UNDERSTANDING MATURITY: INSIGHTS INTO THE MECHANISMS UNDERPINNING MATURITY IN GADOIDS

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

Marked shifts in the life history traits of fish have been reported in many exploited fish stocks, with a particular trend towards decreasing size and age at maturity. Though other environmental and behavioural factors have been implicated, the key driver of these changes links to fishing pressure, through both the direct selective effects of fishing itself, and indirectly through the manipulation of important biological and environmental factors. Although reproduction itself has been well described in teleosts, the mechanisms of environmental and endogenous entrainment of maturation remain unclear and it was the principal aim of this thesis to improve current understanding of these systems in gadoids.

Photoperiod has been identified as the strongest environmental cue for entraining seasonal behaviours, including seasonal reproduction. Over the last decade, several of the key drivers involved in the photoneuroendocrine cascade have been elucidated in mammals and birds, with the *Eya3* pathway emerging as an important mechanism for entraining maturation. However, little is yet known of their influence on maturation in fish. In the first study, the photoperiodic regulation of the *Eya3-Tshβ-Dio2* cascade was analysed in Atlantic cod exposed to either continuous light (reproductive inhibition) or simulated natural photoperiod (reproductive stimulation) from July to December. Monthly expression was measured through QPCR, demonstrating a strong activation of pituitary *Eya3* under declining photoperiod. As this coincided with the onset of secondary gametogenesis, these results suggest that *Eya3* may play a stimulatory role in the photoneuroendocrine cascade of Atlantic cod.

Although photoperiod represents the most reliable and noise free proximate signal to entrain the reproductive process, it is clear that a minimum growth and energetic state

must be reached for maturation to progress. This directed the second line of study – a series of diet restriction trials on haddock and cod designed to investigate the influence of naturally occurring lipid levels in the diet on growth and reproduction, define the "critical window" in which fish assess their energetic state and how this is analysed before commencing secondary gametogenesis, and to assess whether *Eya3* is regulated by the growth axis in cod. The results of these experiments indicate that overall size around the autumn equinox is the most accurate indicator of maturation commitment in cod. Additionally, *Eya3* expression was elevated in maturing fish indicating a role for this marker in linking the energetic signal with entrainment of the reproductive axis.

Finally, an analysis of the physiological and genetic stock structure of cod from the North Sea IVa stock region and westward into VIa was performed. The results of this analysis support previous genetic studies, indicating further fine-scale structuring of these stocks, reflective of the structure indicate by the differences in maturation strategies of the component populations. The results identified both environmental and harvest related pressures which may be driving the current stock structure.

The results of these studies greatly improves our understanding of the key drivers and mechanisms regulating maturation in cod, highlighting new avenues for future research.

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GLOSSARY OF ABBREVIATIONS AND ACRONYMS

aa	amino acid
AIC	Akaike information criterion
ANOVA	analysis of variance
BLAST	basic local alignment search tool
bp	base-pair
BPG	brain-pituitary-gonad
CA	cortical alveolus
cDNA	complimentary DNA
cds	coding DNA sequence
cm	centimetre
CNR	circumnuclear ring
d	day
ddRAD	double-digest restriction-site associated DNA sequencing
Dio1	deiodinase 1
Dio2	deiodinase 2
Dio3	deiodinase 3
DNA	deoxyribonucleic acid
DSTs	data storage tags
E_2	17β-estradiol
Ef1α	elongation factor 1 alpha
Eya2	eyes-absent homolog 2
Eya3	eyes-absent homolog 3
FAO	Food and Agriculture Organisation of the United Nations
FCE	food conversion efficiency
Fsh	follicle stimulating hormone
g	gram/grams
GH	growth hormone

GLM	general linear model
GnRH	gonadotrophin releasing hormone
GSI	gonadosomatic index
GVBD	germinal vesicle breakdown
HAD	haloacid dehalogenase
HL	high to low
HR	high ration
hrs	hours
HSI	hepatosomatic index
ICES	the International Council for the Exploration of the Sea
ICV	intracerebroventricular
IGF-1	insulin-like growth factor-I
IUCN	International Union for the Conservation of Nature
K	Fulton's condition factor
kb	kilo base-pair
Kn	relative condition
L	litre
LC	leading cohort
LD	long day
Lh	luteinising hormone
LH	low to high
LL	continuous light
LMM	linear mixed-effects model
LR	low ration
m	meter
M	mole
mm	millimetres
MBH	medio-basal hypothalamus
MERL	Macrahanish Marine Environmental Research Laboratory

mg	milligram
mins	minutes
MIQE	minimum information for publication of real-time PCR experiments
ml	millilitre
mM	millimoles
mtDNA	mitochondrial DNA
MU	management unit
NBF	neutral buffered formalin
ng	nanogram
NGS	next generation sequencing
nM	nanomole
nRNA	nuclear RNA
Pan 1	pantophysin 1
PCR	polymerase chain reaction
PIT tag	passive integrated transponder tag
PMAT	proportion mature
PMRN	probabilistic maturation reaction norm
PN	perinuclear
PNES	photoneuroendocrine system
ppm	parts per million
PT	pars tuberalis
QC	quality control
QPCR	quantitative PCR
RAD	restriction-site associated DNA sequencing
REs	restriction enzymes
RFLPs	restriction fragment length polymorphisms
RLM	relative liver mass
RNA	ribonucleic acid

rRNA	ribosomal RNA
ScIW	Scotland inshore west
SCN	suprachiasmatic nucleus
ScOW	Scotland offshore west
SE	standard error
SD	short day
SG A	spermatagonia A
SG B	spermatagonia B
ShIE	Shetland inshore east
ShIW	Shetland inshore west
ShOW	Shetland offshore west
SNP	simulated natural photoperiod
SNPs	single nucleotide polymorphism
SSB	spawning stock biomass
stdv	standard deviation
t	tonne
T	testosterone
TACs	total allowable catches
TGC	thermal growth coefficient
TL	total length
Tsh	thyroid stimulating hormone
T4	thyroxine
T3	triiodothyronine
µl	microlitre
UV	ultra-violet
VGLL3	vestigial-like family member 3
VIT	vitellogenesis
^{o}C	degrees centigrade
11KT	11-ketotestosterone

GLOSSARY OF COMMON AND SCIENTIFIC NAMES

Atlantic cod	*Gadus morhua* (Linnaeus, 1798)
Haddock	*Melanogrammus aeglefinus* (Linnaeus, 1798)
European sea bass	*Dicentrarchus labrax* (Linnaeus, 1798)
Japanese quail	*Coturnix japonica* (Temminck & Schlegel, 1846)
House mice	*Mus musculus* (Linnaeus, 1798)
Syrian hamsters	*Mesocricetus auratus* (Waterhouse, 1839)
Siberian hamsters	*Phodopus sungorus* (Pallas, 1773)
Rat	*Rattus norvegicus* (Berkenhout, 1769)
Sheep	*Ovis aries* (Linnaeus, 1798)
Saanen goats	*Capra hircus* (Linnaeus, 1798)
Masu salmon	*Oncorhynchus masou masou* (Brevoort, 1856)
Three-spined stickleback	*Gasterosteus aculeatus* (Linnaeus, 1798)
Fugu	*Takifugu rubripes* (Temminck & Schlegel, 1846)
Japanese medaka	*Oryzias latipes* (Temminck & Schlegel, 1846)
Nile tilapia	*Oreochromis niloticus* (Linnaeus, 1798)
Seabass	*Dicentrarchus labrax* (Linnaeus, 1798)
Red drum	*Sciaenops ocellatus* (Linnaeus, 1798)
Zebrafish	*Danio rerio* (F. Hamilton, 1822)
Mexican cave fish	*Astyanax mexicanus* (De Filippi, 1853)
Tongue sole	*Cyanoglossus semilaevis* G.
Atlantic salmon	*Salmo salar* (Linnaeus, 1798)
Spotted gar	*Lepisosteus oculatus* (Winchell, 1864)
Red jungle fowl	*Gallus gallus* (Linnaeus, 1798)
Guinea pig	*Cavia porcellus* (Linnaeus, 1798)
Common carp	*Cyprinus carpio* (Linnaeus, 1798)
Fathead minnow	*Pimephalus promelas* (Rafinesque, 1820)
European eel	*Anguilla anguilla* (Linnaeus, 1798)

Japanese eel	*Anguilla japonica* (Linnaeus, 1798)
Halibut	*Hippoglossus hippoglossus* (Linnaeus, 1798)
Chum salmon	*Oncorhynchus keta* (Walbaum, 1792)
Sockeye salmon	*Oncorhynchus nerka* (Walbaum, 1792)
Chinook salmon	*Oncorhynchus tshawytscha* (Linnaeus, 1798)

CHAPTER 1: GENERAL INTRODUCTION

Long-term shifts in life history traits of fish, particularly a trend towards decreasing size and age at maturity, have been reported in many exploited fish stocks (Trippel 1995, Law 2000). These changes can affect the overall biomass of fish stocks, as individuals allocate a greater proportion of their energy to reproduction rather than somatic growth (Blanchard et al. 2003, Davie et al. 2007b, 2007c, Conover et al. 2009); thus, the harvestable yield of these stocks decreases causing concern for fisheries. Although maturation trends have occasionally been correlated to long term changes in environmental conditions (van Walraven et al. 2010, Morgan et al. 2013), their occurrence has most commonly been linked to exploitation itself, both directly, through selection (Rochet et al. 2000, Conover and Munch 2002, Stenseth and Dunlop 2009, Conover et al. 2009), and indirectly, through the manipulation of important biological and environmental factors (Law 2000). The possibility of genetic changes in life history parameters such as age and size at maturity is well accepted, as exploitation is selective and life history characteristics are heritable (Rijnsdorp 1993). Several controlled selection experiments have confirmed the potential for harvest induced genetic changes in life history traits (Conover and Munch 2002, Conover et al. 2005, 2009, Conover and Baumann 2009). However, such experiments have been restricted to using fish with short generation times and small body size (Rijnsdorp 1993), and are not readily available for commercial species, which in general are characterised by large body size and long generation time. In addition, expressed life history traits will be influenced not only by genetics but also by environmental variation through phenotypic plasticity (Marshall and Mcadam 2007, Dieckmann and Heino 2007). Teleosts are well known for their phenotypic plasticity, and changes in reproductive patterns may also be due to plastic responses to an enhanced availability of food at reduced population sizes (Law 2000). Consequently, distinguishing between the effects

of genetic selection and phenotypic plasticity on maturation in wild fish stocks is a major challenge. Maturation changes in two commercially important Gadidae, Atlantic cod (*Gadus morhua* L.) and haddock (*Melanogrammus aeglefinus* L.) have been key to the debate about the causes of change in maturation schedules (Devine et al. 2012, Wright et al. 2014b). Both of these fisheries have a long history of exploitation, and declines in the age and size at maturation have been observed in exploited stocks across their distribution (Yoneda and Wright 2004, Harrald et al. 2010, Neuheimer and Taggart 2010, Wright et al. 2011b, Wright and Tobin 2013). While many studies have observed and described these changes in the wild, the underlying mechanisms driving shifts in this life history trait remain unclear. Fishing may have effects on stocks that are not easily reversed if selection targets genetic variation in phenotypic traits, and it is clear that a greater understanding of the interplay between the endogenous entrainment of maturation and the environmental pressures driving current shifts is needed to improve management of exploited stocks.

1.1. OVERVIEW OF SPECIES ECOLOGY AND COMMERCIAL INTEREST

1.1.1. Atlantic cod, *Gadus morhua*

Taxonomy and Habitat Range

The Atlantic cod is an omnivorous marine fish species of the Gadidae family (Cohen et al. 1990). This species is widely distributed across the North Atlantic, ranging from the Gulf of Maine northward along the North American coast to Greenland, and eastward across the North Atlantic to its north eastern limit in the Barents Sea, where is extends south into the Bay of Biscay (Figure 1.1). The species is exposed to a wide range of salinities (10 to 35 ppm) and temperatures (-2˚C to 20˚C) across this range, but is typically found at higher salinities and between 0˚C and 12˚C (Cohen et al. 1990). Atlantic cod are

generally considered a demersal species, primarily occupying continental shelf regions to depths of around 150 - 200m (though they have also been found at depths > 600m) (Hislop et al. 2015).

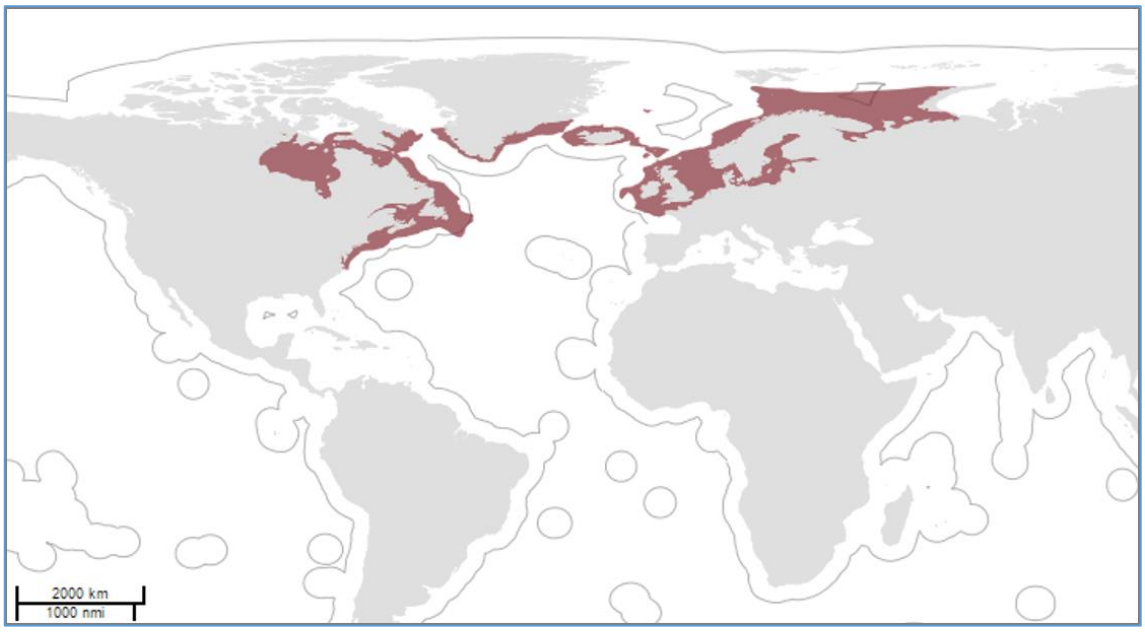

Figure 1.1. Distribution of Atlantic cod, adapted from FAO 2016

Life History

Atlantic cod are iteroparous, group-synchronous, batch-spawners (Hislop 1984). Reproduction is seasonal, with annual batch spawning typically occurring between January and April – though this is dependent on the stock. Atlantic cod are a remarkably fecund species with females, producing ~ 500 000 eggs per kg body weight over a spawning season, and large females have been known to produce up to 9 million eggs over a typical spawning season (Cohen et al. 1990). The size of eggs is variable, differing between stocks and batches in a given spawning season, but are ~1.4 mm in diameter on average. Eggs size is known to increase with female size, and survival of offspring has been positively correlated with egg size (Trippel 1998). Cod eggs are buoyant and remain suspended within the water column prior to hatch. Hatching is temperature dependant but typically occurs between 80 and 100 ˚days post-fertilisation. These newly hatched pelagic

larvae have a limited yolk reserve which is fully absorbed within 6 - 8 days after which exogenous feeding is required to maintain development. Larvae remain mostly pelagic for 2 – 3 months' post hatch, feeding on zooplankton. At about 4 – 5 months these juveniles adopt a more demersal lifestyle, feeding mainly on crustaceans, polychaetes and, increasingly, fish. There is still little information on the movements of these juveniles, though it is thought that they utilise relatively shallow inshore areas as nursery grounds for the first year of life, before migrating offshore to feeding grounds inhabited by mature fish.

Onset of maturity is highly variable between stocks, with northern and northwest Atlantic stocks typically maturing from age 4, while many eastern stocks are found to mature as early as 2 (Wright et al. 2014b). Although the mechanisms underpinning this variation are not clear, further consideration is given to the environmental (Section 1.2.1) and endogenous (Section 1.2.2) mechanisms regulating maturation later in this chapter. Maturation commitment is heralded by the beginning of secondary gametogenesis, approximately 6 months prior to spawning (Kjesbu et al. 2010). The following increase in gonad weight continues for a period of 4 – 5 months before spawning commences in early spring. Spent individuals then return to the feeding grounds to recover, subsequently maturing each year as energy reserves allow (Kjesbu 1989).

Although cod have been found to reach sizes up to 1.8 m total length and 65 kg weight, and can live as long as 25 years, the average lifespan is 4 – 8 years, and sizes greater than 1 m total length and 15 kg weight are uncommon (Cohen et al. 1990)

Commercial Exploitation and Management

Targeted since fisheries began, Atlantic cod has been hailed as one of the most valuable and exploitable marine species, earning the title "beef of the sea" (Kurlansky 1999). Early

fisheries around Europe expanded and grew with the colonisation of North America and the adoption of new fisheries methods and technologies, reaching a peak in global capture in 1978 of almost 4 million tonnes (t) (Cohen et al. 1990). These yields proved unsustainable resulting in a dramatic decline in production and tapering to an all-time low of just over 750 000 t in 2008 (FAO, 2016). The reduction in global capture fisheries production and associated increases in market value spurred an interest in cod aquaculture in the early 00's. However, difficulties associated with early development and on-growing severely impacted aquaculture production with peak production reaching 23 000 t in 2010 – just 13% of the targeted production for that year. Recently, capture fisheries production has begun to increase with 2014 estimates in the region of 1.3 million t. This has led to a decrease in commercial value which, combined with the economic downturn and unresolved production challenges, has resulted in the collapse of the cod aquaculture industry.

This species is listed as "Vulnerable" by the IUCN red list (IUCN 2015). Cod fisheries have been managed by total allowable catches (TACs) and quotas since 1975, and these management efforts have largely been ineffective in maintaining or creating a sustainable level of exploitation. This was largely due to delays in integrating scientific advice into management strategies. However, there has been progress in this regard over the last decade with the reform of the Common Fisheries Policy (Fernandes and Cook 2013). Since these changes to the management in the Northwest Atlantic, focussing on reduced effort and other measures, there has been a recovery in some stocks.

1.1.2. Atlantic haddock, *Melanogrammus aeglefinus*

Taxonomy and Habitat Range

Atlantic haddock belongs to the Gadidae family, within the order Gadiformes. Like cod, Atlantic haddock inhabits the North Atlantic, along the north-eastern coast of North American and southern coasts of Greenland in the west, and from the Bay of Biscay to the Barents Sea in the east (Figure 1.2). This marine fish occupies a range of demersal habitats from 10 m to 450 m depth, but is commonly found between 80 – 200 m over sandy, muddy or gravel substrate with an optimal thermal niche of 4 – 10 ˚C (Cohen et al. 1990).

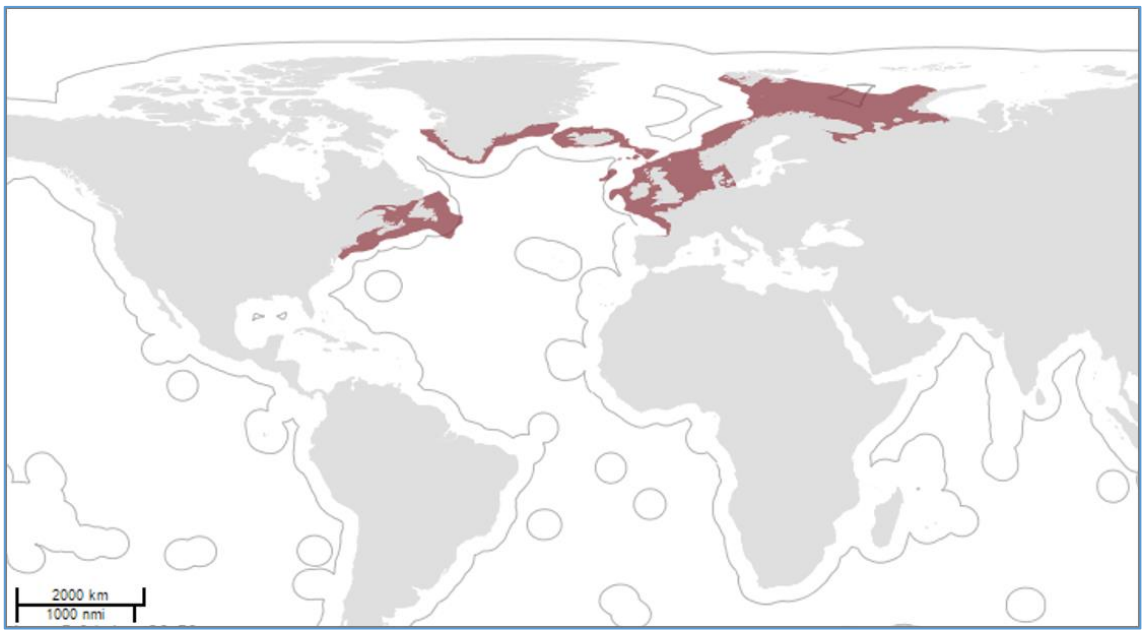

Figure 1.2. Distribution of Atlantic haddock, adapted from FAO 2016

Life History

Although the life histories of haddock have not been studied as extensively as cod, these closely related species share many similarities. Like cod, the reproductive cycle of haddock is seasonal (Hislop 1984). This species is iteroparous and group-synchronous, with females producing a number of batches during the spawning period (Robb 1982). The spawning period is largely stock specific, with peak periods between April and May

in the Northwest Atlantic stocks, and between March and April in the eastern stocks (Cohen et al. 1990, Wright and Gibb 2005). Large females have been known to produce ~1.8 million eggs within a given season, but on average this species produces ~500 000 eggs per kg body weight of the female over the spawning period (Cohen et al. 1990, Wright et al. 2014b). Larvae metamorphose into pelagic juveniles around 2-3 months' post-hatch. These juvenile begin settling at 4 – 5 months at a total length of 4 – 8 cm. Again, as with cod, it is thought that these juvenile haddock utilise nursery grounds during their first year before migrating to the adult feeding grounds (Cohen et al. 1990).

Onset of maturity varies between stocks. In most regions, haddock mature first from age 4 onwards, but several stocks, including the North Sea stock are now found to mature from age 2 (Wright et al. 2014b). There are also considerable differences in growth rate between stocks for this species. Haddock generally do not exceed 1 m in length and the life expectancy is 14 years.

Commercial Exploitation and Management

Atlantic haddock are an important target species in the North Atlantic, particularly in the east. At its peak in 1969, this fishery produced over 950 000 t of haddock, but typical yields for this species are between 200 000 - 500 000 t (FAO, 2016). Severe declines have been noted for some stocks (Rochet 2000, Frank et al. 2001), but overall production has remained relatively stable, bolstered by exceptional recruitment in some years. There has been some interest in aquaculture of this species, but production has never exceeded 72 t since it was first cultured in 2003, and production has since dropped off entirely (FAO, 2016).

Management measures mirror those adopted for cod stocks with similar noted caveats. This species is listed as vulnerable by the IUCN red list (Sobel 1996).

1.1.3. Consequences of exploitation to reproduction/maturation

As discussed, both cod and haddock fisheries have a long history of exploitation, particularly in the Northwest Atlantic where intense fishing during the mid-to-late 1900s culminated in a dramatic stock collapse in the late 1980s and early 1990s. Cod stocks from the Scotian shelf, Newfoundland and Labrador suffered declines as high as 99% during this period, while east Scotian shelf haddock stocks also suffered severe reductions (Frank et al. 2001, Hutchings and Reynolds 2004). Elsewhere, cod and haddock stocks have exhibited similar declines due to fishing pressure. In the North Sea, the spawning stock biomass (SSB) of cod has dropped by almost 90% since the 1970s and although this has recently recovered to precautionary limits the recovery has been limited to the northern half of the stock (Holmes et al. 2014).

Reduced density arising from stock declines would be expected to reduce competition for resources, allowing fish to grow faster and therefore mature earlier. Indeed, several studies have related fast growth to early maturation (Eliassen and Vahl 1982, Holdway and Beamish 1985, Godø and Haug 1999, Yoneda and Wright 2005b). However, this compensatory growth response cannot explain most observed trends in maturation at size. Rather, there is now extensive evidence for a shift in maturation reaction norms consistent with an evolutionary response. This evidence is mostly due to the development of the probabilistic maturation reaction norm (PMRN) that models the probability of maturing within a year-class based on the proportion of immature and mature individuals at a given size and age (Heino et al. 2002). By accounting for size at age, the probability of maturing is independent of variations in growth and survival that confound maturation-size relationships enabling the effect of compensatory growth to be removed (Heino et al. 2002). Using the PMRN approach, many cod and haddock stocks have been shown to exhibit declining trends in size and age at which fish first mature (Barot et al. 2004, Olsen

et al. 2004, 2005, Baulier et al. 2006, Wright et al. 2011a, 2011b) and the rate of maturation change among these stocks was found to be correlated with the level of fishing mortality (Devine et al. 2012).

However, observed changes in the PMRN do not provide sufficient evidence of underlying genetic changes in maturation tendency (Kraak 2007, Law 2007, Wright 2007). A change in a PMRN can be caused by changes in environmental variables that are not directly linked to annual growth (Morita et al. 2009). The PMRN does not account for the effect of growth or lipid stores at the time of developmental decisions which have been found to "gate" the maturation process in salmon (Thorpe 2007), and are likely to influence the maturation decision in other species. Additionally, field applications of the PMRN are limited by the need to characterize gonad development macroscopically, and the estimated maturation probability at age and size reflects the outcome of the fishes continued investment in gonadal development rather than its energetic state during the initial maturation "decision" period (Grift et al. 2003, Barot et al. 2004, Wright 2007). Finally, although the intensity of fishing pressure is likely to have led to a strong selection pressure on life history traits, some estimates of the potential selection differential are too low to explain the high rate of change observed (Andersen and Brander 2009). Hence, the debate over phenotypic plasticity vs. evolutionary response is on-going and disentangling the two has proven difficult (Law 2007, Marshall and Mcadam 2007, Wright 2007). Therefore, further research is required to show the extent to which maturation may be influenced by both plastic and genetic responses to environmental and harvesting related factors. Further, both phenotypic and genetic shifts need to be considered at the population rather than stock level as many studies have demonstrated that present management units do not reflect the level of reproductive isolation and local adaptation.

It is now recognised that an understanding of the processes that regulate temperate fish reproductive physiology is essential in order to accurately and unambiguously interpret changes in maturation trends.

1.2. UNDERSTANDING THE MATURATION PROCESS

1.2.1. Environmental regulation of reproductive physiology

As briefly mentioned, maturation in cod and haddock, like most temperate marine teleosts, is seasonal (Bye 1990). These species are presumed to have evolved a seasonal reproductive cycle to increase the likelihood that offspring encounter favourable environmental conditions for survival (Morgan et al. 2013). However, these species commit to mature long before such conditions can be assessed. In order to time reproductive development so that larvae coincide with favourable conditions, fish must make use of 'proximate' environmental cues, which accurately predict the onset of these 'ultimate' environmental conditions (Baker 1938). Many environmental factors have been implicated as possible proximate cues, including rainfall, food supply, photoperiod and temperature, each of which provide timely indications of pending conditions and can be utilised to provide the animal with specific entrainment to the prevailing local conditions (Bromage et al. 2001). However, in temperate regions, photoperiod has long been heralded as the "zeitgeber", entraining seasonal biological rhythms, including reproduction (Migaud et al. 2010). For cod and haddock, this is evidenced by multiple studies which have altered or inhibited maturation through photoperiod manipulation (Davie et al. 2004, 2007a, 2007b, 2007c, 2008, Norberg et al. 2004, Martin-Robichaud and Berlinsky 2004, Levesque et al. 2005, Taranger et al. 2006, Karlsen et al. 2006a, van der Meeren and Ivannikov 2006, Cowan et al. 2011, 2012).

Photoperiod

The daily and yearly rhythms of light, termed photoperiod, provides a consistent, "noise free" signal, giving the most accurate indication of seasonal conditions. Thus, photoperiod is widely perceived as the master environmental signal entraining seasonal physiologies like reproduction. The physiological basis for 'photoperiodism' is believed to be common to all vertebrates and is rooted in a "central circadian axis" (Menaker et al. 1997) comprising the retina, the suprachiasmatic nucleus (SCN) of the hypothalamus (or an equivalent localised brain centre), and the pineal complex. These have each been shown to be involved to varying degrees in the control and regulation of the photoperiod entrainment rhythms (Menaker et al. 1997, Falcón 1999). In teleosts at present, it is proposed that photoperiodic entrainment is routed somewhere in the non-visual photoreceptor system. In cod, this includes both the retinal and pineal organs (Migaud et al. 2010). Both organs are directly photosensitive and contain a large number of photoreceptor cells, similar to those found in mammals, which react to light stimulation and release neural signals (Migaud et al. 2010). In mammals, the principal neural signal output is the indoleamine hormone, melatonin. Melatonin has a key role in the control of seasonal reproduction in mammals. However, although numerous studies have been conducted, as yet there is little direct evidence of melatonin playing a role in photoperiodic control of reproduction in cod or other teleosts (Migaud et al. 2010). At the interface between the environmental signal and the reproductive axis lies an as yet undetermined pathway in fish. How the light signal is interpreted and integrated is unclear, but possible targets are emerging from the more advanced mammalian and avian models. Of these targets, the *Eya3-Tsh-Dio2/Dio3* pathway has shown potential as a mediator of the photoperiod cue and will be discussed further in Section 1.2.2.

Energetic determinants of maturation

In teleosts, reproduction is typically a long lasting and highly energetic process requiring the division of resources away from somatic growth (Hislop et al. 1978a, Kjesbu and Holm 1994). Consequently, the spawning window is usually highly restricted with the need to optimize the partitioning of available energy. This is achieved through precise co-ordination of gonadal recruitment and development with the prevailing environmental and ecological signals. Crucial to the success of reproduction is the coincidence of these environmental signals with a permissive physiological state or "gate", which in temperate fish species would appear to be based around suitable nutrition and energetic status. To successfully initiate a reproductive cycle following the correct environmental signal within the right window, there is now a well-accepted hypothesis stating that a threshold of size, growth rate and/or energy storage must be surpassed during this critical period for sexual maturation to initiate and succeed, as observed in mammals (Thorpe 1989, Rowe et al. 1991, MacKenzie et al. 1998, Thorpe et al. 1998, Taranger et al. 1999, Migaud et al. 2010). This concept is clearly evident in salmonids (where maturation rate is closely linked to whole body lipid content), though less well characterised in other temperate species (Begtashi et al. 2004). In aquaculture, feed deprivation a year ahead of spawning reduces both fecundity and maturation rate (Shearer and Swanson 2000), highlighting the potential role of food availability in reproduction. However, experimental studies in gadoids have yet to influence maturation rates through diet (Karlsen et al. 1995, Nanton et al. 2001)

There is now substantial evidence that maturation in gadoids is not necessarily size dependant but rather is sensitive to an animal's growth and energetic status at particular times of year. The importance of time is evident from the ability to shift or even inhibit maturation by means of photoperiod manipulation (Skjæraasen et al. 2004, Davie et al.

2007a, 2007b, 2008, Karlsen et al. 2014). Photoperiod appears to control the timing of the period when the physiological threshold must be exceeded for maturation to continue (Bromage et al. 2001). However, it is clear that earlier cues conveying energetic status are also important in this process. The relative importance of body energy stores in permitting maturation to occur and the endocrine mechanisms through which they influence the reproductive system are not well understood in gadoids. In addition, the period when nutritional status influences maturation has not been clearly defined, though evidence suggests that it must fall between the decline in photoperiod from the summer solstice and the rise in gonadotropin production and gonad development marking the initiation of secondary gametogenesis (Davie et al. 2007c, Tobin et al. 2010). Although the endocrine pathways are as yet unclear, gathering evidence from other teleost and vertebrate models would suggest that growth hormone (GH) (Pierce et al. 2001), insulin-like growth factor-I (IGF-1) (Grier et al. 2009), leptin (Schmitz 2013, Trombley et al. 2014), ghrelin (Tena-Sempere 2013), thyroid hormones (Duarte-Guterman et al. 2014) and vestigial-like family member 3 (VGLL3) (Barson 2014, Ayllon et al. 2015b, Barson et al. 2015) may have significant roles in relaying energetic status to the brain-pituitary-gonad (BPG) axis.

Temperature

Although temperature is considered an important cue in regulating reproductive cycles in gadoids, it is more likely to affect the pace of development (Migaud et al. 2010). Experimental evidence supports this idea, as reproduction in cultured fish under photo-thermal regimes which are out of phase, still appear to be entrained by photoperiod (Norberg et al. 2004). Changes in food intake and metabolic rate are related to temperature (Jobling 1988), and unfavourable temperature could reduce energy investment in growth and reproduction indirectly by reducing food consumption (Yoneda

and Wright 2005a). Temperature can also directly affect the rate of oogenesis independent of somatic growth (Tobin and Wright 2011). This is probably because temperature also directly affects hormone synthesis and secretion which may influence many stages of the reproductive cycle, particularly spawning (Kraak and Pankhurst 1997, Suquet et al. 2005). It has also been found that a 1˚C drop in temperature will delay spawning by 8 to 10 days in Norwegian coastal cod (Kjesbu 1994). Thus, temperature may influence reproductive potential of cod and timing of both the decision to mature and spawning (Yoneda and Wright 2005a, 2005b).

Other Factors

Other environmental factors, such as salinity, lunar/tidal cycles, water quality, rainfall, current and stress, that may not act as cues can nonetheless contribute and impact on growth and reproductive performance in fish (Ross 2000, Pankhurst and Porter 2003). In seasonal species these factors may be involved in the timing and synchronizing of the final stage of reproductive development and spawning time (Pankhurst and Porter 2003), and may influence other life histories underpinning reproduction.

1.2.2. Endogenous regulation of reproductive physiology

The Brain-Pituitary-Gonad axis

In teleosts, as in most vertebrates, the neuroendocrine brain-pituitary-gonad (BPG) axis is the key system involved in the regulation and control of puberty and adult reproductive cycles (Zohar et al. 2010). This axis is organised around the hypothalamus of the brain, the pituitary and the gonads. Each of these key regulators integrate with growth/energy pathways and environmental cues to control reproductive processes so that spawning occurs in synchrony with favourable conditions for survival and proliferation (Migaud et al. 2010).

In vertebrates, the neuropeptide gonadotrophin releasing hormone (GnRH), produced in the brain, is known to regulate reproductive activity by stimulating pituitary gonadotropin secretion (Zohar et al. 2010). This has traditionally been described as the starting point of the BPG axis, controlling the onset of puberty, where puberty is defined as the period during which a sexually immature animal acquires the capacity to reproduce (Okuzawa 2003). In fish, puberty is characterised by the first onset of secondary gametogenesis – specifically, vitellogenesis in females and spermatogenesis in males. Many studies have focused on the characterisation of the GnRH system, and it has emerged to be more complex and diverse than originally thought, with eight variants in teleosts alone (Lethimonier et al. 2004, Kah et al. 2007, Kraak 2009). The diversity of GnRH forms in teleosts is suggested to be due to at least two genomic duplication events (Sherwood and Adams 2005). Phylogenetic analysis has identified three distinct types of GnRH variants (White and Fernald 1998, Lethimonier et al. 2004). The first branch - GnRH-1, is considered the major hypophysiotropic hormone (Powell et al. 1994, Holland et al. 1998, Gonzalez-Martinez et al. 2002, 2004a, 2004b) while the significance of the GnRH-2 and GnRH-3 types remains unclear.

In terms of the physiological role of GnRHs, in the European sea bass (*Dicentrarchus labrax* L.), GnRH-3 and GnRH-1 nRNAs have been seen to increase along with the GnRH receptor and follicle stimulating hormone gene expression during sexual differentiation (Espigares et al. 2015) indicating that these GnRHs may play a role in BPG axis during sexual differentiation. In cod, GnRH ligand cDNAs have been isolated for all three forms. A series of experiments looking at expression of these variants in relation to maturation, using photoperiod treatments to inhibit or stimulate maturation, found that GnRH-3 expressed in the brain along with expression of the receptor GnRH-R2a in the

pituitary were strongly associated maturation, suggesting that the role of GnRH-3 is conserved among teleosts (Hildahl et al. 2011a, 2011b, 2012).

As in other vertebrates, the gonadotroph cells of teleosts are located in the anterior lobe (pars distalis) of the pituitary. These cells are innervated by neurosecretory fibers such as GnRH neurons, which release neurohormones originating in the hypothalamus of the brain (Zohar et al. 2010). It is well known that the pituitary gonadotropins play a key role in the regulation of sex steroids and subsequent production of gametes in both male and female teleosts, with the loss of reproductive function being observed following the removal of the pituitary in fish and mammals (Khan et al. 1986, Krishnamurthy et al. 2000).

There are two main forms of gonadotropins in teleosts as in most other vertebrates (Redding et al. 1993) follicle stimulating hormone (*Fsh*) and luteinising hormone (*Lh*). These have been shown to share a common α subunit and a hormone specific β subunit (Yaron et al. 2003). The action of these hormones is executed through specific membrane receptors present on the cell membranes of target tissue. High concentrations of these receptors are found in the steroidogenic cells in the gonads of both male and female teleosts, as this is the main target site for *Lh* and *Fsh* where they stimulate steroid production. *Fsh* has commonly been found as the main driver of vitellogenesis in female fish, while *Lh* is more commonly associated with spermiation in males and final oocyte development in females (Swanson et al. 2003, Yaron et al. 2003, Weltzien et al. 2004).

Expression of pituitary gonadotropin β subunits during puberty has been investigated in a number of fish species (Hassin et al. 2000, Gen et al. 2003, Mittelholzer et al. 2009a, 2009b, Tobin et al. 2010, de Almeida et al. 2011). In females spawning single batches of eggs such as rainbow trout (*Onchorhynchus mykiss* W.), gonadotropic regulation of

gametogenesis is generally characterised by an elevation in *Fsh* during early oocyte growth and vitellogenesis, whereas *Lh* is associated with final oocyte maturation and ovulation (Prat et al. 1996, Gomez et al. 1999). Though there is some evidence of alternative expression patterns for batch spawners (Sohn et al. 1999, Kajimura et al. 2001, Weltzien et al. 2003a, 2003b), both cod and haddock exhibit similar patterns of expression, showing an up-regulation of *Fsh* during early oocyte development, and *Lh* later in the cycle (Tobin et al. 2010, Karlsen et al. 2014). In male fish, *Fsh* has been described to play a role in spermatogenesis whereas *Lh* is associated with spermiation (Mittelholzer et al. 2009b, Carrillo et al. 2010, Tobin et al. 2010, Karlsen et al. 2014).

Gonads represent the final step of the reproductive axis and are responsible for the development of germ cells, and the production of steroids and growth factors. Steroids are involved in stimulating and regulating these processes within the gonads, but also play an important role at the brain and pituitary levels through feedback (Zohar et al. 2010).

There are three main types of steroids: androgens, oestrogens and progestogens. Androgens, including testosterone (T) and 11-ketotestosterone (11KT), are produced in the Leydig cells within the testis, and are the dominant sex steroids involved in spermatogenesis in male teleosts (Borg 1994, Kime 1995, Weltzien et al. 2004). In particular, 11KT is considered as the main androgen (Borg 1994), and has been shown to induce all stages of spermatogenesis *in vitro* (Miura et al. 1991). The oestrogen 17β-estradiol (E_2) plays an important role in the female teleosts reproductive cycle (Kime 1995). As ovarian recrudescence begins, E_2 is secreted by the ovarian follicle and stimulates the liver to produce vitellogenin, which is sequestered by the oocyte (Nagahama 1994). Oestrogens are considered ‘female’ hormones but are also found in male teleosts, and it has been suggested that they have an important role in regulating gene expression in the testis (Scott et al. 2006). Progestogens have been shown to play a

major role during advanced stages of gametogenesis in both male and female teleosts. In females, progestogens have been found to play an important role in final oocyte maturation, namely germinal vesicle breakdown (GVBD) (Nagahama 1994), which is an essential process prior to ovulation. In males, high plasma levels of progestogens have been found in salmonid species during spermiation and is thought to be involved in the acquisition of sperm motility (essential for fertilisation) by increasing the pH of the sperm duct (Miura et al. 1991). Furthermore, precocious spermiation can be induced by injection of progestogens (Nagahama 1994).

As well as their role in gametogenesis, sex steroids involved in the feedback control of reproductive development and provide an indicator to the brain and pituitary as to the reproductive state of the animal (Zohar et al. 2010). Both the brain and pituitary have been found to contain high densities of oestrogen and androgen receptors. Whether feedback is positive or negative depends on the physiological status of the individual and the species itself (Zohar *et al*., 2010). They are also important for the control of sexual behaviour.

While the cascades of hormones and growth factors that regulate gonad growth and gamete maturation in fish have been well characterized in many fish species as shown above, the triggers and mechanisms that initiate this cascade remain unclear.

The Photoneuroendocrine System

Evidence of a pathway linking the environmental factors to the BPG is somewhat lacking in teleosts, and there is certainly no clear pathway proposed for cod. This field of research is still in its infancy among fish species. However, inspiration can be taken from mammalian and avian models where these pathways are better understood. The photoneuroendocrine (PNES) system responsible for decoding and integrating the light

signal has been well described for mammals and birds, and begins with transduction of the light signal into the pituitary. In mammals, this light signal is relayed exclusively by melatonin, while avian models exhibit direct innervation of the pathway through deep brain photoreceptors (Dardente et al. 2014, Follett 2015). This light signal regulates production of the transcriptional co-activator and enzyme, eyes-absent homolog 3 (*Eya3*) in the *pars tuberalis* of the pituitary. Expression of this marker has been positively associated with day length, and is up-regulated under a long day (LD) photoperiod. Induction of this response is surprisingly rapid, with expression of *Eya3* being induced within 14 hours following a switch between short day (SD) and LD photoperiods in Japanese quail (*Coturnix japonica*) (Ono et al. 2009b) and mice (*Mus musculus* L.) (Masumoto et al. 2010). As a transcriptional coactivator, *Eya3*, modulates thyroid stimulating hormone (*Tshβ)* expression, up-regulating *Tshβ* production in the *pars tuberalis*. *Tshβ* is then transported into the medio-basal hypothalamus (MBH), up-regulating type 2 iodothyronine deiodinase (*Dio2*) and supressing type 3 iodothyronine deiodinase (*Dio3*) expression. *Dio2* is responsible for converting thyroxine (T4) into the biologically active thyroid hormone, triiodothyronine (T3). Contrastingly, *Dio3* acts as a thyroid hormone inactivating enzyme converting T3 and T4 into inactive metabolites. As a result, differential expression of *Tshβ* under seasonally altering photoperiods creates a reciprocal switching mechanism which modulates thyroid hormone production. The *Eya3-Tshβ-Dio2/3* pathway thus provides a clear seasonal signal to the BPG, with the potential to consolidate the PNES and BPG axes (Dardente et al. 2014). Indeed, photoperiod manipulation studies in aves have identified both *Eya3* and *Tshβ* in the first wave response to the SD to LD switch, followed by an up-regulation of *Dio2* and down-regulation of *Dio3*, preceding a surge in gonadotropin secretion, indicating stimulation of the BPG (Yoshimura et al. 2003, Nakao et al. 2008b, Ono et al. 2009a, 2009b).

Furthermore, intracerebroventricular (ICV) administration of TSHβ to quail held under continuous SD up-regulates *Dio2* expression, stimulating the reproductive axis, while administration of TSHβ antibodies to quail exposed to continuous LD elicited the opposite response (Nakao et al. 2008a). Similar correlations have been shown in mammals; Syrian (*Mesocricetus auratus* W.) and Siberian (*Phodopus sungorus* P.) hamsters exhibit gonadal reduction in association with an up-regulation of *Dio3* under SD stimulus, while the up-regulation of *Dio2* under LD stimulus is strongly linked to gonad development (Revel et al. 2006, Barrett et al. 2007). In mice, targeted disruption of the *Tshβ* receptors supresses the *Dio2* response to LD stimulus, inhibiting the reproductive response (Ono et al. 2008).

While the pathway appears to be conserved across the vertebrate clade, there are some differences in the upstream drivers and the resultant down-stream affects. In the case of all long-day breeding birds and mammals studied to date, including Japanese quail (Nakao et al. 2008a, Ono et al. 2009b), mice (Ono et al. 2008, Masumoto et al. 2010), hamsters (Watanabe et al. 2004, Yasuo et al. 2010) and rats (*Rattus norvegicus* B.) (Yasuo et al. 2007, Ross et al. 2011), which initiate their reproductive cycle under an increasing photoperiod, the *Eya3-Tshβ-Dio2/3* pathway is positively stimulated following an increasing photoperiod or LD light signal. However, for short-day breeding sheep (*Ovis aries* L.) and Saanen goats (*Capra hircus* L.), the pathway is less straightforward. In sheep it would appear that the pathway is initiated by a LD light signal, as is the case for long-day breeders (Dupré et al. 2010, Sáenz de Miera et al. 2013) which is possibly a reflection of the key stimulatory role of LD on sheep reproductive cycle irrespective of their SD breeding behaviour (Dardente 2012). For Saanen goats however, a LD stimulus inhibits *Dio2* production, suggestive of a reversal of the pathways role to reflect the seasonal entrainment of reproduction (Yasuo et al. 2006).

Studies of this pathway in teleosts are still in their infancy, but there is some evidence to suggest that this pathway is conserved across the vertebrate clade (O'Brien et al. 2012, Nakane et al. 2013, Lorgen et al. 2015). Further work is now needed to test this theory, and similar analyses in other teleost species like cod would greatly improve our current understanding of the mechanisms controlling seasonal reproduction.

1.3. ACCOUNTING FOR STOCK STRUCTURE

It is clear that the reproductive strategy adopted by fish is intrinsically linked to the environment they inhabit (Bromage et al. 2001, Pankhurst and Porter 2003, Yoneda and Wright 2005a, Morgan et al. 2010). However, fishing pressure also influences reproduction by altering the environmental and life history traits underpinning this highly complex life event (Law 2000, Heino and Dieckmann 2004). Much like the environment, fishing pressures vary spatially, and in combination, these pressures create regional differences in many life history traits such as growth and maturation strategy, which may result from either phenotypic plasticity or genetic evolution driven by selection (Wright 2007). Though disentangling the root cause has proven difficult (Law 2000, Wright 2007), such regional variation is thought to reflect the underlying population structure – whether functional or genetic.

Regional variation in maturation schedules allows us to explore possible physiological and genetic drivers of maturation in wild fish, and greatly improves our understanding of the environmental and life history parameters which influence the onset of puberty. Such variation also has potential as a tool for helping to define stock structure – an exercise crucial to the management of stocks.

1.3.1. Mismatch between biological populations and management units

The primary objective of fisheries management is to ensure sustainability of fish stocks. However, as discussed, both cod and haddock have suffered severe declines over the last 50 years and recovery has often been slow or failed completely (Ruzzante et al. 2001, Beacham 2002, Horwood et al. 2006, Hilborn and Litzinger 2009, Schindler et al. 2010, Holmes et al. 2014), leading many to question the suitability of management strategies for these stocks. Importantly, it has been recognised that management measures need to be applied at the appropriate scale – the biological population scale.

Management of fisheries is complex, incorporating biological, economic, social and political factors, and often required to conciliate a range of national and international stakeholders. As a result, the management boundaries used to define management processes – known as a management unit (MU), often fail to account for the underlying population structure (Reiss et al. 2009). This mismatch can result in reduced productivity, local depletion or extirpation, and can even lessen resilience to environmental pressures due to loss of genetic diversity (Hilborn et al. 2003, Hutchinson 2008, Waples et al. 2008, Reiss et al. 2009, Schindler et al. 2010). It has become clear that management strategies need to account for population structure in order to manage these stocks appropriately, and evidence of stock structuring is gradually being incorporated into fisheries legislation (Jakobsen 1987, Dahle 1991, Ralston and O'Farrell 2008, Botsford et al. 2009, Armstrong et al. 2013, ICES 2015). But with the continued exploitation of marine stocks comes an ever increasing need for such rational management, and resolution of stock structure is crucial.

1.3.2. Analysing stock structure

Many approaches have attempted to characterise the population structure of stocks. For cod stocks around Scotland – our study area, methods such as tagging (Robichaud and Rose 2004, Neuenfeldt et al. 2013), otolith chemistry (Wright et al. 2006a, Gibb et al. 2006), otolith shape analysis (Galley et al. 2006), and analysis of variable life history traits (such as age and size at maturity - Wright *et al.* 2011) have been widely used, and their respective roles in deciphering the stock structure in the region are discussed further in Chapter 5. These methods utilise spatially discrete environmental variables to provide a snapshot of an individual's movements or environment at a given life stage, or analyse variations in life history strategies likely to reflect underlying physiological differences. However, while these methods provide essential information on the environmental and behavioural factors and processes underpinning stock structure, alone, they cannot infer an adaptive or evolutionary basis for the structures identified. This can only be achieved through genetics.

Genetic markers are intimately linked to reproduction. As such, patterns of dispersal and intermixing can be inferred at all life history stages through genetic analysis, and this method has been widely used to determine population structure as a result. Again, the application of genetic techniques to determine stock structure in our study area are broadly discussed in Chapter 5. This has proven a fruitful area for population genetics research, with the first genetic analyses occurring as early the 1960s for North Sea cod (Sick 1965). Over the years, as the genetic techniques have improved, so too has our understanding of the stock structure in this region (Hutchinson et al. 2001, 2003, Case et al. 2005, Reiss et al. 2009, Nielsen et al. 2009, Poulsen et al. 2011). Spatial definitions have become more discrete, as the analysis methods have advanced, highlighting the apparent mismatch between management boundaries and the population structure, with

recent work by Heath et al (2014) identifying at least two populations in the North Sea management unit, and alluding to further fine scale structuring in this region. However, this work only accounts for some of the apparent structuring observed using other techniques (Neat et al. 2006, 2014, Wright et al. 2006b, 2011b, Gibb et al. 2006), indicating that further research is needed, and there is a hanging question mark over the structure of the cod stock west of Scotland, where little work has focussed. Physiological evidence indicates a significant difference in the age and size at maturity between cod aggregates within this area which are likely to reflect underlying genetic differences, but confirmation of this structure through genetics is needed.

Single nucleotide polymorphism (SNPs) are fast becoming the marker of choice for population genetic analyses. These markers are widely and densely distributed throughout the genome, but until recently the development of genome wide panels of these markers has been limited to species with pre-existing genomic information (Kess et al. 2015). With the advent of next generation sequencing (NGS) and subsequent development of reduced representation methods, it is now possible to identify and genotype thousands of SNPs, with comparably lower costs and higher coverage across a large number of individuals compared with traditional methods. Double-digest restriction-site associated DNA sequencing (better known as ddRAD), an elaboration of the earlier "single digest" RAD sequencing method (Baird et al. 2008), developed by Peterson *et al.* (2012). includes combinatorial multiplex indexing (primers with different coding), allowing several hundred individuals to be pooled in a single sequencing lane making it ideal for low cost SNP discovery for population genetics.

1.4. EXPERIMENTAL AIMS

The ultimate intention of this research was to improve our understanding of the environmental and endogenous drivers of maturation in cod and haddock with a view to interpreting apparent shifts in the maturation life histories of these species and the phenotypic and genotypic drivers underpinning these changes. The principal aims were; 1) to assess the role of the *Eya3* pathway in the entrainment of maturation in cod (Chapter 3); 2) to investigate the energetic determinants of maturation – specifically, the nature of the energetic cue and critical time frame in which a fish is sensitive to this signal (Chapter 4); and 3) to define the stock structure of cod in IVa and westward into VIa through genetic and physiological analysis (Chapter 5).

CHAPTER 2. GENERAL METHODS

2.1. EXPERIMENTAL PROCEDURES

All animals were held in accordance with, and experiments performed under, the licencing of the Home Office Animals (Scientific Procedures) act (1986).

2.1.1. Maintenance

All fish used in experiments were obtained from the wild, and transferred to the Marine Laboratory in Aberdeen. Fish were held in enclosed tank facilities where filtered seawater was supplied at a flow rate of 6 $L.min^{-1}$ and drained to waste. Additional aeration was provided. Lighting was supplied by artificial illumination. Temperature was maintained at 9 ºC for all experiments.

2.1.2. Anaesthesia

Experimental animals were anaesthetised prior to any handling or experimental procedure. Fish were starved for 24 hours prior to any procedure to ensure full evacuation of the gut. Fish were immersed in a bath containing MS-222 and seawater at a concentration of 100 $mg.L^{-1}$, which induced loss of equilibrium within 3 minutes. Following the experimental procedure, fish were placed in clean aerated seawater and allowed to fully recover before returning to their original housing.

2.1.3. Tagging and Identification

In order to track individual growth rates it was essential to identify individual fish. This was achieved by tagging each fish with a unique Passive Integrated Transponder (PIT) tag. These tags were inserted into the peritoneal cavity approximately 2 cm above the anal pore using a tagging gun. Tags were read when required using a PITtag reader passed within 10 cm of the tag.

2.1.4. Experimental Growth Assessment

Measurements of fish length and weight were made throughout the experiments. Once anaesthetised, total length was measured to the nearest millimetre and fish were weighed to the nearest 0.1g in all cases.

2.1.5. Fish sacrifice

At the end of each trial fish were sacrificed to allow for dissection and removal of tissues. This was achieved by over-exposure to MS-222.

2.2. MATURITY ASSESSMENT

2.2.1. Gross gonadal morphology

Gonad developmental stage was assessed macroscopically through visual inspection of the gonads using the 6 point staging system defined by the ICES Workshop on Maturity Staging of Cod, Whiting, Haddock and Saithe (Bucholtz et al. 2007).

2.2.2. Histology

Gonad samples collected from both experimental and wild fish were fixed in 10 % neutral buffered formalin (NBF) (6.5 g disodium hydrogen phosphate (VWR International ltd, Poole, UK), 3.5 g sodium dihydrogen phosphate (VWR), 100 ml of 40 % formaldehyde (VWR) and 900 ml deionised water) and stored for later analysis.

<u>Embedding and Sectioning</u>

Fixed samples were trimmed and placed individually into cassettes. These cassettes were then placed in an automated tissue processor (Thermo Shandon Excelsior, Thermo Shandon Cheshire, UK) which was programmed to dehydrate, clear and impregnated each sample with paraffin wax as follows:

1. 10 % NBF	30 min
2. 75 % EtOH	60 min
3. 85 % EtOH	90 min
4. 95 % EtOH	90 min
5. 100 % EtOH	90 min (x3)
6. Xylene	40 min (x3)
7. Molten wax	90 min (x3)

Samples were then embedded using a histoembedder (Leica UK Ltd, UK). Blocks were left to harden and cooled on a cool-plate before trimming and finally sectioning. Three serial sections of 5µm thickness were sliced using a rotary microtome (Leica UK Ltd, Milton Keyes, and UK), placed on glass slides and dehydrated in an incubator for a minimum of 60 minutes at 60 ºC.

Staining

The sections were stained with Mayer's haematoxylin and eosin Y using a modification of the procedure of Bancroft and Stevens (1991), as follows;

1. Xylene, 2 mins	
2. Absolute Alcohol I	2 mins
3. Absolute Alcohol II	2 mins
4. Absolute Alcohol III	2 mins
5. Running tap water	30 sec
6. Haematoxylin Z	10 mins

ter	5 mins
8. 1% Acid Alcohol	3-5 quick dips
9. Running tap water	5 mins
10. Scott's tap water substitute	2 min
11. Running tap water	2 mins
12. Eosin	5 mins
13. Running tap water	2 – 5 mins
14. Absolute alcohol I	2 mins
15. Absolute alcohol II	1 mins
16. Absolute alcohol III	1 mins
17. Xylene (Clearing)	until cover-slipping

Samples were cover slipped immediately after staining and left to dry overnight before being read.

2.3. AGE ASSESSMENT

Preparation of otoliths for age estimation for Chapter 5 was undertaken following the standard protocols developed by Easey and Millner (2008).

2.3.1. Embedding in resin

Prepared otoliths were embedded in black polyester resin to allow for easy sectioning using otolith moulds developed specifically for this purpose (Figure 2.1a). A layer of resin (50 g per mould) was added to these mounds and allowed to set (~ 12 hrs). Meanwhile, a sheet was prepared to record otoliths IDs and corresponding sample details, their relative

positions within the resin block and the unique number of the mould used. To ensure that otoliths are sectioned through their core, moulds were aligned using a mounting jig (Figure 2.1b) with camera system and monitor attached. Two lines marked on the monitor indicate the dimensions of the blade used to cut the otoliths. These marks are aligned to grooves on the otolith mould used to mark the final block, indicating where to cut the final block. There are four rows indicated by these grooves on each otolith mould and each must be aligned individually as otoliths are added. Once aligned, a bead of resin is applied to left hand side of each mould and a piece of spaghetti the same length as the mould is fixed to this to allow for future orientation of the sections and blocks. A bead of resin is then added perpendicular to this along the first row. Otoliths are then added, concave side down, and orientated using the monitor so that the marks indicating the blade width are aligned with the "waist" or "v" of the sulcus (Figure 2.1c). For larger otoliths, ~ ⅓ of the thin end is removed to enhance stability, and otolith position is added to the table after each addition. Otoliths are added in this manner, leaving 2-3 mm between each otolith, for the first row and alignment is double checked before moving to the next row. When all rows are filled, alignment is again checked before these otoliths are allowed to set in place (Figure 2.1d). Once the otoliths are set in place, the moulds are filled with resin (~ 120 g) on a level platform and allowed to harden overnight.

2.3.2. Sectioning

To indicate the alignment of otoliths, resin blocks are marked using the same grooves used to align the otolith moulds with the mounting jig system. A line is etched using a scalpel between these grooves for each row and blocks are marked with a unique identifier as are the rows before the resin blocks are removed from the moulds. Blocks are then sectioned with a mechanical saw using these lines as a guide for the blade. A section of

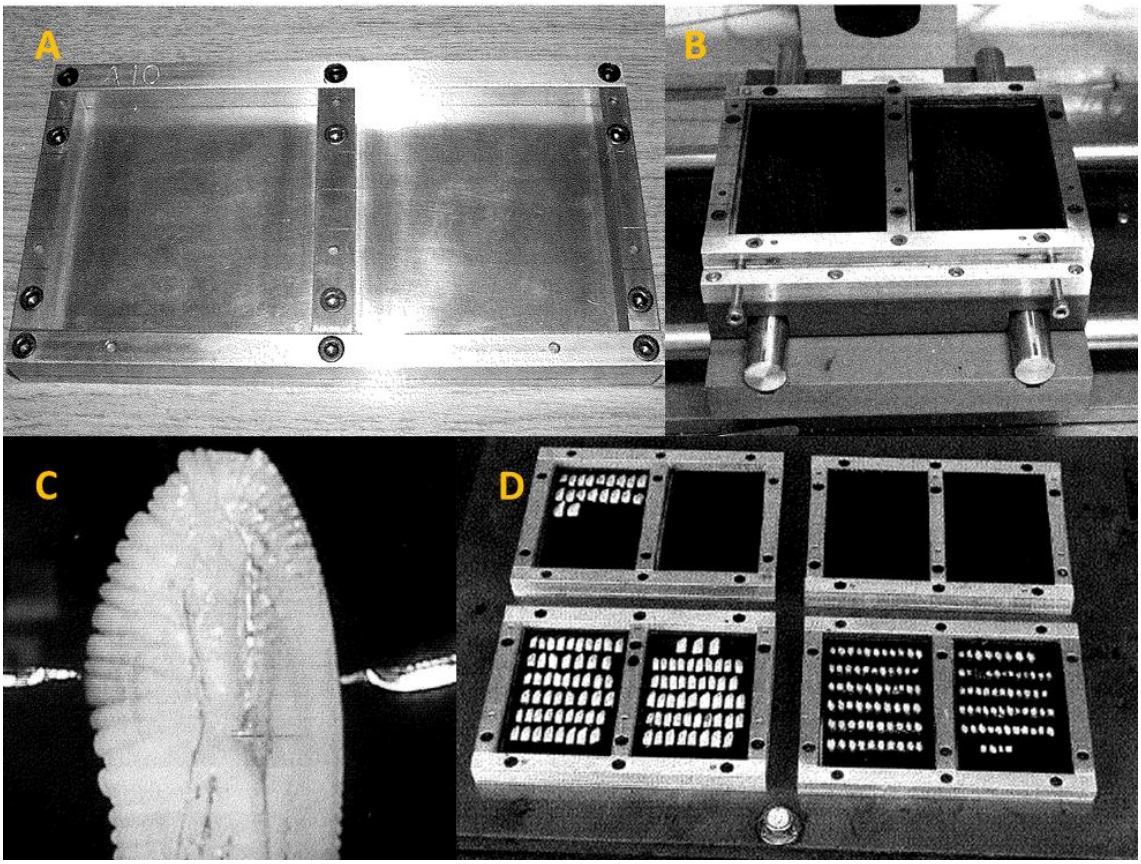

Figure 2.1. Figure shows otolith embedding equipment including the otolith mould (A), and the mounting jig (B). Image C shows the alignment of the blade guides with the "V" or "waist" of the otolith sulcus, and image D shows the positioned otoliths being left to dry on a level surface.

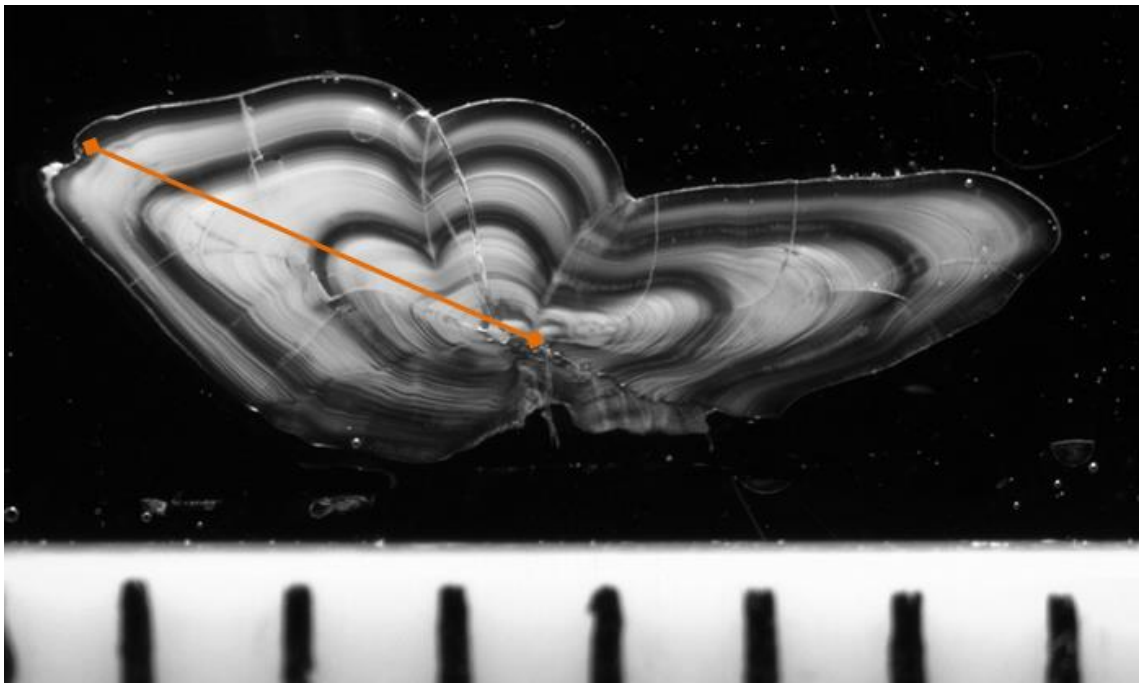

Figure 2.2. Sectioned otolith under transmitted light. The line indicated the reading axis used, and the scale bar is in mm. This cod is two years old as there are two opaque rings present.

no more than 0.5 mm is cut for each row, and labelled appropriately to allow for cross referencing with the position sheet.

2.3.3. Age determination

Sectioned otoliths were analysed under a light microscope using transmitted light. Age was determined by counting the number of opaque rings along one axis of the otolith (Figure 2.2). For august samples, an opaque band was beginning to form at the edge of some otoliths but this is discounted.

Otoliths were read twice each by two independent readers. All readings were blind (no a priori knowledge of previous age estimates). The age estimates were then collated for each otolith (4 estimates per otolith). Where there was disagreement between readings, the otoliths were read once more by both readers. If there were still disagreements in the age, these age estimates were not included in any formal analysis.

2.4. QUANTITATIVE REAL-TIME PCR

Target-gene expression was analysed using quantitative real-time PCR assays. These assays were developed and executed using the methods outlined below.

2.4.1. Total RNA extraction

RNA was extracted from the pituitary and whole brain samples for each fish by thawing in 1ml TRIzol® reagent (Invitrogen, UK) for 10 minutes. Samples were then homogenized using a mini bead beater and allowed to rest at room temperature for 5 mins. 100 µl of BCP was then added and samples were vortexed briefly and left to rest at room temperature for 10 minutes. Samples were then centrifuged at 12 000 g, for 15 minutes at 4 ºC. The aqueous layer was carefully removed to a fresh tube along with 250 µl each of isopropanol and RNA precipitation solution, briefly mixed, and incubated at room

temperature for 10 mins. Again, samples were centrifuged at 12 000 g and 4 ºC for 10 mins. Being careful not to disturb the newly formed RNA pellet at the base of the tube, the aqueous phase was removed and 1 ml of ice cold 75 % Ethanol (EtOH, Sigma-Aldrich, UK) was added to remove impurities. Samples were washed for 5 minutes before centrifuging once more at 7 500 g for 5 mins, removing all traces of EtOH and eluting in 15µl or 50 µl of MilliQ water for pituitary or brain samples respectively.

2.4.2. RNA quality assessment

RNA quality checks were performed with a Nanodrop spectrophotometer (Labtech Int., UK) to evaluate the absorbance ratio at 260/280 (a sample with an absorbance ratio at 260/280 >1.8 indicates a high level of purity, not contaminated by protein (McKenna et al., 2000)), and by gel electrophoresis to assess RNA degradation. 2 µg of denatured total RNA by electrophoresis, on an agarose gel. 500 ng of RNA, made up to a reaction volume of 5 µl with MilliQ water was heat treated for 10 minutes at 70 ºC. 1 µl loading dye was then added, and the samples were loaded onto a 1 % agarose gel and run for 40 minutes at 70 V in TBE buffer. The gel was visualized on a UV trans-illuminator and the total RNA quality was subjectively assessed based on the integrity of the rRNA bands (18s and 28s) as they appeared after separation by gel electrophoresis (Figure 2.3).

2.4.3. DNAse treatment

After the extraction of the RNA and quality checks, samples were treated to remove any contaminating DNA. This was done by using a commercial DNase treatment kit (DNA-free, Applied Biosystems, UK). The treatment was performed by adding 2.5 µl buffer and 0.5 µl DNAfree to 5 µg of total RNA made up to 19.5 µl with MilliQ water. Samples were then incubated at 37 ºC for 30 minutes. After incubation 2.5 µl of re-suspended DNase inactivation reagent was added and mixed well, incubated for 2 mins at room

temperature, and centrifuged at 10 000 g for 1.5 mins. The total RNA supernatant was then transferred to fresh tube.

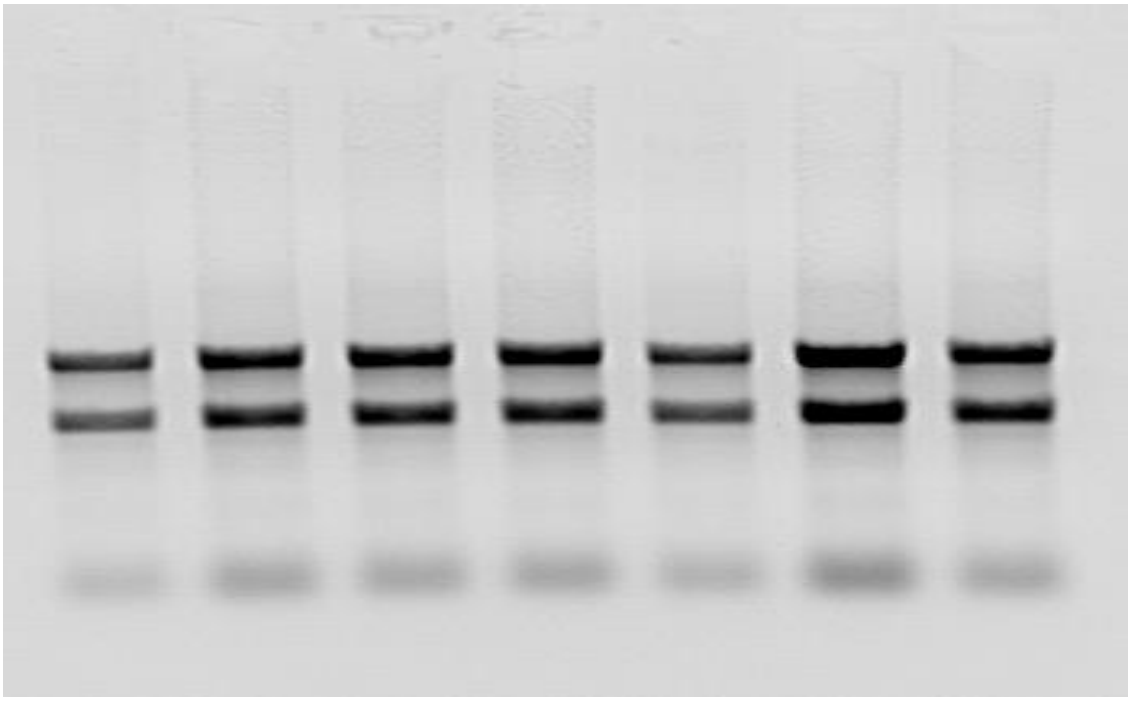

Figure 2.3. Typical total quality showing sharp rRNA 18s and 28s bands after separation by gel electrophoresis

2.4.4. cDNA synthesis

Complimentary DNA (cDNA) was generated from RNA through reverse transcriptase, using a High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems) as follows: 10 µl of total RNA was added to a master mix containing 2 µl of RT buffer, 0.8 µl dNTPs mix, 2 µl RT Random Primer, 1 µl Reverse Transcriptase and 4.2 µl of MilliQ water, to create a final reaction volume of 20 µl. Samples were then placed in a thermocycler set on a program of 25 ºC for 10 mins, 37 ºC for 120 mins, and 85 ºC for 5 mins. Samples were then stored at 4 ºC until required.

2.4.5. Primer design

Partial cDNA sequences for each target gene were generated using primers designed with primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast). One primer pair was designed for each gene of interest based on gene sequences identified within the Atlantic cod Genebuild, available from the Ensembl Genome Project (http://www.ensembl.org/Gadus_morhua). For each gene, the candidate transcripts were next checked by BLAST analysis to identify conserved regions across the Gadidae family. Primers pairs were then designed based on these conserved regions. The house-keeping gene, Elongation factor 1 alpha (*Ef1α*), was selected as the reference gene for normalisation of the QPCR quantification data. One primer pair for this gene was also designed, following the same process described above.

Each primer pair was verified by Polymerase Chain Reaction (PCR). These PCR reactions were performed using; 1µl reaction buffer, 0.8 µl of forward and reverse primers, 0.45 µl $MgCl_2$, 0.25 µl dNTPs, 0.04 µl *Klear Taq* DNA polymerase (KBioscience), 5.66 µl MilliQ and 1 µl of synthesised cDNA. The thermal cycling program consisted of a 15 min initial denaturation at 95 ºC, followed by 35 cycles of 95 ºC for 30 s, X ºC (primer specific annealing temperature, detailed in Section 3.2.3) for 30 s, and 72 ºC for 60 s, with a final extension at 72 ºC for 4 mins. PCR products were then checked on a 1 % agarose gel to verify size and purity of the products, following which, the products were purified using a NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL), to remove excess primers, dNTPs and enzymes. The concentration of these purified PCR products was then measured using a NanoDrop (Thermo Scientific) spectrophotometer.

2.4.6. Cloning

Stable QPCR standards were created by cloning PCR products for each target gene into a plasmid vector using a PGEM®-T easy vector kit (PGEM®-T easy vector systems, Promega, UK). The amount of purified PCR product to be used was first calculated to give a ratio of ~ 1:3 vector to insert using the following equation:

$$ng\ Insert = (ng\ Vector \times kb\ Insert)/kb\ Vector$$

The ligation of PCR product onto the vector was performed according to the PGEM®-T kit protocol, but using half of the recommended quantities (i.e.- 5 µl instead of 10 µl reaction), then mixed gently by pipette and incubated overnight at 4 ºC. Following this step, 25 µl of High-Efficiency Competent Cells (HECCs) were then added to 1 µl of the ligated plasmid and left on ice for 20 mins. These were then heat-shocked for 45-50 s at 42 ºC, to transform the plasmid into the HECCs, then immediately returned to ice for 2 mins. 475 µl of SOC medium was then added and the mixture was then incubated (with shaking at ~ 150rpm) at 37 ºC for 90 mins.

Two LB agar plates were created for each transformed plasmid sample, with each plate containing ampicillin, X-gal and IPTG, in accordance with the kit guidelines. Each transformed plasmid sample was then spread between two pre-heated plates, under sterile conditions, and incubated (sealed and inverted) overnight at 37 ºC.

The following day, white colonies from each plate were selected and transferred under sterile conditions to tubes containing 4 ml LB broth. These were incubated overnight at 37 ºC (with shaking). White colonies only were chosen as these represent colonies in which successful ligation of the PCR product has occurred. Following incubation, the plasmid-vector was purified using a GenElute™ Plasmid Miniprep kit (Sigma-Aldrich) following the kit guidelines, eluting with 50 µl of MilliQ water.

Plasmids were then quality checked by PCR using the appropriate primers for each plasmid sample, and by restriction digest, using restriction enzyme EcoR1, to cut the vector before and after the ligation site. Both products were then quality checked by gel electrophoresis. Plasmids were then linearised by restriction digest using the enzyme PST1 which has a single cut site located on the plasmid. This enzyme was selected following examination of the manufacturer's supplied vector map, as well as checking the inserted sequences for cut sites for our selected enzyme using Bioedit software. Products from this digestion step were again checked by gel electrophoresis to verify that a single product of uniform size was produced. The product concentration was then measured using a NanoDrop spectrophotometer.

2.4.7. QPCR

cDNA for each sample was synthesised from RNA using the methods described above. The gene, elongation factor 1 alpha (*Ef1α*) was used in all cases as a housekeeping reference gene.

The standard for each assay was prepared from the previously cloned, purified and linearised plasmid containing the ligated target gene fragment. The preparation of plasmid based standards for each target gene was calculated according to the supplier's guidance notes, creating an initial stock standard of 10^8 copies per µl in a final volume of 1 ml, by adding a calculated quantity of linear plasmid to λ-TE buffer. Serial dilutions were then created from this initial stock giving standard concentrations of 10^7 copies per µl, 10^6 copies per µl, all the way to 10^1 copies per µl. Following their preparation, a test assay was run including every second plasmid standard (10^8, 10^6, 10^4 & 10^2), alongside a number of cDNA samples. This assay was used to identify the detection range and

amplification efficiency of the assay, as well as identifying the three standards to be selected for subsequent gene expression quantification.

Each QPCR plate was prepared, including, in duplicate, three chosen standards for that particular gene, one non-template control (MilliQ water), and four internal controls consisting of four random cDNA samples from the target tissue which were included in every plate to control for inter-assay variation. The remainder of each plate was filled with unknown samples in duplicate.

The QPCR assay was performed using 0.7 µl of both forward and reverse primer, 5 µl of diluted cDNA, 10 µl of SYBR-green QPCR buffer, and 3.6 µl of MilliQ water to a final reaction volume of 20 µl. The reactions were run on an Eppendorf Mastercycler® ep *realplex* thermocycler using the following programme; 95 ºC for15 mins, followed by 45 cycles of 95 ºC for 15 s, X ºC (annealing temperature of primers) for 15 s, 72 ºC for 30 s, and finally a temperature ramp from 65 ºC to 95 ºC with fluorescence being measured every 0.5 ºC was included to create a melt curve. The copy numbers of each gene were automatically calculated by the *realplex* software by comparison to the standard curve created by the serial plasmid dilutions. QPCR efficiency was calculated for each plate run, with efficiency being greater that 90 % in all cases. A melt curve was also created to verify the specificity of the primers used, and as an indication of assay contamination.

Transcript levels of each target gene in brains and in pituitaries were normalized between plates using internal controls, and all samples were normalized against the house-keeping gene to account for individual variability, thus calculating the absolute copy number per µg of total RNA.

2.4.8. Assay validation

A melt curve analysis was built into the QPCR protocol to determine the temperature at which the PCR products dissociate and thus the fluorescence dropped to background levels. The Eppendorf software then plotted this dissociation curve as a negative derivative of the fluorescence vs temperature. If the peaks (i.e. melting temperature) of all samples and standards were at the same temperature this indicated that the primer pairs used for the QPCR were highly specific and producing a single product. If, however additional and/or shifted peaks were detected this would be an indication of primer dimers, mispriming or some form of contamination and the assay would have to be redesigned or the sample set repeated to remove this ambiguity.

2.5. POPULATION GENETIC TECHNIQUES

Samples of Atlantic cod gill tissue were collected for genetic population analysis from sites around the Scottish mainland and the Northern Isles. ~ 2–3 gill filaments were removed from each fish and stored in 100 % EtOH at 4 º C prior to analysis.

2.5.1. DNA extraction

A 1-2 mm^2 section of gill tissue, blotted and briefly air-dried to remove excess ethanol, was incubated overnight at 55 ºC in a solution of SSTNE buffer (60 µl) and proteinase K (10µg). The following day, temperature was increased to 70 ºC for 15 minutes to deactivate the proteinase K. Samples were cooled to room temperature before adding 4 µl of RNAse (2µg/µl) and incubating for 1 hr at 37 ºC. 46 µl of 5M NaCl solution was then added, the samples mixed, and left on ice for 20 mins. Samples were then centrifuged at high speed and the supernatant was retained. An equal volume of isopropanol was added and mixed by 5-6 sharp inversions followed by a 20 min incubation on ice. Again samples were centrifuged at ~ 2 000 g to pellet the DNA. Supernatant was carefully

removed and 180 µl EtOH (72%) was added and left overnight to eliminate any salt impurities. The following day, samples were centrifuged to re-pellet the DNA and all traces of EtOH were removed before re-suspending in 20 µl of 5mM Tris.

2.5.2. ddRAD library creation

The ddRAD library construction protocol followed a modified version of the protocol described by Peterson et al. (2012). In summary, each sample (0.2µg DNA) was digested at 37°C for 30 minutes using the high fidelity restriction enzymes (REs) *SbfI* (specific for the CCTGCA|GG motif) and *SphI* (specific for the GCATG|C motif; New England Biolabs, UK). Following this, reactions were heat inactivated at 65°C for 20 minutes. Samples were returned to ambient temperature (5 minutes), after which 3 µl of unique combinations of RAD-specific P1 (6 nM) and P2 (72 nM) paired-end adapters that included 5 or 7 bp barcodes were added to each sample and incubated at ambient temperature for 10 minutes. The individual reactions were then incubated for 2 hours at ambient temperature with 3 µl of ligase mastermix (1mM rATP (Promega, Mannheim, Germany) and 2000 cohesive-end Units per µg of DNA of T4Ligase (New England Biolabs) in 1x CutSmart buffer). Ligase activity in each individual reaction was stopped by the addition of 30 µl PB buffer (pH indicator added) from a MinElute PCR purification kit (Qiagen, Manchester, UK) and the individual samples then pooled into one tube. The pooled reaction was pH adjusted with ~ 2 µl, 3M sodium acetate, pH 5.4 and sequentially loaded, in 550 µl aliquots, onto a single MinElute spin column and processed as per manufacturer's instructions. The pooled library was eluted from the column in 2× 36 µl warmed EB buffer (60°C) and stored on ice.

Size selection (~ 400 bp - 700 bp) of DNA fragments was performed by chilled electrophoresis. The excised gel slice was processed through a spin-column (MinElute

Gel Clean Up kit; Qiagen), with the library template DNA being eluted from the column in 2 × 35 µl warmed EB buffer (60°C) and stored on ice (final volume ~ 65 µl).

Following optimisation of PCR conditions, to identify the minimum number of PCR cycles required to produce sufficient product for sequencing, a bulk amplification of the library (400 µl) was undertaken. This large scale PCR comprised 32 µl library template, 9.6 µl combined 10 µM Illumina compatible P1 and P2 adapter specific primers (Peterson et al. 2012), 200 µl Q5 Hot Start HF 2X Master Mix (New England Biolabs) and 160 µl nuclease-free water. After thorough mixing of the components, this mastermix was split into 32 × 12.5 µl separate reactions for PCR. Cycling conditions were: 98°C for 1 minute; then 12 cycles of 98°C for 10s, 65°C for 15s and 72°C for 40s; a final 72°C for 3 minutes. Following PCR, the 32 aliquots were recombined. To confirm successful amplification, 5 µl of the reaction was checked by electrophoresis while the remainder was mixed with 3 volumes PB buffer, acidified with 2.5 µl of 3M sodium acetate, and loaded in 550 µl aliquots onto a single MinElute PCR purification spin-column. The column was processed as per manufacturer's instructions; the library being finally eluted in 2 × 28 µl warmed EB buffer (60°C). The library was further cleaned, to maximise removal of small fragments (< c. 200 bp), using an equal volume of Agencourt AMPure XP paramagnetic beads (Beckman Coulter, High Wycombe, UK) following the manufacturer's instructions. The purified library was eluted in 18 µl EB buffer. For QC purposes 1 µl of purified library was visualised and sized by electrophoresis. DNA concentrations of both the purified library and the PCR template were measured by fluorimetry (Qubit dsDNA high sensitivity Assay Kit, Life Technologies, Paisley, UK). Finally, by taking into account the library size range and proportion of template present in the bulk PCR reaction, the purified library was diluted to 10 nM (amplicon equivalent) in EB buffer, 0.1% Tween20, and stored at -20°C until sequenced.

Sequencing was performed on the Illumina MiSeq platform (162 paired end reads using a 300 base, v2 chemistry kit; Illumina, Cambridge, UK). Raw reads were processed using RTA 1.18.54 (Illumina). and data was then compiled and processed using STACKS (Catchen et al. 2013). Following data processing, loci with a minor allele frequency ≥ 0.15 in at least one test population/sampling area and an F_{st} value ≥ 0.03 were further considered.

2.6. ANALYTICAL CALCULATIONS

2.6.1. Condition

Condition – a measure of the weight-length relationship, is widely used to provide an indication of the relative health and well-being of an individual or group. Though several formulae have been proposed to calculate condition in fish (Bolger and Connolly 1989), Fulton's condition factor (K) is the most consistently applied in the literature. Previous studies on Atlantic cod indicate that K closely reflects energetic reserves, which have previously been used as an indication of resilience. However, a key assumption of Fulton's K is that fish exhibit isometric growth, with the length-weight relationship having a regression coefficient of 3, which is not always the case. To validate our use of Fulton's method, the log transformed length-weight regression coefficient of all datasets was checked. In all cases this value fell within the acceptable range (2.5-3.5) and Fulton's K was calculated for each individual as follows:

$$\text{Condition factor (K)} = \frac{\text{Weight (g)}}{\text{Total length (cm)}^3} \times 100$$

2.6.2. Thermal Growth Coefficient

Similar to Fulton's K, the Thermal Growth Coefficient (TGC), which calculates the rate of growth as a measure of weight gained (g) per degree day, assumes a regression

coefficient of 3 for the length-weight relationship. To ensure our data met this assumption, the regression coefficient of length-weight was first calculated for all datasets. Thermal Growth Coefficient (TGC) was then calculated as follows;

$$TGC_{weight} = \frac{(\sqrt[3]{weight_{end}} - \sqrt[3]{weight_{start}})}{\text{Degree days}} \times 1000$$

2.6.3. Relative Liver Weight

Where liver weights (0.0001 g) were available, liver weight expressed as a percentage of the somatic body weight, referred to as the hepatosomatic index (HSI), was calculated as follows;

$$HSI = \frac{\text{Liver weight (g)}}{Weight_{somatic}(g)} \times 100$$

From this, the relative liver weight (RLM) was calculated as the relationship between HSI of the individual fish and the mean HSI of all fish using the following calculation;

$$RLM_{fish} = \frac{HSI_{fish}}{HSI_{mean}}$$

2.6.4. Gonadosomatic index

Where gonad weights (0.0001 g) were available, gonad weight expressed as a percentage of the somatic body weight, referred to as the gonadosomatic index (GSI), was calculated as follows;

$$GSI = \frac{\text{Gonad weight (g)}}{Weight_{somatic}(g)} \times 100$$

2.7. STATISTICAL ANALYSIS

Where statistical analyses were calculated by hand, Microsoft® Excel 2016 was used to aid data manipulation.

2.7.1. Estimation of the mean

Sample mean ($\overline{X}$) was calculated for growth and analytical parameters as;

$$\overline{X} = \frac{\sum X}{n}$$

where $\sum X$ is the sum of the observed sample values and n is the number of observations. In all cases, mean values were presented with the standard error of the mean (± 1 SE) to give a representation of the sample distribution. SE was calculated as;

$$SE = \frac{stdv}{\sqrt{n}}$$

and stdv, the standard deviation is calculated as;

$$stdv = \sqrt{\frac{\sum x^2 - \frac{\sum x^2}{n}}{n\text{-}1}}$$

2.7.2. Testing for Normality and Homogeneity of Variance

When using parametric tests, it is assumed that observations are taken at random and that their variance is independent. Data must also exhibit a normal (Gaussian) distribution and homogeneous variability. To assess whether our data fit these assumptions, all data were examined for normality and homogeneity before further statistical analysis was performed.

Normality was assessed visually for variables with a large number of observations through careful scrutiny of the plots of the residuals. For variables with a small number

of observations a Kolmogrov-Smirnov test was employed to assess the distribution. This test quantifies the discrepancy between the experimental distribution and that of an ideal Gaussian model.

Homogeneity was assessed using an F-test.

2.7.3. Multiple comparisons

All parametric tests which compared three or more samples were performed using the Analysis of Variance technique (ANOVA). Where appropriate, these calculations were manipulated by the use of General Linear Models (GLMs) or Linear Mixed-effects Models (LMMs). Model formulae which take into account numerous factor levels, including replication (with replicate as a factor nested within treatment) and repeated measures (with individual as a random variable), were constructed. These were simplified through backward selection, eliminating non-significant variables. Post hoc analysis of all significant interactions was performed using Tukey tests. All statistical analyses performed in this thesis were undertaken in R, version 3.2.2.

CHAPTER 3. INVESTIGATING NOVEL PATHWAYS LINKING PHOTOPERIODIC CUES TO THE BRAIN PITUITARY GONAD AXIS IN ATLANTIC COD

3.1. INTRODUCTION

It has clearly been shown that the process of maturation and subsequent gonadal development in teleosts is under the neuroendocrine control of the brain-pituitary-gonad (BPG) axis (Zohar et al. 2010). Although the BPG axis has been well described in vertebrates, the external factors and associated mechanisms which initiate secondary gametogenesis and regulate reproduction remain to be clearly defined in fish (Migaud et al. 2010). In temperate regions, many species utilise seasonal changes in the environment to entrain reproduction to an appropriate season for offspring survival. While a number of environmental cues have been shown to influence the timing of reproduction (Wright and Trippel 2009), photoperiod has long been acknowledged as the strongest proximate environmental cue for initiating and entraining seasonal reproduction (Sumpter 1990, Migaud et al. 2010). In fact, the power of photoperiod to entrain reproduction can be evidenced through the numerous studies which show that maturation can be artificially regulated and even inhibited through photoperiod manipulation in a range of species (Imsland et al. 2003, Begtashi et al. 2004, Davie et al. 2007a, Felip et al. 2008, Carrillo et al. 2010, Taranger et al. 2010). However, the performance of photoperiod manipulation in commercial settings can be variable (Taranger et al. 2006) which in part can be due to technical failings in the systems used (Cowan et al. 2011) but is also due, in part, to our lack of understanding of the photoneuroendocrine systems (PNES) which governs how fish perceive their environment to then entrain maturation (Migaud et al. 2010).

Like many temperate species, Atlantic cod is a capital breeder exhibiting group-synchronous spawning in batches over several weeks (Kjesbu and Kryvi 1989) in the winter, spring and early summer (Wright 2013). Classified as short-day breeders, the

reduction in day length following summer solstice initiates secondary gametogenesis which continues through the autumn and winter months (Davie et al. 2007c). Concomitantly it has been observed that there is increased expression of the gonadotropin, follicle stimulating hormone (*Fshβ*), in male (de Almeida et al. 2011) and female (Cowan et al. 2012) cod from September onwards, marking the initiation of the BPG cascade. Importantly if cod are maintained in a continuous illumination from the summer solstice, to mask the reduction in photoperiod, secondary gametogenesis and the associated autumnal gonadotropin surge are completely inhibited (Cowan et al. 2012). This would suggest that between the reduction of photoperiod from the summer solstice in June, and the gonadotropin gene surge seen from September onwards, lies an as yet unidentified signalling pathway linking the proximate photoperiod cue to the initiation of the BPG cascade in cod.

One such pathway has emerged over the last decade linking sexual development and photoperiod in seasonal vertebrates. There is increasing evidence to suggest that the pathway itself appears to be conserved across the vertebrate clade with examples emerging in mammalian, avian and most recently some teleost models (Follett 2015). This pathway begins with transduction of the light signal into the pituitary. In mammals, this light signal is relayed exclusively by melatonin, while avian models exhibit direct innervation of the pathway through deep brain photoreceptors (Dardente et al. 2014, Follett 2015). This light signal up-regulates expression of eyes-absent homolog 3 (*Eya3*) in the *pars tuberalis* (PT) of the pituitary. As a transcriptional coactivator, *Eya3*, modulates thyroid stimulating hormone (*Tshβ)* expression, up-regulating *Tshβ* production in the PT. *Tshβ* is then transported into the medio-basal hypothalamus (MBH), up-regulating type 2 iodothyronine deiodinase (*Dio2*) and supressing type 3 iodothyronine deiodinase (*Dio3*) expression. *Dio2* is responsible for converting thyroxine

(T4) into the biologically active thyroid hormone, triiodothyronine (T3). Contrastingly, *Dio3* acts as a thyroid hormone inactivating enzyme converting T3 and T4 into inactive metabolites. As a result, differential expression of *Tshβ* under seasonally altering photoperiods creates a reciprocal switching mechanism which modulates thyroid hormone production. The *Eya3-Tshβ-Dio2/3* pathway thus provides a clear seasonal signal to the BPG, with the potential to consolidate the PNES and BPG axes (Dardente et al. 2014). Indeed, photoperiod manipulation studies in aves have identified both *Eya3* and *Tshβ* in the first wave response to the SD to LD switch, followed by an up-regulation of *Dio2* and down-regulation of *Dio3,* preceding a surge in gonadotropin secretion, indicating stimulation of the BPG (Yoshimura et al. 2003, Nakao et al. 2008b, Ono et al. 2009a, 2009b). Furthermore, intracerebroventricular (ICV) administration of TSHβ to quail held under continuous SD up-regulates *Dio2* expression, stimulating the reproductive axis, while administration of TSHβ antibodies to quail exposed to continuous LD elicited the opposite response (Nakao et al. 2008a). Studies in quail particularly have highlighted how rapidly this pathway can be induced, with initial induction of *Eya3* and *Tshβ* occurring within 14 hours of the switch to LD, followed by *Dio2* induction after 18 hours, and a subsequent up-regulation of luteinising hormone (*Lh*) within 22 hours of the switch (Nakao et al. 2008a). Similar correlations have also been shown in mammals. Hamsters for instance exhibit gonadal reduction in association with an up-regulation of *Dio3* under SD stimulus, while the up-regulation of *Dio2* under LD stimulus is strongly linked to gonad development (Revel et al. 2006, Barrett et al. 2007). In mice, targeted disruption of the *Tshβ* receptors supresses the *Dio2* response to LD stimulus, inhibiting the reproductive response (Ono et al. 2008).

While the pathway appears to be conserved across the vertebrate clade, there are some differences in the upstream drivers and the resultant down-stream affects. In the case of

all long-day breeding birds and mammals studied to date, including quail (Nakao et al. 2008a, Ono et al. 2009b), mice (Ono et al. 2008, Masumoto et al. 2010), hamsters (Watanabe et al. 2004, Yasuo et al. 2010) and rats (Yasuo et al. 2007, Ross et al. 2011), which initiate their reproductive cycle under an increasing photoperiod, the *Eya3-Tshβ-Dio2/3* pathway is positively stimulated following an increasing photoperiod or LD light signal. However, for short-day breeding sheep and Saanen goats, the pathway is less straightforward. In sheep it would appear that the pathway is initiated by a LD light signal, as is the case for long-day breeders (Dupré et al. 2010, Sáenz de Miera et al. 2013) which is possibly a reflection of the key stimulatory role of LD on sheep reproductive cycle irrespective of their SD breeding behaviour (Dardente 2012). For Saanen goats however, a LD stimulus inhibits *Dio2* production, suggestive of a reversal of the pathways role to reflect the seasonal entrainment of reproduction (Yasuo et al. 2006).

Studies of this pathway in teleosts are lacking and they are limited to species which initiate secondary gametogenesis in response to a LD stimulus. Nakane et al. (2013) first reported in Masu salmon (*Oncorhynchus masou masou* B.) that a stimulatory LD photoperiod upregulated *Tshβ* and *Dio2* expression in the *saccus vasculosus* within the caudal hypothalamus of the brain. Moreover, removal of this organ appeared to inhibit maturation, which would strongly suggest a role in mediating photic perception and the subsequent regulation of the BPG axis (Nakane et al. 2013). In the three-spined stickleback (*Gasterosteus aculeatus* L.), *Tshβ* expression again echoes the mammalian model, being upregulated under LD stimulus, and was strongly linked to the maturation response (O'Brien et al. 2012). These studies both support an overall conservation of the pathway in vertebrates, but are a long way from truly defining it and linking the PNES and BPG axes in fish, highlighting a need for additional research. In order to expand on our current understanding of the mechanisms controlling seasonal physiology in

vertebrates and more specifically driving maturation in fish, the current study compared the expression patterns of *Eya3, Tshβ* and *Dio2* in the pituitary and brain of the short-day breeder, Atlantic cod, under maturation inhibiting and maturation stimulating photoperiodic regimes. The aim of this study was to investigate if a pathway similar to that seen in other vertebrate models also exists in cod, developing our understanding of the PNES-BPG network and potentially highlighting early biomarkers for maturation commitment in Atlantic cod.

3.2. METHODS

3.2.1. Fish Husbandry & Sample Collection

This study was conducted at Macrahanish Marine Environmental Research Laboratory (MERL, Scotland 55.44 º N, 5.44 º W). Immature mixed-sex Atlantic cod were randomly allocated between two fully covered and light-proof tanks. Fish were acclimated for 5 weeks prior to initiation of the trial. A baseline sample of 13 fish (6 male, 7 female) was collected in July, following which the experimental treatments commenced. Two treatments were set up; 1) a simulated natural photoperiod (SNP) to stimulate maturation, and 2) a constant light treatment (LL) to inhibit maturation. Fish were sampled monthly over the course of 13 months from July to the subsequent August, with up to 12 fish being euthanized each time via lethal anaesthesia (MS-222, 80 ppm, Pharmaq, Fordingbridge, UK). Alongside individual biometric data (total weight and length), whole brains and pituitaries were dissected from each fish and individually snap frozen over liquid nitrogen vapour and stored at – 70 ºC. For further details on the husbandry, experimental setup and sampling protocols used see Cowan et al. (2012).

3.2.2. RNA Extraction & cDNA Synthesis

The molecular techniques used follow the methods described in Section 2.1 of this thesis. Briefly, RNA was extracted from the pituitary and whole brain samples for each fish by thawing in 1 ml TRIzol® reagent (Invitrogen, UK) per 100 mg of tissue. Samples were then homogenized over ice, and RNA was extracted from the solution following the manufacturers' protocol, and eluted in 15 µl or 50 µl for of MilliQ water for pituitary or brain samples respectively. Sample quality was checked using a ND 100 Nanodrop spectrophotometer (Labtech Int., East Sussex, and UK). Complimentary DNA (cDNA) was generated from RNA through reverse transcription, using a High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, USA) as follows: 1µg of total RNA in volume of 10µl MilliQ water was added to a master mix containing 2 µl of RT buffer, 0.8 µl dNTPs mix, 2 µl RT Random Primer, 1 µl Reverse Transcriptase and 4.2 µl of MilliQ water, to create a final reaction volume of 20 µl. Thermocycling conditions were 25 ºC for 10 mins, 37 ºC for 120 mins, and 85 ºC for 5 mins. Samples were then diluted with MilliQ water to a final volume of 200 µl (1:10) and stored at -20 ºC until required.

3.2.3. Primer Design & Molecular Cloning

Partial cDNA sequences for each target gene were generated using primers designed with primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast). One primer pair was designed for each gene of interest based on gene sequences identified within the Atlantic cod genome, available from the Ensembl Genome Project (http://www.ensembl.org/Gadus morhua) (Table 3.1). For each gene, an *in silico* analysis was performed for quality assurance of the chosen sequences whereby the cod specific transcripts were compared against previously described vertebrate sequences to compare sequence identity and putative conserved domains using BLAST and ClustalW analysis while MEGA6® (Tamura et al. 2013) was used to deduce and bootstrap phylogenetic

trees using the maximum likelihood method (Felsenstein 1981). The house-keeping gene, Elongation factor 1 alpha (*Ef1α*), was selected as the reference gene for normalisation of the quantitative PCR (QPCR) data. One primer pair for this gene was also designed, following the same process described above.

Each primer pair was verified by Polymerase Chain Reaction (PCR). These PCR reactions were performed using; 1µl reaction buffer, 0.8 µl of forward and reverse primers (10 pmol μl^{-1}), 0.45 µl $MgCl_2$, 0.25 µl dNTPs, 0.04 µl *Klear Taq* DNA polymerase (KBioSciences, UK), 5.66 µl MilliQ and 1 µl of synthesised cDNA. The thermal cycling program consisted of a 15 min initial denaturation at 95 ºC, followed by 35 cycles of 95 ºC for 30 s, X ºC for 30 s, and 72 ºC for 60 s, with a final extension at 72 ºC for 4 mins where the annealing temperature, denoted X ºC, varied with each specific primer pair (Table 3.1). PCR products were then checked on a 1 % agarose gel to verify size and the presence of a single product, before being extracted and purified from the gel using a NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL, Germany). The purified PCR products were then cloned into a PGEM®-T easy vector (PGEM®-T easy vector systems, Promega, UK). Plasmid insertion was checked by PCR amplification using the appropriate primers and by restriction digest, as well as sequencing (GATC Biotech, London, UK). Upon confirmation of correction product insert a serial dilution of linearised plasmid sample was used for the subsequent QPCR assay.

3.2.4. Quantitative PCR

cDNA for each sample was synthesised from RNA using the methods described above. The genes measured included *Eya3, Tshβ* and *Dio2*. As *Eya3* is found exclusively in the PT of the pituitary in mammals and birds, this gene was quantified only in pituitary samples. *Tshβ* is known to cross from the PT into the MBH in mammals and birds and so expression in both brain and pituitary tissue was investigated for *Tshβ* in cod. *Dio2* is not

known to occur in the pituitary in other vertebrate models and so expression was investigated in the brain tissue alone for this gene. *Ef1α* was used in all cases as a housekeeping reference gene and expression was measured in both brain and pituitary samples.

Where practicably possible, all sample extraction and QPCR assays were conducted according to MIQE (minimum information for publication of real-time PCR experiments) guidelines (Bustin et al. 2009). In order to validate QPCR assays, a serial dilution of linearised plasmid containing the ligated target gene fragment for each gene was tested, from which three dilutions were taken forward as standards to enable absolute quantification of mRNA levels. Each QPCR plate was prepared, including in duplicate, three chosen standards for that particular gene, one non-template control (MilliQ water), and four internal control samples consisting of four random cDNA samples from the target tissue which were included in every plate to control for inter-assay variation.

The QPCR assay was performed using 0.7 µl of both forward and reverse primer (10 pmol ul^{-1}), with one fortieth of the original cDNA reaction, 10 µl of ABsolute™ QPCR SYBR-green QPCR master mix (Thermo Scientific, St. Leon-Rot, Germany), and 3.6 µl of MilliQ water to a final reaction volume of 20 µl. The reactions were run on an Eppendorf Mastercycler® ep *realplex* thermocycler using the following programme; 95 ºC for 15 mins, 45 cycles of 95 ºC for 15 s, X ºC (annealing temperature of primers, see Table 3.1.) for 15 s, 72 ºC for 30 s, followed by a temperature ramp from 65 ºC to 95 ºC with fluorescence being measured every 0.5 ºC to create a melt curve. The copy numbers of each gene were automatically calculated by the *realplex* software by comparison to the standard curve created by the serial plasmid dilutions which following correction was then expressed as the absolute copy number per µg of total RNA. QPCR efficiency was calculated for each plate run, with efficiency being greater that 90 % in all cases. A melt

curve was also created to verify the specificity of the primers used, and as an indication of assay contamination.

3.2.5. Data Analysis

Statistical analysis was undertaken using R version 3.2.2. Normality and homogeneity of variance were tested using Kolmogorov-Smirnov and Bartlett's tests, and all data were transformed as appropriate prior to analysis. Temporal and treatment variations in target gene expression were analysed using a general linear model;

$$Log_n(X) \sim Month*Treatment$$

where X denotes the expressed copy number of the target gene. In all cases, males and females were analysed separately to account for differences in the level and pattern of expression observed between the genders for each of gene being quantified. A significance level of $p < 0.05$ was used and all significant interactions were explored *post hoc* using Tukey's test to identify when significant differences occurred within our dataset.

Table 3.1. Primer name, sequence, predicted amplicon size, annealing temperature and Ensembl transcript ID for each gene of interest.

Name	Sequence	Product size	Annealing temperature (X°C)	Ensembl Transcript ID
*Eya3*F	5'-TCCCTGCTGCTGATCCAGTCC-3'			
		109bp	61.5	ENSGMOT00000014154
*Eya3*R	5'-AGACCTCTCCCAGGCCGTAGA-3'			
*Tshβ*F	5'-AGCGAGGGCAGCTCTCTGTTC-3'			
		116bp	61.5	ENSGMOT00000018765
*Tshβ*R	5'-GTACACGTGGGCCTGTTGCTG-3'			
*Dio2*F	5'-GTTCCTCGCGCTGTACGACTC-3'			
		130bp	61.5	ENSGMOT00000022279
*Dio2*R	5'-CCAGATGGAGCGCATCCCC-3'			
*Ef1α*F	5'-TGAACCACCCTGGCACCATCT-3'			
		84bp	60	ENSGMOT00000013187
*Ef1α*R	5'-GCTCGTTGAACTTGCAGGCGA-3'			

3.3. RESULTS

3.3.1. Atlantic cod *Eya3*, *Tshβ* and *Dio2* partial cDNA sequence and phylogenetic analysis

BLAST analysis of the cod genome (assembly gadMor1, released August 2011) identified transcript sequences for each of the chosen targets. For *Eya3*, we identified a single sequence with high homology to other teleost *Eya3* sequences. This consisted of a partial cds (ENSGMOT00000014154) of 1446bp which translated into a 482 amino acid (aa) deduced partial protein fragment. Importantly, this sequence included the conserved C-terminal "EYA" domain, which is highly conserved across the *Eya* genes and is thought to be critical for *Eya* gene activity and protein-protein interactions. *Eya3* operates as both a transcriptional coactivator and an enzyme of the haloacid dehalogenase (HAD) hydrolase family. The EYA domain contains the signature HAD-motifs which define this family of enzymes (Figure 3.1). A tblastx search of the genome sourced cds found that the Atlantic cod *Eya3* sequence shared high identity with other teleost *Eya3* sequences. The EYA domain in particular shared >90% sequence homology with other fish including fugu (*Takifugu rubripes* T.&S.), Japanese medaka (*Oryzias latipes* T.&S.) Nile tilapia (*Oreochromis niloticus* L.), and the three-spined stickleback, with a reduced identity (<70% ID) with other vertebrates, as was reflected in the phylogenetic analysis (Figure 3.2).

We identified two *Tshβ* paralogs in the cod genome, confirming previous results suggestive of a teleost-specific genome duplication event (Postlethwait et al. 2004); *Tshβ1* (ENSGMOT00000018765) located on GeneScaffold 2156, and *Tshβ*2 (ENSGMOT00000012090) on GeneScaffold 2185. As *Tshβ1* has previously been described as the most highly conserved paralog among teleosts (Kitano et al. 2010, O'Brien et al. 2012) and our own exploratory analysis confirmed this (Figure 3.3 &

Figure 3.4), only the photoperiodic response of *Tshβ1* was analysed. The *Tshβ1* transcript sequence consisted of a partial cds of 387 bp, which translated into a deduced aa sequence of 129 aa. This partial fragment was located within the TSH specific β subunit and included all 12 Cys residues of the conserved cysteine-knot domain common to all gonadotropin β subunits, as well as a partial signal peptide (Figure 3.3). The sequence showed comparable identity (>70%) with *Tsh*β sequences from fugu, medaka, seabass (*Dicentrarchus labrax* L.), and red drum (*Sciaenops ocellatus* L.) with a reduced identity with non-teleost vertebrate *Tsh*β sequences (<55%) and other teleost derived gonadotropins (<47%) (Figure 3.3). Phylogenetic analysis of the deduced aa sequence reflected these findings (Figure 3.4).

A partial cds sequence, 228bp long, for *Dio2* (ENSGMOT00000022279) was also identified from the cod genome which translated into a deduced aa sequence of 76 aa. This fragment covered the entire signal peptide for DIO2 as well as a large section of the conserved DIO specific domain, iodothyronine deiodinase (Figure 3.5). The deduced aa sequence showed greater identity with other teleost DIO2 sequences (>70%), including zebrafish (*Danio rerio* H.), fugu, three-spined stickleback, Mexican cave fish (*Astyanax mexicanus* de F.), and Atlantic salmon (*Salmo salar* L.) compared with other non-teleost vertebrate species (<70%) and zebrafish derived DIO1 (<20%; Figure 3.5). This was reflected in the phylogenetic analysis (Figure 3.6).

A

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1     CCC GCA GTG ACC TCC TAC GGC AAC CAG GCG GCG TTC TCT CCC CTG   45
1      P   A   V   T   S   Y   G   N   Q   A   A   F   S   P   L    15

46    GCC CAG TCT AGC GTG TAC TCC TTC CCC CAG GCA GGC CAG ACC TAC   90
16     A   Q   S   S   V   Y   S   F   P   Q   A   G   Q   T   Y    30

91    GGG CTC CCA CCG TTT GGT GCT ATG TGG CCA GGC ATA AAA ACA GAG   135
31     G   L   P   P   F   G   A   M   W   P   G   I   K   T   E    45

136   ACA GGG CTG CCT GAG GCG CCC TCT GTT GGT CAG CCG GGG TTT CTC   180
46     T   G   L   P   E   A   P   S   V   G   Q   P   G   F   L    60

181   AGC TTC AGC AGC GCA TAC ACG TCC ACG CAG TCC GGC CAG CTT CAC   225
61     S   F   S   S   A   Y   T   S   T   Q   S   G   Q   L   H    75

226   TAT TCC TAC CCC AGC CAA GGC TCA AGC TTC ACC ACG GCC AGC GTT   270
76     Y   S   Y   P   S   Q   G   S   S   F   T   T   A   S   V    90

271   TAC TCC AAC ATC CCC GCT ACC ACG GCC AGC ACT GTC CCC GCC CCA   315
91     Y   S   N   I   P   A   T   T   A   S   T   V   P   A   P   105

316   GCG CCC AAT CAG GAG TTC AGC AGC TAC TCC TCG CTG GGA CCC CAG   360
106    A   P   N   Q   E   F   S   S   Y   S   S   L   G   P   Q   120

361   GCC CAG TTC TCC CAG TAC TAC GCG CCC CTG CCA GGC TAC GCG CCC   405
121    A   Q   F   S   Q   Y   Y   A   P   L   P   G   Y   A   P   135

406   CCC GGC CTG CCG AGC AGC GAC AGT CAC GGT ACG GAC GCT GCG GGC   450
136    P   G   L   P   S   S   D   S   H   G   T   D   A   A   G   150

451   GTG GCC GGC TAC CCG GCC GTC AAG TCG GAG AGC GCC GTG TCC GCC   495
151    V   A   G   Y   P   A   V   K   S   E   S   A   V   S   A   165

496   GGG CTG ATC GCC CTG CCG GCC GGG CTG GCC CTC CCC ACG GGG GCC   540
166    G   L   I   A   L   P   A   G   L   A   L   P   T   G   A   180

541   CGG GAG CTG GAG GAC GCG GGC CGC AGG AAC TCT GTG GGG AAG GCC   585
181    R   E   L   E   D   A   G   R   R   N   S   V   G   K   A   195

586   AAA GGC AAG GCC AAG AAG CCC GAC GGA TGC CCC TCC ACG GAC TCG   630
196    K   G   K   A   K   K   P   D   G   C   P   S   T   D   S   210

631   GAC CTG GAG CGG GTC TTT CTG TGG GAC CTT GAC GAG ACC ATC ATC   675
211    D   L   E   R   V   F   L   W   D   L   D   E   T   I   I   225

676   ATA TTC CAC TCG CTT CTC ACC GGC TCC TTC GCA CAG AAG TTC GGC   720
226    I   F   H   S   L   L   T   G   S   F   A   Q   K   F   G   240

721   AAG GAC CCA GCC ACT GTG CTG AAC CTG GGC CTT CAG ATG GAG GAG   765
241    K   D   P   A   T   V   L   N   L   G   L   Q   M   E   E   255

766   CTG ATC TTT GAA CTG GCC GAC ACA CAC CTG TTC TTC AAC GAC CTG   810
256    L   I   F   E   L   A   D   T   H   L   F   F   N   D   L   270
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271    E   E   C   D   Q   V   H   V   E   D   V   A   S   D   D   285

856   AAC GGA CAG GAC CTC AGT AAC TAT AAC TTC CTG GCC GAC GGC TTC   900
286    N   G   Q   D   L   S   N   Y   N   F   L   A   D   G   F   300

901   AGC GGC TCC AGC GGG GGG GGG GCT CCG GGC GCC GGC GTC CCC GGT   945
301    S   G   S   S   G   G   G   A   P   G   A   G   V   P   G   315

946   GGC GTG GAG TGG ATG CGC AAG CTA GCC TTC CGC TAC CGC CGG CTA   990
316    G   V   E   W   M   R   K   L   A   F   R   Y   R   R   L   330

991   AAG GAG ATC TAC AAC GCC TAC AAG ACC AAC GTC GGA GGC CTG CTG  1035
331    K   E   I   Y   N   A   Y   K   T   N   V   G   G   L   L   345

1036  AGT CCT ATG AAG AGG GAT CTT CTG CTG AGG CTT CAG TCC GAG ATC  1080
346    S   P   M   K   R   D   L   L   L   R   L   Q   S   E   I   360

1081  GAG AAC GTG ACG GAC GCC TGG CTC AGC ACG GCC CTC AAG TCC CTG  1125
361    E   N   V   T   D   A   W   L   S   T   A   L   K   S   L   375

1126  CTG CTG ATC CAG TCC AGG GGG AAG TGT CTG AAC ATC CTG GTC ACC  1170
376    L   L   I   Q   S   R   G   K   C   L   N   I   L   V   T   390

1171  ACC ACC CAG CTG GTG CCA GCC CTG GCC AAG GTG TTG CTC TAC GGC  1215
391    T   T   Q   L   V   P   A   L   A   K   V   L   L   Y   G   405

1216  CTG GGA GAG GTC TTC TCC ATC GAC ACC ATC TAC AGC GCC ACC AAA  1260
406    L   G   E   V   F   S   I   D   T   I   Y   S   A   T   K   420

1261  ATA GGG AAG GAG AGC TGC TTC GAG AGG ATC GTC TCG CGC TTC GGC  1305
421    I   G   K   E   S   C   F   E   R   I   V   S   R   F   G   435

1306  AAG AAG GTG ACC TAC GTG GTG ATC GGG GAC GGC CGC GAC GAG GAG  1350
436    K   K   V   T   Y   V   V   I   G   D   G   R   D   E   E   450

1351  TTT GCC GCC AAA CAG CAC AAC ATG CCT TTC TGG AGG ATC TCC ACC  1395
451    F   A   A   K   Q   H   N   M   P   F   W   R   I   S   T   465

1396  CAC GGG GAC CTG GTG TCC CTG CAC CAG GCG CTG GAG CTG GAC TTC  1440
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1441  CTG TAG   1446
481    L   *    482
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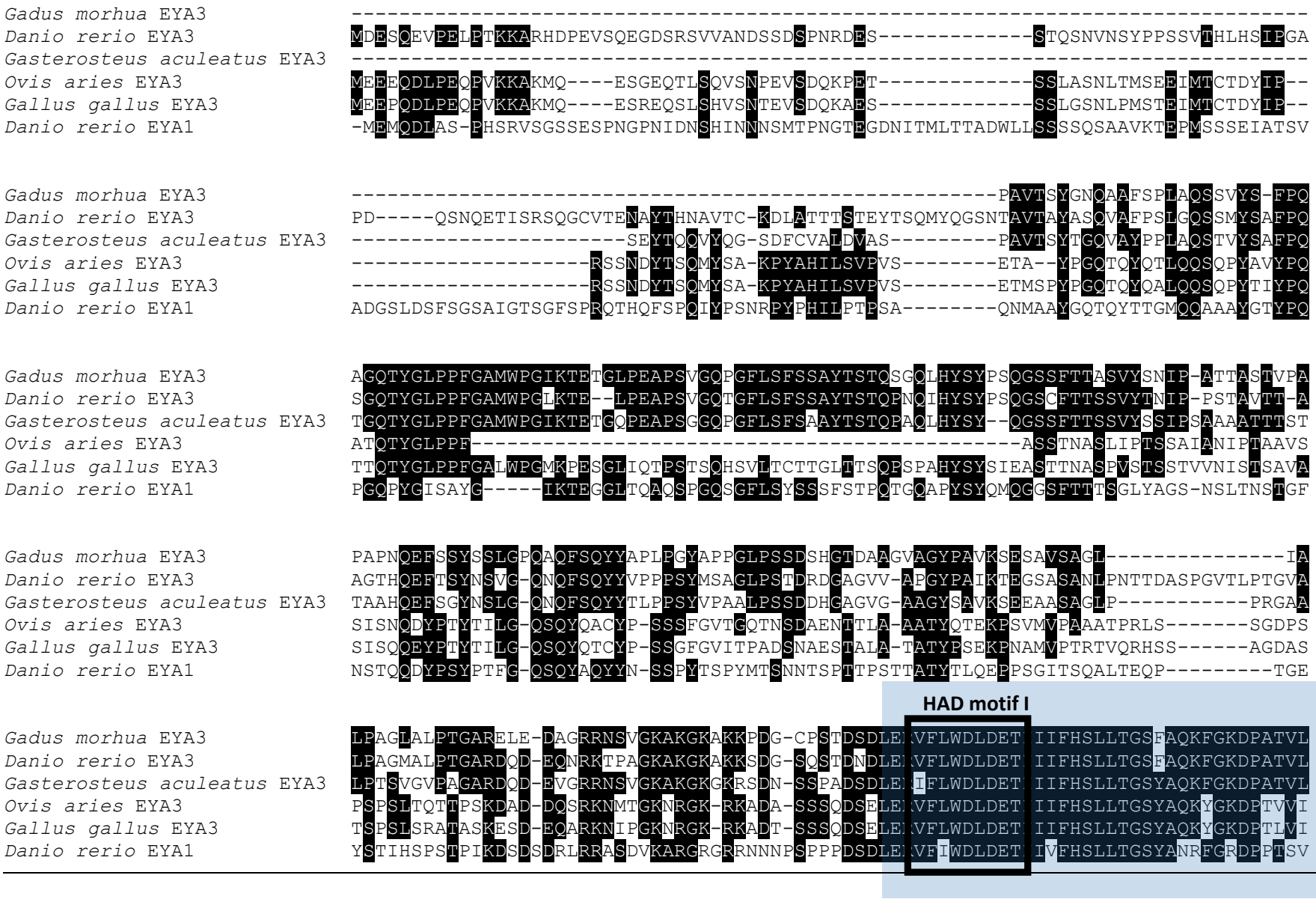
B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Danio rerio EYA3
Gasterosteus aculeatus EYA3
Ovis aries EYA3
Gallus gallus EYA3
Danio rerio EYA1
HAD motif I

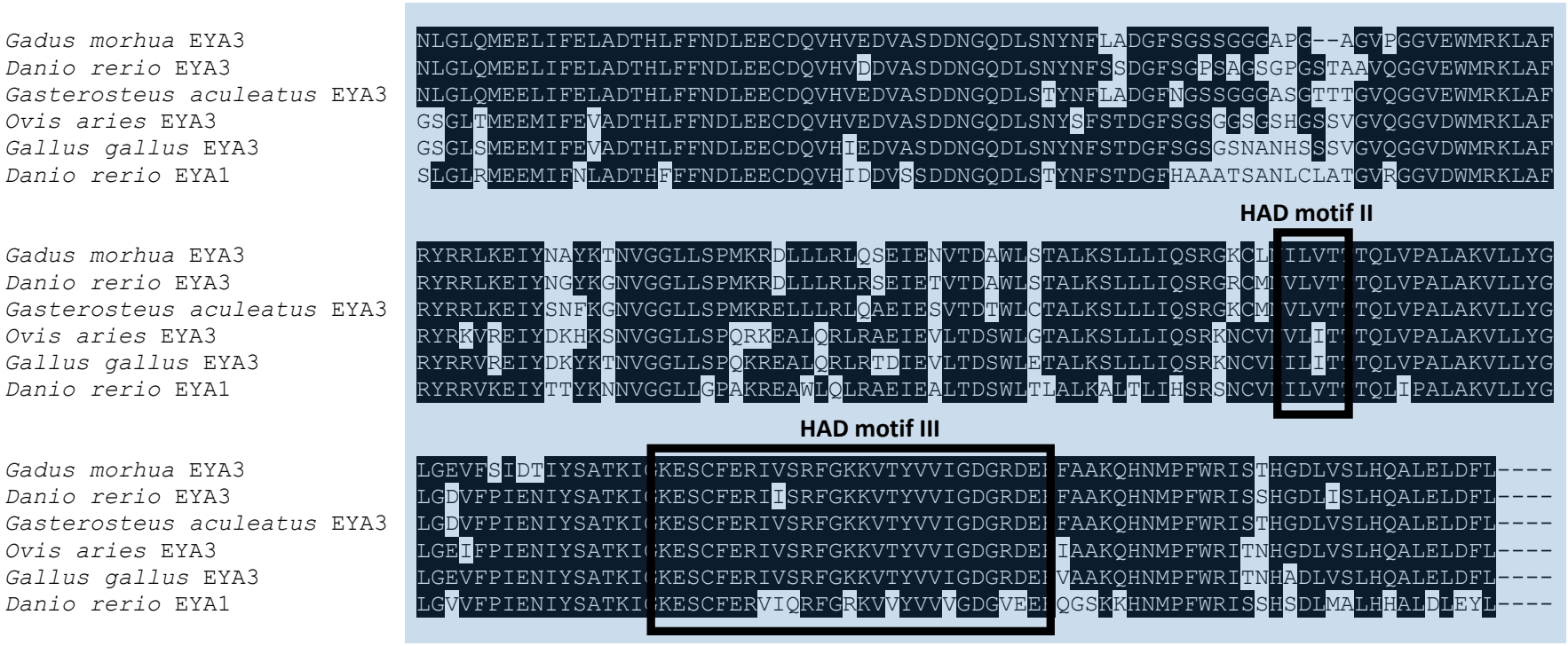

Figure 3.1. A) Nucleotide and deduced amino acid sequence of Atlantic cod *Gadus morhua Eya3* transcript (Accession number: ENSGMOT00000014154). Predicted EYA domain is underlined in black with the proposed HAD-motifs indicated by a broken blue line above the aa sequence. B) Alignment of the deduced aa sequence for vertebrate EYA3 compared with zebrafish *Danio rerio* EYA2. Conserved amino acid residues are shaded. The EYA domain is highlighted in blue and the proposed HAD-motifs are outlined in black.

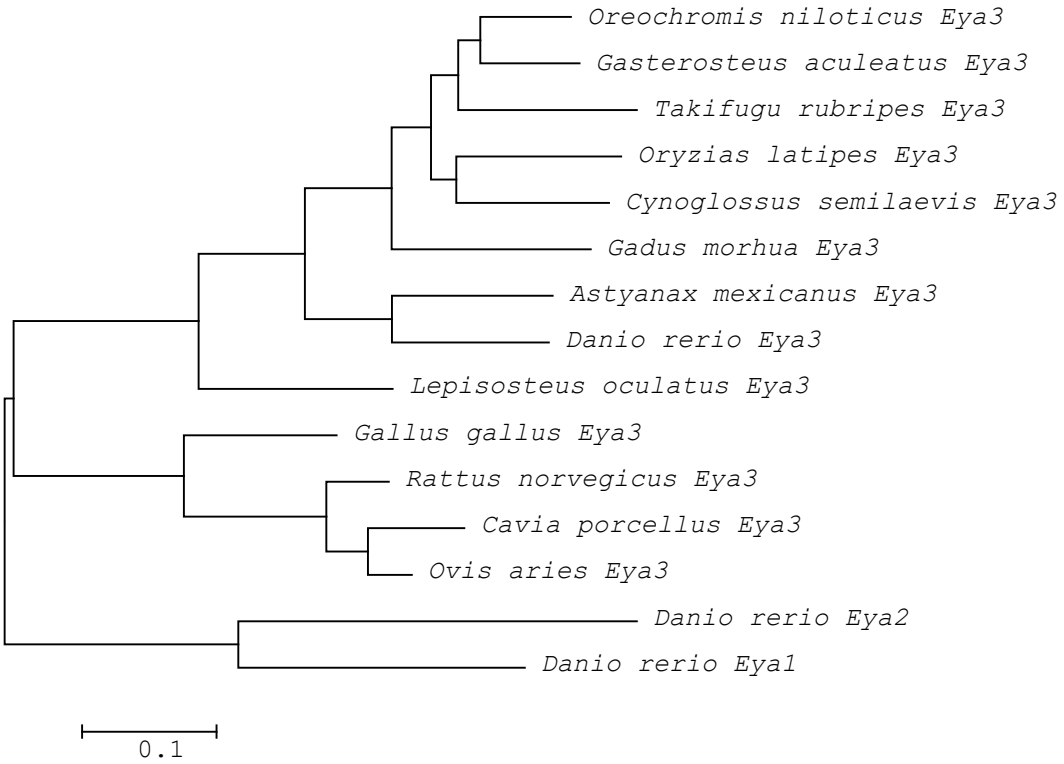

Figure 3.2 Phylogenetic tree analysis of the vertebrate *Eya3* gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-12589.9852) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 15 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1204 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Accession numbers: Nile tilapia *Oreochromis niloticus* XM_003447243.3, three-spined stickleback *Gasterosteus aculeatus* ENSGACT00000003106, fugu *Takifugu rubripes* ENSTRUT00000029672, Japanese medaka *Oryzias latipes* ENSORLT00000011779, tongue sole *Cyanoglossus semilaevis* XM_008331068, Atlantic cod *Gadus morhua* ENSGMOT00000014154, cave fish *Astyanax mexicanus* ENSAMXT00000017928, zebrafish *Danio rerio* (*Eya3*; ENSDART00000049840, *Eya2*; ENSDART00000011652, *Eya1*; ENSDART00000136443), spotted gar *Lepisosteus oculatus* ENSLOCT00000005087, red jungle fowl *Gallus gallus* ENSGALT00000001127, rat *Rattus norvegicus* ENSRNOT00000056170, Guinea pig *Cavia porcellus* ENSCPOT00000012631, sheep *Ovis aries* NM_001161733

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1      TTC AGC CCA GCT GCT CCG ATG TGC GTC CCA ACG GAC TAC ACG CTC   45
1       F   S   P   A   A   P   M   C   V   P   T   D   Y   T   L   15
46     TAC GTG GAG AAG CCC GAG TGT AAC TTT TGT GTG GCT ATC AAC ACC   90
16      Y   V   E   K   P   E   C   N   F   C   V   A   I   N   T   30
91     ACC ATC TGC ATG GGA TTC TGT TAC TCT CGG GAC AGC AAC ATC GGA   135
31      T   I   C   M   G   F   C   Y   S   R   D   S   N   I   G   45
136    GAC CTG GTG GGC CTG CGC TTT CTG CTC CAG AGA GGC TGC ACC TAC   180
46      D   L   V   G   L   R   F   L   L   Q   R   G   C   T   Y   60
181    AAC CAG GTG GAG TAC CGC ACG GCC ATC CTG CCC GGC TGC CCC AGC   225
61      N   Q   V   E   Y   R   T   A   I   L   P   G   C   P   S   75
226    GAG GGC AGC TCT CTG TTC AGC TAC CCC GTG GCC CTC AGC TGC CAC   270
76      E   G   S   S   L   F   S   Y   P   V   A   L   S   C   H   90
271    TGC GGA GCC TGC AAC ACC GCA GTC GAT GAG TGC GCC CAC AGA GCC   315
91      C   G   A   C   N   T   A   V   D   E   C   A   H   R   A   105
316    AGC AGC AAC AGG CCC ACG TGT ACC AAG CCC GTC AGA CAC ATC TAC   360
106     S   S   N   R   P   T   C   T   K   P   V   R   H   I   Y   120
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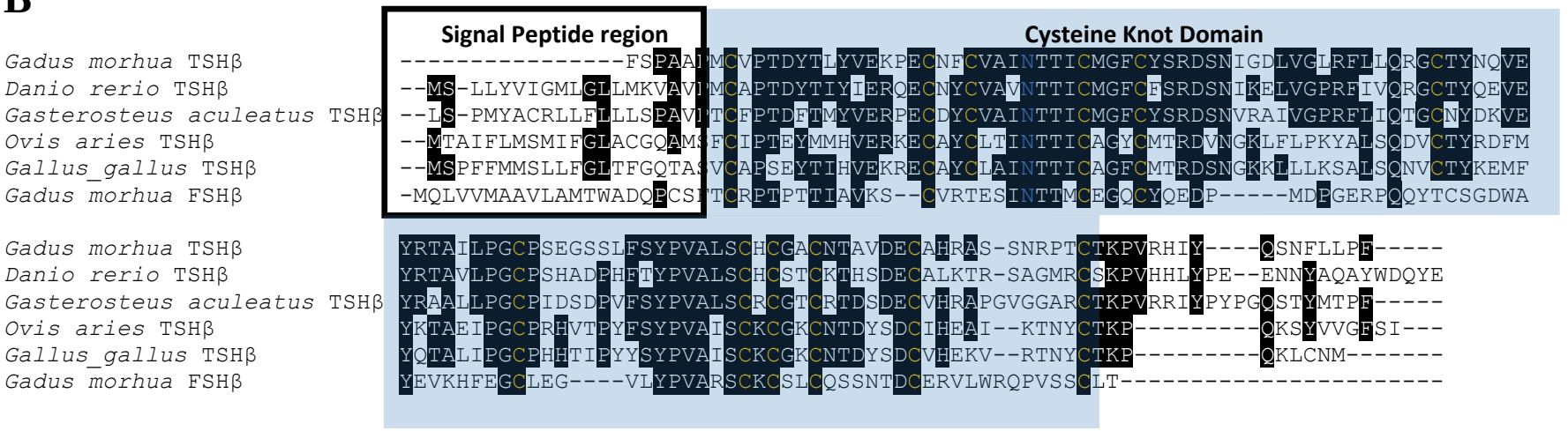
B



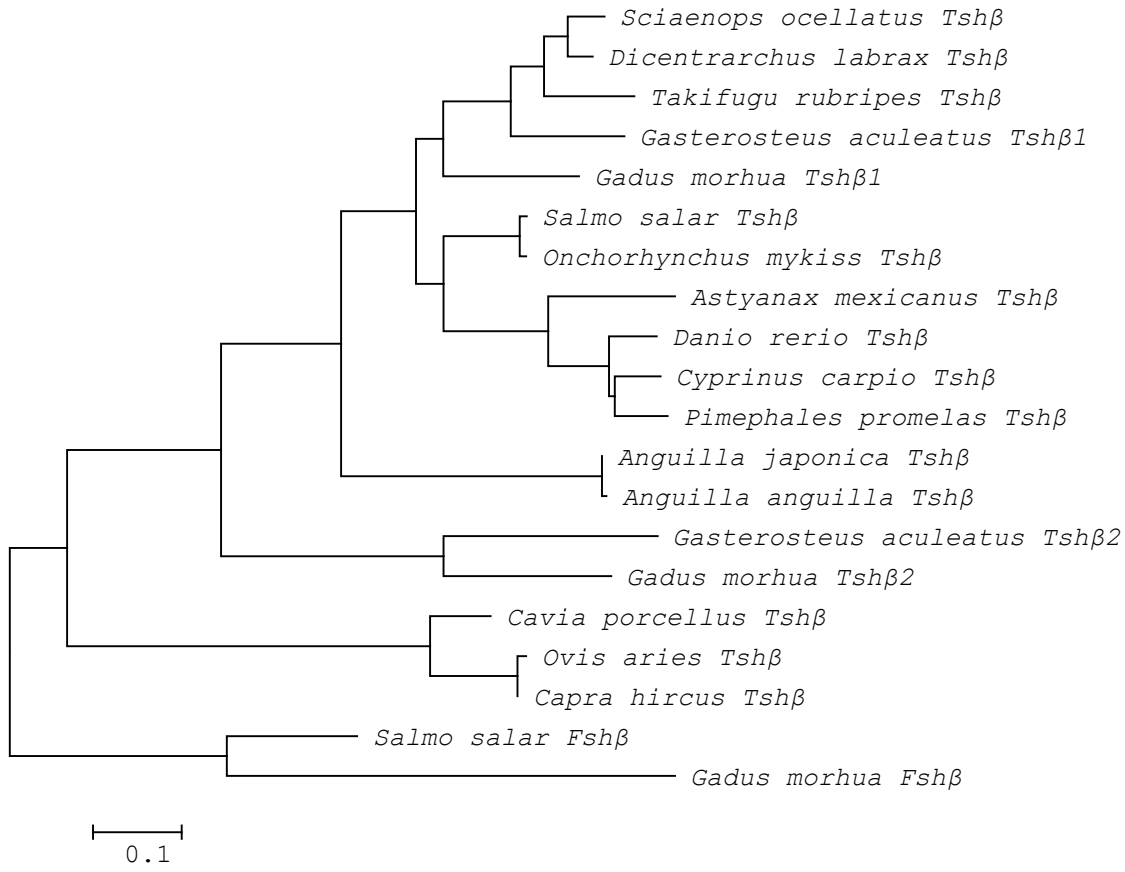


Figure 3.3. **PREVIOUS PAGE.** A) Nucleotide and deduced amino acid sequence of Atlantic cod *Gadus morhua Tsh*β1 transcript (ENSGMOT00000018765). Predicted cysteine-knot domain is underlined in bold. Presumptive hairpin loops are indicated by a broken black line above the aa sequence, the long-loop is indicated by a double line above the aa sequence, and the seatbelt sequence is highlighted by a broken blue line above the aa sequence. B) Alignment of the deduced aa sequence for vertebrate TSHβ compared with Atlantic cod and three-spined stickleback paralogs (TSH*β*2) and the gonadotropin FSHβ protein in Atlantic salmon *Salmo salar* and cod. Shaded regions indicate conservation of the amino acid residues. The predicted signal peptide is outlined in black, the cysteine-knot region is highlighted in blue, conserved cysteine residues are coloured yellow and the conserved glycosylated asparagine residues are coloured blue.

Figure 3.4. **CURRENT PAGE.** Phylogenetic tree analysis of the vertebrate *Tsh*β gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-2589.7105) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 196 positions in the final dataset. Evolutionary analyses were

conducted in MEGA6 (Tamura et al. 2013). Accession numbers: red drum *Sciaenops ocellatus* GU144513, seabass *Dicentrarchus labrax* KJ095101, fugu *Takifugu rubripes* ENSTRUT00000046825, three-spined stickleback *Gasterosteus aculeatus* (*Tshβ1*; ENSGACG00000005276, *Tshβ*2; ENSGACG00000009897), Atlantic cod *Gadus morhua* (*Tshβ1;* ENSGMOT00000018765, *Tshβ*2; ENSGMOT00000012090, *Fshβ;* ENSGMOT00000005957), Atlantic salmon *Salmo salar* (*Tshβ;* NM_001123528, *Fshβ;* AF146152), rainbow trout *Onchorynchus mykiss* NM_001124543, cave fish *Astyanax mexicanus* ENSAMXT00000011999, zebrafish *Danio rerio* ENSDART00000131080, common carp *Cyprinus carpio* AB003585, fathead minnow *Pimephalus promelas* EF590263, European eel *Anguilla anguilla* X73493, Japanese eel *Anguilla japonica* AB175833, quail *Cortunix cortunix* AF541922, red jungle fowl *Gallus gallus* ENSGALT00000004024, Guinea pig *Cavia porcellus* ENSCPOT00000008449, sheep *Ovis aries* ENSOART00000021912, and goat *Capra hircus* XM_005677829.

A

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46     CCC GGG TTC TTC TCC AAC TGC CTG TTC CTC GCG CTG TAC GAC TCG   90
16      P   G   F   F   S   N   C   L   F   L   A   L   Y   D   S   30
91     GTG CTG CTG CTG AAG CGC GCT GTG TCG CTG CTC CGT GCC TCC CGC   135
31      V   L   L   L   K   R   A   V   S   L   L   R   A   S   R   45
136    TCA GCG CGG GGA GGC GGA GAG TGG CAG CGC GCG CTC ACC TCG GAG   180
46      S   A   R   G   G   G   E   W   Q   R   A   L   T   S   E   60
181    GGG ATG CGC TCC ATC TGG AAG GGC TTC CTG CTG GAC GCC AAC AAG   225
61      G   M   R   S   I   W   K   G   F   L   L   D   A   N   K   75
226    CAG
228
76      Q
```

Figure 3.5. A) Nucleotide and deduced amino acid sequence of Atlantic cod *Dio2* transcript (ENSGMOT00000022279). Predicted iodothyronine deiodinase domain is indicated by a broken blue line above the aa sequence. The predicted signal peptide is underlined in bold, with transmembrane domain indicated by a double underline. B) **CONTINUED ON NEXT PAGE**. Alignment of the deduced aa sequence for vertebrate DIO2 compared with DIO1 in zebrafish. Shaded regions indicate conservation of the amino acid residues. The predicted iodothyronine deiodinase domain is highlighted in blue, with the thioredoxin-like fold indicated by a black outline, and the conserved active site residues coloured yellow.

B

```
Gadus morhua DIO2                MG-TGPEDLLVALQILPGFFSNCLFLALYDSVLLLKRAVS-LLRASRSARGGGEWQRALTSEGMR-SIWKGFLLD-ANKQ
Danio rerio DIO2                 MG-LLSVDLLVTLQILPGFFSNCLFFVLYDSIVLVKRVVS-LLSCSG-STG--EWQRMLTTAGVR-SIWNSFLLD-AYKQ
Gasterosteus aculeatus DIO2      MG-MASGGLRVTLQILPGFFSNCLFLALYDSVVLLKAVVS-LLSCSR-AAGRGARRRMLTSAGLR-SVWRSFLLD-AYKQ
Salmo salar DIO2a                MG-AGSVDLLVTLQILPGFFSNCLFLALYDSVVLVKRLVS-LLSCSG-SGG-GEWQRMLTSAGLR-SIWNSFLLD-AYKQ
Salmo salar DIO2b 2b             MG-AASVDLLVTLQILPGFFSNCLFLALYDSVVLVKRVVS-LLSCPG-GGG-GEWQRMLTSAGLR-SIWNSFLLD-AYKQ
Gallus gallus DIO2               MG-LLSVDLLITLQILPVFFSNCLFLALYDSVILLKHMVL-FLSRSKSARG-QTWRR--SSE-LQ-CNPHS-------QG
Ovis aries DIO2                  MG-ILSVDLLITLQILPVFFSNCLFLALYDSVILLKHVVL-LLSRSKSTRG-Q-WRRMLTSEGMR-CIWKSFLLD-AYKQ
Danio rerio DIO1                 MGSAVGFALRKLFVYISAVLMVCAAILRMSMLKLLSFISPGRMRKIHMKMG--ERTTMTQNPKFRYEDWGPAFFSLAFIK

Gadus morhua DIO2                ---------------------------------------------------------------------------------
Danio rerio DIO2                 VKLGEAAPNSKVVKVTGINRCWSISGKTHN----------QCHLLDFESPDRPLVVNFGSAT*PPFISQLPVFRRMVEEF
Gasterosteus aculeatus DIO2      VK-------------------------------------------------------------------------------
Salmo salar DIO2a                VKLGFEAPNSKVVKVPGS-----FRRRSSLTTTMGPHPGDECRLLDFESSDRPLVVNFGSAT*PPFISHLPAFRRLVEEF
Salmo salar DIO2b 2b             VKLGSEAPNSKVVKVPGSGGNHGFRRRSSVTTTAR-HPGDECRLLDFESSERPLVVNFGSAT*PPFISHLPAFRRLVEEF
Gallus gallus DIO2               Q*-------------------------------------------------------------------------------
Ovis aries DIO2                  QVK------------------------------------------------------------------------------
Danio rerio DIO1                 TLFF-----------------------------------------------------------------------------

Gadus morhua DIO2                ---------------------------------------------------------------------------------
Danio rerio DIO2                 SDVADFLLVYIDEAHPSDGWVGPPMENFSFEVRKHRNLEERMFAARTLLEHFSLPPQCQLVADCMDNNANIAYGVSYERV
Gasterosteus aculeatus DIO2      ---------------------------------------------------------------------------------
Salmo salar DIO2a                SDVADFLLVYIDEAHPSDGWVAPAMGPRSFEVRKHRSLEERVVAAKKLIESFSLPSQCQLVADCMDNNANVAYGVSNERV
Salmo salar DIO2b 2b             SNVADFLLVYIDEAHPSDGWVAPTMGPCSFEVRKHRSLEERVVAAMKLTEAFSLPPQCQLVADCMDNNANVAYGVSYERV
Gallus gallus DIO2               ---------------------------------------------------------------------------------
Ovis aries DIO2                  ---------------------------------------------------------------------------------
Danio rerio DIO1                 ---------------------------------------------------------------------------------

Gadus morhua DIO2                -----------------------------------
Danio rerio DIO2                 CIVQKNKIAYLGGKGPFFYNLKDVRRWLEKCYGK**
Gasterosteus aculeatus DIO2      -----------------------------------
Salmo salar DIO2a                CIVQRRKIAYLGGKGPFFYNLKDVRQYLEQSYGKR*
Salmo salar DIO2b 2b             CIVQRRKIAYLGGKGPFFYNLKDVRQYLEQRYGKR-
Gallus gallus DIO2               -----------------------------------
Ovis aries DIO2                  -----------------------------------
Danio rerio DIO1                 -----------------------------------
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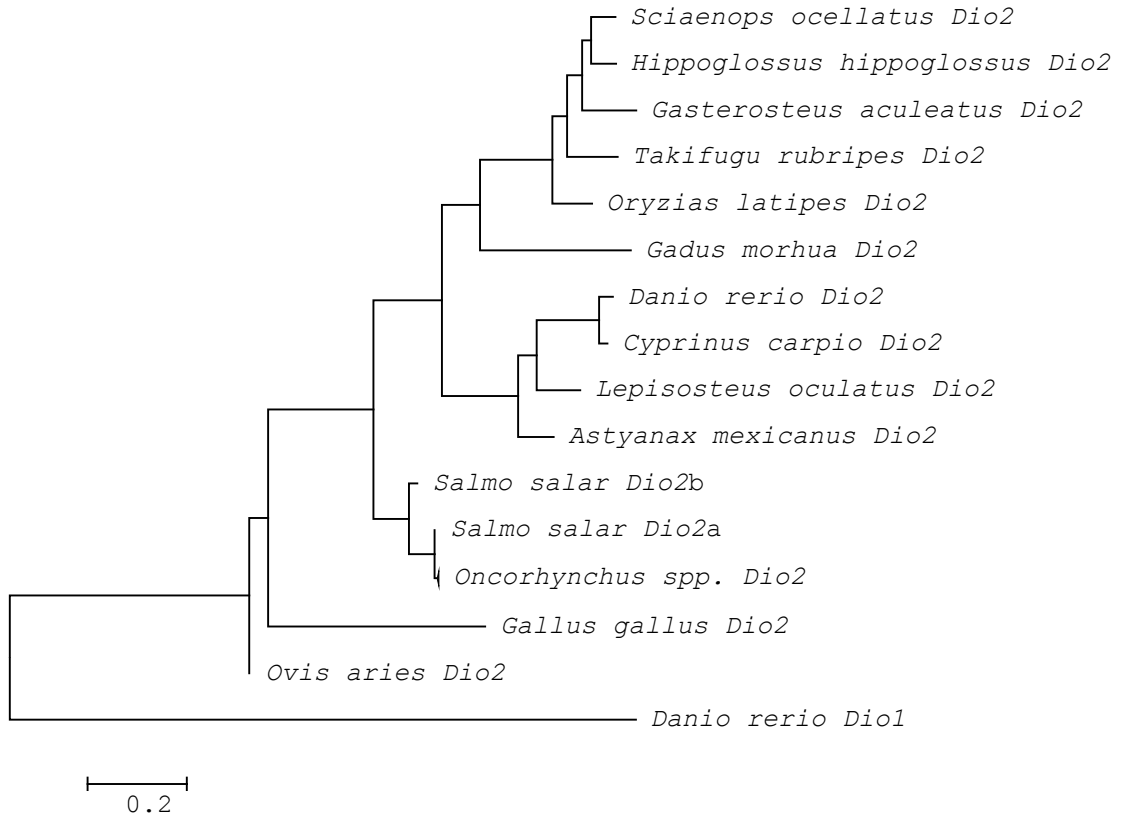

Figure 3.6. Phylogenetic tree analysis of the vertebrate *Dio2* gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-2262.8587) is shown. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 204 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al. 2013). Accession numbers: red drum *Sciaenops ocellatus* KC999978, halibut *Hippoglossus hippoglossus* DQ856304, three-spined stickleback *Gasterosteus aculeatus* ENSGACT00000026661, fugu *Takifugu rubripes* ENSTRUT00000012441, Japanese medaka *Oryzias latipes* NM_001136521, Atlantic cod *Gadus morhua* ENSGMOT00000022279, zebrafish *Danio rerio* (*Dio1;* ENSDART00000132596, *Dio2;* ENSDART00000146276), common carp *Cyprinus carpio* HE580224, spotted gar *Lepisosteus oculatus* ENSLOCT00000010823, cave fish *Astyanax mexicanus* ENSAMXT00000019169, Atlantic salmon *Salmo salar* (*Dio2a;* KP851704, *Dio2b;* KP851705), *Onchorynchus spp.* (*keta*; AB772421, *mykiss*; NM_001124268, *nerka*; AB772420), red jungle fowl *Gallus gallus* ENSGALT00000044669, and sheep *Ovis aries* ENSOART00000003032.

3.3.2. Brain expression of *Dio2* and *Tshβ*

Within the period July to December there was no significant interaction of time and photoperiod treatment on whole brain *Dio2* expression for male (P = 0.235) or female (P = 0.258) Atlantic cod (Figure 3.7). Differences were observed in the overall mean expression levels between treatments (P = 0.027) for females, with higher overall expression observed in the SNP treatment (Figure 3.7). Similarly, there were no significant difference in the temporal expression between July and December of *Tsh*β in the brain between treatments, for males (P = 0.789) or females (P = 0.454). Overall mean expression of *Tsh*β was elevated in the LL treatments for females (P = 0.012) (Figure 3.8). Due to the lack of significant temporal variation with respect to treatment no further samples were analysed.

3.3.3. Pituitary expression of *Eya3* and *Tshβ*

Expression of *Tsh*β in the pituitary did not significantly differ between treatments over time between July and December for either males (P = 0.867) or females (P = 0.141) (Figure 3.9). However, females did exhibit a significant overall higher mean expression level under the LL treatment (P = 0.007). *Eya3* exhibited highly significant differences in temporal expression between treatments for both males (P < 0.001) and females (P < 0.001), for this reason the sample set was extended to a full annual cycle from July to the subsequent August. Cod held under the SNP stimulus exhibited an increase in pituitary *Eya3* expression from August, with significantly elevated expression in November, December, February and March for females, and in October, November, December, February and March for males (Figure 3.10). Expression levels then returned to baseline levels from April to August mimicking the natural change in day length from SD to LD. In contrast, LL individuals showed little temporal variation in expression.

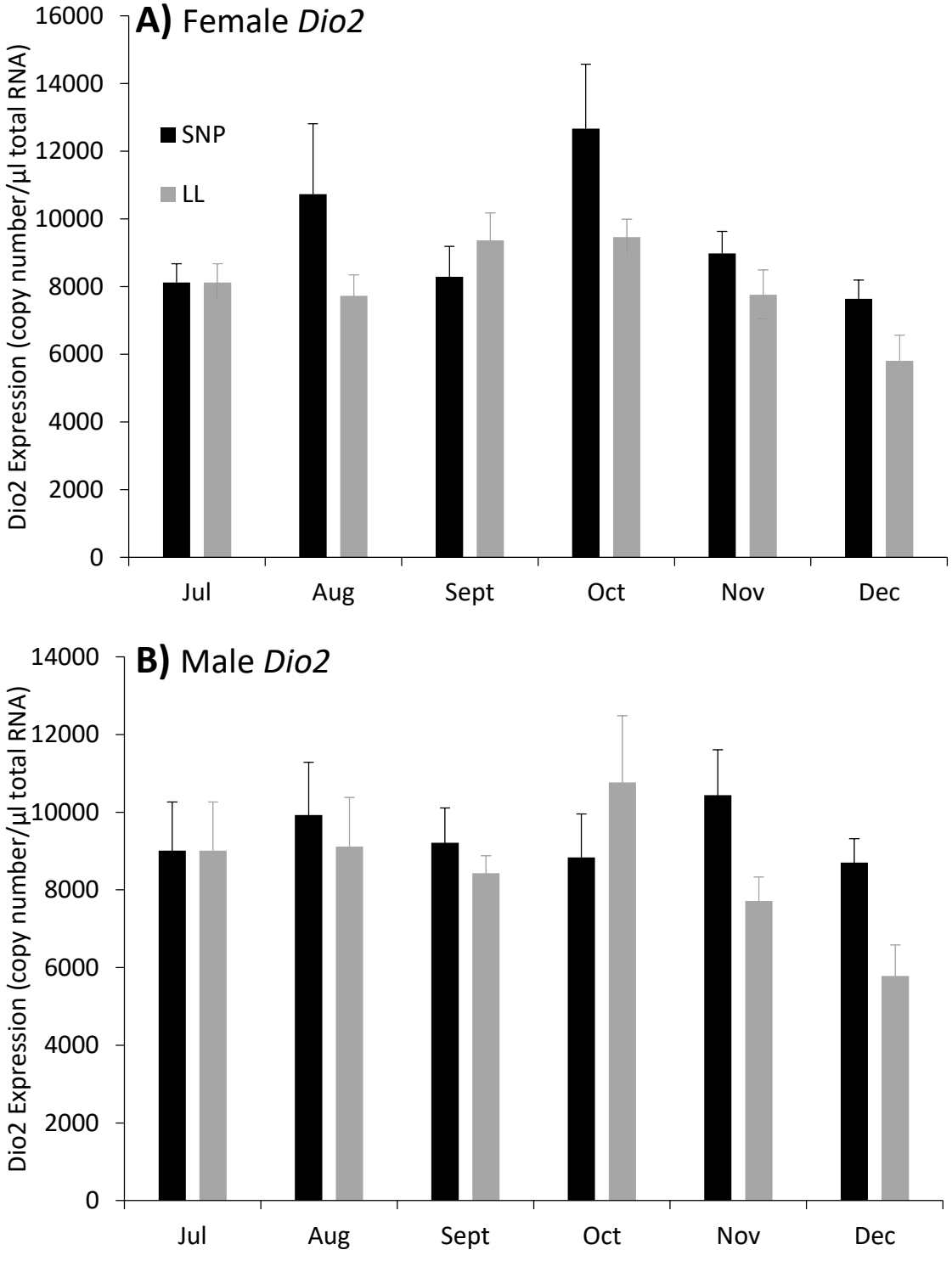

Figure 3.7. Absolute mRNA expression levels of *Dio2* in the brain of **A**) female and **B**) male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL) from July to December 2008. Data presented as mean ± SE.

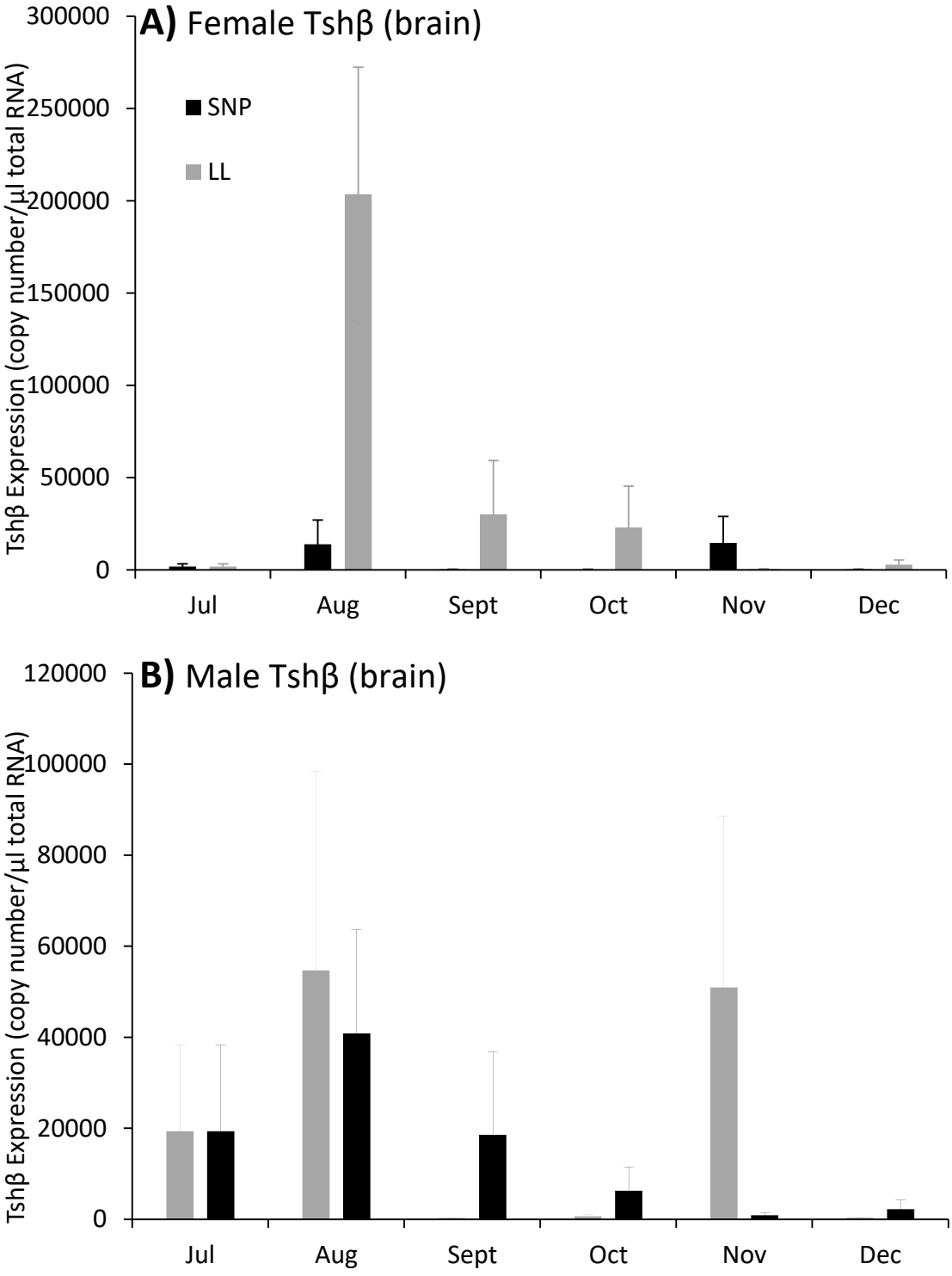

Figure 3.8. Absolute mRNA expression levels of *Tsh*β in the brain of **A**) female and **B**) male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL) from July to December 2008. Data presented as mean ± SE.

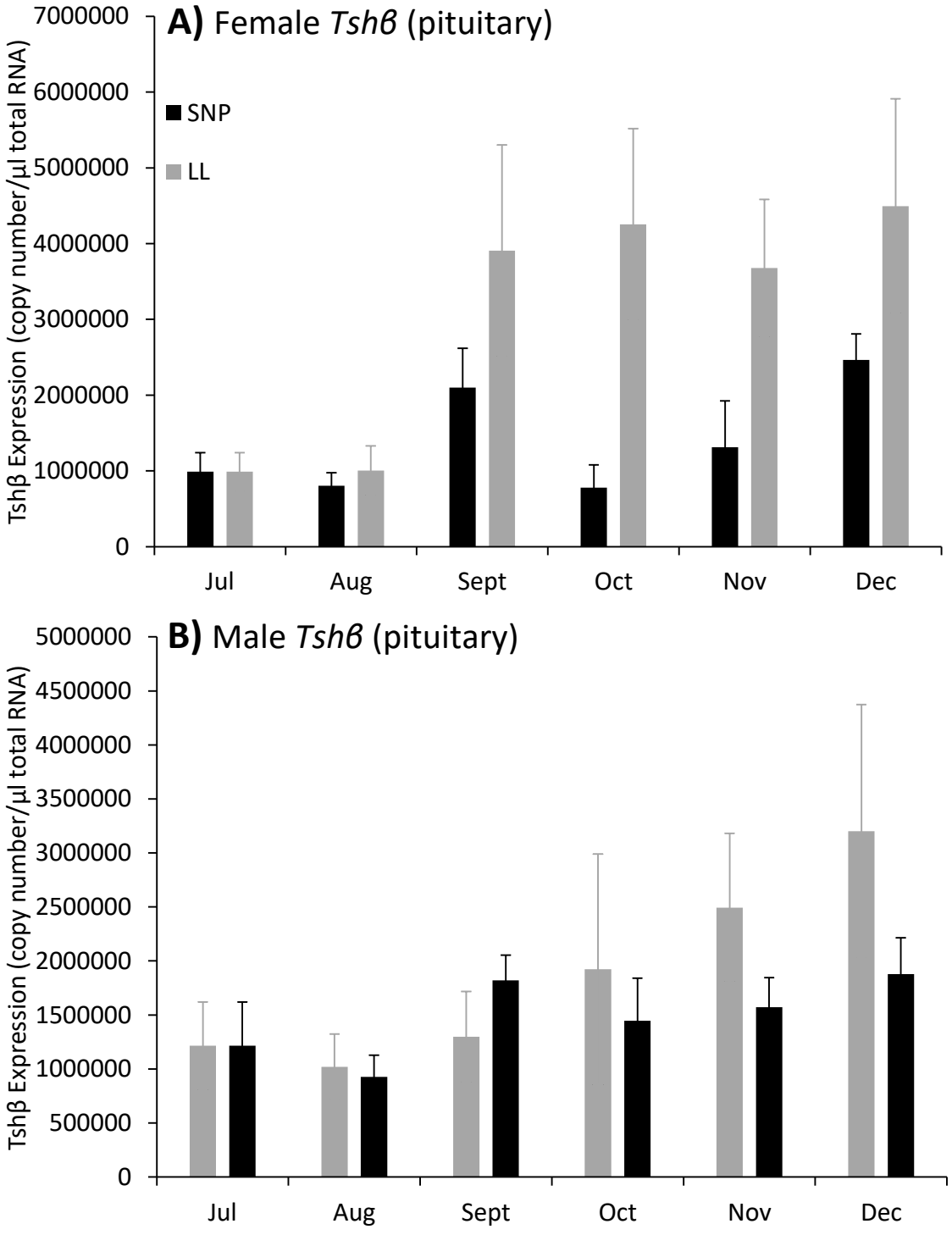

Figure 3.9. Absolute mRNA expression levels of *Tsh*β in the pituitary of **A**) female and **B**) male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL) from July to December 2008. Data presented as mean ± SE.

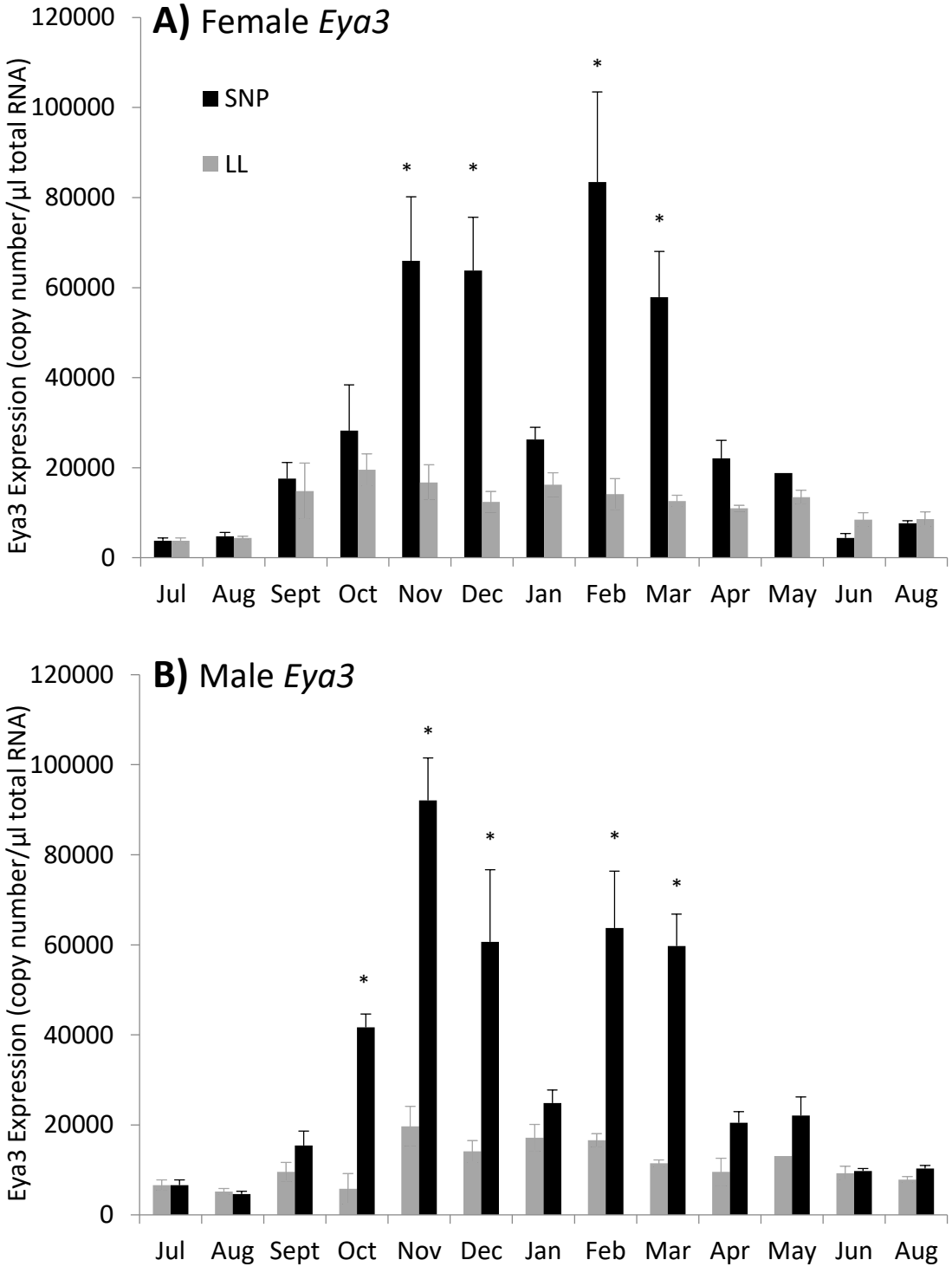

Figure 3.10. Absolute mRNA expression levels of *Eya3* in the pituitary of A) female and B) male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL) from July 2008 to August 2009. * denotes a significant difference between treatments in a given month. Data presented as mean ± SE.

3.3.4. Physiological & endocrine response to SNP and LL

Data on the gonadal development and gonadotropin expression has previously been described by Cowan et al. (Cowan et al. 2011, 2012) for cod used in this study. All individuals held under SNP displayed a typical reproductive cycle with the majority of individuals spawning in March. Female GSI values increased steadily from September, showing a significant increase in November, and reaching a maximum mean value in March (Figure 3.11). Correspondingly, an increase in *Fshβ* expression was observable from September onwards, signifying the onset of secondary gametogenesis, with a significant difference observed in January when *Fshβ* reached its peak (Figure 3.12). Male GSI increased from September on, with significantly differences observed from December to February when male GSI values reached their peak (Figure 3.11). Though no significant differences in *Fshβ* expression were observed for male cod in this study due to high individual variability, males did exhibit a ~3-fold increase in *Fshβ* from September to December indicating gamete development (Figure 3.12). In contrast, females and males from the LL treatment failed to reach maturity, exhibiting no temporal variation in GSI or *Fshβ* expression.

For both males and females from the SNP treatment, the surge in *Fshβ* and GSI strongly corresponded to patterns of *Eya3* expression. All three biomarkers increased following the autumn equinox to varying degrees, and it is difficult to pinpoint which preceded which, though significant differences between the LL and SNP treatments were first indicated through *Eya3* expression patterns in all instances for males, and through elevations in both GSI and *Eya3* in November for females.

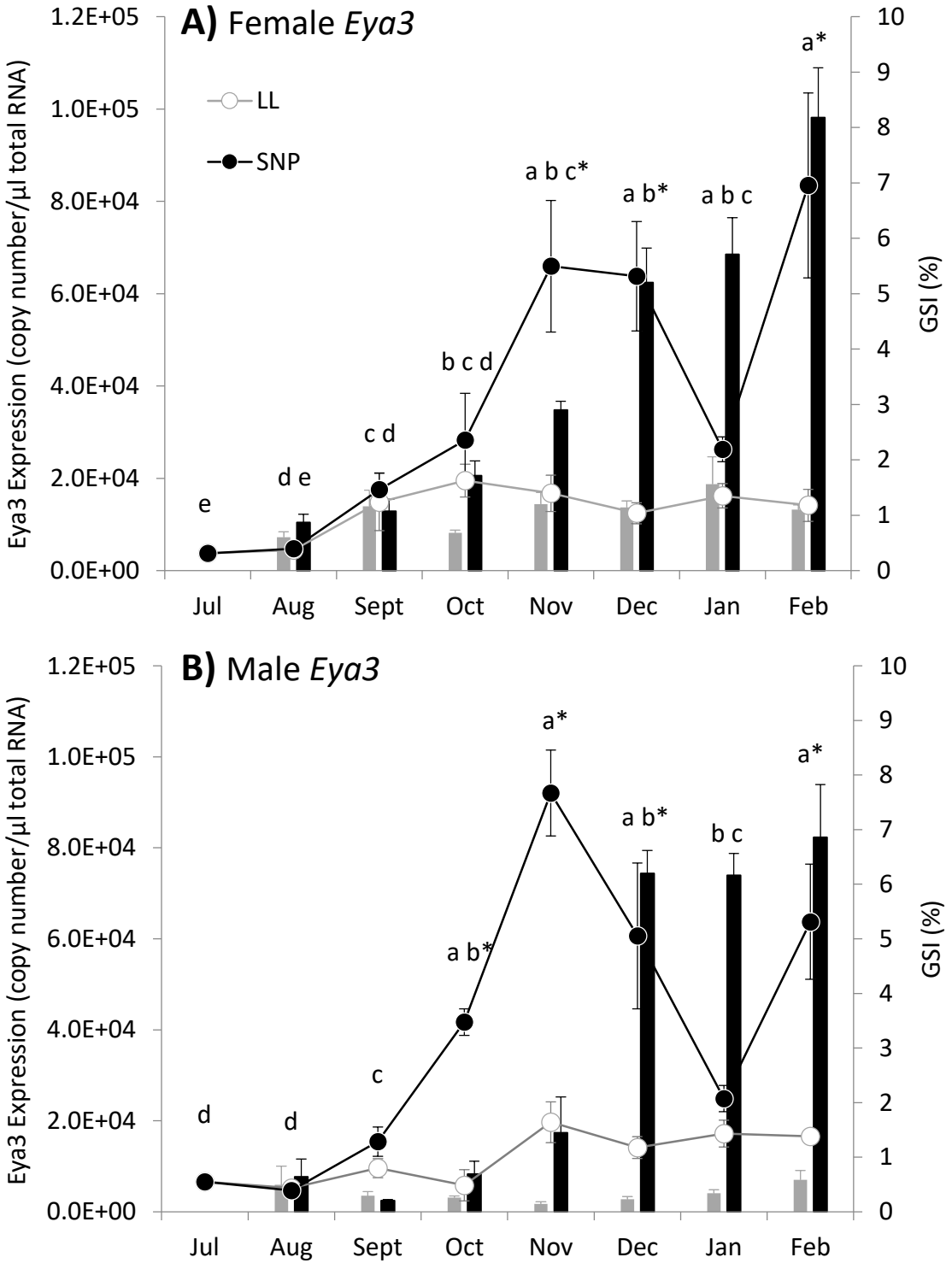

Figure 3.11. Absolute mRNA expression levels of *Eya3* (lines) in the pituitary and GSI (bars) for A) female and B) male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL) from July 2008 to February 2009. * denotes a significant temporal difference between treatments. Significant differences in *Eya3* expression between months for the SNP treatment are denoted by lowercase lettering. Data presented as mean ± SE.

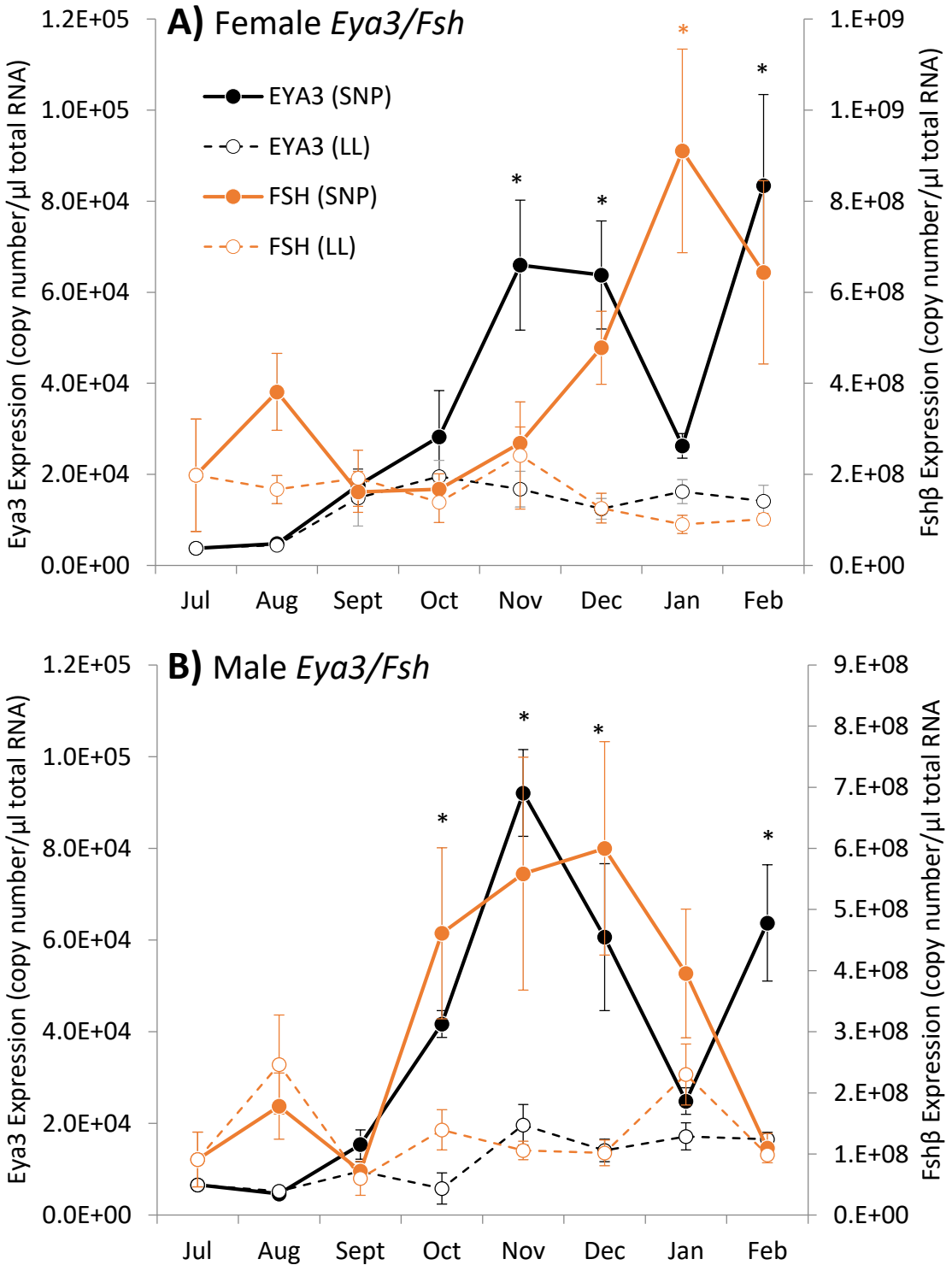

Figure 3.12. Absolute mRNA expression levels of *Eya3* (primary axis) and *Fshβ* (secondary axis for A) female and B) male Atlantic cod exposed to simulated natural photoperiod (SNP) or constant light (LL) from July 2008 to February 2009. * denotes a significant temporal difference between treatments. Data presented as mean ± SE.

3.4. DISCUSSION

Photoperiod entrainment of seasonal physiology and in particular reproduction, is a fundamentally basic mechanism evident in most temperate vertebrates. Recently there have been a number of studies collectively suggesting that, at its core, there is a conserved neuroendocrine pathway that ultimately results in the stimulation of thyroid hormones to initiate the BPG cascade (Follett 2015). Though evidence of this pathway in teleosts is limited, studies performed to date suggest an up-regulation of the proposed pathway in response to a stimulatory photoperiod (O'Brien et al. 2012, Nakane et al. 2013). In both studies, this up-regulation was in response to a LD stimulus which triggers secondary gametogenesis for both the species studied, being three spined stickleback (O'Brien et al. 2012) and Masu salmon (Nakane et al. 2013). The current study is the first to examine this pathway in a teleost species which initiates secondary gametogenesis following a SD stimulus. We demonstrate that the transcriptional coactivator *Eya3* that initiates the pathway shows a clear seasonal expression profile, being upregulated by a stimulatory SD stimulus, and that furthermore this upregulation can be supressed under a photoperiod manipulation that concomitantly inhibits maturation (Cowan et al. 2011, 2012).

In silico analysis of the cod genome clearly identified cod specific partial cDNA sequences for *Eya3*, *Tsh*β and *Dio2*. In all cases the deduced aa sequence shared high sequence identity with other fish species and conformational conservation of the relevant functional domains, indicative of gene homology across the clade. Although Lorgen et al. (2015) have identified two *Dio2* paralogs (*Dio2a* and *Dio2b*) in Atlantic salmon, phylogenetic analysis indicates that salmon *Dio2a* and *Dio2b* diverged during the salmon specific whole-genome duplication, and the sub-functionalisation theory they discuss represents an adaptation of the salmonid family alone. Only one *Dio2* gene has yet been identified among other teleost species. Similarly, O'Brien et al. (2012) identified a *Tshβ*

duplication in the three-spined stickleback. *Tshβ* paralogs have also been identified in zebrafish, and phylogenetic analysis suggests a teleost specific duplication. Our own analysis of the cod genome identified two *Tshβ* sequences from different GeneScaffolds. Synteny analysis (data not shown) of these scaffolds identified several paralogous genes up- and down-stream of the *Tshβ* sequences, indicating that these fragments were indeed paralogs of *Tshβ,* identified as *Tshβ1* and *Tshβ2*. Phylogenetic analysis confirmed a teleost specific divergence, placing the cod *Tshβ2* with the stickleback *Tshβ2,* and *Tshβ1* with all other teleost *Tshβ* including stickleback *Tshβ1*. As O'Brien et al. suggested, and our own analysis confirmed, *Tshβ1* represents the most conserved *Tshβ* paralog among teleosts and was therefore used in this study. Though clearly, further work should be undertaken to definitively sequence the full length of each gene, the high level of identity observed for *Eya3, Tshβ* and *Dio2* would suggest evolutionary conservation, reflective of an important functional role for these genes in a vital pathway in cod.

The working model of the neuroendocrine pathway sees the light signal being relayed to the pituitary where *Eya3* expression upregulates *Tshβ* production which subsequently stimulates *Dio2* expression in the third ventricle of the brain (Follett 2015). Of the three genes analysed in the current study, *Eya3* alone exhibited a significant seasonal response. This was observed as a surge in expression from August until March in the SNP treatment, exhibiting a maximal 17-fold difference in February for females, and a 19-fold difference in November for males, before return to basal levels as day length increased. By comparison, the LL treatment showed little variability, and expression was significantly different between treatments during November, December, February and March for females, and October, November, December, February and March for males. It is clear from these results that expression of the transcriptional coactivator *Eya3*, follows a distinct seasonal cycle, and furthermore this pattern can be inhibited through photoperiod

manipulation that masks the seasonal change in day length. Neither pituitary *Tshβ*, nor whole brain *Dio2* or *Tshβ* showed clear evidence of differential temporal expression with respect to the photoperiod treatments. It should however be recognised that whole-brain homogenates were used to look at expression of both *Dio2* and *Tshβ* in the current study. In mammals and birds, *Tshβ* is known to exhibit localised expression within the ependymal cells of the PT under LD stimulus (Nakao et al. 2008a, Yasuo et al. 2010). Although fish have a more primitive brain structure and do not possess a PT, previous studies in masu salmon have isolated photoperiod induced *Dio2* expression to a region known as the *saccus vasculosus* (Nakane et al. 2013). The same study also found significant differences in DIO2 and TSH protein expression in this region between salmon exposed to LD and SD stimulus, but no concurrent differences at the mRNA level, suggesting that post-transcriptional mechanisms may be more important in regulating patterns in expression for these genes and modulating the cascade of events which follow. It is possible that localised patterns of both *Dio2* and *Tshβ* in this study were confounded due to the sampling strategy, or that the mechanisms by which these genes regulate the PNES of fish occurs at the protein level. Clearly the application of western blotting to quantify protein expression and *in situ* expression measurement would be advantageous to provide spatial context, potentially revealing localised photoperiod response for these targets, and to investigate the relative importance of post-transcriptional mechanism in regulating the function of these genes.

The response of *Eya3* highlighted by this study, a response to SD stimulus, has never before been described in other vertebrate models. In long-day breeders such as hamsters, rats, mice and quail, upregulation of *Eya3* occurs in response to a LD stimulus and typically plays a permissive role in the initiation of the reproductive cycle. Long-day breeders which experience the transition from SD to LD exhibit an initial increase in

Eya3, which stimulates *Tsh*β production. This in turn induces the expression of *Dio2*, which is responsible for converting the prehormone thyroxine T4 into its bioactive form triiodothyronine T3. Increased expression of T3 appears to regulate GnRH production, thus commencing the reproductive cycle (Yoshimura 2004, Barrett et al. 2007, Hanon et al. 2008, Yasuo and Yoshimura 2009). In short-day breeding Soay sheep, the same expression cascade is observed, with stimulation of *Eya3* expression following a LD stimulus. Though studies suggest that this cascade plays a prohibitive role in sheep, curtailing the breeding season and initiating gonadal reduction (Wagner et al. 2008, Hanon et al. 2008, Dupré et al. 2010, Dardente et al. 2010, Sáenz de Miera et al. 2013), there is strong evidence to suggest that short-day breeding sheep rely on this LD signal to initiate the reproductive cycle the following winter (Dardente 2012). This would suggest that although sheep are considered short-day breeders, initiation of their seasonal reproductive cycle is dependent on a permissive LD stimulus, and therefore the pathway as it exists in sheep shares more in common with that of long-day breeders (Dardente et al. 2010). For short-day breeding Saanen goats however, the LD signal suppresses *Dio2* expression in the brain, in direct contrast to the expression profile in sheep. Initiation of the pathway in goats appears to follow a SD stimulus, suggesting a switch in the initiating of the pathway from LD to SD between these closely related species (Yasuo et al. 2006). Saanen goats therefore represent the only other comparable example of SD stimulated secondary gametogenesis among all vertebrate examples studied to date. Although this study focused on *Dio2* expression, the study showed positive stimulation of the pathway following a SD stimulus. It is clear that in the current study, *Eya3* expression is initiated by a SD cue in Atlantic cod, and provides a clear signal of seasonally altering photoperiod. We would therefore conclude that the upstream drivers which initiate the *Eya3*-*Tsh*β-*Dio2* pathway differ among vertebrates according to their breeding strategies, but the

pathway itself and its role in the reproductive cascade is conserved across the vertebrate clade.

In relation to other fish species, this study is the first investigation of *Eya3*, *Tshβ* or *Dio2* expression in a short-day breeding teleost species which shows stimulation of the pathway under a SD stimulus. Though Masu salmon, like Atlantic cod, are widely considered to be short-day breeders (Nakane et al. 2013), Masu salmon utilise a LD cue to initiate secondary gametogenesis, much like sheep; LD induced *Tshβ* expression in the *saccus vasculosus* is thought to act as the main upstream driver governing the induction of season-associated *Dio2*, and the resultant stimulation of the BPG (Nakane et al. 2013). Similarly in long-day breeding sticklebacks, acute induction of *Tshβ* in conjunction with *GnRH* and *Lh* under a LD stimulus, support the idea of conserved pathway linking the PNES and BPG axes in long-day breeders (O'Brien et al. 2012). As neither study has considered *Eya3* expression, it is difficult to draw conclusions about the conservation of this pathway in fish. Indeed, no clear relationship has been shown between *Eya3*, *Tshβ* or *Dio2* in teleosts, current study included. While Nakane *et al.* (2013) reported a significant photoperiod response in *Dio2* expression levels in Masu salmon, the same authors did not observe statistically significant differences in the expression of *Tshβ*. O'Brien *et al.* (2012) observed differences in *Tshβ* expression relative to photoperiod stimuli, but did not consider *Eya3* or *Dio2* expression in their study. Although the current study did attempt to look at the relationship between each of these genes, no significant differences in either *Tshβ* or *Dio2* expression were found. Individually, *Eya3*, *Tshβ* and *Dio2* have each been identified as important messengers of the seasonal photoperiod in teleosts, but it is clear that further work is required for us to fully understand if/how these genes interact before we can conclude whether or not the pathway is conserved across the vertebrate clade.

As is the case for many temperate fish species, initiation of maturation in cod is limited to a specific "window of opportunity", during which the fish which have achieved a permissive physiological state will commit to reproduce the following season (Thorpe et al. 1990, Bromage et al. 2001). Previous experiments in cod indicate this window spans between the summer solstice and mid-autumn (Davie et al. 2007b) when the beginning of secondary gametogenesis is measureable by the increased expression of gonadotropins (Cowan et al. 2012). This window represents a gap in our current understanding of the maturation cycle in cod. The current study has identified *Eya3* as a potential messenger of the proximate photoperiodic cue, showing a clear up-regulation of the gene from around the autumn equinox onwards, following a seasonal expression pattern closely linked to the prevailing photoperiod. This potentially narrows down our search for the specific photoperiod cue which initiates secondary gametogenesis to this period, when day length declines to around 12 hrs. Increasing *Fshβ* mRNA expression and GSI values appear to coincide with the up-regulation of *Eya3* observed, indicating that the seasonal response initiated by *Eya3* may occur over a very short time span. As acute induction of *Eya3* has been shown to occur following < 48 hrs under a LD stimulus in sheep, it is possible that *Eya3* induces a seasonal response in this short period and that our temporal sampling strategy may have been too ambiguous to detect the "critical day length" for initiation of the pathway. Future work should focus on the use of square wave seasonal photoperiods to help identify this period.

The aim of this study was to investigate if the *Eya3-Tshβ-Dio2* pathway, which is known to consolidate the PNES and BPG axis in other seasonal vertebrates, exists in Atlantic cod. Of these three genes of interest, *Eya3* was found to clearly show potential as a seasonal messenger in Atlantic cod. This is also the first account of *Eya3* induction in a vertebrate which initiates secondary gametogenesis following a SD cue, and this SD

induction of the pathway has not previously been observed in teleosts. However, Atlantic cod do share this SD induction of the pathway with short-day breeding goats. This would suggest an overall conservation of the pathway function in vertebrates, and that differences in the up-stream signals which induce this pathway are likely to have evolved as a result of the reproductive strategy adopted by the species.

CHAPTER 4. THE EFFECTS OF DIET MANIPULATIONS ON GROWTH AND MATURATION IN GADOIDS

4.1. INTRODUCTION

As discussed in Chapter 3, photoperiod is a key driver in the initiation and entrainment of seasonal reproduction in fish (Sumpter 1990, Migaud et al. 2010). As such, photoperiod determines the period or "window" when a fishes' state and environment can influence maturation commitment (Aubin-Horth et al. 2005). This window usually relates to the time of year when an individual must decide whether to commit to the most energetically demanding phase of gonad development. For female fish this would be vitellogenesis (Tyler and Sumpter 1996, Burton et al. 1997); for males it is spermatocyte production (Rowe and Thorpe 1990). As reproduction is typically long lasting, and these phases of gonad growth require a considerable provision of resources, it is crucial to the success of reproduction that some permissive physiological state is attained to coincide with the photoperiod "window". This physiological state, in temperate fish species, would appear to be based around a suitable energetic state (Rowe and Thorpe 1990, Thorpe et al. 1990, Rowe et al. 1991), and there is a well-accepted hypothesis stating that a threshold of size, growth rate and/or energy storage must be surpassed during an initial assessment period in order for sexual maturation to initiate and succeed (Eliassen and Vahl 1982, Rowe and Thorpe 1990, Bromage et al. 2001, Metcalfe et al. 2007). However, studies have yet to identify either the time in which energetic state influences maturation or the threshold for energy accumulation or growth which must be surpassed for maturation to proceed.

The importance of lipids, being the main endogenous energy reserve, in the initiation of puberty has been well studied in a range of vertebrates. The ratio of body weight to fat has been linked to puberty in rats, mice and humans for over 50 years (Frisch and Revelle

1970, Young 1976, Katz et al. 1985). Similarly, teleost studies have long focused on the role of lipid reserves in the onset of maturation (Alm 1959), particularly among salmonids (Thorpe et al. 1990, Silverstein et al. 1998, Davies et al. 1999, Shearer and Swanson 2000, Begtashi et al. 2004). Puberty in salmonids is closely linked to lipid reserves. Manipulations of diet and ration have been shown to influence maturation commitment and fecundity in Atlantic salmon (Rowe and Thorpe 1990, Thorpe et al. 1990, Duston and Saunders 1999) and Chinook salmon (*Oncorhynchus tshawytscha* L.) (Silverstein et al. 1998, Shearer and Swanson 2000), suggesting that lipid energy accumulation may be more influential than absolute size in the decision to mature.

Both field and experimental investigations in gadoids show that sexual maturation may also be dependent on accumulated lipid reserves, suggesting that individuals who fail to exceed a threshold lipid energy reserve do not mature (Eliassen and Vahl 1982, Harrald et al. 2010). In gadoids, the liver represents the major organ for lipid storage (Love 1970, Shevchenko 1972) and liver size and composition has been linked to maturation commitment (Eliassen and Vahl 1982). However, intuitively lipid energy would have to be assessed prior to commitment, but many studies have examined liver size long after the commitment has already been made and are therefore not representative of the threshold reserves required for maturation to commence.

Large size and high growth rate has also been linked to maturation in gadoids, with several studies illustrating that fast growing (and subsequently larger) gadoids mature earlier than their slow growing counterparts (Eliassen and Vahl 1982, Holdway and Beamish 1985, Godø and Haug 1999, Yoneda and Wright 2005b, Tobin et al. 2010, Tobin and Wright 2011). However, whether this growth influence is linked to lipid accumulation or some other associated factor is not known. Nevertheless, a threshold size or growth rate may exist which must be surpassed before fish commit to maturing in a given year.

Diet manipulation studies to establish the influence of diet on the reproductive traits of gadoids have largely had an aquaculture focus. As such, the diets, feed rates and size of experimental fish are not particularly representative of wild conditions. Most of these experiments have been designed to define optimal feeding regimes for increased fecundity (Lehmann et al. 1991, Kjesbu and Holm 1994), and though these studies highlighted the potential effects of diet manipulation on growth, they could not define any critical window or threshold levels of growth or energy acquisition, as the fish used were too large and the feed rates too high to inhibit or otherwise influence the decision to mature. One study on Atlantic cod attempting to delay maturation through periodic starvation treatments which varied in timing and duration between July and November, failed to inhibit maturation (Karlsen et al. 1995). All fish matured regardless of treatment suggesting that energy levels and/or growth rates were not reduced below any putative threshold. However, as this study also used cultured fish, it is likely that their advanced size and condition eroded any possible effect of diet restriction on maturation commitment.

Following the stock collapse in the north-west North Atlantic, there was a growth in interest into the potential mariculture of haddock. Consequently, many of the previous investigations into the influence of diet on haddock have focussed on perfecting feed composition for aquaculture purposes, and have rarely investigated the influence of diet and dietary constituents on maturation commitment. Similar to cod, many of these studies are not comparable with conditions in the wild, as neither the growth rates, nor the dietary protein and lipid levels reflect natural conditions (Hislop et al. 1978b, Jones and Hislop 1978, Kim and Lall 2001, Nanton et al. 2001, 2006, Peck et al. 2003, 2005, Treasurer et al. 2006). Nanton *et al*. (2006) did attempt to link dietary lipid level to maturation commitment, but with similar results to Karlsen *et al.* (1995), in that all fish matured

because energy levels and/or growth rates were most likely not reduced below the threshold requirements, and again the quantity of lipid and protein in the diet did not reflect the levels which occur naturally in the prey of wild haddock. To date, there have been no studies which specifically look at the influence of diet and diet composition on maturation commitment in haddock. The influence of diets containing variable lipid quantities (similar to the variability observed in the wild) on growth, and the permissive role that lipid energy reserves may play in the reproductive cycle, remain to be demonstrated.

In Atlantic cod and haddock, it has been shown that the declining photoperiod following summer solstice initiates secondary gametogenesis which continues through the autumn and winter months (Davie et al. 2007c). Concomitantly it has been observed that there is increased expression of the gonadotropin, follicle stimulating hormone (*Fshβ*), in male (de Almeida et al. 2011) and female (Cowan et al. 2012) cod and haddock (Tobin et al. 2010) from September onwards, marking the initiation of the BPG cascade. Importantly if either species are maintained in a continuous illumination from the summer solstice, to mask the reduction in photoperiod, secondary gametogenesis and the associated autumnal gonadotropin surge are completely inhibited (Davie et al. 2007c, 2008, Cowan et al. 2012). This would suggest that between the reduction of photoperiod from the summer solstice in June, and the gonadotropin gene surge seen from September onwards, lies an as yet unidentified signalling pathway linking the proximate photoperiod cue to the initiation of the BPG cascade in cod. In the previous chapter (Chapter 3), precursor signalling pathways within the PNES (e.g. *Eya3*), in cod, were demonstrated to be differentially reactive to photoperiod stimulus in close association with the autumnal equinox and the subsequent wave of gonadotropins expression, adding further credence to the narrow window of maturation commitment. This period marks a "critical window"

in which cod and haddock commit to mature the following year, and they are therefore likely to assess their own growth and/or energetic status in the period leading up to this window. When exactly within this period of assessment occurs, and how long it lasts, is currently unknown; the failure of numerous manipulation studies to influence maturation in gadoids (Kjesbu and Holm 1994, Silverstein et al. 1997, 1998, Yoneda and Wright 2005b, Karlsen et al. 2006b, Harrald et al. 2010), serves to highlight the complexity of the system. While it is clear that accumulated energy reserves, growth rate and size must play an important role in the endogenous reproductive axes further work is clearly needed to assess the relative importance of these factors to maturation commitment and when and how they are assessed. Such studies will greatly improve our understanding of the internal and external drivers of maturation which explain natural variations in this complex life history. With this in mind, three diet manipulation trials were developed and run, to address some of these knowledge gaps.

The first trial was developed to investigate the influence of dietary lipids on growth, accumulated energy reserves and maturation, in haddock. Previous trials investigating the effects of lipid on growth and maturation in haddock have largely had an aquaculture focus, and as a result have failed to provide insight into the effects of lipid energy in the wild. Thus, the diet treatments used for this trial were developed to mimic natural diets with variable lipid levels, that haddock would be expected to encounter in the wild. In this way, it was hoped that we might provide an accurate insight into how natural fluctuations in food availability or diet composition could create differences in maturation and growth as we see among wild haddock stocks.

The second trial was developed to investigate the effect of growth rate and accumulate energy reserves at different periods prior to maturation commitment, in our main study species, cod. This trial applied diet restrictions over two different periods prior to the

commitment window, in the hopes of identifying the period in which cod are most sensitive to their own nutritional status, and provide some insight into how they assess this – whether growth rate, accumulated energy reserves, overall size, or a combination of these endogenous indicators are used. The intention was to create two groups for both periods – one fast growing with high accumulated energy stores and therefore likely to make the commitment to mature, the other slow growing with low stores and unlikely to mature, mimicking slow growth rates and low liver reserves previously linked to non-maturing fish (as seen in the previous trial), thus allowing us to identify which time-period represents the "critical period" when cod commit to maturation and which parameters of growth influence this decision.

Developing on the previous trials, the third study was developed to explore the importance of size and growth as permissive drivers of maturation in cod, and to investigate potential endogenous markers linking this growth/accumulated energy axis to the reproductive axis. As discussed in Chapter 3, *Eya3* has been identified as a potential link between the PNES and BPG axes. However, previous studies have focussed on photoperiod manipulation, making it impossible to assess whether this marker acts as a driver of maturation, or simply acts as a photoperiodic messenger. Therefore, developing on the results from the previous trials, this study used diet manipulation to create two groups which differ in both their maturation commitment and their size. Maintaining both groups under the same simulated natural photoperiod and temperature, allowed us to assess the role of *Eya3* in the reproductive axis of cod.

4.2. EXPERIMENT I: INVESTIGATING THE EFFECT OF DIETARY LIPIDS ON GROWTH AND DEVELOPMENT IN HADDOCK.

4.2.1. Aims

To investigate the influence of dietary lipid on growth parameters and maturation commitment in haddock.

4.2.2. Methods

Fish Collection and Husbandry

Juvenile 0-group haddock were collected from the wild off Stonehaven on the east coast of Scotland (56˚57'50'' N, 02˚12'04'' W) in June and July 2011. These haddock were transferred to the Marine Laboratory in Aberdeen, where they were housed in a cylindrical tank (3000 L) under a partial flow-through system. Photoperiod was simulated (SNP) to reflect the ambient photoperiod (57˚16' N), and temperature was kept at 9 ˚C. Prior to the trial, fish were fed *ad libitum* with a wet feed consisting primarily of chopped squid and sandeel.

Experimental Design

On the 6th June 2012, 45 individuals (mean weight 149 ± 5 g) were anaesthetised and a Passive Integrated Transponder (PIT) tag (Trovan Ltd, Hull, UK) was implanted into the peritoneal cavity to allow for future identification. These fish were then weighed (0.01 g), total length measured (0.1 cm), and allowed to recover in clean aerated water. There were no mortalities associated with tagging. Once recovered, fish were split between two replicate experimental tanks (7 x 3 x 1 m, 3000 L). Each tank was further split into two compartments using a plastic mesh separator, creating two replicate compartments for each tank. The fish were size matched between tanks to eliminate the risk of any potential size effects, and assigned (n=11-12) to each compartment. Using chillers, the temperature

in these tanks was maintained at 9 ºC during the trial, and a Simulated Natural Photoperiod (SNP) was used. All fish were aged 1 at the start of the trial.

Fish were split between two diet treatments, with two replicates for each treatment. The first treatment group, here-after referred to as the high lipid treatment, were fed a high-lipid wet diet of sandeel (lipid content: 7.8 %.g^{-1}). A total of 21 fish were maintained under this diet, with n =10 in one replicate, and n = 11 in replicate two. The second treatment group were maintained on a low-lipid wet diet of squid (lipid content: 2.4%.g^{-1}), and are here-after referred to as the low lipid treatment. A total of 22 fish were maintained on this diet and fish were equally split (n = 11) between replicates. Diets were calorically matched to provide twice the maintenance requirements for each fish (M_{ind}), as described in Jones, 1974 as:

$$M_{ind} = 0.009(exp0.081.T).M_t^{0.8} \ (x\ 2)$$

where T is temperature (ºC) and M_t is the total body weight (g) of the fish. This provided a calculation of the caloric requirement for twice the maintenance ration for each fish in Kcal's per day (Kcal.d^{-1}), which has been shown to maintain a similar growth rate to wild fish (Jones, 1972; Jones, 1974). These values were pooled for individuals in each group to give an estimate of calorie requirements per group (M_{pop}). The weight (g) of feed (W_f) required was then calculated for each group depending on diet treatment. For squid:

$$W_f = M_{pop}.0.97^{-1}$$

where M_{pop} is the total Kcal.d^{-1} requirement of the group, and 0.97 is Kcal.g^{-1} for squid (Rosen & Trites, 2000). For sandeel:

$$W_f = M_{pop}.1.44^{-1}$$

where 1.44 is Kcal.g^{-1} for sandeel (adapted from Hislop *et al*., 1991). Feed weight averaged at approximately 1% (0.02% from lipid) and 0.7% (0.06% from lipid) body weight.day^{-1} for the low lipid and high lipid treatments respectively. Details of the feeding regime are given in Table 4.1.

Table 4.1. Summary of the dietary rations for each replicate (replicates 1 and 2 for each treatment) of the Low Lipid and High Lipid treatments in g per day of feed offered, being exclusively squid or sandeels for the Low and High Lipid treatments respectively. Changes in ration following sampling are shown for each period.

	Ration (g.day^{-1})			
Period	**Low Lipid (1)**	**Low Lipid (2)**	**High Lipid (1)**	**High Lipid (2)**
6th June - 3rd July	21.9	21.4	14.3	14.1
3rd July - 1st Aug	23.2	22.6	15.1	14.9
1st Aug - 29th Aug	25.7	24.8	15.9	15.8

Monitoring

To obtain individual growth profiles, fish were measured every 28 days over the course of the experiment. Prior to each measurement session, feed was withheld for approximately 24hours to ensure evacuation of the gut (Jones, 1974). Under anaesthesia (MS-222, 100mg l^{-1}), fish were identified and measures of total length (±0.1 cm) and weight (±0.01 g) were obtained.

Terminal sampling

On the 29th August, all fish were sacrificed. Final measures of length and weight were taken before fish were dissected to obtain liver weight (±0.0001 g), gonad weight (±0.0001 g), and somatic weight (±0.01 g). Gender was assessed visually, and gonad samples were taken and stored in 10% neutral buffer formalin (NBF) for histological analysis.

The overall population sex ratio (males to females) was 1:0.39. For the low lipid treatment the sex ratio was 1:0.24, compared with 1:0.57 for the high lipid treatment. One fish died over the course of the experiment due to exophthalmia, and so data for this fish were not included in this study. One further fish exhibited abnormalities in its growth due to a spinal deformity and was removed from further analysis to satisfy the statistical modelling assumptions. There were no significant differences in length or weight between treatments at the beginning of the trial (Table 4.2).

Table 4.2. Summary of the distribution of animals by sex for each replicate (replicates 1 and 2 for each treatment), with initial mean weight [0.1 g (range)] and mean length [0.1 cm (range)]. There were no significant differences in Length ($p = 0.676$) or Weight ($p = 0.439$) at the start of the trial (ANOVA).

Treatment	N	Length [mean (range)]	Weight [mean (range)]
Low Lipid	21	25.3 (20 - 27.5)	155.3 (81 - 199.6)
Low Lipid 1	11	25.5 (22.5 - 27.5)	154.3 (101 - 199.6
Low Lipid 2	10	25.1 (20 - 26.5)	156.4 (81 - 183.6)
High Lipid	22	25 (21.5 - 28.6)	147.3 (89.2 - 214.2)
High Lipid 1	11	25.2 (22.5 - 28.6)	146.5 (89.8 - 214.2)
High Lipid 2	11	24.9 (21.5 - 27)	148.1 (89.2 - 197.2)

Other data sources

To show how growth parameters from this study compared with growth in both wild fish and other experimental fish, comparable data were obtained from two sources. For wild fish, we used data collected through the Scottish Government observer programme on haddock stocks from the North Sea and north-east Atlantic. This data included measurements of total length and total weight from haddock sampled between May and September, from 1994 to 2004. Measurements from a total of 188 male and 137 female haddock were included in this dataset.

For experimental fish, we used data from the Tobin et al., (2010) study on the timing of the maturation transition in haddock. This dataset included total length, total weight, liver weight, and gonad weight measurements, as well as estimates of maturity stage and mean values of the leading cohort (defined as the largest 10% of oocytes measured) for females. Measurements from 41 females and 34 males were included.

Statistical analysis

Relative weight indices were calculated according to the methods described in Section 2.6. For this experiment, the influence of diet on the gonadosomatic index (GSI), and relative liver weight (RLM) was analysed using a general linear model (GLM), with both sex and treatment as factors, and replicate as a factor nested within treatment. For comparison of the leading cohort, and GSI between experimental fish from the current dataset and the dataset developed by Tobin *et al*. (2010), a GLM was also used, with the dataset as a factor and calendar day as a continuous variable to account for potential differences between the sampling dates.

To account for repeated measures, changes in length, weight, thermal growth coefficients (TGC), relative condition (Kn), and Fulton's condition factor (K) over the course of the study, were analysed using a linear mixed-effects model. Again, sex and treatment were treated as factors with replicate nested within treatment, and time was treated as a continuous variable. Individual variability over time was treated as a random variable (~ time | fish). Assumptions of normality and homogeneity of variance were achieved through log transformation. A significance level of $p<0.05$ was set in all cases and significant interactions were analysed by Tukey's *post hoc* test. All statistical tests were undertaken in R (version 3.2.2).

4.2.3. Results

Influence of Diet on Growth Parameters

Positive growth was observed in 90% of all measurements taken throughout the trial period with four individuals from the high lipid treatment exhibiting weight loss in more than one month. Food conversion efficiency (FCE) was calculated for each group as a ratio of weight gained to Kcals provided, as rations were matched between treatments in terms of Kcal and not weight. Differences were observed in the overall FCE between treatments; fish from the low lipid treatment exhibited an overall FCE of 0.25, which was 7.25% higher than the FCE of fish from the high lipid treatment (FCE = 0.17; Table 4.3).

Table 4.3. Food conversion efficiency for each replicate (replicates 1 and 2 for each treatment) presented as the mixed-sex replicate and treatment means for each sampling period.

Treatment	FCE1-2	FCE2-3	FCE3-4	FCEtotal
Low Lipid	0.23	0.36	0.16	0.25
Low Lipid 1	0.22	0.37	0.15	0.25
Low Lipid 2	0.23	0.34	0.17	0.25
High Lipid	0.22	0.20	0.12	0.17
High Lipid 1	0.22	0.19	0.12	0.18
High Lipid 2	0.21	0.20	0.11	0.17

All groups exhibited positive growth over the course of the trial. There was no significant effect of sex observed for any variable, hence data presented are those of the mixed-sex treatment means ± the standard error (SE). Length increased significantly between time points ($p<0.01$), and there was an interactive effect between treatment and time ($p<0.02$), indicating that the difference in mean length observed between the high lipid and low lipid treatments increased over time (Table 4.4). Haddock from the low lipid treatment increased in length by 9.8% over the course of the trial, compared with 7.4% for the high lipid treatment (Figure 4.1).

Similarly, total weight increased significantly over the trial period (Figure 4.2). There was a significant interactive effect of time and treatment observed ($p < 0.01$). Again, individuals fed the low lipid diet weighed more than their high lipid counterparts, and this trend increased over time (Table 4.5). Haddock from the low lipid treatment increased in weight by more than 22% over the course of the trial, compared with less than 17% for fish from the high lipid treatment.

To compare somatic condition among experimental fish, a weight-length relationship was derived after log transforming both variables. A regression analysis (Figure 4.3) of this relationship was used to calculate the relative condition (Kn). Fulton's condition factor (K) was also calculated to enable comparison with other studies. Fulton's K decreased significantly over the duration of the trial ($p < 0.01$), as did Kn ($p < 0.01$), but neither variable differed between treatments (Table 4.6 and Table 4.7, respectively). This difference over time equated to no more than a 0.07 difference in K and a 0.01 difference in Kn. Variance at each time point was low, and the significant difference may have little biological value.

Growth rate, expressed as the Thermal Growth Coefficient (TGC) differed significantly between treatments ($p = 0.01$), and increased over the duration of the trial ($p < 0.01$), though no interaction was observed (Table 4.8). For the low lipid treatment, TGC was double that of the high lipid treatment between the July and early August time points (growth period 2-3), but declined to comparable levels thereafter (Figure 4.4).

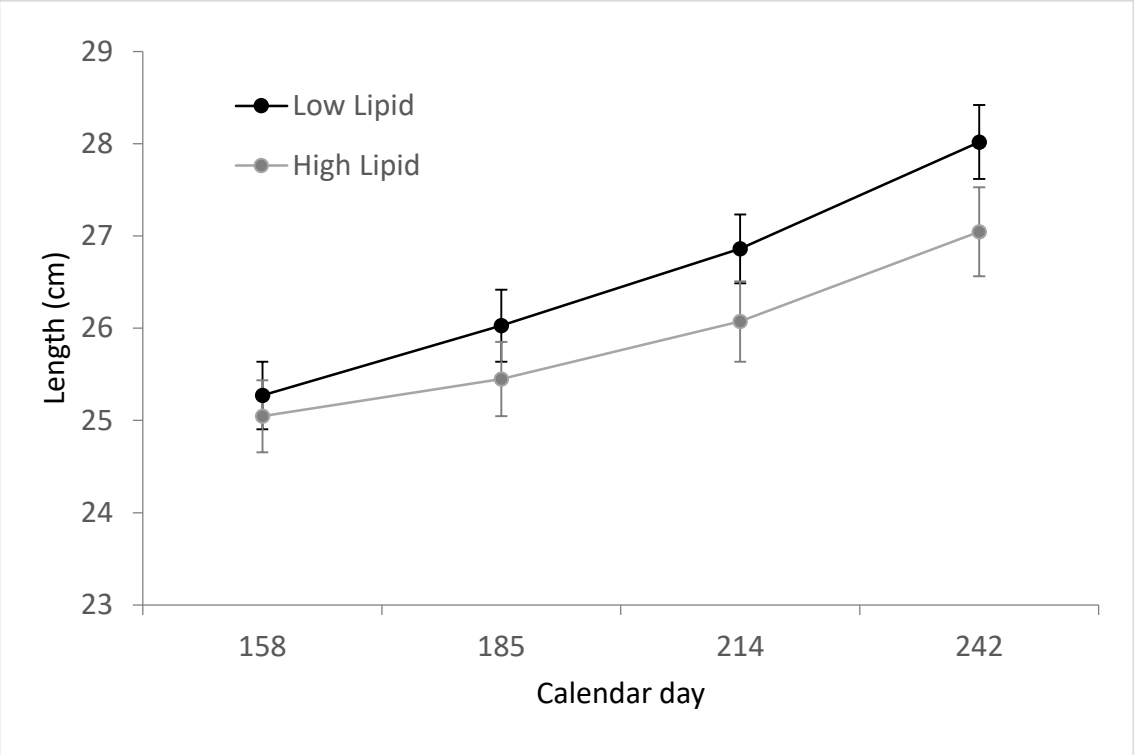

Figure 4.1. Changes in mean length between sampling periods. Data are presented as the mixed sex treatment means (± SE).

Table 4.4. Linear mixed effects model analysis of changes in length (L) relative to both TIME and TREATMENT, with TREATMENT as a factor, TIME as a continuous variable, and individual as the random variable to account for repeated measures over time.

ANOVA: L ~ TIME * TREATMENT				
	numDF	denDF	F-value	p-value
(Intercept)	1	127	8636.83	0.000
TIME	1	127	242.95	0.000
TREATMENT	1	41	0.17	0.684
TIME:TREATMENT	1	127	5.81	0.017

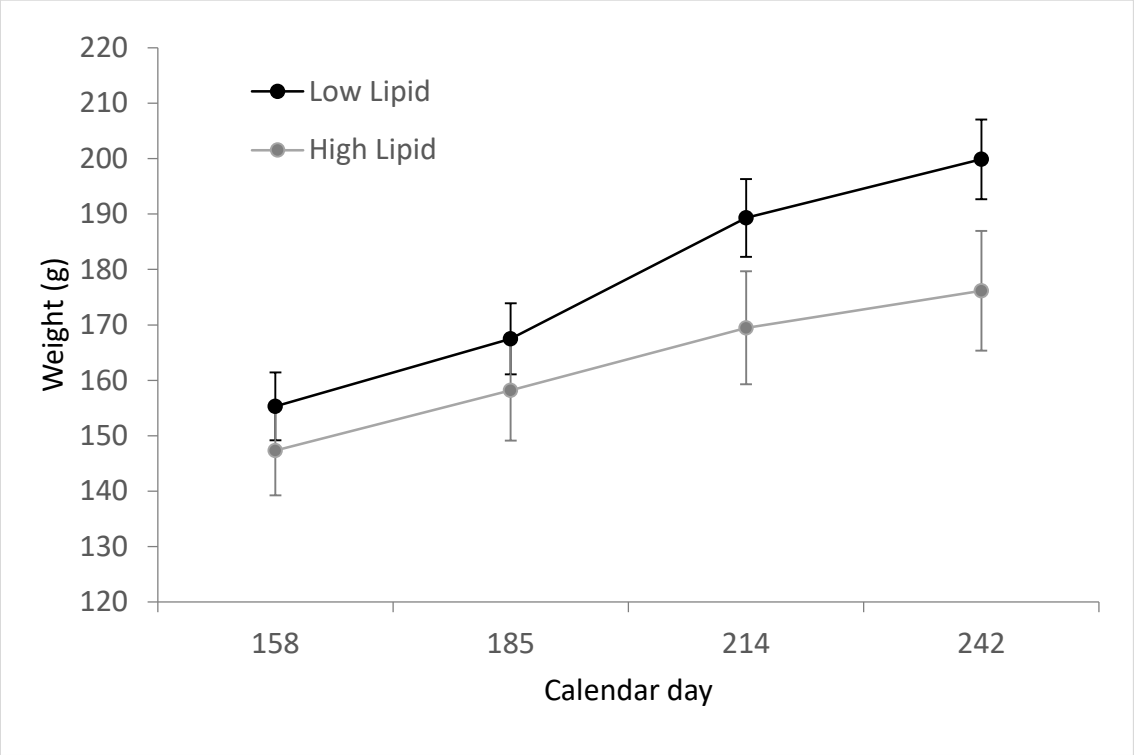

Figure 4.2. Changes in mean weight between sampling periods. Data are presented as the mixed sex treatment means (± SE).

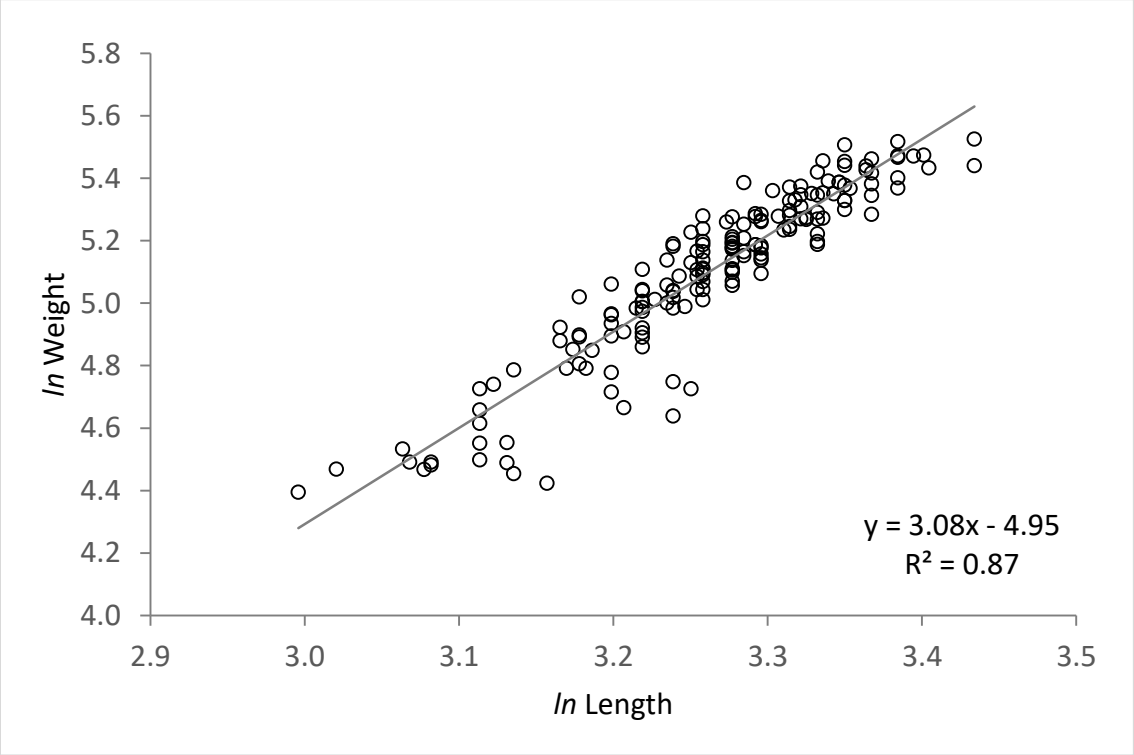

Figure 4.3. Regression of all log transformed lengths and weights for all fish from the current study

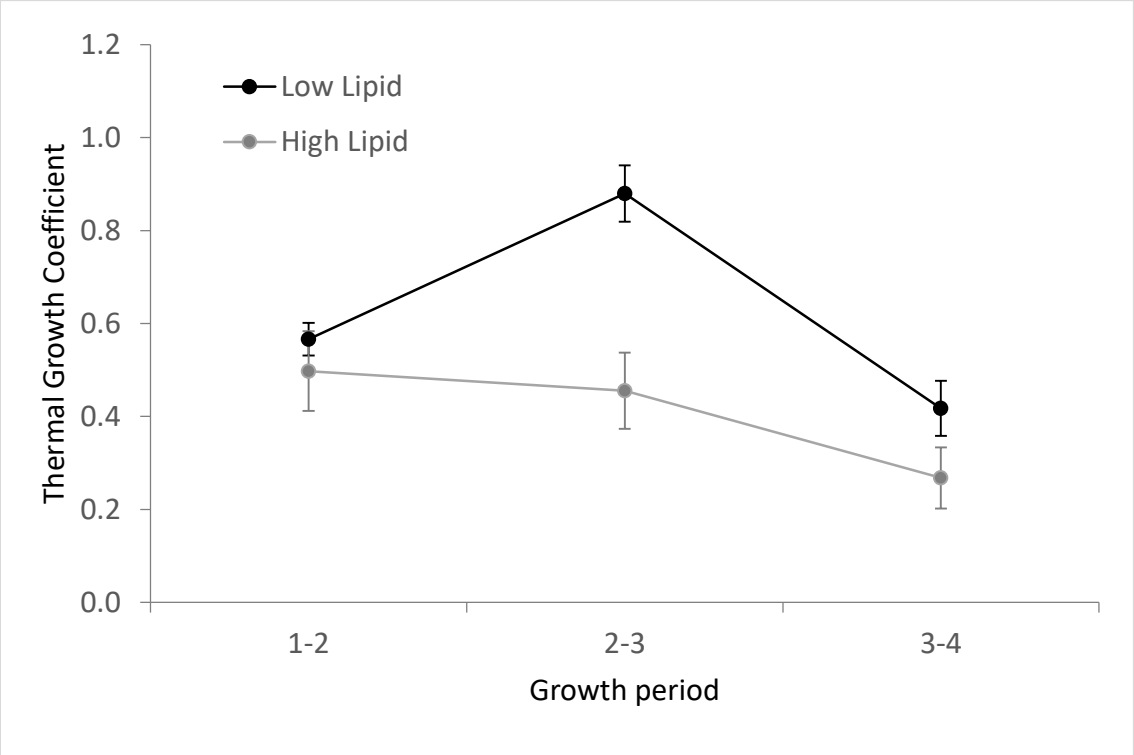

Figure 4.4. Changes in thermal growth coefficients between sampling periods. Data are presented as the mixed sex treatment means (± SE).

Table 4.5. Linear mixed effects model analysis of changes in weight (W) relative to both TIME and TREATMENT, with TREATMENT as a factor, TIME as a continuous variable, and individual as the random variable to account for repeated measures over time.

ANOVA: W ~ TIME * TREATMENT				
	numDF	denDF	F-value	p-value
(Intercept)	1	127	655.78	0.000
TIME	1	127	210.65	0.000
TREATMENT	1	41	0.53	0.472
TIME:TREATMENT	1	127	11.07	0.001

Table 4.6. Linear mixed effects model analysis of changes in Fulton's condition factor (K) over TIME, with TIME as a continuous variable, and individual included as a random variable to account for repeated measures over time.

ANOVA: K ~ TIME				
	numDF	denDF	F-value	p-value
(Intercept)	1	128	7049.62	0.000
TIME	1	128	13.29	0.000

Table 4.7. Linear mixed effects model analysis of changes in relative condition (Kn) over TIME, with TIME as a continuous variable, and individual included as a random variable to account for repeated measures over time.

ANOVA: Kn ~ TIME				
	numDF	denDF	F-value	p-value
(Intercept)	1	128	157640.64	0.000
TIME	1	128	15.21	0.000

Table 4.8. Linear mixed effects model analysis of changes in Thermal Growth Coefficient (TGC) relative to TIME and TREATMENT, with TREATMENT as a factor, TIME as a continuous variable, and individual included as a random variable to account for repeated measures over time.

ANOVA: TGC ~ TIME + TREATMENT				
	numDF	denDF	F-value	p-value
(Intercept)	1	85	167.80	0.000
TIME	1	85	10.65	0.002
TREATMENT	1	41	7.36	0.010

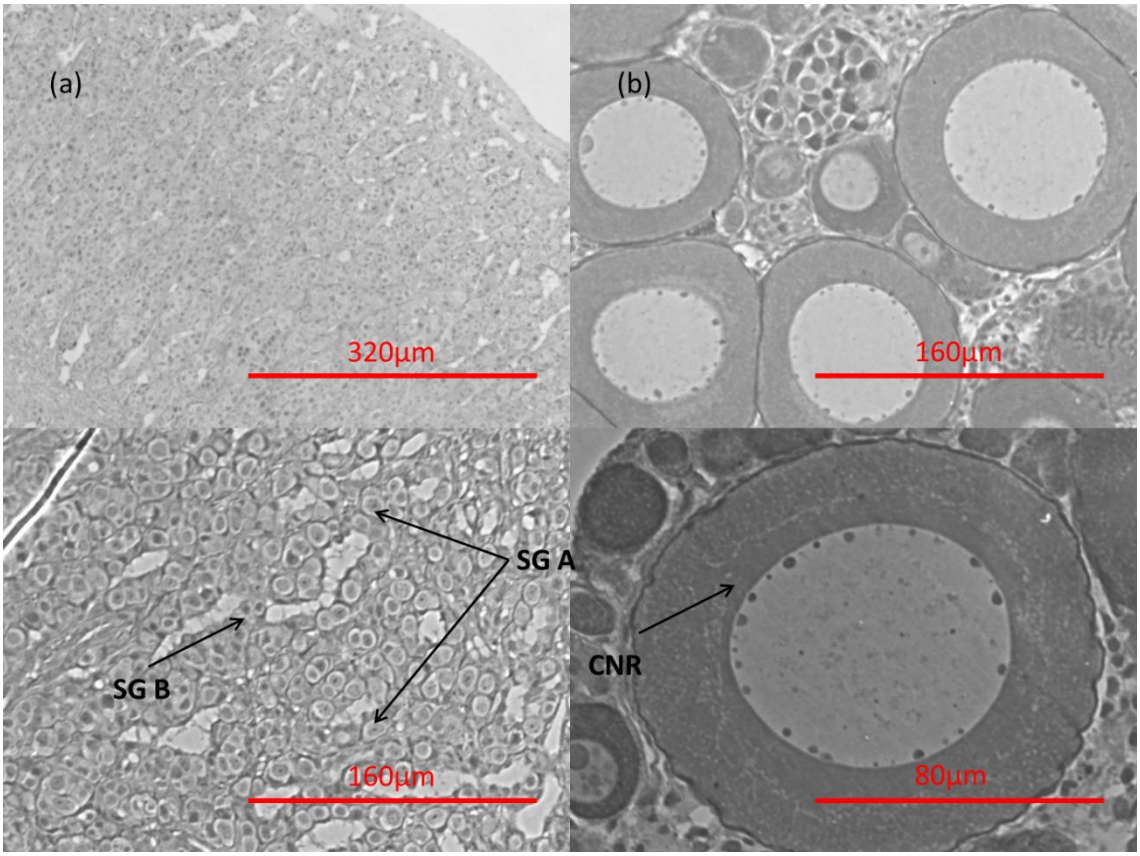

Figure 4.5. Histological analysis revealed similar developmental stages for all experimental fish, with early (SG A) and late (SG B) stage spermatagonia present in all male samples, and CNR stage oocytes present in all female samples.

Influence of Diet on Biometric Indices

RLM and GSI (males and females) were calculated and analysed at the end of the trial for all fish. However, despite difference in growth rate and overall size, there were no differences observed between treatments for any of these indices, owing to high variability within treatments.

Influence of Diet on Maturation

No gross signs of gonad development were observed during final sampling as illustrated by the low GSI values for both males (< 0.05%) and females (< 0.25%). Further, histological analysis of the gonads revealed that although cytological changes were occurring, neither oogenesis nor spermatogenesis had yet commenced for any of our experimental fish (Figure 4.5). Analysis of the leading cohort (LC) of oocytes for females revealed no significant differences between treatments with an overall mean of 150.9 ± 2.2 µm ($p = 0.919$). Thus all fish were classified as immature (juvenile) and no further analysis to compare maturation commitment between the treatments could be performed.

Comparison of Growth and Maturity Parameters with other Experimental Fish

Values for RLM, GSI (females only), leading cohort (LC) and condition (K and Kn) were compared with values for experimental haddock sampled at a similar time period from Tobin et al.'s (2010) study. Measures of GSI, LC and RLM from the 15th August (calendar day 228) and 13th September (calendar day 257) sampling points of this study were chosen for comparison as they straddled the sampling point from the current study (29th August, calendar day 242). Significant differences were observed between datasets for each variable, indicating that these fish were on different developmental trajectories and exhibiting different energetic states. A comparison of the LC of oocytes from females sampled in August (calendar day 228), and September (calendar day 257) from the Tobin

dataset and the LC of oocytes sampled in the present study (calendar day 242) was performed. Analysis revealed a significant difference between the two experiments ($Pr_{(>CHI)} = 0.02$), with larger oocytes observed for experimental fish in the current study (Figure 4.6).

Again, to allow for a comparison of somatic condition between experimental fish, a weight-length relationship was derived after log transforming both variables. A regression analysis (Figure 4.7) of this relationship was used to calculate the relative condition (Kn). Fulton's condition factor (K) was also calculated. There was a significant difference between datasets in both cases, with K ($Pr_{(>CHI)} < 0.01$) and Kn ($Pr_{(>CHI)} = 0.01$) being highest in the current dataset (Figure 4.8).

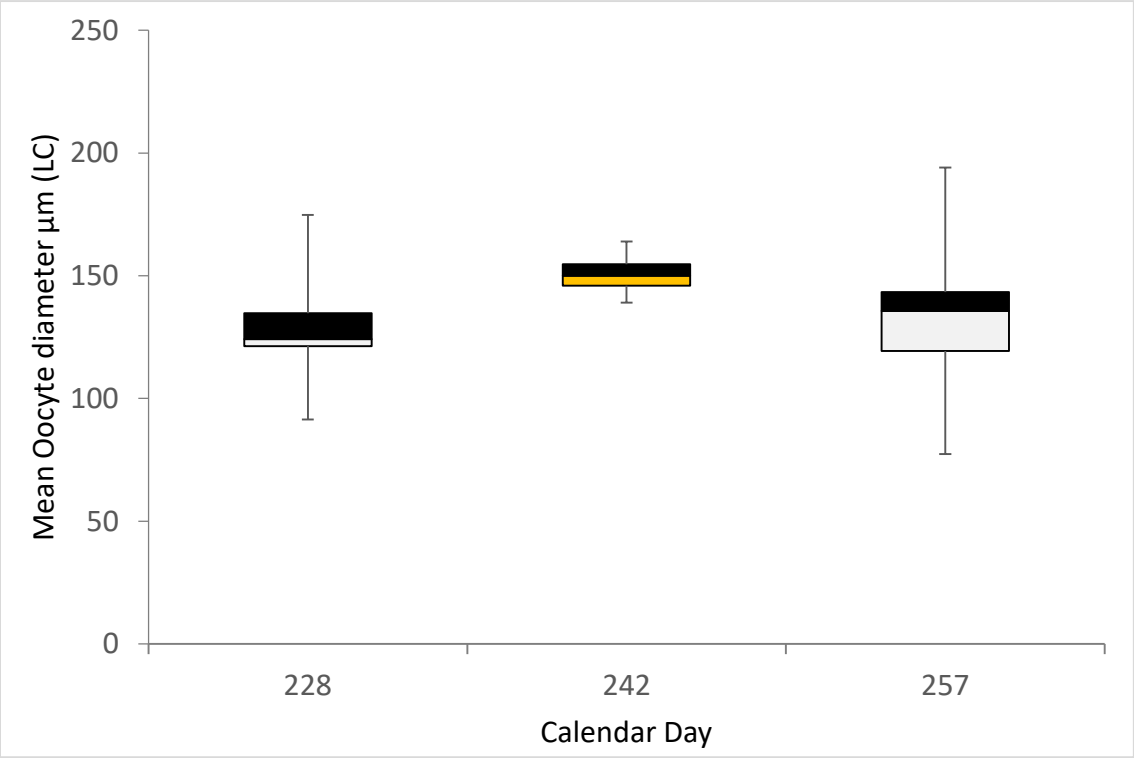

Figure 4.6. Boxplots illustrating the variation in leading cohort size for all female experimental fish in the current study (yellow) and female experimental fish from Tobin et al. 2010. Boxes are plotted against the day of sampling. Box represents the 25th and 75th percentiles and whiskers denote min and max values.

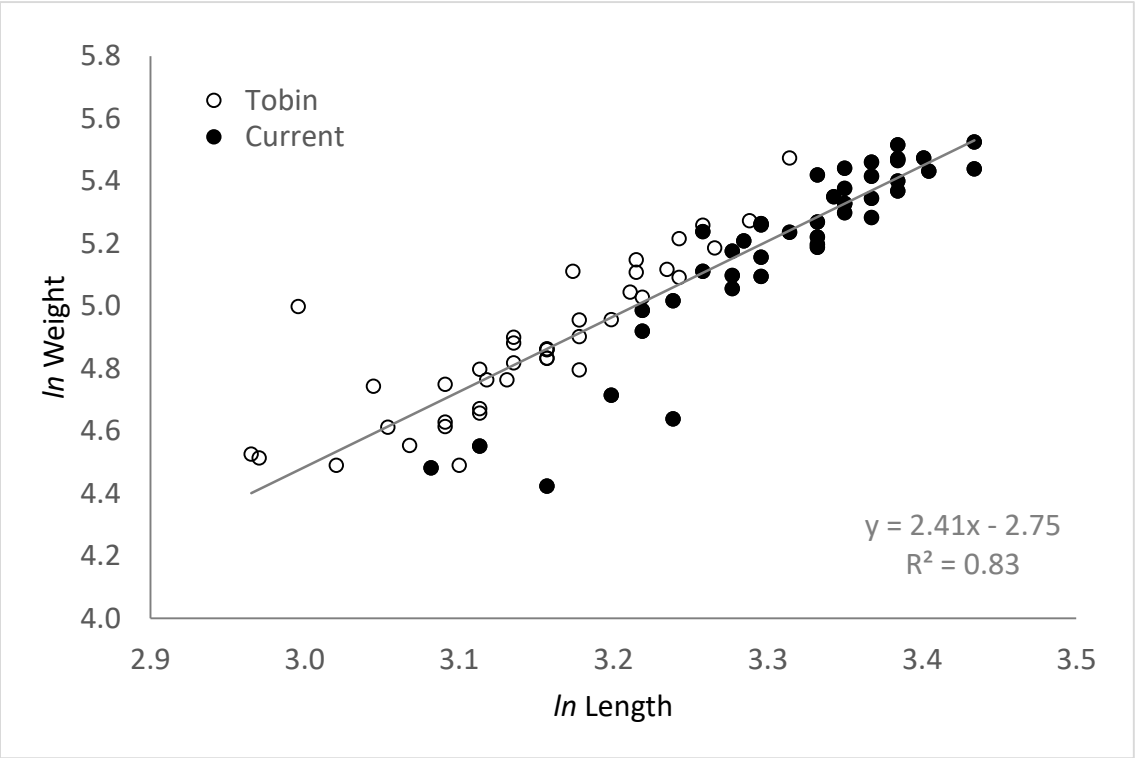

Figure 4.7. Regression of all log transformed lengths and weights for all fish from the current study and from the Tobin et al. 2011 dataset.

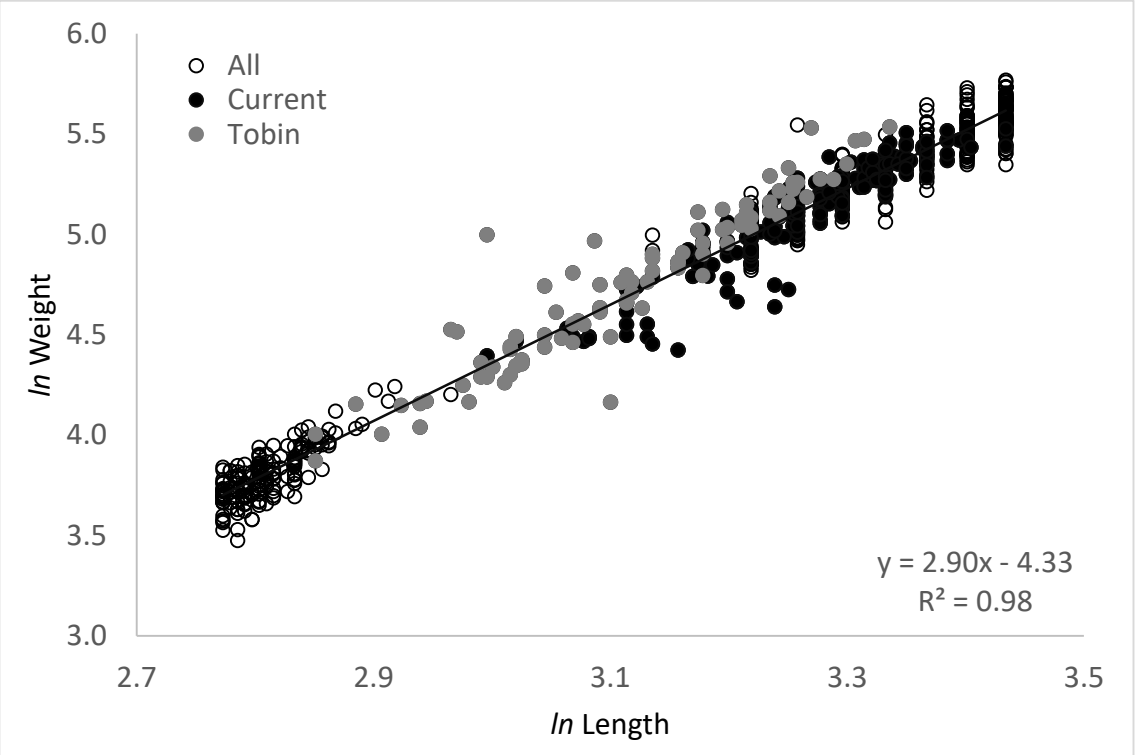

Figure 4.8. Regression of all log transformed lengths and weights for all fish from the current, Tobin et al. 2011, and wild datasets.

Comparative Condition of Experimental and Wild Fish

The K and Kn of experimental fish was compared with values for wild fish to assess the performance of the current diet. Once more a weight-length relationship was derived after log transforming both variables, and a regression analysis (Figure 4.8) of this relationship was used to calculate Kn. Significant difference were observed between datasets for both K ($Pr_{(>CHI)} < 0.01$) and Kn ($Pr_{(>CHI)} < 0.01$). Condition was highest in the current dataset.

4.3. EXPERIMENT II: IDENTIFYING THE CRITICAL PERIOD WHEN GROWTH LIFE-HISTORY INFLUENCES MATURATION COMMITMENT

4.3.1. Aims

This experiment was designed to identify the nutritionally sensitive "critical" period in maturing Atlantic cod, and investigate the nutritional status required to mature.

4.3.2. Methods

Fish Collection and Husbandry

70 juvenile (0-group) Atlantic cod were collected from the Firth of Clyde on the west coast of Scotland (55˚49' N, 04˚57' W) during Oct/Nov 2012. These cod were transferred to the Marine Laboratory in Aberdeen, were they were housed in a cylindrical tank (3000L) under a partial flow-through system. Temperature was maintained at 9˚C over the course of the trial, and photoperiod followed an ambient simulation (SNP, 57˚16'N). Prior to the trial, fish were fed *ad libitum* with a wet feed consisting primarily of chopped squid, sandeel and sprat.

Experimental Design

On the 6th March 2013, 73 individuals (mean weight 101 ± 4 g) were anaesthetised and a Passive Integrated Transponder (PIT) tag (Trovan Ltd, Hull, UK) was implanted into the peritoneal cavity to allow for future identification. These fish were then weighed (0.01 g), total length measured (0.1 cm), and allowed to recover in clean aerated water. Once recovered, fish were split between two replicate experimental tanks (7 x 3 x 1 m, 3000 L). Each tank was further split into two compartments using a plastic mesh separator, creating two replicate compartments for each tank. The fish were size matched between tanks to eliminate the risk of any potential size effects, and assigned (n=18-19) to each compartment. There were 3 mortalities (4%) associated with tagging. The remaining fish

were allowed to recover for three weeks prior to the commencement of the diet trial. Temperature in the experimental tanks was maintained at 9ºC during the trial, and a Simulated Natural Photoperiod (SNP) was used. All fish were aged 1.

The trial itself commenced on the 26th March 2013, 12 weeks prior to the Summer solstice (June 21st). A total of 70 fish were split into two treatment groups, with replicates, and subject to ration manipulation covering a 24-week period from March to September. This 24-week period represented two proposed "nutritionally sensitive" periods – the first covering the time period prior to the Summer solstice (increasing day length), and the second covering the 12 weeks following the solstice (decreasing day length). The first treatment group were fed a high ration diet of squid for the first 12 weeks, followed by a low ration diet for the remaining 12-week period, and are hereafter referred to as the high to low (HL) treatment. A total of 34 fish were maintained under this treatment with n = 16 in replicate 1 and n = 18 in replicate 2. Conversely, the second treatment group were fed a low ration diet for the first 12-week period, followed by a high ration diet for the remainder of the trial, and are here-after referred to as the low to high (LH) treatment. This treatment group consisted of 32 fish, with n = 16 in each replicate. Due to fish exhibiting weight loss early in the trial, these diet treatments were altered on three occasions prior to the switch in June. To account for this, the same rations were mimicked in the second half of the trial in an attempt to match growth patterns between the two periods. A detailed account of the feeding regime is given in Table 4.9.

Monitoring

To obtain individual growth profiles, fish were measured every 21 days over the course of the experiment. Prior to each measurement session, fish were deprived of food for ~24 hrs to ensure evacuation of the gut (Jones, 1974). Under anaesthesia (MS-222, 100 mg l^{-}

^{1}), fish were identified and measures of total length (± 0.1 cm) and weight (± 0.01 g) were obtained.

Table 4.9. Summary of the dietary rations for each replicate of the Low to High (LH) and High to Low (HL) treatments in % body weight per day. Changes in ration following sampling are shown for each period.

	Ration (%BW.day^{-1})	
Period	**High to Low**	**Low to High**
26th Mar - 19th Apr	2	1
19th Apr - 10th May	2.4	1.4
10th May - 22nd May	2.8	1.8
22nd May - 21st June	3.2	2.2
21st June - 12th July	1	2
12th July - 2nd Aug	1.4	2.4
2nd Aug - 14th Aug	1.8	2.8
14th Aug - 22nd Oct	2.2	3.2

Table 4.10. Summary of the distribution of animals by sex for each replicate, with initial mean weight [0.1 g (range)] and mean length [0.1 cm (range)]. There were no significant differences in Length (p = 0.963) or Weight (p = 0.860) at the start of the trial (ANOVA).

Treatment	N	Length [mean (range)]	Weight [mean (range)]
High to Low	32	22.8 (18.9 - 27.1)	104.6 (50.2 - 158.6)
High to Low 1	16	22.8 (19.9 - 26.5)	103.8 (66.4 - 158.2)
High to Low 2	16	22.9 (18.9 - 27.1)	105.5 (50.2 - 158.6)
Low to High	34	22.7 (17.8 - 28.6)	105.5 (43.4 - 190)
Low to High 1	16	22.8 (17.8 - 28.6)	105 (43.4 - 190)
Low to High 2	18	22.6 (19 - 26.7)	106 (59.4 - 159.4)

Table 4.11. Food conversion efficiency for each replicate (replicates 1 and 2 for each treatment) presented as the mixed-sex replicate and treatment means between for each sampling period.

Treatment	FCE 1-2	FCE 2-3	FCE 3-4	FCE 4-5	FCE 5-6	FCE 6-7	FCE 7-8	FCE 8-9	FCE total
High to Low	0.05	0.08	0.08	0.16	-0.24	0.03	0.24	0.15	0.09
High to Low 1	0.05	0.09	0.09	0.18	-0.20	-0.03	0.19	0.17	0.10
High to Low 2	0.06	0.07	0.06	0.15	-0.29	0.08	0.28	0.13	0.09
Low to High	-0.10	-0.02	0.04	0.10	0.05	0.11	0.30	0.19	0.12
Low to High 1	-0.13	-0.02	0.05	0.13	0.07	0.08	0.29	0.22	0.12
Low to High 2	-0.08	-0.01	0.02	0.07	0.03	0.13	0.31	0.17	0.11

Terminal sampling

On the 22nd October 2013, all fish were sacrificed. Final measures of total length and weight were taken before fish were dissected to obtain liver weight (± 0.0001 g), gonad weight (± 0.0001 g), and somatic weight (± 0.01 g). Gender was assessed visually, and gonad samples were taken and stored in 10% neutral buffer formalin (NBF) for histological analysis.

The overall population sex ratio (males to females) was 1:0.74. For the HL treatment the sex ratio was 1:0.52, compared with 1:1 for the LH treatment. 4 fish exhibited excessive weight loss during the trial and were euthanized. Data for these fish were not included in this study. There were no significant differences in length or weight between treatments at the beginning of the trial (Table 4.10).

Other data sources

To show how condition in the current study compared with condition in wild fish we used data collected through the Scottish Government observer programme on cod stocks from the North Sea and north-east Atlantic. This data included measurements of total length and total weight from cod sampled between March and October, from 1994 to 2004. Measurements from a total of 157 male and 125 female cod, of a comparable size/age, were included in this dataset.

Statistical analysis

Relative weight indices were calculated according to the methods described in Section 2.6. For this experiment, the influence of the diet manipulations on measures of relative weight indices (GSI, RLM) and condition (Kn, and K) were analysed through GLM, with both sex and treatment as factors, and replicate as a factor nested within treatment.

To account for repeated measures, changes in length, weight and thermal growth coefficients (TGC) over the course of the study were analysed using a linear mixed-effects model. Again, sex and treatment were treated as factors with replicate nested within treatment, and time was treated as a continuous variable. Individual variability over time was treated as a random variable (~ time | fish). Normality and homogeneity of variance were improved where necessary through log transformation. A significance level of $p<0.05$ was set in all cases and significant interactions were analysed by Tukey's *post hoc* test. All statistical tests were undertaken in R (version 3.2.2).

4.3.3. Results

Influence of Diet on Growth Parameters

Due to weight losses in ~22% of the weight measurements taken, the dietary rations were adjusted on three occasions during the first half of the trial and then mimicked in the second half. Details of the resulting ration manipulations are given in Table 4.9. Food conversion efficiency (FCE) was calculated for each group as a ratio of weight gained to feed weight provided, as rations were matched between treatments in terms of % body weight. Differences were observed in the overall FCE between treatments with fish from the LH treatment exhibiting higher overall FCE (Table 4.11).

As there was no significant effect of sex for any variable or any tank effects observed, data are presented as the treatment means ± the standard error (SE). Length increased significantly between time points ($p<0.01$; Table 4.12), but there was no effect of treatment on this variable or any significant interactive effect observed. The HL treatment increased in length during the first 12-week period, with a length increase 3% higher than the LH treatment over this period. However, LH treatment increase in length during the second 12-week period, surpassing the HL treatment by the end of the trial with an overall

length increase of over 15 % compared with just 12.5 % for the HL treatment (Figure 4.9).

Similarly, total weight increased significantly over the trial period (Figure 4.10). There was a significant change in weight over time ($p < 0.01$) and an interactive effect of time and treatment observed ($p < 0.01$), but no significant difference between the overall treatment mean weight values ($p = 0.62$; Table 4.13). Mean weight was almost 20% higher in the HL treatment by the end of the first 12-week period, but weight gain slowed over the following 12-weeks to a final total increase of 33 %, which was 7% lower than the overall weight gain by the LH treatment.

Table 4.12. Linear mixed effects model analysis of changes in length (L) over TIME, TIME as a continuous variable, and individual as the random variable to account for repeated measures over time.

ANOVA: L ~ TIME				
	numDF	denDF	F-value	p-value
(Intercept)	1	527	14352.49	0.000
TIME	1	527	304.60	0.000

Table 4.13. Linear mixed effects model analysis of changes in weight (W) relative to both TIME and TREATMENT, with TREATMENT as a factor, TIME as a continuous variable, and individual as the random variable to account for repeated measures over time.

ANOVA: W ~ TIME * TREATMENT				
	numDF	denDF	F-value	p-value
(Intercept)	1	526	1569.93	0.000
TIME	1	526	358.62	0.000
TREATMENT	1	64	0.25	0.620
TIME:TREATMENT	1	526	9.11	0.003

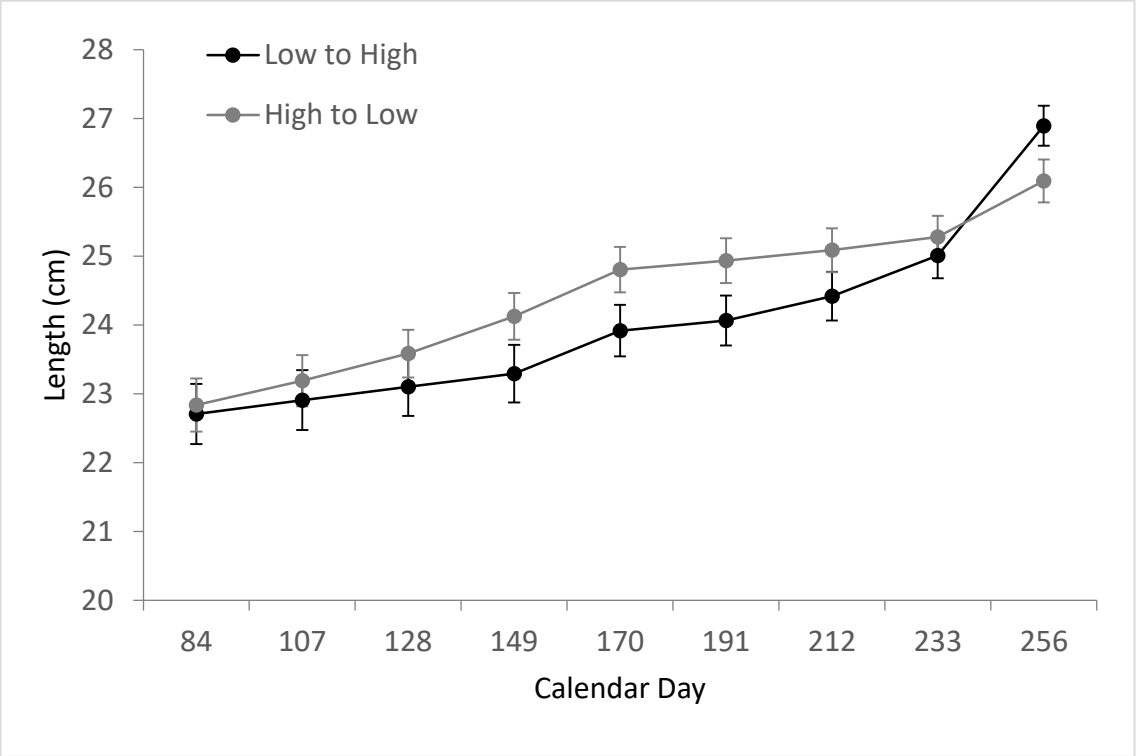

Figure 4.9. Changes in mean length between sampling periods. Data are presented as the mixed sex treatment means (± SE).

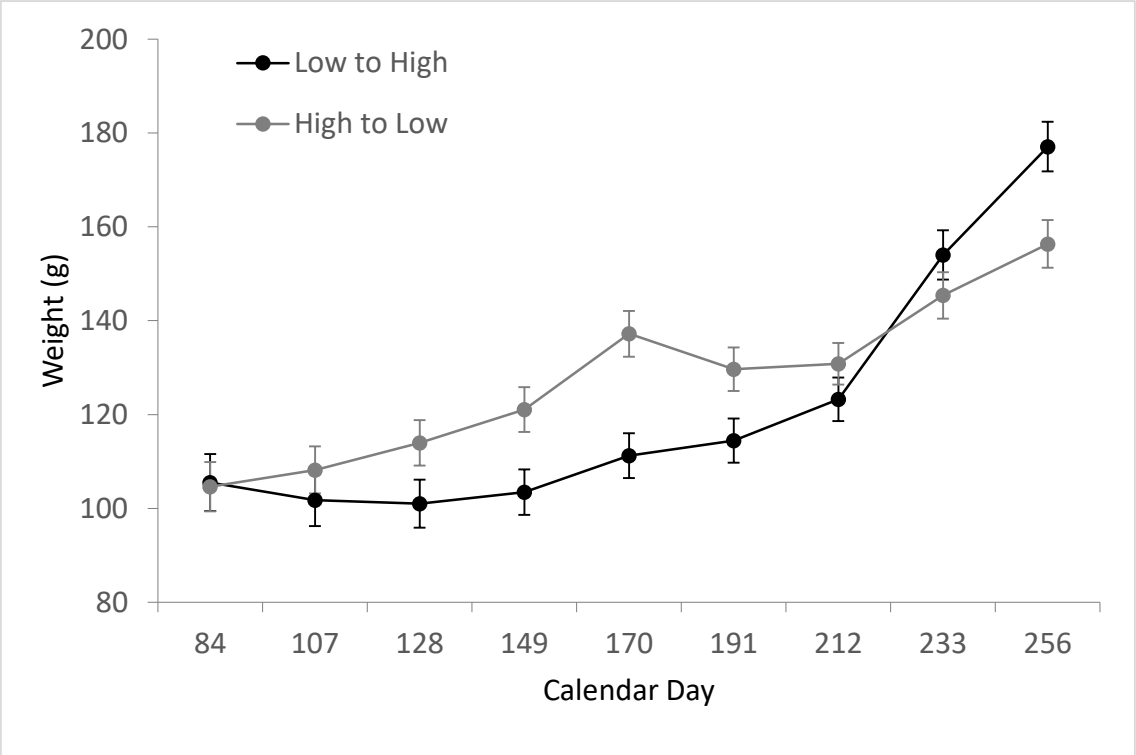

Figure 4.10. Changes in mean weight between sampling periods. Data are presented as the mixed sex treatment means (± SE).

To compare somatic condition among experimental fish, a weight-length relationship was derived after log transforming both variables. A regression analysis (Figure 4.11) of this relationship was used to calculate Kn. Fulton's condition factor (K) was also calculated to enable comparison with other studies. K did change significantly over the duration of the trial ($p < 0.01$), and significant differences were observed between treatment ($p = 0.02$). This treatment effect also changed with time, observed as a significant interaction ($p < 0.01$; Table 4.14). Initially, condition decreased among the LH fish and increased in the HL treatment, creating a difference in the mean values between these treatments at the switch in June of 0.09. However, following the switch, condition in the LH treatment increased by 0.1, reaching a final mean value of 0.9. K in the HL treatment decreased following the switch to finish at a mean value of 0.87 (Figure 4.12).

Kn also changed significantly over the duration of the trial ($p < 0.01$), but there was no difference in the mean Kn between treatments ($p = 0.1$). The treatment mean values did change over the time period, observed as a significant interaction ($p < 0.01$; Table 4.15), and like K, Kn was greatest in the HL treatment prior to the switch in June, with an observed difference of 0.02 between the mean values. However, following the switch, Kn increased for the LH treatment with both treatments finishing the trial period with comparable mean Kn values (Figure 4.13).

Growth rate, expressed as the thermal growth coefficient (TGC) differed significantly between treatments ($p = 0.01$), and increased over the duration of the trial ($p < 0.01$), and again there was a significant interaction observed ($p < 0.01$; Table 4.16), with growth being 1.78 times higher in the HL treatment prior to the switch. Following the switch, growth rate dropped in the HL treatment and at the end of the trial, TGC was 1.82 times higher in the LH treatment (Figure 4.14).

Table 4.14. Linear mixed effects model analysis of changes in Fulton's condition factor (K) relative to both TIME and TREATMENT, with TREATMENT as a factor, TIME as a continuous variable, and individual as the random variable to account for repeated measures over time.

ANOVA: K ~ TIME * TREATMENT				
	numDF	denDF	F-value	p-value
(Intercept)	1	526	22259.09	0.000
TIME	1	526	44.10	0.000
TREATMENT	1	64	5.54	0.022
TIME:TREATMENT	1	526	23.72	0.000

Table 4.15. Linear mixed effects model analysis of changes in relative condition (Kn) relative to both TIME and TREATMENT, with TREATMENT as a factor, TIME as a continuous variable, and individual as the random variable to account for repeated measures over time.

ANOVA: Kn ~ TIME * TREATMENT				
	numDF	denDF	F-value	p-value
(Intercept)	1	526	502576.00	0.000
TIME	1	526	46.50	0.000
TREATMENT	1	64	2.80	0.096
TIME:TREATMENT	1	526	18.70	0.000

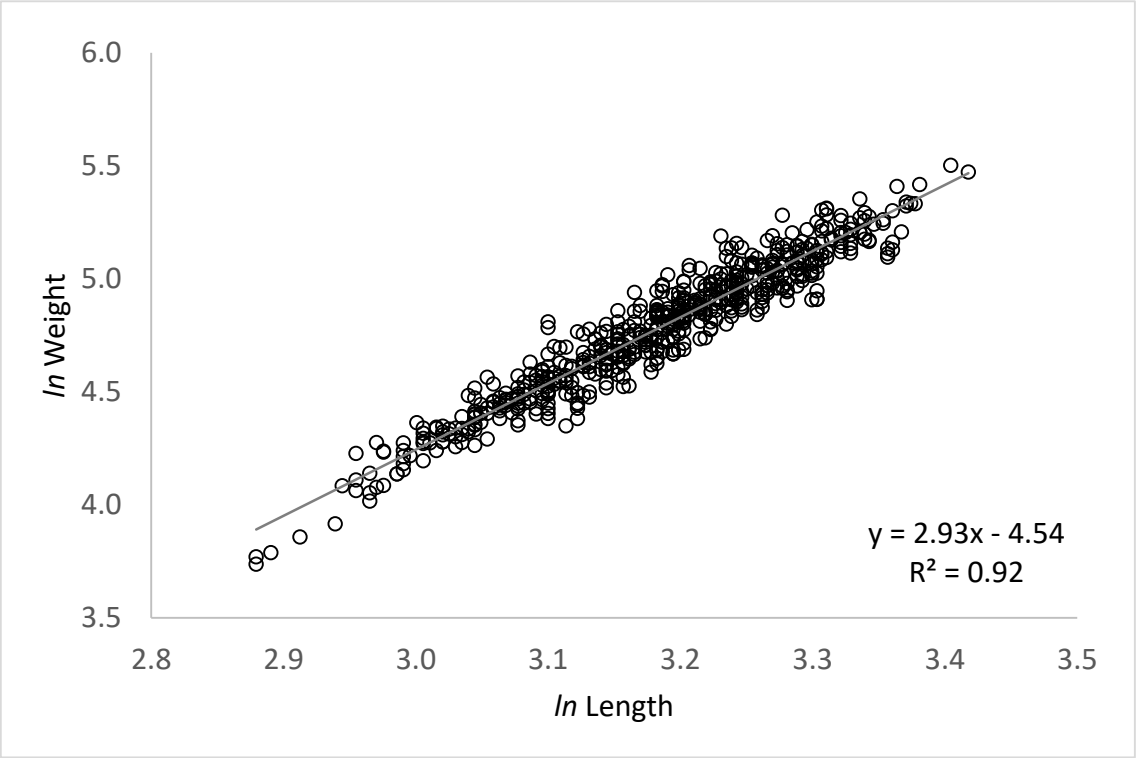

Figure 4.11. Regression of all log transformed lengths and weights for all fish from the current study.

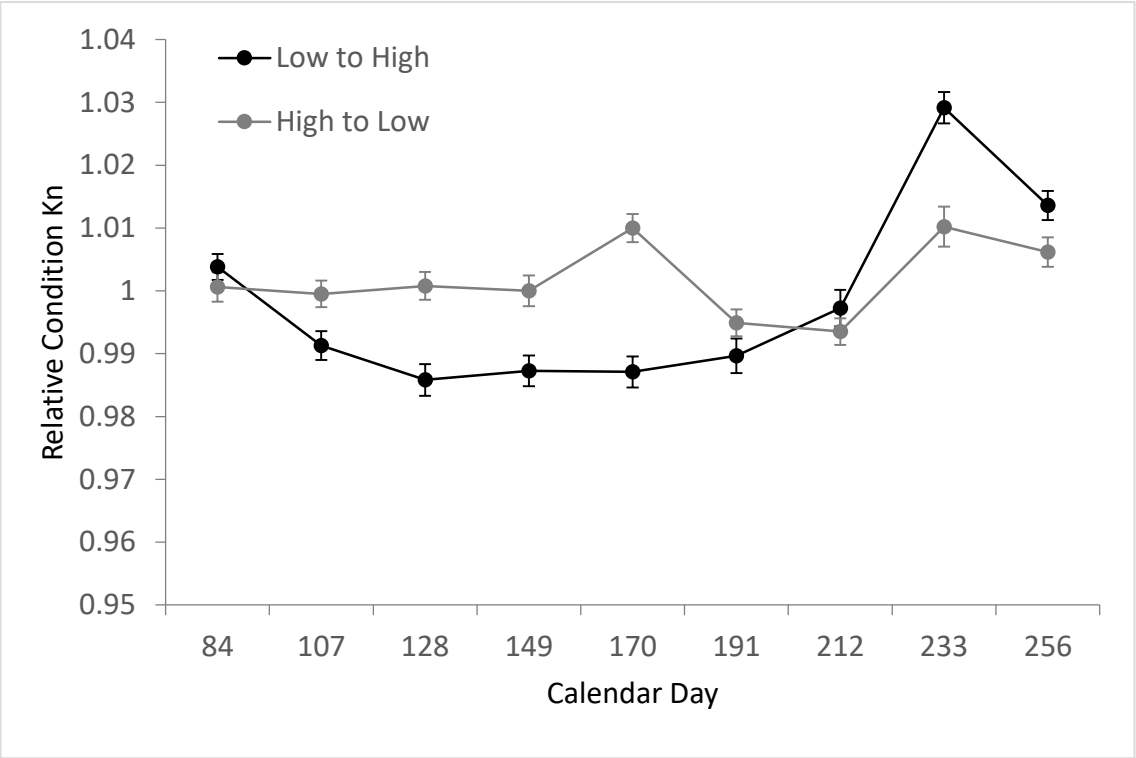

Figure 4.12. Changes in mean relative condition (Kn) between sampling periods. Data are presented as the mixed sex treatment means (± SE).

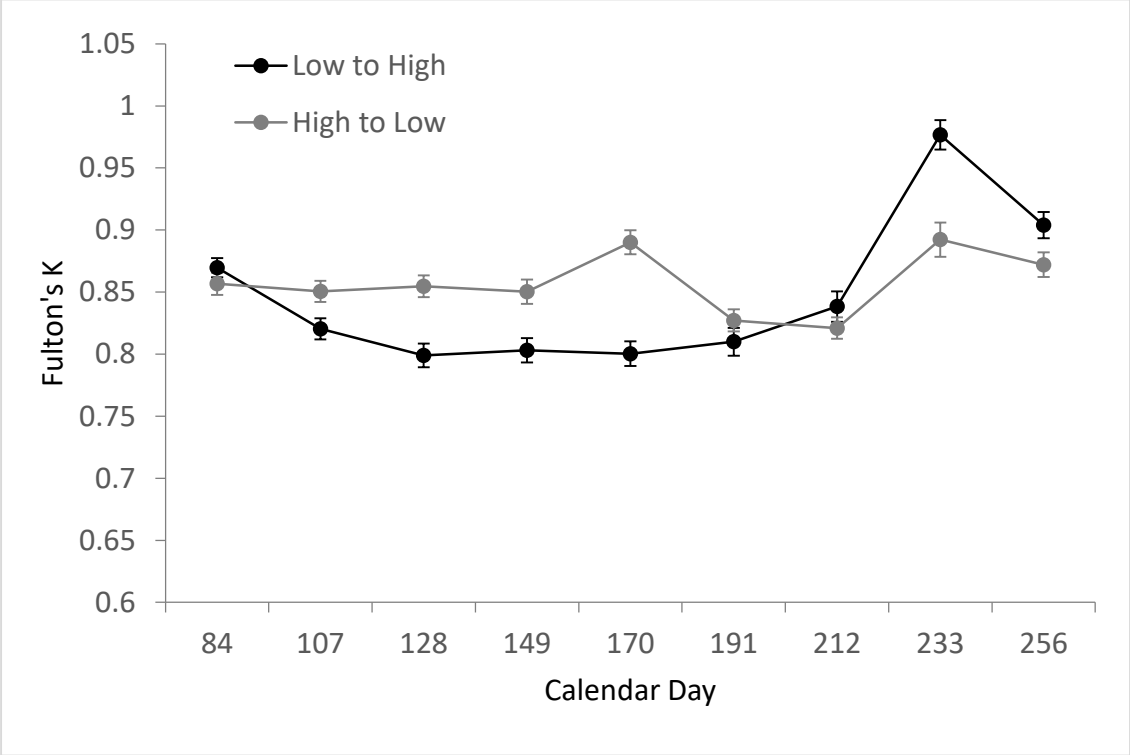

Figure 4.13. Changes in mean Fulton's condition factor (K) between sampling periods. Data are presented as the mixed sex treatment means (± SE).

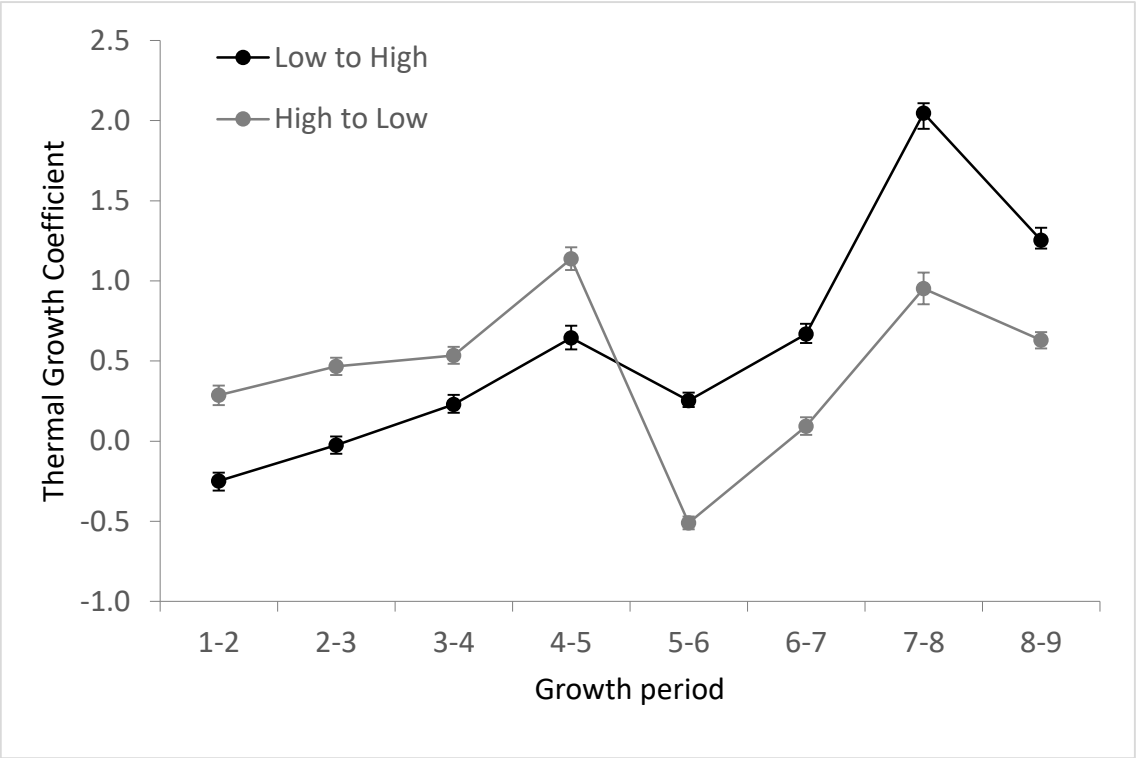

Figure 4.14. Changes in mean thermal growth coefficient between sampling periods. Data are presented as the mixed sex treatment means (± SE).

Table 4.16. Linear mixed effects model analysis of changes in the Thermal Growth Coefficient (TGC) relative to both TIME and TREATMENT, with TREATMENT as a factor, TIME as a continuous variable, and individual as the random variable to account for repeated measures over time.

ANOVA: TGC ~ TIME * TREATMENT				
	numDF	denDF	F-value	p-value
(Intercept)	1	460	353.83	0.000
TIME	1	460	197.24	0.000
TREATMENT	1	64	7.45	0.008
TIME:TREATMENT	1	460	133.01	0.000

Table 4.17. General linear model analysis of differences in the mean Leading Cohort (LC) size between TREATMENTs.

ANOVA: LC ~ TREATMENT					
	Df	Dev. Resid.	Df	Resid. Dev	Pr(>Chi)
NULL			37	9318.000	
TREATMENT	1	66.500	36	9251.500	0.521
TREATMENT:TANK	2	3753.900	34	5497.600	0.000

These results reflect the design of the experiment well, with comparable start and end points for all variables, but significant differences being observed relative to the changes in ration.

Influence of Diet on Biometric Indices

RLM and GSI values were calculated and analysed at the end of the trial for all fish. However, despite difference in growth rate and overall size, there were no differences observed between treatments for any of these indices. Overall mean values (± SE) at the end of the trial were 1 (± 0.04) for the RLM, 0.07 % (± 0.01) for male GSI and 0.27 % (± 0.01) for female GSI.

Influence of Diet on Maturation

Fish were classified as maturing or immature through histological analysis of the gonads. Maturing males were identified by the presence of spermatids, females by the presence of cortical alveoli. Three fish – one male (no. 47) from the HL treatment, and one male (no. 65) and one female (no. 4) from the LH treatment, were classified as maturing in the present study. These numbers were too low to analyse statistically, however there was an observable trend for mature fish to be larger than average by the end of the trial. Fish number 65 was one of the heaviest fish (top 8%) within the LH treatment at the start of the trial. This fish did not increase in size prior to the switch, but grew following the switch to achieve a final weight of 182.3 g. In contrast, fish number 4 from the same treatment was one of the smallest at the start of the trial (bottom 27%). However, this fish continually increased in size over the course of the trial reaching a final size of 189.8 g. Fish number 47 from the HL treatment, like fish number 65, started large, falling within the top 21 % in terms of weight. This fish increased in size continually over the course of

the trial, particularly in the final 21-day stretch when this individual increased in weight by almost 30 % reaching a final weight of 205.1 g (Figure 4.15).

Analysis of the mean LC values for females in each treatment revealed a significant difference between replicates ($Pr_{(>CHI)} < 0.01$) but not treatments ($Pr_{(>CHI)} = 0.52$; Table 4.17), owing to a high level of variability within treatments (Figure 4.16).

Comparative Condition of Experimental and Wild Fish

The K and Kn of experimental fish was compared with values for wild fish to assess the performance of the current diet. Once more a weight-length relationship was derived after log transforming both variables, and a regression analysis (Figure 4.17) of this relationship was used to calculate Kn. Significant difference were observed between datasets for both K ($p < 0.01$) and Kn ($p < 0.01$). Condition was lowest in the current dataset.

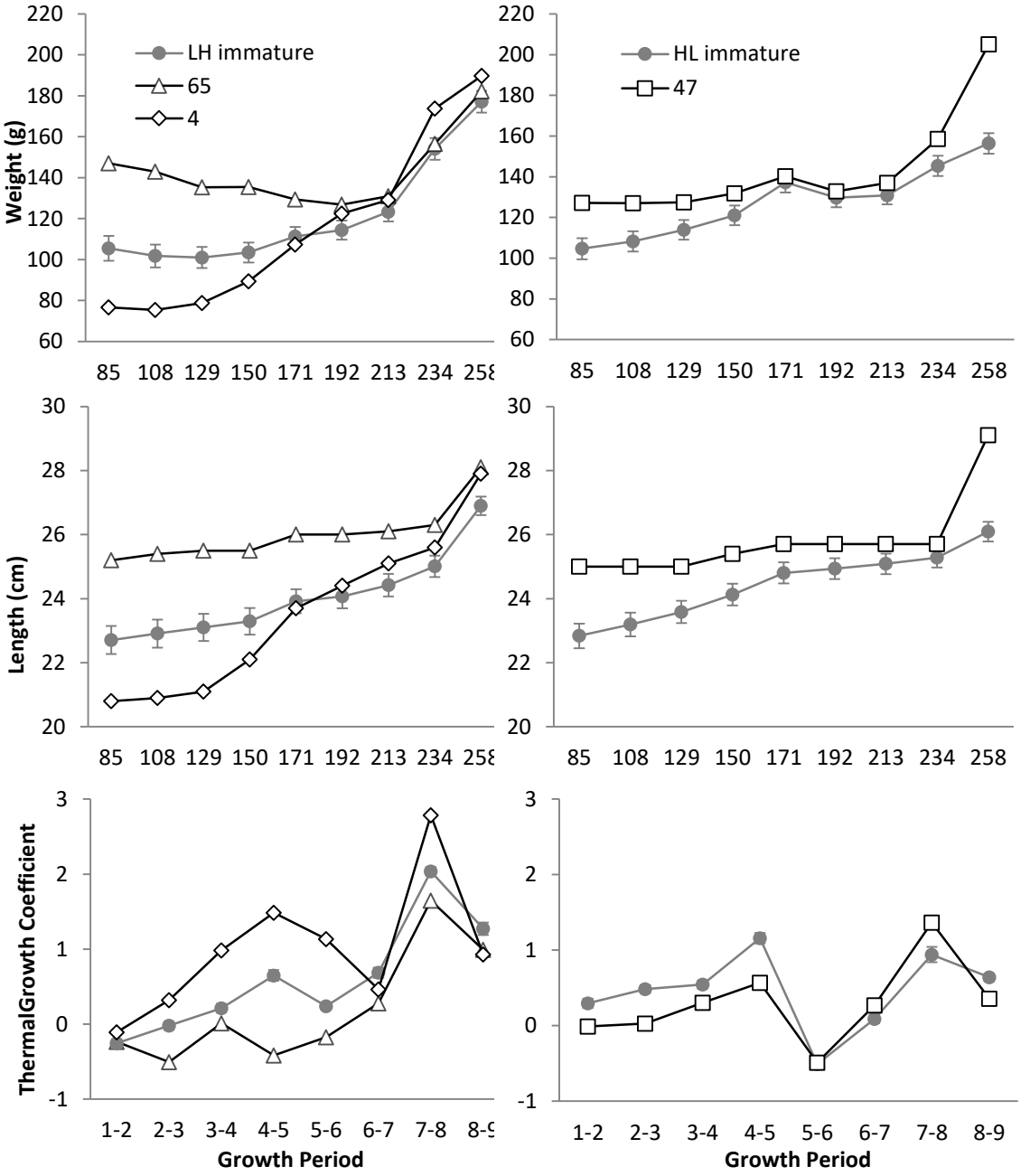

Figure 4.15. Comparison of changes in the mean length, weight and TGC between mature and immature fish from the LH and HL treatments. Data are presented as the mixed sex group means (± SE).

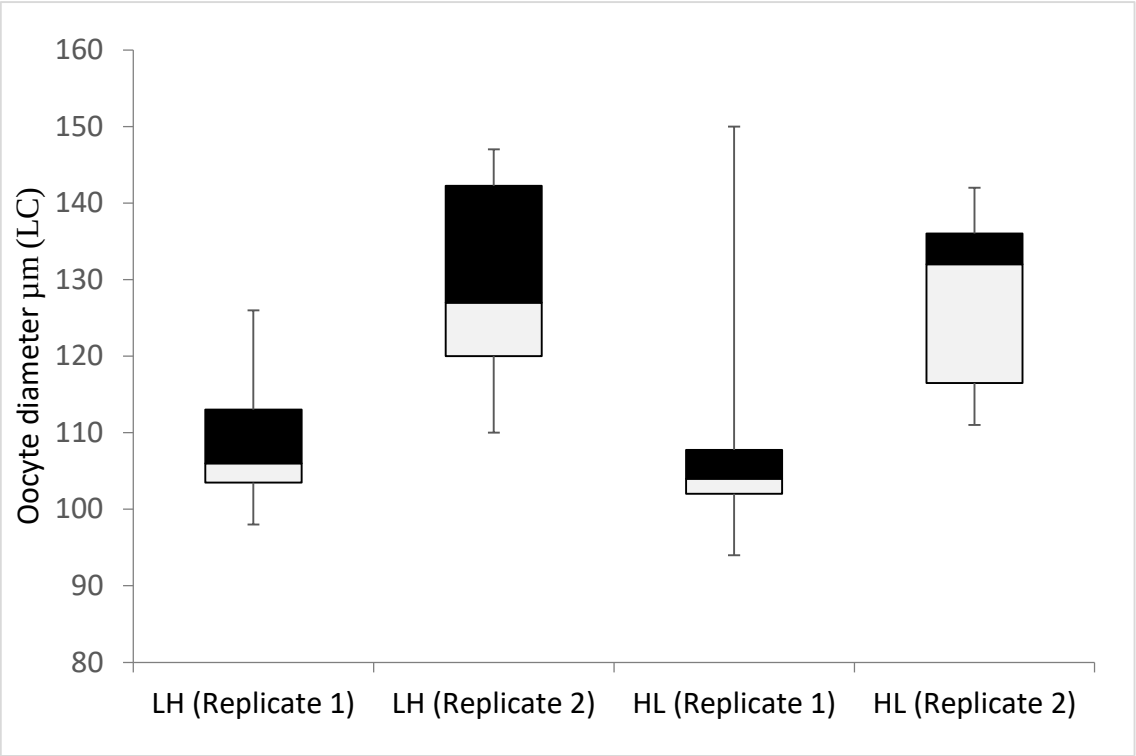

Figure 4.16. Boxplots illustrating the variation in mean leading cohort size females from each replicate of the HL and LH treatments. Box represents the 25th and 75th percentiles and whiskers denote min and max values.

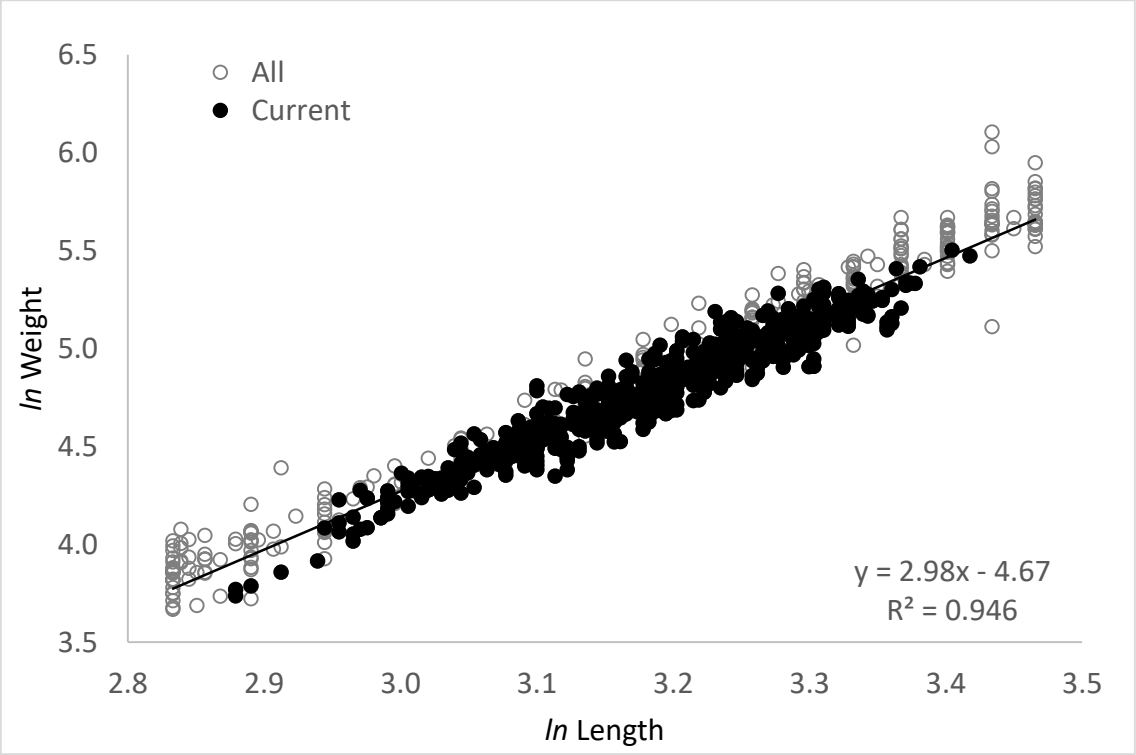

Figure 4.17. Regression of all log transformed lengths and weights for all fish from the current study and for cod from the wild dataset.

4.4. EXPERIMENT III: TO INVESTIGATE THE REGULATORY PATHWAYS RESPONSIBLE FOR INTEGRATING SIZE/ENERGETIC STATUS RELATED INFORMATION INTO THE BPG

4.4.1. Aims

To explore the influence of dietary restriction on maturation commitment as assessed through biomarkers of the PNES system.

4.4.2. Methods

Fish Collection and Husbandry

Juvenile 0-group cod were collected from the wild off Stonehaven on the east coast of Scotland (56˚57'50'' N, 02˚12'04'' W) between August 2013 and March 2014. These cod were transferred to the Marine Laboratory in Aberdeen, where they were housed in a cylindrical tank (3000 L) under a partial flow-through system. Photoperiod was maintained at ambient (57˚16' N), and temperature was kept at 9 ˚C. Prior to the trial, fish were fed *ad libitum* with a pellet feed (Vitalis 5mm; Skretting UK, Cheshire), and supplemented with wet feed.

Experimental Design

On the 9th April 2014, 165 individuals (mean weight 40 ± 1.6 g; Table 4.18) were anaesthetised and PIT tagged (Trovan Ltd, Hull, UK) to allow for future identification. These fish were then weighed (± 0.01 g), total length measured (± 0.1 cm), and allowed to recover in clean aerated water. There were 8 mortalities associated with tagging and a further 34 died over the course of the experiment and were excluded from analysis. This high mortality rate was attributed to aggression and associated cannibalism by larger individuals.

The trial commenced on the 15th May. Fish were anaesthetised, weighed (± 0.01 g) and measured (TL; ± 0.1 cm) and allowed to recover. Once recovered, fish were split between the four compartments of the same two replicate experimental tanks previously detailed in above. These fish were split according to size with one treatment containing the larger fish and one treatment with the smaller fish. The larger fish treatment group was fed *ad libitum* on pellet feed supplemented with wet feed when available. This treatment, hereafter known as the high ration (HR) treatment featured 81 fish, split between two replicates (n = 41 and n = 40). The smaller fish were fed a restricted diet, adapted from previous trials to inhibit maturation. These experimental fish, hereafter referred to as the low ration treatment (LR), were split between two replicates (n = 42 each) and were fed a low ration of pellet feed, calculated as a percentage biomass (% B) using the formula:

$$W_f = B_t.R.100^{-1}$$

where B_t is the biomass of the treatment group and R is the ration expressed as % B. The ration for this group was 2.25 % B but was increased to 3 % B as fish in this treatment were exhibiting poor health. Details of the feeding regime are given in Table 4.18. Throughout the trial, temperature was maintained at 9ºC and photoperiod followed an ambient simulation (SNP, 57˚16'N).

Monitoring

To obtain individual growth profiles and assess fish health, fish were monitored every 21 days following trial commencement. Prior to each measurement session, feed was withheld for approximately 24 hrs to ensure evacuation of the gut (Jones, 1974). Under anaesthesia (MS-222, 100mg l^{-1}), fish were identified and measures of total length (± 0.1 cm) and weight (± 0.01 g) were obtained.

Terminal sampling

Fish were terminally sub-sampled on the 12th September, 23rd October and the 21st November. On these dates, 10 individuals from each replicate were randomly chosen and euthanized. Final measures of length and weight were taken before fish were dissected to collect brain and pituitary tissue. These were snap frozen over liquid nitrogen and subsequently stored at -80 ºC. Measures of liver weight (± 0.001 g), gonad weight (± 0.001 g), and somatic weight (± 0.01 g) were also taken. Gender was assessed visually, and gonad samples were taken and stored in 10% neutral buffer formalin (NBF) for histological analysis. Samples from the 21st of November were assayed for *Eya3* expression following the QPCR protocol detailed in Section 2.1.7.

The overall population sex ratio (males to females) was 1:1. For the HR treatment, the sex ratio was 1:1.1, compared with 1:0.9 for the LR treatment.

Statistical analysis

Relative weight indices (GSI and RLM), and *Eya3* levels were analysed using a general linear model (GLM), as described for Experiments I and II above. Maturity was analysed using a binomial GLM, with logit link to account for the binomial nature of this response. Sex, and treatment were treated as factors, with time as a continuous variable. Tank was treated as a nested factor within treatment, and measures of length and weight at key time points, as well as final liver size, were also included in the full model, which was then simplified by forward selection of variables based on decreasing residual deviance and Akaike information criterion (AIC) allowing for all possible interactions.

To account for repeated measures, changes in length, weight, K, Kn, and TGC over the course of the study were analysed using a linear mixed-effects model, with individual variability over time set as a random variable, as described previously for Experiments I

and II. Assumptions of normality and homogeneity of variance were achieved through log transformation. A significance level of $p<0.05$ was set in all cases and significant interactions were analysed by Tukey's *post hoc* test. All statistical tests were undertaken in R (version 3.2.2).

4.4.3. Results

Due to observed loss of weight among some of the LR individuals, the dietary rations were adjusted on two occasions during the trial period. Details of the resulting ration manipulations are given in Table 4.19. The HR diet remained the same throughout. The mortality rate was relatively high for this experiment, with 20 – 29 % of fish lost from each treatment (Figure 4.19) over the course of the trial. Measurements from these fish were omitted from subsequent analysis.

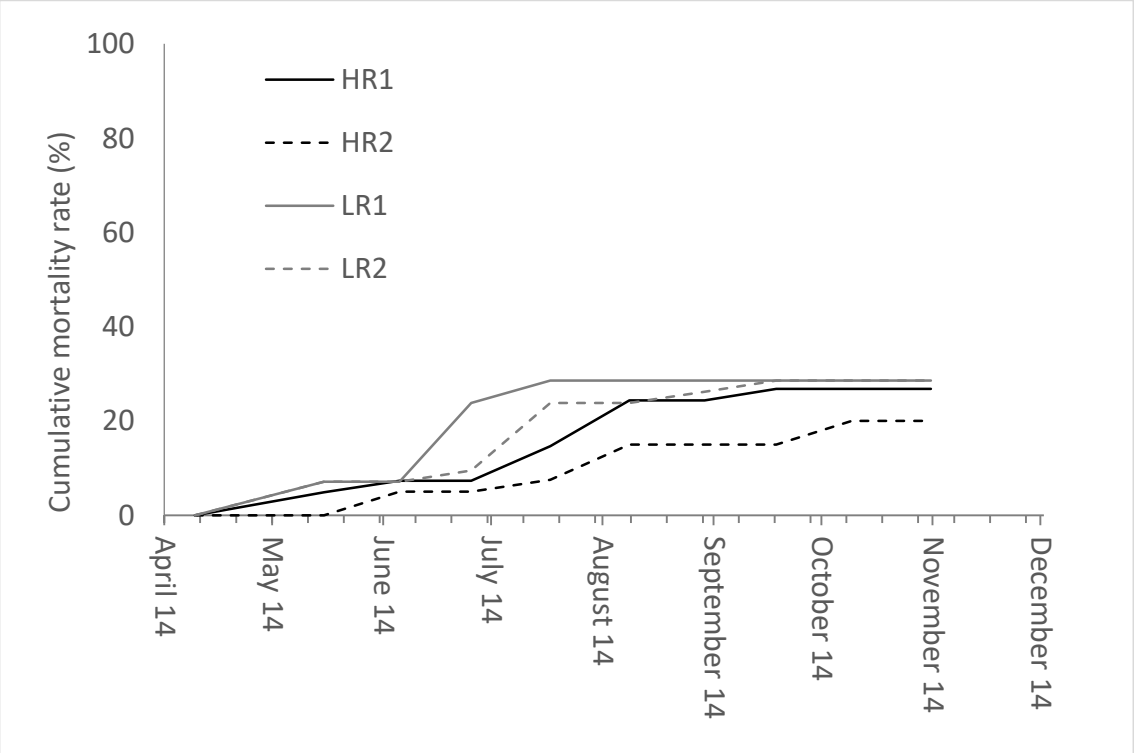

Figure 4.18. Line graph illustrating the cumulative mortality rate over the course of the trial. Data are presented as proportions.

Table 4.18. Summary of the distribution of animals by sex for each replicate, with initial mean weight [0.1 g (range)] and mean length [0.1 cm (range)].

Treatment	N	Length [mean (range)]	Weight [mean (range)]
Low Ration	60	16.3 (13.3 - 20.1)	34.3 (21.1 - 62.4)
replicate 1	30	16.3 (13.3 - 18.9)	34.3 (21.1 - 53.2)
replicate 2	30	16.3 (14.6 - 20.1)	34.4 (23.1 - 62.4)
High Ration	62	19.2 (16.1 - 23.9)	56.2 (30.4 - 98.9)
replicate 1	30	19.4 (17.1 - 23.4)	58 (37.7 - 98.9)
replicate 2	32	19 (16.1 - 23.9)	54.4 (30.4 - 96.6)

Table 19. Summary of the dietary rations for each replicate of the High Ration (HR) and Low Ration (LR) treatments in % body weight per day. Changes in ration following sampling are shown for each period.

	Ration (% Body Weight.day^{-1})	
Period	**Low Ration**	**High Ration**
15th May - 26th June	2.25	*ad libitum*
26th June - 8th Aug	3	*ad libitum*
8th Aug - 21st Nov	2.25	*ad libitum*

Influence of Diet on Growth Parameters

As there was no significant effect of sex for any variable or any tank effects observed, data are presented as the treatment means ± the standard error (SE). Length increased significantly between time points ($p < 0.01$), and there was a significant difference between treatments ($p < 0.01$), though this was clear from the start biometrics (Table 4.20). Significant interaction between treatment and time ($p < 0.01$) does indicate that the initial differences increased with time. The difference in mean length between the treatments increased from 17.5% at the start of the trial to 29 % by the end of the trial, with the HR treatment out-measuring the LR treatment at each monitoring point (Figure 4.20).

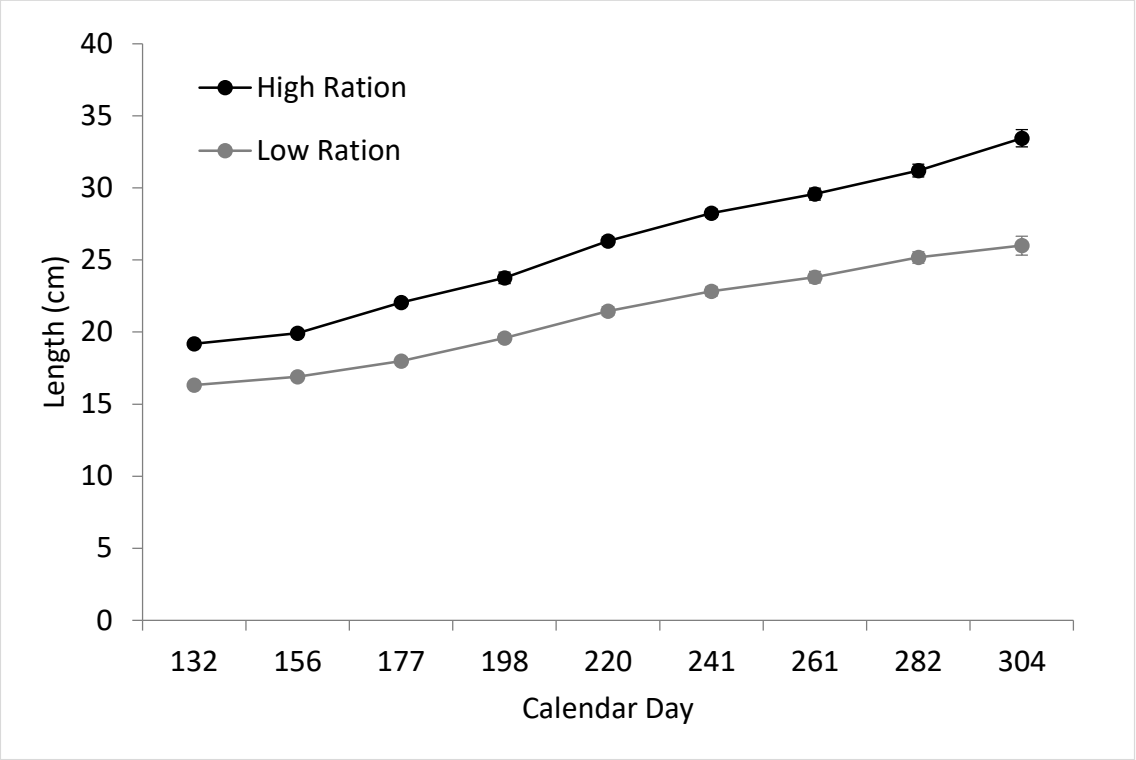

Figure 4.19. Changes in mean length between sampling periods. Data are presented as the mixed sex treatment means (± SE).

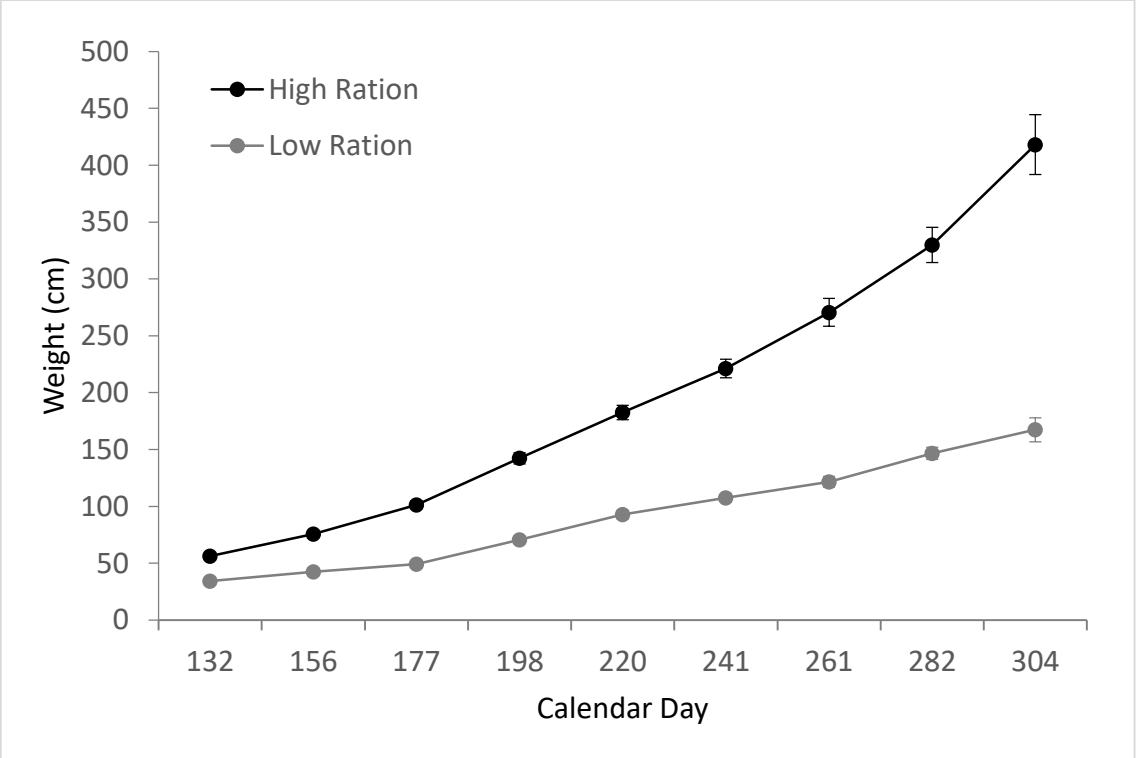

Figure 4.20. Changes in mean weight between sampling periods. Data are presented as the mixed sex treatment means (± SE).

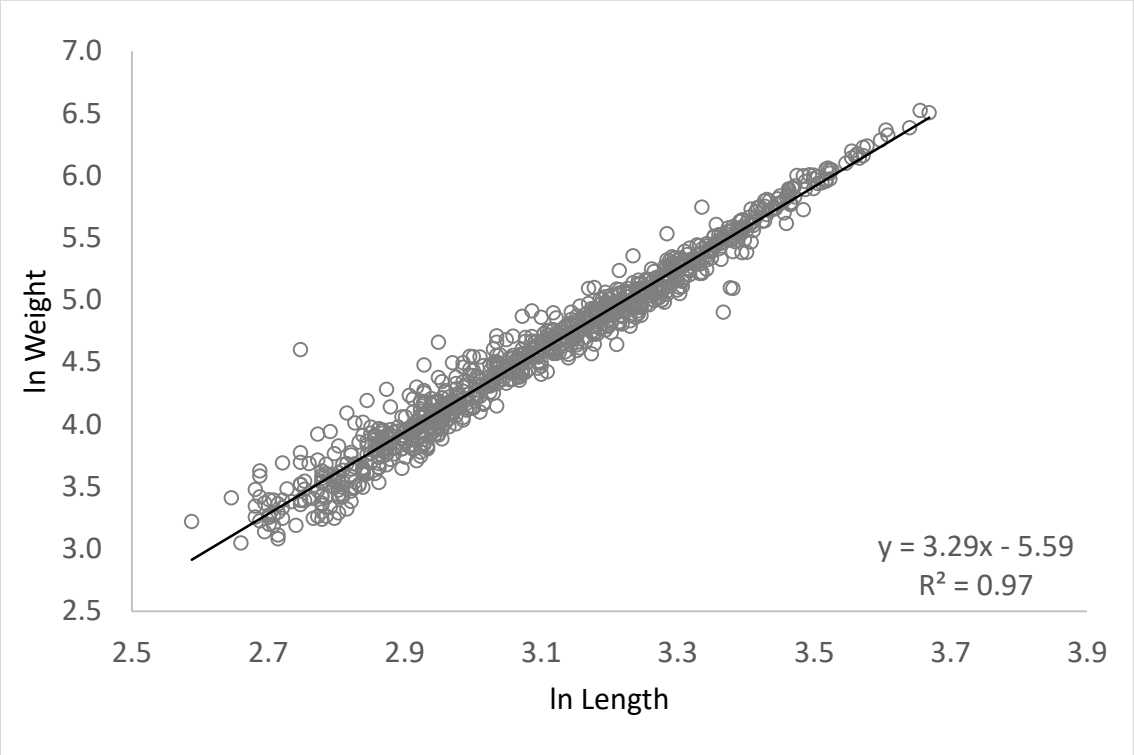

Figure 4.21. Regression of all log transformed lengths and weights for all fish from the current study.

Analysis of weight measurement were identical to those for length. Significant differences over time ($p < 0.01$), between treatments ($p < 0.01$), and between treatments over time ($p < 0.01$) were observed (Table 4.21). Again, the initial difference between the treatments increased over the course of the trial, with the HR group initially weighing 73% more than the LR group, increasing to a difference of over 160% by the end of the trial period (Figure 4.21).

To compare somatic condition among experimental fish, a weight-length relationship was derived after log transforming both variables. A regression analysis (Figure 4.22) of this relationship was used to calculate Kn. Fulton's condition factor (K) was also calculated. However, there was no significant difference between treatments ($p = 0.19$) or over time ($p = 0.79$) for K, or Kn (Treatment; $p = 0.28$, Time; $p = 0.96$), indicating that the diet

Table 4.20. Linear mixed effects model analysis of changes in (log transformed) length (L) relative to both TIME and TREATMENT, with TREATMENT as a factor, TIME as a continuous variable, and individual as the random variable to account for repeated measures over time

ANOVA: L ~ TIME * TREATMENT				
	numDF	denDF	F-value	p-value
(Intercept)	1	814	148309.47	0.000
TIME	1	814	200.71	0.000
TREATMENT	1	814	3261.74	0.000
TIME:TREATMENT	1	814	15.43	0.000

Table 4.21. Linear mixed effects model analysis of changes in (log transformed) weight (W) relative to both TIME and TREATMENT, with TREATMENT as a factor, TIME as a continuous variable, and individual as the random variable to account for repeated measures over time.

ANOVA: W ~ TIME * TREATMENT				
	numDF	denDF	F-value	p-value
(Intercept)	1	814	28573.64	0.000
TIME	1	814	375.65	0.000
TREATMENT	1	814	5171.12	0.000
TIME:TREATMENT	1	814	46.42	0.000

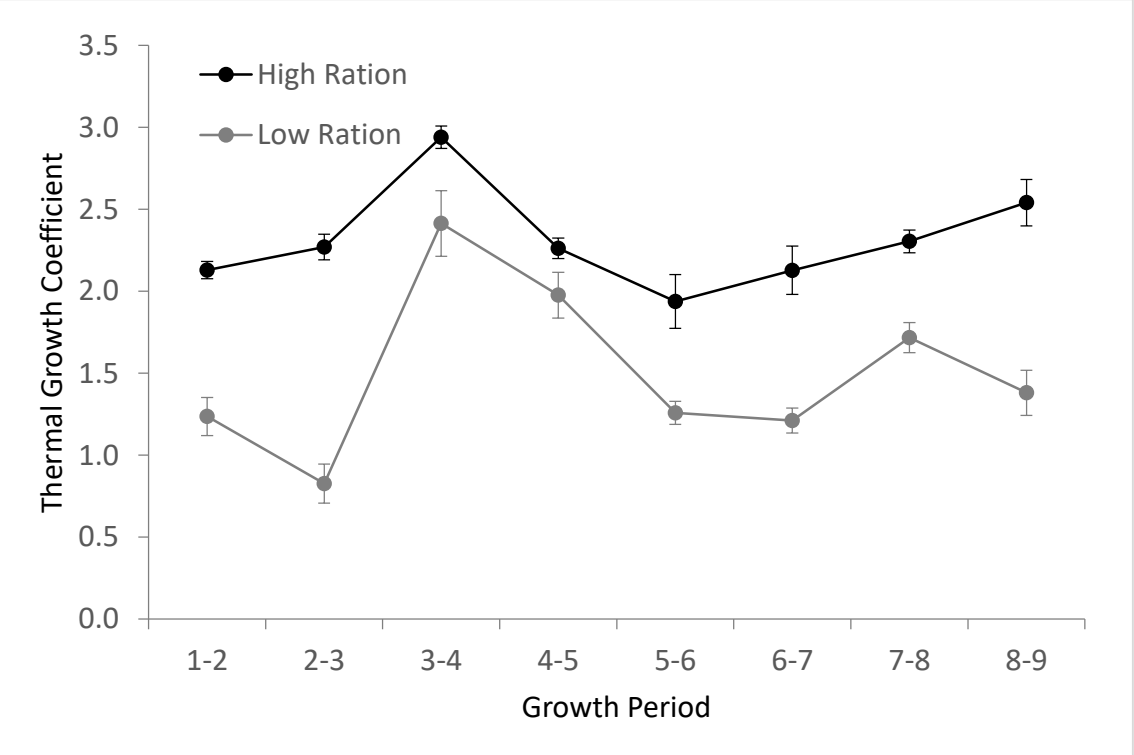

Figure 4.22. Changes in mean thermal growth coefficient between sampling periods. Data are presented as the mixed sex treatment means (± SE).

restriction created differences in overall size, but individuals maintained condition throughout the trial.

Growth rate, expressed as the thermal growth coefficient (TGC) differed significantly between treatments ($p = 0.01$), and varied significantly over the duration of the trial ($p < 0.01$), but the difference between treatments did not increase over time. There was a mean overall difference in growth rate between the HR and LR groups of 46%. (Figure 4.23).

Influence of Diet on Biometric Indices

RLM was strongly associated with final size (as a log transformed measurement of weight) and treatment (Table 4.22). Significant differences in RLM were explained by final weight ($Pr_{(>CHI)} < 0.01$), and by treatment ($Pr_{(>CHI)} < 0.01$). The LR fish were smaller at the end of the trial, but the diet restriction also influenced energy reserves, with lower liver to somatic weight ratios being observed in the LR treatment (Figure 4.24).

Influence of Diet on Maturation

Fish were classified as maturing or immature through histological analysis of the gonads. Maturing males were identified by the presence of spermatids, females by the presence of cortical alveoli. 95% of fish from the HR treatment reached maturity by the final sampling point. Only one fish from the LR treatment matured over the course of the trial (Figure 4.25).

GSI differed significantly between treatments for males ($Pr_{(>CHI)} < 0.01$) and females ($Pr_{(>CHI)} < 0.01$). Differences were also observed between the three terminal sampling periods for both males ($Pr_{(>CHI)} < 0.01$) and females ($Pr_{(>CHI)} < 0.01$) – GSI values increasing over time on both counts. There was also an interactive effect of time and treatment (Table's 4.24 & 4.25) and once again, this was the case for both males ($Pr_{(>CHI)} < 0.01$) and females ($Pr_{(>CHI)} < 0.01$). GSI increased rapidly between the sampling points

for the HR treatment, especially for males, but very little increase was observed for either males or females of the LR (Figure 4.26).

Maturity was strongly associated with final weight (expressed as a log transformation). This variable explained the majority of the variance between maturing and immature fish ($Pr_{(>CHI)} < 0.01$; Table 4.26), and there was a very obvious divide between mature and immature fish in terms of size. All but one mature fish came from the HR treatment, and only 1 fish from this treatment was found to be immature at the end of the trial. All fish over 400g were mature with only 1 fish over 300 g remaining immature (Figure 4.27).

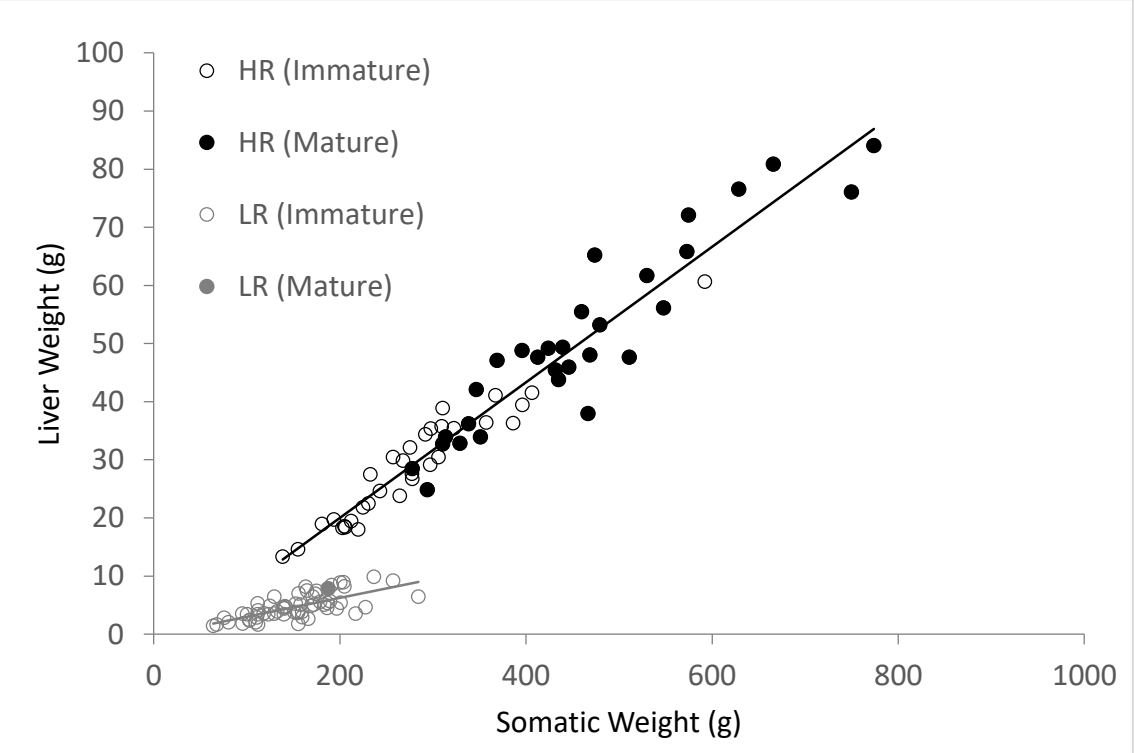

Figure 4.23. Regression of liver and somatic weight for all experimental fish. Regressions are split by treatment.

Table 4.22. General linear model analysis of Relative Liver Weight (RLM) relative to both final (log transformed) weight (Wf) and TREATMENT.

ANOVA: RLM ~ Wf +TREATMENT

	Df	Dev. Resid.	Df	Resid. Dev	Pr(>Chi)
NULL			121	39.060	
Wf	1	24.391	120	14.669	0.000
TREATMENT	1	11.968	119	2.701	0.000

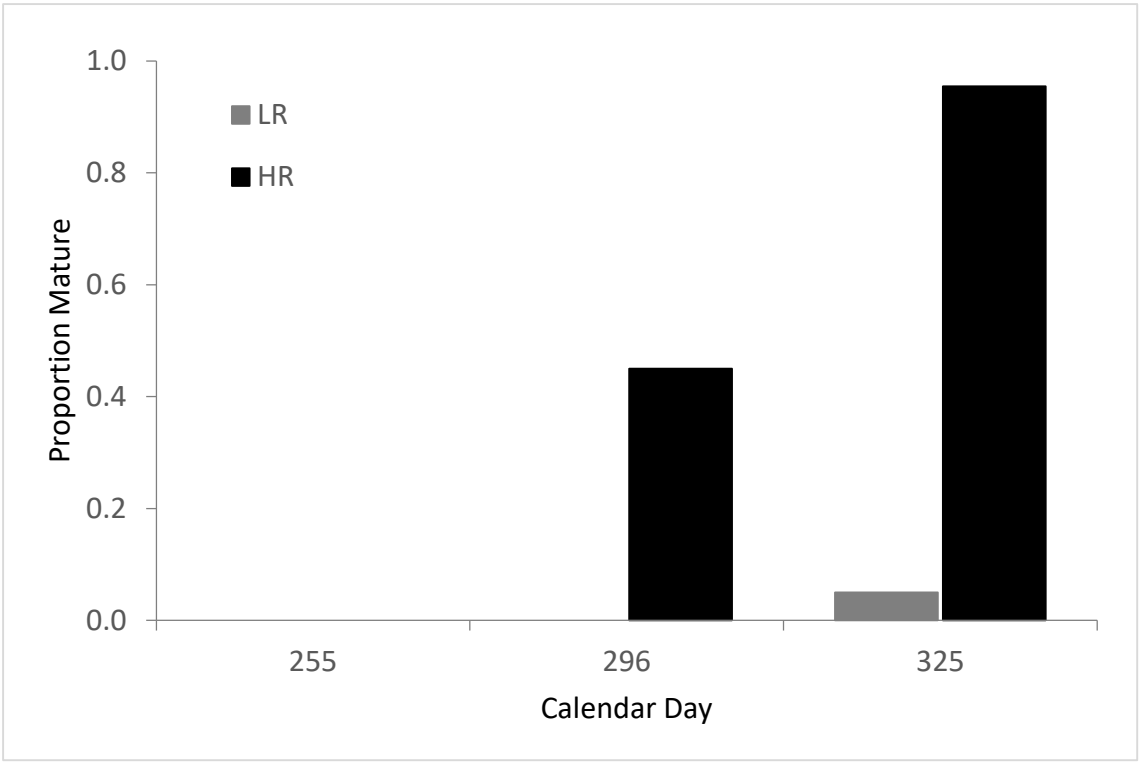

Figure 4.24. Histogram illustrating the differences between treatments and time points in the proportion of fish which are maturing.

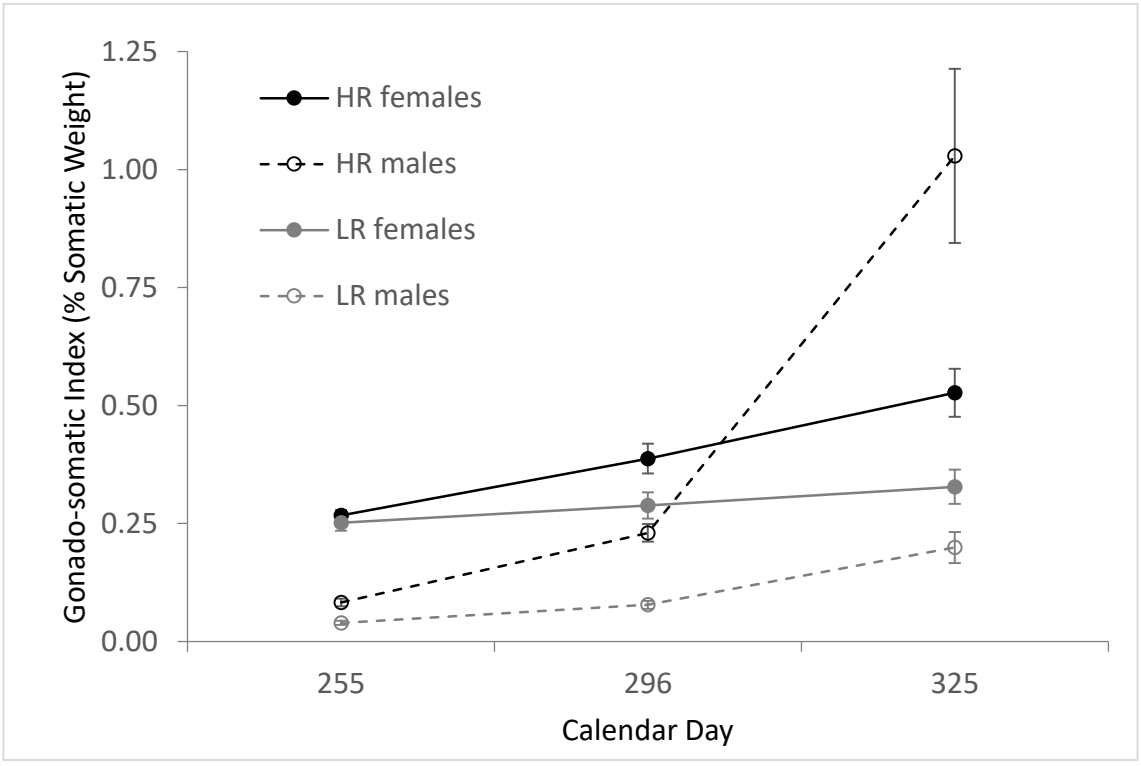

Figure 4.25. Changes in mean GSI values between terminal sampling periods for both males and females from both treatments. Data are presented as the treatment mean values (± SE).

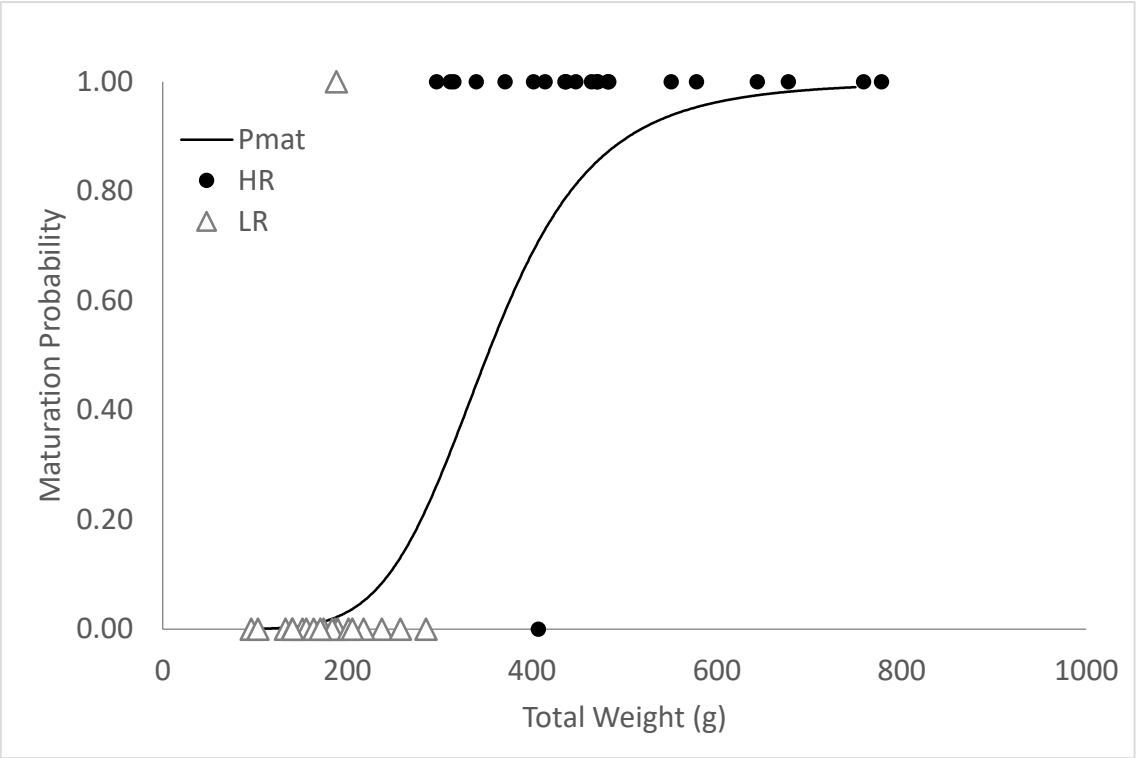

Figure 4.26. Changes in maturity with weight. Predicted proportion mature at weight values were calculated from the GLM outputs. (Table 4.27).

Table 4.23. General linear model analysis of differences in the Gonadosomatic index (males) (GSIm) relative to both the TIME sampled and TREATMENT, with both TIME and TREATMENT as factors.

ANOVA: GSIm ~ TREATMENT*TIME					
	Df	Dev. Resid.	Df	Resid. Dev	Pr(>Chi)
NULL			59	1.012	
TREATMENT	1	0.133	58	0.880	0.000
TIME	2	0.281	56	0.598	0.000
TREATMENT:TIME	2	0.083	54	0.515	0.013

Table 4.24. General linear model analysis of differences in the Gonadosomatic index (females) (GSIf) relative to both the TIME sampled and TREATMENT, with both TIME and TREATMENT as factors.

ANOVA: GSIf ~ TREATMENT*TIME					
	Df	Dev. Resid.	Df	Resid. Dev	Pr(>Chi)
NULL			61	14.051	
TREATMENT	1	2.500	60	11.551	0.000
TIME	2	4.236	58	7.315	0.000
TREATMENT:TIME	2	1.880	56	5.436	0.000

Table 4.25. General linear model analysis of the difference in maturity (MAT) relative to final (log transformed) weight (Wf).

ANOVA: MAT ~ Wf					
	Df	Dev. Resid.	Df	Resid. Dev	Pr(>Chi)
NULL			121	138.297	
Wf	1	79.067	120	59.231	0.000

Eya3 expression

As we were confident of their developmental status (i.e. Mature v. Immature), *Eya3* expression was analysed among fish sampled in the final sampling period (November). There was no significant difference between males and females and so samples were pooled by treatment, with 12 mature (HR) and 12 immature (LR) fish included in the overall analysis There was a significant difference in expression between mature and immature fish ($Pr_{(>CHI)} < 0.01$; Figure 4.28), with expression being ~1.6 times higher in the mature group.

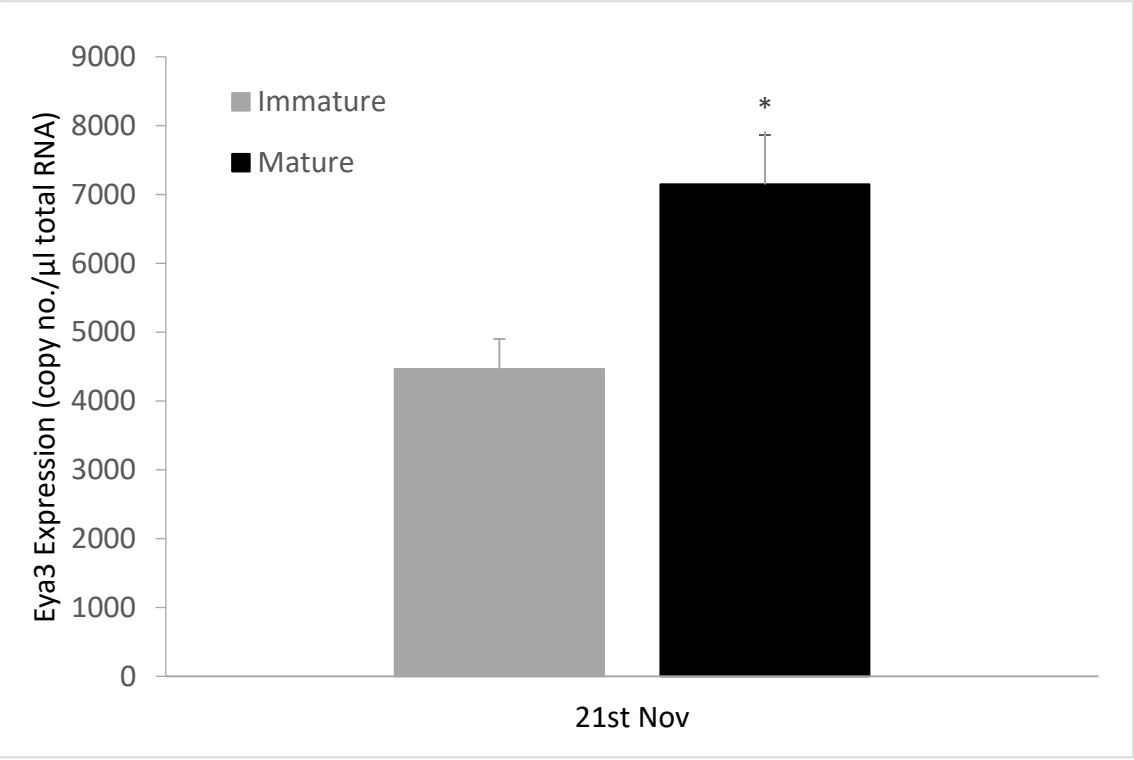

Figure 4.27. Expression of *Eya3* in mature vs immature cod for the high ration and low ration treatments, respectively. *denotes a significant difference. Data are presented as the treatment mean values (± SE).

Table 4.26. General linear model analysis of the difference in mean (log transformed) Eya3 expression (EYA) between TREATMENTs.

ANOVA: EYA ~ TREATMENT					
	Df	Dev. Resid.	Df	Resid. Dev	Pr(>Chi)
NULL			25	6220.500	
TREATMENT	1	1962.700	24	4257.800	0.001

4.5. SUMMARY OF EXPERIMENTAL RESULTS

4.5.1. Experiment I

From this experiment, it would appear that food availability rather than food quality, is the major determining factor effecting growth in haddock. The high lipid diet had no apparent influence on lipid energy acquisition, with haddock from both the low lipid and high lipid treatments exhibiting similar liver-weight ratios. Fish from the low lipid treatment had a higher overall growth rate, reaching significantly larger sizes in terms of both length and weight. No fish had initiated maturation by the end of the trial, and there were no significant differences in gonad development between the treatments to suggest any influence of dietary lipid levels on maturation.

In comparison with the Tobin *et al*. (2010) dataset from the August-September period, haddock from the current study were larger, had a higher relative liver weight and gonadosomatic index, and the leading cohort of oocytes were larger for females in the current study regardless of when sampled. Condition was also higher in the current study. When compared with wild fish, condition in the current study was again higher, but comparable.

4.5.2. Experiment II

There was no consistent effect of diet restriction prior to or after the summer solstice on maturation in Atlantic cod. Thus it cannot be concluded that treatment had any effect on maturation in this study. Though three fish were identified as maturing through histological analysis, overall the diet regimes had no effect on LC or GSI, suggesting that all fish were at a similar developmental stage when sampled. All three maturing fish exhibited different growth patterns over the study period, with the only common factor

being that they all reached a large size by the end of the study. When compared with wild cod, condition was slightly higher in the current study.

4.5.3. Experiment III

The low ration diet set forth in this experiment (2.25 – 3 % body weight) was capable of maintaining condition while also inhibiting sufficient growth/energy acquisition thus preventing maturation from proceeding, with only one fish from this treatment initiating maturation by the end of the trial. By comparison, 95% of the individuals maintained on the high ration matured by the trial end. Evident in this study, size was a clear determinant of maturity, suggesting that size around the autumn equinox is the best predictor of maturity in cod. Analysis of *Eya3* expression suggests differential in the expression pattern between maturing and immature fish with apparent suppression of the gene in immature fish. As the role of this gene in the reproductive axis of fish has already been highlighted in Section 3.4 of this thesis, these results signify a role for this marker in linking the growth and reproductive axes in cod

4.6. DISCUSSION

The overarching aims of this chapter were to investigate the influence of dietary lipids on growth, accumulated energy reserves and maturation, and to investigate the effect of growth rate and accumulate energy reserves at different periods prior to maturation commitment. This was achieved through the application of diet manipulations on wild-caught juvenile haddock and cod. Specifically, these trials looked at the effect of high lipid and low lipid diets on growth and maturation, and a variable ration was employed to investigate *how* and *when* fish assess their energetic state. Building on the results of these investigations, a further experiment was designed, which aimed to assess the importance of size and growth as permissive drivers of maturation, and to investigate

potential endogenous markers linking the growth and reproductive axes in cod. The results of these investigations suggest that size is a strong predictor of maturity. This was most evident in experiment III when differences in feeding regime led to mostly large mature and mostly small immature groups of cod. Attempts to examine dietary lipid, liver energy and growth effects on maturation commitment and timing gave more equivocal results.

Experiments 1 and 2 were undertaken before we investigated the molecular pathway described in Chapter 3 and it became clear after this study that the decision period when fish assess their state, may in fact closely linked to the switch between long and short days around the autumn equinox. It would appear that fish make this assessment quite close to this time point, which is when all currently identified cytological and molecular changes occur marking the initiation of maturation. While we were able to influence various aspects of the growth axis, terminal sampling was planned to coincide with a presumed "early marker" for maturation, somewhere between the June solstice and the beginning of the reproductive cascade, and as such, the sampling events were too early for either gross or histological assessment of maturity, and no subsequent early markers for maturity were found to allow us to assess how these size related factors might influence maturation commitment. However, both experiments did provide some insight into modes of growth and energy assessment in cod, and how these are linked with variation in diet, and crucially, provided insight into the influence of these factors are likely to have on maturation. Importantly, these experiments provided *a prior* knowledge of diet manipulations to inhibit maturation in cod for use in future studies.

In the first experiment (Experiment 1) it was anticipated that growth or lipid accumulation might be highest in the high lipid treatment. However, the low lipid diet proved superior in terms of growth, and there was no observable difference in the RLM, or condition of

fish between these treatments. The diets developed for this experiment were designed to be isocalorific. As a result, fish from the low lipid diet were fed a higher quantity of feed in terms of wet weight, as squid has a lower calorific value compared with sandeel. This disparity between the treatments may have resulted in high competition for resources in the high lipid treatment, leading to stress and lower overall food intake in terms of weight. These diets were designed to mimic naturally occurring variability in food resources in the wild. Wild haddock have a varied diet which often includes squid and sandeel, and fluctuates with changes in available resources (Kim and Lall 2001, Nanton et al. 2001, Treasurer et al. 2006). The results of this experiment suggest that the level of lipid variation naturally occurring in the wild is not enough to influence lipid accumulation or growth. Rather, it would appear that food availability is the limiting resource influencing growth in wild haddock. Growth in the wild is therefore likely to be strongly influenced by prey density/food availability but not by differences in foraging behaviour.

The lack of an effect of lipid level on liver weight contradicts previous theories developed from aquaculture perspectives. Excessively high liver weights observed among aquaculture fish are thought to reflect the high lipid content of aquaculture feeds (Kim and Lall 2001, Nanton et al. 2001, Treasurer et al. 2006). This study suggests that the large livers developed under aquaculture conditions are an artefact of artificial diets and excessive feeding.

When compared with both wild samples and experimental haddock from Tobin *et al*. (Tobin et al. 2010), condition of haddock from experiment 1 was slightly reduced. However, individuals from this experiment did exhibit higher values for GSI and RLM compared with the haddock sampled at similar time points from the Tobin study, and the leading cohort of oocytes were significantly more advanced. There was a considerable size difference between haddock from these studies. The mean weight of maturing fish

from the Tobin study at the September sampling period was 144.7 g (± 6.4 g). Haddock from Experiment 1 had a mean weight of 149 g (± 5 g) before the trial even commenced. It is likely, given their size and the level of gonadal development, that had sampling occurred at a later date, we may have found that all these fish matured. Haddock did not increase in size much over the course of the trial, with a mean increase of 23 % over the 84-day trial period, compared with a 97 % increase over a similar period for the Tobin *et al*. fish. This would suggest that low growth does not inhibit maturation, and size may be the over-riding determining factor. By extension of my previous point, differences in maturation in the wild may be linked to size, which in turn is more closely linked to differences in food availability than to differences in diet.

The proposed design of Experiment II was expected to produce groups of fish with comparable initial and final measures of size, which differed in their growth trajectories over the course of the trial. These different growth trajectories were expected to influence the pattern of energy accumulation, ultimately effecting biometric indices, condition and maturation commitment. Indeed, growth parameters did follow the expected trajectory with comparable values between treatments at the beginning and end of the trial, but significant differences observed between treatments prior to the switch in June. However, the diet regimes had no apparent effect on liver weight or reproductive development, indicating that there is no specific "window" in which growth rate is an important determining factor for maturation commitment. Among the three fish identified as maturing, growth was highly variable over the entire trial period. However, these fish did share one commonality – size. All three fish were among the largest at the end of the trial.

Condition of these fish was slightly reduced compared with wild cod, indicating that the diet manipulations maintained a natural condition. As three fish did mature, if could be assumed that the size range exhibited in this experiment was bordering the threshold for

maturation inhibition in cod, though this is by no means definitive, and as growth rates and size at maturation are variable in the wild, reflecting local variations in environmental and selective pressures, this must be taken into account in interpreting these data. Fish from this study ranged in weight from 214 – 291 g, with the smallest maturing fish weighed 180 g at the end of the trial in October. This threshold for weight has not previously been identified for cod, with other diet manipulations all failing to inhibit maturation. Even the smallest cod measured in June from the Karlsen *et al*. (1995) study was over 200 g, which likely explains why the diet restrictions in this study had no effect on maturation. This led to the development of a third diet restriction trial to test this theory and assess if size – as indicated from the previous two experiments, is the main growth/energy dependant factor influencing maturation onset in cod.

The results of experiment III clarified that size is indeed a crucial indicator of maturation commitment in Atlantic cod. There was a significant difference in total weight between treatments at the end of the trial. However, it is more likely that weight around the autumn equinox, when (as described) cod are likely to assess their energetic status and initiate reproduction, represents the threshold for growth/energy theorised by Bromage *et al*. (2001). The smallest fish to subsequently mature measured just 136 g mid-September, and on average, maturing fish measured 281 g (± 15 g) at this time. By comparison, immature fish had a mean weight of 122 g (± 9.6 g) with the largest immature fish weighing 185 g during this period. This suggest a minimum likely threshold weight of ~ 130 g must be reached prior to the switch from long day to short days around the autumn equinox, and that all fish that reach a weight above ~190 g are likely to make to commitment to mature. Thus a size range of 130 – 190 g is theorised to be the critical determining threshold range for growth/energy acquisition in cod.

Although size is the clearest indicator of maturation commitment observed, liver size also had a significant association with maturity in this study. There is a clear positive relationship between somatic weight and liver size. However, the relationship observed in the current study between the HR and LR treatment could not be explained by size alone. Fish from the HR treatment exhibited significantly higher RLM values. This further supports the previous theory that food availability rather than food quality is a more important determinant of liver size. It is clear that size acts as the major determinant of maturation commitment, but lipid reserves may have an additional permissive role in this crucial decision.

The analysis of *Eya3* expression in Experiment III is, to the authors knowledge, the first study to provide evidence for growth/energy related regulation of this gene, linking photoperiod entrainment with the growth and reproductive axes. As this gene is strongly associated with the photoperiodic signal, previous work has utilised photoperiodic manipulations to assess the function of this gene in relaying the photoperiodic message to the BPG axis (Ono et al. 2009b, Masumoto et al. 2010). Such studies have not attempted to assess the potential role of size or energetic state and would be ill equipped to do so given the confounding maturation response to photoperiod. However, with the ability to inhibit maturation through diet, samples from Experiment III provided a perfect opportunity to assess the possible interaction between the growth axis and this gene under the same photoperiodic conditions. The role of *Eya3* as a driver of light entrainment of the reproductive axis has already been discussed in Chapter 3, Section 3.4, and it was key objective of the current Chapter to investigate its potential additional role as mediator of the energetic state response within this system. In a thrilling conclusion to these two chapters, there was a significant positive correlation between maturation and *Eya3* expression, indicating that the growth axis (via a sized linked indicator) may indeed

regulate *Eya3* production, providing a mechanism for the integration of environmental and endogenous drivers of maturation in cod. This work is still in its infancy, and though this preliminary study greatly improves our understanding of the mechanisms shaping the maturation response in cod, further work is need. The nature and level of this regulation needs to be further assessed before firm conclusions can be drawn.

Overall, these experiments indicated that size is the major determining factor regulating maturation commitment in gadoids. Although liver may have a role to play, this may simply be as a buffer to the size response. The window in which the decision to mature is made occurs closer to the autumn equinox than previously thought, and evidence from Experiment III suggests that this decision is mediated by the incorporation of a permissive indicator of size with the reproductive cascade via *Eya3*. This work greatly expands our current understanding of the environmental and endogenous regulation of puberty in cod and is the first study to provide evidence of a molecular link between the growth, reproductive and PNES axes in vertebrates.

CHAPTER 5. DETERMINATION OF THE RESIDENT INSHORE AND OFFSHORE MIGRATORY COD POPULATIONS AROUND SHETLAND (IVa) AND WESTWARD INTO VIa

5.1. INTRODUCTION

Although, as discussed, both photoperiod and diet are important environmental factors linked to maturation in cod, many other factors also have a role to play, underpinning this highly complex life history event. The reproductive strategy adopted by fish reflects the environment they inhabit combined with other selective pressures they endure. These pressures and environmental factors vary geographically and over time, resulting in distinct differences in maturation schedules being observed across these scales (Yoneda and Wright 2004, Wright et al. 2011b). This phenotypic variation is interesting, as it allows us to explore possible physiological and genetic drivers of maturation, but ultimately it is also a potentially useful way of defining stock structure.

For many commercially important fish stocks, there has been a significant shift in the age and size at first maturity linked to the selective pressures associated with fishing, which has resulted in fish from heavily exploited areas maturing earlier and at smaller sizes (Law 2000, Wright et al. 2011b). Atlantic cod has experienced such shifts, with precocious maturation being observed among stock components throughout its range (Yoneda and Wright 2004, Wright et al. 2011b). Historically, this species supported one of the largest marine demersal fisheries, but has since suffered severe declines in many areas due to over-exploitation resulting in the complete closure of some major fishing grounds (Myers et al. 1997, Fu et al. 2001). Precocious maturation is known to reduce the growth potential of fish and diminishes the recruitment potential of the affected stock, and so represents an additional concern to fisheries management.

Management measures have been imposed in an attempt to mitigate the effects of fishing, however at present, the management boundaries used to delineate a stock (known as management units), often do not account for population structure within those boundaries (Reiss et al. 2009). Management units may encompass a number of reproductively distinct populations, or conversely, a single population may span a number of these units. Such patterns may also vary seasonally as populations may inter-mix outside the spawning season, or migrate between different management areas (Ruzzante et al. 2000). Where management units span multiple reproductively distinct sub-populations the less productive populations are vulnerable to extirpation from overfishing (Hutchinson 2008, Waples et al. 2008, Reiss et al. 2009) resulting in a loss of population diversity. This has been shown to cause reduced resilience to environmental change and can cause delayed recovery following stock collapse (Hilborn et al. 2003, Schindler et al. 2010), as seen in Newfoundland cod (Ruzzante et al. 2001, Beacham 2002). It has become clear that management strategies need to account for population structure in order to manage these stocks appropriately, and evidence of stock structuring is gradually being incorporated into fisheries legislation (Jakobsen 1987, Dahle 1991, Ralston and O'Farrell 2008, Botsford et al. 2009, Holmes et al. 2011, Armstrong et al. 2013, ICES 2015). But with the continued exploitation of marine stocks comes an ever increasing need for such rational management, and resolution of stock structure is therefore key.

A variety of approaches have been widely adopted to help characterise and define the population structure of Atlantic cod. Many of these methods utilise spatially discrete environmental variables to provide a snapshot of an individual's movements or environment at a given life stage, while others infer population segregation based on trait variation. Evidence from egg surveys and scientific survey data suggest that, although widely distributed, Atlantic cod aggregates annually at particular geographic locations to

spawn, and evidence from tagging and genetic studies suggest that these spawning aggregates may represent discrete groups of fish that return yearly to the same location (Robichaud and Rose 2004, Wright et al. 2005, 2006a, Svedäng et al. 2007). With advances in tagging methods and the continued development and improvement of data storage tags (DSTs), tagging studies have allowed us to follow the migratory behaviours of these spawning aggregates, offering insight into the timing of migration to spawning sites, patterns of migration, range and area use, and even natal homing (Robichaud and Rose 2004, Neuenfeldt et al. 2013). Atlantic cod exhibit a range of migratory behaviours, with evidence of "sedentary residential" groups which remain in close proximity to their spawning sites year round, such as those spawning in the coastal waters west of Shetland (Neat et al. 2006), and wide ranging "migratory" groups which travel in excess of 100km out-with the spawning season to feeding grounds, like the northeast Arctic cod (Godø 1995). Evidence that migration to the spawning grounds prior to the spawning season is both rapid and direct has also been shown through tagging studies (Wright et al. 2006a), and there is much evidence of natal homing across the North Atlantic, throughout the species range (Neuenfeldt et al. 2013). This kind of data has enabled the identification of environmental and behaviour boundaries influencing movement within and between populations and as such, has helped characterise the population structure of Atlantic cod, identifying potentially distinct populations and considerable sub-structuring within management units.

Otolith elemental composition is increasingly being used as an aid to stock component identification. Chemical signatures contained within the otolith reflect the chemical characteristics of the environment inhabited by the fish and are continually deposited as the fish moves through the environment, representing an "elemental fingerprint" reflective of the individual's movements. Otolith chemistry has thus been used to identify

the spatial life-histories of a number of marine species, including Atlantic cod. Such studies have largely supported the idea of natal homing and recruitment of spawners from local nursery grounds and have identified distinct populations around the north and east coast of Scotland, and along the Swedish southeast coast (Wright et al. 2006a, Gibb et al. 2006, Svedäng et al. 2010). Differences in otolith shape have also been used as an indicator of environment. Studies in cod have identified a high level of spatial segregation between spawning stocks from around the UK (Galley et al. 2006), and between central and northeast Atlantic stock components (Higgins et al. 2010) but this methodology is not widely used alone and is more frequently used to support other methodologies (Higgins et al. 2010).

Phenotypic variation has also been used as an indicator of population structure. Differences in traits and behaviours often reflect underlying differences in life histories and provide insight into the biological and environmental processes involved in local adaption which ultimately effect stock structure and should be recognised by management. These studies have been particularly useful in distinguishing between sympatric populations based on their different life histories (Sherwood and Grabowski 2010, Grabowski et al. 2011, McAdam et al. 2012). Morphometric analysis has been used successfully to distinguish between cod ecotypes in the Gulf of Maine (Sherwood and Grabowski 2010), and around Iceland (Grabowski et al. 2011, McAdam et al. 2012), but has proven less reliable in discerning between geographically separated stocks (Higgins et al. 2010), but including additional biological markers has allowed for greater discrimination in such cases (Higgins et al. 2010). Indeed, many studies combine methodological approaches to improve the resolving power of the structure analysis. As briefly discussed, differences in maturation schedules are widely observed in exploited stocks, reflecting local environmental and anthropogenic pressures, and as such offer

great potential as a biological marker of population structure. Assessments of length and age at maturity, in conjunction with tagging and genetic marker analysis, has proven a useful tool in population assignment for cod and other species along the Norwegian Skagerrak coast (Olsen et al. 2004, 2008, Knutsen et al. 2015).

Though these methodologies provide much insight into the various ecological, behavioural and geographical factors and processes involved in shaping population structure and when combined have proven powerful in resolving population structure (Olsen et al. 2004, 2008, McAdam et al. 2012, Knutsen et al. 2015, André et al. 2016), these approaches cannot distinguish between evolutionary distinct populations and behaviourally distinct ecotypes. This can only be achieved through genetic analysis.

Genetic markers have the advantage of being intimately linked to reproduction allowing for the detection of dispersal and inter-mixing at all life history stages, and as such have been widely used in population structure analyses since their development in the early 1960's (Sick 1961). Much of this work has focused on Atlantic cod, providing increasingly refined definitions of stock structure as the methodologies have progressed. Early studies which used protein (Mork et al. 1985) and mtDNA (Carr and Marshall 1991, Carr et al. 1995) markers found little genetic variability, suggesting a single panmictic stock across the species range. However, with the development of more variable markers, including Restriction Fragment Length Polymorphisms (RFLPs) (Jónsdóttir et al. 2003) and microsatellites (Hutchinson et al. 2001, O'Leary et al. 2007), came further resolution, separating the eastern and western Atlantic stocks. In the Northwest stock, considerable sub-structuring around the Gulf of Saint Lawrence (Ruzzante et al. 2000), Newfoundland and Labrador (Bentzen et al. 1996, Ruzzante et al. 2001, Beacham 2002), and Greenland (Pampoulie et al. 2011) has been identified through microsatellite analysis. Similar sub-structuring has been identified in the Northeast component, with evidence of isolated

populations around the Icelandic coast (Pampoulie et al. 2006), in the North Sea (Hutchinson et al. 2001, Nielsen et al. 2009), and along the Swedish and Norwegian coasts (Jorde et al. 2007, Skarstein et al. 2007) again using microsatellites. The RFLP *Pantophysin* 1 (*Pan* 1), a non-neutral marker first described by Pogson (1995), has shown strong discriminatory capabilities for cod populations and has frequently been used in tandem with other neutral and non-neutral markers to increase resolving power. This has increased definitions of cod stocks around Georges Bank (Lage et al. 2004) and Greenland (Pampoulie et al. 2011) in the west, and around Iceland (Pampoulie et al. 2006, McAdam et al. 2012) and Norway (Pogson and Fevolden 2003) in the east, though no such definitions have been identified using *Pan 1* in the North Sea (Case et al. 2005). More recently still, analysis of cod population structure using single nucleotide polymorphisms (SNPs) analysis has added to these definitions (O'Leary et al. 2006, Wirgin et al. 2007, Moen et al. 2008) particularly in the North Sea (Heath et al. 2014) and around the Faroe Islands (Peterson, unpublished). With the advent of next-generation sequencing, comes the ability to discover and accurately genotype thousands of novel SNPs. The cost of developing such datasets is continually decreasing while the speed and coverage is increasing, making this an attractive route for large scale analyses of population structure.

It is important to note that genetic studies in themselves do not explain the mechanisms shaping population structure or the resultant differential life-history strategies observed among populations. Equally, genetic techniques are not practicably possible in the field and therefore not a useful tool for the quick assignment of individuals to a given population. To provide a more accurate picture of stock structure and dynamics, a combined definition incorporating both genetic and physiological evidence is best.

The North Sea cod stock is typical of many with a spawning stock biomass that has remained below the "safe biological limit" until very recently (ICES 2013, 2015). Around the UK, there are three management units for cod – one to the north and east, including the North Sea stock (IV), the Eastern Channel (VIId) and the Western Skagerrak (IIIa) ICES regions, one to the west, encompassing ICES region VIa, and one to the south and west, incorporating the English Channel and parts of the Celtic and Irish seas. However, there is a growing wealth of evidence indicating sub-structuring within these stocks, giving rise to a need for better understanding of the stock dynamics in order to accurately inform management. Tagging studies have been undertaken around the UK for over 50 years providing the basis for population structure analyses which has followed. These conventional tag-recapture studies suggested that the migratory limits for cod in the North Sea were unlikely to exceed 100km, concluding that the stock may exhibit structuring at such a scale (Wright et al. 2006a, Righton et al. 2007). With advances in tagging technologies and the development of data storage tags (DSTs), deduction of the individual home range is now possible based on the geo-located movement history of tagged fish (such as reviewed in Neat *et al.* 2014). Such tagging studies have subsequently been utilised to identify mixing between the southern North Sea and English Channel stocks (Righton et al. 2007, Neuenfeldt et al. 2013). A division between southern and northern North Sea groups has also been observed, and an apparent division between offshore and coastal cod has also been suggested for northern North Sea cod (Wright et al. 2006a, Neat et al. 2014). It has been suggested that this division represents a behavioural preference for deeper, cooler waters > 100m by the off-shore northern North Sea cod (Righton et al. 2010). This structure has been supported by recent genetics studies. Early microsatellite work identified distinct groups in the north-eastern and central North Sea (Hutchinson et al. 2001, 2003). This was further defined by Heath *et al.* (2014) through analysis of SNPs.

This study, much like the tagging studies, suggested depth/temperature as a defining feature, separating inshore (<100m) cod from deeper offshore cod centred around Viking Bank (Heath et al. 2014). The inshore component identified spanned much of the eastern and northern UK coasts, however the study alludes to further fine-scale structuring, which tagging studies would strongly support (Neat et al. 2006, 2014, Heath et al. 2014). A highly residential group has been identified through DST analysis in the coastal waters west of Shetland (Neat et al. 2006). Similar highly residential groups observed along the Norwegian coast have been identified as distinct populations, with spatial segregation occurring over as small a scale as 30km (Knutsen et al. 2003, Jorde et al. 2007, Olsen et al. 2008), thus there is a need for further analysis to tease apart the stock dynamics in this region. Little is known of the population dynamics in the offshore waters west of Shetland and Scotland. Electronic tagging of the Viking population is known to expand northward following the continental shelf edge but the western extent of this population is unknown. Additionally, although the inshore and Viking stocks aggregate in different areas to spawn, they are known to inter-mix as juveniles off Shetland's east coast, and the migratory patterns of both groups suggest mixing out-with the spawning period (Wright et al. 2006a, Neat et al. 2014).

Regional differences in length-at-age and maturation schedules within the North Sea (Wright et al. 2011b) appear to correspond to the scale of population differences observed through genetics and tagging (Nielsen et al., 2009; Heath et al., 2014), although no simultaneous comparison of phenotypic and genotypic differences has been made. This would suggest differentiation in the life-history strategies of these groups, and as a result, their resilience to environmental and anthropogenic pressures. Applying the same management strategies to multiple stocks that differ in their resilience to exploitation inevitably results in the overfishing and likely collapse of weaker components (Schindler

et al. 2010). Disentangling the population structure around the coastal and offshore waters of mainland Scotland and the Northern Isles is therefore hugely important for informed management of these stocks.

To this end, the objective of this study was to use a combined approach, incorporating both genotyping and phenotyping methods, to improve our understanding of variability in size and age at maturation and resolve the spatial and temporal population structure exhibited by Atlantic cod in this region. Specifically, the aims were; 1) to apply newly developed NGS SNP screening methods to identify an enriched panel of SNPs which are spatially informative across the study area, 2) apply these to investigate further the population structure of cod inhabiting this area, 3) to investigate population mixing out-with the spawning season, 4) to determine the temporal stability of the population structure, and finally 5) to investigate to relationship between phenotypic variability of maturation schedules and the observed genetic structure. It is hoped that this study will contribute towards the further development of appropriate management strategies for cod populations around Shetland (IVa) and westward into VIa.

5.2. METHODS

5.2.1. Sample collection

Gill, gonad and otolith samples along with measures of length, sex and macroscopic maturation stage were collected on board research and commercial fishing vessels from six areas (Figure 5.1 & 5.2); Shetland east coast – inshore and offshore (ShIE, Viking), Shetland west coast – inshore and offshore (ShIW, ShOW), and Scottish west coast – inshore and offshore (ScIW, ScOW). Samples were taken during autumn (2013), when mixing among populations is thought to occur, and again in February and March (2014) during the spawning season (Table 5.1). Gill samples were preserved in 100% ethanol,

gonad samples in 10% neutral buffered formalin (NBF), and otoliths were cleaned and stored dry in labelled envelopes. Archived gill samples taken on board research and commercial cruises during the autumn and spawning periods in 2002 and 2003, provided by Marine Scotland Science were also analysed to provide a temporal comparison (Figure 5.3).

Table 5.1. Details of the length range, mean length and standard error, and proportion mature (PMAT) for males and females from each area sampled during both the "autumn" and "spawning" time periods.

Sampling period	Area	Sex	N	Length Range (cm)	Mean Length (cm)	SE	PMAT
"autumn"							
Nov 2nd - Jan 6th	ScIW	Male	25	30-79	49.2	2.47	0.72
		Female	25	33-96	55.1	3.54	0.84
	ScOW	Male	100	36-99	64.4	1.40	0.94
		Female	186	34-102	61.0	0.82	0.72
	ShIE	Male	31	38-79	61.3	2.18	0.58
		Female	57	42-89	70.5	1.49	0.79
	ShIW	Male	50	39-77	62.4	1.23	0.80
		Female	150	40-99	66.0	0.89	0.90
	Viking	Male	19	49-91	69.4	2.99	0.68
		Female	29	32-91	65.8	3.06	0.62
	ShOW	Male	21	36-81	59.7	2.23	0.86
		Female	27	45-74	58.8	1.25	0.52
	total		720	30-102	63.1	0.47	0.79
"spawning"							
Feb 1st - Apr 15th	ScIW	Male	92	33-92	58.7	1.57	0.76
		Female	137	35-103	64.0	1.77	0.68
	ScOW	Male	12	39-63	54.2	2.12	1.00
		Female	61	42-84	64.6	1.36	1.00
	ShIE	Male	50	41-82	65.7	1.52	0.74
		Female	150	36-95	66.7	1.05	0.88
	ShIW	Male	50	33-81	54.9	1.48	0.72
		Female	150	38-98	59.7	0.96	0.55
	Viking	Male	23	33-86	49.2	3.51	1.00
		Female	27	33-110	57.9	4.45	0.37
	ShOW	Male	25	42-75	56.7	1.99	1.00
		Female	25	41-85	56.6	2.29	0.96
	total		802	33-110	61.4	0.53	0.76

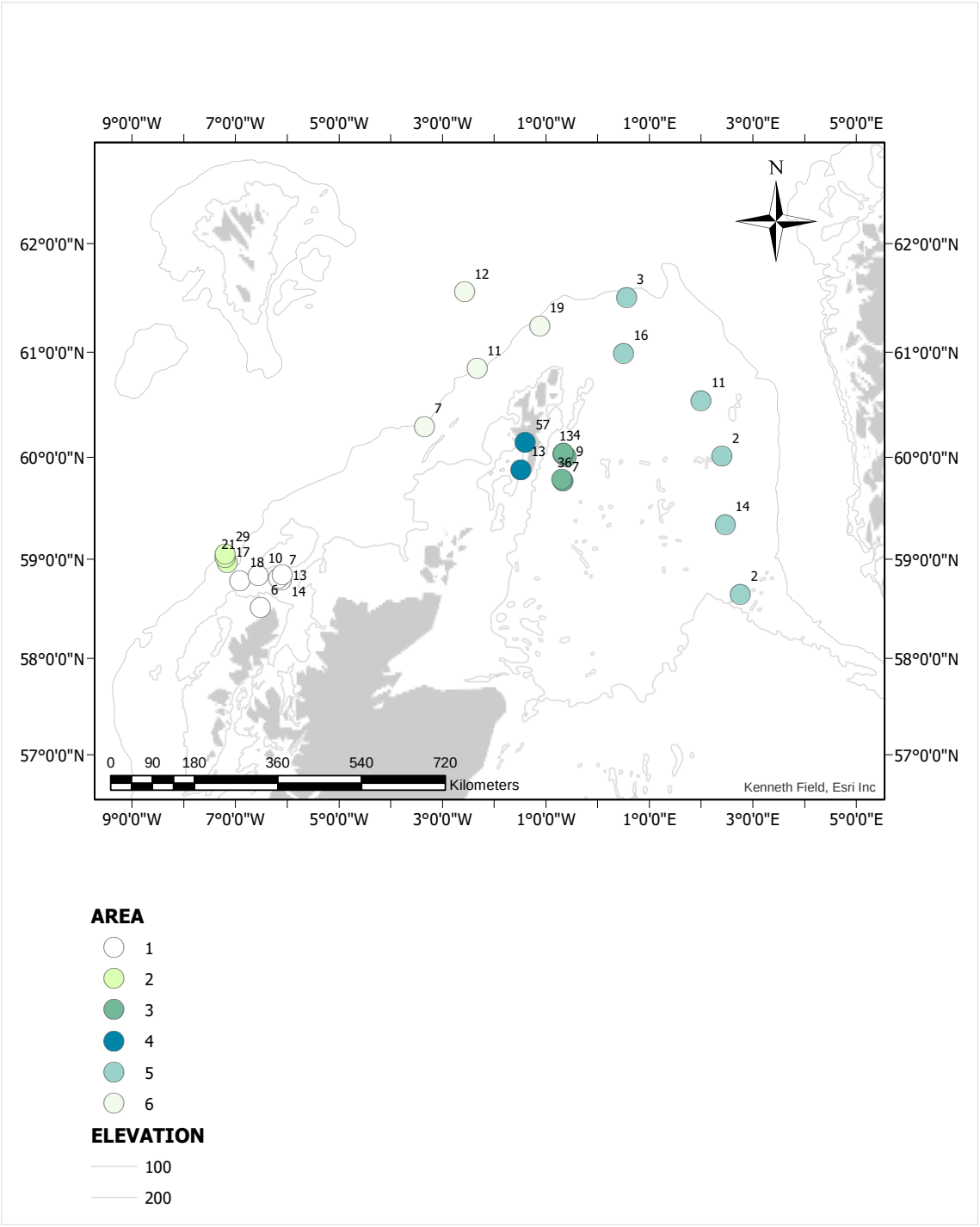

Figure 5.1. Haul locations and area designations for samples collected during the spawning period (Feb/Mar 2014). Points are labelled with the number of fish sampled for each haul. 1) ScIW, 2) ScOW, 3) ShIE, 4) ShIW, 5) Viking, 6) ShOW

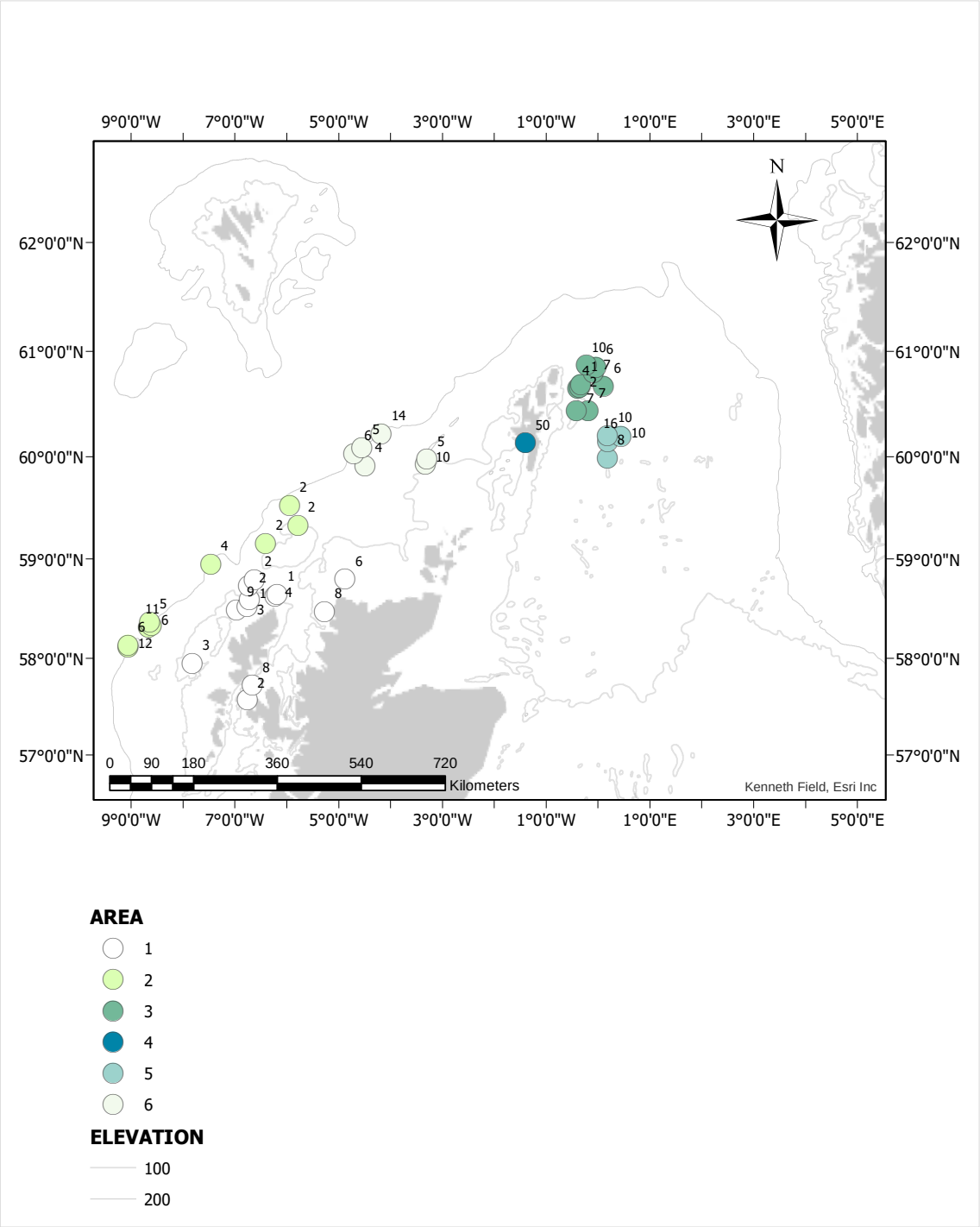

Figure 5.2. Haul locations and area designations for samples collected during the autumn period (Nov/Dec 2013). Points are labelled with the number of fish sampled for each haul. 1) ScIW, 2) ScOW, 3) ShIE, 4) ShIW, 5) Viking, 6) ShOW

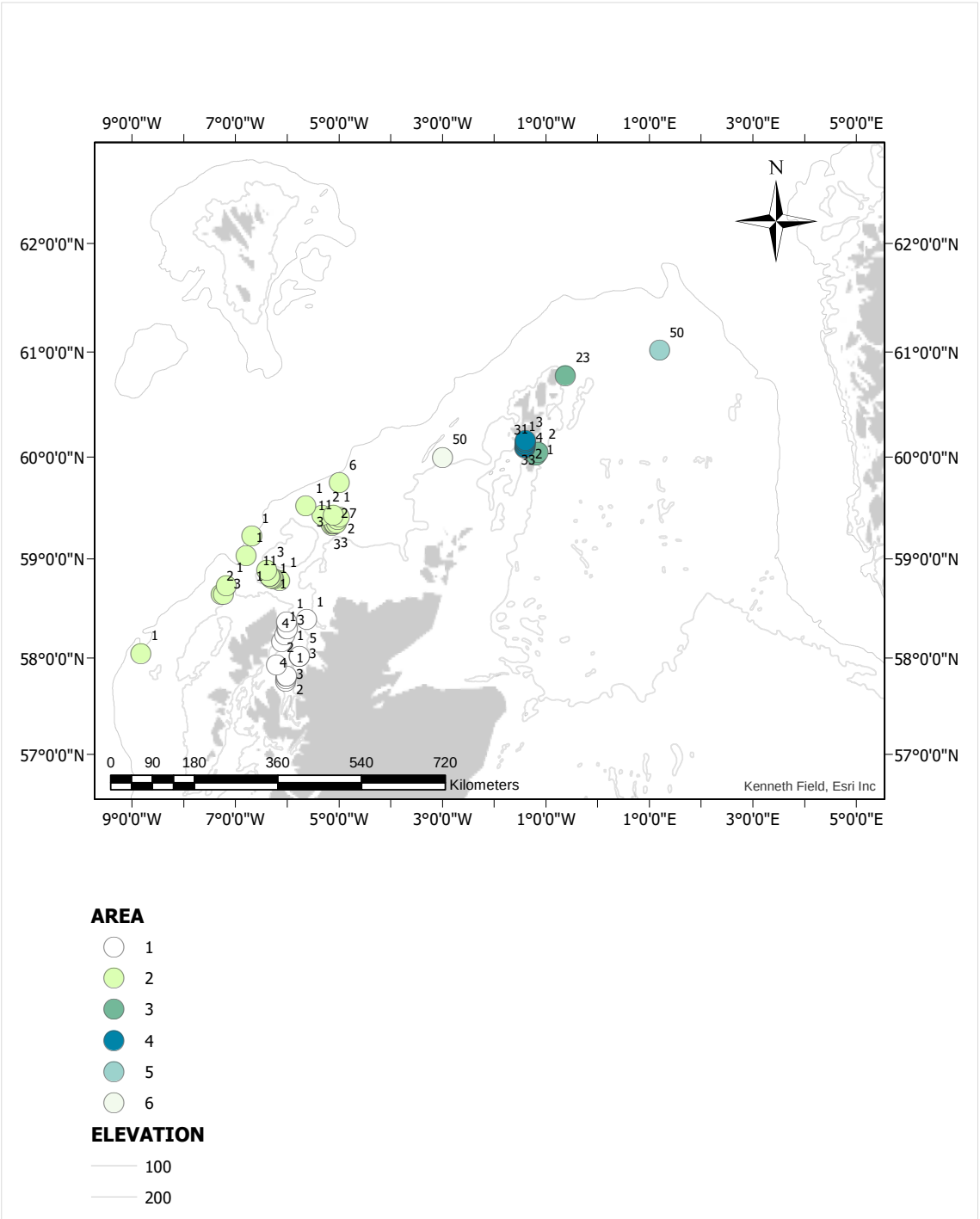

Figure 5.3. Haul locations and area designations for "historic spawning" samples collected during February and March of 2002 and 2003. Points are labelled with the number of fish sampled for each haul. 1) ScIW, 2) ScOW, 3) ShIE, 4) ShIW, 5) Viking, 6) ShOW

5.2.2. Age estimation

Age was estimated by counting the annual increments from sectioned otoliths. Due to breakages and severe decalcification of a small number of otoliths, it was impossible to determine the age of ~ 10% of cod sampled, with 1363 fish aged in total. Otoliths were embedded in black polyester resin and sectioned (0.5 mm) following standard protocols developed by Easey and Millner (2008), as described in Section 2.4. Age was then estimated from the sectioned otoliths at 10 X magnification using transmitted light by two readers, independently. Age estimates from both readers were then compared for each fish, with an overall agreement of > 80%. Where age estimates differed between readers for a given fish, otolith sections for these fish were read again until a consensus was reached.

The autumn-to-spawning period spans January 1st – the assumed birth-date for all Atlantic cod, meaning that cod from the same year-class would be aged as 1 year older in the spawning season compared with the autumn. To account for this, all statistical analysis involving age was assessed and presented in terms of year-class. As < 6% of cod aged were > 5 (2008 year-class or earlier; n = 80), and there were no fish aged 1 (2013 year-class), all analysis was restricted to age 2 - 5 cod (2009 – 2012 year-classes, inclusive).

5.2.3. Determination of maturation stage

For all cod sampled during the spawning period and all male cod from the autumn period, maturation stage was determined macroscopically during initial processing using the staging system defined by the ICES Workshop on Maturity Staging of Cod, Whiting, Haddock and Saithe, (Bucholtz et al. 2007). All female cod from the autumn sampling period (n=418) were staged histologically (Section 2.3.2) to ensure that early maturation commitment, visible at the cellular level, was identified. Early ovarian development was

classified based on the developmental stage of the most advanced oocytes, using Wallace and Selman's (1981) classification system; perinuclear (PN), circumnuclear ring (CNR), cortical alveolus (CA), and vitellogenic (VIT) (Figure 5.4). Only sections with at least 10% of oocytes at the CA stage were considered to be maturing individuals.

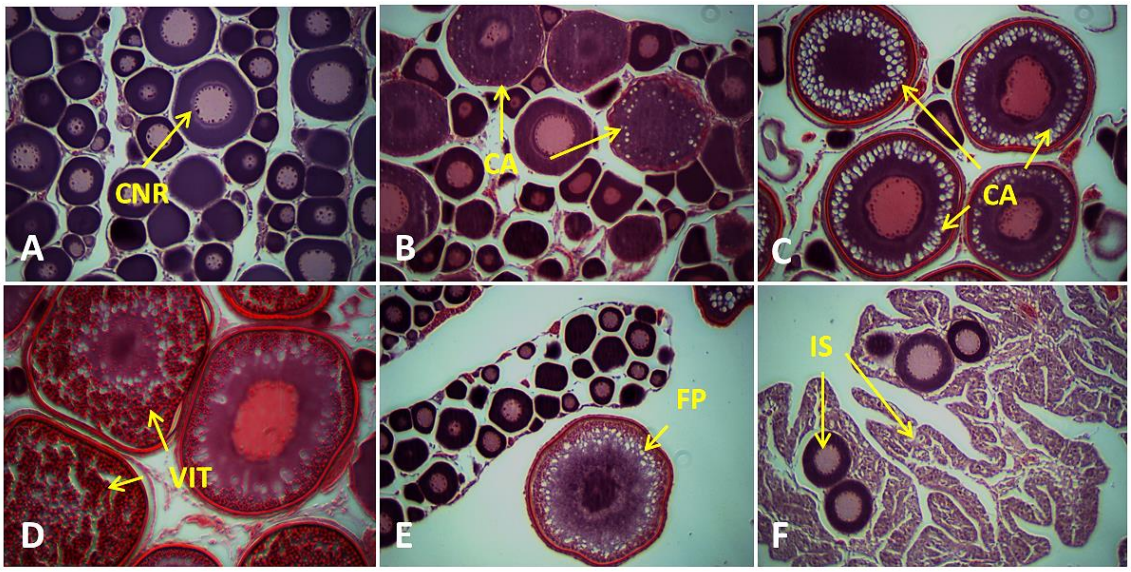

Figure 5.4. Histology stages present within autumn samples (x100 magnification). A) early CNR stage oocytes, B) late CNR and early CA oocytes, C) late stage CA, and D) shows the vitellogenic (VIT) stage of oocyte development. Image E) highlights a false positive (FP) where a small number of oocytes appear to be developing (< 10 %). This may be caused by carry over during sectioning. Image F) shows an unusual individual with both male and female cells. Macroscopically these individuals are usually classified as female (see Bucholtz *et al*., 2007). However, intersex individuals accounted for < 0.05 % of the dataset and were omitted from any analyses.

5.2.4. Length-at-age analysis

Differences in length-at-age among areas were examined in both the autumn and spawning season datasets using a generalized linear model (GLM) with a Gaussian distribution. Length was the response variable with age, expressed as year-class to allow for comparison between autumn with spawning fish, treated as a continuous variable and sex and area as factors. Although the two sample times did not allow consideration of growth rate of year-classes the interaction between age and area was used to infer if there were area specific differences in length-at-age.

5.2.5. Maturity analysis

Maturation was modelled for each sampling period using a binomial GLM according to;

$$logit(PMAT) \sim L * factor(age) * factor(area) * factor(sex)$$

where PMAT is the proportion mature and area, age (year-class) and sex are treated as factors. As each area assessed used data from multiple hauls, a quasibinomial link function was used to account for spatial autocorrelation caused by within-haul association, where variance is given by the dispersion parameter multiplied by the mean.

Model selection for the GLMs was through backward step-wise selection of variables based on model deviance compared using ANOVA. All analysis was undertaken in R (v3.2.2), using the mgcv and MASS libraries.

5.2.6. Genetic analysis

DNA extraction

DNA was extracted using a SSTNE/salt extraction method modified from Aljanabi and Martinez (1997) and detailed in Section 2.2. Each sample was quantified by spectrophotometry (NanoDrop™ (ND 1000), Thermo Fisher Scientific Inc., USA) and a subsample were further quantified by fluorescence (Qubit® 2.0, Invitrogen, Paisley, UK) to validate. Quality was assessed by agarose gel electrophoresis.

Marker Development using ddRAD

Double-digest Restriction-site Associated DNA sequencing (ddRAD) was used to investigate evidence of population structuring and thus identify and prioritise novel markers within the cod genome that were potentially informative with respect to sampling areas. DNA samples from 20 individuals from each of the six areas sampled during the spawning period were taken forward for this analysis. Samples were chosen based the

quality of the extracted DNA and were gender balanced and size matched (in terms of Total Length, TL) where possible (see Table 5.2 for further details), but due to high levels of degradation, gender balancing/size matching was not possible for all areas.

Table 5.2. Sample breakdown for all SNP genotyping approaches

		ddRAD samples	KASP Spawning samples	KASP Autumn samples	KASP Historic samples
ScIW	female	10	25	25	8
	male	10	25	24	4
ScOW	female	18	37	26	13
	male	2	10	25	7
ShIE	female	10	27	40	6
	male	9	29	20	9
ShIW	female	12	27	30	8
	male	8	28	28	9
ShOW	female	13	12	23	28
	male	7	17	21	21
Viking	female	12	13	25	13
	male	8	15	19	6

The ddRAD library construction protocol followed a modified version of the protocol described by Peterson *et al.* (2012), and described in Section 2.2. Sequencing of this library was performed on the Illumina MiSeq platform (162 paired end reads using a 300 base, v2 chemistry kit; Illumina, Cambridge, UK). Raw reads were processed using RTA 1.18.54 (Illumina), and data was then compiled and processed using STACKS (Catchen et al. 2013). Following data processing, loci with a minor allele frequency ≥ 0.15 in at least one test population/sampling area and an F_{st} value ≥ 0.03 were further considered. Due to variability in the sequencing outputs, two data processing scenarios were run, as described below.

Scenario 1 – All locations panel:

In this scenario, all areas and all individuals were considered and loci which were present in at least 17 individuals from each area were used. A total of 40 loci matching these criteria were considered informative (F_{st} > 0.03) and were taken forward for population structure analysis.

Scenario 2 – High stringency panel:

For this scenario, a higher stringency in-data pre-processing was applied, filtering out individuals with relatively low numbers of individual reads in order to improve confidence in the detection of heterozygous loci. This dataset ultimately included a total of 60 individuals from 5 areas (ScIW = 17, ScOW = 15, ShIW = 6, ShIE = 10 and Viking = 12) in subsequent analysis. A total of 50 informative loci were identified and taken forward for the population structure analysis. The Shetland inshore samples were initially pooled for loci identification, but both pooled and segregated scenarios were considered in the analyses which followed.

Analysis of ddRAD datasets

Initial marker description, including Hardy-Weinberg equilibrium, Fixation index (F_{st}) and pairwise population differentiation, was performed in GENEPOP v 4.2 (Rousset 2008). ARLEQUIN v 3.5 (Excoffier and Lischer 2010) was used to examine for signatures of directional selection by implementing a hierarchal island model (20 000 simulations, 100 demes per group, 10 groups). A Bayesian clustering analysis was performed in STRUCTURE v 2.3.4 (Pritchard et al. 2000) using an admixture model and correlated allele frequencies among populations, as well as providing sampling information as a prior in order to improve accuracy in detecting population structure. To achieve this, an initial K value optimisation trial was run with the number of iterations at

each K value set to 5, an initial burn-in of 50 000, and 100 000 MCMC repetitions for the range of K values being assessed. The K range values were set according to the scenario being assessed, ranging from 1 to the maximum number of areas/populations present. These results were then assessed using CLUMPAK software (Kopelman et al. 2015). The most likely K was calculated for each scenario according to both the log probability of the data and the Evanno method. Thereafter, each dataset was re-run in STRUCTURE with the number of iterations set to 10 for the most likely K value, an initial burn-in of 250 000, and 500 000 MCMC repetitions. Results were then compiled in CLUMPAK. The dataset was also processed for each scenario using a discriminant analysis of principal components using the ADEGENET program (Jombart 2008).

SNP genotyping – KASP

Of the 90 total loci analysed from the ddRAD dataset, 13 loci - 3 from the "all locations" panel and 10 from the "high stringency" panel, were taken forward for a more in-depth genotyping analysis. Selection of the loci was prioritised based on the potential resolving power in relation to the study areas (F_{st} value), the number of SNP's observed within the ddRAD sequence fragment (≤3 SNPs within a *circa* 150bp fragment), the physical position of the informative SNP in relation to available sequence information (>50bp either side of SNP) and the outcome of a blast search of the publicly available cod genome sequence (Assembly: gadMor1, released Jan 2010) seeking to determine that the fragment returned a clear single high identity return (Table 5.3). These SNPs were translated into genotyping assays designed using the KASP on demand genotyping system (KASP™ v4.0, LGC Genomics, UK) and a total of 832 samples were genotyped by LGC genomics. This included samples from the spawning period (included a reanalysis of the ddRAD sample set), as well as samples from the autumn period. It was the aim of this study to analyse a minimum of 50 individuals from each area for each time point, and to keep

samples gender balanced, but samples were limited in some areas and this was not always possible. For additional comparison, this KASP sample set also included 132 samples from the 2002/2003 spawning period, with between 12 and 49 samples from each site. Further details of this breakdown can be found in Table 5.2.

Analysis of KASP dataset

Analysis of the KASP dataset, similar to the ddRAD analysis, consisted of an initial assessment of the markers in GENEPOP. Hardy-Weinberg equilibrium tests, Fixation index estimates and pairwise exact tests of population differentiation were calculated as described above. Clustering analysis was also performed using the parameter setting described above for the initial K optimisation and subsequent Bayesian clustering analysis in STUCTURE. Results were compiled using CLUMPAK.

Table 5.3. (a) and (b) show the sequence and SNP information for all 13 loci used in the KASP analysis. TBLASTX results are included showing all associated genes.

(a)

Locus Name	Genomic Location	Nucleotides	Overlapping Gene(s)	Orientation	Query Length	Score	E-val	%ID	Fst
GM_ALL120_1219	GeneScaffold_3030	29106 - 29249	gpsm2l	Forward	144	277	1.60E-74	99.31	0.0567
GM_ALL120_1400	GeneScaffold_4111	323803 - 323946	ctnna2	Reverse	144	278	6.40E-75	99.31	0.0311
GM_ALL120_2904	GeneScaffold_375	79033 - 79176	nmnat2	Reverse	144	281	1.00E-75	100.00	0.063
GM_HS60_276	GeneScaffold_1603	82750 - 82893		Forward	144	277	1.40E-74	99.31	0.1004
GM_HS60_2938	GeneScaffold_4034	445 - 588	elfn1b (1 of 2)	Forward	144	275	6.30E-74	98.61	
GM_HS60_2938	GeneScaffold_3719	482571 - 482592	NPR2	Reverse	22	43	3.20E-04	100.00	0.074
GM_HS60_3664	GeneScaffold_3173	114907 - 115050		Reverse	144	276	2.30E-74	99.31	0.0713
GM_HS60_4684	GeneScaffold_1266	60796 - 60939		Forward	144	281	6.80E-76	100.00	0.1129
GM_HS60_4814	GeneScaffold_1378	90489 - 90632		Forward	144	273	2.10E-73	99.31	0.1728
GM_HS60_5331	GeneScaffold_3302	4102 - 4245		Reverse	144	281	5.70E-76	99.31	0.1436
GM_HS60_5602	GeneScaffold_2478	255576 - 255719	supt7l	Reverse	144	280	1.10E-75	100.00	0.1628
GM_HS60_6695	GeneScaffold_3295	15184 - 15327		Forward	144	276	2.80E-74	100.00	0.0621
GM_HS60_7086	GeneScaffold_1780	1458538 - 1458681	mrpl47	Forward	144	278	6.80E-75	100.00	0.078
GM_HS60_7185	GeneScaffold_2373	67695 - 67838	mpv17	Forward	144	280	1.30E-75	100.00	0.0401

(b)

Locus Name	Loci Sequence (SNP annotated)
GM_ALL120_1219	TGCAGGACTATGAAAGAGCCATCGATTACCATCTCAAACACCTC**[A/C]**TCATCGCCCAGGACCTTGAAGACCGGTACTTACCTCAAACGCAGTGTTTTGTTGTTGTGGTTTCCTCCCAAAGGAGGCTTAGTAACATAGGGAGAATAT
GM_ALL120_1400	CATGCTTGTTAAGCAGGATTCCCAAAGCG**[T/C]**ACACAGAGTTCAATGGGGCATGCACGTGGACTGTTTTAAAGTCATCGCAGGCCTTTTAGCTTGATTTCCCGCTGCTTGCACTGGTTCACTTATTTATTTACGTTTTTCTGCCTT
GM_ALL120_2904	CATGCTAATTTCATATATTTGTGCACTTTAATTAGAGCATATTGAGCATATATTTCCATTAGCATGTAGCTTGGTGTC**[G/T]**TACCTTCATGAGGTCGCGGTGGTGTTCCAGGACACTGCAGGTGGTCTGCCAGGTCTGCTGGTAGC
GM_HS60_276	CATGCCAATGTATCACGTCCACTTATTAAAATACCTGCGACAGC**[G/A]**AAAGTGCTACACAGTGCAAGCTACAAAAAAAAATCATCTAAACTATGTTTCACATGCCACACAAAAATATCTAAAGGGGTGTTACCCCATCAAGCGGTCG
GM_HS60_2938	TGCAGGCCAACCTCATCGAGAGCGTCAGCCCAAACACCTTCTGGGAGTGTGTCAACATCGAGAACATAGACCTCTC**[C/A]**ATGAATAGGTACTGGCATGGCAGCATGTGTGAAGCATGTGTGTGATGAGAAAACATAGAATCCTGAG
GM_HS60_3664	CATGCAGGCCTACATATAGAAAATACAGAAAACAGA**[T/C]**GGCTCACAGAGAAAAGGTGAACAACCGATTGAAATGACATGGAATCTGAGCGTATTCATAAAATGTCAAACAGTGTGATAAAGTTAAAACCCACGGGTAAGTTATCT
GM_HS60_4684	CATGCAAG**[G/A]**CCCCTTGAGAGTTTGACAATTTGTACTTTCTACCTAGCTTTGTGCTATTGTTGCTATGGTCCACTCTGGTCTCCTCATATCACAATGAGTGGATGTTCAGGTGCAGGTCCGACCAGAGCAGAACACGAGCTATAC
GM_HS60_4814	CATGCATTCTTTTCTTTAAATGGAGGTATTCAGATGATGAATTATTTCAACGCCAAGTATAGTTTTT**[A/C]**GCCTTTATCTCTAACTTGTGAATGATACCCTTCTTAAGTGTGTTGAAACTTTGTTAAAACTGAGAAACATGTGTGT
GM_HS60_5331	TGCAGGTGCTCCAGAGCAGGCGCTCCAGAGCTGGCAAACCAGATATTTGCCAGCCTCTTGAGGGAGCCTATACCTTTTGCGAGGCAAAGGGCCCCTCCTGG**[A/G]**CATGCAGGCAGTCAATTCCATCTACCAGGCCCTTGTGGCTTC
GM_HS60_5602	CATGCATTTTTTTAACTCTCT**[A/C]**ATCAAAACAAATAAAAATATCATCCCTCATTTTGACAGCACGCGGCGGTGCTGGAGGATTGGGTCCTTGATGGCTCTATAAGGCTACCAGTCTGCCTTGGTTTGTTTGGACTGCGCTTTGGG
GM_HS60_6695	CATGCTTGTATTAAAGAATAAAAAAGAAAACATGAATCCTTGTCGTAATTTTTTCACGCATAATTTTAATAAACAAGATAGTAAG**[A/C]**CCTTGTAAGTCCACATCTGTATTTCATATAGGCCTGTATTAAATACATATAGTTATGA
GM_HS60_7086	TGCAGGCGAGATGAATCCAAAGAAAAATAAATATACATCATAGTAGTTATCA**[A/T]**GCAACATATATCAATATAGCCATAGAACAATTGTATTAACCCAGTTACTGAGGAGGTTCACGATGAACTTCAAATTCCCATCTTCTTGACC
GM_HS60_7185	TGCAGGGTTTGTGTTAGTGTTGGGGTGGTTATTCAAAAATC**[G/A]**GACCTTGAATTTGGGTATTGTGGTAGCAGCCATCAGAGCATCCTTTCTATCCAAGATGGAAGCGAATCTGTGTTTGATCGCCTGTCATATGAAATAGTAGGC

5.3. RESULTS

5.3.1. Population variation in length-at-age

For all areas sampled during the autumn and spawning periods, mean length and proportion mature ranged from 49.2 – 110 cm and 0.37 – 1, respectively (Table 5.1). Both sex ($p < 0.001$) and sampling period ($p < 0.001$) had a significant effect on length-at-age for all areas combined (Figure 5.5). Thus all data were analysed and presented separately for males and females from each sampling period. Age ($p < 0.01$) and area ($p < 0.01$) had a significant effect on length of female cod from the spawning period, and there was a significant interaction suggestive of differences in growth rate ($p < 0.01$; Table 5.4). Female fish from the ShIW and ShOW spawning period were largest at age 2, but smallest at age 5. In contrast, females from both the Viking area and ScIW were relatively small at age 2 but largest at age 5 (Figure 5.6). The same effects and interactions were observed for males from the spawning period, with age ($p < 0.01$), area ($p = 0.01$) and their interaction ($p = 0.04$; Table 5.5) each exhibiting significant differences in mean length. Again, within the spawning period, males from Viking and ScIW were comparatively small at age two but were the largest fish at age 5 (Figure 5.6). Individuals from the autumn sampling period also displayed significant differences in mean length, with differences observed among females (Table 5.6) in terms of age ($p < 0.01$), areas ($p < 0.01$), and the age-area interaction ($p < 0.01$; Figure 5.6) and for males (Table 5.7) in terms of age ($p < 0.01$) and area ($p < 0.01$; Figure 5.6).

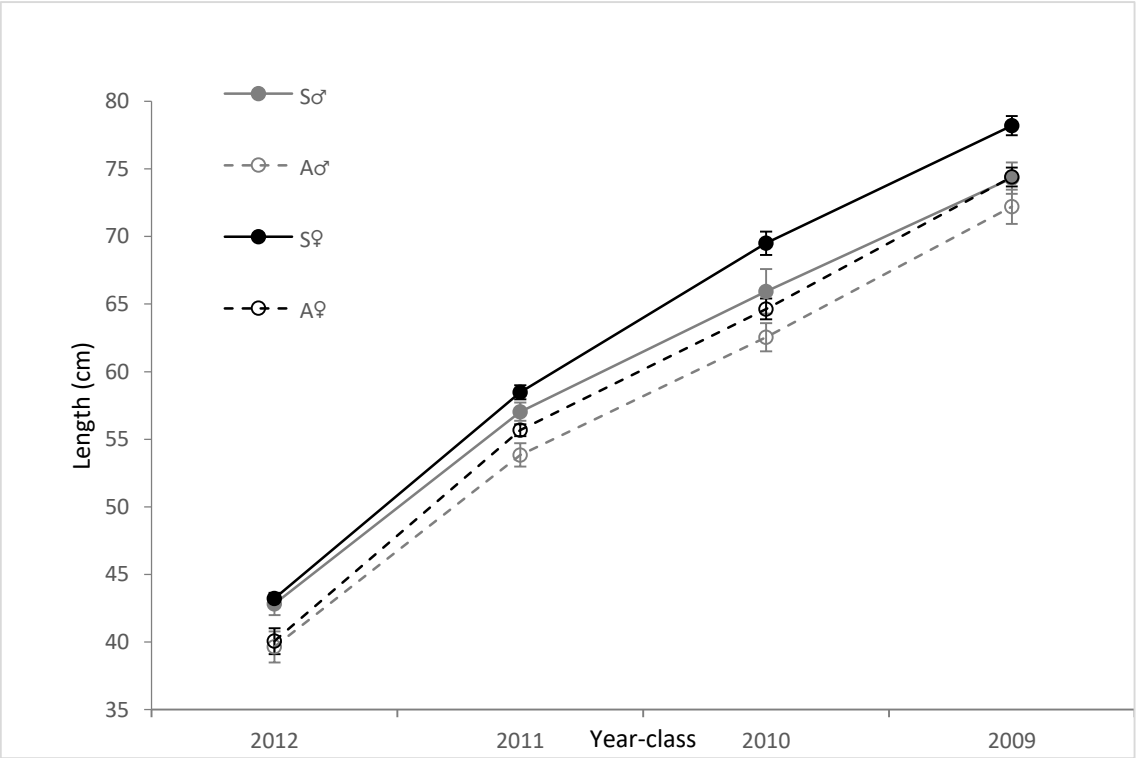

Figure 5.5. Mean length at age for all females and males from the spawning and autumn sampling periods. Data are presented as the mean ± SE.

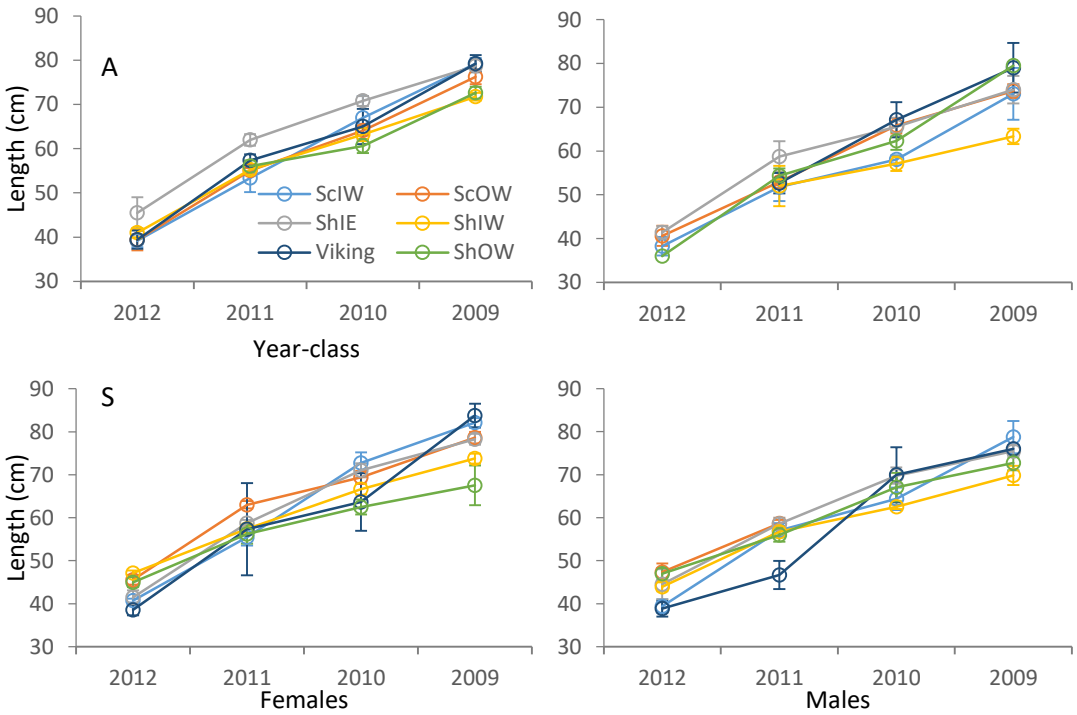

Figure 5.6. Mean length at age for cod from each area sampled during the spawning period. Data are presented as the mean ± SE. **A)** fish mean length in the autumn period and **S)** fish mean length in the spawning period. Female fish are present to the left; males, to the right

Table 5.4. Generalized linear model (GLM) results (a) and Analysis of Deviance (ANOVA) output (b) showing the significant effects of age, area and the age:area interaction of length of female cod from the spawning period.
(a)

Term	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	13.161	1.654	7.957	**0.000**
AGE	14.031	0.454	30.876	**0.000**
ScOW	17.183	3.710	4.632	**0.000**
ShIE	12.325	2.781	4.432	**0.000**
ShIW	16.811	2.387	7.042	**0.000**
Viking	-3.524	3.999	-0.881	0.379
ShOW	18.573	4.382	4.239	**0.000**
AGE:ScOW	-4.011	1.038	-3.864	**0.000**
AGE:ShIE	-3.186	0.738	-4.315	**0.000**
AGE:ShIW	-5.102	0.677	-7.541	**0.000**
AGE:Viking	0.557	1.245	0.447	0.655
AGE:ShOW	-6.545	1.278	-5.122	**0.000**

(b)

Term	Df	Deviance	Df.Res	Deviance. Res	Pr(>Chi)
(null)			515	111145.0	
AGE	1	84903.0	514	26242.0	**0.000**
factor(AREA)	5	1320.0	509	24923.0	**0.000**
AGE:factor(AREA)	5	3384.0	504	21538.0	**0.000**

Table 5.5. Generalized linear model (GLM) results (a) and Analysis of Deviance (ANOVA) output (b) showing the significant effects of age, area and the age:area interaction of length of male cod from the spawning period.
(a)

TERM	ESTIMATE	S.E.	t value	Pr(>|t|)
(Intercept)	18.962	3.378	5.614	**0.000**
AGE	12.389	1.139	10.889	**0.000**
ScOW	3.069	2.069	1.483	0.140
ShIE	2.343	1.638	1.143	0.155
ShIW	0.412	1.495	0.276	0.783
Viking	-5.950	1.958	-2.937	0.003
ShOW	1.545	1.794	0.861	0.390

(b)

Term	Df	Deviance	Df.Res	Deviance. Res	Pr(>Chi)
(null)			132	10951.0	
AGE	1	6243.4	131	4707.6	**0.000**
factor(AREA)	5	696.4	126	4011.1	**0.000**

Table 5.6. Generalized linear model (GLM) results (a) and Analysis of Deviance (ANOVA) output (b) showing the significant effects of age, area and the age:area interaction on length of female cod from the autumn period.
(a)

TERM	ESTIMATE	S.E.	t value	Pr(>|t|)
(Intercept)	12.214	4.727	2.584	**0.010**
AGE	13.587	1.519	8.947	**0.000**
ScOW	10.079	5.203	1.937	0.053
ShIE	20.611	6.454	3.194	**0.002**
ShIW	16.833	5.486	3.069	**0.002**
Viking	3.950	6.382	0.619	0.536
ShOW	22.911	8.721	2.627	**0.009**
AGE:ScOW	-2.823	1.634	-1.728	0.085
AGE:ShIE	-4.263	1.859	-2.294	**0.022**
AGE:ShIW	-5.032	1.650	-3.049	**0.002**
AGE:Viking	-0.929	1.886	-0.493	0.623
AGE:ShOW	-6.789	2.537	-2.676	**0.008**

(b)

Term	Df	Deviance	Df.Res	Deviance. Res	Pr(>Chi)
(null)			385	51643.0	
AGE	1	34485.0	384	17158.0	**0.000**
factor(AREA)	5	1745.0	379	15414.0	**0.000**
AGE:factor(AREA)	5	804.0	374	14609.0	**0.001**

Table 5.7. Generalized linear model (GLM) results (a) and Analysis of Deviance (ANOVA) output (b) showing the significant effects of age and area on length of male cod from the autumn period.
(a)

TERM	ESTIMATE	S.E.	t value	Pr(>|t|)
(Intercept)	22.989	3.488	6.590	**0.000**
AGE	9.531	0.739	12.898	**0.000**
ScOW	2.985	2.439	1.224	0.223
ShIE	5.294	2.769	1.913	0.058
ShIW	-4.383	2.618	-1.674	0.096
Viking	5.426	3.016	1.799	0.074
ShOW	2.495	2.877	0.867	0.387

(b)

Term	Df	Deviance	Df.Res	Deviance. Res	Pr(>Chi)
(null)			160	18014.8	
AGE	1	8409.6	159	9605.2	**0.000**
factor(AREA)	5	1764.0	154	7841.2	**0.000**

5.3.2. Population variation in maturity

Spawning

Among the six sample locations sampled in the spawning period there were qualitative differences in the age and size at maturity (Table 5.8). Over 80 % of fish sampled during this period were mature. Almost all males (> 99 %) from sampling areas ScIW, ScOW, Viking and ShOW were mature. In contrast, females sampled during this period exhibited greater variability in the age and size at which they matured. Only one female from ScOW and ShOW was immature at age 2, with ~ 94 % mature in these areas, while all age 2 females from ShIW and Viking were immature, and only 12 % from ShIE and ScIW were mature at age 2. All fish, regardless of sex or area, were mature by age 5. Due to differences in maturation relative to age and length between sexes, data were analysed for males and females separately for each sampling period.

Females from areas ScOW and ShOW, sampled during the spawning period, were omitted from formal analysis due to the high proportion of mature fish (> 0.99). Length ($p < 0.01$), age ($p < 0.01$) and area ($p < 0.01$) each had a significant effect on the PMAT (Table 5.9), but no interactive effects were found. Female cod from the Viking area were considerably larger and later to mature than female cod from other areas (Figure 5.7), with an Lp50 of 73.5 cm at age 3, compared with 44 cm, 49.5 cm and 56.5 cm for females from ScIW, ShIE and ShIW respectively, and 63 cm at age 4, compared with 33.5 cm, 39 cm and 46 cm (ScIW, ShIE and ShIW respectively).

Due to the high proportion (> 0.99) of mature males at most sites, only areas ShIE and ShIW from the spawning period were included in the formal analysis. Length and area had a significant effect on the PMAT (both; $p < 0.01$), but there was no significant effect

of age, and no interactive effects were found (Table 5.10). Male cod from ShIE matured at a larger size than male cod from ShIW (Figure 5.8).

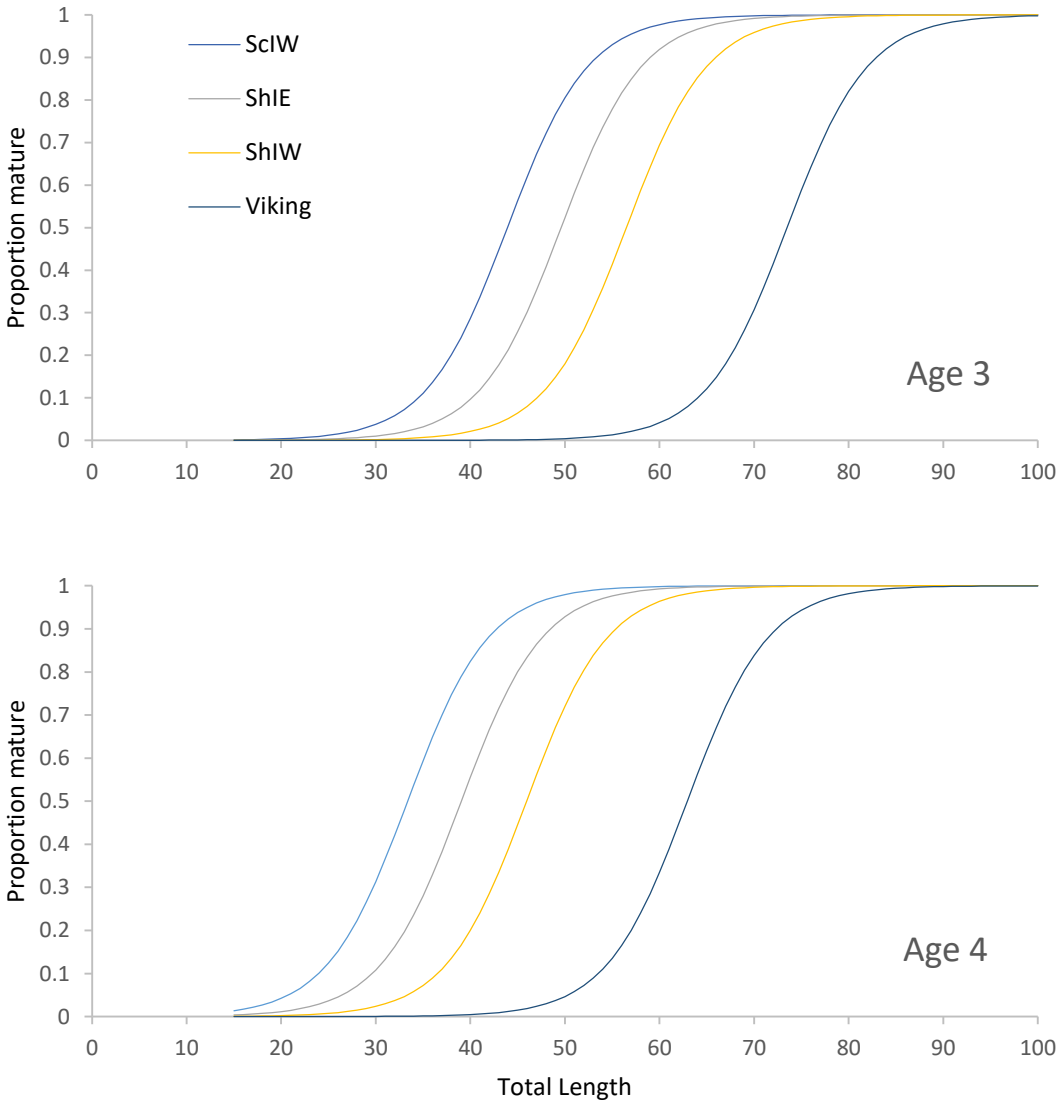

Figure 5.7. Predicted proportion mature at age 3 and 4 for female cod from the spawning time period, based on model coefficients in Table 5.9. ScOW and ShOW were excluded as all individuals were mature at age 2.

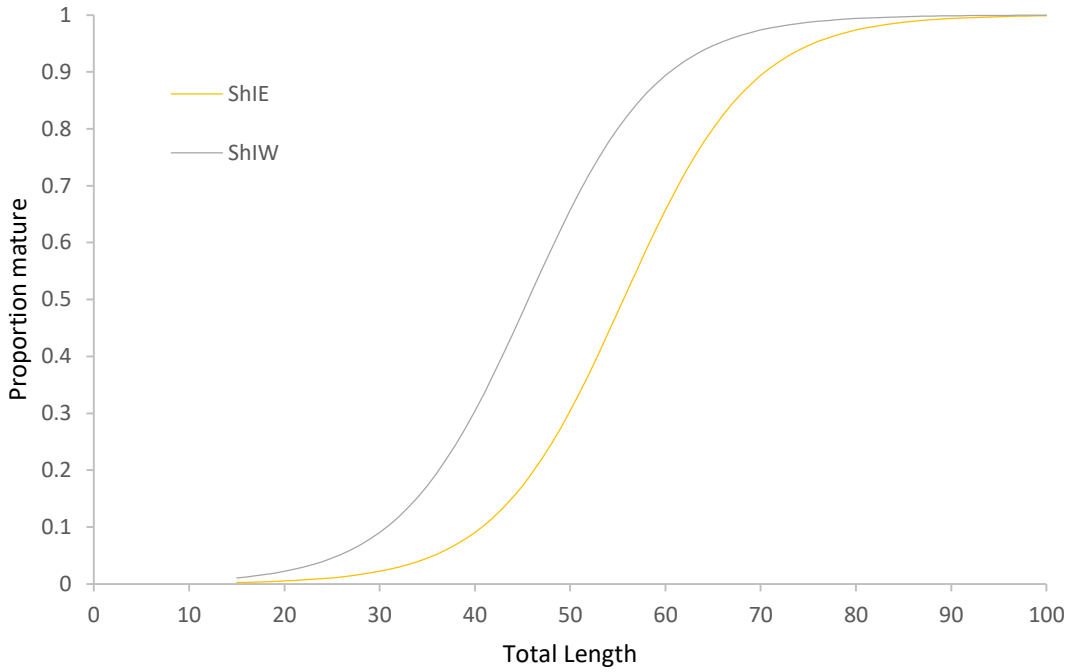

Figure 5.8. Predicted proportion mature at length for male cod from the spawning period, based on model coefficients from Table 5.10. ScIW, ScOW, Viking and ShOW were omitted as all individuals were mature. Age did not have a significant effect on the proportion mature.

Table 5.8. Details of the mean length, and proportion mature for all cod sampled according to sampling period, sex, area and year-class (age).

Sampling period	Sex	Area	Year-class	N	PMAT	Mean Length (cm)	SE
"spawning"							
	Females	ScIW	2012	49	0.14	40.7	0.44
			2011	15	0.87	55.4	1.92
			2010	17	1.00	72.8	2.43
			2009	40	1.00	82.1	1.09
		ScOW	2012	9	1.00	45.6	1.13
			2011	24	1.00	63.0	0.85
			2010	20	1.00	69.4	1.43
			2009	8	1.00	78.6	1.37
		ShIE	2012	9	0.11	41.4	1.63
			2011	61	0.85	58.7	0.76
			2010	31	1.00	71.0	1.65
			2009	40	0.98	78.2	1.27
		ShIW	2012	42	0.00	47.1	0.56
			2011	53	0.53	57.4	0.87
			2010	22	1.00	66.6	1.28
			2009	29	1.00	73.8	1.15
		Viking	2012	13	0.00	38.5	1.22
			2011	3	0.33	57.3	10.73
			2010	3	0.33	63.7	6.69
			2009	4	1.00	83.8	2.75
		ShOW	2012	8	0.88	45.0	1.15
			2011	8	1.00	56.2	2.19
			2010	3	1.00	62.5	1.76
			2009	5	1.00	67.5	4.62

Table 5.8 (cont.). Details of the mean length, and proportion mature for all cod sampled according to sampling period, sex, area and year-class (age).

Sampling period	Sex	Area	Year-class	N	PMAT	Mean Length (cm)	SE
"spawning"							
	Males	ScIW	2012	5	1.00	39.4	1.61
			2011	18	1.00	57.1	1.68
			2010	15	0.93	64.4	2.61
			2009	4	1.00	78.8	3.74
		ScOW	2012	3	1.00	47.3	2.03
			2011	8	1.00	58.7	0.78
			2010	1	1.00	39.0	
		ShIE	2012	2	0.00	44.5	3.50
			2011	23	0.57	58.6	1.41
			2010	9	0.89	69.8	1.91
			2009	13	1.00	75.5	1.41
		ShIW	2012	17	0.35	43.9	1.06
			2011	23	0.87	56.7	1.05
			2010	2	1.00	62.5	0.50
			2009	7	1.00	69.9	2.24
		Viking	2012	13	1.00	38.8	1.82
			2011	3	1.00	46.7	3.28
			2010	4	1.00	70.0	6.39
			2009	1	1.00	76.0	
		ShOW	2012	8	1.00	46.9	1.35
			2011	10	1.00	56.0	1.56
			2010	5	1.00	67.1	3.31
			2009	2	1.00	72.8	1.75

Table 5.8 (cont.). Details of the mean length, and proportion mature for all cod sampled according to sampling period, sex, area and year-class (age).

Sampling period	Sex	Area	Year-class	N	PMAT	Mean Length (cm)	SE
"autumn"							
	Females	ScIW	2012	7	0.71	39.2	1.45
			2011	5	0.80	53.4	3.16
			2010	2	1.00	67.0	4.95
			2009	2	0.50	79.3	1.25
		ScOW	2012	6	0.00	39.4	2.19
			2011	96	0.69	55.0	0.53
			2010	28	0.86	64.1	1.11
			2009	29	0.97	76.3	1.68
		ShIE	2012	2	0.00	45.5	3.50
			2011	11	0.64	62.0	1.29
			2010	14	0.86	70.8	1.15
			2009	15	0.87	78.7	1.51
		ShIW	2012	2	0.00	41.0	1.00
			2011	30	0.80	55.3	1.13
			2010	31	0.90	63.2	1.51
			2009	62	0.98	71.8	0.87
		Viking	2012	5	0.00	39.4	2.06
			2011	6	0.33	57.3	1.36
			2010	4	0.50	65.0	3.98
			2009	8	1.00	79.3	1.92
		ShOW	2011	12	0.33	56.1	1.26
			2010	8	0.75	60.6	1.59
			2009	2	0.50	72.7	1.35

Table 5.8 (cont.). Details of the mean length, and proportion mature for all cod sampled according to sampling period, sex, area and year-class (age).

Sampling period	Sex	Area	Year-class	N	PMAT	Mean Length (cm)	SE
"autumn"							
	Males	ScIW	2012	8	0.38	38.3	2.14
			2011	6	1.00	51.8	3.20
			2010	2	1.00	58.1	0.90
			2009	2	1.00	73.1	5.95
		ScOW	2012	6	0.33	40.6	2.29
			2011	23	0.96	53.0	0.72
			2010	18	1.00	65.8	1.79
			2009	29	1.00	73.7	1.58
		ShIE	2012	5	0.00	41.4	1.47
			2011	8	0.50	58.8	3.47
			2010	8	0.63	65.5	2.08
			2009	4	1.00	74.0	3.11
		ShIW	2011	4	0.75	52.0	4.64
			2010	19	0.58	57.1	1.64
			2009	9	0.89	63.3	1.76
		Viking	2011	3	0.00	52.7	2.33
			2010	7	0.71	67.1	4.00
			2009	3	1.00	79.0	5.69
		ShOW	2012	1	0.00	36.0	
			2011	8	0.75	54.4	1.68
			2010	7	1.00	62.3	2.04
			2009	1	1.00	79.5	

Table 5.9. Generalized linear model (GLM) results (a) and Analysis of Deviance (ANOVA) output (b) showing the significant effects of length (L), area and age on the PMAT of female cod from the spawning period.

(a)

TERM	ESTIMATE	S.E.	t value	Pr(>|t|)
(Intercept)	-10.243	1.508	-6.793	**0.000**
L	0.233	0.028	8.337	**0.000**
ShIE	-1.326	0.638	-2.079	**0.039**
ShIW	-2.934	0.634	-4.631	**0.000**
Viking	-6.899	1.139	-6.058	**0.000**
(AGE)4	2.460	0.796	3.090	**0.002**
(AGE)5	0.849	0.745	1.140	**0.256**

(b)

Term	Df	Deviance	Df.Res	Deviance. Res	Pr(>Chi)
(null)			172	194.5	
L	1	92.8	171	101.7	**0.000**
factor(AREA)	3	25.3	168	76.5	**0.000**
factor(AGE)	2	6.3	166	69.8	**0.000**

Table 5.10. Generalized linear model (GLM) results (a) and Analysis of Deviance (ANOVA) output (b) showing the significant effects of length (L) and area on the PMAT of male cod from the spawning period.

(a)

TERM	ESTIMATE	S.E.	t value	Pr(>|t|)
(Intercept)	-8.219	2.154	-3.815	**0.000**
L	0.148	0.035	4.210	**0.000**
ShIW	1.478	0.657	2.249	**0.028**

(b)

Term	Df	Deviance	Df.Res	Deviance. Res	Pr(>Chi)
(null)			62	95.1	
L	1	22.4	61	72.7	**0.000**
factor(AREA)	1	5.9	60	66.8	**0.017**

Autumn

There were a number of qualitative differences between the autumn and spawning sampling period datasets. For simplicity, wherever age is stated for autumn samples, it refers to the age these fish would be at spawning to allow for comparison of the same year-class between the spawning and autumn periods. During the autumn period, there were no males aged 2 sampled in the ShIW and Viking sampling areas, and no females at this age sampled in ShOW. Although the overall proportion of maturing fish in the autumn sample (78%) was comparable to the spawning total (80%), there was considerably more variation in the autumn sample set – particularly among males. Age 2 males were mostly immature, with no maturing males found at ShIE or ShOW, and < 35 % were maturing in the ScIW and ScOW areas. Age 3 males also showed considerable variation among areas, with no maturing fish found in Viking samples, compared with > 95 % maturing in the ScIW and ScOW areas. All but one male was maturing at age 5. Of the age 2 females sampled in autumn, 75 % were maturing in the ScIW area, compared with no maturing females of this year class found in the ScOW, ShIE and Viking areas. The proportion of mature females may have differed among areas and year-classes, but over 90 % were mature by age 5. Interestingly, for females from the ScOW and particularly the ShOW areas, which were mostly mature (99%) by age 2 for the spawning samples, the proportion of immature fish aged 2 and 3 was higher in the autumn period, with many large immature fish being found in these areas (Table 5.8).

Analysis of the mean PMAT for females sampled during the autumn period showed that length ($p < 0.01$) and area ($p = 0.02$), but not age, had significant effects on the PMAT. No interactive effects were found (Table 5.11). Female cod from the ShIE (Lp50 = 59.5 cm), Viking (Lp50 = 62 cm) and ShOW (Lp50 = 59 cm) area matured at a considerably

greater size than female cod from ScIW (Lp50 = 38 cm), ScOW (Lp50 = 49.5 cm) and ShIW (Lp50 = 44 cm) during the autumn sampling period (Figure 5.9).

For males sampled during the autumn period, analysis of the mean PMAT found significant effects of both length ($p < 0.01$), and area ($p < 0.01$), but not age, and again no interactive effects were found (Table 5.12). Male cod from the ShIE and Viking areas exhibited almost identical maturity ogives (Lp50 = 60.5 cm & Lp50 = 61 cm, respectively), differing significantly from the ScIW area (Lp50 = 39.5 cm), and matured at the largest size. Both ShOW (Lp50 = 42.5 cm) and ShIW (Lp50 = 54 cm) areas also differed significantly from ScIW, maturing at a larger size than both Scottish west coast areas, but smaller than ShIE and Viking (Figure 5.9).

Table 5.11. Generalized linear model (GLM) results (a) and Analysis of Deviance (ANOVA) output (b) showing the significant effects of length (L) and area on the PMAT of female cod from the autumn period.

(a)

TERM	ESTIMATE	S.E.	t value	Pr(>|t|)
(Intercept)	-5.473	1.748	-3.131	**0.002**
L	0.143	0.033	4.388	**0.000**
ScOW	-1.586	1.207	-1.313	0.191
ShIE	-3.030	1.498	-2.023	**0.044**
ShIW	-0.844	1.292	-0.653	0.515
Viking	-3.362	1.581	-2.127	**0.035**
ShOW	-2.969	1.422	-2.088	**0.038**

(b)

Term	Df	Deviance	Df.Res	Deviance. Res	Pr(>Chi)
(null)			203	278.1	
L	1	77.3	202	200.8	0.000
factor(AREA)	5	34.832	197	166.0	0.020

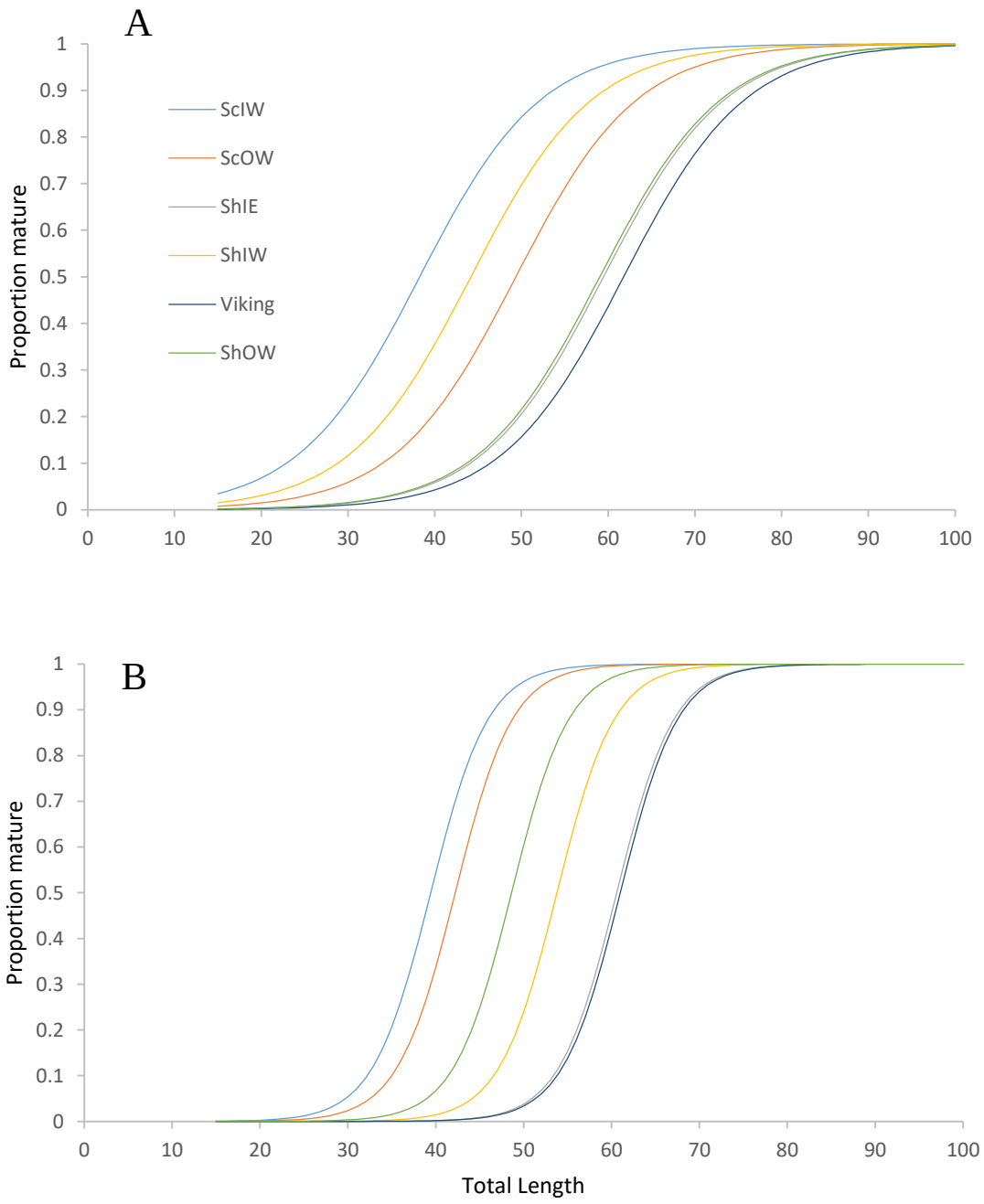

Figure 5.9. Predicted proportion mature at length for female (A) and male (B) cod from the autumn period, based on model coefficients from Tables' 5.11 and 5.12 respectively**.** Age did not have a significant effect on the proportion mature.

Table 5.12. Generalized linear model (GLM) results (a) and Analysis of Deviance (ANOVA) output (b) showing the significant effects of length (L) and area on the PMAT of male cod from the autumn period.

(a)

TERM	ESTIMATE	S.E.	t value	Pr(>|t|)
(Intercept)	-12.038	1.755	-6.859	**0.000**
L	0.305	0.041	7.436	**0.000**
ScOW	-0.850	0.819	-1.039	0.301
ShIE	-6.466	1.139	-5.676	**0.000**
ShIW	-4.388	0.930	-4.720	**0.000**
Viking	-6.590	1.247	-5.283	**0.000**
ShOW	-2.809	1.000	-2.808	**0.006**

(b)

Term	Df	Deviance	Df.Res	Deviance. Res	Pr(>Chi)
(null)			140	176.3	
L	1	60.1	139	116.1	**0.000**
factor(AREA)	5	51.4	134	64.7	**0.000**

5.3.3. Population Structure Analysis - ddRAD

Scenario 1 – All locations panel

Among the 40 loci analysed in for the "All Locations" panel, single locus F_{st} values ranged from 0.028 to 0.087 (Table 5.13). No loci were found to deviate from the Hardy-Weinberg equilibrium across all areas with only one locus (GM_ALL120_4488) observed to deviate in the majority of areas (4 of 6). No loci were deemed to be outliers based on the Arlequin analysis, thus the markers included in this panel are considered neutral (Figure 5.10). Using these markers, population segregation was possible using Fischer's probability test (Table 5.14). The results of this test suggested three discernible groups consisting of a "common cluster" including ScOW, ShIW, ShOW and ShIE, which was flanked on either side by ScIW and Viking.

Table 5.13. Summary of locus F_{st} frequency distributions for each scenario.

F_{st}	All Locations	High Stringency (pooled)	High Stringency (segregated)
> 0.10	-	4	8
0.08 –	2	3	1
0.06 -	3	6	9
0.04 -	10	14	10
0.02 -	25	23	21
0.00 -	-	-	1

Table 5.14. Summary of the Fischer's exact probability test of pairwise comparisons of populations using the "All Locations" panel according to Raymond & Rousset (1995). Significant pairwise differences are indicated in **bold**.

POPULATION PAIR			CHI^2	DF	P-VALUE
ScIW	&	ScOW	160.7238	78	**0.000**
ScIW	&	ShIE	146.3031	480	**0.000**
ScOW	&	ShIE	81.23598	76	0.319
ScIW	&	ShIW	136.4423	380	**0.000**
ScOW	&	ShIW	92.32437	80	0.163
ShIE	&	ShIW	95.0154	78	0.092
ScIW	&	Viking	155.6585	878	**0.000**
ScOW	&	Viking	132.1057	680	**0.000**
ShIE	&	Viking	175.2926	380	**0.000**
ShIW	&	Viking	137.6236	380	**0.000**
ScIW	&	ShOW	153.0484	480	**0.000**
ScOW	&	ShOW	92.40974	80	0.162
ShIE	&	ShOW	80.3864	76	0.343
ShIW	&	ShOW	97.11887	76	0.052
Viking	&	ShOW	127.1095	480	**0.001**

Analysis of the initial K value optimisation revealed a most likely K value of 4 by both the log probability and the Evanno method, thus the clustering method described above was run with a K range of 4. Similar to the Fischer's output, the clustering analysis results, compiled in CLUMPAK, revealed genetic heterogeneity in the ShIE, ShIW, ShOW and ScOW cluster, flanked by the distinct clusters of ScIW and Viking (Figure 5.11a). Equally, the discriminant analysis of principal components found a comparable clustering, with both Viking and ScIW being isolated from the main "common cluster" when comparing PC1 to PC2 and PC3 respectively (Figure 5.11b). As a whole, the

proportion of the total dataset variance explained by the model was 75.5%, with 8.2% for PC1, 6% for PC2 and 5.6% for PC3.

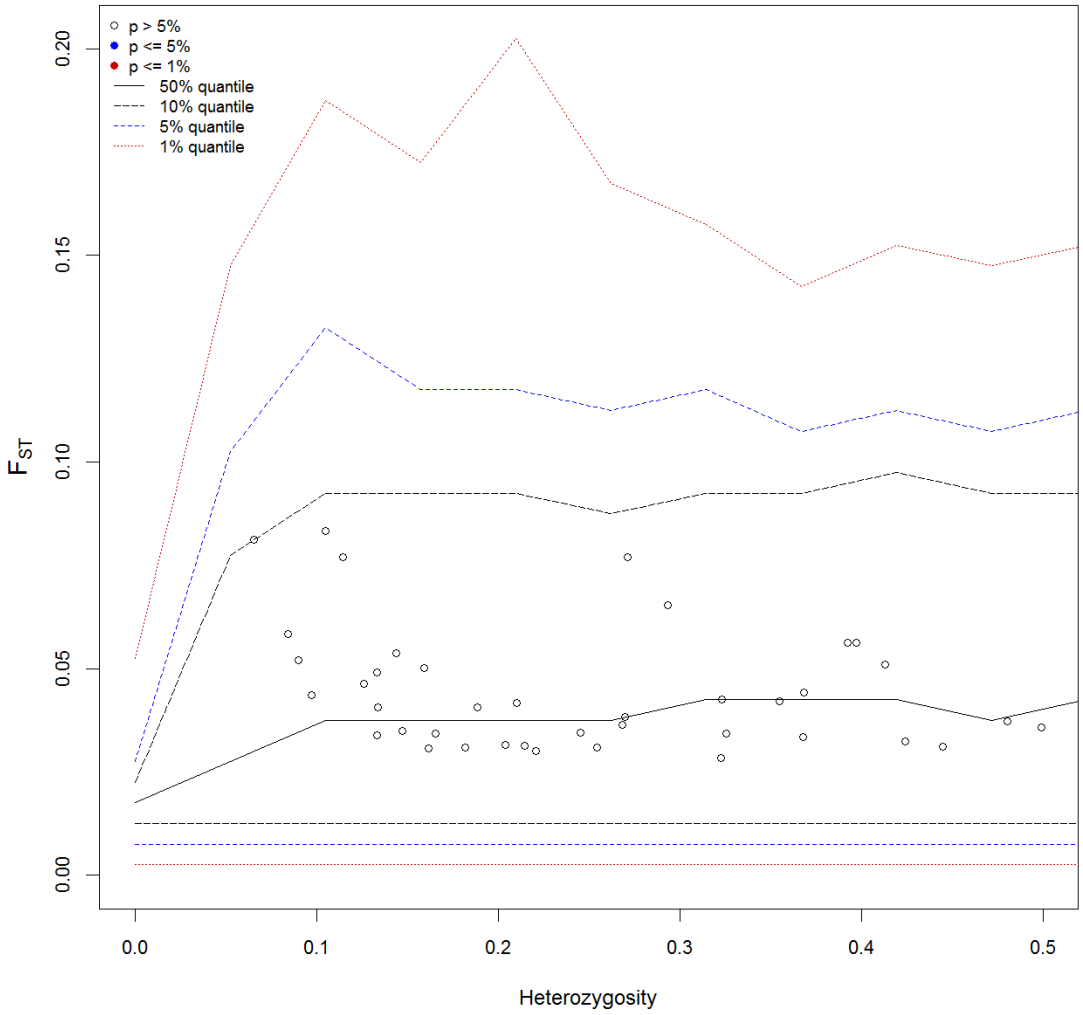

Figure 5.10. Outlier test to demonstrate the presence of selection using a hierarchical island model-based test using ARELQUIN v3.5.1.2 for the "All locations" panel.

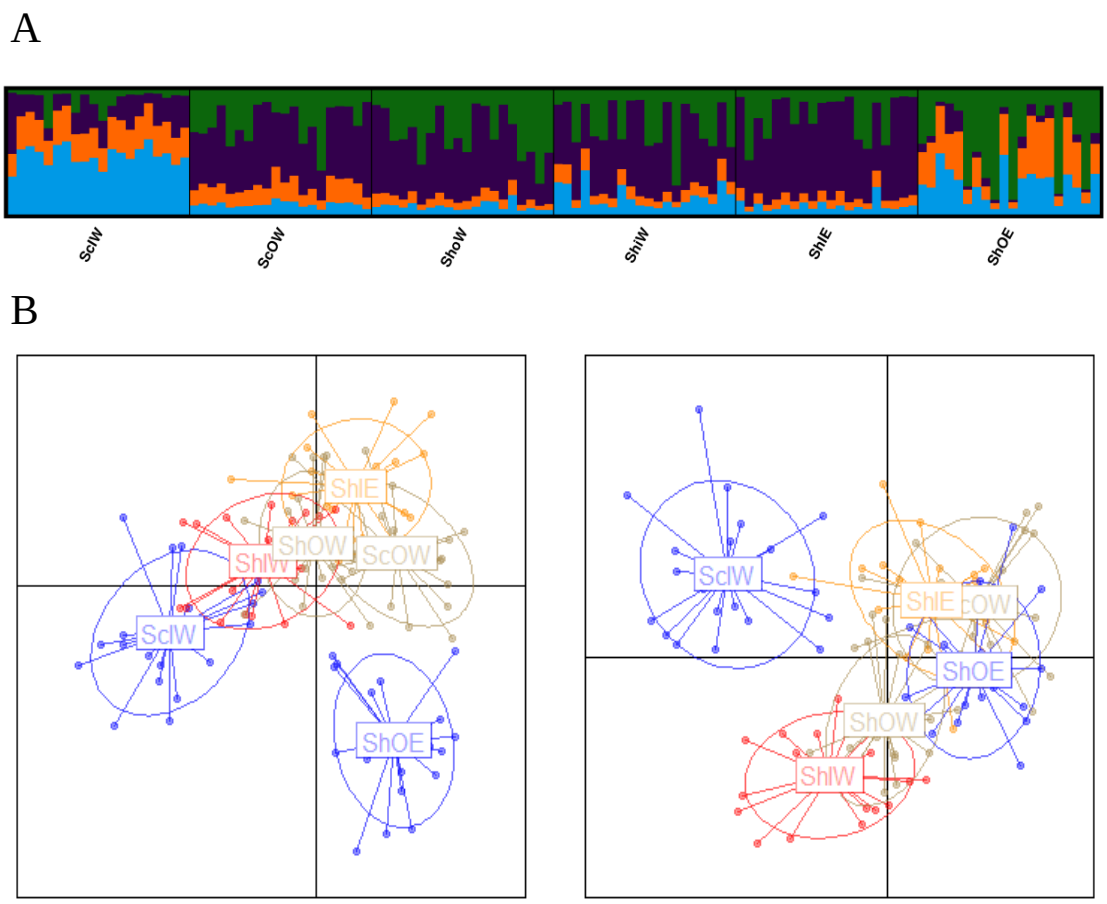

Figure 5.11. Results from clustering analysis of the "All locations" panel using a) STRUCTURE with a K value of 4, samples are ordered to reflect geographical connectivity and b) discriminant analysis of principal components using ADEGENET showing PC1 vs PC2 (left) and PC1 vs PC3 (right).

Scenario 2 – High stringency panel

Within this panel, single locus F_{st} values ranged between 0.017 and 0.176 with 8 SNPs showing an $F_{st} > 0.1$ (Table 5.13). One locus (GM_HS60_5331) showed departure from the Hardy-Weinberg equilibrium in all populations suggesting that this locus may be under selection. However, no loci were identifiable as outliers based on the Arlequin analysis, thus the panel is thought to be neutral (Figure 5.12). Population segregation by Fischer's probability tests with the Shetland inshore samples pooled identified all 4 areas as distinct populations (Table 5.15a). With the Shetland samples segregated, ShIW

samples were comparable with ScIW, Viking (p = 0.058) and ShIE, while all other comparisons were distinct (Table 5.15b). Analysis of the initial K value optimisation revealed a most likely K value of 4, thus the clustering method was run with a K range of 4 for this dataset. This analysis showed, as with the "all locations" panel, that ScIW and Viking were distinct. However, in this scenario, there is evidence of finer scale structuring within the previously described "common cluster" (Figure 5.13a). When the dataset was processed using discriminant analysis of principal components, ScIW and ScOW were considered isolated when comparing PC1 to PC2, while Viking was isolated in PC3 (Figure 5.13b). As a whole, the model explained 86% of the total dataset, with PC1, PC2 and PC3 accounting for 7.3%, 6.1% and 5.4% respectively.

Table 5.15. Summary of the Fischer's exact probability test of pairwise comparisons of populations using the "High Stringency" panel, with Shetland inshore samples pooled (a) and separated (b). Significant pairwise differences are indicated in **bold**.

(a)

POPULATION PAIR			CHI^2	DF	P-VALUE
ScIW	&	ScOW	150.172	100	**0.001**
ScIW	&	ShI	146.206212	100	**0.002**
ScOW	&	ShI	187.914851	96	**0.000**
ScIW	&	Viking	149.854158	100	**0.001**
ScOW	&	Viking	179.891341	98	**0.000**
ScI	&	Viking	187.699068	100	**0.000**

(b)

POPULATION PAIR			CHI^2	DF	P-VALUE
ScIW	&	ScOW	150.0697	100	**0.001**
ScIW	&	ShIE	135.442555	100	**0.011**
ScOW	&	ShIE	183.122713	96	**0.000**
ScIW	&	ShIW	93.822794	100	0.655
ScOW	&	ShIW	131.379119	94	**0.007**
ShIE	&	ShIW	85.332275	88	0.561
ScIW	&	Viking	150.706878	100	**0.001**
ScOW	&	Viking	180.01549	98	**0.000**
ShIE	&	Viking	168.077326	100	**0.000**
ShIW	&	Viking	120.981845	98	0.058

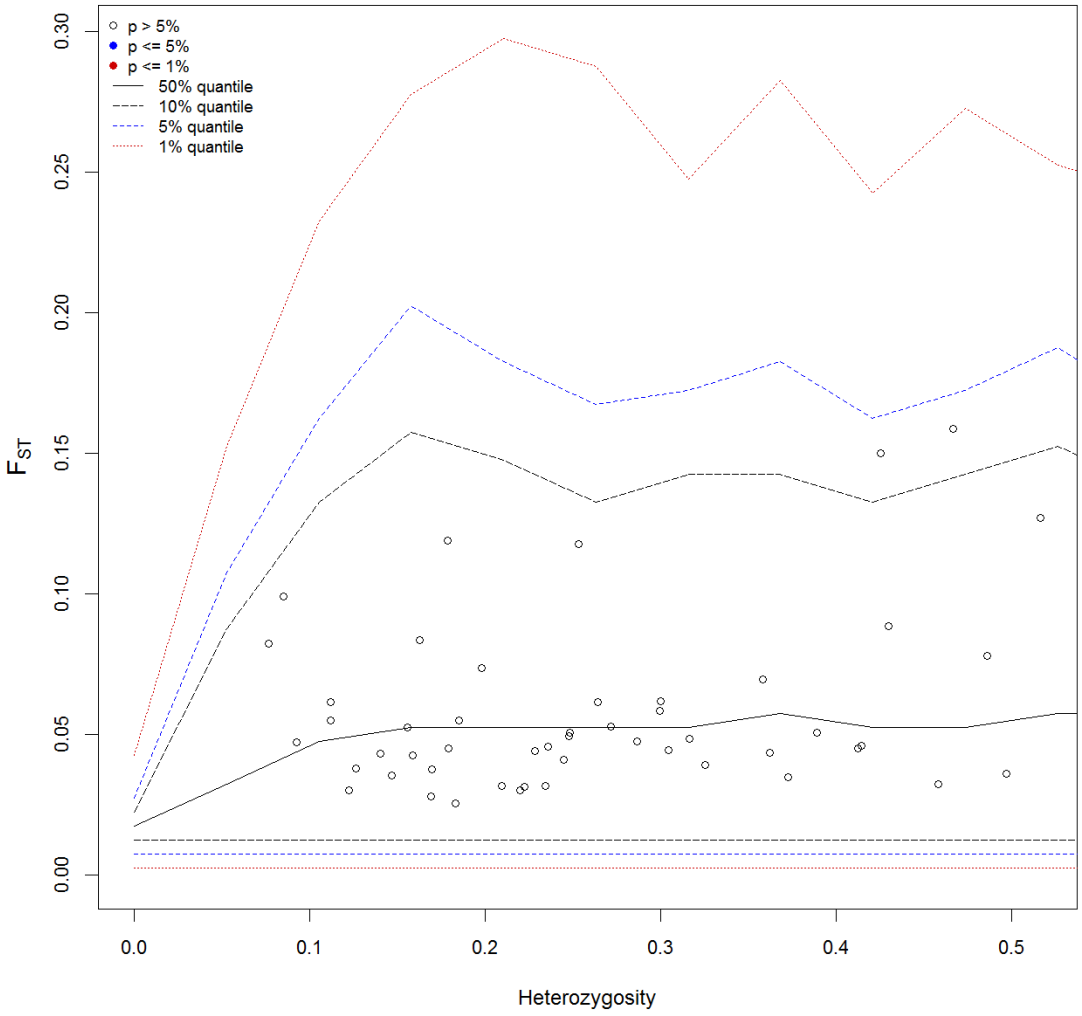

Figure 5.12. Outlier test to demonstrate the presence of selection using a hierarchical island model-based test using ARELQUIN v3.5.1.2 for the "High stringency" panel.

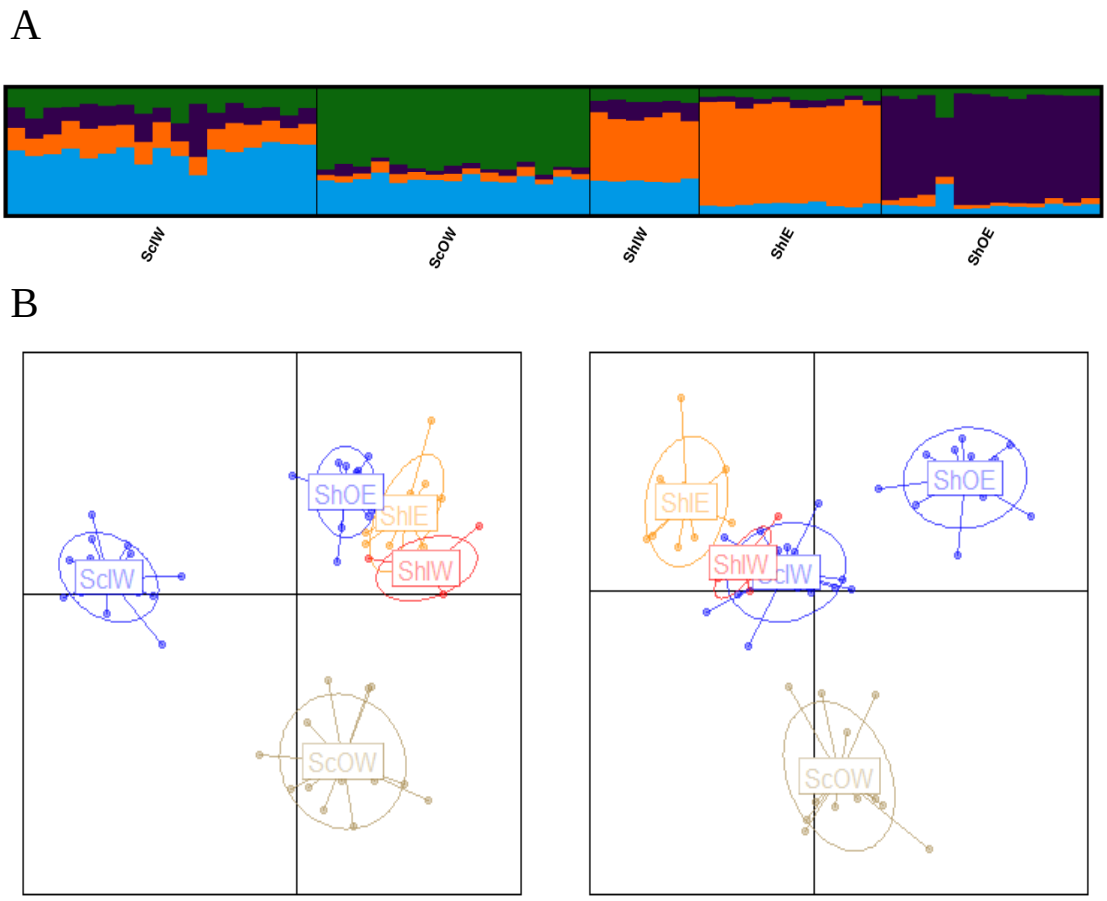

Figure 5.13. Results from clustering analysis of the "High stringency" panel using a) STRUCTURE with a K value of 4, samples are ordered to reflect geographical connectivity and b) discriminant analysis of principal components using ADEGENET showing PC1 vs PC2 (left) and PC1 vs PC3 (right).

Table 5.16. Summary of the differences in F_{st} observed between the raw and corrected ddRAD datasets, and compared with the KASP output for the same dataset (n = 120).

Locus	F_{st} ddRAD	F_{st} ddRAD (corrected)	F_{st} KASP
GM_ALL120_1219	0.048	0.048	0.036
GM_All120_1400	0.023	0.023	0.021
GM_ALL120_2904	0.066	0.066	0.053
GM_HS60_276	**0.079**	**0.083**	0.024
GM_HS60_2938	0.063	0.063	0.063
GM_HS60_3664	0.073	0.073	0.060
GM_HS60_4684	0.109	0.109	0.102
GM_HS60_4814	**0.187**	**0.088**	0.043
GM_HS60_5331	**0.172**	**0.075**	0.069
GM_HS60_5602	0.169	0.169	0.153
GM_HS60_6695	0.065	0.065	0.040
GM_HS60_7086	0.082	0.082	0.020
GM_HS60_7185	0.058	0.058	0.061

5.3.4. Marker Validation

As a method of validation, the genotyping results obtained through RAD for the original 120 individuals (20 individuals per area sampled during the spawning period), were compared with the KASP genotyping output for the same individuals. A number of discrepancies between these datasets led us to manually assess the allelic variation for several of our RAD loci, where we found some errors which were over-looked by the processing software. These loci were subsequently updated, but in some cases this resulted in a reduced allelic diversity and therefore a reduced F_{st} value. There were no changes to the individual or overall F_{st} values for loci from the "All Locations" panel when they were compared with the corresponding KASP samples. However, the "High Stringency" overall loci F_{st} value was reduced by 24 %, mainly due to reduced allelic diversity in loci GM_HS60_4814 (53 % F_{st} reduction) and GM_HS60_5331 (56.3 % F_{st} reduction), though loci GM_HS60_276 did exhibit a slight increase in F_{st} (4.6 %). As a result, loci from both the "All Locations" and "High Stringency" panels showed reductions in F_{st} (Tables 5.16) when assessed by KASP.

5.3.5. Population Structure Analysis – KASP

<u>Spawning</u>

Among the 13 loci applied to the spawning period dataset, single locus F_{st} values ranged from 0 – 0.033 (Table 5.17). None of the loci analysed were found to deviate from the Hardy-Weinberg equilibrium. Population segregation by Fischer's probability tests distinguished Viking from all areas except ShIE, though this comparison was near significant ($p = 0.086$). ScIW was also considered isolated from the Shetland offshore areas as well as ShIE (Table 5.18), but could not be distinguished from the ScOW ($p = 0.109$) and ShIW ($p = 0.064$) areas.

Table 5.17. Summary of the F_{st} output for each dataset assessed by the KASP loci.

Locus	F_{st} Spawning period (n = 371)	F_{st} Spawning & Autumn (n = 658)	F_{st} Spawning & Historic (n = 625)
GM_ALL120_1219	0.0329	0.0418	0.0957
GM_All120_1400	-0.0017	-0.0029	-0.0017
GM_ALL120_2904	0.0165	0.0079	0.0066
GM_HS60_276	0.004	-0.0009	-0.002
GM_HS60_2938	0.0052	0.0036	0.0053
GM_HS60_3664	-0.0039	0.0075	0.0042
GM_HS60_4684	0.0132	0.0064	0.0123
GM_HS60_4814	-0.0027	-0.0037	0.003
GM_HS60_5331	0.0283	0.0177	0.0263
GM_HS60_5602	-0.0041	-0.003	-0.004
GM_HS60_6695	-0.0017	0.0004	0.0091
GM_HS60_7086	0.0022	0.0095	0.0502
GM_HS60_7185	0.0043	0.0017	0.01
All:	0.0075	0.008	0.0215

Table 5.18. Summary of the Fischer's exact probability test of pairwise comparisons of populations from the spawning period. Significant pairwise differences are indicated in **bold**.

POPULATION PAIR			CHI^2	DF	P-VALUE
ScIW	&	ScOW	35.14217	26	0.109
ScIW	&	ShIE	49.074322	26	**0.004**
ScOW	&	ShIE	37.494085	26	0.067
ScIW	&	ShIW	37.777335	26	0.064
ScOW	&	ShIW	33.392896	26	0.151
ShIE	&	ShIW	19.987422	26	0.792
ScIW	&	ShOW	39.167656	26	**0.047**
ScOW	&	ShOW	18.711906	26	0.848
ShIE	&	ShOW	15.508253	26	0.947
ShIW	&	ShOW	25.452188	26	0.494
ScIW	&	Viking	Infinity	26	**0.000**
ScOW	&	Viking	50.089517	26	**0.003**
ShIE	&	Viking	36.342655	26	0.086
ShIW	&	Viking	45.99028	26	**0.009**
ShOW	&	Viking	40.932504	26	**0.032**

For the clustering analysis, initial K value optimisation suggested a most likely K value of 2 by both the log probability and the Evanno method, thus STUCTURE analysis was run with a K range of 2. Results compiled from CLUMPAK again showed a "common

cluster" exhibiting genetic heterogeneity. This cluster included the ShIE, ShIW, ShOW and ScOW areas, and was flanked by ScIW and Viking which appear subtly distinct (Figure 5.14a), supporting the Fischer's pair-wise exact test results (Table 5.18).

Evidence of mixing

In order to investigate the population structuring out-with the spawning period, genotyping data from the spawning and autumn sampling periods, generated through KASP, were compared. When applied to the combined spawning and autumn dataset, single locus F_{st} values ranged from 0 – 0.042 (Table 5.17). All loci were considered neutral based on the Hardy-Weinberg equilibrium analysis. The Fischer's probability tests identified differences between the time points for both Viking ($p = 0.01$) and ScIW ($p = 0.026$). During the autumn sampling period, both Viking and ShIE differ significantly from ScIW and ScOW, indicating some segregation between the east and west even out-with the spawning season (Table 5.19). Comparing ShIE and Viking shows that there is less differentiation between these areas during the autumn ($p = 0.766$, compared with 0.086 during the spawning period), suggestive of mixing.

A most likely K value of 2, suggest by both the log probability and the Evanno method, was used for the clustering analysis which was run as previously described. Results compiled from CLUMPAK showed some evidence of mixing during the autumn period, with the ScOW autumn cluster exhibiting a similar structure to the ScIW samples, and the ShIE autumn cluster sharing similarities with the Viking samples (Figure 5.14b). This would appear to support the results of the Fischer's tests, indicating mixing in the Viking and ShIE areas, and ScIW and ScOW areas outside the spawning season.

Table 5.19. Summary of the Fischer's exact probability test of pairwise comparisons of populations from the spawning period compared with the autumn period. Significant pairwise differences are indicated in **bold**.

POPULATION PAIR			CHI^2	DF	P-VALUE
ScIW_A	&	ScIW_S	41.805254	26	**0.026**
ScOW_A	&	ScOW_S	30.809872	26	0.235
ShIE_A	&	ShIE_S	20.528777	26	0.766
ShIW_A	&	ShIW_S	27.776739	26	0.370
ShOW_A	&	ShOW_S	14.561239	26	0.965
Viking_A	&	Viking_S	45.529636	26	**0.010**
ScIW_A	&	ScOW_A	35.79355	26	0.096
ScIW_A	&	ShIE_A	Infinity	26	**0.000**
ScOW_A	&	ShIE_A	41.560361	26	**0.027**
ScIW_A	&	ShIW_A	28.944874	26	0.314
ScOW_A	&	ShIW_A	24.331858	26	0.557
ShIE_A	&	ShIW_A	23.49552	26	0.605
ScIW_A	&	ShOW_A	38.810744	26	0.051
ScOW_A	&	ShOW_A	25.787291	26	0.475
ShIE_A	&	ShOW_A	22.97599	26	0.634
ShIW_A	&	ShOW_A	17.455311	26	0.895
ScIW_A	&	Viking_A	Infinity	26	**0.000**
ScOW_A	&	Viking_A	41.821069	26	**0.026**
ShIE_A	&	Viking_A	20.519745	26	0.766
ShIW_A	&	Viking_A	27.470753	26	0.385
ShOW_A	&	Viking_A	28.17945	26	0.350

Temporal Structure

In order to evaluate the temporal stability of the population structuring observed, the contemporary samples obtained during the spawning season were compared with samples collected over 10 years' prior during the spawning period in 2002 and 2003. When applied to the combined spawning and historic spawning dataset, single locus F_{st} values ranged from 0 – 0.096 (Table 5.17). As with previous datasets, no loci were found to deviate from the Hardy-Weinberg equilibrium. The Fischer's probability tests identified differences between the contemporary and historic Viking samples ($p < 0.01$), but all other samples appear unchanged (Table 5.20). In the historic dataset, both Viking and ScIW areas were significantly different from all other areas, which formed a "common cluster" between the two areas. Though a similar structure exists in the contemporary

dataset, the Viking area is less distinct compared with the historic structure. This is supported and illustrated by the clustering analysis. Results of the clustering analysis was again suggestive of a common cluster of ScOW, ShIW, ShOW and ShIE, flanked by the distinct ScIW and Viking clusters, apparent in the contemporary dataset, was more obvious in the historic dataset (Figure 5.14c).

Table 5.20. Summary of the Fischer's exact probability test of pairwise comparisons of populations from the contemporary spawning period compared with the historic spawning dataset. Significant pairwise differences are indicated in **bold**

POPULATION PAIR			CHI^2	DF	P-VALUE
ScIW_H	&	ScIW_S	11.509615	26	0.994
ScOW_H	&	ScOW_S	29.525115	26	0.288
ShIE_H	&	ShIE_S	35.364623	26	0.104
ShIW_H	&	ShIW_S	30.852127	26	0.234
ShOW_H	&	ShOW_S	32.228918	26	0.186
Viking_H	&	Viking_S	Infinity	26	**0.000**
ScIW_H	&	ScOW_H	49.41902	26	**0.004**
ScIW_H	&	ShIE_H	42.712509	26	**0.021**
ScOW_H	&	ShIE_H	41.113313	26	**0.030**
ScIW_H	&	ShIW_H	40.055289	26	**0.039**
ScOW_H	&	ShIW_H	43.811319	26	**0.016**
ShIE_H	&	ShIW_H	16.378448	26	0.927
ScIW_H	&	ShOW_H	39.829505	26	**0.041**
ScOW_H	&	ShOW_H	28.016635	26	0.358
ShIE_H	&	ShOW_H	22.320079	26	0.671
ShIW_H	&	ShOW_H	25.086897	26	0.514
ScIW_H	&	Viking_H	Infinity	26	**0.000**
ScOW_H	&	Viking_H	Infinity	26	**0.000**
ShIE_H	&	Viking_H	53.76881	26	**0.001**
ShIW_H	&	Viking_H	Infinity	26	**0.000**
ShOW_H	&	Viking_H	Infinity	26	**0.000**

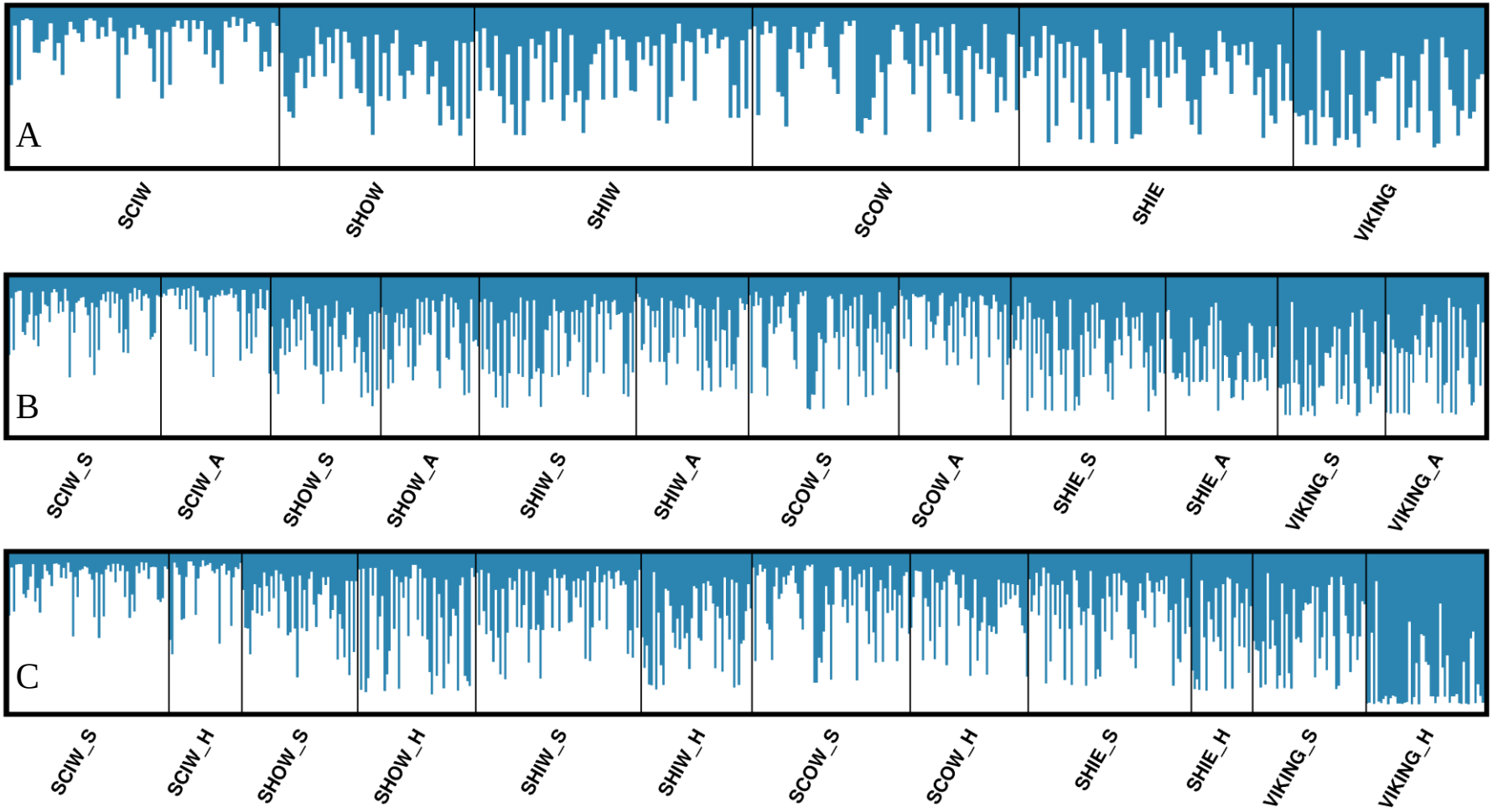

Figure 5.14. Results from clustering analysis of the spawning (A), combined spawning and autumn (B), and combined spawning and "historic" spawning (C) dataset using STRUCTURE with a K value of 2. Samples are ordered to reflect geographical connectivity.

5.4. DISCUSSION

Understanding stock dynamics and the underlying stock structure is fundamental to the development of appropriate management measures for mobile marine species like the Atlantic cod. Increasingly, evidence of stock structure is being incorporated into management strategies, and these strategies are continually developing as better definitions unfold (Jakobsen 1987, Dahle 1991, Ralston and O'Farrell 2008, Botsford et al. 2009, Armstrong et al. 2013, ICES 2015). However, progress is slow as there are numerous knowledge gaps to be filled. Around the UK, several populations of Atlantic cod have been identified, but there is a mismatch between the stock structure observed and the relative management boundaries. Further structuring within these boundaries has also been alluded to through numerous studies, but has yet to be confirmed. For inclusion in future management strategies the stock structure needs to be resolved and these knowledge gaps filled, and it was the aim of this study to address this. Through analysis of SNP data, the current study provides evidence which is suggestive of fine scale structuring in cod stocks around Shetland (IVa) and westward into VIa, which is further supported by the physiological evidence. This considerably expands our current knowledge on the stock structure and concurrent phenotypic traits, and will feed into further development of management for these stocks.

Of the 6 sites analysed through KASP, highly significant differences were observed between Viking and every other site except ShIE, where there is evidence of some mixing. ScIW was also identified to be differentiated from other sites. Though the analysis scenarios assessed using the SNP panels for the smaller RAD sample set allude to finer scale structuring among these site, it is clear from the KASP results that there is some level mixing at all sites, but that mixing is low in both the Viking and ScIW suggestive of an underlying population difference. Conservatively, the genetic analyses suggest a

population structure containing two discernible groups – the Viking and ScIW groups, which flanked a common central cluster where mixing of these genotypes was apparent. The coastal populations around Shetland and the offshore west populations exhibited higher variance in the allelic frequency compared with either the Viking or ScIW sites, but share similarities with both. This analysis is supported by the current phenotypic analysis, which shows a significant difference in the maturation strategies of Viking fish compared with others. Viking cod in the present study were found to mature later and at larger sizes that cod from the other regions which further illustrates the potential isolation of this group.

The Viking Bank cod have previously been recognised as a distinct population based on many studies of genetic relatedness (Heath et al. 2008, Nielsen et al. 2009, Poulsen et al. 2011), tagging (Wright et al. 2006b, Neuenfeldt et al. 2013, Neat et al. 2014), otolith shape (Galley et al. 2006) and as inferred through life-history traits (Yoneda and Wright 2004, Wright et al. 2011b), which supports the current interpretation. Heath et al. (2014) identified a strong differentiation between cod sampled from the Viking area, and cod from the southern and central North Sea, around Shetland and along the Scottish West coast. However, this study did not find a difference between fish sampled in inshore areas west of Scotland and other areas, as was apparent in the current study. Thus, the identification of this inshore group is evidence of further fine-scale structuring of cod in VIa and IVa. Heath's study did not include samples from offshore sites on the west coast; These were included in the current study to analyse the potential western extent of the Viking population. Viking cod appear to have an environmental preference, primarily inhabiting deeper (>200m) waters of the northern and north-eastern North Sea, around Viking Bank (Neat et al. 2014). It was theorised that perhaps these cod might expand along the continental shelf edge into northern and western areas; however, this was not

apparent from the analysis. The lack of any obvious physical barriers to mixing in the North Sea would suggest that behavioural and/or environmental factors are responsible for maintaining the relative discreetness of the Viking group. Considerable tagging evidence would suggest that limited home range may have a part to play (Wright et al. 2006b, Neuenfeldt et al. 2013, Neat et al. 2014). Indeed, genetic and tagging studies from the Skagerrak and Kattegat regions have identified juveniles with a North Sea origin in nursery areas along the coast, but no corresponding adults which would suggest that the Viking population may utilise the Norwegian coastal areas as nursery habitats but juveniles return to their natal spawning grounds upon maturation (Knutsen et al. 2004, Svedäng et al. 2007, André et al. 2016). Investigations of life-stage connectivity suggest that this isolation may also have arisen through oceanographic barriers to early life-stage transport (Heath et al. 2008). Evidence of mixing out-with the spawning season has been observed for Viking and ShIE cod through tagging (Wright et al. 2006b, Neat et al. 2014) and biophysical models of early life-stage transport (Heath et al. 2008). Conversely, the same models of egg and larval transport indicate low connectivity between Viking and the Scottish west coast and there is no tagging evidence for such exchange. Within the central cluster region tagging and otolith microchemistry indicate that settled juveniles mostly recruit to local spawning grounds and adults have a home range of < 100 km (Neat et al. 2006, Wright et al. 2006b). However, otolith microchemistry does suggest that some limited exchange of juveniles may occur between the west of Shetland and the Scottish west coast consistent with the lack of genetic difference observed.

The discovery of a second potentially distinct population centralised around ScIW expands our current knowledge of the population sub-structuring in this region. This group has not previously been identified through genetic analysis, though otolith microchemistry studies and tagging have alluded to it (Wright *et al.* 2006a;b; Gibb *et al.*

2006). Geographically, the ScIW and Viking groups are separated by the greatest distance, and there would appear to be considerable barriers to gene-flow and migration between them. However, similar barriers are not apparent between ScIW and other areas – particularly between ScIW and ScOW. Adults from the ScIW group are known to be highly residential (Wright et al. 2006a), and the limited association with ShIW and ScOW observed through the KASP analysis suggests a low level of mixing during earlier life stages. It is also possible that the apparent separation between ScIW and ScOW represents a behavioural preference for deeper offshore waters by ScOW cod, similar to that observed in the Viking group. This apparent sympatric divergence of ecotypes has previously been observed in southern Icelandic cod populations (Grabowski et al. 2011), and could explain how these apparently over-lapping populations may be distinct.

From the analysis of the autumn and spawning dataset, we see clear evidence of mixing out-with the spawning season. Statistical analysis of the genotyping data identified a significant difference between the spawning and autumn samples from Viking. In addition, the same analysis no longer differentiates between the Viking and ShIE, with the observed allelic variation being similar for these groups. In support of this, there is evidence of large immature fish in the ShIE area, and the other offshore areas to the west. As Viking is the only population where large immature fish were found during the spawning period, these may represent Viking fish. Mixing was also apparent on the west coast with the genotyping analysis detecting a significant difference between the ScIW spawning and autumn samples. This analysis also grouped the west coast sampling areas during the autumn period. Tagging studies have identified similar overlapping in home range out with the spawning season for the Viking and Shetland populations (Neat et al. 2014), but this study is the first to illustrate this through genetics.

The population structure indicated in the current study is clearly evident in the 2003/2004 ("historic") sample set with a central cluster flanked on either side by the highly distinct ScIW and Viking groups. However, there is also a highly significant difference observed between the historic and contemporary Viking groups. The historic Viking group is easily and significantly distinguishable from all other sites in the historic sample set. By comparison, the contemporary Viking group appears to share a greater element of genetic overlap with the central cluster. It is unlikely in the timescale (10yrs) that this difference could represent a genuine evolutionary shift, or even a selective response to pressure. It may however represent an increase in mixing between groups in the study area. Cod stocks in this region have historically been overfished, but substantial improvements to their management have resulted in a steady increase in the spawning stock biomass (SSB) in these regions since 2003 (Holmes et al. 2014, ICES 2015). As a result the cod stocks around Shetland and west of the Scottish mainland have grown (ICES 2015). With this increased density, these cod may have expanded their home range to decrease competition for resources and in doing so, increased the probability of mixing with the contemporary Viking group. If this is the case, it is curious that the maturation phenology of cod in the Viking region has remained consistent with historic maturation trends. It may be that environmental factors have the greatest influence on maturity in cod, and this adoption of later maturing phenotypes by mixing fish highlights the reproductive plasticity of this species.

Of course, the difference observed between the current and historic Viking samples could have arisen through some technical error. However, these sample sets have been checked and re-checked many times to confirm labelling and sampling locations. An additional investigation of the current dataset using the markers described in Heath *et al*. (2014) was recently undertaken by a student at the University of Stirling to further define the

structure observed. Interestingly, this investigation obtained similar results to ours, with a clear switch in genotype assessment of Viking cod between the 2003/2004 dataset and the current one. To provide some confidence in these results, future work should ultimately include a more extensive analysis of cod in this region with a particular focus on the temporal changes. Recent studies have brought to light a new resource in this respect. Methodologies for extracting ancient DNA from otoliths have been developed, allowing us to make use of an extensive temporal resource in the form of otolith archives. The DNA obtained from such studies is generally highly degraded and therefore not appropriate for whole genome sequencing methods such as RAD. However, these samples could be screened for markers developed in this study and others. Analysis of an extended temporal range would allow us to follow the genetic trajectory of this population over the last number of decades, providing an important insight into historic structure of this population.

The level of structure identified in the ddRAD scenarios was considerably more defined than when the chosen SNP panel was applied to the wider sample set through KASP assay. The SNP panels used for these ddRAD scenarios utilised between 40 and 50 informative loci (depending on the processing method), showing high heterogeneity and divergence between the sample sites. In these scenarios, there was clear definition between both ScIW and Viking and all other sites and further fine-scale structuring was observed depending on the in-data pre-processing method applied. Though the most informative SNPs were taken forward for KASP, numbering only 13, the level of coverage and variability between samples was reduced, likely causing the decrease in definition observed. Another reason for this difference may be explained simply. A caveat of the method used for SNP discovery concerns ascertainment bias. The initial RADseq used for SNP discovery, included 20 individuals from each of the 6 sites of interest. While

this ensured that the panel of SNPs chosen for analysis exhibited variability within each of these sites, it is apparent upon comparison with the KASP results, that this variability was not as high when applied to the larger sample set. This is clearly shown by the change in F_{st} values, with several loci exhibiting lower F_{st} values (signifying less heterozygosity) when assessed by KASP and applied to the wider sample set. Had all samples been included in the original RADseq library, the SNP dataset generated would have had greater resolving power, but the costs of such coverage would have been prohibitive. This ascertainment bias also limits the application of these markers to other stocks for similar reasons (Carlsson et al. 2013).

Another point to note, is that the data processing software STACKS, incorporated into the methodology, uses a likelihood model hypothesis testing approach to generate SNP and genotype calls (Catchen et al. 2013). This is a conservative approach and therefore not particularly good at identifying rare alleles, or assessing genotypes when the read numbers for an individual are low. As a result, some discrepancies were observed in the RAD genotyping database when compared with the more robust KASP dataset. This also resulted in a reduction in F_{st} value for some loci when moving from the RAD to KASP sample sets. The ddRAD methodology is relatively new and the associated data processing programmes are still being developed (Peterson et al. 2012, Catchen et al. 2013). It has been indicated that future iterations of STACKS may be able to account for this bias using a Bayesian approach, improving the robustness of the methodology (Catchen et al. 2013).

The SNP discovery method described here (ddRAD) identified over 1,000 putative SNPs for Atlantic cod from the west coast of Scotland and the coastal and offshore waters around Shetland. This approach can be used for rapid and comparatively low cost SNP discovery and validation, even in non-model organisms as it is not dependent on prior

knowledge of the genome. Although the cost is comparable with other traditional methods of marker identification, this approach has the added benefit of identifying large numbers of SNPs. The SNP library generated for use in this study was mined specifically for SNPs exhibiting variability between sampling areas. However, this library could equally be used to discover variable SNPs relating to other divergent characteristics present in the dataset, such as markers for sex, markers for fast growth, or markers for different maturation phenotypes (early v late maturing). Although in theory the RAD sequencing method is thought to mainly identify selectively neutral SNPs (Peterson et al. 2012, Carlsson et al. 2013), many of the SNPs identified and the majority of those used in the current study were from genic regions and therefore non-neutral. However, there was no divergence from Hardy-Weinberg equilibrium for any of the SNPs used, so these markers were unlikely to be under selection. While the use of both neutral and non-neutral markers may show divergence of populations based on selective pressures as well as evolutionary differences, this is unlikely to be the case in this study

There has been a significant shift in age/size at maturity for cod within the study area with a substantial decline in the size and age at which cod first mature in some regions (Yoneda & Wright 2004; Wright *et al.* 2011) Differences in the maturation probabilities observed between regions included in the current study area reflect these shifts, with the majority of fish in the study region maturing at age 2/3 (particularly males). For Viking cod, maturation probability has remained relatively stable between the 1970s and 2000s (Wright et al. 2011b) and is consistent with reports in the early 1900s. This was still evident in the current study. Relative to the other groups, female Viking cod were significantly larger and older at first maturation with an Lp50 of 73.5 cm at age 3. Corresponding Lp50 values from the Wright et al (2011) study indicate that maturity at age/size has changed very little over the last 40 year period, and reflect historic estimates.

These results support the genetic structure evident in the current study and demonstrate that reported differences in maturation schedule may be related to population divergence. It is likely that the difference observed in the Viking group links to their preference for the relative stability of deep water environments and the slower growth associated with such environments (Neat et al. 2014). The lack of similar maturation trends in corresponding environments off the west of Shetland and Scotland might therefore seem curious. Indeed, similar trends in length and age at maturity were once observed for these stocks (Wright et al. 2011b). The current trend towards early maturation in these areas and indeed all other areas around Shetland and off the Scottish west coast most likely reflect local adaptation to fishing pressure. Unlike the Viking population and until very recently, cod from the west coast and around Shetland have been fished beyond the safe biological limits (Holmes et al. 2005). As discussed in the general introduction, this fishing pressure exerts a strong selective force for fast growth and early maturation, which is evident in the current study. The high level of mixing observed between these stocks might suggest a level of resilience and phenotypic plasticity, but it remains to be seen whether the effects of over fishing in these areas will have long-term implications at the genetic level. It should also be noted that, although fishing pressure is likely to be the main driver for small size/young age at maturation observed among these areas, warmer temperatures along the west coast have also likely had an influence, by promoting faster growth (Tobin and Wright 2011, Wright and Tobin 2013). As discussed extensively in Section 4.6, size at the onset of maturation is a key permissive driver of puberty in cod. By extension, the higher growth rate observed among warm-water inhabiting cod will increase the chance that a fish will reach such a permissive state in a given year, decreasing the age at maturity for such groups. This highlights the need to

incorporate environmental factors, such as temperature into the development of PMRNs for such stocks (Grift et al. 2007).

Though similar trends in age/size at maturation were observed in the autumn sample, the high proportion of immature fish at many of the sites suggests that migratory mature fish may not yet have returned to these areas. This would support tagging studies which have shown that migration to the spawning sites is both rapid and direct (Wright et al. 2006a). However, the presence of large immature fish, particularly in offshore sites possibly indicates mixing with Viking fish, as the Viking group was the only one with large immature fish present during the spawning period. This might indicate that Viking cod do travel along the continental shelf out-with the spawning season into deep water regions west of Scotland. Males sampled during the autumn period from the Viking area were all immature, though they ranged in age up to 5 years, and large immature males were found at many of the offshore sites. This is in contrast to the high proportion of young immature males found at all sites during the spawning period. Why these large immature males were not also found in the offshore samples during the spawning period is unknown. It is possible that the influx of mature fish considerably outnumbered these immature fish and our sampling did not account for them as a result. It is equally likely that the cause is behavioural, with the immature fish actively avoiding the spawning aggregates or utilising different habitats. Further work is needed to assess the behaviours of these cod before a conclusion can be made. DST tagging of juvenile fish has not previously been attempted, as mortality rates are high and early tags were too large; But with developments in this area, tags are becoming increasingly smaller and less expensive, presenting a possible avenue to assess the behaviours of immature cod during the spawning season in future.

Differences in the length-age relationship have previously been used to characterise sub-populations within a stock (Knutsen et al. 2015). The results shown here for length-at-age show differences in this characteristic which support the population structure evident through genetic analysis. Both Viking and ScIW – the most genetically distinct groups, exhibited differences in length-at-age compared with the other groups. Where mixing is high, it tends to erode differences in such traits, which would explain why the length-age relationship of areas from the central cluster was similar. Significant differences between this cluster and the Viking and ScIW areas would indicate that mixing between these areas was low. It should be noted however, that this study analysed the size range at a given age for each site, rather than plotting the growth trajectory of a given year-class. It is impossible to assess whether the differences observed represent actual differences in growth or whether they represent natural fluctuations over time, and to properly assess differences in the length-age relationship between these sites, a time series following the growth trajectory of given year-classes should be analysed in future.

The key objective of this study was to assess the population structure of Atlantic cod within the study region using a combination of genetic and physiological assessment techniques. The current genetic analysis alludes to a finer scale structure of Atlantic cod stocks in the IVa and VIa stock regions than has previously been reported by Heath *et al.* (2014). This is supported by the physiological evidence. Together, the genetic assessment and the assessments of growth and maturation identified two potentially distinct groups – the late maturing Viking group, and the early maturing ScIW group. These groups exhibit limited mixing during the spawning season but mix freely with neighbouring areas during the autumn. Both the genetic and physiological evidence points towards high mixing among all other areas. The lack of discernible differences in maturation schedules, and size at age for these areas is indicative of this. The key driver of these differences

appears to be linked to fishing pressure, though temperature along with other behavioural and biophysical factors may explain the apparent divergence of the ScIW group as well as the relative stability of the Viking group. However, disentangling the phenotypic response from potential genetic shifts requires a clear understanding of these factors and how they interrelate. Though clear advances have been made in this respect within this thesis, further work is needed to advance our understanding of driving forces which influences maturation strategies in the wild.

The growing evidence for fine scale population structuring in VIa and IVa, indicated by this study and many others, has important implications for fisheries management. The spawning stock biomass (SSB) in the North Sea has doubled in the last decade, and Viking cod are the biggest contributors to this (Holmes et al. 2014). However, this population matures later signifying a lower overall population growth rate which should be considered in developing sustainable yield targets. If this population becomes depleted it may put pressure on the less productive populations in the inshore and western areas, which may result in local depletion and a reduction in the overall genetic diversity of the stock. As genetic diversity contributes towards stock resilience, maintaining this diversity is a key concern in the face of increasing exploitation and should be acknowledged by fisheries management. While this work and the work of others indicate that Viking should be managed as a separate stock, further work is needed to define this stock and understand the underlying dynamics. Evidence of mixing presented in the current study through genetic analysis indicates that this population may be more resilient than once thought, and that cod in the North Sea exhibit plasticity in their life-history traits. However, further work is needed to assess the level of mixing present and the over-riding effects on population resilience. Further, this work highlights the need to include physiological measures when assessing population structure. The physiological evidence presented here

strongly indicates a difference in the life-history strategy of Viking cod, in spite of potential mixing. The would suggest that environmental factors have a key role to play in driving the observed differences between groups. While these differences may or may not have a genetic basis, they nevertheless require important consideration in the development of management strategies for such stocks.

CHAPTER 6. GENERAL DISCUSSION

According to life history theory the optimal life history strategy of an individual is ultimately a trade-off between traits such as growth, body condition and reproductive investment, and between investment in current and future years (Roff 1984, Stearns 1992). Maturation schedules of many commercial species, especially Atlantic cod and haddock have changed in recent decades with increasing reproductive investment at a cost to somatic investment (Harrald et al. 2010, Wright and Tobin 2013). Whilst it is clear that changing maturation schedules will reflect changes in response to 'proximate' environmental cues, this requires a better understanding of the physiological regulation of maturation/puberty. Photoperiod is generally key to the seasonal timing of reproductive 'decisions' a fish makes. However, the mechanisms of light interpretation and integration are not well understood. To address this, one of the primary aims of this thesis was to improve our understanding of this mechanism, taking inspiration from other vertebrate models. This thesis therefore investigated the role of the *Eya3* pathway (an important mechanism involved in light perception and integration in mammalian and avian species, with a presumed role in seasonal entrainment of reproduction) in the entrainment of maturation in cod (Chapter 3). The photoperiod cue itself is a clear seasonal signal, but the energetically demanding nature of gonad development engenders a need to reach sufficient energetic state before committing to this energetically costly life event. At a particular time or time window driven by the photoperiodic cue, some signal of energetic state plays a permissive/inhibitory role, determining if maturation can proceed. Possible time windows and energetic signals were therefore explored in a series of diet restriction experiments, to determine the nature of the energetic cue and critical time frame in which a fish is sensitive to this signal (Chapter 4). Despite commonalities in the important drivers of maturation, which were further clarified by this research, there are differences

in maturation schedules, implicating other factors in the maturation strategies of cod and haddock. Importantly, these differences indicate a population structure not currently accounted for by management, with costly implications if not addressed. Comparison of population level differences in maturation schedules may provide insight into why temporal changes in maturation probability have arisen, while also providing insight into the physiological population structure, thus inspiring the third line of research described in this thesis (Chapter 5). As a whole, this research was undertaken to improve our understanding of the environmental and endogenous drivers of maturation in cod and haddock with a view to improving our interpretation of the mechanisms driving shifts in the age and size at first maturity in the wild.

A key objective of this thesis was to expand our current understanding of the mechanisms regulating seasonal reproduction in Atlantic cod. Photoperiodic entrainment of seasonal behaviours, reproduction in particular, is evident in most temperate vertebrates, and much work has focussed on describing this system (Migaud et al. 2010). Although the photoreceptive pathways may differ, the neuroendocrine pathway appears to be conserved among vertebrates, and ultimately results in the stimulation of thyroid hormones to initiate the BPG cascade (Follett 2015). At its core, the *Eya3*-*Tshβ*-*Dio2/3* pathway has emerged as a potential mechanism for integrating the photoperiod signal into the reproductive axis, and thus directed our first line of investigation (see Chapter 3).

To the authors' knowledge, this is the first description of *Eya3*, *Tshβ* and *Dio2* genes in Atlantic cod. Both *Tshβ* and *Dio2* are emerging as important markers in the PNES of mammals and other vertebrates, and appear to be highly conserved within the vertebrate clade, suggesting that these markers must have an important functional role in cod. Localised expression of both *Tshβ* and *Dio2* has been observed in other vertebrate models (Nakao et al. 2008a, Yasuo et al. 2010), and although teleost brain structure is

comparatively primitive, it is possible – even likely, that expression of *Tshβ* and *Dio2* is similarly restricted to discrete regions of the brain. Both genes have been characterised in a limited number of teleosts, with current research in salmon suggesting that expression might be contained within the *saccus vasculosus*, a small circumventricular organ of the hypothalamus, surrounding the base of the pituitary on the ventral surface of the brain (Nakane et al. 2013). The same study found significant differences in expression of these genes at the protein level when comparing salmon held under LD and SD stimulus, when expression at the mRNA level was not observed. This result might suggest that post-transcriptional mechanisms for *Tshβ* and *Dio2* regulation of this pathway may be more important than regulation at the transcription level. Clearly, further research is needed to investigate both the localised expression of these genes in cod, and the potential protein-level mediation of this pathway. Though not presented in this thesis, an attempt to look at the localised expression of these markers along with *Eya3* through *in situ* hybridisation was made, but due to fish health issues, the photoperiod trial designed to produce appropriate brain samples failed to create any discernible difference in *Eya3* expression (assessed through QPCR) between treatments and had to be stopped, thus the analysis could not be performed. Nevertheless, this remains an important avenue for future consideration and the application of *in situ* expression measurement would provide spatial context and potentially reveal localised photoperiod response for these targets. Further, future research should not ignore the potential importance of post-transcriptional mechanisms regulating this pathway in vertebrates.

Eya3, exhibits a clear seasonal pattern of expression in cod, being up-regulated under a declining photoperiod, closely matching patterns of gonadotropin expression and increasing GSI, and has been correlated with maturity commitment within this thesis (Chapter 4, Section 4.5.3). As discussed in Chapter 3 (Section 3.4), *Eya3* expression is

clearly regulated by the declining photoperiod and appears to be suppressed when daylight exceeds ~13 hrs, around the spring equinox. The following surge in expression closely corresponds to the internal rhythms of gonad development and *Fsh* secretion which mark the beginning of secondary gametogenesis, highlighting a potential mechanism for the photoperiodic entrainment of maturation around this time point. Secondary gametogenesis has previously been noted as the most energetically costly period in cod (Section 4.1), and it is theorised that a mechanism must exist by which an individual assesses their energetic state before initiating this costly phase of development. Evidence of such a mechanism was highlighted in Chapter 4 (Sections' 4.4 – 4.6) of this thesis, with differential expression of *Eya3* being linked to maturation commitment. As such, the *Eya3* pathway represents an obvious mechanism integrating both the environmental photoperiod signal and the endogenous energetic state signal, for entraining the reproductive axis. As evidence presented in Chapter 3, supported by other teleosts studies, indicate that the pathway itself appears to be conserved among teleosts, but the mechanisms of light perception and interpretation clearly differ between species, further investigation into the upstream mechanisms modulating the *Eya3* response is needed to understand this process. In addition, much could be learned from investigating this pathway in other species which initiate maturation under a declining photoperiodic cue. Some work has been performed on goats indicating that they may exhibit similar mechanisms of light perception and integration (Yasuo et al. 2006). However, Atlantic cod do share this short-day entrainment trait with Seabass (Carrillo et al. 2015), and exploring this pathway in such a species would greatly expand our understanding of the driving mechanisms of photoperiodic entrainment of maturation in fish.

Initially, it had been hoped that this investigation might unearth an early biomarker for maturation commitment, linking the proposed photoperiod cue with the beginning of

secondary gametogenesis, exhibiting a discernible pattern somewhere between the June solstice and the autumn equinox. This was the original proposed window in which Atlantic cod assess their "energetic state" prior to commitment (Davie et al. 2007c, Cowan et al. 2012). However, it is clear from this study that the cascade of events initiating the reproductive axis in cod occurs over a much tighter timescale, with the up-regulation of *Eya3* marking the integration of the photoperiodic cue around the autumn equinox, in conjunction with a surge in the gonadotropins marking initiation of the BPG. Acute induction of *Eya3* has been shown following transition to a permissive photoperiod in quail (Nakao et al. 2008a), mice (Masumoto et al. 2010) and sheep (Dupré et al. 2010) and subsequent responses in the BPG have been observed within days of this response (Yoshimura et al. 2003, Nakao et al. 2008a, Ono et al. 2009a, 2009b). Recent evidence in seabass also suggest that the photosensitive window is much narrower than originally thought, having identified the reducing photoperiod in September (13hrs to 12 hrs daylight) to be the proximate cue entraining reproduction in this species (Espigares et al. 2015). As Atlantic cod exhibit similar short-day entrainment, it is possible that this window also applies to cod. As a result, the original sampling regime developed by Cowan *et al.* (2012), may have not been at high enough a sampling resolution to detect the "critical day length" for initiation of the pathway, and the subsequent cascade of events resulting in the up-regulation of *Fsh*, thus linking the *Eya3* pathway with the onset of maturation. Future studies should focus on; 1) determining the speed of the seasonal cascade, and 2) identifying the critical day length. The first option would ideally assess expression patterns following a switch from LD to SD stimulus with a high resolution sampling regime incorporating several daily sampling sessions over several days following the switch. Identifying the critical day length is likely to be a bit more ambiguous as it is not necessarily a specific day length that initiates the cascade and could

be a relative difference between day lengths. To assess this, fish could be maintained under a constant LD photoperiod which is subsequently and sequentially dropped through different day lengths (13 hrs, 12 hrs, 11 hrs, etc.…), taking samples after each drop. Both research routes would also benefit from incorporating diet manipulations to assess the interplay between the growth and light axes.

The diet manipulation trials described in Chapter 4 were specifically designed to investigate the apparent physiological threshold of size or energetic status that must be met before an individual can enter puberty. Results from these diet manipulations suggest that overall size (weight) is a key factor determining maturation commitment. This was clear from the third experiment where there was a significant difference in the final weight of mature fish compared with their immature counterparts. All fish over 190 g prior to the autumn equinox matured, and no fish below 130 g at this time was found to mature. Recent studies on seabass have equally identified a positive correlation between weight and maturation, suggesting a threshold size is required at the onset of maturation in order to initiate gametogenesis (Carrillo et al. 2015). This is in agreement with the gating theory suggest by Bromage et al. (2001) which states that fish must reach a critical size at a specific stage of maturation development in order to initiate a reproductive cycle.

While the endocrine mechanisms mediating this size effect are currently unknown, recent work by Espigares *et al.* (2015) noted that, although the hormonal rhythms expressed by large mature and small immature seabass were similar, the amplitude of expression was greater in large fish, suggesting that size is a permissive condition, allowing fish to synthesis and release functional levels of the key endocrine drivers initiating maturation. It is therefore possible, that the difference in *Eya3* expression observed in Section 4.4.3 could have resulted from differences in expression linked to size rather than maturity. Further research is clearly needed to disentangle these competing views, and would

benefit from a combined approach, using nested photoperiod and diet manipulations to produce combined and isolated effects of both photoperiod and size on *Eya3* expression and maturation commitment.

The apparent threshold size described in the current study is not definitive – studies in this field have suggests critical sizes of ~100g and lower for cod (Hislop 1984; Saborido-Rey & Junquera 1998; Cook *et al.* 1999). Moreover, the evident population differences from Section 5.3 and reported by Harrald et al. (2010) point to local selection for this threshold. Future research could further define this threshold by utilising photoperiod manipulations - introducing fish to permissive photoperiods at progressively larger sizes to determine the minimum size at which maturation will proceed under such conditions but must also account for population of origin.

Age and size at maturity can be modulated by both genetic and environmental factors and as such, present differences in these life-histories between individuals in the wild may describe a selective adaption to local pressures or may simply reflect genetic drift in response to behavioural or environmental isolation. Knowledge of genetic structure is therefore crucial in understanding the driving mechanisms of contemporary maturation variation in the wild. Thus my final line of investigation was established to determine how maturation phenotype differed in relation to genetic structuring in cod exposed to different exploitation histories (Wright et al., 2011) and environments (Neat et al., 2015). Results of this study indicate that differences in maturation phenotype within our study region correspond with the genetic structure observed as has only previously been inferred. This adds to the increasing evidence linking population structure and maturation thresholds in cod (Olsen et al. 2009). Tentatively, the results gathered here from both the phenotypic data and the genetic analysis, suggest that the Viking and Scottish inshore west sites are relatively discreet. This would largely support the previous genetic analysis

of Heath et al. (2014), with the exception of the Scottish inshore population designation. Viking cod are characterised by a late maturing phenotype. Fishing is likely to be an important contributing factor influencing the genetic population structure observed (Trippel 1995, Law 2000, Jørgensen and Fiksen 2005, Wright et al. 2011b), though temperature and environment also have a role to play. As the Viking region has, historically, been subject to low exploitation and the environment is relatively stable (annual temperature fluctuations of just 1.5 ºC; Wright et al. 2011b), the maturation phenotype has remained stable over the last century (Wright et al. 2011b). Conversely, areas on the west coast of Scotland and around Shetland have, until recently, been heavily exploited. Fishing is a selective process, harvesting fish based on size, and there are a number of theories suggesting both a genetic and an ecological basis for the associated decline in size and ages at first maturity (Grift et al. 2003, Law 2007, Wright 2007, Heino et al. 2002, Rijnsdorp et al. 2010, Wright et al. 2014a). Between inshore and offshore waters, and indeed between the North Sea and the west coast, there are differences in the thermal environment, with offshore deep habitats like Viking being more stable than inshore areas which are comparably more variable thermal environments fluctuating greatly on daily and yearly cycles. Temperature can have a significant influence on growth driving differences in maturation phenology. Reflecting this, the distinction between the ScIW and Viking areas may also represent a behavioural preference for inshore and offshore environments, similar to differences observed between cod populations off Norway (Heino et al. 2002, Olsen et al. 2009) and Iceland (Grabowski et al. 2011, McAdam et al. 2012).

Currently, cod in the study region are managed as a West Coast stock (ICES area VIa) and a North Sea stock. However, these management units do not accurately reflect the population structure identified in the current study. Although some mixing is evident in

all areas, particularly outside the spawning season, it is unclear if this level of gene flow would be enough to sustain these populations in the event of heavy exploitation, or maintain genetic diversity within the stocks. Tentatively, the results gathered here from both the phenotypic data and the genetic analysis, suggest that additional management measures should be implemented to ensure conservation of the Viking and Scottish inshore west cod populations.

This study used the recently developed ddRAD sequencing methodology to prioritise candidate SNPs markers for population structure analysis of cod in our study area. This SNP dataset is an important resource with the potential to be mined further for trait associated markers linked to length and age at maturity. In addition, the sample set itself can be reanalysed as informative markers for growth and reproduction emerge. Very recently, one such marker, vestigial-like family member 3 (*VGLL3*), has been closely linked to age at maturity in Atlantic salmon (Ayllon et al. 2015a, Barson et al. 2015). This gene, an adiposity regulator, was found to describe 39.4% of the variation in age at puberty for salmon (Barson et al. 2015), and has potential as a marker for early maturation. As the gene is also linked to body size and age at maturation in other vertebrate models (Elks et al. 2010, Cousminer et al. 2013, Halperin et al. 2013, Perry et al. 2014), it appears to be highly conserved, playing an important functional role in the initiation of puberty. Adiposity is known to have a key role in modulating puberty in mammals as well as salmon, and it is likely that this gene may serves as an important mechanism linking lipid reserves and maturation in vertebrates, thus this may have an important role in the pathways described above for *Eya3*. If this gene can be found to link with the pathway described above, and this gene is also found to fluctuate between early and late maturing cod in the wild, this would significantly increase our understanding of

the interplay between genetic and environmental regulation of maturity and help disentangle the genetic and phenotypic response to fishing pressure.

Future research should explore this further both in the field and through targeted experimentation. As a first step, the relative importance of this marker in cod should be assessed. The resource already exists to compare *VGLL3* in the North Sea in the form of our Chapter 5 sample set. This sample set includes both late maturing and early maturing phenotypes and would be an excellent first point of call to assess *VGLL3* divergence in cod. If similar results are obtained in cod as are observed among salmon stocks, future work should delve further into the endogenous regulation of this gene in cod and other teleosts.

CONCLUSION

The results of this study greatly increase our understanding of the drivers and mechanisms which entrain the maturation process in gadoids. *Eya3* has been identified as a potential early marker linking the environmental cue with the endogenous axes of growth and reproduction. Further support for the role of size and size related traits in modulating puberty was also shown through diet restriction. Finally, the interaction between these physiological traits and the genetic population structure of wild cod was analysed, hinting that Atlantic cod exhibit a high level of phenotypic plasticity in these traits. Work thus far has largely focussed on the influence of individual factors on maturation commitment in cod, but it is clear that a more holistic approach is needed to get a true understanding of the interaction of traits which influence this highly complex life-history parameter.

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