ASPECTS OF THE INTERRENAL FUNCTION, STRESS RESPONSE, SEXUAL DIMORPHISM AND GROWTH PERFORMANCE OF THE ATLANTIC HALIBUT, HIPPOGLOSSUS HIPPOGLOSSUS

Nigel Robert Jordan

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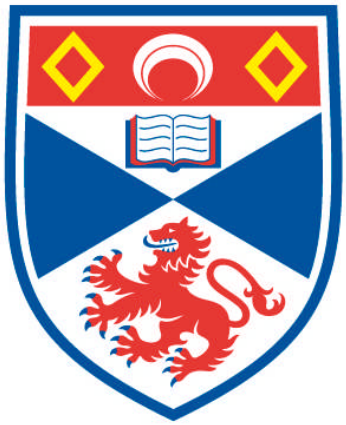
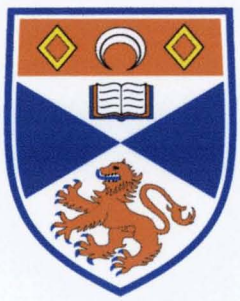
at the
University of St Andrews



2005

Aspects of the interrenal function, stress response, sexual dimorphism and growth performance of the Atlantic halibut, *Hippoglossus hippoglossus*.



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ABSTRACT

Abstract

Chapter 1
Growth rates between individually tagged Atlantic halibut, from a single batch of farm produced eggs, on-grown in sea cages and pump ashore tanks for three years were significantly different. The tank reared fish 405g – 5992g showed a 29% premium in growth (final weight) compared to the cage reared fish 444g -4640g.
Females in both systems reached a greater size (7352g tanks, 5836g cages) than males. Males that matured early (3819g tanks, 2877g cages) had a lower mean end weight than males maturing a year later (4326g tanks, 3086g cages). Early maturing males had the largest initial size. Seasonal variations in growth were observed for all groups. Major divergences in growth between males and females only became apparent when the males first matured at around 1.5 – 2 kg. No female maturation was observed during the trial. Halibut growth was determined to be positively allometric with growth of males being more linear then females. Condition factor increased with time whilst there was a decrease in Specific Growth Rate (SGR) from approximately 0.5%day^{-1} to 0.1%day^{-1} throughout the trial. Concentrations of plasma cortisol, osmolality, chloride and glucose measured through the trial provided no evidence of chronic stress at either site.

Chapter 2
Acute confinement stress (2, 12 and 30 minutes) was shown to elicit both primary and secondary stress responses in accordance with other marine teleosts. Increases in plasma cortisol, osmolality, Cl^-, Na^+ and glucose were observed, reaching maximum concentrations within 80 minutes, although there was no effect on plasma K^+. The duration of the confinement appeared to have no effect on the magnitude of the response. Following repeat confinements (4 days later) there was no evidence of either habituation or a cumulative effect in terms of cortisol or glucose whereas the effects on osmoregulatory function (Na^+, Cl^- and osmolality) appeared to be longer lasting. The results provided the first information regarding the stress response of the Atlantic halibut and enabled a better interpretation of the vales measured in the fish reared in tanks and cages (chapter 1).

Chapter 3
In vitro cortisol production (% above basal secretion), measured by radioimmunoassay, from perifused interrenal tissue of the Atlantic halibut was significantly stimulated by porcine adrenocorticotrophic hormone (ACTH) (0.01-1.0μM) and [Asn^1, Val^5] angiotensin II (AII) (0.1-10μm). No significant increase in cortisol production resulted from physiological levels of potassium (K^+) although non-physiological levels (10mMK^+) did elicit a mild response in comparison to the effects of ACTH and AII. Maximum steroid production was in response to 0.01μM ACTH (1351% above basal secretion) and 1.0μM AII (397% above basal secretion). With increased concentrations above these levels of both ACTH and AII there was a reduction in the degree of cortisol stimulation. The results show that the interrenal tissue of the Atlantic halibut responds in accordance to that of other teleosts to classical steroidogenic peptides.

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CHAPTER 1

INTRODUCTION: THE ATLANTIC HALIBUT, *HIPPOGLOSSUS HIPPOGLOSSUS*

1.1 Introduction

Of the Pleuronectiformes (flatfish) the Atlantic halibut *Hippoglossus hippoglossus* is the largest. It is a member of the sub family Pleuronectinae that is within the family Pleuronectidae, one of the five families of the suborder Pleuronectoidei (Ahlström, Amaoka et al., 1984; Hensley and Ahlström, 1984). Other members of the Pleuronectidae family include the plaice, dab, flounder, and lemon sole (Scott and Scott, 1988; Haug, 1990). The family Pleuronectidae comprises of flatfish that primarily have their eyes located on the right side of their body. Individual exceptions to this do exist however. Both in the wild and within cultured populations of halibut a small percentage of sinistral specimens show a reversal of this dextral predisposition.

Investigation of the karyotype of the Atlantic halibut shows that it consists of 24 pairs of chromosomes (2n =48) and that the number and morphology of the chromosomes is similar to most other pleuronectid fish that have been studied (Brown, Bromage et al., 1997)

In general descriptive terms the Atlantic halibut has an elongated body that is strongly laterally compressed. It lies on its left hand side (apart from sinistral exceptions previously mentioned) with both eyes located on the uppermost right hand side. There is a steep arch in the clearly visible lateral line above the pectoral fin. Small cycloid scales that are surrounded by rings of smaller supplementary scales cover the body. The caudal fin is large and concave. The upper surface colour is variable and ranges from shades of brown to green with mottled patterns present. The lower surface is usually white although mottled variations to this have been noted. (Andriyashev, 1954; Scott and Scott, 1988) (Figure 1.1).

The Atlantic halibut is visually sexually monomorphic. Morphological sexual dimorphic traits exist only as statistical dimensional differences that manifest themselves part way through the life cycle.

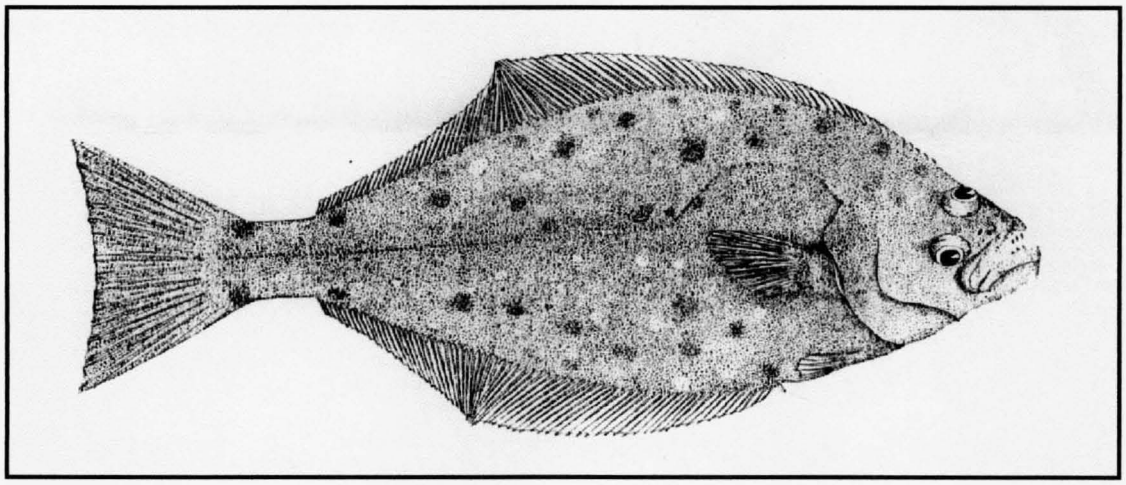

Figure 1.1 The Atlantic halibut, *Hippoglossus hippoglossus* (Scott and Scott, 1988)

The Atlantic halibut is distributed along both the eastern and western seaboards of the Atlantic. Along the eastern coastline the distribution ranges from around 36° to 57° north and along the western coast from about 42° to 65° north. Populations are also found around the southern coasts of Greenland, Iceland and the Faroes (Haug, 1990). However, despite the wide distribution of these populations a number of reports indicate that the North Atlantic halibut populations from the Faros, Iceland and Greeenland region as a whole, in terms of genetic variation are considerably homogeneous (Haug and Fevloden, 1986; Fevloden and Haug, 1988). Exceptions to this were fish sampled from northern Norway, which stood out as representatives of a separate breeding population (Foss, Imsland et al., 1998). It is postulated that this low level genetic variation is due to significant migratory patterns and dispersion of both eggs and larvae.

As previously mentioned the Atlantic halibut is the largest of all flatfish Captured females have weighed as much as 333kg and males up to 50kg (Kjørsvik and Holmefjord, 1995). Otolith ageing data from large specimens indicates that they are in the range of 30–35 years old. Heavy fishing pressure has however imposed limits on the number of large fish now being caught. Historically wild stocks of the Atlantic halibut have been commercially exploited. Catch records from the Gulf of Maine and Georges Banks began in 1893, although heavy fishing occurred prior to this, and they show that the annual landings have generally decreased since the 1890's. From 1893-1940 the annual average was 662mt yr^{-1} declining to an average of 144mt yr^{-1} during 1941-1976. Since 1977 the landings have averaged 95mt yr^{-1}. For 1999 the reported landings were 20mt (Figure 1.2)(NEFSC, 2002).

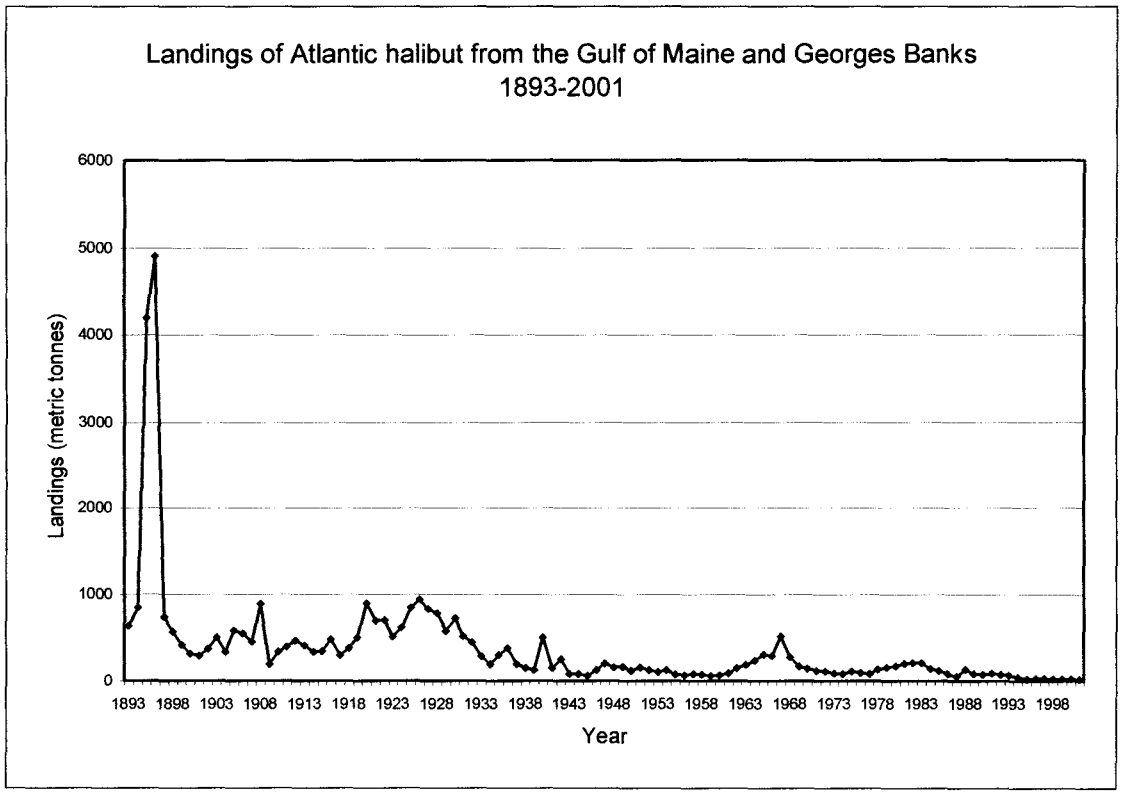

Figure 1.2 Landings of Atlantic halibut from the Gulf of Maine and Georges Banks 1893-2001 (NEFSC, 2002)

In terms of world landings for Atlantic halibut Canada and Iceland lead the table landing 866mt yr^{-1} and 884mt yr^{-1} respectively in 1995. This however was a reduction on the 1986 figures when 3691mt yr^{-1} and 1614.5mt yr^{-1} were landed (Forster, 1999). Table 1.1 shows the 1986 and 1995 annual landings for Canada, Iceland, Faroe Islands, Norway and all other countries combined.

Table 1.1 Landings (metric tonnes) of Atlantic halibut in 1986 and 1995 by country (Forster, 1999)

Country	**1986 Landings (mt)**	**1995 Landings (mt)**
Canada	3691.6	866.2
Iceland	1614.5	884.4
Faroe Islands	639.5	653.0
Norway	784.6	548.8
All other (28)	1288	721.1
Total Atlantic halibut	7746	3673.5

Landings by the UK fleet both into the UK and foreign ports from 1995 – 2000 also show a similar pattern of decline (table 1.2) going from 424mt yr^{-1} to 210mt yr^{-1} (MAFF, 1995, 1996, 1997, 1998; DEFRA, 1999, 2000).

When the UK landings of halibut are compared to the UK landings of other species such as cod and haddock (table 1.2) it is clear that a relatively small amount of the species is available for the consumer. This combined with the seasonality of the supply and the high quality of the flesh means that halibut can command a high price. This scenario is akin to that of wild salmon before large scale commercial farming operations began in the 1970's and as with wild salmon at that time halibut is supplied

to a niche market. Considering the high market value (compared to the now low market value of farmed salmon which, has further sparked interest in species diversification) and good flesh quality it is perhaps inevitable that halibut became of interest to the aquaculture industry. Allied to this its adaptability to a captive environment, good growth rates and food conversion efficiency, a fillet yield in the region of 60%, large egg batches and its relative resistance to many diseases and parasites common to the aquaculture industry then it becomes clear why there has recently been so much focus on rearing Atlantic halibut across the world.

Table 1.2 Landings of Atlantic halibut, cod and haddock (metric tonnes) by the UK fishing fleet to UK and foreign ports by year (MAFF, 1995, 1996, 1997, 1998; DEFRA, 1999, 2000).

Year	Halibut landings by UK fleet to UK and foreign ports (mt)	Cod landings by UK fleet to UK and foreign ports (mt)	Haddock landings by UK fleet to UK and foreign ports (mt)
1995	424	78690	86339
1996	397	79830	89680
1997	367	74636	83389
1998	203	77182	83437
1999	256	51699	72007
2000	210	41750	50657

Much of the early work on Atlantic halibut was carried out on wild caught fish (Prince, 1916; Øiestad and Haugen, 1980) and gametes (Blaxter, Danielsen et al., 1983). The exception to this being (Rollefsen, 1934) who obtained eggs from fish held in the aquarium housed at Trodheim's biological station. The majority of this early work was based on fertilisation and incubation methodologies with investigations being carried out into the effects of temperature variations (Blaxter,

Danielsen et al., 1983) and incubation apparatus (Øiestad and Haugen, 1980) (Solemdal, Tilseth et al., 1974). It was not until the establishment of captive broodstocks, initially in Norway and then subsequently in Scotland, Iceland, Canada and others (Holmefjord, 1996) that any substantial advancements in rearing techniques were made. Eggs from newly captured fish displayed buoyancy problems, which made working with them extremely difficult. This was primarily attributed to stress caused by the catching process and the stripping of the newly caught fish (Haug, 1990). Captive broodstock caught on long line and now spawning farm reared fish have led to improvements in the provision of good quality eggs.

Atlantic halibut are batch spawners with only a proportion of the maturing oocytes undergoing hydration and being discharged at any one time. Despite the ability to produce enough eggs to supply the industry there were, and still are, albeit with some improvements, high mortalities throughout the larval stage, which is universally regarded as the main bottleneck in the rearing process (Holmefjord, 1993; Kjørsvik and Holmefjord, 1995). High mortalities during the long yolk-sac stage and problems associated with first feeding have meant that much of the research so far has been directed at these areas.

The rearing cycle of halibut, as with many marine fish is fairly complex. Broodstocks of Atlantic halibut are usually maintained in circular tanks 5 – 15m in diameter. Water depth is in the region of 1 – 2m and there is usually the facility to drop the water level and maintain it at between 0.5m and 1m in order to allow entry to the tanks for the purpose of stripping eggs and milt during the spawning season. Entry to the tank is also required for assessment of the developmental status of the broodstock, primarily visually and by touch but also in some cases using non-invasive ultrasonography. The broodstock are held in full strength seawater at a temperature of $< 8^{o}C$ (Olsen,

Evjemo et al., 1999). In one season a female halibut may produce up to 16 batches of eggs at a mean ovulatory interval of 70 – 90 hours (Holmefjord, 1991; Norberg, Valkner et al., 1991; Jordan, 1997). At the time of stripping the halibut are lifted clear of the water onto a trestle bench. Gentle pressure along the ovary and testes releases the eggs and milt, which are collected into separate containers, care being taken not to allow contact with any water, which would activate the sperm. Egg quality is usually assessed, visually by examination of both the chorion and blastomere morphology (Shields, Brown et al., 1997) and by determination of fertilisation rates, with only high quality eggs being retained. In such cases around 90% of the stripped eggs will be fertilised and 75 – 80% of the fertilised eggs will hatch (Olsen, Evjemo et al., 1999). The timing of stripping has been found to be critical for the production of viable eggs as egg quality, due to over ripening, is greatly reduced 6-12 hours after the time of ovulation (Norberg, Valkner et al., 1991; Bromage, Shields et al., 1994). Whilst the spawning season of captive fish is normally around February it is, however, common for hatcheries to hold temperature and phase shifted photoperiod manipulated stocks in order to have continuous egg production (Smith, Bromage et al., 1991).

After fertilisation the 3mm eggs are incubated in cylindroconical tanks until hatching, which normally takes around 16-19 days at 5°C (Kjørsvik and Holmefjord, 1995). Part way through the incubation period it is common for the eggs to be surface-disinfected and then stocked into larger cylindro-conical tanks or silos (Olsen, Evjemo et al., 1999; Shields, Gara et al., 1999; Shields, 2001) in advance of the yolk sac stage of development. Following hatching the pelagic larvae are approximately 6mm in length and have a large yolk sac. Resorption of the yolk sac is prolonged and it is this stage that has proved to be the main bottleneck in the production cycle of the Atlantic

halibut. During this phase, while the halibut are surviving on their endogenous reserves, there is a husbandry requirement to provide optimum water quality whilst causing as little mechanical damage as possible to the sensitive larvae. Damage to neuromasts has been shown to be caused by handling prior to 174DD (Nairn, Batty et al., 2002) this has negative implications for the mechano-reception abilities of the larvae and hence predator detection, rheotaxis, feeding and obstacle avoidance. The transfer from endogenous to exogenous nutrition occurs once the majority of the yolk sac has been absorbed. The timing of the removal of the larvae from the silos to the illuminated "green water" rearing tanks depends on the particular protocol employed. Addition of food to the rearing tanks takes place either on transfer or shortly after at around 220 – 270 DD post hatch (Shields, 2001). Ideally just prior to transfer the temperature is gradually stepped up from 5-6°C to 9-12°C. Live feed is presented to the halibut, as there is no suitable commercially manufactured alternative at present. Due to the size of halibut at the onset of the feeding phase (12mm) the use of rotifers is not essential although they may be incorporated in the diet. Enriched *Artemia* or marine calanoid copepods are the two main types of live feed used in hatcheries. Although calanoid copepods are regarded as the benchmark diet by which to compare *Artemia* based feed strategies (Shields, Gara et al., 1999) there exist problems in maintaining a consistent supply when harvesting from the wild. Also parasitic and pathogenic contamination associated with wild caught copepods can cause significant mortalities. Investigations into the beneficial effects of incorporating calanoid copepod (*Eurytemora* spp. and *Temora longicornis* into the diet have been carried out (Witt, Quantz et al., 1984; Naess, Germain-Henry et al., 1995; Rønnestad, Helland et al., 1998; Rønnestad, Hemre et al., 1998). Some significant efforts have also been made on a commercial scale (Otter Ferry Seafish in Scotland) to culture calanoid

copepods for inclusion in the feeding regime. Investigations have also taken place into the use of harpacticoid copepods: e.g. *Tisbe* spp. (Støttrup and Norsker, 1997; Nanton and Castell, 1998). The use of *Artemia*, whilst enabling good growth and survival, has however been associated with poor pigmentation of the larvae due to insufficient uptake of Essential Fatty Acids (EFA). The EFA's of particular interest in terms of amount and ratios are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) (Naess, Germain-Henry et al., 1995; McEvoy, Navarro et al., 1996; McEvoy, Naess et al., 1998; Shields, Gara et al., 1999; Shields, 2001; Bell and Sargent, 2003). The digestibility of copepods by halibut larvae also appears to be greater in comparison to that of *Artemia* (Luizi, Gara et al., 1999). Whilst it is clear that there are significant benefits to be gained from incorporating copepods into the feeding regime the obstacles in doing so have meant that the enriched Artemia strategy has been adopted by most halibut hatcheries.

Live feed production is a costly and time-consuming operation so it is advantageous to wean the halibut onto a commercially prepared inert diet as soon as possible. Weaning takes place once the larvae have metamorphosed and become demersal (Shields, Gara et al., 1999). The metamorphosed halibut are at this stage of development around 150mg and the process occurs between day 40 and 60 post first feeding or approximately 90 days after hatching. Water temperatures through weaning are ideally increased to 13-14°C and the fish grow rapidly to about 1g. Rapid growth will continue through the nursery phase with the halibut reaching 5-10g approximately 150 days after hatching. Through this stage of development and up to 100-200g it is common for the fish to be grown on in land based tanks. The time taken to reach 200g is approximately 1 year at which stage it would is common to transfer the fish to either sea cages or larger pump ashore tanks for on-growing.

It is only in recent years, with better understanding of both broodstock and larval nutrition and also rearing conditions and apparatus that significant numbers of fish have been weaned and are now being on-grown. (Olsen, Evjemo et al., 1999) reports that in 1985 in Norway the first two halibut survivors were produced. In the UK the first tentative steps towards investigating the feasibility of halibut farming were taken by Seafish Aquaculture at the Ardtoe Marine Farming Unit in 1983. Financial assistance provided by the Highland and Islands Development Board enabled the collection of a small number of wild broodstock (Seafish, 1996) Following some initial success the British Halibut Association (BHA) (later re-named British Marine Finfish Association) was formed in 1987 to support halibut research and encourage the commercialisation of the results. In 1995 the output of the 3 BHA member hatcheries was around 10,000 fry. Subsequently there has been fairly rapid growth throughout the nineties and in 1997 it was reported that a combined figure from Norway, Scotland and Iceland of around 370,000 weaned fry were taken through to the nursery stage (Olsen, Evjemo et al., 1999).

Commercial farming of halibut now takes place in a number of countries around the world. These include the UK, Norway, Iceland, Canada, Ireland, Chile, and the USA with it even being reported that Atlantic halibut will be commercially bred and on-grown in Hawaii (Sing, 2002).

Production figures for 2002 from Norway (Rosenlund, 2002) estimate that 600-650,000 juveniles were produced from 11 hatcheries with a further 400,000 juveniles being imported for on-growing. In the UK in 2002 there were approximately 120,000 juveniles produced from 4 commercial hatcheries and that on-growing of these fish would be at 7 halibut sea cage sites located in; Uist, Lewis, Shetland, Orkney, Lochaber (2) and Argyll (Slaski, 2002). In addition to these sites, part funding for a

recirculating land based on-growing facility in North Wales has been granted to the commercial aquaculture company Selondia although no production is yet underway. Confirmation that the culture of Atlantic halibut has joined the ranks of other globally distributed aquaculture products came with the announcement that the first shipment of farmed Scottish Atlantic halibut to New York was made in March 2003 by Marine Harvest Scotland (Anon, 2003).

Despite the successes achieved there is however considerable scope for improvement in many areas of the production process.

In terms of on-growing, whilst it may appear to be the simplest step biologically it inherently requires the greatest inputs both in terms of time and financially. Decisions made at this stage will ultimately determine the profitability of the entire production process. Due to large numbers of fish only recently becoming available relatively little work has been carried out either on the biology or culture of juvenile fish through the on-growing process.

Prior to 1999 Marine Harvest Scotland, The British Marine Finfish Association (BMFA) and Otter Ferry Seafish Ltd identified a number of key unresolved issues facing the farming of halibut on a commercial scale. A primary avenue of investigation was the suitability of sea cages for the on-growing of halibut and to compare growth rate and performance with that achieved in pump ashore tanks. This issue was also highlighted by (Forster, 1999). It is known that halibut adapt well to living in onshore tanks (Seafish, 1996) but due in part to the economic pressures of large-scale production the mainstay of the UK halibut industry is at present cage culture. There are a number of contributory factors that have driven the Scottish halibut farming community along this route: -

- In addition to the high capital costs associated with establishing new pump ashore on growing facilities there are only 4 existing large-scale shore based farms in Scotland all differing in status. At the start of this study one was primarily producing turbot (Tayinloan). The largest has been mothballed since an outbreak of VHS (Gigha) and of the other 2 smaller units one holds cod (Ardtaraig) and the other at Otter Ferry Seafish holds a few broodstock halibut, some juveniles and supplied the tank space for this project.
- The majority of cages currently being used for on-growing halibut are primarily based on existing salmon farming technology, albeit with modifications. This limited investment in development of new cage specifications specifically for halibut only serves to widen the difference in capital costs between onshore tanks and sea cage systems.
- There is a large amount of expertise and experience in growing salmon in cages in Scotland and with falling prices there is also a desire to diversify into new species. Given this scenario a transfer of similar technology is perhaps inevitable.
- Site availability for land-based farms is a finite resource although conversely the availability of suitable locations for sea cages is also limited. However land costs in many areas may prove to be prohibitive.
- There is still at present no legislative requirement to locate farms on land in order that the treatment of their waste will comply with enforced discharge consents. Indeed sheltered sites that may not normally be suitable for large numbers of salmon could perhaps profitably support lower numbers of higher value species.

The last point is perhaps the most compelling in terms of advocating land based farms in favour of sea cages out with any decision based on fish performance. If discharge consents and legislation regarding waste management from sea cages was further tightened, then aquaculturists would be forced to deal with the waste produced from production sites and make the decision to either clean up or dilute/disperse. Cleaning or treatment of wastewater is really only a feasible option if the farms are land based and if the dilute/disperse option is taken then this means locating cages further off shore. Off shore site development in turn would require significant investments in cage technology to provide halibut with a suitable rearing environment. Halibut do not appear to respond well to weather/wave induced disturbances (Martinez-Cordero, 1994 (a); Martinez Cordero, 1994 (b)) and presently one of the main site selection criteria for cage on-growing is a relatively sheltered location.

The second area of interest that needed elucidation was the relative growth rates of male and female halibut and exact timing of maturation. It has been known for some time that females have higher growth rates than males and that this is associated with early maturation (Bjornsson, 1995). Depending on the ratios of males to females the profitability of on–growing individual batches of fish can vary considerably and detailed knowledge of the maturation patterns and growth rates of males and females provides a useful management tool.

1.2 Aims and Objectives

The main aims of the project were: -

- To determine the magnitude of any differences in growth rates between halibut held and ongrown in commercial sized land based tanks and in sea cages.

- To determine the magnitude of sexual dimorphic differences in weight for a representative batch of hatchery produced halibut during a complete rearing cycle.

- To investigate the patterns of growth and maturation within a typical population of hatchery produced halibut during a complete rearing cycle.

- To investigate whether there were any chronic disturbances and differences in terms of primary and secondary indicators of stress occurring between halibut held in land based tanks and in sea cages.

- To qualify any field measurements of primary and secondary indicators of stress by establishing their profile following a typical aquacultural stress in a controlled setting.

- To investigate in-vitro the stimulatory effects of ACTH, Angiotensin II and Potassium on cortisol production (a primary indicator of stress) from the interrenal tissue of Atlantic halibut.

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CHAPTER 2

GROWTH AND SEXUAL DIMORPHISM OF THE ATLANTIC HALIBUT IN TWO TYPES OF REARING SYSTEM

2.1 Introduction

With increasing numbers of halibut juveniles becoming available to the market there is increased interest from commercial ongrowers around the world in developing halibut as an aquaculture product. Decisions made regarding the type of ongrowing process employed have great financial implications. Cage culture and land based culture systems have their inherent benefits and limitations and whilst system choice often involves a complex trade off between financial, logistical and biological factors, the correct choice of system can potentially offer increased growth and production. Compared to salmonids relatively little work has been undertaken on halibut ongrowing to optimise the rearing process. Some studies do provide some preliminary information on the factors affecting the growth processes of the Atlantic halibut under farmed conditions.

2.1.1 Cage culture

The use of cages, in some form or another, either as temporary holding facilities or as longer term rearing units has been practised for many centuries (Beveridge, 1996). However, the development of sea cages and in particular open sea cages that are commonly used in modern aquaculture has only occurred relatively recently. Commercial cage fish culture of the yellowtail (*Seriola quinqueradiata*) first took place during the 1950's in Japan. The cage rearing of salmon began in the early 1960's in Norway and in 1965 the White Fish Authority (later Seafish) began trials in the UK (Beveridge, 1996; Abbors, 2000). The result of these developments is that nearly all salmon on-growing now takes place in sea cages as opposed to land based tanks. Within the time frame of these developments the on-growing of halibut is even more recent. Combined with the fact that many of the companies now involved with

halibut on-growing have previously had experience of salmon farming, means that the majority cages being used for halibut have been adapted from those used for salmon. Prime examples of this in the UK are Marine Harvest (Scotland) Ltd. who are the largest halibut on-growers and salmon farming company and Kames Fish Farming Ltd who developed the first commercial designs for the square wooden framed "Kames cages" and who now operate one of the few cage based halibut on-growing facilities.

As with most flatfish both in the wild and under farmed conditions halibut spend the majority of their time on a substrate rather than in the water column (Martinez Cordero, 1994 (b)). It is this requirement that has been responsible for the major adaptation to any cage system used for flatfish in general and more specifically for halibut i.e. to provide a flat and stable base on which the fish can lie. A number of approaches to this have been taken ranging from the installation of a solid base, the attachment of a tarpaulin to the interior of a tensioned mesh and more simply just the provision of a mesh base alone tensioned by either weights or attachment to a submerged frame.

For the wide range of species held in cages there are a multitude of cage designs, which according to (Kerr, Gillespie et al., 1980; Beveridge, 1996), can be classified in four different ways (Figure 2.1.1)

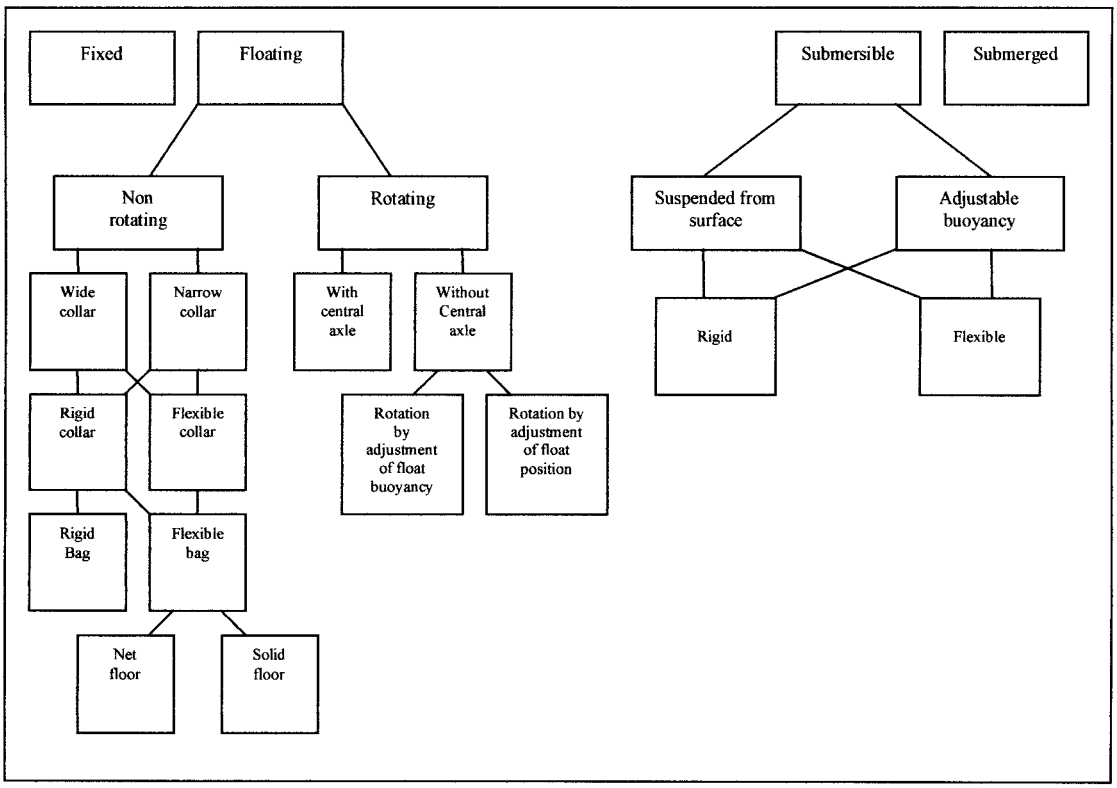


Figure 2.1.1 A classification system for cages developed by (Beveridge, 1996) from (Kerr, Gillespie et al., 1980).

From figure 2.1.1 it can be seen that there are 4 basic categories of cages; fixed, floating, submersible and submerged and within each type there can be a number of alternative configurations such as collar type, bag / net type and base type. At present according to the above classification floating, non rotating, wide collared cages with a flexible net and a tensioned base are those most commonly used for the on growing of halibut (Figures 2.1.2 –2.1.3).

Figure 2.1.2 12-meter square pens used for halibut on-growing at Loch Teacuis on the west coast of Scotland.

Figure 2.1.3 5-meter square pens used for halibut on-growing at Loch Teacuis on the west coast of Scotland.

The design choice of cages currently employed in the on-growing process of halibut means that the tensioned bases, and hence the fish in contact with them, are susceptible to the transfer of wave-induced motion from the floating collar. This has placed inherent limitations on site selection. (Martinez Cordero, 1994 (b)) showed that cage disturbance resulting from strong wave action increased the swimming activity of halibut held in square salmon pens with a tarpaulin base and those fish remaining on the base adopted an arched body posture. This arched body posture has also been observed following acute crowding stress experiments conducted by (Van Ham, 2003) who postulated that this behaviour was directly related to stress. Whilst growth rate in the cages was not reported by (Martinez Cordero, 1994 (b)) it was concluded that halibut adapted well to modified salmon cages although behaviour during adverse weather conditions may necessitate further technological developments. There is a large amount of anecdotal evidence from established halibut farms that feeding is suppressed following episodes of rough weather and, although little published data on the subject is available, it is reasonable to conclude that there would be a corresponding reduction in growth rates. Due to these design characteristics of the cages and the subsequent effects on the fish during episodes of rough weather, the current practice is to position halibut cages in sheltered locations.

Transfer of cages to offshore locations, in particular for flatfish, requires careful consideration. Although large offshore floating cages that are suitable for salmon have been developed and are successfully used world-wide, their successful use is only facilitated by the position within the cage that salmon occupy. In order for flatfish to be transferred to offshore cages the supporting mesh would need to be removed from the pitch and roll effects of wave action. The effects of wave action can be reduced by

either employing damping devices or by positioning the floatation devices below the critical area of wave action.

A number of cages, some specifically designed and some adapted, for flatfish have been developed for offshore use. Brief descriptions of the most prominent systems are described below.

Fiskeriforskning & Refa, A.S. Flat bottomed net cage

A Norwegian partnership of Refa and Fiskeriforskning has developed a cage based on an earlier design by another Norwegian company, AMY. The design employs the principle of dampers in order to reduce the effects of wave action and was the result of a three-year R&D project. Patented in several countries and introduced commercially in 1999 the system has been successfully used for species such as halibut, sole and plaice. The system has also been used for receiving newly caught cod, where a flat bottom is required for acclimation (Figures 2.1.4 –2.1.5).

The cage consists of three main parts: flotation, mesh and base.

Floatation: Two PEH pipes forming a circle with a circumference of 50m provide floatation

Mesh: Knotless mesh is used to hold the fish

Base: The base also has a circumference of 50m and a total surface area of 180m^2. It is constructed from DYNEEMA ™ net, which is pulled taut on a rigid frame comprising of aluminium tubes.

The base of the cage can be raised or lowered pneumatically to facilitate husbandry operations and, when raised, it is possible for workers to walk on the suspended mesh (Midling, 1998). The PEH-floatation ring is secured to the cage base by guiding ropes and supporters, which also keep the cage base in a horizontal position whilst being raised and lowered. The damping units are reported to be capable of reducing wave

motion by between 0.5m and 1.0m. In trials carried out during the design of this system stocking densities of between 50-60 kg/m^2 were achieved without any reported adverse effects. Given these stocking densities a single cage would offer a holding capacity of approximately 11,000kg. The manufactures of these cages claim that for routine husbandry operations no more than one person per cage is required.

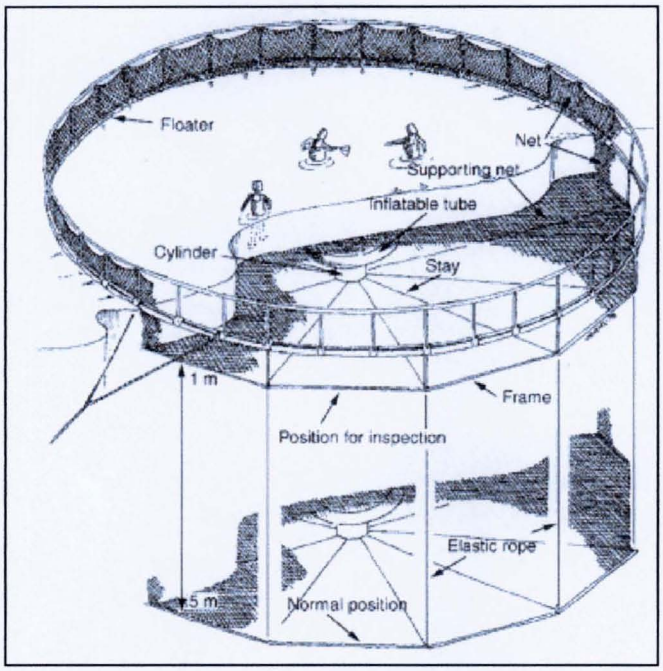
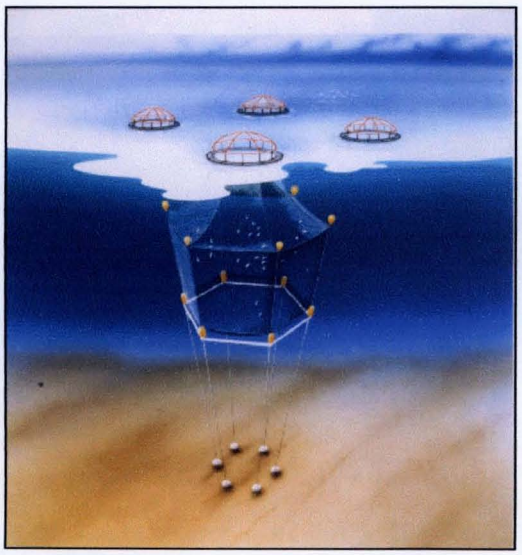


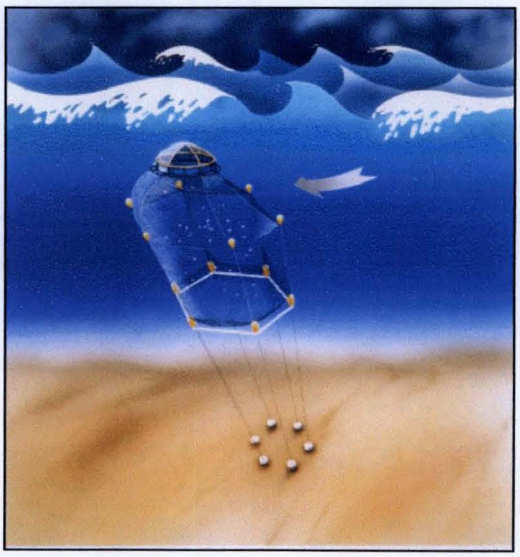
Figure 2.1.4 Drawing of Refa flat base cage

Figure 2.1.5 View of Refa flat base cage

REFA Tension leg cage

The Tension Leg Cage (Figures 2.1.6 – 2.1.7) was originally designed for pelagic fish, in areas where high wave action is encountered has been modified for use with flatfish. The main difference this cage has as opposed to other designs is that the rigid portion of the cage along with the majority of the floatation is located at the base. The sides of the pen are stretched upwards from the base by a number of buoys. This has the advantage that the shape of the base is constantly maintained which suits flatfish applications. The cage is moored by six anchor lines, which allow for horizontal movement of the cage without disturbing the vertical position of the cage base.





Figures 2.1.6 & 2.1.7 Representation of Refa Tension Leg Cage under different weather conditions.

Shelves have been installed in the cages in order to increase the holding capacity of the cages. However, problems with excessive biofouling and initial logistical problems associated with its removal have meant that the cages have not yet been used on a full commercial basis.

Ocean Spar flatfish Sea Station™

Ocean Spar Technologies have developed the flatfish ***Sea Station™***, a submersible sea cage designed specifically for flat fish. The system is based on their earlier ***Sea Station™*** submersible cage system designed for holding pelagic fish in open ocean conditions (Figures 2.1.8 –2.1.9)

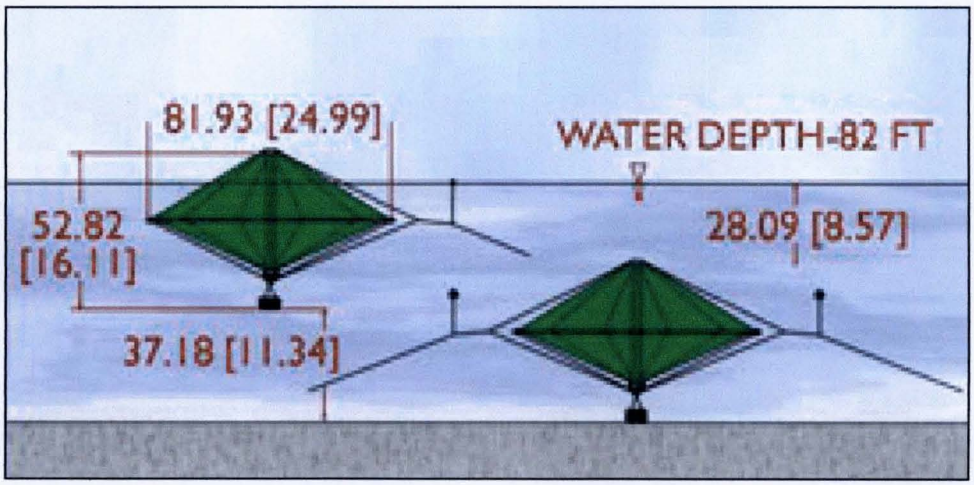


Figure 2.1.8 Schematic view of Ocean Spar Sea Station™ in operation

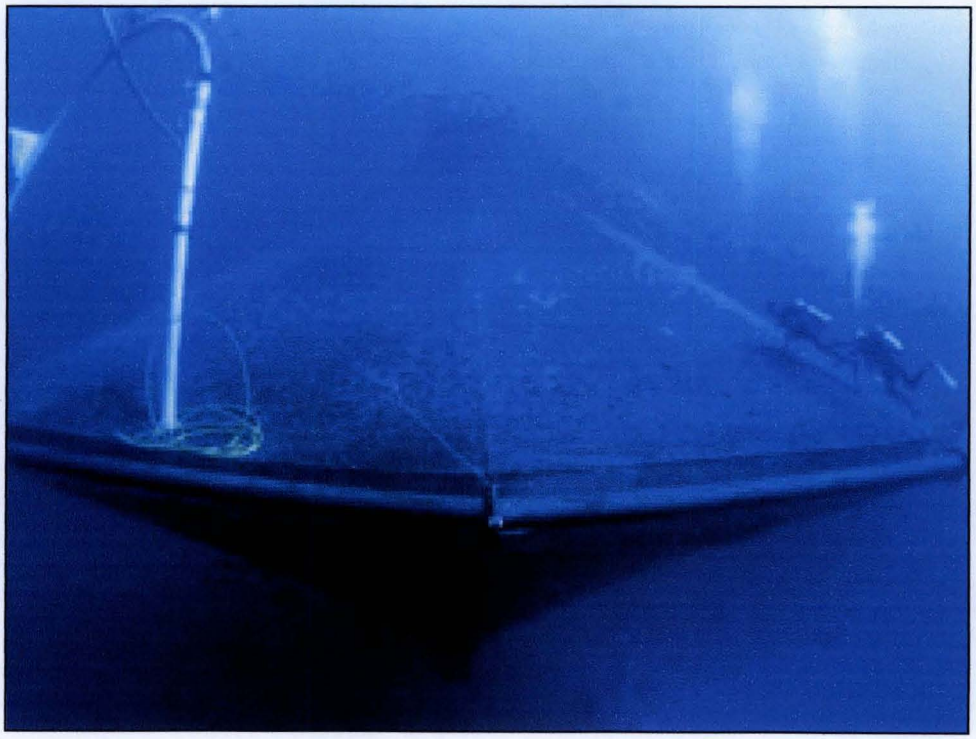


Figure 2.1.9 Underwater view of Ocean Spar Sea Station™

The main feature of this cage system is the 15m long vertical steel spar buoy. From the steel spar the net is stretched to a 25m diameter steel ring constructed from eight individually sealed sections of steel pipe and back again to the steel spar. Radial bars from the buoy to the steel ring offer support to the net. A single cage offers a surface area of approximately 490m^2. Depending on the prevailing weather conditions the position of the cage in the water column can be adjusted using compressed air. The position of the cage relative to the seabed is maintained with the use of up to 3 anchors. Sea Station cages have been used commercially for the production of flounder in North America.

SADCO-SHELF submersible cages

SADCO-Shelf is a Russian company that manufacture a range of submersible sea cages: SADCO 1200, 2000 and 4000 with the numbers denoting the cage volume (Figure 2.1.10). In addition to these the company has designed submersible cages that they believe would be suitable for both demersal species and flatfish (Figure 2.1.11)

Figure 2.1.10 View of floating SADCO-SHELF 4000 submersible cage.

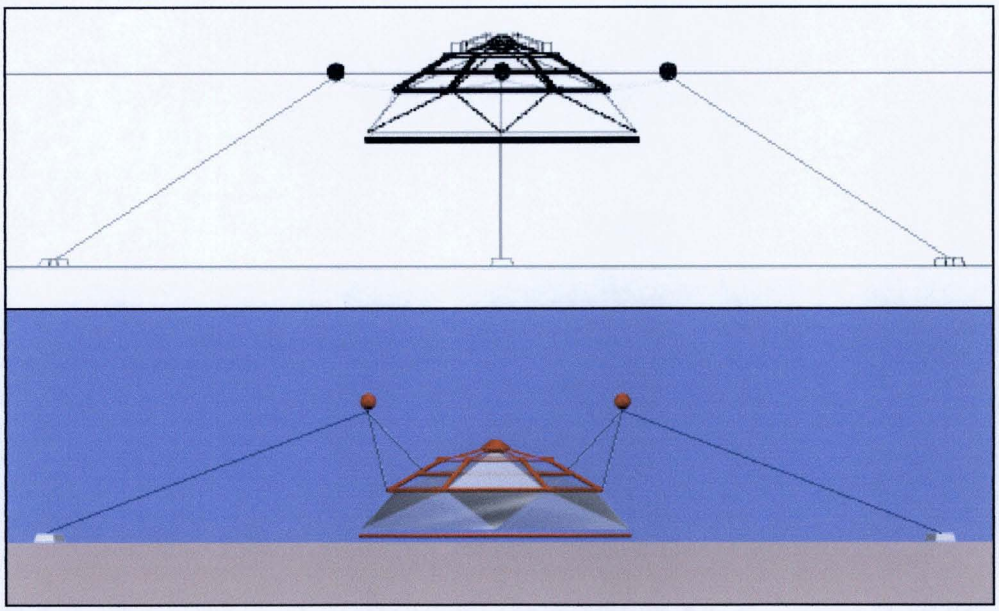


Figure 2.1.11 Schematic representation of SADCO-SHELF demersal and flatfish cages

The SADCO-SHELF flatfish cages offer a surface area of approximately 530m^{2}and the horizontal plane is maintained with a three-way anchor system.

2.1.2 Land based Marine Fish Farms

Flow through, land based systems operate on the basic principal that water is pumped from the sea or seawater well, distributed around the fish holding system and is then returned back to the sea. At each stage of the water supply and distribution process it is possible that a varying number of treatments can occur which aid in both the management of the farmed biomass and of the waste discharge. However, in the simplest design of flow through systems the water remains untreated in any way either before entering or after leaving the holding tanks. The nature and complexity of possible treatments are dependent on the interaction of a number of technical, biological and financial constraints and requirements. The type of treatment employed

can range from filtration, oxygenation, sterilisation, and temperature control. The majority of decisions on the nature and degree of each particular treatment will usually be dependent on both the species being cultured and the life stage.

Whilst pump ashore systems allow for varying levels of control over the rearing environment, the greater the control required the greater the likelihood that financial constraints will begin to dictate the design of the system. For example, if a warm water species was to be cultured in a locale with a sub-optimal ambient temperature profile, economics would dictate that water reuse / recirculation systems would firstly need to be installed, otherwise heating of the flow through water would be financially unfeasible.

As pump ashore systems can range from simple flow through to fully recirculating hatcheries, design choices regarding the different treatment systems available are numerous and outside the scope of this introduction. A large number of texts covering the subject are however available (Wheaton, 1977; Lawson, 1995; Timmons and Losordo, 1997; Huguenin and Colt, 2002; Timmons, Ebling et al., 2002).

For an on-growing unit for flatfish in an area with a suitable ambient temperature and with no constraints on the amount of water available for pumping as in the current study, the required system is relatively simple.

The primary stage in the water supply network is the pumping set, which may have some degree of filtration on the inlet that offers some protection to the pump from large solid objects rather than enhancing water quality.

The type and layout of the seaward pumping system can vary from floating / suspended pumps, submerged pumps to onshore pumps and again the exact configuration chosen for the pump set is dependant on a number of factors such as site

topography, financial constraints, local weather conditions and location in relation to other water users.

From the pump set the water is usually directed either filtered or unfiltered to a header tank arrangement. A header tank (Figure 2.1.12) is a common component of seawater systems (Huguenin and Colt, 2002) and they are generally employed as they fulfil a number of functions: -

- Buffer transients between supply and demand
- Storage for aging, treatment or part time pumping
- Provide time after power failure
- Provide constant head (flow control)
- Acts as sedimentation tank
- Distribution system (large header)

Figure 2.1.12 View of a large flow through facility (tanks and raceways) on the west coast of Scotland used for both salmon and turbot. The large blue tank in the foreground is one of two header tanks supplying the rearing tanks.

From the header tank water can be distributed to the fish tanks by either pipes or an open channel (Figures 2.1.13 –2.1.14). If oxygenation / aeration is employed it can be added either in the header tank, to the supply pipe / channel or directly into the fish rearing tanks.

Figure 2.1.13 View of a large pump ashore facility on the west coast of Scotland showing an open distribution channel running between the tanks.

2.1.2.1 Tank design

In terms of tank design, criteria such as shape, size and configuration of both the inflow and outflow can have numerous effects on the hydrodynamic characteristics of a tank. This can, in turn, affect water quality either throughout the tank or in localised areas which impacts on fish performance and ultimately the profitability of a system. The basic design principles for any tank in terms of water quality should be good mixing, good solids removal, minimal stagnant regions and even distribution of

dissolved oxygen, metabolites and food (Cripps, 1992). Shape is a criterion that may be selected for in order to maximise the utilisation of available floor space within a facility. Square or rectangular tanks and raceways make better use of floor space than circular tanks, although for certain applications water quality may be unsuitable due to either waste build up in corners or uneven flow patterns and oxygen concentrations (Lawson, 1995). Better hydraulic profiles may be found in circular tanks (Wheaton, 1977) although compromises between optimising floor space and greater water quality can be achieved with the use of rounded corners in either square or rectangular tanks.

Figure 2.1.14 View of a pump ashore facility in Scotland showing the water distribution pipe running centrally between the circular 5m diameter tanks.

Incorrect design of circular tanks can lead to torus shaped irrotational zone about the central drain which can lead to a localised build up of waste (Timmons, Summerfelt et al., 1998). The majority of work on tank design has been based on round fish

production and little work has been carried out on tank design for flatfish. Results from all studies show that tanks design is highly species and age specific. (Cripps, 1992) did however, conclude from available data that for on-growing turbot large, carefully managed, shallow circular tanks with a central drain, multiple inlets and a slightly sloping base appeared most suitable. The majority of these features are in place in the 24m-diameter tank pictured in Figure 2.1.15 that, by the removal of a wall panel, was adapted for turbot having previously been used for salmon. There is no evidence to suggest that similar tanks would not be the most suitable for halibut, with the exception of perhaps a greater depth, as halibut spend a greater percentage of time in the water column.

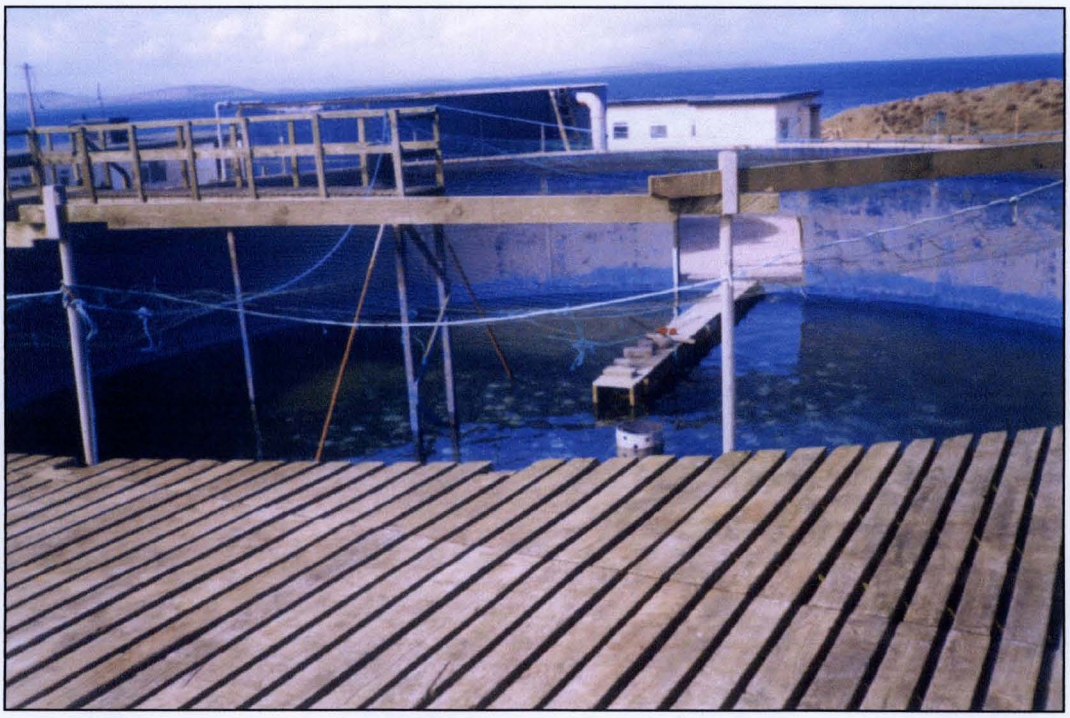


Figure 2.1.15 View of a large 24m-diameter, shallow circular tank with a central drain, adapted for turbot having previously been used for salmon

2.1.3 Advantages and disadvantages of tanks and cages

Some of the respective advantages and disadvantages of cage and pump ashore rearing facilities are presented in the Table 2.1.1

Table 2.1.1 Advantages and disadvantages of tanks and cages

Cages	Tanks
More likely to be adversely affected by poor weather conditions	Less likely to be affected by poor weather conditions
More difficulty in observing fish and monitoring their welfare	Ease of observing fish and their welfare
Husbandry operations more difficult	Husbandry operations more simple
Site availability at present limited. With advances in cage technology and the possibility of offshore sites being available site selection may become less of an issue	Site availability limited. Land prices may prove restrictive
With good site selection water quality should be constant. May however be affected by runoff	Pipe line inflow depth critical in providing good quality water
Capital costs lower than pump ashore site (although dependant on type of cages used)	Capital costs high
No reliance on electricity. Can be moored in more remote locations	Reliance on electricity and therefore a higher level of infrastructure than cages
More risk from predators	Predator risk low
Little scope for effluent treatment. May become more of an issue if discharge regulations are tightened	Effluent treatment relatively easy to facilitate
No or little control over water quality parameters	Water quality parameters can be controlled more fully
Theoretically lower running costs – dependant on replacement time of mesh and type of maintenance vessels required	High running/pumping costs

2.1.4 Growth

Growth of fish, taken in the context that growth can be the increase or decrease of body mass, is a contributory part of an energy budget. The energy budget can take the form:

GE = FE + UE + ME where GE is gross energy, FE and UE are faecal and non-faecal energy losses and ME is metabolisable energy. ME can be further divided into maintenance energy requirements (ME_m) and those that lead to growth (ME_p). By due process growth potential can be described by ME_p/ME_m (Van Weerd and Komen, 1998) which is under the control of an array of parameters and interactions including environmental, endocrine, sexual, developmental and genetic factors (Weatherley, 1972; Bagenal and Tesch, 1978; Brett, 1979; Ricker, 1979; Pankhurst and Van der Kraak, 1997; Wendelaar Bonga, 1997).

2.1.4.1 Growth rates

There are a number of different indices of growth that are commonly employed in the expression of individual and population growth rates. Of these, specific growth rate (SGR or G_W), or more correctly instantaneous relative growth rate and condition factor are the most commonly used: -

$$SGR\ (\%day^{-1}) = (\log_e W_2 - \log_e W_1)\ /t \times 100$$

Where W_1 and W_2 are the initial and final weights (g) of the fish and t is the period of growth (Ricker, 1979)

In aquaculture, % growth/day or SGR has previously been the basis for commercial growth tables, used for stock management. For the purposes of estimating future

growth and for comparing different stocks of fish such use of SGR is however problematic. Within a particular growth period the occurrence of changes in size class and temperature can lead to considerable variation between observed growth and calculated growth.

The underlying reasons for such discrepancy lie within the nature of the SGR calculation and both biotic and abiotic factors that effect the growth process.

SGR does not remain constant throughout the entire growth period. Few metabolic processes are independent of size and the process of growth conforms to this (Brett, 1979). Metabolic processes generally proceed at a declining rate with increasing size. As a result, there is a gradual decrease in growth rate of animals with increasing size. Taking this factor into account direct comparisons of SGR should be limited to fish of the same size.

This relationship between decreasing SGR (G_w) and increasing weight (W) can be described mathematically by the power function of the form:

$$G_w = aW^b$$

or

$$\log_e G_w = \log_e a - b \log_e W$$

where G_w specific growth rate and W is fish weight in grams (Brett, 1979; Jobling, 1983a, b).

An extension of this problem of size occurs when fish of different size are held under different temperature regimes. The effect of size on temperature/growth interactions has been documented for a range of species and it is common that the optimum temperature for growth tends to become lower as the fish increase in age and size (Pedersen and Jobling, 1989; Fonds, 1992; Bjornsson and Tryggvadóttir, 1996; Jonassen, 1999). Due to the problems associated with using SGR for comparative

purposes alternative measures, based on the analysis of changes in the cube root of weight ($^3\sqrt{W}$) with time, have been developed (Holmefjord, 1995; Jobling, 2004)

Assuming length increases during the growth period are linear, modelling of growth in terms of the cube root of body weight can be represented by the form:

$$^3\sqrt{W_2} = {}^3\sqrt{W_1} + G_s(t2-t1)$$

where G_s is the slope of the regression of $^3\sqrt{W}$ against time, which represents the growth index.

Temperature variations between treatments and sites play an important role in determining the growth rate of fish and to account for any differences in water temperature the growth index can be modified by the inclusion of a thermal growth coefficient (*TGC*):

$$[(^3\sqrt{W_2} - {}^3\sqrt{W_1})(\Sigma T)^{-1}] \times 1000$$

where ΣT is the sum day-degrees Celsius. The modified growth model with the *TGC* included is given by :

$$W_2 = [\,^3\sqrt{W_2} + [(TGC/1000) \times (\Sigma T)]]^3$$

With detailed information regarding average previous local water temperatures it is possible to forecast future growth utilising *TGC*. However, problems occur when fish are held under ambient conditions and large fluctuations from expected temperatures occur. Various assumptions are made regarding the use of the *TGC,* i.e. the temperature range is not out side that which is optimal for the fish under investigation and, although invalid, there is a steady increase in growth rate with increasing temperature. Thus, despite having to exercise some caution when predicting fish growth the use of *TGC* or GF3 it has some advantages over the use of SGR. Predictions of growth in hatchery reared salmon have been made using the *TGC* by

(Iwama, 1996) and it has, under the title of GF3, been used as a comparative tool by (Holmefjord, 1995)

A derivative of growth, which is commonly used, and has also been used as a tertiary, structural indicator of stress (Barton and Iwama, 1991), is Fulton's condition factor, also referred to as the ponderal index or k factor: -

Fulton's condition factor (k) is calculated from $k = \frac{100w}{L^3}$

where w and L are the observed total weight and length. If the growth is approximately isometric, that is the regression coefficient b=3 in the length weight relationship represented by : -

$$w = aL^b$$

Condition factor can successfully be used to compare differences related to sex, season or location (Weatherley, 1972). Even with allometric growth and whatever the value of b, Fulton's condition factor may be used if the fish are approximately the same length.

To calculate b, log weight against log length is plotted and the regression line calculated by the method of least squares. The regression coefficient is b and log a is the intercept on the line of the y axis (Ricker, 1979)

2.1.4.2 Growth models

Growth, as a result of its sensitivity to differing environmental parameters and phylogenetic constraints is fundamentally an indicator of performance. With adequate information modelling of this performance can be therefore used to compare actual rates with predicted values for optimal conditions. Additionally it allows comparisons to be made between different stocks or groups of fish. Also inherent is the predictive capability, which allows future weights or lengths to be calculated for various given conditions. There are a variety of approaches that have been taken, the differentiation between descriptive and more mechanistic growth models made by (Mooij, 1998) indicates the differences in the biological basis of the parameters employed in the various commonly applied approaches to modelling of growth

Descriptive mathematical models for expressing the growth of fish have been primarily applied to data collected in fisheries research. The techniques have, however, been transferred to the more recently developed field of aquaculture and are useful stock management tools. Commonly used models include the Gompertz, Logistic and Johnson's growth curves.

However, the most widely used growth model has been the Von Bertalanffy (VBGC) or Brody – Bertalanffy curve that was developed from the initial work conducted by Putter, 1920 (Ricker, 1979; Pitcher, 1982)

The Von Bertalanffy model can be written, for length, as:-

$$L_t = L_T(1 - \exp[-K(t - t_0)])$$

or for weight:-

$$W_t = W_T(1 - \exp[-K(t - t_0)])^3$$

Growth curves can be fitted to either length or weight data but length is more commonly used. Ultimately, the quality and nature of the data will determine which parameter is more applicable but difficulties in adequately fitting a curve can occur when weight data is used rather than length as the inflection point of the curve, i.e. the point at which the curves shape alters, may occur at a later point than when length is used. This therefore increases the complexity of the curve that is to be described by the formula. If however, the relationship between length and weight is known, as determined by the power function: -

$$w = aL^b$$

then one value can be calculated from the other.

It is possible to accommodate seasonal differences of growth into expansions of the VBGC. The seasonal peaks and troughs are usually accounted for with additions of either cosine or sine functions into the exponent part of the model (Ricker, 1979; Fontoura, 1996).

There has been much discussion regarding the appropriate means of comparison of curves fitted with the von bertalanffy model (Misra, 1980; Bernard, 1981; Misra, 1986; Cerrato, 1990, 1991; Chen, 1992; Jones, 2000) however most methods are complex and require the development of dedicated programs. A relatively simple comparative method is however available which provides growth performance indices in terms of either weight (phi, Ø) or length (phi prime, Ø′)(Munro, 1983; Pauly, 1984; Aripin, 2000) where: -

$$Ø = \log_{10}(K) + 2/3 \log_{10}(W_T)$$

$$Ø' = \log_{10}(K) + 2 \log_{10}(L_T)$$

The more mechanistic approach to growth modelling has been taken by a number of authors with much of it based on the models developed by (Elliott, 1975; Elliott, 1995). The methodology has been widely used to investigate the growth of various populations of brown trout in streams and rivers see (Elliott, 1995)). The generic model has been applied to other species including immature stone loach, *Barbatula barbatula* (Elliott, 1996), parr of Atlantic salmon, *Salmo salar* (Elliott, 1997; Forseth, 2001) Arctic charr (Larsson, 1998). The model is based on a temperature dependent power function of weight, which can be expressed in terms of specific growth rate or instantaneous relative growth rate, Gw expressed as % at temperature T°C at time t when weight is Wt:

$$Gw = 100\ (a + b_2\ \mathrm{T})\ W_t^{-b1}$$

from this, fish weight at the end of a given period could be calculated

$$Wt = [(a+b2\ \mathrm{T})\ b_1 t + W_o^{b1}]^{1/b1}$$

Later improvements to the model (Elliott, 1995) where all the parameters, as opposed to only one in the earlier funtion, can be defined in biological terms resulted in the form:

$$Gw = cW_t^{-b}\ (T\text{-}T_{LIM})/(T_M\text{-}T_{LIM})$$

where c is the growth rate of a 1g trout at the optimum temperature and b, the weight exponent is the power transformation of weight that produces linear growth. T_M, T_L and T_U are the lower and upper temperatures at which growth rate is zero and

$$T_{LIM} = T_L \text{ if } T7\ T_M \text{ or } T_{LIM} = T_U \text{ if } T>T_M.$$

Similarly, fish weight at the end of a given period of time can be calculated using:

$$W_t = [W_o^b + bc(T\text{-}T_{LIM})\ t\ /\ \{100\ (T_M - T_{LIM})\}]^{1/b}.$$

Alterations to the notation of the earlier model have been made (Mooij, 1990, 1998) although they used essentially the same methodology to (Elliott, 1975) to analyse the growth of six major European freshwater fish species (Mooij, 1998).

2.1.4.3 Stress and growth

Growth is under the control of multiple endocrine systems, though primarily via the brain neuroendocrine-growth hormone (GH)-insulin-like growth factor (IGF) axis (Pankhurst and Van der Kraak, 1997). Biological actions of IGF in mammals include multiple metabolic, mitogenic and growth related effects (Lowe, 1991). In teleosts (salmonids) GH in concert with IGF is the major endocrine promoter of growth (McCormick, Shrimpton et al., 1998). For teleosts (at least in carps) GH release and the regulation of its secretion from the pituitary is under multifactorial control with inhibitory effects of somatostatin (SRIF), seratonin (5-HT) and Noradrenaline (Norepinephrine, NE) and stimulatory effects of GH-releasing factor (GRF), gonadotrophin-releasing hormone (GnRH), cholecystokinin (CCK), dopamine (DA), neuropeptide Y (NPY) and thyrotrophin releasing hormone (TRH) being reported (Peter and Marchant, 1995). GH, a 188-amino acid peptide with 5 cysteine residues, stimulates growth through direct action on some tissues as well as by the stimulation of production of insulin-like growth factor (IGF) (Peter and Marchant, 1995), primarily in the liver (Holly and Wass, 1989) which has a large number of GH receptors. In addition to GH, growth has also been shown to be stimulated by insulin although there may be species-specific differences in efficacy (Duan and Hirano, 1992; McCormick, Tsai et al., 1992; Peter and Marchant, 1995). Additionally thyroid hormones T_4 and T_3 and gonadal steroids can have growth promoting effects (Pankhurst and Van der Kraak, 1997). GH levels and IGF levels, although little work

has been done on the latter, have been shown to be disturbed as a result of stress. Under acute stress plasma GH levels have been shown to decrease in the rainbow trout and Atlantic salmon (Pickering, 1991; McCormick, Shrimpton et al., 1998) and plasma IGF levels increase (McCormick, Shrimpton et al., 1998) indicating elevated IGF levels may be maintained after a reduction in circulating GH levels. Although stress is generally accepted to facilitate reduced growth in fish, limited documentation is available and is again based primarily on salmonids. Crowding stress has been shown to reduce growth in coho salmon (Fagerlund, McBride et al., 1981), brown trout (Pickering and Stewart, 1984) and Atlantic salmon (McCormick, Shrimpton et al., 1998).

2.1.5 Environmental Effects on growth

2.1.5.1 Stocking density

Under intensive rearing conditions such as those experienced in most of the commercial finfish aquaculture operations currently operating in the British Isles, stocking density plays an important role in determining how the fish perform and hence on the economics of the individual rearing system. Obvious problems will occur if stocking densities are increased so greatly as to have a detrimental effect on water quality. Even below such levels, incorrect stocking densities for a particular species/size group can lead to a reduction in the overall biomass gain and increased variation between individuals within groups of cultured fish.

Investigations into increased variation within populations has been carried out and is considered, by a number of authors, to be indicative of the establishment of hierarchies and the suppression of growth of certain individuals (Brett, 1979; Purdom, 1974; Zoccarato, Benatti et al., 1992). In marine flatfish, variations in individual

growth rates are reportedly related more to social interactions than to food availability (Purdom 1974).

Optimum stocking density has been shown to be both species and age/size specific. In response to varying stocking densities different species exhibit different reactions. Some species exhibit a negative response to increasing stocking density (Danielssen and Hjertnes, 1991; Bjornsson, 1994) whilst others show no negative effects and others even exhibit positive effects (Papst, 1992). (Irwin, O'Halloran et al., 1999) concluded that a significant negative effect of increasing rearing density in juvenile turbot populations resulted in heterogeneous growth rates and the depression of growth of some individuals. (Martinez-Tapia and Fernandez-Pato, 1991) whilst not finding any significant differences in growth of turbot at differing stocking densities noted that growth rate (SGR) and food conversion rate (FCR) show an improvement in those fish stocked in highest density groups. Although this may appear to conflict with the findings of (Irwin, O'Halloran et al., 1999) the fish investigated had a higher initial weight, which suggests that turbot, *Scopthalmus maximus*, is similar to other species in that optimum stocking density, or at least tolerance to higher stocking densities, may increase with size.

Few works on the effect of stocking density on the growth of the Atlantic halibut exist. (Bjornsson, 1994) showed however, that for halibut, when the stocking density increased above certain threshold levels corresponding to 100% coverage of the tank bottom (start wt 1.8-3.2 kg), growth rates were significantly affected. Therefore for 2kg fish this equates to the optimal density being between 25 and 50 kg/m^2 and for a 10kg halibut between 50 and 100 kg/m^2. These figures are in agreement with a range of size specific stocking densities presented by (Englesen,

1995) reported that for fish of: 0.45 – 1.5kg, 1.5 – 2.5kg, 2.5 – 4.25kg, 4.25 – 6.4kg and 6.4kg + the respective stocking densities should be 30,40,50,60 and 70kg/m^2

2.1.5.2 Temperature

Due to the poikilothermic nature of fish temperature plays an important role in the determination of growth rate. Temperature governs the rate of metabolic reactions affecting all physiological processes including metabolism, food intake and nutritional efficiency. Assuming that fish are in a healthy state, are fed to satiation and that the genetic nature of the fish does not serve to inhibit growth, then temperature has perhaps the greatest effect of all external factors on the rate of growth of a particular stock (Shepherd and Bromage, 1992). As with much of the earlier work on Atlantic halibut investigations into the effects of temperature have focussed mainly on the survival and performance of eggs and larvae (Blaxter, Danielsen et al., 1983; Bergh, Opstad et al., 1989; Pittman, Skiftesvik et al., 1989). The optimal temperature for larval development has been found to be 6°C which is also the temperature commonly used for holding broodstock.

For juvenile halibut there is a reported interaction of temperature, fish size and growth with the optimal temperature for growth declining as weight increases (Aune, 1994; Aune, Imsland et al., 1997; Jonassen, 1999). This ontogenetic relationship has also been demonstrated for turbot (Imsland, Sunde et al., 1996; Imsland, Foss et al., 2001) and cod (Pedersen and Jobling, 1989). Temperature optimums for 5 – 90g halibut have been reported to be between 12 and 15°C, for 100 – 500g fish 11.4°C and for 2900 – 5000 g fish 9.7°C (Aune, 1994; Hallaråker, Folkvord et al., 1995; Bjornsson and Tryggvadóttir, 1996; Aune, Imsland et al., 1997; Jonassen, 1999). Ontogenetic downshifts in optimum temperature requirements are common in many species of fish

and, if economically possible, it is desirable to actively control the temperature of the rearing environment either by choice of location or by utilisation of available technologies.

2.1.5.3 Sexual dimorphism

The growth rate of many species of fish is known to vary between sexes. These differences are particularly evident following maturation and are more pronounced if the timing of the maturation process varies. Such differences can lead to large inter sex variations in growth rates. The nature of sexual dimorphic differences in growth rates is species specific. In salmonids, including the rainbow trout *Oncorhynchus mykiss* and Chinook salmon *Oncorhynchus tshawytscha*, males become larger in size than the females (Peterson, Winkelman et al., 1992; Elvinson and Johansson, 1993). In contrast to this, females become larger than males for a range of flatfish species including turbot (Nijhof, 1994; Imsland, 1995, 1997), the dab *Limanda limanda* (Lozán, 1992) and the Atlantic halibut (Jákupstovu and Haug, 1988; Haug, 1990; Bjornsson, 1995; Norberg, Weltzien et al., 2001). Size at maturation for halibut varies with sex with males maturing earlier than females. Respective size at maturation for males and females was found to be 3.2kg and 12.7kg (Bjornsson, 1995). This difference in growth rates between males and females can have profound commercial implications in terms of grading and grading,, forecasting of future stock value and harvesting strategies.

2.1.6 Aims

The general aims of the present section of the study were to investigates the effects of rearing system type on the performance of a standard commercial, farm reared, batch of Atlantic halibut and also to determine when the effects of sexual dimorphism began to become apparent in terms of growth.
More specifically the aims were: -

To determine the magnitude of any differences in growth rate, condition factor and maturation rate between Atlantic halibut on-grown in either sea cages or pump ashore tanks.

To determine any differences in growth rate between farm-reared male and female Atlantic halibut on-grown under commercial conditions, and to establish the point at which growth rates of males and females begin to diverge.

To determine whether all males from a single year class mature in the same year and ascertain the magnitude of any differences in final weight between different groups of maturing males.

To determine the exact patterns of growth during a commercial rearing cycle for males, maturing males and females reared in different commercial scale systems.

To carry out a preliminary investigation into the effects of being on-grown under different rearing conditions on primary and secondary indicators of stress in the Atlantic halibut.

2.2 Materials and Methods

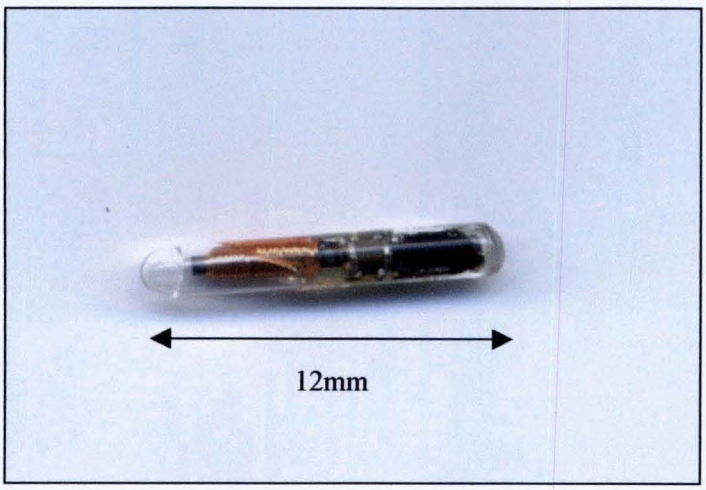
2.2.1 Trial fish

The fish used in the study were juvenile Atlantic halibut, *Hippoglossus hippoglossus* (L.), produced from 1998 fertilized eggs by Otter Ferry Seafish, Argyll, Scotland. All fish for the study were from a standard batch produced using the normal commercial practices of the farm. The eggs and milt from which the trial fish were produced were pooled from a number of broodstock (size unrecorded by hatchery), which, initial results suggest, provided up to 8 combinations of siblings and half siblings. At the present time further results regarding the exact genetic background of all the fish are however unavailable. The batch of fish used was randomly selected from an un-graded group prior to the commencement of the study.

Prior to conducting the main trial an initial feasibility study into the use of Passive Integrated Transponder (PIT) tags injected intramuscularly into the shoulder region of approximately 400g halibut was conducted (Figures 2.2.1 & 2.2.2). For the feasibility study 20 halibut were anaesthetized on the 19th October 1999 using 2-Phenoxy-Ethanol (Sigma Chemical Company, St Louis, USA) at a concentration of 1.0ml l^{-1} raw unfiltered ambient temperature seawater. Each fish was injected with a 12mm PIT tag (Fish Eagle International, Lechlade, Gloucestershire, UK) in the shoulder region on its upper surface longitudinally parallel to the lateral line. After tagging the fish were allowed to recover in a 5m diameter, 1m deep, circular rearing tank supplied with raw unfiltered, ambient temperature seawater. Tag retention and fish survival after 1 week was 100% and on the 25th October 1999 a further 1629 fish were tagged in the same manner. All tag numbers were recorded along with individual weights (nearest 2g) and fork lengths (nearest mm). After PIT tagging, all fish were allowed to

recover in aerated seawater before being transferred to a 12m diameter, 2m deep, rearing tank.

Following PIT tagging and an acclimation period of 1 month the fish were graded into two size groups of equal numbers around the mean weight calculated after the initial tagging and weighing (mean wt = 413g). Each size group was then divided randomly in half with one half of the larger size group and one half of the smaller size group



Figures 2.2.1 & 2.2.2 Site of PIT tag insertion (red oval) in the shoulder region of a halibut and a 12mm PIT tag

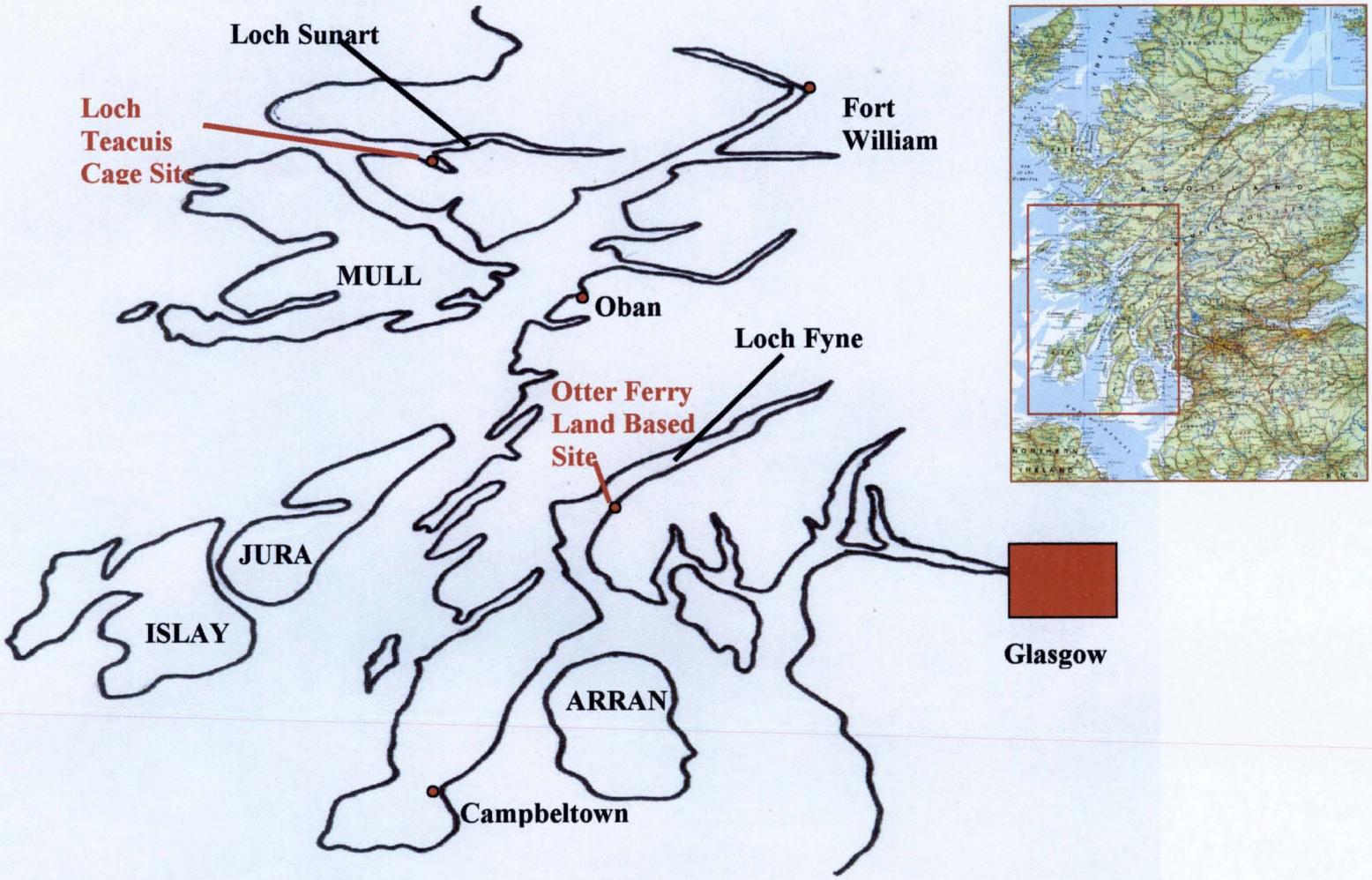


Figure 2.2.3 Location of trial cage site and tank sites on the West Coast of Scotland

then being transferred by well boat to two 5m x 5m, 3m deep square cages located at a Marine Harvest Scotland halibut production site at Loch Teacuis, Argyll, Scotland (Figure 2.2.3 and 2.2.4). The remaining half of the larger and smaller size groups were transferred to two 5m diameter, 1 m deep circular tanks at Otter Ferry Seafish, Argyll (Figures2.2.3 and 2.2.6). Therefore at each site, two equivalent experimental groups were created and these will be referred to as 'Initially Small' and 'Initially Large' respectively (Table 2.2.1). The fish were graded into the 'Initially Small' and 'Initially Large' groups at each site in order to simulate the normal grading practices of the farms and thus reduce the possibility of aggression and other size related hierarchical effects that may have impacted on growth. Due to the number of tanks the experimental procedure had to be performed on an unreplicated basis at each site. The data from the tanks at each site was pooled which gave a single mean weight for each sex at both sites on each sample date. The mean weights of each sex at the time of PIT tag insertion are presented in Table 2.2.2 (see also Table 2.3.5).

Table 2.2.1 Initial weights (g) of fish from different size groups at Otter Ferry and Loch Teacuis 6 weeks prior to the transfer date when fish were moved from the main holding tank at Otter Ferry to the experimental tanks at Otter Ferry and the cages at Loch Teacuis

	Otter Ferry Initially Large	Otter Ferry Initially Small	Loch Teacuis Initially Large	Loch Teacuis Initially Small
Mean Weight (g) (± SE)	486 ± 4.5	327 ± 3.2	505 ± 4.4	343 ± 3.7

Table 2.2.2 Initial weights (g) (pooled tank data) of fish from different sex groups at Otter Ferry and Loch Teacuis 6 weeks prior to the transfer date when fish were moved from the main holding tank at Otter Ferry to the experimental tanks at Otter Ferry and the cages at Loch Teacuis

Fish Group	Otter Ferry	Loch Teacuis
All fish	405 (n=629)	444 (n=478)
Females	399 (n=358)	445 (n=276)
Males maturing in the 1st year	452 (n=70)	472 (n=79)
Males maturing in the 2nd year	400 (n=201)	425 (n=123)

2.2.2 Loch Teacuis cages

The individual cages in the study were part of a larger cage group, which consisted of 20-24 (depending on usage at any particular time) 12x12m square and 4m deep cages. All of the cages at the site were used throughout the study for on-growing halibut. The site had a holding capacity of approximately 120 mt of halibut.

All the cages in the group had previously been used for rearing salmon but had undergone modifications for use with halibut. The net pens at Loch Teacuis were constructed from a cuboid tubular steel frame, which supported the mesh and formed the walkway structure. Hollow plastic buoys below the main walkway structure provided floatation. The cage groups were anchored to the seabed to maintain position. Hanging from the main frame, set at a level below the base of the net was a square, steel, base-tensioning frame, which helped to ensure that the base of the net was as flat as possible. Attached to the base of the net, on the inner surface, was a white tarpaulin that provided a more solid base for the fish. The tarpaulin also allowed the halibut to feed on pellets that had not been taken in the water column and would have otherwise passed through the base mesh. The tarpaulin remained in place for 9 months until the pellet size was large enough to prevent passage through the base mesh. In order to maintain an acceptable stocking density that was within a commercially applicable range over the 3 year study period, the fish were first held in two 5m square and 3m deep cages. After the 5th weighing (29/09/00) the fish were then divided equally between four 5m cages. The Initially Large group of fish was divided between 2 cages as were the Initially Small group of fish in an attempt to reduce the effects of size hierarchies. A final transfer was made after the 7th sampling date, when for economic reasons all of the fish had to be combined into one 12m

square, 4m deep cage (Figure 2.2.5). 100 fish were tagged and added to the initially small size group of fish after the 3rd sampling date to replace those lost to seals shortly after the initial transfer of all the fish by well boat from Otter Ferry Seafish. The replacement fish were not used in any of the analysis but were present only to reinstate the original numbers and hence stocking density.

The cages were located in Loch Teacuis, which forms a basin off Loch Sunart on the West Coast of Scotland. The water depth below the cages was approximately 22m. Entry to the loch is via 2 narrow channels and the topography of the basin limits any fetch in the approaches to the cages thus minimizing the effects of poor weather on the cage structure. A predator net was suspended over each pen but no shade netting was in place over the tanks. No additional artificial lighting was employed.

Figure 2.2.4 5 metre square trial cages located at Loch Teacuis

Figure 2.2.5 12 metre square trial cages located at Loch Teacuis

2.2.3 Otter Ferry Tanks

The rearing tanks at Otter Ferry are located on the southerly shore of Loch Fyne, on the West Coast of Scotland. Two sizes of tanks were used in the study. In order to maintain an acceptable stocking density within a commercial range over the 3-year period the fish were first held in two 5m diameter and 1m deep fibreglass tanks. After the 5th weighing (12/09/00) the fish were divided between 4 of the 5m tanks. A final transfer was made after the 7th weighing (26/02/2001) when for economic reasons all of the fish had to be combined into 1 x 12m diameter and 2m deep tank (Figure 2.2.7). Raw, unfiltered seawater at ambient temperature and pumped from a depth of approximately 25m was supplied to all the trial tanks. Additional aeration was supplied to the tanks via in-tank diffusers. Each of the tanks in the study was individually covered with a dark, but not light proof, cover. No artificial lighting was used.

Figure 2.2.6 5 metre diameter trial tank at Otter Ferry Seafish

Figure 2.2.7 12 metre diameter trial tanks at Otter Ferry Seafish during weighing procedure.

2.2.4 Feeding

Fish at each site were fed a commercial marine fish diet supplied to the trial by Trouw Aquaculture (Renfrew, Scotland, UK)(for composition see table 2.2.3). Fish were hand fed twice a day for the duration of the trial and at a rate slightly exceeding that suggested in the supplied feed tables. Slight excess feeding was employed to minimize any feed distribution differences inherent between the two types of rearing systems and also between different personnel feeding the fish. Pellet size was increased as the trial progressed according to the manufacturer's recommendations. Feed amount and pellet size were adjusted based on data obtained during each weight and length sampling.

Table 2.2.3 Composition of Trouw Marine Fish diet fed to trial fish at both sites.

Content	Amount
Protein	48.0 %
Oil	26.0 %
Ash	10.0 %
Fibre	1.0 %
Phosphorus	1.50 %
Copper	10mg/kg
Vitamin A	12000 iu/kg
Vitamin D3	2000 iu/kg
Vitamin E (as alpha-tocopherol acetate)	300 iu/kg

2.2.5 Sampling procedure

Prior to each sampling the fish were starved for 24 hours and not fed again until the entire sampling procedure was completed.At each sampling point all fish were weighed and measured (fork length) with all the information being recorded against each fish's individual PIT tag number. Both sites were sampled at intervals of approximately 12 weeks. The sampling dates for Otter Ferry were: 24/01/00, 21/03/00, 12/06/00, 12/09/00, 7/12/00, 26/02/01, 26/06/01, 10/10/01, 16/01/02, 28/05/02 and 18/09/02. At the Loch Teacuis cage site the sampling dates were: 18/01/00, 23/03/00, 21/06/00, 29/09/00, 13/12/00, 13/03/01, 12/06/01, 25/09/01, 27/02/02, 08/05/02, and 12/09/02. Variation in interval duration was due to staff

availability at each site and the severity of the weather, which at times prohibited sampling at the Loch Teacuis cage site. Every attempt was made to minimize the delay between sampling Loch Teacuis and Otter Ferry at each data collection point. To facilitate removal of fish from the 5m diameter tanks at Otter Ferry the water level in each tank was lowered and batches of fish were hand netted into a harvest bin in which they were anaesthetized with 2-phenoxy ethanol ($1mgl^{-1}$). After being weighed and measured the fish were returned to another harvest bin with clean, oxygenated seawater to recover before being returned to a new 5m tank. As the experiment proceeded and the fish were moved to a 12m diameter tank the sampling procedure was modified. The water level in the tank was again lowered. The fish were crowded together and batches were hand netted into a small area of the tank divided off with a plastic walled crowding frame into which anaesthetic was added. The fish were then netted out, weighed and measured and returned immediately to a new 12m-diameter tank.

At the cage site in Loch Teacuis the sampling procedure was identical when fish were held in either $25m^2$ pens or $144m^2$ pens. The tensioned mesh holding the fish was loosened and the fish were crowded by a combination of lifting a portion of the net out of the water and pulling a row of buoys under the base of the net. The fish were then hand netted into a black plastic dustbin in which they were anaesthetized with 2-phenoxy ethanol ($1mgl^{-1}$). After being weighed and measured the fish were returned to a new, clean cage. Following data collection Specific Growth Rate (SGR) and Condition Factor and GF3 values (see section 2.1.4.1) were calculated for each fish. Following sexing (next section) and determination of the timing of maturation for the males, linear regression was performed on the log values of mean SGR and mean intermediate weight (g) (mid point between samples) to determine values for a and b

in the form $SGR(\%day^{-1})={}_aW^b$. Von Bertalanffy Growth curves for the different groups of fish were fitted and growth parameters were estimated using a non linear method (FiSAT package) (Sparre, 1989). Regressions were performed in order that growth type and condition factor indices could be determined.

2.2.6. Sexing

All the halibut in the trial were sexed on two separate occasions. The fish held in the cages at Loch Teacuis were sexed in the first instance (13/03/01) using non-invasive ultrasonography. At the end of the experiment, following harvesting, a second definitive, visual sexing of the Loch Teacuis fish was carried out in the processing factory during the gutting process. The fish held in the tanks at Otter Ferry were sexed on both occasions (26/02/01 and 15/10/02) using non-invasive ultrasonography, as harvesting schedules did not allow for the culling of any fish at the end of the trial. A Sonovet 600 ultrasound with a 7.5MHz linear probe was used. After weighing and measuring the fish the linear scanhead was placed on the surface of the halibut approximately over the anterior end of the ovaries or testes, directly posterior to the gut region (see figure 2.2.8). The scanhead was then slowly moved towards the posterior of the fish. Acoustic energy emitted by the transducer at megahertz (MHz) frequencies is absorbed or reflected as it passes through tissues. The reflective properties of different tissues vary; denser tissues give a lighter image as they reflect more energy than more fluid filled organs, which correspondingly produce a darker image. Determination of sex was based on the shape, size and texture of the gonadal image, converted from the acoustic signals, on the ultrasound display screen.

In addition to the sexing data obtained from either the ultrasonography or direct observations of the removed gonads, a record was made of any males that were producing milt at the time of sampling. As expected no females were seen to be producing eggs during the experiment.

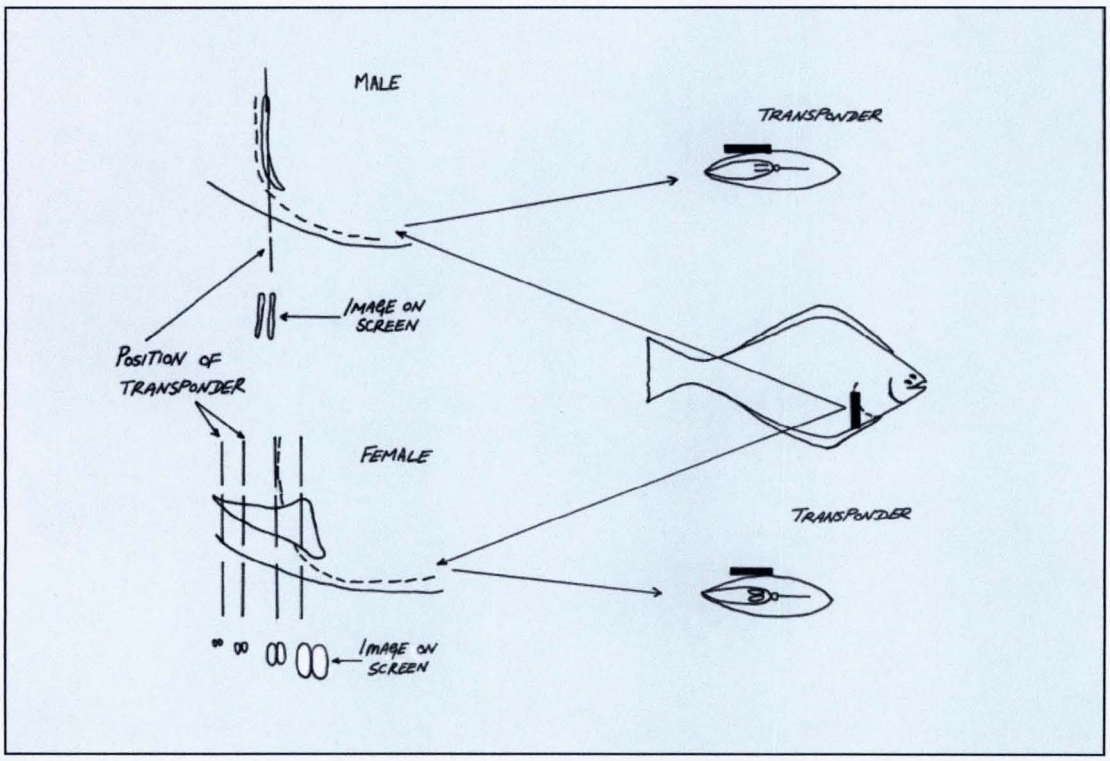


Figure 2.2.8 Initial position of ultrasound transponder on the upper surface of the halibut and the corresponding image types in relation to it anterior movement.

2.2.6.1 Visual sexing of Loch Teacuis fish.

At the end of the study the fish that had been held in the cages at Loch Teacuis were all harvested. The fish were transported on ice to a Marine Harvest (Scotland) Ltd. processing plant for gutting and cleaning. Prior to gutting the PIT tags were located

and removed. Visual inspection of the gonads as they were removed gave definitive information on the sex of the fish, which was compared to the information obtained using ultrasound.

2.2.7 Blood sampling

In order to establish if any chronic disturbances or differences in plasma parameters; cortisol, plasma osmolality, chloride, sodium, potassium and glucose concentrations existed between fish held in the 2 systems blood samples were taken from fish at both sites at each sampling point.

At the start of each sampling, before water levels were dropped in the tanks or nets were lifted on the cages to facilitate the removal of fish, blood samples were taken from between 5 and 15 fish netted from each cage or tank. All blood samples were taken in the morning on arrival at each site between 8 and 10 am. The variation in number of fish sampled was dependent on how agitated the remaining fish in each holding system became. Every attempt was made to remove fish as quickly as possible and without causing disturbance to the remaining fish. Following removal of approximately 1ml of blood by caudal veinipuncture to a heparinized syringe the fish were held in a separate container filled with clean seawater until all blood samples had been taken. The fish were then returned to the rearing tank or cage. Once all blood samples had been taken the main weighing and measuring procedure could begin. The blood samples were transferred to numbered Ependorf collection tubes and stored on ice prior to being centrifuged at 13000 rpm for 3 minutes. Plasma samples were removed to clean Ependorf tubes and frozen at -20 awaiting further analysis.

2.2.8 Plasma Analysis

2.2.8.1 Plasma Osmolality

Plasma osmolality was measured in duplicate by freezing point depression (Roebling osmometer, Camlab, Cambridge).

2.2.8.2 Plasma Cl^-

Plasma chloride concentration was measured in duplicate by automatic chloride titration (Chloride Analyser 925, Corning Ltd., Essex).

2.2.8.3 Plasma glucose

Plasma glucose concentration was measured in duplicate using a Sigma Diagnostics enzymatic glucose assay kit (Procedure N° 510). Sample absorbance (A test) was read at 450 nm (Amersham Pharmacia Biotech Ultrospec 3300pro UV/visible spectrophotometer) using a Milli-Q® blank as a reference and compared with a known standard value (A standard). The following calculations were used to determine the glucose concentration:

A (test) / A (standard) x 100 = Plasma glucose (mg/dL)

SI units e.g. (mmol/L) = Plasma glucose / 18

2.2.8.4 Plasma cortisol

Plasma cortisol concentration was determined by Radioimmunoassay (RIA) using the method employed by (Birrell, 1998) and adapted from (Waring, Stagg et al., 1996).

2.2.9 Cortisol Extraction

75µl of plasma was added to 600 µl of ethanol in a 1.5ml Eppendorf tube. All samples were shaken for 1 minute and centrifuged (Sanyo Microcentaur) at 13000 rpm for 3 minutes. The supernatant was poured into a numbered plastic LP4 tube (Denley Instruments Ltd.). The remaining pellet was re-suspended in 600µl of ethanol and centrifuged at 13000 for a further 3 minutes. The supernatant was combined with initial supernatant in the LP4 tube. The supernatants in the LP4 tubes were then dried down in a centrifugal evaporator (GL11 Gyrovap and Crist CT 02-50 chiller) set at 30°C.

2.2.10 RIA Components

2.2.10.1 Assay Buffer

Before each assay a new volume of assay buffer was made up. Assay buffer stock, stored at room temperature, consisted of 1L H_20 + 71.6g $Na_2HPO_4.2H_2O$ (40mM) + 15.3g $NaH_2PO_4.2H_2O$ (10mM).

To make up 100ml of assay buffer 10ml of buffer stock was added to 90mlmilliQ® + 0.9g NaCl + 0.5g BSA. pH was adjusted to 7.4.

Throughout the assay the assay buffer was either stored on ice or refrigerated.

2.2.10.2 Cortisol standards

The cortisol standard consisted of inert hydrocortisone (11β, 17α, 21-trihydroxypregn-4-ene,3,20-dione, 98%, Sigma) stored at 2mg/ml in 99.7-100% ethanol at –20°C. A 10ng/ml solution was prepared by serial dilution with ethanol prior to use in a series of triplicate standards. Standards ranged from 19.5pg/tube in 2.0µl to 5000pg/tube in 500µl in plastic LP4 tubes (Denley Instruments Ltd.)(Table 2.2.4). The standards were dried in a centrifugal evaporator in the same manner as the unknown samples and either stored on ice, if being used immediately, or sealed and stored at –20 for later use.

Table 2.2.4 Volumes and concentrations of cortisol standards

500	µl	5000	pg
250	µl	2500	pg
125	µl	1250	pg
62.5	µl	625	pg
31.3	µl	312.5	pg
15.6	µl	156	pg
7.8	µl	78	pg
3.9	µl	39	pg
2.0	µl	19.5	pg

Triplicate sets of tubes were also required for both total counts and zero counts.

2.2.10.3 Tritiated cortisol

A stock solution of [1,2,6,7-^{3}H] cortisol (^{3}H-cortisol) in toluene:ethanol (9:1 v/v), with a specific activity ranging from 80-105Ci/mmol (Amersham Life Science Ltd.) was stored at –20°C. A working stock solution, again stored at –20oC was prepared by diluting 50µl of the stock solution (250µCi in 250µl) in 4.95ml of 9:1 v/v toluene:ethanol (i.e. 50µl stock + 4.455ml toluene + 0.495 ethanol). This working

stock solution contained approximately 20,000 dpm/µl. For each assay an aliquot of working stock was dried down and resuspended in a volume of assay buffer to give a final count of approximately 20,000 dpm per 200µl. The volume of tubes to be processed in each assay determined the volume of the working stock aliquots and also the volume of assay buffer used (Table 2.2.5).

Table 2.2.5 Volumes of 3H-cortisol and assay buffer required for varying numbers of tubes.

Number of tubes	^{3}H-cortisol (µl)	Assay buffer (ml)
50	85	10
100	170	20
150	255	30
200	340	40

2.2.10.4 Cortisol Antibody

The anti-cortisol serum was obtained from Diagnostics Scotland. The serum was a pool of selected antisera obtained from a single sheep injected with cortisol –3-0 (carboxymethyl) oxime-bovine albumin conjugate. The supplied vials contained the lyophilised residue from 1ml of anti-cortisol serum diluted 1 in 5 with a 0.04M phosphate buffer, containing 0.5%(v/w) bovine serum albumin. The lyophilised vials were stored at 4°C until required. The reagent was reconstituted by the addition of 1ml MilliQ® and left to stand for 1 hour at room temperature. 40µl aliquots were then stored at –20°C prior to use in each assay. On day 1 of each assay the antibody was

diluted 1 in 300 in the ^{3}H-cortisol-assay buffer mix giving a final antibody concentration of 1 in 5000.

2.2.10.5 Dextran coated charcoal

Dextran coated charcoal was prepared on day 1 of the assay and refrigerated overnight before use on day 2. 250mg of charcoal (BDH) was added to 25mg of dextran (Sigma) in 50ml of assay buffer. The solution was stirred on ice for 2 hours and for a further 30 minutes the following day prior to being used.

2.2.11 Determination of Cortisol by Radioimmunoassay

2.2.11.1 RIA, day 1

All sample and standard LP4 tubes were removed from the freezer and allowed to thaw on ice. The appropriate amount of cortisol antibody was added to the ^{3}H cortisol previously resuspended in assay buffer immediately before addition to the LP4 tubes. 200µl of the antibody - 3H cortisol – assay buffer solution was added to each LP4 tube, including total count tubes and zero tubes which contained no cortisol. All tubes were vortex mixed for 10 seconds and then incubated at 37°C in a water bath for 1 hour. The samples and standards were then removed from the water bath and stored over night at 4°C.

2.2.11.2 RIA, day 2

200µl of chilled, stirred DCC was added to each of the sample, standard and zero tubes. The total count tubes had 200µl of assay buffer added to ensure that total tube

volumes were maintained. As the total counts tubes received no DCC they therefore showed the total amount of radioactive counts that were added to all the other tubes.
All tubes were vortex mixed for 10 seconds and held on ice for 15 minutes before being centrifuged at 2500 rpm and 4oC for 15 minutes (MSE Mistral Centrifuge, Fisher Scientific Ltd). The supernatant from each tube was carefully removed with a Pasteur pipette to a PONY vial (Packard Bioscience BV). To each PONY vial was then added 4ml of Emulsifier Scintillator plus™ liquid scintillation cocktail (Canberra Packard Ltd.). The tubes were then shaken and counted for 5 minutes with a 2000 Tri-Carb® Scintillation Analyser (Canberra Packard Ltd.)
Sample values were subsequently calculated from a standard curve of % bound plotted against pg cortisol/tube using the Curve Expert v 1.34.

2.2.12 Temperature measurement

Temperature data was supplied by the staff at each site. Weekly recordings of the temperature at the depth of the fish in the cages and in the individual holding tanks at Otter Ferry were made throughout the trial.

2.2.13 Growth curve fitting

The Von Bertalanffy growth curves were fitted to the age-at-time data using the FISAT suite of fisheries tools (Sparre, 1989, Gayanilo, 1995). Curves were initially fitted to length-at-age data as this provided a better it as a result of lower inflection points for the length data as opposed to weight data. Values for the growth rate k and

time zero (t_0) remain the same between Von Bertalanffy curves in terms of length and weight. The asymptotic weight (WT) was calculated from the asymptotic length (LT) by the length weight relationship $W_t = aLt^b$ where the coefficients a and b were established from length/weight data collected for each group (females, 1st year maturing males and 2nd year maturing males at each site). Growth performance indices in terms of length (Ø′) and weight (Ø) were then calculated.

2.2.14 Statistical analysis

All statistical analysis was performed using STATISTICA™ Version 7 (Statsoft®) and SPSS for windows Version 10(SPSS Inc, Illinois). To assess normality of distributions a Kolmogorov-Smirnov (Zar, 1984) test was used and heteroscedasticity was tested for using the Levene's *F*-test. Data was Log transformed if required.

If not otherwise stated, a significance level of 0.05 was used.

Growth trial – size-at-age data

A GLM one-way repeated measures ANOVA was carried out with a subsequent Tukey –Kramer post hoc test. To provide multiple comparisons for each time point in the size-at-age analysis a one-way ANOVA followed by a Tukey-Kramer *post hoc* test was used.

GF3

A GLM one-way repeated measures ANOVA was used to compare GF3 values for the levels sex and site.

SGR

Regression lines for Specific Growth Rates (SGR) were compared using Analysis of covariance (ANCOVA). A full factorial one-way ANCOVA with weight as a covariate was subsequently applied to investigate differences between the individual groups of fish (Sokal and Rohlf, 1995).

Condition factor

Condition factor against length was analysed using Log linear regression. Homogeneity of slopes were tested for using covariance analysis (ANCOVA) and further tested using a full factorial one-way covariance analysis (ANCOVA). Factor levels were site and sex. (Sokal and Rohlf, 1995)

Mortality

Data on mortality / survival between sites was tested using a χ^2 test (Zar, 1984).

Growth type

Isometry/allometry was determined by confirming whether *b* values, the allometry coefficient of $SGR(\%day^{-1})=aW^b$ were significantly different from the isometric value (3), a *t*-test (H_o : b=3) with a confidence level of 95% (p= 0.05) was applied, expressed by the following equation:

$$t_s = \frac{b-3}{s_b}$$

where t_s is the *t*-test value, *b* the slope and s_b the standard error of the slope (*b*)(Sokal and Rohlf, 1995; Santos, Gaspar et al., 2002).

Size Ranking

Initial size *vs* final size was tested by Spearman's rank correlation (Zar, 1984)

Coefficient of Variation (CV)

CV=100*(Standard deviation/mean weight) was regressed against mean weight and analysed by means of log regression.

Plasma Parameters

Differences in total mean plasma parameters (cortisol, osmolality, chloride and glucose were compared using a t-test. Temporal variations of the same parameters between site were compared using a GLM repeated measures ANOVA. Temporal trends were determined with linear regression.

2.3 Results

2.3.1 Temperature profiles

Weekly temperature readings for both Loch Teacuis and Otter Ferry (Figure 2.3.1) followed a similar pattern throughout the duration of the experiment. The main differences occurred between the two sites when temperatures were at their minimum and maximum. Between the temperature extremes, differences were minimal. Maximum and minimum temperatures at each site occurred during late August to early September and early March respectively. Maximum temperatures at Loch Teacuis were consistently higher than at Otter Ferry whilst no such pattern occurred with minimum temperatures. The Maximum temperature observed at Loch Teacuis was 15.4°C compared to 13.8°C at Otter Ferry. The minimum temperature observed at Loch Teacuis and Otter Ferry was 6.4°C. During the entire trial the mean overall temperature at Otter Ferry was 9.8 °C and the sum of day degrees was 10408(dd). At Loch Teacuis the overall mean temperature was 10.1°C and the sum of day degrees was 10919(dd)

2.3.2 Sex ratios

The observed female: male sex ratio of all fish sexed at Otter Ferry was 53.1 %: 46.9 $\chi^2 = 2.8$, $P = 0.09$ at Loch Teacuis the ratio was 55.2%: 44.8% $\chi^2 = 6.4$, $P = 0.012$. The sex of the 100 (approx.) fish lost at Loch Teacuis in a seal predation incident, between transfer and the first sampling at Loch Teacuis, were unknown. Therefore it could not be determined whether the ratio of males to females at the start of the study would have been significantly different from that observed in the population of sexed fish. Complete sex ratio results of females, males, males observed maturing first in February 2001, males observed maturing first in February 2002 and males that were

not observed producing milt in either year of the study are presented in Table 2.3.1 and Figures 2.3.2- 2.3.3. The major difference between the populations was in terms of the numbers of males observed maturing first in 2001 and 2002. A greater percentage of males were observed maturing first in 2001 at Loch Teacuis (17.5%) than at Otter Ferry (11.1%). It is unclear whether, from the observations made, this difference is a true representation of the actual numbers of fish reaching maturity or whether more inspections of the fish around the time of spawning would have resulted in the ratio of males observed producing milt being different. The population structure of the fish, with a full set of readings for each sample point and that were used in the final analysis is presented in Table 2.3.2.

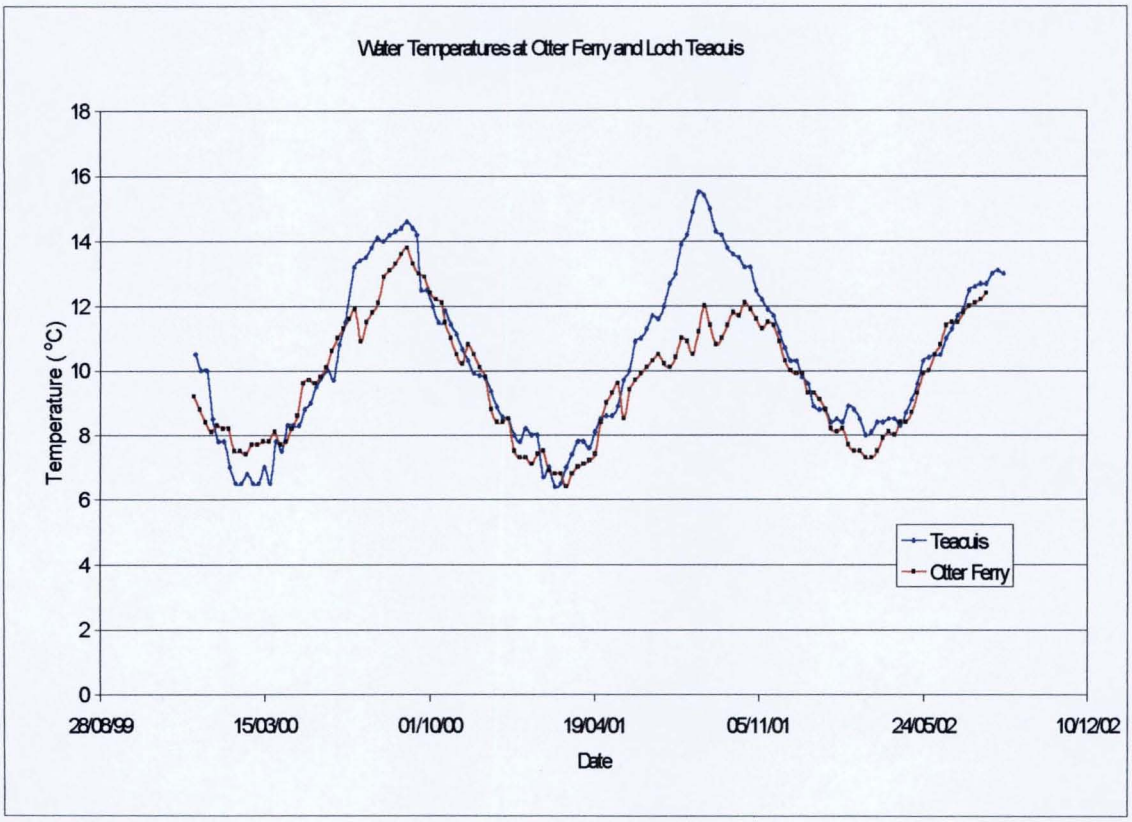


Figure 2.3.1 Temperature profiles at Otter Ferry and Loch Teacuis based on weekly readings taken at each site from December 1999 to October 2002.

Table 2.3.1 Population structures at Otter Ferry and Loch Teacuis as a percentage of the entire sexed population at each site and as a percentage of the male population at each site in terms of males reaching maturity in 2001, 2002 and males not observed producing milt in either year

Otter Ferry Fish	Total Number	% of sexed fish	% males
All sexed	754	100	
Females	400	53.1	
Males (total)	354	46.9	100
2001 maturing males	84	11.1	23.7
2002 maturing males	233	30.9	65.9
Males not releasing milt	37	4.9	10.4
Loch Teacuis fish	Total Number	% of sexed fish	% males
All sexed	601	100	
Females	332	55.2	
Males (total)	269	44.8	100
2001 maturing males	105	17.5	39.1
2002 maturing males	150	25	55.8
Males not releasing milt	14	2.3	5.1

Table 2.3.2 Population structure of sexed fish at Otter Ferry and Loch Teacuis used in the final analysis

Otter Ferry Fish	Total Number	% of sexed fish	% males
All sexed	629	100	
Females	358	56.9	
Males (total)	271	43.1	100
2001 maturing males	70	11.2	25.8
2002 maturing males	201	31.9	74.2

Loch Teacuis Fish	Total Number	% of sexed fish	% males
All sexed	478	100	
Females	276	57.7	
Males (total)	202	42.3	100
2001 maturing males	79	16.5	39.1
2002 maturing males	123	25.7	60.9

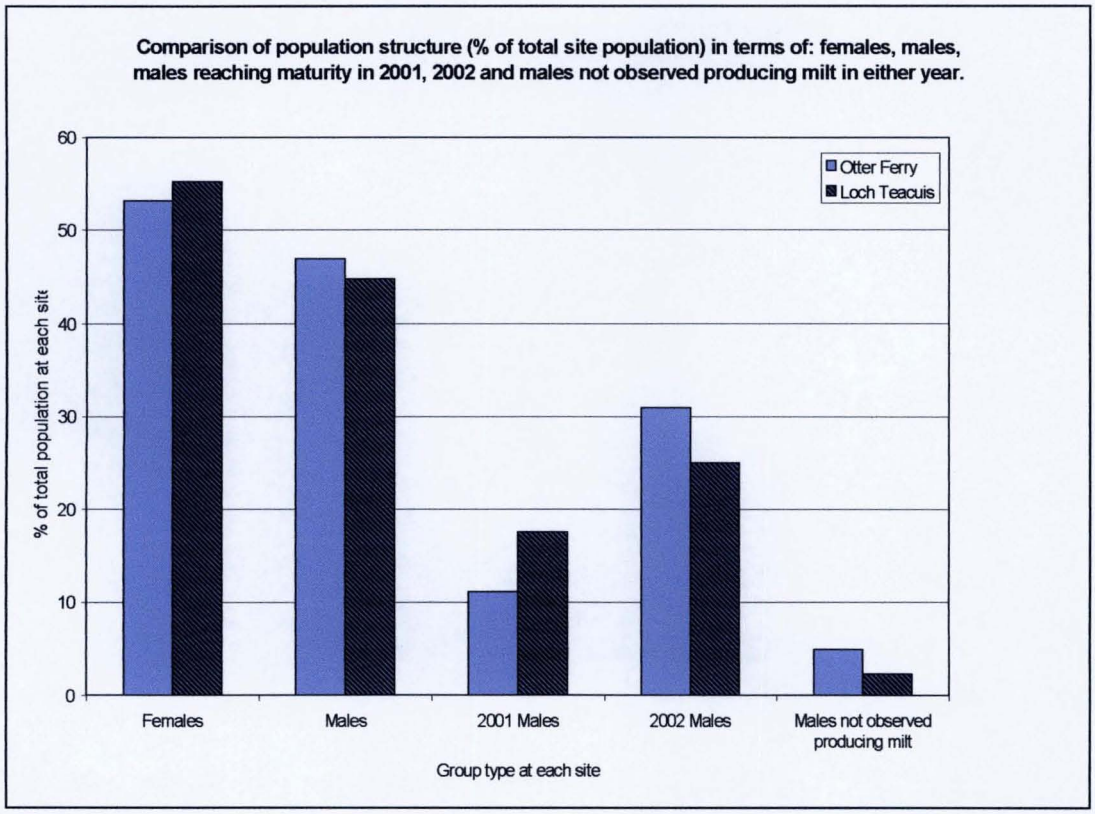


Figure 2.3.2 Observed sex ratios at Otter Ferry and Loch Teacuis of different group types as a percentage of the total population at each site in terms of: females, males, males first reaching maturity in February 2001 or February 2002 and males not observed producing milt in either year

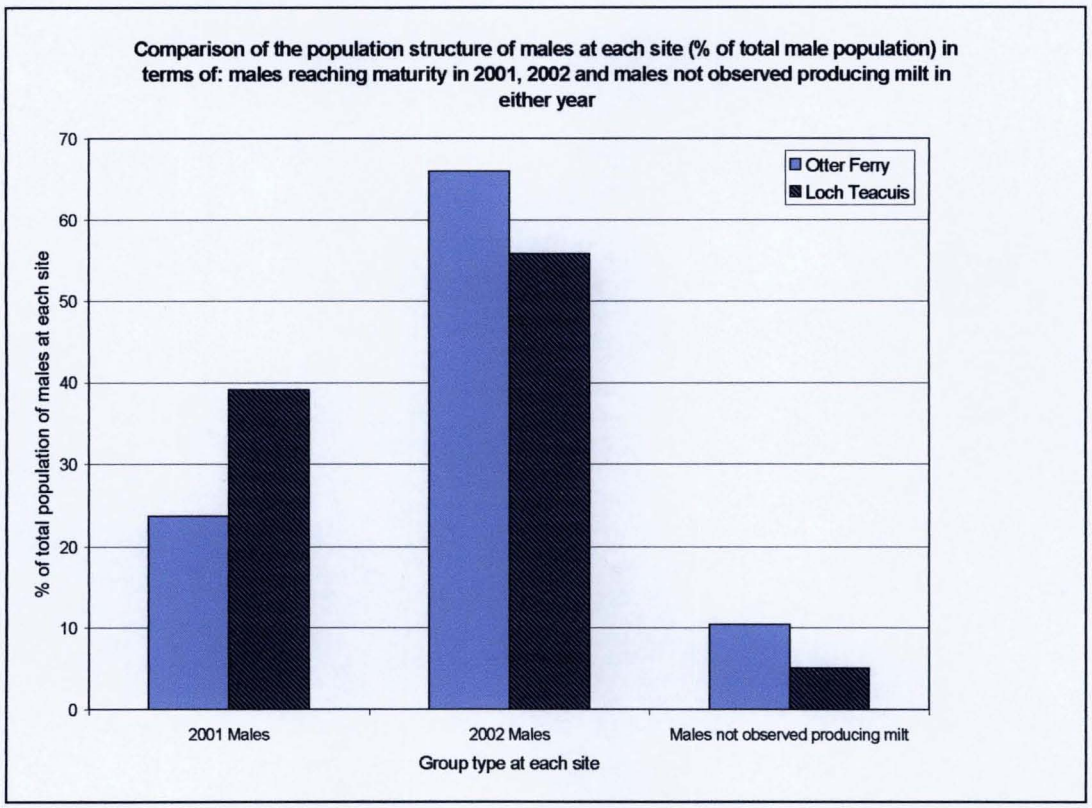


Figure 2.3.3 Observed sex ratios at Otter Ferry and Loch Teacuis of different group types as a percentage of the total population of males at each site in terms of: males first reaching maturity in February 2001, February 2002 and males not observed producing milt in either year

2.3.3 Stocking density

Stocking density (kgm^2) increased at each site prior to the redistribution of fish. At Otter Ferry fish were transferred from 2 x 5m tanks to 4 x 5m tanks (09/2000)(sample point 5) and from 4 x 5m tanks to 1 x 12m tank (02/2001)(sample point 7). At Loch Teacuis fish were redistributed from 2 x 5m cages to 4 x 5m cages (09/2000)(sample point 5) and from 4 x 5m cages to 1 x 12m cage (03/2001)(sample point 7) (Figure 2.3.4). Following each redistribution there was a corresponding decrease in stocking density (kgm^2) for each size group. Greater growth rates in the tanks at Otter Ferry and slight inherent differences in the surface area of each type of system (cages>tanks) meant that stocking density was slightly higher at Otter Ferry than Loch Teacuis at each sample point. However, no fish were removed from the tanks at Otter Ferry in order to balance the stocking densities at each site, as this would have compromised fish numbers in the sexual dimorphism study. Maximum stocking densities were 38.43kgm^2 at Otter Ferry and 27.32kgm^2 at Loch Teacuis.

2.3.4 Mortality

At Otter Ferry from the 2nd sampling date to the last the total number of fish decreased from 833 to 732 (Figure 2.3.5). Survival was therefore 87.9% (Figure 2.3.6). Maximum loss between sample dates was 24 (between the 4th (12/06/00) and 5th (12/09/00)).

At Loch Teacuis between the transfer date and the 1st sampling date (24/01/00) an estimated 102 fish were lost due to seal predation from the Initially Small group of fish. 102 halibut from another cage containing fish of the same size at Loch Teacuis were tagged and added to the cage as replacement fish These replacement fish served only to maintain a similar stocking density and were not used in the final analysis..

Another loss of fish occurred at Loch Teacuis when 39 fish were trapped in a fold in the net during crowding as part of the sampling procedure (12/06/2001, sample date 8). Apart from these two events the largest decrease in fish numbers between sample dates was 21 (between the 10th (10/10/01) and 11th (26/02/01)). If these two events are included from the 2nd sampling date to the last the total number of fish effectively decreased from 810 to 585 therefore survival was 72.2%. This survival rate was significantly lower than at Otter Ferry ($\chi^2 = 61.62$, $p < 0.001$). Discounting the two large losses at Loch Teacuis numbers decreased from 805 (after addition of replacement fish) to 723 (including fish killed during sampling) therefore survival was 89.8% (no significant difference between Loch Teacuis and Otter Ferry, $\chi^2 = 1.361$ $p > 0.05$)(Figure 2.3.6).

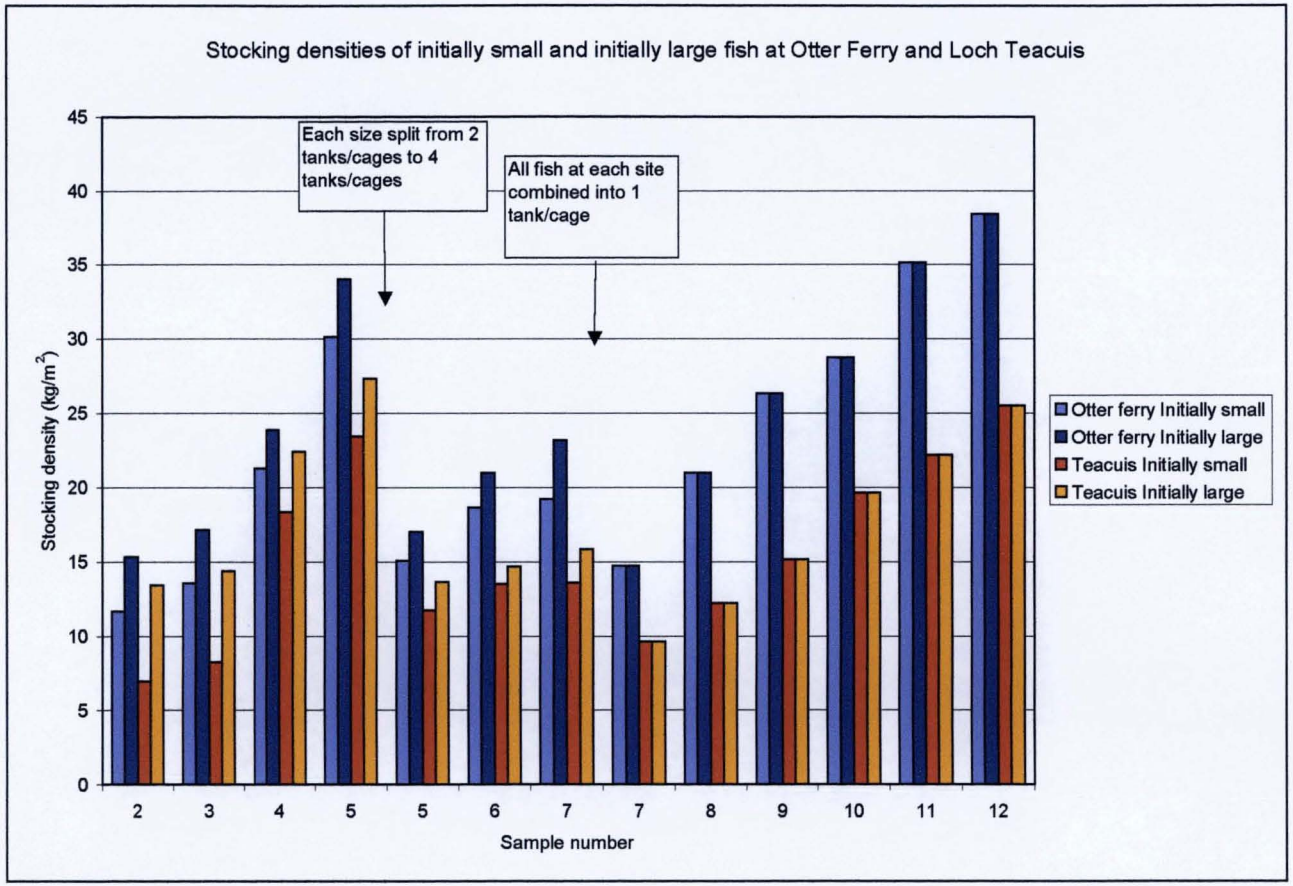


Figure 2.3.4 Stocking densities of fish at Otter Ferry and Loch Teacuis at each sample point between 18/01/00 and 18/09/02. Arrows indicate point at which fish were transferred from 2 – 4, 5m tanks/cages and 4, 5m – 1, 12m tank cage.
Sample date numbers 2-12 equate to 24/01/00, 21/03/00, 12/06/00, 12/09/00, 7/12/00, 26/02/01, 26/06/01, 10/10/01, 16/01/02, 28/05/02, 18/09/02 at Otter Ferry and 18/01/00, 23/03/00, 21/06/00, 29/09/00, 13/12/00, 13/03/01, 12/06/01, 25/09/01, 27/02/02, 08/05/02, 12/09/02 at Loch Teacuis

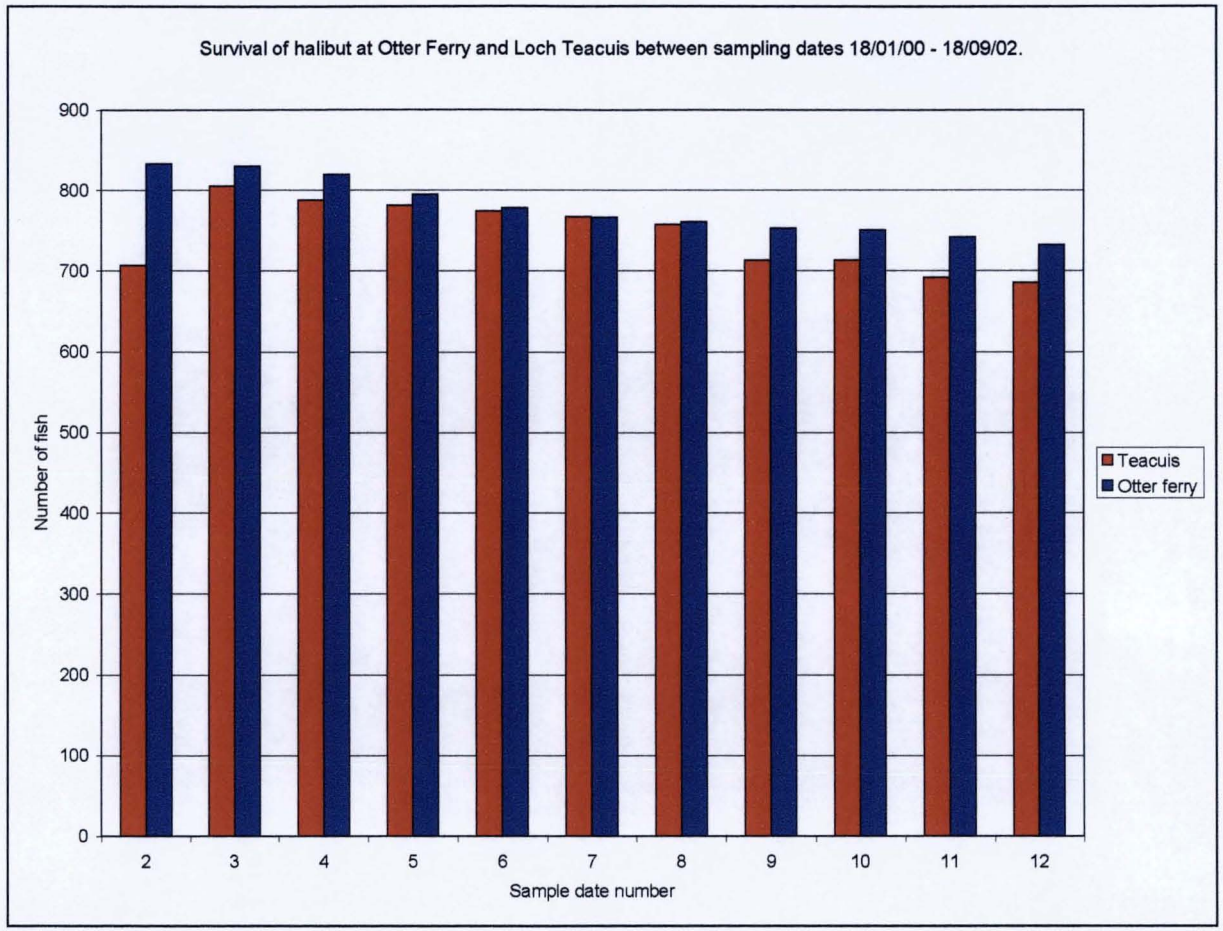


Figure 2.3.5 Survival between sample periods of halibut at Otter ferry and Loch Teacuis Sample date numbers 2-12 equate to 24/01/00, 21/03/00, 12/06/00, 12/09/00, 7/12/00, 26/02/01, 26/06/01, 10/10/01, 16/01/02, 28/05/02, 18/09/02 at Otter Ferry and 18/01/00, 23/03/00, 21/06/00, 29/09/00, 13/12/00, 13/03/01, 12/06/01, 25/09/01, 27/02/02, 08/05/02, 12/09/02 at Loch Teacuis

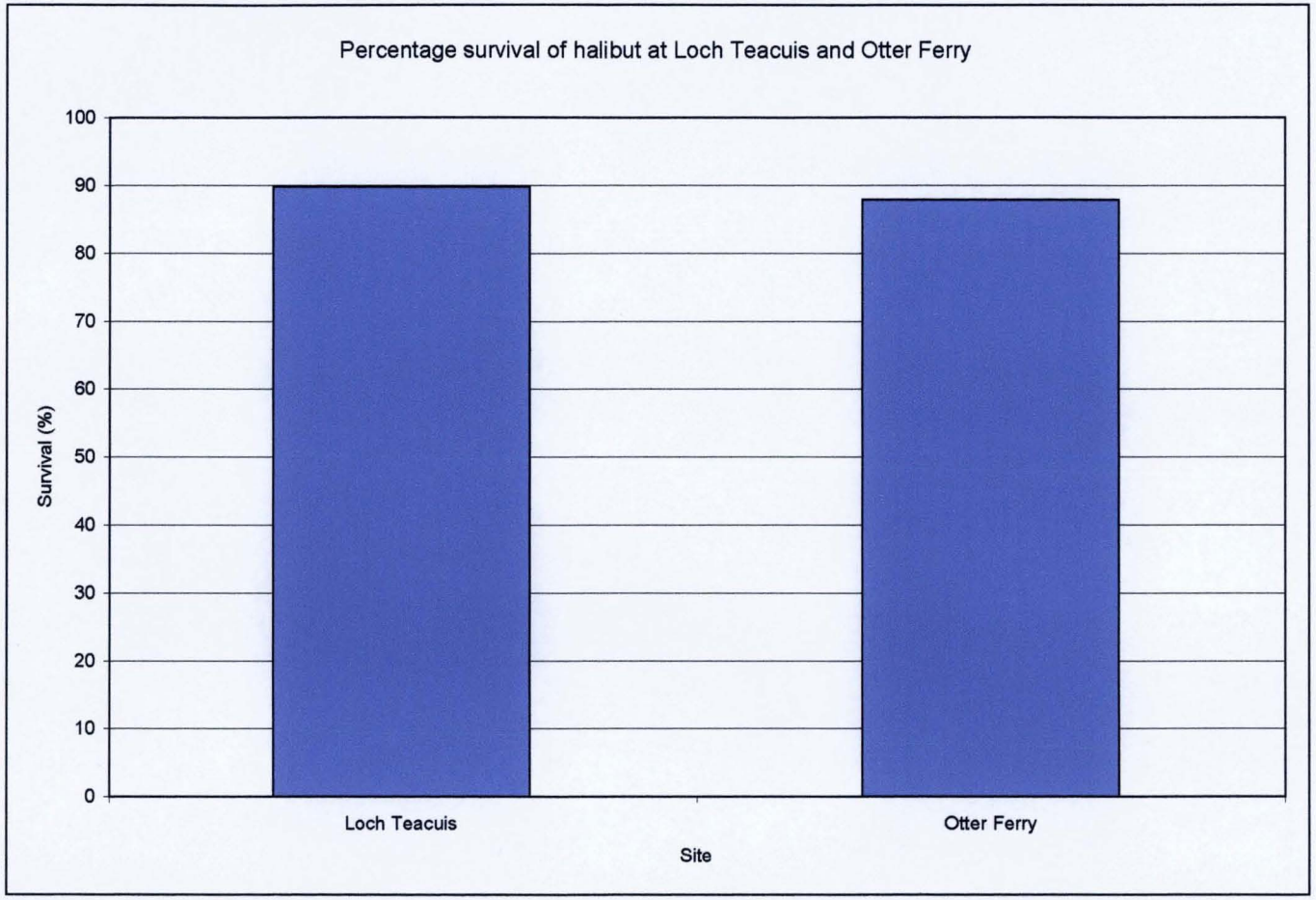


Figure 2.3.6 Survival (%) of halibut ongrown in tanks at Otter Ferry and in cages at Loch Teacuis between 18/01/00 and 18/09/02.

2.3.5 Inter site variation in mean weight (g) between all fish combined, females and maturing male year classes

At the time of PIT tag insertion, the mean weight of all fish, randomly selected and destined to be transferred, from the holding tank at Otter Ferry to the Loch Teacuis cage site (444g) was slightly greater than the group of fish that subsequently remained at Otter Ferry (405 g) (one-way ANOVA, $P<0.001$).

During the remaining period of the trial, following transfer, the mean weights for the entire population at each site increased at each sample point. From the first sampling point after transfer onwards, with the exception of sampling date 4 where no significant difference was observed, the mean weight of the fish at Otter Ferry was, and remained significantly higher than at Loch Teacuis (one-way ANOVA, $P<0.001$). Overall, the mean individual growth trajectories between all fish combined at each site during the entire trial differed significantly (Site effect, GLM repeated measures ANOVA, $P < 0.001$, Table 2.3.3) Each group of fish at Otter Ferry; females, males maturing in the 1st year and males maturing in the 2nd year all performed significantly better than their counterparts in the cages at Loch Teacuis, (GLM repeated measures ANOVA $P < 0.01$, followed by Tukeys *post hoc* test, Table 2.3.4)

The mean weight of the population in cages at Loch Teacuis increased to 4640g by the end of the trial. At Otter Ferry the mean weight increased to 5992g at the end of the trial (Figure 2.3.7). Taking the Teacuis weight to be 100% the final difference equated to a 29% premium in mean weight for the fish at Otter Ferry. The mean weights (g) for all groups at each sample date are presented in Table 2.3.5. Again, taking the weights at Loch Teacuis to be 100%, the final between site differences for the females, 2001maturing males and 2002 maturing males were 26, 32,and 40% respectively.

Growth throughout the trial varied with season, and corresponded to changes in temperature, day length and sexual maturation. Minimal increases in weight occurred from around September through to March. This corresponded to a decrease in water temperature, a decrease in day length and the onset of spawning. Maximal increases in weight occurred through the spring and summer, which corresponded to increasing water temperature, increasing day length and cessation of spawning.

Table 2.3.3 GLM repeated measures ANOVA of mean weight (g) for date, site and sex (factors)

Source of variation	SS	d.f	MS	*F*-ratio	*P*
SITE	19.24	1	19.24	145.7	<0.001
SEX	20.25	2	10.13	76.7	<0.001
SITE*SEX	0.23	2	0.11	0.9	0.424
Error	145.41	1101	0.13		
DATE	898	11	81.64	23753.0	<0.001
DATE*SITE	7.61	11	0.69	201.3	<0.001
DATE*SEX	35.47	22	1.61	469.1	<0.001
DATE*SITE*SEX	0.36	22	0.02	4.8	<0.001
Error	41.62	12111	0		

2.3.5.1 Mean weights of males not observed reaching maturity in either 2001 or 2002

A small group of fish at each site, identified by ultrasound (Otter Ferry) and visual examination (Loch Teacuis) as males, were not observed producing milt in either 2001 or 2002. From examination of the mean weight data for these groups of fish it was concluded that the pattern was consistent with that of males who had been observed first producing milt in 2002. In addition to this, larger S.E.M. values and the small size of the two groups (Table 2.3.1) meant it was uncertain whether the two groups truly represented males that had not produced milt. It is possible that the fish could have had produced milt either late or early in the spawning season and so

missed detection on the sample date or their status could have been wrongly recorded. For these reasons their inclusion in any further analysis was omitted.

2.3.6 Sexual dimorphism and inter sex variation in mean weight (g) at Otter Ferry and Loch Teacuis

Significant differences occurred between the weight-at-age trajectories of the different sexes over both sites ($P<0.001$, Table 2.3.3) The non significant site*sex interaction ($P= 0.424$) indicates that the performance of each sex group, in relation to the others was, the same at each site. These differences in weight-at-age were further investigated by a repeated measures comparison of all the six, separate, groups at both sites (Table 2.3.4). *Post hoc* comparisons revealed that there were similarities ($P<0.001$) between the performance of the males maturing in both years at Otter Ferry (OFM1 and OFM2) and the females at Loch Teacuis (LTF). The performance of both groups of males (LTM1 and LTM2) at Loch Teacuis were also similar. The performance of the females at Otter Ferry (OFF) was significantly different ($P<0.001$) from all other groups. This information is however, due to the growth profiles of some groups crossing those of others, somewhat misleading. The final weights of some of the statistically similar groups varied greatly and, with additional growth, would probably diverge further. A one-way ANOVA was performed followed by a Tukeys *post hoc* multiple comparison to determine the significance of the differences between the groups at each sampling date (Figure 2.3.8)

Table 2.3.4 GLM repeated measures ANOVA of mean weight (g) group and date

Source of variation	SS	d.f	MS	*F*-ratio	*P*
Group	45.96	5	9.19	69.6	>0.001
Error	145.41	1101	0.13		
Date	898	11	81.64	23753	>0.001
Date*Group	49.12	55	0.89	259	>0.001
Error	41.62	12111	0.00		

2.3.6.1 Performance at Otter Ferry

All groups of fish, females (OFF), males maturing first in 2001 (OFM1) and males maturing first in 2002 (OFM2) increased in weight throughout the trial (Figure 2.3.8). The females (399g start – 7352g end mean wt) showed the largest weight increase. Males who were observed producing milt first during 2002 exhibited a greater weight increase (400g – 4326g) than the males first observed producing milt in 2001 (452g start – 3819g end mean wt). The mean weight of the 2001 maturing males was greater than that of the other two groups (females and 2002 maturing males) until sample point 5 (12/09/00). From a significant (one-way ANOVA $P<0.01$) weight premium over the females and 2002 males at sample point 5 negative inter sample date growth during the 2001 winter spawning season was observed for the 2001 maturing males. Mean weight of the 2001 males was significantly lower than the other two groups by 16/02/01 (ANOVA $P<0.01$). Negative growth was not observed for the remaining non-spawning males during the 2000-2001 winter. For both 2001 and 2002 maturing males negative growth was observed during the 2002 winter spawning season. Negative growth was not observed for the females in any spawning season. Following negative or minimal growth by the males, weight increase was observed through the

summer months until the next winter. There was however, a continued divergence between both groups of males and the females.

2.3.6.2 Performance at Loch Teacuis

All groups of fish: females (LTF), males maturing first in 2001 (LTM1) and males maturing first in 2002 (LTM2) increased in weight throughout the trial (Figure 2.3.8). The females (445g start – 5836g end mean wt) showed the largest weight increase. Males who were observed producing milt first during 2002 exhibited a greater weight increase (425g – 3086g) than the males first observed producing milt in 2001 (472g start – 2877g end mean wt). The mean weight of the 2001 maturing males was significantly greater (ANOVA $P<0.05$) than that of the 2002 maturing males sample until sample point 5 (28/08/00) although differences between the 2001 males and the females during the same period were not significant. From a significant (one-way ANOVA $P<0.01$) weight premium over the 2002 males at sample point 5 (28/08/00) negative inter sample date growth during the 2001 winter spawning season by the 2001 first spawning males was observed. Significant differences (one –way ANOVA $P<0.01$) between all groups were observed, with female mean weights being the greatest and 2001 first spawning males the lowest, from sample point 7 (13/03/01) until point 10 (27/02/02). From point 10 onwards no significant differences were observed between both groups of males who maintained a low level of weight increase. Negative growth was not observed for the 2002 first spawning males during the 2000-2001 winter. For both 2001 and 2002 maturing males negative growth was not observed during the 2002 winter spawning season although weight increase was minimal. Negative growth was not observed for the females in any spawning season.

Following negative or minimal growth by the males over the winter months subsequent weight increase was observed through the summer months until the following winter. There was however, a continued divergence between both groups of males and the females.

Table .2.3.5. Mean weights (g ± SEM) of all groups of fish at Otter ferry and Loch Teacuis at each sample date

Group/date	Date 1	Date 2	Date 3	Date 4	Date 5	Date 6	Date 7	Date 8	Date 9	Date 10	Date 11	Date 12
All fish OF	405±5	648±7	742±6	1098±10	1615±13	2009±15	2179±16	3117±23	3981±34	4382±63	5414±78	5992±92
Females OF	399±6	640±9	734±10	1093±14	1618±22	2059±31	2254±36	3286±54	4378±70	5189±89	6705±118	7352±132
1st year males OF	452±13	717±19	825±22	1179±32	1699±48	1840±53	1813±54	2433±76	3041±95	2983±92	3375±105	3819±126
2nd year males OF	400±7	636±11	726±13	1080±19	1581±29	1979±40	2171±47	3054±68	3602±78	3430±76	3825±82	4326±94
All fish LT	444±5	589±7	659±6	1074±10	1338±13	1498±15	1575±16	2037±23	2649±34	3507±63	4004±78	4640±92
Females LT	445±7	591±9	659±8	1085±13	1351±17	1529±19	1634±21	2163±29	2889±45	4291±75	5031±89	5836±107
1st year males LT	472±12	619±18	686±15	1108±23	1383±29	1473±33	1403±31	1718±39	2134±50	2310±53	2514±60	2877±79
2nd year males LT	425±10	565±13	639±13	1028±21	1281±27	1444±31	1552±34	1960±44	2441±57	2516±57	2655±60	3086±74

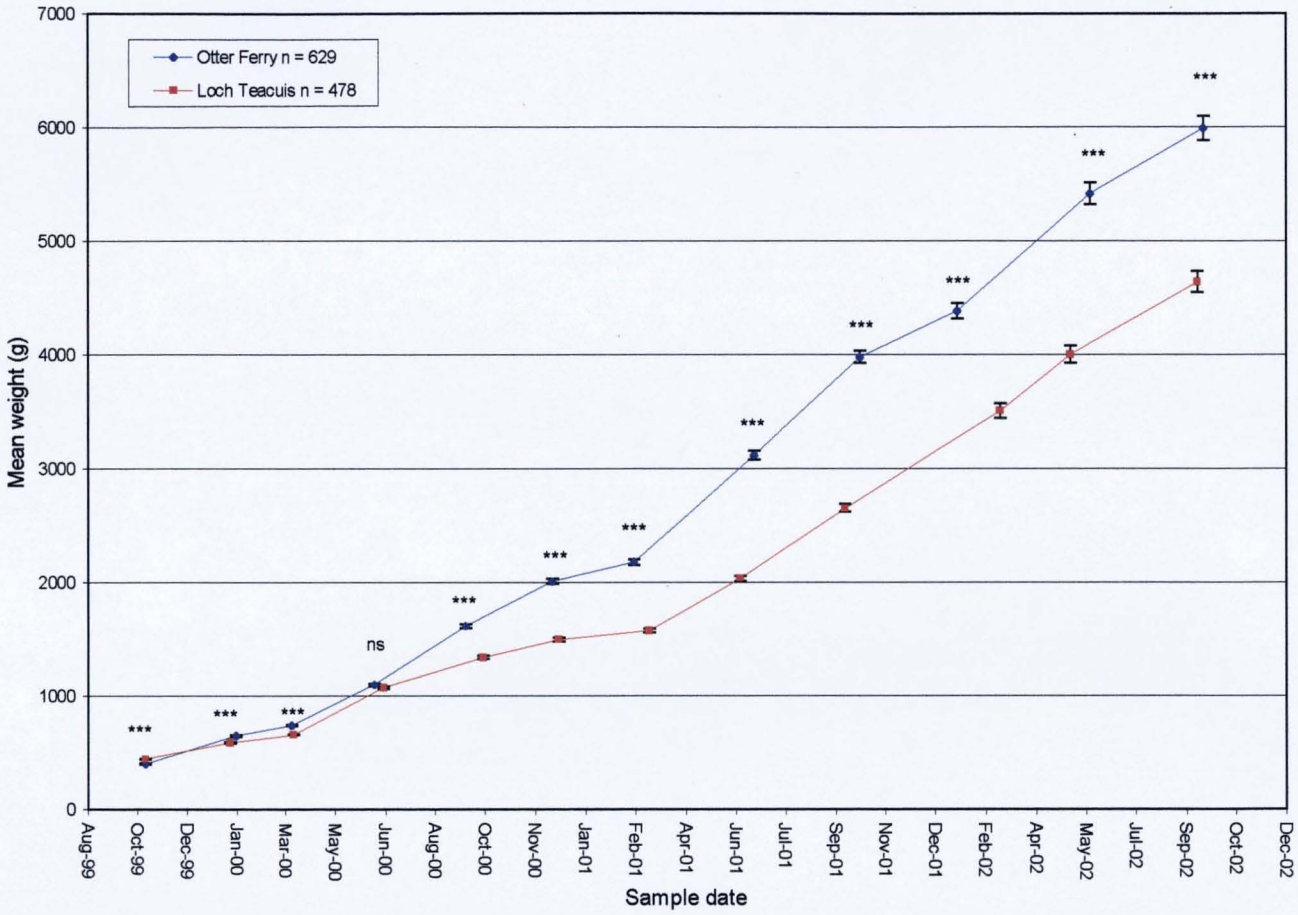


Figure 2.3.7 Mean weight (g) vs sample date of all fish at Otter Ferry and Loch Teacuis. Vertical lines indicate standard error of mean (S.E.M.). *Indicates significant differences in weight (one-way ANOVA) between corresponding sampling dates at each site.

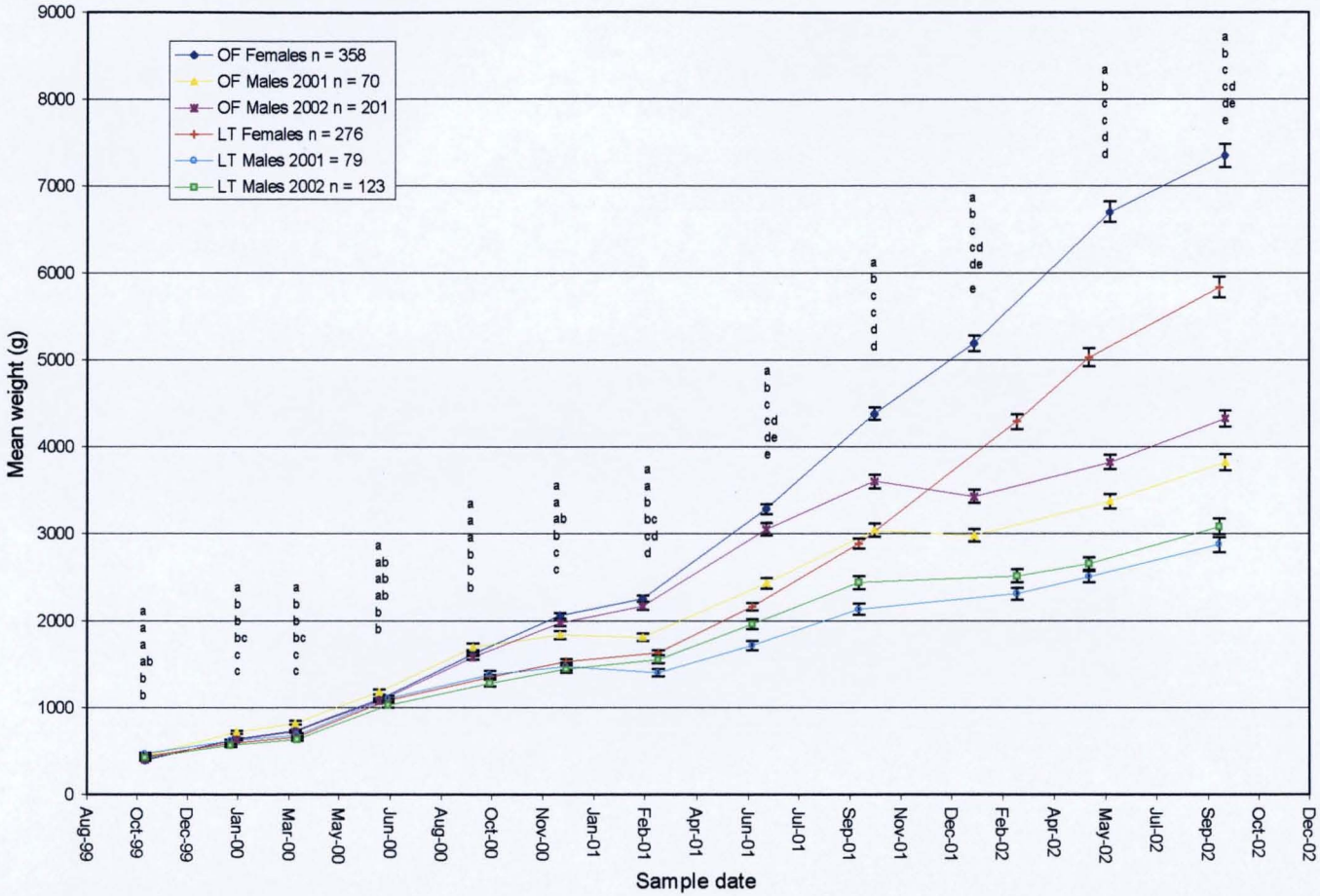


Figure 2.3.8 Mean weight (g) against time of all fish at Otter Ferry and Loch Teacuis. Vertical lines indicate standard error of mean (S.E.M.). Different letters denote significantly different groupings (one-way ANOVA) at each weighing period.

2.3.7 Specific Growth Rate (SGR) for all groups of fish at Otter Ferry and Loch Teacuis

SGR decreased with increasing weight for all groups throughout the trial (Figures 2.3.9 and 2.3.10). Growth decreases were all significant (Linear log regression P <0.05) with the exception of the females at Loch Teacuis, (P = 0.3) (Figure 2.3.11). The overall regression slopes (log SGR *vs* log intermediate weight) for each group were not significantly different (ANCOVA, $F_{5\ 50}$ = 1.308, $P.$ = 0.276) (Table 2.3.7). At the beginning of the trial all groups had SGR values of above between 0.3 and 0.6% day^{-1} and by the end of the trial the SGR value for all groups was around 0.1% day^{-1} (Figures 2.3.9 and 2.3.10). However, during the trial there were seasonal fluctuations in SGR between the different groups at each site

SGR between sexes and between different year class spawning groups of males varied with season and with maturation status. A drop in SGR values for all groups occurred during the winter months. Additionally during these months the lowest and, in some cases, negative SGR values were exhibited by the groups of males that were observed spawning.

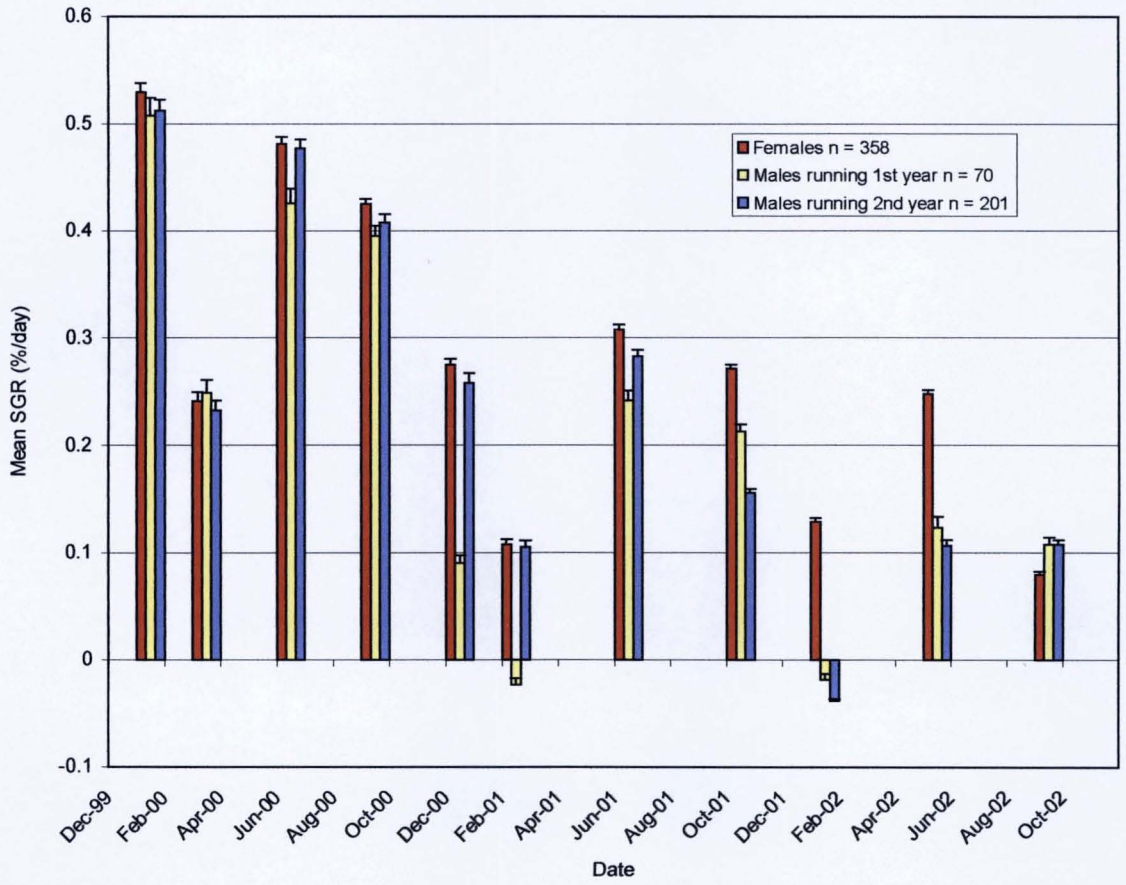


Figure 2.3.9 Mean Specific Growth Rates (SGR) at each sample date for females, males first observed maturing in 2001 and males first observed maturing in 2002 at Otter Ferry. Vertical lines indicate S.E.M.

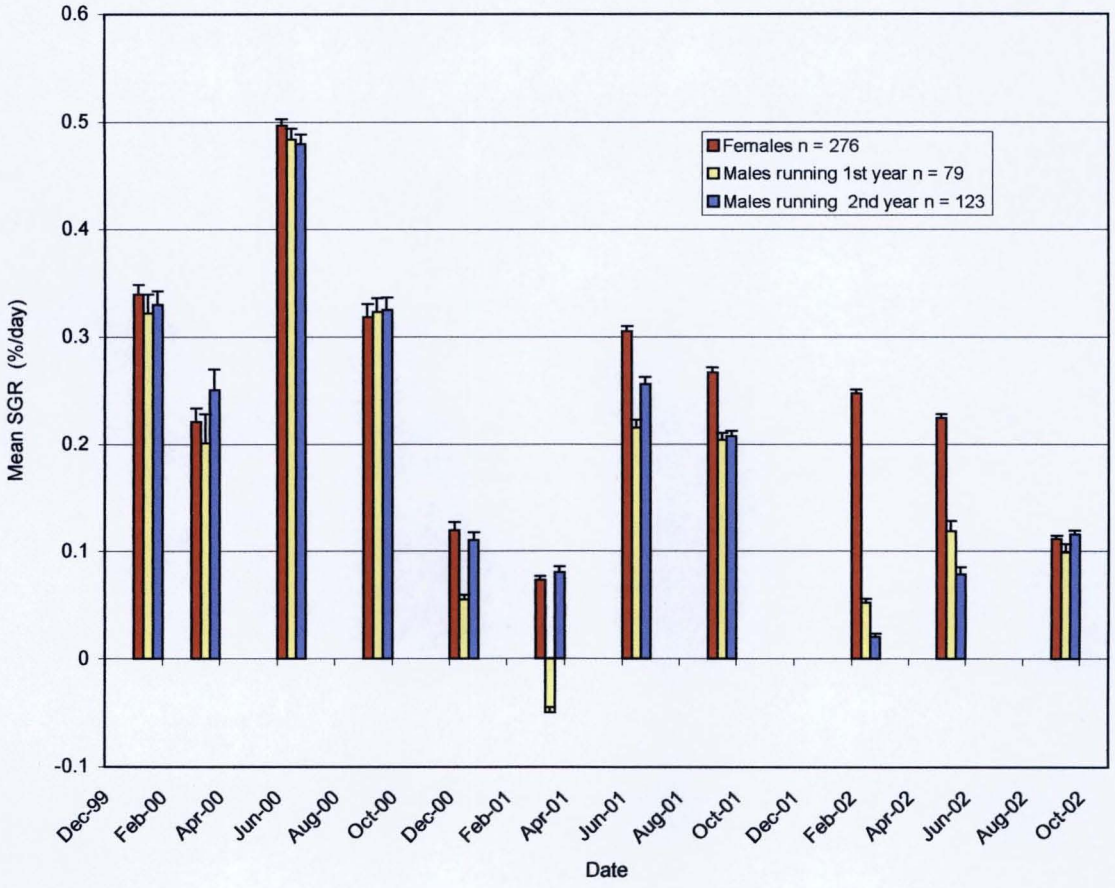


Figure 2.3.10 Mean Specific Growth Rates (SGR) at each sample date for females, males first observed maturing in 2001 and males first observed maturing in 2002 at Loch Teacuis. Vertical lines indicate S.E.M.

2.3.7.1 Interrelationship of size and specific growth rate

In order to determine the relationship between size (wt) and specific growth rate for halibut, SGR and mean intermediate weight data for all the groups at both sites, females, males first observed reaching maturity in 2001 and males first observed reaching maturity in 2002, was employed for curve fitting of the power form

$$SGR\ (\%day^{-1}) = aW^{b}$$

equivalent to the linearized form of the model:

Log SGR (% day-1) = Log a + b Log W (see Figure 2.3.11)

Calculated values for the slope coefficient b were found to be negative for all groups at each site (Table 2.3.6) There was no significant difference in the slopes for all the groups (ANCOVA, $F_{5\ 50}$ = 1.308, $P.$ = 0.276, Table 2.3.7). A fully factorial ANCOVA was then performed indicating that group had no significant effect on the change of SGR with increasing weight. (ANCOVA, $_{F5\ 50}$ = 2.291, P = 0.058, Table 2.3.8). As a result pooled values for all the fish at both could be used to calculate values of a and b. The derived values were : (a) = 11.35 ± 4.68 SE and (b) = -0.53 ± 0.21 SE. The near significant result is however, reflected by the differing trajectories of both female groups compared to all the male groups (Figure 2.3.11). The resulting mathematical relationship of SGR ($\%day^{-1}$) = $11.35W^{-0.53}$, when used to predict weight increase with time for the individual groups, slightly underestimated the final weight on every occasion. A more accurate fit for the individual groups was obtained by applying a von Bertalanffy growth curve to the weight-at-age date (Section 2.3.8)

Table 2.3.6 Value of the parameters a and b from the mathematical form:- log SGR = log a + b log W

Site	Sex	Year Matured	Mean weight range (g)	(a)	SE	(b)	SE	r^2	*P* (slope)
OF	All	n/a	398–7352	24.49	± 5.00	-0.63	± 0.21	0.49	<0.05
OF	F	n/a	398–7352	8.52	± 3.71	-0.47	± 0.17	0.45	<0.05
OF	M	2001	452-3819	293.76	± 10.76	-0.99	± 0.32	0.58	<0.05
OF	M	2002	400-4326	123.59	± 8.77	-0.86	± 0.29	0.53	<0.05
LT	All	n/a	425-5836	3.68	± 7.56	-0.40	± 0.27	0.19	>0.05
LT	F	n/a	444-5836	1.36	± 5.61	-0.25	± 0.23	0.11	>0.05
LT	M	2001	471-2876	96.38	± 15.35	-0.89	± 0.37	0.41	<0.05
LT	M	2002	425-3086	178.24	± 16.44	-0.98	± 0.38	0.42	<0.05
OF +LT	All	n/a	398-7352	11.35	± 4.68	-0.53	± 0.21	0.43	<0.05

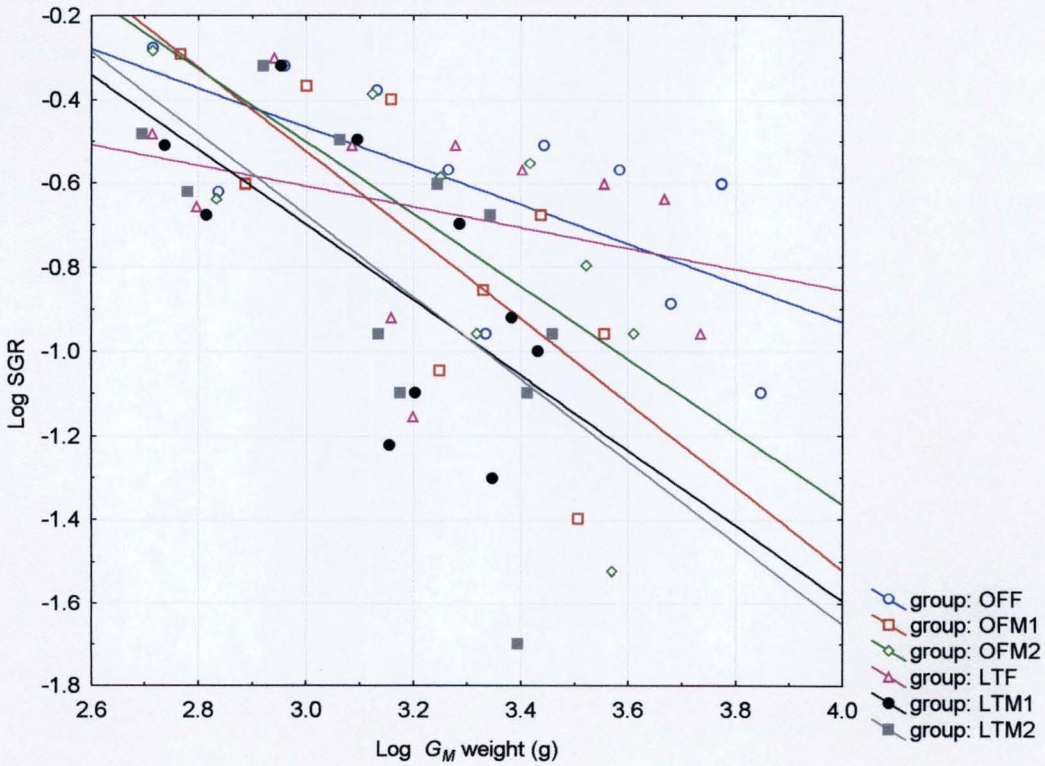


Figure 2.3.11

Log specific growth rate *v* geometric mean G_M weight of all groups of halibut at Otter Ferry and Loch Teacuis.
(OFF) logSGR= 0.93-0.46log*W*, r^2 = 0.45, *P*=0.02, n=11,, (OFM1) logSGR= 2.46-0.99log*W*, r^2 = 0.58, *P*=0.01, n=10, (OFM2) logSGR= 2.09-0.86log*W*, r^2 = 0.52, *P*=0.01, n=11, (LTF) logSGR= 0.13-0.24log*W*, r^2 = 0.11, *P*=0.3, n=11, (LTM1) logSGR= 1.98-0.89log*W*, r^2 = 0.41, *P*=0.04, n=9, (LTM2) logSGR= 2.25-0.97log*W*, r^2 = 0.42, *P*=0.03, n=10

Table 2.3.7 Analysis of covariance (ANCOVA) for the regression of specific growth rate (SGR, % d ay^{-1} against Log geometric mean weight (G_M g) for all groups of fish) for the determination of homogeneity of slopes

Source of variation	Type III sum of squares	d.f.	Mean square	*F*-ratio	*P*
Main effect Group (G)	.379	5	0.076	1.091	.377
Covariate Log G_M (W)	2.567	1	2.567	36.948	<0.01
Interaction (G x W)	.455	5	0.091	1.308	.276
Error	3.474	50	0.069		

Table 2.3.8 Analysis of covariance (ANCOVA) for the regression of specific growth rate (SGR, % d ay^{-1} against Log geometric mean weight (G_M g) for all groups of fish

Source of variation	Type III sum of squares	d.f.	Mean square	*F*-ratio	*P*
Main effect Group (G)	.818	5	0.164	2.291	0.058
Covariate Log G_M (W)	2.277	1	2.277	31.871	<0.01
Error	3.929	55	0.071		

2.3.8. GF3 Growth Factor

Temperature and weight differences can be better accounted for using the GF3 calculation: $W_2 = [\sqrt[3]{W_2} + [(TGC/1000) \times (\Sigma T)]]^3$

Large variations in GF3 growth rate were observed during the trial period for all groups which corresponded to both seasonal fluctuations (females and non spawning groups of males) and maturation status (spawning male groups at each site) Values ranged from a maximum of 2.0 to a minimum of –0.26(Figure 2.3.12).

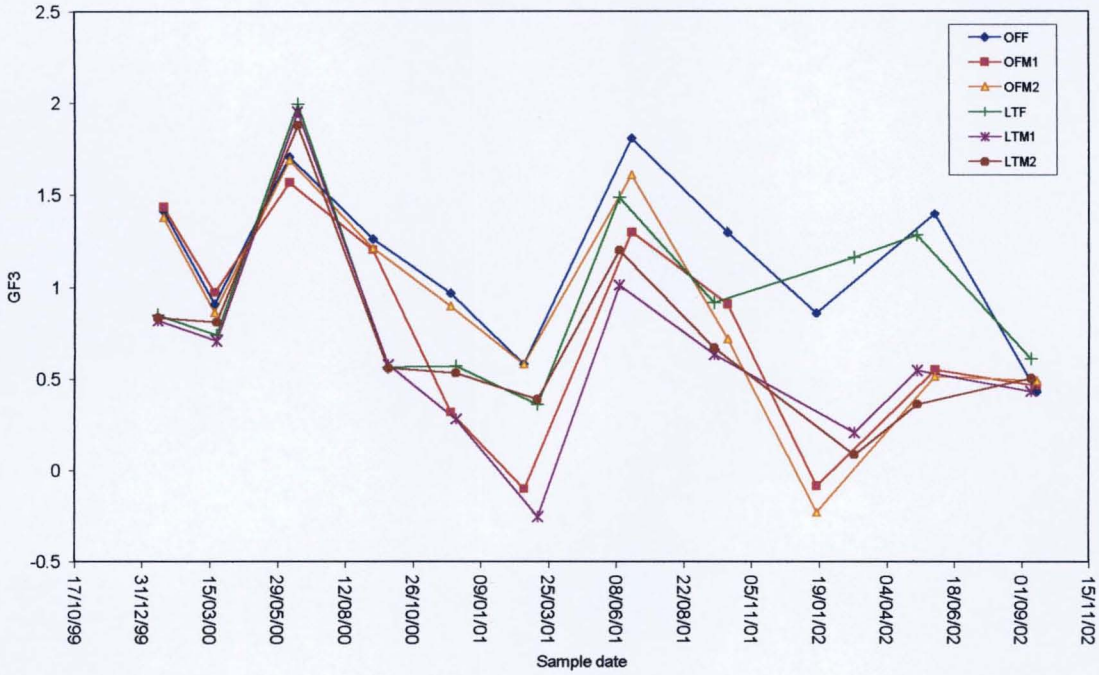


Figure 2.3.12 GF3 profiles for each group of fish n= 358, 70 & 201 for OF females, 1st yr males and 2nd year males. n= 276, 79 & 123 for LT females, 1st yr males and 2nd year males.

Significant differences ($P<0.001$) in the GF3 profiles were observed between both site and sex (repeated measures ANOVA). There was however no interaction between the two factors (Table 2.3.9). Post hoc comparisons revealed a similarity between the profiles of the LTM2 and the OFM1 groups. All other groups were significantly different.

Table 2.3.9 Repeated measures ANOVA for GF3 with sex and site as factors

Source of variation	Type III sum of squares	d.f.	Mean square	*F*-ratio	*P*
Effect Sex (Sx)	247.7	2	123.86	410.95	<0.001
Effect Site (S)	61.51	1	61.51	204.08	<0.001
Site * Sex	0.701	2	0.351	1.16	0.31
Error	331.84	1101	0.301		

2.3.9. Fitting of VonBertalanffy growth model.

Von Bertalanffy growth curves were fitted to age at length data. Values for the asymptotic length (L_T), the growth coefficient (K) and the theoretical age at zero length (t_0) were determined using the FISAT II program. The asymptotic weight (W_T) was subsequently determined from the from the length–weight relationship $W = aL^b$ The resulting parameters (Table 2.3.10) were used to fit the von Bertalanffy curve in terms of weight:

$$Wt = W_T (1-\exp[-K(t-t_0)])^3$$

to all groups at each site (Figures 2.3.13)

Growth performance indices Φ and Φ' were calculated for length and weights of all the groups of fish (Table 2.3.10): -

$$\Phi = \log_{10} (K) + 2/3 \log_{10} (Wt\ T)$$

$$\Phi' = \log_{10} (K) + 2 \log_{10} (LT)$$

Table 2.3.10 Parameters for Von Bertalanffy growth curves and growth performance indicators Φ' and Φ; based on length and wt.

Parameters	Otter Ferry females	Otter Ferry males yr 1	Otter Ferry males year 2	Teacuis females	Teacuis males yr 1	Teacuis males yr 2
Wt T (g)	40633	5558	6874	48263	3987	4463
LT (cm)	1354 ± 285	727± 37.2	775± 46.5	1406± 822	657± 38.5	682± 31.2
K	0.21± 0.08	0.52 ± 0.1	0.53 ± 0.1	0.15 ± 0.14	0.51± 0.12	0.52 ± 0.09
$t_{(0)}$ (yr^{-1}.)	0.39 ± 0.19	0.42 ± 0.17	0.63 ± 0.15	-0.04 ± 0.5	0.21 ± 0.24	0.38 ± 0.15
r^2	0.98	0.98	0.97	0.97	0.97	0.98
Φ' *(for L)*	5.585	5.439	5.503	5.487	5.343	5.384
Φ *(for wt)*	2.395	2.213	2.282	2.315	2.108	2.149

Whilst not used in this study for any statistical analysis the magnitude of the values Φ' and Φ were in general accordance with the actual final weight rankings of the groups in the trial.

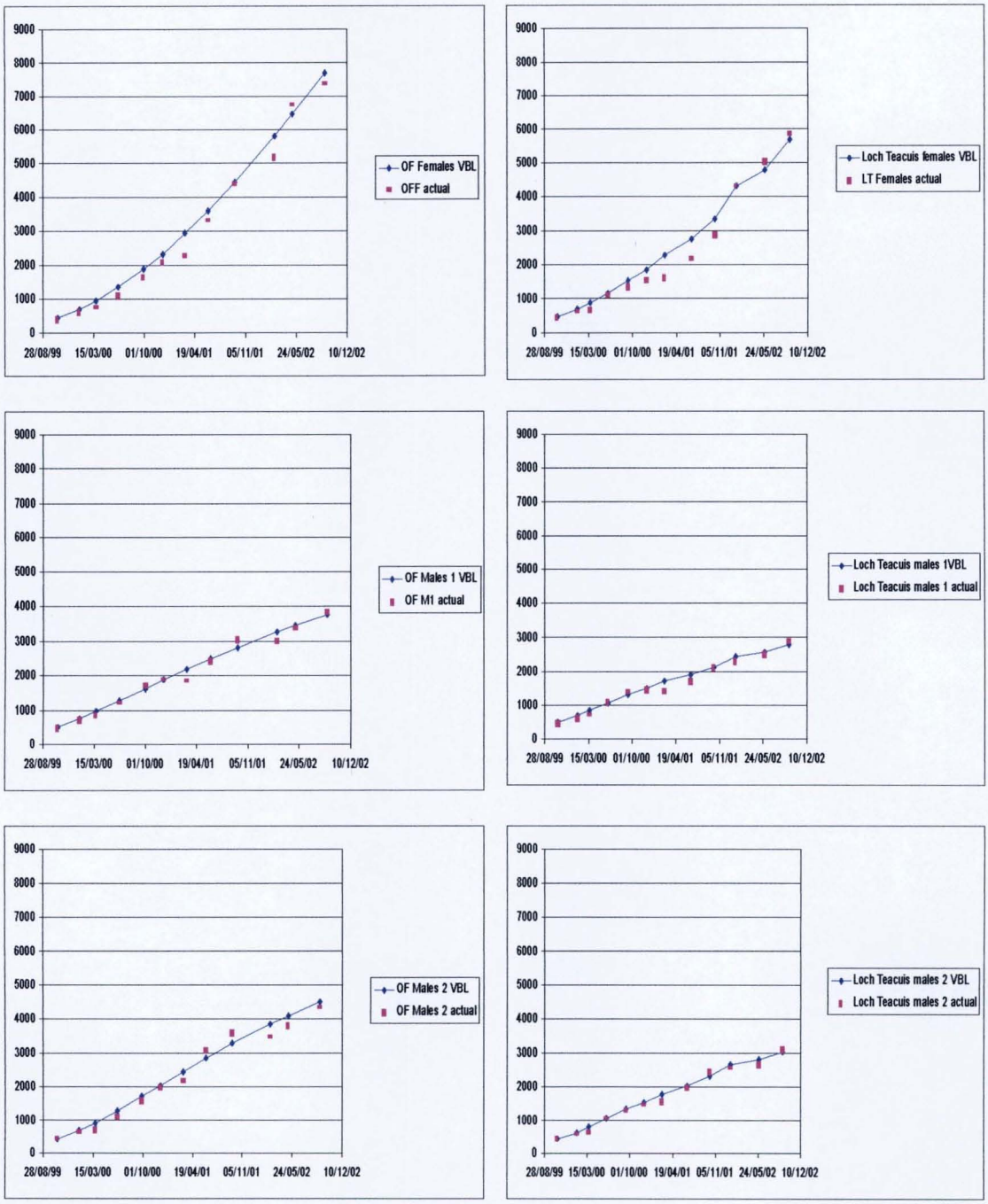


Figure 2.3.13 Von Bertalanffy growth curves fitted to weight data for all groups at Otter Ferry and Loch Teacuis. Each point represents the mean weight at each sample date. n= 358, 70 & 201 for OF females, 1st yr males and 2nd year males. n= 276, 79 & 123 for LT females, 1st yr males and 2nd year males.

2.3.10 Length weight relationship

Collected weight – length data was fitted to the mathematical form:

$$W = aL^b$$

or in its Logarithmic form:-

$$\text{Log } W = \text{Log } a + b \text{ Log } L$$

The derived value of b (slope), the growth coefficient was calculated for each group of fish at each site. The regression coefficients for all groups of fish, both males and females, at each site were not significantly different (ANCOVA, P=0.358)(Table 2.3.11 Figure 2.3.14). Thus a common value of a: -5.334 (±0.048 SE) and b: 3.16 (±0.003SE), could be calculated from the regression line of log wt *vs* log length of all fish at both Otter Ferry and Loch Teacuis at all sample dates (n = 16416, r^2 = 0.99) which gave the power weight : length relationship:-

$$W = 0.0000046\, L^{3.16}$$ (see Figure 2.3.15)

Table 2.3.11 Analysis of covariance (ANCOVA) for the regression of Log length against Log weight for all groups of fish to the determination of homogeneity of slopes

Source of variation	Type III sum of squares	d.f.	Mean square	*F*-ratio	*P*
Main effect Group (G)	.0.00065	5	0.0001	1.14	.351
Covariate Length (L)	6.539	1	6.539	57334.93	<0.01
Interaction (G x L)	.0.00064	5	0.00013	1.12	0.358
Error	0.007	60	0.00011		

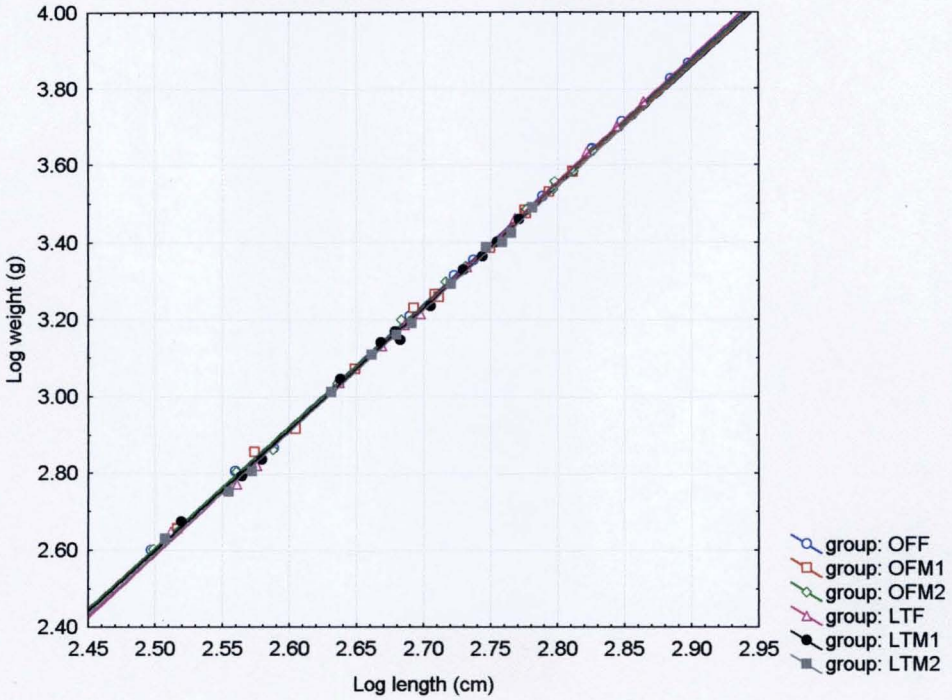


Figure 2.3.14 Log weight vs log length plot and regressions for all groups at both sites

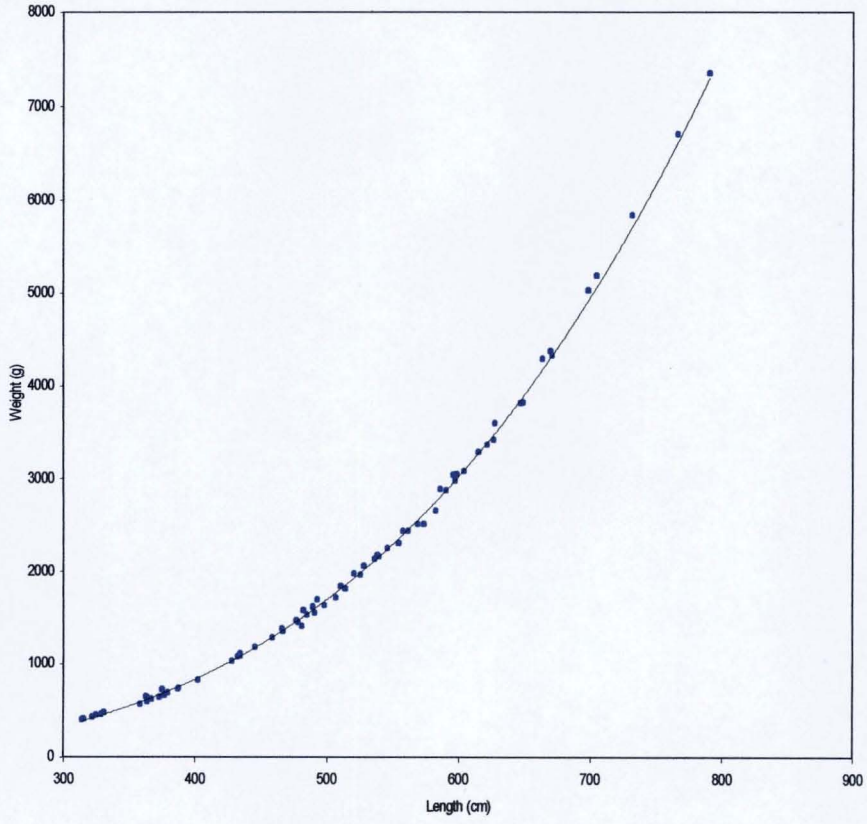


Figure 2.3.15 Power form $W = 0.0000046\,L^{3.16}$ fitted to actual weight – length data for all groups

2.3.11 Growth type

The isometry/allometry exponent b (3.16) was found to be significantly different from the isometric value 3 (t test $P<0.05$) therefore the growth type was determined as being positively allometric.

2.3.12 Condition factor

All groups exhibited an increase (linear regression, $P<0.05$, Figure 2.3.16) in condition factor with increasing length throughout the trial. Confirming the uniformity in length/weight data for all the groups there was no significant difference in the regressions of length and condition factor for all the groups (ANCOVA, $F_{5\ 60} = 0.875$, $P = 0.504$) (Table 2.3.12). A fully factorial ANCOVA was then performed indicating that site had a significant effect on the relationship between condition factor (ANCOVA, $_{F2\ 65} = 4.73$, $P = 0.03$). There was no overall significant effect of sex on condition factor (ANCOVA, $_{F2\ 65} = 1.5$, $P = 0.23$). Additionally there was no significant interaction between site and sex (Table 2.3.13) however, *post hoc* comparisons did reveal differences between the LTM2 fish and all other groups except the LTM1 fish. The LTM1 group also differed from the OFF group.

The males maturing in the 1st year had the lowest r^2 values followed by the 2nd year males, with the females having the highest values. These differences are a reflection of the seasonal and maturation status differences in condition factor between the groups. Prior to spawning the males had their highest condition factor with the lowest occurring post spawning. Seasonal based differences were observed for the females with the lowest values corresponding to the periods when water temperatures were lowest (Figures 2.3.17-18).

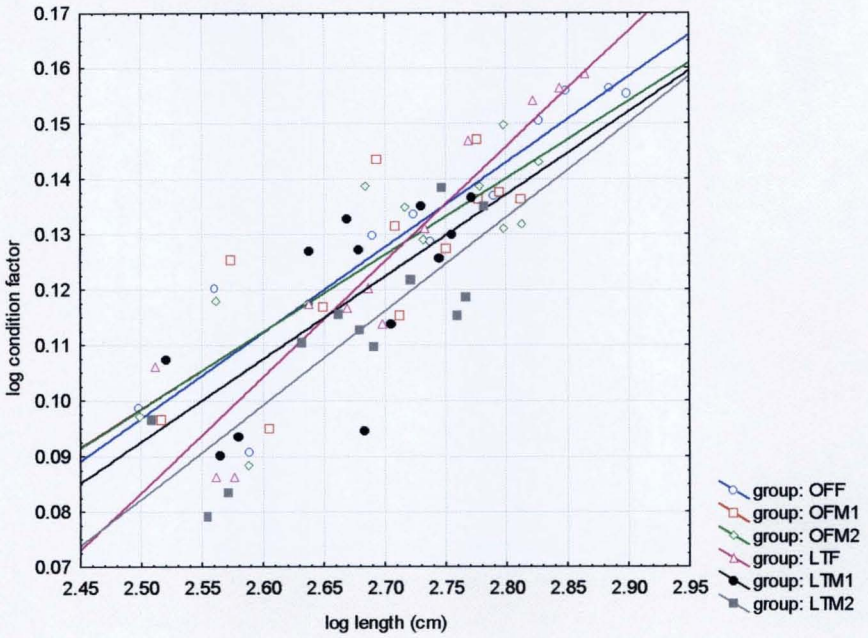


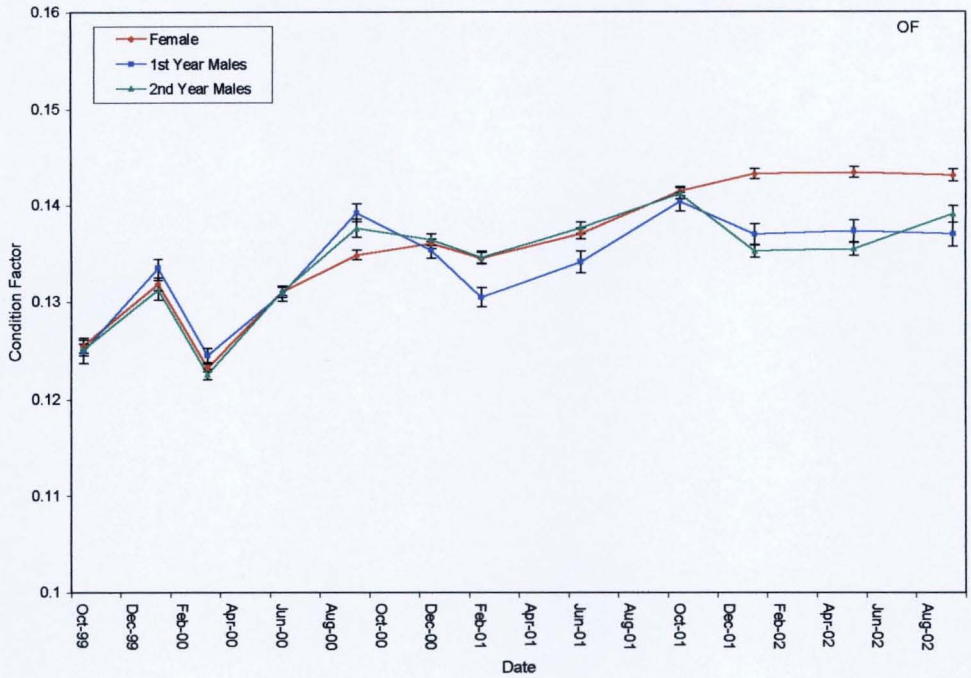
Figure 2.3.16 The relationship between log length and log condition factor (*K*) for all groups (OFF) K=-0.28+0.154L, $r^2 = 0.87$, (OFM1) K=-0.248+0.138L, $r^2 = 0.58$, (OFM2) K=-0.25+0.139L, $r^2 = 68$, (LTF) K=-0.437+0.208L, $r^2 = 0.87$, (LTM1) K=-0.279+0.149L, $r^2 = 0.48$, (LTM2) K=-0.34+0.169L, $r^2 = 73$. n = 12 all groups

Table 2.3.12 Analysis of covariance (ANCOVA) for the regression of condition factor against length for all groups of fish for the determination of homogeneity of slopes

Source of variation	Type III sum of squares	d.f.	Mean square	*F*-ratio	*P*
Main effect Group (G)	0.0053	5	0.0011	1.058	.393
Covariate Length (L)	0.146	1	0.146	145.05	<0.01
Interaction (G x L)	0.0044	5	0.00088	0.875	.504
Error	0.06	60	0.001		

Table 2.3.13 Analysis of covariance (ANCOVA) for the regression of condition factor against length for all groups of fish

Source of variation	Type III sum of squares	d.f.	Mean square	*F*-ratio	*P*
Covariate Log Length (L)	0.0188	1	0.0188	170.63	<0.01
Effect Sex (Sx)	0.00033	2	0.000166	1.50	0.231
Effect Site (S)	0.00052	1	0.000522	4.73	0.033
Site * Sex	0.00021	2	0.00011	0.96	0.39
Error	0.065	65	0.001		



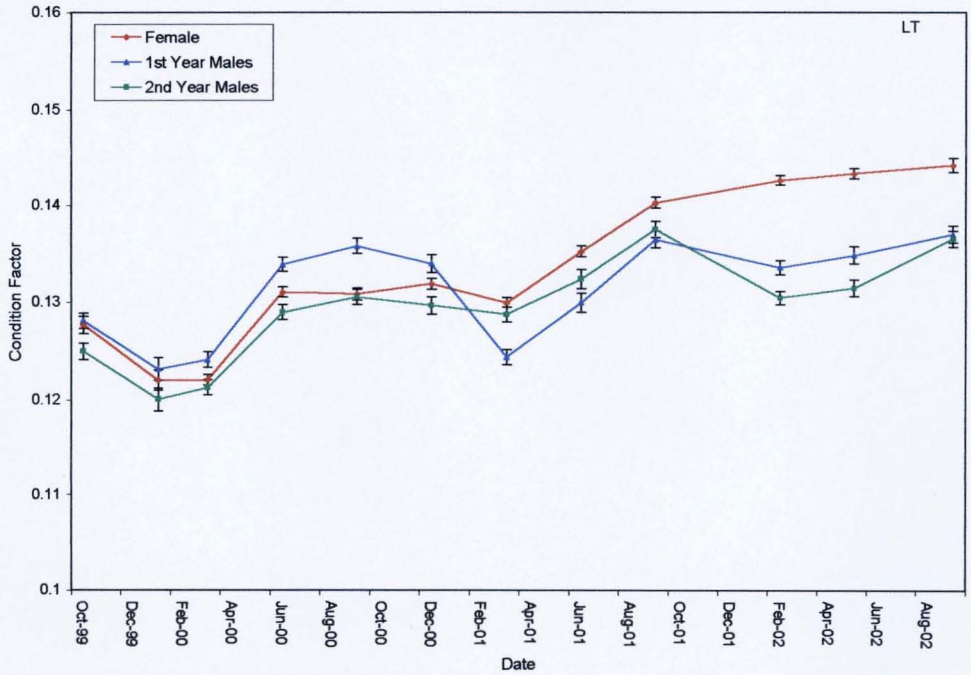


Figure 2.3.17 and 18 Mean condition factors for females, males first observed reaching maturity in 2001 and males first observed reaching maturity in 2002 at Otter Ferry and Loch Teacuis. Vertical lines indicate S.E.M. n= 358, 70 & 201 for OF females, 1st yr males and 2nd year males. n= 276, 79 & 123 for LT females, 1st yr males and 2nd year males.

2.3.13 Size distributions and ranks

There were slight increases (log regression) in the coefficient of variation (CV) (CV = ratio of standard deviation to the mean wt expressed as a percentage) for all groups of fish at both sites except for the Loch Teacuis males maturing in the 1st winter, which exhibited a slight decrease ($P<0.05$ for all Otter Ferry groups and females at Loch Teacuis)(Figure 2.3.19).

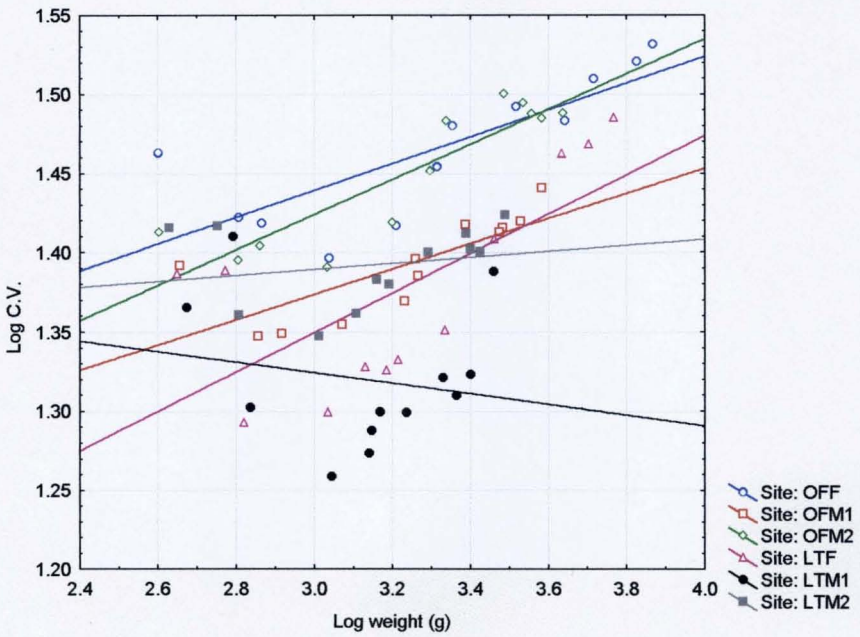


Figure 2.3.19 Log regression of C.V.against weight for all groups of fish. (OFF) CV=1.18+0.085 log *W*, r^2=0.63, P<0.05, (OFM1) CV=1.14+0.08 log *W*, r^2=0.58, P<0.05, (OFM2) CV=1.09+0.111 log *W*, r^2=0.78, P<0.05, (LTF) CV=0.98+0.124 log *W*, r^2=0.47, P<0.05, (LTM1) CV=1.42-0.033 log *W*, r^2=0.034, P>0.05, (LTM2) CV=1.33+0.019 log *W*, r^2=0.05, P>0.05, (n=12 all groups).

There was a moderate degree of size rank correlation (final weight against initial weight) within all groups at each site (Spearman rank correlation, $r_s > 0.33$, $P<0.01$). A strong size rank correlation, $r_s > 0.5$, $P<0.01$, became apparent for all groups when sample date 3 mean weights were compared to the final weights.

2.3.14 Blood Plasma Parameters

2.3.14.1 Site differences between cortisol, osmolality, chloride and glucose

All blood samples were taken within the same time period, between 8am and 11am on each sampling date. Identical sampling practices were maintained throughout the trial and the method of storage of blood samples from each date at each site was identical. For each parameter measured there was little variation between sites for the mean of all samples taken during the course of the trial (Figure 2.3.20).

No significant difference (t test, $P>0.05$) was found for total mean cortisol (19.76±1.22ng/ml Otter Ferry and 22.96±1.28 ng/ml Loch Teacuis) or total mean osmolality (389.5±5.3 mOsm/kg Otter Ferry and 387.1±5.6 mOsm/kg Loch Teacuis). Significant differences (t test $P<0.05$) between both total mean chloride (155.8±1.5 mmol/l Otter Ferry and 163.6±2.17 mmol/l Loch Teacuis) and total mean glucose (28.6±1.33mg/dL Otter Ferry and 43.7±2.39mg/dL Loch Teacuis) levels at each site were however found.

Comparisons of site over time revealed no significant differences in cortisol, osmolality or chloride ($P>0.05$). A significant difference in glucose levels between the sites was in evidence ($P<0.05$)(GLM repeated measures ANOVA) (Figures 2.3.21 – 22)(Tables 2.3.14-17)

Table 2.3.14 Repeated measures ANOVA, Cortisol levels at Otter Ferry and Loch Teacuis

Effect	SS	df.	MS	*F*	*P*
Site	12.38	1	12.38	0.36	0.56
Error	343.84	10	34.38		

Table 2.3.15 Repeated measures ANOVA, Osmolality levels at Otter Ferry and Loch Teacuis

Effect	SS	df.	MS	*F*	*P*
Site	1293	1	1293	1.17	0.304
Error	11061	10	1106		

Table 2.3.16 Repeated measures ANOVA, Chloride levels at Otter Ferry and Loch Teacuis

Effect	SS	df.	MS	*F*	*P*
Site	362	1	362	2.56	0.14
Error	1412	10	141		

Table 2.3.17 Repeated measures ANOVA, Glucose levels at Otter Ferry and Loch Teacuis

Effect	SS	df.	MS	*F*	*P*
Site	4584.73	1	4584.73	8.90	0.02
Error	3604.80	7	514.97		

2.3.14.2 Cortisol, osmolality, chloride and glucose levels for fish in tanks at Otter Ferry.

Linear regression of Figure 2.3.21 revealed no significant trends in cortisol, osmolality, chloride or glucose (P>0.05) during the trial at Otter Ferry. However, the intermittent and one off nature of the blood sampling procedure makes it difficult to draw any firm conclusions regarding long or short term trends. Mean cortisol concentrations ranged from 38.95 – 6.58 ng/ml. Mean osmolality ranged from 479.2 – 334.6 mOsm kg^{-1}. Mean chloride concentrations ranged from 180 – 136.4 mmol/l. Mean glucose concentrations ranged from 55 – 18.5 mg/dL. As expected variations in chloride concentrations were associated with variations in osmolality.

2.3.14.3 Cortisol , osmolality, chloride and glucose levels for fish in cages at Loch Teacuis

Linear regression analysis of Figure 2.3.22 revealed there to be a slight increase in cortisol levels with time P<0.05 whereas slight decreases were noted for osmolality and glucose P<0.05 although r^2 values were low (0.56, 0.4 and 0.4 respectively). Chloride showed no change with time P=0.219. As with Otter Ferry, the intermittent and one off nature of the blood sampling procedure again make it difficult to draw any firm conclusions regarding long or short term trends. Mean cortisol concentrations ranged from 52.96 – 6.6 ng/ml. Mean osmolality ranged from 485 – 329.85 mOsm kg^{-1}. Mean chloride concentrations ranged from 207 – 133.3 mmol/l. Mean glucose concentrations ranged from 86.86 –19.68 mg/dL. As expected variations in chloride concentrations were associated with variations in osmolality.

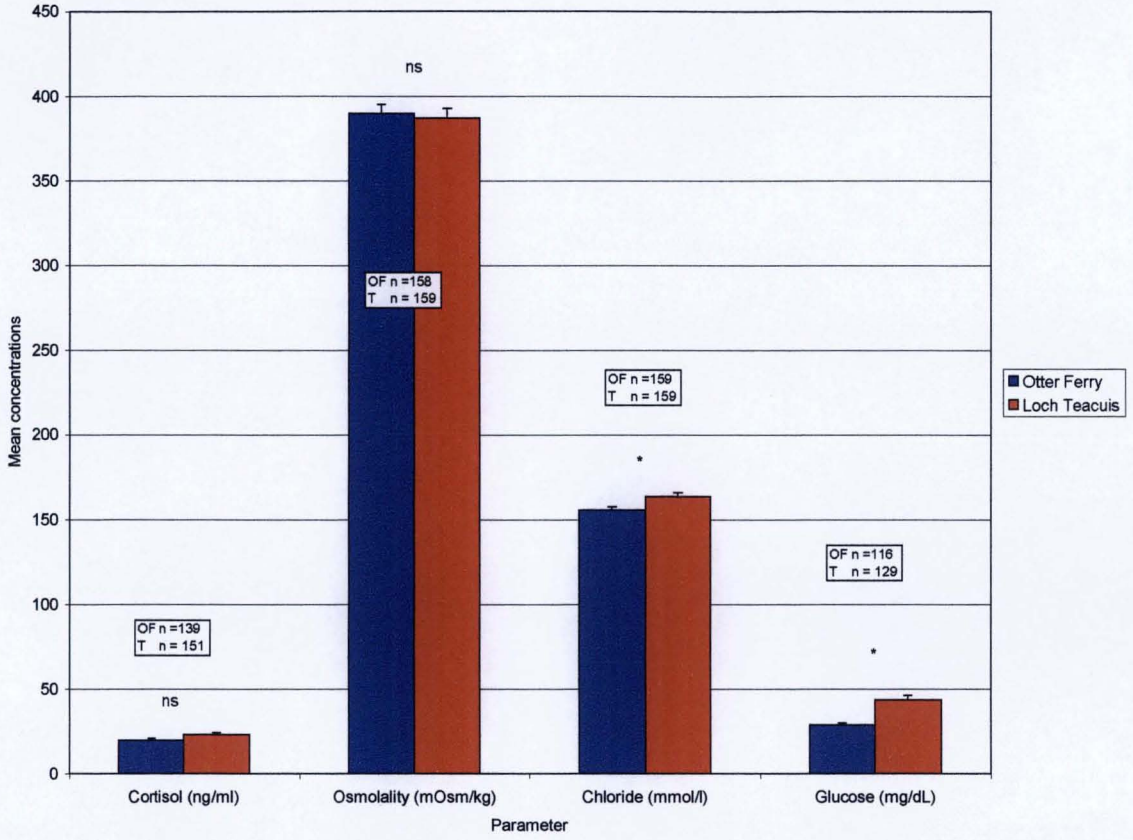


Figure 2.3.20 Comparison of mean cortisol, Osmolality, chloride and glucose concentrations from all blood samples taken at Otter Ferry and Loch Teacuis. between 18/01/2000 and 18/09/2002. Vertical lines indicate S.E.M. * indicates significant a difference ($p<0.05$) between values at each site

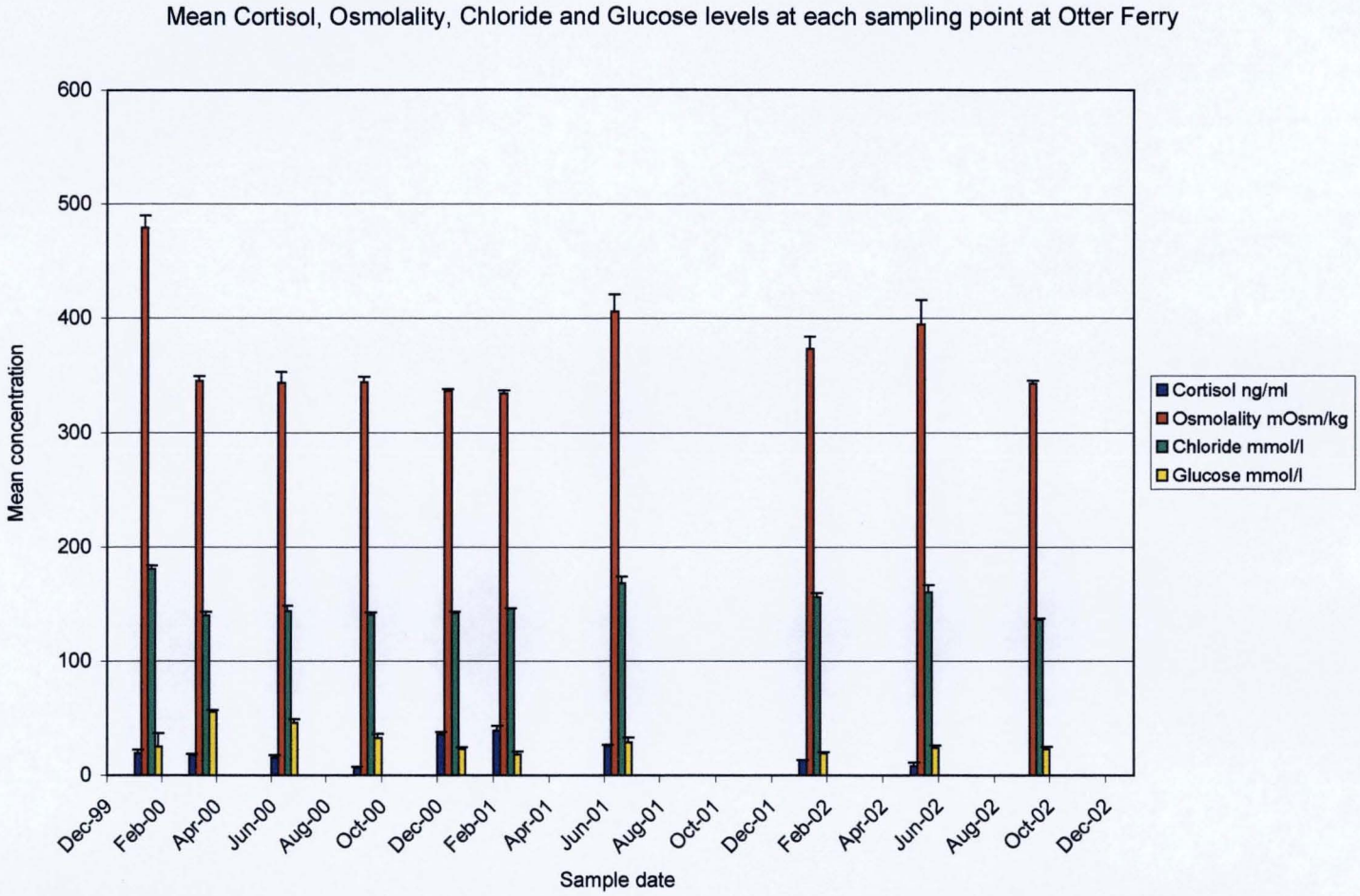


Figure 2.3.21 Mean cortisol, osmolality, chloride and glucose concentrations from fish at Otter Ferry at each sampling point between 24/01/2000 and 18/09/2002. Vertical lines indicate S.E.M. n values at each respective sample point were: for cortisol – 18,5,4,12,10,10,17,15,12, osmolality and chloride- 18,5,4,12,10,10,17,15,12,10. and glucose – 6,5,4,12,10,9,16,15,12,10.

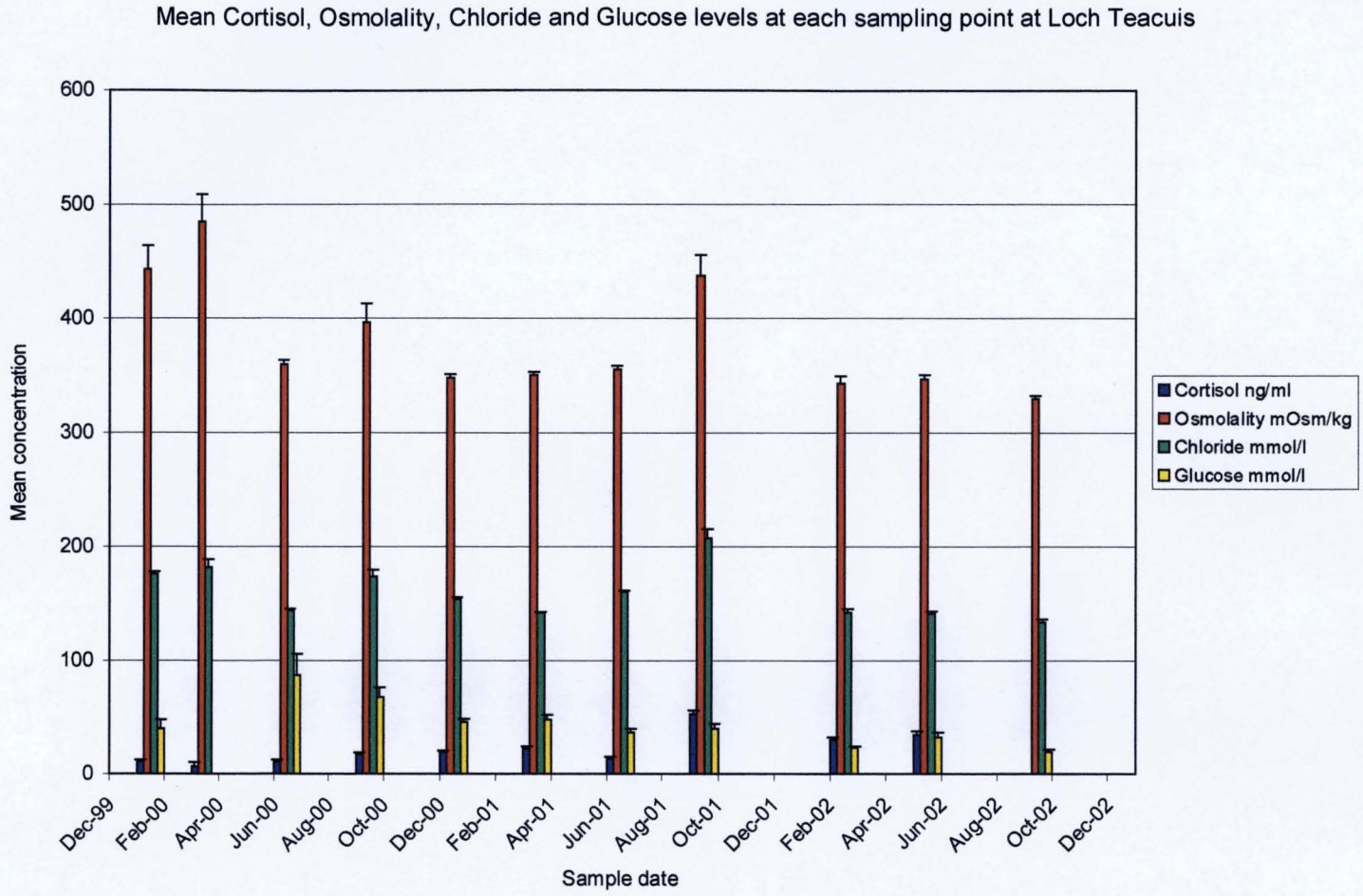


Figure 2.3.22 Mean cortisol, osmolality, chloride and glucose concentrations from fish at Loch Teacuis at each sampling point between 18/01/2000 and 12/09/2002. Vertical lines indicate S.E.M. . n values at each respective sample point were: for cortisol – 24,8,8,20,10,19,20,20,10, osmolality and chloride- 24,8,8,20,10,19,20,20,10,10 and glucose – 5,na,7,19,10,19,20,19,10,10,10.

2.3.15 Ultrasound

It was relatively easy to differentiate between the ultrasound images of the ovaries and testes through a combination of differences in shape, shade and texture. The ovaries, particularly in the older larger halibut, developed further in an anterior direction along the body than the male testes (Figures 2.3.23, 2.3.24 & 2.3.25). The resulting cross-section visible on the screen decreased steadily in size the further back the transducer was moved along the body (Figures 2.3.26 – 2.3.29). With the males, the cross-section of the testes finished much more abruptly.

The shade and texture of the ovaries were considerably lighter and more granular then the much darker immature testes due to the greater density of the ovaries and the higher water content of the testes. Mature male testes were considerably larger than the immature testes and lighter due to the presence of spermatozoa (Figures 2.3.30 & and 2.3.31)

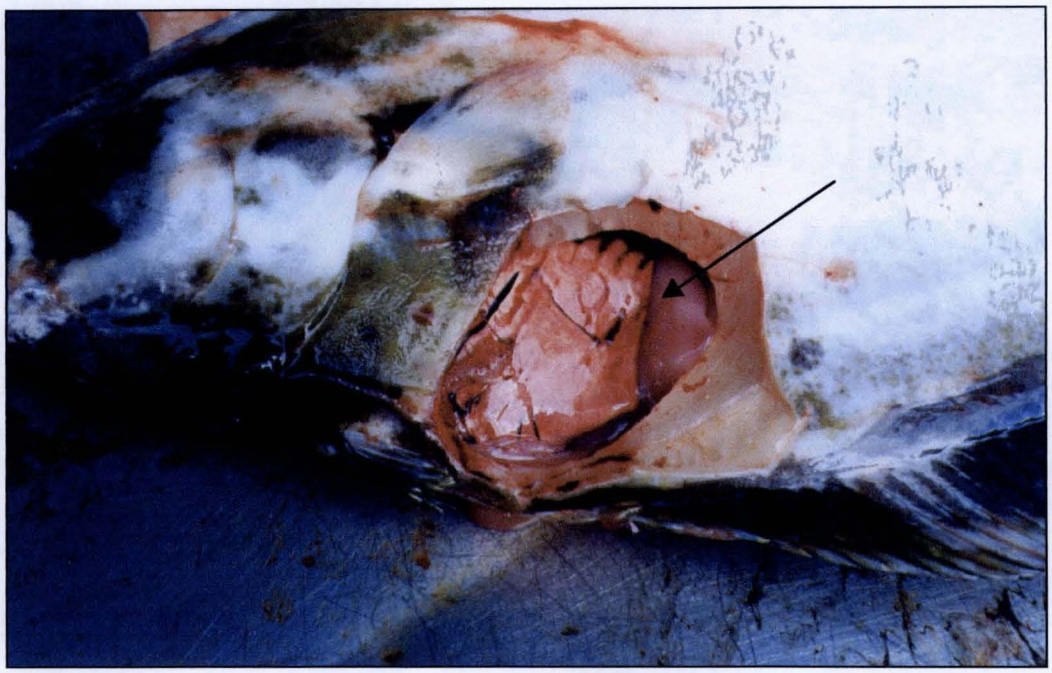


Figure 2.3.23 Testis of halibut (arrowed) showing extent of anterior ingress.

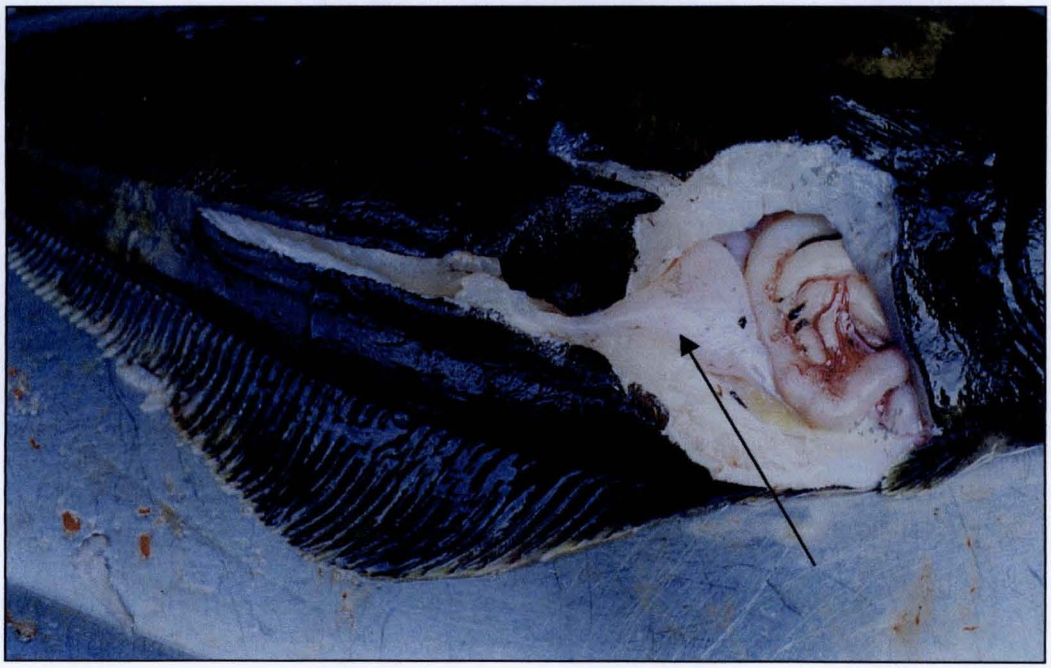


Figure 2.3.24 Ovary of halibut (arrowed) showing a greater degree of anterior growth than testis (Figs 2.3.23 & 25).

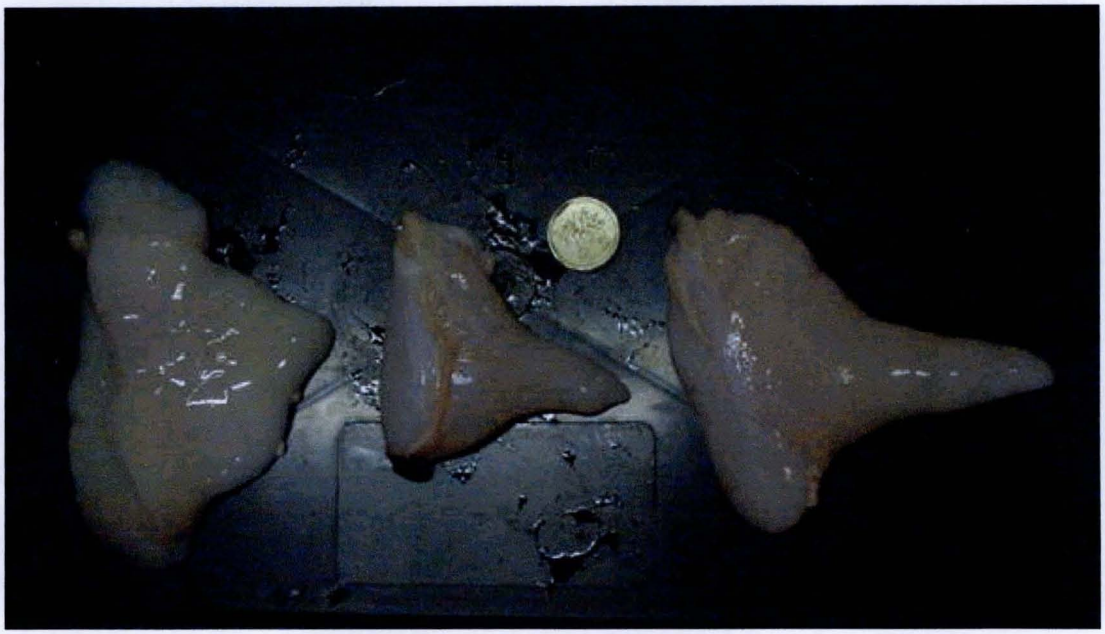
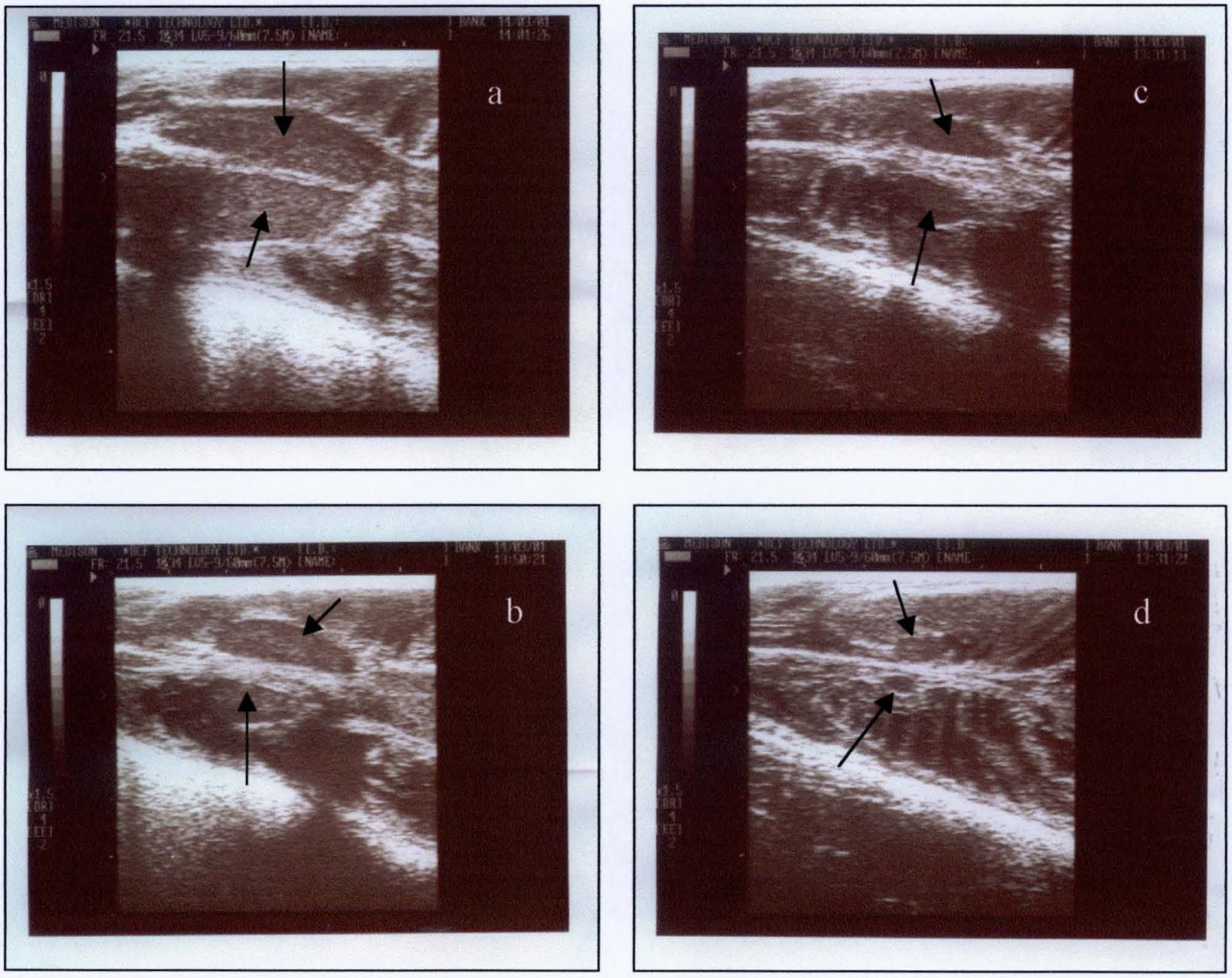
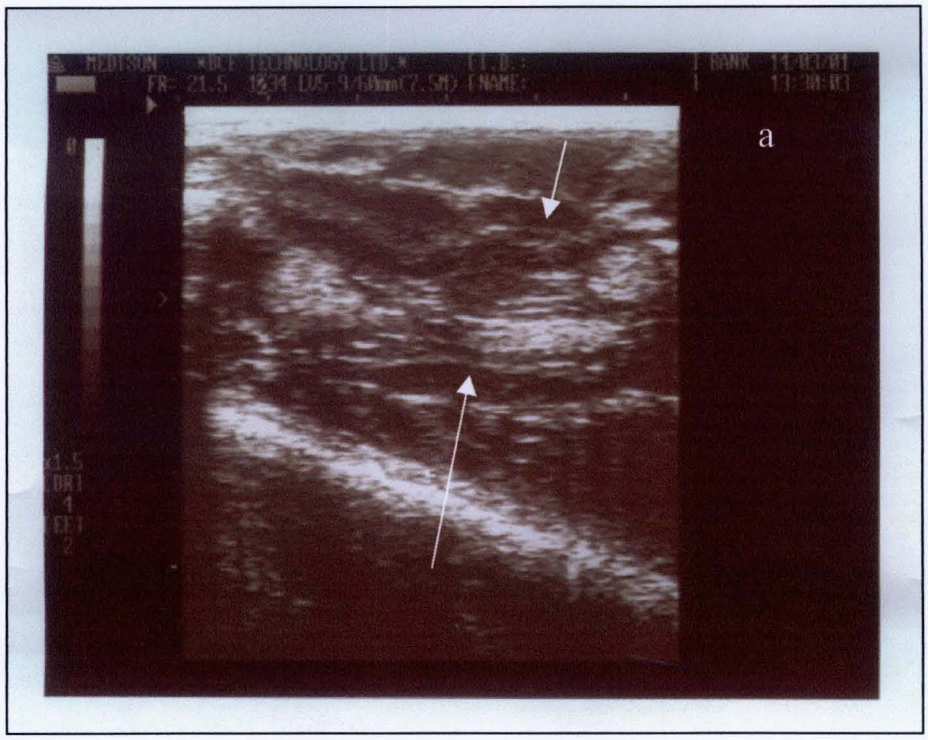
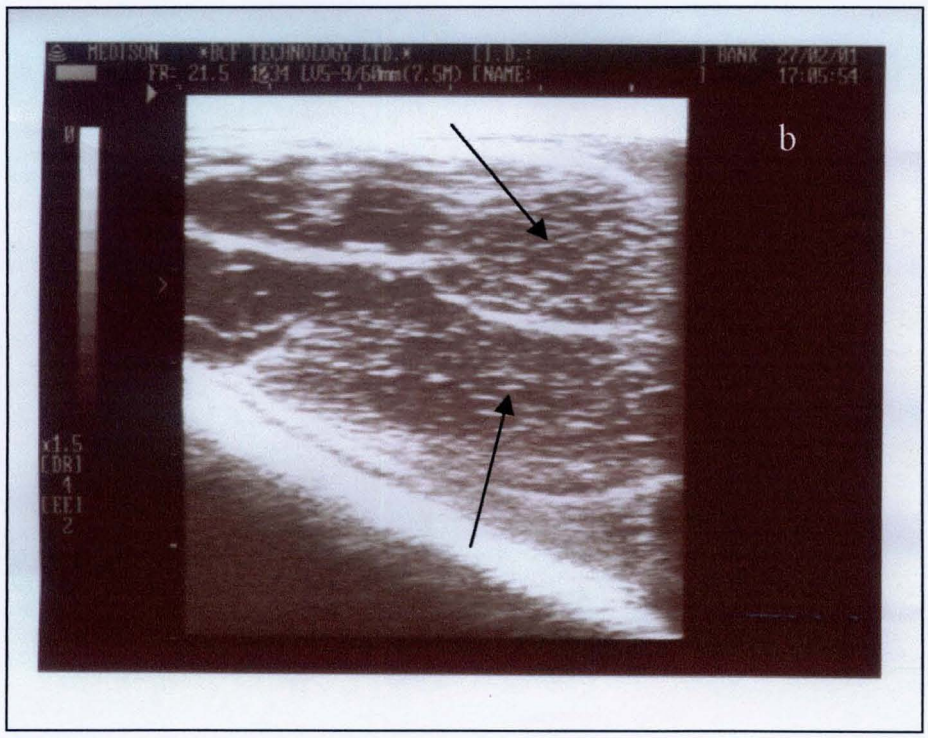


Figure 2.3.25 Mature (prior to spawning) halibut testis (left) and immature ovaries (from two different females) centre and right showing anterior lobes. Diameter of coin used for scale = 22.5mm.



Figures 2.3.26 – 2.3.29 Progressively smaller images of halibut ovaries (a-d) as the ultrasound transponder is moved further along the body of the halibut in an anterior direction.





Figures 2.3.30 – 2.3.31 Ultrasound images of immature testes (a) and mature testes (b).

2.4 Discussion

From the present study a number of major features were immediately apparent. Over the entire duration of the study, in both types of rearing system females exhibited better long-term growth than either the early or late maturing groups of males. The late maturing (2002) males also showed a similar long-term pattern of increased performance over the early maturing (2001) males. All groups of fish performed better in the tanks than the cages for the majority of the study and the final weights for all groups were significantly higher for the tank reared fish than the cage reared fish.

Female growth premiums were expected prior to the commencement of the study. Increased growth of females has also been noted for a number of species including turbot (Deniel, 1990; Imsland, 1997) and sea bass (Saillant, Fostier et al., 2001). The findings of the present study were in accordance with those of previous studies on the performance of halibut (Jákupstovu and Haug, 1988; Bjornsson, 1995). However, whilst these two previous studies were in agreement on the general pattern of increased performance by females over males there were, however, discrepancies in the growth rates achieved by males and females. Such differences are due mainly to the differences in the size of fish used but also to the genetic stocks that the fish originated from. (Jákupstovu and Haug, 1988) reported high annual growth rates of 8 kg/yr (size range 7-110kg) and 2 kg/yr (size range 3-32kg) for females and males respectively. These figures are much greater than the figures of 3.2kg/yr (size range 2-12kg) for females and 1.4kg/yr (size range 2-7 kg) for males reported by (Bjornsson, 1995). The figures given by (Bjornsson, 1995) are closer to those found in the present study which ranged from 1.8 – 2.3 kg/yr for females and 0.8 – 1.3 kg/yr for males and are also in accordance to figures given by (Rabban,

1986; Haug, Huse et al., 1989). Differences in performance in terms of growth and disease resistance between individuals as a result of family genetic history and more widely the stock are to be expected (Jonassen, Imsland et al., 2000; Imsland, Jonassen et al., 2002). The differences between the present study and that of (Bjornsson, 1995) are greater for females and may be accounted for by the smaller fish used in the present study and therefore lower annual weight gain. The growth rates of the males would appear to be broadly similar which would be in agreement with the linear growth proposed by Bjornsson. Growth curves of males in the present study approached a more linear relationship than those of the females. In the mathematical form SGR = aW^b the parameter a relates to environmental factors affecting growth such as temperature whilst b indicates how rapidly SGR declines with weight (Brett and Shelbourn, 1975; Bjornsson, 1995). Where b is 0 SGR does not change with weight i.e. exponential growth and where b is –1 then absolute growth (kg/yr) does not change with weight i.e. linear growth. The figures in the present trial indicate that growth of male halibut in the range 400g to 4500g approaches a linear relationship whilst for the females a less linear growth relationship is evident. The slight non-significant result for SGR and the significant result for GF3 indicate that the observed growth rate differences are affected to a high degree by both sex and rearing system.

From the growth curves of females, males first observed spawning in 2001 and males first observed spawning in 2002 (Figures 2.3.8) at Otter Ferry and Loch Teacuis, it is clear that there were a number of mechanisms affecting the growth rates of each group throughout the duration of the trial. All the groups followed the same general pattern of

slower growth during the winter of 1999-2000. This was followed by an increase in growth rate during the following summer. All groups again had reduced growth during the winter of 2000-2001. The males that matured in the spring of 2001 had the greatest growth reduction with negative growth observed at both Otter Ferry and Loch Teacuis. During the summer of 2001 all groups showed increased growth from the previous winter. During the winter of 2001-2002 however, no decrease in growth rate was observed for the females whereas a decrease was observed for both spawning year groups of males. This pattern would seem to indicate, given that winter temperature at each site was similar each year, female growth, prior to maturation, is modulated primarily by temperature and that the optimum temperature requirement for halibut decreases with increasing size. Similar findings to this were observed for halibut by (Aune, 1994; Aune, Imsland et al., 1997; Jonassen, 1999) cod (Pedersen and Jobling, 1989) and turbot (Imsland, Sunde et al., 1996; Imsland, Foss et al., 2001).

For the males it is evident from the different growth curves between males that matured in 2001 and males that matured in 2002 that, in addition to temperature (when within an acceptable range), maturation processes had the greatest effect on growth. It would appear that once the males have matured and devoted energy reserves to gonadal growth, any subsequent direction of energy into somatic growth is insufficient to ensure that weights recover to match those of the females or non-maturing males. Compensatory growth is in evidence when considering the SGR, GF3 and condition factor data. Leading up to spawning SGR rates (Figures 2.3.9 and 2.3.10) and GF3 rates (Figure 2.3.12) decreased but subsequently recovered in part to the levels observed in the female population. Condition factor (Figures 2.3.17 and 2.3.18) of maturing males also

decreased during the spawning season and were followed by a similar pattern of recovery as that for SGR. This process was in evidence at each site. Similar seasonal condition factor patterns were observed for halibut by (Haug, Huse et al., 1989). Related to the condition factor is the length-weight relationship. Given the non-significant effect of group on condition factor at the same length a common exponent b in the relationship: $W=aL^b$ is not unexpected. A pooled value of 3.6187 for b was found by (Haug, Huse et al., 1989) conforming to the positively isometric value of 3.16 determined in the current study.

Apart from the differences in growth modulated by temperature and by the process of maturation, there would appear to be additional factors involved. From the start of the study the precocious males i.e. those destined to spawn in 2001 as opposed to 2002 showed increased growth compared to the other two groups although significant differences were noted at Otter Ferry although not at Loch Teacuis. In addition the females showed increased growth over the late maturing males during the same period.

Increased growth by precocious males prior to spawning compared to that exhibited by non maturing males has been demonstrated for the first time in halibut. The process is however known to exist in a number of other species including Arctic charr (*Salvelinus alpinas* (Damsgard, Arnesen et al., 1999), Chinook salmon (Heath, Devlin et al., 1996) and Atlantic salmon (Thorpe, Talbot et al., 1990; Kadri, Mitchell et al., 1996). Currently it is hypothesised that maturation processes are controlled ultimately by external environmental cues, primarily photoperiod and light (Duston and Bromage, 1988;

Bromage, Porter et al., 2001) and that the fish are reliant on this information for the synchronisation of maturation with the correct seasonal conditions.

In addition to this requirement of seasonal information, it is also generally regarded that there is a minimum or threshold developmental stage that must be reached before maturation can begin (Imsland, 1999; Bromage, Porter et al., 2001). As the maturation process can be lengthy, it is the accumulation of surplus energy in the year prior to maturation that determines the response of an individual to timing cues in the year before spawning (Adams and Huntingford, 1997; Godo and Haug, 1999). Increased whole body lipid levels have been linked to this satisfactory maturation condition in the Atlantic salmon (Rowe, Thorpe et al., 1991) and Chinook salmon (*Oncorhynchus tshawytscha*) (Shearer and Swanson, 2000). Increased lipid reserves lead to a higher condition factor and in the present study the condition factor of the fish maturing early in the present trial was also greater than the non-maturing fish from the point of initial measurement in 1999 to spawning in 2001 than the non-maturing fish.

The question arises whether increased growth is a trigger for the maturation process or is a result of the onset of maturation. Once the threshold size/stage or minimum energy reserve has been met, does increased growth result from the commencement of the maturation process and, if so, what triggers one group of males to begin to mature and not another? Alternatively does further increased growth trigger maturation? There are indications for salmonids that initiation of the complex endocrine control of maturation occurs before the maturation related growth premium (Taranger, 1993) and that growth results from at least some of these hormonal changes (Heath, Heath et al., 1991). It has

also been suggested that growth premiums exhibited by precocious males are a result of hormonally induced increases in feeding behavior (Metcalfe, Huntingford et al., 1988; Rowe, Thorpe et al., 1991). In the present study, the difference between mean weight of the 2001 maturing males and 2002 maturing males at the initial weighing was approximately 12.5% or 50g. This difference, present more than a year before any of the fish spawned was likely to have been apparent for some time prior to the first weighing. This would seem to indicate in the Atlantic halibut, that if weight increase is a result of the maturation process that the maturation mechanism is initiated some time in the second year before first spawning.

Whether the differences between spawning time of groups observed in the current study (2001 spawning males and 2002 spawning males) may be linked to the genetic heritage of the individuals involved is at present unknown. Blood samples were taken from the fish for investigation of family traits and the information when available should help elucidate the present findings and give an indication as to whether timing differences vary between families or whether they are a result of differences at meiosis. Initial indications are that there were 8+ different groups including half siblings although the full results are not yet available.

In terms of the differences during the first year to 18 months of the trial between the females and the males maturing in 2002, although there was a difference in mean weights it was rarely significant at Otter Ferry but significant differences were present at Loch Teacuis. Food intake differences have been suggested as a mechanism by which female/male growth differences may be influenced (Lozán, 1992; Imsland, 1997). A

difference of 73% more food intake by females in comparison to males of the same size (20cm) was observed for the dab, *Limanda limanda* which was linked to the females having a larger digestive tract (Lozán, 1992). However in the present study, precocious male growth was greater than that of both the non-maturing males and the females for a period approaching a year and may have been so for some time prior to the commencement of the trial. It would seem unlikely that this degree of growth premium could be supported by such a marked reduction in food intake from that of the females and that the timing of any inter sex food intake differences may be more complex. If such differences do exist, their effect may be greater after spawning. In an evolutionary sense one advantage of sexual dimorphism in growth is to limit the competition males pose to the food supply of the females (Bromley, 2000).

Energy allocation for spawning females has been shown to be greater than that of males in the plaice (Rijnsdorp and Ibelings, 1989; Bromley, 2000) and turbot (Imsland, 1997; Bromley, Ravier et al., 2000) which may indicate that the food intake of maturing and mature males is less than that of females.

Many physiological parameters in fish are either directly affected by growth hormone or indirectly through insulin-like growth factor (IGF-I). The effects of GH on parameters such as growth, condition factor, feed conversion, flesh quality, metabolism, osmoregulation, behaviour and long term survival have been revealed via exogenous treatment, see (Bjornsson, 1997). Studies conducted on naturally occurring levels of GH have given an indication of the role GH plays in reproduction and associated growth rates (Holloway, 1997, 1998; Gomez, 1999; Einarsdottir, Sakata et al., 2002). The overriding

conclusion from the available data is that GH is an important multifunctional hormone and that the relationship between the parameters affected is exceedingly complex. Although the majority of the work has been carried out on salmonids and sparids and cyprinids there is growing evidence that similar responses are exhibited in other species including the halibut (Einarsdottir, Sakata et al., 2002).

Whilst circulating GH levels were not measured in the current study its involvement at various levels in the growth and overall performance of the halibut can be undeniably. Whether the slight differences in temperature or the stress levels observed - albeit generally not significant - contributed to significant differences in growth hormone between the different populations can only be speculated

It has been found previously that major gender differences in GH exist in adult, sexually mature, halibut (Einarsdottir, Sakata et al., 2002). Males had levels an order of magnitude greater than females. The reverse of this has been found in rainbow trout (Holloway, 1998) and goldfish (Marchant, 1986), although no such differences were found in Atlantic salmon (Bjornsson, Taranger et al., 1994)

Additionally male halibut were shown to display annual rhythms relating to spawning period. Concentrations of GH peaked at the end of the spawning period and then dropped rapidly. This is in accordance with the supposition that decreased appetite and food intake associated with either stress or reproduction and subsequent decreased metabolic clearance rates lead to increased levels of GH. Females exhibited no clear annual rhythms in Plasma GH.

In salmonids photoperiod has been found to be a major cue in regulating circulating plasma GH concentrations (Bjornsson, Taranger et al., 1994). However, in halibut no such relationship was found to exist (Einarsdottir, Sakata et al., 2002).

Temperature, and its effects on GH concentrations, has been studied to a lesser degree. Of interest to the current study, where there were short periods when temperatures differed slightly, may be the relationship between fish (salmon) being exposed to low temperatures and a subsequent delay in expected spring rises of GH concentrations (Schmitz, 1994; Einarsdottir, Sakata et al., 2002).

The complex relationship of the effects and interactions of GH when further investigated will no doubt yield improvements in both the product quality and profitability of aquaculture.

2.4.1 Sexual dimorphism and grading

The size of halibut at sexual maturity is of great interest to commercial on-growers, in particular males as they mature within the duration of the farming cycle. In the present study it has been shown that there were two main groups of spawning fish. The main indications are that between 23.7% and 39.1% of the males (Table 2.3.1) will mature in the first phase of spawning and by the second spawning year nearly 100% of the males will have reached maturity. Maturation of the females is less of a problem as harvesting will usually take place before any weight loss associated with the female maturation affects production. In the present study none of the females at either site were observed

producing eggs and their condition factor was not seen to be decreasing at the end of the trial. The disparity observed between sites in terms of the percentage of male fish spawning in the first season (2001) was not expected. Large numbers of environmental factors are involved in regulating and synchronizing the maturation process, primarily temperature and photoperiod (Van der Kraak and Pankhurst, 1997; Bromage, Porter et al., 2001). The fact that only one group (initially small fish at Otter Ferry) had a greatly reduced percentage of fish maturing compared with all other groups at either site suggests that there was an event or events occurring that affected individuals in some cages / tanks more than others. Light levels between tanks and cages, at least day length rather than intensity would have been similar at each site as was temperature apart from short periods during the warmest and coldest periods which occurred more than 6 months prior to the spawning period (spring 2001). At each site a greater proportion of the initially large fish spawned for the first time during the 2001 season which is in support of the threshold theory for maturation (Adams and Huntingford, 1997). Given the greater growth at Otter Ferry it would perhaps also be expected that a greater percentage of males would have reached maturity at Otter Ferry than at Loch Teacuis but the reverse of this situation was observed. As greater numbers of fish than expected reached maturity in the cages for the first time in the 2001 season it could be postulated that some stimulatory factor, or event exclusive to the cage site, was a contributory factor in more initially smaller fish spawning. There is some evidence to suggest that pheromones are involved in priming actions influencing oocyte maturation and ovulation in females and spermiation in males (Van Weerd and Richter, 1991; Stacey, Cardwell et al., 1994). If stimulatory actions do exist, it is feasible that they may be more apparent in fully stocked cage sites in sheltered

sites (reduced flows) with mixed populations (including older fish), than pump ashore facilities where the inflow water does not contain any of the outflow water from the other tanks.

The timing of maturation affects a number of management issues including grading feeding and harvesting. Grading out of males is desirable for a number of reasons. Anecdotal evidence suggests that feeding rates of maturing male halibut drop through the winter period approaching spawning whilst the females feed intake may continue-albeit at a reduced rate. Having both males and females with different feed requirements in the same enclosure increases the difficulty of optimising the food conversion ratio (FCR) hence profitability.

From the mean weight data in the current study grading to separate males and females based on weight prior to maturation of the first maturing group of males would not be feasible. Grading in the spring when the first spawning groups of males are producing milt followed by a grade based on size in the following autumn when the weight of the late maturing males begins to decrease would allow females to be ongrown separately for a minimum of one year.

Harvesting strategies are of great importance in maximizing the profitability of the rearing process of any fish. This is particularly the case with fish that are normally seasonal in supply. Harvesting farmed fish when a large amount of wild fish is available to the market is counter productive in obtaining the maximum price. In terms of the timing of harvesting males, it is clear that given good market conditions it is cost efficient to harvest the fish before any maturation related weight loss occurs. From the maturation

data it is evident that there is little point in harvesting the males from October to April/May when weights are at a minimum. It is also clear that careful consideration must be given to the question of whether it is efficient to utilize valuable space ongrowing males past maturity or whether it would be better to remove the males and allocate the space to new batches of fish.

Differences in growth rates between the fish grown in the sea cages and the fish grown in the tanks proved to be significant from the first sample point onwards. It is likely that the transfer by well boat from the initial holding tanks at Otter Ferry to the cage site at Loch Teacuis was a significantly stressful event for the fish involved. However, the stress of transport is likely to be significant for only a short period of time (days rather than weeks) (Robertson, Thomas et al., 1987; Iversen, Finstad et al., 1998) and it is unlikely to have had a major effect on a long-term growth trial.

Whilst growth rate and condition factor can be used as an indicator of stress (Morgan and Iwama, 1997), no blood plasma parameters were measured during or after transfer. It is therefore difficult to conclude, despite lower initial SGR values and condition factors for the fish at Teacuis, in comparison to those at Otter Ferry, between the October 1999 and January 2001 sample points whether the initial decreased growth rate at Loch Teacuis was stress induced.

Using comparisons of SGR at each site as an indication of fish performance through the rest of the study is problematic due to the increasing difference in size of fish at each site and that dates that fish were sampled on at each site varied. As SGR decreases with size comparison of the inter site values maybe misleading. Also although the majority of the sample dates at each site were fairly close, weather related delays meant intervals between some of the sample dates were greater than originally planned. Comparisons would then be made of growth during periods with not just differences in temperature but also daylength. The use of the GF3 calculation is therefore advised (Holmefjord, 1995; Jobling, 2004)

Comparisons are however useful for identifying general trends and gross differences at each site. A clear correlation between season and SGR was apparent at Otter Ferry and Loch Teacuis. Similar differences were observed when GF3 calculations were employed. Differences in growth at each site were likely to be a result of a combination of factors including temperature, physical disturbance, predation stress and intrinsic husbandry practices. The water temperatures at each site whilst remaining similar for large amounts of the study varied in particular at maximum and minimum points of the annual cycles. Minimum winter temperatures of between 6.4 and 9°C appeared to have had a negative effect on growth at each site

A pronounced increase in the coefficient of variation (*CV*) over time indicates that the fish involved may be growing at different rates and that some of the fish may not be growing to their full potential. The causes of such disparities may be due to competition for food due to underfeeding, the establishment of hierarchies or genetic growth

differences although the effects have been found to be species specific. (Purdom, 1974; Jobling, 2004) The relatively low increase in *CV* over the course of the trial and the initial decrease over the period of the first 5 sampling dates indicates that the grading process employed was successful in disrupting any early establishment of hierarchies. Additionally it indicates that there was no suppression of growth due to poor water quality and that the feeding regime provided sufficient access to food for the entire population.

High size-rank correlations ($r_s > 0.65$) have been previously noted for juvenile halibut (Hallaråker, Folkvord et al., 1995) and for turbot (Imsland, Sunde et al., 1996) Slightly lower values were observed in the current trial although still significant. The variation may simply be due to the differences in the age, sex and size of the fish that were analysed and the significantly longer length of the present study.

For the purposes of calculating either the weights or lengths of halibut at any point in time the fitting of the Von Bertalanffy growth curve provided the best results. The r^2 values (0.97-0.98) indicate a high degree of fit to the data. Slight differences due to maturation and season were however still evident between the predicted and actual values. The parameters for the different groups can be used to provide a range of figure to which future growth data can be compared against.

Although the biological significance of the parameters of the Von Bertalanffy model have been questioned (Ricker, 1979; Cerrato, 1991), curves with positive t_0 values indicate that juveniles is slower compared to older fish (King, 1995). In this case juveniles are taken to be from the point of hatching. The positive t_0 values for all but one of the groups is perhaps a reflection of the longevity of the yolksac stage for halibut. .

2.4.2 Stress and blood plasma parameters

During the course of the trial there appeared to be no apparent seasonal patterns in any of the plasma parameters measured, although a more regular sampling regime may have elicited more information. Daily and seasonal patterns of fluctuating levels of plasma parameters have been demonstrated for a number of species (Laidley and Leatherland, 1988) (Biron and Benfey, 1994; Wendelaar Bonga, 1997) and it is not clear whether fluctuations observed as part of the present study are as a result stress or naturally occurring. The similar mean (over entire trial period) values for plasma cortisol, plasma osmolality, plasma chloride and plasma glucose were recorded at each site although significantly higher levels were observed for both chloride and glucose levels in samples taken from the Loch Teacuis cage fish.

2.4.2.1 Plasma cortisol

During an acute stress response the circulating cortisol concentrations rise rapidly with detectable increases occurring within 5-10 minutes (Sumpter, 1997). Acute stress may lead to elevated levels of cortisol lasting in the order of hours whilst prolonged or chronic stress may lead to elevated cortisol lasting in the order of days or weeks (Pickering and Pottinger, 1989). Every attempt was made to sample fish within 30 seconds of initial netting and therefore any increases in reported plasma cortisol were not due to handling stress.

The levels of cortisol measured during the trial were within the boundaries of resting levels reported for other species (Barton, Schreck et al., 1986; Benfey and Biron, 2000) and certainly below reported post stress levels (Sumpter, Dye et al., 1986; Pottinger, 1998; Ruane, Huisman et al., 2001)

2.4.2.2 Plasma glucose

There is a high degree of variability for resting and post-stress plasma glucose levels in the available literature. Despite the significant difference between mean plasma glucose levels for the tank fish at Otter Ferry and the cage fish at Loch Teacuis the maximum and minimum levels at each site were within the boundaries of resting levels found in some species (Sumpter, Dye et al., 1986) (Pottinger, 1998) although the maximum levels also equate to reported post stress values for some other species (Barton and Schreck, 1987; Vijayan and Moon, 1992). Conversely considerably higher post stress glucose levels have also been reported (Rotllant and Tort, 1997; Benfey and Biron, 2000; Ruane, Huisman et al., 2001).

Given the similarity between mean plasma cortisol levels at each site it would seem unlikely that the differences in plasma glucose levels could be accounted for by sampling stress CA induced liver glycogenolysis. Whether higher levels of plasma glucose in the cage fish at Loch Teacuis represent a genuine longer-term cortisol mediated gluconeogenic post stress response is unclear. However, when this information is combined with the generally reduced specific growth rates and lower condition factors at

Loch Teacuis, it is possible but unlikely that that stress might have been a causative factor.

2.4.2.3 Plasma osmolality and chloride

The overall levels for plasma osmolality and plasma chloride at both sites were slightly higher than the basal and post stress levels found for halibut held in aquarium tanks (chapter 3 this thesis) and by (Van Ham, 2003). In the present growth trial initially high plasma osmolality levels increased the overall mean values, which were for the majority of sampling points more similar to other reported basal levels. In addition it has been shown that there is a positive correlation of plasma osmolality and chloride levels with external salinity (Imsland, Gunnarsson et al., 2003) which makes comparisons difficult. The values obtained in chapter 3 were from fish held at slightly reduced salinities ranging from $28^{o}/_{oo}$ to $34^{o}/_{oo}$ whilst although salinity was not measured year round during the growth trial random measurements at each site all showed the water to be full strength seawater. Whilst a slight but significant difference between plasma chloride levels was observed between sites, equivalent osmolality was not. This the similarity in overall plasma osmolality levels between sites it would again appear to indicate that at the time of sampling the effects of any regularly occurring site specific stressors were not in evidence. Through the majority of the trial the plasma osmolality and chloride levels reported provide no definitive evidence of regular site specific stress induced hydromineral disturbances.

2.4.3 Financial Implications

The financial analysis of large-scale aquaculture operations is a complex operation and subject to a great number of variables. For both land based systems and cage systems any analysis of total cost, covering both capital and running costs is inherently site specific. Differences in location lead to varying land costs, installation costs, equipment choices, labour costs, utilities costs, all of which will affect the overall profitability of any system. Also cost structures are constantly changing with the refinement of existing and the development of new technologies. Better information regarding the availability of suitable sites, possibly obtained from GIS surveys, would certainly aid in the financial planning process of any future developments. At present though the range of alternative combinations of all these parameters make an accurate, all-encompassing, analysis unfeasible. However, the growth data available from the present study is useful in that it can be incorporated into the project planning process and any sensitivity analysis of prospective halibut farming operations. The growth data, both overall and individually for each sex in each type of system, is useful as it provides a range of performance figures. This range provides targets and when combined with data from previous works it enables a more realistic range of expected growth, which can be used when carrying out any financial analysis.

The major difference between the two systems will probably be the capital costs and running costs. This difference is widely perceived within the industry to be a major downside to the future development of land-based systems. However, the development of more sophisticated and therefore more expensive cage structures and mooring systems

and the requirement for service vessels may reduce any such differences. Additionally, the useful working life of the cage equipment may prove to be significantly less than that of land based equipment. Marine cages, nets and other miscellaneous plant and equipment for cod culture were given replacement times of 7, 3 and 4 years respectively (Nautilus, 1997). For both systems feed costs and labour prices should be similar and these, along with seed prices generally contribute the greatest expense of aquaculture operating costs. In a study of the offshore aquaculture of Pacific threadfin, *Polydactylus sexfilis*, in Hawaii feed costs were found to be 30%, labour 17% and stocking 12% of the total (Kam, Leung et al., 2003). Economies of scale in the US may however result in access to lower priced feeds than is possible in Europe and so a figure of between 40 and 50 % may be more realistic. The high cost of halibut juveniles (between £3 and £5 per individual) means that stocking costs may be nearer to 20%. Pumping costs of flow through systems and the need for additional oxygenation at higher stocking densities are an additional major outgoing for land based sites. However, the difference this causes between costs of running a cage site and a land-based site is reduced to some degree by boat maintenance and additional vehicle requirements and transport costs (wellboat or helicopter charter). In terms of other major considerations, both systems will require some form of land based office/storage facility and the associated administrative support and vehicles etc so again differences are likely to be minimal.

The differences in weight observed in the current study were however significant and some form of comparison in terms of financial outcomes is useful. By means of a simple example, if the difference in terms of percentage mean weights obtained at each site for all groups (both males and females) is as found 29.2% this can be interpreted as a

difference of 6 months for the stock to reach a mean market size of 4.5kg. I.e. the market size is reached in 2.5 years rather than 3 years of ongrowing after transfer from the nursery. Given that effectively 6 months is saved per farm cycle then it can be said that an extra cycle could be accommodated per every 6 cycles. If the theoretical capacity of the halibut ongrowing unit is taken to be 100,000 fish and a market price (farm gate) of £7.00 is used then this represents a an extra £3,150,000 per 6 cycles. Additional feed costs (@ £650 per mt and FCR of 1.1 = £307,450) and extra juvenile costs (@ £4.50 per juvenile = £450,000) need to be deducted from this figure which would equate to extra income of approximately £2,392,550 over 6 cycles = 18 years, or around £132,919 yr^{-1}.

This figure can be put into perspective if it is compared to the annual profits of £469,000 yr^{-1} in the normal operating years of a model for a 250 tonne per annum flow through halibut farm (Slaski, 1997). With the combined annual costs of pumping and oxygen of the model being £145,000 it is clear that the magnitude of differences in growth such as those observed in the current study could be the deciding factor of the profitability of one system over the other. Considering this, if the running costs of a cage unit can be brought down to within the amount caused by the difference in growth it is difficult to foresee a scenario where a greenfield land-based operation could be supported.

The IRR of the Slaski model was 15%, which, for a new operation, may be regarded by investors as simply not attractive enough to warrant future investment. An existing land-based site turned over for use with halibut may however prove to be financially acceptable given the closeness of the relationship between improved growth related profits and higher operating costs.

Another aspect of future financial planning is the impact of the differential growth rates between individuals in the same batches. Sexual dimorphism data is therefore useful as the harvest price of different size groups varies considerably. It will enable harvesting strategies, based on accurate male/female population and projected growth calculations, to be tailored to meet the demands of a market supplied with a variable supply of wild halibut allowing smaller, and therefore least valuable, males to be sold when market prices are highest. By taking the expected size ranges and numbers of fish within each size range into account a far greater degree of accuracy can be obtained when undertaking any financial analysis.

CHAPTER 3

EFFECTS OF ACUTE STRESS ON PRIMARY AND SECONDARY STRESS RESPONSES IN THE ATLANTIC HALIBUT

3.1 Introduction

Whilst aquaculturists strive to provide conditions that allow their fish to perform optimally there are inevitable compromises being made to fulfil the economic considerations necessary in the production of a commercially viable end product. Along with these factors such as water quality, stocking density, size grade composition and feeding patterns there is the inherent design of the rearing system and the day to day husbandry activities all of which can act as stressors on the fish. These disturbances experienced by the fish, held under artificial rearing conditions, can lead to various stress responses.

A number of definitions of "stress" have been put forward by different workers to explain what initially may seem to be a relatively simple concept. Problems arise however in trying to develop an all encompassing definition that is applicable to all the disciplines in which the term stress is used. Previous definitions have ranged from any physiological, biochemical or behavioural response to various factors of the physical, chemical and biological environment to only those adaptive responses that extend beyond the normal range of the organism such that the chances of survival are significantly reduced (Barton and Iwama, 1991). Barton, (1997) summarises a number of different definitions and concludes that the common theme running through them is one of a biological response to a stimulus at some level. A more precise definition, and one that is applicable to aquaculture, is given by Wedemeyer, Barton et al., (1990). They conclude that an environmental or biological challenge (stressor) that is severe enough to cause stress is one that requires a compensating response by a fish, population or ecosystem. They add that acclimation may be possible if the compensatory stress response can re-establish a satisfactory relationship between the changed environment and the fish or higher-order biological system. In addition they also state that the

cumulative effects of even sub-lethal stress factors may eventually lead to deaths even though the factors may not individually exceed physiological tolerance levels.

A great deal of discussion has emanated from the search for a definitive answer as to what exactly constitutes stress but perhaps even more has been focused on the quantification of stress responses. There are a large number of methods that have been used to evaluate stress levels in fish and they are documented in several papers (Adams, 1990; Wedemeyer, Barton et al., 1990; Barton and Iwama, 1991; Iwama, Morgan et al., 1995; Morgan and Iwama, 1997; Wendelaar Bonga, 1997). All of the methods used to evaluate stress levels have one factor in common, that is, they measure a change of a particular biological parameter.

Several workers have evaluated stress responses and one of the first was Seyle, (1950) who developed the "General Adaptation Syndrome" which divided stress responses in three levels: Alarm, Resistance and Exhaustion. This model was subsequently modified by Wedemeyer, Barton et al., (1990) who used the terms primary, secondary and tertiary to describe the different levels of biological change that constitute a stress response. The primary response is the activation of the neuro-endocrine release of stress hormones, namely catecholamines and corticosteroids, following the perception a stimulus by the central nervous system. The secondary response involves the response to, and effects of, the stress hormones at a cellular/tissue level. The tertiary response involves the effects that the secondary response has on the entire animal and, thus also at a population/community level if survival and reproduction are affected (Barton and Iwama, 1991). Examples of primary, secondary and tertiary features have been used as indicators of stress are presented in Table 3.1.1. The primary and secondary indicators of stress used in the present study are discussed further below.

Table 3.1.1 Primary, secondary and tertiary features that have been used as indicators of stress (adapted from (Barton and Iwama, (1991))

Primary Neuroendocrine and Endocrine	Secondary				Tertiary
	Metabolic	Haematological	Hydrominerral	Structural	
Plasma Catecholamines	Plasma Glucose	Immunological response	Plasma chloride	Interrenal cell size	Growth
Plasma Corticosteroids	Plasma Lactic acid	Haematocrit	Plasma sodium	Interrenal nuclear diameter	Metabolic rate
Plasma ACTH	Liver & muscle glycogen	Leucocrit	Plasma potassium	Gastric tissue morphology	Disease resistance
	Liver & muscle adenylate energy charge	Erythrocyte (RBC) numbers	Plasma protein	Organosomatic indices	Thermal tolerance
	Plasma cholesterol	Lymphocyte numbers	Plasma osmolality	Condition factor	Tolerance to hypoxia
		Lymphocyte : RBC ratio			Swimming performance
		Thrombocyte numbers			Reproductive capacity
		Blood clotting time			Behaviour
		Haemoglobin			Colour changes

3.1.1 Primary Stress Response

3.1.1.1 Catecholamines

The primary stress response involves the actions of two axis; the Hypothalamic-Sympathetic-Chromaffin Cell Axis (HSC axis) and the Hypothalamic-Pituitary-Interrenal Axis (HPI axis) (Wendelaar Bonga, 1997). The former is involved with the secretion of the catecholamines (CAs) adrenaline and nor-adrenaline (epinephrine and nor-epinephrine) and the later with the secretion of corticosteroids.

Following stimulation by the sympathetic nervous system CAs are released from the chromaffin tissue of the head kidney (Reid, Bernier et al., 1998) (Fabbri, Capuzzo et al., 1998) which, in most teleosts is closely associated with the corticosteroidogenic cells around the posterior cardinal veins (Chester Jones and Mosley, 1980; Abelli, Gallo et al., 1996) (also see section 4.1 this thesis). Following a stressor the increase in CAs occurs rapidly within seconds to minutes and following an acute stress can return to pre stress levels within 10 minutes (Wendelaar Bonga, 1997). Given this rapid rise in particular, the short duration of the response following an acute stress, and also the technical difficulty in measuring catecholamines (Gamperl, Vijayan et al., 1994c) it is clear why the measurement of CAs is seldom used as an indicator of stress (Wedemeyer, Barton et al., 1990). Resting levels and post stress levels are however more achievable when fish are cannulated (Brown and Whitehead, 1995). Resting levels of plasma adrenaline and nor-adrenaline as low as 1-5nM have been reported (Witters, Van Puymbroeck et al., 1991; Butler and Day, 1993; Brown and Whitehead, 1995) rising to post stress levels >1000nM (Fabbri, Capuzzo et al., 1998).

The functions of catecholamines are numerous although the major effects involve the stimulation of cardiovascular and respiratory processes. This serves to optimise the oxygen

levels within the blood by enhancement of the affinity and the capacity of haemoglobin to bind oxygen (Fabbri, Capuzzo et al., 1998) and the mobilisation of glucose, primarily from the liver (glycogenolysis) in response to increased energy demands (Wendelaar Bonga, 1997, Morata, Vargas et al., 1982). Conflicting reports exist regarding the mobilisation of free fatty acids (FFAs) by CAs from lipid reserves with nor-adrenaline having a positive effect as opposed to none being evident following administration of adrenaline (Sheridan, 1987; Scott-Thomas, Ballantyne et al., 1992). In marine fish the relationship between FFA mobilisation and CAs is unclear (Wendelaar Bonga, 1997) although post confinement stress peaks have been observed in the turbot (Waring, Stagg et al., 1997).

3.1.1.2 Cortisol

The development of radioimmunoassay and similar techniques has allowed the accurate determination of trace amounts of a biomolecule in small samples of biological fluids. In the study of stress in finfish the ability of RIA to accurately determine levels of hormones, particularly cortisol, has provided workers with the singly most utilised tool. With these developments and the previously mentioned problems associated with measuring CA levels, plasma cortisol measurements have been used more widely to index primary stress responses (Wedemeyer, Barton et al., 1990).

Corticosteroid release, in particular cortisol, is stimulated primarily by the action of adrenocorticotrophin (ACTH) controlled in turn by corticotrophin-releasing hormone (CRH). ACTH and CRH are synthesised as part of a larger precursor. The precursor, termed

proopiomelanocortin (POMC), is the common precursor of not only ACTH, but also β-endorphin, the melanotrophins (MSHs) and other peptides which have corticosteroiogenic properties. POMC is synthesised in two cell types of the pituitary gland, the corticotrophs of the pars distalis and the melanotrophs of the neurointermediate lobe (Sumpter, 1997). This cascade of hormones is termed the HPI axis and is responsible for the effects of stress. ACTH is secreted rapidly in response to stress and acts on the interrenal tissue releasing cortisol (see also section 4.1, this thesis).

Cortisol is not stored, but synthesised *de novo* from cholesterol thus there is a lag period before levels begin to rise, which allows for basal or resting levels to be measured (Henderson, 1997, Kime, 1987). Additionally the peak response time is longer than that of the CAs with times of up to 4 hours being reported (Vijayan and Moon, 1994) although times of between 30 minutes to 1hr are more common (Cech, 1996, Waring, Stagg et al., 1996b).

In teleosts, cortisol has combined mineralocorticoid and glucocorticoid actions, given that its major roles are the regulation of hydromineral balance and energy metabolism (Wendelaar Bonga, 1993; Sumpter, 1997; Wendelaar Bonga, 1997). Whereas adrenaline is responsible for the immediate post stress production of glucose, it has been shown that cortisol has a direct and positive effect on gluconeogenesis and is important in the long-term control of glucose (Vijayan, Ballantyne et al., 1991; Vijayan, Reddy et al., 1994). Plasma FFA stimulation and reduction in muscle and liver lipid stores may also be under the control of cortisol (Sheridan, 1988). In addition to the hydromineral and metabolic functions of cortisol previously mentioned, longer-term exposure to high circulating cortisol concentrations may have immunosuppressive effects (Balm, 1997; Fletcher, 1997). Additionally, cortisol inhibits

growth due to stimulation of energy consumption (Pickering, 1990) and by possible interactions with other growth regulating hormones (Wendelaar Bonga, 1997).

The majority of work on primary stress responses in finfish, and particularly in relation to aquaculture has been focused on salmonids with little work having been carried out on the Atlantic halibut. In order to interpret values recorded in chapter 1 it is necessary to determine, in a controlled environment, basal and post stress concentrations of plasma parameters as comparison to levels recorded from other species would be unreliable. Intra species variation has been demonstrated by Davis and Parker, (1986) and Audet, Besner et al., (1993) as well as variations in stress response between diploid and triploid fish (Biron and Benfey, 1994; Benfey and Biron, 2000; Sadler, Pankhurst et al., 2000). Summaries of documented changes in plasma cortisol levels for number of species have been compiled (Barton and Iwama, 1991; Gamperl, Vijayan et al., 1994c; Van Ham, 2003) and, whilst experimental protocols vary, therefore not allowing direct comparisons, some indication of the variability of the response to different stressors can be ascertained.

The degree of variability in terms of response between species was highlighted by Davis and Parker, (1986) when the plasma corticosteroid response of fourteen species of freshwater fish to transportation was compared. Plasma corticosteriod levels were measured directly after the capture by electrofishing and again after transportation for two hours. Of the fourteen species four showed no corticosteroid response, eight had an intermediate response and two had a high response. The difference in plasma corticosteroid response to different stimuli can be attributed to a variety of factors and whilst some stressors appeared to elicit no response other types of stressor provoked a stress response in the same species. Similar varying results were also found for turbot when subjected to different stressors (Mugnier, Fostier et al., 1998).

This only serves to highlight the care that must be exercised when comparing the results from different investigations and species.

3.1.2 Secondary Stress Responses

The secondary responses of interest in the present study were changes in plasma electolytes and plasma osmolality (hydromineral disturbances) and plasma glucose changes (metabolic disturbances). As mentioned both are closely related to the primary stress responses involving increased CAs and cortisol.

Although aquaculture practices such as crowding, netting and transportation evoke a simple stress response, additional physiological disturbances compound those evoked by adrenaline and cortisol, the result is compound stress. Compound stress can be caused by vigorous activity, exposure to sub-optimal water quality or by physical injury (McDonald and Milligan, 1997). Increased lactic acid release, damage to gills or interrenal organs can also lead to extracellular ion and or acid-base disturbances (Cech, Bartholow et al., 1996).

However, following acute stress under conditions of normal water quality it is the effects of a simple stress response that will normally lead to the observed changes in the secondary indicators of stress (McDonald and Milligan, 1997).

3.1.2.1 Metabolic indicators of stress

Plasma glucose and plasma lactate concentrations are the most frequently measured metabolic indicators of stress (Wedemeyer, 1990). The main sources of plasma glucose increases are glycogenolysis, mediated by CAs, and gluconeogenesis which is primarily under the control of cortisol (Vijayan, Ballantyne et al., 1991; Wendelaar Bonga, 1997; Fabbri, Capuzzo et al., 1998). Plasma lactate (the dissociated form of lactic acid) increases occur under anaerobic metabolic conditions (Lehninger, Nelson et al., 1993). Increased plasma lactate levels have been recorded following various stressors including; confinement (Vijayan, Pereira et al., 1997; Sadler, Pankhurst et al., 2000; Sadler, Wells et al., 2000), exercise (Van Ham, Van Anholt et al., 2003) and handling (Pickering, Pottinger et al., 1982). Despite the differing causes of increased plasma lactate and glucose and the lack of involvement of cortisol in lactate production there is however a relationship between the two responses in that lactate and amino acids have been shown to be preferred substrates for gluconeogenesis (Suarez and Mommsen, 1987; Vijayan, Pereira et al., 1994). There is an additional relationship between lactate and other secondary, hydromineral, stress responses as lactoacidosis can cause more complex electrolyte disturbances than those mediated by adrenaline in a simple stress response. In rainbow trout elevated lactate concentrations in muscle may increase muscle osmotic pressure which leads to a net shift of fluid from the extracellular to the intracellular compartment (Milligan and Wood, 1986). The impact of a transcellular osmotic gradient on blood is to cause haemoconcentration, which is manifested as increases in haematocrit, plasma protein and osmolarity and the effects of H^+ions on gill funtion and therefore ionic, osmotic and acid-base regulation (Evans, 1993; McDonald and Milligan, 1997).

As a result of plasma glucose levels being modulated by both CAs and cortisol, glucose concentration has been more widely used as a secondary indicator of stress per se whereas plasma lactate has been used as an indication that fish have been subjected to conditions eliciting anaerobic metabolism (Neilsen, Boesgaard et al., 1994).

The actions of CAs, which stimulate increased plasma glucose levels, lead to depletion of hepatic glycogen. Cortisol has been shown to increase the hepatocyte use of the amino acid alanine which, is a preferred substrate for gluconeogenesis.(Vijayan, Pereira et al., 1994; Vijayan, Pereira et al., 1997). Additionally cortisol has been shown to enhance amino acid mobilisation (Vijayan, Pereira et al., 1997). When employing plasma glucose as a secondary indicator of stress it is therefore necessary to consider other parameters in order to establish the degree of involvement of CAs and cortisol in the mediation of any observed response.

3.1.2.2 Hydromineral Balance/Control

Teleost fish generally maintain their blood osmotic concentration at approximately 250-500 mOsm/kg regardless of whether they inhabit freshwater which may be <0.1 mOsm/kg or seawater which is approximately 1000 mOsm/kg (Parry, 1966). In order to maintain a relatively stable osmotic concentration freshwater and marine fish osmoregulate differently although the gills are the common site for the majority of passive ion and water flux. Freshwater hyperosmotic fish face a volume load and salt loss whereas hypo-osmotic marine fish are potentially volume depleted and salt loaded (Evans, 1993). Freshwater fish produce copious amounts of dilute urine thus removing water and conserving salts whilst there is active uptake of ions over the gills. Conversely seawater fish imbibe water, produce small

amounts of urine and actively excrete monovalent ions (Na^+ and Cl^-) via branchial chloride cells and divalent ions (Mg^{2+} and SO_4^{2-}) by renal and intestinal excretion.

This close relationship between the body fluids, the control mechanisms and the surrounding water means that disturbances in any of these factors may have important effects on the physiology of the fish. The effects of various stressors on the control mechanisms can result in a disturbance of water/ion homeostasis and is a highly characteristic indicator of stress. In simple stress responses the increased levels of adrenaline circulating in the blood cause disturbances in blood Na^+ and Cl^- concentrations. Adrenaline causes ionic/osmotic disturbance by increasing aortic blood pressure resulting from vasoconstriction and increased cardiac output (Mazeaud and Mazeaud, 1981). Adrenaline also increases gill diffusing capacity due to increased perfusion of lamellae and an increase in the number of lamellae perfused and an increased flux of water across the gills (Fletcher, 1992; Nilsson, 1996), in addition increased flux of electrolytes across the gills and decreased drinking (Mazeud, 1977). In the seawater mullet adrenaline has been shown to block Na^+ and Cl^- excretion across the gills (Pic, Mayer-Gostan et al., 1974).

The result is that in marine fish, following an acute simple stress, the general pattern is an increase in plasma osmolality, Na^+ and Cl^- with possible increases of plasma K^+ (Waring, Stagg et al., 1996a). Additionally muscular contraction can cause an efflux of K^+ ions from the myoplasma into the extracellular fluid (Sjogaard, 1990) and has been found to be related to the intensity of exercise in rainbow trout (Thomas, Poupin et al., 1987). Recovery from stress induced ionic disturbances is primarily mediated by cortisol. The restorative role is facilitated by increasing the ion-transporting capacity of the gills by activation of branchial ion pumps (Flik and Perry, 1989), increasing the number of chloride cells (Foskett, Bern et al., 1983) and increased branchial Na+/K+- ATPase activity(McCormick, 1995).

3.1.3 Modifying factors

When employing the use of primary, secondary or tertiary indicators of stress it should be considered that various biotic and abiotic factors are involved in determining the severity of any response. The differences in response between species and between stressor have been mentioned. Seasonal and diel variations of plasma glucose levels were reported for Atlantic cod (*Gadus morhua*) and American plaice (*Hippoglossoides platessoides*)(Audet, Besner et al., 1993) and carp (Malinovskaya, 1992). Temperature and salinity have been shown to affect both interrenal and carbohydrate stress responses in Chinook salmon (Barton and Schreck, 1987) and turbot (Van Ham, Van Anholt et al., 2003) as has water quality (Hontela, Daniel et al., 1997) (Brown and Whitehead, 1995)and cortisol response can vary with strain (Fevloden, Refstie et al., 1991; Pottinger and Moran, 1993; Pottinger, Moran et al., 1994).

3.1.4 Aims

In the present study the effects 3 different timed confinements 2, 12 and 30 minutes followed by repeated confinements of the same duration after an interval of 4 days were investigated. Confinements of the nature employed in the study are common practice in commercial aquaculture operations when fish are sample weighed or transferred between tanks. The effects investigated were plasma cortisol, osmolality, chloride, sodium, potassium and glucose plasma concentrations. Little information regarding the basic stress response of the Atlantic halibut is available in the literature. Therefore in order to interpret plasma parameters measured in halibut held in commercial aquaculture systems (chapter 1, this thesis) information on basal and post stress plasma parameters is required. The experiments reported in this chapter aim to provide this information to the aquaculture of Atlantic halibut.

3.2 Materials and methods

3.2.1 Holding of fish prior to experiment

Prior to the commencement of the experiment juvenile Atlantic halibut, *Hippoglossus hippoglossus*, supplied by Mannin Seafarms Ltd., Isle of Man, were held in 1m diameter 70 cm water depth tanks at the Gatty Marine Laboratory aquarium, St Andrews. The tanks were supplied with raw un-filtered seawater at ambient temperature. The water was aerated by means of 2 air stones located on the floor of each tank. The tanks were covered with blacked out lids to minimise any disturbances from passing personnel and to isolate the tanks from the main aquarium lighting. 25W internal lamps located on the underside of the lids provided 24hr continuous illumination.

3.2.2 Holding of fish during experiment

In order to acclimatise the fish to the experimental system 5 weeks prior to the 1st sampling date 90 juvenile Atlantic halibut were randomly distributed between 3 tanks, 30 fish per tank. During the experiment each tank was fed a 50g maintenance ration of Trouw Halibut diet on alternate days. Before and during the experiment temperature, salinity (American Marine Inc, Pinpoint salinity meter) and dissolved oxygen (YSI 55 dissolved oxygen meter) levels in each tank were measured twice daily.

3.2.3 Sampling procedure

Each of the 3 tanks of fish was subjected to a single timed crowding stress. 3 different crowding durations were investigated, 2,10 and 30 minutes. A repeat of the crowding stress of the same duration as the first event was then carried out on each batch of fish 4 days later.

To facilitate each crowding event, 30 fish were netted from the holding tank and placed in a black bin containing 30 litres of seawater sourced from a spare holding tank.

The netting procedure and use of the 30litre bin replicated activities commonly employed on commercial farms during sample weighing.

The timed crowding began after the last fish had been netted into the bin. For the duration of the crowding event an air stone was placed at the base of the bin. At the end of the crowding period the fish were randomly distributed among five 200litre tanks each containing aerated, unfiltered ambient temperature seawater and supplied with a small inflow of new seawater (0.5-1l/min).

3.2.4 Blood sampling

Following each crowding stress both primary and repeat, six sets of blood samples from 5 fish were taken at timed intervals after the last fish was distributed into the 200litre tanks (the only exception to this was following the primary 2 minute crowd when blood samples were taken from six fish at the 5, 20 and 40 minute samplings and from 4 fish at the 80, 180 and 360 minute samplings). The 1st set of blood samples was taken after five minutes. The subsequent sets were taken after 20, 40, 80, 180 and 360 minutes. In order to reduce any further sampling stress on the remaining

fish only one fish per 200litre tank was removed at each timed interval. The netting procedure was also carried out as gently as possible again to reduce the stress effect on the remaining fish.

Immediately after each fish was removed from the water a 1ml blood sample was taken from the caudal vein with a heparinised needle and a 2.5ml syringe. Blood samples were stored on ice until the completion of the sampling procedures.

Following blood sampling each fish was placed in a separate numbered bucket and anaesthetised in 100ppm 2-phenoxy- ethanol (Sigma). Once lightly sedated each fish had fork length (FL) measured and was weighed to the nearest 0.1g.The fish were then returned to the initial holding tank for recovery.

The blood samples being stored on ice were transferred to numbered 1.5ml Eppendorf tubes and centrifuged at 13000rpm for 3 minutes (Sanyo Microcentaur). Four aliquots of plasma from each sample were removed to numbed 1.5ml Eppendorf tubes. The determination of plasma osmolality, plasma chloride levels and the extraction process for plasma cortisol were carried out using fresh aliquots of plasma. The remaining aliquots of plasma were stored at –20°C.

3.2.5 Length / Weight data

Following the blood sampling procedure and prior to being returned to the holding tanks all fish were individually measured (FL) and weighed.

3.2.6 Plasma Analysis

3.2.6.1 Plasma Osmolality

Plasma osmolality was measured in duplicate by freezing point depression (Roebling osmometer, Camlab, Cambridge).

3.2.6.2 Plasma Na^+ and K^+

Plasma sodium and potassium concentrations were measured in duplicate by flame emission spectrophotometry using a Bayer 3M lithium standard (Flame Photometer 480, Corning Ltd., Essex).

3.2.6.3 Plasma Cl^-

Plasma chloride concentration was measured in duplicate by automatic chloride titration (Chloride Analyser 925, Corning Ltd., Essex).

3.2.6.4 Plasma glucose

Plasma glucose concentration was measured in duplicate using a Sigma Diagnostics enzymatic glucose assay kit (Procedure N° 510). Sample absorbance (A test) was read at 450 nm (Amersham Pharmacia Biotech Ultrospec 3300pro UV/visible spectrophotometer) using a Milli-Q® blank as a reference and compared with a known standard value (A standard). The following calculations were used to determine the glucose concentration:

$$A\ (test) / A\ (standard) \times 100 = Plasma\ glucose\ (mg/dL)$$

$$SI\ units\ e.g.\ (mmol/L) = Plasma\ glucose / 18$$

3.2.6.5 Plasma cortisol

Plasma cortisol concentration was determined by Radioimmunoassay (RIA) using the method employed by Birrell (1998) and adapted from Waring (1996).

3.2.7 Cortisol Extraction

75µl of plasma was added to 600 µl of ethanol in a 1.5ml Eppendorf tube. All samples were shaken for 1 minute and centrifuged (Sanyo Microcentaur) at 13000 rpm for 3 minutes. The supernatant was poured into a numbered plastic LP4 tube (Denley Instruments Ltd.). The remaining pellet was re-suspended in 600µl of ethanol and centrifuged at 13000 for a further 3 minutes. The supernatant was combined with initial supernatant in the LP4 tube.

To ensure that the sample cortisol levels would be within the range of the standard curve the 12000µl samples were effectively diluted at this stage before drying. The combined supernatants were vortex mixed (IKA MS1 Minishaker) for 10 seconds prior to being divided. For the 2 minute stress, duplicate 400µl aliquots (3 x dilution) were removed to separate, labelled LP4 tubes. For the 10 and 30 minute stress duplicate 200µl aliquots (6 x dilution) were used. The supernatants in the LP4 tubes were then dried down in a centrifugal evaporator (GL11 Gyrovap and Crist CT 02-50 chiller) set at 30°C.

3.2.8 RIA Components

3.2.8.1 Assay Buffer

Before each assay a new volume of assay buffer was made up. Assay buffer stock, stored at room temperature, consisted of 1L H_20 + 71.6g $Na_2HPO_4.2H_2O$ (40mM) + 15.3g $NaH_2PO_4.2H_2O$ (10mM).

To make up 100ml of assay buffer 10ml of buffer stock was added to 90mlmilliQ® + 0.9g NaCl + 0.5g BSA. PH was adjusted to 7.4.

Throughout the assay the assay buffer was either stored on ice or refrigerated.

3.2.8.2 Cortisol standards

The cortisol standard consisted of inert hydrocortisone (11β, 17α, 21-trihydroxypregn-4-ene,3,20-dione, 98%, Sigma) stored at 2mg/ml in 99.7-100% ethanol at –20°C. A 10ng/ml solution was prepared by serial dilution with ethanol prior to use in a series of triplicate standards. Standards ranged from 19.5pg/tube in 2.0µl to 5000pg/tube in 500µl in plastic LP4 tubes (Denley Instruments Ltd.)(Table 3.2.1). The standards were dried in a centrifugal evaporator in the same manner as the unknown samples and either stored on ice, if being used immediately, or sealed and stored at –20 for later use.

Table 3.2.1 Volumes and concentrations of cortisol standards

500	µl	5000	pg
250	µl	2500	pg
125	µl	1250	pg
62.5	µl	625	pg
31.3	µl	312.5	pg
15.6	µl	156	pg
7.8	µl	78	pg
3.9	µl	39	pg
2.0	µl	19.5	pg

Triplicate sets of tubes were also required for both total counts and zero counts.

3.2.8.3 Tritiated cortisol

A stock solution of [1,2,6,7-^{3}H] cortisol (^{3}H-cortisol) in toluene:ethanol (9:1 v/v), with a specific activity ranging from 80-105Ci/mmol (Amersham Life Science Ltd.) was stored at –20°C. A working stock solution, again stored at –20oC was prepared by diluting 50µl of the stock solution (250µCi in 250µl) in 4.95ml of 9:1 v/v toluene:ethanol (i.e. 50µl stock + 4.455ml toluene + 0.495 ethanol). This working stock solution contained approximately 20,000 dpm/µl. For each assay an aliquot of working stock was dried down and resuspended in a volume of assay buffer to give a final count of approximately 20,000 dpm per 200µl. The volume of tubes to be processed in each assay determined the volume of the working stock aliquots and also the volume of assay buffer used (Table 3.2.2).

Table 3.2.2 Volumes of 3H-cortisol and assay buffer required for varying numbers of tubes.

Number of tubes	^{3}H-cortisol (µl)	Assay buffer (ml)
50	85	10
100	170	20
150	255	30
200	340	40

3.2.8.4 Cortisol Antibody

The anti-cortisol serum was obtained from Diagnostics Scotland. The serum was a pool of selected antisera obtained from a single sheep injected with cortisol –3-0 (carboxymethyl) oxime-bovine albumin conjugate. The supplied vials contained the lyophilised residue from 1ml of anti-cortisol serum diluted 1 in 5 with a 0.04M phosphate buffer, containing 0.5%(v/w) bovine serum albumin. The lyophilised vials were stored at 4°C until required. The reagent was reconstituted by the addition of 1ml MilliQ® and left to stand for 1 hour at room temperature. 40µl aliquots were then stored at –20°C prior to use in each assay. On day 1 of each assay the antibody was diluted 1 in 300 in the ^{3}H-cortisol-assay buffer mix giving a final antibody concentration of 1 in 5000.

3.2.8.5 Dextran coated charcoal

Dextran coated charcoal was prepared on day 1 of the assay and refrigerated overnight before use on day 2. 250mg of charcoal (BDH) was added to 25mg of dextran (Sigma) in 50ml of assay buffer. The solution was stirred on ice for 2 hours and for a further 30 minutes the following day prior to being used.

3.2.9 Determination of Cortisol by Radioimmunoassay

3.2.9.1 Day 1

All sample and standard LP4 tubes were removed from the freezer and allowed to thaw on ice. The appropriate amount of cortisol antibody was added to the ^{3}H cortisol previously resuspended in assay buffer immediately before addition to the LP4 tubes. 200µl of the antibody - 3H cortisol – assay buffer solution was added to each LP4 tube, including total count tubes and zero tubes which contained no cortisol. All tubes were vortex mixed for 10 seconds and then incubated at 37°C in a water bath for 1 hour. The samples and standards were then removed from the water bath and stored over night at 4°C.

3.2.9.2 Day 2

200µl of chilled, stirred DCC was added to each of the sample, standard and zero tubes. The total count tubes had 200µl of assay buffer added to ensure that total tube volumes were maintained. As the total counts tubes received no DCC they therefore showed the total amount of radioactive counts that were added to all the other tubes.
All tubes were vortex mixed for 10 seconds and held on ice for 15 minutes before being centrifuged at 2500 rpm and 4°C for 15 minutes (MSE Mistral Centrifuge, Fisher Scientific Ltd). The supernatant from each tube was carefully removed with a Pasteur pipette to a PONY vial (Packard Bioscience BV). To each PONY vial was then added 4ml of Emulsifier Scintillator plus™ liquid scintillation cocktail (Canberra

Packard Ltd.). The tubes were then shaken and counted for 5 minutes with a 2000 Tri-Carb® Scintillation Analyser (Canberra Packard Ltd.)

Sample values were subsequently calculated from a standard curve of % bound plotted against pg cortisol/tube using the Curve Expert v 1.34.

3.2.10 Statistics

Statistical analysis was performed with STATISTICA™ Version 7 (Statsoft®), and GraphPad Instat® (GraphPad Software, San Diego, California, USA). All data was tested for normality using the Kolmogorov-Smirnov test.

A Tukey multiple comparison post-hoc test followed all one-way ANOVAs that showed significant differences ($P<0.05$).

A repeated measures ANOVA was used to compare the response profiles of plasma parameters following initial and repeated confinements.

3.3 Results

3.3.1 Holding and sampling conditions.

The mean weight of the fish used in the experiment was 335.9 ±6.8g S.E.M. with no significant difference between treatment groups ($p>0.05$, ANOVA). Prior to the experiment the fish were held in flow through tanks with in tank aeration for a one-month acclimation period prior to the experiment and water temperatures were between 7 and 9°C (twice-daily measurements). Ambient salinity fluctuations were between 28 and 34°/oo (mean = 33°/oo). Dissolved oxygen concentrations were maintained between 6 and 9 mg/l.

During the experimental procedure the handling time of the fish prior to and during the blood sampling process was kept to a minimum. Removal of blood samples from individual fish took no longer than 40 seconds from netting and all groups of five fish were sampled in less than 3 minutes.

3.3.2 Basal Plasma Levels

Loss of fish just prior to the commencement of the experiment meant that there were insufficient numbers available for basal parameters to be measured prior to each confinement experiment. As a result basal parameters were measured in samples taken from an equivalent controlled unstressed fish group. Basal values used were; Cortisol 12.85 ± 1.13 ng ml^{-1}; Osmolality 328.2 ± 1.88 mOsm kg^{-1}; Cl- 150.5 ± 0.8 mmol l^{-1};

Na+ 158.7 ± 2.5 mmol l^{-1}; K+ 3 ± 0 mmol l^{-1} and Glucose 19.3 ± 1.7 mg dL^{-1}.

3.3.3 Plasma Cortisol

Plasma cortisol concentrations were significantly elevated above basal levels ($P<0.001$, one-way ANOVA) after all confinements (Figure 3.3.1). Maximum values were reached within 80 minutes following all crowding durations. Concentrations did not return to basal levels within the 6-hour sampling period following any of the confinements and significant differences ($P<0.001$, one-way ANOVA) between basal levels and final levels existed following all but one stress (30-min confinement). A reduction in concentration from the maximum values was observed during the time course of the experiment for all treatments. Maximum cortisol concentrations following the 2 min and repeat 2 min confinements were 182±21.9 and 163±28.2 ng ml^{-1}; 12 min and repeat 12 min confinements were 163±16.6 and 206.5±24 ng ml^{-1}; 30 min and repeat 30 min confinements were 200.3±8.6 and 166.5±14 ng ml^{-1}. No significant difference ($P>0.05$, one-way ANOVA) was observed between maximum values following any of the confinements. Trajectories following initial confinement and repeated confinements were not significantly different (repeated measures ANOVA $P>0.05$) for any of the durations (Tables 3.2.3-3.2.5)

3.3.4 Plasma Osmolality

Elevated plasma osmolality levels above basal concentrations were observed following all confinement durations (Figure 3.3.2). Maximum values were reached within 80 minutes following all crowding durations. Concentrations returned to basal levels or below within 6 hours for all confinements except the initial 2 minute group. Maximum concentrations were significantly greater ($P<0.05$, one-way ANOVA) than basal levels in all groups apart from those subjected to the second (repeated 4 days after 1st confinement) 12 minute confinement. Maximum plasma osmolality levels

were lower for all repeated confinements than for the initial confinements although this difference was only significant ($P<0.05$, one-way ANOVA) for the 12 minute confinement. Maximum plasma osmolality concentrations following the 2 min and repeat 2 min confinements were 363±6.9 and 349.9±8.6 mOsm kg^{-1}; 12 min and repeat 12 min confinements were 360±2.6 and 334±6.4 mOsm kg^{-1}; 30 min and repeat 30 min confinements were 368.9±2.7 and 360.5±3.5 mOsm kg^{-1}. Trajectories following initial confinement and repeated confinements were significantly different (repeated measures ANOVA, $P<0.05$) for all durations (Tables 3.2.6-3.2.8)

3.3.5 Plasma Cl^-

Elevated plasma Cl^- levels above basal concentrations were observed following all confinement durations (Figure 3.3.3). Maximum values were reached within 80 minutes following all crowding durations. Following the maximum levels, concentrations generally continued to fall during the 6 hr monitoring period although for the initial 2 minute crowd and the repeat 12 minute crowd there was an increase in Cl^- concentration for the last sample point above the penultimate measurement. Maximum concentrations were significantly greater ($P<0.05$, one-way ANOVA) than basal levels in all groups apart from those subjected to the second (repeated 4 days after 1st confinement) 12 minute and 30 minute confinements. Maximum plasma Cl^- levels were lower for all repeated confinements than for the initial confinements with significant differences between maximum values ($P<0.05$, one-way ANOVA) occurring between the initial and repeat 12 minute confinements and the initial and repeat 30 minute confinements. Maximum plasma Cl^- concentrations following the 2 min and repeat 2 min confinements were 173±2.8 and 172.7±3.3 mmol l^{-1}; 12 min and repeat 12 min confinements were 174.4±2.3 and 155.8±2.7 mmol l^{-1}; 30 min and

repeat 30 min confinements were 170.4±2.3 and 151.7±1.3 mmol l^{-1}. Trajectories between the initial and repeat 2 minute confinements were not significantly different (repeated measures ANOVA, $P>0.05$) whereas significant differences were observed between the initial and repeated confinements for both the 12 minute and 30 minute durations (Tables 3.2.9-3.2.11).

3.3.6 Plasma Na^+

The time course of the plasma Na^+ response was similar following all crowding durations except for the repeated 30 minute treatment group which displayed a significant ($P<0.05$, one-way ANOVA) decrease in plasma Na^+ concentration below basal levels followed by a general increase throughout the remaining time course of the experiment (Figure 3.3.4). In all other groups elevated plasma Na^+ levels above basal concentrations were observed following all confinement durations. Maximum values were reached within 80 minutes following all confinement durations. Following maximum levels, concentrations generally continued to fall during the 6 hr monitoring period. Maximum concentrations were significantly greater ($P<0.01$, one-way ANOVA) than basal levels in all initial confinement groups. In all the repeat (4 days after initial stress) confinement groups any increases above basal rates were not significant ($P>0.05$, one-way ANOVA). Maximum plasma Na^+ levels were lower for all repeated confinements than for the initial confinements with significant differences between maximum values ($P<0.05$, one-way ANOVA) occurring between the initial and repeat 2 minute confinements and the initial and repeat 30 minute confinements. Maximum plasma Na^+ concentrations following the 2 min and repeat 2 min confinements were 204.5±2.9 and 167.4±2.9 mmol l^{-1}; 12 min and repeat 12 min confinements were 172.8±2.8 and 168±1.64 mmol l^{-1}; 30 min and repeat 30 min

confinements were 178.8±0.7 and (no peak) mmol l^{-1}. Trajectories between the initial and repeat 2 minute confinements were significantly different (repeated measures ANOVA, $P<0.05$) whereas no significant differences were observed between the initial and repeated confinements for both the 12 minute and 30 minute durations (Tables 3.2.12-3.2.14).

3.3.7 Plasma Glucose

Elevated plasma glucose levels above basal concentrations were observed following all confinement durations (Figure 3.3.5). Maximum values generally took longer to occur than other parameters measured and were reached within 180 minutes following all crowding durations. Maximum levels were reached sooner after all repeat confinements than after all initial crowdings. Following the maximum levels, concentrations generally continued to fall during the 6 hr monitoring period although for the initial 2 minute crowd and the initial 12 minute crowd there was an increase in glucose concentration for the last sample point above the penultimate measurement. Maximum concentrations were significantly greater ($P<0.05$, one-way ANOVA) than basal levels in all groups and were lower for all repeated confinements than for the initial confinements although the differences were not significant ($P>0.05$, one-way ANOVA). Maximum plasma glucose concentrations following the 2 min and repeat 2 min confinements were 54.7±8.6 and 51.9±11.1 mg dL^{-1}; 12 min and repeat 12 min confinements were 46.2±4.4 and 45.7±9.2 mg dL^{-1}; 30 min and repeat 30 min confinements were 69.7±4.5 and 52.5±6.8 mg dL^{-1}. Trajectories between the initial and repeat 2 minute and initial and repeat 12 minute confinements were not significantly different (repeated measures ANOVA, $P>0.05$) whereas significant

differences were observed between the initial and repeated confinements for the 30 minute duration (Tables 3.2.15-3.2.17).

3.3.8 Plasma K^+

There was no elevation in plasma K^+ following any of the confinement durations. Measured levels remained at 3 mmol l^{-1} at all points both before and after stress.

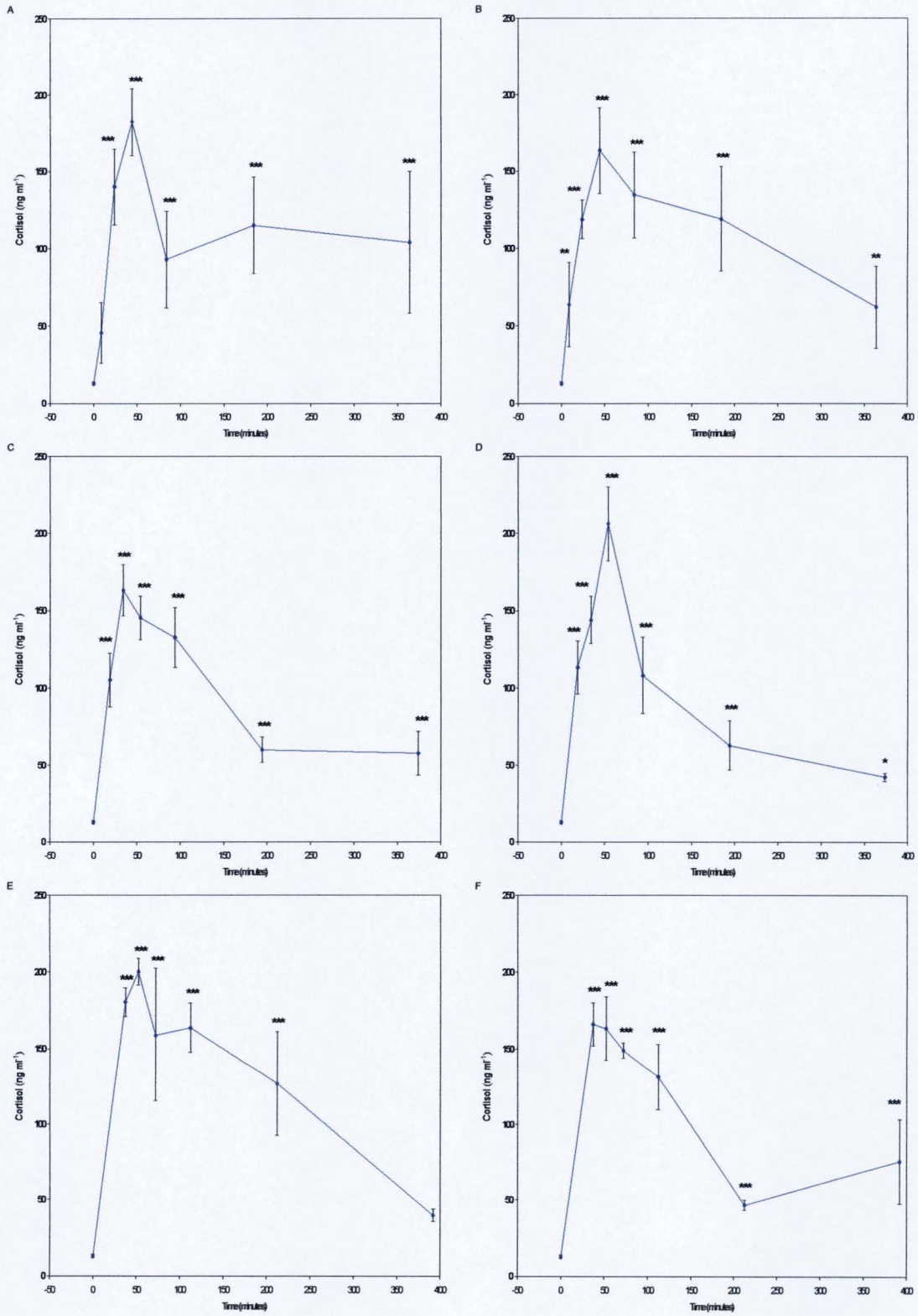


Figure 3.3.1. Plasma cortisol (ng ml^{-1}) changes with time (minutes) from Atlantic halibut (each point n=5) confined underwater for: 2 minutes (A), 2 minutes (same group 4 days later (B)), 12 minutes (C), 12 minutes (same group 4 days later (D)) and 30 minutes (E), 30 minutes (same group 4 days later (F)). *p<0.05, **p<0.01 and ***p<0.001 compared to 0 h basal values (ANOVA). Vertical lines represent S.E.M.

Table 3.2.3 Repeated measures ANOVA for cortisol, stress 1 and stress 1 repeated

Effect	SS	df	MS	*F*	*P*
Stress	331.7	1	331.7	1.4	0.718
Error	18934.4	8	2366.8		

Table 3.2.4 Repeated measures ANOVA for cortisol, stress 2 and stress 2 repeated

Effect	SS	df	MS	*F*	*P*
Stress	59.9	1	59.9	0.62	0.810
Error	7721.14	8	965.14		

Table 3.2.5 Repeated measures ANOVA for cortisol, stress 3 and stress 3 repeated

Effect	SS	df	MS	*F*	*P*
Stress	6716.7	1	6716.7	2.33	0.166
Error	23094.15	8	2886.77		

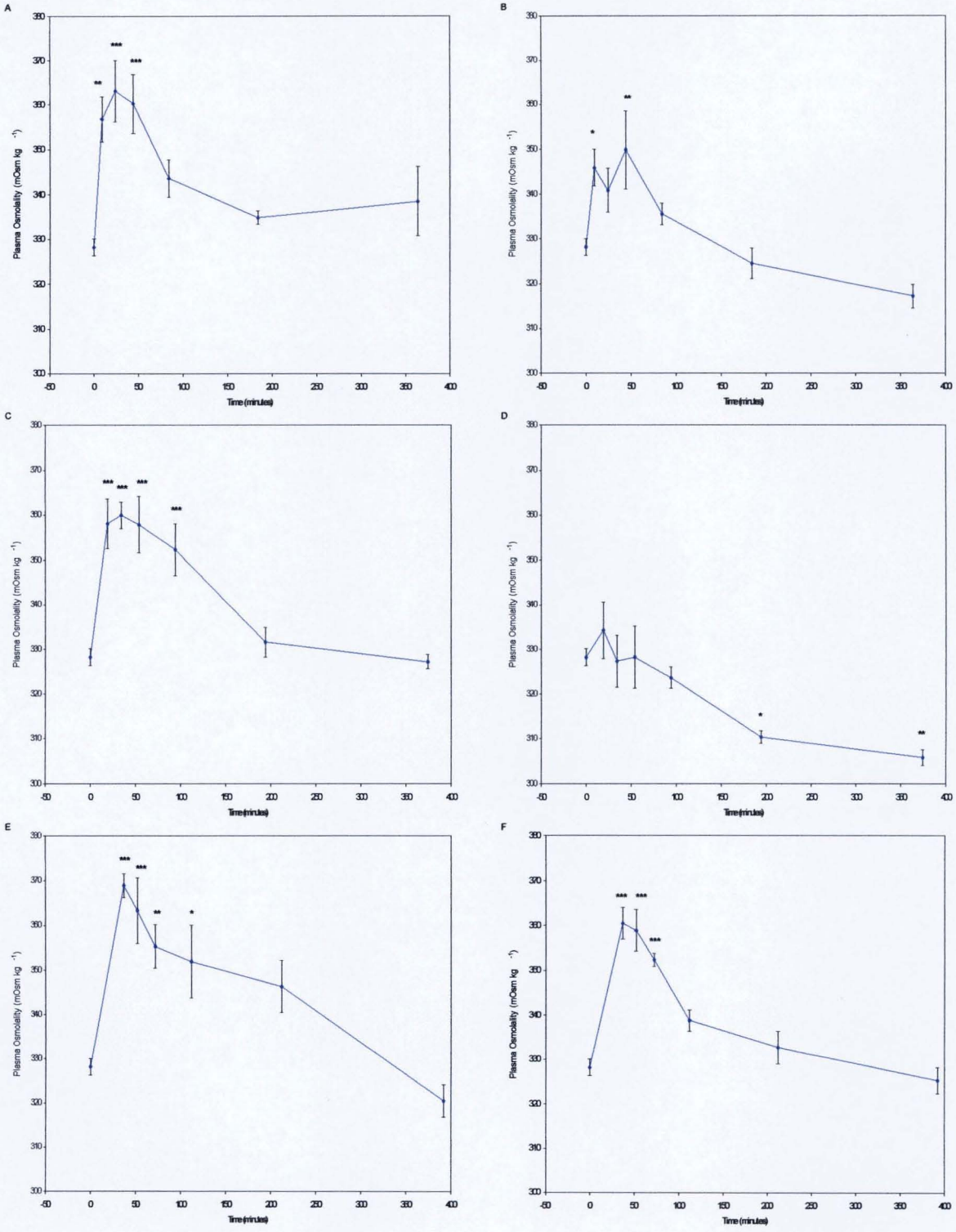


Figure 3.3.2. Plasma osmolality (mOsm kg^{-1}) changes with time (minutes) from Atlantic halibut (each point n=5) confined underwater for: 2 minutes (A), 2 minutes (same group 4 days later (B)), 12 minutes (C), 12 minutes (same group 4 days later (D)) and 30 minutes (E), 30 minutes (same group 4 days later (F)). *p<0.05, **p<0.01 and ***p<0.001 compared to 0 h basal values (ANOVA). Vertical lines represent S.E.M

Table 3.2.6 Repeated measures ANOVA for osmolality, stress 1 and stress 1 repeated

Effect	SS	df	MS	*F*	*P*
Stress	2084.63	1	2084.63	34.6	0.0004
Error	482.31	8	60.28		

Table 3.2.7 Repeated measures ANOVA for osmolality, stress 2 and stress 2 repeated

Effect	SS	df	MS	*F*	*P*
Stress	8825.66	1	8825.66	136.4	<0.0001
Error	517.63	8	64.7		

Table 3.2.8 Repeated measures ANOVA for osmolality, stress 3 and stress 3 repeated

Effect	SS	df	MS	*F*	*P*
Stress	506.26	1	506.26	16.35	0.0037
Error	247.7	8	30.96		

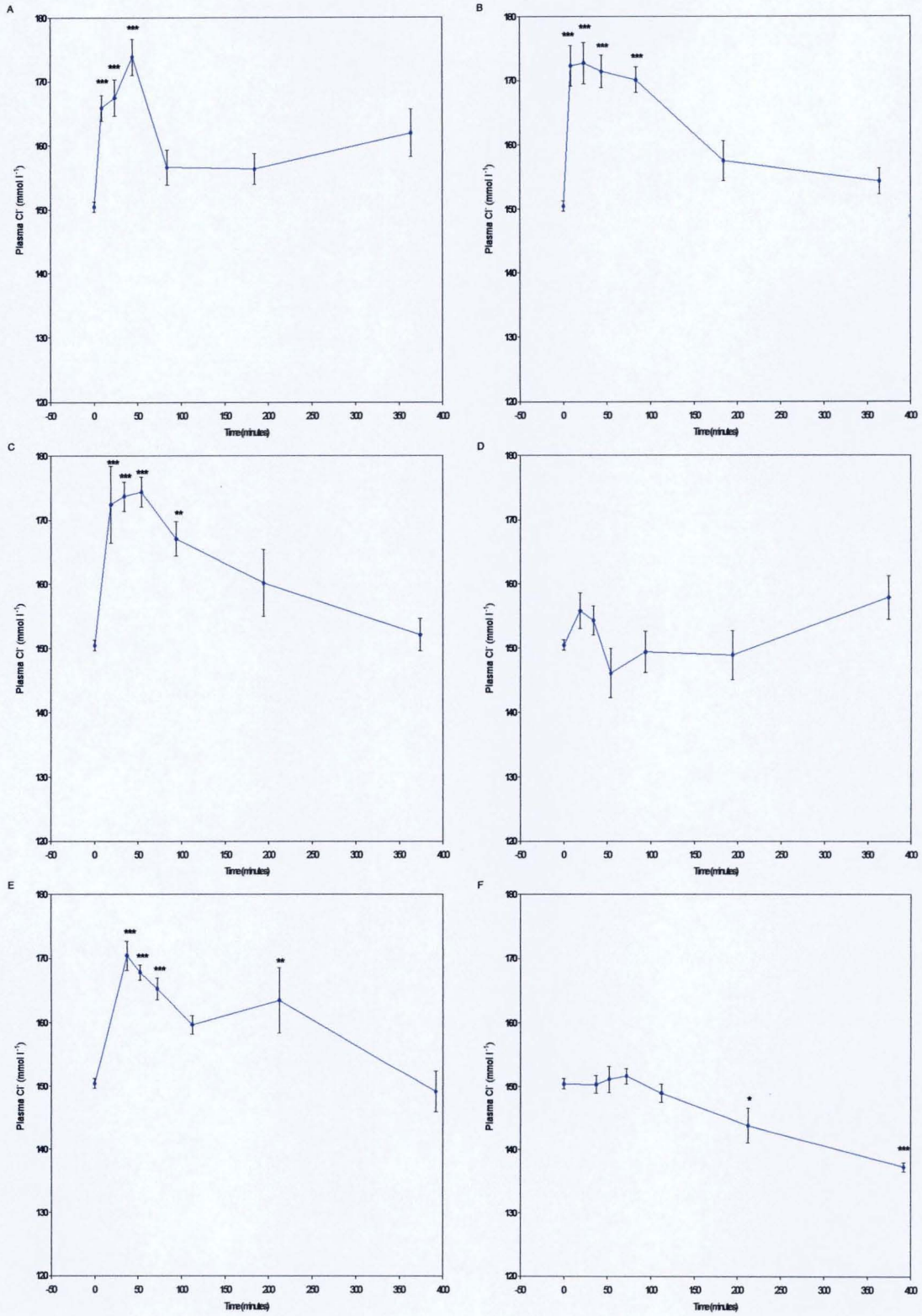


Figure 3.3.3. Plasma Cl^- (mmol l^{-1}) changes with time (minutes) from Atlantic halibut (each point n=5) confined underwater for: 2 minutes (A), 2 minutes (same group 4 days later (B)), 12 minutes (C), 12 minutes (same group 4 days later (D)) and 30 minutes (E), 30 minutes (same group 4 days later (F)). *p<0.05, **p<0.01 and ***p<0.001 compared to 0 h basal values (ANOVA). Vertical lines represent S.E.M

Table 3.2.9 Repeated measures ANOVA for chloride, stress 1 and stress 1 repeated

Effect	SS	df	MS	*F*	*P*
Stress	89.16	1	89.16	1.55	0.249
Error	461.47	8	57.68		

Table 3.2.10 Repeated measures ANOVA for chloride, stress 2 and stress 2 repeated

Effect	SS	df	MS	*F*	*P*
Stress	2734.38	1	2734.38	48.07	0.001
Error	455.06	8	56.88		

Table 3.2.11 Repeated measures ANOVA for chloride, stress 3 and stress 3 repeated

Effect	SS	df	MS	*F*	*P*
Stress	3054.98	1	3054.98	187.96	<0.0001
Error	130.03	8	16.25		

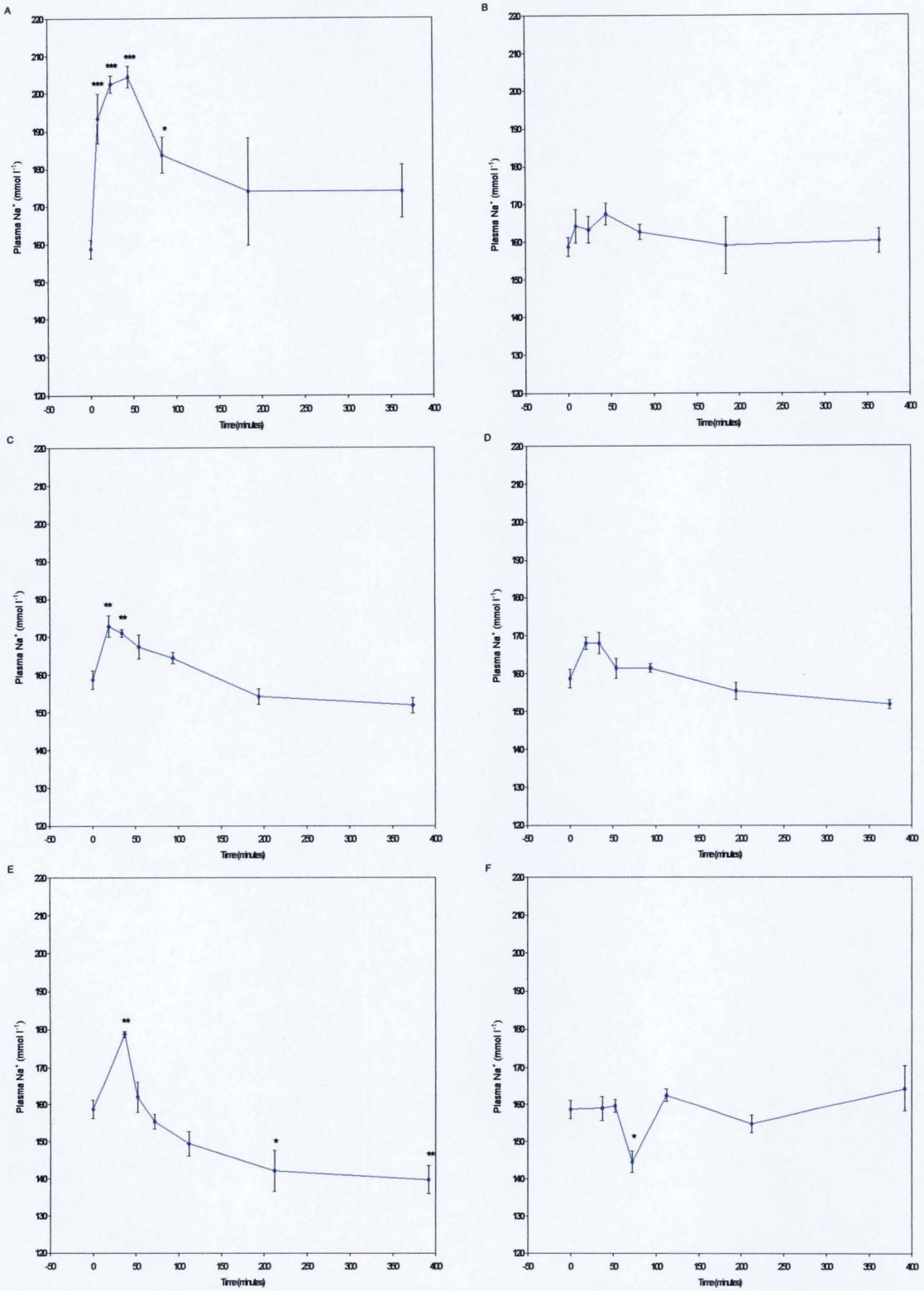


Figure 3.3.4. Plasma Na^+ (mmol l^{-1}) changes with time (minutes) from Atlantic halibut (each point n=5) confined underwater for: 2 minutes (A), 2 minutes (same group 4 days later (B)), 12 minutes (C), 12 minutes (same group 4 days later (D)) and 30 minutes (E), 30 minutes (same group 4 days later (F)). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to 0 h basal values (ANOVA). Vertical lines represent S.E.M

Table 3.2.12 Repeated measures ANOVA for sodium, stress 1 and stress 1 repeated

Effect	SS	df	MS	*F*	*P*
Stress	8619.15	1	8619.15	58.53	<0.0001
Error	1178.18	8	147.27		

Table 3.2.13 Repeated measures ANOVA for sodium, stress 2 and stress 2 repeated

Effect	SS	df	MS	*F*	*P*
Stress	86.91	1	86.91	4.881	0.058
Error	142.46	8	17.81		

Table 3.2.14 Repeated measures ANOVA for sodium, stress 3 and stress 3 repeated

Effect	SS	df	MS	*F*	*P*
Stress	108.13	1	108.13	1.143	0.316
Error	757.03	8	94.63		

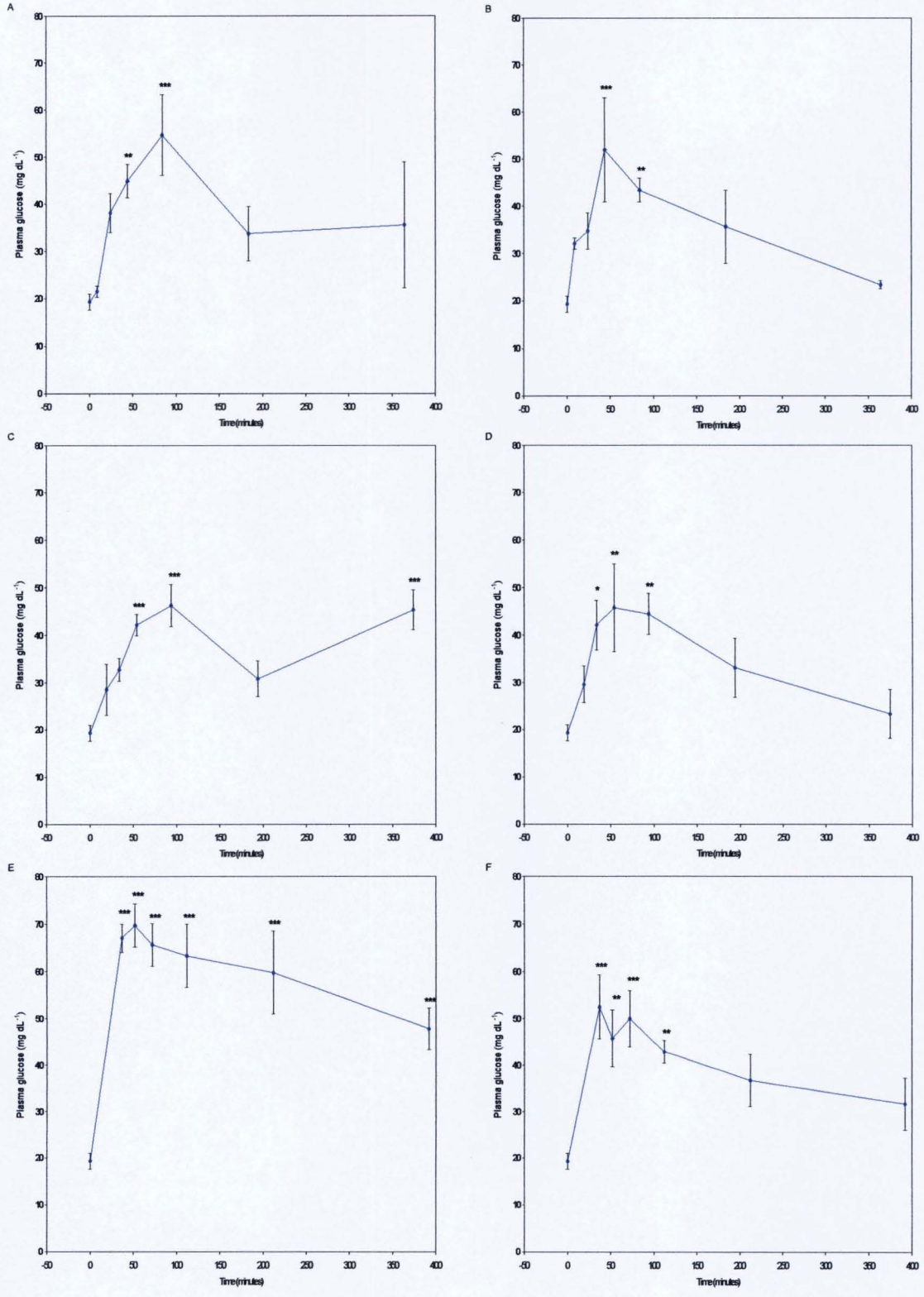


Figure 3.3.5. Plasma glucose (mg dL^{-1}) changes with time (minutes) from Atlantic halibut (each point n=5) confined underwater for: 2 minutes (A), 2 minutes (same group 4 days later (B)), 12 minutes (C), 12 minutes (same group 4 days later (D)) and 30 minutes (E), 30 minutes (same group 4 days later (F)). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to 0 h basal values (ANOVA). Vertical lines represent S.E.M

Table 3.2.15 Repeated measures ANOVA for glucose, stress 1 and stress 1 repeated

Effect	SS	df	MS	*F*	*P*
Stress	21.92	1	21.92	0.131	0.727
Error	1341.13	8	167.64		

Table 3.2.16 Repeated measures ANOVA for glucose, stress 2 and stress 2 repeated

Effect	SS	df	MS	*F*	*P*
Stress	19.88	1	19.88	0.231	0.644
Error	689.56	8	86.19		

Table 3.2.17 Repeated measures ANOVA for glucose, stress 3 and stress 3 repeated

Effect	SS	df	MS	*F*	*P*
Stress	4611.9	1	4611.9	49.07	0.0001
Error	751.89	8	93.99		

3.4 Discussion

Under conditions normally experienced in commercial aquaculture practices a wide range of stressors can be experienced by the fish during the course of a rearing cycle. Whether the stressor is chronic or acute in nature or whether the stressor occurs sporadically or on a more frequent basis will determine the nature and severity of any particular stress response that the fish may exhibit.

Periodic acute crowding of fish in commercial operations is often necessary during a number of commonly occurring management practices including fish transfer, sample weighing, prophylactic treatments, grading and counting. The effects of these practices on the fish in terms of performance and general health can be deleterious if the stress is too severe, prolonged or frequent. Monitoring of the primary and secondary indicators of stress during both initial and repeated acute crowding events provides an insight into the effects that a particular stressor has on the fish and can also be used as management tool for refining the rearing process.

3.4.1 Cortisol

The post stress cortisol concentration profiles measured during the current trial conformed to classical responses previously reported for other species subjected to various stressors including confinement, handling, exercise and tank water level reduction (Pickering, Pottinger et al., 1982; Biron and Benfey, 1994; Neilsen, Boesgaard et al., 1994; Einarsdottir and Nilssen, 1996; Waring, Stagg et al., 1996b, 1997).

Peak levels of plasma cortisol following acute stress are both stress and species specific. Prior to Rotllant, Balm et al., (2001) and Arends, Mancera et al., (1999) who

reported levels in excess of 175 and 450 ng ml^{-1} in the gilthead sea bream no records of cortisol levels exceeding 100 ng ml^{-1} had been reported for any Sparid *sp* (Rotllant and Tort, 1997). In salmonids levels exceeding 200 ng ml^{-1} have however been frequently reported (Sumpter, Dye et al., 1986; Benfey and Biron, 2000).

Higher levels still, in excess of 700 ng ml^{-1} have been reported for the striped bass and the roach (Noga, Kerby et al., 1994; Pottinger, Yeomans et al., 1999). Additionally, within the limited information regarding post stress plasma cortisol levels in the Atlantic halibut, similar peak levels of plasma cortisol after intense stress to those measured in the present study have been found (Van Ham, 2003). Clearly there is great variation in the reported post stress concentrations of plasma cortisol and whilst the maximum peak values reported in the present study fall within what could be regarded as a commonly expected range they are perhaps nearer the lower end of the scale. There was no evidence of any cumulative effects or habituation of the cortisol response following the repeat stress for either the 2 minute crowd or the 30 minute crowd when maximum values were slightly lower after the repeat stress than after the initial stress. The maximum value after the 12 minute crowd was slightly higher following the repeat stress than the initial stress. However, there were no significant differences between any of the maximum values recorded in either the initial stress or the repeat stress for any of the crowding durations ($P<0.05$ ANOVA) thus no clear cumulative pattern was in evidence. Cumulative cortisol responses have been reported for both salmonids *Oncorhynchus tshawytscha* and the marine flatfish *Scopthalmus maximus* (Barton, Schreck et al., 1986; Mesa, 1994; Waring, Stagg et al., 1996b, 1997). The lack of any such effect in the present study is probably a reflection of the time difference between the initial and the repeat stress. Time differences

between stressors in part help determine whether a stress response is cumulative, habituating or compensatory in nature (Schreck, 2000). For a response to be cumulative it would seem likely that the time interval would need to be less than in the current study (Waring, Stagg et al., 1997). Conversely, habituation of the cortisol response following various different stressors has been noted by a number of authors. (Einarsdottir and Nilssen, 1996; Tanck, Booms et al., 2000; Flodmark, Urke et al., 2002). It would appear likely that for the cortisol response to be habituating there needs to be multiple stressors of a single type rather than an initial stress and a single repeat stress (Tanck, Booms et al., 2000; Flodmark, Urke et al., 2002).

The physiological level at which the cortisol response becomes habituating has been the focus of some investigation. It is possible that rather than negative feedback of cortisol regulating ACTH hyposensitivity, it is circulating concentrations of ACTH at the level of the hypothalamus that regulate the habituating interrenal response (Balm and Pottinger, 1995). Further, the system of gene expression of arginine vasotocin (AVT), one of the hypothalamic neropeptides, which is involved in ACTH stimulation (Moons, Cambre et al., 1991) during acute and chronic stress may habituate (Gilchriest, Tipping et al., 2000).

3.4.2 Glucose

Basal glucose levels were 19.3±1.7mg dL^{-1} which are within the range of reported basal levels for other species (Vijayan and Moon, 1994; Waring, Stagg et al., 1996a, 1997). Many authors have however reported higher basal levels of plasma glucose which are frequently in excess of 3 times the value found in the present study (Vijayan, Pereira et al., 1997; Pottinger, 1998; Ruane, Huisman et al., 2001). The basal values determined in

the present study are however, corroborated by similar results obtained for halibut following 30 minutes of net confinement (Van Ham, 2003). The post stress maximum response, depending on the duration of the stress in the current study ranged from 45.7±9.2 to 69.7 ±9.2 mg dL^{-1}. These values were in the range of previously reported post stress maximum values found in other species subjected to differing types of stressors (Barton and Schreck, 1987; Carragher and Rees, 1994; Vijayan and Moon, 1994) and more specifically for halibut following a 30 minute net confinement stress (Van Ham, 2003). Higher plasma glucose levels have however been frequently reported in other species (Davis and Parker, 1990; Biron and Benfey, 1994; Benfey and Biron, 2000).

The pattern of the stress response followed that which has been previously reported (Pickering, Pottinger et al., 1982; Waring, Stagg et al., 1996a; Van Ham, Van Anholt et al., 2003). There was no clear difference in the rate of recovery between each confinement duration. After all of the differing stress durations the values returned towards basal levels although glucose levels did not return to pre-stress levels in any of the groups during the 6 hour sampling period. Elevated concentrations of plasma glucose have been recorded 12 – 48 hours following a number of stressors in different species (Barton, Schreck et al., 1986; Vijayan and Moon, 1992; Rotllant and Tort, 1997; Arends, Mancera et al., 1999; Ackerman, Forsyth et al., 2000), thus it is not unexpected that recovery in the present trial was incomplete after 6 hrs.

An apparent decrease in the maximum response of circulating plasma glucose levels between the initial stress and the repeated stress for all crowding durations was recorded. However, none of the differences were significant ($p<0.05$ ANOVA). The largest reduction was between the initial 30 minute stress and the repeat 30 minute stress

when the plasma glucose levels were 69.7±4.5 and 52.5±6.8 mg dL^{-1}. The lack of any significant difference between the maximum values in any of the initial or the repeated crowdings would suggest that there was no habituation or impairment of the mechanisms controlling glucose mobilization as a result of the initial confinement.

The timing of the maximum response after all of the repeat stressors was faster by one sample point than after the initial stressor. Again, as with the cortisol results there was no definitive evidence that there was either a cumulative response or habituation occurring.

No sustained elevation of plasma glucose concentrations occurred although following the initial 12 minute confinement there was an unexpected peak at the last point. Following both the initial and repeat 30 minute confinements the levels remained elevated for slightly longer than the other groups. This slight increase in recovery time may indicate the activity of gluconeogenesis whereas the response shown following the 2 minute crowd may be due only to catecholamine induced glycogenolysis (Vijayan, Pereira et al., 1997; Wendelaar Bonga, 1997).

3.4.3 Plasma Ions

Disturbances in plasma ion concentrations were evident following all durations of both the initial and repeat stresses. The initial phase of the hydromineral stress responses for all groups (except the Na+ values following the repeated 30 minute crowding when there was an unexpected post stress temporary drop) were in accordance with the findings of other studies on halibut (Van Ham, 2003) and turbot(Waring, Stagg et al., 1996b, 1997). However, in the present study the recovery period was far shorter with levels returning to

basal values or often below within 6 hours as opposed to 24 – 48 hours (Waring, Stagg et al., 1997; Van Ham, 2003). Post stress recovery values at a sub basal level are in accordance with the findings from halibut subjected to net confinement of (Van Ham, 2003) but this was not observed in either of the studies on turbot subjected to net confinement and handling by (Waring, Stagg et al., 1996b, 1997). These differences may in part be due to specific species differences in response to stress or to the different nature of the stressors.

In fresh water fish where an opposite pattern is in evidence recovering plasma ion concentrations can exceed the previous basal levels (McDonald and Milligan, 1997).

Whereas there were no significant differences in the plasma cortisol and glucose responses recorded with successive crowding stressors there were differences between the maximum osmolality and Na^+ and Cl^- plasma concentrations. A reduction in the maximum circulating levels of osmolality, Na^+ and Cl^- after the repeat stress in comparison to the initial stress was noted for all three durations. Acute stress can lead to increased circulating levels of catecholamines which cause an increase in the oxygen uptake capacity of the gills by raising blood flow, recruitment of new, and the distension of existing, lamellae. As a result of these factors the available surface area of the gills that allows for the diffusion of water and ions is increased leading to an increase in the flux of ions. In seawater fish there is a passive influx of ions from the hyperosmotic surroundings (with the reverse situation occurring in freshwater fish) thus an increase in the surface area available for diffusion will increase circulating plasma ion concentration. Catecholamine secretion into the circulation may only occur under conditions of extreme physiological impairment (Perry and Bernier, 1999) and without measurement of plasma

CA's their involvement can only be speculated. The short term nature of the plasma ion response in the present study may imply that a CA derived response can be questioned and that an alternative causal factor such as haemoconcentration may be responsible for the observed disturbance (Wendelaar Bonga, 1997). However, given the nature of the stressor which in effect combined netting, brief air exposure and confinement it would seem plausible that the stress was severe enough in nature to elicit a catecholamine response which subsequently led to the observed ionosmotic disturbances.

Where there was no evidence of impairment, or habituation of the cortisol and glucose responses between the initial and repeat stresses, reductions in maximum post stress levels of osmolality Na^+ and Cl^- after the repeat stress may indicate that the effect of the stresses on osmoregulatory function was longer lasting. Whether the reductions in maximum values were a result of down regulation of catecholamine activity (Gamperl, Vijayan et al., 1994a; Gamperl, Wilkinson et al., 1994b) or whether perhaps hormonally mediated recovery mechanisms and newly activated transport sites in the gills (McDonald and Milligan, 1997) may have remained "active" remains to be established.

In summary the results clearly show that the Atlantic halibut is affected by stressors in a similar manner to round fish previously studied. The degree of the response in terms of circulating plasma cortisol and plasma glucose when compared to other species may be at the lower end of values previously reported (Barton and Iwama, 1991; Barton, 1997; Van Ham, 2003) perhaps indicating a marginally reduced stress response in the Atlantic halibut. Duration didn't appear to effect the magnitude of the response and there appeared to be no signs of either habituation or a cumulative response to a repeated stress 4 days

after an initial stress in terms of plasma cortisol or plasma glucose. However, the recovery from osmotic disturbances may be longer lasting. Given these results it would seem prudent to let fish recover for at least four days before being subjected to further husbandry stressors to allow a full recovery of the osmoregulatory mechanism.

CHAPTER 4

THE IN-VITRO RESPONSE OF ATLANTIC HALIBUT INTERRENAL TISSUE TO ACTH, AII AND K^+ STIMULATION

4.1 Introduction

The steroid glucocorticoid hormone cortisol is the primary corticosteroid in teleost fish (Henderson and Garland, 1980; Sumpter, 1997). Cortisol production in teleost fish is located in the interrenal tissue, which unlike the mammalian adrenal cortex does not form a distinct gland. Distribution of teleost interrenal tissues, whilst its morphological location varies to some extent with species is generally located in groups, chords or strands around the posterior cardinal veins and the dorsal aorta (Figure 4.1.1). Within the kidney the interrenal tissues are usually confined to the anterior section or 'head kidney' (Figure 4.1.2) intermingled to varying degrees also, depending on species, with other cell types such as chromaffin, pigment and haemotopoietic cells (Chester Jones and Mosley, 1980). Increased plasma cortisol is responsible for the osmoregulatory and metabolic physiological responses to stress and its production is the culmination of the hypothalamo-pituitary-interrenal axis.

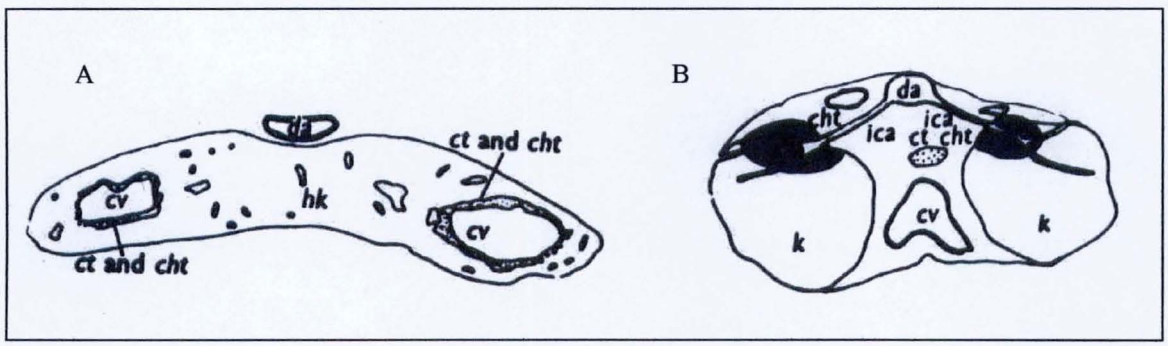


Figure 4.1.1 Diagrammatic representation of the relationship between chromaffin and adrenocortical tissues in A; teleosts (salmon) and B; elasmobranchs (dogfish). In the teleost the chromaffin and adrenocortical tissues are intermingled and in the elasmobranch they are separated completely. Stippled, cortical tissue; black, chromaffin tissue; k, kidney; hk, head kidney; cv, cardinal vein; vc, vena cava; cht, chromaffin tissue; da, dorsal aorta; ica, intercostal artery; ct cortical tissue (Balment, Henderson et al., 1980)

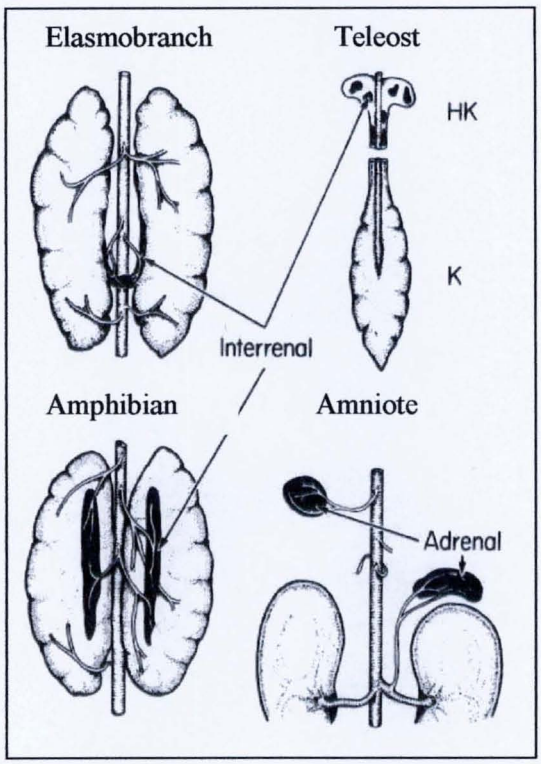


Figure 4.1.2 Position of interrenal tissue in Elasmobranchs, Teleosts, Amphibians and adrenal in Amniotes (reptiles, birds and mammals). HK, head kidney; K Kidney (Balment, Henderson et al., 1980).

Cortisol is under complex multifactorial control although within the HPI axis adrenocorticotrophic hormone (ACTH) plays the central role in controlling the secretion of the corticosteroids from the interrenal tissue (Sumpter, 1997). Additionally the peptide hormones α-MSH and β-endorphin, synthesised and secreted from melanotrophic (MSH) cells in the pars intermedia of the adenohypophysis (Figure 4.1.3) (Henderson, 1997) possibly interact with ACTH and have a controlling role in the regulation of cortisol (Balm, Hovens et al., 1995). ACTH, α-MSH and β-endorphin are synthesised from

peptides, which originate from a common precursor molecule, proopiomelanocortin (POMC). In the ACTH cells the end products are an N-terminal peptide, ACTH and lipotrophin (β-LPH) and in the MSH cells these peptides are processed further: ACTH yields α-MSH and corticotrophin-like intermediate lobe peptide, while β-LPH is cleaved to give β-endorphin and γ-LPH. The latter may be processed further to β-MSH and the N-terminal fragment to γ-MSH (Wendelaar Bonga, 1993). These processes are schematically represented in Figure 4.1.4. In the halibut 8 distinct immunoreactive cell types have been identified and localized in the adenohypophysis and the general cellular organization is similar to that of other evolved teleosts (Weltzien, Norberg et al., 2003).

In addition to ACTH, α-MSH and β-endorphin, which share the same precursor other unrelated peptide hormones including angiotensin II, urotensin I and urotensin II have been shown *in vitro* to have stimulatory effects on cortisol production from isolated teleost interrenal tissue (Arnold-Reed and Balment, 1991, 1994). Also, elevated levels of potassium albeit out with the normal physiological range have been shown to elicit a mild corticosteroidogenic response *in vitro* from the isolated interrenal of the dogfish *Scyliorhinus canicula,* (O'Toole, Armour et al., 1990) although in the rainbow trout, *Salmo gairdineri*, no effect was apparent (Decourt and Lahlou, 1986). In the present study the steroidogenic effects of increasing doses of ACTH, AII and K^+ on isolated interrenal tissues from the Atlantic halibut were of particular interest and are discussed further below.

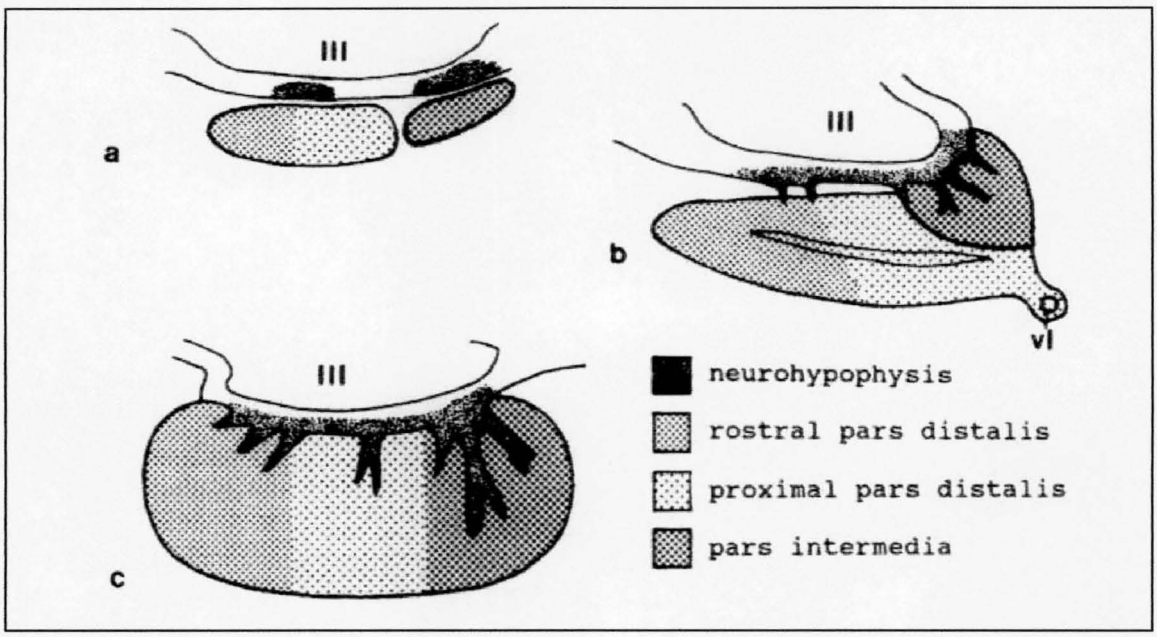


Figure 4.1.3 Representation of a midsagittal section of the pituitary glands of fishes: (a) agnathan (lamprey; in the hagfishes a pars intermedia is absent); (b) chondrichthyan (shark), vl, ventral lobe; (c) actinopterygian (teleost) (Wendelaar Bonga, 1993).

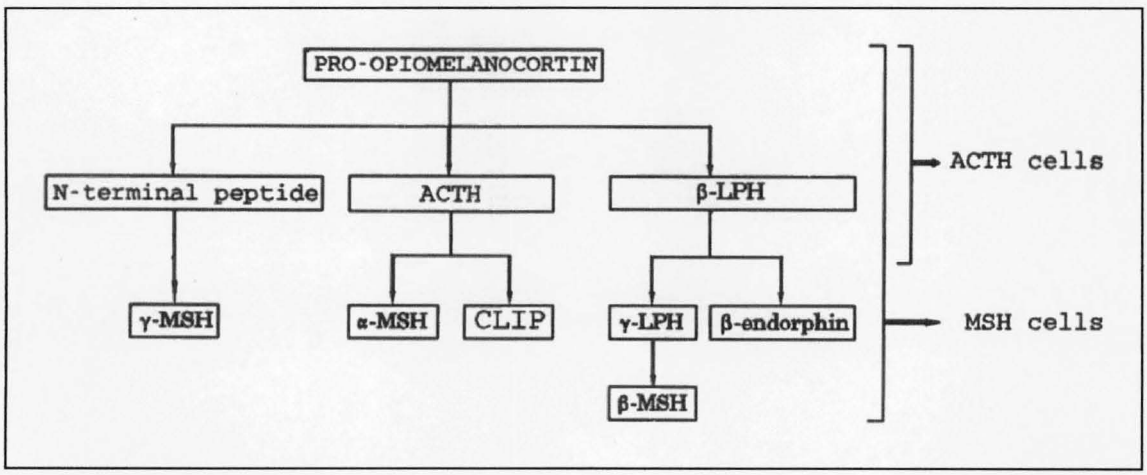


Figure 4.1.4 Schematic representation of the fate of proopiomelanocortin (POMC) in ACTH and MSH cells of the vertebrates; ACTH, adrenocorticotrophic hormone; LPH, lipotropin; MSH, melanophore stimulating hormone; CLIP, corticotropin-like intermediate lobe peptide (Wendelaar Bonga, 1993).

4.1.1 Adrenocorticotrophic hormone (ACTH)

Within the hypothalamo-pituitary-interrenal hormonal cascade the regulation of ACTH secretion in teleosts is generally assumed to be under the control of corticotrophin-releasing hormone (CRH). Demonstrations of *in-vitro* stimulation of ACTH production from isolated pituitary glands by CRH have been carried out for a number of species both freshwater and marine (Baker, Bird et al., 1985; Fryer and Lederis, 1986; Olivereau and Olivereau, 1991; Balm, Pepels et al., 1994; Rotllant, Balm et al., 2001). Although CRH plays the central role in the modulation of ACTH secretion a number of other factors such as the inhibitory role of the neuropeptide melanin-concentrating hormone (MCH) and the stimulatory actions of arginine vasotocin (AVT), isotocin (IT) neuropeptide Y, and Urotensin I (Fryer, 1989; Sumpter, 1997) have been shown.

Cortisol itself via both short and long loop negative feedback mechanisms can also affect ACTH stimulation by CRH and at the pituitary level (Rotllant, Arends et al., 2000) (Donaldson, 1981; Pickering, Pottinger et al., 1987). It has also been shown to act more directly on the interrenal tissue (Bradford, Fitzpatrick et al., 1992).

ACTH is one of a variety of hormones, growth factors and other regulatory molecules that act by altering intracellular levels of adenosine 3′, 5′ - cyclic monophosphate (cAMP) and subsequently the activity of cAMP-dependant protein kinase. ACTH binds to specific receptors in the interrenal tissue, activating adenylate cyclase and raising the intracellular cAMP concentration. Cyclic AMP-dependant kinase then phosphorylates and activates specific enzymes required for the synthesis of cortisol (Lehninger, Nelson et al., 1993). In addition Ca^{2+} influx has been shown to be stimulated by ACTH and implicated as an

additional second messenger (Kojima, Kojima et al., 1985 (a)). When intracellular Ca2+ levels rise activation of calmodulin-dependant enzymes regulate steroidogenesis.

4.1.2 Angiotensin II (AII)

Angiotensin II results from the renin-angiotensin system that is common to all vertebrate groups including teleost fish (Wendelaar Bonga, 1993).

Angiotensin II stimulates a wide range of effectors in teleosts and is associated with, maintenance of body fluid volume, arterial blood pressure (Olson, 1992; Kobayashi and Takei, 1996) and electrolyte balance/osmoregulation (Eckert, Hirano et al., 2003). In the kidney AII produces renal vasoconstriction, antidiuresis and reduces the number of filtering glomeruli (Gray and Brown, 1987; Brown, Gray et al., 1990). AII also has well documented dipsogenic effects both in the teleosts and elasmobranchs (Olson, 1992; Kobayashi and Takei, 1996; Anderson, Takei et al., 2001)

In mammals AII is a major hormonal regulator of aldosterone secretion from the adrenal glomerulosa cells. In mammalian systems when aldosterone secretion is stimulated by AII there is a rapid but transient mobilization of calcium from an intracellular pool and a sustained increase in the influx of calcium in adrenal glomerulosa cells (Kojima, Kojima et al., 1985 (b)). Similar mechanisms for calcium control of steroidogenesis have been shown in the dogfish (Armour, O'Toole et al., 1993). Functional AT_1-like AII receptors coupled to cellular calcium signalling have been demonstrated in the glomeruli of the rainbow trout, Oncorhynchus mykiss (Cobb, Williamson et al., 1999).

AII receptors have been located, amongst other tissues (Russell, Klemmer et al., 2001), in the interrenal tissue of the teleost kidney (Cobb and Brown, 1992) and corticosteroid

stimulation by AII has been shown *in vivo* in elasmobranchs (Hazon, Decourt et al., 1987) and teleosts (Perrott and Balment, 1990). *In vitro* techniques have also demonstrated a similar stimulation of cortisol by AII in teleosts and, 1α-hydroxycorticosterone (1α-OH-B) the cortisol homologue in elasmobranchs, from isolated interrenal tissue (Arnold-Reed and Balment, 1994) (O'Toole, Armour et al., 1990).

AII was however found to be less potent than ACTH in stimulating corticosteroid production from both the perifused interrenal tissue of the trout and the dogfish (Armour, 1991; Arnold-Reed and Balment, 1994).

4.1.3 Potassium (K^+)

Potassium is essential for a number of physiological mechanisms including active co transport and ion channels and the requirement by pyruvate kinase in catalyzing the transfer of the phosphate group from phosphoenolpyruvate to ADP during the final step of glycolysis (Lehninger, Nelson et al., 1993). In mammalian systems K^+ has been shown to stimulate aldosterone and, similar to both ACTH and AII, the mechanism involves an increase in the influx of calcium across the plasma membrane of the adrenal glomerulosa cells (Kojima, Kojima et al., 1985 (a)). In the elasmobranch *Scyliorhinus canicula* K+ was shown to significantly stimulate the in vitro secretion of 1α-OH-B although the effect was only evident at a concentration far exceeding the normal physiological range. In the teleost *Salmo gairdneri* no stimulation of cortisol by K^+ was observed (Decourt and Lahlou, 1986).

4.1.4 In vitro perifusion techniques.

In vitro perifusion techniques have been employed to investigate the steroidogenic effects of various stimuli including ACTH, AII and K^+ on the interrenal tissue from a number of species of teleost fish including carp (Ilan and Yaron, 1976), cichlids ,(Ilan, 1980), turbot (Quabius and Brown, 2000), Chinook salmon (McQuillan, Lokman et al., 2003) and trout (Rance and Baker, 1981; Gupta, Lahlou et al., 1985; Decourt and Lahlou, 1986; Arnold-Reed and Balment, 1994; Brodeur, Daniel et al., 1998). The technique has also been used with elasmobranchs (dogfish) (O'Toole, Armour et al., 1990; Armour, O'Toole et al., 1993).

4.1.5 Aims

The aim of the current study was to verify that Atlantic halibut respond in a similar manner to other teleosts in their response to classical corticosteroid stimuli. Little or no previous information is currently available on the HPI axis and stress response of the Atlantic halibut. Therefore the establishment of basic physiological parameters and responses was necessary in order to interpret the plasma parameters measured in halibut held in commercial aquaculture systems (chapter 1 and chapter 5 this thesis). The effects of varying concentrations of ACTH, AII (AII) and Potassium (K+) on cortisol production in vitro from the interrenal tissue of the Atlantic halibut (Hippoglossus hippoglossus) were investigated.

4.2 Materials and Methods

The effects of varying concentrations of Porcine Adrenocorticotrophic hormone ACTH (Sigma), [Asn1, Val5]-Angiotensin II (Sigma) and K^+ on cortisol secretion *in vitro* from the perifused head kidney of Atlantic halibut was investigated. The method used was based on that previously employed for elasmobranch interrenal glands (O'Toole, Armour et al., 1990; Armour, O'Toole et al., 1993). Similar techniques have been previously used with teleost head kidney preparations (Gupta, Lahlou et al., 1985; Decourt and Lahlou, 1986; Arnold-Reed and Balment, 1991, 1994).

4.2.1 Perifusion Medium preparation

The perifusion medium used was teleost Ringer solution (Table 4.2.1). A 3l stock solution of fresh Ringer solution without glucose was made and refrigerated at 0-4°C in a sealed glass flask. The stock Ringer solution was stored for no longer than 3 days. For each perifusion one litre of Ringer was sufficient. Prior to being used in the perifusions the appropriate amount of glucose was added and the pH was adjusted to 7.4 with HCl or NaOH. On addition of the glucose and adjustment of the pH, the Ringer solution was stirred on ice until used.

Table 4.2.1 Teleost Ringer Recipe as used for isolated interrenal perifusion

Constituents	mM	g l^{-1}
Calcium chloride	2.12	0.312
Magnesium sulphate	0.37	0.091
Magnesium chloride	0.86	0.175
Potassium phosphate	1.61	0.219
Sodium bicarbonate	25	2.10
Sodium chloride	136.78	7.99
Glucose	5.55	1.00

4.2.2 Perifusion Protocol

4.2.2.1 Removal of Head Kidneys

Two perifusions were carried consecutively. Two fish were removed from their holding tank and placed in a 10-litre bucket containing 5 litres of seawater and 10ml of 2-phenoxyethanol. The anaesthetized fish were then sacrificed by an approved Home Office Schedule 1 technique; a blow to the head and severing of the spinal chord. Blood samples were taken by caudal venepuncture with a heparinized needle and stored on ice prior to centrifugation (13000rpm for 3 min). Plasma was removed to a clean eppendorf and stored at –20 degrees C prior to analysis of cortisol, plasma osmolality, chloride, sodium, potassium and glucose levels.

The kidneys were then dissected from each fish in their entirety and placed on ice in a separate glass petri dish containing Ringer solution. The head kidney portion was separated from the remainder of the kidney structure with a razor blade (Figure 4.2.1), gently shaken to remove as much Ringer solution as possible and weighed to the nearest 0.01g. After recording the weight, each head kidney was then returned to the ice cold Ringer solution in the appropriate petri dish.

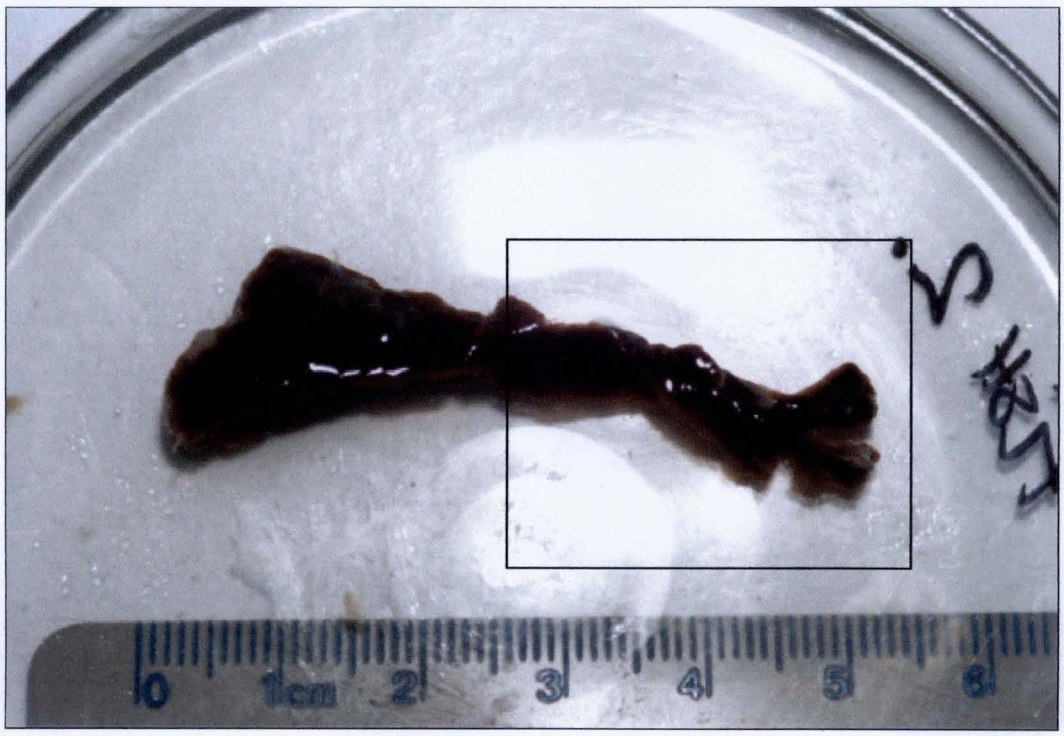


Figure 4.2.1 Dissected halibut kidney showing head kidney region.

4.2.2.2 Perifusion of Head Kidneys

Immediately after weighing each head kidney was diced into roughly 1mm square pieces and placed into the perifusion chambers (one per fish). Each chamber consisted of the needle end of a 5ml syringe cut down to 2cm and fitted with a lid. The diced head kidney was placed between 2 gauze shelves inside each Ringer filled chamber. The chamber was sealed whilst being immersed in Ringer solution which prevented air bubbles from being trapped on the underside of the lid.

The Ringer solution used for the perifusion medium was placed in a glass flask in a water bath at 14°C. 95% O_2 5%CO_2 was applied to the solution via an airstone. The Ringer solution was administered to each chamber with a peristaltic pump (Watson Marlow 205CA) at a rate of 0.5ml/min. The solution entered the chambers via a needle through the lid and exited via a needle attached to the lower end of the syringe. The chambers were held in a vertical position by test tube clamps above a fraction collector (Ultrorac 7000, L.K.B., Broma, Sweden) loaded with glass test tubes (Figure 4.2.2). The exchange rate of the fraction collector was set at 12 minutes. Prior to collection of the first sample the perifusion medium was run through the chambers to waste for 4 hours in order to attain a constant basal secretory rate of cortisol from the interrenal cells.

Prior to application, each challenge substance (ACTH and [Asn1, Val5]-Angiotensin II) was dissolved to the required concentration in Ringer solution that had been maintained at 14°C. Elevated K^+ concentrations were achieved by increasing the amount of potassium phosphate added to an aliquot of normal Ringer solution.

The dose response cycle was initiated immediately after tube number 10 of 20 had moved into the collection position. The peristaltic pump tubes were removed from the Ringer

solution and placed into the test substance. Each test substance was applied for 12 minutes after which the tubes were returned into the flask containing the Ringer solution. To allow for the full response to the stimulus to be determined 12-minute samples were collected for a further 2 hours.

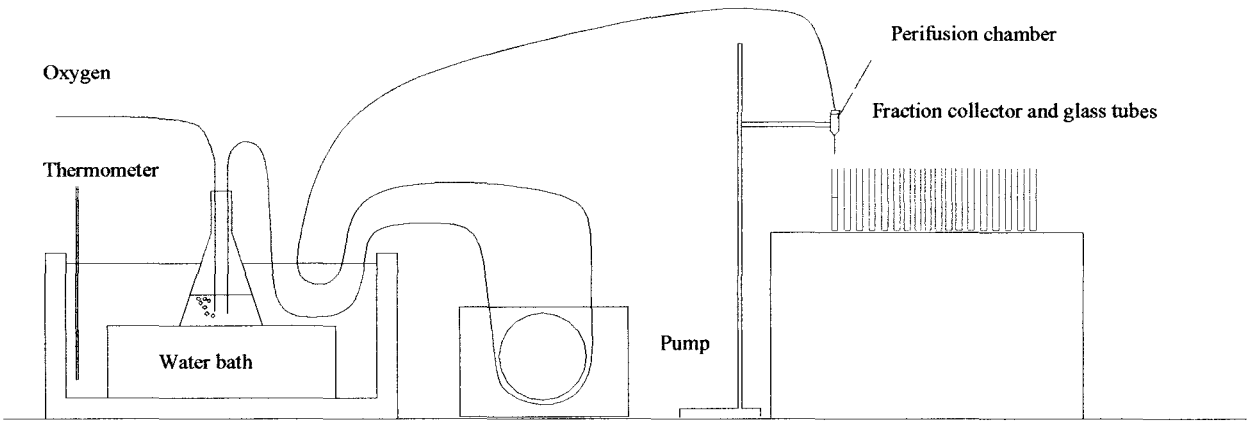


Figure 4.2.2 Schematic representation of interrenal perifusion apparatus

4.2.3 Cortisol extraction procedure

Cortisol was extracted from the samples using SEP PAK C18 cartridges (Waters Corporation, Milford, Massachusetts, USA). The SEP PAK cartridges were attached to 25ml syringes and constant infusion rates were facilitated by an infusion / withdrawal pump (PHD 2000, Harvard Apparatus, MA, USA).

Prior to the extraction of the cortisol the cartridges were primed with 3ml of methanol applied at 20ml/min followed by 5ml distilled water also applied at 20ml/min. The 6ml perifusion samples were then applied at rate of 1.5ml/min. The cartridges were then washed with 5ml of distilled water at 20ml/min after which the cortisol was eluted at 3ml/min with 4ml of methanol and collected in plastic LP4 tubes (Denley Instruments).

A range of different aliquot sizes was taken from each tube to ensure that the concentration of cortisol would fall within the confines of the standard curve when analysed. The aliquot sizes ranged from 250µl to 2ml depending on the concentration of the test substance. All aliquots were labeled and dried in a vacuum centrifuge (GL11 Gyrovap and Crist CT 02-50 chiller) set at 30°C and then frozen at –20°C awaiting further analysis.

The SEP PAK cartridges were reused according to the manufactures instructions up to 4 times. After the cortisol had been eluted 5ml of 8M Urea was applied at 20ml/min followed by washing with 25ml of distilled water at 20ml/min. After this last step the process could begin again with the priming of the cartridges with methanol.

4.2.4 Radioimmunoassay (RIA) procedure

The procedure for the RIA was the same as that employed in chapter 2 Sections 3.2.7-3.2.9

4.2.5 Statistical analysis

Statistical analysis was performed using STATISTICA™ Version 7 (Statsoft®) A Kolmogorov Smirnov test was used to assess the normality of distributions. A paired t test was used to compare maximum vales with basal rates of cortisol secretion. A one-way ANOVA was used to compare differences in response time between test substance dose. Significant ANOVA's were followed by a Tukey-Kramer multiple comparison test. Correlation between dose and response time was determined by linear regression followed by ANOVA. If not otherwise stated, a significance level of 0.05 was used.

4.3 Results

4.3.1 Basal cortisol secretion

Prior to the commencement of the collection process there was a demonstrable washout phase (Figure 4.3.1) where cortisol secretion was high and variable. Prior to the collection phase (after 4 hours) the perifused Ringer solution was passed to waste. During the washout phase a steady basal state was reached and recorded for the first 2 hours of the collection phase prior to the administration of each test substance. The basal secretory rates for each preparation are listed for each experiment in the appropriate figure legends.

4.3.2 Dose dependent effects of Porcine ACTH on cortisol secretion.

A dose dependent response in cortisol production from perifused head kidney preparations was observed for concentrations of 0.0001, 0.001 and 0.01μM ACTH. A decline in stimulation from the maximal value was observed for concentrations of 0.1 and 1.0 μM ACTH (Figure 4.3.2). The maximum increase in stimulation above the basal rate was 1351±789% (S.E.M.) by 0.01μM ACTH. Increases above basal secretion rates following 0.0001, 0.001, 0.1 and 1.0μm ACTH treatments were 72±23, 211±71, 1201±391 and 984±323% respectively. Typically the peak response (Figure 4.3.6) to ACTH usually occurred within 12-72 minutes (mean times for 0.0001, 0.001, 0.01, 0.1 and 10μM ACTH were 21±5, 31±3, 52±8, 48±4 and 41±7 minutes ±SE respectively) and along with duration was dose dependant (Figures 4.3.5 and 4.3.6). Significant differences ($P < 0.05$, ANOVA) were observed between the maximum response time (0.01μM) and the response times following stimulation with both 0.0001 and 0.001μM ACTH. The

responses following treatment with 0.01, 0.1 and 1.0μM ACTH were significantly greater than the basal secretion rate ($P<0.05$, paired t test)

4.3.3 Dose dependent effects of Angiotensin II on cortisol secretion.

The stimulatory effect of angiotensin II on cortisol production was less than that of ACTH. The increase in cortisol production above basal rates was greatest following the addition of 1.0μM AII. An increase of 397±99% was observed whilst treatment with 0.1 and 1.0μM AII resulted in increases of 165±102% and 271±96% above the basal rate respectively (Figure 4.3.3).

The peak response time (Figure 4.3.7) ranged from 24 minutes to 120 minutes (mean times for 0.1, 1.0 and 10μM AII were 46±11, 66±12 and 52±15 minutes ±SE respectively) and was correlated with the % increase above basal secretory levels ($P<0.05$ ANOVA from linear regression of %increase against time). There was no significant difference ($P>0.05$, ANOVA) between peak response times for all treatments. All responses were significantly higher than the basal secretory levels ($P<0.05$, paired t test).

4.3.4 Dose dependent effects of potassium on cortisol secretion.

A mildly dose dependent steroidogenic effect was found when $3mMK^+$, $6mMK^+$ and $10mMK^+$ were applied to the perifusion chamber (Figure 4.3.4). Only a non-physiological concentration of 10mMK elicited a significant effect above basal levels ($p<0.05$, paired t test) The changes caused to the basal levels of cortisol secretion by all K^+ concentrations were slight and there were no significant differences in the timing of the peak response ($P>0.05$, ANOVA). The timing of the peak response time varied

between 48 and 72 minutes. The maximum mean increase expressed as a percentage above the basal level of secretion was 47.2±26.7% S.E.M. following the addition of 10mMK. 3mMK and 6mMK elicited responses of 5.99±5.52% and 20.28±4.7% respectively.

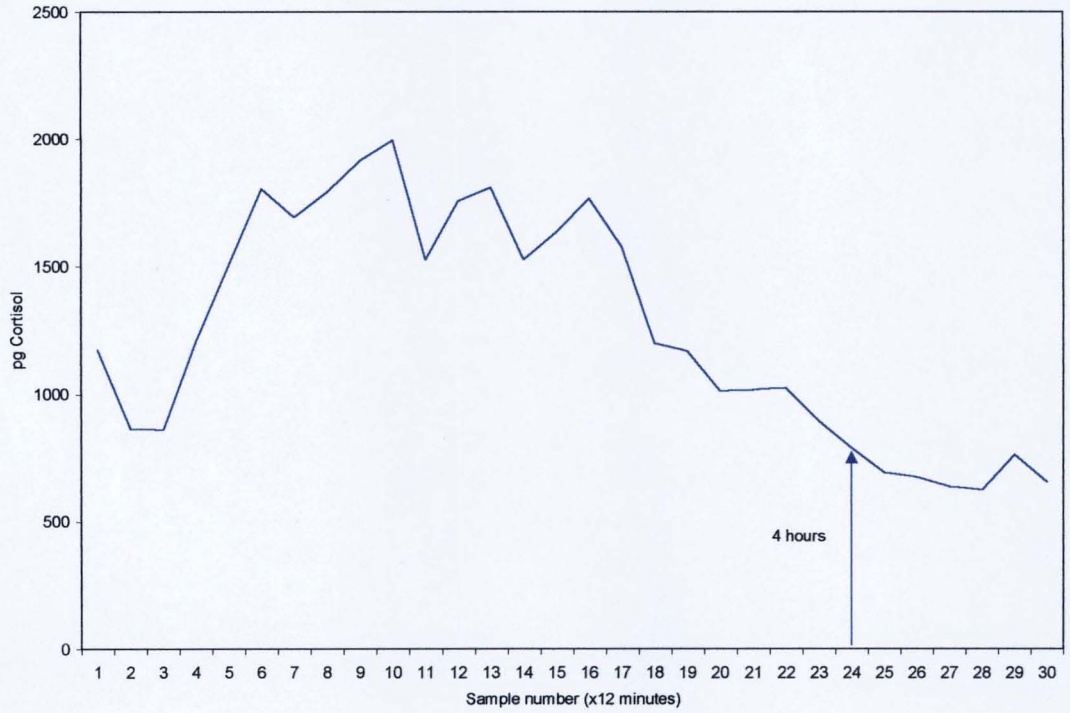


Figure 4.3.1 Basal cortisol secretion from interrenal tissue of Atlantic halibut over a 6 hour period. Arrow indicates 4 hours, after which collections were made during all perifusion experiments.

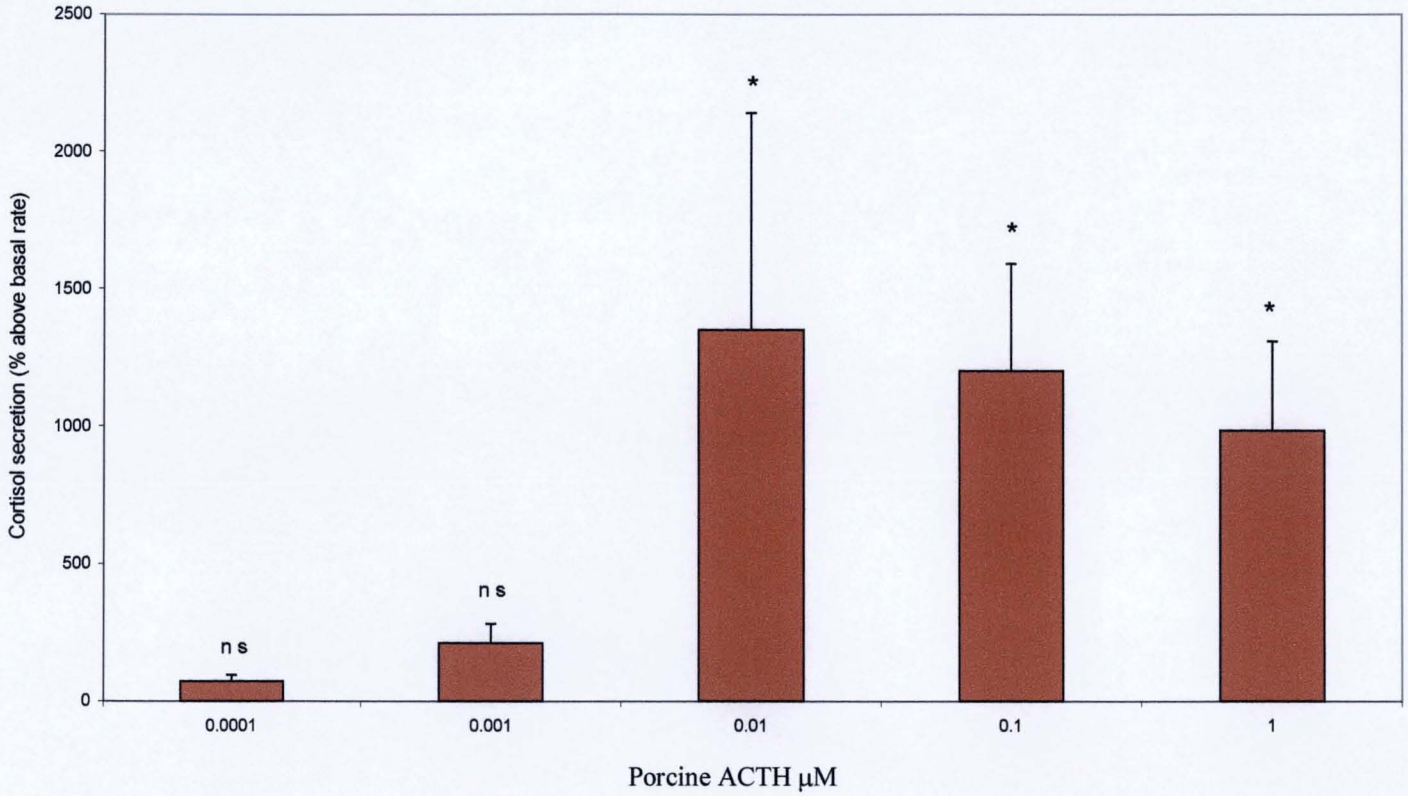


Figure 4.3.2 Comparative effects of 0.0001, 0.001, 0.01, 0.1 and 1.0μm porcine ACTH stimulated cortisol production by Atlantic halibut interrenal tissue. Results are means (±S.E.M) of cortisol production expressed as percentage increases above basal secretory rates. Mean basal secretory rates (n=5) for 0.0001μM group were 766±19, 482±26, 963±29, 91±4 and 1157±149 fmol/mg per 12min. Mean basal secretory rates (n=5) for 0.001μM group were 904±33, 2295±58, 1015±147, 378±68 and 2549±633 fmol/mg per 12min. Mean basal secretory rates (n=6) for 0.01μM group were 22.9±17, 161±4.6, 304±42, 204±44, 19.5±4 and 63±8 fmol/mg per 12min. Mean basal secretory rates (n=10) for 0. 1μM group were 71.4±8.9, 70±15, 8.6±6.3, 1505±159, 48.9±7.11, 1612±32.3, 29±3.5, 395±19, 89±4.6 and 267±30 fmol/mg per 12min. Mean basal secretory rates (n=8) for 1.0μM group were 3558±102, 191±38, 1085±38, 310±50, 87±10, 518±47, 94±13 and 248±22 fmol/mg per 12min (*$P < 0.05$ compared to basal production rate).

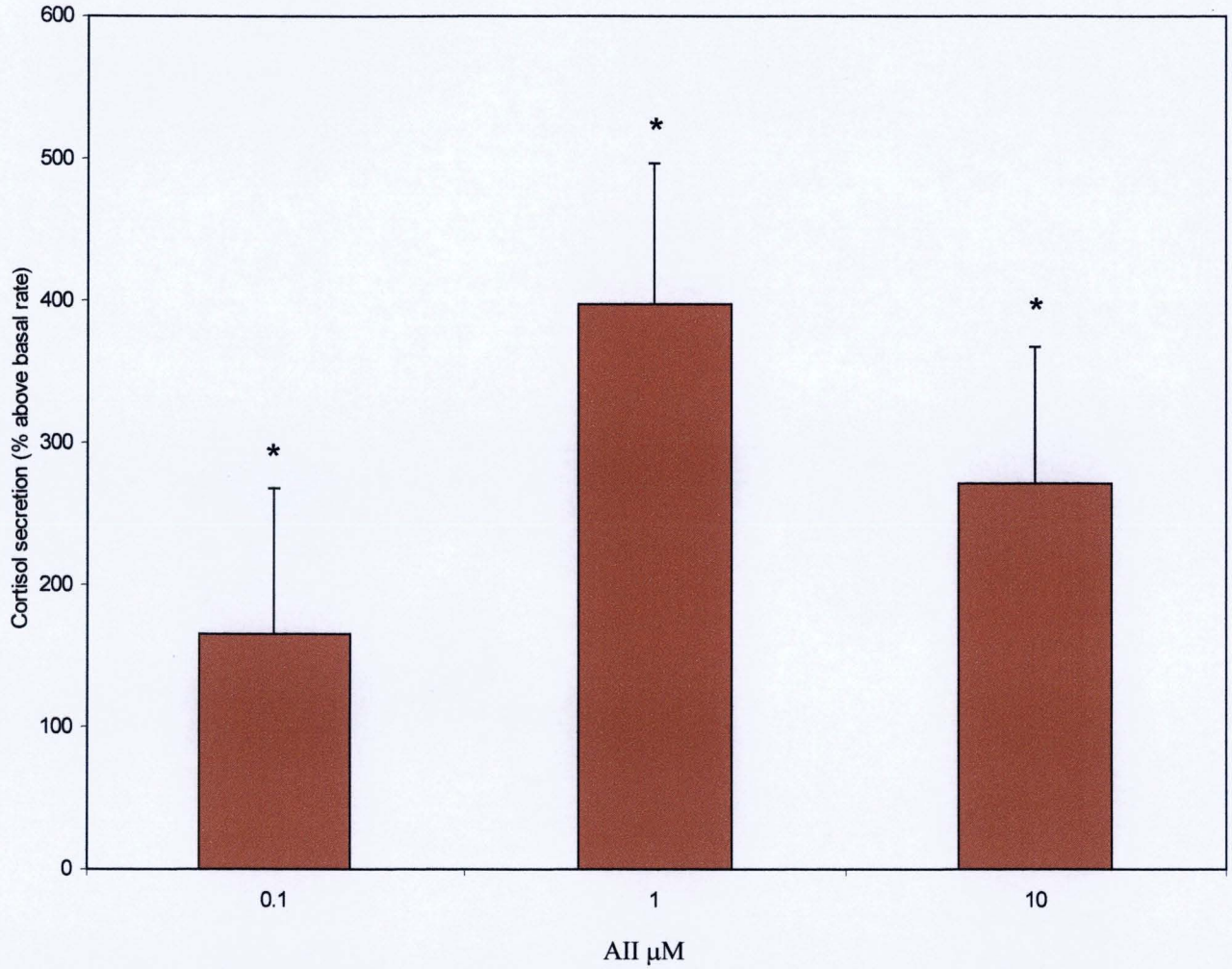


Figure 4.3.3 Comparative effects of 0.1, 1.0 and 10μM angiotensin II stimulated cortisol production by Atlantic halibut interrenal tissue. Results are means (±S.E.M) of cortisol production expressed as percentage increases above basal secretory rates. Mean basal secretory rates (n=6) for 0.1μM group were 740±26, 96±28, 605±23, 317±22, 124±4.5 and 1347±57 fmol/mg per 12min. Mean basal secretory rates (n=6) for 1.0μM group were 226±51, 383±85, 1308±97, 3227±318, 110±110 and 2297±284 fmol/mg per 12min. Mean basal secretory rates (n=5) for 10μM group were 243±29, 232±61, 922±12 and 421±12 fmol/mg per 12min (*P < 0.05 compared to basal production rate).

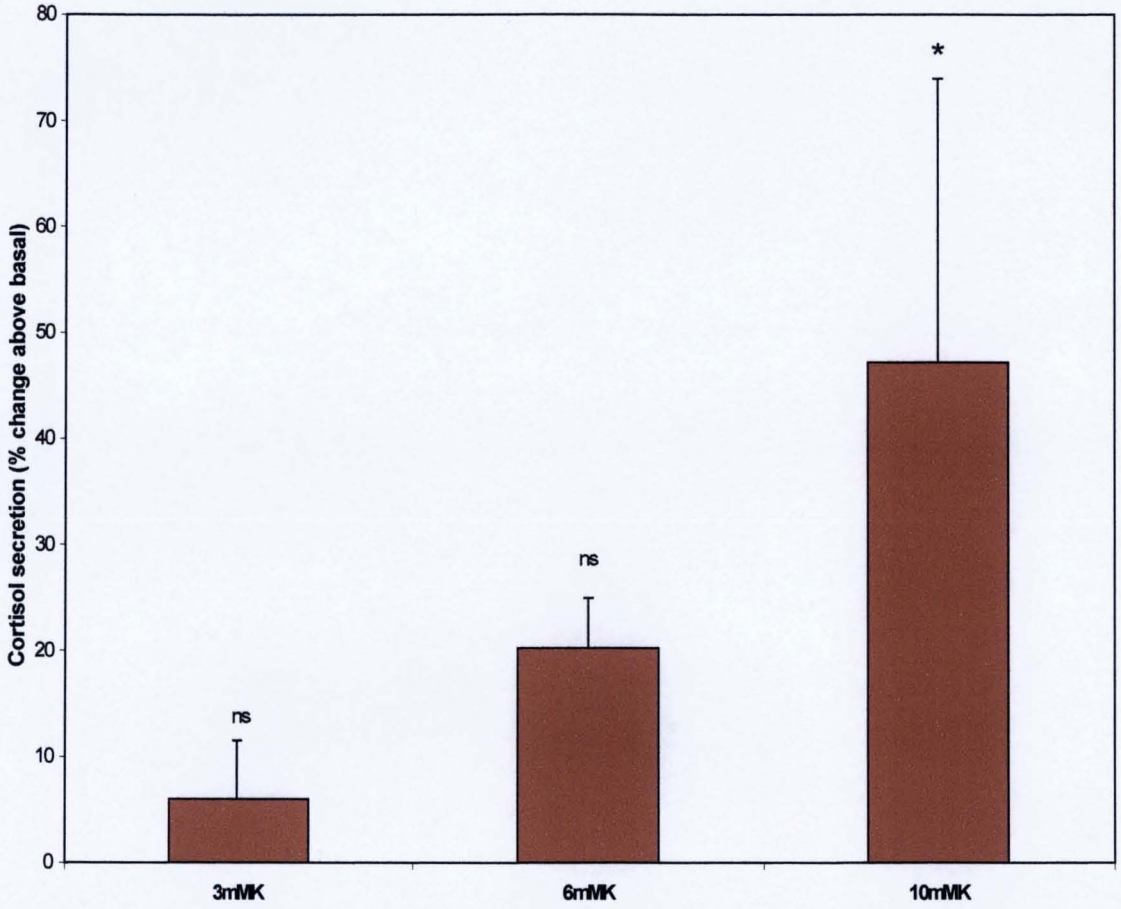


Figure 4.3.4 Comparative effects of 3mMK, 6mMK and 10mMK on cortisol production by Atlantic halibut interrenal tissue. Results are means (±S.E.M) of cortisol production expressed as percentage increases above basal secretory rates. Mean basal secretory rates (n=5) for 3mMK group were 529±24, 1400±64, 129±5.8, 103±3.8 and 295±7.3 fmol/mg per 12min. Mean basal secretory rates (n=5) for 6mMK group were 155±11, 623±35, 174±3.9, 163±26 and 164±5.2 fmol/mg per 12min. Mean basal secretory rates (n=5) for 10mMK group were 165±33, 178±15, 219±10, 274±5 and 1669±28 fmol/mg per 12min (* $P > 0.05$ compared to basal production rate).

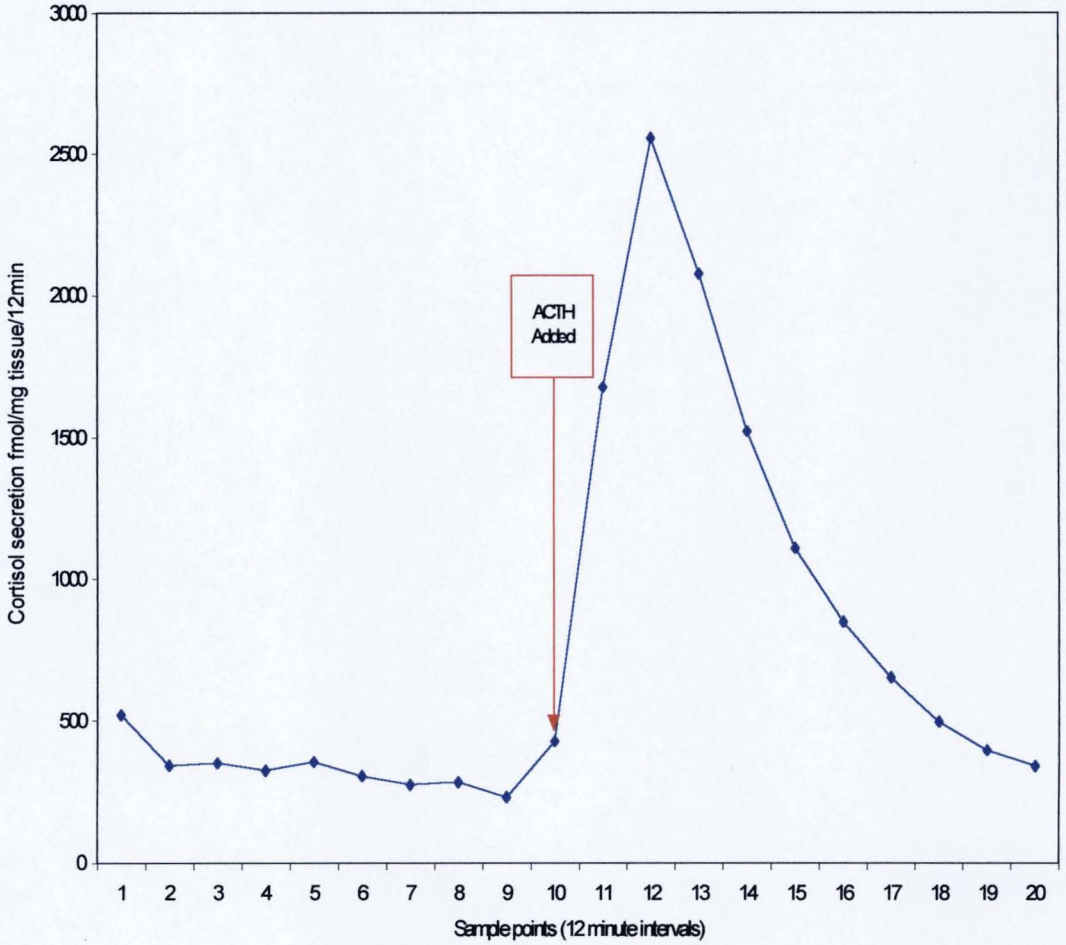


Figure 4.3.5 Typical cortisol secretory response of perifused Atlantic halibut interrenal tissue to porcine ACTH (0.01μM) applied from point 10 to point 11.

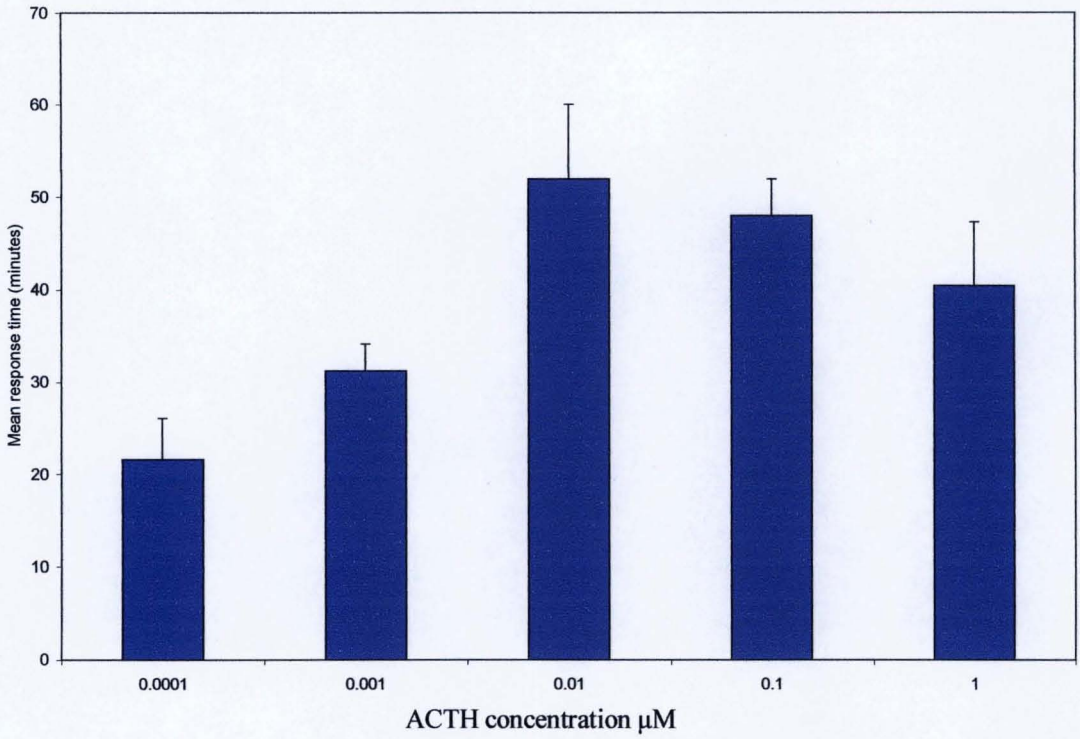


Figure 4.3.6 Dose dependent peak response times for ACTH stimulated cortisol release from Atlantic halibut interrenal tissue Comparative effects of 0.0001, 0.001, 0.01, 0.1 and 1.0μm porcine ACTH stimulated cortisol production by Atlantic halibut interrenal tissue. (n=5) for 0.0001μM group, (n=5) for 0.001μM group, (n=6) for 0.01μM group, (n=10) for 0.1μM group, (n=8) for 1.0μM group, (vertical lines indicate S.E.M.)

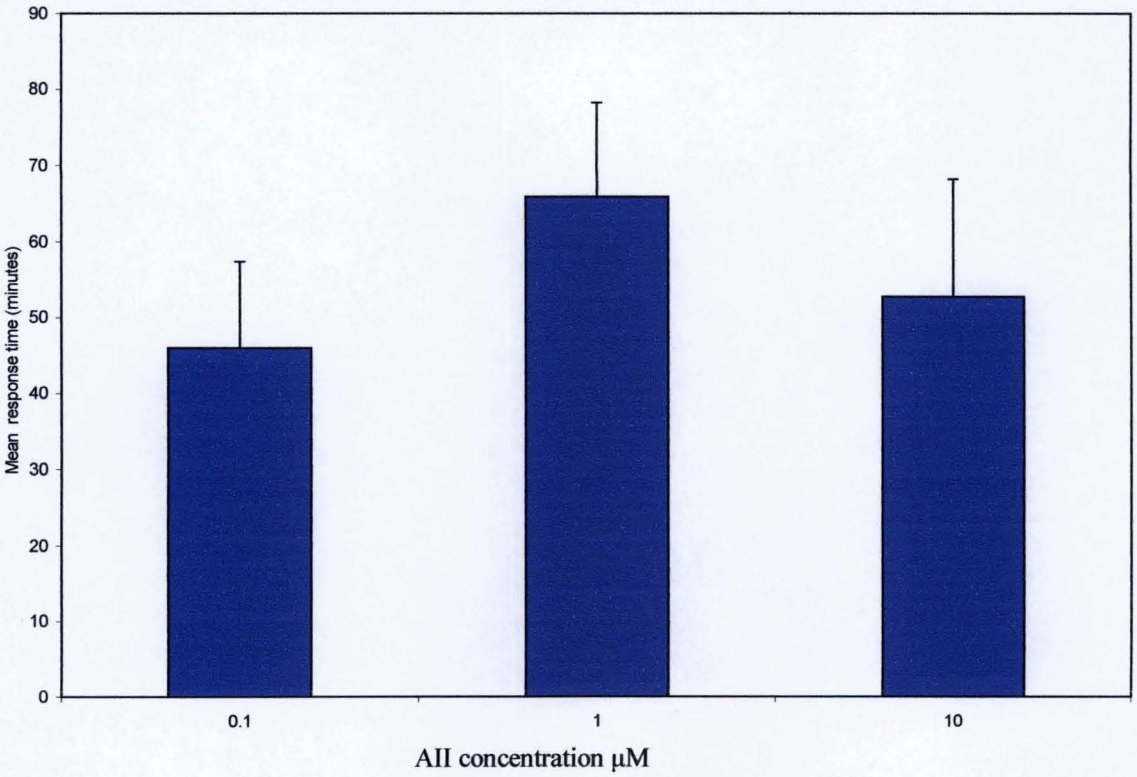


Figure 4.3.7 Dose dependent peak response times for AII stimulated cortisol release from Atlantic halibut interrenal tissue (n=6) for 0.1μM group, (n=6) for 1.0μM group, (n=5) for 10μM group. (vertical lines indicate S.E.M.)

4.4 Discussion

Due to the inherent nature of the teleost head kidney with the intermingling of the interrenal cells with chromaffin cells and lymphomyeloid tissue, cell heterogeneity in the dissected tissues is a potential source of variation between fish (Rance and Baker, 1981). One of the reasons that the dogfish is so suitable for such preparations is that they posses an interrenal gland consisting of pure cortical tissue with no intermingling of medullary tissue (Armour, O'Toole et al., 1993). In the present study considerable variation, in particular at higher doses, between responses from individual fish was apparent. At lower doses there was less variability and more repeatable results were achieved. At higher doses it was possible that, in addition to tissue heterogeneity, complex binding site dynamics associated with allosteric modification would have been responsible for some of the variation. Alternatively lower responses associated with the higher doses may possibly be evidence of ultra-short loop negative feedback mechanisms in operation (Bradford, Fitzpatrick et al., 1992).

4.4.1 ACTH

A dose dependant cortisol secretory response of isolated interrenal tissue to varying concentrations of ACTH was found for 0.0001, 0.001 and 0.01μM (10^{-10} *M*-10^{-8} *M*) At concentrations above this from 0.1 to 1.0 (10^{-7} *M* – 10^{-6} *M*) there was a decrease in maximal cortisol production. A dose dependent response for at least a proportion of the varying concentrations is in accordance with the findings of Rance and Baker, (1981); Gupta, Lahlou et al., (1985); O'Toole, Armour et al., (1990) and Arnold-Reed and Balment, (1994). The observed decrease in maximal cortisol secretion above 0.01 μM

(10^{-8} *M*) was similar to the responses noted by Rance and Baker, (1981) and Gupta, Lahlou et al., 1985) following administration of similar concentrations. No such decreases in maximal cortisol stimulation were however found in the trout or dogfish (O'Toole, Armour et al., 1990; Arnold-Reed and Balment, 1994). The responses in the present study were markedly higher than those recorded by other authors perhaps indicating a greater sensitivity of the halibut interrenal tissue or that lower levels of ACTH are required to elicit a stress response. If this holds true then it could perhaps be possible that control at the Hypothalamus/pituitary level of the HPI axis secretion is down regulated.

4.4.2 Angiotensin II

From the results of the present study it is not immediately clear whether a dose dependent response was being observed for the doses employed. However, although the response following the 10 μM AII challenge is lower than the response following the 1.0 μM AII challenge they are both greater than the response observed following the 0.1μM AII challenge. It may be possible that the profile being observed is similar to that following stimulation by the higher doses of ACTH where there appeared to be a threshold concentration above which no further, and indeed lower than maximal, cortisol secretion was observed.

Whilst it would have been desirable to have applied a further challenge at a lower dose than 0.1μM to test this hypothesis a lack of available fish prevented this work being carried out. Regardless of the nature of the dose dependent response it is however clear

that AII has a significant steroidogenic effect on the interrenal tissue of the Atlantic halibut.

The percentage increase of AII stimulated cortisol production above basal levels was approximately the same as that reported by O'Toole, Armour et al., (1990) for 1α-OH-B, the principal corticosteroid in elasmobranchs.

AII was less potent than ACTH in its ability to stimulate cortisol from the interrenal tissue of the halibut. Similar findings were observed for 1α-OH-B secretion in the dogfish (Armour, 1991) and can be interpreted as a reflection of the importance of ACTH in the control of cortisol secretion as part of the HPI axis.

4.4.3 Potassium

The lack of steroidogenic activity of physiological levels of potassium is in accordance with the findings of O'Toole, Armour et al., (1990) and Decourt and Lahlou, (1986). Whilst in the present study and that by O'Toole, Armour et al., (1990) a stimulatory effect of potassium at concentrations above normal physiological levels was observed no such effect even at concentrations of up to 20 mM was noted by Decourt and Lahlou, (1986). In other vertebrates (amphibians) there is a similar lack of responsiveness in terms of corticosteroid stimulation to increased levels of potassium (Maser, Janssens et al., 1985). In mammalian systems aldosterone but not cortisol secretion has been shown to increase when extracellular potassium levels are raised (Hanning, Tait et al., 1970). In humans a 10% increase in plasma potassium concentration stimulates aldosterone synthesis and release (Lee and Laycock, 1978). The lack of responsiveness of the Atlantic halibut interrenal to increases in potassium in the current study when compared to the elevation

elicited by administration of both AII and ACTH serves to indicate the specificity and sensitivity of the perifusion protocol employed.

4.4.4 Timing of maximum response

The timing of the maximum response elicited by both ACTH and AII was positively correlated with the dose rate (Figures 4.3.6 and 4.3.7) ($P<0.05$ ANOVA). As dose rate increases the duration of the response is liable to change and become longer. The decrease in peak response time observed at high dose rates further implies that some feedback or inhibition process is involved. Allosteric and inhibition mechanisms (Lehninger, Nelson et al., 1993) have been implicated in previous works (Rance and Baker, 1981).

4.4.5 Summary

Both ACTH and AII have been shown to be potent stimulants of cortisol production from Atlantic halibut interrenal tissue. Conversely increased potassium concentrations within a normal physiological range showed no significant steroidogenic activity. At equal concentrations ACTH elicited a greater steroidogenic effect than AII possibly reflecting its primary mediating role in cortisol production. A dose dependent response was in evidence for the ACTH challenges although at high concentrations there was a decrease in peak cortisol secretion below maximum values possibly implicating negative feedback mechanisms. A probable dose dependent response was shown for the AII challenges

although further work would have confirmed this. Again a decrease in maximum cortisol production associated with a higher stimulus concentration similar to the ACTH responses was noted. There was a significant correlation between maximum response time and percentage increase, which served to confirm the possibility of either negative feed back mechanisms, or that a modification of receptor site behaviour was in evidence at higher stimulus concentrations.

CHAPTER 5

DISCUSSION AND SUMARY

5.0 Discussion

From the present study a number of factors regarding several different levels of organisation of the Atlantic halibut and its culture have been ascertained. The individual experiments have given an insight into basic modulating mechanisms of the HPI axis, the stress response following an acute stressor, and the growth and performance of halibut under commercial ongrowing conditions. The laboratory experiments, (chapters 3 and 4) when combined, serve to substantiate the findings and thus allow greater interpretation of the field-based work (chapter 2) which covers an area that has not previously been fully investigated.

In chapter 4, factors modulating the cortisol secretion by the interrenal cells of the Atlantic halibut head kidney were investigated. At a molecular level halibut interrenal cells have been found in-vitro, to respond to the classical stimuli ACTH and AII in a manner similar to that reported for other teleosts. Differences in the degree of response above that noted for other species may indicate that there are however subtle differences in the manner by which cortisol stimulation, as a result of the HPI axis, is modulated or perhaps in the distribution of interrenal cells within the halibut head kidney. The high sensitivity shown to ACTH by the interrenal cells, although no further work was carried out to substantiate the claim, may be indicative of cortisol mediation processes occurring at the hypothalamus – pituitary level.

The ecological niche within which the halibut exists in the wild means there are few natural predators. This, in association with its behaviour, may perhaps be a reason for any down regulation of the stress response. This theory has been postulated by (Van Ham, 2003) based on his work on halibut and turbot and the findings of previous studies

investigating different types of coping stress response between individuals and species. It is suggested that, in terms of stress response, halibut may occupy a position between fish such as turbot which are more sedentary, sit and wait predators, that were shown to have a low degree of activity of the brain sympathetic-chromaffin cell axis and roundfish species that have been studied. In chapter 3 the stress response of the halibut, following three acute confinements, each of a differing duration, was investigated. Again the halibut responded in a manner similar to that of other teleosts although the magnitude of the response for the parameters measured, in particular cortisol, was relatively low on the scale of values previously reported. With the possible high sensitivity of the interrenal cells, ascertained in chapter 4, and the relatively low values of cortisol measured following acute confinements the case for hypothalamus-pituitary level mediation or negative feed back of ACTH by low levels of cortisol is perhaps further strengthened. Clearly further work needs to be carried out to establish the exact nature of the relationship along with clearance rates in the Atlantic halibut.

That Atlantic halibut respond in a classical manner to an acute stress, and by determination of the degree of the response, the use of primary and secondary plasma parameters to quantify stress in a farming situation for Atlantic halibut is justified. The degree of the response to an acute stress in chapter 3 means that the field work data (chapter 2) can now be put into context and interpreted accordingly. Prior to the current study there were no baseline or post stress figures available for the Atlantic halibut.

The collection of plasma samples from the fish in the long-term growth study (chapter 2) was infrequent and provided only snapshots of the long term stress status of the fish. However, relative to the post acute stress values for the primary and secondary stress

responses determined in chapter 3, there proved to be no real indication that, at the times of measurement, the fish held in either of the rearing systems (chapter 2) exhibited a clear stress response. For a full understanding of the nature of any chronic or regularly occurring stressors though, a more frequent monitoring program would be beneficial in any future studies, as would an investigation into natural diel and annual rhythms in plasma parameters for halibut.

Whilst growth and condition factor have been used as tertiary indicators of stress, and lower values for these parameters were observed for the fish held in cages, it would seem probable that differences in the primary and secondary indicators of stress would have been greater between the two sites had the cage fish been subjected to chronic stress. Whether a number of acute stressors, as a result storm induced wave action, throughout the three year sample period elicited stress responses whose cumulative effect was detrimental to growth of the cage fish can only be speculated. It is perhaps more likely that the cage fish, whilst occasionally subjected to wave induced stressors sufficient to induce observable primary and secondary stress responses, utilise energy reserves in a different manner than the tank fish in order to maintain position on a less solid substrate or the inherent nature of the cage rearing system causes them to swim more. Swimming activity has been previously used as an indicator of low growth in relation to stocking density of Atlantic halibut (Kristiansen, Ferno et al., 2003) and therefore examining any differences in swimming behaviour between fish held in different rearing systems may provide more information on system suitability. Additionally, and in relation to swimming activity it may be of interest to investigate whether any differences in activity between systems affects flesh quality.

The observed differences between growth rates of the females and the males and the variation in year of maturation for the males are of particular importance for the halibut farming industry. The information gained in the present study serves to highlight some of the areas of research that need to be focussed on in order to improve the profitability of farming Atlantic halibut. Regarding improvements to the growth rate of both males and females the importance of rearing system type has been discussed, with future work on cage design being crucial for the improvement of growth rates in an offshore-orientated business. If, as previously mentioned, discharge regulations force farming operations into land-based systems then, given that the market price of halibut remains high, temperature control through employment of recirculation technology, which would greatly enhance growth, may prove to be financially viable. However, under present circumstances this method of improving growth rates currently appears to be prohibitively expensive.

The most realistic options presently available, which are able to improve growth in any type of system, address the differences between male and female growth. The first option is production of monosex (all female) stocks, as this would negate the problem of early maturation in male fish. Monosex production is possible via either direct hormonally induced feminisation commonly using 17β-estradiol or by indirect feminisation commonly using either 17α-methyl-testosterone or 17α-methyldihydrotestosterone (Shepherd and Bromage, 1992; Pandian and Sheela, 1995). The former method is unlikely to be accepted by consumers whilst the latter method is successfully used with rainbow trout producing 100% female fish (Shepherd and Bromage, 1992). Treatment of postlarval Atlantic halibut with 17α-methyldihydrotestosterone has yielded 97-100%

males (Hendry, Martin-Robichaud et al., 2003) but no information is as yet available regarding any crosses between sex reversed females and normal females that may yield all female progeny. The time taken for the production of all female halibut, beginning with the initiation of a breeding program will however be in the order of years and so improvements via this method are not likely to become a reality in the near future. The same sort of delays are associated with the development of genetic breeding programs selecting for either late maturation or greater growth.

The most promising method therefore and also the simplest in terms of equipment required is the delaying of maturation and the associated growth premiums achieved via photoperiod manipulation. Photoperiod manipulation has been successfully shown to improve growth in a number of species including juvenile Atlantic halibut (Simensen, Jonassen et al., 2000) and has been used to advance smoltification and reduce early maturation (grilsing) in salmonids (Porter, Randall et al., 1998; Bromage, Porter et al., 2001). Timing of spawning of Atlantic halibut has also been manipulated via photoperiod (Bjornsson, Halldorsson et al., 1998). Successful manipulation of melatonin, the peptide responsible for transferring daylength information to the reproductive axis, in cage reared Atlantic halibut via the provision of a submerged lightsource has been demonstrated (Davie, Jordan et al., 2002). Additionally, transference from a continuous light regime to a simulated natural photoperiod regime, 60^{O}N in that particular trial, has been shown to reduce maturation levels in male halibut (Norberg, Weltzien et al., 2001). It is clear that this method of reducing the effects of early maturation and subsequently improving growth is the most accessible to commercial ongrowers and also the most likely to provide results in the near future.

5.0 References

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