Research into lumpfish (*Cyclopterus lumpus*) broodstock management and gamete quality.

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DECLARATION OF ORIGINALITY

This thesis is the result of my own work and composed solely by myself except where specifically indicated in the text. It has not been previously submitted, in part or whole, to any university of institution for any degree, diploma, or other qualification.

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Abstract

The lumpfish (*Cyclopterus lumpus*) has been proposed as a sustainable solution to sea lice infestations in Atlantic salmon (*Salmo salar*) aquaculture. Current production is reliant on wild caught broodstock and closed life cycle management is essential to improve sustainability and allow for stock improvement. This thesis aimed to address knowledge gaps in the broodstock management of the species. Holding temperature for broodstock during the spawning season should be kept below 10° C to improve egg quality and spawning performance. In order to extend the spawning season, temperatures can be safely lowered to 6° C without impeding egg quality. Oocyte histology and distribution data suggests that lumpfish are a batch spawning species and provided the first histological oocyte development scale for the species. Subsequent research, aimed to address challenges associated with gamete management to improve artificial fertilisation protocols. E*x vivo* egg ageing suggested that the window of overripening for lumpfish is 24 hours before significant reduction in egg quality. Sperm concentration was also affected by high broodstock holding temperature for this species. Research into milt quantification and storage identified two extender solutions and two rapid methods for assessing sperm concentration to aid milt management. Research into egg quality determinants identified several candidates within egg composition which strongly associated with hatching success within lumpfish. Increased levels of essential fatty acids such as EPA and DHA as well as minerals such as calcium and total levels of pigment within eggs correlated with high hatching success. Analysis of the lumpfish ovarian fluid proteome also identified several protein biomarkers for egg quality through analysis of wild, captive “good” and “bad quality eggs, it also Identified several potential biomarkers for overripe eggs in lumpfish. Overall, this research provides important baseline data on the management

of broodstock and the optimisation of hatchery protocols to close the life cycle of the species.

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ETHICS STATEMENT

All experiments were reviewed and approved before work was allowed to proceed. All work conducted in this study was Animal non ASPA work subject to internal ethical reviews. All experimentation performed at the Institute of Aquaculture (IoA) was subject to an ethical review process carried out by the University of Stirling Animal Welfare and Ethical Review Board (AWERB) prior to the work being approved. Experiments which were conducted in Norway were subject (where necessary) to Norwegian ethical review. Samples sent away to the University of Highlands and Islands for proteomic analyses were also subject to ethical review at their institution.

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List of abbreviations and acronyms

1-D SDS-PAGE	1-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis
ACN	Acetonitrile
ANOVA	analysis of variance
ARA	Arachidonic acid
AWERB	Animal welfare and ethical review body
BCA	Bicinchoninic acid assay
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
©	Copyright
CASA	Computer aided sperm analysis
CFU	Cleanerfish unit, NOFIMA
Circa (italics)	Latin approximately
Da	Daltons
DAG	Diacylglycerol
DD	Degree days
DHA	docosahexaenoic acid, 22:6(n-3)
EFA	essential fatty acids
e.g.	Exempli gratia (for example)

ELISA	enzyme-linked immunosorbent assay
EPA	eicosapentaenoic acid 20:5n-3
Ex vivo	Latin “out of the living”
F1	First generation
FA	Fatty acids
FAME	Fatty acid methyl esters
FDR	False discovery rate
FFA	Free fatty acids
g	Grams
g (AV)(italicised)	relative centrifugal force
gL^{-1}	Grams per litre
G.O	Gene ontology
GLM	General linear model
GSI	Gonadal somatic index
Hh:mm:ss	Timing format hours: minutes: seconds
HIS	Hepato somatic index
HNO3	Nitric acid
HPLC	High performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
hr	Hour

HRS	Herring ringers solution
ICP-MS	Inductively coupled plasma mass spectrometry
i.e.	id est, latin. “That is”
In vivo (italics)	Latin, “within the living”
IoA	Institute of aquaculture, university of Stirling
iTRAQ	Isobaric tag for relative and absolute quantitation
kDa	Killodaltons
Kg	Kilogram
L	Litre
LC-MS/MS	Liquid chromatography tandem mass spectrometry
MASTS	Marine alliance for science and technology scotland
mg g^{-1}	Milligram per gram
mg L^{-1}	Milligrams per litre
mL	Millilitre
mm	Millimeter
mM	MilliMole
mOsm	milliosmole, a function of osmolarity
MRM/SRM	Multiple reaction monitoring/ Selective Reaction Monitoring
MS222	Tricaine mesylate. Anaesthetic
MTE	Modified turbot extender

m/z	Mass-to-charge
n	Number or individuals
nL	Nanolitres
nm	Nanometres
NOFIMA	Norwegian institute of food, fisheries and aquaculture research
OM	Oocyte maturation
OMegv	Oocyte maturation eccentric germinal vesicle
OMgvm	Oocyte maturation germinal vesicle migration
OMpov	Oocyte maturation preovulatory
OSD	Oocyte size distribution
p	P Value
PCA	Principal component analysis
Pers obs	Abbreviation, personal observation
Pers com.	Abbreviation. Personal Communication
PGmn	Primary oocyte maturation multiple nucleoli
PGod	Primary oocyte maturation oil droplets
PGon	Primary oocyte maturation one nucleolus
PGpn	Primary oocyte maturation perinucleolar
pH	“potential of hydrogen” Measure of acidity/ alkalinity
PIT tag	passive integrated transponder tag

POA	Post ovulatory ageing
PPM	Parts per million
PPT	Parts per thousand
PUFA	polyunsaturated fatty acids
r^2	Regression coefficient
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Seconds
SD	Standard deviation
SDS	sodium dodecyl sulfate
SEM	Standard error Mean
SGe	Secondary oocyte growth, early
SGfg	Secondary oocyte growth, final growth
SGl	Secondary oocyte growth, late
sperm.ml^{-1}	Sperm per ml
TAG	Triacylglycerol
TEAB	Tetraethylammonium bromide
TECEP	tris(2-carboxyethyl)phosphine
TL	Total lipid
TMT	Tandem mass tag

µL	Microlitres
µm	Micrometres
UV	Ultraviolet light
Vol	Abbreviation of volume
V:V	Ratio, volume to volume
V:W	Ratio, volume to weight
WW	Wet weight

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Chapter One

General introduction

1.1 **The use of cleaner fish in aquaculture**

In the application of biological control of sea lice in Atlantic salmon (*Salmo salar*) farming there are two main cleaner fish species currently in use, the ballan wrasse (*Labrus bergylta*) and the lumpfish (*Cyclopterus lumpus*) (Treasurer. 2018). The cleaner fish species are introduced into salmon cages and naturally predate on the ectoparasitic sea lice (*Lepeophtheirus salmonis & Caligus sp.*) (Darwall et al. 1992). This delousing behaviour is believed to be less stressful to salmon, than other mechanical methods of delousing. As a result it is considered a vital component of the integrated pest management toolkit available to farms to control the lice infestations along with alternative control measures like therapeutants, physical barrier systems e.g. skirts or snorkel nets, physical delousing methods e.g.thermo/hydrolicers etc. (Costello. 2009, Jackson et al. 2018). The efficacy of delousing is highly dependent on the species used; however, a recent review has highlighted limited evidence of efficacy at farm level trials despite an explosion in use (Overton et al. 2020). The authors also not the lac of comparisons with other forms of delousing.

The use of cleaner fish as a biological control of sea lice was first demonstrated in Norway in 1987, where three species of wild caught wrasse were shown to successfully remove sea lice from farmed salmon (Bjordal. 1991). Ballan wrasse have been used to clean salmon for 30 years, however they exhibit a dormancy during winter, and a more effective alternative has been sought. In 2014 evidence of cleaning ability in lumpfish was documented (Imsland et al. 2014), especially during the vital winter months. In Norway alone, 30 million juvenile lumpfish were deployed in salmon farms in 2017 (Mortensen et al. 2020) with 1.9 million lumpfish in the UK in 2016 (Brooker et al. 2018) and worldwide, production is forecast to exceed 50 million by 2020 (Powell et al. 2018) (Figure 1.1). Implementation of new anti-louse chemicals in

the 1980’s saw the industry turn away from cleaner fish in favour of the chemical therapeutants (Skiftesvik et al. 2013). However, the use of cleaner fish has become a viable alternative again due to the increased resistance to anti-sea louse treatments (Igboeli et al. 2012), reduced consumer acceptance of chemotherapeutic drug use in food and a need for a sustainable and effective anti-sea louse control in salmon aquaculture (Bjordal. 1992). Cleaner fish treatments potentially offer both a low cost and low management strategy when compared to other treatments (Bjordal. 1991. Powell et al. 2018). Initially wild caught juveniles and juveniles from wild caught broodstock have been used. With a large fishing pressure on the wild stocks considered unsustainable as well as potential biosecurity risks (Skiftesvik et al. 2013) the salmon industry has recognised the need for a sustainable, disease free, supply of farmed cleaner fish as a vital production goal.

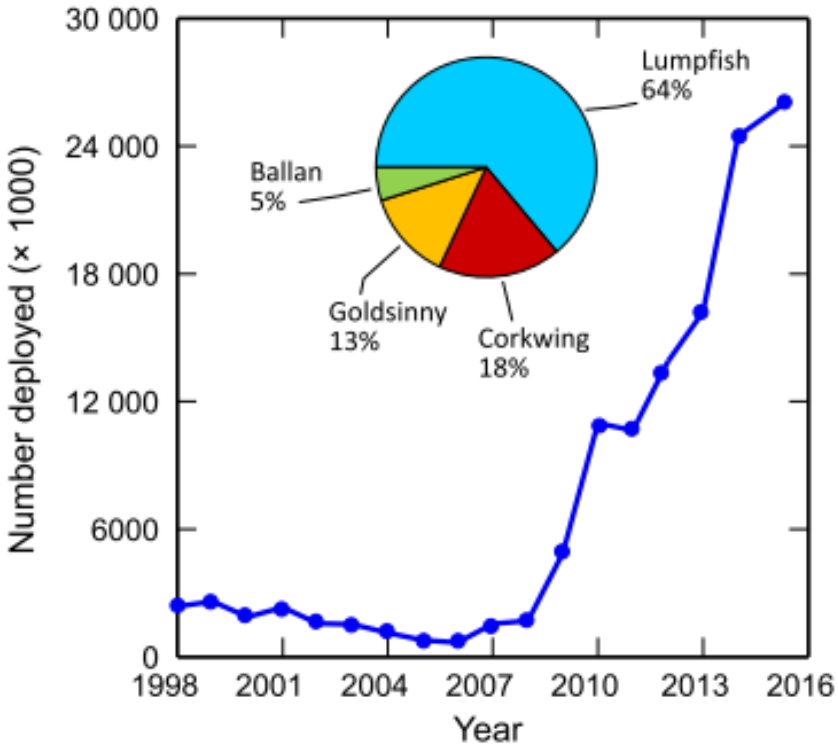

Figure 1.1: Deployment of cleaner fish (×1000) in Atlantic salmon sea pens between 1998 and 2015. Including a breakdown of species during 2015 (inset), (Source: Powell et al 2018).

While ballan wrasse are used effectively at low stocking densities and work well as delousing agents, industry experience suggests they are less effective in colder waters (Leclercq et al. 2014). Salmon farms in the northern regions of Norway, Scotland and Canada regularly experience thermal conditions that may be inappropriate for ballan wrasse and as a result require a suitable alternative for these cold periods or for the entire production cycle. The lumpfish has been shown to be an effective alternative for cold water production in Norway, Iceland and the Faeroe Islands and winter alternative in Scotland (Imsland et al. 2015, Powell et al. 2018). Lumpfish have been observed actively feeding on the gravid female louse (Imsland et al. 2014a), suggesting they

could potentially be an effective cold-water replacement for the ballan wrasse. They require a higher stocking density (10-15 % vs 5 % for ballan wrasse) within the pens to effectively reduce the louse population (Imsland et al. 2014b), however there have been no observed effects on the production of salmon with this increased stocking level despite being observed competing with the salmon for food. The production of lumpfish is a very new industry, with there being very limited knowledge of the species and its requirements for successful production in captivity. As such there are many production challenges that need to be overcome to realise a sustainable supply of captively produced lumpfish. Many of these challenges, which are discussed further below, are made harder to overcome due to the lack of understanding of the species unique basic biology.

1.2 Biology of the Lumpfish

1.2.1 Distribution of the lumpfish

The lumpfish is a pelagic/semi-demersal fish belonging to the family Cyclopteridae (Davenport. 1985). Lumpfish are short globiform, scale-less fish with a high dorsal crest which covers the first dorsal fin entirely and possesses no swim bladder (Davenport. 1985). Lumpfish have a modified pair of pectoral fins, which form a suction disk, commonly used to adhere to surfaces. Much of the anatomical interest in the lumpfish has centred around this sucking ventral disk as well as the large sexual dimorphism displayed within the species (size and colour during spawning season), and cartilaginous skeletal tissue (Davenport. 1985). Davenport & Kjørsvik (1986) noted little skeletal calcification through X-ray's. This reduced calcification of skeletal tissue, as well as reduced muscle density, and thick subcutaneous gelatinous tissue

is thought to enable the lumpfish to maintain buoyancy in its semi-pelagic lifestyle without a swim bladder (Davenport & Kjorsvik. 1986).

The lumpfish is considered a sub-Arctic species, which inhabits the colder waters of the north Atlantic. Eriksen et al. (2014) found that lumpfish were distributed mainly in the 4 – 7 °C water masses within the Barent sea (60% of juveniles, 70% of adults caught during study) indicating association with Atlantic water in the region. Lumpfish fish are widely distributed on both sides of the north Atlantic along the Icelandic, Norwegian, United Kingdom, and Canadian coastlines (Davenport. 1985) (Figure 1.2). It has been suggested that there are three distinct lumpfish populations: west Atlantic north American population (Canada and Greenland), an Icelandic-Norwegian population, and a Baltic sea population (Pampoulie et al. 2014, Garcia-Mayoral et al. 2016, Jónsdóttir et al. 2018, Whittaker et al. 2018).

Juveniles in their first year, post-hatching, can be found adhering to fronds of seaweed in shallow tidal pools and in the sub littoral zone (Daveport. 1985, Ingolfsen & Kristjansson. 2002). After one-year juveniles are recruited to the open ocean community (Davenport. 1985). However little information is known about the juveniles during this stage. It is theorised that juveniles post one year adopt the semi-pelagic lifestyle of the adult (Davenport. 1985).

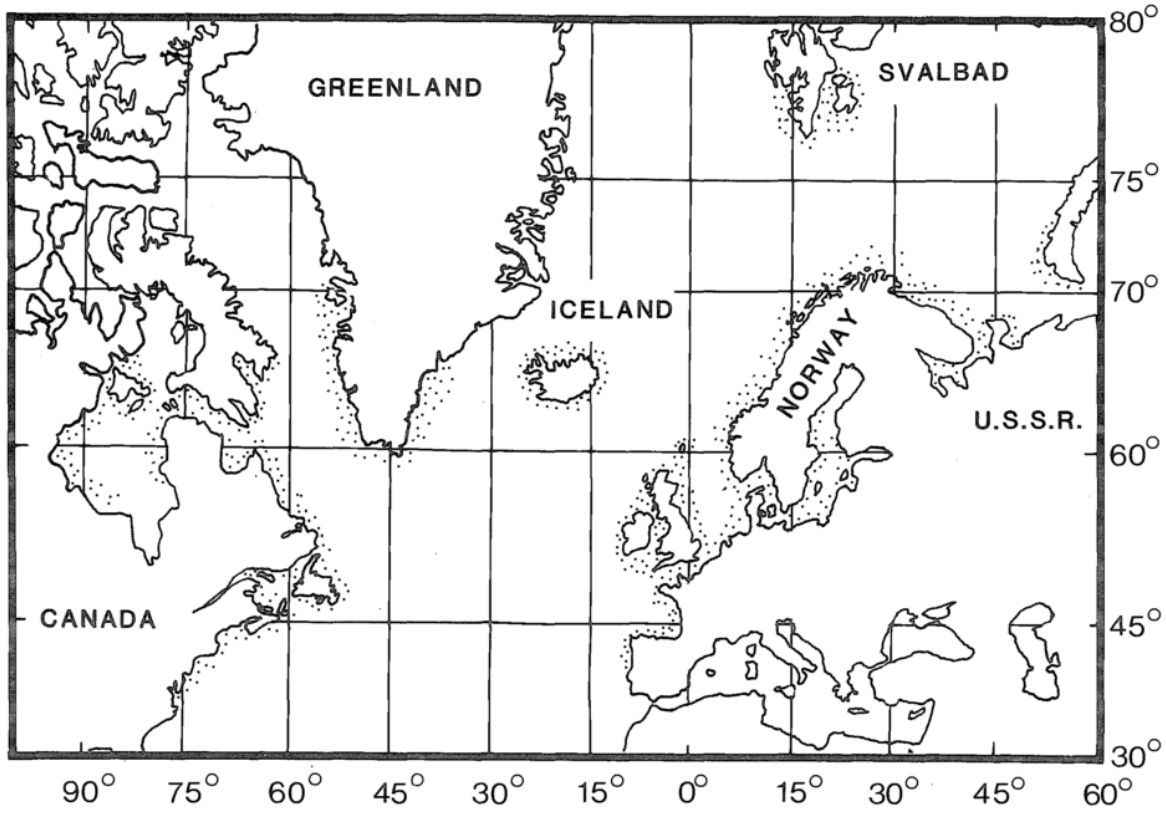

Figure 1.2: Distribution of Lumpfish in the north Atlantic and Arctic seas. (Source: Davenport. 1985).

1.2.2 Reproductive development in lumpfish

Before spawning, in spring and summer, the lumpfish can migrate great distances to shallow coastal waters (Mitamura et al. 2012). They reach maturity in the wild after five to six years (Davenport. 1985, Haatuft. 2015). However captive reared lumpfish have spawned after one to two years (Imsland et al. 2014b, Powell et al. 2018), with experimental broodstocks spawning around 18 months post hatch (*Pers obs*). It has been reported that lumpfish in sea cages start to sexually mature between 450g and 500g (Imsland et al. 2014b). The author suggests a possible link between weight and maturation within lumpfish. However, this weight threshold has not been confirmed as a factor in maturation (Imsland et al. 2014b). During the spawning season, sexually mature males become bright pink or red in colouration with females remaining green,

grey, blue and black (Davenport. 1985, Davenport & Thorsteinsson. 1989). Lumpfish spawning takes place in the shallow sub littoral zone, usually on rocky substrate, Davenport (1985) reported that lumpfish lay 2-3 egg batches over a period of two weeks and Kennedy (2018) supported this with oocyte size distributions displaying two clear cohorts. Kennedy reported bimodal distribution was only present in some females with the assumption that unimodal females had spawned previously. The precise drivers of the extent of batch spawning in lumpfish needs further investigation. The above studies brings into question whether all individuals are truly batch spawning, which of course has impact on the captive management and the estimates of spawning performance in commercial hatcheries that needs closer consideration. The sex ratio for wild caught lumpfish shows a high bias towards the female, with values reported around 75% within the population (Davenport. 1985, Hoenig & Hewitt. 2005, Hedeholm et al. 2014, Kasper et al. 2014). However, fishing effort is heavily biased towards the female of the species, with gear designed to target the largest, slow-moving gravid females it is possible that males are under-represented in the populations (Kasper et al. 2014). Sex ratios in captive reared stocks do not appear to show any sexual bias, a recent study by Imsland et al (2020) showed female percentage in captive stocks ranged between 44% to 59%. Males appear to be able to spawn multiple times and will regularly guard egg masses within their “territory”. There is significant parental care from the male, who plays the main role in rearing (Goulet et al. 1986). Males can often guard two to three egg masses in their territory and it is not uncommon for them to remain with eggs while uncovered at low tides (Davenport. 1985, Goulet et al. 1986). Eggs are laid in a large spongy mass with the number of eggs varying per spawn between 100,000 and 400,000, depending on the size and age of the female (Davenport. 1985).

Fertilisation of the eggs, like most fish species, is external and upon contact with the water the eggs form a sticky egg mass which is ovoid in shape (Davenport. 1983, Davenport et al. 1983, Davenport. 1985). The eggs are bound together by a viscous ovarian fluid, and as it hardens it excretes large amounts of ammonia in the first 24 hours post spawn (Davenport et al. 1983). The fertilised eggs range between 2.2 mm and 2.6 mm in diameter and contain several oil globules which merge to form one single globule through development (Daveport. 1983, Davenport. 1985). There is a wide variation of colours displayed between egg masses, ranging from green and blues to pinks and reds, all of which diminish as development progresses (Davenport. 1985, Davenport & Thorsteinsson. 1989). The incubation period lasts between six to eight weeks (300-350 degree days) and is dependent on external factors such as water temperature and available oxygen (Davenport. 1983). Eggs within the egg mass have been observed to develop at different rates, with length of development increasing towards the centre of the egg mass (Davenport. 1983).

1.2.3 Lumpfish juveniles

Newly hatched lumpfish juveniles are approximately 5.5 mm - 6 mm in length. They hatch with a fin which runs continuously from the back through the tail and underneath to the vent (Davenport. 1985). The ventral sucker is present at this stage allowing immediate adhesion to a surface (Benfey & Methven. 1986). The lumpfish juveniles hatch with a notable mouth and well-developed digestive system that allows them to begin feeding before the disappearance of the yolk sac (Davenport. 1985, Ingolfsson & Kristjansson. 2002). They spend their first year in shallow coastal waters feeding off various zooplankton species with an emphasis on copepods, however, feeding is selective, and they often ignore slow moving or sessile prey items (Davenport. 1985, Ingolfsson & Kristjansson. 2002). Juvenile lumpfish do not use typical larval like

feeding behaviours such as pelagic grazing on prey, instead the majority of feeding occurs while adhered to an available surface (Brown. 1986). After one-year, lumpfish feeding behaviour changes to become more pelagic orientated (Brown. 1986, Ingolfsson & Kristjansson. 2002). Although uncommon, instances of cannibalism have been documented in wild studies (Davenport. 1985, Ingolfsson & Kristjansson. 2002).

Growth in the wild differs significantly between data published for growth in aquaculture. Initial reports by Davenport (1985) suggests that wild lumpfish at one year of age were less than 55 mm in length. A more recent study by Hedeholm et al. (2014) suggest that wild lumpfish reach 10 cm within the first year. Benfrey & Methven (1986) measured initial growth rates in a pilot rearing scheme and post hatching juveniles grew by 7.1 mg and 1.3 mm in the first four weeks of initial feeding. Production data from the UK records captive reared lumpfish reaching over 20 cm within the first year (Unpublished data), however, there is great variation within the populations between spawn groups and season. Growth rates in published works also reflect these faster growth rates (Nytro et al. 2014). Often growth rates in captive aquaculture is faster than those individuals within the wild. However, the high levels of variation within the reporting of wild stocks can be attributed to both sampling methods, and difficulty in assessing lumpfish in the wild (Davenport. 1985)

1.2.4 Lumpfish nutrition

Lumpfish nutrition has been poorly studied throughout its life history, and there is very little on the larval feed preferences in lumpfish, There is data from wild catch gastric lavages as early as 1910 (Davenport. 1985). Mysid and ctenophores composed the main portion of the lavages, however, sand eels and seagrass also made up a small portion of the diet (Davenport. 1985). Catches in Norway by Ingolfsson & Kristjansson.

(2002) have mainly found gut contents of small crustaceans such as gammaridae, copepods and marine worms but the majority of lumpfish's samples had empty or fluid filled guts. During spawning the male lumpfish will stop feeding until the larvae have hatched and it is not uncommon for the male to be stranded at low tide (Davenport. 1985). More recent studies are beginning to shed some light on captive juvenile preferences with more work needed to develop lumpfish nutrition (Ingolfsson & Kristjansson. 2002, Willora et al. 2021).

1.2.5 Lumpfish fisheries and cleanerfish

Traditional industries for lumpfish has mainly been for caviar, with fisheries in Norway, Sweden, Iceland, and Denmark (Davenport. 1985). Lumpfish caviar is commonly used as a substitute for higher quality caviars (Davenport. 1985). In the early half of the 20th century lumpfish was considered a staple fish for consumption in Iceland and Denmark, both with dedicated fisheries (Davenport. 1985). Early production statistics are unreliable as lumpfish catches often came under a "mixed fish" category or collected roe mixed in with other species before landing (Davenport. 1985). Overall fishing effort for lumpfish has increased since 1950 and peaked in 1996 with 73 thousand tonnes. The total catch has remained relatively stable since, between 1996 and 2014 at 73 and 67 thousand tonnes respectively. The fishing effort for lumpfish in recent years has increased due to the increased demand for cleaner fish. Because of this lumpfish are considered to have a moderate to high vulnerability level and have been considered near threatened (NT) by the IUCN red list (Powell et al. 2018).

At present all lumpfish are derived from wild stocks. Skjerneset Fisk in central Norway is currently one of the largest suppliers of lumpfish eggs to northern Europe. However, there are independent fisheries in Iceland, Scotland, Norway and Ireland supplying

local markets. Wild adults are typically captured during the spawning season using gill nets deployed in shallow waters (up to ~30 m deep) (Powell et al. 2018). Fish are held until stripped, both males and females are sacrificially stripped at present due to the desire for disease screening of wild broodstock (*Pers. Com* Tor Otterli Skjerneset Fisk).

Fertilisation of eggs is conducted manually, mixing the sperm with eggs and adding seawater to activate the sperm. Males have been shown to be difficult to strip with only small volumes being collected (Nordberg et al. 2015), most males are sacrificed to obtain milt by dissection of the testes, which are then macerated and passed through a sieve to obtain the sperm (Figure 1.3).

Lumpfish eggs possess a unique problem within aquaculture in that they form a large solid mass upon contact with seawater (Davenport. 1983, Davenport. 1985.) in the wild they are tended to by the males (Goulet et al. 1986). In aquaculture, eggs are moulded into flat pancakes to allow for optimal surface area reducing fungal growth (Powell et al. 2018). there has been some preliminary work in degumming lumpfish eggs, however, this is not yet common practice on farm (Pooley et al. 2019). Eggs are commonly treated with anti-fungal treatments such as Pyceze© or various iodine-based products (*Pers. Obs*) at multiple points during development.

Juveniles are capable of feeding at hatching and are fed on live feeds such as artemia before weaning onto pelleted feed, but juveniles will readily accept commercial diets from hatch (Nytro et al. 2014). Growth rates in captivity are rapid, but variable, with deployment size juveniles (50g) reached in 3 - 4 months (Nytro et al. 2014). Stocks from production can be held back in an attempt to isolate a broodstock for production,

however, a limited number have successfully been maintained. Figure 1.4 displays current production cycle of lumpfish.

The creation of a captive broodstock is paramount in solidifying production in the lumpfish aquaculture sector. At present there have been several attempts at isolating broodstocks by various hatcheries and research institutes. None have successfully created a commercially viable broodstock to date. Stripping of captive stocks have been attempted on farms with experimental broodstocks and through lack of experience and knowledge results in high mortality and low egg quality. Some farms have attempted to use the “natural” spawning broodstock management, similar to that conducted for ballan wrasse (Grant et al. 2016) also with low production success. Powell et al. (2018) suggests that there is 10% survival post spawning and that lumpfish are semelparous, citing the high gonadal investment. This is also suggested by Kennedy et al. (2018) during a study in Iceland, which suggested that survival rates for wild lumpfish is 10% post spawning. However more recent evidence from tag and recapture studies and tracking in Norway and Iceland suggest that there is a high level of female survival post spawning (Kennedy et al, 2020). Experimental broodstock being held at NOFIMA CFU were retained for multiple spawning seasons with low stock mortality (<10 %) (*Pers. Com* Ingrid Lein NOFIMA). This could be due to a more selective stripping process (ultrasound and vent assessment), better handling techniques, water conditions (stable, colder, highly filtered) or a wide range of factors which could allow multiple year broodstocks.

Figure 1.3 Typical images of broodstock stripping. Stripping of females is conducted in a dry environment by gently applying pressure to the abdomen and vent (Left). Stripping of males in conducted by dissection and maceration of the testis (Right).

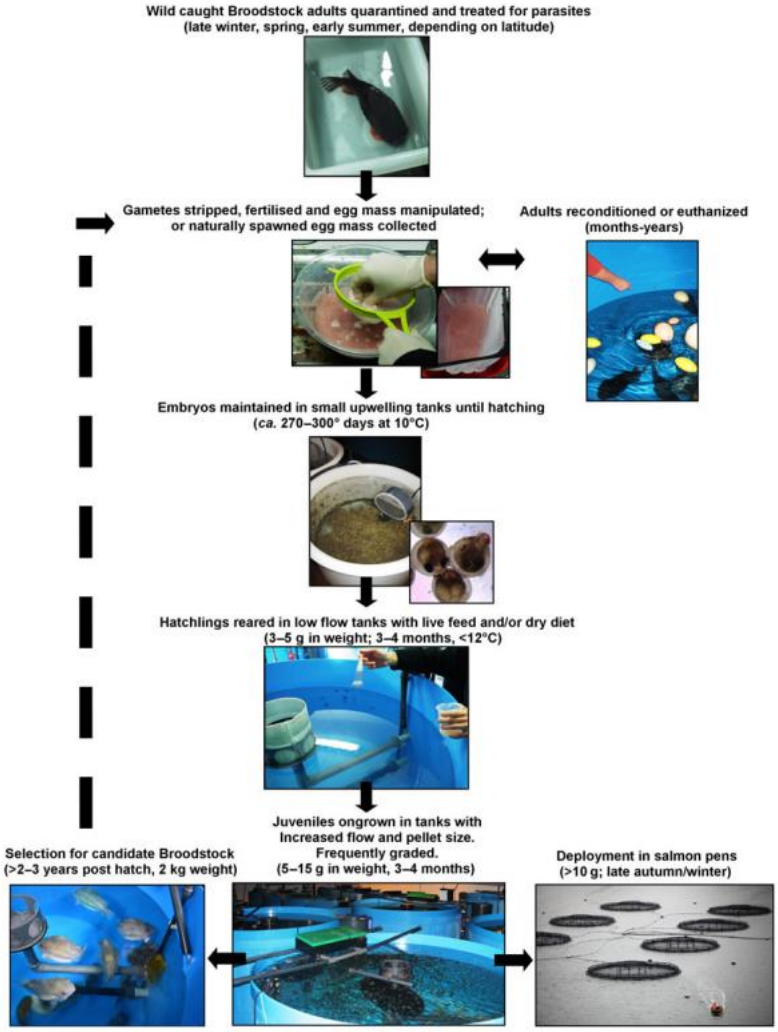

Figure 1.4: Hatchery management and lifecycle of the lumpfish. (Powell et al. 2018)

1.3 Broodstock management of a new aquaculture species

The number of fish species currently domesticated, or under domestication has been increasing rapidly due to the development of commercial aquaculture (Huston et al. 2020). A requirement for domestication and maintaining a sustainable aquaculture product is the development of a captive broodstock from which high-quality seed can be obtained (Mylonas et al. 2010). For many farmed species there is still reliance on the collection of wild broodstock, eggs or juveniles, such as bluefin tuna (*Thunnus spp*), freshwater eel (*Anguilla spp*) and indeed lumpfish(Mylonas et al. 2010). This reliance is both environmentally destructive and unsustainable and decreases in wild populations are commonly seen after intensive fishing efforts (Bene et al. 2015). The successful establishment and domestication of a new species to aquaculture requires understanding of the reproductive biology of the species, the development of tools to monitor performance and a set of protocols to optimise productivity and quality (Migaud et al. 2013). The process of domestication is broadly common for all fish species, although there are species-specific reproductive strategies, which means that different issues must be addressed to enable production to meet commercial viability.

1.3.1 Maturation and spawning

A key stage in closing the life cycle of a new aquaculture species is determining the process and controls of maturation and spawning within the species. Identifying maturation of both males and females is important for both aquaculture and fisheries management of species (Mylonas et al. 2010). While understanding the reproductive strategy of a given species, such as spawning length, batch or non-batch spawning, and reproductive windows is also important in defining how to manage the species (Kjesbu.1994, Tveiten & Johnsen. 1999, Migaud et al. 2013). Once information on

gonadal development has been determined for the species, it can allow for the creation of protocols for reproductive management within hatcheries as well as allow for environmental manipulation of reproduction (Brooks et al. 1997, Van Der Meeren & Ivannikov. 2006).

Oocytes develop within the ovaries through different stages, while there are many species-specific differences, the sequence of oocyte developmental stages can be generalised (Murua & Saborido-Rey. 2003). Based on the dynamics of organisation of the ovaries, species can be classified into three categories of ovarian development (Murua & Saborido-Rey. 2003, Babin et al. 2007, Grier et al. 2009) synchronous, group synchronous and asynchronous.

Within Synchronous species all oocytes develop and mature at the same time. This is common in many anadromous species such as salmonids (Murua & Saborido-Rey. 2003). Spawning for these species occurs in a singular event. In Group synchronous species at least two populations of oocytes can be defined in the ovary at any one time (Murua & Saborido-Rey. 2003). There is a synchronous population of larger oocytes, along with a more heterogeneous population of smaller oocytes from which another batch is recruited. This form of development is common in iteroparous species with relatively small spawning seasons where yolk deposits depend on body reserves such as Atlantic halibut (*Hippoglossus hippoglossus*) (Norberg et al. 1991) and Atlantic cod (*Gadus morhua*) (Kjesbu et al. 2010). The spawning season for group synchronous species usually occurs over a number of weeks or months with distinct periods between spawn dates, such as 3-5 days for Atlantic Halibut (Norberg et al. 1991). Asynchronous species display all stages of oocyte development within the ovaries at any one time, differentiation between cohorts is only distinguishable when hydration occurs. This method of development is common in iteroparous species where oocyte

development and spawning frequency relies on food availability within the environment at a given moment, such as the European hake (*Merluccius merluccius*) (Murua, & Motos. 2006) and Atlantic horse mackerel (*Trachurus trachurus*) (Ndjaula et al. 2006). Egg size within a species is affected by nutritional status of the females during development (Brooks et al. 1997), this can affect asynchronous spawners where eggs ovulated in the later batches during a spawning season display a reduction in quality, associated with the diminishing resources of the female (Kjesbu et al. 1996). The reproductive strategy and process of ovarian development dictates the manner in which the stock is managed in captivity *e.g.* feeding strategy and environmental management, will have to be adjusted accordingly. But if the strategy is not known the characterisation of ovarian development using methods like histological analysis are vital in determining the process of ovarian development and in turn dictating how broodstock should be managed.

In all teleost species studied, oocytes appear to undergo a similar basic pattern of growth (Babin et al. 2007, Grier et al. 2009). Microscopic studies of the stages of oocyte growth have been conducted in many commercially important species such as Atlantic halibut (Hendry et al. 2003), Atlantic cod (Kjesbu & Kryvi. 1989), and common snook (*Centropomus undecimalis*) (Rhody et al. 2013). There is a number of classification systems proposed to document the development of oocytes within the ovary (Babin et al. 2007). Within this area there is the requirement to standardise measurements within a single classification system (Grier et al. 2009, Rhody et al. 2013) to allow for comparisons between and within species. These systems of classification incorporate physiological, biochemical, morphological and histological criteria, including follicle size, somatic tissue types and yolk presence (Grier et al. 2009). The major developmental stages are as follows: oogenesis, primary oocyte

growth, cortical alveolus stage, vitellogenesis, maturation, and ovulation (Murua & Saborido-Rey. 2003). Two or three of these phases can be present within the gonad simultaneously during development (Babin et al. 2007).

The determination of histological definitions can be utilised to interpret the impacts of management strategies on the reproductive development of the new species. This has been effective in closing the life cycle of other marine finfish such as, European seabass (*Dicentrarchus labrax*) (Mayer et al.1988), Atlantic cod (Kjesbu & Kryvi. 1989) or common snook (Rhody et al. 2013) for example. While it is common for oocyte development to typically follow a common cellular development process, there are species specific definitions of size for each developmental stage which is key in interpreting ovarian development. Protocols which enable the rapid characterisation of the diversity of ovarian development in a given individual like oocyte size distribution (OSD) can be very informative (Kjesbu & Kryvi. 1989). Use of such a protocol can allow assessments of the developmental stage of the individual sampled, by placing the individual into defined maturation categories. This can be used on farm for stock management, but also experimentally to determine the effects of treatments on reproduction and maturation (Kjesbu. 1994, Brown et al. 2006, Migaud et al. 2013).

Reproduction imposes considerable metabolic demands in fish. In synchronous spawners, the investment in the gonads is usually considerably higher than asynchronous spawners. The gonadosomatic index (GSI = gonad weight/(body weight- ovary weight) x 100) in synchronous spawning females is usually high with large investments in gonads (Babin et al. 2007). GSIs in mature asynchronous spawners are generally lower than those seen in synchronous spawners (Rinchard & Kestemont. 1996). This lower levels of gonadal investment at a given time point does not reflect the total oocyte investment with GSI varying over the spawning seasons

and may not be comparatively high but is reached several times during a spawning season (Babin et al. 2007). This is important for future broodstock management of the species as methods of gonadal development and investment are species specific and have important impacts on the management of the species.

Ovulated egg size varies between populations and individuals of the same species (Kjesbu et al. 1996). Age at maturity may also affect egg size in fish (Sargent et al. 1999), larger body sizes tend to correlate to larger egg size (Bromage et al. 1992). This is important for management of repeat spawning stocks, where egg quality, and maternal investment in earlier maturation may lead to poor egg quality in initial spawning years (Sargent et al. 1999).

1.3.2 Environmental manipulations

The reproductive cycle of teleosts resident in temperate latitudes is highly seasonal (Van Der Meeren, & Ivannikov. 2006, Kjesbu et al. 2010). In captivity, the accurate simulation and manipulation of environmental stimuli of broodstock is key to synchronising broodstock production and assuring good quality eggs and juveniles for subsequent on growing (Migaud et al. 2013). Environmental management can also be used to extend, shorten or delay maturation and spawning within the species allowing the accurate timing of production to when juveniles are required in hatcheries (Norberg et al. 2001, Tveiten et al. 2001, Norberg et al. 2004). In most temperate marine species, it is apparent that both the seasonally changing day length as well as temperature play important roles in the regulation of reproductive development, timing of the spawning season, productivity and quality (Brooks et al.1997).

Photoperiod plays a strong role in the early stages of maturation and has been shown to affect the seasonal timing of maturation in many species including salmonids

(Bromage. 2001), Atlantic halibut (Norberg et al. 2001) and Atlantic cod (Van der Meeren and Ivannikov, 2006). Manipulating the photoperiod to mimic different seasonality can advance or delay maturation within broodstock groups (Migaud et al. 2013). This is important for matching juvenile production to meet the demand for juveniles as well as reducing hatchery effort over a longer period of spawning. The effects of photoperiod on maturation is highly species specific and thus investigations into the effects on new aquaculture species is important in advancing broodstock management (Watanabe et al. 2006). Determining the effects of such manipulations is reliant on accurate oocyte and sperm development histological data for the species.

Temperature is a well-studied cue in the sexual development of many fish species, it plays two key roles within reproductive development and spawning. During gametogenesis, temperature manipulations can influence the pace of development and the timing of spawning windows as demonstrated in the Common wolffish (*Anarhichas lupus*) (Tveiten & Johnsen. 1999) and to a lesser degree influence fecundity (Kraus et al. 2000) as well as subsequent gamete quality, as demonstrated in Atlantic cod (Rideout et al. 2000). However, during the spawning season itself, temperature plays the stronger regulatory role in determining spawning windows (Kjesbu. 1994; Tveiten et al. 2001), ovulation cycles (Brown et al. 2006) and most importantly gamete quality (Migaud et al. 2013). Within the range of suitable temperature conditions, higher temperatures usually accelerate maturation and spawning, whereas lower temperatures typically slow down and widen the spawning window (Mylonas et al. 2010). For example, in Atlantic cod, Kjesbu (1994) demonstrated that exposure to elevated temperatures resulted in shorter spawning windows than those held at lower temperatures. As such, temperature management, combined with photoperiod manipulations are commonly used to manage the

spawning of broodstocks in numerous captive stocks including Atlantic cod (Davie et al. 2007) and Atlantic halibut (Brown et al. 2006).

1.3.3 Egg quality determinants

The production of good quality eggs is the first stage in ensuring sustainable hatchery production. Egg quality is defined as the characteristics of an egg which determines its capacity to be fertilised and survive (Bobe & Labbe. 2010, Migaud et al. 2013). The characteristics which affect egg quality can be external environmental factors, such as temperature (Migaud et al. 2013) in combination with intrinsic factors, such as nutritional status (Izquierdo et al. 2001) and underlying genetics (Huston et al. 2020).

Good egg quality is essential for maximising productivity of hatcheries as well as advancing on to closing the life cycle of the species before allowing genetic selection to be implemented (Bobe & Labbe. 2010). Underpinning egg quality is the requirement for descriptions of oocyte maturation, spawning windows and environmental controls within the species, which can aid management choices, inform on effects of nutritional composition. Environmental manipulations documented above have been shown to have significant effects on egg quality (Migaud et al. 2013).

Nutritional composition of broodstock diets also has a significant effect on egg quality (Izquierdo et al. 2001). Broodstock nutrition is fundamental in producing good quality eggs and directly influences egg quality. Good broodstock nutrition allows the fish to provide sufficient nutrients into the egg yolk, which is used by the egg and larvae until first feeding (Izquierdo et al. 2001, Migaud et al. 2013). Studies attempting to characterise the effect of broodstock nutrition on egg quality have been conducted in species for which nutritional requirements were not fully identified, thus leading to reproductive performances lower than what had been reported using natural diets

(Mazorra et al. 2003). This is especially true for newly domesticated species where direct comparison of wild and captive stocks display significant failings in reproductive success seen in Atlantic halibut (Mommens et al. 2018a) and white sea bream (*Diplodus sargus*) (Cejas et al. 2003). Thus. broodstock diets need to be formulated to ensure all essential requirements are met for the target species requiring dedicated focus on individual species requirements (Izquierdo et al. 2001). In most marine species, the main differences in egg quality between farmed and wild broodstocks are driven mostly by the fatty acid composition eg. Atlantic cod (Salze et al. 2005), Atlantic halibut (Mazorra et al. 2003) and white seabream (Perez et al. 2007). However, more recent work has demonstrated significant effects on other nutritional components such as mineral content, vitamins, amino acids and pigments on egg quality (Palace & Werner. 2006, Sawanboonchun et al. 2008).

A lack of key nutritional components can have deleterious effects on reproduction, and deficiencies in essential vitamins and minerals can reduce overall egg quality, hatching and juvenile survival (Migaud et al. 2013). Fish fed on areduced inadequate diet can display lower fecundity and delayed, or stalled maturation (Kjorsvik et al. 1990). This is particularly relevant to new aquaculture species, where nutritional requirements may not be as well studied. Lipids and fatty acid composition of broodstock feeds have been identified as major dietary factors that influence successful reproduction and survival of offspring (Izquierdo et al. 2001). Lipids are the most widely documented determinant of egg quality (Rainuzzo et al. 1997, Izquierdo et al. 2001) and are the chemical constituent which have the greatest affects the composition of eggs (Wantanabe, 1985). Most fish preferentially utilise lipids for somatic growth, but they also provide a source of essential fatty acids (EFA) required for the formation of cell membranes (Sargent et al. 2003). It is vital that levels of lipids and essential fatty acids

are present in excess to levels required within the eggs, in broodstock diets to allow for sufficient uptake by the females. Egg quality and juvenile survival have been directly linked to the content of polyunsaturated fatty acids (PUFAS), in particular n-3 fatty acids such as DHA and EPA (Mazorra et al. 2003, Perez et al. 2007). However, the importance of each fatty acid is highly species specific and requires species specific investigations.

In addition to fatty acids, fish eggs contain micronutrients and growth regulators that are essential for development (Kjørsvik et al. 1990, Brooks et al. 1997). These micronutrients include minerals such as zinc, calcium, iron, copper and magnesium that are acquired through vitellogenin (Ghosh & Thomas. 1995). Eggs can import some levels of minerals into the eggs; it is possible this is for osmotic reasons as well as active transport of minerals into the eggs (Lall. 2003). However, some minerals are prevented from entering the egg by the chorion, such as cadmium and selenium (Lall. 2003). Phosphorus has been shown to have an effect on Cod reproductive performance (Lanes et al. 2012). Phosphorus, however, has a relationship with lipid levels, due to their incorporation in phospholipids and their role in quality could be controlled by their inclusion within lipid fraction. The role of calcium has been identified in the hardening of eggs such as Atlantic cod and the lumpfish (Loning et al. 1984).

The effect of pigments, and their role in egg quality is less well documented, and many captive eggs have been shown to be lacking in carotenoids (Slaze et al. 2005) where the difference in egg pigments was correlated to the difference in egg quality. However, there has been some work in recent years and carotenoids are now commonly supplemented in fish diets (Lim et al. 2018). Within salmonids, astaxanthin is thought to have a role as a pro-Vitamin A as well as protective properties for the egg and skin during early development (Christiansen & Torrissen. 1997). Other theories

on the function of carotenoids suggest possible UV protection and improved respiratory function (Mikulin. 2000). It has also been suggested that they may play a role in photoreception within the embryo (Ronnestad et al. 1998). Determining the egg compositional factors which effect egg quality is key in providing sufficient robust juveniles for on growing. It provides a guideline for the creation of broodstock diets which better address the nutritional requirements for good gamete quality.

1.3.4 Gamete management

Hatchery management techniques of fish gametes are important procedures for improving the survival of eggs and juveniles (Bobe & Labbe. 2010). While most captive broodstock species will spawn freely in captivity (Mylonas et al. 2010) there remains some species e.g. salmonids (Kincaid, & Stanley. 1989), flatfish sp. including Atlantic halibut (Holmefjord et al. 1993) as well as lumpfish (Powell et al. 2018), where natural spawning will not reliably occur in captivity and thus stripping and subsequent artificial fertilisation of gametes is required. Thus, management of eggs during this period is key, and mismanagement can have detrimental effects on egg quality. Stripping of fish allows for the surface disinfection of eggs to reduce the linear spread of diseases from broodstock (Salvesen & Vadstein. 1995), improve fertilisation protocols (Suquet et al. 1995, Butts et al. 2009) manage egg masses (Grant et al. 2016a) and open up these species to genetic manipulations, for delousing ability or reduced growth rates (Huston et al. 2020). Underpinning this management is the accurate assessment of the maturation stages of the broodstock to ensure that the fish are stripped shortly after ovulation as eggs held in the body for longer periods of time can result in deterioration of egg quality and eventual reabsorption into the maternal tissue (Craik & Harvey. 1984). Norberg (1991) found that there was a need to closely monitor ovulation in individual Atlantic halibut females to optimise egg quantity and quality with an increase

of 120% yield and 220% fertilisation rate with good monitoring programs. This latency period in which egg reabsorption occurs depends on the species of fish, water temperature (Yaron. 1995) any hormonal manipulation to induce spawning or ovulation, as seen in other species (Mylonas et al. 2010) and the life history of the fish, *eg* temperature experienced, photoperiod *etc* (Van Der Meeren & Ivannikov. 2006). Once eggs have been stripped there is a limited window that they are viable for fertilisation before hatching success is reduced (Poon & Johnson. 1970). Any transporting of both male and female gametes need to be conducted quickly with minimum stress.

The process of egg degradation following ovulation is called post ovulatory aging (POA) but can also referred to as egg aging or over-ripening (Mommens et al. 2015b). Increased POA, either due to eggs being held within the body or following striping while eggs are being held *ex vivo* due to hatchery protocols, can lead to major morphological and biochemical changes in the egg. Interestingly, this depression in quality appears to be similar in eggs retained within the body cavity and stripped eggs, suggesting it is a process endogenous to the eggs themselves possibly in interaction with the ovarian fluid (Bromage et al. 1994). POA has been shown to lead to decreased viability in several species including Atlantic salmon (Mommens et al. 2015b), rainbow trout (*Oncorhynchus mykiss*) (Aegerter & Jalabert. 2004), Atlantic halibut (Bromage et al. 1994) and turbot (McEvoy. 1984). The POA process can last between hours and days, to weeks in the case of salmonids. This ultimately means that more intensive management of broodstock is required in those species which have shorter windows of POA though increased frequency of stock assessments.

The importance of milt management strategies such as cryopreservation, cold storage, and information regarding quantification, fertilisation ratios *etc.* can significantly

improve the efficiency of hatchery production especially if males are a limiting factor in production. Both cold storage and cryopreservation can be used for milt preservation in aquaculture (Migaud et al. 2013). Cold storage requires storing milt diluted in extenders at low temperatures, typically 4 °C (Gallego and Asturiano. 2019) to reduce spermatozoa metabolism allowing them to be stored for longer periods of time without significant changes in milt quality (Chang. 2002). For longer term storage, between spawning seasons (Scott and Baynes. 1980) or for creating genetic storage banks (Gausen. 1993), cryopreservation is the only effective method, keeping milt diluted in a cryoprotectant solution at ultralow temperatures between -79 °C and -196 °C in liquid nitrogen. This method requires specific infrastructure to enable a precise freeze and thawing of the milt. Practically, cold storage of milt is the most useful technique available to support hatchery production by providing a low cost and technically simple solution to the challenge of male availability. The purpose of chilled storage is to allow farms to perform artificial fertilisation in a controlled manner using desirable males. Artificial fertilisation protocols must be standardised, and gamete quality assessed when crosses are made (Jenkins-Keeran and Woods. 2002, Beirão et al. 2019). Simple and accurate methods for milt quantification are important in this context for two reasons. Firstly, it allows standardisation of egg to sperm ratios which have been shown to influence fertilisation success in many species including turbot (*Scophthalmus maximus*) (Suquet et al. 1995), and Atlantic halibut (Tvedt & Benfey. 2001). Secondly, it enables the quantification of total sperm being held in storage which allows farms to accurately plan the volume of eggs that can be fertilised (Cabrita et al. 2014). Thus, methods of identification of sperm concentration are key in developing these strategies.

The above section has summarised some of the key factors influencing success of broodstock management in aquaculture species. However, it continually highlights the need for species specific information, to realise optimal hatchery performance. The success of the newly developing lumpfish hatchery depends on identification of lumpfish specific requirements. Where some information can be derived from the current available scientific literature many of the requirements will need to be determined experimentally.

1.4 Challenges in the broodstock management of lumpfish

Production of lumpfish juveniles has increased dramatically over the past few years, Powell et al. (2018) reported 0.8 million and 1.1 million juveniles in the UK and Norway respectively for 2015. Brooker et al. (2018) reported 1.9 and 15.9 million lumpfish were produced in the UK and Norway respectively and Mortensen et al. (2020) reported 30 million juvenile lumpfish deployed in Norway alone in 2017. At present the majority of this production is derived from wild caught broodstock, which are stripped, and juveniles raised until deployment sizes. Such a wild harvest has brought some to question the sustainability of the process (Powell et al. 2018), however, the long term goal for the sector is closed life cycle management (Huston et al. 2020) given that there is a push to determine genetic effects of delousing and cage performance (Imsland et al. 2020), reduce disease challenges (Einarsdottir et al. 2018, Skoge et al. 2018) and control production timing to optimise deployment (Imsland et al. 2019b).

There has been little work on the broodstock management of lumpfish to date, with the majority of work being focused on the performance and robustness of juveniles (Klakegg et al. 2020). However, recent years have seen an increased interest in closing the lifecycle of the species (Imsland et al. 2018). Within Scotland there have

been several attempts to create a commercial captive lumpfish broodstock (*Pers com*, Alastair Barge, Otter Ferry Sea Fish Ltd). However, the availability of wild caught broodstock, disease issues within isolated broodstocks and poor survival and spawning performance has made large scale investment in a commercial captive broodstock undesirable without further evidence of success. At present broodstock creation has been from production stocks (*Pers com*. David Patterson Otter Ferry Seafish Ltd), with numbers of (usually) fast growing juveniles held back from deployment and on-grown in tank-based facilities as prospective broodstock, though success has been poor to date.

Fundamentally this lack of success is routed in the lack of knowledge regarding the basic process of reproductive development of the species. Furthermore, there is equally a lack of understanding of the environmental parameters that will dictate spawning entrainment and gamete quality in lumpfish. The lumpfish reach maturity in the wild after five to six years (Davenport. 1985, Haauft. 2015); however, lumpfish can spawn after one to two years in captivity (Imsland et al. 2014a, Powell et al. 2018). Definition of the natural spawning season for the lumpfish is highly variable in the literature. Davenport (1985), described the lumpfish spawning season as occurring between April and July with no geographic reference. A recent report by Kennedy (2014) noted that spawning season in Iceland was between January and March, which is the focal window for commercial exploitation. However, fishing for mature lumpfish in central Norway occurs between September and June (*Pers. Com*, Tor Otterlei Skjerneset Fisk) with the main catch window from October to May, with this fishery being the principal supplier of lumpfish eggs to Norwegian and UK based hatcheries. Photoperiod could be considered the driving factor behind maturation within the species, however, data from Imsland et al. (2018a) as well as fisheries data suggests

that photoperiod is not the main driving factor behind the entrainment of maturation in lumpfish. The study (Imsland et al. 2018a) clearly shows that male maturation is not regulated by photoperiod as, spawning colouration and running milt was seen for males in all three photoperiod groups from September onwards. With regards to the females under “natural photoperiod” conditions the authors report no spontaneous female spawning within their study and within all alternative photoperiod stocks both spawned in June and July, independent of photoperiod treatment. Imsland et al. (2014a) notes that cage deployed lumpfish at 400g displayed visible gonads on dissections suggesting a possible weight threshold for maturation in the species. Central Norway spawning of lumpfish, as described above occurs 10 months out of 12 with July and August not seeing spawning behaviour. It is possible, therefore, that temperature plays an important role in the gonadal development, however, there is currently no information on this. Therefore, clearly there is a critical need to determine temperature requirements of lumpfish broodstocks, the interplay of temperature and photoperiod in determining the entrainment and pace of reproductive development as well as the timing and quality of spawning itself (Migaud et al. 2013).

Hatchery experience to date suggest that natural spawning, similar to that conducted for ballan wrasse for example (Grant et al. 2016b) has low production success, and thus striping and artificial fertilisation will be the management approach adopted going forward. Survival post stripping in captivity has been reported to be low (*circa* 10%) (Hedeholm et al. 2014, Powell et al 2018). However, there is a disparity between reports with captive broodstock being held at NOFIMA cleaner fish unit, Sundalsorra, Norway, reportedly being retained for multiple spawning seasons with low stock mortality (<10 %) (*Pers Com.* Ingrid Lein NOFIMA). Factors that influence such a success could be many, but the case highlights that there is scope for significant

improvement in broodstock survival and thus performance, if handling protocols can be optimised. As explained previously, key to the reproducible success of stripping and artificial fertilisation as a production method, is a clear understanding of the reproductive development of the species to ensure gametes are harvested and manipulated optimally. However, information on the reproductive development of lumpfish is currently very limited. Kennedy (2018) provided the first documentation of oocyte development at the macroscopic level for the species, describing them as being a determinate batch spawning species with oocyte development taking up to 8 months. Further work by Kennedy et al. (2020) identified variations in fecundity and egg sizes with broodstock sizes and ages around Iceland. However, these studies are limited by the lack of histological definitions as acknowledged by the authors. For other species, precise histological definitions helped interpreting the impact of management interventions on reproductive development, such as in European seabass (Mayer et al.1988) and Atlantic cod (Kjesbu & Kryvi. 1989). The creation of histological definitions of oocyte development in lumpfish can aid in the description of treatment effects on broodstock, information which is currently lacking in work for this species (Imsland et al. 2018a, Kennedy. 2018, Kennedy et al. 2020).

Male management is also in its infancy, current milt collection methods require sacrificial sampling (Powell et al. 2018) as stripping of milt produces very small volumes that are not commercially viable to use (Nordberg et al. 2015). This could be considered to be a limiting factor in productivity where available males are rapidly used up. Therefore, in the long term, there could be some merit to the development of an enhanced and non-lethal method of stripping that results in the production of relevant volumes of viable milt. In the short term, however, the development of protocols to aid the more effective management of milt, extending windows of availability of viable milt

and thus reducing the number of males required will have significant commercial value (Powell et al. 2018). Nordberg et al. (2015) found that cryopreservation of lumpfish milt was effective at storing sperm between spawning years with no effect on fertilisation potential. However, hatcheries would greatly benefit from the development and application of technologically simple protocols that would improve the windows of available milt (e.g. chilled storage solutions) and improve the efficiency with which limited milt supplies are used (e.g. sperm quantification methods). More accurate concentration measurements can improve milt use through more accurate fertilisation, through optimal sperm to egg protocols as seen in other species (Suquet et al. 1995, Butts et al. 2009).

Egg management equally is an important aspect of hatchery management and is often a bottleneck for many marine species (Brooks et al. 1997). Due primarily to a lack of understanding, mature broodstock within a given spawning season are inspected, through examination of the vent, with varying regularity with several days between inspection not being uncommon. Under these conditions, it is conceivable that eggs can be left in the body cavity for long periods of time and result in over-ripening of eggs and, therefore, a reduction in egg quality. To confound this further, hatcheries are prone to hold unfertilised eggs within their ovarian fluid for lengths of time while they batch process multiple individuals, with further delays potentially added due to degumming and disinfection activities (Powell et al. 2018). Together, this means that there can be highly variable and extended periods of times between ovulation, stripping and subsequent fertilisation of egg batches all of which could affect subsequent quality depending on the process of POA for lumpfish. Clearly the management approach for female lumpfish would benefit from informed refinement and to this end the characterisation of simple aspects of gametogenesis e.g. ovulation

cycles and time frame of post ovulatory ageing, would have the potential to significantly improve hatchery management and productivity.

Ultimately it is important to realise that the quality of eggs (i.e. likelihood to create a viable larvae) is dictated largely by the proximate composition of the egg itself (Bobe & Labbe. 2010). Optimisation of gamete handling protocols and rearing conditions serve to reduce the proportion of potentially viable embryos that are lost, but if an egg does not have the required biochemical composition, optimised rearing conditions will not change the egg fate. To this end, while eggs derived from wild lumpfish broodstock are generally considered good, it has been reported to be highly variable, which could be due to the unusually long spawning season for this species (*Pers. Com*, Tor Otterlei Skjerneset Fisk). Furthermore, the limited production from captive broodstock can be summarised as being generally of poor quality or non-viable. As such on-farm reports suggest that egg quality as it stands in captive stocks is prohibitive to further investment in the development of the industry (*Pers. Com*, David Patterson Otter Ferry sea fish LTD). Despite the widely held importance of broodstock nutrition and association with egg composition and subsequent viability across species (Bobe & Labbe. 2010) there is no information on the composition of lumpfish eggs and their potential association with egg quality. This needs to be addressed urgently to set the benchmark to provide quality assurance in hatchery operations and enable the development of species-specific feed development, which has been a critical development in assuring commercial viability of hatchery production of numerous new species.

1.5 Aims of the thesis.

It is clear that the lumpfish is an important addition to the management toolbox of sea lice within Atlantic salmon farming. However, the current drive for cleaner fish is unsustainable if derived from wild caught broodstock. Recently there has been a push to close the life cycle of this species. The overarching goal of this thesis is to undertake a body of work which addresses critical bottlenecks in broodstock management which will underpin the establishment of a commercially viable closed life cycle production of lumpfish. To this end the specific aims of the thesis are to address key hurdles in creating a commercially viable broodstock by:

1) Describing the effects of rearing temperature on timing of spawning and egg productivity, and quality across a thermal range associated with wild mature lumpfish. The secondary aim being to histologically describe oocyte development for the species. This work provides guidance for optimal rearing of captive broodstock, which is key in realising the industries aspirations to work with closed life cycle management. Chapter 2

2) To identify egg compositional factors effect on egg quality in lumpfish. By conducting comparisons of egg compositions between wild and captive derived eggs to determine current deficiencies in egg quality in captive stocks. Identifying compositional components which associate with egg quality paraments within wild and captive stocks through a large-scale component analysis. And to identify how composition of eggs change throughout development to further inform egg quality information. At its conclusion this work will help prioritise nutrients which associate with egg quality in lumpfish to support the development of species specific broodstock feeds in the future. Chapter 3

3) To identify the effects of storage at cold temperatures in a range of milt extender solutions on the activation of lumpfish milt. To validate rapid and accurate methods for estimating sperm concentration. Both of which will provide much needed information for a limiting factor of hatchery management. Chapter 4

4) To identify the effects of prolonged *ex vivo* post ovulatory aging on lumpfish egg performance for both wild and captive derived eggs. To determine the composition of the ovarian fluid proteome, how the composition differs between wild and captive derived eggs and to assess the effect of ovarian fluid proteome on egg quality within lumpfish and to identify protein markers associated with overripe and poor-quality eggs. Chapter 5

Chapter Two

High temperature is detrimental to captive lumpfish (*Cyclopterus lumpus*, L) reproductive performance.

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2.1 Introduction

As outlined in the previous chapter the lumpfish (*Cyclopterus lumpus*) is a new species to aquaculture with the current supply chain reliant on wild caught broodstock to meet the increasing demand for juveniles. However, the capture tonnage is low in the context of the roe fishery, approximately 0.05 % of the 15,000-tonne annual harvest (Kennedy et al. 2018). In Norway alone, 30 million juvenile lumpfish were deployed in salmon farms in 2017 (Mortensen et al. 2020) with 1.9 million lumpfish in the UK in 2016 (Brooker et al. 2018) and worldwide, production is forecast to exceed 50 million by 2020 (Powell et al. 2018). While the annual harvest of mature brood fish to meet this demand is low in the context of the roe fishery (Kennedy et al. 2018), key advantages in moving towards closed life cycle management for this species include biosecurity, predictable egg supply and the potential to improve farmed stocks through genetic selection (Huston et al. 2020). The first step in closing the life cycle and securing reliable captive broodstock production is to define optimal environmental conditions for egg productivity and quality.

The lumpfish reaches maturity in the wild after five to six years (Davenport, 1985; Haauft, 2015); however, lumpfish can spawn after one to two years in captivity (Imsland, et al. 2014; Powell et al. 2018). Females spawn in shallow coastal waters and have been shown to migrate great distances to spawning sites (Mitamura et al. 2012). Males guard the egg masses prior to hatch and commonly maintain several egg batches from different females in a single location (Davenport, 1985). Female lumpfish remain within the spawning area for a short period of time, possibly moving to alternate spawning areas but then proceed to migrate offshore (Mitamura et al. 2012). There remains a lack of clarity in the natural spawning season in this species,

Davenport (1985) described the lumpfish spawning season as occurring between April and July with no geographic reference. More recently, Kennedy (2014) noted that spawning season in Iceland was between January and March, which is the focal window for commercial exploitation. However, fishing for mature lumpfish in central Norway occurs between September and June (*Pers. Com*, Tor Otterlei Skjerneset Fisk) with the main catch window from October to May, with this fishery being the principle supplier of lumpfish eggs to Norwegian and UK based hatcheries. This is suggestive that reproductive entrainment in the species is not strictly controlled by photoperiod, inferring that temperature may play an important role in regulating reproduction. Interestingly, there are reports that across this broad spawning season there is notable variation in egg quality, with eggs derived from broodstock captured at low temperatures in winter/spring having the highest hatch rates compared to those caught at the start or end of the season when the water temperature was around 14-15 °C.

At present, there is limited published work on the reproductive physiology of lumpfish. Kennedy (2018) has reported oocyte development at the macroscopic level for the species, describing them as being a determinate batch spawning species with oocyte development taking up to 8 months. However, the authors acknowledge the limitations in their analysis due to the lack of histological descriptions of ovarian development. Precise histological definitions helped interpreting the impact of management interventions on reproductive development in many other marine fish species including European seabass (*Dicentrarchus labrax*) (Mayer et al. 1988), Atlantic cod (*Gadus morhua*) (Kjesbu & Kryvi, 1989) or common snook (*Centropomus undecimalis*) (Rhody et al. 2013) for example.

Rearing broodstock within optimal environmental conditions is essential for the reliable production of good quality gametes and subsequent offspring in any commercial hatchery (Migaud et al. 2013). In most marine species, it is apparent that both the seasonally changing day length as well as temperature play important roles in the regulation of reproductive development, timing of the spawning season, productivity and quality (Brooks, et al.1997). Imsland et al. (2018a) suggested that spawning could be influenced by photoperiod, with the simulation of long day to short day returning to long day photoperiod appearing to trigger maturation of lumpfish broodstock. In contrast, while there is anecdotal evidence suggestive of the impact of temperature on spawning in the species, there are no published studies with respect to the impact of temperature on reproductive development and gamete quality for lumpfish. Anecdotal evidence from hatcheries in Scotland correlated broodstock mortality with increasing sea temperatures. Based on past work in temperate marine species there are two key regulatory aspects of temperature in the context of reproductive development and spawning. During gametogenesis, temperature manipulations can influence the pace of development and the timing of subsequent spawning windows as demonstrated in the Common Wolfish (*Anarhichas lupus*) (Tveiten & Johnsen, 1999) and to a lesser degree influence fecundity (Kraus et al. 2000) as well as subsequent gamete quality, as demonstrated in Atlantic cod (Rideout et al. 2000). During the spawning season itself temperature plays a stronger regulatory role, determining spawning windows (Kjesbu, 1994; Tveiten et al. 2001), ovulation cycles (Brown et al. 2006) and most importantly gamete quality (Migaud et al. 2013). While wild survey data suggest that lumpfish occupy a thermal range between 4°C and 15°C, mature adults tend to be associated with the lower end of this range (Mitamura et al. 2012).

This current study was designed to explore the effects of temperature during the spawning season on broodstock performance in captive lumpfish. This study aimed to describe the effects of rearing temperature on timing of spawning and egg productivity, and quality across the observed thermal range associated with wild mature lumpfish and spawning in this species. A secondary objective was to histologically describe oocyte development for the species. Such work provides guidance for optimal rearing of captive broodstock, which is key in realising the industries aspirations to work with closed life cycle management.

2.2 Materials & Methods

2.2.1 Animals and experimental design

All fish used in the experiment were captive reared stock derived from gametes stripped from wild caught parents (3♀:5♂) caught by Skjerneset Fisk at Averøy, Norway and maintained at NOFIMA, AS, Sunndalsora from fertilisation. Prior to the start of the experiment, fish had been maintained at ambient temperature regimes for Sunndalsora, Norway ranging between 5°C in the winter to 13°C in the autumn. They were maintained on a low intensity, 24hr light photoperiod and fed on Skretting Silk (Skretting, Nutreco N.V, Netherlands) pellets at the appropriate size range during grow out. Individual morphometric (weight ±0.1 g and total length ±1 mm), gender and stage of maturity were recorded on the 1st of May 2017 when the stock (*n* = 513) were approximately 15 months old. At this point all fish were tagged with a passive integrated transponder (PIT) tag to enable individual traceability throughout the experiment. Fish were randomly assigned to one of three treatment groups (*n* = 166 - 169 per treatment) with a balanced sex ratio at 1♂:2♀.

Table 2.1: Summary of descriptive data for lumpfish used in the present study.

Treatment	Mortality (%)	Number of males	Number of females	Average weight (start) female (g)	Average weight (start) male (g)
High	5%	58	111	1224.7 ±353	744.7±201
Medium	10%	56	110	1208.9 ±448	659.4±165
Low	1%	56	112	1306.4 ±398	653.3±289

The three stocks were held in three 7000L tanks, fed to satiation using a commercial formulated feed (Silk 4.5mm, Skretting, Nutreco N.V, Netherlands) at 7.6 ± 0.3°C under 24hr low intensity lighting conditions. Following monitoring of the ovarian development of females (both external assessment of the female swelling and ultrasound scanning of a random selection of individuals (n=30 per treatment), water temperatures were changed to experimental levels between the 1st and 7th of June at a rate of 1 ± 0.5 °C/day and were maintained for the duration of the experiment. At the time of applying the temperature treatments, stocks were not significantly different in weight, length, maturation score and sex ratio (Table 2.1). Thereafter, temperatures were kept constant throughout the experimental period being 5.9 ± 0.3 °C, 9.2 ± 0.7 °C and 14.3 ± 0.2 °C, for the low, medium and high temperature treatments respectively (Figure 2.1).

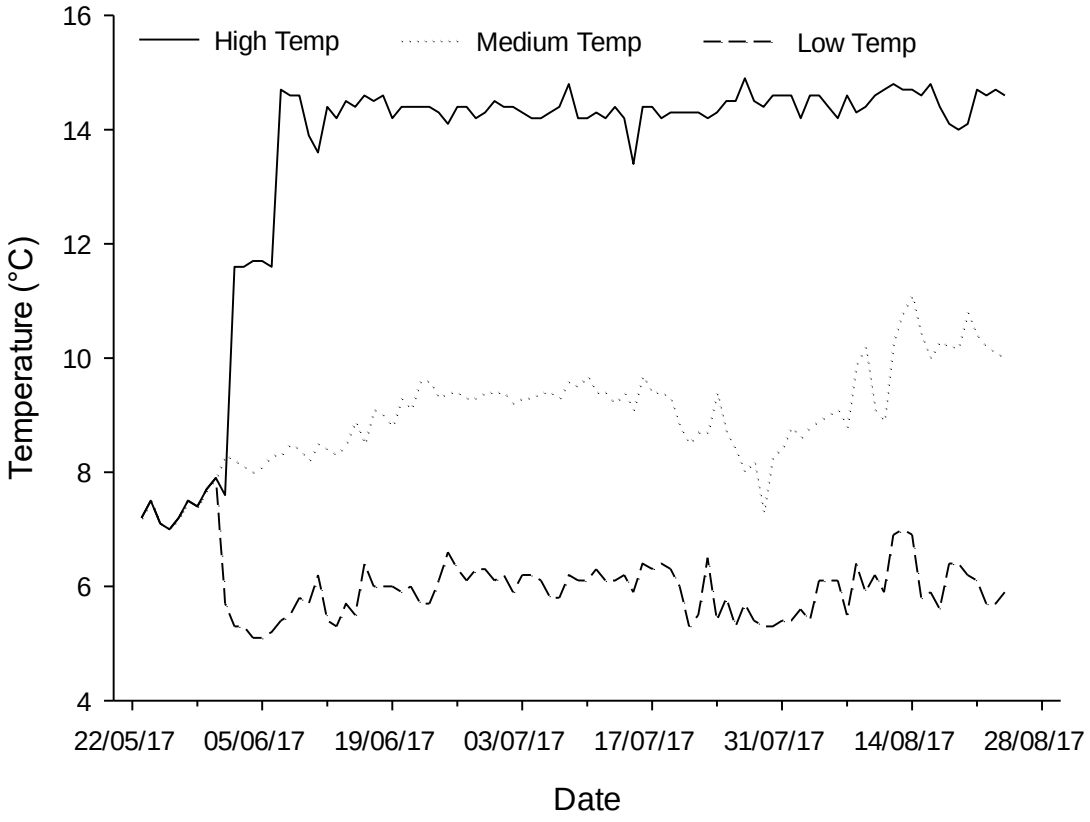

Figure 2.1: Daily temperature (°C) recorded within the three treatment groups.

Following the alteration in temperature all fish were visually inspected every two weeks, when morphometrics were recorded and maturation was assessed both visually and using ultrasound imaging (6.5mHz, Log.Q book XP vet, GE medical systems, USA) of the body cavity. With respect to the ultrasound imaging, female ovarian development was classified using a subjective five point scale; 1.) Immature: Individual with no visible gonads, 2.) Immature: Small gonads, both ovarian lobes are apparent within the image, 3.) Maturing: Significantly enlarged gonads, single ovarian lobe fills the image, at later stages of development individual hydrating oocytes may become apparent within the ovarian tissue (classified 3.5), 4.) Spawning: Significantly enlarged gonads, single ovarian lobe fills the image, free hydrated oocytes apparent on the dorsal region of the ovarian lobe. Following first spawning, open regions filled with ovarian fluid became apparent within the lobe (classified 4.5), 5.) Spent: Ovarian lobes collapsed with small proportion of ovarian tissue left, no free eggs apparent. At the point of inspection if gametes were being freely released this was recorded. In addition, daily inspections of the tanks allowed the recording of spawning events with egg masses collected and weight recorded (±0.1 g). The point at which first spawning occurred and spawning ceased has been used to define “spawning window” for each treatment stock, in this study.

Following temperature change, on four subsequent samplings (weeks 4, 6, 10 & 13 following temperature change), 17 individuals from each treatment, 10 females and 7 males were sacrificed, with females in the late maturing/spawning category being selected based on the maturation assessment. Following euthanasia, (Overdose of MS-222 (1 g L^{-1}), individual weight and total length were recorded and the gonads as

well as livers were dissected and weighed. Samples of the ovaries were preserved in 10% buffered formalin for later image analysis of oocyte size and histological confirmation of oocyte development. For all males, milt samples were placed on ice for subsequent spectrophotometric assessment of sperm density.

2.2.2 Histological characterisation of oocyte development

In order to develop a histological scale of oocyte development for the species a total of 28 ovarian samples previously preserved in 10 % neutral buffered formalin were analysed. All fish came from the study population and included samples taken from prior to the study as well as individuals from the first two sampling dates. This pool of individuals had a range of GSI's from 0.95% to 37.4% to capture the diversity of ovarian development. Fixed ovarian samples were embedded in paraffin with 5 µm sections then mounted and stained using haematoxylin (Shannon) and Eosin. Slides were digitised using Axio Scan.Z1 slide scanner (Zeiss, Oberkochen, Germany), and photographs were then analysed using digital image analysis (Image Pro Plus, Media Cybernetics, USA).

Oocyte development was classified in accordance with Rhody et al (2013) and were as follows: Primary Growth- The primary growth oocyte stage is characterised by 4 stages; one nucleolus (PGon); multiple nucleoli (PGmn); perinucleolar (PGpn); and oil droplets (PGod). Secondary Growth- Secondary growth includes three steps: early (SGe), late (SGl), and full-grown (SGfg). Oocyte maturation (OM) includes three steps: eccentric germinal vesicle (OMegv), germinal vesicle migration (OMgvm), and preovulatory (OMpov). A minimum of 50 individual oocytes from a minimum of 10 individuals were identified for each developmental stage. Oocyte diameter was

measured by digital image analysis and calculated as the average of two diameters perpendicular to each other measured through the nucleus.

2.2.3 Oocyte size distribution analysis

Oocyte size distribution (OSD) was measured according to a protocol adapted from Kjesbu & Kryvi (1989). Briefly, a digital image (Nikon 1, NIKON, Japan) was taken of dissociated oocytes, with individual oocyte diameter (n =100 oocytes per individual) measured by digital image analysis (Image Proplus, Media Cybernetics, USA). Lead cohort oocyte diameter of the population was calculated based on the mean of the largest 10 oocytes following initial imaging and sorting of population size data (Thorsen & Kjesbu 2001). To confirm uniform ovarian development within the species prior to subsequent analysis, OSD was measured in 4 discrete samples (samples excised towards the posterior and anterior end of both ovarian lobes) within six independent females. Both lead cohort oocyte diameter and oocyte size frequency distributions were compared between the four different lobe sections within each individual.

2.2.4 Fecundity estimates

The combined gravimetric and automated particle counting method (Murua et al. 2003) was used to estimate individual total fecundity from a random selection of 10 individuals (1614 ± 245.9 g, 275.1 ± 30.9 mm) from pre-treatment sampled females. Briefly, for each individual, three weighed samples of ovarian tissue were preserved in 10% neutrally buffered formalin, a digital image (Nikon 1, NIKON, Japan) of dissociated oocytes was taken, with the total number of viteliogenic oocytes (oocytes > 370 µm as determined by previous histological examination of oocyte development) determined by digital image analysis (Image Proplus, Media Cybernetics, USA).

2.2.5 Egg quality assessment

Following approximately four weeks of temperature treatments, a gamete quality assessment study was performed. Eggs were stripped from six females from each of the three treatments, with females being selected based on ultrasound assessment of ovarian development to assure they were ready to be stripped and had not previously spawned. For each individual, the egg batch volume was recorded and then eggs were held in chilled storage covered in ovarian fluid prior to the quality test. Males (6 from each treatment) were killed using an overdose of MS222 and the whole testis was dissected, ground, and mixed in a 1:1 ratio with a commercial sperm extender solution (Sperm Coat™, Cryogenetics, Norway). Sperm density was assessed by spectrophotometry and activation in response to seawater was confirmed in an aliquot under a light field microscope. In addition, milt from wild mature male lumpfish (n = 3) was obtained from Skjerneset Fisk at Averøy and processed in the same manner. The gamete quality test was performed in a manner to allow both individual egg viability as well as individual milt viability to be tested. To test individual egg batch viability, each of the six egg batches from the three treatments (n= 18 total egg batches) were fertilised in triplicate by four different pools of milt from the high, medium, low temperature treatments and wild) which contained equal contributions of milt from 6 individual males under each treatment. To test individual milt sample viability, each of the six individual milt samples from the three treatments (n= 18 total milt samples) were used to fertilise eggs, in triplicate, from the high, medium and low temperature treatments with equal contributions of eggs from the 6 individual females from each respective treatment group. For each replicate test, 1 ml of eggs (*circa* 100 eggs) were wet fertilised with 300,000 sperm per egg in a petri dish using 20 ml of 0.2 µm-filtered seawater. Following fertilisation 20ml of water was exchanged with the further addition

of penicillin/streptomycin (Sigma,USA) at 100 units per ml of penicillin and 0.1 mg per ml of streptomycin within the petri dish and these were maintained in a temperature controlled room at 8°C thereafter. Water was exchanged every two days within the petri dishes, with the eggs incubated until 150 °C days at which point the proportion of eyed eggs was recorded.

2.2.6 Sperm density assessments

Sperm density was assessed just prior to the temperature treatments and at each sampling point thereafter. Gonads were excised from six sampled males at each time point, ground and sieved to collect milt. Milt samples were diluted 1:400 in a physiological salt solution (9 gL^{-1} NaCl in deionized water) in a cuvette and analysed by spectrophotometry at 546 nm (SDM6, Cyrogenetics, Norway) with data presented as sperm per ml, samples were calibrated against the physiological solution.

2.2.7 Statistics

All statistical analyses were conducted using Minitab 18 software. A Kolmogorov-Smirnov test for normality was performed on all data sets to assess for normality of distributions. A General Linear model was used to test the effects of the treatments and time on milt density and egg quality. An ANOVA with a post hoc Tukey's T test was used to assess the differences in gonadal development, oocyte histogram populations and oocyte lead cohort population differences. Significance was set at $p<0.05$.

2.3. Results

2.3.1 Oocyte development, size distribution and fecundity estimates.

Histological analysis confirmed that oocyte development was typical for a marine teleost with the primary growth oocytes ranging in size between 82 µm and 216 µm (as defined by population mean diameters), while secondary growth oocytes ranged between 370 µm and 529 µm and oocyte maturation occurred in oocytes between 624 µm and 1398 µm (Table 2.2, Figure 2.2). Typical histological sections are displayed in figure 2.3. To aid the interpretation of subsequent oocyte size distribution analysis the following arbitrary thresholds were set: Primary growth oocytes ≤ 216 µm; Secondary growth & oocyte maturation: ≥370 µm and ≤ 1616 µm (*n.b.* as oocyte development is continuous in this phase there was no clear segregation that could be applied based on size alone) and hydrated oocytes ≥ 1616 µm. The OSD analysis of four independent samples extracted from six pre-treatment females confirmed comparable development in all, with no difference in total oocyte distribution or lead cohort diameter oocytes between four independent gonad sections within individuals (data not shown). When a minimum threshold of 370 µm was applied (*i.e.* all oocytes in secondary growth or greater), the mean relative fecundity in ten pre-treatment females was estimated to be 40,440 ± 12,434 oocytes per Kg body weight with no apparent difference between females.

Table 2.2: Oocyte size range in relation to developmental stage for lumpfish according to Rhody et al. (2013). Values represent mean ± SD of a minimum of 10 individuals in which 50 oocytes were measured for a given developmental stage.

Stage	Step	Diameter (µm)	Number of fish
Primary growth (PG)	**Multiple nucleoli (PGmn)**	82.2 ± 7.6	11
	Perinucleolar (PGpn)	119.9 ± 29.2	12
	Oil droplets (PGod)	216.5 ± 15.2	14
Secondary growth (SG)	**Early (SGe)**	370.4 ± 6.2	10
	Late (SGl)	467.8 ± 60.2	10
	Final (SGfg)	528.5 ± 39.4	10
Oocyte maturation (OM)	**Eccentric germinal vesicle (OMegv)**	623.7 ± 39.0	12
	Germinal vesicle migration (OMgvm)	740.5 ± 88.9	15
	Preovulatory (OMpov)	1398.2 ± 87.3	15

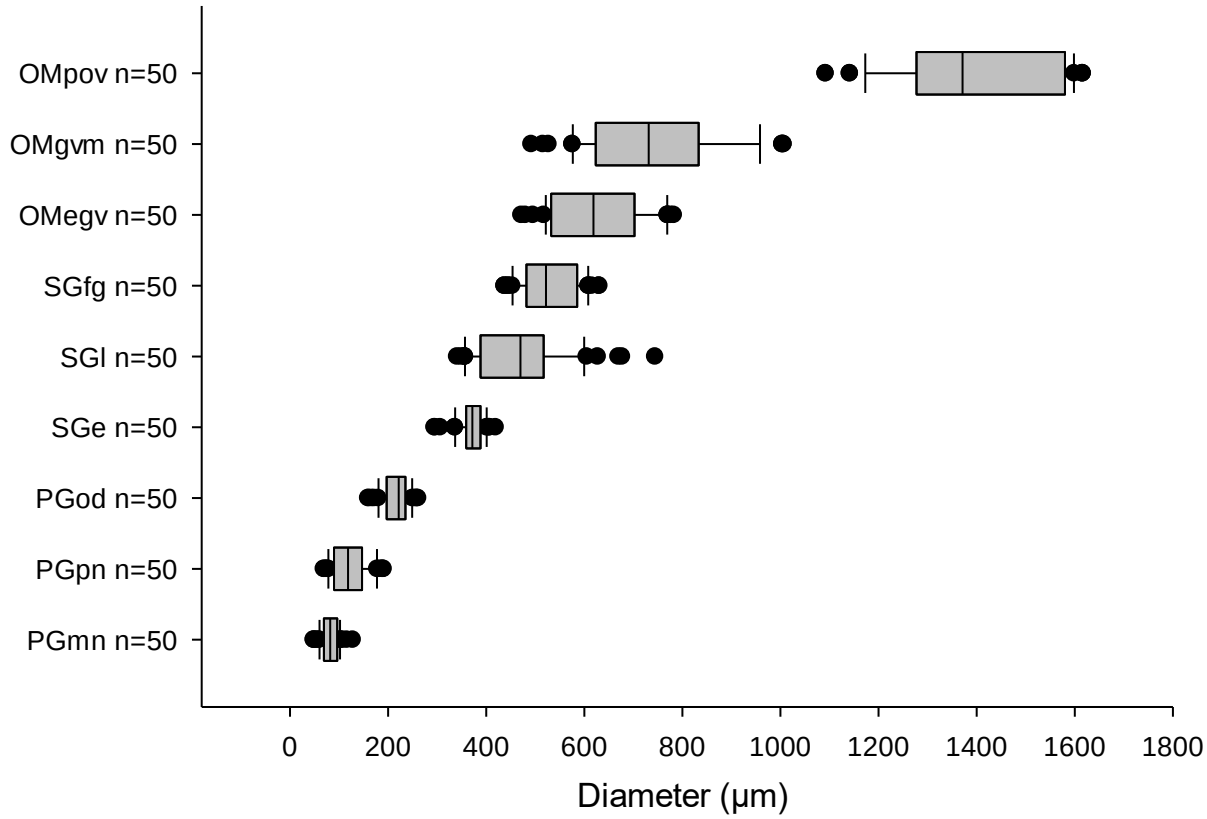

Figure 2.2: Box and whisker plot displaying the diversity in oocyte size (*n*= >10 individuals per stage) observed within developmental stages (Primary (multiple nuclei (PGmn), perinucleolar (PGpn) and oil droplet (PGod)), Secondary (early (SGe), late (SGl) and final (SGf)) and Oocyte Maturation (eccentric germinal vesicle (OMegv), germinal vesicle migration (OMgvm) and pre-ovulatory (OMpov))). Box represents 25th and 75th percentiles while whiskers represent 5th and 95% percentiles, with mean value is indicated as the vertical line within the box. Individual values out with these range are indicated as •.

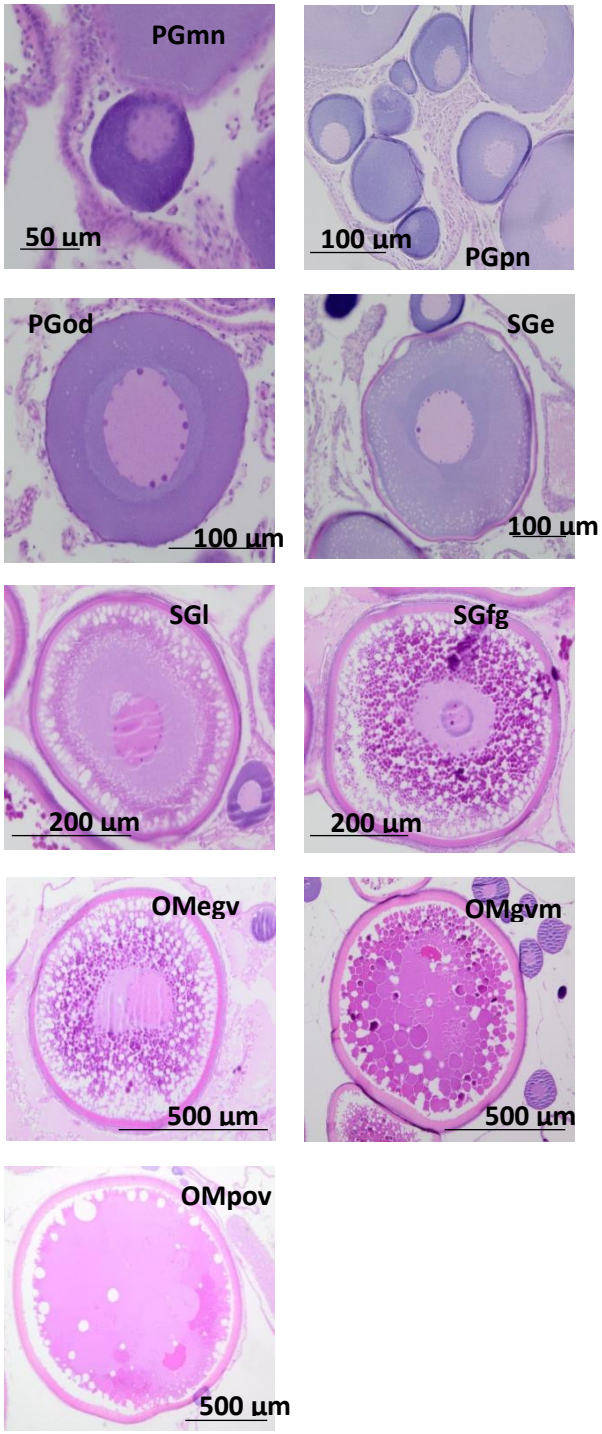

Figure 2.3: Typical examples of histological sections of Primary (multiple nuclei (PGmn), perinucleolar (PGpn) and oil droplet (PGod)), Secondary (early (SGe), late (SGl) and final (SGf)) and Oocyte Maturation (eccentric germinal vesicle (OMegv), germinal vesicle migration (OMgvm) and pre-ovulatory (OMpov)) oocytes of lumpfish with scale bar shown.

All females sacrificed during the study were selected based on ultrasound screening to ensure there were no immature females nor previously spawned or spent individuals. These samples do not inform on treatment effects but rather provide a snap shot of oocyte development in final oocyte maturation. For all female samples (n= 90), lead cohort oocyte diameters ranged from 708 ± 4.6 µm to 2310 ± 7.6 µm indicating that all individuals were in the later stages of oocyte maturation or with ovulated eggs. The majority of individuals (92%) had a leading cohort in final oocyte maturation (OMgvm, OMpov) (15%) or free hydrated oocytes (77%) and no apparent differences in overall oocyte size distribution or lead cohort oocyte diameter in relation to time or treatment were observed.

Assessment of oocyte diameter frequency histograms for each individual showed that, from the low and medium temperature treatments, 20 out of 40 (50%) individuals presented a bimodal oocyte distribution as opposed to a unimodal distribution (Figure 2.4). Typically, this bimodal distribution included one population at the hydrated oocyte stage and one population at the oocyte maturation stage (OMgvm, OMpov) stage. Within the high temperature treatment, the proportion of individuals with a bimodal oocyte distribution was apparently reduced being recorded in 7 out of 40 sampled individuals (i.e. 18% of individuals sampled).

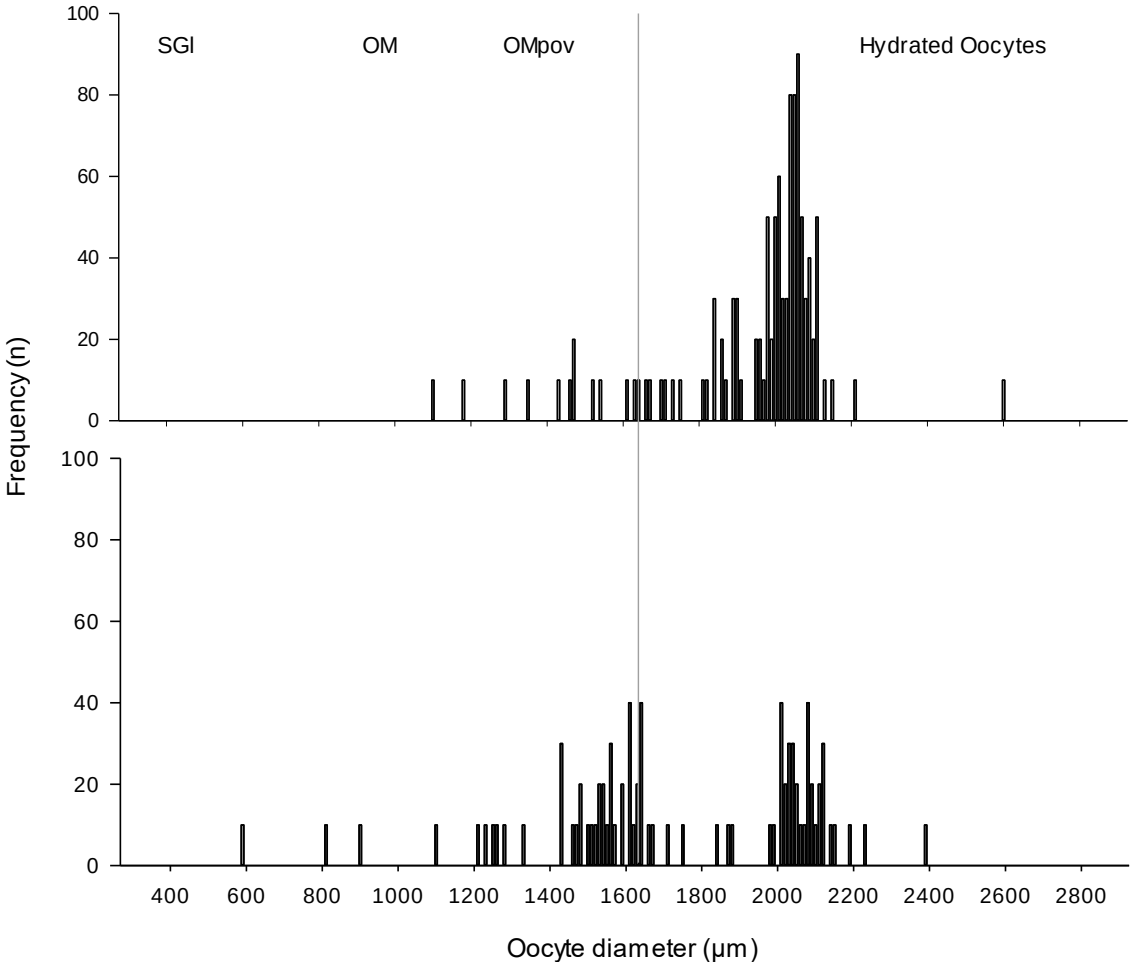

Figure 2.4: Oocyte size distribution histograms showing typical example of individual females within 6 and 9 degree treatments with either unimodal (top) or bimodal (bottom) development. Boundaries of stages of development for hydrated oocytes, OMpov, Oocyte maturation (OM), and Late Secondary Growth phase (SGl) are indicated by vertical lines.

2.3.2 Temperature effects on female maturation and spawning

First spawning was recorded on 3rd June with the last ovulating female recorded on 14th August in the 6°C treatment. Overall, the length of the spawning season appeared to be inversely related to holding temperature lasting 11, 28 and 72 days for the high, medium and low temperature treatments respectively (Figure 2.5). Total productivity in terms of the number of naturally spawned batches was comparable in the low and

medium temperature treatments (n = 25 and 20 respectively) but notably reduced in the high temperature treatment (n = 3) (Figure 2.5). Furthermore, mean batch weight was comparable in the low (144 ± 81 g) and medium temperature treatment (165 ± 105 g) but significantly reduced (>50% reduction) in the high temperature treatment (65 ± 15 g).

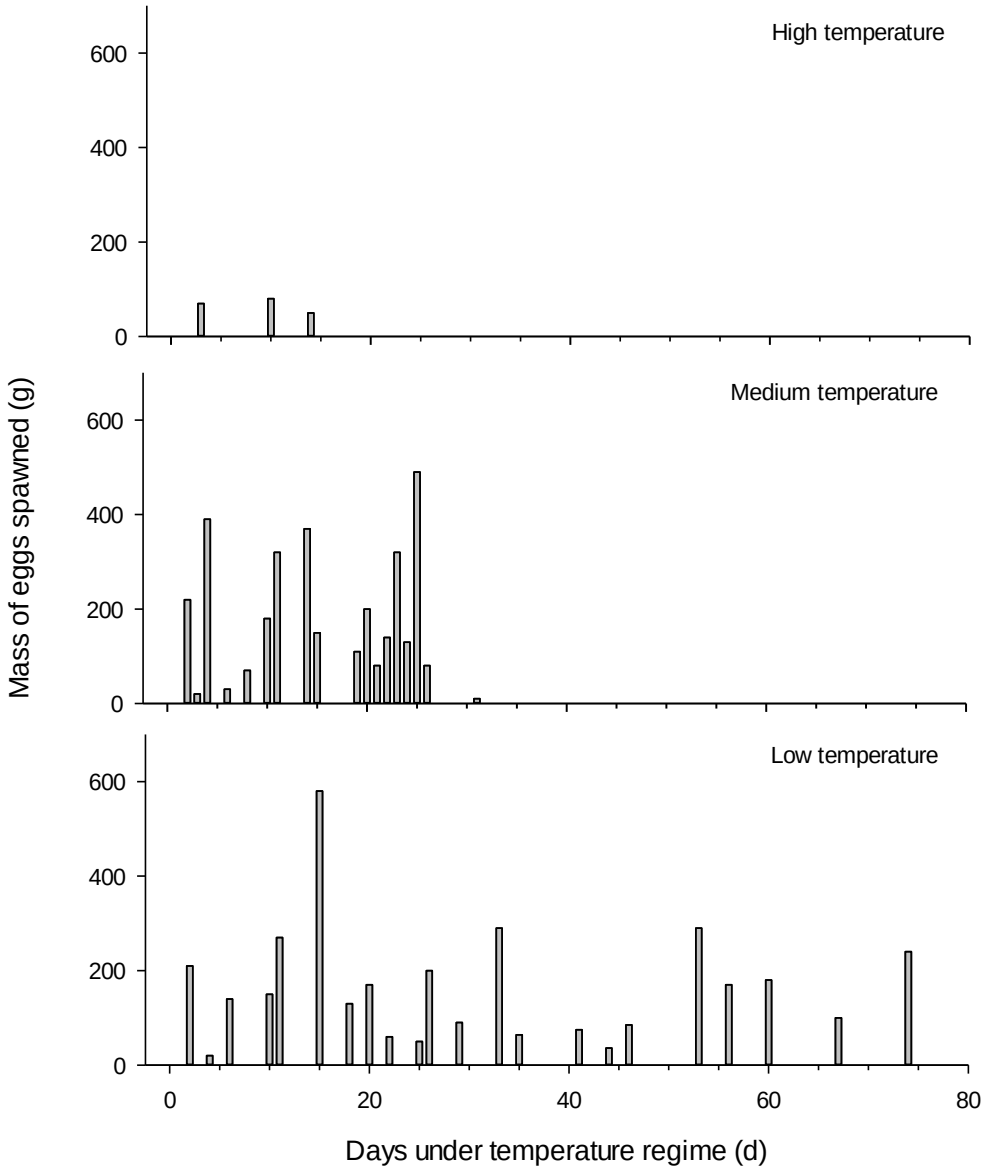

Figure 2.5: Frequency of natural spawning events during the study period for each treatment group. Bars represent total mass of eggs released during that day (g) for the respective treatment.

In each treatment there was a core population not sacrificed during the length of the study (n = 48, 31 and 45 in the high, medium and low temperature treatments, respectively). Based on the repeat assessment of individuals with ultrasound three groups could be distinguished: females, which had spawned (*i.e.* attained a score of 4.5 or 5 during the study), females, which were progressing towards final maturation but did not spawn during the season (*i.e.* did not pass scores of 3-4), and non-maturing females (*i.e.* did not pass scores of 1-2). In all treatments, 15.7± 4.1% of fish were not maturing. In the low and medium temperature treatments, 84.7± 5.8% of females were spawning individuals against only 12% in the high temperature treatment with 68% maturing but not spawning. At the point of tagging (3 months prior to start of spawning), non-maturing females were significantly smaller (454.3 ± 15 g, 242.4 ± 3.1 mm) compared to maturing females (879.4 ± 51.89 g, 279.5 ± 5.8 mm) irrespective of treatments. However, no significant difference in size was observed between females that spawned or not in the high temperature treatment.

2.3.3 Temperature effects on egg quality

There was a significant effect of the temperature treatment on egg quality with no egg batches (either as individuals or as pools) from the high temperature treatment reaching the eyed stage of development (Table 2.3 & 2.4) and will be excluded from subsequent analysis. With respect to the individual egg batch test, there was a high level of variation within and between individual females. However, eyeing rate was comparable in all treatment groups (overall mean eyeing rate = 31.9 ± 10.0%) with the exception of low temperature eggs and milt pool, where eyeing was significantly reduced by almost 90% in comparison to all other milt pool crosses (Table 2.3). With respect to the individual milt quality tests, as stated previously, no eggs from the high

temperature treatment were viable, thereafter eyeing rate in all other tests were statistically comparable ranging from 22.1 ± 15.8 % (low temp. milt vs. low temp. eggs) to 43.0 ± 9.6 % (medium temp. milt vs. medium temp. eggs) (Table 2.4).

Table 2.3: Proportion (%) of eyed embryos in individual egg batch assessments (n= 6 individuals per treatment) compared to pools of milt derived from high, medium and low temperature treatment groups and wild mature lumpfish (each pool contained an equal contribution from 6 males). Superscripts denote significant differences.

	HIGH TEMP. MILT	**MEDIUM TEMP. MILT**	**LOW TEMP. MILT**	**WILD MILT**
High temp. eggs	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]	0 ± 0.0[a]
Medium temp. eggs	33.3 ± 29.6[b]	30.3 ± 29.1[b]	41.8 ± 27.9[b]	40.8 ± 31.5[b]
Low temp. eggs	33.3 ± 33.7[b]	39.3 ± 30.4[b]	4.3 ± 7.0[a]	37.4 ± 33.2[b]

Table 2.4: Proportion (%) of eyed embryos in individual milt assessments (n= 6 individuals per treatment) compared to pools of eggs derived from high, medium and low temperature treatment groups (each pool contained an equal contribution from 6 females). Superscripts denote significant differences.

	HIGH TEMP. EGGS	**MEDIUM TEMP. EGGS**	**LOW TEMP. EGGS**
High temp. milt	0 ±0.0^{a}	39.5 ±8.6^{b}	30.9 ±13.0^{b}
Medium temp. milt	0 ±0.0^{a}	43.0 ±9.6^{b}	30.2 ±17.8^{b}
Low temp. milt	0 ±0.0^{a}	30.9 ±14.1^{b}	22.1 ±15.8^{b}

2.3.4 Temperature effects on sperm density

Prior to the initiation of temperature treatment, there was no difference in sperm density between populations (11.9 ±1.5 $\times 10^{9}$ sperm.ml^{-1}). Following four weeks of thermal treatment, and for the remainder of the study thereafter, sperm density was significantly reduced (*circa* 50% of the pre-treatment value) in the high temperature treatment compared to the pre-treatment level (Figure 2.6). Furthermore, sperm density was also significantly lower in the high temperature treatment compared to that measured for the medium and low temperature treatments after one month of exposure to the temperature treatments (Figure 2.6). There was no significant impact of the low or medium temperature treatments on sperm density with respect pre-treatment levels during the study.

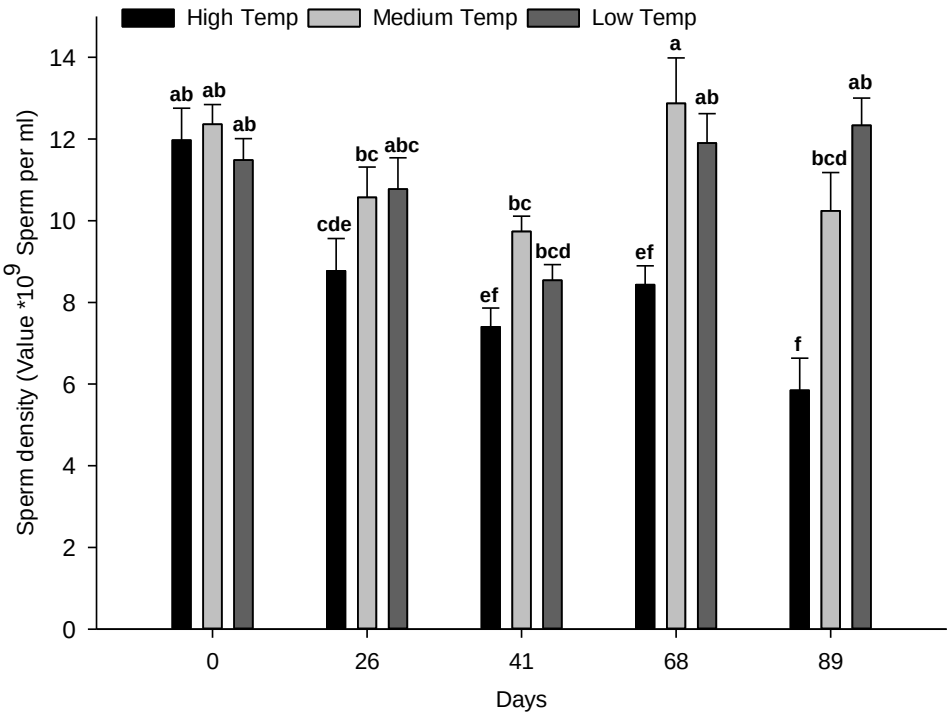

Figure 2.6: Mean sperm density ($\times 10^9$ sperm.ml^{-1}) ± SD for males (*n* = 7 per sample) maintained under either low (6 °C), medium (9°C) or high (14°C) temperature treatments. Superscripts denote significant differences.

2.4. Discussion

Closed life cycle management is an important milestone for the production of lumpfish and biological control of sealice. If this milestone is to be realised at the commercial scale, it is essential to determine the optimal environmental conditions that will assure reliable production of good quality gametes. To this end, this study is the first to consider the impacts of temperature during the spawning window on the length of the spawning season and egg quality within captive reared lumpfish broodstock. The work demonstrated that a higher holding temperature (*circa* 14 °C) significantly reduced spawning activity and had a significant negative effect on egg quality in lumpfish. This higher holding temperature also saw a significant reduction in milt density, although sperm viability appeared not to be impacted.

Despite the continuous photoperiod, the study population demonstrated a clear synchrony in reproductive development with <16% of all fish not maturing (as assessed by ultrasound examination) in the study. Temperature treatments were applied once it was apparent that the study population were ready to spawn with the intention to assess thermal impacts during the spawning season itself not on gametogenesis *per se*. To this end, there was a stricking inverse relationship between temperature and length of the subsequent spawning window. The cessation of spawning within 11 days in the high temperature treament in conjunction with the 50% reduction in egg production and the presence of over $^{2}/_{3}$ of maturing females showing developmental regression during the study period, all indicate that final oocyte maturation and spawning are temperature sensitive in the species. This agrees with findings in other temperate species such as Arctic charr (*Salvelinus alpinus* L.) (Gillet, 1991), common wolfish (Tveiten et al. 2001), pollack (*Pollachius pollachius*) (Suquet et al. 2005) or Atlantic halibut (*Hippoglossus hippoglossus*) (Brown et al. 2006), where

elevated temperatures inhibited spawning and, in the case of European seabass, was associated with gonadal regression (Carillo et al.1995).

Following four weeks of temperature treatment there were clear effects observed on gamete viability. The high temperature had a significant detrimental effect on egg quality with all egg batches tested being non-viable. When considered in context with the significantly reduced spawning activity and the reduced proportion of observed spawning females, it implies that this elevated temperature suppressed spawning and led to oocyte regression in ovarian development following the elevation of temperature. The change from viable to non-viable eggs in the range of 5 °C is quite abrupt though it could be assumed that there will be a proportional response to elevated temperature and egg viability between the 9°C and the 14 °C conditions tested. Such a graded response to increasing temperature has been reported in other species like common wolfish (Tveiten et al. 2001), Atlantic cod (Van Der Meeren & Ivannikov, 2006) and Atlantic halibut (Brown et al. 2006).

The impact of temperature treatments on sperm quality was not as evident. While the high temperature significantly reduced milt density over the treatment period, there was no negative impact on measured viability when sperm density was standardised during the gamete quality assessment. Environmental factors such as temperature have been shown to have an effect on milt volume produced (Kowalski & Cejko, 2019) and overall fertilisation success (Brown et al. 2006). There are very few studies which document sperm density in marine fish broodstock. In common wolfish, elevated temperatures during gametogenesis was shown to reduce spermatocrit (and by inference sperm density) (Tveiten & Johnsen., 1999), while exposure to repeat stressors in rainbow trout (*Oncorhynchus mykiss*) broodstock equally reduced sperm density (Alavi & Cosson., 2005). Given that male lumpfish spend more time in shallow

coastal waters during the spawning season compared to females (Davenport, 1985; Goulet et al.1986; Mitamura et al. 2012), it could explain their need to have a greater temperature tolerance.

It was surprising to observe a significant reduction in embryo viability in the low temperature treatment cross (6°C individual egg batches against a pool of 6°C milt). This suggests that there is also a lower thermal limit for the species, which is supported indirectly by the embryo viability results of Imsland et al. (2019a). The fact that the impact in the current study was only significant in the gamete quality test using the individual egg batches (where individual variability is controlled for) as opposed to the egg pools (where a reduction is apparent though not significant) implies again the effect is mediated primarily by the oocytes themselves.

Ultimately these data suggest an optimal thermal window (>6°C and <14 °C) for holding lumpfish during the spawning season to assure reliable and good quality productivity. There is anecdotal evidence from wild lumpfish fisheries suggesting that the capture of mature lumpfish ceases when sea temperatures reach 14°C (*Pers com.* David Patterson, Otter Ferry Seafish Ltd). Furthermore, in mid-Norway mature fish are caught almost year round at very different temperatures (5-7°C during winter/spring and up to 15°C during the autumn), however autumn caught broodfish produce much poorer eggs than winter/spring caught broodfish. Combined, this information points towards a narrow thermal window required to assure optimal egg quality in the species that needs to be incorporated in future captive broodstock holding.

In addition to defining thermal windows for optimal broodstock performance, this study has provided data on important elements of basic reproductive physiology for the species. While oocyte development typically follows a common cellular development process, species-specific definitions of size at stage of development are very important

in interpreting ovarian development using methods like oocyte size distribution (Kjesbu & Kryvi, 1989). Such detail is lacking for lumpfish with Kennedy (2018) acknowledging that interpretation of oocyte size distribution in the species was "…hindered by the lack of histological examination of the ovaries…". Classification of oocyte development is variable throughout the literature, meaning that direct comparisons between species can be difficult (Brown-Peterson et al. 2011; Rhody et al. 2013). However, size ranges for primary, secondary and oocyte maturation were comparable to those published in cod (Kjesbu & Kryvi, 1989). The current work and histological data reinforce the viteliogenic size ranges suggested in Kennedy (2018) and provides a scale that can be applied in future reproductive studies in this species.

Understanding the reproductive strategy of a given species is important in defining how to manage the species. While not the main focus of the study, results indicate that lumpfish should be considered a determinate batch spawning species. In both the histological examination and a wider OSD survey there was no evidence of continuous recruitment of oocytes and as such the potential annual fecundity should be considered fixed prior to the start of the spawning season. The current estimate of potential annual fecundity at 40,440 ± 12,434 oocytes per Kg body weight is the first accurate estimates for the species. Davenport (1985) suggested that batch fecundity for most females would average 100,000 eggs possibly reaching 400,000 eggs per batch and estimations by Hedeholm et al. (2017) suggested a range between 51,000 to 208,000, although both authors failed to correct for individual size. If the current estimate is extrapolated to a 4 kg weight, which was typical of the Davenport (1985) study, then absolute fecundities of 160,000 ± 48,000 oocytes is in alignment with their realised fecundity estimates. It also became apparent from the OSD that individuals

display both unimodal and bimodal OSD. This did not apparently influence total fecundity estimates, but it does suggest that the species can be batch spawning in nature. Davenport (1985) reported that lumpfish lay 2-3 egg batches over a period of two weeks and Kennedy (2018) supported this with oocyte size histograms displaying two clear cohorts; however, this was only present in some females with the assumption that unimodal females had spawned previously. During the current study, the proportion of individuals with bimodal oocyte development ranged from 18% to 50% with the lower abundance being evident in the high temperature treatment. The precise drivers of the extent of batch spawning in lumpfish needs further investigation. The current study brings into question whether all individuals are truly batch spawning, which of course has impact on the captive management and the estimates of spawning performance in commercial hatcheries that needs closer consideration. A final important definition for setting up broodstock populations is size at which animals are capable of spawning. The demonstration that sibling females < 500 g did not mature during the course of the current study supports Imsland et al. (2014) who suggested that females began to mature at around 450 g (non-spawning individuals mean for the current study was 454.3 g). This requires further investigation as such definitions play an important role in defining production cycles, generation times and predict egg productivity from captive broodstocks.

At present, the management of lumpfish broodstock is in its infancy, with little published guidelines on best management practices. This study provides the first definitions for broodstock thermal management. Future work must build on these definitions to include the optimisation of holding temperatures during early gametogenesis, which has been demonstrated to be a key determinant of egg quality in other species such as Atlantic halibut (Brown et al. 2006), and common wolfish

(Tveiten & Johnsen. 1999). This study also highlights other issues with broodstock management in lumpfish, with egg quality being a key issue. With eyeing rates in this study lower than those reported in industry. A focus needs to be placed on the determination of nutritional factors in lumpfish egg quality, which the subsequent chapter aims to address. This study also identified milt management as an important step in hatchery management, which is also affected by broodstock holding temperature, and reliant on limited quantification and storage techniques, which chapter 4 aims to address. If such work is combined with ongoing work on the photoperiod entrainment of spawning in the species (*e.g.* Imsland et al. 2019b) it is realistic to consider that reliable and effective environmental management of lumpfish broodstock will be a commercial reality soon.

Chapter Three

Egg quality determinants in lumpfish

3.1. Introduction

The production of larval fish, subsequent growth and development, health, and potential productivity as future broodstock is highly dependent on the quality of eggs available to the industry (Migaud et al. 2013). Thus, reliable egg quality is a critical issue when domesticating a new species and the nutritional components effecting egg quality need to be assessed. In fish, egg quality is determined by the composition of the egg and is affected by several factors including broodstock nutrition given that essential nutrients required by the egg, and subsequent larvae before the start of exogenous feeding are provided maternally and incorporated into the oocyte during vitellogenesis (Brooks et al. 1997; Bobe & Labbe. 2010). In most marine species, the main differences in egg quality between farmed and wild eggs associates with differences in the fatty acid composition *e.g.* Atlantic cod (*Gadus morhua*) (Salze et al. 2005) Atlantic halibut (Hippoglossus hippoglossus) (Mommens et al. 2015a) and white seabream (Diplodus sargus) (Perez et al. 2007). However, egg quality is also impacted by other nutritional components such as minerals, vitamins, amino acids and pigments (Palace & Werner. 2006, Sawanboonchun et al. 2008). It is important to identify nutritional factors effecting egg quality in order to inform the creation of captive broodstock diets and husbandry regimes for spawning (Mazorra et al. 2003).

Lipids and overall fatty acid composition of eggs have been identified as major nutritional factors which influence the survival of eggs and subsequent juveniles in Common snook (*Centropomus undecimalis*) (Yanes-Roca et al. 2009), Atlantic cod (Salze et al. 2005) and Atlantic halibut (Bruce et al. 1993). All vertebrates have a specific dietary requirement for polyunsaturated fatty acids (PUFA's) which are essential fatty acids (EFA's) including both n-3 and n-6 PUFA's, which cannot be synthesised *de novo* by the fish. In general, the main EFA's of marine fish include

eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), because marine fish are unable to elongate and desaturate n-3 fatty acids in sufficient amounts to meet the minimum requirements for embryonic development (Sargent et al. 2003). Good egg quality has been directly linked to increased levels of both DHA and EPA (Mazorra et al. 2003, Perez et al. 2007). The ratios of EFA's are also important such as in Atlantic cod where low ratios of EPA/ARA were associated with better hatching rates (Tocher & Sargent. 1984, Salze et al. 2005). An ARA:EPA ratio of 3-4:1 is recommended in Atlantic halibut broodstock diets to improve hatching rate of eggs (Mazzora et al. 2003). Ratios of DHA: EPA above 2 resulted in greater reported egg performance, viable eggs and hatching rate, in sea bass (*Dicentrarchus labrax*) (Navas et al. 1997).

Beyond the total fatty acid abundance within the lipid it is important to realise that there are two fractions of lipids, polar and neutral. Polar lipids are amphiphilic lipids characterised by a hydrophylic head and hydrophobic tail (Zheng et al. 2019). While neutral lipids are hydrophobic lipids lacking a charged group (Athenstaedt. 2010). Within the polar lipid fraction phospholipids play an important role in biological functions (Sargent et al. 2003). Phospholipids are structural components of the lipid bilayer in cell membranes making up to 50 percent of the lipids in cell membranes and affecting membrane fluidity. Despite the ability to synthesise phospholipids, supplementary inclusion of phospholipids within broodstock, larval and juvenile diets have been shown to increase performance in several fish species (Gallagher et al. 1998). Levels of phospholipids and the inclusion of EFA's within the phospholipid fraction have also been correlated with improved hatching rates within Atlantic cod (Lanes et al. 2012), halibut (Mommens et al. 201a) and sea bass (Bell et al. 1997). Neutral lipids are primarily used for storage of energy as excess fatty acids often in

lipid globules. In fish which have high neutral lipid content, neutral lipids are utilised, mainly triacylglycerols as the major source of lipid generated energy (Sargent et al. 2003). Therefore, in the context of marine broodstock management the focus is primarily on the polar lipid fractions due to their key role in formation of cell membranes.

In addition to fatty acids, fish eggs contain micronutrients and growth regulators that are essential for development (Kjørsvik et al. 1990, Brooks et al. 1997, Antony Jesu Prabhu et al. 2016). These micronutrients include minerals such as zinc and calcium, that are deposited during the vitellogenesis process (Ghosh & Thomas. 1995). There are two groups of minerals, major and trace determined by their abundance. The major minerals are present in comparably high levels and are needed in a large continuous supply, such as sodium, calcium and phosphorus. The trace minerals are present in small amounts and are supplied at very low levels such as selenium, cobalt and iron. Both trace and major minerals are essential for life and the amounts of the minerals that are required are not an index of importance (Lall. 2003). Egg quality associations with mineral content is not as well researched as lipid composition. Iron has been shown to increase hatching success in rainbow trout eggs (Hirao et al. 1955), where developmental fluctuations in iron levels were correlated with development of erythrocytes. Higher levels of manganese have also been shown to increase brown and rainbow trout hatching success, with these trace elements thought to play a role in many co-enzymes such as cytochromes (a,b,c) (iron), Cytochrome oxidase (copper), Carbonic anhydrase (zinc) and Pyruvate carboxylase (manganese) (Lall. 2003). However, there is currently less information on the role of minerals within marine fish. Other micronutrients have been shown to impact on reproductive performance such as levels of dietary phosphorus in gilthead sea bream (*Sparus*

aurata) (Lahnsteiner & Patarnello. 2003) and Atlantic cod (Lanes et al. 2012). Phosphorus levels are correlated to lipid levels due to their inclusion in the phospholipid fraction and its role in egg quality could be correlated to that lipid fraction. The role of calcium has been identified in the hardening of eggs such as in cod and lumpfish (Loning et al. 1984), as well as the formation of the skeleton in many bony fish (Lall. 2003). Low levels of dietary selenium have been correlated with reduced growth in channel catfish (*Ictalurus punctatus)* (Wang and Lovell. 1997). Selenium plays a pre-antioxidant role and can reduce the oxidative stress in high PUFA diets (Saleh et al. 2014). In terms of the availability of the mineral to the eggs, while maternal transfer during oocyte development is the primary source, it has been suggested that eggs can import minerals both through active transport and via passive osmotic transfer (Lall. 2003). However, some minerals cannot enter the egg through the chorion, such as cadmium and selenium and have been shown to be limiting factors in developing larvae (Lall. 2003; Pacitti et al. 2015).

The role of pigments in egg quality are poorly documented, and captive derived eggs have been shown to be deficient in total carotenoids compared to eggs from wild stocks such as Atlantic cod (Salze et al. 2005). Cod eggs with increased levels of astaxanthin were associated with increased percentage of fertilised eggs (Sawanboonchun et al. 2008), with Hansen et al. (2016) further reporting a positive correlation between egg pigmentation and hatching in the species. Within salmonids, astaxanthin is thought to have a role as a pro-vitamin A as well as protective properties for the egg and skin during early development (Christiansen and Torrissen. 1997). Other suggested roles of carotenoids include UV protection and improved respiratory function (Mikulin. 2000). It has also been suggested that they may play a role in

photoreception within the embryo (Ronnestad et al. 1998). As such, carotenoids are now commonly supplemented in broodstock diets (Lim et al. 2018).

In terms of the lumpfish, as outlined previously the sector aspires to close the life cycle (Brooker et al. 2016). Ensuring good egg quality derived from captive produced broodstock is essential to enable the reliable supply of robust juveniles for the salmon industry (Powell et al. 2018). Wild derived eggs generally are reported to have better overall quality, however, hatcheries have reported high levels of variation over the fishing season. This concern over egg quality is further supported by the current work with there being a high level of variation in egg quality observed in chapter 2. There is currently no information on the role of nutritional components within egg quality in lumpfish. The main nutritional analysis focus for the species has been conducted on juveniles, either to improve growth, health, and robustness (Willora et al. 2020) or to improve delousing efficiency (Imsland et al. 2018b). However, egg nutritional work has been focused on the caviar trade for human consumption (Stevenson and Baird. 1988).

Lumpfish eggs display a huge diversity of pigmentation and it has been theorised that this variation could be related to egg quality (Davenport. 1985). Previous studies on lumpfish pigmentation have focused on juvenile and mature males (Davenport and Bradshaw. 1995). A more recent study documented liver carotenoids within deployment size lumpfish that could be used as a welfare indicator (Eliasen et al. 2020). That study identified 8 carotenoids present, astacene, astaxanthin, asteroidenone, beta carotene, canthaxanthin, echinenone, and xanthophylls. There has been limited work on pigmentation within lumpfish eggs (Mikulin & Soin 1975, Mikulin et al. 1978), which appear to be crude identification of pigments through absorbance measurements at varying wavelengths, however this author was limited

in translation of these Russian language papers, a review into carotenoids by Craik (1985) noted that English translations of these papers had not been published.

Due to the lack of specific information on lumpfish egg composition, the present study aimed to analyse the proximate composition of wild, at the start and end of the fishing season, and captive derived lumpfish eggs and also to determine how levels of fatty acids, minerals and pigments vary in association with the quality of lumpfish eggs. A further aim of this work was to subsequently measure how levels of fatty acids, minerals and pigments change through embryonic development. At its conclusion this work will help prioritise nutritional factors which associate with egg quality in lumpfish to support the development of species specific broodstock feeds in the future.

3.2. Materials and methods

Both wild and captive derived eggs have been used for this study, methods for collection and incubation can be found in seconds 3.2.1 and 3.2.2 for wild and captive broodstocks respectively.

3.2.1 Wild egg collection, fertilisation and incubation.

Wild broodstock were obtained from Skjerneset Fisk at Averøy, Norway and processed at the farm. On two separate dates (31st October 2018 and 15th May 2019), ten females and three males were randomly sampled from that days catch. Each fish was anaesthetised with MS-222 (200 mg L^{-1}) (Pharmaq AS, Norway) before wet weight (± 1 g) and total length (± 1 mm) were recorded (Table 3.1). The females were then stripped before being killed by an overdose of MS-222 (1 g L^{-1}). Males were killed before wet weight (± 1 g) and total length (± 1 mm) were recorded (Table 3.1). Testis were then dissected out and grounded, sieved and mixed 1:1 with AquaBoost sperm coat© (Cryogenetics, Norway). Eggs and milt were then transported to NOFIMA cleaner fish unit at Sunndalsora in a temperature-controlled box.

Milt was tested for motility, using a light microscope, and density using a spectrophotometer at 540 nm (SDM6, Cryogenetics, Norway). Milt samples which were classified as “poor” based on reduced motility (displaying low sperm activation, abnormal swimming) or low sperm density (< 7×10^{9} / mL) were rejected. A milt “pool” mixing 3 individuals was created and sperm density was re-assessed following pooling to determine milt volume for and accurate standardisation of sperm to egg at the point of fertilisation (minimum 300,000 sperm per egg).

All egg manipulation was conducted in a temperature-controlled room (8 ° C). For each individual female, three replicate batches of six 10 ml (approximately 1000) of eggs

from the stripped egg masses were placed in disposable plastic beakers, creating small pancakes 1-2 eggs thick. These were then fertilised with a volume of milt to give 300,000 sperm per egg, mixed in 50 ml of 0.2 µm-filtered seawater and left for 15 minutes. Then, 1 ml of fertilised eggs from each individual was sampled and placed on a 30 ml petri dish, flattened using a sterile spatula and rinsed with autoclaved seawater. The petri dish was filled with 30 ml of 0.2 µl filtered seawater and an antibiotic solution made of penicillin/streptomycin (1 ml/ 100 ml) (Sigma, USA) and placed on a slowly moving shaker in a climate-controlled room (6 °C). All egg masses were transferred to an incubator and covered with a lid and held at 7.4 °C ± 0.8 °C (Oct) or 7.7 °C ± 0.6 °C (May) until hatching (Figure 3.1).

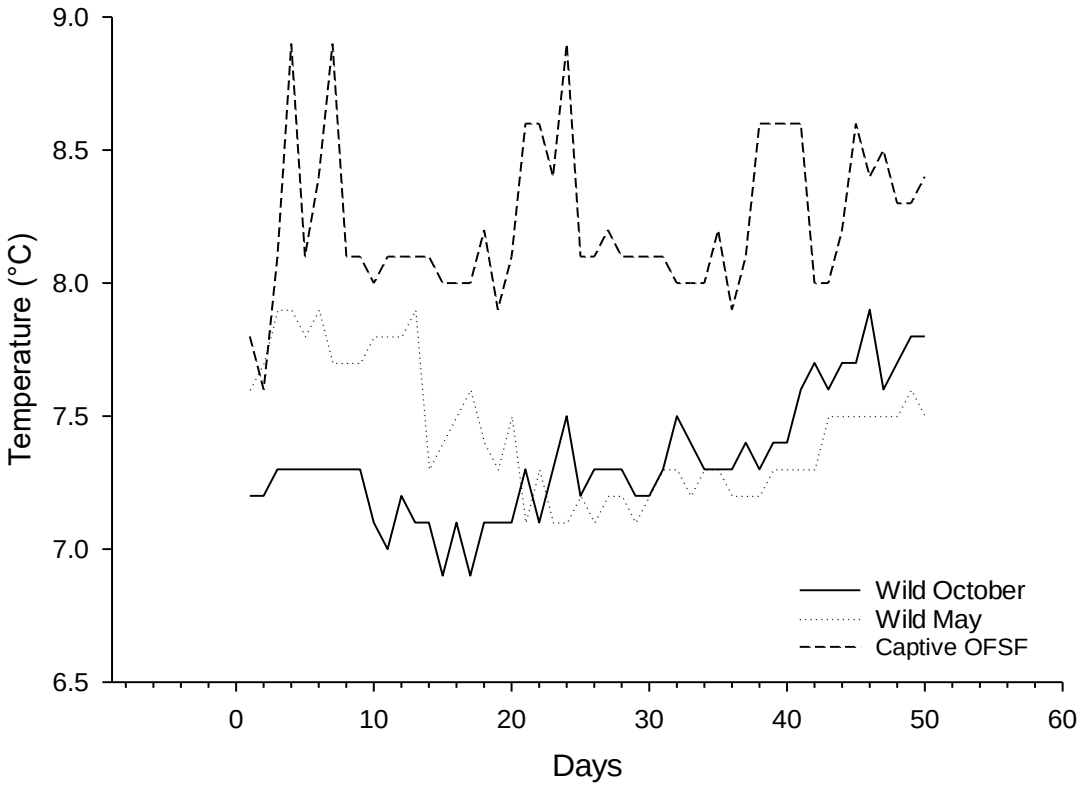

Figure 3.1: Daily temperatures recorded over the incubation period for October (wild), May (wild) and Scottish captive runs.

3.2.2 Captive egg collection, fertilisation and incubation

Eggs used in the study were obtained from captive F1 broodstock reared at Otter Ferry Sea Fish Ltd. (Scotland). Fish were 17 months old and raised from eggs derived from broodstock captured on the west coast of Scotland around the Lochaber coastline. Lighting was maintained at a low intensity under a 24 hr photoperiod for the entire grow out period. Prior to sampling, fish were held on an altered temperature regime (from hatch, 9.4 °C ± 0.8 °C), in accordance with the conclusion of chapter 2, with holding temperatures not exceeding 10 °C to assure minimal impact of detrimental thermal holding conditions. Fish were fed to satiation on Samaki Marine Pellet (World Feeds James A Makie (agricultural), UK). Fish started maturing in December 2018 with natural spawning taking place in mid-January 2019. Fish were monitored by ultrasound every 3 days to assess suitability for stripping. Fish were stripped between January and March 2019; 10 females were used for this study. At the point of stripping, wet weight (± 1 g) and total length (± 1 mm) were recorded (Table 3.1). Males (*n*= 7) were killed with an overdose of MS-222 before wet weight (± 1 g) and total length (± 1 mm) were recorded (Table 3.1) before testes were dissected out and ground, sieved and mixed 1:1 with AquaBoost sperm coat© (Cryogenetics, Norway).

Milt density from the captive stock was assessed by haemocytometer counts and activation response of sperm was observed using a light microscope. Milt which was "poor" in motility (displaying low activation and/or abnormal swimming), or low in sperm density (> 7×10^{9} spermatozoa / mL) was rejected. A milt "pool" was created with three good quality males and the density was re-measured to allow for an accurate standardisation of sperm to egg at the point of fertilisation (300,000 sperm per egg).

All egg manipulation was conducted in a temperature-controlled room (8 ° C). For each individual female, three replicates of, three 5 ml batches (Approximately 500) of eggs were placed in petri dishes creating a small pancake 1 egg thick. These were then fertilised with a volume of milt to give 300,000 sperm per egg, mixed in 20 ml of 0.2 µm-filtered seawater and left for 15 minutes in the incubator. The eggs were then flattened using a sterile spatula and rinsed with autoclaved seawater, placed in a petri dish filled with 30 ml of 0.2 µl filtered seawater and placed in a climate-controlled incubator (8 °C).

3.2.3 Fertilisation and eyed egg measurements

Fertilisation rate was not assessed in the wild October batches due to difficulty in assessing fertilisation as lipid globules impede visibility within the egg. Fertilisation rates were calculated from the pancakes incubated in petri dishes for the wild May and captive stocks. At 24 hours post fertilisation, the fertilisation rate was assessed by counting the number of fertilised eggs and the number of unfertilised eggs and providing a number of total eggs and a fertilisation percentage. Due to difficulty identifying development through the lipid globules, fertilisation was assessed at 24 hours where the Morula stage was clearly visible.

Wild stocks petri dish eggs were monitored until the eyed egg stage at 150-degree days (DD). At this point, each petri dish (n = 3 per individual, n = 10 individuals per stock) was photographed using a digital camera (Nikon 1, NIKON, Japan) and total eggs, eyed eggs and non-eyed eggs were counted.

For the captive stocks, fertilised eggs were transferred to 48 well micro-well plates (Fisher Scientific, UK), each well was filled with 5 ml of 0.2 µl filtered seawater and sealed with eyeing rate being calculated at 150 DD as described above.

3.2.4 Hatching assessments

For the wild batches, hatched juveniles were killed with an overdose of MS-222 (Pharmaq, UK) and transferred to a tray. Egg shells and debris were removed, and photographs taken. The number of hatched juveniles were counted, and hatching percentages were calculated from the number of eggs estimated from the egg per ml data × 20 ml.

For the captive stock the number of hatched juveniles and non-hatched juveniles were used to calculate plate hatch percentages for each replicate.

3.2.5 Embryonic development sampling

Samples of embryos during development were only collected from the wild October and wild May stocks as large incubation facilities were not available for the captive stock eggs. Egg samples (1 × 10ml pancake) were removed and frozen from each replicate at set time points during the experiment. Pancakes were collected from each replicate (*n* = 3 per individual) at 24 hours post fertilisation, 60 DD, 150 DD, 200 DD as well as hatched larvae and were preserved at -20°C.

Samples collected in Norway were shipped back to the University of Stirling on dry ice for analysis.

3.2.6 Lipid analysis

Total lipid (TL) was extracted according to Folch et al. (1957). For each individual egg sample, approximately 1 g of eggs (*n*= 3 biological replicates, *n*= 3 technical replicate per biological repl) were homogenised in 20 mL chloroform: methanol (2:1, v:v) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough), Unless stated otherwise all reagents were analytical grade purchased from Sigma-Aldrich, USA, or

Fisher Scientific, UK. Lipids were separated from debris by adding 5 mL of potassium chloride (KCl; 0.88 % w:v) and left on ice for 1 hr. The upper layer was aspirated, and the lower layer was dried under nitrogen. TL content of each sample was determined gravimetrically after 12 hrs in a vacuum desiccator (Fisher Scientific, UK).

Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalysed transesterification at 50 ˚C for 16 hrs. (Christie, 2003), and FAME extracted and purified as described by Tocher & Harvie (1988). FAME were separated and quantified by gas-liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a flame ionisation detector. Data were collected and processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). Individual FAME were identified by comparison to known standards (RESTEK, UK) and published data (Tocher & Harvie, 1988). The addition of a 17:0 standard at a known concentration (0.1mg/L) enabled absolute quantification of fatty acids.

3.2.7 Polar and neutral fatty acid identification

Polar and Neutral lipid classes were determined by high-performance thin-layer chromatography (HPTLC) using 20 × 20 cm plates (VWR, Lutterworth, England). Extraction of polar lipids were conducted where the plate was fully developed in a neutral solvent; isohexane: diethyl ether: acetic acid (85:15:1.5, by vol.). Extraction of neutral lipids where the plate was fully developed in a polar solvent; methyl acetate: isopropanol: chloroform: methanol: 0.25 % aqueous KCl (25:25:25:10:9, by vol.). Non-eluted material as removed from the plate and, FAME's were then prepared and analysed using the above method (Section 3.2.6).

3.2.8 Lipid Class determination

Lipid class composition was determined by high-performance thin-layer chromatography (HPTLC) using 20 × 10 cm plates (VWR, Lutterworth, England) (Christie. 2003). Approximately 2 µL of total lipid (*n* = 3 per sample, *n* = 3 samples per individual) alongside polar lipid and neutral lipid standards were applied as single spots. Plates were first left in a polar solvent; methyl acetate: isopropanol: chloroform: methanol: 0.25 % aqueous KCl (25:25:25:10:9, by vol) until the solvent had reached halfway up the plate. After drying for 15 mins, the plate was fully developed in a neutral solvent; isohexane: diethyl ether: acetic acid (85:15:1.5, by vol.). The lipid classes were visualised by charring at 160 °C for 25 mins. After spraying with 3 % (w:v) aqueous cupric acetate containing 8 % (v:v) phosphoric acid and quantified by densitometry using a CAMAG-3 TLC Scanner (VWR International, Dorset, UK) (Henderson and Tocher, 1992). Scanned images were recorded automatically and analysed by computer using winCATS Planar Chromatography Manager (version 1.2.3).

3.2.9 Mineral composition

Mineral compositions were determined from whole eggs using the nitric acid (HNO3) digestion technique. Samples, approximately 0.1g, (*n*= 3 biological replicates, *n*= 3 technical replicates per biological rep) were digested in Kjeldahl digestion tubes with 69 % nitric acid using a MARS microwave digestion system (CEM MARSXpress, CEM ltd., Buckingham, UK) using the following program: 10 mins. Heating phase to 190 °C, maintain 190 °C for 20 mins cooling phase to 21 °C for 60 mins. Samples were then diluted with distilled water to 2 % HNO3 and analysed for mineral content via

Inductively Coupled Plasma Mass Spectrometry (ICP-MS; Thermo X series II; Collision cell technology).

3.2.10 Pigment identification

Pigments were analysed by the routine University of Stirling synthetic pigment method (Barua et al. 1993) utilising the methods recommended volume 1 g of egg. Using this method, the resultant HPLC traces did not pass the quality control checks, where the total measured absorbance, as determines by HPLC, did not match total absorbance measured by spectrophotometer (Figure 3.2). During initial optimisation work the HLPC run was extended to 30 minutes from the standard 18 mins. This extension revealed a peak at 22-24 minutes which explained the difference between absorbances for the quality control. Several attempts were conducted to attempt to identify the unknown peak. Isolation of the peak was conducted by fractioning off elutant from the HPLC column, and peak absorbance was measured across the visible wavelength (300-800 nm) and was identified at 326 nm. Identification of this compound was attempted by mass spectrometer, isolated peak samples were analysed, producing a m/z trace characterised by Triacylglycerol (TAG) peaks, removal of the Triacylglycerol was conducted and re-ran, the resulting trace was characterised by Diacylglycerol (DAG). A number of possible peaks were identified; however, no identification was possible within desirable parameters. The same artefact was identified in whole lumpfish flesh, salmon flesh and salmon eggs, utilising both the standard pigment extraction method and Folch total lipid extraction method (section 3.2.6) (Data not shown).

In response extraction of pigments was adapted using the method below, with an increased mass of eggs (5 g instead of 1 g). To assess total carotenoid content and

individual pigment composition of the samples, 5 g of eggs was homogenised and carotenoids were extracted in three-phases using ethyl acetate: ethanol (1:1, v:v) (30 ml), ethyl acetate (20 ml), and iso-hexane (20 ml). After evaporation, samples were re-suspended in iso-hexane and the absorbance measured on a spectrophotometer (CE 2021 2000 series, Cecil 105 Instruments Ltd., Cambridge, UK) at 470 nm against an iso-hexane blank. All samples were subsequently transferred to auto-sampler vials and processed through high performance liquid chromatography (HPLC; Waters Alliance System, Waters Corporation, MA, USA). Identification of carotenoids present was determined using retention time against a known standard of astaxanthin and canthaxanthin. To quantify the outputs, the area of each peak was measured using Empower 2 chromatographic processing software (Waters Corporation, MA, USA).

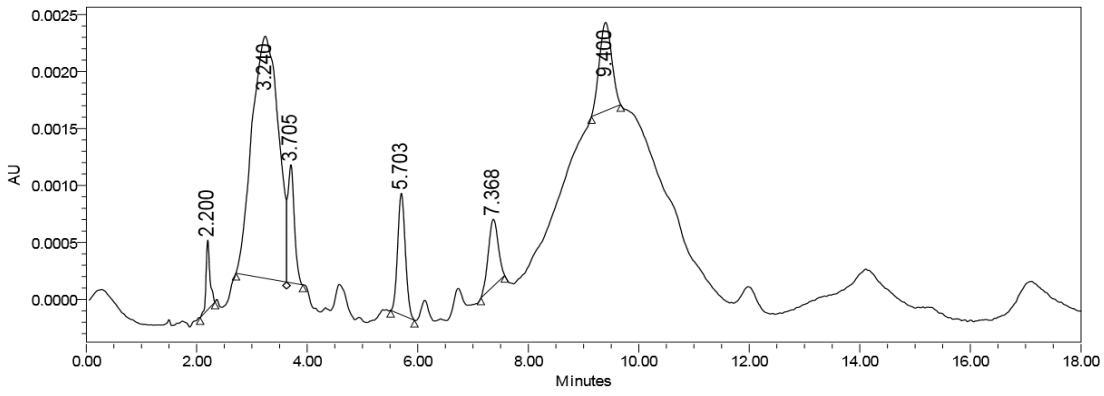

Figure 3.2: A typical HPLC trace for Lumpfish eggs extracted using the University of Stirling synthetic pigment method run at the standard 18-minute runtime. L-R βcarotene, echinenone, canthaxanthin, astacene, axtazanthin, leutin.

3.2.11 Statistics

For gross stock comparisons statistics were conducted using Minitab18 software. Percentage data was arcsine transformed before testing. All datasets were tested for

normality before analysis using a Kolmogorov–Smirnov test for normality. Comparison between stocks was conducted using and ANOVA with a post hoc Tukeys test to assess difference between populations.

Principal component analysis was conducted to determine associations of egg nutrients with egg quality, using SPSS (V23, IBM statistics). The definition of meaningful loading used within this study is set at a loading within a component greater than 0.5 for a positive loading or lower than -0.5 for a negative loading (Peres-Neto et al. 2003).

Assessment of compositional changes over development was conducted using Minitab18 software. A General linear model was conducted to assess the changes in nutritional composition over embryonic development.

3.3 Results

3.3.1 Gross stock comparisons

Wild caught broodstock were larger than the captive stocks, with regards to weight and length for both males and females (Table 3.1). Stripped egg volumes from the wild stocks were significantly larger than those from the captive stock. Furthermore, the number of eggs per ml of the stripped volume was significantly higher in the captive broodstock. In response, there was no significant difference in estimated batch fecundity within this study with batch fecundity for wild broodstocks being 13,396 ± 5216, to 14,445 ± 5330 eggs per Kg for wild 13,920 ± 5532 eggs per Kg respectively compared with 19939 ± 9426 eggs per Kg for the captive stock. Egg diameter was significantly lower in the captive stocks (1.91 ± 0.56 mm) than those in the two wild stocks (2.43 ± 0.48 mm, and 2.58 ± 0.61 mm). There was no significant difference between sperm densities for the three populations, which fall within previously documented levels (chapter 2) (Table 3.1).

Females used for the developmental changes study (see 3.3.4 below) were a subset of the wild population (samples could not be incubated in the same manner in the captive stocks to allow this analysis to be performed), the top performing 8 batches as determine by hatching rate were selected. These individuals were equally taken from the wild October and wild May samples (4: 4) and did not differ significantly from the respective wild populations in weight (3255 ± 149 g) and length (442 ± 11.2 mm). They did not significantly differ from the wild populations in fertilisation (98.0 ± 0.13 %), eyeing rates (88.28 ± 1.79 %) and hatching rate (63.08 ± 4.91 %).

Table 3.1: Summary of descriptive data for lumpfish broodstock used in this study, *n* = 10 for females, males *n* = 3 (± SEM). Different superscript denotes significant difference between stocks.

	wild October	wild May	Captive
Females			
Weight (g)	3370 ± 72 [a]	2840 ± 141 [a]	1716 ± 150 [b]
Total length (mm)	429 ± 5 [a]	419 ± 8 [a]	321 ± 11 [b]
Stripped volume of eggs (ml)	461 ± 38 [a]	380 ± 26 [a]	321 ± 44 [b]
Eggs per ml	97 ± 3 [b]	102 ± 5 [b]	167 ± 20 [a]
Egg Diameter (mm)	2.43 ± 0.48 [a]	2.58 ± 0.61 [a]	1.91 ± 0.56 [b]
Batch Fecundity (Oocytes per kg)	13396 ± 5216	14445 ± 5330	19939 ± 9426
Fertilisation rate (%)	N/A	97.5 ± 0.5 [a]	49.08 ± 4.6 [b]
Eyeing rate (%)	77.3 ± 6.8 [a]	74.2 ± 5.9 [a]	17.1 ± 10.3 [b]
Hatching rate (%)	46.9 ± 7.2 [a]	45.9 ± 5.9 [a]	6.74 ± 6.1 [b]
Males			
Weight (g)	1460 ± 56 [a]	1350 ± 45 [a]	638 ± 39 [b]
Total length (mm)	350 ± 21 [a]	347 ± 23 [a]	228 ± 5 [b]
Sperm density ($\times 10^9$ ml^{-1})	10.9 ± 2.8	12.1 ± 1.4	12.3 ± 2.1

3.3.2 Egg quality parameters

Wild derived eggs performed better in all three measured quality stages. Fertilisation, eyeing, and hatching rates were all significantly higher than those derived from captive stocks (Table 3.1). There was no significant difference in quality between wild eggs derived from fish caught in October and those from May.

3.3.2.1 Egg lipid content and fatty acid composition

Analysis of total lipid content between wild and captive derived eggs shows a clear reduction, 39 % in total lipid levels (by wet weight) within the captive eggs compared to the wild October and May samples which were comparable (Table 3.2).

Captive eggs were significantly lower in terms of absolute levels of fatty acids (µg mg^{-1} of tissue), with the total absolute level of fatty acids being 53 % and 58 % lower than that of the wild derived October, and May broodstocks respectively (Table 3.2), with there being no significant difference between wild populations. With respect to the relative proportion of all fatty acid groups there was no difference between wild October and May samples, however the wild samples differed consistently from the captive eggs. Total saturates were significantly higher in the wild October and wild May stocks than the captive stock. However, the proportional levels of total saturates were significantly lower than those in the captive stock. Absolute levels of total monounsaturated were significantly higher in the wild October and wild May stocks compared to those from captive eggs. Despite this there was no significant difference in proportional levels of monosaturates between the three stocks. Absolute and proportional Levels of Total PUFA were significantly lower in the captive stocks than both the wild October and wild May stocks. However absolute and proportional levels of total n-6 PUFA were significantly higher in the captive derived eggs than those from the wild October and May eggs. While absolute and proportional levels of total n-3 PUFA were significantly higher in the wild October and Wild May stocks than those from the captive stock. Both proportional and absolute levels of DHA and EPA are significantly higher in the wild October and wild May stocks than those in the captive stock. There was no significant difference in the absolute levels of ARA between the three stocks, however the captive stock was significantly higher in proportional levels than both wild October and wild May stocks. The proportional ratios of n-3: n-6 PUFA's was significantly higher in both Wild October and Wild May than those in the captive stock. For the DHA: EPA ratios there was no significant different between proportional levels in the captive stock. While the absolute ARA: EPA ratio was not significantly

different between the three stocks there was a significant increase for the proportional levels within the captive stocks compared to the wild October and wild May stocks.

Table 3.2: Egg fatty acid profiles for the three stocks used in this trial (wild October, May and captive) reported in µg per mg of lipid and proportional levels. Values indicate Mean (*n*= 10) ± SEM, Different letter superscripts denotes significant differences between stocks.

	WILD OCTOBER		**WILD MAY**		**CAPTIVE**	
Total Lipid (mg g^{-1} WW)	3.53 ± 0.54[a]		3.62 ± 0.49[a]		2.15 ± 0.68[b]	
Total FA (µg per ml of lipid)	625.39 ± 26.32[a]		571.01 ± 28.71[a]		334.31 ± 29.69[b]	
	µg/mg Lipid	Proportional (%)	µg/mg Lipid	Proportional (%)	µg/mg Lipid	Proportional (%)
14:00	10.11 ± 0.92 [a]	1.04 ± 0.08 [a]	11.87 ± 1.11 [a]	1.06 ± 0.06 [a]	4.93 ± 0.32[b]	0.65 ± 0.04 [b]
15:00	3.245 ± 0.16 [a]	0.33 ± 0.01 [a]	3.47 ± 0.24 [a]	0.31 ± 0.01 [a]	2.02 ± 0.11 [b]	0.27 ± 0.01 [b]
16:00	156.08 ± 4.20 [a]	14.79 ± 0.27 [b]	156.18 ± 6.50 [a]	14.15 ± 0.22 [b]	125.36 ± 5.96 [b]	16.48 ± 0.24 [a]
18:00	42.51 ± 3.66 [a]	4.07 ± 0.30 [b]	43.44 ± 2.17 [a]	3.93 ± 0.12 [b]	42.53 ± 3.52 [a]	5.81 ± 0.68 [a]
20:00	0.686 ± 0.38	0.11 ± 0.04	1.043 ± 0.39	0.09 ± 0.03	0.71 ± 0.04	0.09 ± 0.01
22:00	0.02 ± 0.02	-	-	0.00 ± 001	0.03 ± 0.03	-
24:0	0.12 ± 0.07	0.01 ± 0.01 [a]	-	-	0.35 ± 0.07	0.03 ± 0.01 [b]
Total saturated	212.76 ± 6.27 [a]	20.35 ± 0.49 [b]	216.02 ± 9.52 [a]	19.54 ± 0.25 [b]	175.97 ± 7.16 [b]	23.33 ± 0.55 [a]
16:1n-9	5.18 ± 0.85 [a]	0.62 ± 0.07 [a]	5.30 ± 0.51 [a]	0.49 ± 0.05 [ab]	2.75 ± 0.16 [b]	0.35 ± 0.01 [b]
16:1n-7	12.23 ± 0.99 [ab]	1.32 ± 0.07 [b]	16.53 ± 1.81 [a]	1.47 ± 0.11 [ab]	12.68 ± 0.70 [b]	1.69 ± 0.09 [a]
18:1n-9	182.19 ± 8.12 [a]	17.46 ± 0.38 [b]	201.15 ± 10.42 [a]	18.15 ± 0.28 [ab]	143.94 ± 6.31 [b]	19.26 ± 0.37 [a]
18:1n-7	31.60 ± 2.54	3.17 ± 0.20	37.51 ± 2.60	3.39 ± 0.14	32.38 ± 1.12	4.39 ± 0.11
20:1n-11	1.46 ± 0.36	0.03 ± 0.03 [a]	0.79 ± 0.48	0.08 ± 0.05 [a]	1.78 ± 0.19	0.21 ± 0.03 [b]
20:1n-9	31.57 ± 2.61 [a]	3.49 ± 0.19 [a]	40.18 ± 4.15 [a]	3.57 ± 0.24 [a]	10.11 ± 0.57 [b]	1.35 ± 0.08 [b]
20:1n-7	4.58 ± 0.25 [a]	0.46 ± 0.03 [a]	5.09 ± 0.49 [a]	0.46 ± 0.03 [a]	0.81 ± 0.07 [b]	0.11 ± 0.01 [b]
22:1n-11	7.26 ± 0.65 [a]	0.70 ± 0.06 [a]	8.51 ± 1.04 [a]	0.75 ± 0.06 [a]	3.12 ± 0.25 [b]	0.39 ± 0.03 [b]
22:1n-9	5.31 ± 0.21 [a]	0.53 ± 0.02 [a]	5.61 ± 0.42 [a]	0.50 ± 0.02 [a]	0.86 ± 0.05 [b]	0.11 ± 0.01 [b]
24:1n-9	-	-	0.11 ± 0.12	0.01 ± 0.01	-	-
Total monounsaturated	281.42 ± 11.76 [a]	27.78 ± 0.52	320.83 ± 18.93 [a]	28.87 ± 0.55	208.48 ± 8.85 [b]	27.87 ± 0.61
18:2n-6	11.93 ± 0.54 [b]	1.17 ± 0.04 [b]	12.5 ± 0.87 [b]	1.12 ± 0.04 [b]	94.85 ± 4.85 [a]	11.43 ± 0.51 [a]
18:3n-6	1.13 ± 0.05 [a]	0.11 ± 0.01 [a]	1.21 ± 0.07 [a]	0.11 ± 0.01 [ab]	0.69 ± 0.05 [b]	0.10 ± 0.01 [b]
20:2n-6	2.75 ± 0.13 [a]	0.27 ± 0.01 [b]	2.75 ± 0.13 [a]	0.25 ± 0.04 [b]	2.91 ± 0.12 [a]	0.37 ± 0.01 [a]
20:3n-6	0.76 ± 0.05 [b]	0.08 ± 0.01 [b]	0.92 ± 0.08 [ab]	0.08 ± 0.02 [b]	1.01 ± 0.08 [a]	0.13 ± 0.1 [a]
20:4n-6 ARA	7.19 ± 1.02 [a]	0.76 ± 0.08 [b]	8.50 ± 0.60 [a]	0.77 ± 0.04 [b]	9.16 ± 0.49 [a]	1.17 ± 0.03 [a]

Table 3.2 (Continued): Egg fatty acid profiles for the three stocks used in this trial (wild October, May and captive) reported in µg per mg of lipid and proportional levels. Values indicate Mean (*n*= 10) ± SEM, Different letter superscripts denotes significant differences between stocks.

	WILD OCTOBER		**WILD MAY**		**CAPTIVE**	
	µg/mg Lipid	Proportional (%)	µg/mg Lipid	Proportional (%)	µg/mg Lipid	Proportional (%)
22:4n-6	0.20 ± 0.03 ^{b}	-	0.08 ± 0.05 ^{b}	0.01 ± 0.01 ^{b}	0.68 ± 0.04^{a}	0.09 ± 0.01 ^{a}
22:5n-6	2.91 ± 0.20 ^{b}	0.28 ± 0.02 ^{b}	3.0 ± 0.21 ^{ab}	0.27 ± 0.02 ^{b}	3.70 ± 0.21 ^{a}	0.48 ± 0.01 ^{a}
Total n-6 PUFA	26.91 ± 1.35 ^{b}	2.67 ± 0.09 ^{b}	29.0 ± 1.75 ^{b}	2.61 ± 0.09 ^{b}	113.02 ± 5.42 ^{a}	13.75 ± 0.49 ^{a}
18:3n-3	5.54 ± 0.31 ^{b}	0.54 ± 0.03 ^{b}	6.08 ± 0.49 ^{b}	0.55 ± 0.04 ^{b}	10.50 ± 0.45 ^{a}	1.32 ± 0.04 ^{a}
18:4n-3	7.82 ± 0.37 ^{a}	0.71 ± 0.03 ^{a}	8.71 ± 0.75 ^{a}	0.78 ± 0.05 ^{a}	3.38 ± 0.24 ^{b}	0.45 ± 0.03 ^{b}
20:3n-3	1.21 ± 0.08 ^{a}	0.12 ± 0.01 ^{b}	1.23 ± 0.08 ^{a}	0.11 ± 0.01 ^{ab}	1.05 ± 0.06 ^{a}	0.13 ± 0.01 ^{a}
20:4n-3	8.92 ± 0.43 ^{a}	0.86 ± 0.03 ^{a}	10.03 ± 0.68 ^{a}	0.91 ± 0.04 ^{a}	5.10 ± 0.27 ^{b}	0.68 ± 0.03 ^{b}
20:5n-3 EPA	182.94 ± 5.26 ^{a}	17.36 ± 0.24 ^{a}	189.97 ± 9.97 ^{a}	17.13 ± 0.21 ^{a}	81.10 ± 4.16 ^{b}	10.47 ± 0.31 ^{b}
22:5n-3	14.25 ± 0.89 ^{a}	1.44 ± 0.06 ^{b}	16.37 ± 0.98 ^{a}	1.48 ± 0.04 ^{b}	13.47 ± 0.74 ^{a}	1.73 ± 0.08 ^{a}
22:6n-3 DHA	298.47 ± 10.92 ^{a}	27.33 ± 0.58 ^{a}	301.85 ± 14.31 ^{a}	27.28 ± 0.49 ^{a}	152.84 ± 6.57 ^{b}	19.55 ± 0.43 ^{b}
Total n-3 PUFA	519.18 ± 15.86 ^{a}	48.35 ± 0.65 ^{a}	534.26 ± 25.15 ^{a}	48.24 ± 0.49 ^{a}	267.46 ± 11.02 ^{b}	34.33 ± 0.40 ^{b}
16:2	4.83 ± 0.63 ^{a}	0.45 ± 0.05	3.73 ± 0.81 ^{a}	0.37 ± 0.08	1.92 ± 0.31 ^{b}	0.28 ± 0.05
16:3	3.66 ± 0.14 ^{a}	0.35 ± 0.01 ^{b}	3.88 ± 0.17 ^{a}	0.35 ± 0.01 ^{b}	3.12 ± 0.15 ^{b}	0.41 ± 0.01 ^{a}
16:4	0.47 ± 0.30	0.04 ± 0.03	0.11 ± 0.05	0.01 ± 0.01	0.14 ± 0.05	0.02 ± 0.01
Total 16:X	8.97 ± 0.71 ^{a}	0.84 ± 0.09	7.73 ± 0.70 ^{a}	0.73 ± 0.10	5.20 ± 0.29 ^{b}	0.71 ± 0.07
Total PUFA	555.07 ± 16.66 ^{a}	51.86 ± 0.65 ^{a}	571.00 ± 26.32 ^{a}	51.58 ± 0.58 ^{a}	385.69 ± 15.68 ^{b}	48.80 ± 0.60 ^{b}
ARA:EPA	0.039 ± 0.01	0.04 ± 0.01 ^{b}	0.044 ± 0.01	0.04 ± 0.01 ^{b}	0.11 ± 0.01	0.11 ± 0.05 ^{a}
DHA:EPA	1.64 ± 0.11^{a}	1.57 ± 0.06	1.59 ± 0.15	1.59 ± 0.4 ^{a}	1.90 ± 0.26 ^{b}	1.87 ± 0.4
N-3:N-6	19.44 ± 2.1 ^{a}	18.08 ± 1.06 ^{a}	18.61 ± 1.6 ^{a}	18.45 ± 1.04 ^{a}	2.45 ± 0.26 ^{b}	2.50 ± 0.69 ^{b}

3.3.2.2 Variation in Polar and neutral fatty acids

As with the total fatty acids there are significant differences between the wild and captive stocks in the polar fraction (Table 3.3). The levels of total saturates was significantly different between all three stocks, however captive derived eggs were significantly higher than both the wild October (10%) and May stocks (20 %). Total monosaturates were significantly higher (16 %) in the captive stocks than both the wild October and wild May stocks, however there was no significant difference between the two wild stocks. Proportional levels of total PUFA in the wild May stocks was significantly higher than the captive stock (6 %), but neither wild May nor captive stocks differed significantly from the wild October stocks. Within the PUFA's the levels of n-3 were significantly higher in the wild October and wild May than those in the captive stocks (40 %) within the polar fraction. Where-as levels of N-6 was six to seven-fold higher in the captive stocks than those in the wild October and wild May stocks. The ratio of n-3: n-6 PUFA within the polar fraction was significantly lower in the captive stocks than both wild stocks (*circa* seven to eight-fold reduction). Levels of ARA: EPA and DHA: EPA were significantly higher in the captive stock than the wild May and wild October stocks. Levels of both DHA and EPA are significantly higher in the wild stocks than the captive stock, however, the level of ARA is significantly higher in the captive stock than both wild stocks.

As with the polar and total fatty acid fractions there were consistent significant differences in the levels between the two wild stocks and the captive stock within the neutral lipid fractions (Table 3.3). Levels of total saturates were significantly higher in the wild May stocks compared to those in the captive stock, however, levels of either did not significantly differ from the wild October stocks. Total monosaturates were significantly higher in the captive stocks than the wild October stock, however, neither

wild October nor captive stocks differed significantly from the wild May stocks. Levels of total PUFA, and n-3 PUFA were significantly higher in the wild October stock than those in the wild May and captive stocks. Levels of n-6 PUFA were significantly higher in the captive stocks than both the wild October and wild may stocks. As a result, the captive eggs ratio of n-3: n-6 ratio is significantly lower than both wild stocks. The ARA: EPA ratio for the neutral lipid fraction were significantly higher in the captive stock than both the wild October and wild may stocks. While the DHA: EPA level in the wild May stock is significantly lower than both the captive stock and the wild October stock. Levels of both DHA and EPA within the neutral lipid fraction were significantly higher in the wild stocks than the captive stock, however, the level of ARA was twice as high in the captive stock than both wild stocks.

Table 3.3: Polar and Neutral lipid fractions of lipids for the three stocks used in this trial (Wild October, May and Captive) reported in proportional levels. Values indicate Mean (*n*= 10) ± SEM, Different letter superscripts denotes significant differences between stocks.

	WILD OCTOBER		**WILD MAY**		**CAPTIVE**	
Fatty acid	Phospholipid fraction	Neutral lipid fraction	Phospholipid fraction	Neutral lipid fraction	Phospholipid fraction	Neutral lipid fraction
% of Total Lipids	38.44 ± 11.4^{b}	60.77 ± 11.36 ^{ab}	29.42 ± 3.47^{b}	70.07 ± 3.24^{a}	41.76 ± 11.78 ^{a}	56.66 ± 11.61 ^{b}
% of total FA						
14:0	1.18 ± 0.07 ^{a}	1.51 ± 0.13	1.07 ± 0.05 ^{a}	1.81 ± 0.23	0.68 ± 0.06 ^{b}	1.15 ± 0.24
15:0	0.40 ± 0.01 ^{a}	0.64 ±0.08	0.35 ± 0.01 ^{a}	0.94 ± 0.29	0.22 ± 0.05 ^{b}	0.44 ± 0.01
16:0	17.78 ± 0.52 ^{b}	21.38 ± 0.82 ^{b}	16.42 ± 0.25 ^{c}	27.99 ± 2.12 ^{a}	19.47 ± 0.34 ^{a}	24.59 ± 1.26 ^{ab}
18:0	5.35 ± 0.22 ^{b}	4.21 ± 0.23 ^{b}	4.78 ± 0.18 ^{c}	6.09 ± 0.87 ^{a}	7.04 ± 0.15 ^{a}	6.60 ± 0.50 ^{a}
20:0	-	-	-	-	-	-
22:0	-	-	-	-	-	-
24:0	-	-	-	-	-	-
Total saturated	24.73 ± 0.70 ^{b}	27.66 ± 1.04 ^{ab}	22.63 ± 0.35 ^{c}	34.14 ± 2.37 ^{a}	27.36 ± 0.43 ^{a}	26.80 ± 3.99 ^{b}
16:1n-9	0.39 ± 0.02	1.36 ± 0.36 ^{b}	0.35 ± 0.01	10.02 ± 3.09 ^{a}	0.35 ± 0.05	13.17 ± 8.65 ^{a}
16:1n-7	0.80 ± 0.04 ^{b}	0.97 ± 0.0.08 ^{b}	0.84 ± 0.06 ^{b}	1.47 ± 0.26 ^{a}	1.08 ± 0.06 ^{a}	1.30 ± 0.08 ^{a}
18:1n-9	10.65 ± 0.49 ^{b}	10.75 ± 0.66 ^{b}	10.58 ± 0.46 ^{b}	16.61 ± 1.17 ^{a}	14.08 ± 0.23 ^{a}	15.25 ± 0.91 ^{a}
18:1n-7	3.14 ± 0.13 ^{b}	2.55 ± 0.11 ^{b}	3.22 ± 0.14 ^{b}	2.83 ± 0.08 ^{b}	4.35 ± 0.06 ^{a}	3.63 ± 0.03 ^{a}
20:1n-11	0.15 ± 0.04 ^{a}	0.25 ± 0.07	0.06 ± 0.03 ^{ab}	0.14 ± 0.06	0.02 ± 0.02 ^{b}	-
20:1n-9	2.51 ± 0.16 ^{a}	2.04 ± 0.13 ^{a}	2.83 ± 0.21 ^{a}	2.01 ± 0.07 ^{a}	1.13 ± 0.09 ^{b}	1.09 ± 0.16 ^{b}
20:1n-7	0.32 ± 0.03	0.28 ± 0.01	0.33 ± 0.04	0.26 ± 0.01	-	-
22:1n-11	0.40 ± 0.02^{a}	0.43 ± 0.02	0.35 ± 0.06 ^{ab}	0.74 ± 0.21	0.24 ± 0.06 ^{b}	0.55 ± 0.01
22:1n-9	0.28 ± 0.02	0.28 ± 0.01	0.21 ± 0.04	0.28 ± 0.01	-	0.65 ± 0.01
24:1n-9	-	-	-	-	-	-
Total monounsaturated	18.55 ± 0.57 ^{b}	18.01 ± 0.92 ^{b}	18.82 ± 0.41 ^{b}	25.99 ± 2.75 ^{ab}	21.16 ± 0.39 ^{a}	27.06 ± 6.99 ^{a}
18:2n-6	0.85 ± 0.05 ^{b}	1.01 ± 0.07 ^{b}	0.78 ± 0.02 ^{b}	1.33 ± 0.12 ^{b}	11.34 ± 0.52 ^{a}	12.97 ± 0.74 ^{a}
18:3n-6	0.07 ± 0.02	0.18 ± 0.01	-	-	-	-
20:2n-6	0.27 ± 0.06	0.21 ± 0.01	0.14 ±0.04	0.32 ± 0.01	0.21 ± 0.05	0.36 ± 0.02
20:3n-6	-	-	-	-	-	-
20:4n-6 ARA	1.16 ± 0.06 ^{b}	0.59 ± 0.02 ^{b}	1.18 ± 0.0.04 ^{b}	0.61 ±0.01 ^{b}	1.77 ± 0.05 ^{a}	1.04 ± 0.04 ^{a}
22:4n-6	-	-	-	-	-	-
22:5n-6	0.26 ±0.03 ^{b}	0.38 ± 0.04	0.20 ± 0.05 ^{b}	0.44 ± 0.05	0.51 ± 0.02 ^{a}	-
Total n-6 PUFA	2.51 ± 0.14 ^{b}	1.81 ± 0.13 ^{b}	2.29 ± 0.11 ^{b}	0.79 ± 0.34 ^{b}	13.72 ± 0.52 ^{a}	10.23 ± 2.23 ^{a}
18:3n-3	0.48 ± 0.26 ^{b}	0.38 ± 0.01 ^{b}	0.32 ± 0.02 ^{b}	0.49 ± 0.08 ^{b}	0.91 ± 0.03 ^{a}	1.09 ± 0.05 ^{a}

Table 3.3 (Continued): Polar and Neutral lipid fractions of lipids for the three stocks used in this trial (Wild October, May and Captive) reported in proportional levels. Values indicate Mean (*n*= 10) ± SEM, Different letter superscripts denotes significant differences between stocks.

	WILD OCTOBER		**WILD MAY**		**CAPTIVE**	
Fatty acid	Phospholipid fraction	Neutral lipid fraction	Phospholipid fraction	Neutral lipid fraction	Phospholipid fraction	Neutral lipid fraction
18:4n-3	0.52 ± 0.03 ^{a}	0.61 ± 0.03	0.53 ± 0.03 ^{a}	0.71 ± 0.01	0.25 ± 0.05 ^{b}	0.45 ± 0.03
20:3n-3	-	-	-	-	-	-
20:4n-3	0.51 ± 0.02 ^{b}	0.57 ± 0.02	0.54 ± 0.02 ^{ab}	0.64 ± 0.01	0.60 ± 0.04 ^{a}	0.63 ± 0.03
20:5n-3 EPA	17.49 ± 0.48 ^{a}	18.06 ± 0.51^{a}	18.51 ± 0.45 ^{a}	16.45 ± 0.91 ^{a}	11.60 ± 0.35 ^{b}	10.88 ± 0.44 ^{b}
22:5n-3	1.44 ± 0.12 ^{b}	1.53 ± 0.07 ^{a}	1.68 ± 0.05 ^{b}	5.29 ± 2.34^{a}	2.27 ± 0.04 ^{a}	2.13 ± 0.10 ^{b}
22:6n-3 DHA	26.85 ± 0.66 ^{a}	25.63 ± 0.96 ^{a}	28.41 ± 0.43 ^{a}	19.30 ± 1.92 ^{a}	19.87 ± 0.66 ^{b}	16.51 ± 0.79 ^{b}
Total n-3 PUFA	47.26 ± 1.14 ^{a}	46.29 ± 1.51 ^{a}	49.85 ± 0.11 ^{a}	29.62 ± 5.09 ^{b}	35.35 ± 0.79 ^{b}	21.82 ± 4.73 ^{b}
16:2	0.60 ± 0.05 ^{a}	0.52 ± 0.02	0.67 ± 0.05 ^{a}	0.54 ± 0.01	0.46 ± 0.04 ^{b}	0.52 ± 0.01
16:3	0.11 ± 0.03	0.20 ± 0.01	0.06 ± 0.03	0.22 ± 0.01	0.05 ± 0.03	-
16:4	-	-	-	-	-	-
Total PUFA	49.77 ± 3.78 ^{ab}	48.125± 4.83 ^{a}	52.14 ± 1.15 ^{a}	30.407±16.89 ^{b}	49.07 ± 2.15 ^{b}	35.608±16.01 ^{b}
ARA:EPA	0.066 ± 0.001 ^{b}	0.026±0.018 ^{b}	0.063 ± 0.002 ^{b}	0.015±0.017 ^{b}	0.152± 0.003 ^{a}	0.086±0.041 ^{a}
DHA:EPA	1.53 ± 0.03 ^{b}	1.420 ±0.15 ^{a}	1.53 ± 0.05 ^{b}	1.092 ±0.68 ^{b}	1.712± 0.07 ^{a}	1.528 ±0.55 ^{a}
N-3:N-6	18.78 ± 0.96 ^{b}	26.454±8.23 ^{a}	21.72 ± 1.25 ^{a}	23.952±13.34 ^{a}	2.57± 0.14 ^{c}	2.184±5.60 ^{b}

3.3.2.3 Variation in egg lipid classes

There was no significant difference in any of the identified polar lipid classes (Table 3.4). However, there was a significantly higher level of total polar lipids in the captive stocks when compared to the wild May stocks, but there was no significant difference between the two stocks and the wild October stock.

Within the neutral fraction there was a higher level of pigmented material in the wild October stock than in both the wild May and the captive stocks. There were twice the levels of sterol or waxy esters within the captive stock than both wild stocks. The wild May stock had significantly higher levels of triaclyglycerol than both wild October and captive stock. Levels of total neutral lipids was significantly higher in the wild may stock than those in the captive stocks, however, neither stocks differed significantly from the wild October stock. The ratio of polar to neutral lipid fractions as determined through lipid class (section 2.8) was significantly higher in the wild May stock than those in both the wild October and captive

Table 3.4: Lipid Class data for the three stocks used in this trial (Wild October, May and Captive) reported in proportional levels. Values indicate Mean (*n*= 10) ± SEM, Different letter superscripts denotes significant differences between stocks.

Lipid classes (%)	Wild October	Wild May	Captive
Lysophosphatidylcholine	4.30 ± 0.87	3.31 ± 1.21	2.02 ± 0.29
Sphingomyelin	9.26 ± 1.26	6.39 ± 1.31	8.81 ± 1.09
Phosphatidylcholines	6.45 ± 1.44	5.16 ± 1.31	9.86 ± 1.89
Phosphatidylserine	2.84 ± 0.35	2.68 ± 0.67	6.01 ± 1.76
Phosphatidylinositol	4.62 ± 0.52	2.87 ± 0.82	4.23 ± 0.57
Phosphatidylethanolamines	5.51 ± 0.90	4.72 ± 0.35	5.75 ± 0.63
Glycolipids	5.47 ± 0.61	4.30 ± 0.41	5.07 ± 0.53
Total Polar Lipids	38.44 ± 11.4^{ab}	29.42 ± 3.47 ^{b}	41.76 ± 11.78 ^{a}
Pigmented Material	10.49 ± 0.86 ^{a}	6.58 ± 1.23 ^{b}	7.55 ± 0.82 ^{b}
Diacylglycerol	11.67 ± 0.83	11.10 ± 0.51	10.21 ± 1.38
Sterol/ Wax Esters	7.31 ± 1.25^{ab}	6.66 ± 1.19 ^{b}	12.22 ± 1.72 ^{a}
Free Fatty Acids	20.63 ± 2.50	26.68 ± 4.27	19.43 ± 2.59
Triaclyglycerol	10.66 ± 0.99 ^{b}	19.05 ± 3.47 ^{a}	7.26 ± 1.38 ^{b}
Total Neutral Lipids	60.77 ± 11.36 ^{ab}	70.07 ± 3.24 ^{a}	56.66 ± 11.61 ^{b}
Neutral: Polar ratio.	1.77 ± 0.69 ^{b}	2.42 ± 0.35 ^{a}	1.57 ± 0.84 ^{b}

3.3.2.4 Variation in mineral composition

Mineral levels within the captive stocks were consistently different to those in the wild stocks, with few differences between wild stocks (Table 3.5). The levels of major minerals within the captive stocks were significantly lower than the two wild stocks. With levels of sodium and potassium were higher in the wild may stock than the captive stock, but there was no significant difference between the two stocks and those in the wild October stock. The levels of magnesium were significantly higher in the wild May stocks than those in the wild October and captive stocks. The two wild stocks were significantly higher in Phosphorus and Calcium than the captive stocks.

Within the minor minerals there were less obvious difference between the three stocks. The two wild stocks were significantly higher in Cobalt than the captive stocks. Levels of manganese, nickel and copper were significantly higher in the captive stock than those in the wild stocks. And there was no significant difference between levels of chromium, iron, vanadium, zinc, and selenium between the three stocks.

Table 3.5; Egg mineral levels for the three stocks used in this trial (Wild October, May and Captive) reported as µg per gram of egg. Values indicate Mean (*n*= 10) ± SEM, Different letter superscripts denotes significant differences between stocks.

Minerals µg / g	Wild October	Wild May	Captive
Sodium	2086.41 ± 114.07 [a b]	2496.08 ± 269.74 [a]	1915.09 ± 126.11 [b]
Magnesium	35.39 ± 1.89 [b]	58.59 ± 14.03 [a]	25.36 ± 3.97 [b]
Phosphorus	1164.78 ± 89.14 [a]	1244.23 ± 99.54 [a]	920.06 ± 49.06 [b]
Potassium	2087.47 ± 69.65 [a b]	2243.84 ± 125.19 [a]	1867.96 ± 127.89 [b]
Calcium	185.61 ± 9.34 [a]	217.89 ± 17.27 [a]	147.93 ± 8.83 [b]
Vanadium	0.077 ± 0.01	0.0598 ± 0.01	0.0831 ± 0.01
Chromium	0.0791 ± 0.01	0.149 ± 0.04	0.066 ± 0.05
Manganese	0.285 ± 0.01 [b]	0.249 ± 0.01 [b]	0.365 ± 0.04 [a]
Iron	3.955 ± 0.02	4.4 ± 0.46	4.06 ± 0.44
Cobalt	0.0066 ± 0.0001 [a]	0.0069 ± 0.0001 [a]	0.0044 ± 0.001 [b]
Nickle	0.2208 ± 0.05 [a]	0.274 ± 0.06 [a]	0.0603 ± 0.04 [b]
Copper	0.7008 ± 0.8 [a]	1.004 ± 0.16 [a]	0.37 ± 0.13 [b]
Zinc	12.357 ± 0.91	12.87 ± 1.23	11.05 ± 0.73
Selenium	0.438 ± 0.02	0.412 ± 0.03	0.386 ± 0.01

3.3.2.5 Identification of lumpfish egg pigments and compositional changes

There were five pigments identified within lumpfish eggs at the point of stripping which were consistently present in all sample sources being; canthaxanthin, astacene, astaxanthin, echinenone and leutin (Figure 3.2). β-carotene was identified in the developmental time series egg samples but was not identified in the samples post stripping (Section 3.3.4.3) . Total pigment levels in the wild samples were 3-fold higher than observed in the captive samples being measured at 12.34 ± 5.88 mg kg^{-1} for wild October and 11.07 ± 2.74 mg kg^{-1} for the wild May samples compared to 4.20 ± 0.84 mg kg^{-1} for the captive sample (Table 3.6). Of the five observed pigments only astacene had levels which were significantly different between stocks being significantly lower in the captive sample compared to the wild May sample with the wild October sample being comparable to both (Table 3.6).

Table 3.6. Total Pigment and pigment levels for the three stocks used in this trial (Wild October, May and Captive) reported as mg per kg of egg. Values indicate Mean (*n*= 10) ± SEM, Different letter superscripts denotes significant differences between stocks.

Pigment	Wild October	Wild May	Captive
Echinenone	1.47 ± 0.97	0.39 ± 0.23	0.08 ± 0.02
Canthaxanthin	2.52 ± 1.26	3.84 ± 1.57	0.64 ± 0.17
Astacene	5.20 ± 1.33 ^{a}	4.68 ± 1.65 ^{ab}	1.14 ± 0.3 ^{b}
Astxanthin	0.67 ± 0.1	0.47 ± 0.14	0.27 ± 0.03
Leutin	1.01 ± 0.55	0.64 ± 0.19	1.82 ± 0.44
Total Pigment	12.34 ± 5.88 ^{a}	11.07 ± 2.74 ^{a}	4.20 ± 0.84 ^{b}

3.3.3 Associationwith egg proximate composition and quality criteria.

A principal component analysis of the full nutritional dataset including 139 total possible explanatory variables and using survival to hatch, referred to as “hatching rate” hereafter, as the principal indicator of egg quality, resolved within 23 components explaining 97 % of the total variance in the dataset. Component 1 had the single greatest contribution explaining 32 % of data variance with components 2-5 explaining between 10-5 % of data variance individually with all other components explaining <5 % to the total data variance thereafter. Hatching rate had a meaningful positive loading (0.660) within component 1 along with this there were a total of 37 nutritional factors (26 % of all measured factors) identified as having meaningful positive loadings contributing to the first component. Similarly, 25 nutritional components (17.9 % of the total analysed factors) were identified as having meaningful negative loadings contributing to the first component (Figure 3.3, 3.4, 3.5).

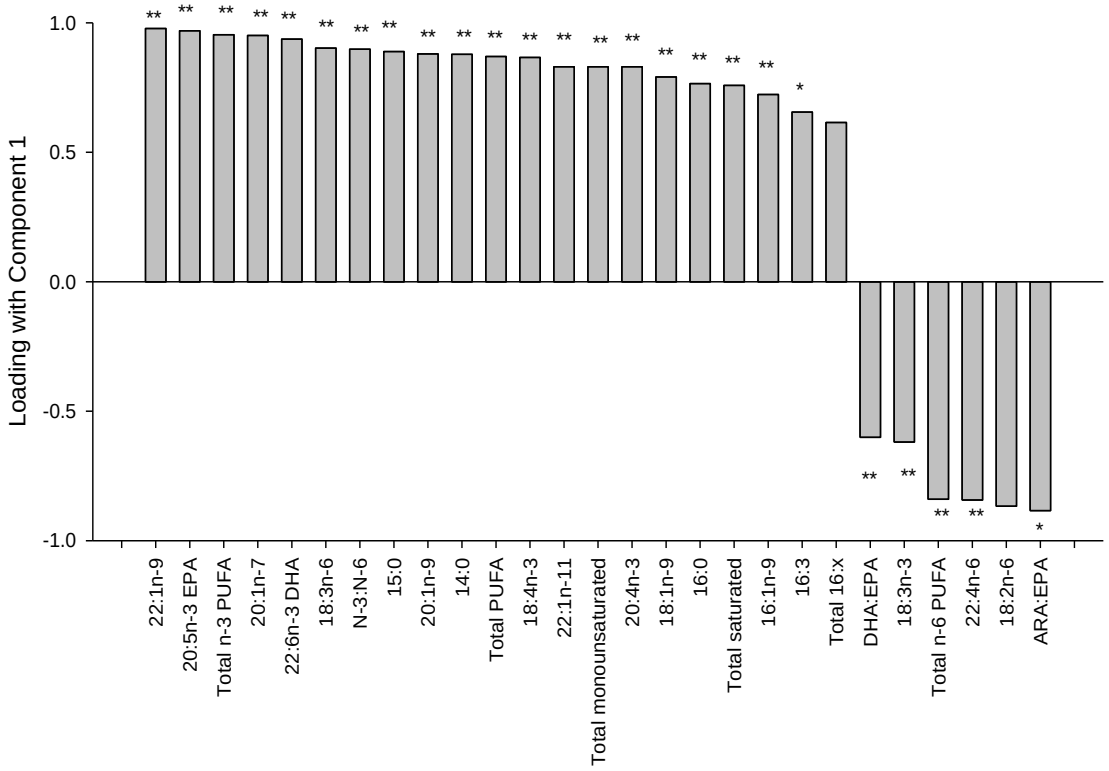

Figure 3.3: Results from the principal component analysis for fatty acids. Values indicate loading against component 1, loading most strongly associated with egg quality, only compounds which displayed meaningful loadings are displayed. ** indicates regression significance ($p < 0.01$), * Indicates regression significance ($p < 0.05$).

3.3.3.1 Association of egg lipid composition and quality criteria

Fatty acids made up the majority (27 of 61 identified significant factors) of identified contributing factors within the first component of the PCA. Fatty acids from the total lipid fraction made up the majority of positive loadings (21/37) while a small number contributed to negative loadings (6/25) (Figure 3.3). The polar fractions made up a smaller number of positive loadings than the total lipid group (8/37), these eight factors, EPA, Total n-3, 20:1n-7, DHA, 20:1n-9, 22:1n-9 and 18:4n-3 were all also identified as having positive associations within the total lipid fraction. Negative loadings in the phospholipoid fraction constituted the majority of meaningful negative loadings (12/35) (Figure 3.4). For the neutral lipid fraction no components reached the positive meaningful threshold, however, five reached the negative meaningful threshold. Of the five identified factors, three also displayed negative loadings within the total lipid fractions, 18:3 N-3, total N-6 PUFA, 18:2 N-6. Within the lipid classes dataset there was two meaningful positive loadings (polar: neutral ratio, total neutral) and two meaningful negative loadings (sterol esters, phosphatidylcholines) associated within the first component (Figure 3.5).

Of the 61 factors identified as having meaningful associations with the first component, 49 had significant linear relationships with hatch rate (Figures 3.4 and 3.5). With respect to the identified fatty acids with the total, phospholipid and neutral fractions, goodness of fit of the linear regressions ranged between 11.1 % and 57.3 % for hatching rate (Table 3.7, Figures 3.5-3.7). The observed r^2 value increased in relationship to the proportion of loading reported within the PCA analysis (Table 3.7). The linear relationship between hatching rate and lipid classes was generally poor ranging between 0.7 % and 9.4 %.

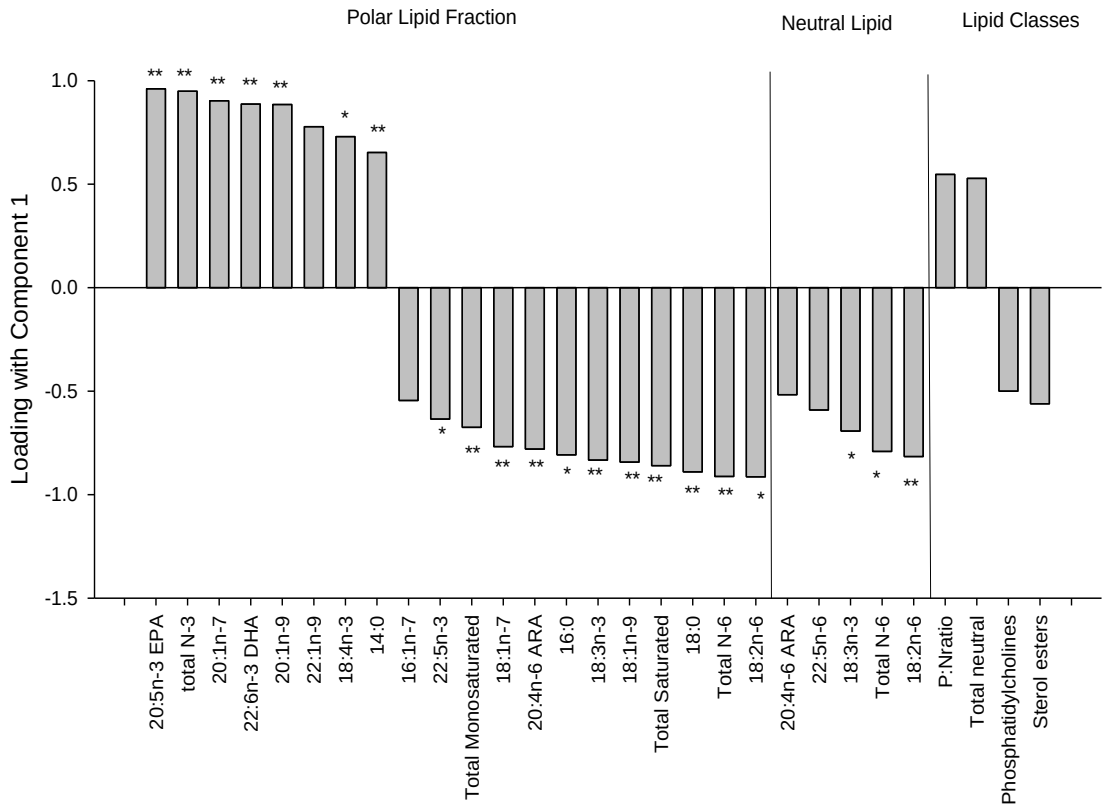

Figure 3.4: Results from the principal component analysis for polar lipid, neutral lipids and lipid classes. Values indicate loading against component 1, loading most strongly associated with egg quality, only compounds which displayed meaningful loadings are displayed. ** indicates regression significance ($p < 0.01$), * Indicates regression significance ($p < 0.05$).

Table 3.7: R-Squared values and loadings within the first component of the principal component analysis for all significant relationships between nutritional component and hatching rate. * indicates P<0.05 ** indicates P<0.01

FREE FATTY ACIDS	LOADING	R^2	PHOSPHOLIPID	LOADING	R^2	NEUTRAL LIPID	LOADING	R^2
22:1n-9	0.978	45.6**	20:5n-3 EPA	0.961	36.5**	18:3n-3	-0.693	16.9*
20:5n-3 EPA	0.969	41.8**	total N-3	0.949	33.1**	Total N-6	-0.791	17.4*
Total n-3 PUFA	0.954	39.9**	20:1n-7	0.903	35.9**	18:2n-6	-0.816	17.5**
20:1n-7	0.951	41.5**	22:6n-3 DHA	0.887	28.2**	**Minerals**		
22:6n-3 DHA	0.937	37.3**	20:1n-9	0.885	44.8	Nickel	0.692	15*
18:3n-6	0.902	40.7**	18:4n-3	0.73	19.6*	Calcium	0.582	15.3*
N-3:N-6	0.898	38.2**	14:0	0.653	32.3**	**Pigment**		
15:0	0.889	41.6**	22:5n-3	-0.635	16.3*	total pigment	0.575	16.7*
20:1n-9	0.88	43**	Total Monosaturated	-0.675	40.8**			
14:0	0.878	42.1**	18:1n-7	-0.768	28**			
Total PUFA	0.87	34.1**	20:4n-6 ARA	-0.78	39.1**			
18:4n-3	0.866	52**	16:00	-0.808	18.8*			
22:1n-11	0.83	39.1**	18:3n-3	-0.833	36**			
Total monounsaturated	0.83	32.1**	18:1n-9	-0.843	57.3**			
20:4n-3	0.83	35.2**	Total Saturated	-0.86	22.7**			
18:1n-9	0.791	27.1**	18:00	-0.89	34.1**			
16:0	0.765	30.7**	Total N-6	-0.912	35.6**			
Total saturated	0.758	26.8**	18:2n-6	-0.914	35.2**			
16:1n-9	0.723	32.6**						
16;3	0.655	19.7*						
DHA:EPA	-0.601	18.9**						
18:3n-3	-0.619	11.1**						
Total n-6 PUFA	-0.84	27.9**						
22:4n-6	-0.843	30.4**						
18:2n-6	-0.867	28.7						
ARA:EPA	-0.884	45.3*						

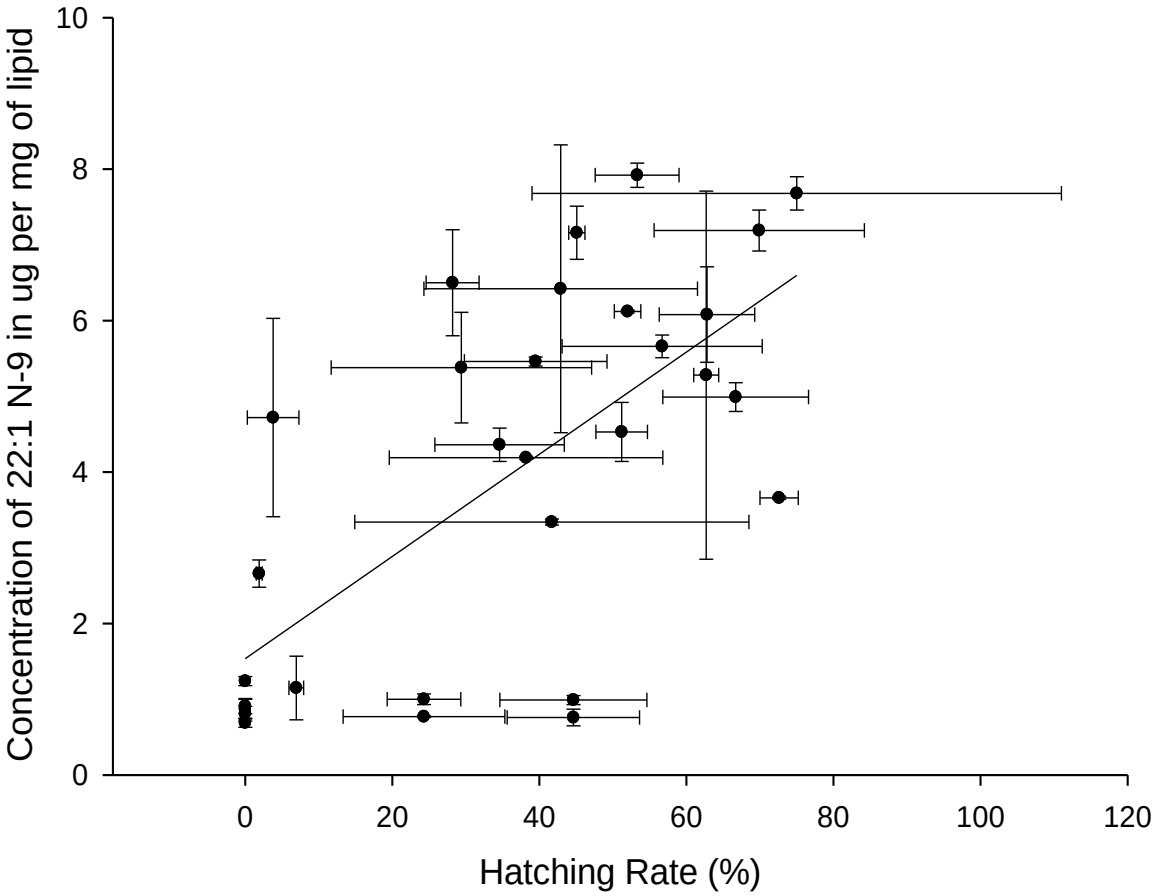

Figure 3.5: Linear relationship between 22: 1 n-9 and hatching rate (*n*= 30) ($p < 0.01$, $r^2 = 45.6$). Solid line represents line of best fit, data points represent mean values for each female ± SEM (*n*= 3).

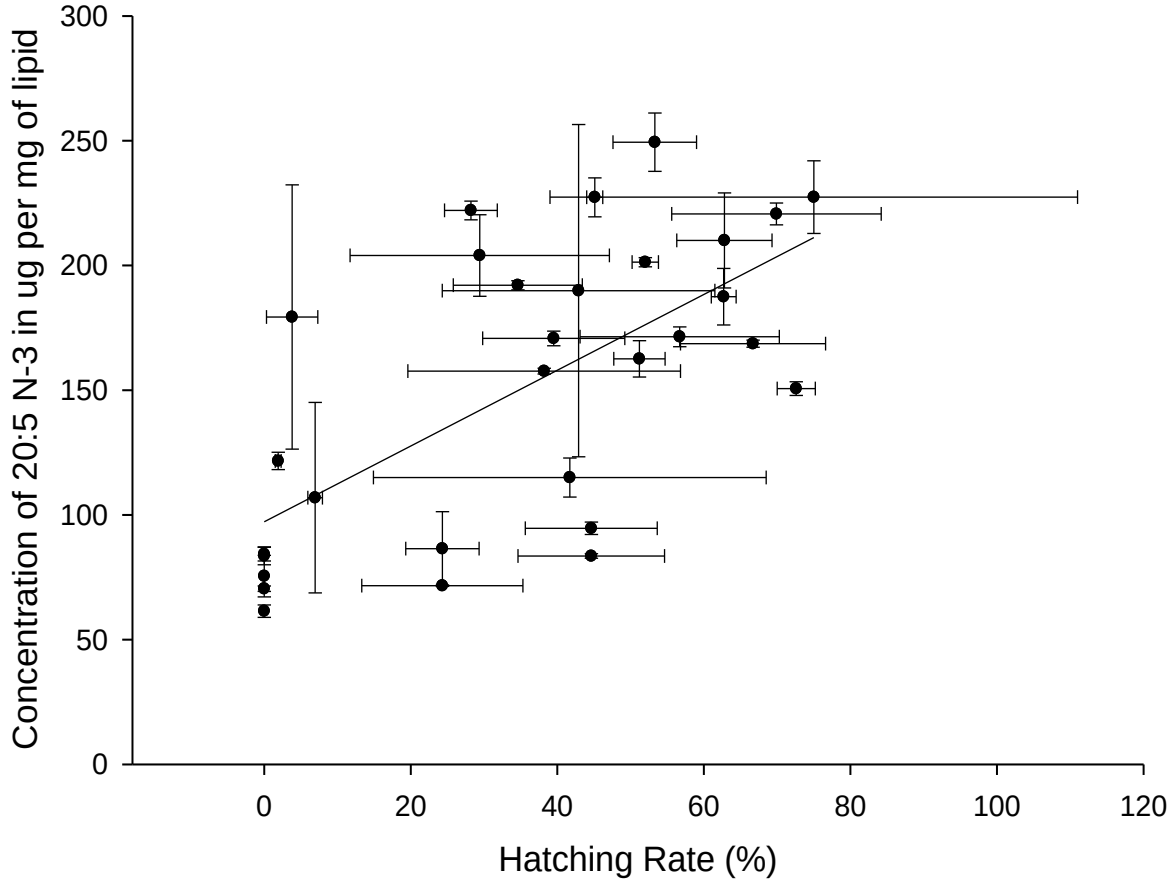

Figure 3.6: Linear relationship between 20: 5 n-3 and hatching rate (*n*= 30) (p <0.01, r^2 = 41.8). Solid line represents line of best fit, data points represent mean values for each female ± SEM (*n*= 3).

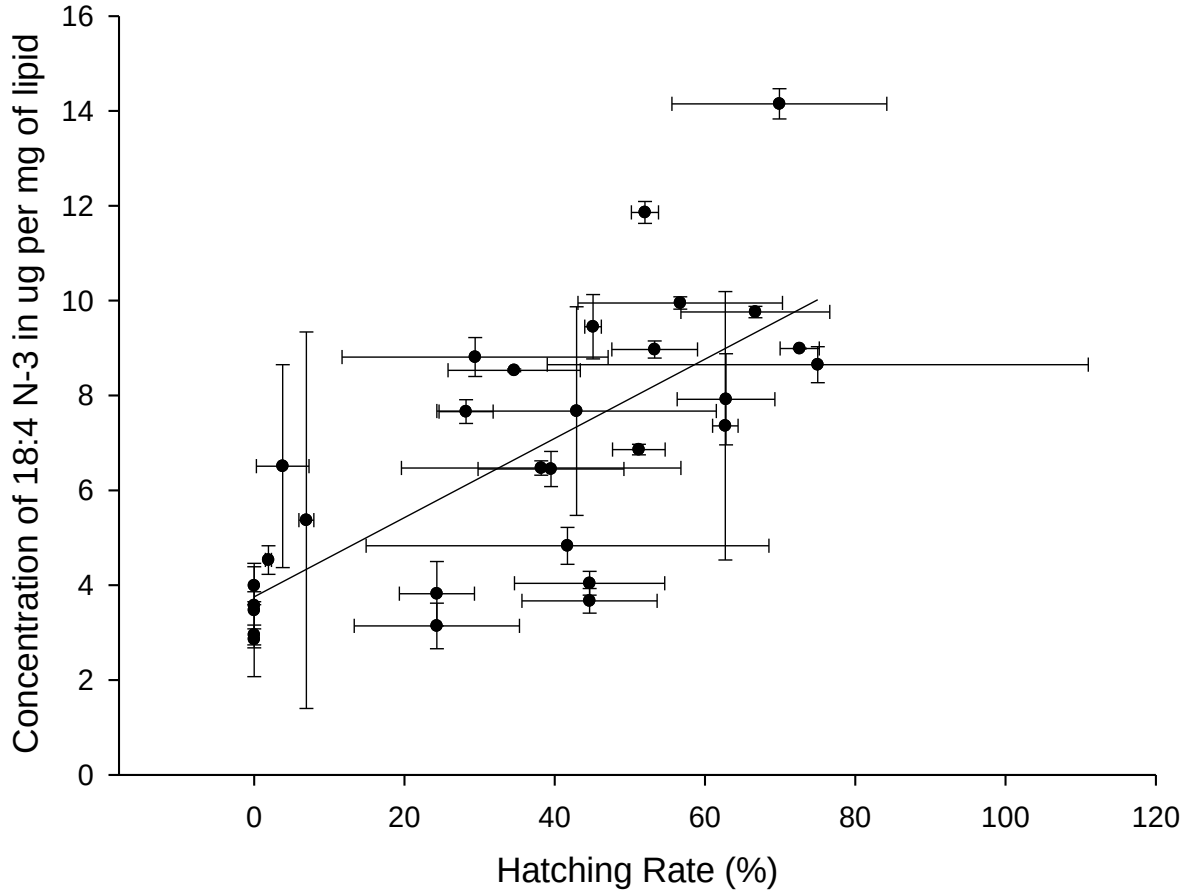

Figure 3.7: Linear relationship between 18:4 n-3 and hatching rate (*n*= 30) ($p < 0.01$, $r^2 = 52.0$). Solid line represents line of best fit, data points represent mean values for each female ± SEM (*n*= 3).

3.3.3.2 Association of egg mineral composition and quality criteria

Of the 14 minerals analysed only four, nickel, copper, calcium and cobalt, were identified as associating with hatch rate, with all four showing a positive association (Figure 3.8). Loading associated with minerals was generally lower than that observed for the fatty acids with the highest loading mineral (nickel) being in the lowest 30 % of contributing factors (27th of 37 positive factors). Of the four identified minerals, only nickel and calcium had significant linear relationships with hatch rate, however, the

goodness of fit was low at 15 % and 15.7 % for nickel and calcium respectively (Table 3.7).

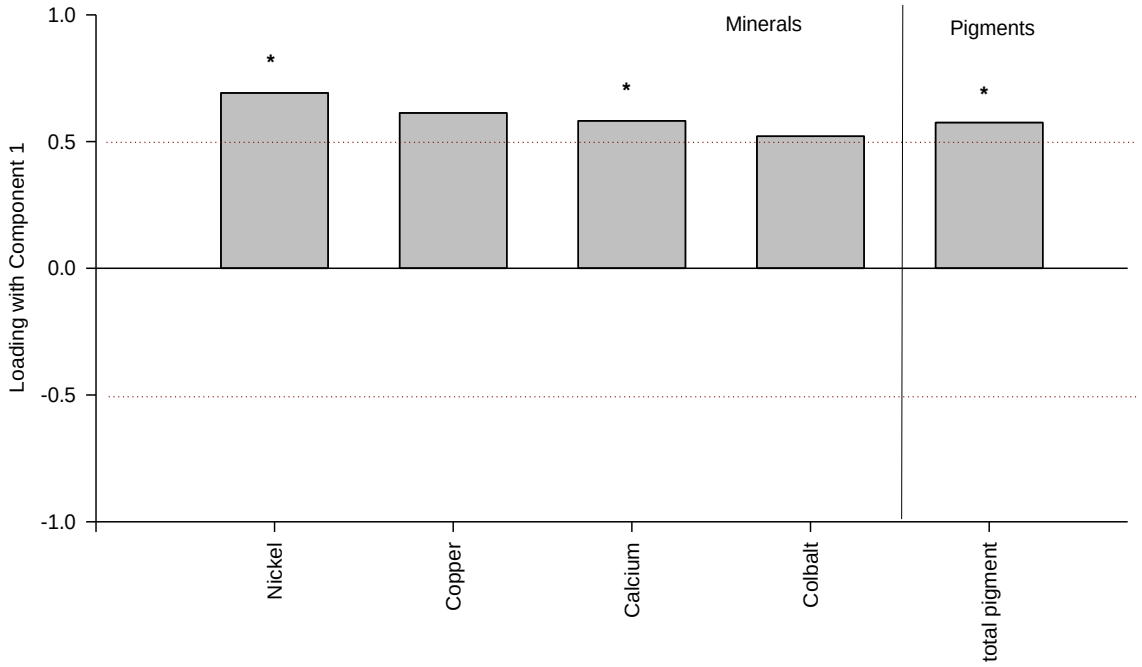

Figure 3.8: Results from the principal component analysis for minerals and pigments. Values indicate loading against component 1, loading most strongly associated with egg quality, only compounds which displayed meaningful loadings are displayed. ** indicates regression significance ($p < 0.01$), * Indicates regression significance ($p < 0.05$).

3.3.3.3 Associations between egg pigment compositions and egg quality

Within the pigments, Total pigment level displayed the only meaningful loading (positive) with the first component, associated with hatching rate. Regression of total pigment levels displayed a significant but low positive linear relationship between concentration and hatching rate (16.7 %) (Table 3.7).

3.3.4 Changes in proximate composition in relation to embryonic development study

In order to investigate the change in egg proximate composition in relation to embryonic development, eight wild batches (four from October and four from May) were selected for time series analysis based on being the batches with highest recorded hatching rates (>50 %). As stated in section (3.3.1) there was no significant difference in the morphometric data for the selected batches parent broodstock compared with the remainder of the wild populations. The fertilisation (98.0 ± 0.13 %), eyeing rates (88.28 ± 1.79 %) and hatching rate (63.08 ± 4.91 %), for the selected batches were not statistically different from the observed means of the two wild populations.

3.3.4.1 Changes in egg FA composition during embryonic development

There was no significant difference in total lipid content at any time point through development. There was a significant reduction in total saturates (28 %), total monosaturates (33 %), and total PUFA (40 %) throughout embryonic development between the point of stripping and hatched larvae (Table 3.8). Within the total PUFAs there was significant reductions in both n-3 (41 %) and n-6 (29 %) between the 0 DD and hatched larvae. There was no significant change in the n-3: n-6 ratio and DHA: EPA ratio over development. However, there was a significant increase in the ARA:EPA ratio between developmental stages and non-fed hatched juveniles. This change is driven by the significant reduction in EPA levels over development. Within the total saturates there were significant reductions in 16:0 (36 %) and 18:0 (20 %) components between the point of stripping and 60DD post fertilisation with no significant change after this point. Levels of 18:0 in hatched juveniles was not

significantly different than 0DD. There were significant reductions in the levels of all identified monounsaturates between the point of stripping and 60DD except 16:1 n-9 where levels were not significantly different over development. There was no significant change in development between 60DD and hatched juveniles. The largest reductions were exhibited in 22:1n-9 (64 %) and 16:1n-7 (58 %). There was a significant reduction in levels of all n-3 PUFA's between the point of stripping and 24hr post fertilisation, except 20:3 n-3 which showed no significant change throughout development. There was also a further significant reduction in the levels of 20:4 n-3, and 18:4 n-3 between 60DD and hatched larvae. There was a significant reduction in 18:2 n-6 and 20:2 n-6 within the n-6 PUFA's between the point of stripping and hatching. With no significant reduction in ARA (20:4 n-6) at any time point. There was also a significant reduction in 16:3 Fatty acid between stripping and 24hr post fertilisation.

Table 3.8: Developmental changes in fatty acid levels for wild lumpfish eggs (*n*= 8) measured at the point of stripping, 24hr, 60DD 150DD, 200DD and Hatched juveniles. Levels are reported at level of fatty acid in µg per mg of lipid. Values are displayed in means ± SEM, differing superscript denotes significance.

FATTY ACID µG/MG LIPID	AT STRIPPING	24HR (8 DD)	60DD	150DD	200DD	HATCHING
14:0	11.17 ± 0.82 ^{a}	10.81 ± 0.71 ^{a}	10.52 ± 0.47 ^{a}	10.38 ± 0.81 ^{a}	9.90 ± 0.46 ^{ab}	7.40 ± 0.38 ^{b}
15:0	3.45 ± 0.19 ^{a}	3.16 ± 0.34 ^{ab}	2.91 ± 0.11 ^{ab}	2.99 ± 0.31 ^{ab}	2.82 ± 0.12 ^{ab}	2.29 ± 0.09 ^{b}
16:0	162.50 ± 7.85 ^{a}	110.89 ± 3.16^{b}	109.98 ± 2.83 ^{b}	105.46 ± 5.28 ^{b}	105.65 ± 4.89 ^{b}	102.42 ± 4.26 ^{b}
18:0	42.35 ± 4.67 ^{a}	32.17 ± 1.83 ^{ab}	30.37 ± 1.72 ^{b}	29.81 ± 2.33 ^{b}	29.94 ± 2.19 ^{b}	33.57 ± 2.41 ^{ab}
20:0	0.70 ± 0.05	0.54 ± 0.16	0.48 ± 0.12	0.52 ± 0.14	0.69 ± 0.12	0.93 ± 0.29
22:0	-	-	-	-	-	0.74 ± 0.74
24:0	0.03 ± 0.03	-	-	0.02 ± 0.02	-	0.02 ± 0.02
Total saturated	220.20 ± 10.33 ^{a}	157.57 ± 3.35 ^{b}	154.28 ± 3.96 ^{b}	149.17 ± 7.70 ^{b}	149.00± 7.04 ^{b}	147.37 ± 6.58 ^{b}
16:1n-9	5.57 ± 0.64	5.87 ± 1.15	6.19 ± 1.11	5.66 ± 1.28	6.33 ± 1.25	5.63 ± 1.00
16:1n-7	14.43 ± 0.75 ^{a}	8.13 ± 1.79 ^{ab}	6.95 ± 1.83 ^{b}	6.86 ± 1.78 ^{b}	6.83 ± 1.62 ^{b}	5.97 ± 1.46 ^{b}
18:1n-9	198.01 ± 11.15 ^{a}	127.72 ± 8.43 ^{b}	129.42 ± 4.85 ^{b}	123.29 ± 6.28 ^{b}	124.25 ± 6.13 ^{b}	117.19 ± 7.09 ^{b}
18:1n-7	33.00 ± 2.95 ^{a}	24.74 ± 1.87 ^{b}	25.07 ± 1.45 ^{b}	24.16 ± 1.27 ^{b}	23.35 ± 1.55 ^{b}	23.41 ± 1.46 ^{b}
20:1n-11	0.45 ± 0.45 ^{a}	3.35 ± 0.50 ^{b}	2.62 ± 0.32 ^{b}	2.78 ± 0.24 ^{b}	3.28 ± 0.30 ^{b}	2.65 ± 0.34 ^{b}
20:1n-9	39.67 ± 4.34 ^{a}	20.59 ± 1.99 ^{b}	20.86 ± 1.79 ^{b}	19.64 ± 1.77 ^{b}	19.75 ± 1.65 ^{b}	19.29 ± 1.63 ^{b}
20:1n-7	4.90 ± 0.44 ^{a}	3.25 ± 0.19 ^{b}	3.20 ± 0.15 ^{b}	3.04 ± 0.24 ^{b}	3.13 ± 0.18 ^{b}	2.87 ± 0.20 ^{b}
22:1n-11	8.52 ± 1.00 ^{a}	4.69 ± 0.53 ^{b}	4.67 ± 0.57 ^{b}	4.43 ±0.53 ^{b}	4.40 ± 0.55 ^{b}	4.17 ± 0.50 ^{b}
22:1n-9	5.71 ± 0.49 ^{a}	2.53 ± 0.58 ^{b}	2.34 ± 0.62 ^{b}	1.99 ± 0.63 ^{b}	2.01 ± 0.62 ^{b}	2.05 ± 0.62 ^{b}
24:1n-9	-	-	-	-	-	-
Total monounsaturated	310.25 ± 16.62 ^{a}	200.88 ± 12.85 ^{b}	201.31 ± 7.68 ^{b}	191.85 ± 9.52 ^{b}	193.32 ± 8.42 ^{b}	183.22 ± 9.12 ^{b}
18:2n-6	12.92 ± 0.81^{a}	8.31± 0.54 ^{b}	8.48 ± 0.29 ^{b}	7.99 ± 0.51 ^{b}	8.01 ± 0.50 ^{b}	7.20 ± 0.38 ^{b}
18:3n-6	1.20 ± 0.07	1.05 ± 0.11	1.06 ± 0.22	1.18 ± 0.31	1.00 ± 0.0.17	0.95 ± 0.21
20:2n-6	2.82 ± 0.26 ^{a}	1.93 ± 0.11 ^{b}	1.80 ± 0.08 ^{b}	1.75 ± 0.13 ^{b}	1.76 ± 0.0.09 ^{b}	1.68 ± 0.09 ^{b}
20:3n-6	0.87± 0.07	0.59 ± 0.12	0.49 ± 0.13	0.60 ± 0.18	0.61 ± 0.17	0.58 ± 0.17
20:4n-6 ARA	7.26 ± 1.15	6.38 ± 0.46	6.07 ± 0.45	5.89 ± 0.52	6.10 ± 0.42	7.51 ± 0.61
22:4n-6	0.13 ± 0.08	0.14 ± 0.03	0.03 ± 0.03	0.03 ± 0.03	0.14 ± 0.07	0.07 ± 0.04
22:5n-6	3.01 ± 0.30	2.26 ± 0.18	2.13 ± 0.15	2.48 ± 0.65	2.18 ± 0.26	2.03 ± 0.18
Total n-6 PUFA	28.23 ± 1.77 ^{a}	20.67 ± 0.92 ^{b}	20.05 ± 0.68 ^{b}	19.92 ± 1.66 ^{b}	19.80 ± 1.13 ^{b}	20.03 ± 1.16 ^{b}
18:3n-3	6.26 ± 0.62 ^{a}	4.03 ± 0.16 ^{b}	3.77 ± 0.18 ^{b}	3.60 ± 0.29 ^{b}	3.69 ± 0.34 ^{b}	3.10 ± 0.30 ^{b}
18:4n-3	9.13 ± 0.77 ^{a}	6.47± 0.44 ^{b}	5.52 ± 0.25 ^{bc}	5.42 ± 0.63 ^{bc}	4.96 ± 0.25 ^{bc}	4.40 ± 0.21 ^{c}
20:3n-3	1.31 ± 0.14	0.76 ± 0.06	0.72 ± 0.07	1.38 ± 0.76	0.71 ± 0.09	0.53 ± 0.12
20:4n-3	10.34 ± 0.78 ^{a}	7.13 ± 0.53 ^{b}	6.03 ± 0.20 ^{bc}	5.68 ± 0.22 ^{bc}	5.64 ± 0.30 ^{bc}	5.10 ± 0.27 ^{c}
20:5n-3 EPA	193.34 ± 9.13 ^{a}	128.27 ± 5.96 ^{b}	120.66 ± 3.64 ^{b}	114.08 ± 5.26 ^{b}	114.57 ± 4.62 ^{b}	112.60 ± 4.85 ^{b}
22:5n-3	14.82 ± 1.33 ^{a}	10.42 ± 0.65 ^{b}	9.59 ± 0.32 ^{b}	9.42 ± 0.64 ^{b}	9.19 ± 0.43 ^{b}	9.02 ± 0.32 ^{b}

Table 3.8 (Continued): Developmental changes in fatty acid levels for wild lumpfish eggs (*n*= 8) measured at the point of stripping, 24hr, 60DD 150DD, 200DD and Hatched juveniles. Levels are reported at level of fatty acid in µg per mg of lipid. Values are displayed in means ± SEM, differing superscript denotes significance.

FATTY ACID UG/MG LIPID	AT STRIPPING	24HR (8 DD)	60DD	150DD	200DD	HATCHING
22:6n-3 DHA	310.78 ± 18.08[a]	206.19 ± 9.50 [b]	193.12 ± 6.63 [b]	181.60 ± 7.61 [b]	181.98 ± 7.50 [b]	186.03 ± 8.71 [b]
Total n-3 PUFA	545.98 ± 28.32[a]	363.26 ± 15.52 [b]	339.41 ± 10.01 [b]	321.18 ± 13.58 [b]	320.73 ± 12.56 [b]	320.77 ± 13.78 [b]
16:2	4.36 ± 0.71	4.68 ± 0.47	5.01 ± 0.55	4.96 ± 0.56	5.06 ± 0.43	5.38 ± 0.45
16:3	3.86 ± 0.17 [a]	2.30 ± 0.32 [b]	2.47 ± 0.38 [b]	2.35 ± 0.39 [b]	2.22 ± 0.31 [b]	2.13 ± 0.22 [b]
16:4	0.59 ± 0.45	0.34 ± 0.19	0.40 ± 0.17	0.65 ± 0.27	0.63 ± 0.30	0.75 ± 0.27
Total PUFA	583.02 ± 29.92 [a]	391.24 ± 16.10 [b]	367.34 ± 10.53 [b]	349.06 ± 15.66 [b]	348.44 ± 13.64 [b]	349.06 ± 14.76 [b]
ARA:EPA	0.04 ± 0.01 [a]	0.05 ± 0.01 [a]	0.05 ± 0.01 [a]	0.05 ± 0.01 [a]	0.05 ± 0.01 [a]	0.07± 0.01 [b]
DHA:EPA	1.61 ± 0.05	1.61 ± 0.03	1.60 ± 0.03	1.60 ± 0.03	1.59 ± 0.03	1.65 ± 0.03
N-3:N-6	19.54 ± 0.85	17.80 ± 0.69	17.04 ± 0.71	16.77 ± 0.89	16.56 ± 0.79	16.26 ± 0.88

3.3.4.2 Changes in egg mineral composition during embryonic development

Changes in the composition in minerals showed significant changes in all major minerals between stripping and 200DD except for phosphorus which was significantly higher in larvae compared to all other developmental stages (Table 3.9). Sodium increased between stripping, early development (24hr to 60DD) and later development (150DD, 200DD), with a significant decrease in levels within hatched juveniles and the later developmental stages (150DD, 200DD). Magnesium increased 16 fold between stripping, early development (24hr to 60DD) magnesium levels at 24hr was significantly lower than levels at 150DD with both 24hr and 60DD were significantly lower than those in 200 DD, with a significant decrease in levels within hatched juveniles to levels comparable to levels reported in development (24hr, 60 DD and 150 DD). Levels of potassium at stripping and early development are twice as high as those in later development (150 DD, 200DD) with levels at hatching comparable to those in early development. Levels for Calcium were 6 times higher at 200DD than levels at the point of stripping. Calcium levels during development were not significantly different, however levels at hatching were 10 time higher than at the point of stripping and almost twice as high as levels during development.

For the minor minerals there is no significant changes through development for Nickle, Copper, Zinc, Chromium and Selenium. There are significant increases in Iron, Cobalt and Vanadium where levels increase throughout development. Vanadium and Cobalt display significant increases between early (24hr to 150DD) and late development (200DD). Iron levels were 25 time higher in hatched juveniles than at the point of stripping. Iron levels were significantly lower in early development (24hr, 60 DD) and hatched juveniles only, with no significant difference between and point in development.

Table 3.9: Developmental changes in mineral levels for wild lumpfish eggs measured at the point of stripping, 24hr, 60DD 150DD, 200DD and Hatched juveniles. Levels are reported as µg per gram of egg. Values are displayed in means ± SEM, differing superscript denotes significance.

	Stripping	24hr (8 DD)	60DD	150DD	200DD	Hatch
Sodium	2158.79 ± 164.93 ^{c}	3900.94 ± 306.38 ^{b}	4533.66 ± 215.15 ^{b}	5611.96 ± 306.52 ^{a}	5584.14 ± 324.74 ^{a}	4667.63 ± 458.9 ^{b}
Magnesium	37.65 ± 2.87 ^{d}	621.15 ± 45.03 ^{c}	694.50 ± 32.33 ^{bc}	836.49 ± 40.83 ^{ab}	948.22 ± 160.84 ^{a}	684.16 ± 55.11 ^{bc}
Phosphorus	1320.60 ± 94.25 ^{b}	1757.57 ± 111.21 ^{b}	1789.34 ± 113.01 ^{b}	1734.01 ± 128.68 ^{b}	1886.94 ± 317.71 ^{b}	2248.26 ± 295.77 ^{a}
Potassium	2194.33 ± 111.42 $^{a\ b}$	2460.58 ± 161.79 ^{a}	2013.23 ± 119.24 ^{ab}	1044 ± 94.59 ^{c}	1148.70 ± 214.81 ^{c}	1988.35 ± 314.68 ^{ab}
Calcium	182.39 ± 12.67 ^{c}	483.01 ± 35.96 ^{bc}	530.64 ± 38.55 ^{bc}	700.36 ± 217.63 ^{bc}	732.99 ± 163.69 ^{b}	1320.45 ± 230.85 ^{a}
Vanadium	0.059 ± 0.01 ^{b}	0.128 ± 0.01 ^{b}	0.174 ± 0.02 ^{b}	0.280 ± 0.03 ^{ab}	0.375 ± 0.12 ^{a}	0.436 ± 0.07 ^{a}
Chromium	0.087 ± 0.02	0.451 ± 0.35	0.590 ± 0.47	1.090 ± 0.93	1.57 ± 1.32	1.73 ± 1.22
Manganese	0.286 ± 0.02 ^{abc}	0.251 ± 0.02 ^{c}	0.345 ± 0.05 ^{bc}	0.384 ± 0.05 ^{abc}	0.443 ± 0.11 ^{ab}	0.523 ± 0.09 ^{a}
Iron	3.87 ± 0.35 ^{bc}	8.81 ± 0.77 ^{b}	18.94 ± 2.33 ^{bc}	45.68 ± 20.51 ^{ab}	51.45 ± 20.20 ^{ab}	76.10 ± 16.19 ^{a}
Cobalt	0.006 ± 0.001 ^{bc}	0.011 ± 0.001 ^{b}	0.041 ± 0.005 ^{bc}	0.135 ± 0.03 ^{a}	0.162 ± 0.04 ^{a}	0.10 ± 0.02 ^{ba}
Nickle	0.211 ± 0.05 ^{b}	1.68 ± 0.67 ^{ab}	2.09 ± 0.68 ^{ab}	2.94 ± 0.96 ^{ab}	3.69 ± 1.76 ^{a}	1.71 ± 0.73 ^{ab}
Copper	0.647 ± 0.11	1.94 ± 1.18	1.49 ± 0.34	1.69 ± 0.41	1.86 ± 0.77	1.81 ± 0.43
Zinc	12.98 ± 0.99 ^{b}	41.35 ± 8.20 ^{ab}	42.44 ± 6.51 ^{ab}	45.35 ± 8.30 ^{ab}	62.92 ± 20.48 ^{a}	46.74 ± 6.71 ^{ab}
Selenium	0.490 ± 0.03	0.642 ± 0.06	0.668 ± 0.07	0.663 ± 0.09	0.743 ± 0.16	0.668 ± 0.13

3.3.4.3 Developmental changes of egg pigments

Along with the five previously identified pigments (section 3.2.4), a new pigment was identified as being present within the developmental progressions. β-carotene was identified in all samples post stripping, with a significant increase through development (Table 3.10). There was a threefold increase in levels of β-carotene between 24 hr and hatching. There is a significant reduction in the levels of canthaxanthin, astacene and Echinenone between stripping (0 DD) and 24hr with levels remaining constant thereafter. Echinenone levels returned to levels comparable to stripping in the hatched larvae. There was no significant variation during embryonic development in either astaxanthin or leutin. As a result, total pigment levels significantly reduced from 0DD to 24hr (23 %) before returning to levels comparable to 0DD at 200DD until the end of the study.

Table 3.10: Developmental changes Pigment levels for wild lumpfish eggs measured at the point of stripping, 24hr, 60DD 150DD, 200DD and Hatched juveniles. Levels are reported as mg per Kg of eggs. Values are displayed in means ± SEM, differing superscript denotes significance.

	Stripping	24hr (8 DD)	60DD	150DD	200DD	Hatch
β Carotene	0	0.78 ± 0.14 ^{b}	0.24 ± 0.0.09 ^{b}	0.41 ± 0.21 ^{b}	1.92 ± 0.32 ^{ab}	3.39 ± 0.81 ^{a}
Echinenone	2.71 ± 0.69 ^{a}	0.45 ± 0.07 ^{b}	0.32 ± 0.09 ^{b}	0.36 ± 0.13 ^{b}	0.67 ± 0.118 ^{b}	1.55 ± 0.37 ^{ab}
Canthaxanthin	9.34 ± 4.16 ^{a}	0.17 ± 0.02 ^{b}	0.20 ± 0.05 ^{b}	0.32 ± 0.08 ^{b}	0.26 ± 0.03 ^{b}	0.20 ± 0.06 ^{b}
Astacene	5.42 ± 3.49 ^{a}	0.34 ± 0.10 ^{b}	0.32 ± 0.08 ^{b}	1.14 ± 0.27 ^{b}	1.49 ± 0.29 ^{b}	0.69 ± 0.189 ^{b}
Astaxanthin	0.99 ± 0.31	1.01 ± 0.19	1.29 ± 0.34	1.14 ± 0.25	1.74 ± 0.49	1.05 ± 0.21
Leutin	1.93 ± 1.35	0.68 ± 1.11	1.09 ± 0.65	0.53 ± 0.22	1.20 ± 0.53	0.33 ± 0.13
Total Pigment	13.23 ± 4.58 ^{a}	3.37 ± 0.53 ^{b}	3.50 ± 0.75 ^{b}	3.77 ± 0.72 ^{b}	7.06 ± 1.21 ^{ab}	7.05 ± 1.2 ^{ab}

3.4. Discussion

Poor egg quality appears to be a significant challenge restricting hatchery production using captive lumpfish, as can be highlighted by the comparisons of wild derived and captive eggs used in chapter 2 and reported egg quality data from industry. In order to close the lifecycle of lumpfish and assure a reliable supply of good quality larvae there is a need to determine effective broodstock and egg management protocols (Powell et al. 2018). While chapter 2 focused on the impact of broodstock rearing temperature on subsequent gamete quality in captive stocks, this current body of work focused on a comparative analysis of egg biochemical compositions in association with measured egg quality as a proxy for broodstock nutritional status. Overall, this study demonstrated that season did not appear to have a major influence on wild egg quality and that both wild origin samples were consistently superior in quality compared to captive derived eggs (46 % vs 6 %, hatching rates respectively). This study profiled the proximate composition of lumpfish eggs for the first time and highlighted large differences between wild and captive eggs at early development stages. There were consistent deficiencies in many of the EFA's associated with good egg development within the captive stocks. This study identified 50 significant relationships between nutritional components and hatching rate as well as considerable PCA loadings associated with egg quality. The strongest relationships were identified between hatching and 22:1 n-9, EPA, Total n-3's and DHA. Significant changes in fatty acids, minerals and pigments over the course of embryonic development were observed in the highest performing egg batches with significant reductions in many n-3 PUFA's, positive and negative changes in both major and minor mineral levels and significant changes in pigment profiles.

To ensure commercial viability, hatcheries must maximise broodstock egg production and numbers of juveniles produced from captive stocks. This productivity is determined by the interplay of the number of eggs and the associated batch quality of the gametes produced by the captive stocks. The broodstocks used in the current study (wild *vs.* captive) differed significantly in body size, with the captive broodstock being *circa* 50% smaller. This is due to a difference in age with the captive stocks being first time spawners at 17 months old while the wild stocks were potentially repeat spawners with an potential age ranging from 5 to 7 years based on Davenport (1985) growth models or 3-5 years based on more recent age estimates by Hedeholm et al. (2014). The captive eggs were notably smaller than the wild sourced eggs with consequently more eggs per ml (>60%) which was related to the differences in broodstock size and/or age (Kjesbu 1989). The outcome being that the batch total volumes observed were comparable between sources. Overall, the batch fecundity estimates reported in this study are lower than those reported in previous studies (Kennedy 2018; chapter 2), however, current estimates correspond to realised fecundity by stripping while previous studies estimated total fecundity. It should be noted that for the captive stocks, stripped females were monitored throughout the reproductive season and at least 35 % ovulated a second batch between 1 and 2 weeks after initial stripping event which were stripped again producing a similar volume of eggs as the first stripping (Data not included). This corroborates data from chapter 2 which estimated approximately 50% of individuals were batch spawners from oocyte frequency analysis, initial work by Davenport (1985) who reported 2-3 batches spawned over a period of two weeks, and Kennedy et al. (2018) who documented two distinct oocyte cohorts within mature and maturing gonads. This work supports previous evidence of batch spawning in the species. In terms of the quality

of the eggs produced, those from wild stocks were significantly better in all egg quality measures in this study, than those in the captive stocks which is common for a new species held in captivity (Salze et al. 2005). Within the captive stocks assessed in this study only 10 egg batches producing eyed eggs and a total of 11% (6 egg batches) producing any hatched juveniles. The overall poor and variable egg quality observed was similar to that observed in chapter 2 demonstrating the challenge to be addressed restricting productivity in hatcheries from both the UK and Norway. It is therefore imperative that the drivers of poor egg quality are identified.

Unreliable egg quality and composition is common in the early stages of closed life cycle management of any marine fish species (Mommens et al. 2015a). In the current study, a significant difference between the biochemical compositions of wild and captive stocks was observed. Lipids are the most common studied macronutrient with regards to broodstock nutrition (Bobe & labbe 2010). Lipids provide the essential fatty acids required for the formation of cell membranes as well as providing energy for somatic growth (Sargent et al. 2003). Levels of total lipid reported within this study are within those reported for the species by Lønning et al. (1988) who reported dry weight lipid content between 21 and 26 % equivalent to between 4.2 and 6.5 % wet weight of the eggs. Levels of total lipid reported in this study are within the range considered normal for marine fish eggs (≈ 5 %) (Sargent et al. 2003). However total lipid was significantly lower in the captive stock eggs (*circa* 40% reduced). The drivers of deficiencies in biochemical components within the eggs are not clear, it could be due to several possible factors including small sized broodstock and eggs, fish spawning for the first time, and insufficient lipid uptake from the diet. Egg quality in other experimental captive stocks fed different diets is also poor suggesting the need for a more specific diet (chapter 2). However, all captive stocks used were first time

spawners and egg quality could be a factor of broodstock size and or age as reported in Atlantic cod (Trippel & Morgan 1994). While environmental conditions were aligned to the findings in chapter 2, possible environmental effects can also not be ruled out. In addition to apparent differences within total lipid content there were also notable differences in the constituent components of the lipid fraction, such as overall free fatty acids and lipid class fractions.

Much of the egg quality research in other species has focused on the EFA's such as DHA, ARA and EPA which are all required for the formation of cell membranes (Valdebenito et al. 2015). Levels of these essential fatty acids within the wild caught eggs are similar to those found in other marine species, proportional levels of DHA and EPA found in this study were 27 % and 17 % respectively. While DHA and EPA proportional levels within the wild stocks for cod have been reported at 30% and 10%, respectively (Salze et al. 2005). Levels of DHA and EPA were found to be significantly lower in the captive broodstock eggs when expressed as both proportional and absolute levels, suggesting that the mechanisms for these deficiencies described above are also acting upon the fatty acid profile of the lipids within the captive broodstocks.

The levels of phospholipids identified within wild lumpfish eggs (30-40%) are generally below the reported range of 60-90% for other marine species (Sargent et al. 2003). However, species which have lipid globules within their eggs such as turbot (Silversand et al. 1996), sea bass (Rønnestad et al. 1998) and Senegal sole (Vázquez et al. 1994) often have a higher level of neutral lipids (generally above 50%). This appears to be the case with lumpfish, where lipid globules occurred in all batches sampled in this study and have been commonly recorded in previous literature (Davenport. 1985; Powell et al. 2018). Neutral lipid levels reported in this study were

between 60-70 % which reflects levels reported in other species with eggs predominantly containing neutral lipids (Silversand et al. 1996; Rønnestad et al. 1998). The role of lipid fractions during embryonic development is species specific and poorly studied (Sargent et al. 2003), however, eggs which have a high level of neutral lipids tend to utilise these as an energy source during embryonic development (Sargent et al. 2003). High levels of neutral lipids and oil globules are suggestive of buoyant eggs; however, this is not the case for lumpfish where eggs have been found to have a higher density than seawater (Davenport & Kjørsvik. 1986).

Identification of nutritional factors effecting egg quality is important for improving egg quality in new aquaculture species (Bobe & Labbe 2010). As explained above there is a clear disparity in egg quality parameters between wild and captive stocks recorded in this current study which reflects commercial experience and explains why egg quality in lumpfish is perceived to be an important hurdle in closing the life cycle of the species (Powell et al. 2018; chapter 2). Numerous studies in “new” aquaculture species such as halibut (Mazzora et al. 2003; Mommens et al. 2015a) and cod (Salze et al. 2005) have attempted to identify compounds which correlate to egg quality though large-scale profiling of egg nutritional profiles. It is vital going forward to identify which of the many proximate components are most strongly associated with egg quality. To this end, principal component analysis was used to reduce and prioritise the number of nutritional factors which are associated with egg quality in lumpfish as previously done in other species *e.g.* brill (*Scophthalmus rhombus*) (Cruzado et al. 2011). As with many marine species (Gallagher et al. 1998; Lahnsteiner & Patarnello. 2003; Mommens et al. 2015a), fatty acids both within the total as well as within the polar and neutral fractions had the greatest apparent influence representing 97% of the total factors prioritised by PCA.

Within the PCA analysis, the hatching rate loaded only 0.666 with the first component explaining 30% of the total variance, which key at determining components to focus on as in previous studies the Pearson's correlation coefficient is used as the indicator of the relationship between nutritional component and hatching rate (Cruzado et al. 2011, Mommens et al. 2015a). Within this study the EFA's in the free fatty acids and phospholipid fractions displayed strong significant ($P<0.01$) associated with hatching rate in lumpfish. These EFA's are commonly associated with egg quality in other marine fish such as Atlantic cod (Salze et al. 2005), Atlantic halibut (Mommens et al. 2015a), and sea bass (Navas et al. 1997). While EPA and DHA, the most commonly studied fatty acids, displayed a higher loading and correlate with egg quality, the strongest relationship found in this study was a non EFA, 18:1 n-9 (Oleic Acid) within the phospholipid fraction. There was a strong negative interaction between the polar fraction Oleic Acid and hatching rate. The levels of this FA was significantly higher in the captive stocks, with primary production mainly terrestrial, with some marine sources (Sargent et al. 2003). The lipid, 18:1 n-9 plays a significant role in the formation of cell membrane bilayers (Rønneberg et al. 1986; Sargent et al. 2003), however it has been shown to have a detrimental effect on egg quality indicators when levels within the egg have been increased in gilthead seabream (Fernández-Palacios et al. 1997) and in Atlantic halibut (Mommens et al. 2015a) and specifically within the polar lipid classes (Almansa et al. 1999). Interestingly, levels of 18:1 n-9 within the free fatty acid group displayed a significantly positive relationship with egg quality in this study. This does not appear to be unusual for this fatty acid as the same trend is seen within sea Bass (Özyurt, & Polat. 2007, Sağglık et al. 2003). The highest loading against the PCA within this study was 22:1 N-9 (Erucic Acid). There is limited information on primary sources of Erucic Acid, however, it has been found to be

present in and polychaetes (Marques et al. 2018) which are common prey items of lumpfish. Erucic Acid has been identified in the eggs of wild (but fed) Atlantic halibut (Mommens et al. 2015a) and wild caught Pacific halibut (*Hippoglossus stenolepis*) (Whyte et al. 1993). Erucic Acid is readily metabolised into shorter chain fatty acids such as 18:1 and 20:1 which play a key role in cell membrane bilayers (Rønneberg et al. 1986; Sargent et al. 2003).

Within the free fatty acids 18:4n-3 correlated positively with hatching rate. The lipid, 18:4 n-3 or stearidonic acid have been shown to play a key role in fertilisation success in Atlantic hallibut (Mommens et al. 2015a), however poor and nonsignificant correlations have been found in other species (Fernández-Palacios et al. 1997, Bruce et al 1993). 18:4 n-3 plays a role in n-3 metabolism pathways into C20 and C22 Fatty acids and plays a key role in eicosanoid regulation (Sargent et al. 2003). The lipid, 16:0 was identified in this study as having a significant positive relationship with egg quality also displayed significant positive relationships with fertilisation and blastomere symmetry in Atlantic halibut (Mommens et al. 2015a). 16:0 has been identified as playing a key role in energy creation for metabolism, it is readily desaturated by fish and is a key constituent of phosphoglycerides which are important components of cell membranes (Sargent et al. 2002). However fatty acid interactions need to be considered when assessing the requirements of fatty acids in new species (Glencross. 2009). Specifically, the interaction between n- 3 and n- 6 PUFA's has been found to be of importance due their competitive actions during eicosanoid synthesis (Sargent et al. 2003). The findings in this study identified that a high ARA: EPA ratio correlated with poor egg quality, as well as a high n-3: n-6 ratio resulting in greater egg quality. Future research into broodstock diets need to consider levels of individual n-3 and n-6 as well as the ratios between them and the relation to egg quality in this species.

There were significant negative relationships with levels of n-6 PUFA's, in both the FFA's and the phospholipid fractions including phospholipid ARA contrasting from Atlantic cod where ARA levels are positively correlated with egg quality parameters (Salze et al. 2005). Where high levels of 18:3 n-3 and 18:2 n-6 were associated with poor egg quality in this study, they also correlated with negative egg quality in Atlantic halibut (Mommens et al. 2015a), however, inclusions of n-6 PUFA's have shown increased hatching in Atlantic cod (Lanes et al. 2012). There is currently a fundamental lack of total lipids within captive lumpfish eggs, which could be a result of several factors outlined above. Should the age or virgin spawning be factors in poor egg quality, current lumpfish broodstock management approaches would need to be reconsidered, however, further work is required to disentangle the significance of these various factors. Lumpfish appear to reflect the requirement for EFA's of many fish species in terms of egg quality, however there are several species-specific fatty acids which appear to drive egg quality in the species. The relatively unknown polar 18:1 n-9 was identified in this study as having the strongest association with egg quality with future work needing to clarify the mechanism for this, as inclusion in the FFA (absolute levels) positively correlated with egg quality. Whereas the highest loading within the PCA relatively unknown 22:1 n-9 was identified in with future work needing to clarify the mechanism for egg quality. The ratios of n-3: n-6 PUFA's appear to be key drivers of poor egg quality in captive stocks currently with ARA:EPA ratio having a significantly negative effect on egg quality in this study.

Levels of minerals within eggs and their role within egg quality has been understudied to date, with little information on the role of minerals in marine species egg quality (Migaud et al. 2013). Minerals levels within captive stocks were found to be lower than those in the wild derived eggs, again typical of early broodstock management (Lanes

et al. 2012). The captive broodstock used in this study were lower in 8 of the 14 identified minerals during this study, and also displayed excess of manganese, compared to wild stocks.

The role of minerals within egg quality is less well documented than fatty acids, with more direct evidence available in freshwater fish where there are differing mineral requirements and osmotic challenges. A study by Kousoulaki et al. (2010) found a positive effect of increased dietary phosphorus supplementation on body growth, morphometry and tissue mineralization in juveniles of Atlantic cod and Lanes et al. (2012) found a significant relationship between dietary phosphorus and hatching rate but found no relationship between levels of iron, calcium or copper. Despite this, information on mineral supplementation for broodstock, and the resulting effect on reproductive performance, egg and milt quality for marine species is limited.

Analysis of minerals in the current study identified no seasonal effects on mineral concentrations within lumpfish eggs except a significantly lower level of magnesium at the start of the season (wild October). This study found significantly lower levels of both major and minor minerals between captive and wild stocks. With regards to mineral effects on egg quality, the PCA identified only 4 of the 14 measured minerals as displaying meaningful loadings in association with hatching rate. Of these only nickel and calcium displayed significant relationships with hatching rates. Kjørsvik & Lønning (1983) suggested that calcium levels are important in the hardening of the chorion, with further work by Lønning et al. (1984) identifying the role calcium plays specifically in hardening of the lumpfish chorion. Calcium is an important macronutrient in fish, it plays a pivotal role in cell signalling during fertilisation, between sperm and egg and within the egg at the point of fertilisation (Webb et al. 2013) as well as affecting bone mineralisation, blood clotting, muscle function, nerve impulse transmission,

osmoregulation and numerous enzymatic processes (Lall 2003). It has also been shown to play a key role in modulating the transcription of number of genes functions gene expression within developing cells (Ermak & Davies. 2002, Saris & Carafoli 2005). There is little published information on the role of nickel in egg quality, with the majority of information on the toxic effects of high environmental nickel on development including inhibiting or delaying hatching in zebrafish (*Brachydanio rerio)* (Dave & Xiu 1991). Overall, there is little known regarding the role of minerals on egg quality in marine fish. This study suggested that minerals play a smaller role in lumpfish egg quality than fatty acids, however despite high levels of variation in fatty acids between egg batches there were still clear associations with hatching rate within the mineral profiles. While future work should focus on the majority of associations in the fatty acid groups (FFA, polar and neutral fractions), minerals and their role in lumpfish egg quality should be clarified in future studies in order to enhance current egg performance.

Many studies have documented the range of lumpfish egg pigmentation and a high level of variation has been reported within this species with egg colours ranging between green, purple, red, pink and orange (Davenport 1985; Davenport & Thorsteinsson 1989; Powell et al. 2018). Total pigment levels reported in this study were similar to those in wild salmon eggs at 10.3 mg g^{-1} (Craik 1985) and approximately one order of magnitude more than that reported for Atlantic cod (Salze et al. 2005). This study identified six pigments within lumpfish eggs; echinenone, canthaxanthin, astacene, axtazanthin, leutin, with β carotene being identified in developing eggs. Identification of pigments through HPLC has not been conducted in lumpfish before, however results appear to be similar to those identified through peak

absorbance spectrophotometry by Mikulin & Soin (1975), Mikulin et al. (1978) despite restricted access to these two papers.

There were significantly lower (33% reduction) levels of pigmentation within the captive stocks than the two wild stocks which is in keeping with studies in cod which reported that carotenoid levels were significantly lower in captive stocks than wild stocks (Salze et al. 2005) but that dietary supplementation can increase levels of pigments within Atlantic cod eggs and increase fertilisation rates, eggs per female (over season), number of floating eggs per female (Sawanboonchun et al. 2008).

Pigments have been shown to have an effect on egg quality in Atlantic salmon (Christiansen & Torrissen 1997), and Atlantic cod (Salze et al. 2005). Dietary levels of Astaxanthin have been shown to improve egg quality in red sea bream (*Pagrus major)* (Watanabe & Miki 1993). However, in the PCA analysis, total level of pigment was the only component of pigment analysis which significantly correlated positively with egg quality within the current study. This suggests that while there is a large biological variation within lumpfish eggs, the role of individual pigments might not play a pivotal role in determining egg quality. However, this study identified an unknown pigmented material, through regular quality control method during HPLC analysis. This compound was later identified as being present in other species and developmental stages (to a lesser extent), which would have not been included in results from studies utilising similar methodologies. Identification of this unknown compound was not fruitful in this case and future work could aim to determine this compound and the role in lumpfish egg quality as it makes up approximately 90% of pigmented material in lumpfish eggs (compared to approximately 30% lumpfish flesh and approximately 5% Atlantic salmon eggs).

Having analysed initial egg composition, documenting compositional changes throughout development can also inform on components that play an active role in supporting embryonic development and thus potentially determining the likelihood of successful development and hatch. Levels of nutritional compounds are known to change over the development of embryos, up to the point of hatching, and during absorption of the yolk sac within many species (Bobe & Lobbe 2010, Migaud et al. 2013). Rapid changes throughout development could indicate a specific requirement for the component at certain stages of development, helping to pinpoint areas of importance for future work. Endogenous reserves in fish eggs provide both a source of energy and biosynthetic precursors to meet the embryonic demands for growth and development. Determining the metabolic transformations and energetics of eggs is important in understanding determinants of egg quality, few studies have examined changes to lipid, mineral and pigments during development of a teleost egg. However similar studies have been performed in other species such as Atlantic halibut (Whyte et al. 1993) and Greenland halibut (*Reinhardtius hippoglossoides*) (Domínguez-Petit et al. 2013).

Levels of all major components of the lipids, saturates, monosaturates, n-6 and n-3's all significantly decreased between the point of stripping and 24 hours post fertilisation. However, n-3 fatty acids significantly decreased over development on top of initial reductions, with significant reductions in 18:4 n-3 and 20:4 n-3 between 24 hours and hatching. The lipid, 18:4 n-3 has been identified specifically in pathways which competes during eicosanoid synthesis (Sargent et al. 2003), differing levels of this FFA over development can have important implications for these signalling molecules. This data suggests that these n-3 PUFAs are being utilised during embryonic development and both fatty acids displayed significant positive relationships with hatching rate

within this study. The ratio of ARA:EPA differed significantly between development and hatch driven by reduction in levels of EPA, suggesting changes to the eicosanoid synthesis pathway requirements post hatching. As discussed above, 18:4 n-3 was the strongest correlation with hatching rate, developmental progression found that levels decreased between the point of stripping and 24 post fertilisations, suggesting that it is a key component in early development. Levels also significantly decreased between 24 hours post fertilization and hatching, suggesting further decreases through development. Further work would identify changes within the two fractions (Free fatty acids and Phospholipids) over development to identify the role 18:4 n-3 plays within each fraction. This data reinforces fatty acid requirements suggested above, as well as suggest higher inclusion of highly utilised 18:4 and 20:4 n-3 fatty acids within broodstock diets. Further work should aim to focus on the developmental pathways utilising these two n-3 PUFA's within lumpfish to identify their role in embryonic development in this species. Identification of polar and neutral lipid fractions, and their changes through development might identify if lumpfish follow other highly neutral lipid containing egg species and utilise neutral lipid fractions for metabolism through development.

Several minerals increased in concentration over the course of embryonic development within this study, of the major minerals sodium, magnesium and calcium all increased between the point of stripping at 200 DD. This suggests there is an import of minerals into the egg through development as suggested in a review by Lall (2003) referring to increases during Atlantic salmon, Japanese rice fish (*Oryzias latipes*) and rainbow trout egg development. This import of minerals has been suggested to be due to changes in osmotic balance as the egg progresses through development (Lall 2003). Within the major minerals, there appeared to be significant changes (+ and -)

between the point of stripping, early development, late development and hatching. This could be related to key developmental stages within the eggs, chosen as markers for this study, such as eyeing. Hardening of the lumpfish chorion has been documented by Lønning et al. (1984), they found that there was significant calcium uptake in the early stages post fertilisation. This study did not find a significant increase in early stages of development for calcium, however calcium levels increased between stripping and 200DD, and was significantly higher at the point of hatching. Import or export of minerals into/ out of the egg suggests that maternal influence of mineral compositions are less vital in the case of some minerals. While species specific, ions such as chlorides, phosphates and sulphates as well as elements such as manganese and selenium often are limiting due to limited ability to be absorbed or transported into the egg (Lall. 2003).

Levels of pigments within this study varied greatly between individuals, a phenomenon which has been documented a number of times for lumpfish within the literature (Davenport 1985; Powell et al. 2018). While there were no significant differences in pigment levels between individuals at any point over development, there were significant change in levels of both total pigment and individual pigment levels through development. Changes in pigmentation coincide with developmental time points for lumpfish and is reflective of hatchery and experimental observations. The dramatic decrease in total pigment levels observed between the point of stripping and 24 hours post fertilisation, is supported my hatchery observations where there is often a massive reduction in egg pigmentation post fertilisation. The increase in total pigment levels post 150DD is also reflected in increased pigmentation after the eyeing stage of development (Chapter 2) where body pigmentation increases to the dark coloured hatched juveniles. Other studies have suggested that pigments within the eggs is a

source of pigments for chromatophores in the juveniles in salmonids (Craik 1985). Carotenoids are one of the most important pigment classes in fish. It has been suggested they have a wide range of functions within fish, as well as developing eggs. These functions include protection from adverse lighting conditions, a provitamin A source, antioxidant functions including singlet oxygen quenching as well as use as an energy source for metabolism (Izquierdo et al. 2001). As the role of individual pigments is hard to define within eggs it is difficult to postulate their role within lumpfish. The wide variety of egg colours documented in previous studies, as well as data from this study suggests lumpfish are able to utilise many different carotenoids during development. However, as in many species, total pigment levels has been associated with hatching rate in this study (Craik 1985; Izquierdo et al. 2001). Dietary inclusion of carotenoids for lumpfish broodstock would likely require high levels of canthaxanthin and echinenone where levels decrease significantly over development suggesting a conversion of both into β carotene as found in red sea bream (*Pagrus major*) (Watanabe and Kiron 1995). Future work should aim to identify the role that the different pigments identified in this study play within lumpfish egg development as well as aim to identify the currently unknown compound indicated above.

In conclusion the present study has confirmed egg quality is significantly poorer in captive derived broodstock than the wild caught stocks, displaying significantly lower fertilisation, eyeing and hatching rates. There was no apparent difference between the start or the end of the wild fishing season in any egg quality parameters. Poor egg quality is a major hurdle in successful commercial production of any new marine species. Through a broad comparative compositional analysis of eggs from both farmed and wild stocks this work has provided the first analysis of egg biochemical composition for the species. This work has demonstrated that while broadly

comparable to other marine species there are some unique aspects that warrant further investigation, such as the strongest correlation of 18:1 n-9 to hatching rate within the phospholipid fraction. The compositional profiles of the captive eggs were significantly lower in key elements in all three major nutritional components measured (lipids, minerals and pigments). This work as well as that from chapters 2, suggests that currently captive broodstock nutrition is not optimal for the species. The natural diet of adult lumpfish in the wild is currently unknown, with 80 % of gastric lavages producing no identifiable food items (Myrseth 1971). Current broodstock diets are not formulated to meet the species needs, this current work has highlighted that diets used should have low levels of phospholipid long chain fatty acids such as 18:1 n-9 as well as high ratios (>10:1) of n-3: n-6 PUFAs while maintaining lower DHA:EPA ratios (< 1.5) and future research should be directed towards resolving the role of the strong relationships identified with hatching success identified in this study 18:1 n-9, EPA, Total n-3 PUFA, DHA and 20:1 n-7. This work has highlighted relationships between hatching rate and mineral concentrations as well as potential mineral uptake by the developing egg, future studies should aim to identify the mechanisms for mineral uptake and limitations of broodstock nutrition and environmental uptake. Initial experimentation through nutritional trial, with varying mineral contents would further improve our understanding of mineral uptake from dietary and environmental sources. Future work should also aim to clarify the pigment profiles within lumpfish eggs, and the role of pigments in egg quality. Ultimately this will help develop broodstock diets that maximise productivity and assure production of robust juveniles for aquaculture, important for lumpfish which are currently perceived to be less robust in sea cages than their wrasse counterparts.

Chapter Four

Short term cold storage and sperm density assessment of lumpfish (Cyclopterus lumpus. L) milt.

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4.1 Introduction

With the increasing demand for lumpfish, and recent findings regarding milt production and management (Powell et al. 2018, Pooley et al. 2019) there is a requirement for the development and validation of reliable hatchery production protocols including the effective management of gametes prior to artificial fertilisation which is the preferred production method. Collection of lumpfish milt is performed post-mortem, as stripping is difficult with at best very small volumes collected (Nordberg et al. 2015). Because of this, males could be considered a limiting resource unless effective milt storage methods can be validated to expand the functional window that male gametes would be available for use in a hatchery setting. The previous two chapters utilised specific sperm to egg ratios, which were standardised throughout each trial, however there are currently no published rapid quantification methods for lumpfish sperm, with haemocytometer counts being the most effective though laborious method.

Both cold storage and cryopreservation can be used for milt preservation in aquaculture (Migaud et al. 2013). Cold storage requires storing milt diluted in extenders at low temperatures (typically 4 °C, (Gallego and Asturiano. 2019)) to reduce spermatozoa metabolism allowing them to be stored from 4 days in turbot, (*Scophthalmus maximus*) (Chereguini et al. 1997) to 56 days in cod, (*Gadus morhua*) (Degraaf and Berlinsky. 2004) without significant changes in milt quality (Chang, 2002). For longer term storage, between spawning seasons (Scott and Baynes, 1980) or for creating genetic storage banks (Gausen, 1993), cryopreservation is the only effective method, keeping milt diluted in a cryoprotectant solution at ultralow temperatures between - 79 and - 196 °C in liquid nitrogen. This method requires specific infrastructure to enable a precise freeze and thawing of the milt. Cryopreservation of lumpfish milt has been shown to be effective in a pilot study by

Nordberg et al. (2015), however the authors acknowledged that the protocol, while effective, still requires optimisation in several key areas.

Practically, cold storage of milt is the most useful technique available to support hatchery production by providing a low cost and technically simple solution to the challenge of male availability. It reduces the need for frequent collections from males, it enables transportation of milt to distant locations (Cabrita et al. 2008) and extends the functional window of availability to allow planned crosses of selected individuals (Jenkins-Keeran and Woods, 2002). For this reason, methods have been developed in a range of marine species like cod (Degraaf and Berlinsky, 2004) and Atlantic halibut, (*Hippoglossus hippoglossus)* (Babiak et al. 2006). However, effectiveness of extenders can be very species specific due to the differences in the biochemical composition of their seminal fluid (Beirao et al. 2019). The composition of the extender solution is an important factor impacting on storage time of the milt (Gallego and Asturiano. 2019). Some extenders are applicable to a range of species, such as Mounib's solution (Mounib. 1978). However, for more effective storage, species specific extender solutions have been developed to mimic their milt compositions, osmolarity, pH and dilution ratio (Beirao et al. 2019). Effective extenders such as Herring Ringers solutions (Pillai et al. 1994), and Modified Turbot Extender (Babiak et al. 2006), are easy to formulate in hatcheries and are easy to adapt to new species.

The purpose of chilled storage is to allow farms to perform artificial fertilisation in a controlled manner using desirable males. Artificial fertilisation protocols must be standardised, and gamete quality assessed when crosses are made (Beirão et al. 2019). Simple and accurate methods for milt quantification are important in this context for two reasons. Firstly, it allows standardisation of egg to sperm ratios which have been shown to influence fertilisation success in many species including turbot (Suquet

et al. 1995), and Atlantic halibut (Tvedt et al. 2001). Secondly, it enables the quantification of total sperm being held in storage which allows farms to accurately plan the volume of eggs that can be fertilised (Cabrita et al. 2014). Absolute sperm counts using either a haemocytometer (Suquet et al. 1992) or Computer Aided Sperm Analysis (CASA) (Kime et al. 2001) are used to determine sperm concentration, with the former being the most common but time consuming, while the later requires specialised microscopy capacity on farms. Alternative indirect estimation methods are possible based on the relationships between spermatocrit (packed cell volume) or spectrophotometric estimation of sperm concentration and haemocytometer cell counts (Tvedt et al. 2001, Rideout et al. 2004). These methods are typically rapid to perform and utilise equipment commonly found in commercial hatcheries. Assessments of sperm quality often relies on subjective assessments to quantify percentage sperm motility, or provide an evidence-based end point when sperm are deemed non-viable (Van der Horst et al. 1980; Jenkins-Keeran & Woods III, 2002) While these methods are less informative than CASA based assessments, they remain the most frequently used indicators of sperm quality in commercial fish hatcheries (Migaud et al. 2013, Valdebenito et al. 2015).

As for many other farmed fish species, artificial fertilisation will be the main production strategy used within commercial lumpfish hatcheries not least because this will enable selective breeding and the possibility of selective enhancement of captive stocks (Houston et al. 2020). Short-term storage of lumpfish milt and the lack of effective management of gametes during artificial fertilisation are two key knowledge gaps that need to be addressed in the optimisation of lumpfish hatchery management (Powell et al. 2018). Therefore, the aim of this study was to test a range of extenders for cold storage of lumpfish milt and validate rapid and accurate methods for estimating sperm

concentration both of which are basic requirements to improve artificial fertilisation protocols to be applied in commercial hatcheries.

4.2. Materials and methods

4.2.1 Lumpfish broodstock

A total of 17 sexually mature males were sampled from a captive broodstock held at Otter Ferry Sea Fish Ltd, Argyll, Scotland. Prior to sampling fish were held on an altered temperature regime (from hatch, 9.4 °C ± 0.8 °C), as recommended by the findings in Chapter 2, with holding temperature not exceeding 10 °C to assure good gamete quality. Lighting was maintained at a low intensity 24 hr photoperiod for the entire grow out period and fish were fed ad libitum a commercial pelleted feed (Samaki Marine Pellet, World Feeds, James A Makie (agricultural), UK). Males initiated sexual maturation from 17 months post hatching in January 2019. Mean weight of males used in this study was 638.3 ± 188.4 g and mean total length was 228 ± 17 mm.

4.2.2. Sampling

Males were killed using an overdose of anaesthetic (MS222, Pharmaq, UK) followed by destruction of the brain. Post mortem, testes were dissected out, weighed (± 0.01 g) and gonadosomatic index (GSI) calculated, before testes were macerated and then placed into fine mesh to strain out milt which was gathered into a petri dish where the volume of milt (± 0.5 ml) was measured using a 1 ml syringe (Fisherbrand, Thermo Fisher Scientific, USA).

For each male, packed cell volume (spermatocrit) calculated as: ((length of cell column/ length of cell column and fluid) ×100) was measured in triplicate using non-heparinised haematocrit tubes (Bris, Modulohm A/S, Denmark) which had been centrifuged for 3 min at 4000 g using a Micro Haematocrit centrifuge (MSE,UK).

A 1:1000 dilution of milt was made using a commercial milt extender (SpermCoat, Cryogenetics, Norway) and three replicate counts were made in a haemocytometer

(Hirschmann, Germany) using an Olympus microscope (Olympus optical, UK) to calculate sperm concentration (sperm per ml of milt). A minimum of 100 grids of 0.25 nl were counted to obtain the average cell count, which was calculated as *Sperm per ml= (Total count/100) × (4000/1000) ×100*.

4.2.3 Cold storage experiment

Five different milt extender solutions, which had previously been reported as being effective in other marine species, were tested: Modified Turbot Extender (MTE)(Babiak et al. 2006), Herring Ringers Solution (HRS)(Pillai et al. 1994), Mounib's solution (Mounib, 1978), and Mounib's with a 1 % BSA inclusion, both of which have been previously tested for cryopreservation of lumpfish milt (Nordberg et al. 2015), and Spermcoat, a commercially available milt storage solution (Cryogenetics, Norway) (Table 4.1). Milt was obtained from 7 males and 1:5 stock dilution (based on commonly identified effective dilution ratios (Beirão et al. 2019)) was created for each extender solution (320 µl of extender and 80 µl of milt) in triplicate wells within 46 well, micro-well plates (Starstedt, USA) which were seam sealed and placed in a fridge (4 °C) between activation tests.

A standardised activation test was performed in triplicate for all samples in a temperature-controlled room (10 °C). A dilution of 1:1000 (milt: activating solution (seawater +1 % BSA)) was created (*n.b.* this equates to 1:200 milt and extender: activation solution) in a 2 ml eppendorf (Eppendorf, Germany). Activated spermatozoa samples were flooded into a haemocytometer well and swimming activity observed under a microscope. Activation time (duration of sperm motility) was measured using a stopwatch and was defined as from the point of activation to the time at which linear movement of spermatozoa were observed to stop similar to the end point used in

Jenkins-Keeran & Woods III (2002). Activation tests were conducted every 7 days until milt was determined as non-activating at 21days, milt was re-tested at day 22 to confirm non activation.

Table 4.1: Chemical composition of the four milt extender solutions prepared and tested in this experiment. No composition data is publicly available for commercial extender tested in this study (Spermcoat, Cryogenetics, Norway). All chemicals were acquired from Sigma Aldrich (Sigma, USA).

	Modified Turbot Extender	**Herring Ringers solution**	**Mounib's solution**	**Mounib's +BSA solutions**
NaCl	4.0908 gL^{-1}	12.0386 gL^{-1}	-	-
KCl	0.1118 gL^{-1}	0.5367 gL^{-1}	-	-
$CaCl_2$	0.2996 gL^{-1}	0.2331 gL^{-1}	-	-
$MgCl_2$	0.5807 gL^{-1}	0.3141 gL^{-1}	-	-
$NaHCO_3$	2.1002 gL^{-1}	0.0840 gL^{-1}	-	-
$KHCO_3$	-	-	1 gL^{-1}	1 gL^{-1}
BSA	10 mgL^{-1}	10 mgL^{-1}	-	10 mgL^{-1}
Sucrose	-	-	42.7875 gL^{-1}	42.7875 gL^{-1}
Glucose	36.032 gL^{-1}	-	-	-
pH	8.1[a]	7.8[a]	7.8[b]	7.8[b]
Osmolarity	400 mOsm/kg[a]	405 mOsm/kg[a]	310mOsm/kg[b]	310mOsm/kg[b]

[a] Babiak et al. 2006, [b] Zilli et al. 2004

4.2.4 Spectrophotometric assessment of sperm concentration

To validate the calculation of sperm concentration from solution optical density, milt was extracted from six males using the method described previously. Milt was then diluted 1:1 in MTE, in three separate aliquots per individual and held in cold storage (4 °C) prior to further manipulation. For each male nine serial dilutions using MTE were made in triplicate (1:20, 1:50, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000, 1:10000). Three replicate counts were made using a haemocytometer to obtain the average

number of sperm per ml for each dilution. A minimum of 100 grids of 0.25 nl were counted to obtain the average cell count, which was calculated as sperm per ml using the following equation *Sperm per ml = ((Total count/ 100) × (4000/1000) ×100)*. Absorbance was measured at 10 nm intervals between wavelengths ranging from 350 nm to 740 nm using a Nanodrop 2000C Spectrophotometer (Thermo Scientific, USA) with a 1 cm path length cuvette (Fisher Scientific, USA) in triplicate for each milt and extender dilution.

4.2.5 Statistics

All data is expressed as mean ± standard error, unless stated otherwise. Statistical analysis was conducted using Minitab18 software. A General Liner Model (GLM) was conducted to test for effects of treatment on milt activation times, with a post-hoc pairwise Tukeys test used to assess differences between treatments and time.

4.3. Results

4.3.1 Experimental animals and milt characteristics

The 17 males processed displayed a mean GSI of 3.5 ± 1.1 % and a mean volume of 5.1 ± 3.6 ml of milt was collected. Sperm concentration was $12.37\times10^9 \pm 2.41\times10^9$ sperm.ml^{-1}, with an average spermatocrit of 87.8 ± 5.7 %. A significant positive relationship between sperm concentration and spermatocrit ($p> 0.001$, $r^2 = 91.8$) was observed (Figure 4.1). However, no relationship was observed between either sperm concentration or spermatocrit and the sampled males GSI or volume of milt recovered (data not shown). Similarly, there was no relationship between GSI and volume of milt recovered.

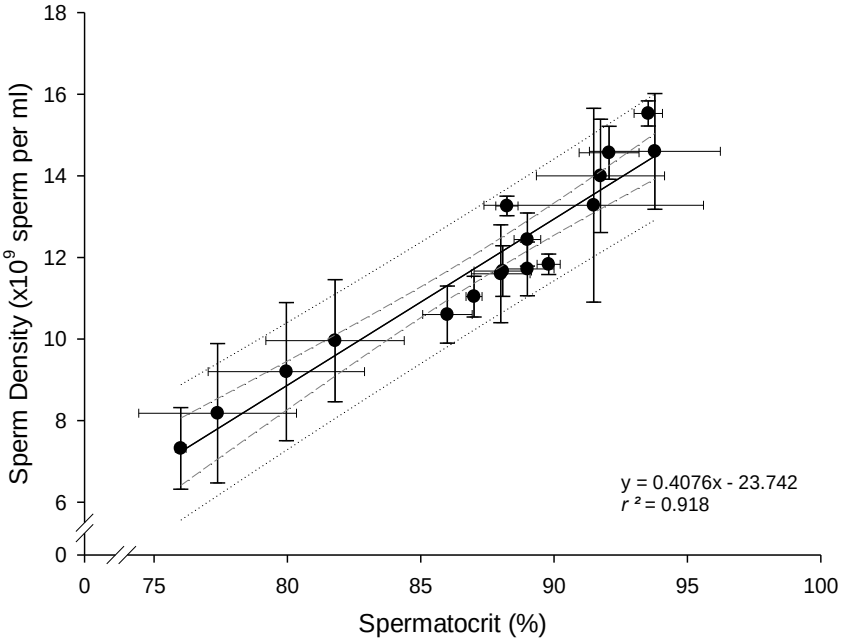

Figure 4.1: Linear relationship between spermatocrit and sperm concentration of milt samples collected from 17 captive reared lumpfish ($p<0.001$, $r^2= 0.918$). Data are presented as mean ± SEM (n=17). Solid line represents best fit linear regression with the 95% confidence intervals for the linear regression indicated by the dashed lines and the 95% prediction intervals for novel values indicated by the dotted lines.

4.3.2 Cold storage experiment

Preliminary testing confirmed that none of the five extenders activated sperm on contact. Following activation, fresh lumpfish spermatozoa remained active for 03:00 ± 00:20 (hh:mm) and no significant difference was found between fresh milt and milt diluted first in the extenders on the day of stripping (Figure 4.2). Following 7 days of cold storage, spermatozoa stored in Mounib's solutions (with and without BSA inclusion) were not motile following activation and activation time decreased significantly in milt diluted with all other extender solutions ($p>0.001$). Spermatozoa stored in MTE, HRS and Spermcoat displayed active swimming response following one week of storage, however they showed a 26.8 %, 50.5 % and a 54.4 % reduction in activation time, respectively. Activation time at 7 days was significantly higher in milt stored in MTE than for Spermcoat but not HRS. Following 14 days in the extenders the MTE, HRS and Spermcoat treatments were statistically comparable with all showing a further significant reduction in activation time representing a 79.8 %, 98.8 % and 93.3 % reduction from the point of collection respectively. At 21 days, sperm could not be activated for any of the extender solutions.

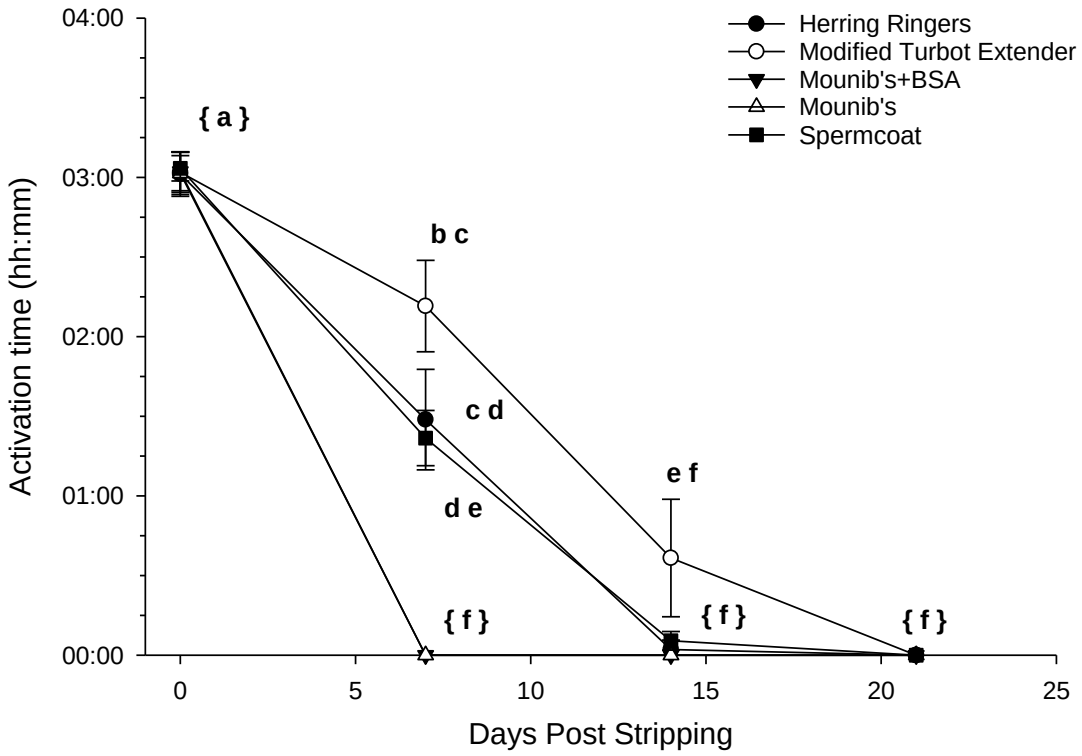

Figure 4.2: Activation time (hh:mm) of spermatozoa from captive lumpfish milt stored in five different milt extenders (HRS, MTE, M, M+BSA and Spermcoat) tested at the point of stripping (0), 7, 14 and 21days post stripping. Time values indicate the time between activation and the cessation of sperm motility. Data are presented as mean ± SEM (*n*=7).

4.3.3 Spectrophotometric assessment of sperm concentration

The spectrophotometric assessment of sperm concentration was performed using MTE, the extender which was shown previously to give the best storage performance. The absorbance spectrum for lumpfish milt diluted in MTE typically shows a steady decrease in optical density from 400 to 700 nm in wavelength (Figure 4.3). The linear relationship between sperm concentration (as measured by haemocytometer) and optical density, was tested for each male dilution curve at 10 nm intervals between 350 and 740 nm. All dilutions of milt in MTE at 1:20 and 1:50 milt to extender ratio

produced measurements outside of the working range of the spectrophotometer and were therefore excluded. Dilution of milt in MTE at 1:10000 milt to extender ratio produced measurements outside the working range of the spectrophotometer at wavelengths greater than 660 nm, as a result the analysed wavelength range was restricted between 350 and 660nm (Figure 4.4). Within this range the 640 nm wavelength produced the highest average r^2 value (97.42%) of 6 male milt dilution curves, with the smallest deviation (± 2.14 (SEM)) between individual regressions (Figure 4.5).

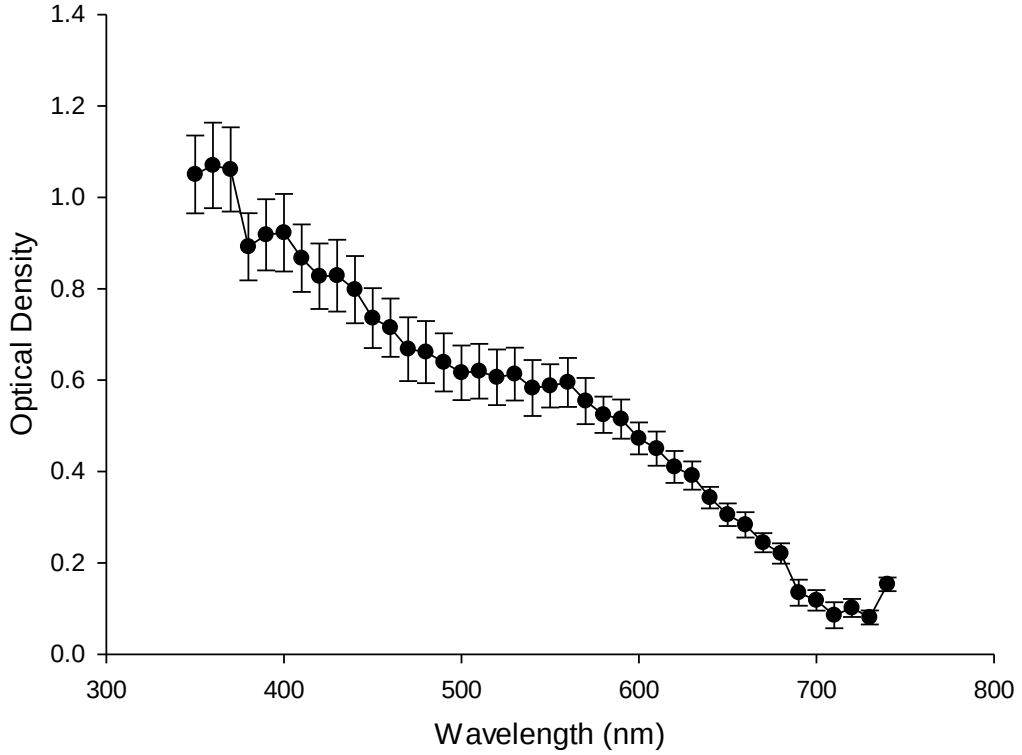

Figure 4.3: Absorption spectrum measured between 350 and 740 nm for captive lumpfish milt diluted 1:100 in Modified Turbot Extender (MTE). MTE was also used as a blank. Data are presented as mean ± SEM (*n*=6).

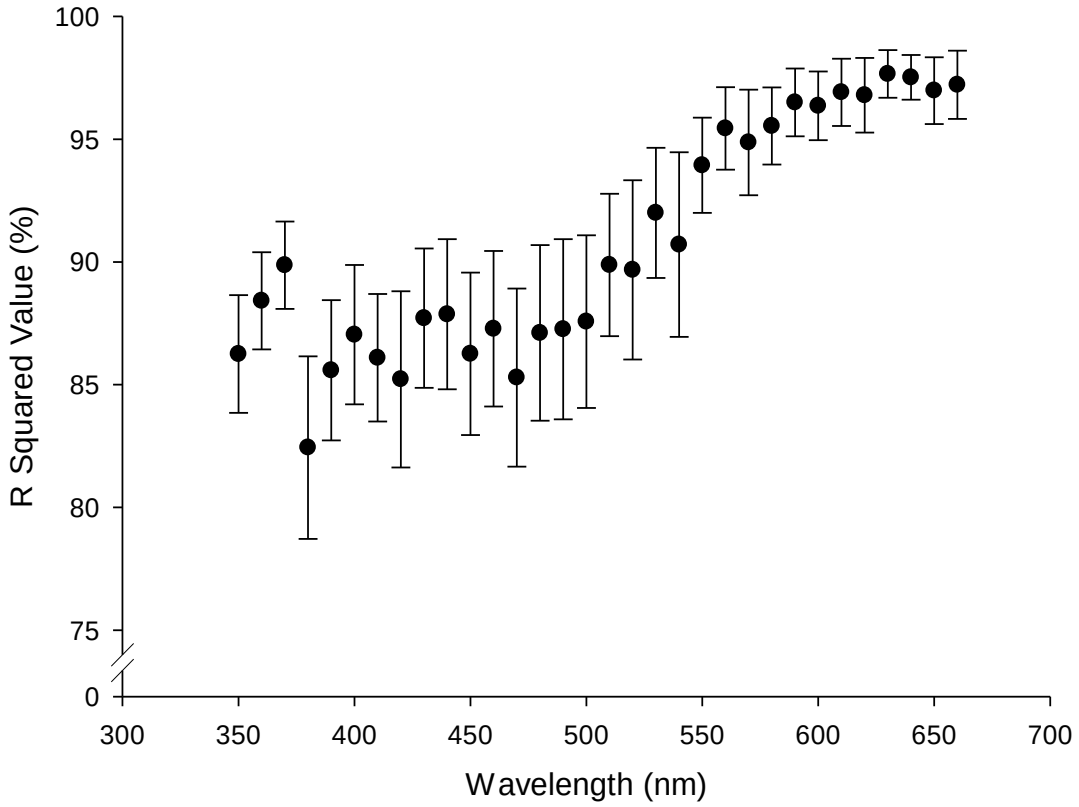

Figure 4.4: Variation in *R-Squared* values for the linear relationship between sperm concentration and absorbance values measured at 10 nm intervals between 350 nm and 660 nm. Values represent mean r^2 value (±SEM) for each individual male dilution curve (*n*=6).

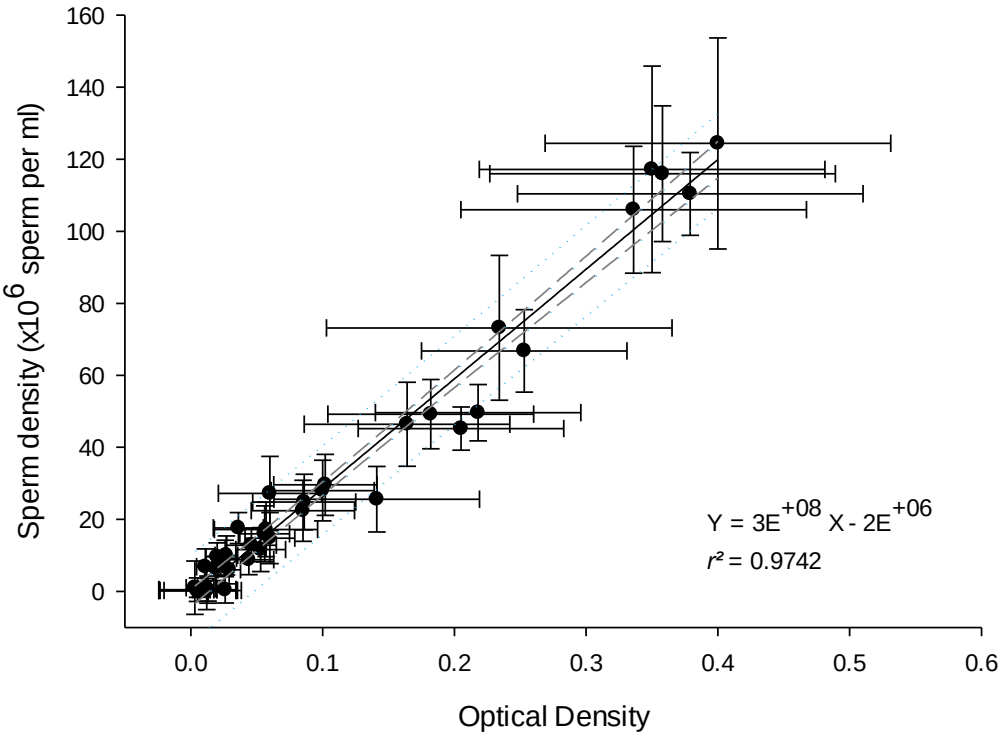

Figure 4.5: Linear relationship (P>0.001, r^2= 0.9742) between optical density measured at 640 nm and sperm concentration following dilution in MTE at a ratio between 1:100 – 1:10000. Absorbance and sperm concentration data are presented as mean ± SEM (*n*=3 replicate measurements per individual with 6 individuals per dilution). Solid line represents best fit linear regression with the 95% confidence intervals for the linear regression indicated by the dashed lines and the 95% prediction intervals for novel values indicated by the dotted lines.

4.4. Discussion

Current reproductive management of captive lumpfish requires artificial manipulation of gametes in order to improve fertilisation, aid disinfection and stock management (Treasurer, 2018). At present, lumpfish males are difficult to strip, and gamete collection requires sacrificing fish which is a limiting factor for production (Powell et al. 2018). Due to these issues more effective milt management is required. The present study aimed to test extender solutions for short term storage of lumpfish milt and provide a rapid and accurate test for sperm concentration which can be conducted in a farm setting.

This study assessed milt quality by measuring the motility window of sperm defined as the period during which sperm were able to move linearly (Jenkins-Keeran & Woods III, 2002). Activation time of lumpfish sperm (≈3 hours) is unusually high compared to many other marine teleosts such as Atlantic halibut (63 – 155 seconds, Tvedt and Benfey, 2001) and turbot (160 seconds, Suquet et al 1992). However, long activation times of sperm have been reported also in sterlet (*Acipenser ruthenus*) with sperm motility maintained for 5-6 hours (Dzyuba et al. 2012) and wolffish, with sperm still active after 2 days (Kime & Tiveten. 2002). Authors suggested this may be due to mixing of urea and seminal fluid upon release whereby sperm is activated at the point of release from the fish. The same may be true for lumpfish but there is no evidence yet available to support this. Importantly, the extended window of sperm motility does not always fully reflect differences in sperm quality as motility itself in terms of velocity matters and this does not always correlate with duration of motility (Valdebenito et al. 2015). Future studies should seek to more clearly define lumpfish sperm quality criteria utilising methods like CASA where possible.

Dilution of milt in extenders can effectively improve the lifetime of the milt over the spawning season. This can allow for more effective stock management; however, extender solutions effectiveness can vary significantly between species (Beirão et al. 2019). In this study the efficacy of five different milt extenders, commonly used in other temperate marine species, was tested. Three extenders (*i.e.* Herring Ringers, Modified Turbot Extender and Spermcoat) significantly extended the life of captive lumpfish milt up to a minimum of 14 days post stripping. Despite the fact Mounib's solutions were shown to be effective cryopreservants (Nordberg et al. 2015), the two Mounib's solutions tested in the present study did not appear to extend the window of viable milt availability. This may suggest that Mounib's solution does not match the composition of lumpfish seminal fluid for short-term cold storage. Osmolality of lumpfish milt has been reported at 463 mOsm / kg (Nordberg et al. 2015) therefore it is possible that the additional hypo osmotic stress the spermatozoa will have experienced in the Mounib's solutions precluded it from being an effective extender solution but has a lesser impact when utilised in cryopreservation. In addition, there was no significant difference between the activation times of milt diluted in MTE and HRS at any time point and while both solutions differ greatly in their chemical constituents they have similar osmolality and pH to that previously reported in lumpfish (Pillai et al. 1994, Vermeirssen et al. 2004, Norberg et al. 2015). Spermcoat displayed significantly lower activation compared with the MTE at 7 days, but not at any other time point. The recommended dilution ratio for Spermcoat was not used in this study (1:1), however it was still effective at storing milt to the expected 14 days according to the supplier. This study showed that MTE was the most effective extender at 7 days post stripping, and still displayed a lower degradation in activation time (79.8%) compared to 93 – 100 % for all other treatments at 14 days. As such this study finds that there are three

available milt extenders which can effectively store lumpfish milt for up to 14 days, MTE, HR and Spermcoat. Due to its lower degradation at 7 days, and 14 days this study continued to use MTE for the remainder of the work. Future work in the species should aim to optimise extender chemical composition for effective short term storage of lumpfish milt.

Sperm concentration reported for lumpfish in the current study are two orders of magnitude lower than those reported in Atlantic halibut (2 - 6×10^{11} spermatozoa/ml) (Tvedt et al. 2001), however they appear to be in line with those reported in other marine teleosts such as sea bass (*Dicentrarchus labrax*) ($4 – 6 \times10^{10}$ spermatozoa/ml) (Fauvel et al. 1999) and Cod ($1.33\times10^{8} \pm 14.5\times10^{8}$ spermatozoa/ml) (Degraaf and Berlinsky, 2004). Sperm densities measured in the current study support previously published data by the same authors (Chapter 2), however these appears to be subtly lower than previously reported data for wild caught fish ($31.44\times10^{9} \pm 8.35\times10^{9}$ sperm ml^{-1}) (Nordberg et al. 2015). Differences observed in sperm concentration could be explained by the methods used to collect milt (stripping in Nordberg et al. (2015) compared to extraction of milt from macerated testis in the current study and Chapter 2. The current study also analysed spermatocrit in captive lumpfish and reported a packed cell volume (76% to 93.5%) which was more consistently at the higher limit than is reported in other temperate species in captivity such as Atlantic halibut (23-97 %, Tvedt et al. 2001), Atlantic cod (18-98.3 %, Rakitin et al. 1999) and common wolfish (*Anarhichas lupus)* (0.5-5.5 %, Tveiten and Johnson,. 1999). This could explain the difficulty in stripping male lumpfish and future work could focus on hormonal manipulations to increase milt production and make stripping a viable option in lumpfish, as shown in Atlantic halibut (Vermeirssen et al. 2004).

Accurately assessing gamete quality is critical in broodstock management and optimising hatchery productivity (Gallego and Asturiano., 2019). There are several common methods for assessing sperm concentration in fish milt including cell counts using a haemocytometer, packed cell volume (spermatocrit), optical density measurements using a spectrophotometer and Computer Aided Sperm Analysis (CASA) (Kime et al. 2001). While CASA is the “gold standard” for sperm quality assessment, it is infrequently used in a hatchery setting due to the requirement for specialised equipment. While cell counts are precise and reliable, they are very time consuming and can be impractical in both a hatchery and lab setting, for example Suquet et al (1992) suggested that to reach an acceptable level of variation it could take 2 hours to assess one fish. Spermatocrit has been successfully used as an effective method of measuring sperm concentration in a range of species (Campbell et al. 1992; Suquet et al. 1995; Gallego et al. 2013) where there is a strong relationship between packed cell volume and sperm concentration. Equally spectrophotometry has been effectively used in several marine species as a reliable method for assessing sperm concentration (Fauvel et al. 2010; Rurangwa et al. 2004). In the present study, both spermatocrit and spectrophotometry were confirmed to be an accurate predictor of sperm concentration in the species. When working with raw milt samples clearly spermatocrit can be used as a rapid method for assessing sperm concentration in lumpfish rather than cell counts using a haemocytometer. However, if the hatchery intends to dilute the milt in an extender then spectrophotometric quantification of sperm concentration can be performed with an equally high level of precision. In the current study utilising MTE as the extender/diluent the best correlation between sperm concentration and measured optical density with the smallest individual variation was found when using a wavelength of 640 nm. However, from 560nm - 660 nm and at

540 nm correlations remained strong i.e r^2 > 95%. A wide range of wavelengths are used to assess sperm concentration's in other species, Fauvel et al (1999) assessed wavelengths in Sea Bass between 200 nm - 500 nm finding the best correlation at 260 nm. While Suquet et al (1992) recommends 420 nm as the optimal measurement for Turbot, having assessed relationships between 350 nm and 750nm. The high level of variation in absorbances is suggested to be due to compositional changes in the associated fluids rather than the sperm themselves (Suquet et al. 1992, Tvet et al. 2001). In terms of practical application of the method, based on experience during the study the authors would recommend a dilution of 1:500 (milt : MTE) to typically reach final sperm concentrations close to the centre of the linear relationship.

In conclusion, this study demonstrates that lumpfish milt can be effectively stored using extender solutions for up to two weeks. The most effective storage medium found in this study was the Modified Turbot Extender using a 1:5 milt to extender ratio. Sperm concentration can be estimated confidently either directly on fresh milt samples using spermatocrit (concentration $(\times 10^9) = 0.4076 \times Spermatocrit(\%) - 23.742$) or with milt samples diluted in MTE using optical density measured at 640 nm (concentration $(\times 10^9) = 3 \times 10^8 \times Optical\ density - 2 \times 10^6$) enabling more standardised and effective use of milt during artificial fertilisation. This work is an important step in generating reliable gamete handling protocols that will play a key role in advancing hatchery management and domestication of lumpfish.

Chapter Five

Impacts of post-ovulatory ageing on egg quality and the proteome of ovarian fluid in lumpfish (*Cyclopterus lumpus*).

5.1. Introduction

A key component in closing the life cycle of any species is improving survival within the egg and early developmental stages. As outlined in the previous chapter (chapter 3) egg quality is a fundamental factor regulating the success when domesticating a new species in aquaculture and needs extensive research to secure a sustainable supply of juveniles. The production of larval fish and their subsequent growth, development and health, and their potential productivity as future broodstock is highly dependent on the quality of eggs available to the industry (Migaud et al. 2013). In fish, egg quality is highly variable and affected by a number of factors including nutrition, stress, egg over-ripening, hormonal induction of spawning, genetics and age of broodstock, in addition to water quality and environmental factors such as photoperiod, temperature and salinity (Bobe & Labbe, 2010). Since all nutrients contained within the egg are derived from the female parent, all of these need to be obtained through broodstock nutrition.

In most cases, post-ovulatory ageing (POA) also known as oocyte aging, over-ripening can induce a significant decrease in egg developmental capacities without any noticeable morphological changes in the appearance of the egg (Aegerter. & Jalabert. 2004). Post-ovulatory ageing has been shown to lead to decreased viability in several species including Atlantic salmon (*Salmo salar*) (Mommens et al. 2015b), rainbow trout (*Oncorhynchus mykiss*) (Aegerter. & Jalabert. 2004) and Atlantic halibut (*Hippoglossus hippoglossus*) (Bromage et al. 1994). The decrease in egg quality occurs differently between species and is highly dependent on external factors and studies have found high levels of female variability (Lahnsteiner et al. 2001). For most fish there is a rapid decrease in the hatch rate of eggs a few hours post ovulation. Within turbot (*Scophthalmus maximus*) there is a significant decrease in fertilisation

and hatching rates between freshly ovulated eggs and 24 hours post stripping where eggs aged 24 hours exhibited no survival to hatching (McEvoy, 1984). In Japanese flounder (*Paralichthys olivaceus*), hatching rate was highest for eggs fertilised 24 hr after ovulation and decreased for ageing times post 24 hr (Hirose et al. 1979). However, salmonids have been shown to be able to hold their eggs without significant effects on egg quality for a longer period of time (Mommens et al. 2015b). Rainbow trout eggs can be held in the body for between 2-4 weeks, however maximum quality is reached by 5 days post ovulation at 10-20 ° C (Aegerter. & Jalabert. 2004). This variation in the period during which eggs remain viable prior to the impact of post-ovulatory ageing is thought to be related to the spawning behaviour of the species, with batch spawners such as Atlantic cod (*Gadus morhua*) (Kjorsvik & Lonning, 1983), Atlantic halibut (Bromage et al. 1994) and turbot (Aegerter & Jalabert. 2004) displaying short ageing times. With single spawners such as many salmonids (Mommens et al. 2015b) having longer over-ripening windows.

Environmental conditions have been shown to be an important factor in ageing due to its role in determining spawning timings and ovulation (Tveiten et al. 2001, Brown et al. 2006). This can result in the eggs being held in the body cavity for longer durations until they can be stripped. Delayed stripping may lead to over-ripening of the eggs and a reduction in egg quality (Aegerter & Jalabert, 2004). Management post stripping, such as transport, can also increase the period of ageing. Best practice for stripping of broodstock requires knowledge of the post-ovulatory ageing process along with reproductive biology and environmental management of the target species (Migaud et al. 2013). Without such information broodstocks will be mismanaged which could have a significant effect on egg quality and stock performance (Mohagheghi Samarin et al. 2015).

Currently little is known about the spawning timings of lumpfish (*Cyclopterus lumpus*), macro-gonadal work by Kennedy et al. (2018), Kennedy et al. (2020) and histological work in chapter 2 (Pountney et al. 2020a) identified lumpfish as batch spawners. This work suggests that the species would have a small over-ripening window, similar to other batch spawning species (Kjorsvik & Lonning, 1983, Bromage et al. 1994) ,however, the reproductive development window is still unknown. There is currently ambiguity over the spawning season for this species with initial reports by Davenport (1985) described the lumpfish spawning season as occurring between April and July with no geographic reference. More recently, Kennedy (2014) noted that spawning season in Iceland was between January and March, which is the focal window for commercial exploitation. However, fishing for mature lumpfish in central Norway occurs between September and June (*Pers. Com*, Tor Otterlei Skjerneset Fisk) with the main catch window from October to May, with this fishery being the principle supplier of wild lumpfish eggs to Norwegian and UK based hatcheries. A Lack of information in broodstock reproductive management spawning season and environmental management can lead to a-synchronous development and long spawning windows (Migaud et al. 2013). Under these conditions, eggs can be left in the body cavity for long periods of time and result in over-ripening of eggs and therefore a reduction in egg quality. Current best practice in Scotland involves monitoring captive broodstock approximately every three days to assess females vents of suitability for stripping. While in Norway wild mature fish are netted and held in a flow through system with the vent regularly assessed to determine suitability for stripping. Despite having demonstrated previously in this work (Section 2.2.1) that ultrasound is an effective tool to assess female maturation and could be a possible used to identify ovulated eggs within the gonads it is, as yet, not being used in this role

commercially to the authors knowledge. To confound these factors further, hatcheries are prone to hold unfertilised eggs within their ovarian fluid for lengths of time while they batch process multiple individuals, with further delays potentially added due to degumming and disinfection activities (Powell et al. 2018). Together, this means that there can be highly variable and extended periods of times between ovulation, stripping and subsequent fertilisation of egg batches all of which could affect subsequent quality if the over-ripening window for lumpfish is short.

Good egg quality is a fundamental factor in the sustainable production of juveniles for aquaculture. All nutritional components required for development are provided for by maternal reserves until the juveniles can begin feeding on external food sources, for lumpfish this is at the point of hatch. The process of egg degradation post ovulation within the body cavity is called post ovulatory aging (POA) (Lahnsteiner. 2000, Rime et al. 2004). Post ovulatory aging occurs at the release of the oocyte from the follicle, where the timing of degradation and resultant effects are species specific. The process of aging can lead to major morphological and biochemical changes in the egg, this degradation in quality appears to be similar in eggs retained *in vivo* or incubated e*x vivo* suggesting it is determined by the eggs themselves potentially in interaction with the accompanying ovarian fluid (Bromage et al. 1994).

Mature oocytes are released at ovulation into the coelomic cavity, they are contained in a semi-viscous fluid known as ovarian fluid. Ovarian fluid is created through filtration of blood plasma, and ovarian epithelial secretions (Matsubara et al. 1985). The composition of ovarian fluid provides components for extension of egg storage and the period where eggs can be effectively fertilised and as long as eggs are maintained within ovarian fluid the process of over ripening follows a natural time course (Bromage et al. 1994). Furthermore, the influence of ovarian fluid appears to extend beyond the

maintenance of eggss with it being shown to affect sperm activity (Turner & Montgomerie. 2002) and ultimately impacting fertilisation success (Lahnsteiner. 2000, Beirão et al. 2015). While evidence suggests that ovarian fluid plays an active role in determining egg quality and fertilisation success, there remains a lack of understanding as to the fluid composition and mode of action to influence these various aspects. It is conceivable that bioactive elements within the ovarian fluid could be used as biomarkers to predict quality of batches within a given species, however this is predicated on identifying and confirming components within the ovarian fluid that determine such aspects (Nynca et al. 2015).

The composition of ovarian fluid varies between species and has been well described within salmonids such as chinook salmon (*Oncorhynchus tshawytscha*) (Rosengrave et al. 2009) rainbow trout and other salmonids (Lahnsteiner et al. 1995). The inorganic composition of ovarian fluid has been identified to have electrolyte concentrations similar to blood plasma in chinook salmon (Rosengrave et al. 2009). For marine species there is less information on the composition of ovarian fluid. However, there is some limited information on the chemical compositions such as pH and osmolarity in turbot (Fauvel et al. 1993), Atlantic halibut (Bromage et al. 1994), and European seabass (*Dicentrarchus labrax*) (Saillant et al. 2001). Increasingly focus is being directed toward the broad proteomic analysis of ovarian fluids to identify candidate biomarkers (Rime et al. 2004, Nynca et al. 2015, Mohagheghi Samarin et al. 2015). This approach brings with it technical challenges including analytical outputs being dominated by a high abundance of certain proteins such as vitellogenin which reduce the resolution of analysis (Rime et al. 2004), and 2) limited access to genomic information (high quality annotated genome sequences corresponding inferred protein libraries) as studies focus on non-model species with limited resources with a 110-

fold reduction in the number of transcribed and identified proteins than the human proteome in the case of rainbow trout at time of their publication (Nynca et al. 2015). However, some evidence for candidate biomarkers and modes of action have been proposed. Rime et al (2004) identified lipovitellin II increases during post ovulatory ageing in rainbow trout, which was correlated with decreasing egg quality. The authors suggest cellular leaking of the eggs into the ovarian fluid due to the increasing abundances of lectin was the mode of action and thus suggested that combinations of these proteins could be used as biomarkers of ageing within the species (Rime et al. 2004).

As stated previously, the commercial production of lumpfish will be reliant on establishing productive stripping protocols, and as such reliable gamete management strategies is essential in order to effectively close the life cycle management of the species (Powell et al. 2018). Once broodstock are maintained under appropriate environmental rearing conditions to allow the natural spawning behaviour to be expressed (chapter 2) and diets are provided to assure the eggs have the optimum biochemical composition (chapter 3) attention must be directed towards the optimum management of the ova post ovulation to maximise fertilisation success. It is clear from all other established finfish species, where artificial stripping is required, that a basic understanding of ovulatory rhythms in combination with an understanding of the over-ripening period for a given species will play a significant role in determining productivity (Migaud et al. 2013). Accurate protocols for stripping and the identification of good quality biomarkers within the ovarian fluid of lumpfish could reduce the high level of reported “female variability” found in both wild and captive stocks, and at least reduce the current production investment in poor quality eggs.

This chapter aims to measure the impact of *ex vivo* post ovulatory aging on lumpfish egg performance for both wild and captive derived eggs to determine the species "over-ripening window". Furthermore, it aims to characterise the ovarian fluid proteome and examine how this change in relation to measured quality, egg origin (wild vs farmed broodstock) and in response to the process of over-ripening itself.

5.2. Methods

5.2.1 Egg samples and broodstock information

For “wild broodstock” eggs used in this study, fish were obtained from Skjerneset Fisk at Averøy, Norway. Ten females and three males were sampled at 15th May 2019. Each fish was anaesthetised with MS-222 (200 mg L^{-1}) (Pharmaq AS, Norway) before physiological data, Wet weight (± 1 g) and Total length (± 1 mm) was measured. Males were killed with an overdose of MS-222 (1 g L^{-1}) before the gonads were dissected out and ground, sieved and mixed 1:1 with AquaBoost sperm coat© (Cryogenetics, Norway). The females were then stripped before being killed by an overdose of MS-222 (1 g L^{-1}). Volume of eggs were measured using a plastic measuring cylinder (± 10 ml), 1 ml of eggs were taken from the batch and counted to get the eggs per ml, with the batch fecundity estimated from body weight and total number of eggs stripped. Eggs and milt were then packaged and returned to NOFIMA CFU, Sunndalsora, AS in a temperature-controlled box. Physiological data found in this study found in table 5.1.

Table 5.1: Broodstock information for the three stocks used within this study (n=10 wild, n=6 captive good, n=6 captive bad) ± Standard error different superscript denotes significant difference in means.

Stock	Weight (g)	Length (mm)	Egg volume (ml)	Eggs per ml	Fecundity (ova per Kg body weight)	Fertilisation (%) "fresh"	Eyeing (%) "fresh"	Hatching (%) "fresh"
Wild	2952.0 ±294.1[a]	426.2 ± 19.9[a]	390.0 ± 74.2[a]	103.6 ± 13.5[a]	117832.9 ±22130[a]	97.6 ±2.0[a]	76.8 ±7.4[a]	54.6 ±8.0[a]
Captive "Good"	2177.0 ± 298[b]	343.3 ± 11.9[b]	235.0 ± 78.3[a]	129.5 ± 47.7[a]	61291.4 ± 21491[b]	77.7 ±8.2[b]	59.0±20.1[a]	31.1 ±12.7[b]
Captive "bad"	1938.3 ±127.6[b]	329.3 ± 9.2[b]	235.0 ± 131.8[a]	152.6 ± 17.9[a]	60986.3 ± 20977[b]	47.9 ±9.5[c]	0.9±1.5[b]	0.0 ±0[c]

Stock	Weight	Length	Sperm Density
Wild	1350 ± 127[a]	347.1 ± 44.5[a]	12.07 ± 1.40[a]
Captive	638.3 ± 188.4 [b]	228 ± 17[b]	12.37 ± 2.14[a]

For “captive broodstock” eggs used in this study came from F1 broodstock which were reared at Otter Ferry Sea Fish Ltd, Scotland. Broodstock were 17 months old and raised from eggs derived from Scottish caught broodstock. Lighting was maintained at a low intensity 24 hr photoperiod for the entire grow out period. Prior to sampling, fish were raised with a reduced summer temperature <10 °C (from hatch, 9.4 °C ± 0.8 °C) based on conclusions from Chapter 2 (Pountney et al. 2020a). Fish were fed to satiation on Samaki Marine Pellet, (World Feeds, James A Makie (agricultural), UK). Fish started maturing in December 2018 with natural spawning taking place in mid-January 2019. Fish were stripped between January and March 2018; 10 females were used for this study. Females were monitored every 3 days for developmental status utilising ultrasound methods described in chapter 2.2.1. Females were stripped when hydrated oocytes were observed in the ovaries. At the point of stripping wet weight (± 1 g) and total length were recorded (± 1 mm) (Table 5.1). Males were killed with an overdose of MS-222 before wet weight and total length and was recorded (Table 5.1) before the gonads being dissected out and ground, sieved and mixed 1:1 with AquaBoost sperm coat© (Cryogenetics, Norway).

5.2.1.2 Characterisation of the impact of *ex vivo* over ripening on egg quality.

For 10 batches of wild and 6 batches of captive stocks a time series study of the impacts of over-ripening of eggs *ex vivo* was established as follows. While a number (45) of egg batches were incubated for the captive runs, only 6 experienced any hatching success in the 4hr post stripping time point. Only these 6 individuals were used for the assessment of impact of *ex vivo* over ripening on egg quality. For all batches, eggs were stripped into a beaker and held in a fridge within their ovarian fluid at 4 °C (± 0.6 °C (Wild), ± 0.4 °C (Captive)) until samples of the eggs were required

between stripping and fertilisation. At the point of fertilisation 1.5 ml of ovarian fluid was withdrawn using a pipette and frozen in a 2 ml Eppendorf (VWR, UK) at – 80 °C.

At 4 hr, 12 hr, 24 hr, 48 hr, 72 hr, 96 hr, 120 hr post stripping, 1ml of eggs were removed using a volumetric scoop and fertilised with a volume of milt to give 300,000 sperm per egg, mixed in 50 ml of 0.2 µm filtered sea water and left for 15 minutes in a petri dish. At the same time a 4 ml sample of ovarian fluid was removed and preserved (-80 ° C) for subsequent proteomic analysis (5.2.2). The petri dish was filled with 30 ml of 0.2 µm-filtered seawater and placed on a slowly moving shaker in a climate-controlled room (8.4 °C ± 1.1 °C (wild), 8.1 °C ± 0.6 °C (captive)). This was conducted in triplicate for each batch of eggs at each timepoint.

Prior to fertilisation wild milt was tested for motility, using a light microscope, and density using a spectrophotometer at 540 nm (SDM6, Cyrogenetics, Norway), for captive milt, motility was assessed via a light microscope and haemocytometer counts as conducted in chapter 4 (Pountney et al 2020b). Briefly: a 1:1000 dilution of milt was made using a commercial milt extender (SpermCoat, Cryogenetics, Norway) and three replicate counts were made in a haemocytometer (Hirschmann, Germany) using an Olympus microscope (Olympus optical, UK) to calculate sperm concentration (sperm per ml of milt). A minimum of 100 grids of 0.25 nl were counted to obtain the average cell count, which was calculated as *Sperm per ml= (Total count/100)* × *(4000/1000)* ×*100*.

Milt which was “poor” in motility (displaying low activation, abnormal swimming), or low in sperm density (> 7×10^{9} / mL) was rejected. A milt “pool” was created with three good quality males and the density was re-measured to allow for accurate sperm

per egg fertilisation number (300,000 sperm per egg). Milt was maintained within the sperm coat at 4 °C for the duration of the experiment.

5.2.1.3 Fertilisation assessments

At 24 hours post fertilisation, eggs were observed under a microscope (Olympus optical, UK) to assess for fertilisation. A subset of fertilised eggs was transferred to 48 well micro well plates (Starstedt, USA), one egg to a well containing 0.2 µm filtered seawater for the duration of development. Fertilisation rate was calculated by identifying fertilised and non-fertilised eggs and dividing by the total number of eggs and expressing as a percentage. Microwell plates were maintained in a climate-controlled room (8.4 °C ± 1.1 °C (wild), 8.1 °C ± 0.6 °C (captive)).

5.2.1.4 Eyeing and hatching assessments

Microwell plates were monitored throughout development, at 150 DD an assessment of eyeing was conducted by counting the number of eggs, eyed eggs and non-eyed eggs to provide a total number and eyed egg percentage. Eggs were monitored daily at the point of first hatch and any hatched juveniles were removed and euthanised using an overdose of MS-222. An assessment of hatching was conducted by counting the number of eggs, hatched juveniles and non-hatched eggs to provide a total number and hatched juvenile percentage.

5.2.2 Ovarian fluid proteomic analysis

In total 10 wild and 10 captive ovarian fluid samples were used. Three separate analysis were conducted;

1) Wild and captive ovarian fluid proteomes comparison.

For this five samples of both wild and captive ovarian fluid were used (Table 5.1, “wild” and “Captive good”)

2) “Good” quality and “bad” quality egg ovarian fluid comparison.

   Determination of “bad” quality eggs within the wild populations was difficult due to a limited range of quality experienced within the wild populations. Thus, captive experimental animals were used within the comparisons for quality determinants. Good quality eggs within the captive samples were those egg which achieved hatching, “bad” quality eggs did not achieve hatching but did develop sufficiently to produce eyed eggs. The “good” ovarian fluid samples were the same samples used in the above analysis. With the 5 “bad” quality eggs from the bad quality stocks described above (Table 5.1, Captive bad).

3) The effects of *ex-vivo* ova ageing on ovarian fluid proteome.

   Comparison of wild “fresh” (*n*= 5) and wild “aged” (96 hr, *n*=5) ovarian fluids. The non-aged wild samples were the same sampled from comparison 1. The aged samples were taken from the same individuals after 96 hr from the point of stripping.

5.2.2.1 Lysis method optimisation

Prior to undertaking the full analysis, a subset of samples (3 captive samples) were used to optimise extraction techniques; the aim was to find a lysis buffer, which would provide the maximum solubilisation and denaturation of all proteins. Three methods were tested; No lysis, lysis method proposed using TMT (tandem mass tagging) protocol (Thermo Scientific, MAN0016969) (Morro et al. 2020) and an acetone precipitation (Thongboonkerd et al. 2002). All reagents were obtained from Sigma Aldrich (UK) unless otherwise specified.

All followed the method below (2.2.2), with the TMT lysis method and acetone precipitation conducted before BCA protein concentration determination.

TMT proposed lysis: Lyse the cells with 5:1 Buffer to sample lysis buffer (200µL of the denaturing reagent (10% SDS) in 1.8mL of 100mM TEAB). The lysate was then centrifuged at 16,000 × g for 10 minutes at 4 ° C. The supernatant was separated and transferred to a new tube. Protein concentration was the conducted by BCA and followed the protein extraction method described below (section 2.2.2).

Acetone precipitation: The samples were fractionated by 50% acetone precipitation for 10 minutes followed by centrifugation at 12,000 ×g for 5 minutes. The pellet was reabsorbed in 1 ml of TEAB and protein concentration assessed by BCA and followed the protein extraction method below (2.2.2).

Following results (see section 3.2, Table 5.2) of this trial the “no lysis” method was adopted for the main experimental work.

Table 5.2: Results from the method optimisation trial, No lysis, TMT method lysis and acetone lysis precipitation. Values are mean (*n*= 3) ± standard error, different superscripts denote significant differences between mean values.

Treatment	Identified individual proteins	Total number of peptides	Number of independent proteins
No Lysis	81 ± 7 ^{a}	212 ± 27 ^{a}	41 ± 14 ^{a}
TMT	46 ± 11 ^{b}	98 ± 26 ^{b}	26 ± 10 ^{b}
Acetone	60 ± 14 ^{ab}	147 ± 58 ^{ab}	28 ± 10 ^{ab}

5.2.2.2 Extraction of protein from ovarian fluid

The protein concentration of ovarian fluid was measured using a Bicinchoninic Acid assay kit (BCA1, Merck Life Science UK Limited, Dorset, England) (Smith et al. 1985) and read using a SPARK multimode microplate reader (Tecan. Männedorf, Switzerland). The following steps, protein reduction, alkylation, precipitation, trypsin digestion and TMT labelling of peptides were performed following manufacturer instructions (TMT10plex™ Isobaric Label Reagent Set, ThermoFisher Scientific), followed by a clean-up of the multiplexed sample using Hypersep SpinTip (Merck Life Science UK Limited, Dorset, England) , according to manufacturer instructions as conducted in Morro et al. (2020). For each individual sample, 100 µg of protein (as identified by BCA) were transferred into a new tube and the final volume was adjusted to 100 µL with 100 mM Triethylamonium bicarbonate (TEAB). Five microlitres of the 200 mM tris(2-carboxyethyl) phosphine (TCEP) were added followed by an incubation at 55 °C for 1 hour. Five microlitres of 375 mM iodoacetamide were added to each sample and incubated for 30 minutes protected from light at room temperature. Six volumes (approximately 600 µL) of pre-chilled (- 20 °C) acetone were added and the protein precipitation proceeded overnight at -20 °C. The samples were centrifuged at 8000 × g for 10 minutes at 4 °C. The acetone was removed by decantation and the pellet was allowed to air dry for 2 - 3 minutes. The acetone-precipitated protein pellets were resuspended in 100 µL of 100 mM TEAB. Sequencing grade trypsin (2.5 µg per tube, Roche, Switzerland) was added to each sample and incubated overnight at 37 °C. Labels were equilibrated at room temperature for 5 minutes and 41 µL of acetonitrile was added to each label vial. Eighty-five microlitres of each sample was added to a specific label vial and incubated for 1 hour at room temperature, information regarding label and tube allocation can be found in Table 5.3. The reaction was

quenched by adding 8 µL of 5 % hydroxylamine followed by a 15-minute incubation at room temperature. One hundred and five microlitres of each tagged sample were combined into a single vial and vortexed and dry using a vacuum drier. Dry multiplexed samples were resuspended in 100 µL of 100 mM TEAB and pipetted into a previously equilibrated Hypersep SpinTip (following manufacturer instructions). Multiplexed samples where vacuum dried once more. All samples were stored at -80 °C.

Table 5.3: Tag and Tube allocation for ovarian fluid samples extracted. Pooled samples contained equal levels of protein from each on the individual female samples as measured by BCA.

TAG	**SAMPLES**		
	Tube 1	**Tube 2**	**Tube 3**
126	Wild (T1)	Captive (G)	Wild (T1)
127N	Wild (T1)	Captive (G)	Wild (T1)
127C	Wild (T1)	Captive (G)	Wild (T1)
128N	Wild (T1)	Captive (G)	Wild (T1)
128C	Wild (T1)	Captive (G)	Wild (T1)
129N	Captive (G)	Captive (B)	Wild (Aged)
129C	Captive (G)	Captive (B)	Wild (Aged)
130N	Captive (G)	Captive (B)	Wild (Aged)
130C	Captive (G)	Captive (B)	Wild (Aged)
131N	Captive (G)	Captive (B)	Wild (Aged)
131C	Pooled Samples	Pooled Samples	Pooled Samples

5.2.2.3 1-D SDS PAGE analysis

The following method was conducted as in (Nynca et al. 2015, Morro et al. 2020). For each ovarian fluid sample (*n* = 15 in total), 10 µg of ovarian fluid was mixed in reducing buffer (13.1 mM Tris—pH 6.8, 2.63 % v/v Glycerol, 0.42 % v/v sodium dodecyl sulfate (SDS), 0.243 % v/v bromophenol blue and 163.5 mM dithiothreitol (DTT)), heated up to 95 ˚C for 5 min and centrifuged at 2,000 g for 30 s. Reduced lysates were loaded into a 1-D SDS polyacrylamide gel (4–15 %, Mini-PROTEAN TGX, BIO-RAD) with a protein ladder reference (5 µl, BenchMark, 10-220 kDa, Bio-rad, MERC UK). Gels

were run using a Mini PROTEAN Tetra Cell System (Bio-Rad) at 200 V (400 mA) for around 50 min. Protein bands were fixed and stained with SimplyBlue Safestain following the manufacturer's instructions and destained overnight in MilliQ water (Millipore, Merck) at RT. 1-D SDS-PAGE pictures were taken using an inGenius LHR Gel Imaging System (SynGene), Gel images for SDS PAGE can be found in figures 5.1 Wild and Captive, Figure 5.2 Good and bad quality eggs, 5.3 Fresh and Aged eggs.

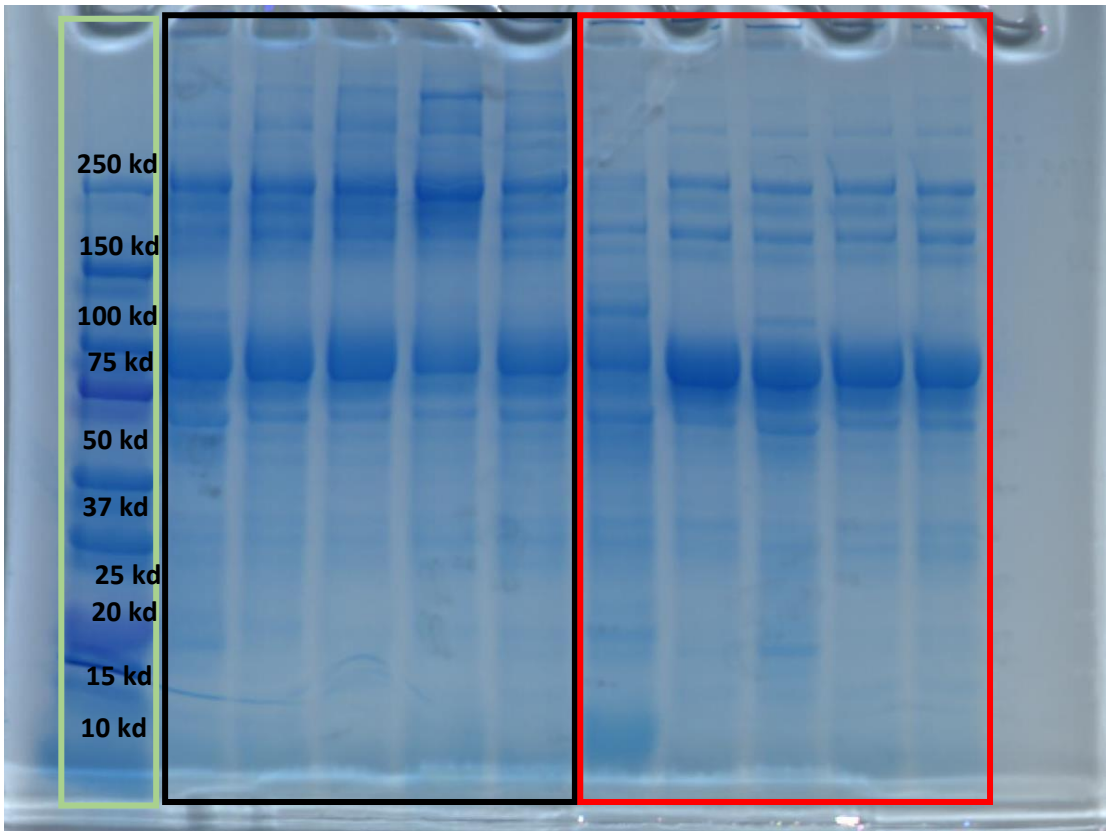

Figure 5.1: Gel electrophoresis of lumpfish ovarian fluid proteins, lanes 1 to 5 (Black) proteins derived from wild lumpfish ovarian fluid, lanes 6 to 10 (Red) proteins derived from captive lumpfish ovarian fluid. Green lane is 10-220 kDa, Bio-rad, MERC UK Standard Ladder.

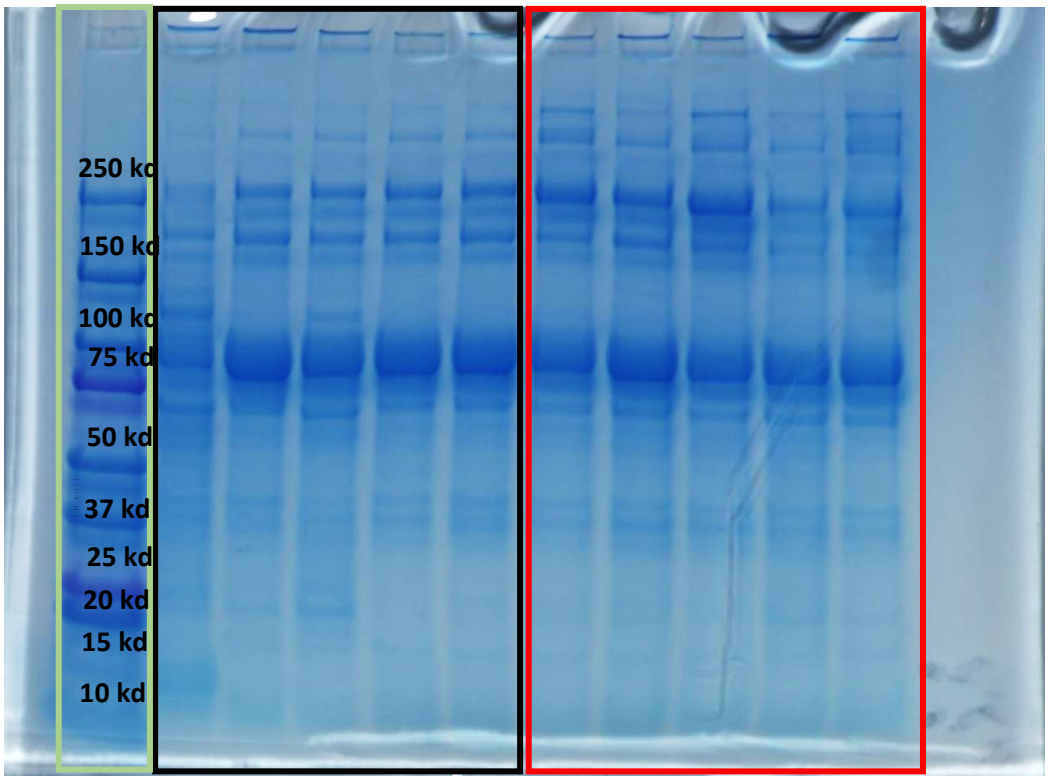

Figure 5.2: Gel electrophoresis of lumpfish ovarian fluid proteins, lanes 1 to 5 (Black) proteins derived from “Good quality” captive lumpfish ovarian fluid, lanes 6 to 10 (Red) proteins derived from “Bad quality” captive lumpfish ovarian fluid. Green lane is 10-220 kDa, Bio-rad, MERC UK Standard Ladder.

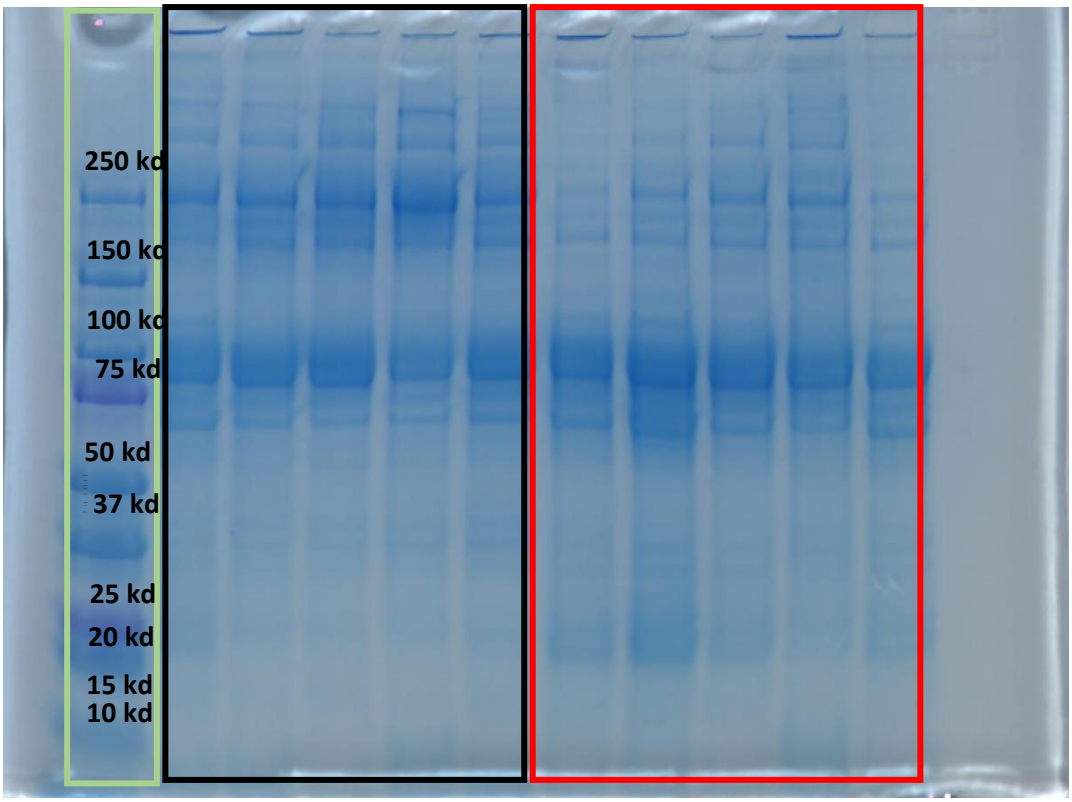

Figure 5.3: Gel electrophoresis of lumpfish ovarian fluid proteins, lanes 1 to 5 (Black) proteins derived from wild lumpfish ovarian fluid taken at stripping, lanes 6 to 10 (Red) proteins derived from “Aged” wild lumpfish ovarian fluid. Green lane is 10-220 kDa, Bio-rad, MERC UK Standard Ladder.

5.2.2.4 LC-MS/MS data analysis and protein identification

Tryptic digests were analysed with an LTQ-Orbitrap XL LC−MSn mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) equipped with a nanospray source and coupled to an Ultra High Pressure Liquid Chromatographer system (Waters nanoAcquity). Initially, 5 µL of sample resuspended in ultrapure water were loaded, desalted and concentrated in a BEH C18 trapping columns (Waters) with the instrument operated in positive ion mode. The peptides were then separated on a BEH C18 nanocolumn (1.7 µm, 75 µm × 250 mm, Waters) at a flow rate of 300 nL/min using an ACN/water gradient; 1% ACN for 1 min, followed by 0−62.5% ACN over 21

min, 62.5− 85% ACN for 1.5 min, 85% ACN for 2 min and 100% ACN for 15 min. MS spectra were collected using data-dependent acquisition in the m/z range 400−2,000 using a precursor ion resolution of 30,000, following which individual precursor ions (top 5) were automatically fragmented using collision induced dissociation with a relative collision energy of 35%. Dynamic exclusion was enabled with a repeat count of 2, repeat duration of 30 s and exclusion duration of 180 s.

Mass spectrometry data was analysed using Progenesis QIP (Nonlinear Dynamics). Each tube was analysed independently and were sampled and run through the mass spectrometer three independent times. The initial search parameters allowed for a single trypsin missed cleavage, carbamidomethyl fixed modification of cysteine residues, oxidation of methionine (variable), acetylation of N-terminal peptides, a precursor mass tolerance of 10 parts per million (ppm), charge of deconvoluted ions of over 1, a fragment mass tolerance of ±0.5 Da, and FDR of 0.01. After normalization, the Hi3 (Top3) method was used for protein quantification (Li et al., 2009); therefore, a minimum of 3 peptides was required for quantification. Moreover, only those proteins identified based on at least one unique peptide were quantified.

Statistical differences were tested by ANOVA in Minitab 18. To be considered differentially abundant proteins (DAPs), a p-value below 0.05 ($p < 0.05$) and a fold change (FC) bigger than 2 was required (Morro et al. 2020). Peptide sequences were matched to a database search against the ballan wrasse (*Labrus bergylta*) SwissProt database, which was downloaded from MASCOT [all databases downloaded in August 2020] and loaded into Progenesis QIP. The ballan wrasse 'predicted' proteome was selected as it was the closest related species-specific proteome with sufficient inputs within the SwissProt database. Those identified as 'uncharacterised' in the ballan wrasse genome were sequentially blasted against the Atlantic salmon,

zebrafish (*Danio rerio*), and whole database of Teleost proteomes UniprotProt databases, in this order of preference. Only homologies of E-value lower than 0.01 were accepted as valid. Where ballan wrasse was not used within the reporting the species is indicated.

5.2.2.5 Further analysis

A total of 105 proteins were uncharacterised using the ballan wrasse proteome database. These proteins were blast searched against the zebrafish proteome, those which remained uncharacterised were then searched against the Atlantic Salmon proteome, those which remained unidentified were blast searched against all teleost data, where this is the case the species used has been highlighted.

The e value for protein identification was set at < 0.001, with priority of identification being set at ballan wrasse > zebrafish > Atlantic Salmon > Teleost (with closest ID's species chosen).

5.2.3 Statistics

All statistics were conducted in Minitab 18 software. Percentage data were arcsine transformed before analysis, in Microsoft Excel. A General linear model was used to determine the effects of ex vivo ageing on egg quality criteria in both wild and captive stocks. A Tukeys t test was used to test for significant difference between wild and captive egg quality criteria and each time point.

5.3. Results

There was significant difference between the wild and captive stock with regards to weight and length (Table 5.1). With regards to egg parameters there was no significant difference between stocks in terms of eggs per ml or stripped egg volume, however batch fecundity was significantly higher (+ 83 %) within the wild stocks compared to the captive stock. There was significantly lower batch success within the captive stocks (also seen in those used in Chapter 3), with 10 batches reaching the eyeing stage and 11 % of all batches (6) producing hatched juveniles.

5.3.1 Characterisation of the impact of *ex vivo* over ripening on egg quality

There was a significant effect of time post stripping on the quality of lumpfish eggs (Figure 5.1 – 5.3). Fertilisation rates started at 97.5 ± 0.3 % for the wild stocks and 77.6 ± 1.6 % for the captive stocks. They decreased to 18.6 ± 5.8 % and 2.9 ± 0.8 % at 120 hours post striping for wild and captive stocks respectively (Figure 5.4). Within the wild stocks there was no significant difference in fertilisation rate until 48 hours post stripping. Within the captive stocks there was a significant decrease in fertilisation rate at 24 hours post stripping. Within the wild stocks there was significant decreases between every time point after 72 hours in fertilisation rate. Whereas in the captive stocks there was no further significant decrease after 24 hours until 96 hours post stripping. At all-time points, mean fertilisation rates were significantly lower in captive egg batches compared to wild egg batches.

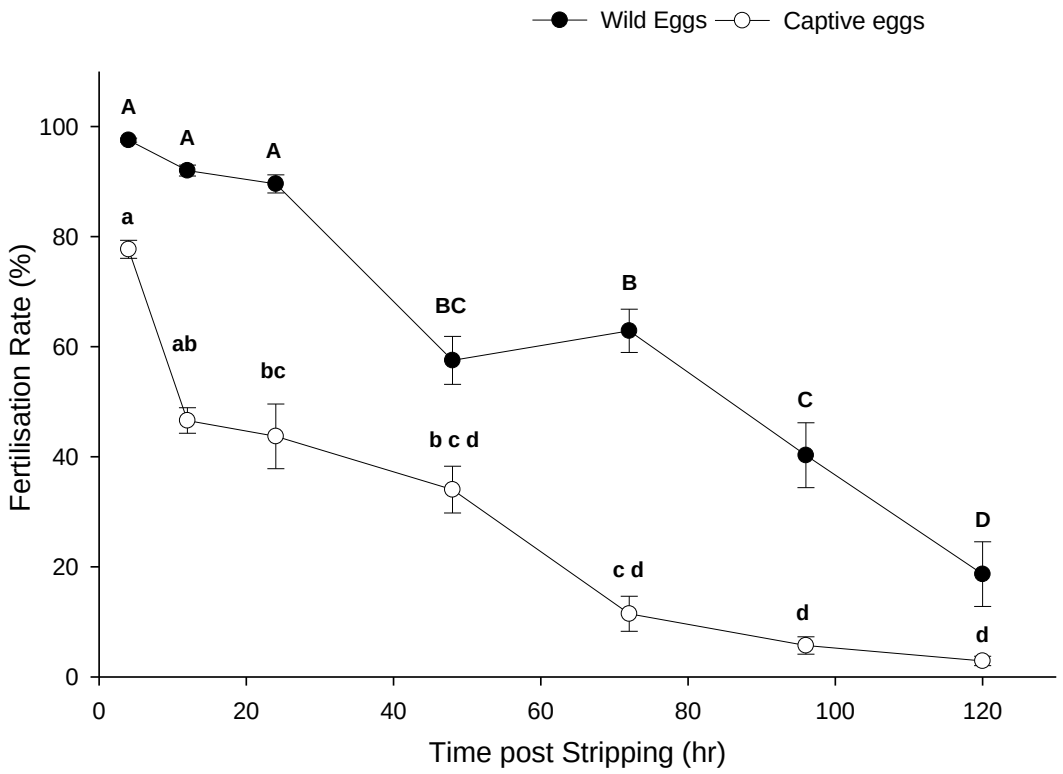

Figure 5.4: Fertilisation rates, measured at 24hr post fertilisation, for lumpfish eggs from wild and captive (*n* = 6) broodstocks, aged for 4, 12, 24, 48, 72, 96, and 120 hours post stripping. Standard error is plotted with different letter superscripts denoting significance within treatment group.

Eyeing rates started at 75 ± 4.1 % for the wild stocks and 49.7 ± 5.1 % for the captive stocks. They decreased to 2.7 ± 1.5 % and 0.4 ± 0.1 % at 120 hours post striping for wild and captive stocks respectively (Figure 5.5). Within the wild stocks there was no significant difference in eyeing rate until 48 hours post stripping. Within the captive stocks there was a significant decrease in eyeing rate at 24 hours post stripping. Within the wild and captive stocks there was no significant decreases in eyeing rate between every time point after 72 hours and 12 hours respectively. With the exception of 120 hours post stripping when both groups were comparable, mean eyeing rate was significantly lower in the captive compared to the wild group.

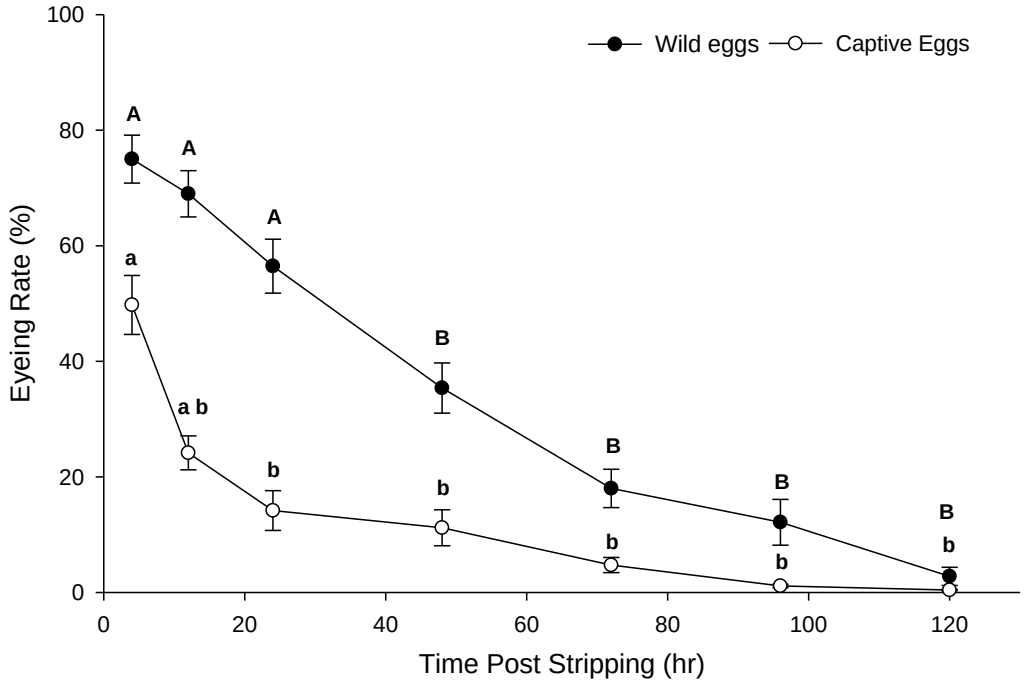

Figure 5.5: Eyeing rates, measured at 150DD, for lumpfish eggs from wild and captive (*n* = 6) broodstocks, aged for 4, 12, 24, 48, 72, 96, and 120 hours post stripping. Standard error is plotted with different letter superscripts denoting significance within treatment group.

Hatching rates started at 53.1 ± 2.0 % for the wild stocks and 28.3 ± 2.9 % for the captive stocks. They decreased to 0 % at 120 hours post striping for both groups (Figure 5.6). Within the wild stocks there was no significant decrease in hatching rate until 24 hours post stripping. Within the captive stocks there was a significant decrease in hatching rate at 12 hours post stripping. Hatching rates in the wild stocks reached 0% at 96 hours post stripping, whereas the wild stocks decreased to 0% at 48 hours post stripping. Mean hatch rate in captive group was significantly lower in all time points up to 72 hours post stripping.

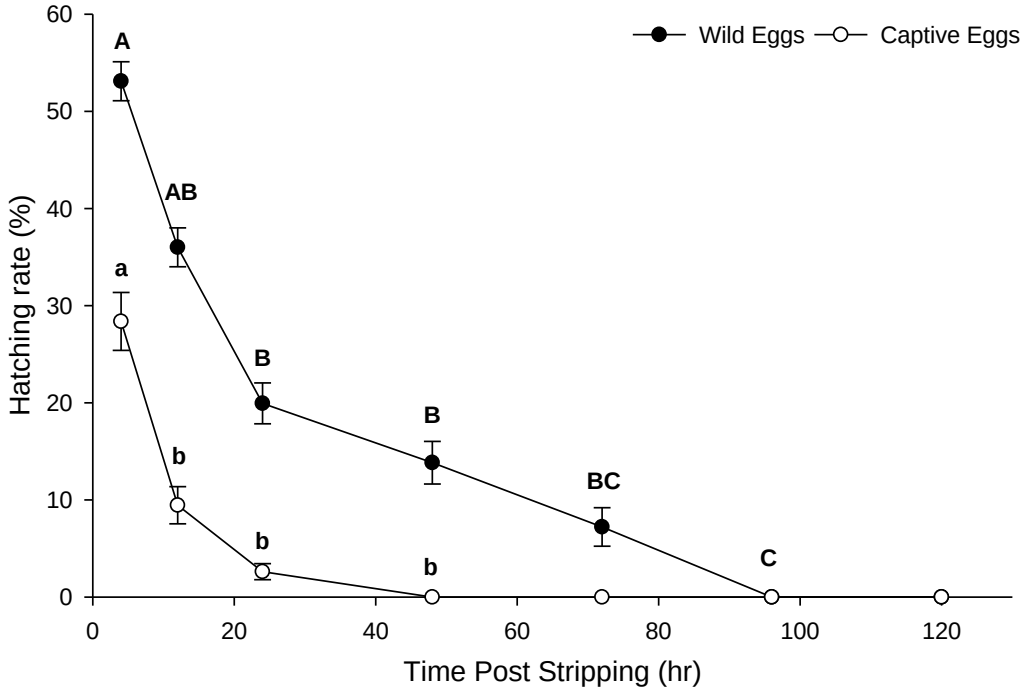

Figure 5.6: Hatching rates for lumpfish eggs from wild and captive (*n* = 6) broodstocks, aged for 4, 12, 24, 48, 72, 96, and 120 hours post stripping. Standard error is plotted with different letter superscripts denoting significance within treatment group.

5.3.2 Ovarian fluid proteome method optimisation

The “No lysis” method and the acetone lysis methods had the most identified number of individual proteins, and the highest level of total identified peptides (Table 5.2). The “No lysis” method was significantly higher in both factors than the TMT method, but was not significantly different from the acetone method. The use of a lysis method in the case did not provide any benefit in the number of proteins identified, or the scope of identification and thus the decision was made not to use any lysis method in the main body of work.

5.3.3 Wild and Captive comparison

Egg quality data for the wild samples used in this analysis fertilisation (97.6 % ±2.0), eyeing (76.8 % ±7.4), hatching (54.6 % ±8.0). For the captive stock's quality data is as follows fertilisation (77.7 % ±8.2), eyeing (59.0 % ±20.1), hatching (31.1 % ±12.7) (Table 5.1).

5.3.3.1 Protein identifications

For the wild and captive comparisons, a total number of 13718 ± 1693 peptides were identified which were mapped onto 820 proteins. Of those 141 ± 8 met the requirements for reliable identification and quantification (i.e. quantified proteins; identified by at least 1 unique peptide and at least 3 peptides).

5.3.3.2 Protein Abundance

There was a high level of variation between runs of the same sample tube, within the top 20 most abundant proteins for each run there were only 4 proteins shared between at least two replicates, and 1 shared with all 3 replicates (Table 5.4). Within the captive groups there was also a high level of variation within the top 20 most abundant proteins for each run there were only 4 shared proteins between at least two replicates, and none shared between all three (Table 5.5). Within each replicate (A, B and C) there was a high number of shared proteins within the 20 most abundant proteins between the wild and captive stocks, 16, 13 and 13 (A, B and C respectively).

Table 5.4: Most abundant ovarian fluid proteins from the wild caught lumpfish (*n*= 5) for each replicate. “ID” Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3E737	Phosphatidylinositol-5-phosphate 4-kinase, type II, gamma a	62.98	A0A3Q3ECA8	Solute carrier family 4 member 11	44.26	A0A3Q3F8Q9	Proteasome 26S subunit, non-ATPase 12	55.90
A0A3Q3FLN3	Transcription elongation factor SPT6 *	54.40	A0A3Q3F9P4	Capping protein inhibiting regulator of actin dynamics *	37.72	A0A3Q3FLN3	Transcription elongation factor SPT6 *	37.72
A0A3Q3GEI5	Src homology 2 domain containing transforming protein D, a	44.12	A0A3Q3E856	Beta 3-glucosyltransferase b	26.15	A0A3Q3FNL3	Keratin 97	21.34
A0A3Q3F836	Histone deacetylase 10	38.98	A0A3Q3GJQ8	Excision repair cross-complementation group 4	24.97	A0A3Q3NA92	Rho guanine nucleotide exchange factor 26	20.68
A0A3Q3G3L2	Heart and neural crest derivatives expressed 2	37.80	A0A3Q3GIU4	Cingulin-like 1	14.99	A0A3Q3FCS3	UHRF1 binding protein 1-like	17.43
A0A3Q3G291	F-box protein 41	37.72	A0A3Q3EJJ8	Spectrin repeat containing, nuclear envelope 1b	13.91	A0A3Q3G106	Leucine rich repeat containing 39	13.35
A0A3Q3MGL4	FA complementation group E	37.72	A0A3Q3G7B8	G_PROTEIN_RECEP_F1_2 domain-containing protein	13.74	A0A3Q3H0X3	TINF2_N domain-containing protein	12.79
A0A3Q3GT17	VPS33B late endosome and lysosome associated	28.26	A0A3Q3H1S6	Unconventional myosin-Id (Myosin 1d) *	13.42	A0A3Q3F9Q7	R3H domain and coiled-coil containing 1-like	10.75
A0A3Q3FY51	Bromodomain adjacent to zinc finger domain, 1A	25.61	A0A3Q3F8I3	Si:ch211-210b2.3	12.86	A0A3Q3EXX5	Si:dkey-224e22.2	9.01
A0A3Q3F5W6	Nuclear cap binding protein subunit 1	17.58	A0A3Q3E0X1	Solute carrier family 35 member B1	12.47	A0A3Q3ND39	Piwi-like RNA-mediated gene silencing 1	8.82
A0A3Q3F9Q7	R3H domain and coiled-coil containing 1-like	16.00	A0A3Q3EKC5	Glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	12.17	A0A3Q3FF52	RAD21 cohesin complex component like 1	7.93
A0A3Q3H272	Tubulin tyrosine ligase-like family, member 12	15.60	A0A3Q3L1E9	Cadherin-2 (Neural cadherin) (N-cadherin) *	11.97	A0A3Q3NPD1	Ecotropic viral integration site 5b	7.83
A0A3Q3MZ45	Nucleolar complex protein 3 homolog	15.49	A0A3Q3EWI9	Zinc finger and BTB domain-containing protein 24 *	11.81	A0A3Q3E320	Pleckstrin homology and RhoGEF domain containing G4B	7.72
A0A3Q3GBP9	Unconventional myosin-Id *	15.19	A0A3Q3F9Q7	R3H domain and coiled-coil containing 1-like	11.79	A0A3Q3L7H1	Inositol 1,4,5-trisphosphate receptor interacting protein	7.68

Table 5.4 (continued): Most abundant ovarian fluid proteins from the wild caught lumpfish (*n*= 5) for each replicate. “ID” Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3GBP9	Unconventional myosin-Id *	15.19	A0A3Q3F9Q7	R3H domain and coiled-coil containing 1-like	11.79	A0A3Q3L7H1	Inositol 1,4,5-trisphosphate receptor interacting protein	7.68
A0A3Q3FJM3	CD166 antigen homolog A *	12.78	A0A3Q3EXX5	Si:dkey-224e22.2	11.67	A0A3Q3GNB6	Vestigial-like family member 2a	7.64
A0A3Q3MF80	Dedicator of cytokinesis 7	12.54	A0A3Q3LW57	Eph receptor A7	10.75	A0A3Q3ETB8	Interleukin 1 receptor accessory protein-like 1a	7.43
A0A3Q3FE21	NAD(P)(+)--arginine ADP-ribosyltransferase	12.49	A0A3Q3M7Q0	Polybromo 1	10.36	A0A3Q3MHD0	Dynamin-type G domain-containing protein	7.30
A0A3Q3E0R8	G-patch domain containing 2 like	12.01	A0A3Q3GE52	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)	10.31	A0A3Q3FG15	Acyl-CoA synthetase long chain family member 4a	7.21
A0A3Q3MTR1	Glycerophosphodiester phosphodiesterase domain containing 5b	11.66	A0A3Q3F5W6	Nuclear cap binding protein subunit 1	10.29	A0A3Q3EF98	Centrosomal protein of 290 kDa (Cep290) *	7.06
A0A3Q3G7S7	Plectin a	11.09	A0A3Q3GP35	Centrosomal protein 72	10.18	A0A3Q3E2T5	Spectrin beta chain	6.88

Table 5.5: Most abundant ovarian fluid proteins from the captive derived lumpfish (*n*= 5) for each replicate. “ID” Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3GEI5	Src homology 2 domain containing transforming protein D, a	45.65	A0A3Q3GJQ8	Excision repair cross-complementation group 4	36.61	A0A3Q3G106	Leucine rich repeat containing 39	52.07
A0A3Q3E737	Phosphatidylinositol-5-phosphate 4-kinase, type II, gamma a	40.30	A0A3Q3ECA8	Solute carrier family 4 member 11	35.24	A0A3Q3FNL3	Keratin 97 OS=Labrus bergylta	48.44
A0A3Q3GT17	VPS33B late endosome and lysosome associated	34.97	A0A3Q3F9P4	Capping protein inhibiting regulator of actin dynamics *	30.35	A0A3Q3F8Q9	Proteasome 26S subunit, non-ATPase 12	32.99
A0A3Q3MTR1	Glycerophosphodiester phosphodiesterase domain containing 5b	34.02	A0A3Q3L1E9	Cadherin-2 (Neural cadherin) *	25.78	A0A3Q3FLN3	Transcription elongation factor SPT6 *	30.35
A0A3Q3G291	F-box protein 41	30.35	A0A3Q3G7B8	G_PROTEIN_RECEP_F1_2 domain-containing protein	23.45	A0A3Q3GPB6	BHLH domain-containing protein	18.94
A0A3Q3MGL4	FA complementation group E	30.35	A0A3Q3E856	Beta 3-glucosyltransferase b	21.55	A0A3Q3NA92	Rho guanine nucleotide exchange factor 26	17.69
A0A3Q3H272	Tubulin tyrosine ligase-like family, member 12	28.72	A0A3Q3GE52	Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)	20.64	A0A3Q3FCS3	UHRF1 binding protein 1-like	16.80
A0A3Q3MZ45	Nucleolar complex protein 3 homolog	20.05	A0A3Q3FTJ5	Dynein axonemal heavy chain 8	20.23	A0A3Q3H0X3	TINF2_N domain-containing protein	14.33
A0A3Q3G3L2	Heart and neural crest derivatives expressed 2	18.19	A0A3Q3FWZ3	Anion exchange protein	18.83	A0A3Q3G950	Prolactin releasing hormone receptor 2b	13.90
A0A3Q3FY51	Bromodomain adjacent to zinc finger domain, 1A	16.66	A0A3Q3EJJ8	Spectrin repeat containing, nuclear envelope 1b	17.87	A0A3Q3G1E3	Ribonuclease P/MRP 30 subunit	11.13
A0A3Q3ESP1	AT rich interactive domain 1Aa (SWI-like)	15.83	A0A3Q3F5W6	Nuclear cap binding protein subunit 1	14.43	A0A3Q3G2P6	Vacuolar protein sorting 13 homolog C	10.63
A0A3Q3FE21	NAD(P)(+)--arginine ADP-ribosyltransferase	15.17	A0A3Q3E837	Histone PARylation factor 1	13.51	A0A3Q3E320	Pleckstrin homology and RhoGEF domain containing G4B	9.26
A0A3Q3FJM3	CD166 antigen homolog A *	12.35	A0A3Q3G278	TATA box-binding protein-like 2 (TBP-like 2) *	11.98	A0A3Q3MHD0	Dynamin-type G domain-containing protein	9.02

Table 5.5 (Continued): Most abundant ovarian fluid proteins from the captive derived lumpfish (*n*= 5) for each replicate. “ID” Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3F9Q7	R3H domain and coiled-coil containing 1-like	11.27	A0A3Q3EL11	RAS p21 protein activator (GTPase activating protein) 1a	11.80	A0A3Q3L1E9	Cadherin-2 (Neural cadherin) *	8.47
A0A3Q3F2M3	PHD finger protein 8	10.86	A0A3Q3M7Q0	Polybromo 1	11.59	A0A3Q3MYK8	Integrator complex subunit 8	7.57
A0A3Q3F9P4	Capping protein inhibiting regulator of actin dynamics *	10.43	A0A3Q3E0X1	Solute carrier family 35 member B1	11.32	A0A3Q3FD36	Rhomboid 5 homolog 1a (Drosophila)	7.41
A0A3Q3FT82	ADP-ribosylation factor-like protein 3 *	10.43	A0A3Q3ET57	WASH complex subunit 5	11.06	A0A3Q3L7H1	Inositol 1,4,5-trisphosphate receptor interacting protein	6.72
A0A3Q3E0R8	G-patch domain containing 2 like	10.08	A0A3Q3GIU4	Cingulin-like 1	10.46	A0A3Q3F9Q7	R3H domain and coiled-coil containing 1-like	6.68
A0A3Q3F5W6	Nuclear cap binding protein subunit 1	10.04	A0A3Q3FI96	Amyloid beta (A4) precursor protein-binding, family A, member 2a	9.88	A0A3Q3NPD1	Ecotropic viral integration site 5b	6.27
A0A3Q3GBP9	Unconventional myosin-Id *	9.87	A0A3Q3F8I3	Si:ch211-210b2.3	8.93	A0A3Q3EXX5	Si:dkey-224e22.2	6.20

5.3.3.3 Treatment comparisons

Within the three replicates there were an average of 141 ± 8 proteins identified with 36 proteins identified in all of the three replicates, and 81 (A), 54 (B) and 75 (C) unique proteins for each replicate (Figure 5.7).

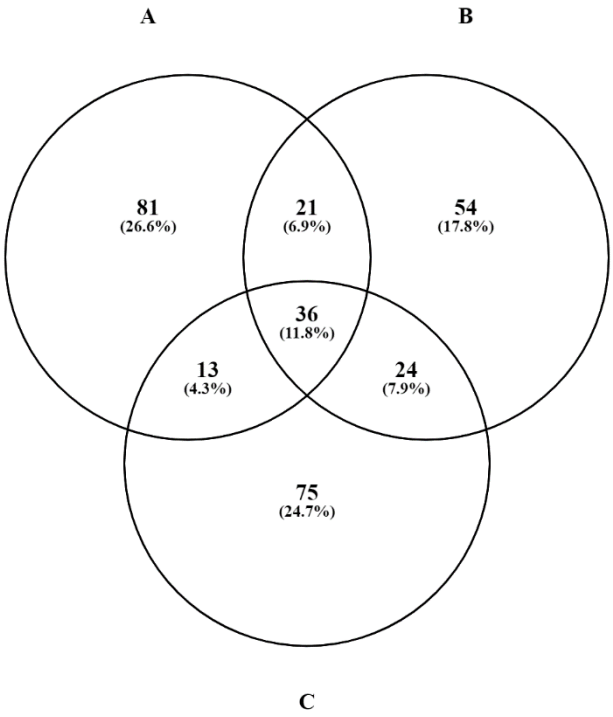

Figure 5.7: Venn Diagram of all identified proteins within each technical replicate conducted for the wild and Captive comparisons. Venny (2.1, Oliveros 2015)

There significant changes in protein abundance identified in all three replicates, 7 (A), 5 (B) and 5 (C), however 9 of these were only identified in one replicate, while still meeting the requirements for identification and quantification (Section 2.2.4), Table 5.6.

A decrease in relative protein abundance was identified in the majority (12 out of 15) of proteins which displayed a significant difference between wild and captive stocks. The greatest reduction was measured in Histone deacetylase 10 (A0A3Q3F836), with

levels in captive stocks only 23% of those in wild derived broodstocks. Only 3 proteins were more abundant within the captive stocks with Keratin 97 (A0A3Q3FNL3) displaying the largest significant increase from wild levels (226%).

Table 5.6: Proteins Identified as showing significant difference between the wild and captive ovarian fluid proteomes. Data is reported as the percentage change between mean of biological replicates (*n* =5) as a percentage of the wild abundance and p value (in brackets). Red cells indicate decreases in relative abundance of the protein, green cells indicate increased relative abundance * indicates initially unknown protein identified in zebrafish.

Protein ID	Name	Replicate A Rate of change (%), (p value)	Replicate B Rate of change (%), (p value)	Replicate C Rate of change (%), (p value)
A0A3Q3GRT1	CULLIN_2 domain-containing protein	59.72 (p = 0.011)	-	-
A0A3Q3GKW4	C2 domain containing 3 centriole elongation regulator]	51.97 (0.027)	-	-
A0A3Q3EB83	Cotranscriptional regulator FAM172A homolog *	54.13 (0.028)	-	-
A0A3Q3FN38	Spectrin repeat containing, nuclear envelope 1a	68.37 (0.038)	-	-
A0A3Q3F836	Histone deacetylase 10	23.44 (0.044)	-	-
A0A3Q3EIR8	RRM domain-containing protein	-	54.38 (0.04)	-
A0A3Q3EDV6	Stromal antigen 2a	-	-	160.89 (0.009)
A0A3Q3FNL3	Keratin 97	-	-	226.96 (0.028)
A0A3Q3EYG9	Coiled-coil_56 domain-containing protein	-	-	55.90 (0.038)
A0A3Q3G1W8	Kazrin, periplakin interacting protein a	65.01 (0.042)	65.96 ns	63.75 (0.049)
A0A3Q3F9Q7	R3H domain and coiled-coil containing 1-like	70.39 ns	66.05 ns	62.14 (0.049)
A0A3Q3F3E0	Pyruvate kinase	57.85 (0.01)	55.32 (0.003)	-
A0A3Q3FMH0	Olfactory receptor	65.05 ns	59.93 (0.041)	-
A0A3Q3EL11	RAS p21 protein activator (GTPase activating protein) 1a	-	196.81 (0.019)	104.45 ns
A0A3Q3ND39	Piwi-like RNA-mediated gene silencing 1	-	56.31 (0.027)	67.88 ns

5.3.4 Egg Quality comparisons

Egg quality data for the Good quality samples used in this analysis fertilisation (77.7 % ±8.2), eyeing (59.0 % ±20.1), hatching (31.1 % ±12.7). For the Bad quality samples quality data is as follows fertilisation (47.9 % ±9.5), eyeing (0.9 % ±1.5), hatching (0 %) (Table 5.1).

5.3.4.1 Protein identifications

Within the “good” quality and “bad” quality comparisons a total of 14952 ± 1386 peptides were identified which were mapped onto 862 proteins. Of those 190 ± 61 met the requirements for reliable identification and quantification (i.e. quantified proteins; identified by at least 1 unique peptide and at least 3 peptides).

5.3.4.2 Protein Abundance

Within the good quality runs within the top 20 most abundant proteins for each run there were only 8 proteins which are shared between at least 2 runs with 1 protein shared between all three groups (Table 5.7). Within the bad quality within the top 20 most abundant proteins for each run there were only 9 proteins shared between at least two runs with only 1 protein shared between all three (Table 5.8). Within each run (A, B and C) there was a high level of shared proteins within the 20 most abundant proteins between the wild and captive stocks, 17, 18 and 16 (A, B and C respectively).

Table 5.7: Most abundant ovarian fluid proteins from the "good" quality eggs derived from captive lumpfish (*n*= 5) for each replicate. "ID" Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3EDU5	CoA synthase	39.63	A0A3Q3F420	Poly [ADP-ribose] polymerase	40.59	A0A3Q3EZ12	Anion exchange protein	73.59
A0A3Q3G7B8	G_PROTEIN_RECEP_F1_2 domain-containing protein	37.71	A0A3Q3GLP9	Coiled-coil domain containing 57	40.49	A0A3Q3G6V6	Microtubule associated serine/threonine kinase 2	48.89
A0A3Q3ERP1	Mitogen-activated protein kinase kinase kinase kinase 3	32.93	A0A3Q3F9P4	Capping protein inhibiting regulator of actin dynamics *	37.71	A0A3Q3G0T6	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	37.71
A0A3Q3GP01	PRP3 pre-mRNA processing factor 3 homolog (yeast)	25.09	A0A3Q3NML4	IF rod domain-containing protein	33.22	A0A3Q3EBE3	Myosin, light chain kinase 5	33.11
A0A3Q3FW63	ELKS/RAB6-interacting/CAST family member 2	21.84	A0A3Q3EU72	Inositol oxygenase (EC 1.13.99.1) *	23.73	A0A3Q3F420	Poly [ADP-ribose] polymerase	31.44
A0A3Q3EVP8	Zinc finger and BTB domain-containing protein 24 *	20.04	A0A3Q3NFX1	RAD50 homolog, double strand break repair protein	21.12	A0A3Q3FWZ3	Anion exchange protein	27.38
A0A3Q3FZC3	Leucine zipper, putative tumor suppressor family member 3b	17.97	A0A3Q3EGS5	Diacylglycerol kinase	18.31	A0A3Q3NGN9	KRIT1 ankyrin repeat containing	26.41
A0A3Q3ENQ1	Collagen alpha-1(XXVII) chain B *	17.05	A0A3Q3EDU5	CoA synthase	15.33	A0A3Q3F321	Ubiquitin-like modifier-activating enzyme 5 *	23.57
A0A3Q3NMU3	Zinc finger protein 654	15.87	A0A3Q3GUY3	Calcium-transporting ATPase	15.01	A0A3Q3MJE2	SH3 domain binding kinase family, member 3	23.01
A0A3Q3GMB3	Chromatin target of PRMT1a	14.35	A0A3Q3GPB6	BHLH domain-containing protein	13.48	A0A3Q3ET57	WASH complex subunit 5	18.29
A0A3Q3G8T6	Si:ch211-272n13.3	12.91	A0A3Q3ENQ1	Collagen alpha-1(XXVII) chain B *	12.87	A0A3Q3NHJ7	GED domain-containing protein	18.02
A0A3Q3L538	Pleckstrin homology domain containing, family A member 7a	12.88	A0A3Q3EZ12	Anion exchange protein	10.94	A0A3Q3FET0	Kinetochore associated 1	17.85

Table 5.7 (Continued): Most abundant ovarian fluid proteins from the "good" quality eggs derived from captive lumpfish (*n*= 5) for each replicate. "ID" Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3NHJ7	GED domain-containing protein	11.93	A0A3Q3GI10	GTP cyclohydrolase 1	10.61	A0A3Q3NI19	Acidic leucine-rich nuclear phosphoprotein 32 family member A *	16.44
A0A3Q3FQZ6	Cystic fibrosis transmembrane conductance regulator *	11.39	A0A3Q3FIB6	Zinc finger, C4H2 domain containing	10.33	A0A3Q3EDU5	CoA synthase	13.43
A0A3Q3FIB6	Zinc finger, C4H2 domain containing	10.49	A0A3Q3L538	Pleckstrin homology domain containing, family A member 7a	10.21	A0A3Q3FLK8	Leucine rich repeat containing 34	12.08
A0A3Q3EJT4	Sad1 and UNC84 domain containing 1	10.33	A0A3Q3E856	Beta 3-glucosyltransferase b	10.04	A0A3Q3EW20	Potassium voltage-gated channel, KQT-like subfamily, member 5a	12.04
A0A3Q3FWN7	Lon protease homolog 2, peroxisomal	10.21	A0A3Q3ELA6	Wu:fc38h03	10.02	A0A3Q3EL81	Protein aurora borealis *	11.74
A0A3Q3F7P2	Fibronectin type-III domain-containing protein	10.04	A0A3Q3FT82	ADP-ribosylation factor-like protein 3 *	9.91	A0A3Q3FCZ6	Laminin subunit alpha 2	11.09
A0A3Q3GKS6	Regulatory factor X, 6	9.46	A0A3Q3LW57	Eph receptor A7	9.49	A0A3Q3EPG4	GDNF family receptor alpha-1	10.41
A0A3Q3EBQ4	Erythrocyte membrane protein band 4.1 like 4B	9.42	A0A3Q3F7P2	Fibronectin type-III domain-containing protein	9.02	A0A3Q3E2T5	Spectrin beta chain	10.01

Table 5.8: Most abundant ovarian fluid proteins from the "bad" quality eggs derived from captive lumpfish (*n*= 5) for each replicate. "ID" Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3EDU5	CoA synthase	37.25	A0A3Q3F420	Poly [ADP-ribose] polymerase	45.81	A0A3Q3EZ12	Anion exchange protein	45.51
A0A3Q3GP01	PRP3 pre-mRNA processing factor 3 homolog (yeast)	31.52	A0A3Q3NFX1	RAD50 homolog, double strand break repair protein	35.32	A0A3Q3EBE3	Myosin, light	40.77
A0A3Q3G7B8	G_PROTEIN_RECEP_F1_2 domain-containing protein	30.34	A0A3Q3NML4	IF rod domain-containing protein	32.86	A0A3Q3F420	Poly [ADP-ribose] polymerase	36.62
A0A3Q3ERP1	Mitogen-activated protein kinase kinase kinase kinase 3	29.71	A0A3Q3F9P4	Capping protein inhibiting regulator of actin dynamics *	30.34	A0A3Q3G0T6	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2	30.34
A0A3Q3EVP8	Zinc finger and BTB domain-containing protein 24 *	20.56	A0A3Q3GLP9	Coiled-coil domain containing 57	30.04	A0A3Q3F321	Ubiquitin-like modifier-activating enzyme 5 *	30.05
A0A3Q3GMB3	Chromatin target of PRMT1a	18.24	A0A3Q3EU72	Inositol oxygenase *	26.93	A0A3Q3FWZ3	Anion exchange protein	25.06
A0A3Q3NMU3	Zinc finger protein 654	17.23	A0A3Q3EDU5	CoA synthase	15.91	A0A3Q3MJE2	SH3 domain binding kinase family, member 3	24.52
A0A3Q3FZC3	Leucine zipper, putative tumor suppressor family member 3b	17.14	A0A3Q3EGS5	Diacylglycerol kinase	15.45	A0A3Q3NHJ7	GED domain-containing protein	21.29
A0A3Q3FW63	ELKS/RAB6-interacting/CAST family member 2	16.66	A0A3Q3GUY3	Calcium-transporting ATPase	14.77	A0A3Q3NGN9	KRIT1 ankyrin repeat containing	20.36
A0A3Q3ENQ1	Collagen alpha-1(XXVII) chain B *	15.84	A0A3Q3ENQ1	Collagen alpha-1(XXVII) chain B *	12.71	A0A3Q3FET0	Kinetochore associated 1	17.01
A0A3Q3NHJ7	GED domain-containing protein	15.39	A0A3Q3FIB6	Zinc finger, C4H2 domain containing	11.04	A0A3Q3NI19	Acidic leucine-rich nuclear phosphoprotein 32 family member A *	16.56
A0A3Q3EZQ6	Unconventional myosin-Id *	13.56	A0A3Q3GI10	GTP cyclohydrolase 1	10.94	A0A3Q3ET57	WASH complex subunit 5	14.91
A0A3Q3G8T6	Si:ch211-272n13.3	13.43	A0A3Q3GPB6	BHLH domain-containing protein	10.91	A0A3Q3F283	Coiled-coil domain containing 85A, like	14.75

Table 5.8 (Continued): Most abundant ovarian fluid proteins from the "bad" quality eggs derived from captive lumpfish (*n*= 5) for each replicate. "ID" Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3FWN7	Lon protease homolog 2, peroxisomal	12.71	A0A3Q3L538	Pleckstrin homology domain containing, family A member 7a	10.78	A0A3Q3FLK8	Leucine rich repeat containing 34	14.43
A0A3Q3GKS6	Regulatory factor X, 6	12.16	A0A3Q3EZ12	Anion exchange protein	9.75	A0A3Q3EDU5	CoA synthase	14.36
A0A3Q3FYI4	P53-induced death domain protein 1	12.14	A0A3Q3FT82	ADP-ribosylation factor-like protein 3 *	9.17	A0A3Q3FCZ6	Laminin subunit alpha 2	12.43
A0A3Q3L538	Pleckstrin homology domain containing, family A member 7a	11.13	A0A3Q3E856	Beta 3-glucosyltransferase b	8.66	A0A3Q3EW20	Potassium voltage-gated channel, KQT-like subfamily, member 5a	11.39
A0A3Q3FQZ6	Cystic fibrosis transmembrane conductance regulator *	10.55	A0A3Q3ELA6	Wu:fc38h03	8.62	A0A3Q3FWN7	Lon protease homolog 2, peroxisomal	10.93
A0A3Q3FE34	Sperm flagellar 2	8.91	A0A3Q3FLK8	Leucine rich repeat containing 34	7.96	A0A3Q3FZK0	PCI domain-containing protein	10.03
A0A3Q3F7P2	Fibronectin type-III domain-containing protein	8.71	A0A3Q3EW20	Potassium voltage-gated channel, KQT-like subfamily, member 5a	7.91	A0A3Q3M292	Dynactin subunit 6 *	9.58

5.3.4.3 Treatment comparisons

Within the three replicates there were an average of 190 ± 61 proteins identified with 49 proteins (19.2%) identified in all of the three replicates, and 149 (A), 38 (B) and 68 (C) unique proteins for each replicate (Figure 5.8).

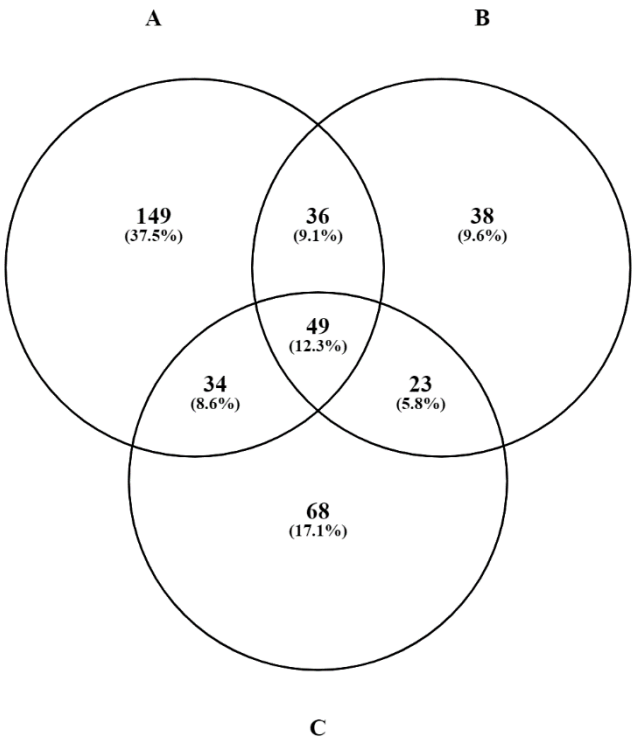

Figure 5.8: Venn Diagram of all identified proteins within each technical replicate conducted for the “good” and “bad” quality eggs derived from captive broodstock, comparisons. Venny (2.1, Oliveros 2015)

There were significant changes in protein abundance identified in all three replicates, 13 (A), 5 (B) and 1 (C), table 5.9. Ten out of 16 proteins which displayed a significance between the “good” and bad” quality groups displayed increased abundance within the “bad quality group, when compared to “good” levels. Abundance of “Calpain 2, (m/II) large subunit, like” was identified as being 501% of abundance levels within the “good”

quality group. Abundance levels of Keratin 97 within the “bad” quality eggs were the largest decrease at only 10% of levels within the “good” quality eggs.

Table 5.9: Proteins Identified as showing significant difference between the “good” quality and “bad” quality eggs derived from captive broodstock, ovarian fluid proteomes. Data is reported as the percentage change between mean of biological replicates (*n* =5) as a percentage of the Good quality eggs and p value (in brackets). Red cells indicate decreases in relative abundance of the protein, green cells indicate increased relative abundance * indicates initially unknown protein identified in zebrafish.

Protein ID	Name	Replicate A Rate of change (%), (p value)	Replicate B Rate of change (%), (p value)	Replicate C Rate of change (%), (p value)
A0A3Q3FEA2	Lin-9 DREAM MuvB core complex component	313.25 (0.002)	-	-
A0A3Q3EJ64	Intraflagellar transport protein 172 homolog	21.81 (0.006)	-	-
A0A3Q3EX26	Carnitine palmitoyltransferase 1Cb	266.80 (0.006)	-	-
A0A3Q3F8Q9	Proteasome 26S subunit, non-ATPase 12	206.50 (0.014)	-	-
A0A3Q3EH51	BTB domain-containing protein	13.97 (0.018)	-	-
A0A3Q3N9D8	MAGUK p55 subfamily member 7 *	147.01 (0.026)	-	-
A0A3Q3GWW5	Caprin family member 2	211.04 (0.033)	-	-
A0A3Q3GNF4	Eukaryotic elongation factor 2 kinase	199.09 (0.04)	-	-
A0A3Q3GIT8	TAO kinase 2b	-	423.64 (0.000)	-
A0A3Q3E7L0	Calpain 2, (m/II) large subunit, like	-	501.49 (0.002)	-
A0A3Q3FNL3	Keratin 97	-	10.33 (0.011)	-
A0A3Q3EDT4	Tripartite motif containing 3a	17.38 (0.018)	119.05 ns	24.94 (0.043)
A0A3Q3E7X7	CULLIN_2 domain-containing protein	58.61 (0.023)	75.37 ns	-
A0A3Q3FH77	Calcium channel, voltage-dependent, alpha 2/delta subunit 1a	16.22 (0.013)	13.83 (0.012)	-
A0A3Q3FYI4	P53-induced death domain protein 1	262.69 (0.007)	269.37 (0.002)	-
A0A3Q3FET0	Kinetochore associated 1	159.17 (0.024)	-	95.28 ns

5.3.5 Ex vivo over ripening

Egg quality data for the fresh samples used in this analysis fertilisation (97.6 % ±2.0), eyeing (76.8 % ±7.4), hatching (54.6 % ±8.0). For the aged stock quality data is as follows fertilisation (40.27 % ± 5.8), eyeing (12.14 % ± 3.9), hatching (0 %) (Table 5.1, figures, 5.4, 5.5, 5.6)).

5.3.5.1 Protein identifications

Finally, within the Wild and wild aged comparisons a total number of 13379 ± 2194 peptides were identified which were mapped onto 749 proteins. Of those 106 ± 11 met the requirements for reliable identification and quantification (i.e. quantified proteins; identified by at least 1 unique peptide and at least 3 peptides).

5.3.5.2 Protein Abundance

There was more variation within the aged “fresh” group within the top 20 most abundant proteins for each run there were only 3 shared proteins between at least 2 runs, with no proteins identified in all three runs (Table 5.10). As with the “fresh” runs, there was a higher level of variation between runs within the top 20 most abundant proteins for each run there were only 2 proteins shared between at least 2 runs, with no proteins identified in all three runs (Table 5.11). Within each run (A, B and C) there was a high level of shared proteins within the 20 most abundant proteins between the wild and captive stocks, with 18, proteins being shared between “fresh” ands “aged” ovarian fluid for all three runs.

Table 5.10: Most abundant ovarian fluid proteins from the “fresh stripped” quality eggs derived from wild lumpfish (*n*= 5) for each replicate. “ID” Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish, with * * indicating it within rainbow trout proteome.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3G9V9	Sorting nexin 24]	87.244304	A0A3Q3NI19	Acidic leucine-rich nuclear phosphoprotein 32 family member A *	87.4003137	A0A3Q3G5W4	ERI1 exoribonuclease 2 (EC 3.1.-.-) *	94.8986743
A0A3Q3FPS1	MLLT6, PHD finger containing	49.28526298	A0A3Q3G7B8	G_PROTEIN_RECEP_F1_2 domain-containing protein	43.9904042	A0A3Q3ELA6	Wu:fc38h03	48.5089158
A0A3Q3EMB8	Unconventional myosin-Id *	42.81845317	A0A3Q3LA06	NUDE_C domain-containing protein	37.7184	A0A3Q3G9T4	Nucleotide-binding oligomerization domain containing 1	45.6782764
A0A3Q3FDD8	C-type lectin domain-containing protein	37.7184	A0A3Q3EW51	Inosine-5'-monophosphate dehydrogenase	37.7184	A0A3Q3E1M6	Oxysterol-binding protein	42.005503
A0A3Q3EW87	Structural maintenance of chromosomes protein	37.7184	A0A3Q3FM25	TRAF2 and NCK interacting kinase	37.7184	A0A3Q3GDG9	Pleckstrin homology domain-containing family A member 7 *	37.7184
A0A3Q3ECM5	Zgc:113279	37.7184	A0A3Q3GL91	VPS50 EARP/GARPII complex subunit	37.7184	A0A3Q3GPJ4	RNA-binding protein with serine-rich domain 1 *	35.7874228
A0A3Q3KWJ9	F-box and WD repeat domain containing 7	35.90049816	A0A3Q3G0S0	Fibronectin type III and SPRY domain-containing protein 1 *	37.3440809	A0A3Q3MJE2	SH3 domain binding kinase family, member 3	28.8226169
A0A3Q3MTC0	DDRGK domain-containing protein 1 *	33.82550703	A0A3Q3FFT4	ATP-dependent 6-phosphofructokinase	30.2992482	A0A3Q3EXX5	Si:dkey-224e22.2	23.9138721
A0A3Q3EZ12	Anion exchange protein	30.82141282	A0A3Q3MF80	Dedicator of cytokinesis 7	28.876924	A0A3Q3EW87	Structural maintenance of chromosomes protein	23.7609991
A0A3Q3KZ04	Cyclin I	29.18262101	A0A3Q3GKS6	Regulatory factor X, 6	27.0526917	A0A3Q3F2V6	Visual system homeobox 2 (Ceh-10 homeodomain-containing homolog) *	23.4942169

Table 5.10 (Continued): Most abundant ovarian fluid proteins from the “fresh stripped” quality eggs derived from wild lumpfish (*n*= 5) for each replicate. “ID” Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish, with * * indicating it within rainbow trout proteome.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3GS75	Palmitoyltransferase	25.78303167	A0A3Q3KWJ9	F-box and WD repeat domain containing 7	24.4319864	A0A3Q3MUP8	SMEK homolog 1, suppressor of mek1 (Dictyostelium)	19.8105719
A0A3Q3GP35	Centrosomal protein 72	25.05011056	A0A3Q3EU72	Inositol oxygenase *	24.0104594	A0A3Q3FFH4	Rho GTPase activating protein 39	16.388321
A0A3Q3E6Z7	NADH:ubiquinone oxidoreductase core subunit S2	24.72320617	A0A3Q3GWN6	DDE Tnp4 domain-containing protein	21.0837121	A0A3Q3LJE4	Collagen alpha-1(XXVII) chain B *	15.7306838
A0A3Q3N113	Ryanodine receptor 2b (cardiac)	22.79649876	A0A3Q3E5V7	Minichromosome maintenance 9 homologous recombination repair factor	20.4608255	A0A3Q3FG15	Acyl-CoA synthetase long chain family member 4a	15.7305215
A0A3Q3FAM3	Integrin_alpha2 domain-containing protein	22.71702389	A0A3Q3GX53	Histone-lysine N-methyltransferase	19.1849933	A0A3Q3G8F2	Unconventional myosin-Id *	13.7246204
A0A3Q3G4E0	Rho guanine nucleotide exchange factor (GEF) 12a	21.87336606	A0A3Q3FL95	Ras interacting protein 1	19.1460189	A0A3Q3FZQ8	Solute carrier family 25 member 45	13.4271876
A0A3Q3FNN6	Cytochrome c oxidase subunit 7A-liver, mitochondrial * *	19.715431	A0A3Q3FZQ8	Solute carrier family 25 member 45	18.8498557	A0A3Q3L538	Pleckstrin homology domain containing, family A member 7a	13.2505953
A0A3Q3FK68	RNA transcription, translation and transport factor	15.92024974	A0A3Q3FE34	Sperm flagellar 2	18.6102889	A0A3Q3LSG6	E3 ubiquitin-protein ligase CBL	12.9791354
A0A3Q3EJJ8	Spectrin repeat containing, nuclear envelope 1b	15.83202174	A0A3Q3MWC6	Si:ch211-225b10.3	16.5987406	A0A3Q3G6X7	Chondroitin sulfate proteoglycan 4	12.7646388
A0A3Q3F2M5	Basic leucine zipper and W2 domains 1a	15.50838433	A0A3Q3FZT7	Coiled-coil domain containing 191	15.9644492	A0A3Q3FDD9	C1q domain-containing protein	11.889118

Table 5.11: Most abundant ovarian fluid proteins from the "aged" quality eggs derived from wild lumpfish (*n*= 5) for each replicate. "ID" Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish, with * * indicating it within rainbow trout proteome.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3G9V9	Sorting nexin 24	95.0273448	A0A3Q3FZ47	NACHT, LRR and PYD domains-containing protein 1 homolog *	98.21211	A0A3Q3G5W4	ERI1 exoribonuclease 2 (EC 3.1.-.-) *	70.71561
A0A3Q3FPS1	MLLT6, PHD finger containing	38.6069806	A0A3Q3NI19	Acidic leucine-rich nuclear phosphoprotein 32 family member A *	87.01226	A0A3Q3GPJ4	RNA-binding protein with serine-rich domain 1 *	44.99216
A0A3Q3FAM3	Integrin_alpha2 domain-containing protein	37.2847251	A0A3Q3GL91	VPS50 EARP/GARPII complex subunit	33.01817	A0A3Q3ELA6	Wu:fc38h03	33.71485
A0A3Q3EMB8	Unconventional myosin-Id *	34.5179351	A0A3Q3G7B8	G_PROTEIN_RECEP_F1_2 domain-containing protein	30.3456	A0A3Q3E1M6	Oxysterol-binding protein	32.76562
A0A3Q3GP35	Centrosomal protein 72	33.7728604	A0A3Q3LA06	NUDE_C domain-containing protein	30.3456	A0A3Q3G9T4	Nucleotide-binding oligomerization domain containing 1	31.37377
A0A3Q3FDD8	C-type lectin domain-containing protein	32.0695399	A0A3Q3EW51	Inosine-5'-monophosphate dehydrogenase	30.3456	A0A3Q3GDG9	Pleckstrin homology domain-containing family A member 7 *	30.3456
A0A3Q3EW87	Structural maintenance of chromosomes protein	30.3456	A0A3Q3FM25	TRAF2 and NCK interacting kinase	30.3456	A0A3Q3EW87	Structural maintenance of chromosomes protein	26.2215
A0A3Q3ECM5	Zgc:113279	30.3456	A0A3Q3G0S0	Fibronectin type III and SPRY domain-containing protein 1 *	28.44795	A0A3Q3LE86	Par-3 family cell polarity regulator	21.30344
A0A3Q3KWJ9	F-box and WD repeat domain containing 7	28.5818106	A0A3Q3MF80	Dedicator of cytokinesis 7	24.09535	A0A3Q3MJE2	SH3 domain binding kinase family, member 3	18.27469
A0A3Q3EZ12	Anion exchange protein	28.1460251	A0A3Q3EU72	Inositol oxygenase *	23.82576	A0A3Q3EXX5	Si:dkey-224e22.2	18.02766
A0A3Q3MTC0	DDRGK domain-containing protein 1 *	25.317145	A0A3Q3FFT4	ATP-dependent 6-phosphofructokinase	23.63549	A0A3Q3MUP8	SMEK homolog 1, suppressor of mek1 (Dictyostelium)	15.94174
A0A3Q3N113	Ryanodine receptor 2b (cardiac)	24.5913531	A0A3Q3GKS6	Regulatory factor X, 6	21.75339	A0A3Q3FG15	Acyl-CoA synthetase long chain family member 4a	15.13499

Table 5.11 (Continued): Most abundant ovarian fluid proteins from the "aged" quality eggs derived from wild lumpfish (*n*= 5) for each replicate. "ID" Indicates the UniProt accession number of the proteins. * indicates initially unknown protein identified in zebrafish, with * * indicating it within rainbow trout proteome.

Replicate A			**Replicate B**			**Replicate C**		
ID	Name	Relative abundance	ID	Name	Relative abundance	ID	Name	Relative abundance
A0A3Q3GVY8	Leucine zipper protein 2	23.7103966	A0A3Q3MWC6	Si:ch211-225b10.3	18.65814	A0A3Q3F2V6	Visual system homeobox 2 *	13.51419
A0A3Q3KZ04	Cyclin I	21.9684617	A0A3Q3FE34	Sperm flagellar 2	18.48848	A0A3Q3G891	Calpain 3b	13.35944
A0A3Q3GS75	Palmitoyltransferase	21.3626761	A0A3Q3KWJ9	F-box and WD repeat domain containing 7	17.94069	A0A3Q3LSG6	E3 ubiquitin-protein ligase CBL	13.29762
A0A3Q3EJJ8	Spectrin repeat containing, nuclear envelope 1b	18.9706763	A0A3Q3GX53	Histone-lysine N-methyltransferase	17.3038	A0A3Q3L538	Pleckstrin homology domain containing, family A member 7a	12.81503
A0A3Q3G4E0	Rho guanine nucleotide exchange factor (GEF) 12a	18.7988444	A0A3Q3GXD4	Peroxisome assembly protein 12	15.92944	A0A3Q3G8F2	Unconventional myosin-Id *	12.40598
A0A3Q3FNN6	Cytochrome c oxidase subunit 7A-liver, mitochondrial **	17.241393	A0A3Q3FZQ8	Solute carrier family 25 member 45	15.74201	A0A3Q3G6X7	Chondroitin sulfate proteoglycan 4	11.98884
A0A3Q3FKQ1	MICOS complex subunit	17.1309593	A0A3Q3GWN6	DDE Tnp4 domain-containing protein	15.63928	A0A3Q3FFH4	Rho GTPase activating protein 39	10.97407
A0A3Q3E6Z7	NADH:ubiquinone oxidoreductase core subunit S2	16.6300916	A0A3Q3E5V7	Minichromosome maintenance 9 homologous recombination repair factor	15.247	A0A3Q3LJE4	Collagen alpha-1(XXVII) chain B *	10.60606

5.3.5.3 Treatment comparisons

Within the three replicates there were an average of 106 ± 11 proteins identified with 21 proteins identified in all of the three replicates, and 53 (A), 61 (B) and 43 (C) unique proteins for each replicate (Figure 5.9).

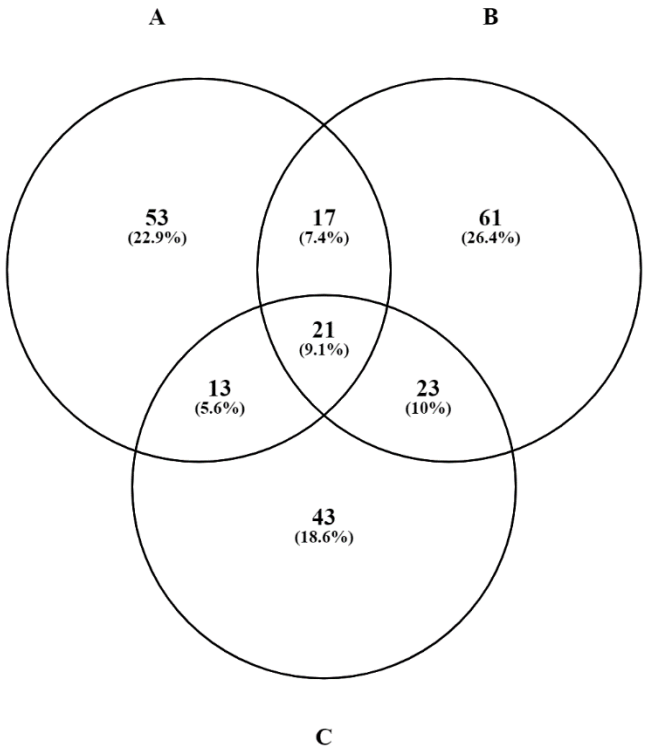

Figure 5.9: Venn Diagram of all identified proteins within each technical replicate conducted for the “fresh” and “aged” eggs derived from wild caught broodstock, comparisons. Venny (2.1, Oliveros 2015)

There were significant changes in protein abundance identified in all three replicates, 8 (A), 9 (B) and 8 (C), however, 13 of these were only identified in one replicate, while still meeting the requirements for identification and quantification (Section 2.2.4), table 5.12

There was a decrease in abundance in 19 out of 23 of the proteins which exhibited significant changes between “fresh” and “aged” groups. The largest decrease was in levels in Syntaxin 3A, which were 39% of abundance levels in the “fresh” group. Levels of the MICOS complex subunit within the A run exhibited “aged” group abundances 549% of those in the “fresh” group significantly higher. Leucine-rich repeats and immunoglobulin-like domains 2, abundance in within the “aged” group was 295% of levels in the “fresh” group.

Table 5.12: Proteins Identified as showing significant difference between the “Fresh” eggs and “aged” eggs derived from wild caught broodstock, ovarian fluid proteomes. Data is reported as the percentage change between mean of biological replicates (*n* =5) as a percentage of the Fresh eggs and (p value in brackets). Red cells indicate decreases in relative abundance of the protein, green cells indicate increased relative abundance * indicates initially unknown protein identified in zebrafish.

Protein ID	Name	Replicate A Rate of change (%), (p value)	Replicate B Rate of change (%), (p value)	Replicate C Rate of change (%), (p value)
A0A3Q3GCI8	Leucine-rich repeat and fibronectin type III domain-containing protein 1 *	53.36 (0.014)	-	-
A0A3Q3F723	PA domain-containing protein	53.15 (0.014)	-	-
A0A3Q3FPS1	MLLT6, PHD finger containing	78.33 (0.019)	-	-
A0A3Q3KZ04	Cyclin I	75.27 (0.028)	-	-
A0A3Q3FLK8	Leucine rich repeat containing 34	62.09 (0.047)	-	-
A0A3Q3EXA8	Syntaxin 3A	-	39.21 (0.001)	-
A0A3Q3FI08	ATP-binding cassette, sub-family A (ABC1), member 4b	-	53.04 (0.003)	-
A0A3Q3EPL8	Leucine-rich repeats and immunoglobulin-like domains 2	-	295.62 (0.047)	-
A0A3Q3G0S0	Fibronectin type III and SPRY domain-containing protein 1 *	-	76.17 (0.05)	-
A0A3Q3H2C9	Wu:fj29h11	-	-	58.49 (0.012)
A0A3Q3GKZ2	Mitochondrial uncoupling protein 2 (UCP 2) *	-	-	64.89 (0.034)
A0A3Q3GMB6	ANK_REP_REGION domain-containing protein	-	-	69.92 (0.035)
A0A3Q3EC93	D-aminoacyl-tRNA deacylase 2 *	-	-	44.21 (0.05)
A0A3Q3ND39	Piwi-like RNA-mediated gene silencing 1	73.37 ns	70.15 (0.002)	62.11 ns
A0A3Q3NF32	Rho GTPase activating protein 45b	112.99 ns	197.56 (0.033)	135.92 ns
A0A3Q3NQ34	RING-type domain-containing protein	85.35 ns	171.29 ns	305.54 (0.027)
A0A3Q3E6Z7	NADH:ubiquinone oxidoreductase core subunit S2	67.26 ns	58.55 (0.029)	-
A0A3Q3FKQ1	MICOS complex subunit	549.49 (0.037)	183.59 ns	-
A0A3Q3G7S7	Plectin a	55.34 (0.017)	117.11 ns	-
A0A3Q3GJD9	Muscleblind like splicing regulator 1	55.98 (0.024)	65.84 ns	-
A0A3Q3EMY9	Mitogen-activated protein kinase kinase kinase kinase 1	-	72.93 ns	73.14 (0.033)
A0A3Q3ET57	WASH complex subunit 5	-	53.62 (0.022)	56.04 (0.017)
A0A3Q3GWB1	MFS domain-containing protein	-	41.82 (0.03)	41.57 (0.013)

5.4. Discussion

This study aimed to quantify the impact of *ex vivo* post ovulatory aging on lumpfish egg performance for both wild and captive derived eggs to determine the species "over-ripening window". Furthermore, it sought to utilise TMT Isobaric tagging of proteins to characterise the ovarian fluid proteome for the first time in the species and examine how this change in relation to measured quality, egg origin (wild vs farmed broodstock) and in response to the process of post ovulatory ageing itself. The applied goal of this research was to inform guidance on the stripping of mature broodstock to minimise the negative impacts of post ovulatory ageing and furthermore identify candidate protein biomarkers that could in the future be used to rapidly confirm batch quality.

There is a distinct lack of information in broodstock reproductive management, spawning season and environmental management in lumpfish aquaculture. Current best practice in Scotland involves monitoring captive broodstock approximately every three days to assess females vents of suitability for stripping, similar to that of halibut. In wild fisheries in Norway wild mature fish are netted and held in a flow through system with the vent regularly assessed to determine suitability for stripping. Often, hatcheries are prone to hold unfertilised eggs within their ovarian fluid for lengths of time while they batch process multiple individuals, with further delays potentially added due to degumming and disinfection activities (Powell et al. 2018). Together, this means that there can be highly variable and extended periods of times between ovulation, stripping and subsequent fertilisation of egg batches. Using current best practice, the typical window between ovulation and fertilisation could be up to 4 days and the potential impact of post ovulatory ageing on the subsequent batch quality remains unknown.

The current study demonstrated that there was a significant negative effect on egg quality for both wild and captive stocks associated with prolonged post ovulatory ageing. While quality (fertilisation, eyeing and hatch rates) were consistently lower in captive vs wild batches the impact of post ovulatory ageing followed a similar pattern in both stocks. Within the fertilisation and eyeing rates at no time point did any of the eggs reach 0 (%) for the measurements of quality. Fertilisation rates for both wild and captive stocks decreased 80 % from freshly stripped eggs to 120hr of ageing, fertilisation rates differed significantly from fresh eggs at 24 hr and 48 hours for captive and wild stocks respectively. For the eyeing rates there was a significant effect of ageing for both wild and captive eggs, with a 97 % and 99.2 % reduction respectively after 120 hours of ageing. The wild stocks were able to withstand a longer period of aging with regards to fertilisation and eyeing rates when compared to the captive eggs. However, the period at which there was a significant effect on egg quality was 24 hours and 12 hours in the wild and captive stocks respectively with reductions in hatching success at 75 % and 78 % respectively. The period of ageing identified in this study is similar to that found in turbot where eggs were not viable after 1 day of ageing (McEvoy. 1983), in ayu (*Plecoglossus altivelis*) where eggs were significantly reduced in hatching at 1-2 days (Hirose et al. 1979) and cod where eggs were significantly affected after 12 hours (Kjorsvik & Lonning. 1983). These findings show that hatching success in lumpfish deteriorates after only 24 hours post ovulation, requiring close monitoring of ovulation in this species. On farm practice in Scotland currently monitors females in 3-day intervals, which based on data in this study is inadequate. Utilisation of ultrasound to monitor ovulation was utilised in Chapter 2, this could be a possible avenue to improve current broodstock assessment at present. The creation of ovarian

fluid biomarkers could be an important tool for rapid stock ovulation and quality assessments.

Biomarkers are important tools that can supply much needed data regarding a particular treatment effect or condition (Yilmaz et al. 2017). A biomarker is a biological characteristic that is measured and evaluated objectively as an indicator of normal biological processes, pathogenic processes, or pharmacologic response to therapeutic intervention. Ovarian fluid biomarkers offer the only predictive marker for egg quality at present. While there are many effective tools for assessing proteomes, Isobaric tags for relative and absolute quantification (iTRAQ) has become the technique of choice in the biomarker discovery field (Casey et al. 2017). Tandem mass tagging (TMT) is another isobaric labelling technique, which follows the same principle as iTRAQ quantitation; however, the labels are slightly different in structure and mass.

To the authors knowledge there are only two previous studies of lumpfish proteomes in which the skin mucus was analysed and not ovarian fluid (Patel et al. 2017, Patel et al. 2019). Prior to the extraction of protein, samples can be subjected to a process of lysis with the aim being to improve the number of detectable peptides within the sample (Walker. 2005). There is a wide range of lysis methods which can be tailored to the specific fluid or tissue, lysis is often applied to tissues (Shehadul Islam et al. 2017), however lysis is also common in fluids such as blood (Bossuyt et al. 1997). Equally in some studies, as was the case for the previous work in lumpfish (Patel & Brinchmann. 2017, Patel et al. 2019), a lysis step can successfully not be used prior to analysis and as such it was necessary to complete a pilot study to confirm if lysis was of value to the current work or not. In this study two lysis methods were tested against a negative control, the acetone lysis produced similar results to that of the no lysis method with regards to identified proteins. With the potential for the lysis method

to add additional error to the analysis with no perceived benefit to the protein coverage the no lysis method was conducted in the main body of the experiment.

In total, 649 proteins were reliably identified and quantified (based on a minimum of three peptides and one unique peptide) in lumpfish ovarian fluid. Proteomic investigations in other fish species reported a variety of protein identification results in ovarian fluid. Nynca et al. (2015) reported 59 proteins in rainbow trout ovarian fluid (identification based on a minimum of two unique peptides). A total of 174 proteins were identified in Chinook Salmon (using two or more peptide hits, (Johnson et al. 2014)). Regarding the previous studies in lumpfish, 82 proteins were reported in the skin mucus (identification based on a minimum of one unique peptide; Patel et al. 2019), with a preliminary study by the same author identifying 40 proteins in skin mucus with the same criteria (Patel & Brinchmann. 2017). These differences in protein identification has been shown to be affected by a number of different biological (fish tissues and fluids used) and technical (sample processing, fractionation strategy, MS-instrument type, data acquisition parameters, data trimming and database identification version and parameters) factors. The studies cited above all used 2D SDS PAGE, with identification based on targeted spots within the gel, whereas this study used Isobaric tagging which is reported to identify a larger number of proteins than other methods (Casey et al. 2016).

There was a high amount of variation in the reporting of proteins in all three experiments, with a low number of shared proteins between replicates and tube runs. Reproducibility over three runs within each tube was 30.9%, 35.8% and 32.1% for the wild captive comparison, egg quality and ageing experiments (proteins shared between at least two replicates). With replication of differentially expressed proteins at 43.8%, 31.4% and 52.7% respectively (proteins shared between at least two

replicates). This reproducibility falls within the published reproducibility rates (23 – 33 % duplicate, 14 - 16 % triplicate (I'D in all three runs)) of isobaric tagging techniques for duplicate and triplicate TMT runs (Casey et al. 2016). The effectiveness of increased runs from multiples tubes have been shown to increase overall protein coverage by 10-15% for duplicates and a further 5% for the third replicate (Casey et al. 2016). Despite this, the data from this study suggests that use of TMT labelling, while effective at characterising a wide number of proteins, requires further investigation using techniques such as immunoblotting, ELISA (enzyme-linked immunosorbent assay), or a targeted mass spectrometry assay such as MRM/SRM (Multiple reaction monitoring/ Selective Reaction Monitoring) to confirm abundance changes (Casey et al. 2016). Due to the non-selective nature of the process, the highest abundance proteins can dominate samples, reducing observable compositional changes (Morro et al. 2020)

As with many proteomic studies many of the proteins identified have not been studied in the species (Nynca et al. 2015, Patel et al. 2019, Morro et al. 2020) as was the case with this study. Much work has been conducted in humans (*Homo sapiens*), mice (*Mus musculus*) and zebrafish where the role of these proteins have been documented (Lucitt et al. 2008, Uhlén et al. 2015). Even within these species many of the G.O functions and proteins have only been described *in-silico* with roles theorised depending on homologous roles in other species. While this study reports documented functions for proteins, their specific role within lumpfish is inferred from analogous proteins and their function may vary depending on how conserved the protein and genes are. The lumpfish genome has been sequenced and proteins and functions may change with *in-silico* translation of the genome.

There was some difficulty in identifying the role of many of the highest abundance proteins within the ovarian fluid proteome, with G.O functions in many of the proteins unidentified. Only 40 % (24/60) of the most abundant proteins within the wild ovarian fluid had any G.O functions associated with them. As in chapter 3, wild samples in this study are used as the benchmark for assessing treatment effects. Utilising information for those protein with G.O functions documented, the lumpfish ovarian fluid proteome appears to be characterised by proteins associated with maintenance such as Plectin a, identified in zebrafish as playing a fundamental role in structural integrity of cells and tissues (Andrä et al. 1997). Solute carrier family 35 member B1 which have been shown to be heavily involved in maintenance of the endoplasmic reticulum in *Caenorhabditis elegans* and has been demonstrated to be vital in larval development in the species (Dejima et al. 2009). It also contains proteins which have been associated with the regulation of steroids such as Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase), which has been associated with the regulation of Glucocorticoids as class of corticosteroids (Draper et al. 2003). Proteins also associated with metabolic processes such as Acyl-CoA synthetase long chain family member 4a which has been associated with the metabolism of long chain PUFA's in zebrafish (Miyares et al. 2013).

Within the wild and captive comparisons there was a high level of shared proteins within the top 20 most abundant, 56.4 % were shared when utilising all three replicates. While there is no comparable ovarian fluid data within fish, common reports within numerous fluids/ tissues and species document the highly conserved nature of the highest abundance proteins within a proteome (Fonslow et al. 2013, Morro et al 2020). There were no unique proteins identified in either the wild or captive ovarian fluids, however, there were relative compositional changes in abundance between the

conditions. Within the top 20 abundant proteins, five displayed significant changes in abundance between the wild and captive stocks. Two proteins displayed significant increases in abundance in the captive stocks when compared to the wild, RAS p21 protein activator (GTPase activating protein) 1a, and Keratin 97. The protein, RAS p21 has been proposed to play a crucial role in signal transduction pathways from activated cell surface receptors controlling cell proliferation, differentiation, and metabolism, in mammals (Geyer et al. 1996). The genes for RAS P21 have been identified in several fish species including rainbow trout, pink salmon (*Oncorhynchus gorbuscha*) and Dover sole (*Solea solea*) and have been documented as being highly variable (Rotchell et al. 2001). Keratin 97 was documented as being highly upregulated (3.8-fold increase) in experimentality androgen reduced zebrafish (Shu et al. 2020), however, the authors offer no explanation to its role. There were three significantly downregulated proteins (when compared to the wild stocks) within the high abundance proteins: Histone deacetylase 10, R3H domain and coiled-coil containing 1-like, and Piwi-like RNA-mediated gene silencing 1. Of these three R3H domain and coiled-coil containing 1-like was identified as being significantly or close to significantly downregulated in all three replicates. The gene ontology annotation for R3H domain and coiled-coil containing 1-like in zebrafish proposes a role within nucleic acid binding (Howe et al. 2013). This protein was also identified in comparable low abundance in the quality comparison experiment, and low abundance within the aged wild groups within the ageing experiment, although between experimental comparisons are difficult to make in this study.

The findings suggest that while there are compositional changes within the most abundant proteins between the wild and captive stocks the majority of changes occur, as in most proteomic work, in the lower abundance proteins (Fonslow et al. 2013). Of

the 16 proteins displaying significant changes within abundance 11 occurred within the lower abundant proteins within the ovarian fluid. Notable proteins within this group are Pyruvate Kinase, significantly downregulated in two replicates, is a key regulatory enzyme within the glycolysis pathway of fish species, carp (*Cyprinus carpio*) (Driedzic & Hochachka. 1976) rainbow trout (Somero, & Hochachka. 1968) and an Antarctic species *Trematomus bernacchii*. Pyruvate kinase is involved in the cell energy metabolism where it catalyses the transformation of phosphoenolpyruvate to pyruvate leading to ADP being phosphorylated into ATP (Rawn, 1983). Pyruvate kinase within the ovarian fluid was directly correlated with fertilisation rates in four species of carp where increased and decreased activity both resulted in poor egg quality (Lahnsteiner et al. 2001). In this study the abundance of Pyruvate kinase in the captive stock, was half that of the wild group which suggests low biological activity of the enzyme within the captive ovarian fluid.

Within the egg quality comparisons there was a higher level of shared proteins within the top 20 most abundant with 77.2 % being shared when utilising all three replicates. There were no unique proteins identified in either the “good” or “bad” quality ovarian fluids, however there were compositional changes within the protein abundances. There were two proteins identified within the most abundant proteins as being significantly different between the good and bad quality egg, ovarian fluid. The protein, P53-induced death domain protein 1 had increased abundance (260% increase) in the “bad” quality eggs when compared to the good quality. While the calcium channel, voltage-dependent, alpha 2/delta subunit 1a was significantly lower in abundance (16% of “good” levels) in the “bad” quality ovarian fluid. The P53 protein has been identified as promoting apoptosis in mammals (Berube et al. 2010) as well as zebrafish (Chen et al. 2009). It is possible that it is associated in a similar role in lumpfish and if

that were the case then this suggests that elevated apoptosis was present in the bad egg batches in the current study. The alpha 2/delta 1a subunit of the calcium channel has been shown to have a modulating effect on the activation kinetics of the calcium channel (Catterall. 2011). A decrease in abundance of this subunit suggests that Ca2+ channels are less easily polarised/ depolarised with decreased abundance of this subunit (Singer et al. 1991, Carvacho et al. 2018). Oocyte maturation is associated with changes in the electrical properties of the plasma membrane and alterations in the function and distribution of ion channels (Nader et al. 2013, Carvacho et al. 2018). Variations in the expression, function or distribution of the transporters during oocyte maturation are key to reproductive success. Calcium channels are important in regulating membrane potential as well as modulating signalling pathways (Singer et al. 1991, Nader et al. 2013). Moderation of the calcium channels during oocyte maturation is key in internal signalling during fertilisation as well as blocking polyspermy and the final phases of meiosis (Nader et al. 2013). Thus, decreases in abundances of key subunits suggest possible issues within these pathways.

The majority of changes occurred in the lower abundance proteins within the ovarian fluid (14/16). Notable proteins within this group includes BTB domain-containing protein, which was 97% reduced in abundance in “bad” quality eggs in this study and has been shown to be key in many biological processes e.g. Wnt-β-catenin (Park et al. 2005), apoptosis (Phan & Dalla-Favera 2004), and ubiquination and protein degradation (Geyer et al. 2003). The gene coding for this protein has been identified to be strongly expressed within oocytes of zebrafish (Smith et al. 2006) suggesting it plays a key role in oocyte development. The “Calpain 2, (m/II) large subunit, like protein” is part of the Calpain proteases which regulate the breakdown of proteins. This protein was increased in abundance (500%) in the “bad” quality eggs compared

to the “good” quality. Expression of genes which code for this subunit in rainbow trout have been increased during muscle atrophy (Salem et al. 2005). Salem et al. (2006) identified that there was an increased expression of Calpain proteases during and after spawning in rainbow trout. The author suggests that they may control protein metabolism during the spawning season and activate the apoptosis pathway (Salem et al. 2006).

Within the *ex vivo* post ovulatory ageing comparisons there was the highest level of shared proteins within the top 20 most abundant with 82.5 % were shared when utilising all three replicates. There were no unique proteins identified in either the wild or captive ovarian fluids, however there were compositional changes within the protein abundances. There were five proteins identified within the most abundant proteins as being significantly different between the “fresh” and “aged” ovarian fluid. Four proteins were identified as being significantly lower in abundance in the aged ovarian fluid when compared to the “fresh” ovarian fluid; MLLT6 PHD finger containing, Cyclin I, NADH:ubiquinone oxidoreductase core subunit S2 and Fibronectin type III and SPRY domain-containing protein 1. With one protein identified in the highest abundance as significantly increasing in the aged ovarian fluid; MICOS complex subunit. Fibronectin type III and SPRY domain-containing proteins have been associated with cell adhesion and immune responses (Perfetto et al. 2013). The genes coding for Fibronectin proteins have been identified in zebrafish and pufferfish (*Takifugu rubripes*) with many appearing to be conserved between species (Boudinot et al. 2011). The gene encoding for Fibronectin proteins have been identified as potential biomarkers (when upregulated) of egg quality with cows (*Bos Taurus*) (Adona et al. 2016). The MICOS (mitochondrial contact site and cristae junction organizing system) complex is only found within the mitochondria, where it forms part of an interaction

between the mitochondria and the endoplasmic reticulum (Kozjak-Pavlovic. 2017). This interaction is comparatively new in mammals, with previous interactions only considered within teleosts and fungi (Copeland & Dalton. 1959, Herrera-Cruz, & Simmen. 2017). The 550 % increase of abundance of this protein suggests significant cell (egg) lysis within the ovarian fluid.

As with the previous two experiments the majority of changes in protein abundances occurred in the lesser documented proteins, 18 out of the 23 identified significant changes in abundance. A total of 15 proteins were significantly lower in abundance, Leucine-rich repeat and fibronectin type III domain-containing protein 1 *, PA domain-containing protein, Leucine rich repeat containing 34, Syntaxin 3A, ATP-binding cassette, sub-family A (ABC1), member 4b, Wu:fj29h11, Mitochondrial uncoupling protein 2 (UCP 2) *, ANK_REP_REGION domain-containing protein, D-aminoacyl-tRNA deacylase 2 *, Piwi-like RNA-mediated gene silencing 1, Plectin a, Muscleblind like splicing regulator 1, Mitogen-activated protein kinase 1, WASH complex subunit 5, and MFS domain-containing protein. While three were significantly higher in abundance in the “aged” ovarian fluid, Leucine-rich repeats and immunoglobulin-like domains 2, Rho GTPase activating protein 45b, RING-type domain-containing protein. Reductions of Piwi Proteins within zebrafish oocytes have led to widespread apoptosis (Houwing et al. 2007), the protein identified within this study, has been identified as being homologous to Piwi RNA-mediated gene silencing. While WASH complex’s have been associated with key functions within the development of oocytes (cytokinesis) and expulsion of the polar body post fertilisation (Wang et al. 2014). WASH complex subunits have been identified in this study as being significantly reduced in aged ovarian fluid when compared to fresh ovarian fluid.

This study aimed to characterise the ovarian fluid proteome of lumpfish, with a total of 649 proteins identified in all four treatments. As with the previous chapter it utilised the fresh wild eggs as a benchmark for good quality in the species, on which treatment differences were compared. This study identified significant difference between wild and captive, “good” and “bad” quality eggs and fresh and aged eggs ovarian fluid proteomes. A total of 54 potential proteins have been identified in this body of work as candidates for quality biomarkers in this species. The TMT isobaric tagging methods have a low documented level of reproducibility (Casey et al. 2017) despite this several proteins within the POA and egg quality runs were identified as displaying a significant change in abundant in multiple replicate runs. These candidates, such as Pyruvate Kinase (Wild and Captive), WASH complex subunit 5 (POA) and P53-induced death domain protein 1 (Quality) could potentially be used as quality biomarkers within the ovarian fluid. Similarly, proteins such as MICOS complex subunit (POA), Calpain 2, (m/II) large subunit, like (Quality) and Keratin 97 (Quality) with large changes in abundance between treatments should also be considered as potential markers. Future work would specifically target these proteins using immunoblotting, ELISA, or a targeted mass spectrometry assay such as MRM/SRM to confirm abundance changes in relation to egg quality and POA.

In conclusion the present study has identified the period of over-ripening within lumpfish as well as identifying potential biomarkers of poor and overripe eggs within lumpfish. The over-ripening of eggs through mismanagement of broodstock can have a significant detrimental effect on egg quality (Bromage et al. 1994, Mommens et al. 2015b). Poor egg quality is a major hurdle in successful commercial production of any new marine species. There appeared to be a significant difference in the performance of the wild and captive eggs at all time points however the rate of degradation followed

a similar pattern, with significant difference between fresh eggs, occurring after 24 hours for both groups. This suggests that even if egg composition improves within the species, there is a small window that these benefits can be lost because of mismanagement of ovulation and stripping in the species. This study clearly shows that intensive management of broodstock is required in order to identify ovulating individuals for stripping. With a short window (24hr) post ovulation until a significant reduction in quality, current broodstock assessments need to be conducted daily, as opposed to 3 days it is currently. The ovarian fluid of wild lumpfish was identified as containing many proteins associated with a variety of processes e.g. maintenance, metabolism and immune response. Three experimental tubes identified several potential biomarkers, within the most abundant proteins and within the lower abundant proteins which significantly change with decreasing egg quality and increased ageing. Proteins such as P53-induced death domain protein 1 and MICOS complex appear to be associated with poor quality and increased ageing in lumpfish. Future work needs to clarify the role of many of the proteins identified in this study in the process of poor quality or ageing within lumpfish. It would also aim to build on the data in the current study to develop individual biomarkers for poor quality or ageing within the species. Development of such can allow rapid on farm testing of ovarian fluid to improve broodstock management, identify good quality individuals and reduce hatchery effort in poor quality eggs.

Chapter 6

General Discussion

The overall aims of this research were to improve the current understanding of lumpfish (*Cyclopterus lumpus*) reproductive biology in order to provide information to close the life cycle of the species and produce a commercially viable captive lumpfish broodstock. The production of lumpfish juveniles has increased dramatically over the past few years, Powell et al. (2018) reported 0.8 million and 1.1 million juveniles in the UK and Norway respectively for 2015. Brooker et al. (2018) reported 1.9 and 15.9 million lumpfish were produced in the UK and Norway respectively. Whilst, Mortensen et al. (2020) reported 30 million juvenile lumpfish deployed in Norway alone in 2017. At present the majority of this production is derived from wild caught females which are stripped, and juveniles raised until deployment sizes. Such a wild harvest has brought some to question the sustainability of the process (Powell et al. 2018), however the long term goal for the sector is closed life cycle management (Huston et al. 2020) given that there is a push to determine genetic effects of delousing and cage performance (Imsland et al. 2020), reduce disease challenges (Einarsdottir et al. 2018, Skoge et al. 2018) and control production timing to optimise deployment (Imsland et al. 2019b). The production of lumpfish is a very new industry, with there being very limited knowledge of the species and its requirements for successful production in captivity. As such there are many production challenges that need to be overcome to realise a sustainable supply of captively produced lumpfish. Many of these challenges, are made harder to overcome due to the lack of understanding of the unique species basic biology. To date research investigating this species has focused on confirming delousing efficacy in salmon pens (Imsland et al. 2018), surveying the status wild broodstock (Kenneday et al. 2018) and improving juvenile management (Imsland et al. 2015). At present published work aiming at closing the life cycle of lumpfish is limited to photoperiod manipulations of broodstock (Imsland et al. 2018, Imsland et al.

2019) and the two papers published by this body of work (chapter 2, chapter 4). This thesis being the first body of work solely dedicated to closing the life cycle of the species.

Chapter two aimed to describe the effects of temperature during the spawning season on broodstock performance in captive lumpfish. This study described the effects of rearing temperature on timing of spawning and egg productivity, and quality across a thermal range associated with wild mature lumpfish. A secondary objective was to histologically describe oocyte development for the species. While teleost oocyte development follows a similar pattern, there are species specific differences which need to be defined for each species to identify treatment effects such as in this study. The new knowledge generated will help to more accurately describe the effects of environmental manipulations on the reproductive biology of captive lumpfish and optimise conditions for spawning and gamete quality. Conditions required for optimal broodstock reproduction in captivity are still largely unknown and the findings from chapter 2 demonstrated thermal regimes have significant effects on both male and female spawning season and gamete quality. Published evidence (Imsland et al. 2018, Imsland et al. 2019) and evidence from fisheries (*Pers. Com*, Tor Otterlei Skjerneset Fisk and Kennedy et al. 2020) suggest a weak photoperiodic entrainment of the spawning window. The impact of photothermal regimes during early gonadal development of lumpfish should be assessed. Methodologies similar to those works in Atlantic cod (*Gadus morhua*) (Van Der Meeren & Ivannikov. 2006) and haddock (*Melanogrammus aeglefinus*) (Martin-Robichaud & Berlinsky. 2004) as environmental controls of spawning are key in closed life cycle management (Wang et al 2010).

Chapter 3 aimed to compare and contrast the proximate composition of wild and captive derived lumpfish eggs and also to determine how levels of fatty acids, minerals

and pigments vary in association with the quality of lumpfish eggs. A further aim of this work was to subsequently measure how levels of fatty acids, minerals and pigments change through embryonic development. Initial descriptions of egg quality made in chapter 3 identified several nutritional components of lumpfish egg composition which warrant further investigation. While broad egg quality investigations are useful in identifying possible nutritional components which effect egg quality, egg quality in fish is multifactorial with their being a considerable number of environmental, nutritional, and management effects (Bobe & Labbe. 2010). This style of investigation can open up a large number of potential areas for future work and is usually the first step in identifying factors effecting egg quality in a new species. The work conducted in chapter 3 identified several important areas for investigation moving forward: determination of optimal levels within lumpfish eggs of compounds which have been well documented to affect egg quality in other species, such as EFA's, and total pigment levels. The role of those which are relatively unknown, such as the minerals (e.g. calcium and nickel) and individual fatty acids which are apparently individual to this species (e.g. 18:1 n-9). The next step in this process is to conduct broodstock nutritional trials to correlate dietary inclusions with egg quality for this species (Bobe & Labbe. 2010). While a number of nutritional trials have been conducted on lumpfish to date, they have all focused on juvenile nutritional requirements (Jonassesn et al. 2017, Imsland et al. 2019) and not broodstock requirements as yet. Chapter 3 has identified key nutritional components which can be controlled in order to clarify their role within egg quality, such as methodologies conducted in Sawanboonchun et al. (2008) where the positive benefits of astaxanthin boosting in Atlantic cod broodstock diets was demonstrated. While a number of nutritional components have been identified by this work as effecting egg quality, the initial focus would be on those

components which loaded highly with in the PCA such as 22:1n-9, EPA, Total n-3PUFA's, as well as those which displayed high r^2 values when correlated with hatching rate, such as 18:4n-3 and Phospholipid 18:1n-9.

The aim of chapter 4 was to test a range of extenders for cold storage of lumpfish milt and validate rapid and accurate methods for estimating sperm concentration. Males are a limited resource in current lumpfish broodstock management, both methods identified in this chapter have been used to improve artificial fertilisation protocols during the 2019 and the 2020 spawning seasons at a commercial hatchery in Scotland. The identification of quantification methods can further inform management effects on male reproductive performance, as seen in chapter 2. The creation of fertilisation protocols *e.g*. standardisation of sperm to egg ratio, have been shown to be beneficial in many other species such as Atlantic cod (Butts et al. 2009) and European eel (*Anguilla Anguilla*) (Butts et al. 2014) and is a possible fruitful avenue of investigation for this species to further reduce the impact of limiting male production.

The management of reproduction in the species appears to be through stripping, rather than spontaneous spawning like in the other cleaner fish species currently farmed, *i.e*. ballan wrasse (*Labrus bergylta*) (Grant et al. 2016). This requires more intensive management of lumpfish ovulation than is current being conducted on farm. Chapter 5 identified that the window for striping good quality eggs for the species is relatively short post ovulation (24 hrs), before eggs see a significant reduction in quality through post ovulatory ageing. Chapter 2 utilised ultrasound to identify female reproductive stages, combining the basic information determined in chapter 2 and 5, can inform stripping protocols for the species. It is possible that manipulation or induction of ovulation become necessary to ensure good gamete quality and improve management in this species through synchronisation of spawning. Future work will

benefit from the tools, such as ultrasound gonad assessment, sperm management developed in chapters 2, 4 and 5 and should investigate the use of hormonal therapy such as GnRH to synchronise ovulation within captive lumpfish broodstock like that conducted in other species (Mylonas & Zohar 2000). This manipulation would allow for more controlled spawning and stripping protocols, as well as provide a wider time window for essential egg management practices such as degumming (Pooley et al. 2019) and disinfection (Powell et al. 2018) might be necessary within this species, however this warrants further investigation. While this work has confirmed that this species is a determinate batch spawning species. The endogenous hormonal regulation of oocyte maturation and ovulation has not yet been described. This work would be useful in the creation of therapies for the wider control of maturation and spawning. Specific management interventions that would be useful would be the ability to induce ovulation in order to coordinate egg production within hatcheries such as that found in turbot (*Scophthalmus maximus*) (Mugnier et al. 2000) and the reduction of spermatocrit or increase in seminal fluid volume to aid non sacrificial stripping of milt from mature males as seen in Atlantic halibut (Martin-Robichaud et al. 2000).

At present the industry aims to utilise first year spawning broodstock, as shown in chapter 2 and 3 these fish are considerably smaller in size at first spawning than wild caught broodstock. Broodstock size has been shown to have an effect on egg quality in other species such as Atlantic cod (Kjesbu et al. 1996) and could be affecting egg quality and juvenile's robustness in lumpfish. Future work is required to document the effects of delayed first spawning similar to work conducted in rainbow trout (*Oncorhynchus mykiss*) (Bourlier & Billard 1984), reconditioning of broodstock which spawn in the first year could also be effective. However, it is possible that with such large investment in gonads for this species that delaying first spawning could improve

stock performance such as found in gilthead seabream (*Sparus aurata*) (Kissil et al. 2001). Investigations into the of 2nd and 3rd year spawning season on egg quality, fecundity and production parameters as conducted in red porgy (*Pagrus pagrus*) (Mylonas et al. 2004) would be beneficial for this species.

The initial intentions of this work were to conduct on farm trials with an experimental broodstock held on site in Scotland. As stated previously there was a significant issue in Scotland at isolating a captive broodstock due to disease challenges thought to be caused by thermal stress. The veterinary request to cull all 7 isolated broodstocks required a re-shaping of the aims for this body of work. The use of captive Norwegian broodstocks and wild caught broodstock allowed the work to identify a key failing within Scottish broodstock practice and improvements were made during the latter stages of the PhD (Pountney et al. 2020a). These improvements allowed the creation and maintenance of a small experimental broodstock to spawning, which were then used moving forward. The justification of lower holding temperatures reduced the reluctance of on farm investment in a broodstock. Implementation of nutritional information from chapter 3 has improved on farm egg quality and hatching success, to a point where the farm may not require wild caught stock to meet demand in 2020/2021. Furthermore, hatchery management techniques developed throughout this PhD, including sperm concentration assessments, cold storage of milt (Pountney et al. 2020b), and ultrasound screening of lumpfish broodstock have been utilised effectively in this new captive broodstock.

While this body of work has made some important strides in the area of broodstock management within this species. It has also highlighted the large body of work which still needs to be conducted for more effective closed life cycle management is still to be achieved. This work has allowed the creation of a productive captive broodstock in

Scotland which means that as of 2020/2021 production season the commercial hatchery may not require (for the first time) any eggs from wild caught broodstocks. Considering that in 2016 at the start of this body of work all broodstocks at this site were non-viable or did not reach spawning demonstrates the real impact this research program has made. A focus should now be directed towards developing further management techniques in photo-thermal entrainment, induction of ovulation, optimisation of stripping, gamete management and storage. This current work will be a cornerstone in further enhancing the management and performance of captive stocks and together this make the industry target of sustainable closed life cycle management viable and open the door to selective breeding for the sector (Huston et al 2020). While areas for selective improvement within the species have been identified, e.g. delousing efficiency, growth rate & robustness these will only be realised when captive broodstock performance is assured.

Overall, the use of cleaner fish as anti-sea lice treatment measures appears to be a tool which is effective and potentially more sustainable than other sea louse treatments. If this “natural” approach to pest management is to be an effective tool, maintaining a good quality supply of broodstock in closed life cycle management is critical, with the research contained within this thesis being one of the first dedicated bodies of work to this end.

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LIST OF PUBLICATIONS

Pountney, S. M., Migaud, H., & Davie, A. (2020). Short term cold storage and sperm concentration assessment of lumpfish (Cyclopterus lumpus. L) Milt. Aquaculture, 735646.

Pountney, S, M., Lein, I., Miguard, H., Davie, A., (2020) High temperature is detrimental to captive lumpfish (Cyclopterus lumpus, L) reproductive performances. Aquaculture. 522 735121

LIST OF CONFERENCES

Pountney, S, M, Lein I, Counter-Selly, S-L, Miguard H, Davie, A**.** Egg quality determinants in lumpfish (*Cyclopterus lumpus*). *MASTS Annual science meeting 5th to 8th October 2020*. Virtual, Scotland. Oral Presentation.

Pountney, S, M, Lein I, Miguard H, Davie, A**.** Don't count your lumpies before they hatch: Egg quality of wild and captive lumpfish (*Cyclopterus lumpus*). *University of Stirling PhD Aquaculture student symposium 24th to 25th October 2019*. University of Stirling. Oral Presentation.

Pountney, S, M, Miguard H, Davie, A**.** One Lump or two? Methods for quality control and storage of lumpfish (*Cyclopterus lumpus*) milt. *University of Stirling PhD Aquaculture student symposium 24th to 25th October 2019*. University of Stirling. Poster Presentation.

Pountney, S, M, Lein I, Miguard H, Davie, A**.** Temperature effects of captive Lumpfish *Cyclopterus lumpus* broodstock spawning season and gamete quality. *Aquaculture 18 (WAS/EAS)* 25th to 29th August 2018. Montpellier France. Oral Presentation.

Pountney, S, M, Lein I, Miguard H, Davie, A**. Plenary session, Student Spotlight competition.** Temperature effects of captive Lumpfish *Cyclopterus lumpus* broodstock spawning season and gamete quality. *Aquaculture 18 (WAS/EAS)* 25th to 29th August 2018. Montpellier France. Oral Presentation.

- Winner Best Presentation

Pountney, S, M, Lein I, Miguard H, Davie, A**.** Temperature effects of captive Lumpfish *Cyclopterus lumpus* broodstock spawning season and gamete quality.

University of Stirling PhD Aquaculture student conference April 17th2018. University of Stirling. Poster Presentation.

List of training and courses

November 2019: Arch- UK, ECR proteomics workshop. Stirling UK

November 2017: **ScotPil Training course**, Edinburgh and Stirling. Modules **E1-L, Pil-A, Pil-B, Pil-K**, Fish, Freshwater and Marine

List of Grants

£11,561 EU Transnational Access Grant (**AE100026**) Lead Applicant. (10th call, 2018).

$500 WAS Student Spotlight competition Aqua2018, Montpellier, France Winner.

$400 WAS Student travel award, for Best Student Abstracts at WAS/EAS 2018

£500 Scottish Aquaculture Innovation Centre (SAIC) Conference travel grant to attend EAS/WAS 2018.

£37,142 EU Transnational Access Grant (**AE040063**) Co Applicant. (8th call, 2017).