Optimising Juvenile Lumpfish (*Cyclopterus lumpus*) Diet Through a Comparative Study of Wild and Farmed Populations of the Faroe Islands

Thesis submitted for the degree of Doctor of Philosophy

Institute of Aquaculture

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by

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Declaration

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted by me, and has not been submitted for any other degree or qualification.

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Abstract

Lumpfish welfare has become a concern as the use of cleaner fish in salmon farms continues to grow. High mortality rates at the deployment phase have raised ethical concerns, highlighting the need to ensure optimal welfare for lumpfish (*Cyclopterus lumpus*). Challenges in addressing lumpfish welfare include infectious diseases, lack of established nutritional requirements, and standardised operational welfare indicators (OWI) for this species. The aim of this PhD was to investigate the nutritional status, health and welfare of farmed lumpfish compared to the wild counterparts in order to optimise feed formulations and enhance welfare and robustness in farming environments. Chapter 1 provided an overview of lumpfish biology in the wild, its role in aquaculture, and key welfare and nutritional considerations. Chapter 2 detailed the general methodology for fish sampling, welfare scoring, and laboratory analyses. Chapters 3 and 4 compared the nutritional and biochemical differences between farmed and wild lumpfish in the Faroe Islands. Chapter 3 focused on body composition, amino acid, and fatty acid profiles, revealing significant differences in lipid content being higher in farmed fish. This difference reflect the impact of high-energy diets, feeding regimes and controlled environments in farming conditions. The fatty acid profile of wild fish reflects the fatty acid composition of their natural prey, while the farmed fish is influenced by the aquafeed composition, mainly the use of vegetable ingredients, and the availability of seasonal preys in the sea cages. The amino acid profile is also influenced by diet and environmental conditions. Even though the amino acids had similar levels in both farmed and wild lumpfish, some differences were found in methionine and cysteine levels. Chapter 4 investigated liver colour and nutritional composition, showing differences in lipid content, lipid classes, fatty acid, and carotenoid levels. The high fat storage of farmed fish, both in whole fish and liver, from land-based hatcheries suggest they have good energy storage and appear ready for deployment. However, the high mortality rates observed post-deployment indicate that the hatchery diet may not adequately prepare lumpfish for the challenges of the sea cage environment. Chapter 5 assessed the impact of farming practices on OWI and liver health of lumpfish compared to wild populations, finding higher levels of physical damage and compromised liver health in farmed lumpfish. Chapter 6 examined the effects of varying levels of EPA and DHA on the growth, health, chemical composition, and stress response of lumpfish. Results showed that diets with higher EPA and DHA levels (2-3% of diet) led to better growth performance, higher survival rates, and lower stress responses. Chapter 7 discussed findings and implications for feed formulation and best practices in husbandry, proposing future research directions. This research highlights the importance of tailored nutritional strategies in promoting lumpfish welfare in aquaculture, providing insights that enhance sustainability and ethical use of lumpfish as cleaner fish.

Keywords: cleaner fish, lumpfish, welfare, nutrition, OWI, EPA and DHA

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List of Abbreviations

ALA – Alpha-Linoleic acid

ANOVA – Analysis of Variance

ARA – Arachidonic acid

bFCR – Biological feed conversion ratio

BHT – Butylated hydroxytoluene

BC – Body condition

CB – Cerebrosides

CHOL – Cholesterol

DAG – Diacylglycerol

DGC – Daily growth coefficient

DHA – Docosahexanoic acid

DMA – Dimethyl acetals

DPA – Docosapentaenoic acid

EPA – Eicosapentanoic acid

FAME – Fatty acid methyl esters

FCE – Feed conversion efficiency

FCR – Feed conversion ratio

FFA – Free fatty acids

GC – Gas chromatography

H&E – Haematoxylin and eosin

HSI – Hepatosomatic index

KO – Krill oil

LA – Linoleic acid

LABWI – Laboratory based welfare indicators

LC-PUFA – Long chain polyunsaturated fatty acids

LPC – Lysophosphatidylcholine

MT – million tonnes

MUFA – Monounsaturated fatty acids

N-3 PUFA – n-3 polyunsaturated fatty acid

N-6 PUFA – n-6 polyunsaturated fatty acid

NBF – Neutral buffered formalin

OA – Oleic acid

OFN – Oxygen free nitrogen

OWI – Operational Welfare Indicators

PC – Phosphatidylcholine

PCA – Principal Components Analysis

PE – Phosphatidylethanolamine

PG – Phosphatidylglycerol

PI – Phosphatidylinositol

PS – Phosphatidylserine

PUFA – Polyunsaturated fatty acids

RO – Rapeseed oil

SAFA – Saturated fatty acids

SE – Sterol esters

SC-PUFA – Short chain polyunsaturated fatty acids

SM – Sphingomyelin

SGR – Specific growth rate

TAG – Triacylgylcerols

TGC – Thermal growth coefficient

VSI – Viscerosomatic index

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Chapter 1. Introduction

1.1. Aquaculture and Salmon production

Aquaculture is the farming of finfish, molluscs, crustaceans and other aquatic organisms like seaweeds and algae (FAO, 2020). Since the late 1980s, capture fishery production has been relatively static. In contrast, aquaculture has not only surpassed capture fisheries, but become the predominant source of fish for human consumption (FAO, 2024). In 2022, production in fisheries and aquaculture set a new record with 223.2 million tonnes (MT), which included 185.4 MT of aquatic animals and 37.8 MT of algae. Globally, the total production of capture fisheries was 92.3 MT (excluding algae), while the total production of aquaculture was 130.9 MT (FAO, 2024).

Due to its continuing impressive growth, aquaculture keeps expanding faster than other major food production sectors (Little et al., 2016). Although the vast diversity of aquatic species are farmed, few species command the majority of production, particularly grass carp (*Ctenopharyngodon idellus*) in inland aquaculture, and Atlantic salmon (*Salmo salar*) in marine aquaculture (FAO, 2022). Atlantic salmon is one of the major farmed species with a production of over 2.7 MT in 2022 (FAO, 2024) (Figure 1.1), and has become an internationally marketed commodity product (Bostock et al., 2010). The salmon sector, notably, also led the way in establishing contemporary and industrialised aquaculture (Anderson et al., 2017), and today stands as one of the most profitable and technologically sophisticated industries (FAO, 2022).

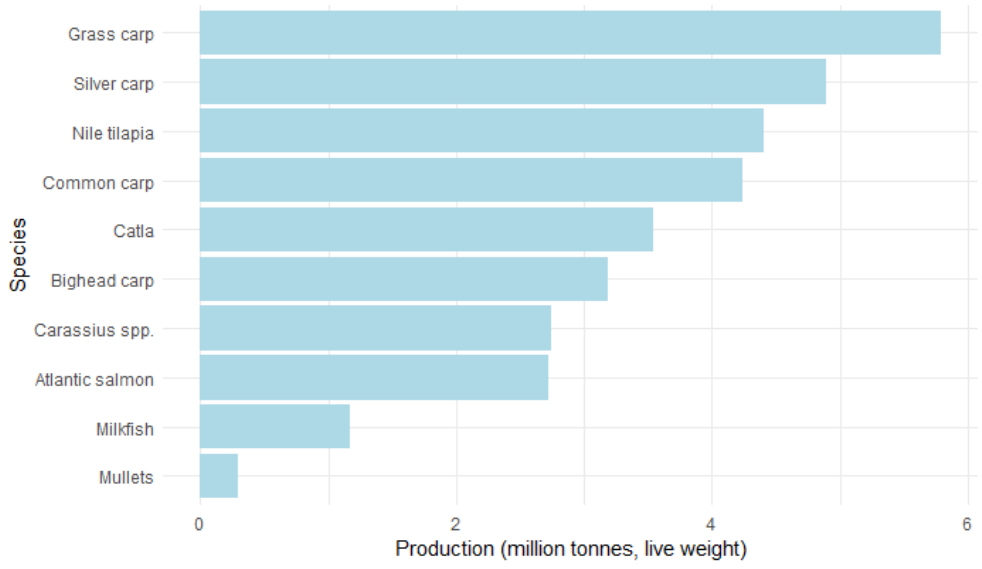


Figure 1.1. Production of the ten major species in world aquaculture in 2020 (FAO, 2022).

The two largest salmon producing countries are Norway and Chile, with a production of 1.56 MT and 0.72 MT in 2021, respectively. There are other small producers in Europe, North America and Australia that make up for the rest of the production, such as United Kingdom, Canada, Faroe Islands and Tasmania. After Norway and Chile, the United Kingdom is the largest producer of farmed salmon amongst all European countries, reporting a production of 0.18 MT in 2023 (Crawford, 2003|; FAO, 2024). Salmon production in United Kingdom developed during the late 1970s, mainly in sea lochs and inshore waters on the West Coast of Scotland (Kenyon & Davies, 2018; Whitmarsh & Wattage, 2006). The Faroe Islands also have a noteworthy production of Atlantic salmon, reaching 0.1 MT in 2023 (FAO, 2024). Both the Faroe Islands and Scotland had similar relative growth trends since 1998 (Figure 1.2).

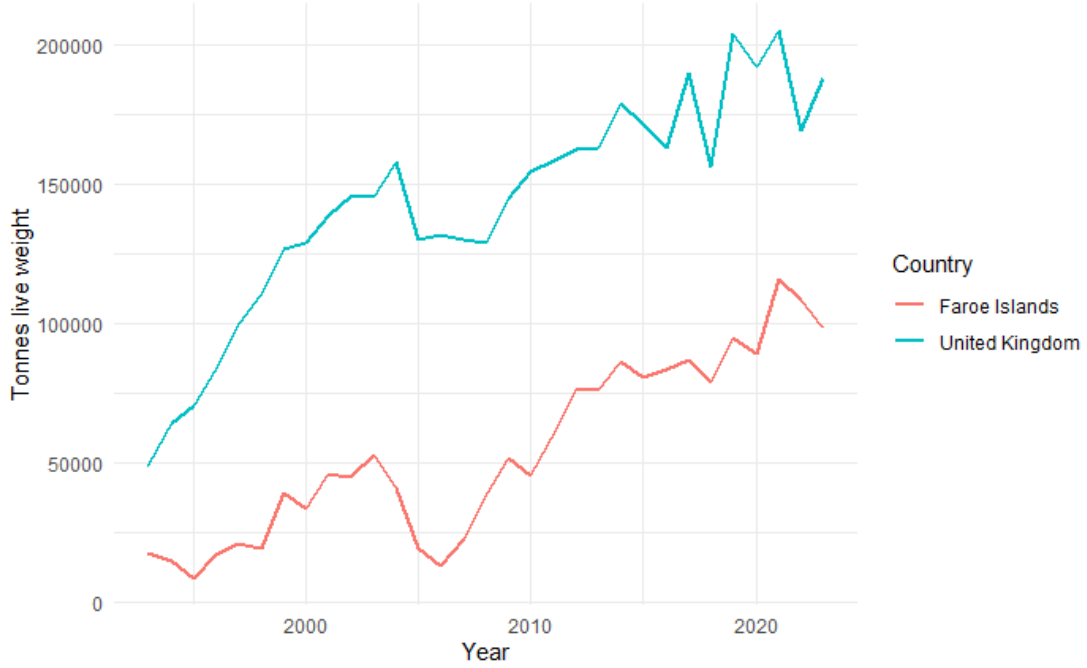


Figure 1.2. Tonnes of salmon live weight produced each year in Scotland and Faroe Islands (1993-2023). Data are from FAO databank (1993-2021), Scottish Fish Farm Production Survey (2022), Hagstova.fo (2022-2023).

1.2. Aquaculture in the Faroe Islands

The Faroe Islands ("Føroyar" in Faroese) is an archipelago of 18 islands situated in the North Atlantic, situated about half way between Scotland and Iceland, with a population of approximately 55 thousand people (Føroya landsstýri, 2024) (Figure 1.3). They are an autonomous region of Denmark since the first democratic constitution of 1849 (Kočí & Baar, 2021). These islands are made mainly of basalt and tuff, and they are characterised by high cliffs, deep fjords, and steep mountains, typical of volcanic archipelagos (Føroya landsstýri, 2024).

Like many isolated and coastal communities in the North Atlantic, the Faroe Islands rely heavily on fishing, fish processing, maritime activities, and offshore industries (Hayfield, 2018). The fishing industry targets mainly cod (*Gadus morhua*), haddock (*Melanogrammus aeglifinus*) and saithe (*Pollachius virens*) (Hegland & Hopkins, 2014). The geography and the territory, with temperate oceanic waters and the strong currents in the fjords, create the ideal conditions for fish farming in the Faroe Islands. The average sea water temperature is relatively stable, ranging from 6°C in the coldest month to 10 °C in late summer, making it ideal for farming cold-water species. Fish farming in the Faroe Islands began in the late 1960s, and expanded into a significant industry during the 1980s (Simonsen et al., 2014). Atlantic salmon is the primary focus of Faroese aquaculture which is conducted along the coast, using similar practices across different sites. Seaweed farming has also grown steadily (ICES, 2023).

Figure 1.3. Map showing the location of the Faroe Islands, including the 18 islands' names, and the Faroese flag (adapted from https://web.uri.edu/steep/communities/faroe-islands/)

In recent years, aquaculture has expanded, and farmed salmon accounts for half of the country's export value, offering employment to the communities throughout the islands (Hegland & Hopkins, 2014). There are three major farming companies: Mowi and Bakkafrost, both with partial foreign ownership, and Hiddenfjord, which is entirely locally owned (Johannesen et al., 2018).Despite the constant growth of the salmon farming industry in the North Atlantic countries, some of the main bottlenecks affecting production nowadays are disease outbreaks and parasitic infestation, together with sustainable feeds and escaped fish (Lekang et al., 2016). In particular, the parasitic infection by ectoparasitic copepods crustaceans, known as sea lice, continue to be a key limitation to the expansion of salmonid aquaculture industries worldwide (Brooker et al., 2018). With its large production of farmed salmon, the Faroe Islands also encounter challenges related to disease outbreaks and infections such as sea lice, increasing production cost and challenging fish welfare (Kragesteen et al., 2018; Simonsen et al., 2014).

1.3. Sea lice

Sea lice are marine ectoparasitic crustaceans, members of the copepods family, belonging to the family Caligidae. Although there are 37 caligid genera that include roughly 559 species, *Lepeophtheirus salmonis* (Krøyer, 1837) and *Caligus elongatus* (Nordmann, 1832) are the ones which are mainly accountable for serious disease in salmonid aquaculture (Costello, 2006; Pike & Wadsworth, 1999). *L. salmonis* is considered the dominant species in cage-culture in the Northern Hemisphere, whereas *C. elongatus* is distributed in both Hemispheres, and has a seasonal pattern in Norway, Faroe Islands, Ireland and Scotland (á Nordi et al., 2015; Pike & Wadsworth, 1999). Sea lice have flattened bodies and they are able to attach to the surface of the hosts through appendages, which are small paired sucker-like structures positioned anteriorly in their bodies. These appendages also contain a specialised endocuticle which play a critical role in the initial adhesion process (Ohtsuka et al., 2021). Gravid females produce multiple egg strings, each with hundreds of eggs that hatch into the first of three free-living planktonic stages, before settling on a host (Heuch et al., 2000). The lifecycle of *L. salmonis* comprises two initial planktonic stages, Nauplius I and II, followed by one copepodid stage. These stages are free-swimming, but only the copepodite stage is infective to its hosts. These stages are followed by two chalimus stages (Chalimus I-II) that use a frontal filament to be attached to the host, and feed on the host skin (Hamre et al., 2013). These are followed by two preadult immature stages (male or female), and finally an adult stage (Whelan, 2010) (Figure 1.4). *L. salmonis* has a life cycle of eight developmental stages as well as *C. elongatus* (Hamre et al., 2013). *L. salmonis* is a specialist parasite of salmonids, whereas *C. elongatus* can infest over 80 different fish species (Kabata, 1979).

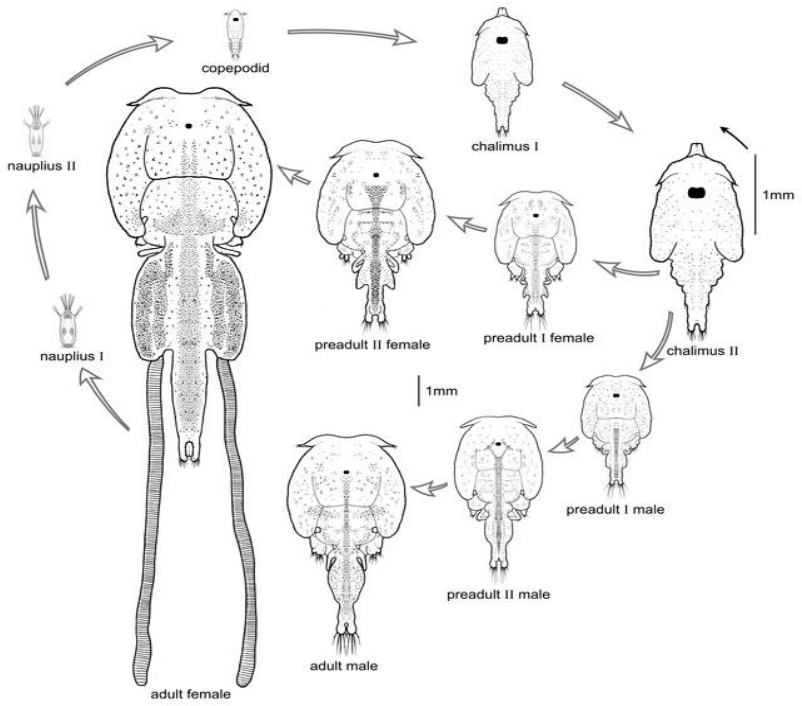


Figure 1.4. Lifecycle of *Lepeophtheirus salmonis* (Sea Lice Research Centre, 2020).

Each stage duration is strongly temperature dependent (Costello, 2009; Pike & Wadsworth, 1999), however, at colder temperatures, they live longer and grow larger. Each adult female is able to produce an average of 500 eggs per egg string at 10°C (Costello, 2006). In the study by Heuch et al. (2000), females that were sampled at lower temperature (8.9°C) contained longer egg strings and overall more eggs than those sampled at higher temperature (12.2°C).Sea lice can be found anywhere on the fish body, but they tend to aggregate mainly just behind the head of the fish and fins, dorsally and anteriorly (Pike & Wadsworth, 1999). Sea lice primarily feed on the host's epidermis, using rasping mouthparts to remove mucus, skin, and underlying tissues. This feeding behaviour results in the loss of epithelium, bleeding, tissue necrosis, and the reduction of physical and microbial protective functions, consequently disrupting the fish osmoregulatory homeostasis (Costello, 2006; Whelan, 2010). Epidermal erosion caused by sea lice has been related to anaemia, reduced lymphocyte levels, and increased cortisol levels, which in turn lead to a compromised immune system. These perturbations make the fish more vulnerable to secondary infections, resulting in disease and high mortality rates. Additionally, fish affected by sea lice exhibit reduced appetite, slower growth, and decreased food conversion efficiency (Costello, 2006; Pike & Wadsworth, 1999). Sea lice can also infect wild salmonids and other farmed fish, with escalating infestations reported over the years (Costello, 2006).

The abundance of sea lice depends on sea water temperature, host density and spatial distribution (Costello, 2006). The first outbreaks of *L. salmonis* in farmed salmon production was in Norwegian farms during 1960s when cage farming began, followed by the Scottish industry in the mid-1970s and

late 1980s in North America with infections of *C. elongatus* (Rae G.H., 1979cited in Pike & Wadsworth, 1999).

In the Atlantic salmon farming industry, sea lice are the most damaging and widespread pathogenic parasites. Because of the negative effects that sea lice have on fish welfare and salmon production, it is necessary to have efficient sea lice control and preventive strategies (Aaen et al., 2015).

1.4. Sea lice control methods

Sea lice are a massive economical and biological obstacle, and their control is a significant limitation on farm profitability. The costs related to sea louse control increase in proportion to production (Aaen et al., 2015; Costello, 2009). The costs for sea lice management include medicine or treatment costs, effects on fish growth, food conversion, staff time, mortality and downgrading of fish after harvest. The most recent estimate of the cost of sea lice to the salmon industry was US$436 million in 2011 (Abolofia et al., 2017). Adjusting for inflation, this cost is estimated to be US$612 million in 2024. This cost accounts for 8.70% of the production value in affected countries and result in a sea lice control cost of US$0.41/kg of harvested salmon (Abolofia et al., 2017). Without any treatment, it would cost the industry four times more due to higher mortalities (Costello, 2009).

There are several treatments to control sea lice. The first methods were drug based: Emamectin benzoate, Cypermethrin, Deltamethrin, Teflubenzuron, Diflubenzuron, Azamethiphos and Hydrogen peroxide are available as a treatment against sea lice, though not all of them are authorised in every country (Treasurer, 2018). These drugs are used either as in-feed or in bath treatments. However, the use of these chemical treatments led to an increase in drug resistance and to the need of alternative control methods (Aaen et al., 2015; Treasurer, 2018). Chemical treatments are toxic to non-target species, particularly crustaceans and their direct release into the marine environment is increasingly concerning (Burridge et al., 2010). Other innovative non-chemical strategies such as brushes or water jets (hydrolicer), warm water or freshwater bath treatments (thermolicer), laser technologies, physical barrier technologies like lice skirts, snorkel or enclosed cages, are being used (Aaen et al., 2015; Holan et al., 2017). Preventive measures have been introduced such as fallow periods, where for 4-6 weeks no fish are present in a farming area, synchronised treatments across geographic areas, deployment of cleaner fish where possible, monitoring of lice abundance through regular sea lice counts, and removal of moribund and sick fish from the cages (Aaen et al., 2015; Costello, 2006). Furthermore, better management of farms that include communicating the health status of fish to nearby farms, the prevention of escaped salmon, and the selection of farm sites, are key to infestation management strategies (Costello, 2006; Treasurer, 2018).

During recent years, technological developments in the industry have been made to better address lice issues. These include the production of larger smolt, so that the longer time spent on land shortens the production cycle at sea. Also, the gradual relocation of marine sites to more exposed areas is driven by the need for better control measures (ICES, 2023). Among the preventive measures, the deployment of

cleaner fish represents a salmon welfare-friendly strategy due to less handling which comes with medicinal bath or mechanical treatment, and a green alternative to the use of chemical treatments (Overton et al., 2020; Treasurer, et al., 2018).

1.4.1. Cleaner fish

The use of cleaner fish is an example of "cleaning symbiosis", as defined by Feder (1966), where cleaner organisms remove ectoparasites, bacteria, diseased or injured tissue from cooperative host organisms. Also, this mutually beneficial behaviour provides a source of food for the cleaner (Feder, 1966). Using cleaner fish lowers the need for medicines to control sea lice, resulting in improved fish health, reduced costs and harvested fish with no medicinal residues (Vaughan et al., 2017).

The first attempt to use cleaner fish to delouse salmon dates back to the 1980s in Norway, where fish, mainly from the wrasse family (Labridae), were reared with salmon and their delousing potential was investigated (Bjordal, 1991; Bjordal, 1988). The use of wrasse both in tanks and sea cages decreased the numbers of sea lice attached to salmon, leading to its widespread use in the salmon industry (Bjordal, 1988). This increased the demand for cleaner fish, and nowadays, the salmon sector widely uses it as a biological control against sea lice, making them a well-integrated part of integrated pest management (Nilsen, 2008; Treasurer, 2018). These fish with a natural delousing behaviour towards other fish are a successful example of biological control (Treasurer, 2018). Four species of wrasse and one species of lumpfish were identified as potential salmon delousers: goldsinny wrasse (*Ctenolabrus rupestris*), corkwing wrasse (*Symphodus melops*), rock cook (*Centrolabrus exoletus*), ballan wrasse (*Labrus bergylta*), and the common lumpfish (*Cyclopterus lumpus*) (Skiftesvik et al., 2014). Despite there being many species of cleaner fish, presently there are two main delouser species that are stocked with salmon in North Atlantic countries, ballan wrasse (*Labrus bergylta*) and lumpfish (*Cyclopterus lumpus*) (Brooker et al., 2018). These two species are different in terms of ecology, physiology and behaviour (Table 1.1), but they are both proven to be efficient at delousing salmon (Imsland et al., 2018; Skiftesvik et al., 2013).

The production cycle for lumpfish is shorter than ballan wrasse, as it needs only 5 to 7 months to reach the deployment size of around 20 g, compared to 18 months needed for ballan wrasse to reach 40-50 g (Erkinharju et al., 2021). Ballan wrasse is deployed with a stocking ratio of 5 % of the salmon stock, whereas lumpfish stocking ratio is 10-15 % (Brooker et al., 2018).

Lumpfish are a cold-water species and are preferably deployed in sea cages when water temperatures are low, as they continue to actively feed at temperatures near 4°C (Nytrø et al., 2014). On the other hand, ballan wrasse is a temperate species which exhibit reduced activity at temperatures below 5-7 °C, going into a winter dormancy where physiological activity is reduced. This has led farmers to prefer to stock ballan wrasse in summer months and lumpfish during winter months or cooler temperatures (Geitung et al., 2020).

Table 1.1. Main differences between ballan wrasse and lumpfish in terms of deployment in the cages.

Species	 **Ballan wrasse (*Labrus bergylta*)[1]**	 **Lumpfish (*Cyclopterus lumpus*)[2]**
Deployment window	Spring/summer Increasing water temperature	Late autumn/winter Decreasing water temperature
Deployment size	40-50 g	15-30 g
Stocking rate	2-10%	7-10%
Time to deployment	1.5 years	5-7 months
Feeding behaviour	Winter dormancy, will not feed < 5-7°C	Will feed as low as 4°C

[1] (Sterry, 2015)

[2] (Marine Stewardship Council, 2020)

Since lumpfish are more efficient in cold water, this species is preferred in Canada, northern Norway, northern isles of Scotland and the Faroe Islands (Imsland et al., 2014). Furthermore, in the Faroe Islands the import of wrasse is banned as there are no native species of wrasses used as cleaners in Faroese waters (Johannesen et al., 2018). Therefore, this Thesis focuses solely on lumpfish as it is the only species of cleaner fish used and native to the Faroe Islands, where the fieldwork was carried out.

1.5. Lumpfish ecology and biology

1.5.1. Species description

The lumpfish, also known as the lumpsucker, was first formally named and described by Linnaeus in 1758, as *Cyclopterus lumpus*, meaning “round fin”. This species is a bony fish (class: Osteichthyes, infraclass: Teleostei) belonging to the order Scorpaeniformes and the family Cyclopteridae. *Cyclopterus lumpus* is morphologically distinct, being the sole species within the genus Cyclopterus (Powell et al., 2018). The name “lumpfish” stems from its strange appearance: it has a compressed body, a short and thick head, a terminal mouth that contains small teeth and a long and high crest. The crest is made of the first dorsal fin which is covered by thick skin and compressed tubercles. These tubercles are also found on the side of the body, forming three longitudinal rows (Davenport, 1985) (Figure 1.5). What gives the species the generic name lumpsucker is the round sucker disc spanning approximately 20%

in total body length, which is a specialised modification of the pelvic fins used to attach to surfaces and substrates (Davenport & Thorsteinsson, 1990; Davenport, 1985) (Figure 1.5).

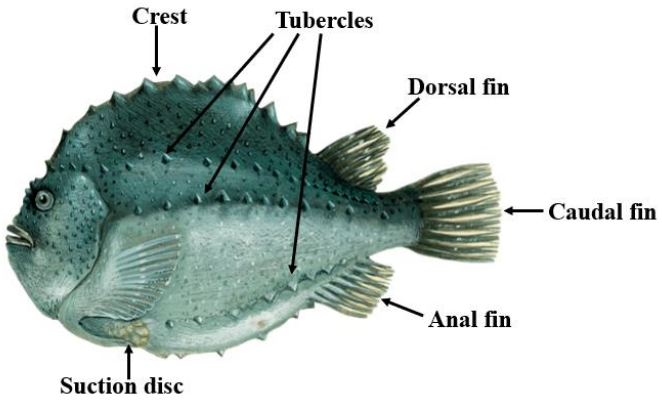


Figure 1.5. Lumpfish, *Cyclopterus lumpus* (Marine Stewardship Council, 2020).

Lumpfish lack a swim bladder and are able to achieve buoyancy thanks to a cartilaginous skeleton, lipid reserves, subcutaneous jelly and various low density fluids (Davenport & Kjørsvik, 1986). Other features are the rubbery, scale less skin texture and the vivid skin colours of the adults. During spawning season, adults show a pronounced sexual dimorphism, especially in size and colour (Figure 1.6). Males are typically smaller than females (30 ±10 and 42 ± 10 cm length, respectively) and display a red and orange coloration of fins, eyes and ventral areas of the body, while females (much larger, 42 ± 10 cm length) are grey or blue-green (Atkinson & Kulka, 2017; Davenport, 1985) (Figure 1.6). In both sexes, juvenile lumpfish display light green or green-yellow skin. Nevertheless, in response to substratum colour or when juveniles are associated with rock pools or weed, they lighten or darken the skin by control of melanophores (Davenport & Thorsteinsson, 1990).

Figure 1.6. Colours difference during spawning of male and female of lumpfish (Atkinson & Kulka, 2017).

1.5.2. Habitat and distribution

Lumpfish are a semi-pelagic species that during winter feed in the open sea, while in spring and summer prefer shallower waters for spawning (Davenport, 1985b; FAO, 2020). Lumpfish usually lay their eggs in shallow water inshore on stones and amongst *Laminaria* beds. Lumpfish exhibit external fertilization, where the female spawns her eggs onto a substrate, typically a rock, and the male then fertilizes them. When eggs get in contact with seawater, they adhere to each other and form masses which are fanned and guarded aggressively by the males (Davenport, 1985; FAO, 2020). During warmer months, temperatures are higher, food is more abundant, and therefore juveniles remain close to the coastal waters during the first year of life, typically found among kelp and in floating seaweed (FAO, 2018; Ingolfsson & Kristjansson, 2002). After approximately one year of age, juveniles (at lengths > 50 mm) will go to deeper water, assuming a semi-pelagic lifestyle (Daborn & Gregory, 1983a).

Lumpfish are well distributed along both sides of the Atlantic Ocean and have been recorded in 24 countries in Europe and North America (Davenport, 1985). On the western North Atlantic coast, they are distributed from the island of Disko (north-western Greenland) southwards to Chesapeake Bay, incorporating most of eastern Canada. On the western side of the Atlantic Ocean, lumpfish are easily found in waters off Iceland, south of Greenland, the Faroe Islands, UK and Norway (Figure 1.7).Other European occurrences are north of Iceland, Svalbard, White and Barents Seas and the Baltic Sea (Kudryavtseva & Karamushko, 2002). Lumpfish have occasionally been reported in lower latitudes such as Spain, and southern Portugal, whereas one vagrant female was recorded in the Mediterranean Sea (Banon et al., 2008; Dulčić & Golani, 2006) (Figure 1.7).

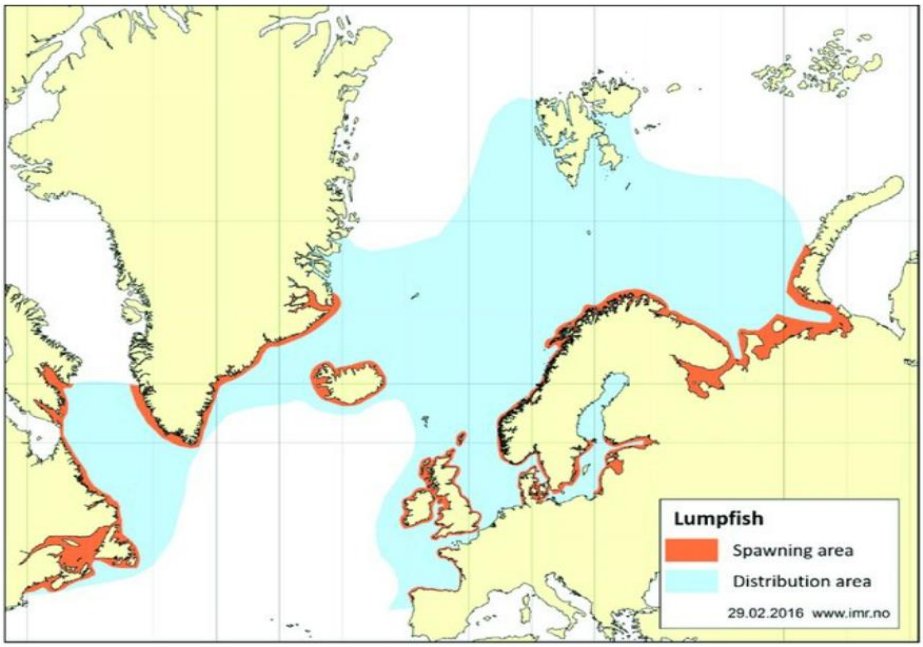


Figure 1.7. Lumpfish distribution map, including spawning area and distribution area (Institute of Marine Research, 2019).

Many reports lack information on the semi-pelagic adult phase outside the spawning season, and this is a problem for the assessment of the total area occupied by the species and the population structure of the lumpfish stock (Davenport, 1985; Eriksen et al., 2014). However, a more recent survey conducted in the Barents Sea reports a mean annual biomass of 48.000-143.000 tonnes and a mean annual abundance of 53-132 million individuals since 1980 (where 40-80% of the total abundance were juveniles) (Eriksen et al., 2014). In the study by Eriksen et al. (2014), most juveniles were found in temperatures of 5–7°C, while the majority of adults were found in temperatures range of 4–7°C.

Traditionally lumpfish were harvested solely for the roe, as lumpfish caviar is marketed as an inexpensive alternative to sturgeon caviar (Powell et al., 2018). Lumpfish fisheries exploit the breeding season, capturing female fish in inshore waters, where only the roe is landed (Davenport, 1985; Kennedy et al., 2019). Currently, the most significant lumpfish fisheries are the Canadian eastern Atlantic–west Greenland region (approximately 70% of the catch) and Iceland (about 23% of the catch), primarily exploited by the Greenlandic and Icelandic fleets, respectively. In 2013, lumpfish was classified as "Near Threatened" in the IUCN Red List (IUCN, 2015), although a recent assessment indicates that the species should be considered "Threatened" in certain areas of the North Atlantic (Atkinson & Kulka, 2017). The decrease of wild adult populations has been attributed to increasing harvest pressure (Hoenig & Hewitt, 2005), and, in recent years, this pressure has been exacerbated by the development of the cleaner fish industry, which initially relied solely on wild broodstock (Powell et al., 2018).

1.6. The use of lumpfish in Aquaculture

1.6.1. Lumpfish production

Interest in lumpfish production raised due to the growing cleaner fish industry and it is chosen by many fish farmers in Norway, Scotland, Faroe Islands and Canada as part of integrated pest management (Skar et al., 2017; Treasurer, 2018). In Scotland, two companies with 3 sites had an estimated production of 16 tonnes of lumpfish and wrasse in 2019, and the production reached 21 tonnes in 2023 (Marine Directorate, 2023). However, while for ballan wrasse the source of ova laid down were own broodstocks, for lumpfish only foreign ova have been laid down to hatch in Scotland, coming mainly from Iceland and occasionally from Norway (Munro, 2018).

In the Faroe Islands, two local companies (Svínoy and Nesvík Marine Centre) locally produce lumpfish, both achieving approximately 800 thousand fish per year in 2022. However, it has been estimated that 2 million lumpfish are needed to fulfil the needs of all the salmon sites in the Faroe Islands (Johannesen et al., 2018). Therefore, most of the deployed lumpfish have been imported from Iceland (Jacobsen, 2021; Johannesen et al., 2018). At the moment there is no production of lumpfish in the Faroe Islands, as the two companies mentioned above ceased lumpfish production in 2023. Nowadays the ones being deployed are solely of Icelandic origin.

1.6.2. Hatchery phase

Before reaching the ideal deployment size for the salmon sea cages, lumpfish are reared in hatcheries. Lumpfish hatcheries can either buy eggs or have their own captive broodstock or a mixture of captive and wild broodstock. For example, in the Faroe Islands, Nesvík Marine Centre stripped eggs from wild females, whereas the milt was mostly obtained from captive males, either by stripping or after killing the fish (Figure 1.8 A,B). Eggs are mixed with a suitable amount of milt and seawater for fertilisation and the egg mass is made into small pancake shapes, before disinfection in 500 ppm glutaraldehyde in seawater. After incubation at ambient temperature, hatching occurs at around 300 to 330 degree days. Larvae are left to swim in a collection tank (Figure 1.8 C) and they are fed live feed (Artemia sp.), approximately 5 days after hatching, for the first two weeks of feeding. Next, they are weaned to 0.5 mm dry pellets and can then be transferred to larger tanks, where pellet size is gradually increased. In the on-growing phase fish are kept in larger tanks at ambient sea temperature (6-11°C) (Figure 1.8 D), with a biomass of below 8 kg/m^3 for fish less than 5 g and around 12 kg/m^3 for fish bigger than 5 g. At least 4 weeks before being deployed in the sea cages, lumpfish are vaccinated. Bacterial infections such as atypical furunculosis (*Aeromonas salmonicida*), vibriosis (*V. wodanis, V. logeii* and *V. splendidus*), *Pasteurella* sp. and *Tenacibaculum* sp. have challenged lumpfish production at Nesvík (Johannesen et al., 2018).

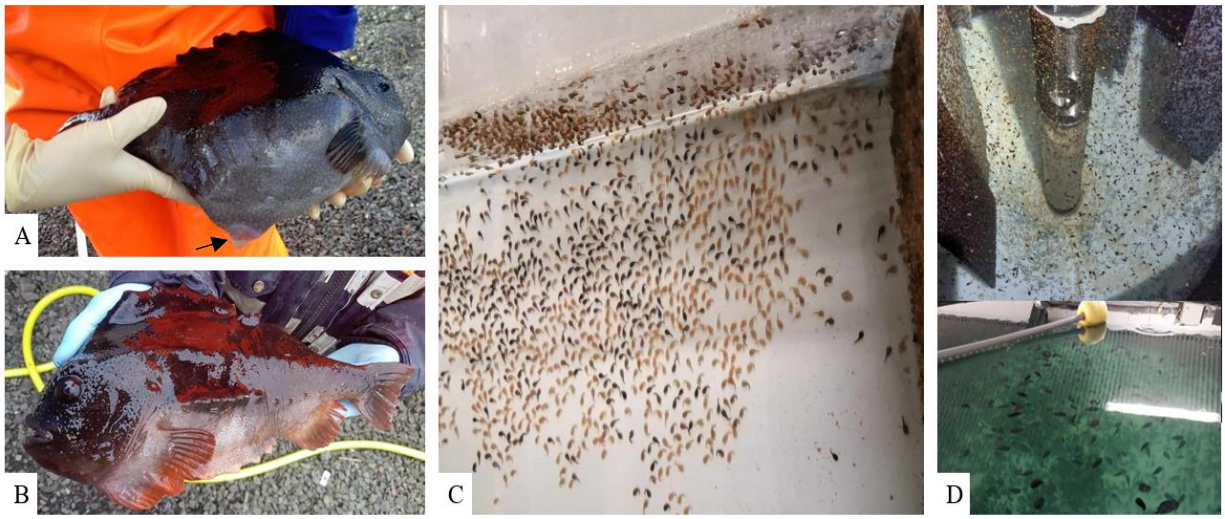


Figure 1.8. (A) Adult female ready for stripping with swelling of area around the genital opening. (B) Adult male ready for milt harvest. (C) Lumpfish larvae left to swim in a collection tank (photo taken in Nesvík Marine Centre, Faroe Islands). (D) Example of two different tank settings for the on-growing phase: tank with black plastic shelters for first feeding larvae (above), tank with on-growing juvenile lumpfish, approximately 10-20 g (below) (photos taken in Nesvík Marine Centre, Faroe Islands).

1.6.3. Deployment phase

The deployment phase represents the most challenging phase in lumpfish production. Lumpfish are transferred into the sea cages when they reach a suitable size, which is usually at least 20 g. Lumpfish are usually starved for 1-3 days before transfer to reduce the risk of stress and digestive issues during transport to marine sites for deployment (Arge, 2022, personal communication). When lumpfish are deployed in the sea cages along with salmon, due to their natural requirements for surface adhesion, they need a substrate or a shelter to attach and rest during periods of inactivity or environmental perturbations (Imsland et al., 2018). Current practices include the use of plastic barrels or artificial kelp made of plastic sheets attached to ropes. Another design deployed in Scotland is a combination of artificial kelp with layflat, which consists of plastic sheeting weighed down with a lead line and ropes for attachment to the sea cage handrail (Imsland & Conlon, 2019). Imsland & Conlon (2019) also demonstrated that recycled materials from the farms such as recycled feed pipes and walkway tubes can be effectively used as a low-cost alternative as substrates and hides for lumpfish in sea cages. Another challenge with deployment is that sea cages, can have different environmental conditions and in some cases be exposed to tidal currents or storms. Lumpfish go from a sheltered hatchery period to being deployed in these environments and this can be a factor influencing their susceptibility to skin damage, stress, and handling during transfer and stocking (Imsland & Conlon, 2019; Treasurer, 2018). Implementing better acclimation protocol for when lumpfish are transferred from hatchery systems to open sea cages, could improve the overall welfare, reducing the stress of transfer (Brooker et al., 2018). Among the best practices for stocking cleaner fish, ensuring there is appropriate nutrition when deployed, health and welfare checks, as well as humane slaughter at the end of production (Treasurer, 2018). Lumpfish cannot solely rely on sea lice when in the sea cages, and a balanced diet is essential to guarantee fish robustness, health and delousing activity (Garcia de Leaniz et al., 2022). The lack of knowledge regarding lumpfish nutritional requirements has been highlighted as one of the challenges in lumpfish production, especially during the deployment phase (Boissonnot et al., 2022; Reynolds et al., 2022) with recent studies focussing on the right balance of macronutrients in order to optimise lumpfish diets throughout the deployment phase (Hamre et al., 2022). Infectious diseases also significantly affect lumpfish health and survival in the sea cages. Infectious diseases can be present in the sea cages, but also be a result of secondary opportunistic infections due to suboptimal environmental and rearing conditions, injuries, wounds and stress (Johannesen et al., 2018). Cleaner fish are susceptible to a wide range of bacteria, viruses, fungi and parasites. Bacterial infections in lumpfish can be caused by *atypical Aeromonas salmonicida*, different species of *Vibrio*, *Pasteurella* sp., *Pseudomonas anguilliseptica*, *Tenacibaculum* spp. and *Moritella viscosa.* In the hatcheries, fungal infection can lead to significant mortalities. Among viruses, Viral haemorrhagic septicaemia virus (VHSV), Ranavirus, *Cyclopterus lumpus* virus (CLuV)/Lumpfish flavivirus (LFV), Nodavirus, *Cyclopterus lumpus* Coronavirus (CLuCV), *Cyclopterus lumpus* Totivirus (CLuTV), and as well as

Infectious pancreatic necrosis virus (IPNV) have been reported to affect lumpfish (Erkinharju et al., 2021). Other challenges in the sea cages such as poor environmental conditions, vaccination, handling, salmon delousing treatments, nutrition and or behaviour can lead to a compromised lumpfish immune system, making them more susceptible to different pathogens, either primary or opportunistic (Erkinharju et al., 2021). The high mortality rates reported throughout the deployment phase has raised ethical concern in the cleaner fish industry. Reynolds et al. (2022) identified the primary causes of mortality in lumpfish production, with the majority being transport, grading, bacteria and mechanical delousing. All these challenges have led to the need of regular health and welfare monitoring of lumpfish in the sea cages.

1.6.4. Lumpfish efficacy

The increasing demand for cleaner fish, where approximately 60 million are deployed worldwide each year, coupled with evidence of poor welfare and high mortality in the sea cages, necessitates robust evidence of the delousing efficacy of cleaner fish (Overton et al., 2020). Several studies have looked at the efficacy of lumpfish at delousing salmon, comparing lice levels with and without lumpfish both in small scale experimental units and large scale with production-size sea cages (Imsland & Reynolds, 2022). Imsland et al. (2014) was the first to show the efficacy of lumpfish to control sea lice in a small-scale experiment with each cage being stocked with 120 salmon and 12 to 18 lumpfish, resulting in a cleaner fish density of 10% to 15%. The results of gastric lavage showed that lumpfish had been grazing on sea lice. However, it is challenging to use small-scale tanks or cages for these kind of studies as they do not reflect large volumes, high densities and deep cages of the commercial scenarios (Overton et al., 2020). Subsequent larger studies were carried out exploring how lumpfish size (Boissonnot et al., 2022), seasonality (Eliasen et al., 2018), and genetic background (Imsland et al., 2016; Imsland et al., 2021) affect the cleaning efficacy of lumpfish. Eliasen et al. (2018), Imsland et al. (2021) and Boissonnot et al. (2022) found that smaller lumpfish (< 100g) were better at lice grazing than larger fish. Furthermore, in Eliasen et al. (2018), the abundance of zooplankton in spring-summer in the Faroe Islands negatively affected the efficacy of lumpfish. A genetic effect correlated with lumpfish behaviour was also investigated, showing a significant family effect on grazing (Imsland et al., 2016; Imsland et al., 2021). Several of these studies mentioned mortality in farming conditions, commonly reaching 45 % as mentioned in Boissonnot et al. (2022).

1.7. Lumpfish welfare

1.7.1. Fish welfare

Cleaner fish farming is still relatively new in the aquaculture industry compared to other species. It is important to ensure the welfare of lumpfish in production for them to be robust and efficient delousers. Additionally, lowering mortality rates of cleaner fish is fundamental to keep defining it as a sustainable and environmentally friendly method against sea lice.

The concept of animal welfare is founded on the Five Freedoms introduced in the Brambell Report (1965). These freedoms include freedom from hunger and thirst, freedom from discomfort through the provision of an adequate environment, freedom from pain, injury, and disease, and freedom to express normal behaviour (FAWC, 2010; Mcculloch, 2013; Segner et al., 2012). In recent years, growing public and scientific concern about the welfare of farmed fish has led to the development of related policies, such as Council Directive 98/58/EC and the Council of Europe recommendation on the welfare of farmed fish in 2005 (Martins et al., 2012). However, despite this increased focus within the scientific community, efforts to assess and improve fish welfare at the farm level are sometimes lacking (Treasurer, 2018). In aquaculture systems, stressors primarily arise from handling, unsuitable conditions like confinement or overcrowding, poor water quality, low dissolved oxygen levels, and the presence of various pathogens (Huntingford et al., 2006).

In order to properly assess fish welfare, it is essential to understand species-specific biology and optimal environmental conditions in order to draw conclusions related to welfare and establish specific welfare indicators. Welfare indicators should be measurable on a commercial farm, science-based and should be be measured over an extended period (Martins et al., 2012).Welfare indicators can be divided into direct animal-based and indirect resource-based welfare indicators. Direct animal-based welfare indicators can be further divided into individual or group based. Individual based generally focus on the external appearance of the fish. Some examples include skin, fin and eyes status, gill integrity, opercular or mouth damage, presence of deformities and condition factor. Group based assess populations such as fish behaviour (location in water column, air-gasping, aggression, activity, feed intake), growth, mortality, health status and disease (Table 1.2).

Table 1.2. Overview of individual and group based OWI for lumpfish.

Individual based OWI	Group based OWI
Skin status	Behaviour
Fin damage	Growth
Eyes integrity	Mortality
Presence of deformities	Health status
Opercular damage	Disease
Snout and mouth damage	Appetite
Condition factor	Blood in water

Indirect resource-based welfare indicators are parameters related to environmental conditions: water quality (oxygen, carbon dioxide, ammonia, salinity, pH, biological oxygen demand, temperature, light, water flow, turbidity, nitrites, and nitrates) (Huntingford et al., 2006; Noble et al., 2018; Treasurer, 2018). Segner et al. (2012) reports that also other parameters should be observed to assess the welfare in the farm, such as the system (fish density, unit size) and the feed quality (composition, essential nutrients, contaminants, lipid oxidation, and immunostimulants).

Though there is a suite of welfare indicators that can be used to assess welfare, not all of them are suitable and applicable on a farm basis as they can be time-consuming and impractical. For example, elevated levels of cortisol in blood, mucus, faeces or in the water are used as an indicator of stress in fish, but it is not feasible during routine monitoring as it requires fish handling and killing in the case of blood, as well as lab analysis (Adams, 1990; Huntingford et al., 2006; Scott & Ellis, 2007).

On the other hand, the use of underwater cameras or sonar systems are non-invasive tools that can detect changes in behaviour through direct observation and can detect early signs of potential welfare issues (Huntingford et al., 2006; Kristmundsson et al., 2023).

A set of techniques have been suggested to provide an assessment of welfare and these vary depending on species, life stages and farming circumstances (Huntingford et al., 2006; Turnbull et al., 2005). These are defined as Operational Welfare Indicators (OWI) and must be easy to use on farm, repeatable, replicable, reliable, tailored to specific systems and husbandry protocols, and accurately reflect the welfare of the fish. Among them, skin or fin damage, abnormalities, body condition, growth, and mortalities represent some examples of OWI (Noble et al., 2012).

While other farmed fish species such as Atlantic salmon or rainbow trout (*Oncorhynchus mykiss*) have well established welfare indicators and OWI, the early nature of the cultivation of cleaner fish has posed challenges in establishing standardised OWI.

1.8. OWI in lumpfish

Though several studies attempting to establish OWI in lumpfish (Garcia de Leaniz et al., 2022; Gutierrez Rabadan et al., 2021; Imsland et al., 2020, 2022; Noble et al., 2019), these should be standardised among farmers and stakeholders, implemented in the guidelines, and regularly used in the farms in order to be effective. Noble et al. (2019) published a fact sheet regarding operational and laboratory based-welfare indicators for lumpfish as part of the Rensvel OWI Fact Sheet series (Noble et al., 2019). The fact sheet provides a concise summary of science-based findings and practical experiences concerning various life-stage and species-specific OWI as well as laboratory based-welfare indicators for lumpfish. The OWI that were covered are direct indicators (individual based, group based, laboratory based) and indirect indicators (environment based) in lumpfish. More recently in the UK, the new version of the RSPCA Assured Standards was expanded to address the welfare of cleaner fish. The RSPCA Assured Standards is dedicated to promote higher welfare standards for farmed animals in the UK, ensuring that farmed animals are reared, transported and humanely slaughtered under better life conditions than minimum requirements. In the case of cleaner fish, the assessment of the potential risks of sea lice treatments, mandatory documentation, and analysis of all causes of mortality, ensuring ample shelters and appropriate feeding, and lowering the density of fish during transport were included in the scheme (RSPCA, 2024). In the Faroe Islands, as part of lumpfish health monitoring in the salmon sea cages, lumpfish are assessed for OWI, liver colour, stomach content and signs of disease (Eliasen et al., 2018; Eliasen et al., 2020), and a new manual with updated guidelines has been recently published and currently in use by the Faroese farmers (Østerø & Eliasen, 2023).

1.8.1. Individual based OWI

Across welfare indicators, there is a range of morphological welfare indicators that can be used to assess lumpfish, from juveniles to broodstock, and in different rearing systems, such as: fin erosion and splitting, skin damage, eye damage, opercular damage, snout and mouth damage, vertebral deformities and suction disc deformities (Noble et al., 2019).

Skin damage can result from infectious diseases, after transport, as well as net cleaning or net collisions in sites exposed to strong currents. Fin damage can affect the caudal, dorsal, anal, and pectoral fins (Noble et al., 2019), with juvenile lumpfish having high level of caudal fin damage due to aggression.

To reduce fin nipping, continuous feeding and a strict grading plan are beneficial during the juvenile phase (Noble et al., 2019; Treasurer et al., 2018). Also, reduced stocking densities along with the provision of sufficient surface area to attach and rest, can be beneficial towards reduced aggression (Treasurer et al., 2018).

Eye damage can be unilateral or bilateral and results from handling, diseases, and collision with nets. Snout and mouth damage can also result from diseases, handling and contact with net, hard surfaces, or sharp edges (Noble et al., 2019). Opercular damage can also result from suboptimal rearing conditions

and health related factors (Scholz et al., 2018).Suction disc deformities are a species-specific OWI for lumpfish, where a normal disc is complete, whereas a deformed one has a discontinuity (Noble et al., 2019). The underlying causes of sucker disc deformities are unclear; however, genetic factors and nutritional causes are highlighted both in Reynolds et al. (2022) and Rabadan et al. (2021). Rabadan et al. (2021) reported a higher percentage of fish from the hatcheries with a sucker disc deformity, compared to the deployed lumpfish. This could be due to familial differences and different batches as reported by Danielsen (2016). All these OWI mentioned are routinely assessed and scored, and do not necessarily require the sacrifice of the fish.

A study by Eliasen et al. (2020) highlighted the use of lumpfish liver colour as a welfare indicator for the nutritional status of the fish. In the study, farmed lumpfish exhibited a wide range of liver colours, ranging from very pale, through orange and bright orange, to dark reddish (Figure 1.9 A). Lumpfish with orange livers had a better nutritional status and overall welfare compared to the fish with a dark reddish liver. The same study showed that lumpfish with dark and red livers have the lowest content of total lipids, triacylglycerols (TAGs) and astaxanthin in their livers, indicating starvation (Eliasen et al., 2020). Yellow pale livers can be a sign of infectious disease or low carotenoid levels in the feeds provided (Imsland et al., 2022). The liver holds a store of energy reserves and is often used through the hepatosomatic index (HSI) to evaluate the energy status of the fish (Brooker et al., 2018; Campbell & Love, 1978). However, assessing and scoring lumpfish liver colour and calculating the HSI cannot be effectively utilised as an OWI. Unlike other non-invasive welfare indicators, liver colour requires euthanising the fish.

Due to their rounded and laterally compressed body shape, condition factor can be challenging to estimate in lumpfish. However, condition factor is a good welfare indicator, which helps to assess the nutritional status of the fish and be an early indicator of suboptimal feeding or compromised nutrition (Treasurer et al., 2018). However, through poor nutrition or infectious diseases, lumpfish may become progressively thinner or emaciated, with the head becoming the widest part of the body (Noble et al., 2019). Studies have looked at the body condition of lumpfish in the sea cages (Eliasen et al., 2020; Engebretsen et al., 2024; Rabadan et al., 2021; Rey et al., 2021) by calculating the weight-length relationship using fitted regression for lumpfish deployed in salmon cages. Through the weight-length relationship, lumpfish were categorised as being in good condition, underweight or emaciated. In contrast, Østerø et al. (2024) employed a new approach to assess the body condition of deployed fish, by using the length-weight relationship of wild lumpfish as a benchmark. This method, which more closely mirrors the natural growth patterns of the fish, resulted in higher percentage of farmed lumpfish being classified as underweight or emaciated, compared to the other models. This resulted in an overestimation of the deployed lumpfish being in good condition.

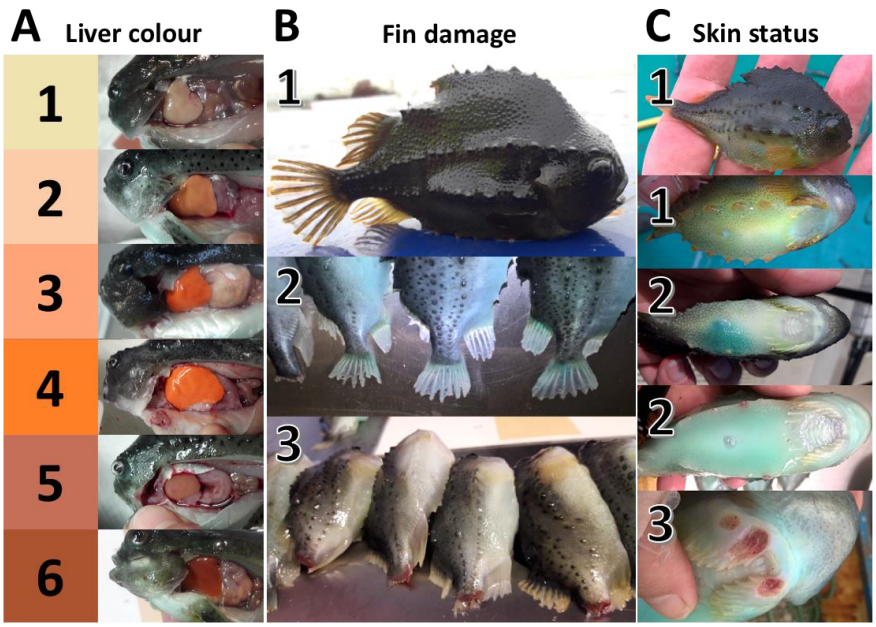


Figure 1.9. Example of the scoring system used to assess liver colour (A), caudal fin damage (B) and skin status (C) from Eliasen et al. (2020).

1.8.2. Group based OWI

Group-based OWI can assess lumpfish at different life stages by examining factors such as mortality, health status, appetite, growth rate, and behaviour. Mortality rates and their variations can serve as both short-term (daily, weekly, monthly) and long-term indicators (over a production cycle) of welfare issues, providing a clear measure of overall welfare (Noble et al., 2019). Another challenge is that multiple bacteria may be present in the same fish (Reynolds et al., 2022).

The impact of certain infectious diseases on the skin, eyes, behaviour, and mortality of lumpfish underscores the importance of regular health monitoring at each life stage. Health status can be monitored on farms using OWI or through diagnostic sample analysis.

Changes in appetite, along with feeding response and growth rate, are strong indicators of potential welfare issues. A loss of appetite can be a sign of an infectious disease and, over the long term, may result in emaciated fish (Noble et al., 2019). Many farmers qualitatively observe short-term changes in fish behaviour as an early warning for potential health and welfare threats (Treasurer et al., 2018). In lumpfish, appropriate behavioural OWI include types of swimming activities, aggression, and ventilation rate (Noble et al., 2019). Swimming activity is limited almost in all life stages: larvae attach to the tank walls most of the time, while in juveniles the swimming activity is increased and maintained until sexual maturation (Treasurer et al., 2018). They can also alternate between 'sit and wait' strategies and more active foraging based on food availability (Killen et al., 2007). However, it could be challenging for the farmers to quantify the behaviour and monitor most of the fish in large scale production, as the data collection and processing can be labour intensive (Treasurer et al., 2018).

Aggression, in the juvenile phase seems to increase with size disparity and it is not correlated to lack of food. Regular grading, reduced light intensity, and the use of enrichment where fish can hide or explore, are strategies that can be used to reduce aggression (Treasurer et al., 2018). Subsequently, aggression can be used as an OWI through the assessment and scoring of fin damage and physical damage (Martins et al., 2012).

1.8.3. Laboratory based OWI

While OWI are welfare indicators that can be measured directly on farms (Noble et al., 2012), laboratory-based welfare indicators (LABWI) are defined physiological welfare indicators sampled either from the animal or its environment, and sent for laboratory analysis (Noble et al., 2018). These indicators are measured through stress responses, including plasma cortisol, glucose, plasma osmolality, magnesium, and chloride levels (Noble et al., 2019).

Elevated plasma cortisol levels indicate a primary stress response in fish and are associated with negative experiences (Ellis et al., 2012), while glucose, lactate, osmolarity, chloride, and magnesium indicate a secondary stress response (Treasurer, 2018). Lumpfish show a lower peak in acute stress compared to ballan wrasse and salmonids (Hvas et al., 2018; Treasurer, 2018). Lumpfish tend to hide from danger rather than swim away, resulting in lower glucose levels in response to stress (Treasurer, 2018). Additionally, no significant effect of acute stressor exposure was found on osmolality levels in lumpfish (Hvas et al., 2018). Some differences can be found in the levels of magnesium and chloride; however, they may reveal both acute and chronic welfare problems (Segner et al., 2012). Physiological welfare indicators are informative, but they can be labour intensive, complex and fish need to be killed to collect blood or other tissues.

1.9. Lumpfish feeding and nutrition

1.9.1. Feeding in the wild

After hatching near the coast, lumpfish larvae and juveniles are typically found among kelp during their first year of life, both attached to it or free-floating (Ingólfsson & Kristjánsson, 2002).

During the first two weeks lumpfish larvae spend more time clinging to substrate rather than swimming, feeding from the cling position and avoiding exposure to potential predators. As larvae grow, they swim more and orient themselves to be able to catch prey (Brown, 1986).

Juveniles and adult lumpfish have a functional mouth, with small, sharp, conical teeth, and a well-developed digestive system. The digestive system includes a sac-like stomach for storage, pyloric caeca and a long intestine, being more than twice the length of the body (Davenport, 1985; Timeyko, 1986; Zhukova & Stroganov, 2022). Juvenile lumpfish can exhibit two different foraging modes depending

on prey availability: they can either actively swim and search for prey when prey is scarce or they can attach with the suction disc, and sit and wait for when prey is more abundant (Killen et al., 2007).
In both Daborn & Gregory (1983) and Ingólfsson & Kristjánsson (2002), the diet of lumpfish changes as they grow, and it is influenced by food availability and circulation patterns in their habitat. When they still have some yolk sac, larvae feed on crustacean larvae and halacarid mites (Ingólfsson & Kristjánsson, 2002b). After completely absorbing their yolk sac, juvenile lumpfish start by feeding on a wide range of near-surface plankton, largely on harpacticoid copepods (Figure 1.10). As they grow larger, their diet shifts to include a broader range of prey. Larger juveniles mainly consume amphipods, isopods, crabs, polychaetes and even smaller co-specifics. Overall, a clear change in food composition is evident as lumpfish grow and these prey become insignificant in fish larger than 25 mm (Daborn & Gregory, 1983; Ingólfsson & Kristjánsson, 2002). Stomach samples also showed that jellyfish, crustaceans, and chaetognaths are present in the lumpfish diet (Hamre et al., 2022; Sharpton, 2023).

Figure 1.10. Example of stomach content of wild lumpfish sampled in this study (from left to right, three different amphipods, one krill or shrimp crustacean) (photos by Di Toro J., 2021).

1.9.2. Feeding in the farms

In hatcheries, first feeding of lumpfish larvae is performed using live feed (Artemia sp.) approximately 5 days after hatching and during the first two weeks of feeding. After that, they are weaned to 0.5 mm dry pellets and the pellet size is gradually increased (Johannesen et al., 2018a). In tanks, lumpfish were observed to consume pellets floating or at the bottom of the tanks (Johannesen et al., 2018b). In the juvenile phase, approximately from 5 to 20 g, lumpfish are fed continuously and graded to reduce aggression and high levels of fin nipping (Noble et al., 2019).

The delousing behaviour of lumpfish is an example of opportunistic feeding when reared with salmon in the sea cages (Imsland et al., 2015). Although they spend a limited amount of time cleaning sea lice off Atlantic salmon and only a small proportion of individuals delouse, this activity is sufficient to reduce sea lice infestation levels in the cages (Imsland et al., 2014).

However, lumpfish cannot rely exclusively on sea lice as a food source, as sea lice provide only a small and temporary contribution to their diet. Appropriate feeding is fundamental to keep their health, welfare and delousing activity (Treasurer et al., 2018). Lumpfish in the sea cages are typically fed either by automated feeders or manual feeding. Automated feeders distribute feed in specific areas, whereas manual feeding is done along the cage net and near the shelters once or twice daily. As an effective strategy it is recommended, where possible, to use a combination of automated feeders and one daily manual feeding, possibly near the shelters (Johannesen et al., 2018b).

Both in hatcheries and salmon pens, lumpfish are fed with pellets (Leeming, 2017). An alternative feeding strategy, which has been tested and used in sea cages are feed blocks, which are deployed near shelters and can last up to several days (Imsland et al., 2018, 2019, 2020).

Lumpfish kept in cages with salmon spend most of the daylight time either foraging for food or resting among seaweed. Notably, no antagonistic behaviour has been observed between lumpfish and salmon by Imsland et al. (2014). The feeding behaviour of lumpfish in the sea cages has been primarily described as highly opportunistic, adapting to spatial and temporal variations in food sources (Imsland et al., 2015). They target various food sources, switching their choices to whatever is available in their close environment, from grazing on nets, to free-swimming organisms to lumpfish or salmon pellets as well as sea lice from salmon (Imsland et al., 2014). However, zooplankton and organisms related to biofouling are mainly available in the summer months as reported in Eliasen et al. (2018). In Imsland et al. (2016), a size effect was found on feeding preference where small size lumpfish (approximately 20 g) showed a higher preference for zooplankton and naturally occurring food such as sea lice, compared to larger lumpfish (70-110 g), that fed mainly on pellets. Despite being offered lumpfish feed in the sea cages, in some cases lumpfish rely solely on prey and can experience difficulties in starting to feed after being transported from the hatcheries into the sea cages. This can lead to poor nutrition and exacerbate emaciation. Also, underfeeding or poor feeding in the cages or sites exposed to strong currents can be additional risk factors for emaciated fish (Noble et al., 2019). Regular monitoring of

body condition, liver colour and feeding responses where possible can help farmers spot welfare issues and take action to solve them (Treasurer et al., 2018). Though research on lumpfish nutrition has progressed in recent years, nutritional requirements, physical properties of the feed, and best delivery protocols in the cages are not well optimised.

1.10. Nutritional requirements of fish

Generally, to determine the dietary requirements for a nutrient, feed trials are carried out, where diets with graded amounts of the nutrient of interest are tested. Accurate determination of the dietary requirement for a nutrient requires a sufficient number of diets or treatments formulated to contain graded amounts of the nutrient under investigation, and that all other nutrients in the experimental diets are provided at levels equal to or in excess of their requirements. In particular, the experimental diets would have a graded range of concentrations of the nutrient, at least five, from significantly deficient to in excess of the anticipated requirement. The nutrient of interest when establishing nutrient requirements can be a macronutrient (protein, lipids, or carbohydrates) or a micronutrient (vitamins or minerals) (NRC, 2011).

1.10.1. Macronutrients

The main macronutrients in fish nutrition are protein and lipid, as fish have a limited ability to metabolise carbohydrates compared to terrestrial animals (Hemre et al., 2002). Fish do not have a requirement for carbohydrates. Fish species vary greatly in their ability to utilise dietary carbohydrates for growth, largely reflecting their feeding habits. Including an appropriate amount of digestible carbohydrates in the diets of species that can utilise them more efficiently is crucial to spare lipids and proteins from being used as energy sources (NRC, 2011). Despite the low nutritional value for fish, carbohydrates are used as an effective binding agent during feed manufacturing, particularly for feeds that are designed to float (Joshi & Aithal, 2021).

Proteins and lipids are the primary energy sources for fish. Proteins and amino acids, which are the building blocks of protein, are organic nitrogen containing compounds. They are essential components of all living organisms, performing numerous structural and metabolic functions (Wu, 2009). Proteins are crucial for every cell type, including muscles, bones, and organs, playing a role in connective tissues (collagen and elastin) and mechanical functions like myosin. Amino acids are also involved in forming coenzymes, structural molecules, metabolic intermediates, neurotransmitters, and hormones. However, the conversion of amino acids into these functional compounds is quantitatively minor compared to their use in protein synthesis or their catabolism (NRC, 2011).

Protein deposition is influenced by genetics, as well as external factors like diet and environment. The amino acid composition of fish is consistent across species and minimally affected by size in juvenile fish (Glencross et al., 2011). Animals must obtain essential amino acids from their diet through consumption of protein or mixtures of amino acids as they cannot synthetise them, while non-essential

amino acids can be synthetised internally from precursors. When essential amino acids are excluded from diets, growth is greatly affected, whereas non-essential amino acids will not affect growth, meaning that they can be synthetised endogenously (Jobling, 2001). For example, a deficiency in tryptophan led to vertebral deformities, fin erosion and cataracts in rainbow trout (Cowey, 1994).

Essential amino acids for marine fish are histidine (His), threonine (Thr), valine (Val), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met) and phenylalanine (Phe). Non-essential amino acids are serine (Ser), alanine (Ala), cysteine (Cys), aspartic acid (Asp), glutamic acid (Glu) and Taurine (Tau). Conditionally essential amino acids are tyrosine (Tyr), glycine (Gly), arginine (Arg) and proline (Pro). For example, Tyr is considered conditionally essential as it is synthetised from Phe, and if provided with the diet, the requirement for Phe will be reduced (NRC, 2011).

Not all dietary protein sources have the same nutritive value, as it depends on their digestibility and amino acid profile. The requirement for protein is related to specific needs for essential amino acids, a general need for amino groups to synthesise non-essential amino acids, and general energy and metabolic needs. Since protein sources are the most expensive ingredients in commercial diets, the goal is to minimise the use of protein as an energy source (Nguyen & Davis, 2009) which can be done by providing an adequate amount of energy from lipids (NRC, 2011).

Fish have evolved to efficiently convert macronutrients into energy, with lipids being particularly important due to their high energy density (Sargent et al., 2003). Lipids can be distinguished into two main groups, neutral and polar. Neutral lipids are soluble in nonpolar solvents, they serve as an energy source, and include triacylglycerols (TAG), wax esters, sterols, steryl esters and free fatty acids (FFA). Polar lipids make up the structure of cell membranes, have nonlipid head groups, and therefore have a wider range of solvent solubility. They include phosphoglycerides, sphingolipids, sulpholypids and glycolipids (Sargent et al., 2003; Turchini et al., 2010).

Lipids are the source of energy intake, they are essential components of cell membranes, precursors of compounds such as eicosanoids, and they are carriers of fat-soluble vitamins A, D, E, and K (Tocher, 2003; Torres et al., 2011). The crucial building blocks of lipids are fatty acids that provide diversity and chemical specificity to complex lipids (Glatz, 2011). Lipid requirements include energy need, functional lipid class, and essential fatty acids. The requirements for specific fatty acids vary based on their specific functional roles and whether the body is able to synthetise them (NRC, 2011). Species that cannot synthetise them from precursors, have to get them through their diet (Tocher, 2010).

The type of polyunsaturated fatty acids (PUFA) required by a fish depends on their biosynthetic capacity. Marine species typically require long-chain PUFA (LC-PUFA) as essential fatty acids due to their limited ability to synthesise these from shorter-chain precursors. Conversely, freshwater species can often meet their PUFA needs with short-chain PUFA (SC-PUFA), as they have a greater capacity to elongate and desaturate short-chain fatty acids into long-chain fatty acids (NRC, 2011).

Marine fish need three essential LC-PUFA for normal growth, development, and reproduction. These essential fatty acids are docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-

3) which are converted from linolenic acid (LNA, 18:3n-3), and arachidonic acid (ARA, 20:4n-6) which is converted from linoleic acid (LA, 18:2n-6) (Sargent et al., 1997; 1999).

All fatty acids serve as vital sources of energy, regardless of the additional roles some PUFA play as essential fatty acids (Tocher, 2003). The degree to which a fatty acid is used for energy depends mainly on its dietary concentration, where higher concentrations result in increased oxidation of all fatty acids. Exceptions include 22:1n-11, which is highly oxidized regardless of concentration, and DHA, which is often conserved due to its inefficiency as a substrate for beta-oxidation (Sargent et al., 2003).

The rapid expansion of the aquaculture industry has driven research on fish lipid metabolism, prioritising the requirements for essential fatty acids, necessary for proper growth and development. Importantly, these requirements can vary quantitatively during different stages of fish ontogeny, so accurately defining these requirements for a fish species involves identifying not only the absolute needs for specific PUFA, but also the optimal balance between different PUFA, and how these needs change throughout various life stages (Tocher, 2010). If a diet is deficient in essential fatty acids long-term, fish experience problems such as reduced growth, high mortality as well as reduced reproduction, fatty liver and intestinal steatosis (Glencross, 2009). The minimal requirement level of essential fatty acids is the dietary levels needed to prevent deficiency-related pathologies. However, when increasing their inclusion levels beyond this minimum, it may enhance growth and survival partly by sparing protein, allowing for the definition of optimal requirement levels (Sargent et al., 2003). However, exceeding an upper limit can result in unwanted lipid deposition in the peritoneal cavity, liver, or other tissues.

Defining requirements can be challenging as lipid, protein, and carbohydrates are sources of energy. Therefore, dietary lipids are influenced by dietary protein and carbohydrates (NRC, 2011).

1.10.2. Micronutrients

Micronutrients in nutrition, which include vitamins and minerals, are essential for the overall health and proper physiological functioning of fish. Among these micronutrients, vitamins play a critical role in numerous physiological processes, despite not supplying energy. They can be categorised into two types: water-soluble and fat-soluble vitamins. Water-soluble vitamins include the B-complex group and vitamin C, which must be regularly supplied as they are not stored in the body. Fat-soluble vitamins, such as vitamins A, D, E, and K, are stored in the body's fatty tissues and liver. Deficiencies in vitamins may negatively affect growth performance, cause skeletal deformities, and impact the survival rate of the fish. Ensuring that fish receive a balanced diet that includes the necessary vitamins helps support their overall health and efficiency in aquaculture settings (NRC, 2011).

Minerals are inorganic elements that are vital for the normal functioning of fish and they can be distinguished into two groups: macro minerals (needed in large amounts) and micro minerals (required in smaller quantities) (NRC, 2011). They form skeletal structures, such as bones and scales, and are involved in various metabolic processes like osmoregulation. Important macrominerals include sodium,

phosphorus, calcium, potassium, magnesium, and sulphur. Important microminerals include selenium, molybdenum, vanadium, zinc, iron, manganese, and copper (Ageeva et al., 2021). Fish can absorb these minerals through their diet as well as directly by the gills from the aquatic medium in which they live (NRC, 2011).

1.11. Nutritional studies in lumpfish

Initial strategies for feeding wild-caught juvenile lumpfish involved the use of commercial feeds for species such as salmon, cod or flatfish. When lumpfish were fed salmon feed, which is high in oil, it caused low survival and fat deposits in the liver and brain (Sayer et al., 2000). This highlighted the necessity of formulating tailored feeds that cover the nutritional requirements of lumpfish (Powell et al., 2018).

Due to the rising ethical concerns regarding lumpfish welfare, several studies have aimed to improve the knowledge of their nutrition (Willora et al., 2021). To find the optimal composition of feed, it is fundamental to address optimal primary nutrients and micronutrients for lumpfish according to fish size, temperature and growth rate. It is unlikely that sea lice itself play a major role in terms of nutrition for lumpfish (Johannesen et al., 2018a), since female lice contains only 1.6% lipids, whereas egg strings 6-7% (Tocher et al., 2010). An analysis conducted at the Institute of Aquaculture (Stirling, UK) examined the nutritional composition of sea lice, although the developmental stages were not separated or identified. The findings revealed that sea lice contained 2.1% ash, 33.0% moisture, 46.% protein, and 11.9% lipids (unpublished data). Also, sea lice infestations can be highly variable and too low for long periods to offer suitable nutrition for lumpfish (Johannesen et al., 2018a). Therefore, feeds for lumpfish should be provided to have adequate nutritional status, welfare, and high survival rates at the same time. Hamre et al. (2022) attempted to elucidate the correct balance of macronutrients and found that optimal growth for lumpfish (1.7-50 g) is achieved with a feed containing 55% protein, 17% lipid, and 6% carbohydrate. High dietary lipid levels (17%) promoted growth and improved welfare scores without increasing cataract frequency, but led to higher lipid accumulation in tissues. Carbohydrates were poorly utilised, negatively affecting growth. While the immune responses were normal in fish on the optimal diet, extreme protein levels (43 and 68%) were suboptimal in terms of immune system.

The use of different raw materials has also been investigated: the replacement of fish meal with plant protein was investigated by Willora et al. (2020, 2022), and the replacement of fish oil by rapeseed oil was investigated by Willora et al. (2021). Replacing up to 50% fish meal with plant protein ingredients affected the structure of the intestine but did not adversely affect growth performance, body chemical composition or muscle fibre cellularity (Willora et al., 2020). Higher replacement level (75%) led to reduced growth and signs of intestinal inflammation, such as shorter mucosal folds and increased number of goblet cells. Also, fish with signs of intestinal inflammation showed lower weight Willora et al., 2022).

In Willora et al. (2021) lumpfish growth was affected by the highest inclusion of rapeseed oil (10% of the diet) that resulted in a reduced growth rate, lower condition factor, increased HSI, and higher lipid deposition in the body and liver. The fatty acid composition of the fish closely mirrored their diets, with significant decreases in essential fatty acids (EPA and DHA) in the 100% rapeseed oil group. The effect on growth in the 100% rapeseed oil group suggests that dietary EPA+DHA levels of 3-4% (of total fatty acids) did not meet the nutritional requirements for these essential fatty acids.

Other studies also investigated the correct feeding frequency (Imsland et al., 2019a) and the physical properties of the feed (Imsland et al., 2018; Imsland et al., 2019b, Imsland et al., 2020), using alternative strategies like the use of feed blocks rather than the conventional pellets.

Feeding lumpfish daily resulted in the highest growth rates, but also led to the highest prevalence and severity of cataracts, along with increased liver vacuolization and gut inflammation. In contrast, feeding three days per week produced the lowest growth rates and the best feed conversion ratio, while significantly reducing cataract prevalence and severity, suggesting that reducing feeding frequency can help control growth and improve eye health in lumpfish (Imsland et al., 2019a). Studies that used feed blocks suggested that while pellets support faster growth, it also increases the risk of cataract development, whereas feed blocks resulted in slower growth and significantly reduced the prevalence and severity of cataracts. Pelleted feed had higher nutrient content, including higher protein, lipid, and essential fatty acids (EPA and DHA) content, compared to feed blocks. Eye cataracts are probably caused by rapid growth in hatcheries and dietary deficiencies regarding levels of specific amino acids in different tissues (Jonassen et al., 2017; Treasurer, et al., 2018). It has welfare implications, as it results in reduced feeding and growth, and as lumpfish detect lice by sight, it also affects the delousing efficiency (Jonassen et al., 2017). However, these studies mainly aimed to achieve a controlled growth either using restricted feeding regimes (Imsland et al., 2019a) or low energy feed blocks (17.3 MJ/kg) (Imsland et al., 2020), since fast growth of lumpfish is not desirable due to the lower delousing activity of bigger size fish (Imsland et al., 2016).

1.12. Gaps in knowledge, research hypothesis and objectives

The aquaculture industry, particularly in the North Atlantic, has grown rapidly and it relies on biological methods such as the use of cleaner fish to fight sea lice infestations. Lumpfish became a preferred choice as delousers due to their adaptability in cold waters and the number of lumpfish deployed both in Scotland and in the Faroe Islands has increased dramatically over the past few years. However, despite their role in integrated pest management, the deployment of lumpfish juveniles is currently hampered by poor welfare, low survival, non-optimised diets, and lack of robustness. High levels of physical damage in farmed lumpfish together with liver colour of recently deployed lumpfish being different than that in wild ones, suggest that fish are under compromised nutritional and welfare conditions when they are deployed. Although OWI for lumpfish have been proposed, they are not yet standardised, leaving farmers without consistent tools to monitor welfare. Addressing these challenges

and lowering mortality rates of cleaner fish is fundamental to keep defining it a sustainable and environmentally friendly method to control sea lice.

In this Thesis wild lumpfish are used as a comparative benchmark to assess the welfare of their farmed counterparts, as they provide a reference for morphology, nutritional and welfare status. However, wild and farmed lumpfish inhabit different environments, and farmed fish are under controlled conditions designed for optimising fish farming. Despite the aim of farming being domestication and adaptation to artificial environments, comparisons to the wild population remain a valuable tool to identify suboptimal farming practices.

This research study aims to fill the gap regarding the optimal nutritional requirements for lumpfish juveniles to improve their welfare and robustness when they are deployed in salmon sea cages.

Therefore, the challenges addressed in this Thesis are:

1) To assess the nutritional status of the wild populations and compare it to the farmed counterparts, as they often exhibit a compromised nutritional status shortly after deployment in salmon sea cages (Chapter 3 and 4).
2) To investigate if a compromised nutritional status is also reflected in OWI like liver colour (Chapter 5).
3) To fill knowledge gap about the nutritional requirements of lumpfish throughout their deployment phase (Chapter 6)
4) To determine the optimal levels of essential fatty acids, particularly EPA and DHA, required for the growth, health, and welfare of juvenile lumpfish (Chapter 6).

By addressing these objectives, this research will provide insights to improve lumpfish management in farming conditions, ensuring their overall welfare while improving sustainable practices in the salmon farming industry.

Chapter 2. Materials and methods

2.1. Ethical statement and fish sampling

Lumpfish analysed in Chapter 3-5, both farmed and wild, were sourced from Atlantic salmon farms, lumpfish hatcheries (Nesvík and Svínoy), and from wild populations in and around the Faroe Islands. Lumpfish used for the feed trial in Chapter 6 were sourced solely from the lumpfish hatchery in Nesvík (Faroe Islands). Fish were analysed for morphometric data, OWI, histology, and nutritional content (Figure 2.3). Experimental procedures in Chapter 3-6 were conducted according to the Directive 2010/63/EU regarding the protection of animals for scientific purposes, approved by the head veterinarian "Landsdjóralæknin" in according to the Welfare act 2018, 10 (DJÓRAVÆLFERÐARLÓGIN - Løgtingslóg 49 apríl 30 2018, Faroe Islands). Experimental procedures for wild and farmed lumpfish (Chapter 3-5) were also reviewed and approved by the Animal Welfare and Ethical Review Body of the University of Stirling (AWERB 19 20 007). The feed trial (Chapter 6) was reviewed and approved by Firum Animal Experimentation Ethics Committee (approval number 12, Torshavn, Faroe Islands) and approved by the Animal Welfare and Ethical Review Body of the University of Stirling (AWERB 2022 7252 5873). During each sampling in the farms, lumpfish were euthanised with an overdose of Finquel (0.8 g/l, MS-222, MSD Animal Health), while wild fish were euthanised through exsanguination via a gill cut (Chapter 3-5). During the samplings (S0, S1, S2, S3) of the feed trial (Chapter 6), lumpfish were euthanised with an overdose of Finquel (0.8 g/L, MS-222, MSD Animal Health). At each sampling occasion, each fish was weighed out to the nearest gram and measured to the nearest millimetre. Measurements included the total length of the fish, from the snout to the final part of the lobe of the tail and the height which is measured from the highest part of the crest to the bottom of the belly (Figure 2.1). In addition, each fish was scored for OWI (section 2.2), tissue samples were stored for histological analyses (section 2.4) while feeds, whole fish and tissues, were stored for nutritional analyses (Section 2.3). Blood (Chapter 6) was withdrawn from the caudal vein for cortisol analysis during S2 and S3 (Section 2.5).

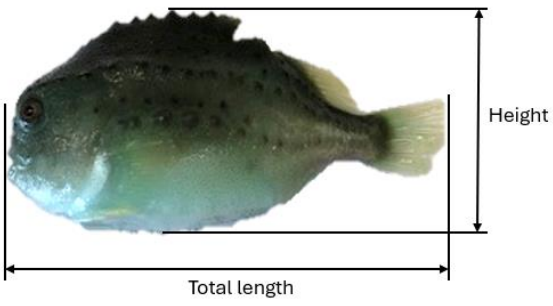


Figure 2.1. Lumpfish showing measurements for total length, from tip of snout to end of the tail fin, and height measured from highest part of the crest to the bottom of the belly.

2.2. Growth parameters/feed performance calculations

The sampling points during the feed trial (Chapter 6) included an initial sampling (S0), intermediate (S1) and a final sampling (S2) where fish weight, number of fish, daily feed intake, daily mortalities and temperature were recorded.

Fish were manually fed twice a day with a feeding rate of 2.5% of their body weight. The daily feed was administered weighed, divided into two equal portions, and the first feeding was supplied in the early morning between 8 and 9 am, and the second feeding in the afternoon between 2 and 3 pm. When feeding, the pellets were dropped slowly to the surface of water close to the shelters and using the tank water flow to ensure an even distribution of the feed within the tank. After every feeding event, uneaten pellets were siphoned from the bottom of the tank using a hose after approximately 30 min to 1 h. Uneaten pellets were weighed out for each tank to record and monitor daily feed intake. To measure daily feed intake, the weight of the wet feed waste is measured for each feeding event for each tank, and converted back to dry weight, using a correction factor. This correction factor was calculated for each feed by placing 10 grams per feed in quadruplicate in 0.5l of seawater for 30 minutes and 1 hour. The wet feed was then collected with a net and weighed out again. The ratio between the dry feed and the wet feed is then called correction factor.

These allowed the calculation of growth parameters such as specific growth rate (SGR), feed conversion ratio (FCR), biological feed conversion ratio (bFCR), daily growth coefficient (DGC), thermal growth coefficient (TGC) and feed conversion efficiency (FCE) using the following equations:

$$SGR = (e^{g} - 1) \times 100$$

where $g = (\ln final\ biomass(g) - \ln initial\ biomass(g)/number\ of\ days$ (Houde, 1981)

$$bFCR = \frac{Feed\ intake\ (g)}{Biomass\ gain\ (g) - Mortality\ biomass\ (g)}$$

where feed intake is the total of feed ingested (g), biomass gain is the final biomass (g) – initial biomass (g), and mortality biomass (g) is an adjustment that accounts for any mortality that accounted over the measured period (Moran et al., 2009)(Moran et al., 2009).

$$DGC = \frac{(final\ biomass^{1/3} - initial\ biomass^{1/3})}{number\ of\ days}$$ (Fournier et al., 2002)

$$TGC = \frac{(final\ biomass^{1/3} - initial\ biomass^{1/3})}{(temperature\ (°C) \times number\ of\ days)}$$ (Lugert et al., 2016)

$$FCE = \frac{Final\ biomass\ (g) - initial\ biomass\ (g)}{feed\ intake\ (g)}$$ (Akand et al., 1989)

During each sampling, fish weight, total length, fish height, liver weight and viscera weight were recorded to calculate body condition (BC), hepatosomatic index (HSI) and viscerosomatic index (VSI) as follow:

$BC = \frac{weight\ (g)}{length\ (cm)} \times height\ (cm)$ (Johannesen et al., 2018a)

$HSI\ = \frac{liver\ weight\ (g)}{fish\ weight\ (g)} \times 100$ (Willora et al., 2021)

$VSI = \frac{viscera\ weight\ (g)}{fish\ weight\ (g)} \times 100$ (Willora et al., 2021)

2.3. OWI

The OWI were scored using visual assessment, following the protocol in use at Firum (Faroe Islands), which is detailed in Østerø & Eliasen (2023), and described in Eliasen et al. (2020). The parameters scored were state of dorsal, anal and caudal fin, skin status, eye damage and sucker disc deformities (Table 2.1-2.6). The scoring system used for the OWI is from 1 to 3, and it is based on the methods established at Firum (Faroe Islands), as the samplings were all carried out in the Faroe Islands, within the lumpfish monitoring checks in place at Firum.

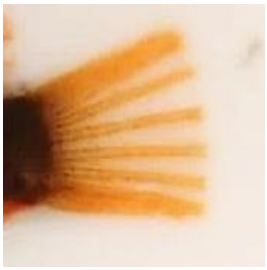
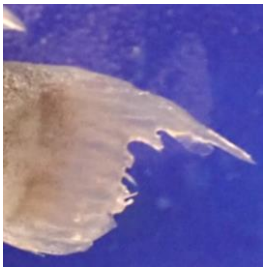
2.3.1. Fin damage

Fin damage (Table 2.1) was given the score 1 when the fin had no discolorations, unusual spots and the edge of the fin was smooth and even. All fin rays were intact, straight, and showed no signs of damage, and the membrane between the rays was free from holes or signs of deterioration. Also, the base of the fin was healthy and showed no signs of rotting or injury.

When the fin was scored 2, the edge of the fin showed irregularities due to small incisions and there was evident disruption in the fin outline. Some of the rays appeared bent and unevenly spaced or frayed, while the membrane showed evident rips or holes, causing discontinuity in the overall structure of the fin.

When the fin was given score 3, the fin was severely damaged, with significant parts missing. The natural shape was completely lost and the area surrounding the damage may have showed darker coloration due to potential infections. The base of the fin could also show additional wounds and signs of detachment. Most of the rays could be broken or entirely missing and the remaining ones unevenly spaced. The membrane between the rays was mostly gone, showing a frayed appearance. In this case, the fin mobility was severely compromised and might affect normal swimming, balance, or direction.

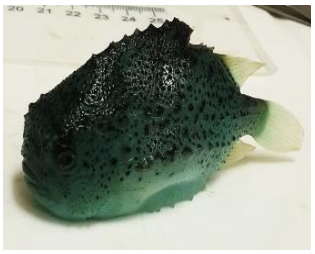
Table 2.1. Semi-quantitative scoring system for measuring dorsal, anal and caudal fin damage in lumpfish (photos by J. Di Toro, 2019-2022).

SCORE	FIN		
	Dorsal	**Anal**	**Caudal**
1	 No visible damage All rays are intact	 No visible damage	 No visible damage
2	 Some damage Small incisions on fin	 Some damage Small incisions on fin Fraying	 Some damage Small incisions on caudal fin and fraying
3	 Severe damage	 Severe damage Missing fin and/or open wounds	 Severe damage Missing fin and open wounds

2.3.2. Skin status

The skin integrity was also scored from 1 to 3 (Table 2.2). When the skin was given score 1, there was no sign of discoloration or unusual spots. The skin was uniform, with no sign of damage, and all the orifices were clear from abnormalities. Also, there were no signs of bacterial infections or parasites. When the skin scored 2, there was moderate damage that could manifest as areas of discoloration, pale spots or darker patches. The area around the anus, might appear red, swollen or both, with clear signs of secretions. Score 3 was given to the skin when there were open wounds and very damaged area. The areas affected could also have unusual secretions and redness, clearly indicating an on-going infection. The skin integrity was assessed based on external appearance and visible signs of damage, evaluating discoloration, wounds and potential infections, whereas body damage would be referring to other forms of damage such as physical trauma and internal injuries.

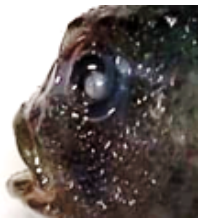
Table 2.2. Semi-quantitative scoring system for measuring skin status in lumpfish.

Score	**1**	**2**	**3**
			
Description	No visible damage	Moderate damage	Severe damage, open wounds

2.3.3. Eyes integrity

Eye damage was scored from 1 to 3, depending on the severity of the damage (Table 2.3). Score 1 was given when both eyes were healthy, displaying a clear and consistent colour and there were no signs of cloudiness or unusual spots. Also, there were no signs of lesions or swelling in the periocular region. When the eyes were scored 2, one eye was healthy, and the other one could display cloudiness, discoloration or lesions. The eye might also bulge out and signs of swelling, lesions or ulcers could be evident in the periocular region. When the eyes scored 3, both eyes displayed severe damage, such as cloudiness, discoloration and lesions, clearly indicating infections, physical injuries and cataracts. Both eyes could bulge out, and the periocular areas could show abnormal discolorations, swelling or lesions. Severe damage to one eye (score 2) was considered less severe than significant damage to both eyes (score 3), since bilateral damage is likely to have a bigger impact on the fish ability to navigate and locate food. However, it does not differentiate between slight damage in both eyes versus severe damage in one eye. When evaluating eye condition, detecting early stages of cataracts was also challenging, therefore only severe cases were recorded.

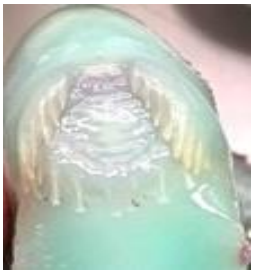
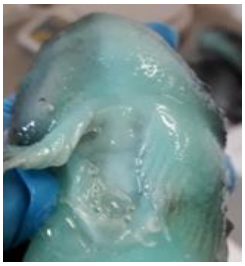
Table 2.3. Semi-quantitative scoring system for measuring eyes integrity in lumpfish.

Score	**1**	**2**	**3**
			
Description	Both healthy	One damaged	Both damaged

2.3.4. Sucker disc

The sucker disc was scored from 1 to 3, depending if there were evident deformities (Table 2. 4). Score 1 was given to a sucker disc which was symmetrical, the ridges allowed attachment to various surfaces and the colour was consistent with the rest of the fish body. When sucker disc scored 2, the disc may appear slightly asymmetric. The ridges normal pattern might be disrupted or absent in some areas and there could be some signs of discoloration. When the score 3 was given, the sucker disc was highly deformed, the disc shape and the ridges were completely compromised, and the fish could not use its sucker disc to adhere to surfaces. In severe cases, the disc could show signs of infections, lesions or abrasions.

Table 2.4. Semi-quantitative scoring system for measuring sucker disc integrity in lumpfish.

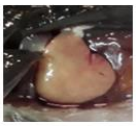
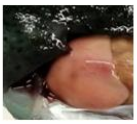
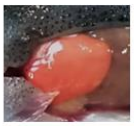
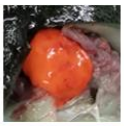
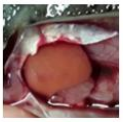
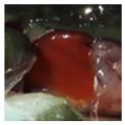
Score	**1**	**2**	**3**
			
Description	Normal Both sides symmetrical	Slightly deformed 25-50% of the disc is deformed	Highly deformed >75% of the disc is affected

2.4. Liver colour and stomach content

After this external assessment, fish were opened with an anteroposterior cut on the left side and liver colour was scored using a scale from 1 to 6 following the method used in Østerø & Eliasen (2023) (Table 2.5). Livers were scored as 1 or 2 when they displayed a pale or yellow coloration, which could indicate disease or life stage changes. Scores of 3 or 4 were assigned to livers ranging from orange to bright orange, indicative of good welfare. Livers that were reddish brown and dark brown were scored 5 and 6, reflecting poor welfare.

The scale used for liver colour was problematic for statistical analysis due to best welfare having intermediate scores (3 and 4). This was taken into account before analysing the data. The transformation process involved assigning scores in a different order. On the new scale, score 1 (good welfare, healthy liver colour) was assigned to the livers that previously scored 3 and 4 (light to bright orange), score 2 (pale liver, compromised welfare) to the livers that scored 1 and 2 (pale yellow), and score 3 (starvation and malnutrition) to the livers that scored 5 and 6 (dark and reddish brown).

Table 2.5. Semi-quantitative scoring system for measuring liver colour in lumpfish.

Score	1	2	3	4	5	6
						
	Very pale yellow	Pale yellow	Orange	Bright orange	Dark red	Reddish brown

After scoring the liver, the stomach was located and cut open, the content poured into a white bowl and diluted with some water for identification of prey and pellets (Figure 2.2 A-B). In other sampling occasions, the stomach content was placed into a petri dish diluted with distilled water and pictures of it were taken using a dissecting microscope to facilitate identification (Figure 2.2 C). The stomach content was classified as detailed in Table 2.6. When the pellets were very smashed and digested, they were recorded as "unidentified" pellet as it was challenging to identify whether it was salmon or lumpfish pellet. Where more than one prey was present, the main food was recorded first, followed by the others.

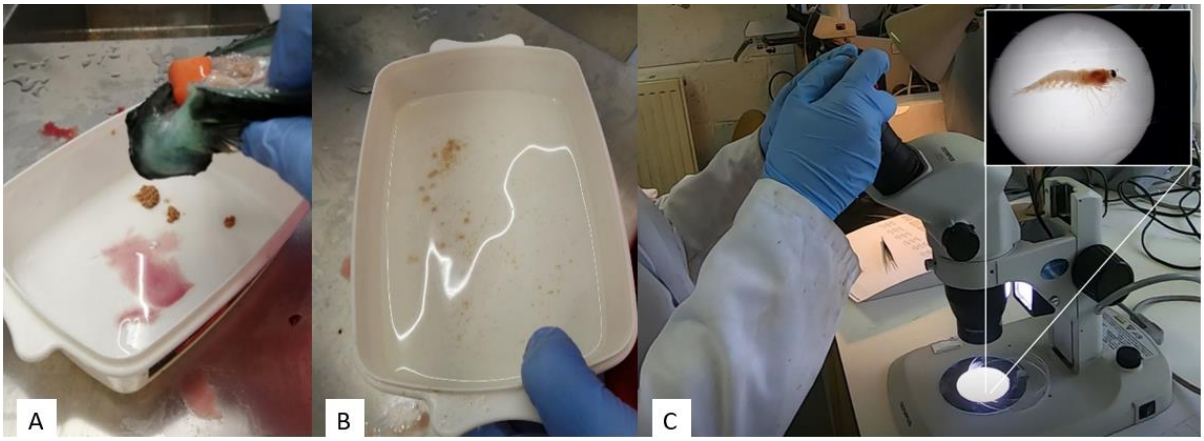


Figure 2.2. Example of stomach content dissection. (A) Lumpfish stomach is dissected, and content is poured into a container. (B) The stomach content is diluted with water to facilitate identification of prey and pellets. (C) The stomach content is observed under a dissecting microscope and pictures are taken to facilitate later identification.

Table 2.6. Stomach content classification used to identify stomach content of lumpfish. Where more than one prey was present, the main food was recorded first, followed by the others.

Stomach content
Empty
Sea lice
Lumpfish pellet
Salmon pellet
Unidentified pellet
Planktonic prey
Benthic prey
Other (seaweed, fish larvae, plastic..)

2.5. Nutritional analyses

2.5.1. Proximate composition of fish and feeds

Farmed and wild whole lumpfish as well as feeds were analysed for proximate composition according to standard procedures (AOAC, 2000).

2.5.1.1. Moisture content

To obtain moisture content and for further processing, samples were homogenised and dried through two different processes depending on the sample type (Figure 2.3).
Feeds were ground in a grinder (Knifetec™ 1095, Foss, Sweden) to obtain a homogenous sample (Figure 2.3 B). To determine the moisture content, 5 g of the homogenized feed were placed in a pre-weighed foil pot in duplicate and placed in the oven (Oven-55S, Sciquip, UK) at 103 °C overnight. Samples were cooled down in a desiccator before re-weighing, and moisture was calculated as follow:

$$\%_{Moisture} = \frac{Sample\ weight\ (g) - Dried\ sample\ weight\ (g)}{Sample\ weight\ (g)} \times 100$$

Fish were chopped into small pieces and blended (Robot coupe Blixer 4V.V, France) until a homogenous paste was obtained (Figure 2.3 A). Fish were blended individually or pooled (3 fish per pool) according to different sampling occasions. Pooling was conducted during the feed trial samplings (Chapter 6) to obtain sufficient sample material for analysis, especially when fish sizes were small or sample availability was limited. To ensure consistency, fish fed the same dietary group and from the same tank were grouped for pooling. A portion of the paste obtained after homogenizing the fish was weighed (wet weight) and placed into a pre-weighed pot. The pot was frozen at -20 °C and finally freeze-dried

at -60 °C (Alpha 1-4 LSC, Christ, Germany) until the sample was completely dried (dry weight). Moisture was calculated using the same formula as detailed above. After freeze-drying them, samples were further ground (Knifetec™ 1095, Foss, Sweden) before further analyses to achieve a homogenous powder due to the presence of skin and bones in whole fish.

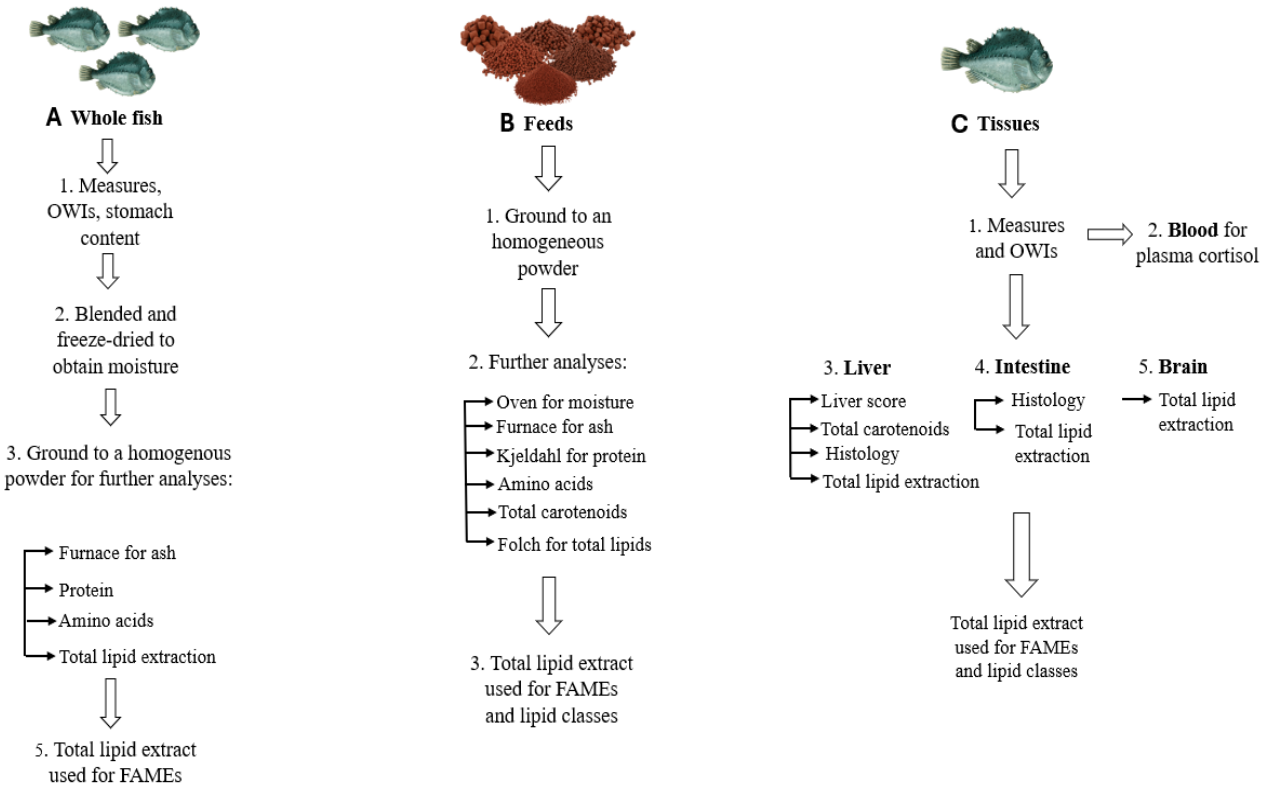


Figure 2.3. Flow chart of the laboratory analyses conducted during the study. (A) Whole fish from different origins were analysed for Chapter 3 (n = 167), and whole fish from the hatchery were used for Chapter 6 (n = 174); (B) Commercial feeds were analysed in Chapter 3, whereas experimental feeds were analysed in Chapter 6;(C) Tissues: liver from fish from different origin were analysed for Chapter 4 and 5 (n = 161), whereas liver, intestine, brain, and blood from the hatchery fish were used for Chapter 6 (n = 106).

2.5.1.2. Ash content

To measure the ash content, 1 g of both feeds and whole fish were weighed into a pre-weighed porcelain crucible in duplicate and placed into a muffle furnace (Carbolite Elf 11/14B, UK) at 600 °C overnight. Samples were cooled down to room temperature in a desiccator before being re-weighed. The ash content was calculated as follows:

$$\%_{Ash} = \frac{Ash\ weight\ (g)}{Sample\ weight\ (g)} \times 100$$

2.5.1.3. Crude protein content

Crude protein content of feed and whole fish samples was measured by determining nitrogen content using automated Kjeldahl analysis (Opsis LiquidLINE KjelROC Analyzer and Opsis LiquidLINE KjelROC Sampler KD-525, Sweden) (Kirk, 1950). Approximately 0.2 g of each sample was weighed to 4 decimal places in duplicate into a folded 42.5 mm circular filter paper (Fisher Scientific, UK). The filter paper containing the sample was then placed into Kjeldahl digestion tube where 12 ml of sulphuric acid and 2 copper Kjeltabs (KT-211-A Missouri Tablet, Opsis LiquidLINE, Sweden) were added. Samples were digested for 1 hour on the digestion block (DI-220-A, KjelROC Digestor Auto 20,, Sweden) at 420 °C. After digestion, tubes were cooled down and distilled using the KjelROC analyser. During the distillation, the nitrogen content of the sample was measured through titration. To calculate the protein content, the nitrogen content measured was multiplied by the conversion factor 6.25. This considers that protein contains 16% nitrogen and that all nitrogen in food protein is protein-bound. The results were converted from a dry to a wet basis as previously indicated with the ash as follows:

$$\%\ Protein_{wet\ basis} = \left(\frac{\%\ Protein_{dry\ basis}}{100}\right) \times (100 - \%_{Moisture})$$

2.5.1.4. Crude lipid content

Crude lipid of freeze-dried whole fish, ground feeds, livers and whole intestine was obtained using the method according to Folch et al. (1957). When two livers were pooled, livers were thawed, blended and homogenised with a spatula before further analysis. When analysing the whole intestine, two intestines from the same treatment were dissected, the content if present was removed and cut into small pieces and mixed thoroughly to homogenise the sample.

Folch method consisted of approximately 0.5 g of sample (dried whole fish, liver or whole intestine) being weighed out to 4 decimal places in duplicate and homogenised in 20 ml of 2:1

chloroform/methanol in a glass tube using an IKA Ultra-Turrax T8 tissue disrupter (Fisher Scientific, Loughborough, UK). After keeping the homogenate on ice for a minimum of 1 hour, 5 ml of KCl aqueous solution (0.88%) were added and mixed on a vortex (2:1, chloroform/methanol and KCl). Samples were left on ice for at least 5 minutes before being centrifuged at 400 g for 5 minutes. After centrifugation, the sample separated into two distinct phases and the aqueous layer was removed by aspiration. The organic phase was filtered through pre-washed (2:1 chloroform/methanol) Whatman No.1 filter paper into new pre-weighed tubes. The solvent containing the lipid extract was evaporated under a stream of oxygen-free nitrogen (OFN) (oxygen free, ≥99.99% nitrogen, 300 bar EVOS Ci 50 L cylinder, BOC Ltd, UK) on a turbovap (TurboVap® LV, Biotage, UK) and desiccated in a vacuum desiccator overnight. To quantify the percentage of lipid contained in each sample, the tubes were re-weighed. The % lipid was calculated as follow:

$$\%\ Lipid = \frac{Weight\ lipid\ (g)}{Weight\ sample\ (g)} \times 100$$

The total lipid extracts were re-dissolved in 2:1 chloroform/methanol containing 0.01% Butylated hydroxytoluene (BHT), used to prevent lipid oxidation, at a concentration of 10 mg/ml. The extracts were transferred into glass vials and stored at -20 °C prior to fatty acid and lipid class analyses.

Because of the properties of the raw materials used in the feeds, lipid in feed samples were extracted with an altered Folch method. Approximately 0.3 g of ground thawed feed were placed into 36 ml of 2:1 (chloroform/methanol) instead of 20 ml. After homogenising the sample using the turrax (IKA Ultra-Turrax T8 tissue disrupter, Fisher Scientific, Loughborough, UK), samples were left in the spark proof freezer at -20 °C overnight instead of 1 hour. After removing the homogenate from the freezer, 9 ml of KCl aqueous solution (0.88%) instead of 5 ml were added to maintain the same proportion (2:1, chloroform/methanol and KCl). The lipid was extracted using the same procedure as described above for whole fish, livers and whole intestine where samples were centrifuged at 400 g for 5 minutes. The sample separated into two distinct phases and the aqueous layer was removed by aspiration. The organic phase was filtered through pre-washed (2:1 chloroform/methanol) Whataman No.1 filter paper into new pre-weighed tubes. The solvent containing the lipid extract was evaporated under a stream of OFN on the turbovap, and desiccated in a vacuum desiccator overnight. In order to quantify the percentage of lipid contained in each sample, the tubes were re-weighed and the % lipid was calculated as before: weight lipid (g)/weight sample (g) x 100.

Although crude lipid of brain was extracted using the method by Folch et al. (1957), some adaptations were carried out due to the small size and fatty nature of the tissue. Samples of brain were stored at -70 °C and were kept frozen before being weighed out into a reactive vial. Two millilitres of 2:1 chloroform/methanol were added to the vial and homogenised using the turrax. The homogenate was then transferred into a 15 ml glass tube and 8 ml of 2:1 (chloroform/methanol) were added and used to rinse the reactive vial and the probe. The final volume of 2:1 chloroform/methanol used was 10 ml

instead of 20 ml to minimise sample loss during the homogenisation process. Samples were left overnight in the spark proof freezer at -20 °C to extract the lipids more efficiently and increase accuracy. After removing the sample from the freezer, 2.5 ml of aqueous KCl (0.88%) were added (2:1, chloroform/methanol and KCl). Samples were centrifuged at 400 g for 5 minutes. After centrifugation, the sample separated into two distinct phases and the aqueous layer was removed by aspiration. The organic phase was filtered through pre-washed (2:1 chloroform/methanol) Whatman® No.1 70 mm circular filter paper. The solvent containing the lipid extract was evaporated under a stream of OFN on a nitrogen evaporator. Dried lipid extracts were redissolved in 1.5 ml of 2:1 chloroform/methanol and transferred into pre-weighed small glass vials. Samples were dried under a stream of OFN on a nitrogen evaporator and stored under vacuum in a desiccator overnight. To quantify the percentage of lipid contained, the tubes were re-weighed, and the total lipid extracts were re-dissolved for fatty acid analysis adding 2:1 chloroform/methanol containing 0.01% BHT, to prevent lipid oxidation, at a concentration of 10 mg/ml. The glass vials were stored at -20 °C prior to fatty acid analysis.

2.5.2. Fatty acid profile

The fatty acid profile of feeds, whole fish, whole intestine, livers and brains were characterized by analysis of fatty acid methyl esters (FAME) using gas chromatography as described by Christie (2003). The analysis was performed on the previous total lipid extracted following Folch et al. (1957). The FAME was prepared by transmethylation where 100 µl of total lipid extract (at a concentration of 10 mg/ml) was mixed with 100 µl of 17:0 fatty acid standard (10 mg/ml) and evaporated under a stream of OFN for a few minutes, until the sample is completely dry. One ml of toluene and 2 ml of 1% sulphuric acid (H_2SO_4) in methanol were added to each sample. Tubes containing the sample and the solvents were incubated in a hot block at 50°C for 16-18 hours after being gassed with OFN for approximately 10 seconds to prevent oxidation and sealed with glass stoppers and a piece of small tissue.

FAME produced were extracted adding 2 ml of 2% potassium bicarbonate ($KHCO_3$) and 5 ml of 1:1 iso-hexane/diethyl ether, containing 0.01% BHT. To obtain two separate phases, tubes were mixed and centrifuged at 400 g for 5 minutes. The upper organic layer was transferred into a new test tube and 5 ml of 1:1 iso-hexane/diethyl ether were added to the remaining layer. The tubes were mixed and centrifuged again to obtain maximum recovery of FAME and the second upper layer was added to the first upper layer. To evaporate the solvent, the organic solvent was dried under a stream of OFN and the extract suspended in 0.5 ml of iso-hexane. The extract was purified using silica clean-up cartridges (Clean-up® silica extraction columns; UCT, Bristol, Pennsylvania, USA). UCT silica clean-up cartridges were pre-conditioned using 5 ml of iso-hexane and samples were pushed onto the pre-conditioned cartridges using an adapted glass syringe. FAME from the cartridges were eluted into a clean test tube by adding 10 ml of 95:5 iso-hexane:diethyl ether. This eluent containing the FAME was

evaporated under a stream of OFN. It was then resuspended in 1 ml of iso-hexane and transferred into vials for gas chromatography (GC) analysis.

FAME were separated and quantified by gas–liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy). The GC was 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 (FID). The carrier gas used was hydrogen. The oven thermal gradient was from 50 °C to 150 °C at 40 °C/min to a final temperature of 230 °C at 2 °C/minute. Data were processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). Individual FAME was identified by comparing the samples profile to known standards (Supelco™ 37-FAME mix; Sigma-Aldrich Ltd., Poole, UK) and published data (Tocher & Harvie, 1988). The addition of heptadecanoic acid (17:0) at a concentration of 10 mg/ml as an internal standard allowed to calculate fatty acid content per g of sample.

2.5.2.1. Fatty acid retention

The fatty acid retention was calculated in the feed trial described in Chapter 6. The retention percentage was calculated using the method described in Glencross et al. (2003), and it represents the incorporation of dietary fatty acids into the fish whole body over the course of the feed trial. The retention (%) was determined by using the average cumulative feed intake per fish in each tank and the average increase in lipid, protein and fatty acids in the fish from S0 to S2. An average for each dietary treatment was calculated. The retention for each fatty acid was calculated as follow:

$$\%_{Fatty\ acid\ retention} = \left(\frac{FA_f - FA_i}{FA_c}\right) \times 100$$

where FA_f is the final amount of a particular fatty acid in the fish body at the end of the study (S2), whereas FA_i is the initial amount of that fatty acid in the fish body at the beginning of the study (S0). FA_c is the amount of the specific fatty acid consumed by the fish throughout the study. FA_i was calculated for each fatty acid as:

$$FA_i = FA_{ri} \cdot (W_i \cdot L_{ri})$$

Where: FA_{ir} is the initial relative amount of the particular fatty acid in relation to all fatty acids in the fish body, and $(W_i \cdot L_{ri})$ represents the absolute amount of lipid in grams, calculated as the weight of the fish at the start of the trial W_i multiplied by the lipid percentage L_i of the fish at the start of the trial. The same method was used to find the final amount for each fatty acid FA_f.

The amount of the specific fatty acid consumed by the fish throughout the studyFA_c was calculated as:

$$FA_c = FA_d \cdot (FI \cdot L_d)$$

Where FA_d is the relative amount of the particular fatty acid in the diet, $(FI \cdot L_d)$ represents the absolute amount of lipid that has been consumed, where FI represents the feed intake, and L_d the lipid percentage of the diet.

2.5.3. Lipid class composition

Lipid class analysis was performed on total lipid extracted from lumpfish livers. Lipid classes were separated by high-performance thin-layer chromatography (HPTLC) using 10 × 20 cm x 0.25 mm plates (VWR, Lutterworth, UK) according to Henderson & Tocher (1992). Before performing lipid classes analysis, plates were cleaned using 2:1 chloroform/methanol, allowing the solvent to evaporate by air drying.

Twelve 3 mm origins were marked with pencil on the plate at a distance of 1.5 cm between them. Total lipid samples in duplicate (1.5 -2 µl), two lipid classes standards (one neutral and one polar) and one blank were applied to each origin through a Microliter™ glass syringe (Hamilton, Bonaduz, Switzerland). To separate polar lipid classes, plates were developed to 5.2 cm in methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.). Excess solvent was evaporated via air drying and vacuum desiccation for 15 minutes. To separate neutral lipid classes, plates were developed to 9.5 cm in the same direction in a solvent mixture containing iso-hexane/diethyl ether/acetic acid (85:15:1.5, by vol.). Excess solvent was evaporated via air drying and vacuum desiccation for 15 min. Lipid classes were visualized by spraying with 3% aqueous cupric acetate containing 8% phosphoric acid and charring plates at 160 °C for 20 min in an oven (Hotbox Oven Size 2, Gallenkamp, UK) (Figure 2.4). Lipid classes were quantified by densitometry using a CAMAG-3 TLC Scanner (version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) with winCATS software (Planar Chromatography Manager, version 1.2.3) to quantify the lipid classes.

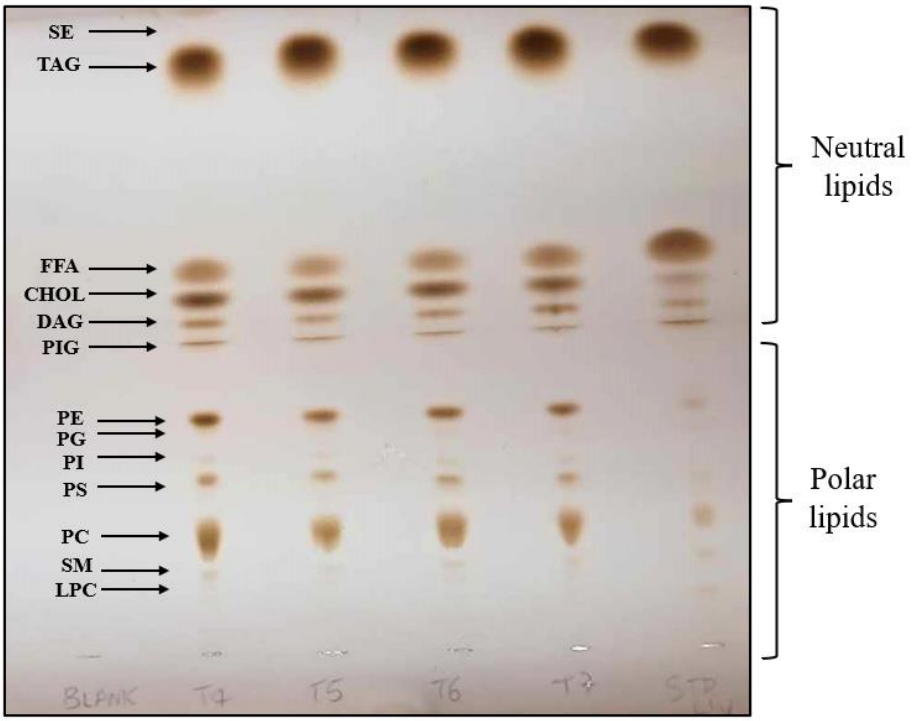


Figure 2.4. Example of silica plates lipid classes composition of lumpfish liver. Each different dark band represent a different lipid class. Polar lipid classes are developed from the bottom of the plate to approximately 5.2 cm and they are lysophosphatidylcholine (LPC), sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). Neutral lipid classes are developed to 9.5 cm to the same direction and they are cholesterol (CHOL), diacylglycerol (DAG), free fatty acids (FFA), triacylglycerols (TAG) and sterol esters (SE).

2.5.4. Total carotenoids of livers

Total carotenoids were extracted from lumpfish livers largely by the methods described by Barua et al. (1993) and Bell et al. (1998). Approximately 1 g of liver from individual fish was homogenized in 10 ml of 1:1 ethyl acetate/ethanol using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK). The homogenate was centrifuged for 5 min at approximately 400 g and the supernatant transferred to a new tube. The pellet was re-homogenized in 5 ml of ethyl acetate, mixed and recentrifuged, and the supernatant was combined with the first supernatant. Finally, the pellet was re-homogenized in 5 ml of iso-hexane and recentrifuged, and the supernatant was combined with the pooled supernatant. The pooled supernatant was dried under a stream of OFN and the residue redissolved in 5 ml of iso-hexane. Total carotenoid was measured first spectrophotometrically at 470 nm using the E1% (w/v) of 2100. Then, it was measured using the HPLC (Waters 2695 Separations Module, UK) equipped with a Roc silica 5µ, 150 x 4.6 mm column, Guard cartridge and a Dual λ Absorbance Detector (Waters 2487, UK). An isocratic solvent system was used containing iso-

hexane/acetone (82:18 by vol.) at a flow rate of 1.2 ml/minute. Astaxanthin and canthaxanthin were detected at 474 nm and quantified using external standards of astaxanthin and canthaxanthin obtained from Roche (Welwyn Garden City, UK).

2.5.5. Total carotenoids of feeds

Total carotenoids were extracted from feeds as follow. Approximately 0.75 g of ground feed were weighed out up to 2 decimal places into a tared 25 ml volumetric flask, and approximately 100 µl of Protex 6L, 25 mg of BHT and 1.5 ml of distilled water were added to the flasks containing samples. After ensuring that the ground feed was covered with water, the flasks were placed in an ultrasonic water bath at approximately 50 °C for 30 minutes. After this, 10 ml of ethanol was added to the warm suspension, agitated, and 12.5 ml of dichloromethane further added and mixed. Samples were left to stand in the dark overnight. Flasks were diluted to volume with dichloromethane, shook vigorously and solids were allowed to settle in the dark for 2 hours, before further processing. One ml of the settled solvent mixture was transferred to a clean tube, and it was dried under a stream of OFN before being redissolved in 1 ml of isohexane: acetone (82:18). Samples were mixed and centrifuged at 7500 g for 5 minutes before being transferred to appropriate vials for HPLC analysis. Total carotenoids were measured using the HPLC (Waters 2695 Separations Module, UK) equipped with a Roc silica 5µ, 150 x 4.6 mm column, Guard cartridge and a Dual λ Absorbance Detector (Waters 2487, UK). An isocratic solvent system was used containing iso-hexane/acetone (82:18 by vol.) at a flow rate of 1.2 ml/minute. Astaxanthin was quantified using external standards of astaxanthin obtained from Roche (Welwyn Garden City, UK).

2.5.6. Amino acid profile

The amino acid profile of individual whole fish (hatchery n=10, wild n=10) and feeds was determined using the Waters ACCQ-TAGTM Ultra Method for hydrolysate amino acid analysis (Waters Corporation, Milford, Massachusetts, USA) as described in Glencross et al. (2021). Approximately 250 mg of samples were weighed out in duplicate into microwave tubes. Ten ml of phenolic 6M HCl was added to each tube and left to stand for 15 minutes before filling with a stream of OFN for 20 seconds. A set of duplicate samples was hydrolysed at 150 °C, and another set of the same samples in duplicate was hydrolysed at 190 °C using a microwave digestion system (MARS 6 240/50, CEM, USA). After digestion, the tube contents were transferred into 250 ml volumetric flasks, rinsed with ultrapure water and diluted up to volume (250 ml) with ultrapure water. Flasks contents were mixed and approximately 1 ml of sample was taken from the flask through a 0.45 µM hydrophilic syringe filter and stored in the fridge prior derivatisation. The derivatisation was performed according to the manufacturer instructions by using a Waters H-Class UPLC fitted with an ACQUITY BEH Phenyl 1.7 µ 2.1 X 100 mm UPLC column (Waters Ltd, Hertfordshire, UK). The calibration standard used was the Waters Amino Acid

Hydrolysate Standard and an isocratic solvent system containing AccQ-Tag Ultra Reagent Diluent (Eluent A) and Ultra Reagen Buffer (Eluent B) was used at a flow rate of 1.2 ml/minute.

The quantification of amino acids was based on the integration of chromatographic peaks obtained during the analysis. The concentration of each amino acid in the sample was determined using the formula:

$$pmol/\mu l = \frac{Sample\ Area \times Dilution\ factor}{Standard\ Area}$$

Where the sample area corresponds to the integrated chromatographic peak area for the amino acid in the sample, the standard area corresponds to that of the calibration standard, and the dilution factor accounts for the sample dilution. The concentration of each amino acid was then expressed as grams per 100 grams of the sample, using the formula:

$$g/100g = pmol/\mu l \times Molecular\ Weight \times Conversion\ Factor$$

The conversion factor accounted for the sample weight, final dilution volume, and unit adjustments.

2.6. Histological analysis

Tissues from individual fish, extracted for histological analysis, were fixed in 10% neutral buffered formalin (NBF) such as liver, spleen, anterior and distal intestine. Tissues were dehydrated through a graded series of alcohols, followed by chloroform as a clearing agent and finally tissues were infiltrated with paraffin wax (Thermo Electron Shandon Citadel 2000 tissue processor, Thermo Fisher Scientific, USA). Tissues were embedded in paraffin wax on the histoembedder (Leica HistoCore Arcadia H, Leica Biosystems, Watzlar, Germany) with an Arcadia C cold plate attachment used to cool down the wax blocks. To expose the surface of the tissues, final blocks were first trimmed at 20 µm thickness on a microtome Leica RM 2035, Leica Instruments, Nussloch, Germany). Trimmed blocks were submersed in distilled water for 20 minutes followed by 5 minutes on a cold plate before sectioning. A ribbon of sections was produced when sectioning at 5 µm thickness on the microtome and placed in a heated water bath containing distilled water. The sections were transferred into clean glass slides and placed in a drying oven at 60 °C for at least 1 hour. Slides were stained with Haematoxylin & Eosin (H&E) (Martoja et al., 1970).

Stained slides were scanned using AxioScan (ZI, ZIESS®, Oberkochen, Germany) and were uploaded to QuPath® v0.2.3 (Bankhead et al., 2017) to visualise and perform the following measurements: liver intracytoplasmic vacuolization, intestine muscular thickness, liver congestion, liver inflammation, liver fibrosis, and liver necrosis.

2.6.1. Liver intracytoplasmic vacuolization

Livers from individual fish were analysed using Fiji ImageJ® (Schindelin et al., 2012) to measure liver intracytoplasmic vacuolization. To do so, five screenshots were randomly taken across each liver, avoiding blood vessels, bile ducts and evident artefacts. Each screenshot was converted to grayscale (8 bit b/w). A threshold was applied to separate the vacuoles from the background and watershed separation to separate connected components (Figure 2.5 A-C). In each picture, fat vacuoles are counted and reported as percentage. The average of the five screenshots was used for each individual fish. The average of the five screenshots was used to represent liver intracytoplasmic vacuolization for each fish, as preliminary analysis showed minimal variability between screenshots. An example of different levels of liver vacuolation, from the lowest (1-2 %) to the highest (27-29 %) is shown in Figure 2.6 (A-C).

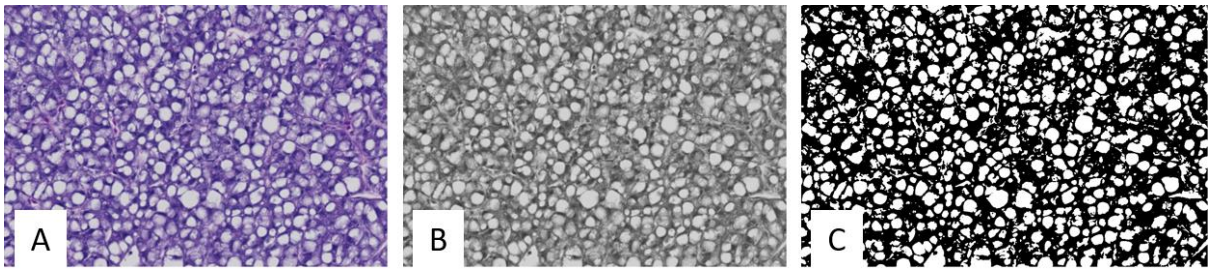


Figure 2.5. Lumpfish liver stained with H&E. Picture shows the process of determination of liver vacuolisation through ImageJ® software. (A) Original screenshot of H&E stained liver. (B) Original image converted to 8-bit. (C) Threshold applied to the grayscale image (photos by Di Toro J., 2023).

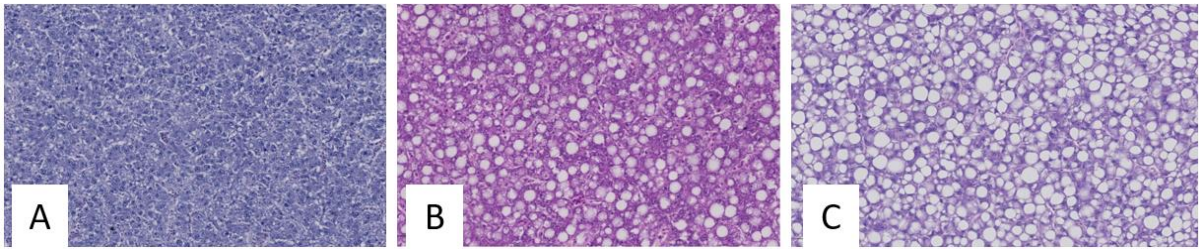


Figure 2.6. Example of different percentages of liver vacuolisations. Images were taken from livers stained with H&E and % were calculated using ImageJ. (A) Liver intracytoplasmic vacuolization of 1-2 %. (B) Liver intracytoplasmic vacuolization of 13-15 %. (C) Liver intracytoplasmic vacuolization of 27-29 % (photos by Di Toro J., 2023).

2.6.2. Liver inflammation, congestion, fibrosis, and necrosis

The whole liver was also examined for health indicators such as the presence of inflammation, congestion, fibrosis, and necrosis, using a semi-quantitative scoring system. Each of these parameters were scored from 0 to 3, as shown in Table 2.7. Score 0 was given when no signs of inflammation or the others was found. Score 1 (mild), when one area of the liver shows signs of inflammation. Score 2 (moderate), when two or three areas are affected by inflammation or the others. Score 3 (severe), when more than three areas are affected by inflammation, congestion, fibrosis and necrosis.

An example of inflammation, congestion, fibrosis and necrosis of livers stained H&E from lumpfish from different origins (land-based hatcheries, sea cages and wild) is shown in Figure 2.7 (A-D).

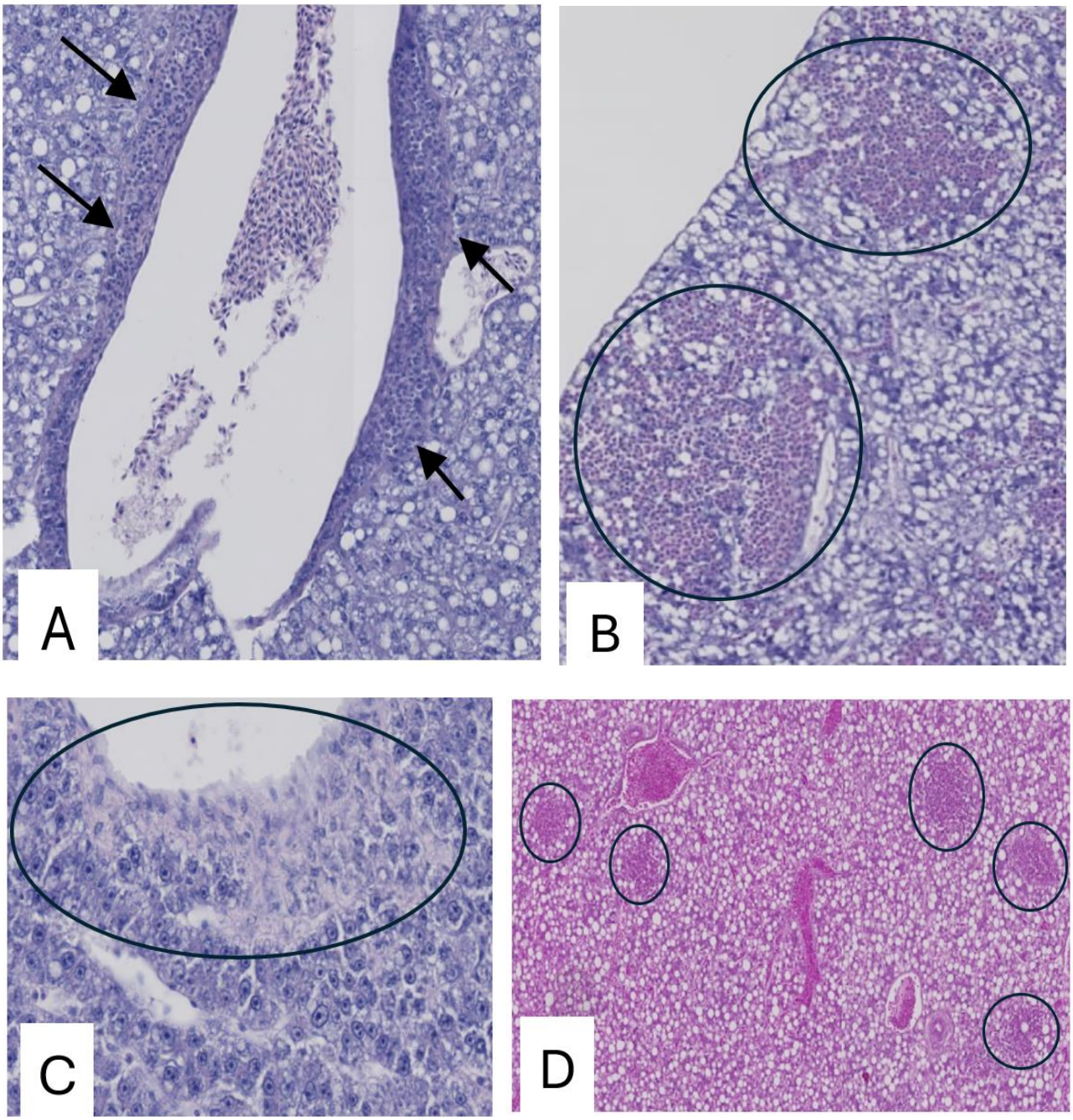


Figure 2.7. Example of (A) inflammation (score 3), characterised by a dense infiltration of inflammatory cells in the peri-tubular regions, including lymphocytes and granulocytes, as indicated by the black arrows, suggesting an active inflammatory response. (B) Example of severe vascular congestion (score 3), the circled regions show areas of vascular stenosis, where blood vessels are significantly narrowed and engorged with blood cells, leading to compromised tissue perfusion. (C) Example of fibrosis (score 2), where circled areas highlights hepatocytes that are replaced with connective tissue. The affected area shows a reduction in normal hepatocyte density, with fibrous connective tissue disrupting the typical architecture of the liver. (D) Example of necrosis (score 3), where black circles are highlighting multiple areas containing dead hepatocytes, loss of cellular

integrity, fragmented nuclei, and cytoplasmic eosinophilia. The necrotic foci are distributed across the tissue, suggesting widespread damage and advanced tissue degradation.

Anterior and distal intestine inner circular muscle thickness were quantified. An average of thirty measurements were taken for each intestinal area, using the line annotation tool in QuPath® v0.2.3 (Bankhead et al., 2017) as shown in Figure 2.8.

Table 2.7. Semi-quantitative scoring system used for assessing signs of inflammation, congestion, fibrosis and necrosis in livers from lumpfish from different origins (land-based hatcheries, sea cages and wild).

Score	Description
0 = Absent	No signs of inflammation/congestion/fibrosis/necrosis
1 = Mild	One area shows sign of inflammation/congestion/fibrosis/necrosis
2 = Moderate	2-3 areas are affected by inflammation/congestion/fibrosis/necrosis
3 = Severe	>3 areas are affected with inflammation/congestion/fibrosis/necrosis

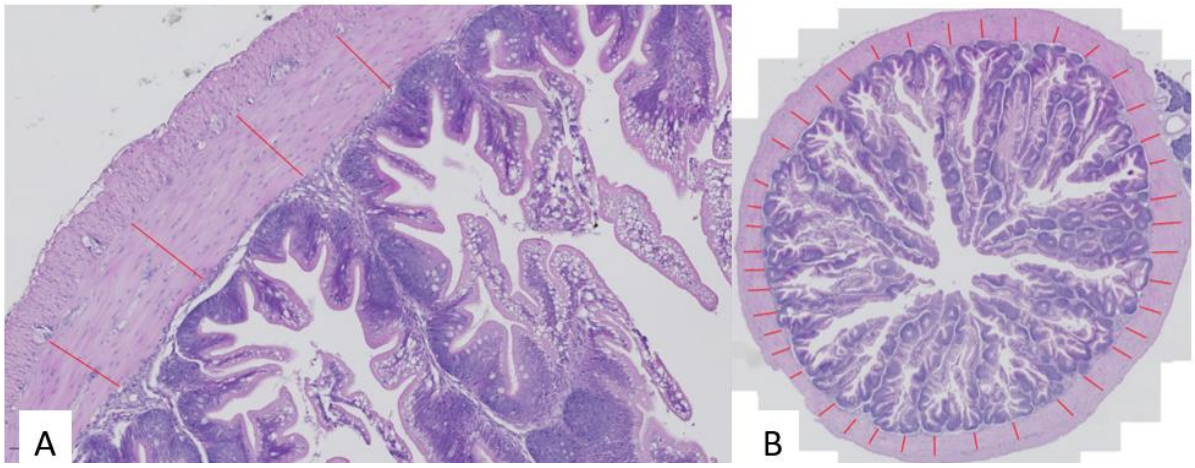


Figure 2.8. Example of anterior intestine inner circular muscle thickness measurement through the line annotation tool of QuPath® v0.2.3. (A) Red lines go from mucosa to lamina propria which represent the thickness of the inner circular muscle layer of intestine. (B) Several measurements (red lines) of the thickness of the inner circular muscle layer of intestine.

2.7. Cortisol analysis

Blood samples were collected as part of the stress challenge of the feed trial (Chapter 6). A total of 198 fish were sampled for this purpose. Each fish was anaesthetised using Finquel (MS-222, MSD Animal Health), and within three minutes, blood was withdrawn with a syringe from the caudal vein into heparinised vacutainers (23G, 0.6 x 25 mm, BD Microlance, Denmark).

The blood was centrifuged for 5 minutes and plasma was stored at -20 °C for cortisol analysis.

To determine cortisol in plasma, 100 µl of plasma were placed into Eppendorf tubes. Fifty µl of internal standard (d4 cortisol, 50 ng/ml, Sigma-Aldrich, UK) were pipetted into each tube, followed by 500 µl of 1% KCl and 500 µl of ethyl acetate. The mix was vortexed and centrifuged for 2 minutes at 16000 rpm. After centrifuge, the upper layer was transferred into new pre-labelled Eppendorf tubes and further 500 µl of ethyl acetate were added to the remaining layer. Samples were vortexed and centrifuged again as described previously. The second upper layer was removed and added to the previous upper layer. This ethyl acetate extract was dried under OFN until completely dry and resuspended in 100 µl of 1:1 methanol/water. To precipitate protein, samples were vortexed and left on ice for 1 hour. Samples were centrifuged at 16000 rpm for 2 minutes and the precipitated material formed a pellet. The supernatant was transferred into clean glass vials prior cortisol determination.

Samples were analysed using the LC-MS and mass spectrometer XEVO® TQ-S coupled to Acquity I-class UPLC (Waters, UK) equipped with a Column ACQUITY UPLC® HSS T3 1.8 µm i.d. 2.1 x 50 mm (Waters, UK). A gradient solvent system was used and contained water/0.1% (w/v) ammonium formate/ 0.1% (v/v) formic acid and methanol/0.1% (w/v) ammonium formate/0.1% (v/v) formic acid.

2.8. Statistics

All statistical analyses were performed using R (R Core Team, 2021) and figures were plotted using “ggplot2” (Wickham et al., 2016), with specific packages employed depending on the analyses conducted in each experimental Chapter. The complete set of annotated R Markdown notebooks used in this Thesis is publicly available at https://zenodo.org/records/15571560. Excel data files were imported into R using the “readxl” package (Wickham & Bryan, 2019), which is part of the “tidyverse” collection of R packages (Wickham et al., 2019). The ”dplyr” package was used for data manipulation (Wickham et al., 2020).

Linear models were carried out in every experimental Chapter using the built-in lm() function in the base R package. When the linear model showed an overall statistically significant result ($P < 0.05$), the F statistic with the degree of freedom and the P value were reported from the linear model summary table, and values having high Cook’s distance scores were considered outliers and checked through the Residuals vs Leverage plot. A post hoc Tukey HSD test was then performed to identify differences between groups. In Chapter 6, polynomial and linear regression models were used to determine the

optimal dietary EPA+DHA levels based on specific growth rate (SGR), survival rates, and cortisol levels. The best-fitting model was selected to provide recommendations for dietary formulations.

Generalised linear models with a binomial family were constructed using the glm() function in R to analyse binary responses between various predictor variables, such as fish origin, diet, weight, and liver score and OWIs. Predictor variables were included as categorical or continuous variables depending on the analysis. OWIs and liver scores were transformed into a binary response (1 for good welfare, 2 for compromised welfare).

Ordinal logistic regression models were used to analyse the effects of categorical predictors, such as fish origin, on various response variables such as histological parameters. This was conducted using the polr() function from the MASS package in R (Ripley et al., 2013). Null models containing only an intercept were constructed as baselines for comparison with full models. Likelihood ratio tests were performed to assess whether the inclusion of predictors significantly improved model fit. For models where significant effects were observed ($P < 0.05$), post hoc pairwise comparisons were conducted using Tukey's method to identify differences between groups.

Simple linear mixed effects models were performed using the package "lme4" to account for potential variation by tank during the feed trial (Bates et al., 2015) and "lmerTest" (Kuznetsova et al., 2017).

To investigate the effect of diet on survival, a survival analysis was carried out using the "coxme" (Therneau & Therneau, 2015) and "survival" packages (Therneau et al., 2015). A Cox mixed-effects model was constructed, where diet is treated as a fixed effect and tank as a random effect.

PCA was performed using the "FactoMineR" package (Lê et al., 2008) to visualise and identify patterns regarding the differences between groups in terms of macronutrient and fatty acid profile. The data for the PCA is mean-centered, subtracting the mean of each variable from the values. PCA was performed with scaling, where each variable was divided by its standard deviation. This is performed by default to give equal weight to all variables. The summary outputs of the PCA also provided information about the eigenvalues, proportion of variance explained and variable contributions. The outputs of the PCA were visualized using the "factoextra" package (Kassambara, 2016). A categorical PCA was performed using the "Gifi" package (Mair et al., 2019) to visualise and identify patterns regarding OWIs. Data in Tables are presented as mean ± standard deviation (SD).

Chapter 3. How Faroese farmed lumpfish (*Cyclopterus lumpus*) differs from wild populations in terms of body composition, amino acid and fatty acid profiles

3.1. Abstract

Juvenile lumpfish are deployed in the salmon sea cages for their delousing activity against sea lice. Despite not being farmed for human consumption, nutritional studies are pivotal to elucidate nutritional requirements, feeding strategies and develop tailored diets for lumpfish. This study investigated the nutritional composition of whole lumpfish from Faroese wild and farmed populations in order to gain better knowledge regarding dietary strategies and feed formulations for juvenile lumpfish. Wild lumpfish served as the ideal model for comparison as their natural diet and environment provide a benchmark for elucidating the nutritional and welfare requirements for farmed counterparts, particularly given the species relatively early stage of cultivation. Significant variations were found between lumpfish from different origins and environments. Farmed lumpfish, both from hatcheries and sea cages, have a higher lipid content compared to their wild counterparts, likely due to the high-energy diets and controlled environments provided in farming conditions. This suggests that overenergetic diets are not recommended, and diets should contain only 10-15% of lipids. Also, the fatty acid profile of whole fish reflected dietary inputs. Farmed lumpfish exhibited higher levels of n-6 PUFA, primarily due to the high levels of LA and ALA present in the feed. On the other hand, wild lumpfish had higher levels of n-3 PUFA, mainly due to the higher levels of EPA and DHA. The protein content of whole fish was also significantly different among the two origins. When comparing the essential amino acids of the farmed fish to the wild counterpart, similar levels were found, showing that the nutritional requirements for essential amino acids were covered by the diets provided in the hatcheries. This study underscores the need for improving tailored diets and feeding strategies for lumpfish, to improve survival and welfare throughout the deployment phase. Based on the body composition of the wild population, diets for juvenile lumpfish should contain 10-15 % of lipids.

Keywords: Lumpfish, farmed, wild, body composition, fatty acids

3.2. Introduction

Lumpfish are currently farmed and widely deployed as cleaner fish in salmon farming in Norway, Scotland, Faroe Islands and Iceland (Imsland et al., 2014). Norway has experienced an increased use of lumpfish over the years, from 10 million in 2015 to 27 million in 2021. However, in 2022 it saw a drastic decrease to 17 million (Fiskeridirektoratet, 2023) due to the mortality rates and a reduction in their deployment by the farmers (Wilcox, 2023). Lumpfish have been used as cleaner fish in the Faroe Islands since late 2014 (Eliasen et al., 2018). Although most of the lumpfish deployed in the Faroe Islands are of Icelandic origin (Steinarsson & Árnason, 2018), there has been a small and increasing local production of lumpfish since 2015 with around 400 thousand lumpfish produced in 2019, 500 thousand in 2020, and 800 thousand in 2021. However, this production has ceased in 2023 (Jacobsen, 2021). Lumpfish production in the UK started in 2013, and about 600 thousand lumpfish and wrasse were reared in Scotland in 2022 (Marine Directorate, 2023).

Juvenile lumpfish are usually deployed when they attain a size of 25-30 g, which generally occurs around 6-8 months post-hatching (Powell et al., 2018), at a stocking density of 8 % to 15 % both commercially and in scientific trials (Imsland et al., 2014; Imsland et al., 2020).

Approximately 2 million lumpfish are needed each year in the salmon farms of the Faroe Islands alone provided all farms use lumpfish (Johannesen et al., 2018a). A significant part of these fish will experience high mortality rates. For instance, in the Faroe Islands, a mortality of 86.2 % was reported from 2021 to 2022 (S.L. Østero, personal communication, November 9, 2023). On the other hand, a mortality rate of 45% per year was reported by the Norwegian Veterinary Institute (Stien et al., 2020), and it is suggested to be higher in some sites. Possible reasons for such high mortalities include handling, infectious diseases, mechanical treatments against sea lice, and dietary effects (Reynolds et al., 2022). However, mortality causes can be multifactorial, where a primary cause can enable secondary factors or infections and be influenced by poor nutritional status, and inadequate feeding strategies (Reynolds et al., 2022). In addition, within few weeks post deployment some lumpfish show a compromised nutritional status and very low lipid reserves as reported in Boissonnot et al. (2022) and Eliasen et al. (2020).

Initial strategies for feeding wild-caught juveniles involved the use of commercial feeds for species such as salmon, cod or flatfish. When lumpfish were fed salmon feed, which is high in oil content, it caused low survival and fat deposits in the liver and brain (Sayer et al., 2000). This highlighted the necessity of formulating tailored diets that cover the nutritional requirements of lumpfish (Powell et al., 2018).

Compared to other species such as Atlantic salmon, the farming of lumpfish as a cleaner fish started quite recently e.g. in Norway only in 2010 (Mortensen et al., 2020) and in the Faroe Islands in late 2014 (Eliasen et al., 2018). Being a more recent addition to the aquaculture industry, there is a need for further research into the biology and ecology of lumpfish, as well as nutrient requirements (Powell et al., 2018).

Despite lumpfish not being farmed for human consumption (Garcia de Leaniz et al., 2022), nutritional studies are pivotal to elucidate nutritional requirements, feeding strategies and develop tailored diets (Willora et al., 2020) to meet the demand for robust cleaner fish.

The body composition of farmed lumpfish is approximately 87-92% water, 5-7% crude protein, 1-2 % ash, and 0.7-1.3% crude lipid (Ageeva et al., 2021). In farmed fish, changes in body mass are mainly due to size/age of the fish, feeding regimes and diet (Jobling, 2001; Sutton et al., 2000), as well as prey availability and composition for wild fish (Eliasen et al., 2018; Guo et al., 2022). In farm environments, fish have access to highly energetic diets, formulated with higher levels of fat compared to the wild prey. This contributes to higher fat deposition as documented by Gélineau et al. (2001). Also, the controlled environment and the limited space in tanks, may reduce the fish overall energy expenditure, exacerbating fat deposition. In the sea cages lumpfish can also access high energy diets, such as salmon feed as well as feed on other preys such as zooplankton, sea lice, and organisms associated to biofouling the sea cages (Eliasen et al., 2018). In this sense, wild lumpfish in floating seaweed ignore prey such as molluscs and nematodes, and mainly eat planktonic organisms such as copepods, amphipods, and isopods and even smaller sized conspecifics (Ingolfsson & Kristjansson, 2002).

A need for better nutrition in deployed lumpfish has been highlighted in several studies, including the lack of knowledge regarding nutritional requirements and optimal feeding strategies (Boissonnot et al., 2022; Garcia de Leaniz et al., 2022; Hamre et al., 2022). Comparing farmed to wild fish is a common practice in aquaculture research, as wild fish serve as a benchmark for developing and optimising feed formulation for their farmed counterparts (Lenas et al., 2011; Orban et al., 2003; Oztekin et al., 2020). To the best of our knowledge, the nutritional composition of wild lumpfish, as well as the comparison to farmed counterparts has not been thoroughly explored.

The aim of this study was to investigate how wild lumpfish composition differ from farmed fish in terms of macronutrients, fatty acids and amino acid content, to formulate a more suitable diet.

To do so, lumpfish were sampled from two origins: farmed and wild. Farmed fish were sampled from the hatcheries and from different sea farm locations, whereas wild fish were sampled from the wild populations around the Faroe Islands.

3.3. Materials and methods

3.3.1. Fish collection and sample preparation

Lumpfish were sampled in the Faroe Islands, according to the Directive 2010/63/EU regarding the protection of animals for scientific purposes and approved by the head veterinarian of the Faroe Islands “Landsdjóralæknin” in according to the Welfare act 2018, 10 (DJÓRAVÆLFERÐARLÓGIN - Løgtingslóg 49 apríl 30 2018, Faroe Islands). Sampling procedures were also reviewed and approved by the Animal Welfare and Ethical Review Body of the University of Stirling (AWERB 19 20 007).

Lumpfish for this study were sourced from salmon farms, lumpfish hatcheries, and from wild populations in and around the Faroe Islands (Figure 3.1). Sixty pre-deployment lumpfish of

approximately 25-35 g were sampled from two hatcheries, Nesvík and Svínoy, in July and September 2020 respectively. Deployed fish (n= 334) were collected from sea cages of seven salmon farming sites across the Faroe Islands from October 2019 to January 2021 as shown in Figure 3.1. Wild lumpfish (n= 169) were captured from October 2019 to August 2021 as by-catch from seaweed farm harvest and annual pelagic research surveys conducted in the summers 2020 and 2021 by the Faroe Marine Research Institute (FAMRI, Faroe Islands). FAMRI conducts surveys of the marine environment each year to inform the Faroese government regarding the status of fish stocks and its relative changes.

Collected lumpfish were categorised according to size classes: < 50 g, 50 - 300 g, 300 g - 1000 g, 1000 - 5000 g, with each fish chemically analysed individually. Fish from 1000 g to 5000 g are mainly wild and can be rarely found in sea cages due to mortalities and harvest timing of salmon. Therefore, the focus of the data analysis is on lumpfish < 50 g to 1000 g, which are the sizes mainly found throughout deployment in the sea cages.

Farmed fish were euthanised with an overdose of Finquel (0.8 g/L, MS-222, MSD Animal Health) and frozen at -20 °C before laboratory analysis. Wild fish were euthanised through exsanguination via a gill cut and frozen at -20 °C. During each sampling, fish were measured to the nearest mm and weighed out to the nearest gram. Measurements included the total length of the fish, from the snout to the final part of the lobe of the tail and the height which is measured from the highest part of the crest to the bottom of the belly. Before dissection, each fish was also scored for OWI and stomach content was identified following the methods detailed in in Chapter 2.

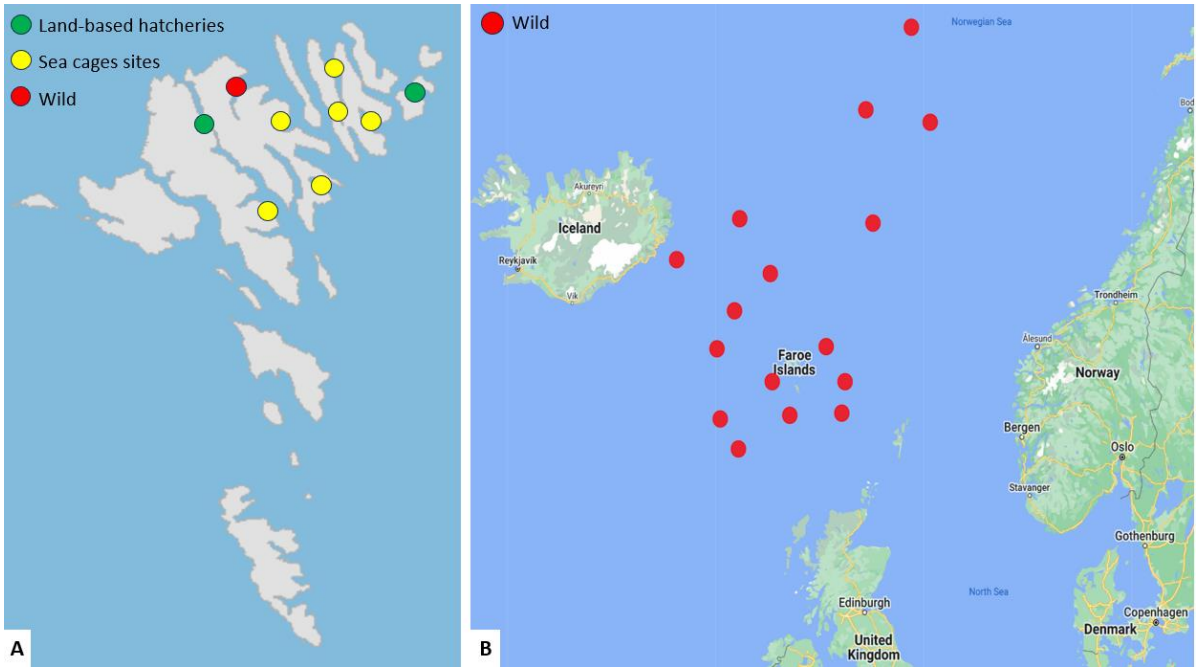


Figure 3.1. Lumpfish sampling sites across the Faroe Islands from October 2019 to August 2021. (A) Green circles show land-based hatcheries, yellow circles are marine Atlantic salmon sites, and red circles are wild fish locations; (B) Wild fish location from the annual pelagic research survey conducted by FAMRI (Faroe Islands).

3.3.2. Biochemical analyses

3.3.2.1. Proximate analysis

Each fish was left to thaw gradually on ice at room temperature, cut into small pieces and homogenised in a blender (Robot coupe Blixer® 4V.V, France) to obtain a homogenous paste. A sample of the homogenised paste was placed in a plastic pot with a lid, the wet weight recorded and subsequently frozen at -20°C. Frozen pots were dried using a freeze dryer at -60 °C (Alpha 1-4 LSC, Christ, Germany) to obtain the moisture content of the fish. Due to the high presence of skin and bones in the paste, the freeze-dried fish were also ground (KnifetecTM 1095, Foss, Sweden) into a fine powder to better homogenise the samples prior to further nutritional analyses. Part of this powder was then weighed and burned in the muffle furnace (Carbolite Elf 11/14B, UK) at 600 °C overnight to determine the ash content (AOAC, 2000).

Protein was measured from the fine powder by determining nitrogen content using an automated Kjeldahl analysis (Opsis LiquidLINE KjelROC Analyzer and Opsis LiquidLINE KjelROC Sampler KD-525, Sweden). Samples were digested using sulphuric acid and distilled (KjelROC analyser, Sweden). During the distillation, the nitrogen content of the sample was measured through titration. The protein content is measured by multiplying the nitrogen content by the conversion factor 6.25. This considers the fact that protein consists of 16% nitrogen and all nitrogen present in food protein is bound within the protein structure.

Part of the powder was also used to extract total lipids, following the method by Folch et al. (1957), using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK) and 20 ml of 2:1 chloroform-methanol as solvent system. Total lipids were gravimetrically calculated. Each fish was analysed for proximate composition in duplicate.

Feeds were ground (KnifetecTM 1095, Foss, Sweden) and analysed for proximate analysis according to standard procedures (AOAC, 2000). Moisture of feeds was determined by oven drying the samples at 103 °C overnight, while ash was measured after burning the ground pellets at 600 °C overnight in the muffle furnace (Carbolite Elf 11/14B, UK). Protein was determined using the automated Kjeldahl analysis as mentioned above. Total lipids of feeds were extracted with the Folch method (Folch et al., 1957), using 36 ml of 2:1 chloroform-methanol for the extraction. Total lipids of whole fish were stored in 2:1 + BHT at -20°C prior to fatty acid analysis. The results of proximate analysis are presented on wet basis. Gross energy was calculated by applying the respective energy conversion factors for protein, fat, and carbohydrates (5.65 kcal/g, 9.45 kcal/g, and 4.2 kcal/g, respectively) to their proportions in the sample. The calculation involved multiplying the percentage composition of each macronutrient by its corresponding energy conversion factor and summing the results (Henken et al., 1986). A more detailed description about the methods used for proximate analysis and lipid extraction can be found in Chapter 2.

3.3.2.2. Fatty acid methyl esters (FAMEs)

The fatty acid profile of whole fish was characterized by analysis of FAME using gas chromatography as described by Christie (2003). The analysis was performed on the previous total lipids extracted using the Folch method (1957). The FAME was prepared by transmethylation at 50 °C for 16 hours, and their extraction and purification was carried out as described in Tocher and Harvie (1988). FAME were separated and quantified by gas–liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy). The GC was 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 (FID). The carrier gas used was hydrogen. The oven thermal gradient was from 50 °C to 150 °C at 40 °C/minute to a final temperature of 230 °C at 2 °C/minute. Data were processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). Individual FAME was identified by comparing the samples profile to known standards (Supelco™ 37-FAME mix; Sigma-Aldrich Ltd., Poole, UK) and published data (Tocher & Harvie, 1988). Heptadecanoic acid (17:0) at a concentration of 10 mg/ml was used as internal standard to calculate fatty acid content per g of sample.

3.3.2.3. Amino acid profile

The amino acid profile of whole fish was determined using the Waters ACCQ-TAGTM Ultra Method for hydrolysate amino acid analysis (Waters Corporation, Milford, Massachusetts, USA) as described in Glencross et al. (2021). A 250 mg sample of freeze-dried fine powder (as per the method mentioned earlier) was taken from each fish. Each sample was digested in 10 ml of phenolic acid (6M HCl) in a microwave digestion system (MARS 6 240/50, CEM, USA). The acid hydrolysis was carried out in duplicate at 150 °C and 190 °C. The digestion at 190 °C was carried out for the hydrolysis of the standard amino acids, while methionine and cysteine were hydrolysed at 150 °C. Tryptophan could not be determined with this method as the acid hydrolysis destroys it.

After digestion, the digested samples were diluted up to 250 ml with ultrapure water. Approximately 1 ml of diluted sample was filtered using a 0.45 μM hydrophilic syringe filter and stored in the fridge prior derivatisation. The derivatisation was performed according to the manufacturer instructions by using a Waters H-Class UPLC fitted with an ACQUITY BEH Phenyl 1.7 μ 2.1 X 100 mm UPLC column (Waters Ltd, Hertfordshire, UK). The calibration standard used was the Waters Amino Acid Hydrolysate Standard, and an isocratic solvent system containing AccQ-Tag Ultra Reagent Diluent (Eluent A) and Ultra Reagent Buffer (Eluent B) was used at a flow rate of 1.2 ml/minute.

3.3.3. Statistical analyses

Percentage data such as moisture, ash, protein, lipid and fatty acids were transformed using the arcsine transformation from (Zar, 2014).

We performed linear models with macronutrients (moisture, ash, protein, lipid) and individual fatty acids or amino acids as the response variable. The categorical predictor was the origin of the fish which was divided in three groups (two groups from the farmed origin: sea cage and land-based hatcheries; one group from the wild); for example, lm(Lipid ~ Origin). The linear models were used to investigate whether there are significant differences between wild, sea cage and land-based lumpfish in terms of body composition, fatty acid, amino acid profiles and stomach content. Model diagnostics were carried out using visual inspection of model plots to examine the model fit. For this dataset, untransformed data were used for the linear models reported. In the case of amino acids, the data was moisture corrected. When the linear model showed an overall statistically significant result ($P < 0.05$), the F statistic with the degree of freedom and the P value were reported. A post hoc Tukey HSD test was then performed to identify differences between groups.

Principal Component Analysis (PCA) on macronutrients and fatty acid profile was performed using the "FactoMineR" package (Lê et al., 2008) to visualise and identify patterns regarding the differences between groups. The data for the PCA is mean-centered, subtracting the mean of each variable from the values. The outputs of the PCA were visualized using the "factoextra" package (Kassambara, 2016).

Linear regression models were used to examine the relationship between weight and lipid content, and between weight and protein content for different origins. The observations were grouped by origin and a separate linear regression model was fitted for each group, where weight was the independent variable and lipid, or protein content of the whole fish was the dependent variable. Results were reported using a scatter plot to visualise these relationships, including R^2 and p-values for each group.

A multinomial logistic regression was performed using the "vglm" function from the "VGAM" package (Yee, 2024) to investigate the relationship between lumpfish weight in the sea cages and stomach content. The stomach content, divided into categories using the main food found, was the response variable, and weight was used as a predictor.

Data were analysed using R (R Core Team, 2021), and figures plotted using "ggplot2" (Wickham et al., 2016). Excel data files were imported into R using the "readxl" package (Wickham & Bryan, 2019), which is part of the "tidyverse" collection of R packages (Wickham et al., 2019). Data in Tables are presented as mean ± standard deviation (SD).

3.4. Results

3.4.1. Feeds composition

The analysed feed composition for both lumpfish deployed in the sea cages and lumpfish reared in the hatcheries is reported in Table 3.1. The protein content varied between the diets, with diet D (hatchery) and diet B (sea cage) having the highest levels (54.3%), and diets A and C had similar levels of crude protein (D=B>A=C). Diets A, C and D had similar levels of crude fat of approximately 15% and only diet B had the highest level of crude fat (21.8%) (B>A=C=D). Ash content was similar in diet A and B (8.1-8.6 %), slightly higher in diet C (9.6%) and the highest in diet D (11.3%) (D>C>A=B). Moisture content was similar across the diets. Diet A and D had levels of 7.8-9 %, and diet C and B slightly lower levels (6.8-7.2 %) (A=D>B>C). Gross energy had similar values across the diets, with diet B being the highest (22.6 MJ/kg) (B>A=C=D).

Table 3.1. Proximate nutrient composition of lumpfish feeds. Lumpfish diet A and B were provided to lumpfish in the sea cages, while lumpfish diet C and D were used in the land-based hatcheries.

	Lumpfish feeds			
Site	*Sea cages*		*Hatcheries*	
Composition	**Diet A**	**Diet B**	**Diet C**	**Diet D**
Pellet size (mm)	2.2	3.0	1.5	4.5
Protein (%)	46.9	50.4	45.7	54.3
Carbohydrates (calculated %)[1]	21.3	12	22.8	11.6
Lipid (%)	15.8	21.8	15.1	15.0
Ash (%)	8.1	8.6	9.6	11.3
Moisture (%)	7.9	7.2	6.8	7.8
Astaxanthin (mg/kg)	34.3	28.7	20.0	27.2
Gross energy (MJ/kg)[2]	21.1	22.6	20.8	20.8

[1] Carbohydrates (%) = 100 – (Moisture (%) + Ash (%) + Protein (%) + Lipid (%))

[2] Gross energy (MJ/kg) = Protein × 5.65 + Lipid × 9.45 + Carbohydrates × 4.2

As lumpfish also have access to salmon feed while in the sea cages, the salmon feeds that were provided were also analysed and reported in Table 3.2. Salmon feed used in the study was generally more energy-dense, due to the higher lipid content (24-31%), and it contained higher levels of astaxanthin compared to lumpfish feeds. Protein levels were similar (40-49%). This is particularly relevant as lumpfish in sea cages have access to salmon feed, which could influence their overall nutritional status.

Salmon diet A had the highest levels of crude protein (49.2%) followed by diet B and F, while diet C, D and E had similar levels (approximately 40%) (A>B>F>C=D=E). The crude lipid was the highest in diet E, C and F (approximately 30%), slightly lower in diet B (27.4%) and the lowest in diet A (23.7%) (E=C=F>B>A=D). The ash content was the highest in diet A (9%), followed by diet B and F, and lower in diet C, D and E (approximately 6%) (A>B=F>C=D=E). Diet A, B and E had similar low moisture levels (6.2-5%), while diet D was slightly higher (7%), and C and F were the highest (8 and 9% respectively) (F>D>A=B=E). Gross energy in salmon feed was the highest in diet E (E>C>B=F>A=D).

Table 3.2. Proximate nutrient composition of salmon feeds. The salmon diets (A-F) were delivered to the sea cages where lumpfish were sampled from.

	Salmon feeds					
Composition	**Diet A**	**Diet B**	**Diet C**	**Diet D**	**Diet E**	**Diet F**
Pellet size (mm)	3	4-6	6-9	6	9	9
Protein (%)	49.2	46.4	40.7	39.6	40.1	44.1
Lipid (%)	23.7	27.4	30.6	23.9	31.4	29.8
Carbohydrates (calculated %)[1]	11.9	11.7	14.0	23.6	15.6	9.0
Ash (%)	9.0	8.0	6.6	5.9	6.5	7.9
Moisture (%)	6.2	6.5	8.1	7.0	6.4	9.2
Astaxanthin (mg/kg)	82.5	98.5	93.0	105.1	100.0	102.0
Gross energy (MJ/kg)[2]	23.1	23.9	24.2	23.0	24.6	23.8

[1] Carbohydrates (%) = 100 – (Moisture (%) + Ash (%) + Protein (%) + Lipid (%))

[2] Gross energy (MJ/kg) = Protein × 5.65 + Lipid × 9.45 + Carbohydrates × 4.2

3.4.2. Fish composition

The weight of farmed lumpfish ranged from 35 g to 1000 g, achieving weights ranging from approximately 500 g to 1000 g while deployed in sea cages, provided they remained in good health throughout the deployment. The PCA of lumpfish < 150 g (Figure 3.3) showed distinct clustering, with PC1 accounting for 45% of the variation mainly due to moisture (-0.91), protein (0.77) and lipid (0.62). PC2 accounted for 28% of the variation which was associated with ash (-0.71). The PCA plot indicates that lipid content contributes to both PC1 and PC2, suggesting that lipid plays a crucial role in differentiating between the origins along both axes. The length of the vectors for lipid, moisture and weight indicate a stronger influence of these variables on the variation among samples. The vector for moisture points in the opposite direction of lipid, indicating a strong negative correlation, whereas lipid content increases, moisture content decreases, and vice versa. The vectors for protein and ash suggest a weak positive correlation between lipid and protein, and a weak or no strong correlation of ash with the other variables. Weight points predominantly along PC2, having a strong influence on it.

Furthermore, a significant negative correlation was observed between moisture and protein content (Spearman's rho = -0.92, $P < 0.001$), as well as between moisture and lipid content (Spearman's rho = -0.71, $P < 0.001$), indicating strong inverse relationships where increases in moisture content are associated with decreases in both protein and lipid content.

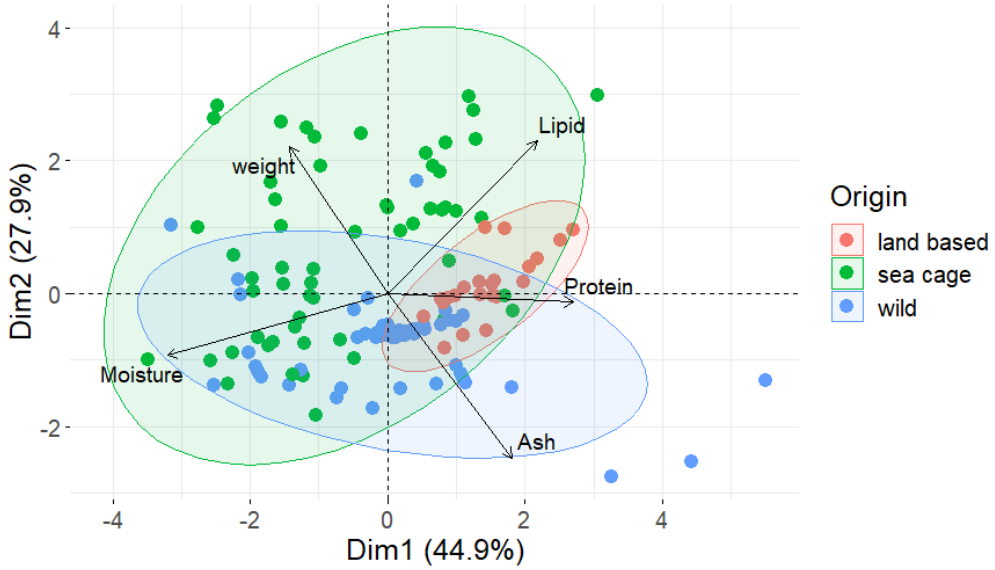


Figure 3.2. PCA biplot of lumpfish body composition (<150 g) showing separation of individuals depending on their origin: land-based hatchery (red, n=20), wild (blue, n=24) and sea cage (green, n=56). Vectors are the macronutrients and their relative influence on the dimensions of the PCA.

The body composition of lumpfish differed significantly among origins (wild, sea cage and land-based; Table 3.3). Wild and sea cage lumpfish had similar moisture levels (87.0 ± 2.5% and 86.7 ± 2.9%, respectively), which were significantly higher than those of land-based lumpfish (85.5 ± 1.2%; one-way ANOVA, $F_{5,237} = 6.52$, $P = 0.020$ for origin). here was also a significant effect of size ($P = 0.001$) and an interaction between origin and size ($P = 0.001$), indicating that moisture content was influenced by both predictors.

Land-based lumpfish had the highest protein content (8.9 ± 0.4%), significantly higher than the levels observed in wild (7.4 ± 1.3%) and sea-cage lumpfish (7.0 ± 2.0%; one-way ANOVA, $F_{5,178}=7.2$, $P < 0.001$ for origin). Protein content was not significantly influenced by size alone ($P = 0.177$), but the interaction between origin and size was significant ($P < 0.001$).

Significant differences were found regarding lipid content in terms of origin (one-way ANOVA, $F_{5,178}=24.5$, $P=0.015$). Sea-cage lumpfish had the highest lipid content (3.6 ± 2.4%), significantly higher than wild lumpfish (2.7 ± 2.1%). Land-based lumpfish had intermediate lipid levels (3.0 ± 0.7%). Lipid content was strongly influenced by size ($P < 0.001$) and by the interaction between origin and size ($P < 0.001$).

Land-based and wild lumpfish had similar ash content (1.8 ± 0.1% and 1.8 ± 0.4%, respectively), which were significantly higher than the ash content of lumpfish from sea cages (1.6 ± 0.2%; one-way ANOVA, $F_{5,178}=7.2$, $P < 0.001$ for origin). Ash content was also significantly affected by size ($P < 0.001$), but the interaction between origin and size was not significant ($P = 0.058$).

The lipid content of the whole fish was also analysed in relation to the predominant food type identified in the stomach and an effect of stomach content type was found (linear model, $F_{4,81} = 6.18$, $P < 0.001$, Figure 3.2). The highest average lipid content was found in lumpfish that had consumed salmon feed, indicating that this feed is higher in lipids (4.6 ± 1.8 %), resulting in higher lipid deposition. Lumpfish that had eaten lumpfish feed and unidentified pellet show similar levels of lipid content (3.2%), which are lower than those that consumed salmon feed (4.6%), but higher than the ones with an empty stomach (2.9%). The stomach content category that has the lowest lipid content (0.8%), is the fish that had mainly preys in the stomach, such as planktonic and benthic preys or sea lice alone.

Also, the stomach content of the fish, divided into categories, was analysed in relation to the fish size. Results from the multinomial logistic regression showed that as the weight of the fish increases, the log odds of the fish to have eaten salmon pellets ($P<0.001$) and lumpfish pellets ($P=0.02$) also increase. A tendency for the likelihood of the fish to have consumed prey was found, but it was not statistically significant ($P =0.083$).

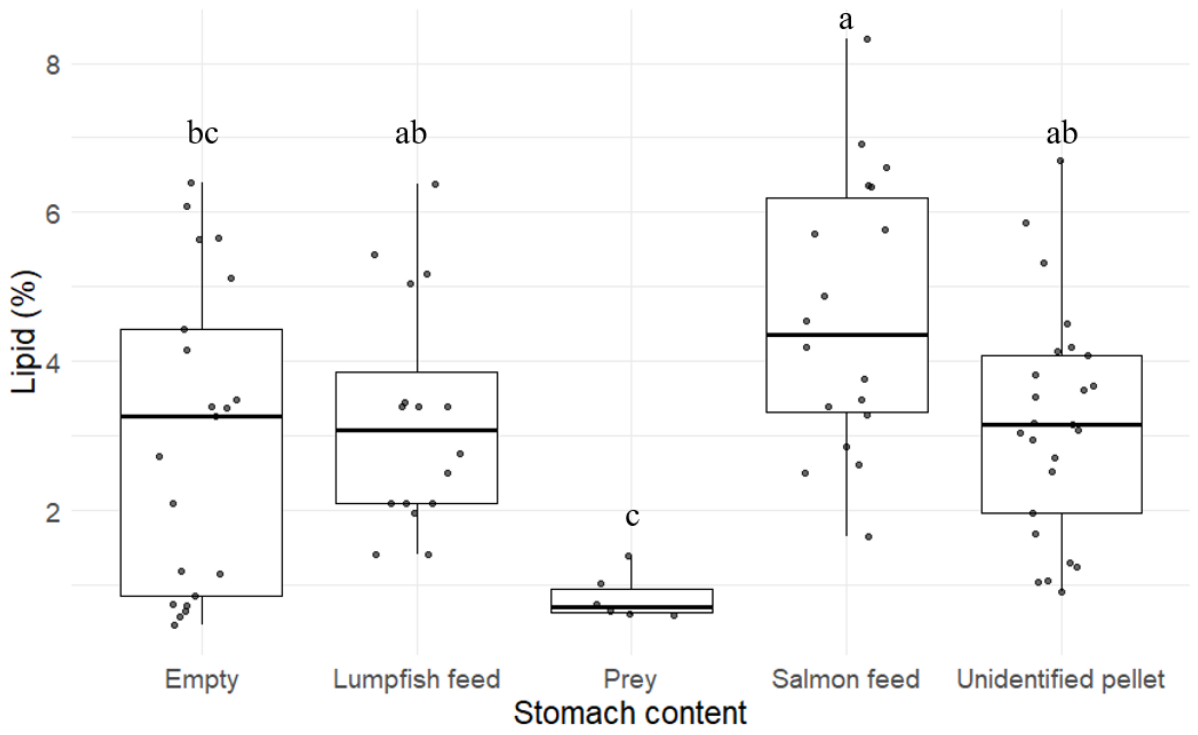


Figure 3.3. Lipid content (%) of whole lumpfish from the sea cages according to the stomach content divided according to five main categories (Empty stomach (n=47), lumpfish feed (n=101), prey (n=25), salmon feed (n=71), unidentified pellet (n=45)). The stomach content used was the predominant food item identified.

3.4.3. Fatty acid profile of whole fish

The fatty acid profile of whole fish < 150 g differed significantly among origins, as shown by the PCA in Figure 3.6. PC1 accounted for 71.4% of the variation which was due to oleic acid (OA) (-0.96), eicosapentaenoic acid (EPA) (0.94) and docosahexaenoic acid (DHA) (0.94), while PC2 accounted for 18.2% of the variation which was associated with SAFA (-0.68) and docosapentaenoic acid (DPA) (0.66).

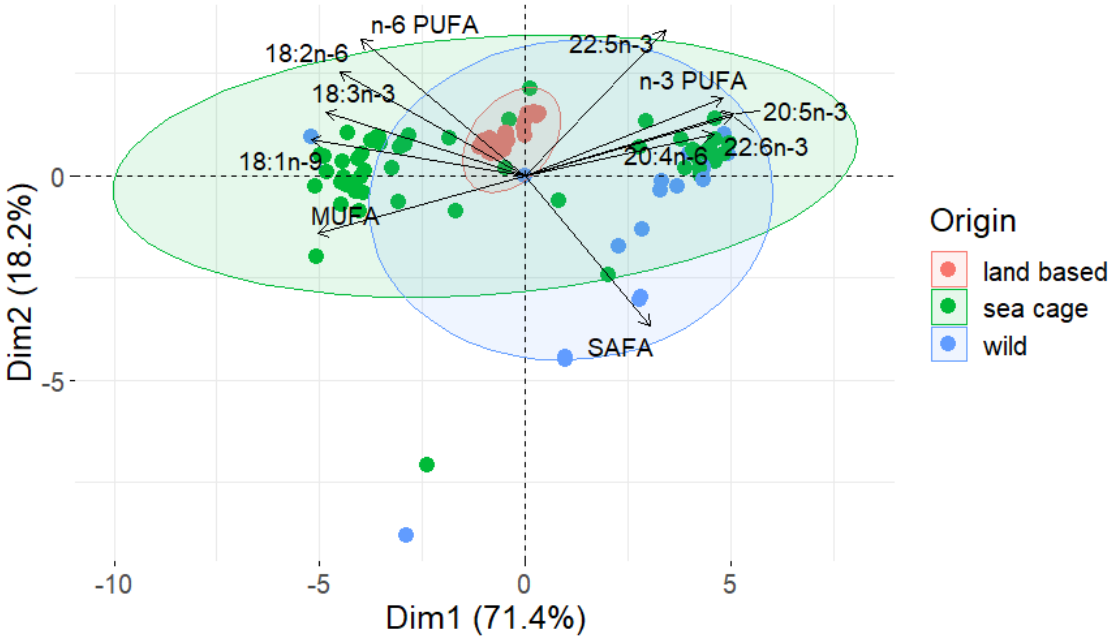


Figure 3.4. PCA biplot of fatty acid profile of whole lumpfish (<150 g) showing separation of individuals depending on the origin (land-based n=20, sea cage=59, wild n=25) and relative influence of different fatty acids.

The fatty acid composition varied significantly among whole lumpfish < 150 g from different origins (wild, sea cage and land-based; Table 3.3).

Total saturated fatty acids (SAFA) were highest in wild lumpfish (29.3 ± 5.0%), intermediate in land-based fish (24.4 ± 0.7%), and the lowest in sea cage fish (21.7 ± 5.2%; one-way ANOVA, $F_{5,177}$=17.4, P<0.001). Palmitic acid (16:0) was the predominant SAFA and showed significantly higher levels in wild lumpfish (17.8 ± 2.9%) compared to sea-cage (13.6 ± 2.4%) and land-based fish (16.2 ± 0.7%; one-way ANOVA, $F5_{,177}$=22.0, P<0.001).

Total MUFA was significantly higher in sea-cage fish (47.0 ± 9.5%) compared to wild (46.2 ± 15.1%) and land-based lumpfish (35.9 ± 2.3%; one-way ANOVA, $F_{5,177}$=22.5, P<0.001), due to the amount of OA (sea cage 27.8 ± 9.9%, wild 18.5 ± 6.5%, and land-based 22.8 ± 2%).

Land-based lumpfish exhibited the highest levels of n−6 PUFA (13.0 ± 0.9%) compared to sea-cage (10.8 ± 4.4%) and wild fish (3.5 ± 2.3%) (one-way ANOVA, $F5_{,177}$=41.2, P<0.001), mainly due to the high levels of linoleic acid (LA).

No significant differences were found in n-3 PUFA among origins. EPA was significantly higher in land-based fish (9.8 ± 0.8%), followed by wild (7.7 ± 5.7%) and sea cage (6.6 ± 4.0%). Absolute levels of EPA and DHA were the highest in land-based fish (one-way ANOVA, $F_{5,239}$= 28.4, P<0.001).

Table 3.3. Proximate nutrient composition and fatty acid profile of whole lumpfish from different origin (land-based, sea cages and wild). Crude lipid, crude protein and ash are reported on wet basis. Different superscript letters denote differences among the dietary groups according to one-way ANOVA and Tukey HSD test.

	Wild (n= 53)	Sea cage (n=110)	Land-based (n=20)	P (Origin)	P (Size)	P (Origin x Size)
Moisture (%)	87 ± 2.5^{b}	86.7 ± 2.9^{b}	85.5 ± 1.2^{a}	0.020	0.001	0.001
Protein (%)	7.4 ± 1.3^{b}	7.0 ± 2.0^{b}	8.9 ± 0.4^{a}	<0.001	0.177	<0.001
Lipid (%)	2.7 ± 2.1^{b}	3.6 ± 2.4^{a}	3 ± 0.7^{ab}	0.015	<0.001	<0.001
Ash (%)	1.8 ± 0.4^{a}	1.6 ± 0.2^{b}	1.8 ± 0.1^{a}	<0.001	<0.001	0.058
16:0	17.8 ± 2.9^{c}	13.6 ± 2.4^{b}	16.2 ± 0.7^{a}	<0.001	0.063	0.459
18:0	5.2 ± 1.3^{b}	3.7 ± 1.5^{a}	4 ± 0.4^{a}	<0.001	<0.001	0.131
∑SAFA[1]	29.3 ± 5.0^{c}	21.7 ± 5.2^{b}	24.4 ± 0.7^{a}	<0.001	0.898	0.680
16:1n-7	4.7 ± 1.7^{b}	3.9 ± 1.2^{a}	4.9 ± 1.7^{ab}	0.002	<0.001	<0.001
18:1n-9	18.5 ± 6.5^{b}	27.8 ± 9.9^{a}	22.8 ± 2^{a}	<0.001	0.009	0.620
18:1n-7	3.8 ± 0.8^{b}	3.9 ± 0.7^{b}	4.6 ± 0.1^{a}	<0.001	0.308	0.061
20:1n-9	7.3 ± 3.4^{c}	4.6 ± 2.6^{b}	1.2 ± 0.1^{a}	<0.001	<0.001	<0.001
22:1n-11	4.4 ± 2.8^{c}	2.8 ± 1.7^{b}	0.3 ± 0.2^{a}	<0.001	<0.001	<0.001
∑MUFA[2]	46.2 ± 15.1^{b}	47 ± 9.5^{b}	35.9 ± 2.3^{a}	<0.001	<0.001	0.002
18:2n-6	2.0 ± 2.0^{c}	9.3 ± 5.0^{b}	11.3 ± 0.6^{a}	<0.001	0.466	0.688
20:4n-6	0.9 ± 0.7	0.9 ± 1.0	0.9 ± 0.2	0.608	<0.001	<0.001
∑n-6 PUFA[3]	3.5 ± 2.3^{c}	10.8 ± 4.4^{b}	13 ± 0.9^{a}	<0.001	0.009	0.087
18:3n-3	0.8 ± 0.6^{b}	2.8 ± 1.7^{a}	2 ± 0.1^{a}	<0.001	0.314	0.994
18:4n-3	1.4 ± 0.9^{b}	1.4 ± 0.5^{b}	1.7 ± 0.2^{a}	0.013	<0.001	0.008
20:5n-3	7.7 ± 5.7^{b}	6.6 ± 4.0^{b}	9.8 ± 0.8^{a}	0.001	<0.001	0.002
22:5n-3	0.6 ± 0.4^{c}	0.7 ± 0.4^{b}	1.2 ± 0.1^{a}	<0.001	<0.001	0.003
22:6n-3	9.2 ± 8.0	8.1 ± 6.0	9.8 ± 1.3	0.158	<0.001	0.078
∑n-3 PUFA[4]	20.4 ± 14.9	20.2 ± 10.0	24.5 ± 3.3	0.070	<0.001	0.022
∑PUFA	24.5 ± 16.3^{c}	30.9 ± 10.4^{b}	38.6 ± 3.8^{a}	<0.001	<0.001	<0.001

∑LC-PUFA[5]	17.5 ± 13.8[ab]	15.3 ± 10.2[b]	20.8 ± 2[a]	0.026	<0.001	0.023
EPA/DHA	1.0 ± 0.2	0.9 ± 0.2	1 ± 0.1	0.356	0.182	0.780
EPA+DHA	16.9 ± 13.5[ab]	14.7 ± 9.9[b]	19.6 ± 2[a]	0.033	<0.001	0.026
EPA (g/100g)	0.02 ± 0.04[c]	0.1 ± 0.1[b]	0.2 ± 0.1[a]	<0.001	0.024	0.020
DPA (g/100g)	0.001± 0.003[c]	0.01 ± 0.02[b]	0 .02± 0.01[a]	<0.001	0.144	0.021
DHA (g/100g)	0.02 ± 0.04[c]	0.1 ± 0.2[b]	0.2 ± 0.1[a]	<0.001	0.066	<0.001
EPA+DHA (g/100g)	0.04 ± 0.1[c]	0.2 ± 0.3[b]	0.3 ± 0.2[a]	<0.001	0.042	0.002
EPA+DPA+DHA (g/100g)	0.04 ± 0.1[c]	0.2 ± 0.3[b]	0.3 ± 0.2[a]	<0.001	0.045	0.002

[1] includes 15:0, 20:0, 22:0, 24:0; [2] includes 16:1n-9, 20:1n-11, 20:1n-7, 22:1n-9, 24:1n-9; [3] includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6, 22:5n-6; [4] includes 20:3n-3, 20:4n-3, 21:5n-3

The body composition of lumpfish varied significantly across seasons (summer, autumn, and winter; Table 3.4). Moisture content was highest in summer (88.6 ± 1.9%) and significantly lower in autumn (85.5 ± 2.9%) and winter (86.2 ± 2.6%) (one-way ANOVA, $F_{5,104} = 13.54$, $P < 0.001$ for season).

Ash content was significantly affected by season, with the highest levels observed in winter (1.7 ± 0.1%) compared to summer (1.7 ± 0.2%) and autumn (1.5 ± 0.2%) (one-way ANOVA, $F_{5,104} = 11.71$, $P < 0.001$ for season). Protein content varied significantly across seasons with the highest levels observed in winter (7.8 ± 1.5%) and autumn (7.6 ± 1.1%), while summer had the lowest values (5.6 ± 2.6%) (one-way ANOVA, $F_{5,104} = 14.25$, $P < 0.001$ for season). Size also had a significant effect ($P = 0.002$), and a significant interaction between season and size was detected ($P < 0.001$). Lipid content did not significantly differ among seasons (one-way ANOVA, $F_{5,104} = 19.95$, $P = 0.370$).

Fatty acid composition showed significant seasonal variations. SAFA were highest in summer (27.3 ± 4.6%), followed by autumn (19.2 ± 3.7%) and lowest in winter (19.3 ± 2.0%) (one-way ANOVA, $F_{5,104} = 48.0$, $P < 0.001$). MUFA were significantly higher in winter (50.5 ± 3.6%) compared to autumn (47.9 ± 8.1%) and summer (42.3 ± 12.9%) (one-way ANOVA, $F_{5,104} = 17.8$, $P < 0.001$).

N-6 PUFA were significantly higher in autumn (13.21 ± 1.87%) and winter (13.65 ± 1.64%) than in summer (5.06 ± 2.62%) (one-way ANOVA, $F_{5,104} = 85.81$, $P < 0.001$). N-3 PUFA were highest in summer (24.5 ± 15.24%) and decreased in autumn (19.98 ± 6.15%) and winter (18.95 ± 3.66%) (one-way ANOVA, $F_{5,104} = 18.5$, $P < 0.001$). A similar trend was also observed in LC-PUFA (one-way ANOVA, $F_{5,104} = 23.89$, $P < 0.001$).

EPA + DHA was significantly higher in summer (21 ± 14.1%) compared to autumn (13.79 ± 5.7 %) and winter (9.27 ± 3.3%) (one-way ANOVA, $F_{5,104} = 24.1$, $P < 0.001$). Absolute levels of EPA levels were lowest in summer 0.03 ± 0.02 g/100g) compared to autumn and winter (0.12± 0.11 g/100g, 0.12± 0.16 g/100g, respectively (one-way ANOVA, $F_{5,105} = 6.85$, $P = 0.013$). DHA followed a similar trend, being significantly higher in autumn (0.18 ± 0.22 g/100g) and winter (0.12 ± 0.19 g/100g), and lower in summer (0.03 ± 0.02 g/100g) (one-way ANOVA, $F_{5,105} = 15.89$, $P < 0.001$).

Table 3.4. Proximate nutrient composition and fatty acid profile of whole lumpfish from sea cages sampled during different seasons (summer, autumn and winter). Lipid, protein and ash are reported on wet basis. Different superscript letters denote differences among the seasons according to one-way ANOVA and Tukey HSD test.

	Summer	Autumn	Winter	P (Season)	P (Size)	P-value (Season x Size)
Moisture (%)	88.6 ± 1.9^{b}	85.5 ± 2.9^{a}	86.3 ± 2.6^{a}	<0.001	<0.001	0.141
Ash (%)	1.7 ± 0.2^{a}	1.5 ± 0.2^{b}	1.7 ± 0.2^{a}	<0.001	<0.001	0.426
Protein (%)	5.6 ± 2.6^{b}	7.6 ± 1.13^{a}	7.8 ± 1.5^{a}	<0.001	0.002	<0.001
Lipid (%)	3.7 ± 3.5	3.8 ± 1.9	3.2 ± 1.5	0.370	<0.001	0.476
∑SAFA[1]	27.3 ± 4.6^{b}	19.2 ± 3.7^{a}	19.3 ± 1.9^{a}	<0.001	0.828	<0.001
18:1n-9	15.9 ± 5.1^{b}	31.7 ± 7.2^{c}	34.9 ± 3.9^{a}	<0.001	<0.001	0.105
∑MUFA[2]	42.3 ± 12.9^{b}	47.9 ± 8.1^{a}	50.5 ± 3.6^{a}	<0.001	<0.001	0.070
18:2n-6	2.7 ± 2.4^{b}	12.1 ± 2.1^{a}	12.7 ± 1.9^{a}	<0.001	0.017	0.068
∑n-6 PUFA[3]	5.1 ± 2.6^{b}	13.2 ± 1.9^{a}	13.7 ± 1.6^{a}	<0.001	0.601	0.002
18:3n-3	0.8 ± 0.7^{b}	3.2 ± 1.3^{c}	4.1 ± 0.9^{a}	<0.001	<0.001	<0.001
20:5n-3	9.2 ± 5.6^{b}	6.0 ± 2.6^{a}	4.6 ± 1.6^{a}	<0.001	<0.001	0.006
22:6n-3	11.8 ± 8.6^{b}	7.8 ± 3.6^{a}	4.5 ± 1.7^{a}	<0.001	<0.001	<0.001
∑n-3 PUFA[4]	24.5 ± 15.2^{b}	19.9 ± 6.2^{a}	15.9 ± 3.7^{a}	<0.001	<0.001	<0.001
∑LC-PUFA	21.8 ± 14.6^{b}	14.6 ± 5.9^{c}	9.5 ± 3.4^{a}	<0.001	<0.001	<0.001
EPA+DHA	21 ± 14.1^{b}	13.8 ± 5.7^{c}	9.1 ± 3.3^{a}	<0.001	<0.001	<0.001
EPA (g/100g)	0.03 ± 0.02^{b}	0.12 ± 0.11^{a}	0.12 ± 0.16^{a}	<0.001	0.0130	0.008
DHA (g/100g)	0.03 ± 0.02^{a}	0.18 ± 0.22^{b}	0.12 ± 0.19ab	<0.001	<0.001	<0.001

[1]includes 14:0. 15:0, 16:0, 18:0, 20:0, 22:0, 24:0;

[2] includes 16:1n-7**,** 16:1n-9,18:1n-9, 18:1n-7, 20:1n-9, 20:1n-11, 20:1n-7, 22:1n-9, 22:1n-11, 24:1n-9;

[3] includes 18:3n-6, 20:2n-6, 20:4n-6, 20:3n-6, 22:4n-6, 22:5n-6;

[4] includes 18:4n-3, 22:5n-3, 20:3n-3, 20:4n-3, 21:5n-3.

3.4.4. Amino acid profile of whole fish

The amino acid profile was measured in lumpfish < 50 g, and it is reported in Table 3.5. The amino acid composition varied between whole lumpfish from different origins (wild and land-based). Total amino acid content was higher in land-based fish (6.49 g/100 g) compared to wild fish (5.92 g/100 g) (one-way ANOVA, $F_{5,557}$=17.46, P<0.001). Methionine was significantly higher in land-based fish (0.27 ± 0.03 g/100 g) than in wild fish (0.23 ± 0.05 g/100 g, one-way ANOVA, F5,25=4.63, P=0.031). Cysteine also showed significantly higher levels in land-based fish (0.15 ± 0.05 g/100 g) compared to wild fish (0.08 ± 0.02 g/100 g; one-way ANOVA, $F_{3,17}$=11.14, P<0.001). Phenylalanine and tyrosine exhibited a trend towards higher concentrations in land-based fish (one-way ANOVA, $F_{3,17}$=3.17, P=0.058 and $F_{3,17}$=4.15, P=0.059, respectively). No significant differences were observed in the other amino acids analysed.

Table 3.5. Amino acid profile (g/100g of sample) of lumpfish < 50 g on wet basis according to different origins (land-based hatcheries, wild). P values are from linear models using moisture corrected amino acids.

	< 50 g		
Amino acid (g/100 g)	**Land-based**	**Wild**	**P**
Essential amino acids	**n=7**	**n=8**	
Histidine (His)	0.15±0.02	0.13±0.03	0.103
Threonine (Thr)	0.28±0.04	0.24±0.06	0.125
Valine (Val)	0.31±0.08	0.31±0.04	0.991
Isoleucine (Ile)	0.25±0.07	0.25±0.03	0.941
Leucine (Leu)	0.46±0.10	0.43±0.06	0.419
Lysine (Lys)	0.41±0.12	0.41±0.06	0.947
Methionine (Met)	0.27±0.03	0.23±0.05	0.031
Phenylalanine (Phe)	0.29±0.04	0.25±0.04	0.058
Conditionally essential amino acids			
Tyrosine (Tyr)	0.23±0.02	0.20±0.04	0.059
Glycine (Gly)	0.69±0.16	0.61±0.14	0.253
Arginine (Arg)	0.49±0.07	0.43±0.08	0.089
Proline (Pro)	0.37±0.07	0.36±0.03	0.533
Non-essential amino acids			
Serine (Ser)	0.34±0.06	0.27±0.10	0.105
Alanine (Ala)	0.40±0.09	0.36±0.05	0.258
Cysteine (Cys)	0.09±0.05	0.02±0.01	<0.001
Aspartic acid (Asp)	0.54±0.13	0.52±0.08	0.727
Glutamic acid (Glu)	0.80±0.19	0.82±0.12	0.762
Taurine (Tau)	0.12±0.07	0.09±0.06	0.294
Total AA	6.49	5.92	
Total crude protein	8.9	7.4	

3.5. Discussion

Understanding dietary influences on lumpfish body composition is essential to optimise husbandry practices. The differences between farmed and wild fish in terms of lipid content reflect the impact of high-energy diets, feeding regimes and controlled environments in farming conditions. The fatty acid profile varied distinctly between farmed and wild fish, with diet and size class playing a crucial role in these differences. A seasonal effect also affected the body composition and fatty acid profile of lumpfish in the sea cages.

While the essential amino acid levels were generally within acceptable ranges for both farmed and wild lumpfish, suggesting adequacy of the hatchery diets, there were few differences in certain amino acids. These differences suggest variations in dietary intake or metabolism, though specific requirements are not yet known. These findings underscore the importance of further research into species-specific dietary needs to enhance nutrition and improve welfare in aquaculture systems.

3.5.1. Whole fish composition

Results obtained (Figure 3.2) suggest a strong differentiation between wild and farmed lumpfish based on their body composition. Wild fish are more distinct, characterised by higher moisture and lower lipid levels, while sea cage fish have the highest lipid content and variability. Lipid content was affected both by origin, size and interaction of these predictors, suggesting the influence of these.

This suggests that factors such as diet composition and environmental conditions determine lipid deposition in lumpfish. The interaction between size and origin implies that the effect of farming conditions on lipid content may be more pronounced at certain life stages.

Similar patterns of increased lipid content in farmed versus wild populations have been reported in yellow perch (*Perca flavescens*), sea bream (*Sparus aurata*) and salmon (González et al., 2006; Grigorakis, 2007; Henriques et al., 2014), where farmed fish accumulate higher lipid reserves due to energy-rich diets and controlled feeding regimens. Factors influencing lipid content in fish can be complex and impacted by diet, environmental conditions, and genetics (NRC, 2011). Environmental conditions, such as water temperature and availability of prey, contribute by affecting metabolic rates and energy expenditure. Additionally, genetic factors can determine an individual predisposition for lipid storage (NRC, 2011). In controlled farming environments, lumpfish receive a consistent and nutrient-rich diet that encourage them to reach their maximum lipid content earlier. In contrast, wild lumpfish experience seasonal variations in food availability. As juveniles, they remain in coastal habitats where food resources may be less energy-dense, delaying lipid accumulation. Only after transitioning to a pelagic lifestyle, they can access larger and more energy-rich prey, do wild lumpfish reach comparable lipid reserves (Cox & Anderson, 1922).Capturing wild lumpfish of the target size for this study proved challenging due to the offshore semi-pelagic nature of this species (Cox & Anderson, 1922). Most of the wild fish analysed were caught in summer, when there is an abundance of

zooplankton and this is reflected in the lipid content of the fish (Eliasen et al., 2018; Wang & Jeffs, 2014). Future studies ought to focus on access to wild fish year-round, however difficult that may be and more directly compare wild and sea cage-based fish season by season.

The protein content of whole fish differed among origins. However, weight itself was not a significant predictor of protein levels. Shearer et al. (1994) found that in salmonids, the protein content of the body initially increases as the fish grows, but eventually plateaus. This plateau in protein content suggests that while early growth requires high protein deposition, later stages shift towards lipid accumulation as an energy reserve. In lumpfish, this pattern may be less pronounced due to species-specific growth and metabolic rates, but the overall trend aligns with findings in other fish species (Shearer et al., 1994). Literature more generally indicates that protein content in fish is highly dependent on endogenous factors such as fish size and species, which results in relative protein content mostly being influenced by the relative increase or decrease of other contents such as lipids and moisture (Glencross et al., 2011; Shearer et al., 1994). As lipid content in farmed lumpfish is highly variable and depends on whether the fish were in tanks with very low energy requirements or whether the fish had access to the highly energetic salmon feed, the more stable protein content is proportionally affected by the lipid content and potentially also the moisture content.

When fish undergo changes in body condition, these changes will result mainly in changes regarding the composition of the body, particularly in lipids and moisture (Jobling, 2001). Changes where an increase in percent lipid results in a subsequently decrease in moisture are more substantial than protein levels changes (Shearer, 1994).

The lumpfish in our study had a high proportion of moisture in their bodies (approximately 85-90%), which is comparable to other studies on lumpfish (Ageeva et al., 2021; Hamre et al., 2022) and much higher than other farmed marine fish such as salmon and ballan wrasse (Betancor et al., 2016; Cavrois-Rogacki et al., 2022). The high water content may be related to their gelatinous body structure and low muscular density, which differs from the flesh composition seen in other commercially farmed fish species. Changes in one component in body composition will affect the relative contents of other components.

Wild fish used in this study were mainly caught in summer and showed a consistent body composition in terms of macronutrients and micronutrients. On the other hand, lumpfish from the sea cages were caught from six farming sites at different seasons and showed a high variability in weight and body composition (Table 3.4). The results regarding the body composition of lumpfish from sea-cages also agree with the results by Ageeva et al. (2021), and is highly influenced by life stage, sex, body mass, feeding regime and season (Davenport & Kjørsvik, 1986; Gélineau et al., 2001; Huntingford et al., 2006; Jobling, 2001).

3.5.2. Stomach content and feeds

Lumpfish have a more variable diet in the sea cages as they have access to a wide range of food, from lumpfish to salmon feed, sea lice, zooplankton, and biofouling (Eliasen et al., 2018). This differs from wild lumpfish, whose diet is more strictly influenced by natural prey availability. Lumpfish from sea cages were also sampled at different seasons, so their access to live prey will have been more variable due to seasonality than that of the wild fish, caught only in one season. This variation in food accessibility may have contributed to the higher variability observed in body composition among farmed lumpfish compared to their wild counterparts. Moreover, medium to big lumpfish more often have salmon pellets in their stomachs, as they are bigger in size and contain higher energy levels compared to the smaller lumpfish feed and zooplankton (Imsland et al., 2015). This dietary shift suggests that lumpfish pellets used in the sea cages may be adequate at the early deployment, but when lumpfish reach bigger sizes the pellets may not have the adequate size. If the pellet size is too small or lacks sufficient energy levels, larger lumpfish may preferentially consume salmon feed, which could lead to higher lipid accumulation, and overall altered body composition.

The feeds that were analysed in this study showed that salmon feeds contain considerably higher lipid levels and marginally lower protein content compared to lumpfish feed. In general, salmon feeds are higher in energy compared to lumpfish feeds and this is due to the differences in metabolic needs of these species where salmon require a lipid rich diet to support rapid growth and muscle development. Lumpfish that had the salmon feed as the main food item in the stomach had also a significantly higher lipid content in their body compared to those containing lumpfish feed and the other food categories analysed (Figure 3.3). This aligns with the higher energy density of salmon feed, which promotes lipid accumulation more effectively than lumpfish feed or live prey. In particular, the fish that consumed only zooplankton as the main food item had the lowest lipid content, reflecting the lower caloric content of these prey. This pattern shows how diet directly affects fat storage in lumpfish, reinforcing the need for optimised feed formulation for lumpfish and cleaner fish.

3.5.3. Fatty acid

In terms of fatty acid profile, it was found to differ between origin and size of fish in several ways, reflecting differences in dietary intake, metabolism and environmental conditions. Wild fish contained more SAFA than farmed, particularly due to the high amount of palmitic acid (17.8% wild, 13.6% sea cage, 16.2% land-based). This corresponds well with the literature, where similar levels have been found in farmed lumpfish (13-16%) (Ageeva et al., 2021). Palmitic acid is a major SAFA which is commonly present in different seafood species and plays an essential role as energy source and a component of cell membrane structure. It is primarily obtained from dietary sources, including plankton, algae, and other marine organisms. In farmed fish, aquafeeds contain fish oil, vegetable oils or marine-derived ingredients rich in this fatty acid. In contrast, wild fish obtain palmitic acid primarily

through natural prey, such as zooplankton and small invertebrates (Durmuş, 2019). MUFA levels were higher in wild fish and sea cage and lower in the land-based fish. The predominant MUFA in our study, OA, which serves as a source of energy, was higher in farmed lumpfish (both land-based and sea cage) than in wild ones, suggesting dietary influence from commercial aquafeeds, particularly those containing rapeseed oil (RO), high in oleic acid and often included in aquafeeds. While OA is naturally present in marine prey (Auel et al., 2002), the higher levels in farmed lumpfish potentially indicate access to salmon feed and lumpfish feed (Miller et al., 2008). As with OA, LA and ALA were higher in farmed fish as these fatty acids are predominately found in vegetable oils used in aquafeeds (Fernandez-Jover et al., 2011; Tvrzicka et al., 2011). Similar trends lead to differences in the content of these fatty acids between wild and farmed fish in other species like brown trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) (Antão-Geraldes et al., 2018; Yeganeh et al., 2012). This is also reflected in our data, where farmed lumpfish contained more of these fatty acid than wild.

Long-chain polyunsaturated fatty acids (n-3 LC-PUFA) such as EPA and DHA play essential roles in fish growth, metabolism, and cellular function (NRC, 2011).

N-3 PUFA were significantly higher in the lumpfish from the land-based hatcheries, reflecting the higher n-3 PUFA content in formulated aquafeeds, which are often enriched with fish oil or alternative lipid sources to maintain optimal fatty acid composition. In other species higher levels in wild fish have been seen like salmon (Henriques et al., 2014), sea bass (*Dicentrarchus labrax*) (Lenas et al., 2011), and meagre (*Argyrosomus regius*) (Saavedra et al., 2017).

Wild lumpfish might have a varied diet rich in marine organisms that are high in n-3 LC-PUFA, especially when they are younger and more active in foraging. As wild fish grow larger, their diet might change, either due to a shift in prey availability, seasonality or a change in dietary preference. Farmed fish, on the other hand, are typically fed formulated feeds that can have high levels of n-3 PUFA depending on the composition of the feed.

As fish grow, their nutritional requirements and efficiencies in converting these fatty acids change (NRC, 2011). As lumpfish grow both in farmed and natural environments, they have higher fat storage, accumulating more SAFA and MUFA relative to PUFA. When fish have lower fat storage, SAFA and MUFA are low, and there is selective retention of PUFA (Bandara et al., 2023). Larger fish might have a slower metabolism and different energy requirements compared to smaller growing individuals and this can also affect their fatty acid profile. To further understand this relationship, absolute values of EPA, DPA and DHA (g/100g) were investigated rather than just expressing them as relative percentages. Wild lumpfish had the lowest absolute EPA+DHA levels despite a higher percentage relative to total lipids, suggesting selective retention of PUFA when lipid content is low. Sea-cage lumpfish had intermediate absolute EPA+DHA levels, reflecting their exposure to both formulated feeds and natural prey. Land-based lumpfish had the highest absolute EPA+DHA values, consistent with the higher lipid content and the n-3 PUFA content in their feeds. Additionally, DPA, intermediate in the metabolic conversion of EPA to DHA, followed a similar trend, highlighting the differences in diet composition,

lipid metabolism, and growth stage on fatty acid deposition in lumpfish. While the fatty acid profile of fish reflects the fatty acid profile of the dietary intake (Betancor et al., 2014; Sharma et al., 2010; Willora et al., 2021), different environmental conditions including water temperature, salinity, and stress levels, may influence lipid metabolism. Wild fish experience natural fluctuations in food availability and energy expenditure, which could lead to differences in fatty acid mobilisation and deposition compared to farmed fish, which live in more controlled conditions. Previous studies showed that environmental conditions and stressors can alter fatty acid metabolism and composition, with colder water temperatures favouring higher PUFA retention in membranes (Henderson & Tocher, 1987). Although environmental fluctuations were not investigated, this could partially explain some variations observed between sea cages and land-based lumpfish.

The higher n-6 PUFA content in farmed lumpfish suggests a strong influence from vegetable feed ingredients, which may affect their overall lipid metabolism and fatty acid balance. While n-6 PUFA play a role in cellular function and energy storage, an excessive n-6 to n-3 PUFA ratio can have physiological consequences, potentially affecting immune function, inflammatory and stress responses, and overall fish health (Hundal et al., 2021). In aquaculture, maintaining an optimal n-6/n-3 balance is critical, as elevated n-6 PUFA levels at the expense of n-3 PUFA may influence stress resilience, growth efficiency, and the effectiveness of lumpfish as cleaner fish.

3.5.4. Amino acid profile

In our study few differences in the amino acid profiles between farmed and wild lumpfish were found. The total amino acid content was slightly higher in farmed fish (6.49 g/100 g) than in wild fish (5.92 g/100 g). One possible explanation for the observed differences is that not all amino acids were quantified, as tryptophan is degraded during acid hydrolysis. Additionally, the protein content is estimated using the Kjeldahl method which applies the conversion factor of 6.25 for fish, which may overestimate the actual protein content. This could result in a lower apparent amino acid sum relative to the total protein estimate. Furthermore, nitrogen in fish muscle is not solely derived from amino acids; other nitrogenous compounds, such as nucleotides, ammonia, urea, free amino acids, and peptides, contribute to the total nitrogen content, which may explain the discrepancy (NRC, 2011).

The amino acid profile in fish is influenced by multiple factors including species, dietary intake, environmental conditions and physiological processes. Among these factors, diet plays an important role in providing the type and quantity of amino acids available to the fish for metabolism, growth and development (Li et al., 2021). The values of essential amino acids reported by Ageeva et al. (2021) were closer to the essential amino acids of wild and farmed fish analysed in this study. This similarity suggests that the diets provided in the hatcheries were well balanced in terms of essential amino acids composition, likely meeting the nutritional requirements of lumpfish.

Methionine is a sulphur-containing essential amino acid that plays a vital role in protein synthesis, methylation reactions, and as a precursor for cysteine and taurine synthesis (Townsend et al., 2004). Deficiencies in methionine can lead to adverse physiological effects in fish, including reduced growth performance, impaired immune function, and can lead to cataract development in lumpfish (Jonassen et al., 2017). In the sampled fish, farmed lumpfish had significantly lower methionine levels compared to their wild counterparts (0.23% vs. 0.27%, $P = 0.031$). Although no cataracts were observed in the farmed fish sampled, this finding suggests that methionine levels in farmed fish might be slightly suboptimal.

Previous studies have suggested that optimal methionine levels are crucial for fish metabolic health, as both deficiencies and excesses can have adverse effects, particularly in species that rely on dietary sources due to limited endogenous synthesis. The replacement of animal protein with vegetable-based alternatives in aquafeeds affects the requirements of sulphur-containing amino acids that are low or absent in plant proteins (Andersen et al., 2016).

Another key finding was the significantly lower levels of cysteine in farmed fish compared to wild fish (0.02% vs. 0.09%, $P < 0.001$). Cysteine, though classified as a non-essential amino acid, it serves as a precursor for glutathione, a key antioxidant involved in oxidative stress regulation (Baker & Dilger, 2009). Since cysteine can be synthesised from methionine, the observed lower methionine could also contribute to lower cysteine availability. The lower cysteine levels in farmed lumpfish might indicate differences in diet composition, metabolic regulation, or environmental factors affecting sulphur amino acid metabolism. Wild fish typically consume a diverse range of natural prey items, which may provide a more complex and bioavailable amino acid profile. In contrast, aquafeeds are formulated to meet nutritional requirements, but may not fully replicate the diversity of amino acid sources available in the wild (Wilson, 2003).

Given the role of these amino acids in immune function and overall fish welfare, optimising dietary sulphur amino acid content could be an important consideration for improving feed formulations by increasing methionine supplementation in hatchery diets.

3.6. Conclusions

To the best of our knowledge, no other studies have looked at the body composition of wild lumpfish. Wild lumpfish serve as a benchmark for understanding the species' nutritional requirements, and the body composition can offer insights into welfare and optimal growth. This knowledge can provide a reference point for evaluating the status of farmed fish, and help identify gaps in current farming practices, such as nutritional deficiencies or suboptimal diets. Farmed lumpfish, especially those in hatcheries and sea cages, exhibited higher lipid content compared to their wild counterparts, suggesting higher lipid deposition due to the farming environment and the diet composition. This suggests that high energy dense diets are not recommended for lumpfish.

The fatty acid profile of wild fish reflects the fatty acid composition of their natural prey, while the farmed fish is influenced by the aquafeed composition and the availability of seasonal preys in the sea cages.

The study also highlights that the amino acid profile in lumpfish is influenced by diet and environmental conditions. Even though the essential amino acids had similar levels in both farmed and wild lumpfish, some differences were found in methionine and cysteine levels.

Based on these findings, tailored diets for farmed lumpfish that mimic the nutritional profile (e.g. optimal lipid levels) of wild lumpfish can be formulated. The production of farmed lumpfish with body composition similar to the wild ones might enhance the production of more robust fish. This is particularly relevant considering the short domestication history of lumpfish. If taking the wild lumpfish body composition as a benchmark, diets' formulations for juvenile lumpfish should contain 10-15 % of lipids.

Chapter 4. Liver colour scoring index and nutritional composition of livers from farmed and wild Faroese lumpfish (*Cyclopterus lumpus*) to understand nutritional requirements and as a proxy for health and welfare

4.1. Abstract

The liver is the central organ in lipid metabolism and storage as well as fatty acid metabolism, managing intake, synthesis, and elimination. This chapter examines the livers of lumpfish from two origins, farmed and wild. The study used fish from different environments: 60 lumpfish from land-based hatcheries (farmed), 334 from salmon sea cages (farmed), and 169 from wild populations in the Faroe Islands. Significant variations were found in lipid content, fatty acid profiles, lipid classes and carotenoid levels across these environments. Further examination of the liver fatty acid profiles reflected the influence of the feed composition. Farmed lumpfish, particularly from hatcheries, displayed higher total lipid and TAG in the liver than their wild counterparts, with dietary differences being a contributing factor. Astaxanthin levels, crucial for liver pigmentation, and indicative of fish welfare, were higher in wild and sea cage lumpfish. The liver colour was used as a welfare indicator of the nutritional status of the fish. Farmed fish had predominantly orange livers. However, darker liver colours were reported in some farmed fish, which were associated with poor nutrition, based on very low levels of total lipid, TAG, and histopathological analyses. Wild lumpfish had predominately bright orange livers presumably due to consuming astaxanthin-rich prey, while those in hatcheries had paler livers. This study reveals differences in liver composition of juvenile lumpfish in terms of lipids and carotenoids, crucial for enhancing feed formulations and welfare practices. These findings underscore the need for improved feed formulations, which includes lower lipid content, sufficient levels of TAG for energy reserves, and an adequate supply of carotenoids, especially astaxanthin. While liver colour was not quantitatively measured in this study, the visual scoring aligned with previously validated correlations between liver pigmentation and astaxanthin levels, supporting its use as a proxy for nutritional status

.**Keywords:** liver, lipids, fatty acids, astaxanthin, fish welfare

4.2. Introduction

Lumpfish are widely used as cleaner fish in salmon farming due to their delousing efficacy against sea lice (Eliasen et al., 2018; Imsland et al., 2014). Juvenile lumpfish are reared in the hatcheries, and then deployed in the sea cages with Atlantic salmon when they reach a size of 25-30 g (Powell et al., 2018). However, high mortality rates in sea cages have raised concerns and constant effort is being made to improve vaccination protocols, welfare procedures, husbandry practices, and identify knowledge gaps(Eliasen et al., 2020; Reynolds et al., 2022). The main causes of lumpfish mortalities have been identified as of infectious origin, handling, and mechanical delousing (Reynolds et al. 2022). Also, dietary effects were identified as a factor influencing mortalities (Reynolds et al., 2022). Scarce information regarding the nutritional requirements of lumpfish was identified among the main challenges for improving the welfare and nutrition of farmed lumpfish by Garcia de Leaniz et al. (2022). Lumpfish reared in hatcheries require a well-balanced feed to promote growth and robustness for the following deployment in the sea cages (Hamre et al., 2022).

While deployed in the sea cages, lumpfish cannot survive solely by grazing on sea lice and an adequate feed is essential to avoid malnutrition (Treasurer, 2018). Challenges in lumpfish nutrition are mainly due to the lack of knowledge of specific life stages requirements (Treasurer, et al., 2018), as well as agreed protocols for feeding regime and delivery method (Garcia de Leaniz et al., 2022). Initial attempts of rearing and feeding lumpfish included the use of feeds for other species such as cod, salmon and flatfish. However, when lumpfish were given a high fat diet, this led to increased mortality rates, which were characterised by fat accumulation in liver and brain (Sayer et al., 2000). The liver plays a pivotal role in fish nutrition, serving as the primary organ for nutrient collection and distribution. It gathers a substantial supply of dietary nutrients via the portal vein, which is directly linked to the digestive tract, and then disperses these nutrients to other tissues in the body (Rust, 2003). In particular, the liver is central to the metabolism of fatty acids, managing their intake, synthesis, and elimination (Hodson & Frayn, 2011).

Different fish species exhibit considerable variation in their liver lipid levels: Atlantic cod (*Gadus morhua*) can store a substantial amount of lipids in the liver, with total liver lipid content exceeding 70% (Karlsen et al., 2006), farmed sea bream from 10 to 40% (Yıldız et al., 2006) while Atlantic salmon maintain liver lipid levels low below 5 % (Bell et al., 2001; Betancor et al., 2018). Following feeding, any surplus dietary fatty acid is transported in the form of lipoproteins from the liver to designated lipid storage sites, where they are accumulated and stored as triacylglycerols (TAG). TAG are primarily stored in adipose tissue that can be utilised by the animal during periods of high energy demand such as reproduction (Tocher, 2003b). When TAG levels are low, it indicates poor energy reserves in lumpfish (Eliasen et al., 2020), as they are mobilised for energy production and depleted in starvation periods (Osako et al., 2003).

Beyond this, the liver is also involved in other functions such as carbohydrate storage, the production of bile for lipid digestion as well as executing detoxification processes (Bruslé & i Anadon, 1996). Liver makes up 1.37 and 2.33% of the total fish weight in wild female and male lumpfish respectively (Davenport & Kjørsvik, 1986), with the liver being the primary tissue for lipid storage in this species (Berge et al., 2023). Indeed, in farmed lumpfish, there is a notable accumulation of lipids in the liver, with levels varying between 19% and 35% (Berge et al., 2023; Hamre et al., 2022).

A study by Eliasen et al. (2020) highlighted the use of liver colour as a welfare indicator for the nutritional status of the lumpfish deployed in farming sites in the Faroe Islands. Lumpfish whose livers were orange had a better nutritional status and overall welfare compared to the fish with a dark reddish liver (Eliasen et al., 2020). The same study also showed that lumpfish with dark and red livers had the lowest content of total lipids and TAG in their livers, indicating starvation (Eliasen et al., 2020).

This study also hypothesises that the diet provided to lumpfish may not be nutritionally optimal, suggesting that farmed lumpfish may reach their deployment phase with a suboptimal nutritional status. By relating the nutritional content of lumpfish livers to the welfare scoring index, this study aims to establish the nutritional and welfare status of lumpfish in a farming environment, in comparison to the wild population. To the best of our knowledge, a comparison of the nutritional content of livers from wild and farmed lumpfish has not been done before.

Data regarding the nutritional status of the wild lumpfish are a reference for the improvement of lumpfish nutrition. This study provides insights regarding the levels of total lipid, fatty acids and the pigments that the diet should contain. By coupling the liver data with the body composition and the liver histology detailed in the other chapters, it is possible to increase knowledge regarding diet formulations for juvenile lumpfish and relate to the welfare of the species.

4.3. Materials and methods

Experimental procedures were conducted according to the Directive 2010/63/EU regarding the protection of animals for scientific purposes, approved by the head veterinarian “Landsdjóralæknin” in according to the Welfare act 2018, 10 (DJÓRAVÆLFERÐARLÓGIN - Løgtingslóg 49 apríl 30 2018, Faroe Islands) and were also reviewed and approved by the Animal Welfare and Ethical Review Body of the University of Stirling (AWERB 19 20 007).

Pre-deployment lumpfish (35.7 ± 9.7 g) were sampled from two hatcheries, Nesvík and Svínoy, in July and September 2020 respectively. Deployed fish (90.5 ± 22.9 g) were collected from sea cages of seven Atlantic salmon farming sites across the Faroe Islands from October 2019 to January 2021. These lumpfish were sampled during regular lumpfish health monitoring carried out by Firum or farming companies as part of their regular husbandry practices. Wild lumpfish (50.4 ± 29.2 g) were captured from October 2019 to August 2021 as by-catch from seaweed farms and pelagic fisheries in addition to being collected by staff from Havstovan (FAMRI) while out on their annual pelagic research survey in

summer 2020 and 2021. Wild fish were euthanised through exsanguination via a gill cut and frozen at -20 °C. A summary of the number of fish sampled for each origin and size class is reported in Table 4.1. All the fish that were not caught by fishing vessels in commercial fishing gear were euthanised with an overdose of Finquel (0.8 g/l, MS-222, MSD Animal Health).

Each fish was dissected with an anteroposterior cut on the left side and liver colour was scored from 1 to 6 according to the classification in Eliasen et al. (2020). This categorisation reflects different underlying causes:

-Pale yellow (scores 1 and 2): these colours were observed in fish with low pigmentation and/or potential disease. The lack of carotenoids (especially astaxanthin) may indicate immune challenges, stress or low levels of carotenoids in the feed provided.

-Orange to bright orange (scores 3 and 4): identified as the optimal condition, reflecting adequate pigmentation and nutrition. These fish generally had sufficient lipid reserves and were associated with better welfare indicators.

-Dark reddish-brown (scores 5 and 6): Associated with poor nutrition and lipid depletion, suggesting energy deficiency and compromised welfare.

Each liver was collected, frozen on dry ice during the sampling and stored at -70 °C for further nutritional analysis. A detailed description of the sampling methods, liver colour scoring, and further analyses can be found in Chapter 2.

4.3.1. Morphometric data

At each sampling occasion, each fish was weighed out to the nearest gram and measured to the nearest millimetre. Measurements included the total length of the fish, from the snout to the final part of the lobe of the tail and the height which is measured from the highest part of the crest to the bottom of the belly.

HSI (Willora et al., 2021) was calculated as follow:

$$HSI = \frac{Liver\ weight\ (g)}{Fish\ weight\ (g)} \times 100$$

4.3.2. Biochemical analyses

4.3.2.1. Total lipid extraction

Total lipids were extracted from thawed livers following the method described by Folch et al. (1957). Approximately 0.5 g of sample was weighed out to 4 decimal places in duplicate and homogenized in 20 ml of 2:1 chloroform/methanol in a glass tube using an IKA Ultra-Turrax T8 tissue disrupter (Fisher Scientific, Loughborough, UK). The homogenate was left on ice for at least one hour

before adding 5 ml of a 0.88% aqueous solution of KCl (w/v). After centrifuging at 400 g for 5 min, the sample separated into two distinct layers; aqueous and organic.
The aqueous layer was removed by aspiration after which the organic phase containing the lipid extract was filtered and evaporated under a stream of OFN and desiccated in a vacuum desiccator overnight. The total lipid content was gravimetrically calculated. The lipid extract was redissolved at a concentration of 10 mg/ml in 2:1 chloroform/methanol containing 0.01% BHT, and stored at -20 °C prior to fatty acid and lipid class analyses.

4.3.2.2. Fatty acid methyl esters (FAME)

The analysis of FAME of lumpfish livers was performed on the previous total lipid extracted using the Folch method (1957), and it was characterized using gas chromatography as described by Christie, (2003). The FAME was prepared by transmethylation, and their extraction and purification was carried out as described in Tocher & Harvie (1988). Briefly, 100 µl of total lipid extract was mixed with 100 µl of 17:0 fatty acid standard and evaporated under a stream of OFN. The extract was incubated with 1 ml of toluene and 2 ml of 1% sulphuric acid (H_2SO_4) in methanol at 50 °C for 16 hours. Two ml of 2% potassium bicarbonate ($KHCO_3$) and 5 ml of 1:1 iso-hexane/diethyl ether containing 0.01% BHT were added to the FAME produced and two separate phases were obtained. The upper layer was transferred and 5 ml of 1:1 iso-hexane/diethyl ether were added to the remaining layer to obtain maximum recovery of FAME. The organic solvent was dried under a stream of OFN and eluted through UCT silica clean-up cartridges by adding 10 ml of 95:5 iso-hexane:diethyl ether.
FAME were separated and quantified by gas–liquid chromatography using a Fisons GC-8160 (Thermo Scientific, Milan, Italy). The GC was equipped with a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), on-column injector and a FID. The carrier gas used was hydrogen. The oven thermal gradient was from 50 °C to 150 °C at 40 °C/min to a final temperature of 230 °C at 2°C/minute. Data were processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Milan, Italy). Individual FAME was identified by comparing the samples profile to known standards (Supelco™ 37-FAME mix; Sigma-Aldrich Ltd., Poole, UK) and published data (Tocher & Harvie, 1988). This eluent containing the FAME was evaporated under a stream of OFN and resuspended in 1 ml of iso-hexane for GC analysis. Heptadecanoic acid (17:0) at a concentration of 10 mg/ml was added as internal standard to calculate fatty acid content per g of sample.

4.3.2.3. Lipid class analysis

Lipid classes analysis was also performed on total lipid extracted with the Folch method from lumpfish livers. Lipid classes were separated by high-performance thin-layer chromatography (HPTLC) using 10 × 10 cm x 0.25 mm plates (VWR, Lutterworth, UK) according to Henderson and Tocher (1992). Plates were cleaned using 2:1 chloroform/methanol, allowing the solvent to evaporate by air

drying. Twelve 3 mm origins were marked on the plate and lipid samples in duplicate (1.5 -2 µl) and two lipid classes standards (one neutral and one polar) were applied to each origin using a Microliter™ glass syringe (Hamilton, Bonaduz, Switzerland). The plate was first developed in methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.) to 5.2 cm for polar lipids, and then developed to 9.5 cm using a solvent mixture containing isohexane/diethyl ether/acetic acid (80:20:1, by vol.) for neutral lipids. Lipid classes were visualized by spraying 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid and charring plates at 160 °C for 20 minutes. Lipid classes were quantified by densitometry using a CAMAG-3 TLC Scanner (version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) and winCATS software (Planar Chromatography Manager, version 1.2.3).

4.3.2.4. Total carotenoids

Total carotenoids were extracted from lumpfish livers by the HPLC method for synthetic carotenoids described by Barua et al. (1993) and Bell et al. (2002). Different carotenoid sources are used in aquaculture feeds, as salmon diets often include synthetic astaxanthin or natural carotenoid sources such as from algae (*Haematococcus pluvialis*), red yeast (*Phaffia rhodozyma*) or bacteria (Panaferd). To extract carotenoids, approximately 1 gram of tissue was homogenized in 10 ml of 1:1 ethyl acetate/ethanol using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK). The homogenate was centrifuged for 5 min (Lê et al., 2008) and the supernatant transferred to a new tube. The pellet was re-homogenized in 5 ml of ethyl acetate, and the resulting supernatant was combined with the previous supernatant. Finally, the pellet was re-homogenized in 5 ml of iso-hexane and recentrifuged, and the supernatant combined with the pooled supernatant.

The pooled supernatant was dried under a stream of OFN and redissolved in 5 ml of iso-hexane. Total carotenoid was measured first spectrophotometrically at 470 nm using the E1% (w/v) of 2100. Then, it was measured using the HPLC (Waters 2695 Separations Module, UK) equipped with a Roc silica Detector (5µ, 150 x 4.6 mm column, Guard cartridge and a Dual λ Absorbance) (Waters 2487, UK). An isocratic solvent system was used containing iso-hexane/acetone (82:18 by volume) at a flow rate of 1.2 ml/min. Astaxanthin and canthaxanthin were detected at 474 nm and quantified using external standards of astaxanthin and canthaxanthin obtained from Roche (Welwyn Garden City, U.K.).

4.3.3. Statistical analyses

To investigate the nutritional differences between wild, sea cage and land-based lumpfish, simple linear models were constructed with liver lipid, fatty acids, lipid classes and carotenoids as response variables and origin of the fish and size as predictors (e.g. lm(Lipid ~ origin*size)). The fish origin is whether fish were farmed or from wild populations. However, due to the substantial difference between fish reared in land-based hatcheries and fish deployed in the sea cages, the origin of the fish

includes three distinct groups (land-based, sea cages, and wild). Percentage data such as lipid, lipid classes and fatty acids were transformed using the arcsine transformation from Zar (2014).

When the linear model showed a significant effect ($P < 0.05$), a post hoc Tukey HSD test was performed to identify differences between groups. F-statistic and p-values were reported from the linear model summary table and values having high Cook's distance scores were considered outliers and checked through the Residuals vs Leverage plot.

PCA was performed using the "FactoMineR" package (Lê et al., 2008) to visualise and identify patterns regarding the differences between groups in terms of fatty acid profile. Before performing the PCA, variables were centered, where the mean of each variable in the data was calculated and subtracted from each data point to ensure that the new data points have a mean of zero. After centering, the PCA was performed with scaling, where each variable was divided by its standard deviation. This is performed by default to give equal weight to all variables. The summary outputs of the PCA also provided information about the eigenvalues, proportion of variance explained and variable contributions. To ensure that differences in composition were statistically significant among fish origins (wild, land-based, and sea cage) and season, a Multivariate Analysis of Variance (MANOVA) was conducted on the fatty acid profiles of lumpfish livers. The outputs of the PCA were visualized using the "factoextra" package (Kassambara, 2016).

A binomial generalised linear model (GLM) was performed to analyse the effect of fish origin (land-based, sea cage, wild) and weight on liver colour. Liver colour scores of 3 and 4 were classified as healthy (score = 1), while scores 1, 2, 5, and 6 were classified as unhealthy (score = 0). The model included site, weight, and their interaction as predictor variables. The model was fitted with glm function in R with the "binomial" family using the packages MASS (Ripley et al., 2013).

The same binary variable (healthy = score 3 or 4, unhealthy = score 1, 2, 5, or 6) was used to evaluate the proportion of lumpfish with a healthy liver. Using the "dplyr" and "ggplot2" packages in R (Wickham et al., 2020), a logistic regression model was fitted to assess the relationship between weight and the probability of achieving a healthy liver score (score 3 or 4). The model used log-transformed weight as a predictor to estimate the probability of a healthy liver score (3 or 4). These probabilities were then visualised in a logistic regression plot, where a fitted curve illustrates how the likelihood of a healthy liver score changes with weight (Wickham et al., 2020).

A generalised linear model with a binomial family was constructed to test if the relationship between the weight of the fish, which was used as predictor, and the likelihood of having an healthy liver (liver colour 3 and 4) is significant (e.g. glm(BrightLiver ~ weight, family = binomial, data = owi150).

Data were analysed using R (R Core Team, 2021), and figures plotted using "ggplot2 (Wickham et al., 2016). Excel data files were imported into R using the "readxl" package (Wickham & Bryan, 2019), which is part of the "tidyverse" collection of R packages (Wickham & Bryan, 2019). Linear models were carried out using the built-in lm() function in the base R package. Data in Tables are presented as mean ± standard deviation (SD).

4.4. Results

4.4.1. Fish sampled per category

The number of lumpfish sampled across different environments (land-based hatcheries, sea cages, and wild populations) and classified by size categories, is summarised in Table 4.1. The majority of land-based fish were in the smallest size class (<50 g), while sea cage fish were predominantly in the 50-150 g and 150-300 g size classes, with only few reaching 1-3 kg. Wild fish showed a broader distribution, with individuals in multiple size categories, including larger specimens (1-3 kg and 3-5 kg).

Table 4.1. Summary of number of fish sampled per category (land-based, sea cage, wild) and size class (< 50g, 50-150 g, 150-300 g, 300g-1kg, 1-3 kg, 3-5 kg).

	< 50 g	**50-150 g**	**150-300 g**	**300 g-1 kg**	**1-3 kg**	**3-5 kg**
Land-based	60	0	0	0	0	0
Sea cage	7	157	70	98	2	0
Wild	62	8	0	34	58	7

4.4.2. Total lipid and fatty acid profile of livers

Total lipid content of lumpfish livers (<150 g) ranged from 2.8 % to 33.1%, and it differed between groups (one-way ANOVA, $F_{5,120}$=2.3, P=0.049). Wild lumpfish and lumpfish from the sea cages had a significant lower lipid content (16.3 ± 7.1% and 16.4% ± 7.6% respectively) in the liver compared to the land-based ones (19.3 ± 7.3 %) (Figure 4.1).

The fatty acid profile of livers also differed among groups (Table 4.2) and this separation can be seen in Figure 4.2. In this PCA, fish <150 g were analysed. The MANOVA revealed a significant effect of origin on the fatty acid composition (Pillai's trace = 0.639, F = 14.08, P < 0.001). PC1 accounted for 64.9% of the variation which was due to EPA (0.87) and DHA (0.85), while PC2 accounted for 11.7% of the variation which was associated with MUFA (-0.70) and n-6 PUFA (0.77).

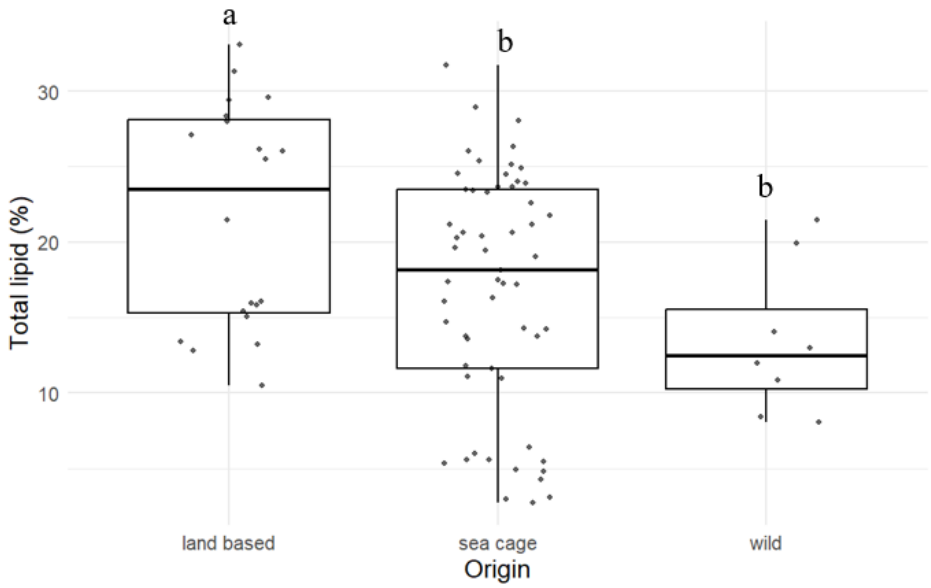


Figure 4.1. Total lipid (%) of lumpfish livers from different origins: land-based hatcheries (n=20), sea cages (n=97) and wild (n=8).

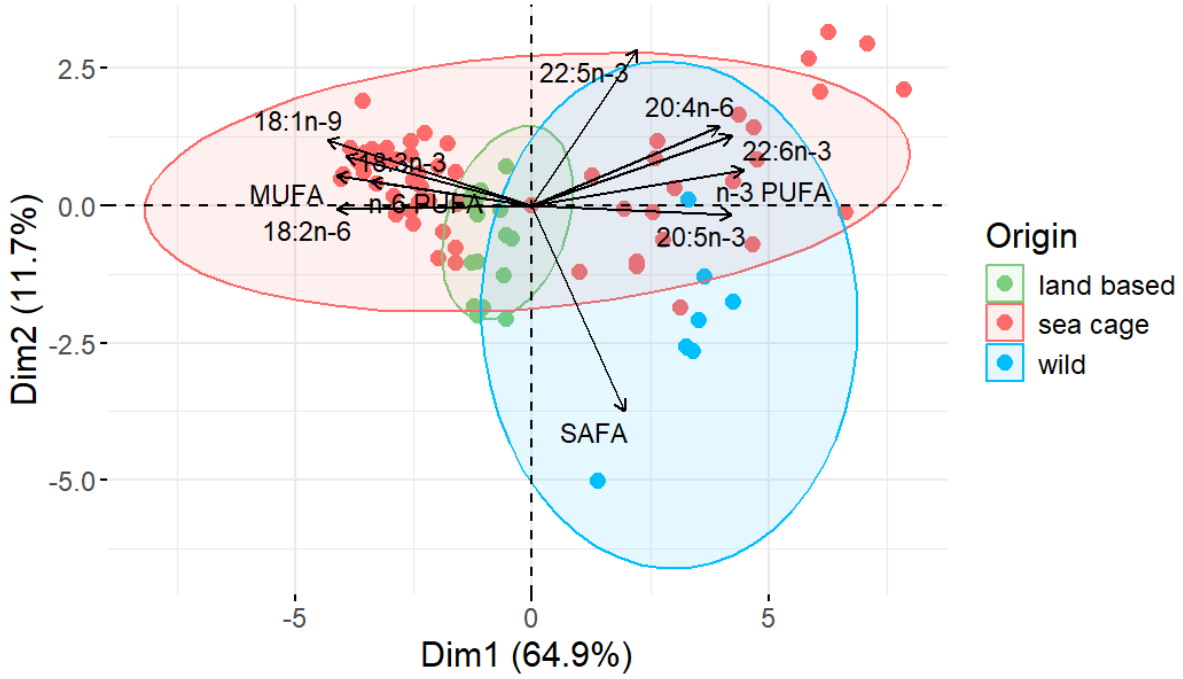


Figure 4.2. Principal Components Analysis biplot of fatty acid profile of livers of fish < 150 g showing separation of individuals depending on the origin (land-based, sea cage and wild) and relative influence of different fatty acid.

Table 4.2. Fatty acid profile (%) of lumpfish livers from different origin (land-based, sea cages and wild). Data are expressed as mean ± SD. Different superscript letters denote differences among the groups according to one-way ANOVA and Tukey HSD test.

Origin	**Land-based (n=21)**	**Sea cage (n=56)**	**Wild (n=8)**	**P**
14:0	2.1 ± 0.4[a]	2.0 ± 0.9[a]	4.3 ± 0.9[b]	<0.001
16:0	13.3 ± 0.9[a]	11.2 ± 1.6[b]	14.6 ± 3.0[a]	<0.001
18:0	4.8 ± 0.4	4.0 ± 1.6	4.0 ± 1.6	0.006
ΣSAFA[1]	20.5 ± 1.1[a]	17.5 ± 2.7[b]	23.6 ± 4.3[c]	<0.001
16:1n-7	4.1 ± 0.9[a]	2.7 ± 1.2[b]	3.0 ± 1.9[ab]	<0.001
18:1n-9	30.8 ± 6.2[a]	29.1 ± 12.4[a]	11.4 ± 1.8[b]	<0.001
18:1n-7	6.6 ± 0.2[a]	4.8 ± 1.1[b]	2.7 ± 0.8[c]	<0.001
20:1n-9	1.1 ± 0.1[a]	3.7 ± 2.3[b]	6.0 ± 0.7[c]	<0.001
22:1n-11	0.3 ± 0.1[a]	1.2 ± 0.9[b]	3.5 ± 0.5[c]	<0.001
ΣMUFA[2]	44.3 ± 5.1[a]	45.8 ± 8.3[a]	36.1 ± 3.1[b]	0.026
18:2n-6	12.9 ± 2.5[a]	9.7 ± 5.7[b]	2.8 ± 0.4[c]	<0.001
20:4n-6	0.7 ± 0.2	1.4 ± 1.8	1.1 ± 0.5	0.449
Σn-6 PUFA[3]	14.7 ± 2.8[a]	12.1 ± 4.3[b]	5.3 ± 0.7[c]	<0.001
18:3n-3	1.9 ± 0.3	2.5 ± 1.5	1.5 ± 0.2	0.149
18:4n-3	1.3 ± 0.4[a]	1.7 ± 1.1[a]	4.8 ± 1.2[b]	<0.001
20:5n-3	8.5 ± 1.9[a]	8.7 ± 4.3[a]	12.8 ± 2.9[b]	0.023
22:5n-3	1.3 ± 0.5[a]	1.2 ± 0.5[a]	0.7 ± 0.1[b]	0.002
22:6n-3	5.4 ± 0.8[a]	8.0 ± 5.1[b]	11.9 ± 3.3[b]	<0.001
Σn-3 PUFA[4]	19.8 ± 2.2[a]	23.6 ± 8.5[a]	33.2 ± 5.9[b]	<0.001
ΣPUFA	35.2 ± 4.8	36.7 ± 7.4	40.3 ± 5.8	0.535
ΣLC-PUFA[5]	15.2 ± 1.6[a]	17.9 ± 9.1[ab]	25.4 ± 5.4[b]	0.004
EPA/DHA	1.6 ± 0.6[a]	1.2 ±0.4[b]	1.1 ± 0.3[b]	0.001
EPA+DHA	13.9 ± 1.8[a]	16.7 ±8.7[a]	24.7 ± 5.3[b]	0.001
EPA (g/100g)	0.40 ± 0.57	0.38 ± 0.53	0.37 ± 0.54	0.157
DPA (g/100g)	0.06 ± 0.12	0.05 ± 0.08	0.06 ± 0.11	0.079
DHA (g/100g)	0.31 ± 0.45	0.32 ± 0.42	0.30 ± 0.43	0.269
EPA+DHA (g/100g)	0.70 ± 1.01	0.70 ± 0.93	0.67 ± 0.96	0.292
EPA+DPA+DHA (g/100g)	0.77 ± 1.12	0.75 ± 1.01	0.72 ± 1.06	0.300

[1]includes 15:0, 20:0, 22:0, 24:0; [2]includes 16:1n-9, 17:01, 18:01, 20:1n-11, 20:1n-7, 22:1n-9, 24:1n-9; [3]includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6, 22:5n-6; [4]includes 20:3n-3, 20:4n-3; 21:5n-3; [5]includes 20:5n-3, 22:5n-3, 22:6n-3.

Significant differences were found in almost all the fatty acids in livers. SAFA were highest in wild lumpfish (23.6 ± 4.3%), intermediate in land-based fish (20.5 ± 1.1%), and lowest in sea cage fish (17.5 ± 2.7%; one-way ANOVA, $F_{5,120} = 6.28$, $P < 0.001$). Among SAFA, 16:0 was the most abundant and it was significantly higher in wild lumpfish (14.6 ± 3.0%) than in land-based (13.3 ± 0.9%) and sea cage fish (11.2 ± 1.6%; one-way ANOVA, $F_{5,120} = 4.88$, $P < 0.001$).

Levels of MUFA were higher in farmed fish (land-based and sea cage, 44.3 ± 5.1% and 45.8 ± 8.3% respectively) than the wild ones (36.1 ± 3.1%, one-way ANOVA, $F_{5,120} = 1.96$, $P = 0.026$), mainly due to OA. OA was higher in land-based and sea cage (30.8 ± 6.2% and 29.1 ± 12.4% respectively) than wild fish (11.4 ± 1.8%).

N-6 PUFA were significantly higher in the land-based hatcheries fish (14.7 ± 2.8%), followed by the sea cages fish (12.1 ± 4.3%), and wild fish (5.3 ± 0.7%, one-way ANOVA, $F_{5,120} = 6.72$, $P < 0.001$), due to the higher levels of LA (land-based: 12.9 ± 2.5%, sea cage 9.7 ± 5.7%, wild 2.8 ± 0.4% (Table 4.2)).

N-3 PUFA were significantly higher in wild fish (33.2 ± 5.9%) than the farmed ones (land-based 19.8 ± 2.2%, sea cage 23.6 ± 8.5%)), mainly due to EPA and DHA (EPA + DHA: wild 24.7 ± 5.3%, sea cage 16.7 ±8.7%, land-based 13.9 ± 1.8%).

Absolute concentrations of EPA and DHA (g/100g) did not differ among origins (one-way ANOVA, $F_{5,150} = 11.28$, $P = 0.292$). Further PCAs were carried out to investigate the effect of season on the fatty acid profile, as shown in Figure 4.3 and Figure 4.4. The MANOVA revealed a significant effect of season on the fatty acid composition (Pillai's trace = 1.167, $F = 25.52$, $P < 0.001$). In Figure 4.3, PC1 accounted for 73.4% of the variation, mainly due to n-3 PUFA (0.95), OA (-0.93) and MUFA (-0.91). PC2 accounted for 9% of the variation, mainly due to SAFA (-0.71), ALA (0.32) and n-6 PUFA (0.32). Fish from winter had a higher amount of n-6 PUFA, while fish from the summer contained more SAFA and n-3 PUFA (Figure 4.3).

The PCA in Figure 4.4 shows the variation in fatty acid profile during summer only in relation to the origin of fish < 150 g. The MANOVA revealed a significant effect of origin on the fatty acid composition of fish from summer (Pillai's trace = 0.925, $F = 12.23$, $P < 0.001$). PC1 accounted for 60% of the variation, mainly due to n-3 PUFA (-0.97), LA (0.88) and ALA (0.85). PC2 accounted for 15.4% of the variation, due to DPA (0.84), SAFA (-0.75) and OA (0.39). The data points of the cluster of the fish from the sea cages in summer are more disperse and show higher variability in the fatty acid profile, compared to the land-based and the wild ones.

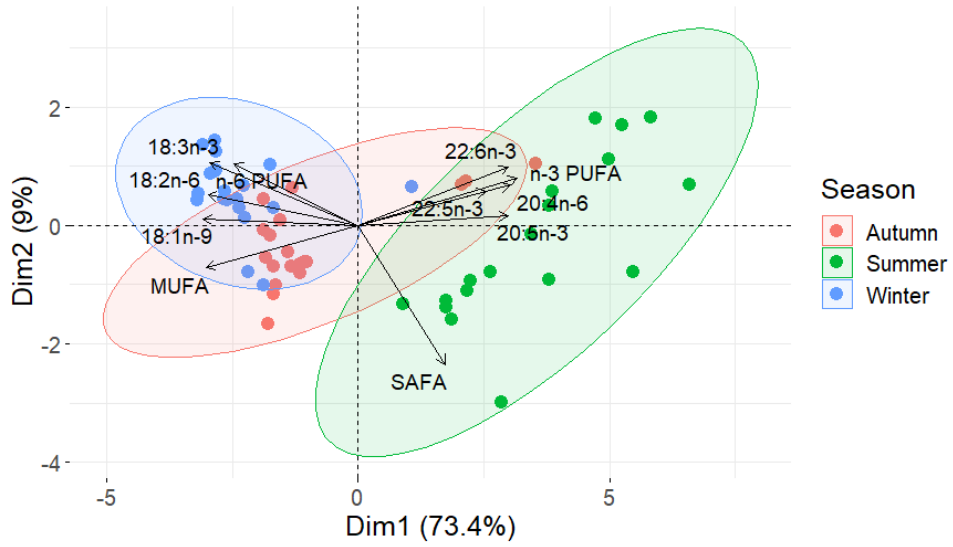


Figure 4.3. Principal Components Analysis biplot of fatty acid profile of livers from fish from the sea cages, showing separation of individuals depending on the season (autumn, summer and winter) and relative influence of different fatty acid.

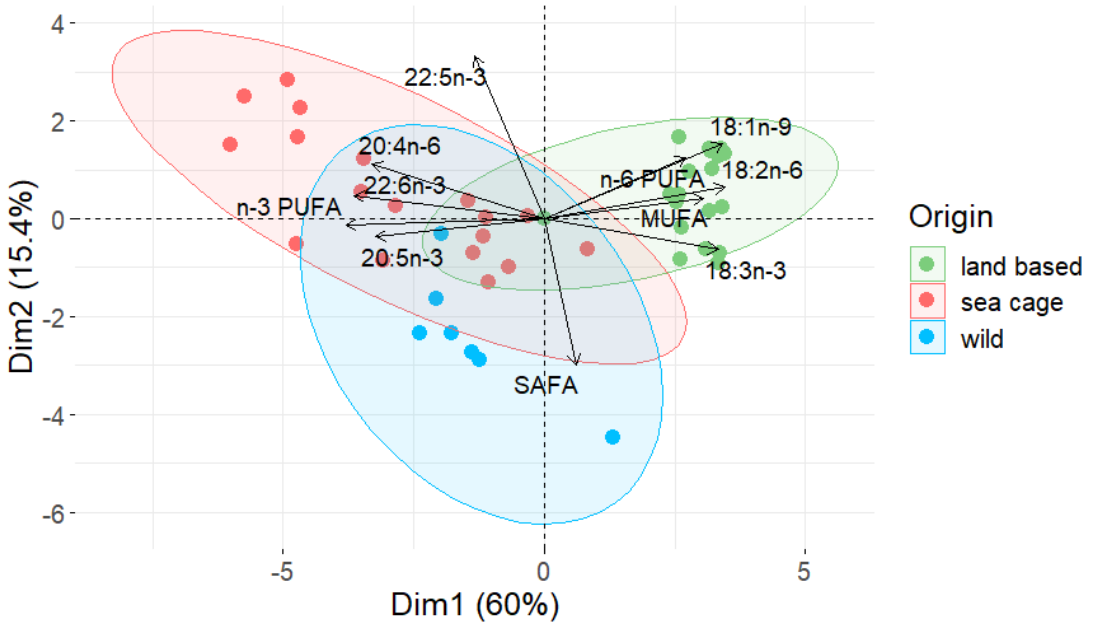


Figure 4.4. PCA biplot of fatty acid profile of livers during summer showing separation of individuals < 150 g according to origin (land-based, sea cage and wild).

4.4.3. Lipid classes

Total neutral lipid was significantly higher in fish from the land-based hatcheries (85.5 ± 3.9%) and the sea cages (78.0 ± 13.0%) compared to the wild group (68.1 ± 7.0%; one-way ANOVA, $F_{5,69}$ = 5.1, P < 0.001), mainly due to the higher levels of triacylglycerols (TAG) and diacylglycerol (DAG) (Table 4.3). DAG levels were elevated in land-based fish (9.2 ± 1.2%) relative to sea cage (6.3 ± 3.8%) and wild fish (5.7 ± 1.4%; one-way ANOVA, $F_{5,69}$ = 2.04, P = 0.010). Although TAG levels tended to be higher in land-based fish (42.4 ± 4.6%), differences were not statistically significant ($F_{5,69}$ = 1.26, P = 0.068). Similarly, free fatty acid (FFA) and sterol ester (SE) levels did not differ significantly among origins. In contrast, total polar lipid content significantly differed across groups ($F_{5,69}$ = 6.15, P < 0.001), with wild lumpfish showing the highest levels (32.0 ± 7.0%) compared to sea cage (20.0 ± 10.2%) and land-based fish (14.4 ± 3.8%). Pigmented material (PIG) followed the same trend, with wild fish having significantly higher concentrations (16.9 ± 8.6%) than sea cage (4.1 ± 2.2%) and land-based fish (4.7 ± 0.9%; $F_{5,69}$ = 21.54, P < 0.001). Among the phospholipids, phosphatidylcholine (PC) was the most abundant and significantly differed among groups, being higher in wild and sea cage fish (6.9 ± 3.2% 6.8 ± 3.4% respectively, one-way ANOVA, $F_{5,69}$ = 3.03, P = 0.017). Phosphatidylethanolamine (PE) also showed significant variation ($F_{5,69}$ = 2.24, P = 0.036), with higher levels observed in wild and sea cage fish (3.5 ± 1.9% and 4.4 ± 2.6% respectively). No significant differences were detected in phosphatidylinositol (PI), phosphatidylserine (PS), or sphingomyelin (SM) content among origins.

Table 4.3. Lipid classes (% total area) of liver from wild and farmed (land-based hatcheries and sea cages) lumpfish < 150 g. Values (%) are mean ± SD. Different letters indicate significant differences (ANOVA, post hoc Tukey´s, P < 0.05) among groups.

Lipid class	Wild (n=9)	Sea Cage (n=47)	Land-based (n=19)	P
SE	5.4 ± 2.2	9.3 ± 8.8	5.2 ± 2.1	0.123
TAG	27.6 ± 11.1	32.5 ± 21.7	42.4 ± 4.6	0.068
FFA	18.9 ± 3.9	20.4 ± 7.3	22.5 ± 1.5	0.278
CHOL	10.5 ± 4	9.2 ± 5.4	6.1 ± 0.6	0.056
DAG	5.7 ± 1.4^{ab}	6.3 ± 3.8^{b}	9.2 ± 1.2^{a}	0.010
Total neutral lipid	**68.1 ± 7^{c}**	**78 ± 13^{b}**	**85.5 ± 3.9^{a}**	**<0.001**
PE	3.5 ± 1.9^{ab}	4.4 ± 2.6^{b}	2.8 ± 0.8^{a}	0.036
PG	1.5 ± 1.7^{b}	1.2 ± 1^{b}	0.1 ± 0.1^{a}	<0.001
PI	1.4 ± 1	0.8 ± 0.8	1 ± 0.3	0.050
PS	0 ± 0	0.4 ± 0.7	0.3 ± 0.3	0.115
PC	6.9 ± 3.2^{ab}	6.8 ± 3.4^{b}	4.5 ± 1.1^{a}	0.017
SM	0.9 ± 0.6	1.1 ± 1.1	0.4 ± 0.1	0.406
LPC	0.8 ± 0.7^{b}	0.3 ± 0.5^{a}	0.6 ± 0.6^{ab}	0.011
PIG	16.9 ± 8.6^{b}	4.1 ± 2.2^{a}	4.7 ± 0.9^{a}	<0.001
Total polar lipid	**32 ± 7^{b}**	**20 ± 10.2^{a}**	**14.4 ± 3.8^{a}**	**<0.001**

4.4.4. Total carotenoids

Astaxanthin, canthaxanthin, lutein, astacene, astaxanthin esters and beta-carotene were identified in lumpfish livers (Table 4.4). Total carotenoid levels were significantly higher in wild (169.3 ± 152.0 mg/kg) and sea cage fish (157.1 ± 128.5 mg/kg) compared to land-based fish (39.7 ± 16.7 mg/kg; one-way ANOVA, $F_{5,106} = 12.0$, $P < 0.001$). Astaxanthin was the main carotenoid responsible for lumpfish liver pigmentation, followed in small quantities by lutein, astacene and canthaxanthin. Higher levels of astaxanthin were detected in both lumpfish livers from the sea cages (152.5 ± 127.6 mg/kg) and the wild (156.6 ± 158.6 mg/kg), while lumpfish from the hatcheries had significantly lower levels (34.6 ± 15.2 mg/kg; one-way ANOVA, $F_{5,106} = 13.41$, $P < 0.001$). Canthaxanthin was higher in wild (0.8 ± 1.0 mg/kg) compared to the sea cage group (0.1 ± 0.3 mg/kg) and land-based fish (0.2 ± 0.1 mg/kg; one-way ANOVA, $F_{5,106} = 9.38$, $P < 0.001$). Lutein showed a similar pattern, with the highest levels in wild fish (6.7 ± 15.6 mg/kg), intermediate levels in land-based fish (3.4 ± 3.3 mg/kg), and lowest in sea cage fish (1.4 ± 1.5 mg/kg; one-way ANOVA, $F_{5,106} = 5.72$, $P = 0.006$). Astacene was higher in the lumpfish livers from the sea cages (2.6 ± 1.8 mg/kg), followed by wild (1.6 ± 0.8 mg/kg) and in the land-based hatcheries (1.1 ± 0.3 mg/kg; one-way ANOVA, $F_{5,106} = 3.03$, $P = 0.001$). Astaxanthin esters were most abundant in wild fish (2.8 ± 6.3 mg/g), but nearly absent in sea cage fish and land-based fish (0.2 ± 0.8 mg/kg and 0 ± 0 mg/kg respectively; one-way ANOVA, $F_{5,106} = 5.39$, $P < 0.001$). Beta-carotene was detected at low levels in wild fish (0.1 ± 0.3 µg/g), and was nearly absent in sea cage and land-based fish (one-way ANOVA, $F_{5,106} = 9.7$, $P = 0.020$).

Table 4.4. Total carotenoids (mg/kg ± SD) in liver of lumpfish from three different origins. Different letters indicate significant differences (ANOVA, post hoc Tukey´s, $P < 0.05$) among groups.

	Wild n=7	**Sea Cage n=41**	**Land-based n=17**	**P**
Astaxanthin	156.6 ± 158.6^{b}	152.5 ± 127.6^{b}	34.6 ± 15.2^{a}	<0.001
Canthaxanthin	0.8 ± 1^{c}	0.1 ± 0.3^{b}	0.2 ± 0.1^{a}	<0.001
Lutein	6.7 ± 15.6^{ab}	1.4 ± 1.5^{b}	3.4 ± 3.3^{a}	0.006
Astacene	1.6 ± 0.8^{ab}	2.6 ± 1.8^{b}	1.1 ± 0.3^{a}	0.001
Astaxanthin esters	2.8 ± 6.3^{b}	0.2 ± 0.8^{a}	0 ± 0^{a}	<0.001
Beta-carotene	0.1 ± 0.3^{b}	0 ± 0.1^{a}	0 ± 0^{a}	0.020
Total carotenoids	169.3 ± 152^{b}	157.1 ± 128.5^{b}	39.7 ± 16.7^{a}	<0.001

4.4.5. HSI

The HSI denotes the ratio between the weight of the liver and the total weight of the fish. Wild fish had a higher HSI (median HSI: 2.14) than the land-based and sea cage fish (median HSI: 1.82 and 1.94 respectively) ($F_{2,148}$=7.12, one-way ANOVA, P = 0.001, Figure 4.5).

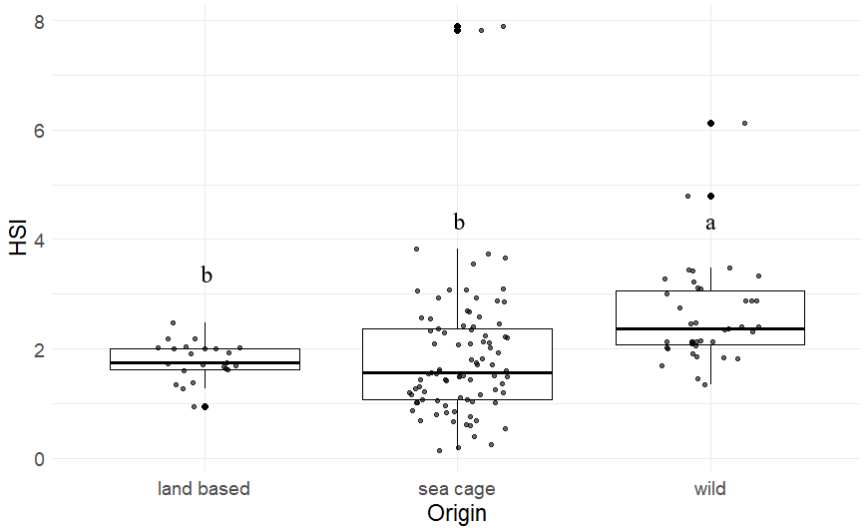


Figure 4.5. Hepatosomatic index (HSI) of lumpfish from different origins: land-based hatcheries n=27, sea cages n=141, and wild n=87. Different superscript letters denote differences among the samplings according to one-way ANOVA and Tukey HSD test (P < 0.05).

4.4.6. Liver colour

There was an effect of the origin of the fish on liver colour (DF=2, X^2=34.43, P < 0.001). Post-hoc analysis showed that sea cage fish had worse liver colour scores than both land-based (estimate = 2.27, P < 0.001) and wild fish (estimate = -1.10, P < 0.001). Land-based fish had slightly fewer orange livers than wild fish (estimate = 1.17, P < 0.001) (Figure 4.6).

Lumpfish livers also predicted astaxanthin levels, with orange livers containing more astaxanthin ($F_{2,122}$ = 10.82, P < 0.001). Lumpfish from the land-based hatcheries had a liver colour mainly ranging from 3 to 4 (orange and bright orange respectively), that in total account for 75% of the observations (Figure 4.6). Twenty two percent of the livers got a score of 2, and 3% got a score of 1 (very pale). There were no fish from the hatcheries that got a liver score of 5 or 6 (Figure 4.6).

The most common liver score for lumpfish from sea cages was 4 (76%), followed by 3 (7%), 2 (6%), 1 (5%), 5 (4%) and 6 (2%). Although the fish from the sea cages had mainly healthy livers (score 4), liver colour 5 and 6 (dark red, brown red) were recorded in this group. Wild lumpfish mainly displayed bright orange livers 4 (75%), followed by 3 (10%), 1 (8%), 2 and 5 (4% each).

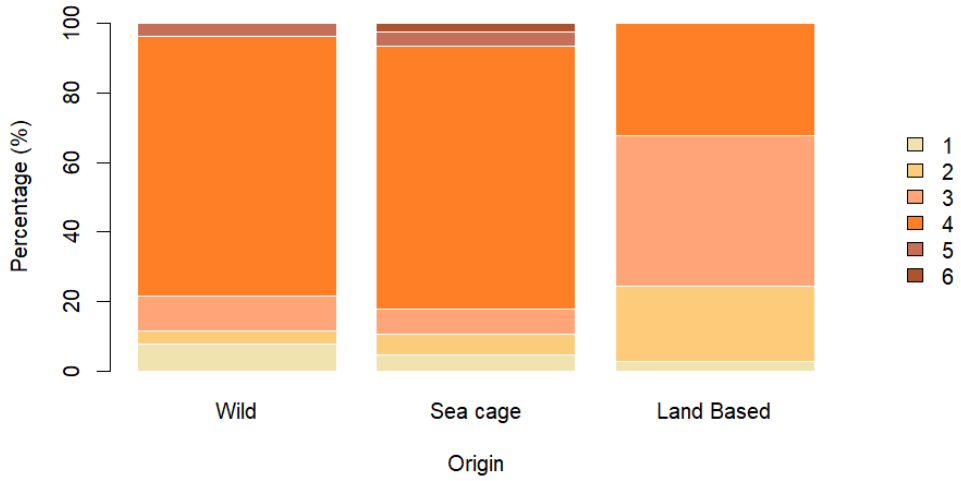


Figure 4.6. Frequency of lumpfish liver colour from different origins (land-based hatcheries, sea cages and wild). Colours are as defined by the liver score chart by Eliasen et al. (2020).

A significant relationship between weight and the probability of having an orange liver was found (binomial general linear model, estimate = 0.005, P=0.002), indicating an increased likelihood of healthy livers with higher weight. However, this effect was stronger in land-based fish than in sea cage fish (estimate = 0.113, P = 0.021).

The proportion of lumpfish with livers scoring a 3 or 4 (healthy livers) changes with the size of the fish (binomial general linear model, estimate = 0.606, P < 0.001) as shown in Figure 4.7. Fish with liver score 4 had a significantly higher amount of TAG ($F_{5,152}$=18.89, P < 0.001) and total lipid ($F_{5,152}$=11.28, P < 0.001) compared to the other scores (Figure 4.8). Additionally, 23% of the sampled fish across all origins had TAG levels indicative of starvation. These fish had liver scores of 5 and 6, with average TAG levels of 20.6 and 2.2, respectively.

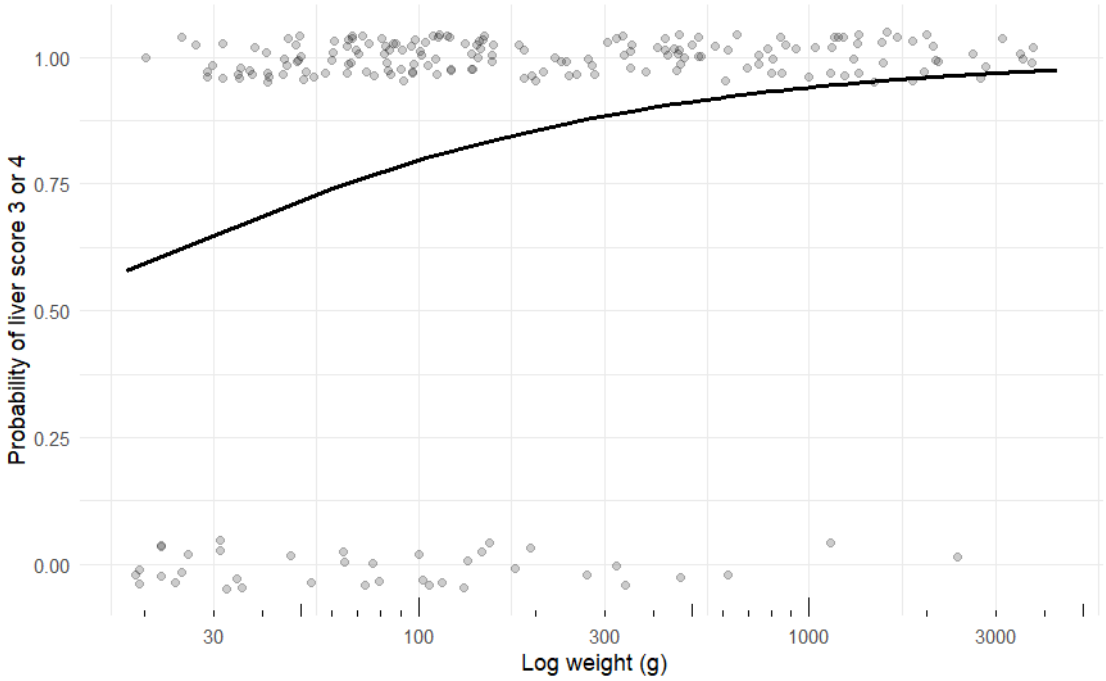


Figure 4.7. Proportion of lumpfish liver scoring 3 or 4 (healthy scores) versus weight.

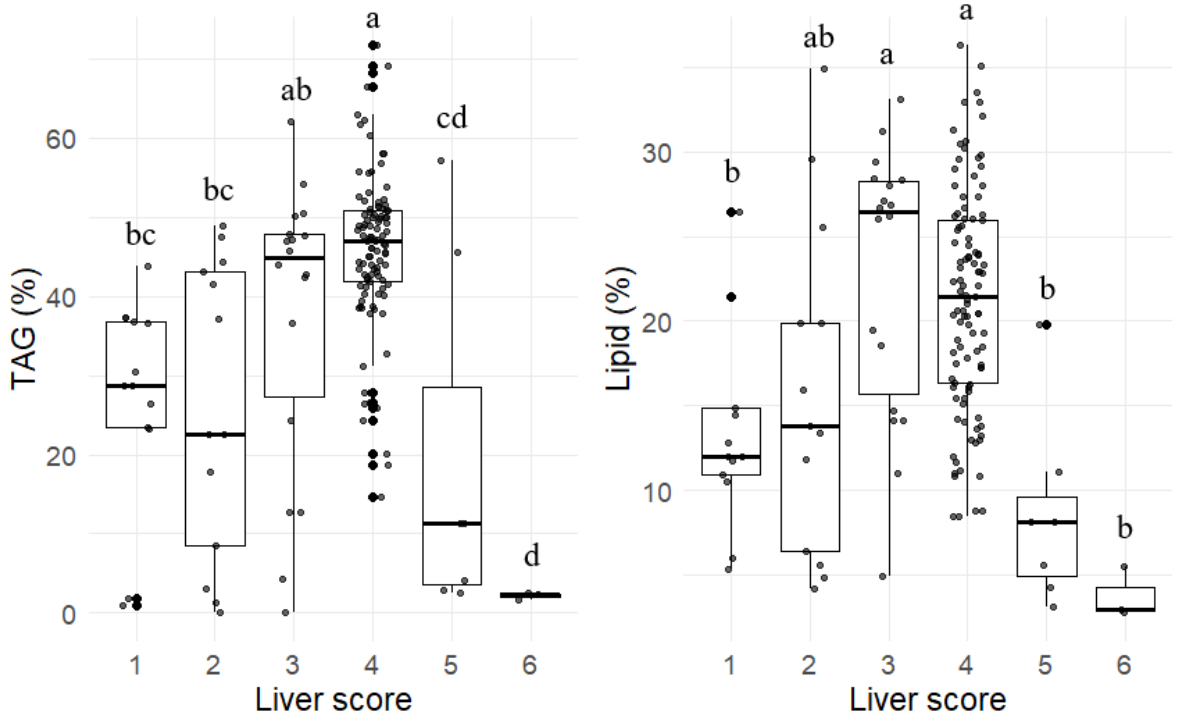


Figure 4.8. TAG levels (%) and lipid content (%) of lumpfish liver according to different liver scores (1-6).

4.5. Discussion

In this study, the nutritional status of lumpfish from different farming environments and from wild populations was established, with a focus on liver. Other studies examined liver composition to understand its impact on the nutritional and welfare status of the fish (Cejas et al., 2004; Eliasen et al., 2020).

In this study total lipid content of liver ranged widely, with farmed fish showing higher levels compared to wild ones. This also agrees with lipid classes composition where higher TAG levels were found in farmed fish compared to wild.

The fatty acid analysis revealed significant variations based on the fish origin and season. The fatty acid analysis highlighted differences among groups, notably higher SAFA and n-3 PUFA in wild fish and higher MUFA and n-6 PUFA in farmed fish. Seasonal changes also influenced fatty acid profiles, with winter fish having more n-6 PUFA and summer fish higher in SAFA and n-3 PUFA. Substantial differences were found also in the lipid classes profile where total neutral lipids, especially TAG and DAG, were higher in hatchery and sea cage fish compared to wild ones. Conversely, wild fish had higher amounts of total polar lipids.

In the study, carotenoid analysis of lumpfish livers also showed distinct variations. Astaxanthin was the predominant carotenoid, contributing significantly to liver pigmentation, with higher levels in livers from sea cages and wild lumpfish compared to those from hatcheries. Additionally, liver colour,

influenced by carotenoid levels, varied across groups, affected by dietary differences, size class and origin of the fish.

4.5.1. Lipid content of livers

It is clear from this study that there is a large variation in the total lipid content of lumpfish livers (2.8-33.1%). Total lipid content of wild and sea caged sampled lumpfish was generally lower than those sampled from the hatcheries, which is in agreement with Passantino et al. (2024), who also documented lumpfish from the hatcheries having higher lipid content in the liver compared to wild fish. The higher lipid content observed in the hatchery fish reflects the intake of energy dense formulated feeds, facilitating rapid growth and energy storage. In contrast, lumpfish deployed in the cages have a more diverse and variable diet, which include also other prey such as sea lice, zooplankton and organisms related to biofouling. These prey usually fluctuate in availability throughout the year, mainly being available during the summer months, potentially influencing overall energy intake (Eliasen et al., 2018). Also, in some cases fish in sea cages experience difficulties starting to feed after being transferred from the hatcheries into the sea cages, resulting in a lower caloric supply and lower lipid accumulation. Similarly, wild lumpfish can encounter periods of reduced feeding or starvation as they rely solely on seasonally available prey, thereby influencing their lipid reserves (Huntingford et al., 2006).
The lower lipid content of lumpfish in sea cages and wild may also be caused by the higher activity levels in the cage environment, compared to the controlled environment of hatcheries. This increased physical activity in both wild and sea cage lumpfish contributes to higher energy expenditure and subsequently lower lipid storage (Imsland et al., 2014). Conversely, lumpfish in the hatchery are reared in tanks, at higher density, with ample availability of feed, and lower activity levels, resulting in higher lipid deposition in fish (Powell et al., 2018).

4.5.2. Fatty acid profile of livers

To ensure a homogeneous comparison of fatty acid profiles across different origins, the PCA analysis included only individuals weighing less than 150 g. This size class reflects the typical size of lumpfish deployed in the sea cages during initial months of deployment. The PCA analysis on the fatty acid profile of the livers suggest that there are distinct patterns in the fatty acid profile of livers from different groups as observed by the spatial distance between the clusters. The clusters for wild and land-based fish showed a high degree of homogeneity, due to the proximity of points within each cluster, reflecting consistent dietary patterns. Specifically, the homogeneity of the hatchery lumpfish may be explained by the controlled environment and type of feed consumed. On the other hand, the sea cage fish cluster displayed a more heterogeneous pattern in fatty acid profiles, indicating a greater variability. This may be explained by varying environmental conditions, the fish being sampled in different seasons, different feeding regimes, sampling across different farms, differing seasonal availability of natural prey

items, and therefore fish with a large variety in food choices and activity levels. Lumpfish in cages also prey on sea lice, but this is a small proportion of their total feed intake regardless of season, suggesting other prey significantly influence their fatty acid composition (Johannesen et al., 2018a).

Wild fish had a significantly higher amount of SAFA, due to the levels of palmitic acid and significantly lower amounts of MUFA and n-6 PUFA. Consequently, this resulted in higher proportion of n-3 PUFA mainly due to EPA and DHA. This fatty acid profile is likely due to the feeding habits of wild lumpfish, feeding mainly on copepods, shrimp and krill which are rich in n-3 PUFA and low in n-6 PUFA (Hellessey et al., 2022; Linder et al., 2010).

This trend in the fatty acid profile between farmed and wild fish was also found in Blanchet et al. (2005) when comparing farmed and wild rainbow trout, with farmed fish typically exhibiting higher n-6 PUFA. However, when comparing farmed and wild Atlantic salmon, the farmed fish had significantly higher absolute amounts of n-6 PUFA, n-3 PUFA, and SAFA, while wild fish had higher levels of MUFA. This variation is due to the use of vegetable oils in farmed fish diets, which differs significantly from the natural diet of wild fish (Blanchet et al., 2005).

Additionally, the fatty acid profile of fish from both the wild and the sea cages is strongly influenced by seasonal variation as seen in the PCA of Figure 4.3. This PCA included only sea cage fish to account for seasonality, as all wild fish were caught during the summer. Fish sampled in winter have a more distinct pattern towards n-6 PUFA, MUFA and ALA, likely due to the scarcity of zooplankton during this season. Under these conditions, sea cage fish primarily rely on salmon and lumpfish feeds, which are rich in vegetable oils, such as RO, which can include significant amounts of oleic acid (Miller et al., 2008), LA and ALA (Tvrzicka et al., 2011). The cluster of fish from autumn overlaps mainly with the fish from winter, showing a similar trend to the winter fish. This is mainly due to the seasonal variation in abundance and availability of zooplankton in the ocean (Eliasen et al., 2018). Liver from summer fish have higher amounts of SAFA, ARA and n-3 PUFA that are found predominantly found in zooplankton, which are abundant in these fatty acids (Auel et al., 2002; Brett et al., 2009).

The fatty acid composition of live prey could have influenced the fatty acid profile of fish livers (Wang & Jeffs, 2014). For instance, the crustaceans *Themisto abyssorum* and *Themisto libellula*, commonly found in the stomach of wild lumpfish, are rich in n-3 PUFA (Auel et al., 2002). Krill and small shrimps also contain high amounts of phospholipids associated with EPA and DHA (Kim et al., 2014; Xie et al., 2019). Consequently, a diet predominantly comprising these crustaceans could lead to higher n-3 PUFA levels in the body and liver composition of these fish. The PCA of Figure 4.4 included fish only < 150 g sampled in summer, revealing similar fatty acid profiles between wild and sea cage fish. This similarity confirms that both groups share similar diets during summer months due to increased zooplankton availability (Eliasen et al., 2018). On the contrary, lumpfish from the land-based hatchery are fed solely on lumpfish feed, and therefore they reflect the fatty acid profile of the feed, which is higher in MUFA, n-6 PUFA and ALA compared to the other groups.

Practical limitations prevented wild fish from being sampled during the bad weather seasons, and therefore the fatty acid profile reflects the composition of the prey only available during spring and summer. Indeed, EPA and DHA levels of wild lumpfish liver are higher than in farmed fish, which could be attributed to two factors: the fatty acid composition of their natural prey and selective retention. In the wild, fish eat less overall, resulting in a higher proportional retention of polar lipid, along with EPA and DHA (Bandara et al., 2023).

4.5.3. Lipid classes of livers

Total neutral lipids were higher in fish from the hatcheries and the sea cages than in wild fish. This resulted in proportionally higher levels of polar lipids in the wild fish compared to the other groups. The total neutral lipids levels were mainly due to the levels of TAG and FFA. FFA accumulation is typically a result of enzymatic hydrolysis of TAG by lipolytic enzymes such as lipases and phospholipases, which are known to be highly active in fish liver tissue (Jangaard & Power, 1966). Given the high TAG content observed, it is likely that TAG was the main source of FFAs, although some contribution from phospholipid breakdown cannot be excluded. The levels of FFA are similar across groups in this study; however, they are higher than the FFA levels of lumpfish livers reported by Eliasen et al. (2020) (on average 21% in this study compared to 3% in Eliasen et al. (2020), suggesting that oxidation may have occurred due to the way samples were collected and processed. In this study, liver samples were collected post-mortem during sampling and stored at –20 °C until further analysis. However, it is possible that delays in freezing during early handling could have caused insufficient inhibition of enzymatic activity causing lipolysis. In future work, lipid preservation could be improved by flash-freezing samples in liquid nitrogen immediately after dissection, storing at –80 °C, or by inactivating enzymes through chemical inhibition (e.g. chloroform:methanol).

Wild fish had lower levels of TAG in their livers compared to the land-based fish and this agrees with the study by (Cejas et al., (2004), where captive white seabream (*Diplodus sargus*) had significantly higher amounts of TAG in both liver and muscle compared to the wild counterparts. TAG serve as fat reserve in the liver and when levels are very low, they are an indicator of low lipid deposition or starvation (Eliasen et al., 2020). Wild fish had on average 27% of TAG and this does not indicate poor lipid reserves. In comparison, Eliasen et al. (2020) reported TAG levels of approximately 16.2 ± 8.6 % in starving lumpfish that also showed very dark reddish-brown livers.

The TAG levels of the livers from lumpfish from the sea cages showed a higher variability that can be due to different nutritional statuses of the fish in the cage environment caused by size differences and food preferences. The fish with liver score 5 or 6 had low levels of TAG, indicating a poor nutritional status, and these levels are in agreement with the observations by Eliasen et al. (2020).

4.5.4. Liver colour

Liver colour is an indicator of welfare and nutritional status of lumpfish deployed in the sea cages, as the different colour are primarily influenced by different levels of carotenoids and lipid content (Eliasen et al., 2020).

Liver pigmentation was mainly due to high levels of astaxanthin, followed by small quantities of lutein, astacene and canthaxanthin. High levels of astaxanthin were also identified by Eliasen et al. (2020), reporting values of 85.4 ± 55.9 mg/kg in healthy livers of lumpfish deployed in the sea cages.

In aquatic ecosystems, microalgae naturally produce astaxanthin, which is then consumed by zooplankton or crustaceans. Fish subsequently ingest zooplankton, acquiring their natural coloration through this dietary intake (Higuera-Ciapara et al., 2006).

In farming systems, salmon feed used in the sea cages contains a considerable amount of astaxanthin (approximately 100 mg/kg of feed), while the lumpfish feed used in the hatcheries and sea cages had a lower amount (approximately 36-38 mg/kg of feed). Astaxanthin is added to salmon feeds to enhance the appealing pink-red colouration of salmon fillets, as consumers associate this pigmentation with freshness, quality, and nutritional value. Additionally, astaxanthin serves an important physiological role due to its antioxidant properties, supporting the immune system of farmed salmon.

Lumpfish from the land-based hatcheries not only had measurably lower levels of carotenoids in their livers, but these differences were also visually apparent, with hatchery lumpfish having paler livers. This variation may be related to the life stage of the lumpfish. Specifically, hatchery-reared lumpfish weighing less than 50 g were found to have a 16% lower likelihood of exhibiting an orange liver, compared to their larger counterparts (50-150 g). The hatchery-reared fish also had less astaxanthin accumulation in the liver due to feed provided in the hatcheries low in astaxanthin, reported in Table 3.2. in Chapter 3. Also, a paler liver is not necessarily a sign of bad nutritional status. However, a sudden appearance of pale livers in captive lumpfish population is an early indicator of disease outbreak (Imsland et al., 2022). In addition, diseases, particularly those affecting the liver or the immune system, might alter how astaxanthin is processed and stored (Chang & Xiong, 2020).

The liver colour of wild lumpfish in this study was mainly bright orange with high levels of astaxanthin. Wild lumpfish feed mainly on copepods as well as shrimp and krill that contain considerable amounts of astaxanthin (Lambertsen & Braekkan, 1971). The pale livers (score 1) reported in the wild group in Figure 4.6 were scored in large mature female lumpfish that were about to spawn. During the breeding season, female lumpfish mobilise their fat reserves (Craig et al., 2000) and deposit their pigments in the roe, resulting in very pale-yellow livers and bright roe, which ranges in colour from purple to red, and orange (Passantino et al., 2024).

In the sea cages, lumpfish had more variable shades of liver score as shown in Figure 4.6, reflecting a higher variability in the nutritional status of these fishes, and this was also found in the study by Eliasen et al. (2020). Fish that had very dark and reddish livers (6 and 5) had very low total lipids in the liver

and very low levels of TAG, indicating malnutrition as shown in Figure 4.8, while fish with orange and bright orange liver (3-4) had the highest levels of lipid and TAG, followed by lower levels in paler ones (2-1). This was also shown by Eliasen et al. (2020) who also described how fish with pale livers also had skin injuries and scored poorly on other welfare indicators. This suggests that fish with dark livers containing very low levels of lipids have a generally compromised health status.

Generally, the liver colours observed in this study were mostly healthy regardless of origins. A liver score of 3 and 4 accounted for at least 75% of the land-based hatcheries. The samples that scored 1 or 2, though they might come across as less than optimal, may be an indication for low levels of pigments in the feed delivered in the hatchery, but may also be related to life stage, as shown in Figure 4.7.

Despite most of the livers from the sea cages scoring a 3 or 4 (83 %), six percent indicated poor nutritional status. This can be caused by several factors, such as non-optimal feeding, exposure to currents and waves, lack of shelters and inadequate acclimatisation to the sea cages leading to poor feeding. Larger fish had healthier liver colours more frequently as shown in Figure 4.7. This could be because fish with poor liver colours, possibly indicating poor nutrition, might struggle to find feed and therefore may not survive. Also, larger fish have access to salmon feed which is rich in astaxanthin (Imsland et al., 2015).

4.5.5. HSI

Wild fish had proportionally larger livers, resulting in higher HSI levels compared to the fish from the hatcheries and farms. The HSI is an indicator of the energy reserves stored in the liver of the fish (Hismayasari et al., 2015) and other authors have reported similar values of HSI in lumpfish: 0.9-1.5 (Imsland et al., 2022) in sea caged fish, 2.2-3.5 in land-based hatchery, and 2.2-2.5 in wild fish by Passantino et al. (2024). On the contrary, the higher HSI levels found in wild lumpfish contradicts the results by Passantino et al. (2024) where wild lumpfish had a significantly lower HSI compared to the hatchery-produced ones. In our study this may be caused by the higher body weight and age of the wild fish sampled compared to the farmed groups (sea cages and land-based). The variation of HSI in hatchery fish was smaller than in the other groups, due to the similarity in weight and feeding.

It is difficult to assess the reasons for the remainder of the liver colours and difference in HSI as one limitations of this study is the low sample size and the size class of the wild fish sampled (Table 4.1) where all wild fish were sampled in the summer, showing a consistent body composition in terms of macronutrients and micronutrients.

4.6. Conclusion

The analyses of livers reflected the feeding habits of the fish, where fish from the hatchery and the wild are the more different ones between them. Fish from the sea cages have intermediate values between wild and hatchery due to the access to both feeds and seasonal prey.

The differential levels of TAG and lipid content observed across the groups point to varying energy reserves in the liver, which are critical for lumpfish survival. Farmed fish from land-based hatcheries had higher lipid content in their livers and elevated levels of TAG, indicating a diet high in lipid and the effect of the tank rearing environment. The high fat storage of farmed fish from land-based hatcheries suggest they have good energy storage and appear ready for deployment. However, the high mortality rates observed post-deployment indicate that the hatchery diet may not adequately prepare lumpfish for the challenges of the sea cage environment.

Also, liver colour in the hatchery fish was affected by the low levels of astaxanthin in the hatchery feeds, reflecting the limitations of their feed in accessing this dietary component.

High levels of astaxanthin in wild lumpfish correlate with their varied diet, rich in natural sources of this carotenoid. The variation in liver colour, particularly the darker liver colours in some fish from the sea cages which results also in lower levels of lipid and TAG, is associated with poor nutrition. Lumpfish in the sea cages need to have an adequate nutrition throughout the deployment to be able to be robust and delouse efficiently when deployed in the sea cages.

Consequently, this study underscores the necessity of optimised nutritional strategies for lumpfish in different rearing environments. Diets for juvenile lumpfish should be formulated to mirror their natural diet, including lower lipid content (10-15%), sufficient levels of TAG for energy reserves, and an adequate supply of carotenoids, especially astaxanthin (≥0.05% of feed).

Chapter 5. Assessment of the impact of farming on Operational Welfare Indicators (OWI) and histological parameters of juvenile lumpfish (*Cyclopterus lumpus*): a comparison with the wild populations.

5.1. Abstract

The use of lumpfish as a cleaner fish in Atlantic salmon farming has become a focal point due to high mortality rates and poor welfare during the deployment phase, raising both ethical and economical concerns. This study aimed to assess the health and welfare of farmed lumpfish, in comparison to their wild counterparts, using Operational Welfare Indicators (OWI) like fin damage, skin status, eyes integrity, sucker disc deformities and Welfare Indicators (WI) such as liver colour score, as well as histomorphometry of liver, assessing parameters such as intracytoplasmic vacuolisation, inflammation, congestion, fibrosis or necrosis. The present study revealed substantial differences in the different parameters evaluated, that highlight the impact of farming systems in lumpfish welfare, compared to a wild environment. Farmed fish showed the highest prevalence and severity in fin damage mainly in the sea cages, possibly due to aggression in the hatcheries and mechanical damage in the sea cages. Wild lumpfish exhibited better welfare in terms of OWI scores as they scored better in all indicators measured. Liver intracytoplasmic vacuolisation was the highest in fish from the hatcheries, reflecting the diet composition and the rearing environment. In the sea cages, liver intracytoplasmic vacuolisation varied greatly suggesting a range of nutritional status, with an effect of seasonality. In addition, other factors or conditions in sea cages might expose fish to liver damage that lead to the development of fibrosis and necrosis, maybe due to long-term stressors. These findings underscore the need for regular monitoring of welfare parameters in lumpfish in the farms, particularly for non-lethal parameters like fin damage and skin integrity. The liver score index, which requires sacrificing the fish, could be used as a secondary WI if needed. In the hatcheries strategies can be implemented to minimise fin damage, by reducing densities, ensuring appropriate feeding regimes and providing enough shelter space to avoid fin nipping. In the sea cages, it would also be appropriate to avoid the deployment of lumpfish in sites with strong currents as well as lumpfish being subjected to mechanical delousing.

Keywords: OWIs, histological assessments, body condition, fish welfare

5.2. Introduction

The use of cleaner fish in salmon farming is hampered by poor health and welfare (Marcos-Lopez et al., 2017). The main concern regarding the welfare of cleaner fish, both lumpfish and ballan wrasse, is the high mortality rates after their deployment in salmon cages (Geitung et al., 2020; Reynolds et al., 2022). Specifically, mortality of lumpfish has been reported to be very high in Norway, Scotland and the Faroe Islands, raising ethical and economical concerns (Marine Directorate, 2023; Røsaeg & Colquhoun, 2014). A recent study by Reynolds et al. (2022) investigated the main causes of mortalities of lumpfish from three different locations in Norway, where handling and grading of salmon represented the highest percentage (21.2%), followed by mechanical delousing (19.7%) and bacterial infections (16.7%). Each of these identified causes can expose the fish to skin injuries and secondary infections, impacting the welfare and overall health of the fish as well as compromising survival rates.

Lumpfish are susceptible to a range of bacterial diseases, including atypical *Aeromonas salmonicida*, *Moritella viscosa*, *Pasteurella* sp., *Pseudomonas anguilliseptica*, *Tenacibaculum maritimum*, *Vibrio anguillarum*, and *Vibrio ordalii*. These bacterial infections have been identified as primary health challenges across lumpfish production (Marcos-López et al., 2017). Although vaccines exist for a number of these disease, their effectiveness varies, highlighting the challenges in developing efficient vaccines for this species (Erkinharju et al., 2017; Onireti et al., 2023). Furthermore, a poor nutritional status can result in a weakened immune system, enhancing the susceptibility to diseases (Han et al., 2021).

Regular monitoring in the farms allows farmers to track the overall welfare status of the fish and gives them the opportunity to react to potential welfare problems that may occur if the welfare status on the farm deteriorates (Garcia de Leaniz et al., 2022). Operational welfare indicators (OWI) are practical indicators, easy to use, that can be applied by the farmers and implemented in husbandry protocols to assess welfare. However, each species requires a set of OWI to meet the species-specific welfare needs of the fish, as well as the life stage and the farming system (van de Vis et al., 2012). The use of OWI specifically developed and validated for lumpfish is essential (Noble et al., 2019). Other farmed species such as Atlantic salmon or rainbow trout have well established OWIs (Noble et al., 2018, 2020), while lumpfish farming is a fairly new field due to the early nature of its cultivation which only started in 2013 (Treasurer, 2018). Though several studies have identified species-specifics OWI for lumpfish Boissonnot et al., 2022; Eliasen et al., 2020; Gutierrez Rabadan et al., 2021; Imsland et al., 2020; Noble et al., 2019), they are not standardised among farmers and stakeholders, implemented in the guidelines, and regularly used in the farms. In UK, the new version of the RSPCA Assured standards were expanded to address the welfare of cleaner fish, by including the assessment of the potential risks of treatments, mandatory documentation and analysis of all causes of mortality, ensuring ample shelters and appropriate feeding, and lowering the density of fish during transport (RSPCA, 2024). In the Faroe Islands, as part of lumpfish health monitoring in the salmon sea cages, lumpfish are assessed for OWI,

liver colour, stomach content and signs of disease(Eliasen et al., 2018; Eliasen et al., 2020; Østerø & Eliasen, 2023).

Evaluating welfare comprehensively, by including also health parameters changes, is beneficial, as it can shed light on various aspects of the welfare status of farmed fish (Segner et al., 2012). While OWI are non-destructive and non-invasive (Barreto et al., 2022), the use of histological tools normally requires the sacrifice of the animals. Liver, together with gills, kidney and skin are the primary tissues used when assessing health status of a fish (Bernet et al., 1999; Ytteborg et al., 2023). Histological evaluation of liver also provides valuable information related to fat deposition, providing insights regarding the nutritional status of the fish (Eliasen et al., 2020).

To the best of our knowledge, the welfare status of wild lumpfish, compared to the farmed counterparts has not been thoroughly explored. The hypothesis is that wild lumpfish will have fewer occurrence of fin damage and other injuries related to handling and farming than farmed lumpfish. However, wild lumpfish will have higher prevalence of starvation or parasite related health problems.

The aim of this study was to evaluate the welfare status of lumpfish from different origins in the Faroe Islands: from hatcheries, deployed in the sea cages, and from the wild populations. Comparisons were made using OWI and histological assessments of tissue samples.

5.3. Materials and Methods

5.3.1. Ethical statement and fish origin

Lumpfish for this study were sourced in the Faroe Islands. Sampling procedures for the fish were conducted according to the Directive 2010/63/EU regarding the protection of animals for scientific purposes, approved by the head veterinarian "Landsdjóralæknin" in according to the Animal Welfare act 2018, 10 (DJÓRAVÆLFERÐARLÓGIN - Løgtingslóg 49 apríl 30 2018, Faroe Islands) and were also reviewed and approved by the Animal Welfare and Ethical Review Body of the University of Stirling (AWERB 19 20 007).

Lumpfish were sampled from two origins (farmed and wild): juvenile lumpfish from land-based hatcheries, lumpfish deployed in Atlantic salmon sea cages, and lumpfish from the wild populations.

Sixty pre-deployment lumpfish (34.4 ± 9.4 g) were sampled from two lumpfish hatcheries (Nesvík and Svínoy, Faroe Islands), whereas 334 deployed lumpfish (93.6 ± 27.3 g) were sampled from seven salmon farming sites. These fish were harvested from the sea cages using a dip net and they were sampled during the health monitoring check at Firum (Faroe Islands), where regular monitoring of deployed lumpfish was carried out at the sea cages farming sites. To investigate whether seasonality might affect the lumpfish deployed in sea cages, these fish were sampled during different seasons.

Wild populations (26.5 ± 24.9 g) were sampled (162 fish) from a seaweed farm and from the annual pelagic research survey conducted by Havstovan during spring and summer months (Faroe Islands) (Table 5.1). More detailed regarding the sampling locations can be found in Figure 3.1.

Pre-deployment lumpfish ranged from 25 to 35 g, while fish from the sea cages ranged from 27 g to 1 kg. Wild lumpfish varied a lot in size, ranging from < 50 g to over 1 kg. Only eight wild fish were within the weight range 50-150 g. To have a more homogenous comparison in terms of size class, lumpfish that weighed < 150 g were analysed for this study (n = 294).

Table 5.1. Summary of number of fish sampled per category (land-based, sea cage, wild) and size class (< 50g, 50-150 g, 150-300 g, 300g-1kg, 1-3 kg, 3-5 kg).

Category	**< 50 g**	**50-150 g**	**150-300 g**	**300-1 kg**	**1-3 kg**	**3-5 kg**
Land-based	60	0	0	0	0	0
Sea cage	7	157	70	98	2	0
Wild	62	8	0	34	58	7

5.3.2. Morphometric data and Operational Welfare Indicators (OWI) scoring

During each sampling of captive fish, the fish were netted and euthanised using an overdose of Finquel (0.8 g/L, MS-222, MSD Animal Health). Wild fish were euthanised through exsanguination via a gill cut and frozen at -20 °C. Each fish was weighed out to the nearest gram and measured to the nearest millimetre. The total length of the fish (in cm) was measured from the snout to the final part of the lobe of the tail as detailed in Figure 2.1. These measurements were used to calculate the predicted weight (W_s) to a certain length (L), according to the length-weight relationship: $Ws = a \times L^b$ (Keys, 1928). The coefficients a (-1.477 ± 0.023 SE) and b (3.094 ± 0.019 SE) were obtained from fitted log-log regression on wild lumpfish data from Østerø et al. (2024).

This approach better reflects the natural growth patterns and may provide a more accurate benchmark for assessing the condition of lumpfish in salmon cages (Østerø et al., 2024).

The body condition of individual lumpfish was evaluated by determining the relative weight (W_r), expressed as a percentage, according to the following formula: $Wr = \left(\frac{W}{Ws}\right) \times 100$ (Blackwell et al., 2000), where W is the actual weight of the fish and W_s is the predicted weight. When W_r > 90%, fish were in good body condition, when W_r = 75-90%, they were underweight, and when W_r < 75%, fish were emaciated (Gutierrez Rabadan et al., 2021; Noble et al., 2019)

Each fish was also scored for OWI which were fin damage (dorsal, anal and caudal fin), skin status, eyes integrity, sucker disc deformities and liver colour according to the method developed by Eliasen et al. (2020). The liver colour results are reported in Chapter 4.

5.3.2.1. Fin damage

Fin damage (dorsal, anal and caudal) in lumpfish is assessed using a scoring system from 1 to 3. Score 1 indicates no visible damage, with fins showing smooth edges, intact rays, and healthy membranes and bases. Score 2 reflects minor damage, evident from irregular fin edges, small incisions, and noticeable rips in the membrane. Score 3 signifies severe damage, with substantial parts of the fin missing, disrupted shape, potential infection signs, fragmented membranes, and impaired fin mobility affecting swimming and balance. A detailed description of the fin damage scoring is in Chapter 2 (Section 2.2.1, Table 1-3).

5.3.2.2. Skin status

Skin integrity in fish is scored from 1 to 3. Score 1 represents healthy skin with no discoloration or abnormalities. Score 2 indicates moderate damage, visible through discoloration, redness, or swelling around orifices, suggesting potential infection. Score 3 is assigned for severe skin damage, characterized by open wounds, unusual secretions, pus, and clear signs of ongoing infection. A detailed description of the skin status scoring is in Chapter 2 (Section 2.2.2, Table 4).

5.3.2.3. Eyes integrity

Eye damage in fish is assessed with scores from 1 to 3, based on severity. Score 1 indicates both eyes are healthy with no cloudiness or spots. Score 2 is assigned when one eye shows symptoms like cloudiness, discoloration, lesion or swelling, while the other remains healthy. Score 3 reflects severe damage in both eyes, evident through cloudiness, discoloration, lesions or swelling, suggesting serious conditions like infections or cataracts. Only advanced cataract stages are recorded due to difficulty in early detection. A detailed description of the eyes´ integrity scoring is in Chapter 2 (Section 2.2.3, Table 5).

5.3.2.4. Sucker disc deformities

The sucker disc in fish is rated from 1 to 3 based on deformities. Score 1 indicates a symmetrical disc with effective ridges for attachment and consistent colour. Score 2 represents slight asymmetry, size irregularities, disrupted ridge patterns, or initial discoloration. Score 3 denotes severe deformation with compromised shape and ridges, impairing the ability to adhere to surfaces, and may include signs of infections or abrasions (Chapter 2, Section 2.2.4, Table 6).

5.3.2.5. Liver colour

Liver colour was scored from 1-6 using the method developed by Eliasen et al. (2020). Livers scored 1 and 2 displayed a pale and yellow coloration, while liver 3 and 4 ranged from orange to bright orange. Livers that were reddish brown and dark brown were scored 5 and 6 (Chapter 2, Section 2.2.5, Table 7).

5.3.3. Histological analysis and scoring system

After measuring the fish and scoring for OWI, the most external part of the liver lobe was sampled, measuring approximately 1 cm in both length and height (Figure 5.1). These samples of livers were fixed in 10% neutral buffered formalin (NBF) and processed for histological analysis. The tissues were dehydrated through a graded series of alcohol, using chloroform as clearing agent and infiltrated with paraffin wax (Citadel 2000, Thermo Fisher Scientific, USA). After being embedded in paraffin wax, the paraffin blocks were trimmed at 20 µm thickness on a microtome (Leica RM 2035, Leica Instruments, Germany) to expose the surface of the tissues. After trimming, blocks were submersed in distilled water for 20 mins, before sectioning. Sectioning was performed at 5 µm thickness on the microtome and slides were stained with haematoxylin and eosin (H&E) (Martoja, 1970). Stained slides were scanned (AxioScan, ZIESS®, Germany) and uploaded to QuPath® 0.4.2 (Bankhead et al., 2017). An average of 5 images at x10 were selected from the livers and analysed for lipid intracytoplasmic vacuolization using Fiji ImageJ® (National Institute of Health, Maryland, USA). Pictures were

converted to black and white, using an 8-bit conversion, a threshold was applied to separate the intracytoplasmic vacuoles from the background and watershed separation to separate connected components. In each picture, intracytoplasmic vacuoles were counted and reported as percentage (% of the total area of the picture).

The liver was also examined for presence of inflammation, congestion, fibrosis and necrosis using a semi-quantitative scoring system. Each of these parameters was scored from 0 to 3, as shown in Table 5.2. Congestion in the liver refers to an accumulation of blood, which results in dilated vessels filled with blood in histological slides. Inflammation in fish liver represents the liver reaction to injury, infection or toxic compounds (Seki et al., 2009). Necrosis can be a result of long-term stressors, including infectious diseases, toxins or poor environmental conditions (Schwaiger et al., 1997). Fibrosis can be an indication of chronic injury or ongoing inflammation as it is a reparative response to chronic damage that leads to fibrous bands or scar tissue, disrupting the normal architecture of the liver (Cao et al., 2023).

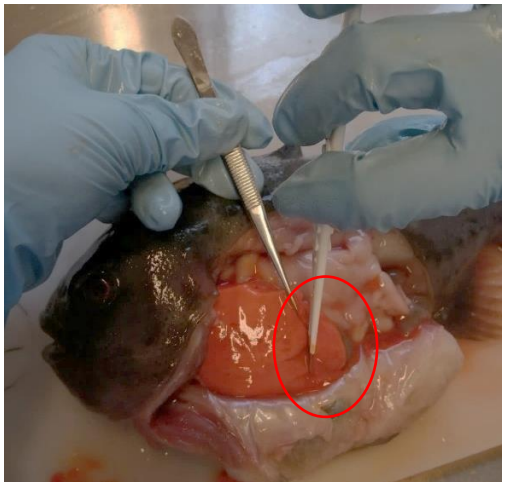


Figure 5.1. Circled area indicates the most external part of the liver lobe sampled and fixed in 10% neutral buffered formalin (NBF) for histological analysis.

Table 5.2. Semi-quantitative scoring system used for assessing signs of inflammation, congestion, fibrosis and necrosis in livers from lumpfish from different origins (land-based hatcheries, sea cages and wild).

Score (qualification)	Description
0 = Absent	No signs of inflammation/congestion/fibrosis/necrosis
1 = Mild	One area shows sign of inflammation/congestion/fibrosis/necrosis
2 = Moderate	2-3 areas are affected by inflammation/congestion/fibrosis/necrosis
3 = Severe	>3 areas are affected with inflammation/congestion/fibrosis/necrosis

5.3.4. Statistical analyses

Percentage data such as liver vacuolization and relative weight were transformed using the arcsine transformation from Zar (2014). A simple linear model was performed with relative weight (W_r) as response variable. The categorical predictor was the origin of the fish which was divided in three groups: two groups from the farmed origin, sea cage and land-based hatcheries; one group from the wild (e.g. lm(W_r ~ group)). They were used to investigate whether there are significant differences between wild, sea cage and land-based lumpfish in terms of body condition. Model diagnostics were carried out using visual inspection of model plots and untransformed data were used for the linear models reported here. When the linear model showed a significant effect ($P < 0.05$), a post hoc Tukey HSD test was performed to identify differences between groups. F-statistic and p-values were reported from the linear model summary table and values having high Cook's distance scores were considered outliers and checked through the Residuals vs Leverage plot.

A categorical Principal Component Analysis (PCA) was performed using the "Gifi" package (Mair et al., 2019) to visualise and identify patterns regarding OWI. To maintain consistency in the data scaling across different OWI in the PCA, the scoring scale was adjusted for liver score. The liver score, which was originally rated on a 1 to 6 scale, was shortened to a 1 to 3 scale as it was used for the other OWI. This transformation ensured that each variable contributed equally to the PCA, eliminating potential biases from differing scales. The transformation process also involved assigning scores in a different order as the original scale was not ordered according to health status. Therefore, on the new scale, score 1 (good welfare, healthy liver colour) was assigned to the livers that previously scored 3 and 4 (light to bright orange), score 2 (pale liver, compromised welfare) to the livers that scored 1 and 2 (pale yellow), and score 3 (starvation and malnutrition) to the livers that scored 5 and 6 (dark and reddish brown). The outputs of the PCA were visualized using "ggplot2" (Wickham et al., 2016).

An ordinal logistic regression analysis to investigate the effect of origin of the fish on fin damage was conducted, using the package MASS (Ripley et al., 2013). The models were performed with fin damage (dorsal, anal and caudal) as response variables, and origin of the fish divided in three groups (sea cage, land-based, wild) as categorical predictor (e.g. polr(Dorsal fin~ Group, data = owi150, Hess = TRUE)). A null model that only contains an intercept and lacks any predictors was also established as a baseline (e.g. nullmodel <- polr(Dorsal fin~ 1, data = owi150, Hess = TRUE). A likelihood ratio test was employed to compare the full model with the null model and a significant p-value from this test would indicate that the model provides a better fit to the data than the null model, suggesting that there is an effect of origin of the fish on the OWI considered. If the p-value is significant ($P < 0.05$), a post-hoc analysis was performed using Tukey's method for pairwise comparisons to further discern differences between the different groups (land-based, sea cages, wild) in relation to the OWI.

A binomial logistic regression analysis was conducted to investigate the effects of origin of the fish (3 groups: sea cage, land-based, wild) on skin status, eyes, and sucker disc deformities as for these OWI,

there was no fish that had a score 3 (high damage) (e.g. glm(formula = Eyes ~ Group, family = binomial, data = owi150).

Regarding the histological parameters, a linear model was conducted using liver intracytoplasmic vacuolization as response variable and origin of the fish as categorical predictor, divided in three groups: land-based, sea cages and wild. A further linear model was conducted using liver intracytoplasmic vacuolization of fish from the sea cages as response variable, and season as categorical predictor. This was used to investigate whether there is a significant effect of seasonality on liver intracytoplasmic vacuolization. The effect of seasonality was analysed only in the fish from the sea cages as wild fish were caught only in summer, and there is no effect of season in the land-based hatcheries which use a controlled environment throughout production. Untransformed data were used for these models. When the linear model showed a significant effect ($P < 0.05$), a post hoc Tukey HSD test was performed to identify differences between groups. F-statistic and p-values were reported from the linear model summary table and values having high Cook's distance scores were considered outliers and checked through the Residuals vs Leverage plot.

An ordinal logistic regression analysis to investigate the effect of origin of the fish on histological parameters too. The models were performed with liver inflammation, congestion, fibrosis and necrosis as response variables, and group (sea cage, land-based, wild) as categorical predictor. A null model that only contains an intercept and lacks any predictors was also established as a baseline as explained above. A likelihood ratio test was employed to compare the full model with the null model and a significant p-value from this test would indicate that the model provides a better fit to the data than the null model, suggesting that there is an effect of origin of the fish on the histological parameter considered. If the p-value is significant ($P < 0.05$), a post-hoc analysis was performed using Tukey's method for pairwise comparisons to further discern differences between the different groups (land-based, sea cages, wild) in relation to the histological parameters assessed.

Data were analysed using R (R Core Team, 2021) and figures were plotted using "ggplot2" (Wickham et al., 2016), which is part of the "tidyverse" collection of R packages (Wickham et al., 2019). Excel data files were imported into R using the "readxl" package (Wickham & Bryan, 2019).

5.4. Results

The body condition was significantly different among the three groups ($F_{2,170} = 14.59$, $P < 0.001$), being higher in the land-based fish, followed by the wild, and lastly sea cage fish. Frequencies of lumpfish body condition are reported in Table 5.3 and the distribution of the relative weight (W_r) in Figure 5.2. The highest number of fish in good condition (85%, 51 out of 60) was observed in land-based hatcheries, where only 2% of the fish were found to be emaciated (1 out of 60) and 13% underweight (8 out of 60). The lowest % of fish in good condition (53%, 175 out of 331) and the highest % of emaciated fish (25%, 83 out of 331) were found in sea cages where 22% were underweight (73

out of 331). Overall wild fish were in good condition (68%, 110 out of 162) and only 6% (10 out of 162) were emaciated and 26% underweight (42 out of 100) (Table 5.3).

Table 5.3. Frequencies of lumpfish body condition from different origins (land-based hatcheries, sea cages and wild) according to their relative weight (W_r). When W_r > 90% fish were in good condition; 75-90% underweight, and < 75% emaciated.

W_r	**land-based (n=60)**	**Sea cage (n=331)**	**Wild (n=162)**
Good condition			
> 90%	85%	53%	68%
Underweight			
75 - 90%	13%	22%	26%
Emaciated			
< 75%	2%	25%	6%

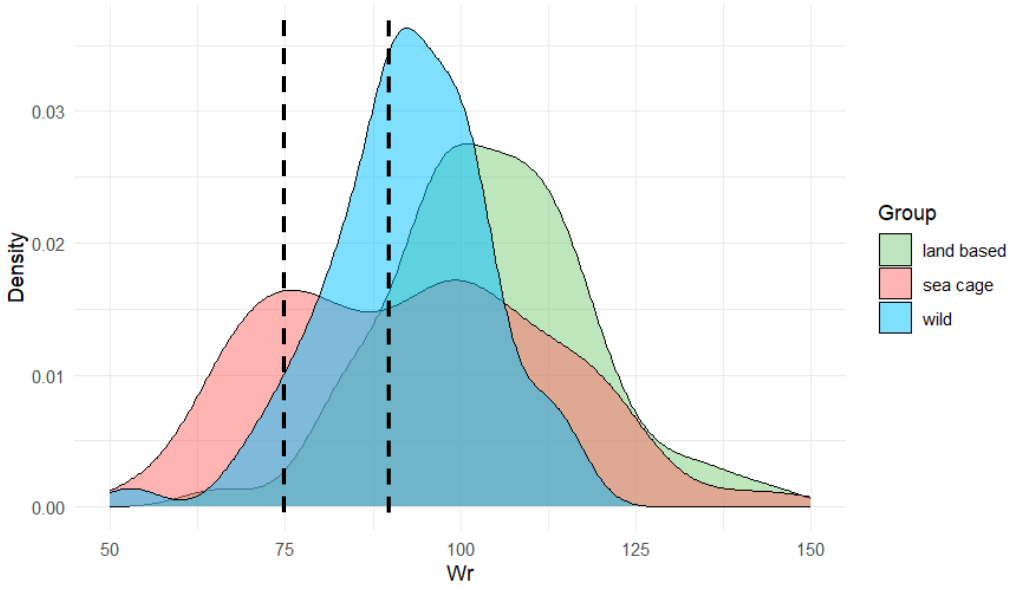


Figure 5.2. Distribution of lumpfish relative weight (W_r) from different groups (land-based hatcheries, sea cages and wild). Dotted lines indicate when W_r < 75%, fish were emaciated; 75-90% underweight, and > 90% fish were in good condition.

Principal component analysis (PCA) was used to identify the parameters that contributed to the highest variability in the OWI scored (Figure 5.3). PC1 accounted for 30.4% of the variation which was due to anal fin damage (-0.88), caudal fin damage (-0.79), and dorsal fin damage (-0.72). PC2 accounted for 17.6% of the variation which was associated with skin status (-0.76) and liver colour (-0.74). Damage in the dorsal, caudal and anal fin were positively correlated, as well as skin status and liver colour (Figure 5.2). However, the combined variance explained by the first two dimensions of the PCA is only 48%, indicating that there could be other factors affecting the OWI that are not captured by the first two components of the PCA. Damage in the fins was the OWI that showed the highest variability as indicated by the PCA (Figure 5.3). The differences between groups are shown in Figure 5.4.

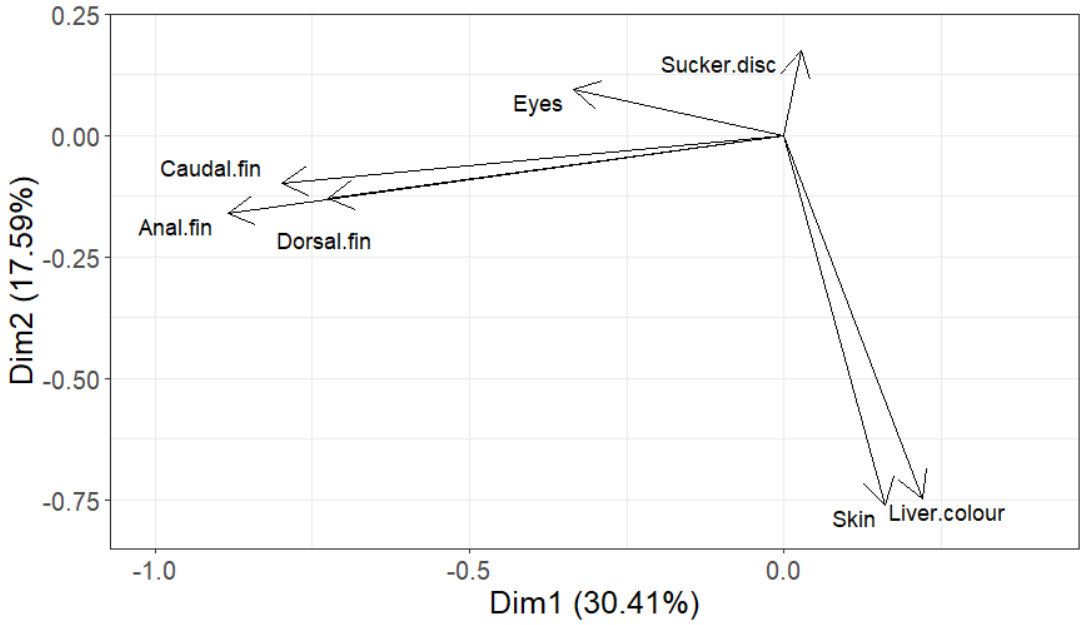


Figure 5.3. Principal Components Analysis biplot of Operational Welfare Indicators (OWI) of lumpfish showing separation of individuals depending on their origin (land-based hatchery, sea cage and wild) and relative influence of different parameters.

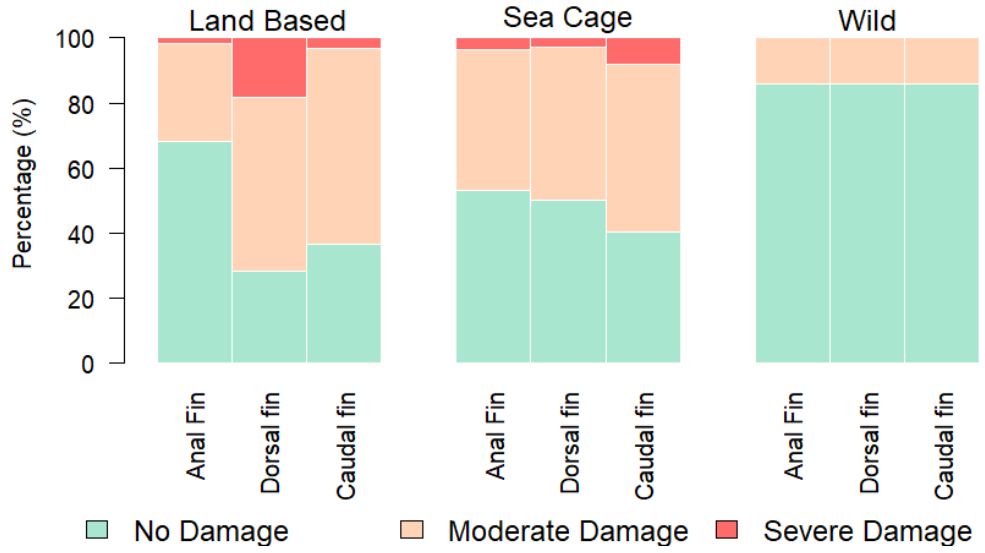


Figure 5.4. Percentage distribution of fin damage in lumpfish < 150 g from different origins (land-based, sea cage and wild). Score 1 indicates no damage, score 2 indicates moderate damage, and score 3 indicates severe damage.

Origin significantly affected all fin damage scores: anal (DF=2, χ^2=13.56, P = 0.001), dorsal (DF=2, χ^2=33.29, P < 0.001), and caudal (DF=2, χ^2=23.32, P < 0.001) (Figure 5.4). Wild fish had the best fin condition overall, with 86% (70 out of 81) showing no damage and none displaying severe damage in any fins.

Lumpfish from the sea cages had a statistically significant higher likelihood of experiencing damage to their anal fin compared to fish from the wild (estimate = 2.03, SE = 0.38, P < 0.001). However, no significant difference was found when comparing land-based fish to the sea cages ones (estimate = -0.68, SE = 0.31, P > 0.05).

In lumpfish from the land-based hatcheries, 18% (11 out of 60) had severe damage in the dorsal fin, whereas the fish from the sea cages had a lower prevalence, with only 3% having severe damage (7 out of 242). Lumpfish from both the land-based hatcheries and the sea cages are more likely to have higher dorsal fin damage scores compared to those from the wild (estimate = 2.59, SE = 0.397, P < 0.001), with the land-based hatcheries lumpfish showing a more pronounced effect (estimate = 1.18, SE = 0.305, P < 0.001).

Both the fish from the land-based hatcheries and from the sea cages have a significantly higher likelihood of experiencing damage to their caudal fin compared to the wild fish, while no difference was found in caudal fin damage between land-based and sea cage fish (P = 0.998).

The other OWI scored such as eyes integrity, skin status and sucker disc deformities showed better trends in severity, compared to fin damage. Eye, skin, and sucker disc scores were all very low regardless of fish origins with no statistical differences found (P > 0.05, Figure 5.5).

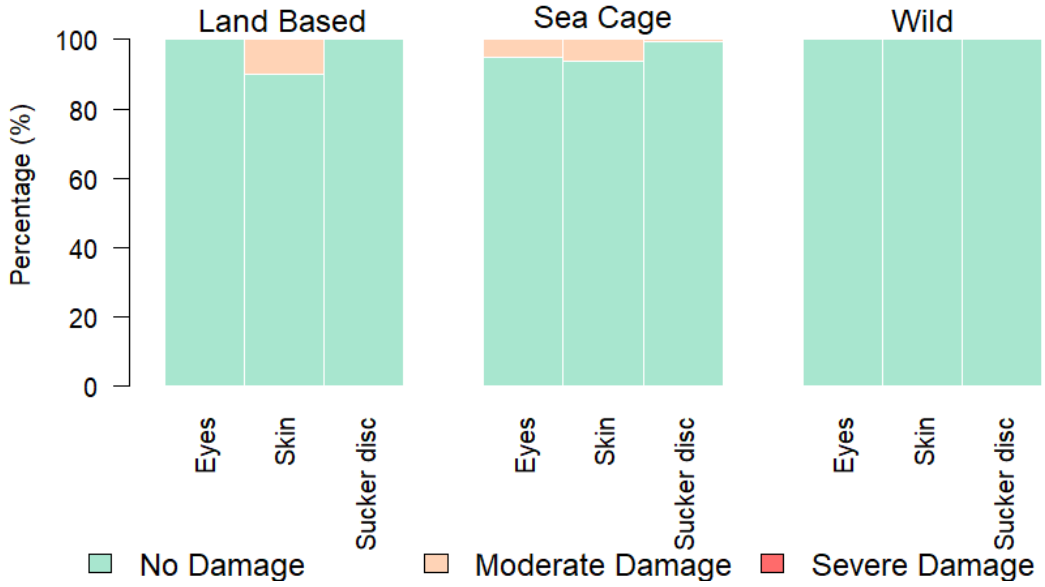


Figure 5.5. Percentage distribution of eyes, skin status and sucker disc deformities of lumpfish from different origins (land-based, sea cage and wild). Score 1 indicates no damage, score 2 indicates moderate damage, and score 3 indicates severe damage.

5.4.1. Histological results

The origin of the fish influenced the liver intracytoplasmic vacuolization in the livers of lumpfish ($F_{2,52}$=11.52, P < 0.001), being higher in the land-based hatcheries fish than in the sea cage or wild ones (Figure 5.6). Liver vacuolization on average was 25.3 ± 2.6 % in the land-based hatcheries (n=10), while the wild fish had a liver vacuolisation of 13.4 ± 2.4 % (n=18). Lumpfish from the sea cages had a higher variation in liver intracytoplasmic vacuolisation, ranging from 1.1 to 24% (average 12.6 ± 7.4 %, n=59). The effect of the season on liver vacuolization was also investigated in the case of the fish from the sea cages, and a significant effect was found ($F_{3,55}$=11.92, P < 0.001). Liver vacuolization was higher in autumn and winter and significantly lower in spring and summer (Figure 5.7).

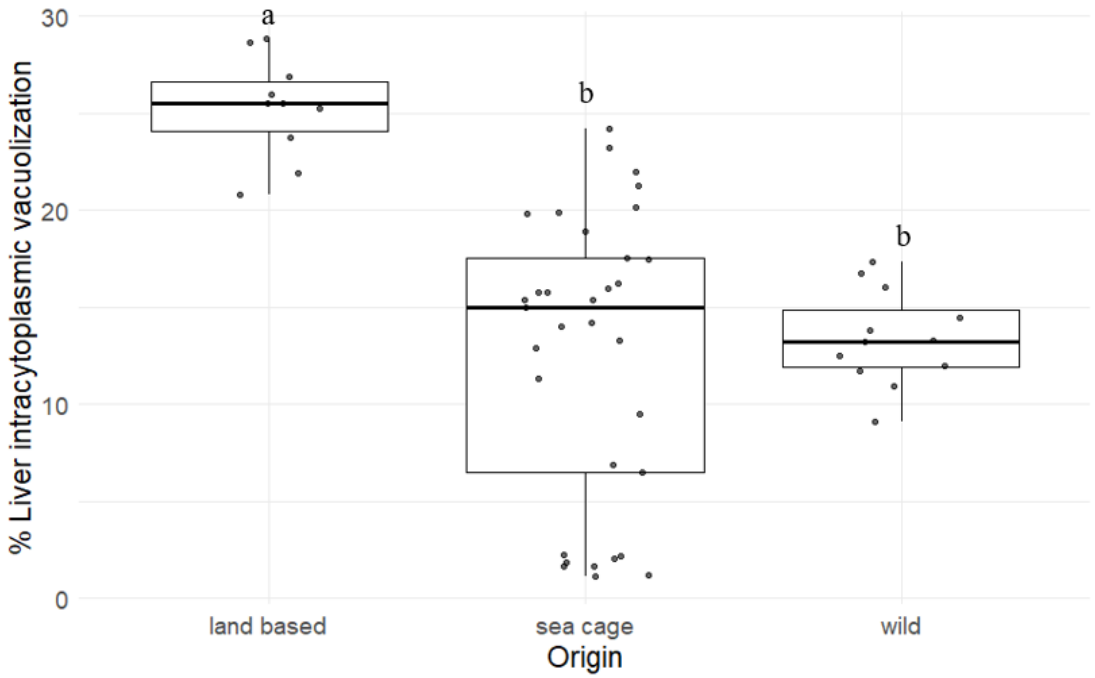


Figure 5.6. Percentage of liver intracytoplasmic vacuolization of lumpfish from different origins. Boxes and whiskers represent quartiles for each treatment group. Different superscript letters denote differences in body condition among the groups according to one-way ANOVA ($P < 0.05$).

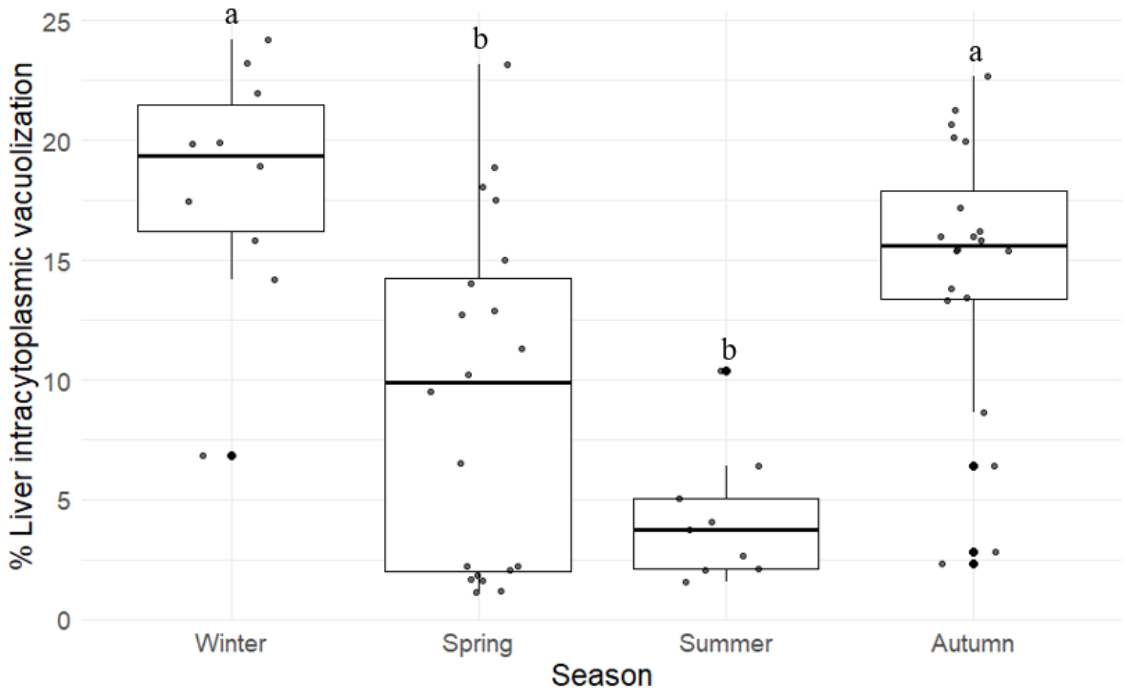


Figure 5.7. Percentage of liver intracytoplasmic vacuolization of lumpfish from the sea cages during different seasons. Boxes and whiskers represent quartiles for each treatment group and dots are outliers. Different superscript letters denote differences in body condition among the groups according to one-way ANOVA ($P < 0.05$).

Livers were also semi-quantitatively assessed for signs of congestion and inflammation, as well as necrosis and fibrosis (Figure 5.8). Although inflammation seemed to be more severe in wild fish, while fish from the sea cages displayed the most varied levels of inflammation, there was no effect of fish origin on liver inflammation (DF=2, X^2=1.03, P = 0.596). Wild fish was the only group displaying moderate to severe cases of congestion. Despite this, the actual percentages were relatively small and there was no effect of the origin of the fish on liver congestion (DF=2, X^2=1.11, P = 0.575).

There was an effect of the origin of the fish on liver fibrosis (DF=2, X^2=14.51, P < 0.001). Lumpfish from the sea cages and the land-based hatcheries had a significant higher likelihood of experiencing liver fibrosis compared to fish from the wild. Fish from sea cages appeared to have more varied fibrosis levels than fish from the other origins, with 45% showing mild to moderate fibrosis (27 out of 59) (Figure 5.7). No significant difference was found when comparing land-based fish to the sea cages for fibrosis. Overall, fish from wild sources exhibited the healthiest liver tissue in terms of fibrosis.

Fish origin significantly also affected the level of necrosis in the livers (DF=2, X^2=11.16, P = 0.004). Lumpfish from both land-based hatcheries and sea cages are significantly more likely to have higher liver necrosis compared to the wild fish, with the sea cage fish showing a more pronounced effect.

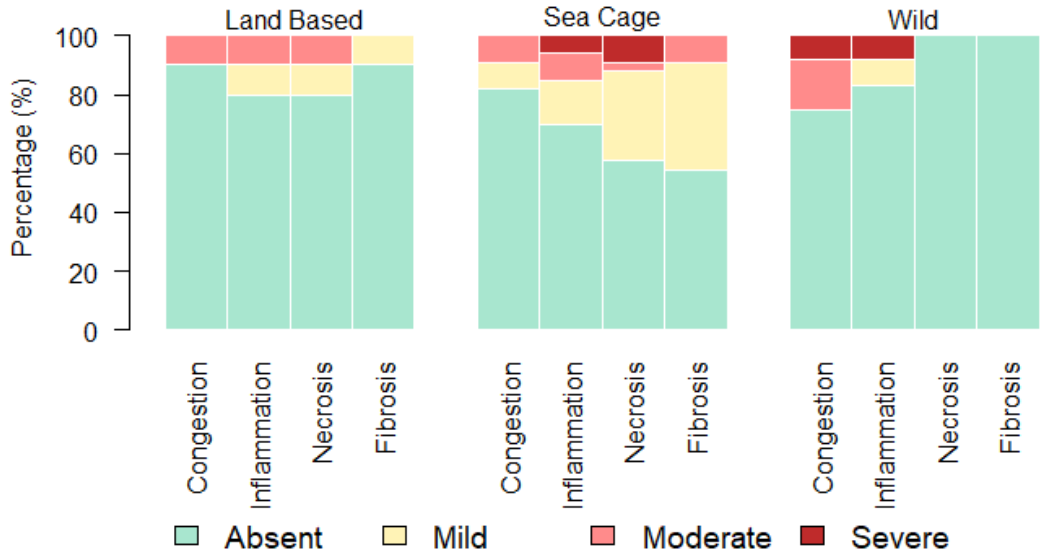


Figure 5.8. Percentage distribution of congestion, inflammation, necrosis and fibrosis of livers from lumpfish from different origins (land-based, sea cage and wild). Score 1 indicates absent, score 2 indicates mild signs where one area is affected, score 2 indicates moderate signs, and score 3 indicates severe damage due to congestion/inflammation/necrosis/fibrosis.

5.5. Discussion

In this study, changes in welfare and health between farmed and wild lumpfish were assessed, highlighting significant differences in body condition, fin damage, and other health and welfare indicators. While wild lumpfish generally maintained good condition, a notable proportion of farmed lumpfish in sea cages exhibit higher rates of being underweight and emaciated, suggesting suboptimal farming conditions and feeding strategies. Additionally, severe fin damage was more prevalent in farming environments, particularly in hatcheries, linked to high density and behavioural stress such as fin nipping. Histological assessments revealed that liver vacuolization was highest in land-based hatchery fish, indicating excess energy intake and fat deposition, while in the sea cages a seasonal effect was found. Liver fibrosis and necrosis were most pronounced in sea cage fish, pointing to possible long-term stress and suboptimal conditions.

5.5.1. Body condition

The present study highlights substantial differences in the welfare and health of lumpfish from farmed and wild origin. Lumpfish from the wild were generally in good condition, where 26% were underweight and 6% emaciated regarding the weight-length relationship, likely due to their natural diet and prey availability. Wild fish can encounter periods of starvation due to factors such as seasonal changes, competition for resources, or shifts in prey availability (Eliasen et al., 2018). This natural scarcity can impact their overall body condition and status of the fish. The likelihood of fish from sea cages being underweight (22%) or emaciated (25%) was higher. This suggests that some specific farming conditions as well as feeding strategy have an impact on the body condition of the sea cage fish. Also, the presence of infectious diseases such as bacterial or viral infections can affect metabolism and nutrient absorptions, resulting in a suboptimal body condition. This likely reflects different nutritional statuses when the fish are deployed in the sea cages, as also found by Boissonnot et al. (2023) and Østerø et al. (2024). Lumpfish from the land-based hatcheries had the highest body condition, and this was due to the frequent feeding regimes of the hatcheries where fish have ample access to feed, and the rearing environment of the tanks. Other studies have looked at the body condition of lumpfish in the sea cages (Eliasen et al., 2020; Engebretsen et al., 2024; Rabadan et al., 2021; Rey et al., 2021). However, these studies calculated weight-length relationship based on fitted regression of lumpfish that were deployed in salmon cages, resulting in lower exponent *b* values: 2.55 (Rabadan et al., 2021), 2.75 hatchery and 2.91 sea cage fish (Rey et al., 2021), 2.50 (Engebretsen et al., 2024). This caused an underestimation of the % of lumpfish categorised as underweight or emaciated compared to the wild model. Using the length-weight relationship of wild lumpfish in our study served as a benchmark to assess the body condition of the fish, mirroring more closely the natural growth patterns of the fish (Østerø et al., 2024).

The age of fish also plays a crucial role in their body condition, changing as they grow (Kamimura et al., 2021). A limitation of this study was the difficulty in determining the precise age of the sampled fish as growth alone is not a good proxy for age in lumpfish (Wilson et al., 2009). This leads to the speculation that farmed lumpfish, both from the hatcheries and the sea cages, being fed energy dense diets, grow faster and might be younger on average than their wild counterparts. A study by Albert et al. (2002) assessed the age of lumpfish through otoliths and identified lumpfish that measured less than 8 cm in total length to be one year old. By comparing this study to our morphometric data regarding the wild lumpfish sampled, we assume that the wild fish analysed should be in their first year of life.

5.5.2. Fin damage

When fish are reared in aquaculture settings, they are exposed to various stressors that can impact their overall welfare (Ashley, 2007). Lumpfish from the land-based hatcheries had severe damage in the anal, dorsal, and caudal fins, with the highest damage percentage (18%) reported in the dorsal fin. This suggests that specific stressors in the hatcheries, such as the high density in the tanks, might lead to varying impacts on different fins (Ellis et al., 2002). Notably, behavioural issues such as tail biting and fin nipping, which have been observed in both lumpfish and wrasse (Erkinharju et al., 2021) in sea cages (Powell et al., 2018) and tanks (Noble et al., 2019), could be contributing factors, particularly in the case of dorsal fin damage. This is consistent with observations in Atlantic salmon where dorsal fin damage is often linked to aggression (Cañon Jones et al., 2010; Turnbull et al., 1998) and in Asian seabass (*Lates calcalifer*) reared at high stocking density (Khan et al., 2022). Fin damage can predispose lumpfish to infectious diseases such as secondary bacterial infections due to fin rot or fungal infections. Other stressors in the farming environment such as crowding, water quality (Santos et al., 2010), competition for resting spaces (Johannesen et al., 2018), and different feeding strategies (Latremouille, 2003) can also affect fin health.

Also, lumpfish from the sea cages had severe damage in all the fins, with the highest percentage (8%) reported in caudal fin, followed by anal fin (4%). Overall, 51% of the lumpfish in the sea cages reported moderate damage in the caudal fin. This result agrees with the study by Boissonnot et al. (2023), where approximately 50% of the deployed lumpfish in Norway presented damages on caudal fin, as well as in deployed wild caught ballan wrasse (Treasurer & Feledi, 2014).

These fin damages, especially in the sea cage environments, might be due to mechanical causes, such as interactions with cage structures, nets and strong currents (Braithwaite & McEvoy, 2005; Latremouille, 2003). Additionally, during the deployment phase in the sea cages, lumpfish may be subjected to mechanical delousing procedures intended for salmon, which can result in skin injuries and fin damage for these fish, leading to increased susceptibility to infectious diseases (Boissonnot et al., 2023; Reynolds et al., 2022). The difference in anal fin damage between sea cages and land-based

hatcheries in our study suggests that environmental and mechanical factors in the sea cages might play a significant role in this kind of damage.

Lumpfish from the land-based hatcheries showed the highest percentage of moderate damage, while those from the sea cages had the highest percentage of severe damage. This could be due to the longer time spent by fish the sea cages compared to the tanks as the welfare deterioration in sea cages can be the result of cumulative damage rather than farming environment. Also, Boissonnot et al. (2023) speculated that the caudal fin damage reported in the lumpfish in the sea cages results from the social aggression in the hatcheries in the pre-deployment.

Lumpfish from the hatcheries had an average weight of 34.4 ± 9.4 g. This size of 20-30 g is usually reached in five to seven months when lumpfish are reared in hatcheries (Brooker et al., 2018). Therefore, the fish sampled in the hatchery were approximately 5-7 months old. Lumpfish sampled from sea cages, with an initial average weight of 93.6 ± 27.3 g, are estimated to be between 8 to 12 months old. This estimation is based on the growth rate where lumpfish roughly double their weight every four weeks, as reported by Johannesen et al. (2018). The extended time that lumpfish spent transitioning from hatchery rearing, through transport to salmon sites, and finally to sea cage deployment, may lead to an increased cumulative damage in OWI. This is particularly evident in the case of fin damage and agrees with the results in rainbow trout by Barrows & Lellis (1999).

In general, the increased damage in farmed fish likely stems from the more challenging environments they encounter in both sea cages and hatcheries. Although fin damage has been reported also in wild populations, it is more common in aquaculture environments (Latremouille, 2003). Indeed, in this study, wild fish had the best fin condition, with a significant majority showing no damage (86%). No lumpfish from the wild population showed severe damage in the fins, suggesting that severe fin issues are not common in natural environment. However, this perspective might be slightly skewed, as wild fish with compromised health status will likely not survive and be predated, making them less represented in the samples. In contrast, sampling fish randomly from a salmon sea cage or hatchery tank will likely give a better representation of the average situation at the site due to the controlled environments.

5.5.3. Eyes, skin and sucker disc status

Other welfare indicators such as the condition of the eyes, the status of the skin and the integrity of the sucker disc are vital for lumpfish survival both in the wild and in farming conditions. Healthy eyes are crucial for navigation and for locating feeds and preys, the skin is the primary barrier against pathogens and environmental threats, and the sucker disc is essential for attachment to substrates to sit and rest. In this study these OWI scored very low in all the sampled groups.

Skin damage together with fin damage were identified as the most useful OWI for farmed lumpfish by Garcia de Leaniz et al. (2022), as open wounds both in the fins and skin expose the fish to further injuries, secondary infections (Aeromonas salmonicida, Vibrio spp. or Flavivirus infections) and other

pathogens. In our study only a small percentage of lumpfish from the sea cages and from the hatcheries had some moderate damage in the skin. This in contrast with the study by Gutierrez Rabadan et al. (2021) and Boissonnot et al. (2023) where almost half of the fish sampled from the sea cages had skin damage. This can be due to the lumpfish in our study being sampled before being exposed to any other delousing treatment, but also less exposure to strong currents on the sites and potentially a shorter deployment time. Eye damage was detected only in the fish from the sea cages, and this also agrees with Rabadan et al. (2021). This can be the result of abrasion and contact with the cage nets due to exposure to currents, and an overall cumulative damage occurring throughout the deployment phase.
Regular observation of the skin appearance, eyes' integrity, and ocular area as well as fin integrity can aid in early detection of health issues in fish, reducing the incidence of infectious diseases (Segner et al., 2012). Despite the skin and eyes damage being low in this study, it would be beneficial in further research to also correlate these data with the mechanical delousing treatments, especially when severe damage is detected.
Sucker disc is essential to lumpfish for attachment to substrates and resting (Davenport & Thorsteinsson, 1990). Sucker disc deformities were detected in this study in a very small percentage only in the fish from the sea cages (0.6%). The underlying causes of sucker disc deformities are unclear. However, genetic factors and nutritional causes are highlighted both in Reynolds et al. (2022) and Rabadan et al. (2021). Rabadan et al. (2021) reported a higher percentage of fish from the hatcheries with a sucker disc deformity, compared to the ones assessed from the sea cages. In our study, the fish sampled from the hatcheries were free from sucker disc deformities. This could be due to early screening for these fish in the hatcheries, but also due to genetic differences as reported by Danielsen (2016).

5.5.4. Liver intracytoplasmic vacuolization

This research indicates that the welfare of lumpfish was significantly influenced by both their origin and the conditions of their farming sites. Significant differences were also found in the histological parameters assessed in lumpfish from farmed and wild origin. The liver intracytoplasmic vacuolization reflects the nutritional status of the fish (Eliasen et al., 2020), where a higher liver vacuolization indicates that the energy intake surpasses expenditure, resulting in a higher fat deposition in the liver, leading to vacuolization (Caballero et al., 2004). In this study, the land-based fish had the highest liver vacuolization and this also agrees with the liver fat content previously described (Chapter 4). This can be due to the supply of energy-dense feeds being delivered in the hatcheries, but also the tank rearing environment that makes the fish less active coupled with regular feeding schedules (Bolla et al., 2011). This result also agrees with the study by Imsland et al. (2019) where lumpfish that were fed more frequently had an increased liver vacuolization, with the highest being the fish that were fed daily. Fish from the sea cages had similar liver vacuolization levels to the wild group. However, the variation in liver vacuolization in the fish from the sea cages could be explained by the type of feed

(salmon or lumpfish feed), and the prey availability in the cages due to seasonality (Eliasen et al., 2020). On the other hand, wild fish were caught during a summer survey, and they display similar vacuolization percentages among them, most likely due to the same environmental conditions of their habitat and preys' availability during the summer period.

Season significantly impacted liver vacuolization in fish from the sea cages, being higher in autumn and winter and lower in spring and summer. This difference in seasonality could be explained by the feed composition as fish during autumn and winter mainly access lumpfish and energy dense salmon feed only, while during spring and summer they have access to other preys such as zooplankton, switching their feeding preferences (Eliasen et al., 2018). Wild fish and those from sea cages during summer and spring experienced similar levels of liver vacuolization likely due to shared dietary sources due to the increased presence of natural prey like zooplankton. This dietary overlap during the warmer months suggests a convergence between the two groups, highlighting how environmental and dietary factors influence liver vacuolization patterns irrespective of the fish living conditions.

5.5.5. Liver fibrosis and necrosis

Incorporating histological examinations in this study has been used as validation for health status, and it provides a more accurate understanding of the physiological changes in fish when faced with various environmental stressors (Schwaiger et al., 1997). Differences were also found in the histological observations of lumpfish livers, in terms of fibrosis and necrosis. Land-based fish mostly had healthy livers, but a small fraction showed mild to moderate necrosis. There were no fish from this group with moderate or severe fibrosis which is a positive indication of the general liver health. Fish from the wild displayed the most robust liver health regarding necrosis and fibrosis, with no fish showing evidence of these changes in their liver. In contrast, fish from sea cages had the most varied responses, with some showing no necrosis, but others displaying severe necrotic changes. This suggests that factors or conditions in sea cages might expose fish to liver damage, leading to the development of fibrosis as the liver undergoes repair processes and necrosis, as a result of long-term stressors. Stressors that affect both liver vacuolization, fibrosis and necrosis include the type of feed, such as a high content of carbohydrates, the use of high levels of vegetable oils or an imbalanced amount of amino acids (Caballero et al., 2004; Raskovic et al., 2011). Also, the presence of infectious diseases such as Infectious Pancreatic Necrosis Virus, Flavivirus, Piscirickettsia salmonis, and bacterial hepatitis can result in different degrees of liver inflammation, fibrotic changes and liver necrosis (Erkinharju et al., 2021; Schwaiger et al., 1997).

Fin damage can negatively impact the health of fish as it is considered a portal for both bacteria and fungi (Brooker et al., 2018; Noble et al., 2012) and in this study fin damage was detected in all the groups. A limitation of this study is that the fish sampled both from farmed and wild origin were not screened for infectious diseases, therefore it is difficult to speculate the aetiology of histological

observations in the liver of these fish as inflammation, congestion, fibrosis and necrosis can be a result of prolonged stressors due to diseases, exacerbated by poor environmental conditions. Flavivirus in lumpfish for example, that results in liver inflammation, can be detected also in fish with no clinical signs as reported in Brooker et al. (2018), making it difficult to have an overall picture of the health status of these fish. Screening the fish for diseases could corroborate these histological assessments.
Another limitation of this study was the sample size of the wild populations, and the limited access to wild lumpfish of appropriate sizes during other seasons than summer. If more fish were sampled, other variations could be investigated, such as seasonal variation and the effect of fish size in the wild populations.

5.6. Conclusions

This study examined the welfare and health status of farmed and wild lumpfish using OWI and histology. This was done to understand the key differences in health and welfare indicators between wild and farmed lumpfish, leading to improvements and better practices in farming procedures. Significant deviations of health and welfare parameters from the wild observations can be caused by environmental, nutritional and handling issues that need to be addressed in the farms.
Regular monitoring is essential to prevent welfare deterioration during the production cycle. Strategies can be implemented in the hatcheries to minimise fin damage, such as reducing density, ensure an appropriate feeding regime, and provide enough shelter space to reduce aggression and fin nipping. In the sea cages, it would be appropriate to avoid the deployment of fish in sites known for strong currents, that could lead to skin abrasions and fin damage due to the contact with the cages net. Also, preventing lumpfish from undergoing through mechanical delousing could have a significant impact on their overall welfare status. The implementation of these procedures has the potential to have farmed lumpfish in a better welfare and health status, resulting in better survival rates in the sea cages as well as better efficiency in delousing.

Chapter 6. Investigating the effects of increasing levels of EPA and DHA on growth, health, chemical composition and stress response in juvenile lumpfish (*Cyclopterus lumpus*)

6.1. Abstract

Optimised diets for lumpfish are essential to guarantee fish robustness from rearing in the hatcheries throughout the deployment phase. Nutritional requirements for juvenile lumpfish are not well established and poor nutritional status has been pinpointed among the causes for the high mortality rates reported when lumpfish are deployed in the sea cages. Chapters 3 and 4 of this thesis investigated the key differences in terms of nutritional status between wild and farmed lumpfish populations of the Faroe Islands. From this dataset, specifically total lipids and EPA+DHA levels were identified as potential main drivers in composition differences among the different stocks of lumpfish and a feed was formulated based on these differences. In particular, the nutritional requirements for EPA and DHA are not known in juvenile lumpfish. EPA and DHA are essential fatty acids required for normal growth, immune response, survival and stress tolerance, among others. The aim of this study was to investigate the effects of increasing levels of EPA+DHA on growth parameters, chemical composition and stress response of juvenile lumpfish. Juvenile lumpfish were fed five experimental diets: a formulated basal pellet coated with krill oil (KO) or rapeseed oil (RO) or a blend of the two oils (0KO, 25KO, 50KO, 75KO, 100KO) to achieve diverging levels of EPA+DHA (5.6-22.8% of total fatty acids, 0.9-3.4% of feed). A commercial diet (COM) was also used as a standard benchmark. At the end of the feed trial, lumpfish were exposed to an acute stressor, which was a combination of chasing for 8 minutes and confinement for 1 minute, to investigate stress responses 1 hour and 6 hours after the stress challenge. The different diets did not influence growth parameters, condition indices and survival. Significant differences were found in the lipid content (%) of whole fish, liver and intestine. The fatty acid profile of these tissues reflected the dietary input, whereas the only difference found in the lipid classes composition was in free fatty acids of intestine which were higher in fish fed 100KO and 0KO. The diet did not affect OWI or histological parameters such as liver intracytoplasmic vacuolization or anterior and distal intestine muscle thickness. Plasma cortisol was higher in fish fed 25KO than the other diets 6 hours after exposure to the acute stressor. To estimate the minimum requirements of EPA+DHA in the diet, a polynomial model was performed using EPA+DHA fed to the fish to achieve a sufficient SGR, higher survival and low cortisol levels. Based on the model results we recommend dietary inclusions of 2-3 % of EPA+DHA (15-18% of total fatty acids) when formulating diets for juvenile lumpfish.

Keywords: juvenile lumpfish, nutritional requirements, EPA and DHA, cortisol, growth, survival

6.2. Introduction

High mortalities of lumpfish have been reported in sea cages in the weeks post-deployment, mainly due to pathogens, transport, salmon grading and mechanical delousing (Reynolds et al., 2022), as well as poor nutritional status (Boissonnot et al., 2022; Eliasen et al., 2020). Powell et al. (2018) reported that one third of the deployed lumpfish die of starvation within a few months, and therefore, lowering the mortality rates and ensuring good welfare is fundamental (Treasurer, 2018).

One of the main bottlenecks of lumpfish production is the availability of balanced diets at the deployment stage. Nutritional requirements of the species, physical properties of the feed and best delivery protocols in sea pens are not well optimised (Boissonnot et al., 2023). Poor nutrition and unbalanced diets will lead to poor health and welfare, consequently leading to high mortality rates. To find the feed optimal composition, it is fundamental to address optimal macro and micronutrients requirements for lumpfish according to fish size, temperature and growth rate (Hamre et al., 2022).

Due to the rising ethical concerns regarding lumpfish welfare, several studies have aimed to improve the knowledge on their nutrition. Hamre et al. (2022) attempted to elucidate the correct balance of macronutrients and recommended that diets for juvenile lumpfish (10-50 g) should contain 55% protein, minimum 10% lipid and maximum 10% carbohydrate. The use of different raw materials has also been investigated such as the replacement of fish meal with plant meals (Willora et al., 2020, 2022) or the replacement of fish oil by rapeseed oil (RO) (Willora et al., 2021). Replacing fish meal with up to 50% plant protein ingredients affected the structure of the intestine but did not adversely affect growth performance, body chemical composition, or muscle fibre cellularity (Willora et al., 2020). In Willora et al. (2021) lumpfish growth was affected by the highest inclusion of RO (10% of the diet), which resulted in a reduced growth rate, suggesting that the dietary eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) level did not meet the nutritional requirements for these essential fatty acids.

Other studies also investigated the correct feeding frequency (Imsland et al., 2019) and the physical properties of the feed (Imsland et al., 2018; Imsland et al., 2020; Imsland et al., 2019), using alternative strategies like the use of feed blocks rather than the conventional pellets. However, these studies aimed to look at achieving a controlled growth either using restricted feeding regimes (Imsland et al., 2019) or low energy feed blocks (17.3 MJ/kg) (Imsland et al., 2020), since f0ast growth of lumpfish is not desirable due to the suspected lower delousing activity of bigger size fish (Imsland et al., 2016). A constraint of all these studies is that the actual feed intake of lumpfish was not monitored due to the limitations in retrieving uneaten pellets. Indeed, Imsland et al. (2018) proposed an estimated bFCR which was based on feed presented rather than feed ingested.

Chapters 3, 4 and 5 of this Thesis investigated the key differences in terms of nutritional status, health and welfare between wild and farmed lumpfish populations of the Faroe Islands. From this dataset, specifically total lipids and EPA+DHA levels were identified as potentially the main drivers in

composition differences among the different stock of lumpfish (see Chapters 3 and 4). The nutritional requirements for EPA and DHA are not known in juvenile lumpfish (Willora et al., 2021). EPA and DHA are essential fatty acids required for many key metabolic and physiological pathways affecting normal growth, immune response and survival rates in marine fish (NRC, 2011). Moreover, it has been proven that dietary DHA increases the tolerance of red sea bream (*Pagrus major*) and marbled sole (*Limanda yokohamae*) to various stressful conditions such as changes in temperature, salinity, exposure to air and low dissolved oxygen (Kanazawa, 1997). Also, in juvenile Japanese seabass (*Lateolabrax japonicus*) when fed levels of 18% n-3 LC-PUFA (of total fatty acids), improved growth rates, immune functions and stress tolerance (Xu et al., 2016). Inclusion of EPA+DHA (0.2 and 0.4% of the diet) in diets for striped catfish (*Pangasianodon hypophthalmus*) resulted in enhanced growth rates, better antioxidant capacity and disease resilience towards different stressors (Kumar et al., 2022).

To the best of our knowledge, no other studies have elucidated the nutritional requirements of EPA and DHA in lumpfish. Therefore, the overarching aim of the present study was to evaluate the effects that increasing dietary inclusion levels of EPA+DHA have on chemical composition, welfare and growth parameters, as well as stress response in juvenile lumpfish.

To do so, five diets with diverging levels of EPA+DHA were fed to juvenile lumpfish in a controlled tank-based feed trial. To investigate stress responses of the fish fed the dietary treatments, lumpfish were also challenged by exposure to an acute stressor at the end of the trial. This study hypothesizes that by assuring that the requirements for lipids and EPA+DHA are met, lumpfish robustness and stress response would be boosted.

6.3. Materials and Methods

6.3.1. Ethical approval

This feeding experiment was conducted according to the Directive 2010/63/EU regarding the protection of animals for scientific purposes, approved by the head veterinarian "Landsdjóralæknin" in agreement with the Welfare act 2018, §10 (DJÓRAVÆLFERÐARLÓGIN - Løgtingslóg 49 apríl 30 2018, Faroe Islands). The trial was also reviewed and approved by Firum ''Animal Experimentation Ethics Committee'' (approval number 12, Torshavn, Faroe Islands), and by the Animal Welfare and Ethical Review Body (AWERB 2022 7252 5873) at the University of Stirling (UK).

6.3.2. Fish

A total of 576 juvenile lumpfish were sourced from Nesvík Marine Centre (Faroe Islands), which uses predominately wild females and captive bred males as broodstock. Fish were reared in a shallow raceway recirculating aquaculture system (RAS) at approximately 9 °C with a photoperiod of 24 h light for juveniles less than 3 g, and 18:6 light:dark cycle for on-growing fish (3-30 g). Larvae

were reared using live feed *Artemia* sp., enriched with Easy Dry Selco® (Inve, Belgium), for four weeks. The fish were then weaned onto feed Larviva Prostart (pellet sizes: 80-125/125-250/250-400 µm; Biomar, Denmark), followed by Larviva Prowean (pellet sizes: 80-200/ 150-400/ 350-600/ 550-800/ 750-1000 µm; Biomar, Denmark). Prior to the trial, fish were fed Lumpfish Grower (pellet sizes: 1.1/1.5/2 mm; Biomar, Denmark) to satiation. The trial was carried out from 14th of May to 8th of July 2022.

6.3.3. Experimental system and culture conditions

Lumpfish that were approximately 5 to 7 months post hatch (average weight 20 ± 2 g) were selected for the trial. They were weighed and visually assessed for welfare (e.g. absence of damage in the fins and eyes, good skin status and body condition) before being transferred from the lumpfish hatchery into an experimental flow-through system, located in Nesvík (Faroe Islands). A total of 576 lumpfish were randomly allocated to 24 tanks (24 fish/each tank, 96 fish/experimental diet), with an average density of 3.2 ± 0.4 kg/m^3 per tank (64 x 64 x 58 cm external measurements, approximately 150 L). Fish were acclimatised to the experimental system for 10 days before the start of the experiment. The flow-through system was gravitationally fed filtered and UV-treated seawater from a nearby seawater intake. The treated seawater was fed into a header tank (145 x 125 x 80 cm) placed 155 cm above ground level. To ensure good aeration and avoid potential gas supersaturation, the header tank was supplied with 2 air stones connected to an air pump (Mistral II 4000, AB Aqua Medic GmbH, Bissendorf, Germany). Turnover time in the header tank was approximately 20 minutes. Tanks were square with rounded corners, black side walls and white bottoms (Figure 6.1 A). Two black shelters made from polyethylene tubes cut in half lengthways (approximately 37-38 cm long and 13 cm wide) were suspended within each tank. A black mesh was used to cover half of the tank to provide more shelter and overhead shade to the fish (Figure 6.1 A).

Time controlled tubular fluorescent lights were placed on the ceiling, in two rows (286.0 lux ± 3.0 at surface, 33 lux ± 3.0 under the mesh), and they were set to a 12:12 light: dark cycle (Figure 6.1 B). The water exchange was 150 l/h, which was increased after 14 days to 200 l/h to ensure oxygen saturation levels above 95% as biomass increased. Temperature and oxygen saturation were measured daily for each tank and salinity was measured weekly. Temperature (Starmon-tilt, Star-Oddi, Iceland) and oxygen saturation (RBR solo^3 DO, Canada) were also logged throughout the trial period using loggers set to record every minute at the tank outflow. Seawater had a mean temperature of 9 °C (min 7.5, max 10.9° C) and oxygen level had a mean saturation of 97% (min 93%, max 103%). Salinity measured 35 ppt and water pH was 8.2 throughout the experiment.

Figure 6.1. (A) Tank setting with two black shelters suspended and a black mesh covering half of the tank. (B) Set up of the trial facility which included twenty-four square tanks (64 x 64 x 58 cm) placed in a fed flow-through system (Nesvík, Faroe Islands).

6.3.4. Feed formulation

Experimental fish were fed either one of five experimental diets or a commercial diet. A 3 mm commercial diet for juvenile lumpfish manufactured by extrusion by Havsbrun (Fuglafjørður, Faroe Islands) was used as a commercial control in the trial (COM).

To evaluate the effects that increasing dietary levels of EPA+DHA (5.6-22.8 % of total fatty acids) have on lipid and fatty acid metabolism as well as health and welfare parameters, a basal extruded diet (Havsbrun, Faroe Islands) was formulated (Table 6.1), and coated with either RO, KO (Qrill Antarctic Phospholipid Oil, QAPO; Aker Biomarine, Norway) or a blend of both oils, where KO was replaced by RO, generating five experimental feeds with diverging EPA+DHA levels as follows: 100% KO (100KO), 75% KO (75KO), 50% KO (50KO), 25% KO (25KO) and 0% KO (0KO). The experimental diets (3 mm) were formulated and coated to be isoproteic and isolipidic, and therefore isoenergetic (Table 6.1).

In the dataset from Chapter 3 of this Thesis, which investigates the key differences in nutritional status between wild and farmed lumpfish populations of the Faroe Islands, it was found that the content of certain amino acids was higher in wild fish compared to farmed ones. Specifically, the levels of amino acids such as lysine, methionine, and threonine were enhanced in the formulation compared to the commercial diet.

Oils were added post-extrusion through vacuum coating, using a vacuum chamber (17.7 x 15.8 x 8.3 inches; High-Capacity 3 Gallon Vacuum Chamber, VEVOR) and a Single Stage Vacuum Pump (VidaXL, 141651.A, 220V, The Netherlands). At each coating occasion, oil was weighed into the vacuum chamber, the basal pellets added, and mixed thoroughly for 5 minutes. To assure full oil absorption and homogeneity, the mixture was put under vacuum for at least 2-3 minutes until no foam developed. The feed was coated in several batches of 160 g for each diet to assure a homogenous distribution of the oils into the pellets. Diets were stored at room temperature before being used. Samples were repeatedly collected from different batches after coating and stored at -20 °C for further nutritional analyses (Table 6.2, 6.3, 6.4 and 6.5).

Table 6.1. Ingredient composition (%) of the experimental diets fed to lumpfish.

Raw material	100KO	75KO	50KO	25KO	0KO
Soy protein concentrate[1]	41.78	41.78	41.78	41.78	41.78
Blue whiting meal[2]	21.23	21.23	21.23	21.23	21.23
Wheat[3]	10.00	10.00	10.00	10.00	10.00
Fish protein concentrate[4]	8.00	8.00	8.00	8.00	8.00
Krill meal[5]	7.50	7.50	7.50	7.50	7.50
Krill oil[5]	11.09	8.32	5.55	2.77	-
Rapeseed oil[6]	-	2.77	5.55	8.32	11.09
Lysine[7]	4.00	4.00	4.00	4.00	4.00
Methionine[8]	2.00	2.00	2.00	2.00	2.00
Histidine[9]	0.34	0.34	0.34	0.34	0.34
Monoammonium phosphate[10]	1.30	1.30	1.30	1.30	1.30
Vitamin and mineral premix[11,12]	1.25	1.25	1.25	1.25	1.25
Asta Pink 10%[11]	0.05	0.05	0.05	0.05	0.05

[1]Aminola, Serbia; [2]Havsbrun, Faroe Islands; [3]Lantmannen, Sweden; [4]Aquarius AS, Norway; [5]Aker Biomarine, Norway; [6]Cargill, UK; [7]Pinnlee, China; [8]Evonik, Belgium; [9]CJ Europe, Indonesia [10]Yara, Norway; [11]DSM, The Netherlands; [12]contains Vitamin A, D_3, E, K_3, C, Complex B vitamins, niacin, choline, inositol, copper, iron, manganese, iodine and zinc.

Table 6.2. Amino acid profile of the experimental diets (g/100 g). CV represents the coefficient of variation between the commercial diet (COM) and the experimental diets (100KO, 75KO, 50KO, 25KO, 0KO).

Amino acid (g/100 g)	Diet COM	Diet 100KO-0KO	CV (%)
Essential amino acids (EAA)			
Histidine (His)	0.7	1.4	47.1
Threonine (Thr)	1.8	1.8	0.8
Valine (Val)	2.4	2.5	3.4
Isoleucine (Ile)	2.2	2.3	4.7
Leucine (Leu)	3.7	3.8	1.3
Lysine (Lys)	3.5	4.0	10.8
Methionine (Met)	1.3	2.0	26.9
Phenylalanine (Phe)	2.2	2.3	3.2
Total EAA	17.8	20.2	0.09
Conditionally essential amino acids			
Tyrosine (Tyr)	1.7	1.7	0.8
Glycine (Gly)	2.9	2.7	7.1
Arginine (Arg)	3.2	3.6	8.0
Proline (Pro)	2.8	2.5	9.4
Total CAA	10.6	10.4	0.02
Non-essential amino acids			
Serine (Ser)	2.2	2.4	5.9
Alanine (Ala)	2.6	2.5	3.6
Cysteine (Cys)	0.5	0.7	20.2
Aspartic acid (Asp)	4.3	4.8	7.7
Glutamic acid (Glu)	9.1	8.1	8.6
Taurine (Tau)	0.3	0.2	31.4
Total NAA	19.0	18.6	0.02
Total AA	47.4	49.1	0.03
Total crude protein	51.1	50.3	0.01

Table 6.3. Fatty acid composition (% total fatty acids) of total lipid from KO (QAPO, Aker Biomarine) and RO sourced at Havsbrun (Faroe Islands).

Fatty acid (%)	QAPO Krill oil	Rapeseed oil
14:0	7.4	n.d.
16:0	20.3	4.2
18:0	1.1	1.8
ΣSAFA[1]	**29.6**	**7.1**
16:1n-7	5.0	0.2
18:1n-9	10.3	59.7
18:1n-7	6.9	3.3
20:1n-9	0.6	1.4
22:1n-11	0.5	n.d.
ΣMUFA[2]	**24.0**	**65.5**
18:2n-6	2.3	19.1
20:4n-6	0.4	n.d.
Σn-6 PUFA[3]	**3.1**	**19.2**
18:3n-3	2.9	8.2
18:4n-3	7.2	n.d.
20:5n-3	18.1	n.d.
22:5n-3	0.5	n.d.
22:6n-3	**11.7**	**n.d.**
Σn-3 PUFA[4]	**41.8**	**8.2**
ΣPUFA	**46.4**	**27.4**
n-3/n-6	**13.3**	**0.4**
EPA/DHA	**1.5**	**n.d.**
EPA+DHA	**29.8**	**n.d.**

EPA (g/100g)	**14.7**	**n.d.**
DHA (g/100g)	**9.5**	**n.d.**
EPA+DHA (g/100g)	**24.2**	**n.d.**

n.d. not detected

[1]Includes 15:0, 20:0, 22:0, 24:0; [2]Includes 16:1n-9,17:1, 20:1n-11, 20:1n-7, 22:1n-9 and 24:1n-9; [3]Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6;[4]Contains 20:3n-3, 20:4n-3, 21:5n-3; SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LC-PUFA, long-chain polyunsaturated fatty acids (sum of 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3)

Table 6.4. Proximate nutrient composition and fatty acid profile (%) of the commercial (COM) and experimental diets (100KO, 75KO, 50KO, 25KO, 0KO).

	COM	100KO	75KO	50KO	25KO	0KO
Moisture (%)	8.7	9.7	9.7	9.4	8.1	9.6
Ash (%)	8.9	8.2	8.3	8.0	8.0	7.9
Crude protein (%)	51.1	50.3	50.5	50.3	50.5	49.9
Crude lipid (%)	21.5	15.1	15.4	15.4	15.6	15.9
Carbohydrates (%)[1]	9.9	16.8	16.1	16.9	17.9	16.8
Gross energy (MJ/kg)	22.3	20.8	20.8	21.0	21.2	21.0
14:0	6.5	8.3	6.5	4.8	3.5	2.0
16:0	14.3	18.6	15.6	13.0	10.7	8.5
18:0	2.0	1.5	1.6	1.7	1.8	1.9
ΣSAFA[2]	**23.8**	**28.9**	**24.5**	**20.2**	**16.7**	**13.2**
16:1n-7	6.2	5.3	4.4	3.5	2.8	2.0
18:1n-9	13.0	15.3	24.2	32.8	39.8	47.2
18:1n-7	3.0	5.6	4.9	4.4	3.9	3.4
20:1n-9	10.5	2.4	2.5	2.5	2.5	2.6
22:1n-11	14.1	2.2	2.2	2.0	1.9	1.8
ΣMUFA[3]	**50.3**	**32.7**	**39.9**	**46.8**	**52.2**	**58.4**
18:2n-6	3.9	4.7	7.7	10.5	12.9	15.2
20:4n-6	0.4	0.4	0.3	0.3	0.2	0.1
Σn-6 PUFA[4]	**4.8**	**5.3**	**8.3**	**10.9**	**13.1**	**15.4**
18:3n-3	1.2	2.6	3.8	4.5	5.8	6.1
18:4n-3	2.4	4.7	3.5	2.5	1.5	0.6
20:5n-3	7.5	13.5	10.5	7.8	5.5	3.1
22:5n-3	0.6	0.4	0.4	0.3	0.2	0.1
22:6n-3	7.7	9.4	7.4	5.6	4.1	2.6
Σn-3 PUFA[5]	**20.2**	**31.7**	**26.4**	**21.3**	**17.4**	**12.6**
ΣPUFA	26.0	38.4	35.7	33.0	31.2	28.5
ΣLC-PUFA	16.8	24.3	19.3	14.8	10.8	6.8
EPA/DHA	1.0	1.4	1.4	1.4	1.4	1.2
EPA+DHA	**15.2**	**22.8**	**17.9**	**13.4**	**9.5**	**5.6**
EPA (g/100g)	**1.2**	**1.3**	**1.0**	**0.8**	**0.6**	**0.4**
DHA (g/100g)	**1.4**	**0.9**	**0.8**	**0.6**	**0.5**	**0.3**
EPA+DHA (g/100g)	**2.6**	**2.2**	**1.8**	**1.4**	**1.1**	**0.7**

[1]Carbohydrates (%) = 100 – (Moisture (%) + Ash (%) + Protein (%) + Lipid (%)); [2]Includes 15:0, 20:0, 22:0, 24:0; [3]Includes 16:1n-9,17:1, 20:1n-11, 20:1n-7, 22:1n-9 and 24:1n-9; [4]Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6;[5]Contains 20:3n-3, 20:4n-3, 21:5n-3; SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; LC-PUFA, long-chain PUFA (sum of 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3).

Table 6.5. Lipid classes (% total area) of the commercial (COM) and experimental diets (100KO, 75KO, 50KO, 25KO, 0KO).

Lipid class (%)	COM	100KO	75KO	50KO	25KO	0KO
Sterol esters (SE)	3.4	4.6	3.6	2.9	0.9	1.6
Triacylglycerols (TAG)	53.3	36.9	42.8	48.2	56.6	63.8
Free Fatty acids (FFA)	13.3	12.4	10.3	9.9	9.4	8.8
Sterols (ST)	10.4	13.0	12.2	11.5	10.0	9.0
Diacylglycerol (DAG)	3.2	4.0	3.7	3.5	3.0	2.5
Total neutral lipid	**83.6**	**70.8**	**72.6**	**76.0**	**79.9**	**85.7**
Unknown glycolipid (GLY)	2.6	2.6	2.3	1.7	0.9	0.6
Phosphatidylethanolamine (PE)	1.7	4.5	3.6	3.6	3.5	2.4
Phosphatidylglycerol (PG)	0.0	0.0	0.2	0.2	0.0	0.0
Phosphatidylinositol (PI)	0.5	0.4	0.5	0.4	0.6	0.7
Phosphatidylserine (PS)	0.0	0.3	0.3	0.3	0.0	0.0
Phosphatidylcholine (PC)	6.3	17.8	16.2	13.7	11.2	7.7
Sphingomyelin (SM)	0.4	0.2	0.3	0.3	0.3	0.3
Lysophosphatidylcholine (LPC)	0.8	2.1	2.3	1.9	1.8	1.3
Pigmented material (PIG)	4.1	1.3	1.9	1.9	1.0	1.3
Total polar lipid	**16.4**	**29.2**	**27.6**	**24.0**	**19.2**	**14.3**

6.3.5. Feed trial

The diets were tested in quadruplicate, with 24 experimental tanks in total. To ensure that all experimental treatments were exposed to the same environmental conditions within the trial facility, diet was assigned in a random block design. The trial facility was divided into four blocks, two front and two back blocks. Each block included 6 tanks. Tanks within each block were randomly assigned to one of the six feeds in the trial (Figure 6.2). Fish were acclimatised to the tanks for 10 days. During these 10 days, fish were fed the same diet they were being fed in the hatchery (Lumpfish Grower 2mm, Biomar), and gradually switched to the treatment diets during the acclimatisation period. This helps to have a smoother transition as the acclimation feed and the experimental feeds had different diameters.

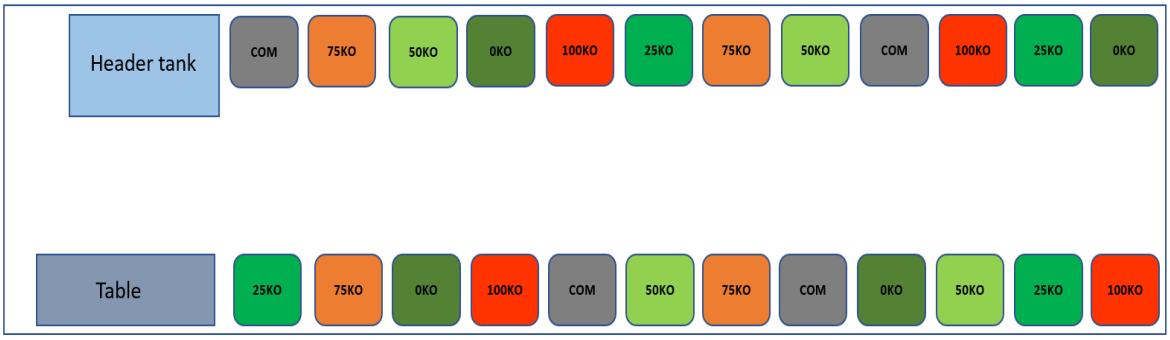


Figure 6.2. Trial facility plan and experiment set up where each tank was randomly assigned a diet: COM, 100KO, 75KO, 50KO, 25KO, 0KO (Nesvik, Faroe Islands).

At the end of the acclimatisation period, a bulk weight was carried out for each tank to assess the initial biomass. The tank with the highest biomass was chosen as reference to establish the starting feeding regime. The average initial biomass was 4.4 ± 0.3 kg/m^3.

Automated feeders tend to dispense feed unevenly for a short period of time, which can result in competition among fish as not all of them have equal access to it (Johannesen et al., 2018). To ensure even feeding, fish were manually fed twice a day with a feeding rate of 2.5% of their body weight and this was adjusted during the trial. The daily feed was divided into 2 equal portions and the first feeding was supplied in the early morning between 8 and 9 am, and the second feeding in the afternoon between 2 and 3 pm. For each feeding occasion, the portion was divided into two, and the first half was fed to the fish. After approximately two minutes, the fish were fed the rest of the portion. When feeding, the pellets were dropped slowly to the surface of water close to the shelters and using the tank water flow to ensure an even distribution of the feed within the tank. Feeding rate was adjusted and predicted on a weekly basis using the following formula in use at the hatchery that predicts the number of days for lumpfish to reach a specific body weight (FBW), based on initial body weight (IBW) and water temperature (T):

$$Days = \frac{\left(\ln \times \frac{FBW}{IBW}\right)}{\left(\ln\left(1 + \frac{\left((0.4908 \cdot T) - 0.4729\right)}{100}\right)\right)}$$

Also, both the initial bulk weight and sampling weights during samplings 0, 1, 2 and 3 were used as reference to adjust feeding portions weekly (Figure 6.3).

After every feeding event, uneaten pellets were siphoned from the bottom of the tank using a hose after approximately 30 min to 1 h. Uneaten pellets were weighed out for each tank to record and monitor daily feed intake. To estimate the daily feed intake of the fish, the weight of the wet feed waste is measured for each feeding event, and converted back to dry weight, using a correction factor (Betancor et al., 2016). To calculate the correction factor for each feed, 10 grams were weighed out in

quadruplicate and placed in 0.5l of seawater for 30 minutes and 1 hour. The wet feed was then collected with a net and weighed out again. The ratio between the dry feed and the wet feed is then called correction factor.

Mortalities were removed daily, weighed, measured, assessed for external damage, scored for OWI, such as fin damage (dorsal, anal, caudal), skin status, eyes integrity, sucker disc deformities, liver colour, and the stomach was dissected to check content. Also, moribund fish that showed obvious signs of distress and/or behavioural abnormalities such as swimming sideways, faster swimming or gasping at surface, as well as severe injuries were euthanised with an overdose of Finquel (0.8 g/l, vet 1000 mg/g MS-222, MSD Animal Health), and samples collected for posterior health analysis. Feed delivery and feed intake were adjusted taking daily mortalities into account.

6.3.6. Samplings

Four sampling points were carried out throughout the trial. A baseline sampling was carried out at transfer to the experimental tanks (S0), two main nutritional samplings (S1 and S2) at approximately 3 weeks intervals, and a stress challenge (S3) at the end of the trial (Figure 6.3).

Figure 6.3. Flowchart of the trial. Fish were sampled upon arrival (S0) and acclimatised for 10 days. The trial lasted 52 days and consisted of a nutritional trial (S1 and S2) and a stress challenge (S2 and S3).

6.3.7. Sampling 0 (S0)

During S0, a total of 30 fish were sampled as follow to have a reference of the stock population: ten fish were stored at -20 °C for proximate analysis; ten fish were dissected for anterior intestine, distal intestine, liver and spleen which were fixed in 10% NBF for histological analyses; ten fish were dissected to collect liver, whole intestine and brain for lipid analysis. Each fish sampled was euthanised with an overdose of Finquel (0.8 /l, vet 1000 mg/g MS-222, MSD Animal Health), weighed out to the nearest gram and total length and height measured to the nearest mm. Also, liver and viscera weight were recorded. Fish were also scored for OWI such as fin damage (dorsal, caudal and tail), skin status, eyes integrity, suction disc deformities and liver colour. These parameters were scored from 1 to 3, depending on the severity of the damage, where 1 is no damage and 3 is severely damaged, according to the scoring system used for OWI which was developed by Firum (Faroe Islands) to monitor welfare during routine lumpfish monitoring samplings at the salmon cages. Liver colour was scored from 1 to 6, following the method by Eliasen et al. (2020). A detailed description of the methods used for morphometric data, liver and viscera weight, and the scoring system used for OWI can be found in Chapter 2. Lumpfish initial morphometric data and condition indices (S0) are showed in Table 6.6.

Table 6.6. Initial morphometric data and condition indices of lumpfish before being fed the experimental diets (S0).

Parameter	Sampling 0 (S0)
Initial body weight (g)	20.0 ± 1.9
Total body length (cm)	7.7 ± 0.3
Body height (cm)	3.3 ± 0.3
Body condition	0.8 ± 0.1
HSI (%)	2.0 ± 0.4
VSI (%)	10.9 ± 1.9

6.3.8. Sampling 1 (S1) and 2 (S2)

To ensure that all the fish were in a similar metabolic state, fish were starved 24 to 48 hours prior to sampling, and by clearing the digestive tract, more accurate measurements of body weight and composition could be achieved. Each fish sampled during S1 and S2 was euthanised, measured for morphometric data, liver weight and OWI as described above for S0. During S1 and S2, seven fish were randomly chosen from each tank and sampled as follows: three fish from each tank were stored at -20 °C for proximate analysis; two fish were dissected for anterior intestine, distal intestine, liver and spleen and fixed in 10% NBF at room temperature for histological analyses; liver, whole intestine and brain were dissected from 2 fish and stored at -20 °C for lipid analysis.

During S2, seven fish were also randomly chosen from each tank and sampled as described. Additionally, in S2, three fish were first anesthetised to collect blood from the caudal vein for cortisol analysis. After that they were euthanised, and stored at -20 °C for proximate analysis.

6.3.9. Stress challenge and sampling 3 (S3)

At the end of the nutritional trial (S2), all the remaining fish (n=144) were counted, evenly redistributed within treatments among the 24 tanks, and then left to recover for three days before the stress challenge. During these three days fish were fed the experimental diets twice a day, according to the feeding regime of 2.5% of their body weight. During the stress challenge, fish were individually netted from two random tanks of the same diet, weighed out and then transferred back into one tank. The challenge involved inducing acute stress through two procedures: chasing and confinement in the net. First, the group of fish within each tank was actively chased using a net for a duration of 8 minutes, and then, the same group was netted and confined in the water for 1 minute (Hvas et al., 2018). Fish were left to recover in the tank after exposure to the acute stress, and blood was sampled quickly 1 hour and 6 hours after returning to the tank as shown in Figure 6.4. Blood samples were collected within approximately 2 minutes after entering the tank and catching the fish to minimise the influence of handling stress on cortisol levels. At each sampling point (time 0, 1 and 2), each fish (time 0: n=72; time 1: n=36; time 2: n=36), was anaesthetised and sacrificed, and blood was drawn from the caudal vein into heparinised vacutainers (23G, 0.6 x 25 mm, BD Microlance, Denmark). Each fish was also weighed, and total length was measured.

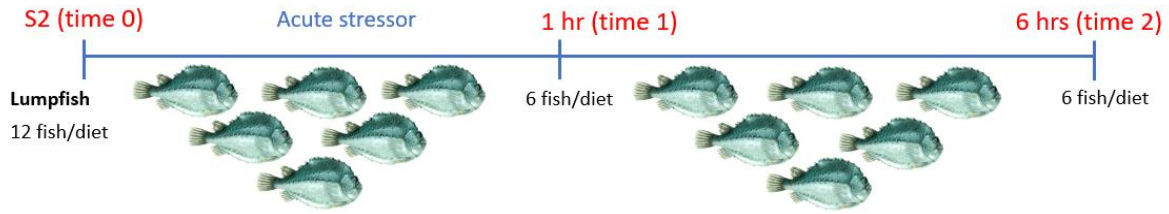


Figure 6.4. Flowchart of the stress challenge. Fish were sampled before being stressed (S2, time 0), 1 h (time 1) after being exposed to the acute stressor and 6 hrs after stress exposure (time 2).

6.4. Analyses

6.5. Growth parameters/feed performance calculations

Throughout the trial, number of fish per tank, daily feed intake, daily mortalities and temperature were recorded. These allowed the calculation of growth parameters as follow:

Specific Growth Rate (SGR) was calculated as:

$$SGR = (e^{g} - 1) \times 100$$

where $g = (\ln(final\ biomass, g) - (\ln(initial\ biomass, g)/number\ of\ days$ (Houde & Schekter, 1980).

Biological Feed conversion ratio (bFCR) was calculated as:

$$bFCR = \frac{Feed\ intake\ (g)}{Biomass\ gain\ (g) + Mortality\ biomass\ (g)}$$

where feed intake is the total of feed ingested (g), biomass gain is the final biomass (g) – initial biomass (g), and mortality biomass (g) is an adjustment that accounts for any mortality that accounted over the measured period (Moran et al., 2009).

Daily growth coefficient (DGC) was calculated as:

$DGC = \frac{final\ biomass\ (g)^{\frac{1}{3}} - initial\ biomass\ (g)^{\frac{1}{3}}}{number\ of\ days}$ (Fournier et al., 2002).

Thermal growth coefficient (TGC) was calculated as:

TGC = ((final biomass (g) $^{1/3)}$ - (initial biomass (g)$^{1/3}$) / temperature (°C) x number of days) (Lugert et al., 2016).

Feed conversion efficiency (FCE) was calculated as:

$FCE = \frac{final\ biomass\ (g) - initial\ biomass\ (g)}{feed\ intake\ (g)}$ (Brett, 1979).

During each sampling point, fish weight, total length, fish height, liver weight and viscera weight were recorded to calculate body condition (BC), hepatosomatic index (HSI), and viscerosomatic index (VSI) as follow:

$BC = \frac{weight\ (g)}{length\ (cm) \times height\ (cm)}$ (Johannesen et al., 2018a).

$HSI = \frac{liver\ weight\ (g)}{fish\ weight\ (g)} \times 100$ (Willora et al., 2021).

$VSI = \frac{visceral\ weight\ (g)}{fish\ weight\ (g)} \times 100$ (Willora et al., 2021).

6.5.1. Nutritional analyses

6.5.1.1. Sample preparation

Three whole frozen fish from S0, S1 and S2 were allowed to defrost at ambient temperature, then cut into pieces and blended (Robot coupe Blixer 4V.V, France) to obtain a homogenous paste. A portion of this paste was weighed in duplicate into a container and frozen at -20 °C. To determine fish moisture, these frozen containers containing the paste were placed in a freeze-dryer at -60 °C (Alpha 1-4 LSC, Christ, Germany) until completely dry. Due to the presence of skin and bones, the dried paste was further ground (KnifetecTM 1095 grinder, Foss, Sweden) into a fine powder to ensure sample homogeneity before further analysis. Feeds were also ground (KnifetecTM 1095, Foss, Sweden), and analysed for proximate analysis.

6.5.1.2. Proximate analysis

The ground powder obtained from whole fish was used to determine ash, protein and total lipids content in duplicate. A portion of the powder was incinerated in a muffle furnace (Carbolite Elf 11/14B, UK) at 600 °C overnight to measure ash content, following standard procedures (AOAC, 2000). Crude protein content was quantified through measuring the nitrogen content, using the Kjeldahl method (Opsis LiquidLINE KjelROC Analyzer and Sampler KD-525, Sweden), where a sample of the fine powder underwent acid digestion with sulfuric acid, and then distillation. During distillation, the nitrogen content was determined by titration. The protein content was measured using a 6.25 conversion factor that considers that protein consists of 16% nitrogen, and all nitrogen present in food protein is bound within the protein structure. Additionally, total lipids were extracted following the method by (Folch et al., 1957), where the ground powder was homogenised using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK), and 20 ml of 2:1 chloroform-methanol as solvent system. Total lipids were gravimetrically calculated. Samples of liver and whole intestine collected from two fish for each tank were pooled and analysed for total lipid following the Folch method described previously for whole fish. Total lipids were also extracted from brain from two fish for each tank, using 10 ml instead of 20 ml of 2:1 chloroform-methanol to minimise sample loss during the homogenisation process. Total lipids of whole fish, feeds, liver, whole intestine and brain were stored in 2:1 + BHT at -20 °C prior to fatty acid analysis.

Ground feed samples were placed in an oven at 103 °C overnight to determine moisture content, while ash content was determined by placing the ground samples at 600 °C overnight in the muffle furnace (Carbolite Elf 11/14B, UK). Crude protein was determined using the Kjeldahl method as described above. Total lipids of feeds were extracted according to the Folch method (Folch et al., 1957) using 36 ml of 2:1 chloroform-methanol as solvent system. Carbohydrate content in feeds was estimated by subtracting from 100 the sum of moisture, protein and lipid content. Gross energy of feeds was calculated using coefficients of 5.65, 9.45 and 4.2 for protein, fat and carbohydrates, respectively

(Henken et al., 1986). Comprehensive details about the methods used for proximate analysis and lipid extraction can be found in Chapter 2.

6.5.1.3. Fatty acid methyl esters (FAME)

The fatty acid profile of whole fish, feeds, liver, whole intestine and brain was determined by analysis of FAME using gas chromatography following the method by Christie (2003) on the total lipids extracted as described earlier (Folch et al., 1957). Transmethylation at 50 °C for 16 h was carried out to prepare the FAME, and their extraction and purification was carried out as described in Tocher and Harvie (1988). FAME were separated and quantified by gas–liquid chromatography (Fisons GC-8160, Thermo Scientific, Italy). The gas chromatograph was equipped with a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), an on-column injector and a flame ionisation detector (FID). The carrier gas used was hydrogen, and the oven thermal gradient was from 50 °C to 150 °C at 40 °C/minute to a final temperature of 230 °C at 2 °C/minute. Data were processed using Chromcard for Windows (version 2.01; Thermoquest Italia S.p.A., Italy). Individual FAME was identified by comparing the samples profile to known standards (Supelco™ 37-FAME mix; Sigma-Aldrich Ltd., Poole, UK), and published data (Tocher and Harvie, 1988). Heptadecanoic acid (17:0) at a concentration of 10 mg/ml was used as internal standard to calculate fatty acid content per g of sample.

6.5.1.4. Lipid classes

Lipid class analysis was performed on total lipid extracted from feeds, liver, whole intestine and brain. Lipid classes were separated by high-performance thin-layer chromatography (HPTLC) using 10 × 10 cm x 0.25 mm plates (VWR, Lutterworth, UK) according to Henderson and Tocher (1992). Twelve 3 mm origins were marked on the plate and total lipid samples in duplicate (1.5 -2 µl), one neutral and one polar standard and one blank were applied to each origin using a Microliter™ glass syringe (Hamilton, Bonaduz, Switzerland). To separate polar lipid classes, plates were developed to 5.2 cm in methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol.). To separate neutral lipid classes, plates were developed to 9.5 cm in the same direction in a solvent mixture containing iso-hexane/diethyl ether/acetic acid (85:15:1.5, by vol.). Lipid classes were visualised by spraying with 3% aqueous cupric acetate containing 8% phosphoric acid, and charring plates at 160 °C for 20 minutes in an oven. Lipid classes were quantified by densitometry using a CAMAG-3 TLC Scanner (version Firmware 1.14.16; CAMAG, Muttenz, Switzerland) with winCATS software (Planar Chromatography Manager, version 1.2.3) to quantify the lipid classes.

6.5.1.5. Amino acid profile

The amino acid composition of feeds was analysed using the Waters ACCQ-TAGTM Ultra Method for hydrolysate amino acid analysis (Waters Corporation, Milford, Massachusetts, USA) as described in Glencross et al. (2021). For this analysis, 250 mg of ground feeds was used and digested

in 10 ml of 6M HCl using a microwave digestion system (MARS 6 240/50, CEM, USA). The digestion was carried out in duplicate, at 150 °C and 190 °C. The higher temperature of 190 °C facilitated the hydrolysis of standard amino acids, while methionine and cysteine were specifically hydrolysed at 150 °C. Tryptophan could not be determined due to its degradation during acid hydrolysis.

Following digestion, hydrolysed samples were diluted to a volume of 250 ml with ultrapure water. Approximately 1 ml of the diluted sample was passed through a 0.45 µM hydrophilic syringe filter and kept refrigerated until derivatisation. Derivatisation was performed as per manufacturer guidelines using Waters H-Class UPLC fitted with an ACQUITY BEH Phenyl 1.7 µ 2.1 X 100 mm UPLC column (Waters Ltd, Hertfordshire, UK). The calibration standard used was the Waters Amino Acid Hydrolysate Standard, and an isocratic solvent system containing AccQ-Tag Ultra Reagent Diluent (Eluent A) and Ultra Reagen Buffer (Eluent B) was used at a flow rate of 1.2 ml/minute.

6.5.2. Histological analyses

Anterior intestine, posterior intestine and liver were fixed in 10% NBF and were processed for histological analyses. Tissues were dehydrated through a graded series of alcohol (Citadel 2000, Thermo Fisher Scientific, USA), with chloroform serving as the clearing agent before being infiltrated with paraffin wax. Once embedded in paraffin wax, blocks were trimmed at 20 µm thickness to expose the tissues, using a microtome (Leica RM 2035, Leica Instruments, Germany). Before sectioning, blocks were immersed in distilled water for approximately 20 minutes to rehydrate the tissues. Sections were cut at a thickness of 5 µm on the microtome, and these sections were subsequently stained with H&E following the protocol by Martoja (1970). Stained sections were digitalised using an AxioScan scanner (ZEISS® Germany) and uploaded to QuPath® version 0.4.2 (Bankhead et al., 2017) to perform measurements like liver intracytoplasmic vacuolization and intestine muscular thickness.

Selected images from the livers were analysed using Fiji ImageJ® (Schindelin et al., 2012) to measure liver intracytoplasmic vacuolization. Briefly, five images at 10x magnification were converted to black and white (8-bit), a threshold was applied to differentiate intracytoplasmic vacuoles from the background, and a watershed algorithm was used to delineate connected structures. Within each image, the intracytoplasmic vacuoles were counted, and their coverage was expressed as a percentage of the total image area. Comprehensive details about the methods used for image analysis can be found in Chapter 2.

6.5.3. Plasma cortisol

Plasma was obtained from blood sampled before fish were challenged (time 0), 1 hour (time 1) and 6 hours after acute stress exposure (time 2). The blood was centrifuged for 5 minutes at 6,000 U/minute (RCF) (MC6 240V, Sarstedt™, Germany), and plasma was stored at -20 °C for posterior cortisol analysis. One hundred ml of plasma and 50 µl of internal standard (d4 cortisol, 50 ng/ml, Sigma-Aldrich, UK) were purified with 500 µl of ethyl acetate and 500 µl of 1% KCl. This mix was

centrifuged, and the upper layer transferred into a new Eppendorf tube, and further 500 µl of ethyl acetate were added to the remaining layer. The second upper layer was added to the previous upper layer and dried under OFN until completely dry, before being resuspended in 100 µl of 1:1 methanol/water. Samples were analysed using the LC-MS and mass spectrometer XEVO® TQ-S coupled to Acquity I-class UPLC (Waters, UK) equipped with a Column ACQUITY UPLC® HSS T3 1.8 µm i.d. 2.1 x 50 mm (Waters, UK). More details of the extraction can be found in Chapter 2.

6.5.4. Fatty acid retention

The fatty acid retention was calculated using the method described in Glencross et al. (2003), where the retention percentage represents the incorporation of dietary fatty acids into the fish whole body over the course of the feed trial. The retention (%) was determined by using the average cumulative feed intake per fish in each tank and the average increase in lipid, protein and fatty acids in the fish from S0 to S2. An average for each dietary treatment was calculated. The retention for each fatty acid was calculated as follow:

$$\text{Fatty acid retention (\%)} = \left(\frac{FA_f - FA_i}{FA_c}\right) \times 100$$

where FA_f is the final amount of a particular fatty acid in the fish body at the end of the study (S2), whereas FA_i is the initial amount of that fatty acid in the fish body at the beginning of the study (S0). FA_c is the amount of the specific fatty acid consumed by the fish throughout the study. FA_i was calculated for each fatty acid as:

$$FA_i = FA_{ri} \cdot (W_i \cdot L_{ri})$$

Where: FA_{ir} is the initial relative amount of the particular fatty acid in relation to all fatty acids in the fish body, and $(W_i \cdot L_{ri})$ represents the absolute amount of lipid in grams, calculated as the weight of the fish at the start of the trial W_i multiplied by the lipid percentage L_i of the fish at the start of the trial. The same method was used to find the final amount for each fatty acid FA_f.

The amount of the specific fatty acid consumed by the fish throughout the studyFA_c was calculated as:

$$FA_c = FA_d \cdot (FI \cdot L_d)$$

Where FA_d is the relative amount of the acid in the diet, $(FI \cdot L_d)$ represents the absolute amount of lipid that has been consumed, whereFI represents the feed intake, and L_d the lipid percentage of the diet.

6.6. Statistical analyses

To investigate the effect of diet while accounting for potential variation by tank, simple linear mixed effects models with diet as predictor and tank as a random factor were constructed using the package "lme4" (Bates et al., 2015) and "lmerTest" (Kuznetsova et al., 2017). This was performed on weight, body condition and HSI. The outcomes of those models show a small variance component for tank,

suggesting that tank effects are minimal (variance < 0.001). Consequently, simple linear models were chosen to investigate the dietary effect with diet as categorical predictor. Morphometric data, condition indices, growth parameters, proximate analysis (moisture, ash, protein, lipid), histological parameters (liver intracytoplasmic vacuolization, anterior and distal intestine muscle thickness), individual fatty acids or lipid classes were treated as the response variable (e.g.: lm(SGR ~ diet)). Proximate analysis data, individual fatty acids and lipid classes were transformed using the arcsine transformation (Zar, 2014). To assess the relationship between individual body weight and whole fish lipid content, linear models were fitted separately for each dietary treatment group.

The retention of protein, lipid and specific fatty acids (%) was determined by calculating the average intake of these nutrients by the fish in each tank along with the average increase in these specific fatty acid within the fish body throughout the trial. These calculations provided unique values for each tank that were used to calculate an average value for each dietary treatment (Glencross et al., 2003). Simple linear models were constructed to investigate whether there was a dietary effect on retentions, with diet as categorical predictor and lipid, protein or individual fatty acid retentions as the response variable (e.g.: lm(Lipid_retention ~ diet)). Before running the linear models, retentions were transformed using the arcsine transformation (Zar, 2014). Model diagnostics were carried out using visual inspection of model plots to examine the model fit. For this dataset, untransformed data were used for the linear models reported. When the linear model showed an overall statistically significant result ($p < 0.05$), the F statistic with the degree of freedom and the P value were reported. A post hoc Tukey HSD test was then performed to identify differences between diets. To test for the effect of diet over time, linear models with proximate analysis, growth and histological parameters as response variable and an interaction between diet and sampling point as predictor variables were constructed (e.g. lm(Lipid whole fish~ sampling*diet)).

To determine EPA+DHA requirement, a polynomial and a linear regression model were performed based on the method described in Houston et al. (2022). The predictor variable was EPA+DHA % of the diet, and the response variables selected were SGR, survival rates and cortisol levels (e.g.: lm(SGR ~ feedEPAplusDHA); lm(SGR ~ feedEPAplusDHA + I(feedEPAplusDHA^2)). These models were compared to investigate the best fit regarding the relationship between the response variables and dietary EPA+DHA. The best fitting model was used for further analysis. Visual inspection of model plots and p-values were used to give recommendations regarding the EPA+DHA levels to use in the formulations. The recommendations were based on higher survival rates, low cortisol levels, and sufficient SGR.

To investigate the effect of diet on survival, a survival analysis was carried out using the “coxme” (Therneau & Therneau, 2015) and “survival” packages (Therneau et al., 2015). A Cox mixed-effects model was constructed, where diet is treated as a fixed effect and tank as a random effect (e.g.: coxme(Surv(Time,Status)~Diet+(1|Tank)).

PCA was performed using the "FactoMineR" package (Lê et al., 2008) to visualise and identify patterns regarding the differences among diets regarding the fatty acid profile of the tissues. Data for the PCA is mean-centered, subtracting the mean of each variable from the values. The outputs of the PCA were visualized using the "factoextra" package (Kassambara, 2016).

The fin damage that was scored 1 to 3 was transformed into a binary response (1 for good welfare, 2 for compromised welfare). The liver score, which was originally rated on a 1 to 6 scale (see Chapter 2), was also transformed to have a binary response. The transformation process involved assigning score 1 (good welfare, healthy liver colour) to the liver that scored 3 and 4 (light to bright orange) and score 2 to the livers that scored 1 and 2 (pale yellow), and 5 and 6 (dark and reddish brown). To investigate the effects of diet on fin damage and liver colour, a binomial logistic regression analysis was conducted. Liver score and fin damage were treated as a binary response and diet as a categorical predictor (e.g. glm(formula = Liver score ~ Diet, family = binomial, data = owi).

Data were analysed using R (R Core Team, 2021), and figures plotted using "ggplot2" (Wickham et al., 2016). Excel data files were imported into R using the "readxl" package (Wickham & Bryan, 2019), which is part of the "tidyverse" collection of R packages (Wickham et al., 2019). Data in tables are presented as mean ± standard deviation (SD).

6.7. Results

6.7.1. Growth parameters

Growth parameters, condition indices, survival rate and feed intake are reported in Table 6.7 for S1 and Table 6.8 for S2. All the experimental diets were well accepted by juvenile lumpfish, according to acclimatisation observations and feed intake. After 21 days of feeding the experimental diets (S1), there were no significant differences in body weight, body length, height, body condition and HSI between fish fed the different dietary treatments (Table 6.7). No significant differences were neither found between the experimental groups on growth parameters in S1 (SGR, DGC, TGC, FCR, bFCR and FCE) (Table 6.7). The dietary treatments influenced the feed cumulative intake per fish at the end of S1 ($F_{5,18}$ = 4.53, P =0.007), with fish fed 100KO and 75KO showing the highest feed intake (6.1±0.9 g and 5.5±0.7 g respectively), whereas fish fed 50KO-0KO had intermediate values (5.1 g). On the contrary, fish fed COM had the lowest feed intake (3.3±0.9 g/fish).

After 47 days of feeding the experimental diets (S2), there were not significant differences between fish fed the different diets regarding body weight, body length, height and body condition (Table 6.8). The COM diet significantly affected the cumulative feed intake at S2 ($F_{5,18}$=6.29, P <=0.001). However, when excluding the COM diet from the mean cumulative intake no differences were found between experimental diets ($F_{4,15}$=1.77, P = 0.120) (Figure 6.5). The hepatosomatic index of the lumpfish changed over time ($F_{2,235}$ = 6.57, P = 0.002) regardless of diet (S1; $F_{5,112}$ = 0.65, P = 0.662, S2; $F_{5,84}$ =

1.10, P = 0.364). Fish fed 75KO had SGR, DGC and TGC significantly higher than 100KO, whereas the other diets showed intermediate values.

Based on the survival analysis, dietary treatments did not have a statistically significant effect on survival over time.

Table 6.7. Growth performance indicators and condition indices in lumpfish reared for 21 days (**S1**) and fed one commercial diet (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO). Data are expressed as mean ± SD (n=4).

Parameter	**COM**	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Body weight (g)	38.5±13.8	47.4±10.9	43.9±11.9	45.0±13.3	44.3±9.4	45.7±12.3	0.380
Body length (cm)	9.8±0.9	10.4±0.8	10.0±0.9	10.1±1.0	9.9±0.7	10.0±0.9	0.626
Height (cm)	4.2±0.5	4.6±0.4	4.4±0.5	4.4±0.6	4.5±0.4	4.5±0.5	0.167
Body condition	0.9±0.2	1.0±0.2	1.0±0.1	1.0±0.1	1.0±0.1	1.0±0.1	0.327
SGR (%)	1.9±0.5	2.8±0.5	2.2±0.4	2.5±0.7	2.5±0.2	2.4±0.2	0.280
DGC (%)	2.1±0.9	3.0±0.5	2.3±0.4	2.7±0.8	2.7±0.2	2.6±0.3	0.298
TGC	2.2±0.7	3.3±0.6	2.5±0.5	2.9±0.9	2.9±0.3	2.8±0.3	0.298
bFCR	0.2±0.1	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.1	0.2±0.0	0.152
FCE	5.3±2.0	4.4±0.3	3.9±0.6	4.6±1.0	4.8±1.1	4.7±1.0	0.080
Cumulative feed intake/fish (g)	3.3±0.9^{b}	6.1±0.9^{a}	5.5±0.7^{a}	5.1±0.7^{ab}	5.1±1.4^{ab}	5.1±0.6^{ab}	0.007
Survival (%)	93.8±5.4	92.7±6.3	91.7±3.4	89.6±8.0	92.7±7.1	88.5±6.3	0.827
HSI (%)	2.1±0.5	2.5±0.9	2.4±0.6	2.3±0.6	2.3±0.5	2.4±0.5	0.662

Table 6.8. Growth performance indicators and condition indices in lumpfish reared for 47 days (**S2**) and fed one commercial diet (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO). Data are expressed as mean ± SD (n=4).

Parameter	**COM**	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Body weight (g)	78.8±21.5	81.3±26.2	89.3±28.3	82.7±19.0	75.7±23.4	75.7±25.7	0.715
Body length (cm)	12.2±1.0	12.2±1.5	12.8±1.2	12.2±1.0	11.6±2.3	11.7±1.3	0.274
Height (cm)	5.3±0.8	5.4±0.7	5.6±0.8	5.4±0.5	5.0±1.0	5.2±0.7	0.707
Body condition	1.2±0.2	1.2±0.2	1.2±0.2	1.2±0.1	1.2±0.3	1.2±0.2	0.620
SGR (%)	2.9 ± 0.8^{ab}	2.0 ± 0.4^{b}	3.0 ± 0.4^{a}	2.5 ± 0.5^{ab}	2.3 ± 0.3^{ab}	2.0 ± 0.4^{ab}	0.033
DGC (%)	3.7 ± 0.9^{ab}	2.6 ± 0.5^{b}	3.9 ± 0.6^{a}	3.2 ± 0.6^{ab}	2.9 ± 0.4^{ab}	2.6 ± 0.5^{ab}	0.042
TGC	3.8 ± 1.0^{ab}	2.7 ± 0.6^{b}	4.1 ± 0.6^{a}	3.4 ± 0.6^{ab}	3.0 ± 0.5^{ab}	2.7 ± 0.5^{ab}	0.042
bFCR	0.1 ± 0.0^{b}	0.2 ± 0.0^{a}	0.2 ± 0.0^{ab}	0.2 ± 0.0^{ab}	0.2 ± 0.0^{ab}	0.2 ± 0.1^{ab}	0.013
FCE	9.4 ± 0.9^{a}	5.0 ± 0.9^{b}	6.7 ± 1.0^{ab}	6.9 ± 1.3^{ab}	5.3 ± 0.7^{b}	6.6 ± 2.4^{ab}	0.009
Cumulative feed intake/fish (g)	10.3 ± 3.8^{b}	19.8 ± 1.6^{a}	18.2 ± 1.7^{a}	16.7 ± 1.6^{a}	18.1 ± 3.0^{a}	16.2 ± 3.0^{ab}	0.001
Survival (%)	90.3±10.5	86.5±9.2	88.5±4.0	87.5±6.8	78.1±5.2	80.2±7.9	0.354
HSI (%)	2.2±0.5	2.4±0.5	2.4±0.5	2.4±0.4	2.4±0.6	2.1±0.7	0.364
VSI (%)	10.7±1.2	11.7±1.4	11.1±2.4	10.8±1.0	10.5±2.8	10.8±1.3	0.128

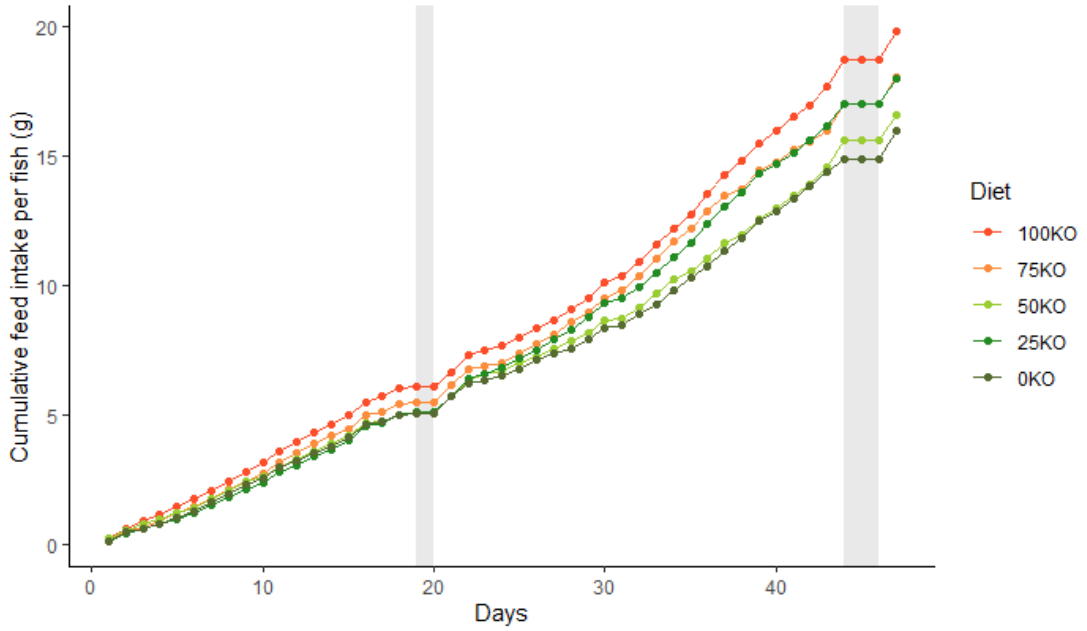


Figure 6.5. Mean cumulative feed intake of individual lumpfish fed the five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) throughout the trial (47 days). Shaded grey lines indicate starvation time before sampling points.

6.7.2. Whole fish chemical analysis

Whole fish composition in S1 and S2 is reported in Table 6.9 and 6.10, respectively. The total lipid of dried whole lumpfish regardless of diet increased over time ($F_{2,147}$= 158.7, P < 0.001). Similarly, the total lipid of whole lumpfish differed between diets in S1 ($F_{5,63}$=13.43, P < 0.001). Fish fed 75KO and 0KO showed a higher lipid content in their body compared to fish fed COM. At the end of the trial (S2), diet COM and 75KO had the highest lipid content compared to the other diets (29.8±1.2% and 28.9±0.5%). A significant positive association regarding weight and lipid content was found in fish fed COM (P= 0.043, R^2 = 0.35), while no significant relationships were observed in the other experimental diets (100KO-0KO, P > 0.05).

Diet and time had interacting effects on the crude protein content of lumpfish ($F_{11,129}$= 20.08, P < 0.001), indicating that diets differed in their effect on body composition over time with the 100KO diet resulting the smallest proportional deposition of protein, and the COM diet resulting in the largest proportional deposition of protein. There were no differences in crude protein at S1 ($F_{5,66}$=1.02, P=0.412), but at S2, fish fed 100KO had the highest amount of protein, and diet COM had the lowest, 59.2±0.7 % and 55.4±1.1 % respectively, due to the changes in lipid content ($F_{5,63}$=15.78, P<0.001). Fish from all diets had lower proportions of protein at S2 than at S1 (P<0.001).

The moisture of lumpfish decreased over time regardless of diet ($F_{2,147}$=72.37, P < 0.001). There was also an interacting effect of diet and time on moisture content of whole fish ($F_{11,129}$=28.38, P < 0.001).

In S1, fish fed diet 0KO had the highest moisture content, albeit not different to that in fish fed COM, 50KO or 25 KO. On the contrary, fish fed diet 75KO the lowest moisture but comparable t that found in groups 100KO and 50KO in S1. Fish fed diet COM and 75KO had the lowest moisture content at the end of the trial (S2), compared to the other diets.

Ash of whole lumpfish decreased over time regardless of diet ($F_{2,147}$= 112.3, P < 0.001). There was also an interacting effect of diet and time on ash content, indicating that ash content changed differently depending on the diet ($F_{11,129}$= 29.48, P < 0.001). In S1, diet COM-fed fish had 13.6% ± 2.2 of ash, which was higher than fish fed diets with inclusion levels of KO ranging from 50-100. In S2, diet 75KO had a slightly lower significant ash content than the other diets, but not different to COM-fed fish.

Table 6.9. Whole fish composition (%) of lumpfish fed one commercial diet (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) (n=4) after 21 days (**S1**). Crude lipid, crude protein and ash are reported on dry matter. Different superscript letters denote differences among the samplings according to one-way ANOVA and Tukey HSD test ($P < 0.05$).

S1	**COM**	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Moisture	88.1±1.3^{ab}	87.4±0.4^{bc}	87.1±0.7^{c}	87.6±0.5^{abc}	88.0±0.5^{ab}	88.2±0.5^{a}	< 0.001
Crude lipid	20.8±0.7^{b}	22.1±1.8^{ab}	23.5±0.3^{a}	22.3±2.1^{ab}	21.9±1.5^{ab}	23.0±3.3^{a}	< 0.001
Crude protein	60.7±2.9	60.5±0.9	59.4±0.7	60.0±1.5	60.2±0.8	60.3±2.3	0.412
Ash	13.6±2.2^{a}	12.1±0.7^{b}	12.0±0.8^{b}	12.3±0.6^{b}	12.5±0.5^{ab}	12.4±0.9^{ab}	0.002

Table 6.10. Whole fish composition (%) of lumpfish fed one commercial diet (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) (n=4) after 47 days (**S2**). Crude lipid, crude protein and ash are reported on dry matter. Different superscript letters denote differences among the samplings according to one-way ANOVA and Tukey HSD test ($P < 0.05$).

S2	**COM**	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Moisture	85.5±0.4^{b}	86.5±0.6^{a}	85.7±0.3^{b}	86.6±0.5^{a}	86.5±0.7^{a}	87.0±0.5^{a}	< 0.001
Crude lipid	29.8±1.2^{a}	26.5±1.0^{b}	28.9±0.5^{a}	26.5±2.2^{b}	27.0±1.5^{b}	26.5±1.5^{b}	< 0.001
Crude protein	55.4±1.1^{d}	59.2±0.7^{a}	56.0±0.5^{cd}	57.8±1.9^{ab}	57.9±1.2^{ab}	56.9±1.6^{bc}	< 0.001
Ash	9.9±0.3^{bc}	10.5±0.5^{a}	9.6±0.1^{c}	10.3±0.7^{ab}	10.4±0.5^{a}	10.8±0.7^{a}	< 0.001

6.7.3. Tissue total lipid content

There was an interaction between diet and time ($F_{11,175}$=61.01, P < 0.001), indicating that some diets (COM and 75KO) increased the lipid content of the fish faster than the others. Figure 6.6 shows the differences found. Lipid content of liver ranged from 21.9 % to 30.7%, where 100KO had the lowest lipid content (average 24.2%), 75KO and COM intermediate values (25.8% and 25.7% respectively), followed by 50KO and 0KO (26.5% and 26.3%) and 25KO which was the highest (28%). Diet affected the lipid content of the liver at the end of the trial ($F_{5,40}$=2.77, P = 0.031, Figure 6.6 A) with fish fed 25KO displaying highest lipid content in the liver compared to 100KO, with the other treatments showing intermediate values (Figure 6.6 B).

The lipid content of whole intestine did not change over time ($F_{1,50}$=1.42, P = 0.239). However, there was an effect of the diet on the lipid content of whole intestine ($F_{5,40}$=4.04, P = 0.004, Figure 6.6 C), with fish fed COM showing the highest lipid content in this tissue, compared to groups 100KO and 0KO. Despite the lipid content of lumpfish brain changing over time ($F_{1,54}$=16.4, P < 0.001), there was no effect of diet on the lipid content of this tissue ($F_{5,40}$=1.69, P=0.158) (Figure 6.6 D).

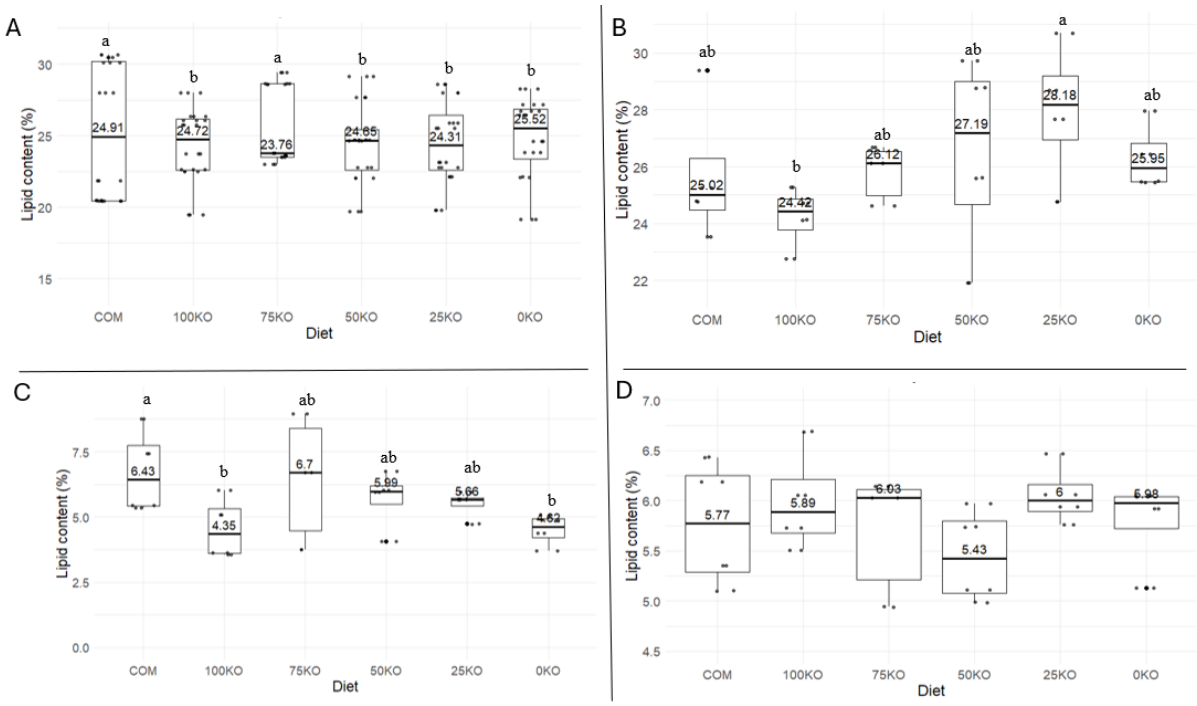


Figure 6.6. Total lipid (%) of dried whole fish (A), liver (B), intestine (C), and brain (D) of lumpfish fed either a commercial control (COM) or five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of (S2) of feeding the experimental diets. Boxes and whiskers represent quartiles for each sampling. Different superscript letters denote differences among the dietary groups according to one-way ANOVA and Tukey HSD test (P<0.05).

6.7.4. Fatty acid profile of tissues

Variations in the fatty acid profile of whole fish, liver, intestine, and brain among dietary treatments can be seen in the PCA biplots in Figure 6.7 A-D. The clusters of the PCAs are widely dispersed and located far apart from each other, indicating a clear separation of the dietary groups in both whole fish and intestine (Figure 6.7 A, C). This spatial distance suggests that there are distinct patterns in the fatty acid profile in these tissues. In the PCA for whole fish (Figure 6.7 A), PC1 accounts for 89.8% of the variation, mainly due to DHA (0.99), OA (-0.99) and LA (-0.98). A similar trend was found in the PCA for whole intestine where PC1 accounted for 84% of the variation which was also due to DHA (0.99), OA (-0.97) and LA (-0.97).

In the case of liver, the clusters for the dietary groups slightly overlapped, and PC1 accounted for 76.3% of the variation which was due to DHA (0.98), DPA (0.95) and EPA (0.93). The analysis of the fatty acid composition of whole fish, whole intestine and liver reflected the fatty acid profile of the dietary treatments due to the blend of KO and RO, reflecting the decreasing levels of inclusion of EPA+DHA. On the other hand, the clusters of the fatty acid profile of brain largely overlapped, except for 0KO, which showed a more distinctive cluster (see Figure 6.7 D). This implies that the fatty acid profile of brain of the different dietary groups shared a higher degree of similarity as we expected. Indeed, PC1 and PC2 for brain accounted for 75.6% of the variation which is lower than the sum of PC1 and PC2 in the other tissues. PC1 accounted for 58.4% of the variation which was due to oleic acid (0.96) and MUFA (0.92), PC2 accounted for 17.2% of the variation which was associated with EPA (0.86) and DPA (0.82).

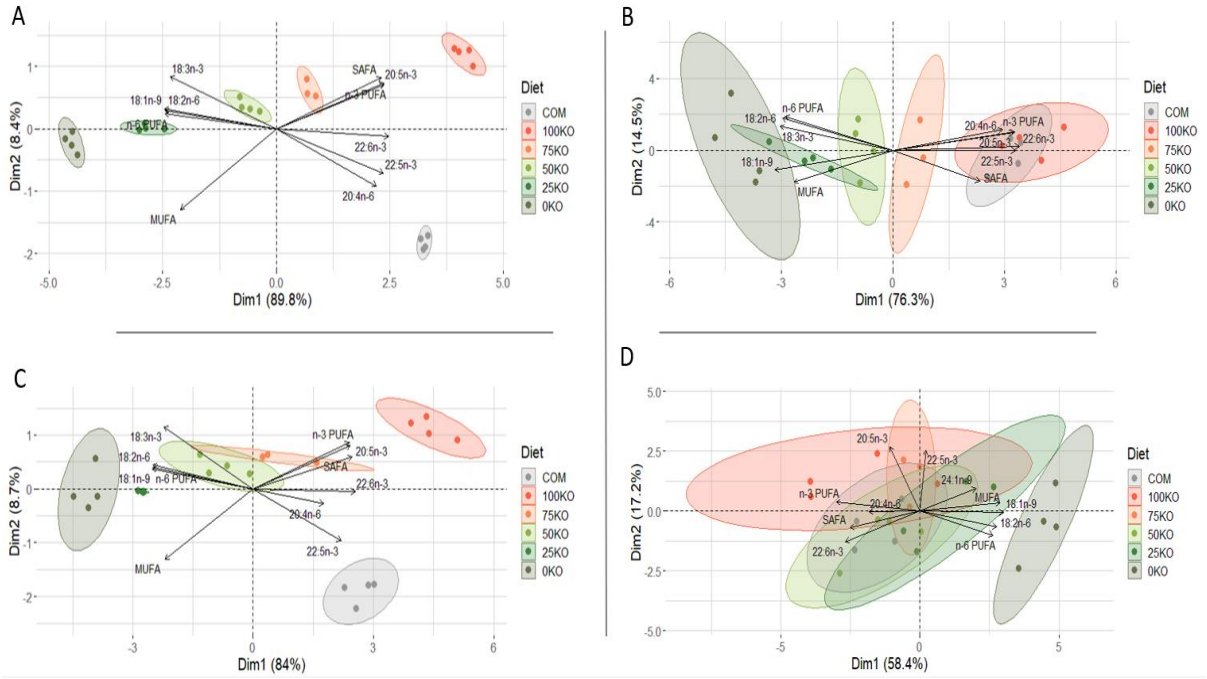


Figure 6.7. Principal Component Analysis (PCA) biplot of lumpfish whole fish (A), liver (B), intestine (C) and brain (D) showing separation of tanks depending on dietary treatments and relative influence of fatty acid profile. Lumpfish were fed one commercial control diet (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO).

6.7.4.1. Fatty acid profile of whole fish

Significant differences were found in all the fatty acids in whole fish (Table 6.11). Fish fed 100KO had the highest amount of SAFA, due to higher levels of 16:0 (100KO>75KO=COM>50KO>25KO>0KO), while fish fed 0KO had the highest amount of MUFA due to the higher amount of OA, which is found in RO (0KO>25KO>50KO>75KO>100KO>COM). Diets with higher inclusion levels of RO had also higher levels of n-6 PUFA (0KO>25KO>50KO>75KO>100KO), mainly due to LA, while diet with higher inclusion of KO had higher levels of n-3 PUFA, due to higher levels of EPA and DHA (100KO>75KO=COM>50KO) (Table 6.11).

Table 6.11. Fatty acid profile of whole fish (n=4) of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of feeding the experimental diets (**S2**). Different superscript letters denote differences among the dietary groups according to one-way ANOVA and Tukey HSD test ($P < 0.05$).

	COM	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
14:0	4.5±0.6^{a}	4.7±0.5^{a}	3.9±0.6^{b}	3.6±0.4^{b}	2.5±0.3^{c}	1.9±0.2^{d}	<0.001
16:0	14.1±0.3^{a}	17.3±0.1^{b}	15.1±0.5^{c}	13.9±0.1^{a}	12.2±0.0^{d}	10.6±0.2^{e}	<0.001
18:0	2.5±0.1^{a}	3.0±0.2^{b}	2.8±0.2^{c}	2.8±0.1^{c}	2.7±0.1^{c}	2.8±0.1^{c}	<0.001
ΣSAFA[1]	**21.6±1.0^{a}**	**25.4±0.5^{b}**	**22.2±0.9^{a}**	**20.7±0.4^{c}**	**17.8±0.4^{d}**	**15.6±0.2^{e}**	**<0.001**
16:1n-7	6.3±0.1^{a}	5.3±0.1^{b}	4.3±0.2^{c}	3.8±0.1^{d}	3.1±0.1^{e}	2.5±0.0^{f}	<0.001
18:1n-9	21.6±0.2^{a}	22.6±0.5^{b}	31.9±0.2^{c}	34.8±0.8^{d}	40.4±0.9^{e}	44.1±0.5^{f}	<0.001
18:1n-7	4.3±0.0^{a}	6.2±0.1^{b}	5.4±0.1^{c}	5.1±0.1^{d}	4.5±0.0^{e}	4.1±0.1^{f}	<0.001
20:1n-9	7.7±0.3^{a}	2.2±0.1^{b}	2.2±0.1^{b}	2.3±0.0^{b}	2.3±0.0^{b}	2.3±0.1^{b}	<0.001
22:1n-11	6.1±0.3^{a}	1.2±0.0^{b}	1.1±0.1^{bc}	1.1±0.1^{cd}	1.0±0.0^{de}	0.9±0.0^{e}	<0.001
ΣMUFA[2]	**49.1±0.4^{a}**	**39.0±0.5^{b}**	**46.4±0.2^{c}**	**48.3±0.8^{d}**	**52.5±0.8^{e}**	**55.1±0.5^{f}**	**<0.001**
18:2n-6	5.4±0.1^{a}	6.1±0.3^{b}	8.4±0.2^{c}	10.3±0.4^{d}	12.5±0.2^{e}	14.6±0.2^{f}	<0.001
20:4n-6	0.5±0.0^{a}	0.4±0.0^{b}	0.3±0.0^{c}	0.3±0.0^{c}	0.3±0.0^{d}	0.3±0.0^{d}	<0.001
Σn-6 PUFA[3]	**6.5±0.1^{a}**	**7.0±0.3^{b}**	**9.1±0.2^{c}**	**11.1±0.5^{d}**	**13.1±0.3^{e}**	**15.3±0.2^{f}**	**<0.001**
18:3n-3	1.3±0.0^{a}	2.4±0.1^{b}	3.1±0.0^{c}	3.6±0.1^{d}	4.3±0.0^{e}	4.9±0.1^{f}	<0.001
18:4n-3	2.2±0.1^{a}	3.6±0.1^{b}	2.5±0.1^{c}	1.9±0.1^{d}	1.2±0.0^{e}	0.7±0.0^{f}	<0.001
20:5n-3	7.8±0.2^{a}	10.6±0.1^{b}	7.8±0.3^{a}	6.6±0.1^{c}	4.9±0.1^{d}	3.6±0.1^{e}	<0.001
22:5n-3	0.9±0.0^{a}	0.8±0.0^{b}	0.6±0.0^{c}	0.5±0.0^{d}	0.4±0.0^{e}	0.4±0.0^{f}	<0.001
22:6n-3	8.7±0.2^{a}	9.1±0.1^{b}	6.8±0.3^{c}	6.0±0.2^{d}	4.7±0.2^{e}	3.8±0.0^{f}	<0.001
Σn-3 PUFA[4]	**22.1±0.5^{a}**	**27.8±0.2^{b}**	**21.7±0.7^{a}**	**19.4±0.2^{c}**	**16.2±0.3^{d}**	**13.8±0.3^{e}**	**<0.001**
ΣPUFA	29.3±0.7^{a}	35.6±0.4^{b}	31.4±0.8^{c}	31.0±0.6^{c}	29.7±0.6^{a}	29.3±0.5^{a}	<0.001
ΣLC-PUFA[5]	12.0±0.1^{a}	16.1±0.1^{b}	11.9±0.1^{c}	10.1±0.1^{d}	7.7±0.1^{e}	5.7±0.1^{f}	<0.001
EPA/DHA	2.5±0.0^{a}	4.5±0.0^{b}	3.9±0.0^{b}	3.5±0.0^{c}	2.8±0.0^{d}	1.7±0.0^{e}	<0.001
EPA+DHA	**16.0±0.0^{a}**	**19.8±0.0^{b}**	**14.6±0.0^{c}**	**12.5±0.0^{d}**	**9.7±0.0^{e}**	**7.4±0.0^{f}**	**<0.001**

[1] Includes 15:0, 20:0, 22:0, 24:0; [2] Includes 16:1n-9,17:1, 20:1n-11, 20:1n-7, 22:1n-9 and 24:1n-9; [3] Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6;[4] Contains 20:3n-3, 20:4n-3, 21:5n-3; [5]Includes 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3.

6.7.4.2. Fatty acid profile of liver

In the liver (Table 6.12), fish fed 100KO showed higher levels of SAFA, albeit not different to those fed 75KO. Fish fed 0KO had the highest levels of MUFA, although not different to 25KO, with OA being the major contributor (0KO=25KO>50KO=75KO>100KO=COM). Fish fed 0KO had the highest levels of LA and n-6 PUFA, but not different to 25KO fish (0KO=25KO≥50KO≥75KO≥100KO=COM). Fish fed 100KO had highest levels of n-3 PUFA and LC-PUFA, due to increasing levels of EPA, DPA, and DHA (100KO=COM>75KO>50KO>25KO>0KO).

Table 6.12. Fatty acid profile of liver (n=4) of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of feeding the experimental diets (**S2**). Different superscript letters denote differences among the dietary groups according to one-way ANOVA and Tukey HSD test ($P < 0.05$).

	COM	100KO	75KO	50KO	25KO	0KO	P
14:0	2.5±0.3^{a}	2.2±0.1^{b}	1.9±0.3^{c}	1.9±0.3^{c}	1.6±0.0^{c}	1.6±0.1^{c}	<0.001
16:0	12.9±0.5^{a}	14.3±0.9^{b}	13.7±0.7^{ab}	13.3±0.8^{ab}	13.1±0.7^{ab}	12.8±1.7^{a}	0.017
18:0	3.4±0.4^{ab}	4.4±0.4^{c}	3.9±0.5^{b}	3.5±0.5^{b}	3.6±0.3^{b}	2.9±0.3^{a}	<0.001
ΣSAFA[1]	**19.0±0.5^{ab}**	**21.1±1.1^{c}**	**19.7±1.0^{ac}**	**18.9±0.9^{ab}**	**18.5±0.9^{ab}**	**17.4±2.1^{b}**	**<0.001**
16:1n-7	4.5±0.4^{a}	3.4±0.1^{b}	2.8±0.5^{bc}	2.7±0.3^{bc}	1.7±1.1^{d}	2.4±0.4^{cd}	<0.001
18:1n-9	34.0±2.6^{a}	35.7±2.3^{a}	41.9±3.6^{b}	43.6±2.8^{b}	47.9±1.1^{c}	49.2±2.8^{c}	<0.001
18:1n-7	6.2±0.2^{a}	7.6±0.5^{b}	6.7±0.3^{c}	6.2±0.2^{a}	5.4±0.1^{d}	5.0±0.3^{e}	<0.001
20:1n-9	6.0±0.7^{a}	1.9±0.1^{b}	1.9±0.1^{b}	1.9±0.2^{b}	2.0±0.2^{b}	1.7±0.1^{b}	<0.001
22:1n-11	2.2±0.4^{a}	0.4±0.0^{b}	0.4±0.0^{b}	0.4±0.1^{b}	0.3±0.0^{b}	0.3±0.1^{b}	<0.001
ΣMUFA[2]	**55.6±1.7^{ab}**	**50.1±1.9^{c}**	**54.8±2.7^{a}**	**55.8±1.9^{ab}**	**58.2±0.4^{bd}**	**59.5±2.4^{d}**	**<0.001**
18:2n-6	6.3±0.9^{a}	7.0±0.9^{a}	8.5±1.8^{ab}	10.2±1.7^{b}	10.7±1.1^{bc}	12.7±2.6^{c}	<0.001
20:4n-6	0.3±0.0^{a}	0.3±0.0^{b}	0.3±0.0^{c}	0.2±0.0^{cd}	0.2±0.0^{e}	0.2±0.0^{de}	<0.001
Σn-6 PUFA[3]	**7.4±1.0^{a}**	**7.9±1.0^{a}**	**9.3±1.9^{ab}**	**11.0±1.8^{b}**	**11.4±1.0^{bc}**	**13.3±2.7^{c}**	**<0.001**
18:3n-3	1.1±0.2^{a}	1.8±0.2^{b}	2.3±0.4^{bc}	2.6±0.3^{cd}	2.9±0.3^{de}	3.3±0.6^{e}	<0.001
18:4n-3	1.3±0.2^{a}	1.7±0.3^{b}	1.2±0.3^{ac}	0.9±0.2^{c}	0.6±0.0^{d}	0.5±0.1^{d}	<0.001
20:5n-3	6.3±0.7^{a}	8.1±0.8^{b}	6.0±0.8^{a}	5.0±0.6^{c}	3.7±0.1^{d}	2.9±0.5^{d}	<0.001
22:5n-3	1.2±0.1^{a}	1.2±0.1^{a}	0.9±0.1^{b}	0.8±0.1^{bc}	0.7±0.1^{c}	0.4±0.0^{d}	<0.001
22:6n-3	6.1±0.4^{a}	5.9±0.6^{a}	4.1±0.4^{b}	3.5±0.2^{b}	2.8±0.4^{c}	2.1±0.3^{d}	<0.001
Σn-3 PUFA[4]	**17.6±1.2^{a}**	**20.6±2.0^{b}**	**15.8±2.0^{ac}**	**14.1±1.1^{c}**	**11.7±0.2^{d}**	**9.7±1.4^{e}**	**<0.001**
ΣPUFA	25.3±2.2^{ab}	28.8±2.0^{a}	25.4±3.8^{ab}	25.3±2.9^{ab}	23.3±0.8^{b}	23.1±4.1^{b}	<0.001
ΣLC-PUFA[5]	14.7±0.4^{a}	16.2±0.5^{b}	12.01±0.5^{c}	10.4±0.3^{d}	8.3±0.2^{e}	6.4±0.3^{f}	<0.001
EPA/DHA	1.0±0.1^{a}	1.4±1.6^{b}	1.5±1.3^{b}	1.4±1.0^{b}	1.3±0.6^{b}	1.4±0.6^{b}	<0.001
EPA+DHA	**12.4±0.1^{a}**	**13.9±1.6^{b}**	**10.0±1.3^{c}**	**8.5±1.0^{d}**	**6.5±0.6^{e}**	**5.0±0.6^{f}**	**<0.001**

[1]Includes 15:0, 20:0, 22:0, 24:0; [2]Includes 16:1n-9,17:1, 20:1n-11, 20:1n-7, 22:1n-9 and 24:1n-9; [3]Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6;[4]Contains 20:3n-3, 20:4n-3, 21:5n-3; [5]Includes 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3.

6.7.4.3. Fatty acid profile of intestine

Also in the intestine (Table 6.13), fish fed 100KO exhibited the highest levels of SAFA, primarily due to increased levels of 16:0. Fish fed higher levels of RO had the highest levels of MUFA, as well as n-6 PUFA, due to increased levels of LA (0KO>25KO>50KO>75KO>100KO=COM). Fish fed diet containing higher inclusions of KO, also had a higher amount of EPA and DHA in the intestine (100KO>COM>75KO>50KO>25KO>0KO).

Table 6.13. Fatty acid profile of intestine (n=4) of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of feeding the experimental diets (**S2**). Different superscript letters denote differences among the dietary groups according to one-way ANOVA and Tukey HSD test ($p < 0.05$).

	COM	100KO	75KO	50KO	25KO	0KO	P
14:0	5.5±0.1^{a}	6.2±0.2^{b}	5.0±0.2^{c}	4.4±0.3^{d}	3.1±0.3^{e}	2.6±0.2^{f}	<0.001
16:0	15.0±0.2^{a}	18.3±0.2^{b}	15.3±0.2^{a}	14.2±0.2^{c}	12.0±0.1^{d}	11.3±0.6^{e}	<0.001
18:0	2.8±0.3^{a}	3.2±0.1^{b}	3.1±0.8^{ab}	3.0±0.2^{ab}	2.9±0.1^{a}	3.0±0.4^{ab}	0.016
ΣSAFA[1]	**23.9±0.3^{a}**	**28.3±0.3^{b}**	**23.9±0.7^{a}**	**22.1±0.5^{c}**	**18.5±0.3^{d}**	**17.3±1.1^{e}**	**<0.001**
16:1n-7	6.5±0.4^{a}	5.3±0.1^{b}	4.7±0.2^{bc}	4.1±0.2^{cd}	3.4±0.2^{d}	2.2±1.4^{e}	<0.001
18:1n-9	19.1±0.1^{a}	19.0±0.3^{a}	27.5±1.3^{b}	31.4±0.5^{c}	37.4±0.3^{d}	40.3±1.4^{e}	<0.001
18:1n-7	4.0±0.0^{a}	6.1±0.1^{b}	5.1±0.0^{c}	4.8±0.0^{d}	4.4±0.1^{e}	4.0±0.1^{a}	<0.001
20:1n-9	7.9±0.2^{a}	2.2±0.1^{b}	2.3±0.0^{bc}	2.4±0.1^{c}	2.4±0.1^{c}	2.3±0.1^{c}	<0.001
22:1n-11	6.3±0.3^{a}	1.3±0.1^{b}	1.3±0.2^{bc}	1.2±0.2^{bc}	1.2±0.1^{bc}	1.1±0.0^{c}	<0.001
ΣMUFA[2]	**46.9±0.8^{a}**	**35.4±0.2^{b}**	**42.4±1.6^{c}**	**45.3±0.8^{d}**	**50.2±0.1^{e}**	**52.0±1.2^{f}**	**<0.001**
18:2n-6	5.8±0.4^{a}	5.8±0.2^{a}	9.7±0.1^{b}	11.1±0.5^{c}	13.0±0.1^{d}	14.9±0.3^{e}	<0.001
20:4n-6	0.6±0.1^{ab}	0.5±0.1^{b}	0.5±0.2^{bc}	0.3±0.2^{c}	0.3±0.0^{c}	0.4±0.1^{ac}	0.002
Σn-6 PUFA[3]	**7.0±0.4^{a}**	**6.8±0.2^{a}**	**10.5±0.4^{b}**	**11.9±0.5^{c}**	**13.7±0.1^{d}**	**15.6±0.3^{e}**	**<0.001**
18:3n-3	1.0±0.6^{a}	2.4±0.1^{b}	3.4±0.3^{c}	3.8±0.1^{c}	4.5±0.0^{d}	4.7±0.3^{d}	<0.001
18:4n-3	2.3±0.1^{a}	3.8±0.2^{b}	2.7±0.4^{c}	2.1±0.1^{a}	1.4±0.1^{d}	0.8±0.1^{e}	<0.001
20:5n-3	7.4±0.1^{a}	11.0±0.4^{b}	7.8±0.5^{a}	6.7±0.3^{c}	4.9±0.1^{d}	3.9±0.2^{e}	<0.001
22:5n-3	0.8±0.0^{a}	0.6±0.0^{a}	0.5±0.0^{b}	0.5±0.0^{b}	0.4±0.0^{b}	0.3±0.2^{c}	<0.001
22:6n-3	8.9±0.5^{a}	9.7±0.2^{b}	7.2±0.5^{c}	6.4±0.4^{d}	5.3±0.2^{e}	4.5±0.3^{f}	<0.001
Σn-3 PUFA[4]	**21.3±0.8^{a}**	**28.6±0.6^{b}**	**22.4±0.3^{c}**	**20.1±0.7^{d}**	**17.1±0.3^{e}**	**14.6±0.3^{f}**	<0.001
ΣPUFA	29.2±0.6^{a}	36.3±0.5^{b}	33.7±0.9^{c}	32.6±0.6^{d}	31.3±0.3^{e}	30.7±0.5^{e}	<0.001
ΣLC-PUFA[5]	18.1±0.2^{a}	22.4±0.2^{b}	16.6±0.4^{c}	14.6±0.3^{d}	11.7±0.1^{e}	9.7±0.2^{f}	<0.001
EPA/DHA	0.8±1.0^{a}	1.1±0.9^{b}	1.1±0.4^{bc}	1.0±0.2^{c}	0.9±0.2^{d}	0.9±0.5^{a}	<0.001
EPA+DHA	**16.3±1.0^{a}**	**20.7±0.9^{b}**	**15.0±0.4^{c}**	**13.0±0.2^{d}**	**10.2±0.2^{e}**	**8.4±0.5^{f}**	**<0.001**

[1]Includes 15:0, 20:0, 22:0, 24:0; [2]Includes 16:1n-9,17:1, 20:1n-11, 20:1n-7, 22:1n-9 and 24:1n-9; [3]Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6;[4]Contains 20:3n-3, 20:4n-3, 21:5n-3; [5]Includes 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3.

6.7.4.4. Fatty acid profile of brain

The lowest levels of SAFA in the brain was observed in fish fed 0KO albeit not different to fish fed 25KO or 75KO (0KO≥25KO≥75KO≥50KO≥100KO≥COM). On the other hand, fish fed 0KO had highest levels of MUFA, with fish from the other treatments displaying similar contents of fatty acids from this group. Fish fed diets with a higher inclusion of KO (50-100KO) had the highest EPA contents, with the remainder of the diets displaying similar values. The lowest DHA was found in fish fed 0KO, where OA and n-6 fatty acids such as LA, increased gradually (0KO>25KO=50KO>78KO=COM>100KO) (Table 6.14).

Table 6.14. Fatty acid profile of brain (n=4) of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of feeding the experimental diets (S2). Different superscript letters denote differences among the dietary groups according to one-way ANOVA and Tukey HSD test ($P < 0.05$).

	COM	100KO	75KO	50KO	25KO	0KO	P
14:0	0.7 ± 0.1^{a}	0.6 ± 0.1^{ab}	0.6 ± 0^{bc}	0.6 ± 0^{bc}	0.5 ± 0.1^{cd}	0.5 ± 0^{d}	<0.001
16:0 DMA	0.4 ± 0^{ab}	0.4 ± 0^{ab}	0.4 ± 0^{a}	0.3 ± 0^{bc}	0.3 ± 0^{c}	0.3 ± 0^{c}	<0.001
16:0	18.5 ± 0.2^{ab}	18.7 ± 0.4^{ab}	18.2 ± 0.1^{ac}	18.9 ± 0.4^{b}	18.6 ± 0.5^{ab}	17.7 ± 0.2^{c}	<0.001
18:0 DMA	1.9 ± 0.1^{ab}	2.1 ± 0.3^{b}	2.1 ± 0^{b}	1.8 ± 0.1^{a}	1.9 ± 0.2^{ac}	2.1 ± 0.1^{bc}	0.003
18:1 DMA	1.8 ± 0.1^{a}	1.7 ± 0.2^{a}	1.9 ± 0.1^{b}	1.8 ± 0.2^{ab}	1.8 ± 0^{a}	1.9 ± 0.1^{b}	<0.001
18:0	7.9 ± 0^{a}	8.3 ± 0.3^{bc}	8.1 ± 0.1^{ac}	8.4 ± 0.3^{c}	8.1 ± 0.2^{ab}	8 ± 0.1^{a}	0.002
ΣSAFA[1]	27.9 ± 0.1^{a}	28.5 ± 0.6^{ab}	28 ± 0^{a}	28.7 ± 0.7^{b}	28.1 ± 0.6^{a}	27.2 ± 0.2^{c}	**<0.001**
16:1n-7	2.2 ± 1.3^{a}	2.5 ± 0.2^{a}	2.5 ± 0.2^{a}	2.2 ± 0.1^{a}	2.2 ± 0.1^{a}	2.2 ± 0.3^{a}	0.648
18:1n-9	17.6 ± 0.4^{ab}	17.4 ± 0.8^{a}	18.2 ± 0.3^{bc}	17.8 ± 0.4^{ab}	18.8 ± 0.6^{c}	20.1 ± 0.2^{d}	<0.001
18:1n-7	3.1 ± 0.1^{ab}	3.4 ± 0^{c}	3.2 ± 0^{d}	3.1 ± 0.1^{ad}	3 ± 0^{b}	3 ± 0.1^{b}	<0.001
20:1n-9	1.1 ± 0.1^{a}	0.7 ± 0^{b}	0.8 ± 0^{b}	0.8 ± 0^{c}	0.8 ± 0^{c}	0.9 ± 0^{d}	<0.001
24:1n-9	2.3 ± 0.2^{ab}	2.2 ± 0.2^{bc}	2.4 ± 0.1^{ac}	2.1 ± 0.2^{b}	2.2 ± 0.2^{bc}	2.5 ± 0.1^{a}	0.002

ΣMUFA[2]	28.3 ± 0.6[ab]	27.4 ± 1.1[ac]	28.3 ± 0.4[bc]	27.3 ± 0.6[c]	28.3 ± 0.9[b]	30 ± 0.2[d]	**<0.001**
18:2n-6	1.9 ± 0.1[a]	1.9 ± 0.2[a]	2.3 ± 0[b]	2.4 ± 0[b]	2.7 ± 0[c]	3.3 ± 0.1[d]	<0.001
20:4n-6	2.8 ± 0[a]	2.6 ± 0.1[b]	2.6 ± 0[bc]	2.7 ± 0.1[c]	2.6 ± 0[b]	2.5 ± 0.1[d]	<0.001
Σn-6 PUFA[3]	5.3 ± 0.2[a]	5.1 ± 0.1[b]	5.4 ± 0.3[a]	5.8 ± 0.2[c]	6 ± 0[c]	6.6 ± 0.2[d]	**<0.001**
18:3n-3	0.2 ± 0[a]	0.3 ± 0[b]	0.3 ± 0[c]	0.4 ± 0.1[c]	0.4 ± 0[d]	0.5 ± 0[d]	<0.001
18:4n-3	0.3 ± 0[ab]	0.3 ± 0[c]	0.3 ± 0[a]	0.3 ± 0[b]	0.2 ± 0[d]	0.2 ± 0[d]	<0.001
20:5n-3	5.6 ± 0.2[a]	6.8 ± 0.3[b]	6.7 ± 0.3[b]	6 ± 0.2[c]	5.9 ± 0.2[c]	5.6 ± 0.3[a]	<0.001
22:5n-3	0.9 ± 0.1[a]	1 ± 0[a]	1 ± 0.1[a]	0.9 ± 0.1[a]	1 ± 0.1[a]	0.9 ± 0.1[a]	0.069
22:6n-3	26.5 ± 0.7[a]	25.7 ± 0.9[ab]	24.9 ± 0.4[bc]	26 ± 0.4[ad]	25.5 ± 0.8[bd]	24 ± 1[c]	<0.001
Σn-3 PUFA[4]	33.8 ± 0.7[ab]	34.4 ± 1.1[a]	33.5 ± 0.3[ab]	33.7 ± 0.5[ab]	33.3 ± 0.7[b]	31.4 ± 0.5[c]	**<0.001**
Total PUFA	43.7 ± 0.6[a]	44.1 ± 0.6[a]	43.8 ± 0.4[a]	43.9 ± 0.2[a]	43.6 ± 0.5[a]	42.8 ± 0.2[b]	<0.001
ΣLC-PUFA[5]	33.1 ± 0.7[ab]	33.5 ± 1[a]	32.6 ± 0.2[ab]	32.9 ± 0.4[ab]	32.2 ± 0.6[b]	30.5 ± 0.6[c]	<0.001
EPA/DHA	0.2 ± 0[a]	0.3 ± 0[b]	0.3 ± 0[b]	0.2 ± 0[ac]	0.2 ± 0[c]	0.2 ± 0[c]	<0.001
EPA+DHA	32.2 ± 0.7[a]	32.5 ± 1[a]	31.6 ± 0.2[ab]	32 ± 0.4[ab]	31.3 ± 0.7[b]	29.6 ± 0.7[c]	**<0.001**

[1]Includes 15:0, 20:0, 22:0, 24:0; [2]Includes 16:1n-9,17:1, 20:1n-11, 20:1n-7, 22:1n-9; [3]Includes 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6; [4]Contains 20:3n-3, 20:4n-3, 21:5n-3; [5]Includes 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3

6.7.4.5. Nutrient retention in whole fish

Nutrient utilisation efficiency is reported in Table 6.15. Values that exceed 100% signify net production, while values below 100% suggest the use of the nutrients for either energy production or metabolic pathways such as other intermediate conversion (Bou et al., 2017). No significant differences were found between dietary treatments regarding protein retention. Diverging levels of EPA and DHA significantly affected lipid retention, with diet 75KO having the highest lipid retention, only different to that of fish fed 100KO. Regarding fatty acids retention, the dietary treatments did not affect the retention of ARA, EPA, DPA and DHA. OA, LA and ALA showed significant differences in retention rates, having the highest retention in the diet 75KO.

Table 6.15. Nutrient utilisation efficiency (% intake) of lumpfish fed 5 experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of feeding the experimental diets (S2). Since diet COM had a different formulation from the experimental diets, it was excluded from the retention analysis. Different superscript letters denote differences among the dietary groups according to one-way ANOVA and Tukey HSD test ($p < 0.05$).

Retention (%)	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Protein	60 ±11	76 ±11	71 ±3	57 ±3	70 ± 20	0.176
Lipid	83 ±17^{a}	132 ±16^{b}	109 ±14^{ab}	88 ±8^{ab}	105 ± 33^{ab}	0.046
OA (18:1n-9)	122 ±29^{a}	181 ±23^{b}	124 ±17^{a}	98 ±9^{a}	108 ± 33^{a}	0.005
LA (18:2n-6)	85 ±19^{b}	134 ±15^{a}	103 ±10^{ab}	86 ±7^{b}	105 ±32^{ab}	0.030
ARA (20:4n-6)	80 ±13	105 ±13	97 ±10	76 ±10	110 ±41	0.172
ALA (18:3n-3)	78 ±16^{ab}	115 ±13^{b}	96 ±10^{ab}	74 ±5^{a}	97 ±30^{ab}	0.043
EPA (20:5n-3)	70 ±14	98 ±12	89 ±12	70 ±7	95 ±36	0.177
DPA (22:5n-3)	151 ±30	220 ±28	191 ±36	144 ±17	195 ±79	0.178
DHA (22:6:n-3)	80 ±15	113 ±14	102 ±15	80 ±8	108 ±42	0.191

6.7.5. Lipid classes

6.7.5.1. Lipid classes of livers

Lipid classes of livers are reported in Table 6.16. No statistically significant differences were found between dietary groups in terms of lipid classes profile for this tissue (Table 6.16). The most predominant lipid classes across all dietary groups in livers were TAG and FFA (10.7-12.8%) among neutral lipids. TAG accounted for over 50% of the lipid classes composition, with values ranging from 56.9 to 59.7%. Among polar lipids, PC (5.2-6.4%) and PE (3.8-4.4%) were the most predominant lipid classes in livers.

Table 6.16. Lipid class composition of livers (n=4) of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of feeding the experimental diets (S2).

Lipid class (%)	**COM**	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Sterol esters (SE)	2.8±3.6	3.2±2.2	3.3±1.6	1.7±1.0	1.8±2.2	2.6±2.9	0.893
Triacylglycerols (TAG)	58.7±2.1	57.8±4.9	59.7±3.6	58.5±5.5	56.7±5.0	56.9±2.7	0.935
Free Fatty acids (FFA)	10.7±1.1	11.0±1.6	11.2±0.5	12.0±3.3	11.2±1.1	12.8±1.5	0.649
Cholesterol (CHOL)	6.5±1.3	6.0±0.3	5.9±0.9	5.6±1.0	7.1±0.3	5.8±0.4	0.124
Diacylglycerol (DAG)	6.7±0.3	5.8±0.8	6.0±0.2	6.5±1.2	6.0±1.1	6.4±0.6	0.702
Total neutral lipid	**86.5±0.4**	**86.7±1.2**	**88.3±2.1**	**87.0±1.3**	**86.2±2.2**	**86.2±1.4**	**0.510**
Phosphatidylethanolamine (PE)	4.2±0.5	3.8±0.3	3.6±0.5	3.6±0.5	4.4±0.9	3.9±0.5	0.353
Phosphatidylglycerol (PG)	0.8±0.5	0.9±0.5	0.9±0.5	1.2±0.2	0.9±0.6	1.0±0.7	0.948
Phosphatidylinositol (PI)	0.4±0.6	0.4±0.4	0.1±0.2	0.1±0.2	0.5±0.7	0.5±0.8	0.768
Phosphatidylserine (PS)	0.1±0.1	0.1±0.1	0.0±0.0	0.0±0.0	0.1±0.1	0.1±0.1	0.857
Phosphatidylcholine (PC)	6.0±0.3	6.0±0.5	5.2±0.8	5.8±0.3	6.4±1.0	6.3±0.9	0.305
Sphingomyelin (SM)	0.3±0.1	0.4±0.1	0.3±0.1	0.3±0.1	0.3±0.1	0.4±0.1	0.310
Lysophosphatidylcholine (LPC)	0.3±0.2	0.4±0.3	0.1±0.2	0.1±0.1	0.1±0.1	0.1±0.1	0.427
Pigmented material (PIG)	1.5±0.4	1.4±0.1	1.5±0.4	1.9±0.2	1.2±0.3	1.3±0.4	0.144
Total polar lipid	**13.5±0.4**	**13.3±1.2**	**11.7±2.1**	**13.0±1.3**	**13.8±2.2**	**13.8±1.4**	**0.510**

6.7.6. Lipid classes of whole intestine

Lipid classes of whole intestine are reported in Table 6.17. The most predominant lipid classes in the intestine were TAG and CHOL (10.7-13.1%) among neutral lipids. TAG accounted for over 45% of the lipid class profile, with values ranging from 45.4% to 54.1% across the different dietary groups. A significant difference was found regarding FFA between the dietary groups, being significantly lower in COM-fed fish compared to 100KO and 0KO fish. Among polar lipids, PC (6.2-9.5%) and PE (5.7-7.0%) were the most predominant lipid classes.

Table 6.17. Lipid class composition of whole intestine (n=4) of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of feeding the experimental diets (S2).

Lipid class (%)	**COM**	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Sterol esters (SE)	2.4±2.1	1.9±1.2	1.6±1.3	1.9±0.8	1.4±1.0	1.1±0.6	0.790
Triacylglycerols (TAG)	54.1±2.6	45.4±6.7	51.6±8.2	45.9±7.3	46.5±4.2	46.4±7.3	0.317
Free Fatty acids (FFA)	8.9±1.3^{b}	12.0±0.7^{a}	11.9±0.4^{ab}	10.7±2.3^{ab}	11.5±0.8^{ab}	13.0±1.1^{a}	0.007
Cholesterol (CHOL)	12.2±0.9	12.9±2.2	11.3±3.5	10.7±1.9	12.7±1.0	13.1±0.7	0.395
Diacylglycerol (DAG)	3.8±0.8	4.2±0.2	4.7±0.1	4.0±0.7	4.6±0.8	4.7±0.9	0.348
Total neutral lipid	**81.4±3.2**	**76.4±4.7**	**81.1±6.0**	**73.2±12.1**	**76.7±2.3**	**78.2±6.1**	**0.507**
Phosphatidylethanolamine (PE)	5.8±1.5	6.7±1.0	5.9±1.9	5.7±1.0	7.0±0.7	6.4±1.3	0.637
Phosphatidylglycerol (PG)	0.7±0.2	1.0±0.4	0.6±0.4	0.6±0.2	0.9±0.9	0.7±0.6	0.933
Phosphatidylinositol (PI)	2.4±0.8	3.3±1.1	2.1±1.9	1.9±0.9	3.3±0.4	3.2±1.3	0.270
Phosphatidylserine (PS)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phosphatidylcholine (PC)	7.4±0.8	9.5±1.7	6.2±4.4	6.5±2.9	9.3±1.2	8.6±2.5	0.299
Sphingomyelin (SM)	0.4±0.2	0.6±0.2	2.3±2.9	2.4±3.9	0.6±0.2	0.6±0.3	0.525
Lysophosphatidylcholine (LPC)	0.0±0.0	0.2±0.3	0.2±0.3	0.2±0.3	0.0±0.0	0.1±0.3	0.678
Pigmented material (PIG)	2.3±0.4	2.4±0.5	1.6±0.3	1.6±0.5	2.2±0.4	2.2±0.4	0.068
Total polar lipid	**18.9±2.7**	**23.6±4.7**	**18.9±6.0**	**18.9±4.0**	**23.3±2.3**	**21.8±6.1**	**0.438**

6.7.7. Lipid classes of brain

Lipid classes of brain are reported in Table 6.18. No statistically significant differences were found between dietary groups in terms of lipid classes profile of brain (Table 6.18). The most predominant lipid classes in brain were polar lipids, with PC and PE being the major contributors (20.7-22.6% and 21-21.8% respectively). Among neutral lipids CHOL was the most predominant lipid class, with values ranging from 24.6 to 27 %.

Table 6.18. Lipid class composition of brain (n=4) of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of feeding the experimental diets (S2).

Lipid class (%)	**COM**	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Sterol esters (SE)	2.0±1.2	2.9±1.5	2.8±2.0	2.6±2.1	4.3±2.9	2.4±0.9	0.756
Triacylglycerols (TAG)	2.2±1.2	3.8±2.1	2.5±0.8	3.8±2.7	3.0±1.1	3.4±2.3	0.827
Free Fatty acids (FFA)	4.9±1.6	4.9±0.5	4.3±0.4	5.1±1.0	5.2±1.4	4.7±1.1	0.901
Cholesterol (CHOL)	26.8±2.6	26.6±2.9	25.4±2.3	26.1±3.4	24.6±1.7	27.0±2.9	0.793
Diacylglycerol (DAG)	1.9±0.5	2.0±0.4	2.0±0.4	2.0±0.3	2.0±0.3	1.9±0.4	0.981
Total neutral lipid	**37.7±3.8**	**40.3±3.6**	**37.0±1.2**	**39.5±2.5**	**39.2±2.9**	**39.4±3.9**	**0.769**
Cerebrosides (CB)	5.5±1.7	5.9±1.3	6.1±1.0	5.1±1.4	5.5±0.9	6.5±0.9	0.705
Phosphatidylethanolamine (PE)	21.8±0.9	21.8±1.5	21.0±0.4	21.1±1.0	21.4±0.6	21.8±0.8	0.777
Phosphatidylglycerol (PG)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phosphatidylinositol (PI)	4.0±0.3	5.4±1.2	5.0±1.8	5.0±1.7	4.3±0.6	4.9±0.9	0.580
Phosphatidylserine (PS)	6.6±1.8	4.1±1.2	7.5±1.5	5.9±1.7	6.6±1.5	4.5±1.8	0.073
Phosphatidylcholine (PC)	22.6±0.9	20.7±2.2	21.9±1.4	21.6±0.6	21.4±2.8	21.1±2.6	0.800
Sphingomyelin (SM)	0.6±0.4	0.4±0.3	0.6±0.6	0.2±0.1	0.4±0.2	0.3±0.2	0.710
Lysophosphatidylcholine (LPC)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Pigmented material (PIG)	1.2±0.3	1.4±0.3	1.0±0.1	1.5±0.5	1.2±0.4	1.5±0.4	0.358
Total polar lipid	**62.3±3.8**	**59.7±3.6**	**63.0±1.2**	**60.5±2.5**	**60.8±2.9**	**60.6±3.9**	**0.769**

6.7.8. OWI

The dietary treatments had no significant effects on the OWI (Table 6.19). Fin erosion (dorsal, caudal and anal fins) was the only damage detected. In S1 and S2 dorsal and anal fin damage had an average of 1 (no damage), while caudal fin damage was on average 1.2 in S1, and 1.1 in S2. Binomial logistic regression analysis confirmed that there were no significant differences in fin damage among the different dietary treatments ($P > 0.05$). No damage was detected regarding eyes integrity, skin status and sucker disc deformities throughout the trial (Table 6.20). Although there were some variations among diets regarding liver score (Figure 6.8), it was not significantly affected by the diet in both S1 and S2.

Table 6.19. Fin damage of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) at S1 and S2. Fin damage was scored from 1 to 3, according to the severity of the damage.

	Dorsal fin		**Anal fin**		**Caudal fin**	
	S1	S2	S1	S2	S1	S2
0KO	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.3 ± 0.4	1.1 ± 0.3
100KO	1.1 ± 0.3	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.2 ± 0.5	1.1 ± 0.3
25KO	1.1 ± 0.3	1.0 ± 0.0	1.0 ± 0.2	1.0 ± 0.0	1.2 ± 0.4	1.0 ± 0.2
50KO	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.2	1.0 ± 0.0	1.3 ± 0.4	1.0 ± 0.2
75KO	1.0 ± 0.2	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.2 ± 0.4	1.1 ± 0.3
COM	1.0 ± 0.2	1.0 ± 0.0	1.1 ± 0.3	1.0 ± 0.2	1.3 ± 0.5	1.1 ± 0.4

Table 6.20. Eyes, sucker disc and skin damage of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) at S1 and S2. Fin damage was scored from 1 to 3, according to the severity of the damage.

	Eyes		**Sucker disc**		**Skin**	
	S1	S2	S1	S2	S1	S2
0KO	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
100KO	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
25KO	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
50KO	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
75KO	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
COM	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0

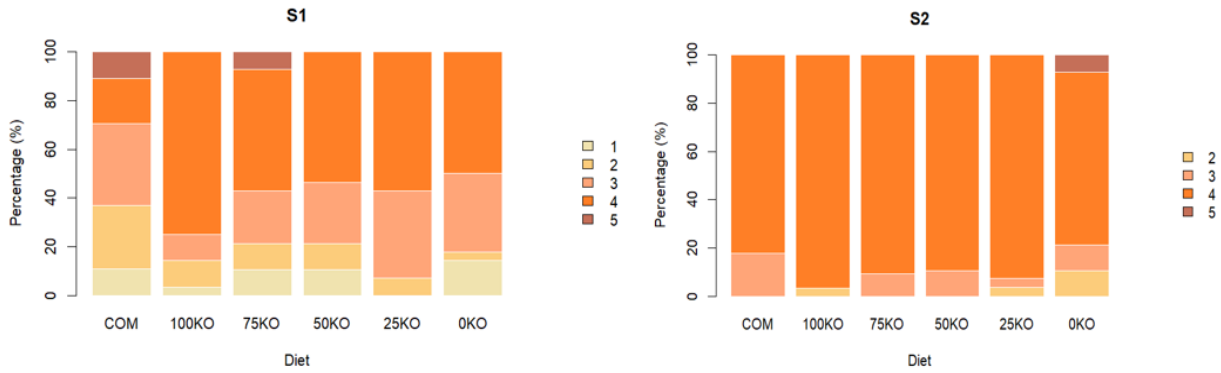


Figure 6.8. Frequency of lumpfish liver colour fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) at S1 and S2. Colours are as defined by the liver score chart by Eliasen et al. (2020), where 1-2 (pale yellow) represent presence of disease or low pigmentation in feeds, 3-4 (orange) is good health and welfare, 5 (reddish brown) is starvation, low fat deposit.

6.8. Histological analysis

The histological parameters assessed in both liver and intestine were not affected by the dietary treatments at either S1 and S2 (Table 6.21 and Table 6.22 respectively).

Table 6.21. Histological analysis of livers and intestine of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 21 days (**S1**).

S1	**COM**	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Liver intracytoplasmic vacuolisation (%)	21.4±5.4	23.0±3.2	21.9±3.1	21.2±3.9	22.3±3.1	23.1±4.8	0.891
Anterior intestine muscle thickness (µm)	180.1±54.1	189.9±45.0	167.3±17.6	203.6±83.8	172.2±47.4	202.2±95.6	0.824
Distal intestine muscle thickness (µm)	123.3±24.5	140.5±72.7	121.8±33.1	135.7±35.4	130.4±20.8	129.5±40.3	0.891

Table 6.22. Histological analysis of livers and intestine of lumpfish fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO, 75KO, 50KO, 25KO, 0KO) after 47 days of feeding the experimental diets (**S2**).

S2	**COM**	**100KO**	**75KO**	**50KO**	**25KO**	**0KO**	**P**
Liver intracytoplasmic vacuolisation (%)	24.1±3.1	22.2±3.2	26.1±2.6	24.4±2.6	25.3±2.3	22.8±5.1	0.276
Anterior intestine muscle thickness (µm)	255.6±144.5	229.8±72.2	159.2±47.8	229.3±67.1	173.5±41.2	186.4±87.7	0.225
Distal intestine muscle thickness (µm)	148.2±35.1	155.2±63.4	130.2±59.7	172.3±32.4	146.1±39.7	145.5±81.7	0.806

6.8.1. Cortisol

There was no effect of the diets on the basal levels of cortisol (on average 2.61 ng/ml) ($F_{5,54}$=1.07, P = 0.383). One hour after being exposed to the acute stressor (T1), cortisol drastically increased (on average 36.2 ng/ml). However, no significant differences were found between dietary treatments during T1 ($F_{5,58}$=0.26, P = 0.928). Significant differences were found between the dietary treatments at the recovery time, 6 hours after the stress challenge (T2) ($F_{5,54}$=4.56, P=0.001). Fish fed the diet 25KO had significantly higher levels of cortisol in T2 (31.5± 16.7 ng/ml), followed by fish fed 0KO (24.7± 10.0 ng/ml). Other dietary treatments (COM, 100KO, 75KO and 50KO) had on average 13.85 ng/ml of plasma cortisol 6 hours after stress exposure (Figure 6.9).

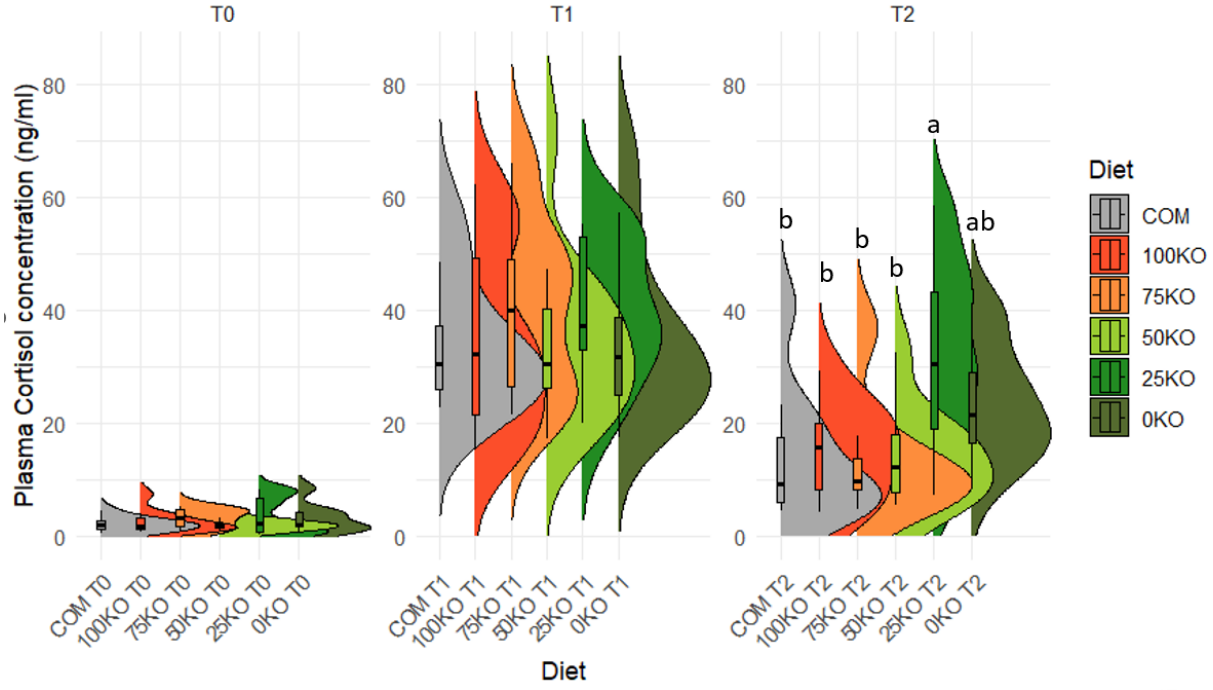


Figure 6.9. Plasma cortisol concentration (ng/ml) of lumpfish at T0 (basal), T1 (1 hour) and T2 (6 hours) after exposure to an acute stressor at the end of the experimental trial. Fish were exposed to a combination of active chasing and confinement during stress exposure. Lumpfish were fed one commercial control (COM) and five experimental diets with decreasing levels of KO (100KO,75KO,50KO,25KO,0KO).

6.8.2. Requirement analysis

To determine EPA+DHA requirement for juvenile lumpfish, a polynomial and a linear regression model were performed. The data (SGR, survival and cortisol) was best described by the polynomial model. The requirements estimate resulted from applying the model to the parameters as shown in Table 6.23. Using the polynomial model to fit survival and cortisol data, the requirement was estimated as 2% EPA+DHA, as percentage of diet, to achieve higher survival and lower cortisol levels. When fitting the model to the SGR data, higher SGR was achieved with 2-3 % EPA+DHA on a feed basis.

Table 6.23. EPA+DHA requirement estimates for juvenile lumpfish from the polynomial model. The estimates of EPA+DHA are expressed as % of feed and as % of total fatty acids.

	EPA+DHA	
Metric	**Requirement (% feed)**	**Requirement (% total fatty acids)**
SGR	≈3	≈18
Survival	≥2	≥15
Cortisol	≥2	≥15

6.9. Discussion

This study investigates the impact of varying dietary inclusion levels of EPA and DHA on the growth, body composition, stress response and overall welfare of juvenile lumpfish. Our findings indicated that different levels of these essential fatty acids did not significantly influence the growth parameters, morphometrics, or survival rates of lumpfish. However, significant differences were observed in the lipid content of the liver and intestine, with higher inclusion levels leading to variations in lipid deposition and fatty acid retentions. Histological analysis revealed no significant differences in liver intracytoplasmic vacuolization nor intestinal muscle thicknesses across dietary treatments, suggesting that lipid content variations did not translate into histological changes. Particularly, the cortisol levels post-stress exposure suggested better stress responses in diets with higher EPA and DHA levels. These results underscore the importance of optimising dietary formulations to enhance the robustness of farmed lumpfish.

6.9.1. Growth parameters and survival

The present study found no significant effect of the experimental diets on morphometric such as body weight, length, height, growth parameters, condition indexes (HSI and VSI) or survival rate of juvenile lumpfish. This indicates that varying levels of EPA+DHA in the diets did not influence the overall growth or survival of the fish. Other studies, in lumpfish (Willora et al., 2021) and Atlantic salmon (Torstensen et al., 2004), found a decreased growth in the groups fed 100% RO (3.1±0.7% and 1.7% of total fatty acids respectively), and this trend was not found in our study regarding diet 0KO. This can be due to the n-3 PUFA requirement for normal growth being met by the EPA+DHA levels in 0KO (5.6 % of total fatty acids), derived from blue whiting and krill meal. This also agrees with the study by Bell et al. (2001) where the highest inclusion of RO (5.4 % EPA+DHA of total fatty acids) did not affect the growth of Atlantic salmon.

SGR in this study was similar to Imsland et al. (2019). On the other hand, TGC ranged from 2.7±0.6 to 4.1±0.6, and it is higher than the values found in Dahle et al. (2020). The observed bFCR of 0.2, though seemingly low, reflects the feed conversion efficiency in the experiment, where low fish density and feed saturation were maintained to ensure ample feed access for all fish. For comparison, the bFCR for Atlantic salmon in a commercial setting is about 1.3 (Frenzl et al., 2014) and for sea bream is about 1.5 (Rimoldi et al., 2018). This difference is not only explained by the difference of the experimental design, but also due to differences in the species. Lumpfish are poor swimmers due to their body composition and morphology (Davenport & Kjørsvik, 1986), and exhibit foraging behaviour that minimizes activity and enables juvenile lumpfish to prioritise growth over active foraging (Killen et al., 2007). Therefore, this bFCR should not be compared to common hatcheries practices, as the setup in this study differs from production. Survival of the fish was not affected by the dietary treatment, and it was on average 85% at the end of the feed trial (S2). The fish fed the COM diet had a significantly lower cumulative

feed intake throughout the trial compared to those fed the experimental diets. This can be due to the higher inclusion of lipid in the commercial diet (21%), compared to the experimental diets (15%). Fish typically eat to meet their energy requirements, and these requirements were met sooner with the energy-dense commercial diet, as also observed in Gélineau et al. (2001) and Li et al. (2016).

6.9.2. Whole fish chemical analysis

The body composition of juvenile lumpfish changed over time, regardless of the diet. This is due to the increase of different body components, such as lipid, as the fish gain mass during growth as also found in Sutton et al. (2000). Increases in lipid percentage were followed by corresponding decreases in moisture content as also detailed in Jobling (2001) in gilthead sea bream (*Sparus aurata*). In S2, fish fed COM and 75KO showed body lipid content which was 3% higher than the other experimental diets. The commercial diet has higher fat inclusion (21.5%) compared to the other experimental diets which resulted in a higher lipid deposition in the body of the fish in the final sampling, and this agrees also with Berge et al. (2023) in lumpfish. The higher lipid deposition and retention found in fish fed 75KO contradicts the antiadipogenic effect of KO (Hwang et al., 2022). This also contravenes the pattern observed in Willora et al. (2021), where juvenile lumpfish fed diets with 100% RO exhibited higher body fat content in contrast to those on diets with greater KO inclusion, which had lower body fat. One possible hypothesis for this discrepancy could be differences in the metabolic response of the fish to varying levels of KO in the diet, potentially influenced by factors such as the interaction of KO with other dietary ingredients, leading to an expected increase in lipid retention. A decrease in protein content was found when the fish increased in size, which was proportionally affected by the increase in lipid content. Protein content in fish is influenced by endogenous factors like fish size and species, which results in changes in relative protein content mostly driven by the relative increase or decrease of other contents such as lipids and moisture (Shearer et al., 1994, Glencross et al., 2011).

6.9.3. Tissue total lipid content and fatty acid profile

Despite the experimental diets being isolipidic, significant differences were found in the lipid content of liver and intestine across the dietary treatments. Liver in lumpfish serves as the main lipid storage organ (Berge et al., 2023), and it highly responds to dietary changes (Hoehne-Reitan & Kjørsvik, 2004), whereas the intestine is primarily responsible for the processes of digestion and absorption (Ray & Ringø, 2014). As the KO content of the feed increased, the lipid content of the liver decreased, except for the 0KO diet, which, despite not including any KO, reached similar levels to diets 75 and 50KO. This trend is consistent with Willora et al. (2021), where fish fed the highest inclusion of RO had similar lipid content to the other dietary treatments containing higher inclusions of krill oil. However, this contradicts findings from Bell et al. (2001), where the total replacement with RO resulted in

significantly higher lipid content in the livers of Atlantic salmon. The observed decrease in lipid content in both the liver and intestine with the 100KO diet can be attributed to the higher inclusion of KO, which resulted in lower lipid deposition due to its antiadipogenic effects (Hwang et al., 2022; Lee et al., 2023).

Despite significant differences in liver lipid content from the dietary treatments, this did not result in differences in HSI, and both liver size and weight remained stable. Furthermore, HSI is influenced by multiple factors, including overall body growth, protein content, and water content in the liver, which might have remained consistent across different dietary treatments, hiding the impact of lipid content on HSI.

The lipid content of the brain was not affected by the dietary treatment in lumpfish as also found in rainbow trout by Roy et al. (2020). The brain is a less plastic organ whose lipid content is relatively stable, and less directly influenced by dietary changes compared to more metabolically active organs like the liver (Mourente et al., 1991).

It is known that the fatty acid profile of fish tissues is significantly influenced by the diet (Xu et al., 2020). The patterns found in the fatty acid composition of whole fish, liver and intestine through the PCAs mirrored the fatty acid profile of the diets, and this trend was also found in Betancor et al. (2014) and Willora et al. (2021). The biplots of the PCAs of whole fish and intestine have distant clusters due to the marked differences in the fatty acid profile of these tissues. On the other hand, in the liver, the clusters slightly overlapped, highlighting the metabolic role of the liver in the fatty acid metabolism, synthesis and disposal (Hodson & Frayn, 2011).

Whole fish and liver fatty acid of lumpfish fed decreasing levels of EPA+DHA resulted in a similar pattern as in Willora et al. (2021), where lumpfish fed higher inclusions of RO resulted in a linear increase of MUFA and n-6 PUFA, and a decrease in SAFA and n-3 PUFA, in both whole body, liver and intestine. However, Willora et al. (2021) found lower EPA levels than DHA in both muscle and whole fish possibly due to EPA being converted into DHA, while in our study EPA and DHA levels maintained similar levels in intestine, liver and whole fish. This could be due to different dietary input of EPA between the two trials as the DHA dietary levels in our study was slightly higher than those used in Willora et al. (2021), and therefore fish had already sufficient DHA in their tissues.

The dietary treatments affected the retention rate of OA, LA and ALA which was the highest in fish fed diet 75KO, likely due to the high retention of lipid of this group. This agrees with the finding of Glencross et al. (2003) where the dietary treatments which resulted in the highest amount of lipid retention, also resulted in elevated retention of OA, and significantly higher retention of LA and ALA in red seabream. Diets 25KO and 100KO had consistently the lowest retention of all fatty acids, possibly due to the low retention of lipid of these dietary groups.

The variations in brain fatty acid profile were less drastic compared to the other tissues, and this was also corroborated by the PCA biplot which showed no separation of the dietary treatments, except for 0KO (Figure 6.7 D), and this agrees with Bou et al. (2017). This means that the dietary treatments had

a small effect on the fatty acid profile of the brain, which tended to conserve the fatty acid levels (Mourente et al., 1991). When fish were fed diets with higher inclusions of EPA+DHA (100-50KO), they tended to incorporate more EPA and DHA in the brain than the other dietary groups (25KO and 0KO), and this agrees with Brodtkorb et al. (1997). Indeed, also our results showed an accumulation of DHA due to the selectivity of the brain towards DHA as also found in Brodtkorb et al. (1997) in juvenile Atlantic salmon and Mourente et al. (1991) in juvenile turbot (Scophthalmus maximus).

6.9.4. Lipid classes of liver, whole intestine and brain

The dietary treatments did not affect the lipid classes composition of liver, intestine and brain as also found in Bou et al. (2017). The only statistically significant difference was found in the free fatty acids of whole intestine, which was significantly higher in fish fed 100KO and 0KO. Higher levels of free fatty acid levels in fish intestine could be a result of the breakdown of triacylglycerol and phospholipid through enzyme activity (Bernárdez et al., 2005), during the digestion and absorption of dietary fats (NRC, 2011).

6.9.5. OWI

The dietary treatments did not significantly affect the OWI scored in this study. Caudal fin damage was the only damage detected, and this also agrees with the findings in Hamre et al. (2022). This could be a result of the tank setting, shelter availability, aggression, and density in the different sampling points. From S0 to S1, 24 fish were in each tank, and most of them were resting in the shelters, possibly leading to some minor intraspecific aggression. Also, there was some variation in the weight within each dietary treatment, and this can affect group hierarchy and therefore aggression levels too (Bessa et al., 2021). However, caudal fin damage improved over time (1.2 in S1 and 1.1 in S2) and this is probably due to the fact that the more severely injured fish died between sampling points.

6.9.6. Histological analysis

Liver intracytoplasmic vacuolization is affected by the nutritional status of the fish, the feeding regime, as well as different dietary lipid sources (Caballero et al., 2002; Eliasen et al., 2020; Imsland et al., 2019b). Despite some dietary treatments resulting in significantly higher or lower liver total lipid content (25KO and 100KO, respectively), this did not correspond to different intracytoplasmic vacuolization. This contravenes studies in other species such as European sea bass juveniles and Atlantic salmon where increased n-3/n-6 ratio resulted in increased hepatic lipid vacuolization (Betancor et al., 2017; Torrecillas et al., 2018). These discrepancies might be due to the use of fundamentally different approaches. The quantification of histological images is subject to resolution, contrast, as well as the threshold settings used in ImageJ, which can introduce variability. The chemical

extraction in the Folch method used to quantify total lipid content may capture a broader range of lipid types, than what is visibly accumulated in vacuoles. Also, histology samples are representative sections, which may not capture the overall lipid content measured by a biochemical extraction that analyse the entire liver sample.

6.9.7. Cortisol

Few studies have investigated the stress responses of lumpfish exposed to acute stressors such as exposure to different temperatures (Hvas et al., 2018), different oxygen saturation levels (Jørgensen et al., 2017), exposure to Atlantic salmon or salmon sensory cues (Staven et al., 2021) or brief air exposure (da Santa Lopes et al., 2023). From these studies, cortisol appears to be the most reliable biomarker for acute stress in lumpfish, while low levels were reported for glucose and lactate in da Santa Lopes et al. (2023), Jørgensen et al. (2017) and Hvas et al. (2018).

In our study, after being exposed to the acute stressor, which was a combination of chasing and confinement, plasma cortisol raised evenly across dietary treatments after one hour (on average 36.2 ng/ml), reaching levels similar to da Santa Lopes et al. (2023) (38.6 ± 2.7 ng/ml). The diets with higher inclusion of KO (100KO, 75KO and 50KO), and therefore higher EPA+DHA levels, regained more quickly basal cortisol levels compared to the other diets (25KO and 0KO), indicating an effect of diverging EPA+DHA levels. Studies in other species have highlighted the effects of EPA and DHA in reducing cortisol levels: in gilthead sea bream (Pérez-Sánchez et al., 2013), in Atlantic salmon (Jutfelt et al., 2007), and in striped catfish (*Pangasianodon hypophthalmus*) (Kumar et al., 2022). Indeed, EPA and DHA play a major role in maintaining physiological balance during stress, as they can affect receptor function and signal transduction pathways, including those involved in stress responses by altering membrane fluidity. This can influence how cells respond to stress signals and potentially lower the activation threshold of the hypothalamic-pituitary-adrenal axis, leading to a moderated release of cortisol (Ganga et al., 2011; Shewchuk, 2014). EPA and DHA are also involved in the production of eicosanoids, which include prostaglandins, playing key roles in the inflammatory and stress response. By reducing the formation of pro-inflammatory eicosanoids and promoting the production of anti-inflammatory and pro-resolving eicosanoids, EPA and DHA can modulate cortisol levels by mitigating inflammation (Ganga et al., 2006). Also, ARA plays a pivotal role in influencing the stress responses in fish, since it is the precursor of pro-inflammatory eicosanoids, contributing to the activation of the stress axis, elevation of cortisol levels and inflammation promotion (Herrera et al., 2019). Therefore, the dietary balance between ARA and n-3-fatty acids in the diet can impact stress responses and overall health in fish.

The cortisol results underscore that assessing fish adaptation to new formulations should not focus only on growth, performance and survival, but also extend to other welfare indicators, like stress resilience, which are relevant for aquaculture practices (Gesto et al., 2021).

6.9.8. Requirement analysis

There are no published requirements for essential fatty acids in lumpfish. Willora et al. (2021) suggested dietary EPA+DHA levels in the range 1.3-2.6% (9-18% of total fatty acids) for 7-40g lumpfish, based on growth performance. In this sense, the diet that only supplied 0.5% of EPA+DHA resulted in reduced growth, suggesting that the requirement for EPA+DHA was not met (Willora et al., 2021).

In our study, we used a polynomial model to estimate the requirement for EPA+DHA in juvenile lumpfish of 20-80 g, in relation to SGR, survival and cortisol, following the method described in Houston et al. (2022). Our results suggest that the diet should contain at least 2% EPA+DHA (15% of total fatty acids) as a minimum requirement to achieve higher survival and lower cortisol, but to achieve a higher SGR, the level is 3% (18% of total fatty acids).

EPA+DHA requirements differ among species, life stage, and different parameters such as maximum performance, health or maintenance could result in different estimates of (Houston et al., 2022). For comparison, recommended levels for juvenile Atlantic salmon is 0.5% (Qian et al., 2020), 2% for gilthead seabream (Houston et al., 2022) or 0.7% for European sea bass (Skalli & Robin, 2004).

6.9.9. Limitations

There are some limitations in this study. The commercial diet (COM) was used to benchmark the experimental feeds and was excluded from the analysis of feed intake, bFCR, fatty acid retention and requirement analysis. This is due to the different chemical-physical characteristics, different production methods, formulation, and energy levels of this diet compared to our experimental diets, which caused discrepancies in the results of these parameters in relation to the experimental diets. The diet 0KO consisted of 5.6% of EPA+DHA, which is higher than other studies with diets deficient in these fatty acids. Further insights could have been made if a dietary treatment with lower levels was used. The survival rate was not as high as in other studies and this could have been due to underlying diseases in the hatchery that evolved throughout the trial in the flow-through system. Further studies regarding gene expression of markers for lipid and fatty acid metabolism could give more insights into fatty acid pathways and corroborate retentions results.

6.10. Conclusions

In this study we investigated the effects that increasing dietary inclusion levels of EPA+DHA have on lipid and fatty acid metabolism, welfare and growth parameters, as well as stress response in juvenile lumpfish. Despite different nutritional studies, there is a need to elucidate the dietary requirements of lumpfish throughout the life cycle in farm conditions. The present study confirms the role of essential fatty acids in supporting optimal health and stress resilience. Based on the results, an inclusion rate of 2-3 % EPA+DHA (18-20% of the total fatty acids), is recommended in the diets for juvenile lumpfish to boost robustness and improve stress responses. Improving the overall welfare of lumpfish by ensuring optimised diets which meet the species nutritional requirements is crucial for the salmon industry, which is currently facing concerns regarding the welfare, the sustainability, and the ethics regarding the use of cleaner fish in salmon cages.

Chapter 7. General Discussion

The aim of this Thesis was to investigate the nutritional status, health and welfare of farmed lumpfish compared to their wild counterparts in order to optimise feed formulation for juvenile lumpfish.

This Thesis tried to address several key challenges:

-Farmed lumpfish often exhibit compromised nutritional status shortly after deployment in salmon sea cages, different from the wild populations.

-A compromised nutritional status is also reflected in OWI like liver colour.

-Other OWI highlight suboptimal farming conditions.

-There is a knowledge gap about the nutritional requirements of lumpfish throughout their life stages.

-To determine the optimal levels of essential fatty acids, particularly EPA and DHA, required for the growth, health, and welfare of juvenile lumpfish.

These challenges were addressed through the following research questions:

1) Does the nutritional status of farmed lumpfish differ from the wild populations?
2) How does the diet composition affect the fatty acid profile of farmed and wild lumpfish?
3) How does the liver score reflect the nutritional status and the welfare of farmed fish in relation to the wild populations?
4) Does farming impact the health and welfare of lumpfish when in the hatcheries and the sea cages? How is the welfare status of the wild population?
5) Do higher levels of EPA+DHA in the diet impact lipid composition, growth and welfare of pre-deployment lumpfish?

1) Does the nutritional status of farmed lumpfish differ from the wild populations?

The main causes of mortality among lumpfish in sea cages include infectious diseases, mechanical treatments against sea lice, and suboptimal diets, as identified by Reynolds et al. (2022). Within few weeks post deployment in the salmon sea cages, many lumpfish exhibit a compromised nutritional status and very low lipid reserves, likely due to inadequate feed composition and poor environmental conditions, according to findings by Boissonnot et al. (2022) and Eliasen et al. (2020). This issue is exacerbated by the scarce information available on the nutritional requirements of lumpfish throughout their life stages, which Garcia de Leaniz et al. (2022) pinpointed as a major challenge in improving the welfare and nutrition of farmed lumpfish. Compared to other species such as Atlantic salmon, the farming of lumpfish as a cleaner fish started quite recently, with significant gaps in knowledge about the best practices for farming this species still present (Powell et al., 2018). Furthermore, when lumpfish are reared in hatcheries, they require a well-balanced feed to promote growth and robustness for the following deployment in the sea cages (Hamre et al., 2022).

By studying the wild lumpfish population (Chapters 3 and 4), we aimed to gain insights into their optimal nutritional requirements, considering they thrive in their natural habitat. To do so, lumpfish were sampled from two origins: farmed and wild. Farmed fish were sampled from hatcheries and from different salmon sea farm locations, whereas wild fish were sampled from the wild populations surrounding the Faroe Islands, both coastal and pelagic.

The focus of Chapter 3 is to evaluate the differences in body composition between farmed and wild lumpfish, with diet, season, and size class playing a crucial role in these differences. The total lipid and fatty acid compositions were identified as the main drivers in the nutritional differences between the two origins. In this sense, farmed lumpfish exhibited higher lipid content than their wild counterparts, reflecting the impact of high-energy diets and controlled farming environments. This trend was also reflected in the lipid content of lumpfish livers which was analysed in Chapter 4, where lumpfish from the land-based hatcheries had a significantly higher lipid deposition in the livers, compared to fish from sea cages and wild fish. This agrees with Passantino et al. (2024) where lumpfish from the hatcheries had significantly higher amount of lipid in the liver compared to the wild counterpart and also when comparing the lipid content of farmed and wild whole fish of other species like yellow perch, sea bream or Atlantic salmon (González et al., 2006; Grigorakis, 2007; Henriques et al., 2014). The lower lipid content of livers of lumpfish in sea cages and in the wild may also be caused by the high activity levels of the fish in the cage environment (Imsland et al., 2014) and the wild. Conversely, lumpfish in the hatchery are reared in tanks, at higher density, with ample availability of feed and lower activity levels (Powell et al., 2018).

The feed delivered in hatcheries is an energy dense formulated feed, which is reflected in the body composition of hatchery fish, which do not have access to any other source of nutrition than the formulated feed. However, farmed lumpfish deployed in the cages also rely on salmon feed, which is

even more energy dense, as well as live prey such as sea lice, zooplankton and other organisms related to biofouling, mainly available in the summer months (Eliasen et al., 2018). Lumpfish from the sea cages were also sampled at different seasons, so that their access to live prey will have been more variable due to seasonality than that of the wild fish caught only in the spring and summer season when there is abundance of zooplankton (Eliasen et al., 2018; Wang & Jeffs, 2014). In some cases, fish in sea cages relied solely on live prey and experienced difficulties to start feeding after being transferred from the hatcheries into the sea cages, resulting in a lower caloric intake. Similarly, as wild lumpfish rely only on the availability of prey, they can encounter starvation periods (Huntingford et al., 2006), and only fully manage to consume enough highly energetic prey once they leave the coastline and develop a pelagic lifestyle (Cox & Anderson, 1922).

In Chapter 3, the stomach content of lumpfish was analysed in relation to the lipid content of whole fish, using the predominant food type identified in the stomach. Lumpfish that had salmon feed as the main food item in the stomach also had a significantly higher lipid content in their body compared to those containing lumpfish feed and unidentified pellets. This is due to the higher levels of lipid and therefore higher energy density of the salmon feeds compared to lumpfish feed, as analysed in Chapter 3. Fish that were found with an empty stomach had intermediate values of total lipid in the body, followed by the fish that consumed prey only. The fish with empty stomachs, but relatively higher total lipid content than those with prey, might have either recently been deployed in the sea cages, retaining fat reserves from the hatcheries, or occasionally managed to feed in the sea cages. The fish that consumed prey only, such as planktonic and benthic prey or sea lice, had the lowest lipid content, reflecting the lower caloric content of these food items, which in turn would result in fat reserve depletion. Moreover, medium to big lumpfish more often had salmon pellets in their stomachs, indicating a preference for the bigger and more energy dense pellets (Eliasen et al., 2018).

Smaller lumpfish were unable to consume large salmon feed pellets, and therefore had to rely on wild prey and lumpfish feed. Given this preference for larger pellets, the standard lumpfish feed pellet (3 mm) may not have an adequate size as the deployed lumpfish grow in the sea cages. When lumpfish are deployed in sea cages with salmon, they are usually provided with only one size of pellet. The chosen pellet size (3 mm) is optimised for the average size of the fish right at the transfer (20-30 g) to ensure that at this developmental stage they can feed efficiently. Using a single pellet size simplifies the feeding process and storage, leading to reduced operational costs. However, increasing the size of the lumpfish feed pellets may encourage the lumpfish to continue consuming lumpfish feed rather than switching to salmon feed as they grow.

Analysing stomach content helps to understand what fish eat, identify their feeding habits, indicate shifts in ecosystem dynamics over time, provide insights into prey selection, and show how prey availability can influence feeding behaviour. However, a limitation of stomach content analysis is that it reflects one feeding event, whereas the lipid content of the fish relates more to the general nutritional history of the fish.

Literature suggests that when fish undergo changes in body condition, these changes will mainly involve the composition of the body, particularly in lipids and moisture (Jobling, 2001). In this study, the protein content of whole fish was relatively stable, showing similar values independently of the origin. Literature more generally indicates that protein content in fish is highly dependent on endogenous factors such as fish size and species, which results in relative protein content mostly being influenced by the relative increase or decrease of other components such as lipids and moisture (Glencross et al., 2011; Shearer et al., 1994). Changes where an increase in lipid results in a subsequently decrease in moisture are more substantial than protein levels changes (Shearer et al., 1994). This might explain the stable protein content in wild lumpfish compared to the decreasing trend in farmed lumpfish. As lipid content in farmed lumpfish is highly variable and depends on whether the fish were in tanks with very low energy requirements or whether the fish had access to the highly energetic salmon feed, the more stable protein content is proportionally affected by the lipid content and potentially also the moisture content.

The amino acid profile in fish is influenced by species, dietary factors, environmental conditions and physiological processes. Among these factors, the diet plays an important role in providing the type and quantity of amino acids available to the fish for metabolism, growth and development (Li et al., 2021). The amino acid profile was analysed in lumpfish < 50 g in this study, and farmed lumpfish had significantly lower methionine levels compared to their wild counterparts. Methionine is a sulphur-containing essential amino acid and methionine deficiencies can lead to adverse physiological effects in fish, including reduced growth performance, impaired immune function, and can lead to cataract development in lumpfish (Jonassen et al., 2017). Although no cataracts were observed in the farmed fish sampled, this finding suggests that methionine levels in farmed fish might be slightly suboptimal.

The replacement of animal protein with vegetable-based alternatives in aquafeeds affects the requirements of sulphur-containing amino acids that are low or absent in plant proteins (Andersen et al., 2016). Another key finding was the significantly lower levels of cysteine in farmed fish compared to wild fish. Since cysteine can be synthesised from methionine, the observed lower methionine could also contribute to lower cysteine availability. The lower cysteine levels in farmed lumpfish might indicate differences in diet composition, metabolic regulation, or environmental factors affecting sulphur amino acid metabolism. Wild fish typically consume a diverse range of natural prey items, which may provide a more complex and bioavailable amino acid profile. In contrast, aquafeeds are formulated to meet nutritional requirements, but may not fully replicate the diversity of amino acid sources available in the wild (Wilson, 2003).

In conclusion, the nutritional status of farmed lumpfish differs from the wild populations. Farmed lumpfish exhibited higher lipid content compared to their wild counterparts smaller than 300 g, suggesting higher lipid deposition due to the farming environment and the diet composition. This suggests that high energy dense diets (20-21 MJ/kg) are not recommended for lumpfish.

2) How does diet composition affect the fatty acid profile of farmed and wild lumpfish?

The fatty acid profile of fish reflects dietary intake (Betancor et al., 2014; Sharma et al., 2010; Willora et al., 2021), thus the fatty acid profile of fish can be used as reliable marker of dietary history, prey composition, and give insights into the feeding habits of the species (Betancor et al., 2014; Sharma et al., 2010; Willora et al., 2021). The fatty acid profile of whole lumpfish and liver from farmed and wild origins were analysed in Chapter 3 and 4, respectively. The fatty acid analysis highlighted differences among origins, seasons and size classes, indicating a difference in diet. However, farmed fish were exposed to different environmental conditions including temperature, salinity, and stress levels, compared to wild fish. These environmental conditions can also influence fatty acid metabolism and composition (Henderson & Tocher, 1987). For instance, changes in salinity have significant effects on the fatty acid profiles of fish, with variations depending on the species (Luo et al., 2021). In Senegalese sole (*Solea senegalensis),* lower salinity resulted in enhanced n-3 LC-PUFA biosynthesis in hepatocytes (Marrero et al., 2021), whereas in silver pomfret (*Pampus argenteus*) acute salinity modulated the expression of PUFA-biosynthesising genes (Luo et al., 2021).

Wild fish had notably higher SAFA and n-3 PUFA, while higher MUFA and n-6 PUFA were found in farmed fish. OA was the predominant MUFA in our data, and it was higher in farmed compared to wild lumpfish in both whole fish and liver. Farmed lumpfish also contained much higher levels of LA and ALA than wild lumpfish, resulting in a higher overall n-6 PUFA content. This difference between wild and farmed fish has also been found in brown trout (*Salmo trutta*) and common carp (Antão-Geraldes et al., 2018; Yeganeh et al., 2012). This indicates that the higher levels of these fatty acids in the farmed lumpfish in our study was most likely related to their access to lumpfish feed only in the case of the hatcheries, and lumpfish and salmon feeds in the case of the fish in sea cages. Aquafeeds are often formulated with ingredients such as fishmeal, fish oil, and vegetable oils. Vegetable oils can include significant amounts of OA (Miller et al., 2008), LA, and ALA, so they can be used as a marker of vegetable oils in the feeds (Fernandez-Jover et al., 2011; Tvrzicka et al., 2011). Oleic acid (OA), which serves as a source of energy, was higher in farmed lumpfish than in wild ones, suggesting dietary influence from commercial aquafeeds, particularly those containing rapeseed oil (RO), high in oleic acid and often included in aquafeeds. While OA is naturally present in marine prey and therefore in wild lumpfish (Auel et al., 2002), the higher levels in farmed lumpfish potentially indicate access to salmon feed and lumpfish feed (Miller et al., 2008).

Wild fish have a significantly higher amount of SAFA, due to the levels of palmitic acid, and significantly lower amounts of MUFA and n-6 PUFA. Consequently, this led to a higher proportion of n-3 PUFA, mainly due to EPA and DHA. The higher relative levels of EPA and DHA in wild lumpfish can be attributed to two factors: the fatty acid composition of their natural prey and selective retention. In the wild, fish consume less overall, leading to a higher proportional retention of polar lipids, along with EPA and DHA. This is because wild fish tend to be leaner, and the total lipid content primarily

reflects phospholipids in cell membranes rather than neutral lipids, which are more associated with energy storage (Bandara et al., 2023).

The fatty acid profile of wild fish in our study gave insights into the feeding habits of wild lumpfish as the fatty acid composition of prey affects the profiles of whole fish and livers (Wang & Jeffs, 2014). The high n-3 LC-PUFA content in the livers and whole fish of wild lumpfish in this study indicate that their diets included an abundance of copepods, shrimp, and krill which contain high amounts of phospholipid bound EPA and DHA (Hellessey et al., 2022; Kim et al., 2014; Xie et al., 2019). These might include the crustaceans *Themisto abyssorum* and *Themisto libellula,* commonly found in the stomach of wild lumpfish, which are rich in n-3 LC-PUFA as reported by Auel et al. (2002). Consequently, a diet predominantly comprising these crustaceans could lead to higher n-3 PUFA levels in the fish. Among MUFA, 20:1n-9 and 22:1n-11, which are known marine zooplankton biomarkers due to their abundance in the wax esters of *Calanus* copepods (Parzanini et al., 2020), were also significantly higher in both wild whole fish and liver in this study.

Wild lumpfish up to 300 g had significantly higher n-3 LC-PUFA due to the contribution of EPA and DHA and this trend was also found in studies comparing wild and farmed Atlantic salmon (Henriques et al., 2014), sea bass (Lenas et al., 2011), and meagre (Saavedra et al., 2017). As fish grow, their nutritional requirements and efficiencies in converting fatty acids change (NRC, 2011). Wild lumpfish might have a varied diet rich in marine organisms that are high in n-3 LC-PUFA, especially when they are younger and more active in foraging. As wild fish grow larger, their diet might change, either due to a shift in prey availability, seasonality or a change in dietary preferences. Farmed fish, on the other hand, are typically fed formulated feeds that can have varying levels of n-3 PUFA depending on the composition of the feed. Lumpfish from the land-based hatchery are fed solely on lumpfish feed, and therefore they reflect the fatty acid profile of the feed used in the hatcheries, which is higher in MUFA, n-6 PUFA and ALA.

Long-chain polyunsaturated fatty acids (n-3 LC-PUFA) such as EPA and DHA play essential roles in fish growth, metabolism, and cellular function (NRC, 2011). N-3 PUFA were significantly higher in the lumpfish from the land-based hatcheries, reflecting the higher n-3 PUFA content in formulated aquafeeds, which are often enriched with fish oil or alternative lipid sources to maintain optimal fatty acid composition. In other species higher levels in wild fish have been seen like salmon (Henriques et al., 2014), sea bass (*Dicentrarchus labrax*) (Lenas et al., 2011), and meagre (*Argyrosomus regius*) (Saavedra et al., 2017).

Wild lumpfish might have a varied diet rich in marine organisms that are high in n-3 LC-PUFA, especially when they are younger and more active in foraging. As wild fish grow larger, their diet might change, either due to a shift in prey availability, seasonality or a change in dietary preference. Farmed fish, on the other hand, are typically fed formulated feeds that can have high levels of n-3 PUFA depending on the composition of the feed.

As fish grow, their nutritional requirements and efficiencies in converting fatty acids change (NRC, 2011). As lumpfish grow both in farmed and natural environments, they have higher fat storage, accumulating more SAFA and MUFA relative to PUFA. When fish have lower fat storage, SAFA and MUFA are low, and there is selective retention of PUFA (Bandara et al., 2023). Larger fish might have a slower metabolism and different energy requirements compared to smaller growing individuals and this can also affect their fatty acid profile. To further understand this relationship, absolute values of EPA, DPA and DHA (g/100g) were investigated rather than just expressing them as relative percentages. Wild lumpfish had the lowest absolute EPA+DHA levels despite a higher percentage relative to total lipids, suggesting selective retention of PUFA when lipid content is low. Sea-cage lumpfish had intermediate absolute EPA+DHA levels, reflecting their exposure to both formulated feeds and natural prey. Land-based lumpfish had the highest absolute EPA+DHA values, consistent with the higher lipid content and the n-3 PUFA content in their feeds. Additionally, DPA, intermediate in the metabolic conversion of EPA to DHA, followed a similar trend, highlighting the differences in diet composition, lipid metabolism, and growth stage on fatty acid deposition in lumpfish. While the fatty acid profile of fish reflects the fatty acid profile of the dietary intake (Betancor et al., 2014; Sharma et al., 2010; Willora et al., 2021), different environmental conditions including water temperature, salinity, and stress levels, may influence lipid metabolism. Wild fish experience natural fluctuations in food availability and energy expenditure, which could lead to differences in fatty acid mobilisation and deposition compared to farmed fish, which live in more controlled conditions. Previous studies showed that environmental conditions and stressors can alter fatty acid metabolism and composition, with colder water temperatures favouring higher PUFA retention in membranes (Henderson & Tocher, 1987). Although environmental fluctuations were not investigated, this could partially explain some variations observed between sea cages and land-based lumpfish.

The higher n-6 PUFA content in farmed lumpfish suggests a strong influence from vegetable feed ingredients, which may affect their overall lipid metabolism and fatty acid balance. While n-6 PUFA play a role in cellular function and energy storage, an excessive n-6 to n-3 PUFA ratio can have physiological consequences, potentially affecting immune function, inflammatory and stress responses, and overall fish health (Hundal et al., 2021). In aquaculture, maintaining an optimal n-6/n-3 balance is critical, as elevated n-6 PUFA levels at the expense of n-3 PUFA may influence stress resilience, growth efficiency, and the effectiveness of lumpfish as cleaner fish.

When fish have a low-fat storage, SAFA and MUFA are low and there is selective retention of PUFA (Bandara et al., 2023). Larger fish might have a slower metabolism and different energy requirements compared to smaller growing individuals and this can also affect their fatty acid profile.

The fatty acid profile of the fish sampled both in the wild and in the sea cages is strongly influenced by seasonal variation as seen in the PCAs of Chapter 4. Seasonal changes impacted the fatty acid profile in such a way that winter fish had overall more n-6 PUFA, MUFA and ALA, while summer fish were

higher in SAFA, ARA and n-3 PUFA. This is mainly due to the seasonal variation in abundance and availability of zooplankton in the ocean which contain SAFA, ARA and n-3 PUFA (Auel et al., 2002; Brett et al., 2009; Eliasen et al., 2018). In winter, when zooplankton is scarce, fish in sea cages primarily rely on salmon and lumpfish feeds, which contain vegetable oils rich in OA, LA and ALA, as mentioned before. The cluster of fish from autumn in the PCA biplot (Figure 4.3) overlaps mainly with the fish from winter, showing a trend more like the winter fish. When investigating the fatty acid profile of fish only sampled during summer months in Chapter 4, it shows that the fish from the sea cages have a similar fatty acid profile to the fish from the wild, showing that in summer both groups have similar diets due to the more abundant zooplankton, available to both wild lumpfish and the ones deployed in the sea cages (Eliasen et al., 2018). One limitation of this study is that all wild fish were sampled during the summer season, and therefore the fatty acid profile reflects the composition of the prey available during spring and summer.

To conclude, wild lumpfish with their diverse and natural diet, contain higher n-3 LC-PUFA than their farmed counterparts. The fatty acid profile of farmed fish is influenced by the aquafeed composition, and the availability of seasonal prey in the case of the sea cages.

3) How does the liver score reflect the nutritional status and the welfare of farmed fish in relation to the wild populations?

The liver plays a pivotal role in fish nutrition, primarily managing nutrient distribution and fatty acid metabolism, managing intake, synthesis and elimination of nutrients including proteins, carbohydrates and lipids (Hodson & Frayn, 2011; Rust, 2003). Following feeding, excess fatty acids are transported as lipoproteins to storage sites, accumulating as TAG that can be use during high energy demand (Tocher, 2003). Also, when energy intake surpasses expenditure, this results in higher liver fat deposition leading to higher liver vacuolization (Caballero et al., 2004). Additionally, the liver stores carbohydrates, produces bile for lipid digestion, and executes detoxification processes (Bruslé & i Anadon, 1996). In lumpfish, the liver constitutes approximately 1.4% and 2.3% of total body weight in females and males, respectively, and is the primary lipid storage tissue (Berge et al., 2023; Davenport & Kjørsvik, 1986). In farmed lumpfish there is a notable accumulation of lipids in the liver, with levels varying between 19% and 35% (Berge et al., 2023; Hamre et al., 2022). Eliasen et al. (2020) established that liver colour in lumpfish could be used as a welfare indicator, where those with orange livers and higher astaxanthin levels had better nutritional status and welfare, than those with dark reddish livers, which indicated starvation, due to low lipid (TAG) content and low astaxanthin levels (Eliasen et al., 2020; Osako et al., 2003). Astaxanthin is the main pigment responsible for lumpfish liver colour and improves flesh pigmentation in salmonids. Apart from contributing to pigmentation, astaxanthin is an antioxidant that protect cells from oxidative damage and enhances the immune system, making fish more resilient to diseases. These properties are crucial for maintaining fish health and welfare,

particularly under stress conditions (Raza et al., 2021). Because of the work from Eliasen et al. (2020), liver colour is now used as an indicator of general welfare in lumpfish as well as an early warning of disease outbreak, due to mobilisation of astaxanthin as part of oxidative stress causing lumpfish livers to lose their orange colouration (Nordgarden et al., 2003).

By relating the nutritional content to the welfare scoring of lumpfish livers in Chapter 4, and by assessing intracytoplasmic vacuolization and liver health through histological parameters in Chapter 5, more knowledge regarding the nutritional and welfare status of farmed lumpfish was gained to improve feed formulation. In Chapter 4, the livers were analysed in terms of lipid content, fatty acid profile, lipid classes, pigment content, and liver colour. In Chapter 5, livers were assessed for liver intracytoplasmic vacuolization, inflammation, congestion, fibrosis and necrosis. In both chapters, farmed fish livers were compared to the wild counterparts, used as a reference.

Total lipid content, TAG, and liver intracytoplasmic vacuolization of lumpfish from the hatcheries were generally higher than the wild and the sea cages fish. The higher fat reserves of the land-based fish can be due to the supply of energy-dense feeds being delivered in the hatcheries, but also the tank rearing environment that makes the fish less active coupled with regular feeding schedules (Bolla et al., 2011). The lower fat reserves of the wild group are most likely due to their natural diets, the environmental conditions of their habitat, and prey availability. High standard deviations in total lipid content, TAG, and liver intracytoplasmic vacuolization among fish from sea cages indicate varying degrees of lipid reserves. This variation suggests a range of nutritional statuses within the group, influenced by factors such as size differences, exposure to currents and waves, insufficient shelter, and in some cases suboptimal feeding practices, which could lead to poor nutrition.

In Chapter 5, an effect of seasonality was also found in the sea cage fish in terms of liver intracytoplasmic vacuolization, which was higher in autumn and winter and significantly lower in spring and summer, probably reflecting access to pelleted feeds only in autumn and winter, and zooplankton in the spring and summer months as reported in Eliasen et al. (2018). During summer and spring, wild fish and those from sea cages experienced similar levels of liver vacuolization, likely due to shared dietary sources due to the increased availability of zooplankton. This dietary overlap during the warmer months suggests a convergence between the two groups, highlighting how environmental and dietary factors influence liver intracytoplasmic vacuolization patterns irrespective of other living conditions.

This variation in the sea cages is further supported by the liver colour, used as an OWI in Chapter 4. A small percentage of darker liver colour was reported in the sea cages, which was associated with very low levels of total lipid and TAG, indicating a poor nutritional status. Eliasen et al. (2020) reported TAG levels of approximately 16.2 ± 8.6 % of starving lumpfish that also showed very dark reddish-brown livers, skin injuries and scored poorly on other OWI. This is an indication that fish with dark livers

containing very low levels of lipids have a generally compromised health status. On the other hand, wild fish had on average 27% of TAG and this does not indicate poor lipid reserves.
Liver colour in lumpfish is primarily influenced by astaxanthin levels, also identified by Eliasen et al. (2020). The liver colour of wild lumpfish in this study was mainly bright orange with high levels of astaxanthin due to the intake of astaxanthin-rich prey, such as crustaceans like krill and small shrimps. Astaxanthin is naturally produced by microalgae in aquatic ecosystems, and subsequently consumed by zooplankton or crustaceans, which then are part of the diet of wild lumpfish (Higuera-Ciapara et al., 2006; Lambertsen & Braekkan, 1971).

On the other hand, a paler liver is not always a sign of bad nutritional status. Pale livers reported in the wild group were scored in large mature female lumpfish that were about to spawn. During the breeding season, female lumpfish mobilise their fat reserves (Craig et al., 2000) and deposit their pigments in the roe, resulting in very pale-yellow livers and bright roe, which ranges in colour from purple to red, and orange (Passantino et al., 2024). However, a sudden appearance of pale livers in captive lumpfish population is an early indicator of disease outbreak (Imsland et al., 2022). Indeed, diseases, particularly those affecting the liver, might alter how astaxanthin is processed and stored (Chang & Xiong, 2020).

Farmed lumpfish receive astaxanthin through formulated feeds: salmon feed used in the sea cages contains a considerable amount of astaxanthin (approximately 100 mg/kg of feed), while the lumpfish feed used in the hatcheries and sea cages had a lower amount (approximately 36-38 mg/kg of feed). Indeed, lumpfish from the land-based hatcheries not only had measurably lower levels of carotenoids in their livers, but these differences were also visually apparent, with hatchery lumpfish having paler livers. This may be due to life stage as it has been shown in Chapter 4, where a significant relationship between weight and the probability of having an orange liver was found, indicating an increased likelihood of healthy livers with higher weight. Lumpfish from the hatcheries (<50 g) had a lower probability of having an orange liver compared to bigger fish (50-150 g), and less astaxanthin accumulation in the liver due to the higher total lipid content. A pale liver, though seemingly less than optimal, may simply be an indication of low levels of pigments in the feed delivered in the hatchery or be related to life stage. Despite deployed lumpfish having access to salmon feed rich in astaxanthin, the presence of pale livers that are not associated with infectious diseases could raise questions about the adequacy of using liver colour as a reliable welfare indicator. For the feed trial (Chapter 6), we selected a 0.05% astaxanthin inclusion in our feed formulation, based on the levels of astaxanthin analysed in the livers of wild lumpfish. This choice accounted for the estimated absorption rate, liver proportion relative to body weight, and the feed intake of the lumpfish over time.

Liver health was evaluated through histological assessment in Chapter 5, and an effect of farming was found in both fibrosis and necrosis, especially in the sea cage fish. This suggests that factors or conditions in sea cages might expose fish to liver damage, leading to the development of fibrosis as the liver undergoes repair processes and necrosis, because of long-term stressors. Stressors that affect both

liver vacuolization, fibrosis and necrosis include the type of feed, such as a high content of carbohydrates, the use of high dietary inclusion level of vegetable oils or an inadequate amount of amino acids (Caballero et al., 2004; Raskovic et al., 2011), but the presence of infectious diseases can also result in different degrees of liver inflammation, fibrotic changes, and necrosis (Erkinharju et al., 2021; Schwaiger et al., 1997). Land-based fish mostly had healthy livers, but a small fraction showed mild to moderate necrosis. Fish from the wild displayed the most robust liver health regarding necrosis and fibrosis, with no fish showing evidence of these changes in their liver. This difference could be due to factors such as diet, water quality, and environmental stressors. Wild fish benefit from a natural diet and cleaner water, while land-based systems may encounter issues with water quality, feed composition, or confinement stress, which can lead to liver damage.

To sum up, results from Chapter 4 and 5 regarding liver health, colour index and fish nutritional status highlighted the necessity of optimised nutritional strategies for lumpfish in different rearing environments as well as improvements in the farming procedures. This study provides insights regarding the diet formulation as diets for juvenile lumpfish should be formulated to mirror their natural diet, including lower lipid content (10-15%), sufficient levels of TAG for energy reserves, and an adequate supply of carotenoids, especially astaxanthin (at least 0.05% of feed). Strategies can be implemented both in the hatcheries and in the sea cages to minimise cases of compromised nutritional status, such as ensuring an appropriate feeding regime and provide enough feed, possibly near the shelters.

4) Does farming impact the health and welfare of lumpfish when in the hatcheries and the sea cages? How is the welfare status of the wild population?

Aquaculture practices significantly impact the welfare of fish throughout their life cycle, influencing their overall health and behaviour, from hatchery to harvest (Ashley, 2007; Rey et al., 2019). The farming of lumpfish for the cleaner fish industry has posed many challenges in terms of best practices due to the relatively new cultivation of this species. Lumpfish are reared in the hatcheries until they reach the juvenile stage, and then are deployed in the sea cages as a biological control method to delouse salmon (Treasurer, 2018). Despite the recent advances in the husbandry procedures, the high mortality rates at the deployment stage raised ethical and economical concerns in Norway, Faroe Islands and in Scotland (Wilcox, 2023). The main causes of mortality were identified by Reynolds et al. (2022), being mostly attributed to salmon handling, mechanical delousing, and infectious diseases. Several studies have looked at improving the welfare of lumpfish by establishing species-specific OWI to use for regular monitoring in salmon farming (Boissonnot et al., 2022; Eliasen et al., 2020; Gutierrez Rabadan et al., 2021; Imsland et al., 2020; Noble et al., 2019). In the Faroe Islands, as part of lumpfish health monitoring in the salmon sea cages, lumpfish are assessed for OWI, liver colour and stomach content (Eliasen et al., 2018; Eliasen et al., 2020).

Chapter 5 investigated the impact of farming on the welfare of lumpfish from the hatcheries, from the sea cages, and from the wild populations in the Faroe Islands. Lumpfish fins (dorsal, anal and caudal) were scored based on damage. The origin of the fish significantly affected the anal, dorsal and caudal fins, with the farmed fish from both hatcheries and sea cages having a higher likelihood of experiencing damage to the fins than their wild counterparts.

Lumpfish from the land-based hatcheries had severe damage in the anal, dorsal, and caudal fins, with the highest percentage reported in the dorsal fin. This suggests that specific stressors in the hatcheries, such as the high density in the tanks, might lead to varying impacts on different fins (Ellis et al., 2002). Notably, behavioural issues such as tail biting and fin nipping, which have been observed in both lumpfish and wrasse (Erkinharju et al., 2021) in sea cages (Powell et al., 2018) and tanks (Noble et al., 2019), could be contributing factors, particularly in the case of dorsal fin damage. Other stressors in the farming environment such as crowding, water quality (Santos et al., 2010), competition for resting spaces (Johannesen et al., 2018), and different feeding strategies (Latremouille, 2003) can also affect fin health.

Lumpfish from the sea cages had severe damage in all the fins, with the highest percentage reported in the caudal fin. This fin damage, particularly in the sea cage environments, might be due to mechanical causes, such as interactions with cages (Braithwaite & McEvoy, 2005; Latremouille, 2003). Additionally, during the deployment phase, lumpfish may be subjected to mechanical delousing procedures intended for salmon, which can result in skin injuries and fin damage for these fish, leading to increased susceptibility to infectious diseases (Boissonnot et al., 2023; Reynolds et al., 2022). The difference in severity of fin damage between sea cages and land-based hatcheries in our study suggests that environmental and mechanical factors in the sea cages might play a significant role. Another factor contributing to the differences in the severity of this damage could be a matter of time spent in the tanks and in the sea cages, as the welfare deteriorates in sea cages due to cumulative damage. If the fish experience a cumulative effect on their fins, this will lead to increased fin damage in the sea cages simply due to these fish having spent more time in the farming systems. The extended time that lumpfish spent transitioning from hatchery rearing, through transport to salmon sites, and finally to sea cage deployment, may lead to an increased cumulative damage in OWI, especially in the case of fin damage.

In general, the increased damage in farmed fish likely stems from the more challenging environment they encounter in both sea cages and hatcheries. Although fin damage has been reported also in wild populations, it is more common in aquaculture environments (Latremouille, 2003). Indeed, in this study, wild fish had the best fin condition, suggesting that fin issues are not common in natural environment. However, this perspective might be skewed, as wild fish with compromised health will likely not survive or be predated, making them less represented in the samples analysed for this study. In contrast, sampling fish randomly from a salmon sea cage or hatchery tank will likely give a better representation

at the site due to regular health and welfare monitoring which is necessary to detect welfare issues before they become mortalities.

Other welfare indicators such as eye condition, skin status and the integrity of the sucker disc are vital for lumpfish survival both in the wild and in farming conditions. Healthy eyes are crucial for navigation and for locating feed and prey, the skin is the primary barrier against pathogens and environmental threats, and the sucker disc is essential for attachment to substrates. Skin damage together with fin damage were identified as the most useful OWI for farmed lumpfish by Garcia de Leaniz et al. (2022), as open wounds both in the fins and skin expose the fish to further injuries, secondary infections and other pathogens.
Eye damage was detected only in the fish from the sea cages, and this also agrees with Gutierrez Rabadan et al. (2021). This can be the result of abrasion and contact with the cage nets due to exposure to currents and an overall cumulative damage occurring throughout the deployment phase.

Sucker disc deformities were detected in this study in a very small percentage only in the fish from the sea cages. The underlying causes of sucker disc deformities are unclear. However, genetic factors and nutritional causes are highlighted both in Reynolds et al. (2022) and Gutierrez Rabadan et al. (2021). Gutierrez Rabadan et al. (2021) reported a higher percentage of fish from the hatcheries with a sucker disc deformity. Compared to our study, where fish sampled from the hatcheries were free from sucker disc deformities. This could be due to early screening for these fish in the hatcheries, but also due to family differences as reported by Danielsen (2016).

Regular observation and monitoring of the skin appearance, eye integrity as well as fin integrity can aid in early detection of health and welfare issues in fish. The use of liver score, investigated in Chapter 4, together with the body condition assessed in Chapter 5, can give insights regarding the feeding and the overall nutritional status of the fish. In our study, the body condition was significantly higher in the land-based fish, followed by the wild, and lastly sea cage fish. Lumpfish from the land-based hatcheries had the highest body condition factor, and this is due to frequent feeding regime and the rearing environment of the tanks as also corroborated by the lipid analysis of whole fish in Chapter 3, and liver lipid content in Chapter 4. Lumpfish from the wild were in good condition, where 26% were underweight and 6% emaciated regarding the weight-length relationship, likely due to their natural diet. Wild fish can encounter periods of starvation due to factors such as seasonal changes, competition for resources, or shifts in prey availability (Eliasen et al., 2018). This natural scarcity can impact their overall body condition and status of the fish, resulting in some wild fish being emaciated or underweight. The size classes of these fish varied slightly: land-based and wild fish were mostly under 50 g, whereas the majority of fish from the sea cages ranged between 50 and 300 grams. Also, lumpfish can exhibit compensatory growth which occurs when normal feeding is resumed after a period of reduced food availability or environmental stress. However, factors such as the duration and severity of

the initial growth restriction, environmental conditions and the feed provided during the compensatory phase, could influence the effectiveness of compensatory growth (D'Arcy et al., 2020).

The likelihood of fish from sea cages being underweight (22%) or emaciated (25%) was much higher. This suggests that the farming conditions and the feeding strategy have an impact on the body condition of the sea cage group. Also, a compromised welfare where fish have fin damage, skin injuries or infectious diseases can affect the overall feed intake. This results in different nutritional statuses in the sea cages, also found in the studies by Boissonnot et al. (2023) and Østerø et al. (2024).

Strategies can be implemented in the hatcheries to minimise fin damage, such as reducing density and providing enough shelter space to reduce aggression and fin nipping. In the sea cages, it is essential to provide shelters for the lumpfish to rest, and to ensure appropriate feeding, possibly near the shelters. It would be appropriate to avoid the deployment of fish in sites known for strong currents that could lead to skin abrasions and fin damage due to the collision with the cage net. Also, preventing lumpfish from undergoing mechanical delousing would have a significant impact on their overall welfare status. The implementation of these procedures has the potential to improve welfare and health status of farmed lumpfish, resulting in better survival rates in the sea cages.

To conclude, lumpfish are a valuable resource in the combat against sea lice, if better deployment strategies and husbandry is used. The Food and Veterinary Authority of the Faroe Islands (Heilsufrøðiliga Starvsstovan) is currently urging better welfare status and lower mortality rates for lumpfish deployed in the sea cages. As part of this, regular welfare checks in the sea cages are in place with follow-up reports by technical and veterinary staff, assessed together with mortality data in each site. Also, lumpfish are not being deployed where mechanical delousing is used or in sites exposed to strong currents. Salmon welfare should be guaranteed during its life cycle in the farms, but it should not come with the additional cost of cleaner fish welfare, in the case of this study, lumpfish.

5) Do higher levels of EPA+DHA in the diet impact lipid composition, growth and welfare of pre-deployment lumpfish?

One of the main bottlenecks of lumpfish production is the availability of balanced diets at the deployment stage. Poor nutrition and unbalanced diets will lead to poor health and welfare, impacting mortality rates. Due to the raising ethical concerns regarding lumpfish welfare, several studies have aimed to improve the knowledge of their nutrition (Willora et al., 2021): Hamre et al (2022) attempted to elucidate the correct balance of macronutrients and recommended that diets for juvenile lumpfish (10-50 g) should have 55% protein, minimum 10% lipid and maximum 10% carbohydrate. The use of different raw materials like plant protein and rapeseed oil was investigated by Willora et al. (2020, 2021, 2022), restricted feeding regimes (Imsland et al., 2019), or alternative feeding types like the use of feed blocks rather than the conventional pellets was investigated by Imsland et al. (2019, 2020). However, such studies used growth to assess the performance of the diets, which does not tell the whole story, so

it is important to include welfare indicators, like stress response and disease resistance (Gesto et al., 2021). Farmed lumpfish are exposed to various stressors, from the rearing in the hatcheries to transportation to the salmon sites, and final deployment in the sea (da Santa Lopes et al., 2023). Therefore, it is important that their diet supports good welfare and a healthy stress response.

From the previous Chapters 3 – 5, the total lipids and EPA+DHA levels were identified as the potential main drivers of composition differences among the different stock of lumpfish. The nutritional requirements of EPA and DHA in juvenile lumpfish are not known (Willora et al., 2021). Therefore, in Chapter 6, we fed lumpfish diets with diverging levels of EPA+DHA, using krill oil, rapeseed oil or a blend of the two. This was done to give recommendations regarding the optimal inclusion levels of these essential fatty acids in feed formulations in terms of fish welfare specifically.

Diverging levels of EPA+DHA did not affect lumpfish growth and survival. Other studies in lumpfish (Willora et al., 2021) and Atlantic salmon (Torstensen et al., 2004) found decreased growth in the groups fed 100% RO (3.11 and 4.2 % of EPA+DHA, respectively), and this trend was not found in our study regarding the diet 0KO. This is because the n-3 PUFA requirement for normal growth was met by the levels of EPA+DHA in 0KO in our study, as it contained 5.6 % of EPA+DHA, derived from marine meal in the formulation of the basal pellet. This also agrees with the study by Bell et al. (2001) where the highest inclusion of RO (5.4 % of EPA+DHA) did not affect the growth of Atlantic salmon when high fish meal diets were used.

At the end of the trial, fish being fed 75KO had the highest lipid content compared to fish on the other experimental diets. The higher lipid deposition and retention found in fish fed 75KO contradicts the antiadipogenic effect of KO. Despite the 75KO diet having a high inclusion of KO (8.3% of the diet), studies have shown that dietary krill oil has antiadipogenic effects. These effects include inhibiting fat deposition by reducing lipogenesis, regulating genes related to lipid metabolism, and providing anti-inflammatory benefits, all due to its high content of n-3 PUFA, particularly EPA and DHA, as well as other components like phospholipids and astaxanthin (Hwang et al., 2022). Despite not being statistically different, 75KO exhibited a slightly higher feed intake compared to the other groups, and this could have led to slightly higher lipid deposition. We expected Diet 0KO, which had no inclusion of krill oil, to have the highest lipid content in the liver due to its higher inclusion of rapeseed oil. However, it reached values like fish fed 75KO and 50KO. This result could be attributed to Diet 0KO still containing n-3 PUFA, particularly 5.6% of EPA and DHA, which could have influenced the liver lipid content.

The patterns found in the fatty acid composition of whole fish, liver and intestine through the PCAs mirrored the fatty acid profile of the diets, as expected (Betancor et al., 2014a; Willora et al., 2021). The biplots of the PCAs of whole fish and intestine had distant clusters due to the marked differences in the fatty acid profile in these tissues. On the other hand, in the liver, the clusters slightly overlapped,

highlighting the metabolic role of the liver in the fatty acid metabolism, synthesis and disposal (Hodson & Frayn, 2011). The fatty acid profile of brain showed no separation of the dietary treatment in the PCA biplot, except for 0KO, highlighting how the dietary treatments had a smaller effect on the fatty acid profile of the brain, which tended to conserve more both the total lipid and the fatty acid levels than the other tissues analysed. Nutrient utilisation efficiency was calculated in Chapter 6 which showed that the dietary treatments affected the retention rate of OA, LA and ALA which was the highest in fish fed diet 75KO, likely due to the high retention of lipid of this group. Diets 25KO and 100KO consistently had the lowest retention of all fatty acids, possibly due to the low retention of lipid of these diets.

In the stress challenge, following exposure to an acute stressor involving a combination of chasing and confinement, plasma cortisol levels initially increased similarly across all dietary treatments. However, six hours after stress exposure fish on diets with higher krill oil inclusion (100KO, 75KO, and 50KO) had quicker reduction in cortisol levels compared to fish on the other experimental diets (25KO and 0KO).

In Chapter 6, a polynomial model was performed to estimate the minimum requirements of EPA+DHA in the diet to achieve a sufficient SGR, higher survival and low cortisol levels. EPA+DHA requirements differ among species, life stage, and different parameters such as maximum performance, health or maintenance could result in different estimates of requirements (Houston et al., 2022). For comparison, recommended levels for juvenile Atlantic salmon are 0.5% (Qian et al., 2020), 2% for gilthead seabream (*Sparus aurata*) (Houston et al., 2022) or 0.7% for European sea bass (Skalli & Robin, 2004). Willora et al. (2021) suggested dietary EPA+DHA levels in the range 1.3-2.6% of the diet (9-18% of total fatty acids). In Willora et al. (2021) the diet that only supplied 0.5% of EPA+DHA resulted in reduced growth, suggesting that the requirement for EPA+DHA was not met. In our study, the results of the model suggest that the diet for juvenile lumpfish (20-100g) should contain at least 2% EPA+DHA (15% of total fatty acids) as a minimum requirement to achieve higher survival and lower cortisol, but to achieve a higher SGR, the level is 3% (18% of total fatty acids) (Figure 7.1).

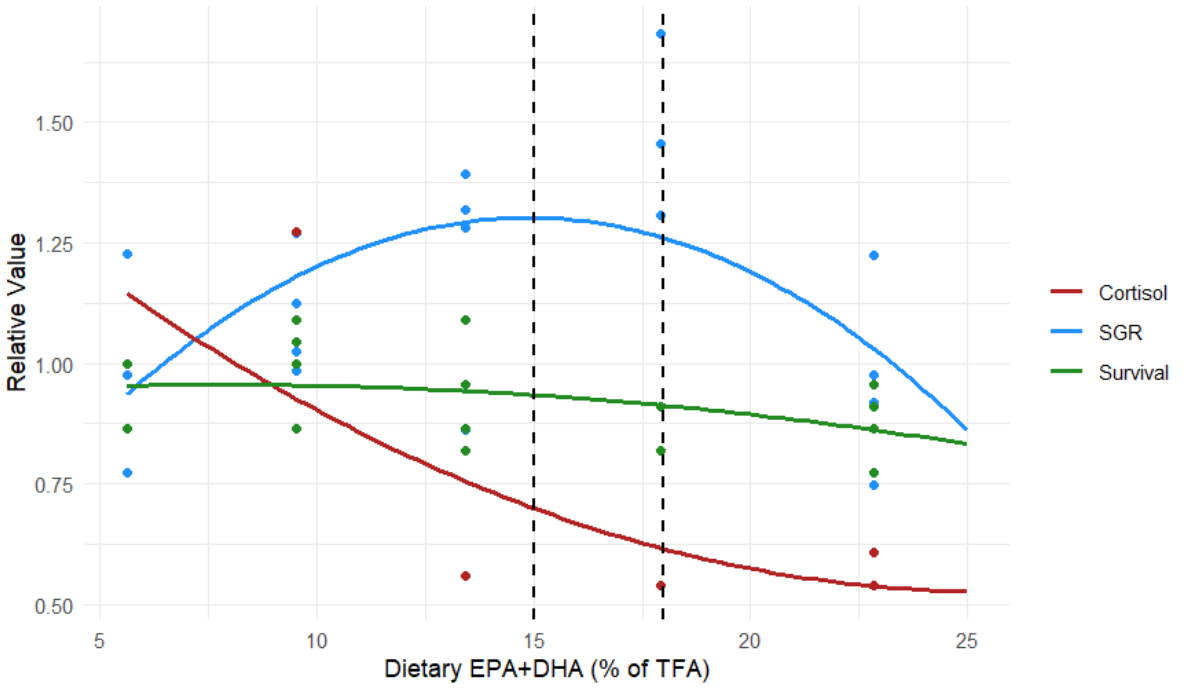


Figure 7.1. Dietary EPA+DHA levels used in the feed trial with juvenile lumpfish from Chapter 6 within this Thesis. Relative value was calculated by normalising the plotted values for SGR, cortisol and survival to the value observed at the lowest dietary EPA+DHA level. The area between the dashed lines indicates the recommended inclusion levels of EPA+DHA in the diet according to cortisol, SGR and survival.

7.1. Summary

In this Thesis, we used wild lumpfish populations, considering they thrive in their natural habitat, as a benchmark to assess the nutritional and welfare status of the farmed counterparts, and to improve feed formulations for the juvenile phase.

The total lipid and the fatty acid composition were identified as the main drivers in the nutritional differences between the two origins. In this sense, farmed lumpfish exhibited higher lipid content than the wild ones, both in liver and whole fish, reflecting the impact of high-energy diets and composition, and controlled farming environments. The lower fat reserves of the wild group are most likely due to their natural diets, the environmental conditions of their habitat, and prey availability. This suggested that high energy dense diets are not recommended for lumpfish. The fatty acid profile of the fish sampled both in the wild and in the farms mirrored dietary intake. Seasonal changes strongly impacted the fatty acid profile in such a way that winter fish had overall more n-6 PUFA, MUFA and ALA, and summer fish higher in SAFA, ARA and n-3 PUFA, mainly due to the seasonal variation in abundance and availability of zooplankton in the ocean. In winter, when zooplankton is scarce, fish in sea cages primarily rely on salmon and lumpfish feeds, which contain vegetable oils rich in OA, LA and ALA. Wild lumpfish with their diverse and natural diet, exhibited higher n-3 LC-PUFA than the farmed counterparts. This effect of seasonality is also corroborated by liver intracytoplasmic vacuolization in sea cage fish, which was higher in autumn and winter and significantly lower in spring and summer, probably reflecting access to pelleted feeds only in autumn and winter, and zooplankton in the spring and summer months.

Farmed lumpfish in sea cages and hatcheries showed higher levels of fin damage and other OWI compared to wild fish, indicating that farming conditions negatively impact their welfare. Mechanical interactions with cage structures, strong currents, and social aggression contributed to fin damage. Another factor contributing to the differences in the severity of this damage could be a matter of time in the tanks and in the sea cages, as the welfare deteriorates in sea cages due to cumulative damage. Also, a higher percentage of fish from the sea cage were underweight or emaciated, suggesting that farming conditions had an impact on the body condition. This was corroborated by the dark liver colours of some sea cage fish, which indicated low lipid reserves and a compromised nutritional status.
In general, the increased damage in farmed fish likely stems from the more challenging environment they encounter in both sea cages and hatcheries. For example, land-based fish mostly had healthy livers, but a small fraction showed mild to moderate necrosis, which could be due to factors such as diet, water quality and environmental stressors. A compromised welfare status where fish have fin damage, skin injuries or infectious diseases can affect the overall feed intake and nutritional status.

To improve feed formulation, a feed trial was performed where juvenile lumpfish were fed diets with diverging levels of EPA+DHA. Higher dietary levels of EPA and DHA influenced lipid retention and

stress response, where fish fed higher EPA and DHA levels showed faster post-stress recovery compared to lower inclusion levels. A dietary inclusion of at least 2% EPA and DHA (15% of total fatty acids) was recommended to enhance survival and welfare, with 3% being optimal for growth (18% of total fatty acids).

In conclusion, the welfare of lumpfish in aquaculture is a major challenge that needs to be addressed. We have shown that the farming conditions affected both nutritional and welfare status of lumpfish. To improve the nutritional condition at deployment, the results of this Thesis provides recommendations in feed formulations for juvenile lumpfish, particularly lipid content and EPA+DHA inclusions.

7.2. Future prospects

It is essential to focus scientific research on optimal rearing conditions, dietary needs and welfare monitoring due to our limited knowledge of lumpfish biology. Findings in this Thesis confirms that farming environment poses challenges in both welfare and nutritional status of lumpfish. The use of wild fish is a common practice in the aquaculture industry as wild fish serve as a benchmark for giving insights into farming practices. The use of wild lumpfish in this study was a starting point to elucidate the welfare status of the fish and give insights into the nutritional requirements of the species in the juvenile phase. Capturing wild lumpfish of the target size for this study proved challenging due to the offshore semi-pelagic nature of this species (Cox & Anderson, 1922). Future studies ought to focus on access to wild fish of the target size year-round, to be able to compare wild and sea cage-based fish season by season.

In this study we focused on the use of OWI in lumpfish, coupled with nutritional status and content. Fish sampled both from farmed and wild origin were not screened for infectious diseases and the organs sampled for histological assessment were liver and intestine. Therefore, it is difficult to speculate the aetiology of histological observations found in liver like necrosis or fibrosis for example, as they can be the result of prolonged stressors due to diseases and poor environmental conditions. In future studies, sampling gills and kidney, both for histology and infectious diseases screening, could give a more complete picture of the health status of the fish both in the farms and in the wild populations, as a high percentage of deployed lumpfish are reported to die from infectious diseases in some sites.

The OWI used in this Thesis were used to provide insights into the external damage and overall welfare of the fish, especially when in farm conditions. However, it would be beneficial in future research to be able to correlate these data with mechanical delousing treatments, disease outbreaks, and mortality data in the sea cages, as they could be the main cause of sudden skin injuries and fin damage. Also, when the welfare of the skin and fins is compromised, it could lead to secondary infections. Trying to correlate these could be beneficial to address best practices. Regular welfare monitoring checks on the same sites coupled with mortality data are essential to see if the welfare of deployed lumpfish deteriorates over time and intervene if necessary. For example, a sudden liver colour change, from bright orange to pale, could be an early sign of a disease outbreak. The sea cages are an integral part of salmon farming and lumpfish were introduced very recently into this farming system. This highlights the need to provide a suitable environment for lumpfish deployed in the sea cages, possibly with the provision of shelters, and with feeds delivered near the shelters.

Recent advances have been made regarding feed formulation for lumpfish juveniles in terms of macronutrients requirements and feeding strategies. More studies would be beneficial to investigate the minimum requirements for minerals and water content of the feeds, mainly because in the wild a relatively large proportion of lumpfish consume large quantities of jellyfish, as mentioned in Hamre et

al. (2022). Also investigating more regarding feed types as alternative strategies like the use of feed blocks are being widely used with some challenges, or the use of functional feeds or inclusion of immunostimulants could have health benefits such as enhancing the immune system prior to deployment. The comparison of farmed and wild lumpfish highlighted how the diet in the sea cages results in large variations in terms of nutritional status, most likely due to highly variable feed and prey availability, and other causes such as infectious diseases and treatments against sea lice used in the farms. In particular, the standard lumpfish feed pellet may not have an adequate size in the sea cages as the deployed fish grow, due to medium to big lumpfish more often having salmon pellets in their stomachs. Optimal pellet size for the deployment phase could be better investigated.

Feed trials where the performance of the diets is mainly assessed by the growth do not tell the whole story. Therefore, it is essential to also include welfare indicators, like stress responses and disease resistance, for a better understanding of how diets impact overall fish health and welfare.

7.3. General conclusions

1. Significant dietary differences were found in the body composition and liver of farmed and wild lumpfish, where factors such as diet, season and size class played a crucial role, particularly affecting lipid content and fatty acid profile.

2. The lower lipid content in wild lumpfish suggests that high energy dense diets are not recommended for lumpfish.

3. Varying degrees of lipid reserves, corroborated by different liver scores and liver damage, indicate that suboptimal feeding practices, size differences, and environmental conditions affect the nutritional status of lumpfish in the sea cages.

4. The fatty acid profile of both liver and whole fish reflected dietary inputs in farmed and wild lumpfish, with the higher levels of n-6 PUFA and slightly higher levels of MUFA, showing the use of vegetable oils in both salmon and lumpfish pellets in the sea cages.

5. Farming conditions, in both hatcheries and sea cages, significantly affected fin health. Stressors like fin nipping primarily resulted in dorsal fin damage in hatcheries, while in sea cages, interactions with cage structures, strong currents and cumulative damage from the hatchery phase led to more tail damage.

6. The differences in body condition between the farmed fish and the wild counterparts underscore the impact of different feeding regimes and environmental conditions between the hatcheries and the sea cages.

7. Higher inclusion of EPA+DHA in diets for juvenile lumpfish resulted in a shorter recovery after exposure to an acute stressor.

8. To boost robustness by achieving higher survival, low cortisol levels and a sufficient SGR, diets formulated for juvenile lumpfish (20-100g) should contain at least 2% of EPA +DHA.

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