segment in whitefish (Rosch and Segner, 1990), catfish and turbot, (Segner *et al.*, 1993) and roach (Dabrowski *et al.*, 1989). Cahu and Zambanino Infante, (1997) investigated the maturation process of the enterocytes in sea bass larvae. Normally the initially high cytosol activity, indicated by the high activity of leucine alanine peptidase decreases as the activity of the brush border enzymes (leucine amino peptidase in this case) increases. When fed on dry diet from day 25 PFF, this maturation process was delayed.

The proportion of total tryptic activity accounted for by that of the second part of the intestine is much more significant in larvae than adults. An increase in trypsin activity occasioned by a transfer to inert diet such as that illustrated in roach larvae by Hofer (1985) may lead to an increased loss of body proteins at a stage when the gut transit time and length of digestive tract are short. Segner *et al.* (1988) demonstrated changes in the morphology and function of the digestive system after weaning onto a dry diet in metamorphosed turbot. The activity of proteolytic enzymes, mucosal volume and surface area all increased substantially.

In the present experiments, once established onto dry feed, halibut larvae exhibited rapid growth and improved vitality indicating that the artificial diet was providing for their nutritional requirements. An increase in growth rate following weaning is widely reported but there is also evidence of physiological benefits from weaning fish larvae from live to formulated diets. Prior to weaning, metabolic disturbances typical of

malnourished fish were observed in the liver of turbot larvae by Segner and Witt (1990). These features, including low glycogen and lipid reserves, swollen mitochondria, nuclear glycogen inclusions and abnormal Golgi morphology were eventually rectified after feeding on dry diet was established. The initial response to weaning however, was an over-compensation in terms of liver lipid content particularly within the cisternae of the endoplasmic reticulum. Weaning onto a dry diet resulted in an increase in brain dry weight and levels of brain DHA in turbot and gilthead sea bream (Mourente and Tocher, 1992, 1993).

Interspecific differences in the ontogeny of the digestive system are critical in determining the efficiency of break-down and assimilation of artificial diets. In a histological examination of two species; striped bass and inland silverside, fed microencapsulated *Artemia*, poor performance of the former species compared to the latter was linked to an incapacity to absorb the particles in the anterior portion of the intestine (Bengtson, 1993). Specific knowledge of the ontogeny of the digestive tract of halibut and its digestive capacity would be helpful in formulating weaning diets and establishing optimum weaning techniques. However, there is no replacement for practical feeding trials such as those carried out in this study.

The presence of exogenous enzymes in live diets has been the subject of discussion by a number of authors. Dabrowski and Glogowski (1977a, 1977b) and Munilla-Moran *et al.* (1990) demonstrated an important supplementation of endogenous proteolytic enzyme activity by that present in the live food. Lauff and Hofer (1984) lent support to these findings by presenting evidence that lower enzyme levels were found in fish fed zooplankton whose enzymes had been denatured than in untreated prey. These authors reported that the role of exogenous enzymes was more significant in young larvae of fish

which developed a stomach at a later stage, e.g. whitefish, than those which possess a stomach from first-feeding, e.g. trout, or those in which a stomach was totally absent, e.g. roach. These exogenously derived enzymes may play a passive role in supplementing endogenous enzyme activity or may be involved in some form of positive feedback mechanism which serves to stimulate endogenously produced enzyme precursors or zymogens (Dabrowski, 1984a). An increased production of trypsinogen was demonstrated at higher copepod prey densities for herring larvae (Pedersen *et al.*, 1987) but trypsin activity was also stimulated in herring larvae following the ingestion of inert plastic spheres (Hjelmland *et al.*, 1988). These authors suggested a possible neural regulation of enzyme production initiated by the presence of bulk in the digestive tract. Stimulation of enzyme secretion following ingestion of food may occur through a positive feedback type mechanism or may be mediated by hormonal and biochemical pathways. Munilla-Moran and Stark, (1989) reported some passive exogenous enzyme activity associated with live prey (rotifers) fed to turbot larvae but proposed that ingestion of the same, led to synthesis of new enzyme proteins, rather than activation of existing zymogens. Person le Ruyet (1989b) presented evidence that the contribution from live prey to total enzyme activity was negligible. The addition of pancreatic enzymes in order to enhance the digestibility of artificial diets has proved successful in some cases. Kolkovski *et al.* (1993) showed that the inclusion of pancreatic enzyme in a microdiet fed to gilthead sea bream larvae enhanced assimilation by 30%.

The enhancement of enzyme activity in the presence of live prey through passive or active pathways may be one reason behind the success reported from some experiments involving co-feeding. Tandler and Kolkovski (1991) demonstrated that co-feeding 20% rotifers along with dry diet improved digestibility and uptake of dry diet with gilthead

sea bream.

Artificial diets for weaning fish larvae are usually composed of high quality raw materials. There is however, evidence that the digestibility of dry diets is lower than live feed. Dabrowski and Kaushik (1984) demonstrated inferior digestibility and lower protein utilisation (NPU) of dry diet than *Artemia* fed to carp larvae. The adequacy of artificial diets with respect to the nutritional requirements of fish larvae is critical. Although these diets can, in theory, be tailored to fulfill nutritional demands, knowledge of dietary needs is first required. There is still relatively little precise information on dietary requirements of fish larvae and many of the studies carried out have been based on the utilisation of nutrients stored in egg and yolk sac. Included in these areas of investigation are some analyses of protein synthesis in marine fish larvae, namely cod (Fyhn and Serigstad, 1987), lemon sole (Ronnestad *et al.*, 1992a), turbot (Ronnestad *et al.*, 1992b) and Atlantic halibut (Ronnestad *et al.*, 1993). Amino acid profiles were investigated by Kanazawa (1988) for Japanese flounder and ayu. A considerable body of research has focused on the role and requirements of some essential fatty acids (EFAs) particularly n-3 HUFAs (Watanabe, 1991; Sargent *et al.*, 1993; Watanabe and Kiron, 1994; Sargent, 1995). Nutritional requirements of fish larvae change progressively from hatching to metamorphosis or transformation and this is of critical importance in determining the suitability of live prey and formulation of enrichments or artificial diets at particular stages during their ontogeny.

The use of stress tests in Experiments 1-3 provided a useful index of fitness which indicated differences between weaned and unweaned fish, between size classes and between larvae of different nutritional status. Stress tests are a useful tool for assessing the relative fitness of a population of larvae. Tests which measure the tertiary effect

(survival in this instance) on the whole animal can be simply performed on a sample of fish in the hatchery. Different stressors can be used to evaluate vitality in fish larvae. In this study, exposure to high salinity was adopted and this eventually causes mortality due to exhaustion of metabolic reserves through an effort to maintain homeostasis. Similar tests have been employed by Dhert *et al.* (1990) for barramundi and by Ashraf *et al.* (1993) for inland silversides. Hayashi (1995) demonstrated an improvement in vitality of red sea bream and Japanese flounder following weaning using a handling stress test where larvae were exposed to air in a hand net for various periods and this technique was also used by Ako *et al.* (1994). Holt (1993) used a low salinity (3ppt) stress test to reveal improved vitality in red drum larvae after weaning.

Water quality is a primary concern during the weaning phase. As far as temperature is concerned, this parameter is not of such critical importance when compared to the earlier life stages. Larvae/fry appeared to feed normally at temperatures which ranged from 11 - 17°C in these experiments. More careful consideration must be given to oxygen concentration, pH and most of all dissolved ammonia concentration when feeding dry diets. During the course of Experiments 4 and 5, rising levels of ammonia, falling pH and oxygen created suboptimal conditions for sensitive young fish larvae and were almost certainly stress inducing. Where earlier weaning and co-feeding were attempted in Experiments 4 and 5 the requirement for green water and low flow rates combined with regular feeding and low consumption of inert diet conspired to reduce water quality and resulted in high ammonia values due to build up of easily degradable wastes.

Halibut larvae tend to show a better feed response during the first-feeding stage in green water (Naas *et al.*, 1992) and this is one of the main obstacles to early weaning since it precludes high flow rates and thus efficient waste removal. The effect of the

addition of algae to culture water is unexplained and may be due to environmental effects i.e. light attenuation and turbidity. Algal cells have been detected as early as 220°Cdays after hatching in the gut of halibut larvae (Reitan *et al.*, 1991) and direct nutritional benefits are possible as well as indirectly through *Artemia* enrichment (Scott and Middleton, 1979; Reitan *et al.*, 1993). It is important to define the earliest possible time at which green water is no longer necessary and this point is most likely related to developmental stage and fitness of the larvae. In Experiment 6, reported in this thesis, green water was unnecessary after day 43 PFF and this enabled weaning to proceed in good quality water using high water exchange rates.

Although aggressive behaviour and cannibalism were noted in the first experiment, grading of fish as carried out in Experiment 3 did not improve survival or growth. An increased disparity in size of larvae within a cohort is invariably a consequence of the transfer to dry diet. This often leads to aggression from larger individuals directed towards smaller conspecifics (Hecht and Pienaar, 1993). Other factors such as stocking density, light regime and feeding rates can also play a role. Cannibalism is a common problem amongst some cultured species. It is encountered in the culture of pike (Giles *et al.*, 1986) and African catfish (van Damme *et al.*, 1990). The latter author stated that the earlier the transfer to dry diet occurs, the greater the mortality from cannibalism which is concurrent with a larger coefficient of variation in size. Similarly Ehrlich *et al.* (1989) found that cannibalism was significantly worse in smallmouth bass fed on dry diet than *Artemia*. Amongst marine fish, problems associated with cannibalism are probably best known for cod (Howell, 1984; Folkvord, 1991; Ottera and Lie, 1991). In an experiment conducted by the last authors, cannibalism accounted for an average of 28% of the total mortality.

The ratio between jaw size of the potential predator and head size of the potential prey is critical (Kusano *et al.*, 1986). Katavic *et al.* (1989) proposed that a body length ratio of 1.9:1 was enough to induce cannibalism in sea bass. Cannibalism is a significant cause of mortality in sea bass, and these authors found that up to 37% of larger individuals had smaller siblings in their stomachs. The maximum wet weight ratios, occurring in Experiment 1 where cannibalism was witnessed were approximately 1:10, whilst this ratio only reached 7.5 in the ungraded groups in Experiment 3 and no aggression was noted. It seems that the halibut is less prone to cannibalism than other culture species and this may be related to their demersal habit following metamorphosis.

As well as mortality, growth rate, particularly in the smaller individuals, may be compromised since feeding competition caused by dominance hierarchies can reduce feed intake among subordinates (Gersanovich, 1983; Koebele, 1985; Katavic *et al.*, 1989). Smaller siblings might also be stressed by the presence of larger individuals (Koebele, 1985). These problems can be alleviated by paying attention to feeding regimes and grading cohorts where necessary. From the results of the grading study and overall observations from all the weaning experiments it is concluded that grading is unnecessary during weaning of halibut larvae. However, it is advisable to grade the fry soon after weaning when large size variations are likely to emerge and the animals are more robust.

The prey requirements of halibut larvae in terms of total intake have until recently been poorly understood. In general, first feeding techniques have improved dramatically in the last few years and as previously mentioned, this has led to increases in growth rate. In the first 3 experiments, *Artemia* rations were fed according to standard regimes practised at SFIA, Ardtoe at the time. In the subsequent experiments the ration was

increased according to updated knowledge and from closer observations on the appetite of the larvae. Thus, larvae were fed to satiation and this yielded important information as to their requirements and relative performance on live and artificial diets. Experiment 6 illustrated that despite being fed to satiation, larvae maintained on *Artemia* after 100 mg grew more slowly than those weaned at 100 mg for which the SGR increased once established on the dry diet. This demonstrates the limit to the growth potential of halibut larvae fed *Artemia* above this weight. There is likely to be a maximal intake reached by halibut larvae of this age, for which the requirement for *Artemia* is in excess of 4000 prey/larvae/day (van der Meeren, 1996), determined by the physical searching, capture and handling times involved in feeding on *Artemia*. Given the choice, halibut larvae of this size will optimally forage for larger *Artemia* as shown by Olsen *et al.* (1997) who fed mixed ages of *Artemia*. Observations of the larvae feeding on the weaning diet revealed that halibut larvae of this age were capable of ingesting relatively large particles (0.5-0.8mm). When comparing the approximate energetic values of dry diets and *Artemia* (see Appendix I) it is clear that on a dry weight basis there is little difference. However, when considering the dietary energy value of ingested feed, assuming the diet is taken soon after presentation i.e. with little increase in water content, volume for volume the dry diet has a much higher calorific content. *Artemia* is typically 91% water whereas the weaning diet has a water content of less than 4%. Assuming similar digestibility, the consumption of dry diet is likely to be much more efficient in terms of the energetics of the larvae.

In all experiments where metamorphosis and pigmentation characteristics were evaluated, there was no discernible benefit derived from early weaning and the incidence of abnormal fry was similar to production batches from the hatchery. This is not

surprising since it is generally accepted that these events are influenced by nutritional factors quite early on in halibut larval development (Harboe *et al.*, 1997) and almost certainly before the animals were weaned in these experiments. Since little or no uptake of dry diet was observed in any of the long term experiments where very early weaning or co-feeding was attempted, there was unlikely to have been any influence exerted by feeding the dry diet at least as far as developmental events were concerned.

The results of these experiments suggest that it is perfectly feasible to wean halibut larvae at an average wet weight of 100 mg and provided that good growth during the first-feeding phase is secured, this can be achieved at around day 40 PFF. It is likely that as techniques are improved, the first-feeding phase can be further shortened. When compared with other marine species such as turbot, sea bass and sea bream for which hatchery production is more well established, the length of the first-feeding stage is not prohibitively long. The total replacement of live feeds remains a primary goal for marine fish producers and this is dependant chiefly on future diet development.

6.9. Conclusions

- Live-feed history has an important influence on weaning success. In particular, high specific growth rates during the live-feeding stage tend to translate to good weaning performance.
- Halibut can be successfully weaned at lower wet weights (about 100 mg) than commonly practiced.
- Behavioural observations and examination of gut contents has shown that halibut will ingest inert food particles at sizes of considerably less than 100 mg. However, attempts to wean at such an early stage will lead to high mortality with conventional diets.
- Early weaning can result in higher growth rates following establishment onto formulated diet compared with a prolonged period on *Artemia*. It is concluded that the energetic demand of halibut at this size is not met with *Artemia* and the growth potential is not realised.
- Coefficient of variation tends to increase during weaning. Although aggressive behaviour and cannibalism was noted in some experiments during weaning, a reduction in size variance achieved by grading did not reduce mortality. However, size specific mortality was clearly demonstrated. This, together with the observation that inert diet uptake is size related, indicates the value of removing smaller individuals from the population for delayed weaning.
- Prolonged presentation of *Artemia* together with inert diet showed no evidence that this method of feeding results in improved survival, growth or conditioning to

weaning diet. Observations suggested that when fed a combination of live and inert diets, halibut larvae showed a clear preference for live prey. As a result, little or no uptake of inert diet occurred. Regardless of fish size, ingestion of dry diet was induced in the majority of the population only when live prey was reduced or totally removed. The nine-day weaning regime used as a standard treatment was employed repeatedly with success.

- Preliminary investigations revealed that halibut larvae of less than 60 mg wet weight will readily ingest immobilised *Artemia*. In the same experimental group, ingestion of a dry diet containing freeze dried *Artemia* was observed. It is suggested that intermediate weaning stages employing these feeding strategies might facilitate early weaning.
- Large savings in *Artemia* consumption are possible when early weaning is achieved during the period when the prey requirements reach levels in excess of 3000/larvae/day.
- When weaning is attempted before the end of the "green water" stage when exchange rates are relatively low, water quality can deteriorate due to a build up of wastes. However, the requirement for algae has been demonstrated to be as short as 43 days and this might be further reduced.
- Halibut appeared able to survive on, and therefore digest and assimilate, inert diet before the end of the pelagic phase, prior to metamorphosis and complete formation of the stomach.
- Metamorphosis and pigmentation characteristics were universally poor in all experimental batches. No treatment effects were observed and it was concluded

that these traits are dependant on physiological events occurring earlier on in the ontogeny of the halibut

- Halibut of > 100 mg are not highly susceptible to handling stress and this was evident from the generally low mortality following the move from larval rearing to experimental weaning tanks.
- The use of autofeeders during weaning seemed to enhance diet uptake due to frequent and regular feeding.

7. General Discussion

The stable supply of high quality gametes is central to the production of any aquaculture species. The pioneering research and development of the halibut aquaculture industry has been dependant upon material derived from a nucleus of wild caught animals. As hatchery reared fish reach maturity and become recruited to the broodstocks, the scarcity of material suffered in these formative years will be eased. To ensure consistency of gamete production and thus optimal use of this valuable resource, a full understanding of the factors involved in determining egg quality is essential.

The investigations described in Chapters 3 and 4 confirmed the fact that halibut egg quality is highly variable among egg batches from a single female, between spawners and between stocks. The technique developed for assessing egg quality based on observations of blastomere morphology, detailed in Chapter 3, provides a method for predicting viability with a relatively high degree of accuracy. This not only enables decisions to be made soon after fertilisation on whether to proceed with incubation, but also provides a useful indicator for broodstock managers and researchers alike. During a spawning season, a female halibut will ovulate several times and as already discussed, it is important that the spawning rhythm is established quickly and as accurately as possible. The observation of blastomere features could potentially aid in this respect. The technique might also be employed as a diagnostic tool in future studies on various topics of reproductive physiology, such as fertilisation protocols, genetic and hormonal manipulation and nutritional or environmental requirements of broodstock. The clear relationship between blastomere anomalies and viability, raises questions as to their causes. Further physiological research into the fundamental causes of cytological

disruption in early embryos may provide a key to our understanding of the nutritional and environmental requirements of broodstock halibut.

An investigation into the role of thermal environment in egg production and quality (Chapter 4), confirmed the importance of maintaining low stable temperature during spawning. There was an indication that temperature profiles several months prior to spawning may impact on egg viability and this should be the subject of further enquiry. The optimum annual temperature profile for halibut broodstock is unknown and this information is essential for broodstock managers, since it has a bearing both on egg production and on the running cost of a broodstock facility. A better knowledge of the relationship between temperature, photoperiod and spawning cycles, will help to secure a regular supply of eggs and larvae for hatchery production. Given the economic implications of controlling temperature, methods for alleviating these costs, e.g. heat reclamation or recirculation, will undoubtedly be explored by commercial operators. Indeed, the feasibility of using recirculation technology for broodstocks is already the subject of current investigation (Shields *et al.*, in press).

The convenience of using microtitre plates as an incubation vessel for separate eggs, allows the researcher to carry out small scale experiments, without the need for fully plumbed incubation facilities and only requires some form of temperature control and light proofing. In this way, comparisons between individual spawners, stocks, sites or year on year comparisons are possible. This technique, used for the work described in Chapters 3 and 4 was also applied successfully for the study on triploidy induction in Chapter 5.

The current work on the use of hydrostatic pressure shock for the induction of triploidy and on the use of temperature shock (Holmefjord and Refstie, 1997),

demonstrate the potential of ploidy manipulation in halibut. Pressure shocking proved an extremely effective agent for the production of triploids. Although further refinement of this technique is required to define more closely the various optima, i.e. level and duration of pressure shock, the experiments on timing of shock have served to identify when, after fertilisation, polar body extrusion takes place. This information will be valuable for future studies on genetic manipulation. Although a preliminary investigation comparing the viability of triploids with diploids suggested that mass production will present problems during the hatchery cycle, this needs to be confirmed with repeat experiments. To evaluate the actual potential of triploid halibut, long term studies are required to confirm the putative advantage of triploidy in terms of sterility and growth. It is also important to know whether there are any competitive disadvantages inherent in triploidy and whether there is any influence on sex ratios. To achieve this, triploids must first be reared to an age at which sexual differentiation occurs (which is unknown) and then to sexual maturity, under normal culture conditions. The sex determining mechanism of the halibut is currently under scrutiny and hopefully, once revealed, will lead to methods for the production of all-female stocks. The first populations of halibut have now been reared through to harvest weight and initial indications are that early maturation and reduced growth rate in males will have an impact on the economics of ongrowing. Once the techniques for genetic manipulation are at our disposal, the possibilities for stock improvement will widen.

The final phase of the hatchery cycle was focused upon in Chapter 6. This work provided an insight into how critical the nutritional and physiological status of the fish is as it enters the weaning stage. Using conventional diets, it is clearly possible to wean halibut off live feeds at a relatively early age after approximately 700 °Cdays on live

feed. This is contingent on the provision that sufficient numbers of nutritionally adequate live prey have been fed during larval rearing and that good growth during this period has been achieved. When given the choice of live prey or weaning diet, halibut larvae select a living food item in preference. For this reason, there seems to be little to gain in prolonging the weaning period, causing wastage of weaning diet and tank fouling, with little significant uptake by the larvae. Behavioural observations suggested that halibut larvae will accept non-living prey or artificial diets at an early age, but whether the digestive capabilities of halibut larvae are sufficiently advanced to utilise dry diets at sizes of less than 100 mg is unknown. Current practices at weaning are inclined towards the slow removal of live diet at a relatively late stage, after the majority of fish have settled to a demersal habit and the stomach is formed. The hatchery operators would rather minimise the losses of valuable juveniles, than reduce the length of the live feed period by a week or two. To make further inroads into the live feeding period, a combined research approach aimed at the development and testing of microdiets along with an investigation of the digestive ontogeny of the halibut is required. A microdiet which is attractive, digestible, water stable and nutritionally sound, would represent a major step forward in the rearing of halibut or any other marine fish with small, pelagic, predatory larval stages.

The work described in this thesis was conducted during a period of concerted exploratory effort aimed at meeting the challenge of mass propagation of a fish species about which little was known. Consequently, there is a fast growing body of literature on the subject of Atlantic halibut biology and culture. More importantly, the industry has a growing skills base of experienced technicians with sufficient knowledge to rear significant numbers and some promise of reward. As Atlantic halibut production gains

momentum, in-house techniques and refinements will inevitably develop, but to secure the future growth and stability of this fledgling industry, continued coordinated research and development programs will be of key importance.

8. References

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9. Appendices

9.1. Appendix I. Proximate composition, gross energy values and fatty acid profiles of dry diet (Nutra Marine) and *Artemia*.

Table 9.1. Comparison of proximate composition of Nutra Marine and typical *Artemia* nauplii (‡from Watanabe *et al*, 1983). Data for Nutra Marine were supplied by Nutreco ARC. Gross energy content is calculated from average values (* from Steffens, 1989).

	Proximate Composition (%)					Energy content (kJ/g)				
	Nutra Marine		‡ <i>Artemia</i> nauplii		*Average values (kJ/g)	Nutra Marine		‡ <i>Artemia</i> nauplii		
	Wet	Dry	Wet	Dry		Wet	Dry	Wet	Dry	
Protein	58.7	60.8	6.0	65.2	23.9	14.03	14.53	1.43	15.58	
Fat	20.0	20.7	1.4	15.2	39.8	7.96	8.24	0.56	6.05	
Carbohydrate	7.0	6.8	0.8	8.6	17.6	1.23	1.20	0.14	1.51	
Ash	10.8	10.4	1.0	10.8	0	0	0	0	0	
Moisture	3.5		90.8		0	0		0		
					Total (kJ/g feed)	23.22	23.97	2.13	23.15	

Ash free dry mass gross energy of *Artemia* from values above = 26.0 kJ/g

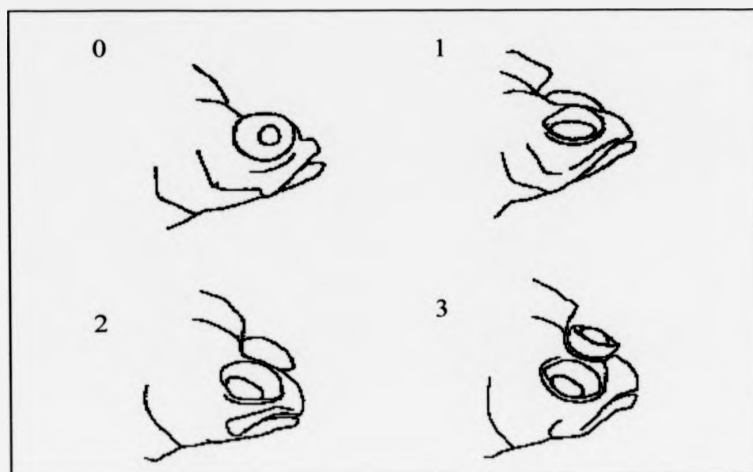
From Emmerson, 1984, ash free dry mass energy content of *Artemia* nauplii = 5.78 cal/mg (24 J/mg or kJ/g). These values are similar.

Table 9.2. Comparison of fatty acid profiles for dry diet (Nutra Marine, T.Skretting AS) and *Artemia* enriched with Algamac 2000 (Biomarine, Aquafauna) and Super Selco (Inve Aquaculture) at 27°C for 18hrs. Data for Nutra Marine were supplied by Nutreco ARC and for the *Artemia* profiles by Seafish Industry Authority and NERC Aquatic Biochemistry Laboratory, University of Stirling.

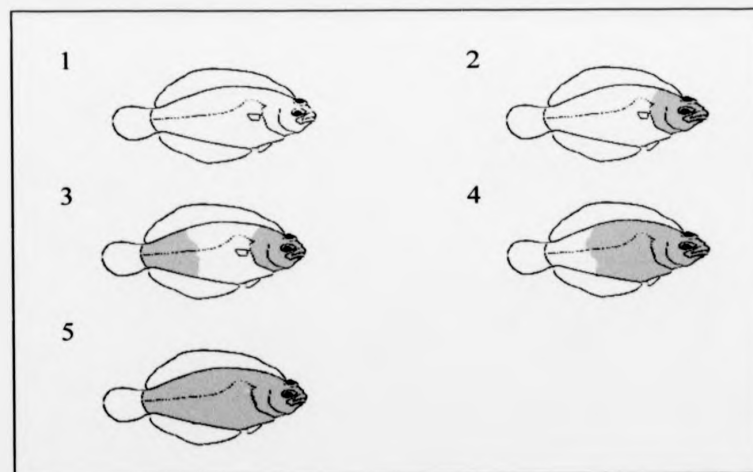
Fatty acid	Area %		
	Dry diet (Nutra Marine)	Algamac enriched <i>Artemia</i>	Super Selco enriched <i>Artemia</i>
C18:2 n-6	9.2	4	5.3
C18:3 n-3	0.2	19.1	19.9
C20:4 n-6 Arachidonic acid (AA)	1	1.5	1
C20:5 n-3 Eicosapentaenoic acid (EPA)	9.8	5.3	13.9
C22:5 n-6	0.4	2.5	0.3
C22:6 n-3 Docosahexaenoic acid (DHA)	12.1	5.1	5.4
Total saturates	27.7	24.2	14.6
Total monoenes	23.9	33.3	29.2
Total n-6 HUFA	11.7	8.1	7
Total n-3 HUFA	26.6	32.3	44.2
DHA/EPA	1.23	1	0.4
EPA/AA	9.8	3.5	13.1

9.2. Appendix II. Classification of eye migration and pigmentation characteristics. (After B. Gara).

A)



B)



A) Eye migration index. Score of 0 (no migration of left eye) to 3 (full, normal migration of left eye) assigned to various stages of eye migration.

B) Pigmentation index. Scores of 1-5 assigned to various pigmentation patterns for both dorsal or ventral surfaces. Normal pattern is dorsal: 5 ventral: 0.

9.3. Appendix III. Common and scientific names of fish species
mentioned in thesis text

Scientific name	Common name	Scientific name	Common name
<i>Abramis brama</i>	Common bream	<i>Morone saxatilis</i>	Striped bass
<i>Acanthopagrus latus</i>	Yellow-finned black porgy	<i>Mugil cephalus</i>	Grey/ striped mullet
<i>Acipenser sturio</i>	Sturgeon	<i>Odontesthes bonariensis</i>	Pejerry
<i>Anarhichus lupus</i>	Wolffish	<i>Onchorhynchus gorbuscha</i>	Pink salmon
<i>Bairdiella icista</i>	Bairdiella	<i>Onchorhynchus keta</i>	Chum salmon
<i>Brachydanio rerio</i>	Zebrafish	<i>Onchorhynchus tshawytscha</i>	Chinook salmon
<i>Carassius auratus</i>	Goldfish	<i>Oncorhynchus mykiss</i>	Rainbow trout
<i>Chanos chanos</i>	Milkfish	<i>Oreochromis mossambicus</i>	Mozambique Tilapia
<i>Clarius gariepinus</i>	African catfish	<i>Oreochromis niloticus</i>	Nile tilapia
<i>Clupea harengus</i>	Herring	<i>Pagrus major</i>	Sea bream or red sea bream
<i>Coregonus lavaretus</i>	Whitefish	<i>Paralichthys olivaceus</i>	Japanese flounder or hirame
<i>Cyclopterus lumpus</i>	Lumpsucker	<i>Phoxinus phoxinus</i>	European minnow
<i>Cyprinus carpio</i>	Common carp	<i>Platichthys flesus</i>	Flounder
<i>Dicentrarchus labrax</i>	Sea bass	<i>Platichthys stellatus</i>	California flounder
<i>Engraulis encrasicolus</i>	European anchovy	<i>Plecoglossus altevelis</i>	Ayu
<i>Engraulis japonicus</i>	Japanese anchovy	<i>Pleuronectes platessa</i>	Plaice
<i>Esox lucius</i>	Northern pike	<i>Psettodes erumei</i>	Indian halibut
<i>Gadus morhua</i>	Cod	<i>Rutilus rutilus</i>	Roach
<i>Gasterosteus aculeatus</i>	Stickleback	<i>Salmo gairdneri</i>	Rainbow trout
<i>Gobius sp.</i>	Gudgeon	<i>Salmo salar</i>	Atlantic salmon
<i>Hippoglossus hippoglossus</i>	Atlantic halibut	<i>Salvelinus alpinus</i>	Arctic charr
<i>Hippoglossus stenolepis</i>	Pacific halibut	<i>Sardina pilchardus</i>	Sardine
<i>Hypophthalmichthys molitrix</i>	Silver carp	<i>Sciaenops ocellatus</i>	Red drum
<i>Ictalurus punctatus</i>	Channel catfish	<i>Scomber scombrus</i>	Atlantic mackerel
<i>Lates calcarifer</i>	Barramundi	<i>Scophthalmus maximus</i>	Turbot
<i>Limanda limanda</i>	Dab	<i>Scophthalmus rhombus</i>	Brill
<i>Limanda punctatissima</i>	Longsnout flounder	<i>Solea solea</i>	Dover sole
<i>Limanda yokohamae</i>	Mud dab	<i>Sparus aurata</i>	Gilthead sea bream
<i>Menidia beryllina</i>	Inland silverside	<i>Sparus barba</i>	Silver sea bream
<i>Menidia menidia</i>	Atlantic silverside	<i>Sprattus brama</i>	Sprat
<i>Micropterus dolomieu</i>	Smallmouth bass	<i>Tilapia aureus</i>	Tilapia
<i>Microstomus kitt</i>	Lemon sole	<i>Tinca tinca</i>	Tench
<i>Misgurnus mizolepis</i>	Mud loach	<i>Trigla corax</i>	Gurnard
<i>Morone chrysops</i>	White bass		