Prifysgol Abertawe
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# Welfare investigations in farmed lumpfish (*Cyclopterus lumpus* L.) used for sea lice control in the salmon industry

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*"En aquel nuevo paisaje, la inteligencia se centraba en encontrar modos de sobrevivir entre pezuñas y cuernos, colmillos y garras. Ese mismo instinto guía el método científico; saber equivale a progreso, progreso equivale a supervivencia."*

JUAN FUEYO

*"In that new landscape, intelligence was focused on finding ways to survive between hooves and horns, fangs and claws. That same instinct guides the scientific method; knowledge equals to progress; progress equals to survival."*

JUAN FUEYO

# Summary

Lumpfish (*Cyclopterus lumpus* L.) are commercially important species used world-wide since 2008 as cleaner fish to control sea lice (*Lepeophtheirus salmonis*), one of the most significant parasites affecting Atlantic salmon (*Salmo salar* L.) and costing the industry over 500M € annually. However, lumpfish mortalities in sea cages can be as high as 100%, raising welfare concerns on whether their use continue to be acceptable. The industry is under scrutiny by both government agencies and non-governmental organisations to improve lumpfish welfare, but they do not provide much direction as welfare standards are not developed yet for this species. This thesis examined novel ways to monitor welfare in farmed lumpfish in order to increase the sustainability and ethical use of lumpfish in aquaculture.

Chapter 1 reviewed the bases of fish welfare as well as lumpfish biology, use as cleaner fish and main challenges within the aquaculture industry. Chapter 2 developed and tested a practical, easy-to-use, validated and highly repeatable (ICC=0.83) scoring index combining six Operational Welfare Indicators to measure lumpfish welfare under farm conditions. Chapter 3 found welfare deterioration in sea cages to be dependent on time spent at sea and to be worse in smaller fish. Welfare monitoring should be particularly regular during the first months post-transfer at sea. Chapter 4 found differences in welfare, growth, feeding preferences and gut microbiota between different genetic stocks of lumpfish, with Icelandic lumpfish growing faster, showing better welfare and ingesting more formulated pellets than Scottish lumpfish. Significant associations in the gut microbiota were identified between compromised welfare and *Candidatus branchiomonas* and high plasma cortisol and *Clostridium*, suggesting these could be used as potential biomarkers. Chapter 5 investigated the effect of sea lice ingestion in lumpfish welfare and gut health and found that sea lice ingestion did not have any influence and is not detrimental for lumpfish welfare.

Welfare investigations under commercial conditions proved that lumpfish welfare status (measured by using individual morphological indicators) deteriorates in time when stocked in salmon net pens, resulting in an increase of these scores, which also differ between commercial sites and populations under the same conditions. The ability of monitoring welfare in regular basis serves as an early warning for health and welfare issues and the application of the index along the knowledge withdrawn from this thesis will help farmers to identify critical periods where lumpfish welfare starts deteriorating, as well as provide scientific reference to policy developers, welfare organisations and NGOs and quality assurance schemes. In this sense, welfare standards for these novel species can be developed, and corrective actions can be taken before any issues progress into mortalities. This will enhance the sustainability of the lumpfish industry and will reduce economic costs of sea lice management, mitigating environmental impacts for not using chemotherapeutants at sea and improving salmon welfare overall.

# Declaration and Statements

I, **Carolina Gutiérrez Rabadán**, declare that this work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Date 21/10/2021

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s). A bibliography is appended.

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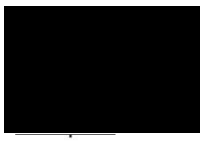
Signed 

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# Authorship Declaration

The following people contributed to the publication of work undertaken as part of this thesis.

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## Contribution

The experiment was conceived and designed by CGL and SC, and CGR contributed with the study idea. CGL, SC and KESS-2 secured funding. Laboratory work was conducted by CGR. CGR and CGL analysed data and prepared tables and figures. CGR, CGL and SC authored and reviewed the manuscript, and all authors approved the final draft.

We, the undersigned, agree with the Authorship Contribution stated above and agree to the inclusion of this work within the current thesis:

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# Table of Contents

Summary… iii

Declaration and Statements. iv

Authorship Declaration… v

Table of Contents… vi

Acknowledgements xi

List of abbreviations xii

List of tables xvi

List of figures xviii

**Chapter 1 General introduction 1**

Animal welfare 1

Fish welfare 2

Aquaculture and welfare of farmed fish 4

Fish welfare assessment 5

Implications of welfare for health, performance and productivity 6

Stress responses in fish 8

Fish microbiome 9

Lumpfish (*Cyclopterus lumpus* L.) 11

Sea lice threatening the salmon industry 12

Sea lice control 14

Use of cleaner fish in the salmon industry 18

Main challenges in the use of lumpfish 18

Thesis aims and objectives 22

**Chapter 2 Development and validation of an Operational Welfare Score Index for farmed lumpfish *Cyclopterus lumpus* L. 25**

Abstract 25

Introduction 26

Materials and methods 28

Development of the Lumpfish Operational Welfare Score Index (LOWSI) 28
Selection and screening of Operational Welfare Indicators (OWIs) 28
Sample collection 28
Welfare Score description 29
Validation of selected individual based OWIs 30
Reliability 31
Construct validity 31
Plasma cortisol analysis 31
Refinement and simplification 32
Pilot trial 32
Statistical analyses 33
Results 34
Selection of Operational Welfare Indicators (OWIs) 34
Utility of welfare indicators for lumpfish 34
Prevalence and variation in OWIs 34
Variation of body condition and incidence of underweight/emaciated fish 35
Variation in plasma cortisol 35
Validation of OWIs 36
Reliability 36
Construct validity 36
Refinement and simplification 36
Pilot trial: LOWSI testing and application 37
Discussion 38
Acknowledgements 42
Tables 43
Figures 50

**Chapter 3 Changes in growth and welfare of lumpfish *Cyclopterus lumpus* L. over time in a longitudinal study 56**
Abstract 56
Introduction 57

Materials and methods 59
Experimental fish 59
Experimental set up 59
Sample collection 59
Stomach content analysis 60
DNA extraction and amplification 60
Microsatellite and genotype analysis 60
Parental assignment 61
Statistical analysis 61
Results 63
Relative abundance of lumpfish of different origins 63
Changes in body size and condition between origins and over time 63
Changes in welfare between origins and over time 64
Changes in the prevalence of sea lice and pellets in stomachs over time 65
Discussion 66
Acknowledgements 69
Tables 70
Figures 71

**Chapter 4 Feeding preferences, growth and gut microbial communities of two stocks of lumpfish Cyclopterus lumpus L. deployed in a common sea cage 73**
Abstract 73
Introduction 74
Material and methods 76
Sample collection and fish tagging 76
Growth rate 77
Welfare scores 77
Plasma cortisol determination 77
Stomach content analysis 78
Microbiome analysis 79
Bioinformatic analysis 80
Statistical analysis 80

Results 83
Comparison between stocks in body size, condition, welfare and cortisol levels 83
Growth and changes in welfare scores of individually tagged lumpfish before and after deployment at sea 83
Stomach content analysis 84
Microbial analyses: diversity and structure 85
Microbial analyses: differential abundance of taxa 86
Discussion 87
Acknowledgements 90
Tables 91
Figures 93

**Chapter 5 Effect of sea lice ingestion on lumpfish *Cyclopterus lumpus* L. welfare and gut health 101**
Abstract 101
Introduction 102
Materials and Methods 105
Fish sampling 105
Welfare scores 105
Sea lice ingestion analysis 105
DNA extraction, 16S rRNA amplicon sequencing and bioinformatic analysis 106
Microbiome network construction and comparison 106
Statistical analysis 107
Results 108
Lumpfish welfare 108
Variation in delousing efficiency 108
Relationship between sea lice ingestion, body size and condition, welfare scores and cortisol response 108
Relationship between sea lice ingestion and diversity of microbiome communities 109

Characterization of lumpfish gut microbial communities and DA analysis 109
Network quantitative comparison 110
Discussion 111
Acknowledgements 114
Tables 115
Figures 119

**Chapter 6 General discussion and conclusions 124**
Lumpfish welfare and stress 125
Lumpfish transfer and deployment at sea 126
Lumpfish microbial communities 127
Sea lice ingestion 128
Stock differentiation 129
Lumpfish underweight and emaciation 129
**Conclusions** 130

**References 131**

**Appendix**
Supplementary tables ..... 154
Supplementary figures ..... 168
Ethics Approval…..... 185
R Scripts ..... 186
Scientific publication ..... 137

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# List of abbreviations

| | |
|---|---|
| %FO | Frequency of occurrence (%) |
| °C | Degree Celsius |
| µM | Micromolar |
| µl | Microlitres |
| 16S rRNA | 16S ribosomal ribonucleic acid |
| 6-FAM | 6-carboxyfluorescein |
| aa | Aminoacetophenone |
| ACTH | Adrenocorticotropin hormone |
| AIC | Akaike Information Criterion |
| AL | Artificial light |
| am | Ante meridiem (before midday) |
| AMR | Antimicrobial-resistant |
| ARI | Adjusted Rand Index |
| ASVs | Amplicon Sequence Variants |
| bp | Base pairs |
| *C. gigas* | *Crassostrea gigas* |
| CI | Confidence intervals |
| CIWF | Compassion in World Farming |
| CNS | Central Nervous System |
| corr | Correlation |
| CpG-ODN | CpG oligodeoxynucleotides |
| CRH | Corticotropin-releasing hormone |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| CSAR | Centre for Sustainable Aquatic Research |
| CV | Coefficient of variation |
| DA | Differential abundance |
| DADA | Divisive Amplicon Denoising Algorithm |
| DESeq | Differential gene expression analysis |
| DNA | Deoxyribonucleic acid |
| dph | Days post-hatch |

| | |
|---|---|
| e.g. | for example |
| ED | Eye darkening |
| ELISA | Enzyme-Linked Immunoassay |
| EST | Expressed sequence tag |
| *et al.* | et alia (and others) |
| EURLFD | European Union Reference Laboratory for Fish Diseases |
| F | Forward |
| FAO | Food and Agriculture Organization |
| FDR | False Discovery Rate |
| FT | Flow-through |
| g | Grams |
| G | Gravid |
| GDs | Gene drives |
| GI | Gastrointestinal |
| GIT | Gastrointestinal tract |
| GLM | Generalized linear model |
| HPI | Hypothalamic-pituitary-interrenal |
| HSP | Heat-shock proteins |
| ICC | Intraclass correlation coefficient |
| IQR | Interquartile |
| IRI | Index of Relative Importance |
| ISO | International Organization for Standardization |
| IUCN | International Union for Conservation of Nature |
| kg | Kilogram |
| kH | Kilohertz |
| Lat | Latitude |
| LM | Linear model |
| LMM | Linear mixed model |
| ln | Logarithm (natural) |
| Long. | Longitude |
| LOWSI | Lumpfish Operational Welfare Score Index |
| LR | Label Rouge |

| | |
|---|---|
| LWR | Length-weight relationship |
| m | Metres |
| M | Million |
| mclr | Modified central log ratio |
| min | Minutes |
| mm | Millimetres |
| MOS | Mannan oligosaccharides |
| MS-222 | Tricaine methanesulfonate |
| N | North |
| NED | 2′-chloro-5′-fluoro-7′,8′-benzo-1,4-dichloro-6-carboxyfluorescein |
| NetCoMi | Network Construction and Comparison for Microbiome data |
| NFSA | Norwegian Food and Safety Authority (Mattilsynet) |
| ng/ml | Nanograms per millilitre |
| NGO | Non-governmental organization |
| NGS | Next-generation sequencing |
| NL | Natural light |
| nm | Nanometres |
| nM | Nanomolar |
| $nmol \cdot l^{-1}$ | Nanomole per litre |
| obs. | Observation |
| OD | Optical density |
| OIE | Office International des Epizooties |
| OTUs | Operational Taxonomic Units |
| OWI(s) | Operational Welfare Indicator(s) |
| $p_{adj}$ | Adjusted p-value |
| PCA | Principal Component Analysis |
| PCR | Polymerase chain reaction |
| PCs | Principal Components |
| PE | Paired-end |
| PERMANOVA | Permutational ANOVA |
| PERMDISP | Permutational analysis of multivariate dispersions |
| pg/ml | Picograms per millilitre |

| | |
|---|---|
| pH | Potential of hydrogen |
| PIT | Passive integrated transponder |
| pm | Post meridiem (after midday) |
| PUFA | Polyunsaturated fatty acids |
| QIIME | Quantitative Insights Into Microbial Ecology |
| R | Reverse |
| R1, R2 | Rater #1, #2 |
| RAS | Recirculating aquaculture system |
| RONC | Remote operated net cleaner |
| rpm | Revolutions per minute |
| RSPCA | Royal Society for the Prevention of Cruelty to Animals |
| s | Seconds |
| SE | Standard error |
| SGR | Specific Growth Rate |
| SIMPER | Similarity of Percentages |
| SP1 | Sampling point 1 |
| SP2 | Sampling point 2 |
| SRUC | Scotland's Rural College |
| SSRs | Simple sequence repeats |
| STR | Short tandem repeat |
| t1, t2 | Time #1, #2 |
| T1,T2 | Time-points 1 and 2 |
| TAG | Triacylglycerol |
| TL | Total length |
| U | Units |
| VIC | 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein |
| W | West |
| WI | Welfare Indicator |
| Wr | Relative weight |
| Ws | Standard weight |
| ZIP | Zero-inflated Poisson |
| ρ | Spearman's correlation coefficient |

# List of Tables

**Table 1.1.** Overview of different sea lice control strategies (preventive and treatments) for *L. salmonis*, unless indicated. Sources: Costello (1993), Rae (2002), Igboeli et al. (2014), Aaen et al. (2015), Holan et al. (2017), Overton et al. (2019), Barrett et al. (2020b).

Table 1.2. Most common welfare indicators used in the aquaculture of different fish species.

**Table 1.3.** Summary of factors affecting delousing behaviour in lumpfish.

**Table 2.1**. Questionnaire given to 53 participants at the 1st Symposium on Welfare in Aquaculture, held on the 14th of May 2019 at Swansea.

**Table 2.2**. Length-weight regression (LWR) coefficients for farmed lumpfish sampled at different life stages (S1-S4), where Ws = standard weight (g) and TL = total length (mm).

**Table 2.3**. Rapid visual scoring of the Lumpfish Operational Welfare Score Index (LOWSI).

**Table 2.4**. Questionnaire given to fish farmers (n=8) to test the practicality of the LOWSI.

**Table 2.5**. Repeatability of different operational welfare indicators (OWIs) and products from the PCA (PC1, PC2), based on the inter- and intra-class correlation coefficients (ICC) of two raters (R1 and R2) that randomly scored the lumpfish.

**Table 3.1**. Summary of body size (BW and TL) and condition (Wr) data according to cage, origin, genetic stock and sampling time point. BW=body weight, TL=total length. Values are represented as mean ± SD=standard deviation. Variance between time-points was significantly different for body weight and total length, but not for body condition (Wr).

**Table 4.1**. Lumpfish diet composition indices by genetic stock (Icelandic, n=26; Scottish, n=30). %FO: frequency of occurrence (absence/presence method); %N: percentage by number (numerical abundance method); %W: percentage by weight (gravimetric method); IRI: Index of Relative Importance.

**Table 4.2**. Bacterial communities (n=15) associated to the Scottish stock with stats and *p* value, using multi-level pattern analysis within the IndicSpecies package (De Caceres et al., 2020) with association function='r.g' (Pearson's phi coefficient) and $\alpha$=0.01. p-values were corrected using the False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995). ASV=Amplicon sequence variant. D5=Genus, D4=Family.

**Table 5.1.** Most representative (strongest) microbial associations within non-delouser lumpfish. Positive (+) associations indicate cooperation while negative (-) associations indicate competition.

**Table 5.2.** Most representative (strongest) microbial associations within delouser lumpfish. Positive (+) associations indicate cooperation while negative (-) associations indicate competition.

**Table 5.3**. Results for quantitative network comparison in non-delouser lumpfish (Compromised welfare, n=10; Good welfare, n=6) on genus-level via permutation tests (n=1000).

**Table 5.4**. Results for quantitative network comparison in delouser lumpfish (Compromised welfare, n=13; Good welfare, n=5) on genus-level via permutation tests (n=1000).

# List of Figures

**Figure 1.1.** Hypothalamic-pituitary-interrenal axis involved in the fish stress response.

**Figure 2.1.** Figure 1.2. Sea lice life cycle (adapted from the Marine Institute). Stages out of the circle represent free swimming stages while stages within the circle are attached to the host.

**Figure 2.1**. Flow chart used to develop an operational welfare score index for farmed lumpfish.

**Figure 2.2**. Diverging stacked bar plot showing the variation in operational welfare indicators (OWI) in lumpfish sampled from hatcheries (A, n=60) and sea cages (B, n=35).

**Figure 2.3**. PCA plot with variables and their contribution (A) and PCA biplot showing variables and individuals (B), separated by stage of development: pre-deployment or hatchery stage (yellow triangles) and post-deployment or sea cage stage (blue circles).

**Figure 2.4**. Variation of body condition (relative weight, %) by stage of development. Lumpfish with normal body condition (Wr>90%) are shown in pink, while underweight (Wr=75-90%) and emaciated (Wr<75%) lumpfish are shown in green and blue, respectively.

**Figure 2.5**. Variation in plasma cortisol (ng/ml) of lumpfish (n=55) sampled from two hatcheries (S3 – Pre-deployment) and one sea farm (S4 – Post-deployment).

**Figure 2.6**. Variation in the Lumpfish Operational Welfare Score Index (LOWSI) pilot trial at six commercial sites (n=245 lumpfish): three hatcheries and three sea farms. The proportion of the lumpfish belonging to each of the welfare classes are represented by colour degradation in the violin plots: green for Class A (Good welfare), orange for Class B (Moderately compromised welfare) and red for Class C (Severely compromised welfare).

**Figure 3.1**. Lumpfish individual body weights **(a)** and total lengths **(b)** of each origin (English in pink, n=84; Norwegian in blue, n=236) at each of the time-point (1: Nov 2018, three weeks post-deployment; 2: Jan 2019, three months post-deployment). Error bars show the distribution of the data (-SD, +SD) with mean trajectories for each origin over time (continuous line for English and dotted line for Norwegian lumpfish). An interaction effect between the predictor variables (origin and time point) is shown.

**Figure 3.2. a)** Body condition (or relative weight, % Wr) distribution in each of the genetic origins (English, n=84; Norwegian, n=236) at each time-point (1: three weeks, 2: three months). An interaction effect between the predictor variables of body condition is shown. **b)** Variation in welfare scores (LOWSI) between lumpfish origins

and time-points (English: 1, n=43, 2, n=41; Norwegian: 1, n=117, 2, n=119).

**Figure 4.1**. Schematic representation of the number of lumpfish used for each analysis within the common garden experiment. Credits: Salmon (clipart-library.com), lumpfish (Philopic).

**Figure 4.2.** Distribution of body size (body weight and total length) and condition (Wr) in each stock of sampled lumpfish (n=142; Icelandic, n=86; Scottish, n=56).

**Figure 4.3**. Spaghetti plots (left) showing individual trajectories in body weight (g), total length (mm) and body condition/relative weight (%) of individually tagged Scottish lumpfish (n=55) before and after deployment at sea. Pink lines show individuals belonging to Class A (n=28), while turquoise lines represent individuals from Class B (n=27). The bar plots (right) summarise the Specific Growth Rate (SGR, %) for body weight (g) and total length (mm) and the specific change of body condition for each individual (green bars for positive SGRs, orange bars for negative SGRs).

**Figure 4.4**. Spaghetti plot showing individual trajectories of welfare scores (LOWSI values) of tagged Scottish lumpfish (n=55) before and after deployment. Pink lines show individuals belonging to Class A (n=28), while turquoise lines represent individuals from Class B (n=27).

**Figure 4.5**. Feeding preferences of Icelandic (n=29) and Scottish (n=31) lumpfish sampled at sea cages, according to food category (expressed as a total number, %). Each number indicates an individual lumpfish. Note that four individuals -15, 34, 37 and 41- (representing 6% of the total population) had no content on their stomachs.

**Figure 4.6**. Non-metric multidimensional scaling (nMDS) ordination plot of gut microbial community structure from two different lumpfish genetic stocks: Icelandic (n=29, green) and Scottish (n=28, orange), based on Bray Curtis dissimilarity distances. Ellipses clustered similar amplicon sequence variants (ASVs) with 95% confidence estimates.

**Figure 4.7**. Relative abundance bar plot showing the 25 most common ASVs, at genus level, in each of the genetic stocks of lumpfish (Icelandic, n=29 and Scottish, n=28).

**Figure 4.8**. Normalized intestinal bacterial counts of **a)** *Candidatus branchiomonas* (Wald test, $p_{adj}$=0.0002) for welfare groups (Good and Compromised) and **b)** *Clostridium sensu stricto 1* (Wald test, $p_{adj}$<0.001) for cortisol range groups (Low or <63 ng/ml and High or >63 ng/ml) within the Scottish lumpfish stock (n=28) sampled at the salmon farm.

**Figure 5.1**. Effect of size (total length, mm) on lumpfish welfare. The grey shadow represents confidence intervals (CI=95%). The green dashed line divides lumpfish from classes A and B, while the red dashed line separates lumpfish from classes B and C.

**Figure 5.2.** Microbial diversity and composition analysis adjusted for body size. **a)** Boxplots of alpha diversity values (Chao's species richness and Shannon diversity), according to non-sea lice eating-lumpfish (No, n=16) and sea lice eating-lumpfish

(Yes, n=18) groups. **b)** Non-metric dimensional scale (nMDS) of beta diversity (Bray-Curtis distances) for the same delousing groups.

**Figure 5.3**. The 25 most representative ASVs (at genus level) according to each delousing group (Sea lice ingestion: No, n=16 and Yes, n=18).

**Figure 5.4**. Microbiome network comparison for non-delouser lumpfish (Compromised welfare, n=10; Good welfare, n=6) on genus-level with 33 taxa per group, using Pearson's correlation coefficient as association measure and Fruchterman & Reingold layout. Positive associations are shown by green lines while negative interactions are shown by red lines. The thickness of the connection lines represents the correlation strength between taxa.

**Figure 5.5**. Microbiome network comparison for delousing lumpfish (Compromised welfare, n=13; Good welfare, n=5) on genus-level with 34 taxa per group, using Pearson's correlation coefficient as association measure and Fruchterman & Reingold layout. Positive associations are shown by green lines while negative interactions are shown by red lines. The thickness of the connection lines represents the correlation strength between taxa.

# Chapter 1. General introduction

## Animal welfare

Animal welfare is a complex concept that has been changing over the years. Welfare was initially defined as '*the state of an individual in relation to its environment*' and it was considered negatively affected if the individual could not cope to control and maintain mental and body stability when facing a challenge (Broom, 1991). It also recognised three key points: basic health and functioning, affective states and natural living (Dahrendorf, 1987). However, the concept of welfare was firstly introduced in the UK by the description of intensive livestock farming practices of the time collected in the book 'Animals Machines' (Harrison, 1964), which prompted the British government to investigate animal welfare in a technical committee chaired by Rogers Brambell (Command Paper 2836, 1965). The *Five Freedoms* framework, born by the combination of the assessment of animal needs and a scientific approach of what matters to animals and how much it matters, has been the basic philosophy of the UK Farm Animal Welfare Committee, established in 1979 (FAWC, 1993, 2009). This framework contained the first principles of good welfare for animals, and was developed to assess animal welfare (Webster, 1994) including:

1. Freedom from thirst, hunger and malnutrition (access to fresh water and a diet to maintain full health and vigour)
2. Freedom from discomfort (access to a suitable environment including shelter and a comfortable resting area)
3. Freedom from pain, injury and disease (access to health care by prevention or rapid diagnosis and treatment)
4. Freedom to express normal behaviour (access to sufficient space, proper facilities and company of another animal's own kind)
5. Freedom from fear and distress (access to conditions which avoid mental suffering)

The Animal Welfare Act (2006) was the first welfare law established that gave responsibility to animal owners and keepers to care for their animals by condemning unnecessary suffering to any animal. Later in 2007, the Welfare of Farmed Animals Regulations 2007 (S.I. 2007 No. 2078), made under the Animal Welfare Act, came

into force to protect the welfare of all farmed animals, avoid animal cruelty and set a minimum of welfare standards (DEFRA, 2013). After this, the concept of welfare adopted a different perspective and resulted in a scientific term within a framework of values. To achieve high animal welfare, actions must be based not only on science, but should also align with the major values that define a good life for animals (Fraser, 2008). According to the Terrestrial Animal Health Code (OIE, 2019a), animal welfare is '*the physical and mental state of an animal in relation to the conditions in which it lives and dies*' suggesting that how animals are culled is also relevant for their welfare.

## Fish welfare

The World Organisation for Animal Health, formerly the Office International des Epizooties (OIE) establishes online standards and provides guidance for aquatic animals worldwide within the OIE Aquatic Animal Health Code. This was the first source to provide guidance in fish welfare, which had been previously omitted in legislation. After 22 editions (1995-2019), the Aquatic Animal Health Code (OIE, 2021), dedicates a thorough section to the welfare of farmed fish. Fish welfare is increasingly drawing attention globally from researchers, stakeholders, retailers, non-governmental organizations (NGOs), quality assurance and policy schemes and consumers (Huntingford et al., 2006), but this has not always been the case. Fish welfare has been a controversial topic as fish had not been considered sentient animals in the past by scientists and researchers, but most importantly by policy developers and the Government. Animal sentience is the ability of animals to experience positive and negative states, feelings or emotions such as contentment and joy or pain, fear and boredom (Harrison, 1964, Command Paper 2836, 1965), extending to the ability to learn from experiences, assess risks and benefits, and make choices (Conradt and Roper, 2005, Sumpter et al., 2008, Brown, 2015). Sentience underlies animal welfare, as without sentience the concept of welfare would be meaningless (Chandroo et al., 2004, Duncan, 2006). Comparative neuroanatomical research of species with different evolutionary levels has shown that the mammalian brain is more complex and comprises more regions than the fish brain (Butler and Hodos, 1996). Hence, some authors have argued that it is unlikely that fish can perceive fear or pain due to the absence of neuro-anatomical regions (or analogous counterparts) or morphological features with such functionality required to feel subjective mental states and claim that fish responses to nociceptive stimuli are limited because they are not even conscious

(Rose, 2002, Rose et al., 2014, Key, 2015). However, others have demonstrated that fish can experience pain. Rainbow trout (*Oncorhynchus mykiss*) possess cutaneous nociceptors that respond to noxious stimuli such as mechanical pressure, extreme temperatures, or poisonous substances with significant adverse behaviour (Sneddon, 2003). Behavioural responses present in goldfish *Carassius auratus* after analgesic administration also showed that similar mammal receptors are present in fish (Ehrensing et al., 1982). Additionally, many of the fish abilities such as navigation, avoiding places where negative experiences previously occurred or recognising social companions are undoubtedly associated with the presence of some degree of emotions and feelings (reviewed by Broom, 2016). Fish are physiologically and behaviourally sentient, and can therefore experience good and bad welfare, just as mammals do (Broom, 2001, Sneddon, 2003, Chandroo et al., 2004, Broom, 2014).

Fish welfare concerns are increasingly growing across the human population: several studies have addressed whether welfare issues are important to consumers and results suggest that they use 'welfare' as a standard indicator of product quality and safety (Harper, 2001), which impacts their food choices. Apparently, when purchasing fish in Spain, environmental perception had more significance than welfare concerns (Honkanen and Olsen, 2009). A later study performed in Denmark showed that important reasons for not buying farmed fish were concerns on medicine residues, environmental impacts and farming conditions; but 48% of the respondents were willing to pay more for trout with a quality label certifying good fish welfare (reviewed by Solgaard and Yang, 2011). This highlights how consumers' perception of fish welfare is changing and gives insights on possible guidelines for standards of good fish welfare to be imposed by the EU in the future.

## Aquaculture and welfare of farmed fish

Aquaculture is defined as the production of aquatic organisms, such as finfish,shellfish (molluscs and crustaceans) and aquatic plants, occurring in an established water environment (inland, coastal or marine areas) that implies individual or corporate ownership of the stock being cultivated (FAO, 1988). Global aquaculture production has grown from less than 20M tonnes in 1950 to near 180M tonnes in 2018 (FAO, 2020). Salmon farming is considered the fastest growing food-production sector worldwide (OIE, 2021), and it is a highly concentrated industry: only four countries namely Norway, Chile, Scotland, and Canada account for 96% of the global production (FAO, 2020, Economics, 2021). Salmon aquaculture has the potential to secure food supply for the continually growing human population (Naylor et al., 2000), but achieving this goal requires a reduction of production costs which generally implies the use of intensive methods. These include the production of fish stocked at high densities, fed with formulated diets in an environment of reduced quality, which promotes the incidence of production-related diseases (Martos-Sitcha et al., 2020). The more intensive aquaculture becomes, the more concerning fish welfare is. For example, in contemporary Atlantic salmon farming, the main welfare issues that have been identified are: closed cage containment (being salmon migratory species) where they cannot express their appropriate behaviour, high mortality rates (due to disease and sea lice), use of antibiotics and sea lice treatments, environmental pollution and degradation, predator control, incidence of deformities, feeds made with vegetal inclusion but also wild-caught fish, inhumane slaughter methods, regular stressful farming practices such as crowding, pumping, handling and grading, high stocking densities and water quality problems (Compassion In World Farming, 2009, Borthwick, 2020, Hvas et al., 2021).

The interest on welfare research and stress of farmed fish has been growing steadily since the early 1990s, with the focus changing from welfare during transport, slaughterhouse, and husbandry systems, to stress management and production optimisation (Barton, 2002, Volpato, 2009, Noble et al., 2012), but the way on how fish welfare should be assessed remained unclear (Huntingford et al., 2006, Ashley, 2007, Huntingford and Kadri, 2009). More recent research has focused on how to define fish welfare through the use of welfare indicators (Segner et al., 2012, Martins et al., 2012, Noble et al., 2012, Noble et al., 2018, Saraiva et al., 2019, Toni et al., 2019).

## Fish welfare assessment

Assessing fish welfare is challenging (Volpato, 2009). In the absence of welfare standards for any fish species, the collection of relevant information is the best option (Turnbull and Kadri, 2007). The '*fish-preference approach*', based on the assumption that a sentient fish does not liberally choose an uncomfortable condition when better conditions are given, provides useful information to improve fish welfare, if used cautiously (Volpato et al., 2007). The use of preference tests (Volpato, 2009) can help to better understand fish needs, but research has focused on the improvement of methods for data collection along with routine observations of the environmental conditions such as water quality parameters (Relić et al., 2010) for instance, and behaviour (Noble et al., 2012, Martins et al., 2012) and the scientific knowledge available of the species. Fish welfare can also be assessed using Welfare Indicators (WIs), which are parameters measured directly (animal-based; focusing on physiology, health, morphology and behaviour) or indirectly (resource-based; focusing on the rearing environment) on the fish (Huntingford et al., 2006, Huntingford and Kadri, 2009, Noble et al., 2018). Not all the WI, such as plasma cortisol levels for instance, are suitable under a farm environment, as they may lack accuracy or be too impractical to use for routine monitoring. Operational Welfare Indicators (OWIs), on the other hand, are WIs appropriate for an on-farm setting, and they must be relevant, practical to use, repeatable and suitable for different life stages, husbandry conditions and rearing systems (Noble et al., 2018, Treasurer, 2018a). For example, fin erosion or damage is a common OWI in many different species (Noble et al., 2012) and more prevalent under aquaculture conditions (Bosakowski and Wagner, 1994). The scoring of OWIs such as eye loss/damage, snout injury, jaw and operculum deformity, fin damage, spine deformity, scale loss/skin damage and sea lice damage are included in the welfare standards for farmed Atlantic salmon (RSPCA, 2021). Additionally, stocking density, disturbance and aggression are also used as welfare indicators (Adams et al., 2007). Darkening of the eye sclera and the body have also been used as indicators of welfare in Atlantic salmon, as well as in other species such as Nile tilapia, *Oreochromis niloticus*; suggesting that darker animals have a lower social rank (submissive) and higher stress levels (O'Connor et al., 1999, Volpato et al., 2003, Vera Cruz and Tauli, 2015). The most common indicators of welfare used in different aquaculture species have been summarised in Table 1.2.

Table 1.2. Most common welfare indicators used in the aquaculture of different fish species.

| Welfare indicators | Specie | Reference |
|---|---|---|
| Eye pathology (damage, exophthalmia, cataracts, loss) | Atlantic salmon<br>Rainbow trout | Noble et al. (2012), RSPCA (2018) |
| Eye and skin darkening | Atlantic salmon<br>Nile tilapia | O'Connor et al. (1999)<br>Volpato et al. (2003), Vera Cruz and Tauli (2015) |
| Snout/mouth injury | Atlantic salmon | Noble et al. (2018), RSPCA (2018) |
| Deformities (jaw, operculum, spinal) | | |
| Opercular damage | | |
| Skin damage/scale loss | | |
| Fin damage | Atlantic salmon<br>Rainbow trout<br>Cleaner fish<br>European sea bass | Noble et al. (2018), RSPCA (2018)<br>Noble et al. (2020), RSPCA (2020)<br>Treasurer and Feledi (2014)<br>Person-Le Ruyet and Le Bayon (2009) |
| Sea lice damage | Atlantic salmon | RSPCA (2018) |
| Stocking density | Atlantic salmon<br>Rainbow trout<br>European sea bass | Adams et al. (2007)<br>North et al. (2006)<br>Person-Le Ruyet and Le Bayon (2009) |
| Aggression (behaviour) | Atlantic salmon | Adams et al. (2007) |

## Implications of welfare for health, performance and productivity

Although welfare and health are closely linked (Ashley, 2007), welfare is not synonymous of health. Good health is crucial to welfare, but welfare is a broader concept and englobes many other aspects as well (Manteca et al., 2012), suggesting that a healthy animal does not necessarily have a good welfare status, but poor welfare can be either a pre-condition or a result of poor health. Good welfare implies that an animal is able to cope with a stressor, maintain its biological functions working properly, and live with good health (Segner et al., 2012). Stressors affect basal

physiological functions as they inflict an allostatic load to the animal, which is the achievement of stability through change (Treasurer, 2018a). The internal homeostasis of an organism is the dynamic equilibrium to maintain stability of vital systems within an optimal range for survival, despite challenges from the external environment (Stott, 1981, McEwen and Wingfield, 2003), and it is also disrupted by stress. Allostasis requires energetic expense, as the fish energy will be reallocated forstress coping and acclimation, instead of being used for other biological functions such as growth, reproduction and immune responses (Wendelaar Bonga, 1997). For instance, chronic stress was proven to decrease food intake and had a negative effect on feed conversion efficiency in European sea bass, *Dicentrarchus labrax* (Leal et al., 2011). Also, females under stress may not invest energy in maturing eggs and spawning and must take a decision between: (1) generating a nutritionally deficient progeny but in high numbers, or (2) saving energy by allowing some eggs to become atretic and maximize reproductive investment into a smaller offspring with higher fitness (Schreck et al., 2016). Although under certain conditions short-term stress can activate the innate immune responses in fish (for instance, the mucosal immune function), in general, chronic stressors lead to immunosuppression and increase the risk of disease susceptibility. In that sense, chronic stress induced pronounced transcriptional differences and caused lasting changes in the genome of stressed Atlantic salmon, *Salmo salar*, fry (Uren Webster et al., 2018b). To prevent fish spending energy on getting through changes in the environment, all potential stressors should be removed, so the allostatic load does not progress to an overload, which would surpass the fish's individual ability to cope. However, farmed fish are constrained to production facilities, and it is practically impossible to eliminate all stressful conditions. For example, injuries and deformities, which are common in standard aquaculture practices, can lead to reduced feeding activity, resulting in poor growth, loss of productivity (Noble et al., 2012), increasing predisposition to infections (Turnbull et al., 1996), reduced market value (Michie, 2001) and increasedmortalities (Miyashita et al., 2000). This negatively impacts the performance (Noble et al., 2012) and the welfare of the fish (Huntingford et al., 2006). Therefore, welfarecan be optimised by reducing the incidence of injuries and deformities, which will concurrently promote good health and reduce the risk of disease, increasing in turn thesuccess and economic sustainability of the business fish farm (Segner et al., 2012).

## Stress responses in fish

Stress responses are reactions fundamentally preserved among all vertebrates (Segner et al., 2012). Considered as an adaptive mechanism (Barton, 2002), they can be beneficial for individual fish, as long as the stressor is not extreme and it lasts for a short period of time, allowing the fish to adapt to the current situation. Contrarily, if the stressor is too intense or lasts for too long, it might result in maladaptation and allostatic overload, and a detrimental state of '*distress*' might follow (Barton and Iwama, 1991, Moberg, 2000, McEwen and Wingfield, 2003). Exposure to a stressor typically triggers a physiological response by the central nervous system (CNS) which is mediated by the hypothalamic-pituitary-interrenal (HPI) axis. Via cholinergic receptors, the chromaffin cells (homologous of adrenal glands in mammals), located in the anterior region of the kidney in teleost fish (Reid et al., 1998), release catecholamine hormones such as adrenaline and noradrenaline immediately into the blood stream (Barton and Iwama, 1991, Barton, 2002), whose plasma levels vary depending on the type and severity of the stressor and the species' response/tolerance to stress (Reid et al., 1998). The release of cortisol in blood plasma is posterior to catecholamine hormones, following activation of the HPI axis and release of corticotropin-releasing hormone (CRH), which stimulates the secretion of adrenocorticotropin hormone (ACTH) (Figure 1.1).

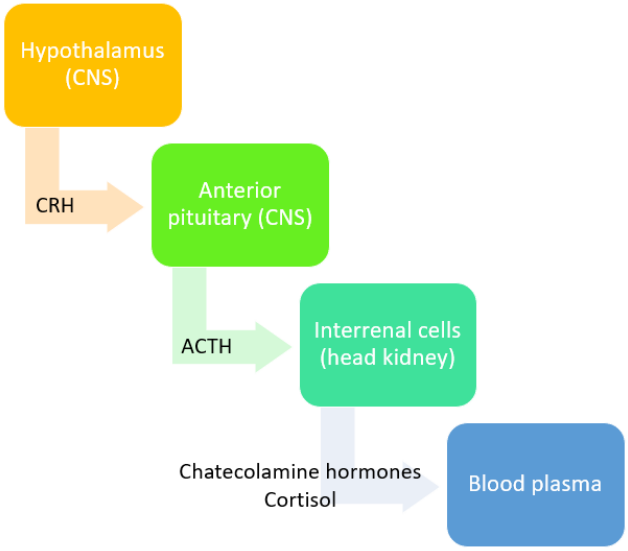


Figure 1.1. Hypothalamic-pituitary-interrenal axis involved in the fish stress response.

ACTH is responsible for the stimulation of interrenal cells to secrete cortisol, which will be distributed to target tissues (Barton, 2002) and whose levels could be maintained longer than catecholamines (Segner et al., 2012). Secondary responses to stress entail

haematological, osmoregulatory, metabolic, immunological and cellular alterations, expressed as changes in blood pressure, increase of glucose and lactate levels, haematocrit, ion composition, production of heat-shock proteins (HSPs), lysozyme activity and antibody production, amongst others (Barton and Iwama, 1991, Wendelaar Bonga, 1997, Barton, 2002). Tertiary stress responses such as changes in feeding activity, decreased growth and condition, disease resistance, metabolic scope for activities like swimming, altered behaviour and lately, survival, are functional and affect the overall performance and welfare of the fish (Barton, 2002, Segner et al., 2012).

There is strong evidence that stressors also modulate changes in gut microbiome (Galley et al., 2014, Uren Webster et al., 2020b). Stress in fish can be controlled with the use of pre- and probiotics. By enhancing the gut microbiota, these can decrease the stress response by lowering CRH and cortisol levels (Forsatkar et al., 2017) and reduce the anxiety behaviour (Davis et al., 2016) in zebrafish, *Danio rerio*. At the same time, stress induces modifications of the gut microbiota, for example, causing a reduction of mucus and removal of autochthonous communities that are protective against potential pathogens (Vatsos, 2017), altering the host immune response as well. Consequently, recent focus has been directed towards the adjustment of the gut microbiota, as well as maintaining health and welfare, so microbiome applications could be an interesting approach to improve host physiological processes (Egerton et al., 2018).

## Fish microbiome

The term *microbiota* comprises the collection or community of microbes that reside within a host or are present in a particular environment, including symbiotic, commensal and pathogenic microorganisms, while *microbiome* refers to the genome of the microbiota (Burokas et al., 2015). Fish microbiome research has grown significantly since the early 1990s with the expansion of the aquaculture industry and the replacement of the Sanger sequencing approach by the revolutionary next-generation sequencing (NGS) platform technologies (Behjati and Tarpey, 2013, Legrand et al., 2020).

Fish have an exceptional and intimate interaction with the aquatic environment they live in (Egerton et al., 2018), as well as with the surrounding coexisting microorganisms. Fish larvae are sterile and microbes originating from the chorion, the

surrounding water and the first feeding, start colonisation of the fish skin, the gills and the digestive tract, right after hatching (Austin, 2006). Microbes can also colonise larvae liver and ovaries (reviewed by Vatsos, 2017). The microbial community of the gastro-intestinal tract (GIT), also known as the gut microbiota, has been extensively studied in fish (Vatsos, 2017). It is classified as *autochthonous* or *indigenous* (adherent), if colonising the epithelial surface and associated with microvilli; or *allochthonous* or *transient* (non-adherent), if present in the lumen and associated with the ingesta (Ringø et al., 2016). Bacteria are the dominant microbiota found in the fish intestine (Rombout et al., 2011) and includes *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Fusobacteria* phyla (Butt and Volkoff, 2019). Intrinsic factors such as the host age (life stage or maturity), gender, genetic genotype, physiological and stress status and pathobiology, or extrinsic like the diet or feeding strategy, the season, the environment (water quality, salinity, temperature, etc.) and the farming system, contribute to shape the composition and the diversity of the gut microbiome, as well as its functionality (Vatsos, 2017, Butt and Volkoff, 2019, Legrand et al., 2020). This creates significant inter- and intra-species variations (Vatsos, 2017). The gut microbiota plays an important role on feeding/digestion/metabolism, stress and immune response, by interacting with the gut-brain axis through a bidirectional communication using hormonal, immune and neural signs (Butt and Volkoff, 2019). This has also repercussion on the behaviour, energy homeostasis and health of the host (Butt and Volkoff, 2019). It is well-known that healthy gut microbiota is essential to promote host health and well-being (Ringø et al., 2016). Knowing how the microbial systems in the host adapt and respond to stressors in farmed species is key to improve health, nutrition and optimise productivity in aquaculture (Legrand et al., 2020). Once the gut microbiome is characterised in healthy species of interest, bacterial communities can be modified to improve host health (Tarnecki et al., 2017). One of the most important applications of fish microbiome research into aquaculture is the use of specific bacterial communities that are beneficial for fish health; for instance, the use of probiotics to avoid the use of antibiotics to mitigate emerging diseases (de Bruijn et al., 2018), and consequently prevent the development of antimicrobial-resistant (AMR) bacteria (Butt and Volkoff, 2019, Yukgehnaish et al., 2020).

## Lumpfish (*Cyclopterus lumpus* L.)

The lumpfish or lumpsucker, Cyclopterus lumpus (Linnaeus, 1758), is a cold-water marine fish belonging to the Cyclopteridae (Scorpaeniforme) family, and the only species within the genus Cyclopterus (Davenport, 1985). Some of their specific anatomical features are a dorsal hump where the dorsal fin grows from, three rows of tubercles organised longitudinally along the main body and a distinctive suction disc (or sucker) located ventrally (Davenport, 1985). Lumpfish are generally solitary benthic species living close or attached to rocky seabed (Davenport, 1985, Stein, 1986), although they are occasionally pelagic and found in open waters (Holst, 1993). The species is widely distributed along both sides of the North Atlantic Ocean (Davenport, 1985), from the Barents Sea, Iceland and Greenland to the Iberian Peninsula in southwestern Europe on the Eastern side (Vasconcelos et al., 2004, Dulčić and Golani, 2006, Bañón et al., 2007, Eriksen et al., 2014, Froese and Pauly, 2014) to Nunavut, Hudson Bay, James Bay and Labrador in Canada to New Jersey and Bermuda in USA on the Western side (Froese and Pauly, 2014). Lumpfish can migrate long distances but show homing behaviour, returning to coastal and shallower areas to breed (Kennedy et al., 2015). Females spawn on rocky substrate and males will fertilize and guard the eggs until fry hatch (Thorsteinsson, 1981) .

Lumpfish females have been targeted since 1950s in Iceland, Norway, Easter Canada and Greenland for the roe market as a cheaper alternative to caviar, producing annually 4M kg (Johannesson, 2006). For example, only in Canada, lumpfish roe landings dropped substantially from an annual average of 349T (1986-2009) to 35T (2010-2015) (Gauthier et al., 2017). After 2013, both Greenland and Iceland accounted for > 94% of the landings (Kennedy et al., 2019), and given that lumpfish return to the same spawning sites every year, local overexploitation is very likely (Gauthier et al., 2017). In recent years, lumpfish are also being fished to supply brood stock for lumpfish hatcheries, being used as cleaner fish in the salmon farming industry (Imsland et al., 2014a), which has also caused a significant reduction in abundance due to intensive fishing of wild stocks (Pampoulie et al., 2014). Unfortunately, due to industry logistics, poor survival and biosecurity reasons, lumpfish lifespan in the aquaculture industry is as short as a single salmon production cycle in sea water (Powell et al., 2018b), and demands cannot be satisfied without oppressing wild stocks (Whittaker et al., 2018). Consequently, the species have been classed as ‘Near Threatened’ by the IUCN Red list (Lorance et al., 2015). Lumpfish commercial production has opened a new market,

abruptly increasing from close to 3.5M in 2014 to almost 16M in 2016, and it has been steadily growing up to 39M in 2019 only in Norway (Norwegian Directorate of Fisheries, 2021b), for their use as a biological control against sea lice (Powell et al., 2018b, Imsland et al., 2018a). Therefore, there is concern about the sustainability of the lumpfish industry and the potential impacts that this escalation in production could have on wild populations (Kennedy et al., 2019).

Lumpfish have been exploited mostly for the roe market as a cheaper alternative to caviar, producing annually 4M kg (Johannesson, 2006). They are also being used as cleaner fish in the salmon farming industry (Imsland et al., 2014a), which has caused a significant reduction in abundance due to overexploitation of some wild stocks (Pampoulie et al., 2014). Consequently, the species have been classed as 'Near Threatened' by the IUCN Red list (Lorance et al., 2015). Lumpfish commercial production has increased abruptly, from close to 3.5M in 2014 to almost 16M in 2016, and it has been steadily growing up to 39M in 2019 only in Norway (Norwegian Directorate of Fisheries, 2021b), for their use as a biological control against sea lice (Powell et al., 2018b, Imsland et al., 2018a).

## Sea lice threatening the salmon industry

The term 'salmon louse' includes diverse species of sea lice from the family Caligidae (Copepoda), the marine parasite with the widest geographical distribution (Costello, 2006). *Lepeophtheirus salmonis* (Krøyer, 1837) and *Caligus elongatus* (von Nordmann, 1832) are the most common species infecting Atlantic salmon (*Salmo salar* L.) in Northern Europe (North Atlantic) and North America, causing the greatest impact to salmonid aquaculture in the Northern hemisphere (Pike and Wadsworth, 1999, Costello, 2006). *L. salmonis*, showing higher incidence and impact than *C. elongatus*, is an obligate ectoparasite and its life cycle involves eight different stages separated by a moult (Hamre et al., 2013). These are divided into two nauplii (Nauplius I and II), one copepodid, two chalimi (Chalimus I and II), two pre-adults (Pre-adults I and II) and an adult stage (male/female) (Figure 2.1). Early stages cause less physiological effectsand damage in the host than mobile (pre-adults and adults) stages (Grimnes and Jakobsen, 1996). High fecundity rate is characteristic of *L. salmonis* since a single mature female can produce up to 11 generations of egg strings within a breeding season, where around 285 eggs/string can hatch into nauplii (Heuch et al., 2000). Copepodids swim primarily to find a host and attach to it, grazing and feeding

on fishmucus and skin and causing epidermal abrasion even in the underlying soft tissues (Pike and Wadsworth, 1999, Tully and Nolan, 2002, Johnson and Fast, 2004). Depending on the severity and extension of these lesions, osmoregulatory disturbances can occur (Wagner et al., 2003), leading to the onset of the primary stress response (Finstad et al., 2000) and potential immunosuppression of the host (Tully and Nolan, 2002). This also contributes to making the host more susceptible to secondary and opportunistic infections (Mustafa et al., 2000), compromising the overall fitness and welfare of salmon and potentially impacting its swimming performance (Wagner et al., 2003). This could culminate in mortalities especially when fish are heavily infested (Grimnes and Jakobsen, 1996, Tully and Nolan, 2002). Considering that the generation time for *L. salmonis* to complete a whole life cycle is around 7.5-8 weeks at 10°C (Johnson and Albright, 1991) or as short as 30 days at 12.2°C (Heuch et al., 2000), and how effortlessly the planktonic stages disperse in some farm environmentsdue to water currents (Krkošek et al., 2005), it is not surprising that sea lice is still themost persisting challenge for salmon aquaculture (Igboeli et al., 2014).

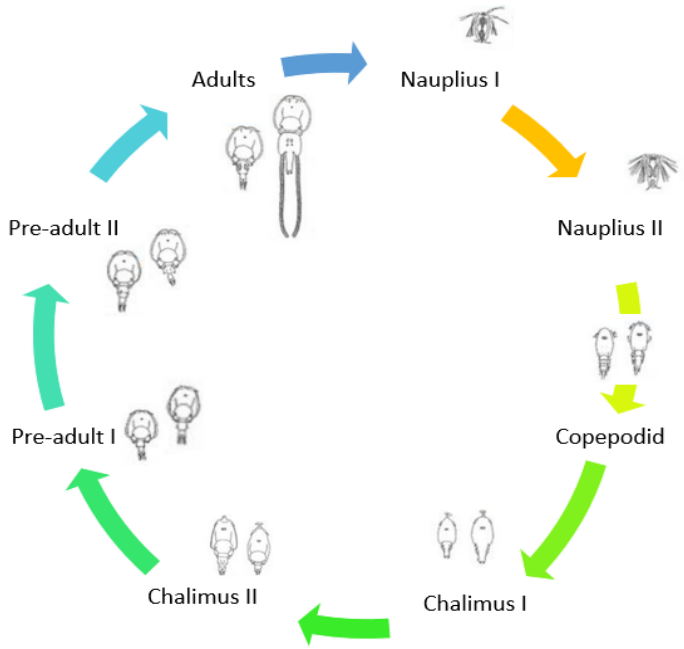


Figure 2.1. Figure 1.2. Sea lice life cycle (adapted from the Marine Institute). Stages out of the circle represent free swimming stages while stages within the circle are attached to the host.

Sea lice inflicts significant economic costs (Abolofia et al., 2017), which includes not only the cost of prevention and management strategies, but also financial losses caused by a reduction in fish growth and poor feed conversion ratio (Mustafa et al., 2001, Rae, 2002). Production losses for mortalities attributed to treatments are also

significant. In 2006, total global sea lice costs were estimated approximately to €300M (US$480M) annually, representing 6% of the product value and an individual cost of €0.1-0.2/kg of fish (FAO, 2008, Costello, 2009a). More than a decade later, sea lice costs are surrounding €490M (NOK 5B) only in Norway (Fletcher, 2019), and are expected to increase in the future. The impact of sea lice has certainly become more evident since salmon farming has intensified, as the increase in population density and the conditions are ideal for the parasitic copepod to proliferate and propagate (Torrissen et al., 2013, Abolofia et al., 2017). This implies a substantial problem not only for local farmed salmon and other neighbouring farms, but also for wild salmonid stocks and other hosts (Johnson et al., 2004, Krkošek et al., 2005, Costello, 2009b, Torrissen et al., 2013). The decrease of some wild salmon populations has been attributed to the presence of greater numbers of sea lice in commercial sea cages (Costello, 2009b).

## Sea lice control

Numerous approaches have been developed to control sea lice infestation in salmon cages: from physical barriers to prevent the encounter between sea lice and salmon, to delousing treatments to remove already settled sea lice. An overview of sea lice control strategies is presented (Table 1.1). Good management practices such as disinfection routines, biosecurity measures, stocking only single-year class smolts, fallowing after each production cycle and daily removal of mortalities (Bron et al., 1993) are essential for efficient sea lice management. Different combined treatments are tactically applied and continually reviewed as an integrated anti-lice strategy. Relying only in one treatment can only be successful as a short-term solution, and treatment rotation is recommended for efficacious control (Igboeli et al., 2014). Perpetuation of thisparasite is linked to two facts: (1) none of the available anti-lice methodologies appearto have 100% efficiency and (2) resistance has developed by evolutionary adaptation and natural selection against different chemotherapeutants (Denholm et al., 2002, Igboeli et al., 2014, Aaen et al., 2015, Coates et al., 2021).

Due to the emergence and spread of sea lice resistance, a current shift has been identified from the use of pharmaceutical treatments to non-medicinal approaches, such as thermal and mechanical delousing (Overton et al., 2019, Sommerset et al., 2021). The latter are responsible for 31% and 25% of all the sea lice treatment mortalities in salmon, respectively, in addition of welfare deterioration (Overton et al.,

2019). Although there has been an increase in mechanical innovation recently, biological innovation and cleaner fish research (19%) still has a significant representation, particularly in Norway (Greaker et al., 2020).

**Table 1.1**. Overview of different sea lice control strategies (preventive and treatments) for *L. salmonis*, unless indicated. Sources: Costello (1993),Rae (2002), Igboeli et al. (2014), Aaen et al. (2015), Holan et al. (2017), Overton et al. (2019), Barrett et al. (2020b). Efficacy scores estimate the effectiveness of sea lice control strategies based on costs applied on Scottish salmon farms under standard efficacy level (£/unit of effectiveness per fish) as suggested by Toma et al. (2020).

| Aim | Approach | Method type | Agent description | Sea lice stage target | Efficacy score | Reference |
|---|---|---|---|---|---|---|
| *Prevention* | Barrier technology | Skirts | Use of 3m tarpaulin around the cage | All stages | 0.58 (0.40-0.90) | Stien et al. (2012) |
| | | Snorkel cages | Roof net and air dome | All stages | Unknown | Stien et al. (2016) |
| | | Full enclosure | Filtered or deep-pumped water | All stages | Unknown | Nilsen et al. (2017) |
| | Behavioural manipulation | On-demand feeding | Use of Aquasmart™ feeding system | Mobile stages | Unknown | Lyndon and Toovey (2000) |
| | | Temporal submergence | AL and NL at different depths | Chalimus | Unknown | Hevrøy et al. (2003) |
| | | | AL (10 m) + deep feeding (5 m) | All stages | Unknown | Frenzl et al. (2014) |
| | Geographic management | Fallowing | Production pause and single-year class | All stages | Unknown | Bron et al. (1993) |
| | | Location | Light, salinity and current speed | Copepodids | Unknown | Genna et al. (2005) |
| | | Firebreaks | Modelling no-farming areas | All stages | Unknown | Samsing et al. (2019) |
| | Traps/filters | Light traps | Photomechanical device | Larval stages and G females | Unknown | Pahl et al. (1999) |
| | | Filtering | Pacific oyster (*C. gigas*) racks at 1,3,6 m | Larval stages | Unknown | Byrne et al. (2018) |
| | Attachment evasion | Repellents and host cue masking | In-water compounds (2-AA) | All stages | Unknown | Hastie et al. (2013) |
| | | | Light modification | All stages | Unknown | Browman et al. (2004) |
| | Incapacitation | Electricity | Direct current electric fence | Larval stages | Unknown | Bredahl (2014) |
| | | Ultrasound | Sound frequencies of 9.3,21,54 kHz | Copepodids | Unknown | Skjelvareid et al. (2018) |
| | | Irradiation | Use of short-wavelength light | All stages | Unknown | No published studies |
| | Population control | Pathogens | Rhabdovirus infecting sea lice | All stages | Unknown | No published studies |
| | | Gene drives (GDs) | CRISPR-based GDs for pest control | All stages | Unknown | No published studies |
| | Functional feeds | Immunomodulation | Diet supplemented with nucleotides | Mobile stages | Unknown | Burrells et al. (2001) |
| | | | Additives (β-glucans and MOS) | Mobile stages | Unknown | Refstie et al. (2010) |
| | | | CpG-ODNs and Aquate® | Copepodids | Unknown | Poley et al. (2013) |
| | | | Sex hormones (17β-estradiol/testosterone) | Copepodids/Chalimus | Unknown | Krasnov et al. (2015) |
| | | | Peptidoglycan extract | Chalimus | Unknown | Sutherland et al. (2017) |
| | | | Skretting Shield (Norway) | Copepodids/Chalimus | Unknown | Bui et al. (2020a) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Repellents/toxins | Phytochemicals | Mobile stages | Unknown | Holm et al. (2016) |
| | Vaccination | Commercial | Providean Aquatec Sea Lice (Tecnovax®) | *C. rogercresseyeri* (Chile) | Unknown | Barrett et al. (2020b) |
| | | Experimental | *my32* recombinant protein | All stages | Unknown | Kumari Swain et al. (2018) |
| | | | P21, P30, P33, P37 recombinant proteins | Chalimus and adult females | Unknown | Contreras et al. (2020) |
| | Breeding | Louse resistance | Resistant salmon families | Mobile stages | Unknown | Holm et al. (2015) |
| *Treatment* | In-feed | Avermectins | Ivermectin (Ivomec®) | Chalimus and mobile stages | Unknown | Palmer et al. (1987) |
| | | | Doramectin (Dectomax®) | Chalimus and mobile stages | Unknown | Roth (2000) |
| | | | Emamectin benzoate (Slice®) | Chalimus and mobile stages | 0.73 (0.50-0.80) | Stone et al. (1999) |
| | | Benzoylphenyl ureas | Teflubenzuron (Ektobann®, Calicide®, Cal-X®) | Larval stages and pre-adults | Unknown | Branson et al. (2000) |
| | | | Diflubenzuron (Lepsidon®) | Larval stages and pre-adults | Unknown | Roth (2000) |
| | Bath | Organophosphates | Dichlorvos (Nuvart®, Aquagard®) | Mobile stages | Unknown | Wootten et al. (1982) |
| | | | Trichlorfon (Neguvon®) | Mobile stages | Unknown | Costello (1993) |
| | | | Carbaryl (Sevin®) | Mobile stages | Unknown | Costello (1993) |
| | | | Azamethiphos (Alfacron®, Salmosan®) | Mobile stages | 0.60 (0.50-0.90) | Roth et al. (1996) |
| | | Pyrethrin/ Pyrethroids | Pyrethrum (Py-Sal®) | Mobile stages | Unknown | Costello (1993) |
| | | | Cypermethrin (Excis®, Betamax®) | Chalimus and mobile stages | Unknown | Hart et al. (1997) |
| | | | Deltamethrin (AlphaMax®) | Mobile stages | 0.60 (0.50-0.90) | Roth (2000) |
| | | Oxidising agent | Hydrogen peroxide (Salartect®, Paramove®) | Chalimus and mobile stages | 0.43 (0.10-0.60) | Treasurer and Grant (1997) |
| | | Freshwater | Low salinity water | Copepodids | 0.64 (0.20-1.00) | Stone et al. (2002) |
| | Mechanical | Flushing | Hydrolicer®, FLS®, SkaMik® | Mobile stages | 0.80 (0.70-0.95) | Gismervik et al. (2017) |
| | Thermal | Warm temperature | Thermolicer® and Optilicer® | Mobile stages | 0.80 (0.70-0.95) | Grøntvedt et al. (2015) |
| | Optical | Automated laser | Stingray Marine Solutions AS™ | Mobile stages | Unknown | Bui et al. (2020b) |
| | Biological control | Cleaner fish (predation) | Wrasse (*Labridae* family) | Mobile stages | 0.72 (0.60-0.90) | Bjordal (1991) |
| | | | Lumpfish (*Cyclopterus lumpus* L.) | | | Imsland et al. (2014a) |
| *Prevention + treatment* | Combined approach | Multiple methods | Skirts + Deep feeding + Lighting + Functional Feed + Cleaner fish | All stages | Unknown | Bui et al. (2020a) |

## Use of cleaner fish in the salmon industry

The use of cleaner fish in aquaculture develops from the biological control of pests extensively used in agriculture. It consists in the control of invertebrate pests using other organisms that can act by different mechanisms such as predation, parasitism, pathogenicity, competition, etc., and generally requires human involvement (Murdoch et al., 1985, Flint and Dreistadt, 1998, Eilenberg et al., 2001). Cleaning mutualism interactions between 'cleaner' and 'client' fish are prevalent and well-studied in the marine environment (Bshary and Côté, 2008). Several wild wrasse species with cleaning behaviour to salmon infested with sea lice were identified as facultative cleaners in the late 80s: goldsinny (*Ctenolabrus rupestris*), corkwing (*Crenilabrus melops*), rock cook (*Centrolabrus exoletus*), cuckoo (*Labrus mixtus*) and ballan wrasse (*Labrus berggylta*); and started being used as a delousing control strategy in commercial salmon farms (Bjordal, 1988, 1990, 1991). Lately, the cunner wrasse (*Tautogolabrus adspersus*) was also trialled to be used as cleaner fish (Costa et al., 2016). Since then, the use of wild wrasse has progressively escalated from 1.7M in 2008 to 20M in 2016 and the production of farmed wrasse from 270K in 2012 up to 1.3M, only in Norway (last update 27/05/2021)(Norwegian Directorate of Fisheries, 2021b). However, wrasse are not active at low temperatures (Morel et al., 2013) and lumpfish have proven to be a suitable alternative in cold water temperatures, reducing the number of sea lice females in trials under a salmon farming set-up (Imsland et al., 2014a).

## Main challenges in the use of lumpfish

Only in Norway, more than 60M cleaner fish are being used annually for the salmon industry (Norwegian Directorate of Fisheries, 2020), but welfare and ethical concerns about the use of live animals for delousing control are currently being raised (Overton et al., 2020, Geitung et al., 2020, Imsland et al., 2020). It is well known that lumpfish experience mass mortalities, especially following deployment in sea cages. Although the exact number is unknown, these can range from 27% up to 100% of the stocked population (Poppe, 2017, OneKind, 2018, Geitung et al., 2020, Stien et al., 2020), with bacterial infections among the main causes (Nilsen et al., 2014, Hjeltnes et al., 2017). Other reasons for lumpfish mortality could be poor husbandry, starvation, extreme environmental conditions (such as high-water temperatures, strong currents, and low

oxygen), and physical damage (Stien et al., 2020, Grefsrud et al., 2019, Anon, 2020a). Recent surveys in Norway indicate that the current situation has not improved in several years. Just as few as 5.3% of respondents reported lower lumpfish mortalities, while most did not know if the situation had improved (Sommerset et al., 2021). Another issue is the unregistered losses, potentially caused not only by mortalities and carcass decomposition, but also by predation (Davenport, 1985) and escapes, which indeed lead to a high risk of disease transmission and genetic contamination of wild populations (Herrmann et al., 2021).

Farmers are being criticized by the Norwegian Food and Safety Authority (NFSA, Mattilsynet) for inadequate care to lumpfish (Stranden, 2020) and the whole industry is being forced to improve lumpfish welfare globally (Garcia de Leaniz et al., 2021), but there is not much guidance available. Individual and grouped-based OWIs have been recently developed for lumpfish, along environmental and laboratory-based indicators (Noble et al., 2019). For example, the individual liver colouration, which informs of the nutritional status of the fish, has been recently used as a welfare indicator in lumpfish (Eliasen et al., 2020). However, as many other laboratory-based OWIs, this requires training, instruments and sacrificial sampling, which may not be practical in a farm environment. It is hence necessary that regulators and policy makers work together and produce agreed standards for the species. In this sense, the RSPCA has recently included 'Cleaner fish' within the last published version of welfare standards for farmed Atlantic salmon. This section covers important guidelines about the husbandry, management/handling, transport, enrichment and feeding aspects, as well as the arrival at the slaughterhouse (RSPCA, 2021), but does not address practical and morphological measurements within the farm.

Stress management is also key to improve welfare and the efficient use of lumpfish in aquaculture (Staven et al., 2021). It is thought that mortalities after deployment in salmon farms could potentially be caused by the struggle in coping with the transition from hatchery to sea cages, where lumpfish are exposed to numerous stressors. Habituation is highly recommended to reduce physiological and behavioural stress responses, such as burst swimming activity, swimming distance from salmon and plasma cortisol levels, which were reduced in experienced lumpfish compared to naïve individuals (Staven et al., 2019). In comparison with species susceptible to stress such as Atlantic salmon and ballan wrasse, lumpfish only show a moderate cortisol stress response with cortisol levels in plasma around 200 nM, while salmon and wrasse

plasma levelsare around 700 nM and 600 nM, respectively; suggesting lumpfish are bolder and moreresilient to stress (Treasurer et al., 2018b). This could be explained by the absence of Mauthner neurons, which are responsible to initiate an escape response (escape reflexor C-start response). Instead, lumpfish would use their suction disc to attach to a solidsurface and hide (Hale, 2000).

Stress also influences the microbiome. Little is known of lumpfish microbiome, but recently published studies focus on characterising bacterial communities in different scenarios, with the main effort on improving health and survival (Klakegg et al., 2020): different life stages (Christie et al., 2018), within healthy populations (Roalkvam et al., 2019), or in different rearing systems with different water treatments (Dahle et al., 2020). Yet, changes in gut microbiota related to their sea lice ingestion ability or a connection between stress and microbial communities have not yet been addressed.

Lumpfish are considered facultative and not 'true' cleaners (Vaughan et al., 2017) as they do not naturally eat sea lice. A recent study, however, showed that lumpfish personality influences the interaction between lumpfish and salmon that promotes the cooperative cleaning behaviour (Whittaker et al., 2021). The delousing efficacy attributable to lumpfish is highly variable, with 10-36% of the population consuming sea lice (Imsland et al., 2014a, Eliasen et al., 2018) and causing a reduction of sea lice levels of 9-73%, although up to 97% for adult female lice (Imsland et al., 2014a, 2014b, 2014c, 2015a, 2015b, 2016a, 2016b, 2018a). It seems that the delousing effect is fairly site-dependant (Barrett et al., 2020a) and potentially larger at big (53-73%) than at a small scale (9-60%) (reviewed by Overton et al., 2020), but there is widespread suboptimal use (Barrett et al., 2020a). Although smaller lumpfish of around 20-30g are thought to be more effective than larger (>75g) (Imsland et al., 2016b), there is evidence that sea lice removal efficiency increases with time spent at sea (Imsland et al., 2014a, 2014b, 2014c), and lumpfish as big as 545g can still ingest sea lice (Eliasenet al., 2018). Sea lice ingestion has a genetic component and appears to be parentally controlled (Imsland et al., 2016a), but many other factors, summarised in Table 1.2, can influence the cleaning behaviour.

Table 1.3. Summary of factors affecting delousing behaviour in lumpfish.

| Group | Factor | References |
|---|---|---|
| Genetic | Parental control | Imsland et al. (2016a) |
| Individual | Stocking density | Overton et al. (2020) |
| | Health/welfare status: disease outbreak or compromised welfare | Brooker et al. (2018), Powell et al. (2018b), Erkinharju et al. (2021) |
| | Skeletal deformities | Fjelldal et al. (2020b) |
| Environmental | Sea lice infection pressure | Imsland et al. (2018a) |
| | Adverse conditions (offshore) | Hvas et al. (2021) |
| | Sea water temperature | Leclercq et al. (2018) |
| Host | Size | Groner et al. (2013) |
| | Depth distribution | Gentry et al. (2020) |
| Husbandry | Acclimatation/habituation | Staven et al. (2019) |
| | Pre-conditioning (use of live feed) | Imsland et al. (2019e) |
| Farm practices | Other anti-lice strategies | Gentry et al. (2020) |
| | Dirty pen nets (food source) | Imsland et al. (2014c), Imsland et al. (2015a), Eliasen et al. (2018) |

# Thesis aims and objectives

This thesis investigated novel ways of monitoring and improving the welfare of lumpfish with the ultimate goal to help increase the sustainability and ethical use of lumpfish as cleaner fish to control sea lice in salmon aquaculture. The primary aim was to develop a tool to measure lumpfish welfare in a practical and efficient manner under farming conditions (Chapter 2), and to use this tool to better understand changes over time at sea (Chapter 3), variation between different genetic stocks (Chapter 4) and the effect of sea lice ingestion on welfare through the assessment of gut microbial communities (Chapter 5).

### Chapter 2. Development and validation of an Operational Welfare Score Index for farmed lumpfish *Cyclopterus lumpus* L.

Lumpfish are increasingly being used by the salmon industry to control sea lice infestations, but their survival in cages is often poor and there are concerns in relation to their welfare (Treasurer et al., 2018b). Some Operational Welfare Indicators (OWIs) have been developed for lumpfish, but the industry needs a practical index that can be routinely used under farming conditions. Chapter 2 developed and validated individual-based OWIs, selected according to their practicality, variability and individuality. The selected OWIs were validated using both reliability and construct validity. By examining correlation between traits and selecting the most sensitive and representative measurable parameters, the index was refined and simplified, and tested in six different commercial sites (hatcheries and sea farms) to assess repeatability and practicality by asking eight different salmon farmers. The translation of this scoring index into a practical platform (Lumpfish Welfare Watcher tool) is currently in progress.

*Gutierrez Rabadan, C., Spreadbury, C., Consuegra, S. and Garcia de Leaniz, C. (2021). Development, validation and testing of an Operational Welfare Score Index for farmed lumpfish Cyclopterus lumpus L. Aquaculture, 531, 735777.*

## Chapter 3. Changes in growth and welfare of lumpfish *Cyclopterus lumpus* L. over time in a longitudinal study

Intensive farming conditions can quickly deteriorate fish welfare. Lumpfish can lose condition in less than four weeks if not optimally reared (Johannesen et al., 2018) and there are indications that their welfare deteriorates in commercial conditions (Gutierrez Rabadan et al., 2021), especially in sea cages. I assessed whether time spent at sea had a negative effect on welfare and whether frequent monitoring could help to identify critical periods. The aim of Chapter 3 was to assess changes in average growth, welfare and feeding preferences over time at two time points (three weeks and three months) after deployment, but also between two different genetic stocks of lumpfish (using microsatellite markers and parental assignment) deployed in the same commercial salmon farm. Feeding preferences were based on the prevalence of ingested sea lice and ingested marine formulated pellets.

## Chapter 4 Feeding preferences, growth and gut microbial communities of two stocks of lumpfish *Cyclopterus lumpus* L. deployed in a common sea cage

Phenotypic variation has been reported between different genetic populations of lumpfish (Whittaker et al., 2018). Due to production logistics, salmon farmers combine lumpfish from different origins in the same commercial cages, so I examined whether different stocks of lumpfish performed differently under a common environment. Lumpfish originated from Iceland and Scotland were stocked at similar ages in a commercial sea cage within a salmon farm under a common garden design. Microbial communities were characterized for each of the stocks using 16S rRNA gene sequencing and differential abundance analyses were performed to investigate possible associations between gut microbial communities and welfare and gut microbial communities and stress. Also, a subsample of individually tagged Scottish lumpfish, scored before and two months after deployment, were used to monitor changes in welfare following deployment at sea.

**Chapter 5 Effect of sea lice ingestion on lumpfish *Cyclopterus lumpus* L. welfare and gut health**

Lumpfish are considered facultative cleaners (Vaughan et al., 2017) and do not eat sea lice under natural conditions; however, they are produced to delouse salmon but the impact of feeding on sea lice on lumpfish gut health and welfare is unknown. Chapter 5 investigates the effect of sea lice ingestion on lumpfish welfare and intestinal microbial communities under commercial conditions. As seen in Chapter 4, different diets can shape the microbial communities in the lumpfish intestine. I hypothesised that sea lice ingestion could influence not only the welfare but also the diversity and composition of the lumpfish gut microbiota, where specific taxa could be promoted/demoted in sea lice eating-lumpfish. To address this hypothesis, the composition of the gut microbiome was characterized according to the presence/absence of sea lice in their stomach contents and microbiome networks were compared and quantified for both delousing groups, also considering their welfare status.

Finally, the key findings of this thesis are compiled and discussed in **Chapter 6**, along with some conclusions.

# Chapter 2 Development and validation of an Operational Welfare Score Index for farmed lumpfish *Cyclopterus lumpus* L.

**Abstract**

Lumpfish (*Cyclopterus lumpus* L.) are widely used for controlling sea lice in salmon farming, but their welfare is often challenged by poor husbandry and disease outbreaks, which compromise their health and ability to delouse salmon. It is necessary to identify when the welfare of lumpfish is being compromised in a fast, simple and effective manner. An Operational Welfare Score Index was developed and validated by visually assessing skin and fin damage, eye condition, suction disc deformities and body condition, indicators that fish farmers considered to be the most informative. Also, percentile length-weight charts are presented to enable the detection of underweight and emaciated lumpfish at different stages of development. The lumpfish welfare score index proved to be repeatable (ICC=0.83±0.05, $p<0.001$), practical (<2min) and easy to be used in a farm environment, according to the results of its application at six commercial sites. Although most lumpfish showed good welfare (71%) and significant differences were found between sites, 28% of lumpfish had lower than expected weights for their length, and 10% were emaciated. The most common welfare problems were suction disc deformities and fin damage in hatcheries, and poor eye condition and body damage in sea cages, conditions that might increase the risk of emaciation. This index, along with the percentile charts, will be particularly useful to assess lumpfish welfare in farm conditions in a quick and accurate manner, which will help to improve their welfare, reduce stress-related mortalities and improve the sustainability of the salmon industry overall.

## Introduction

Fish welfare is a growing area of research due to increasing consumers' demands for ethically produced food (Ashley, 2007). Increasing concern about the welfare of farmed fish has led to the development of welfare standards for a few farmed species (Cooke, 2016). However, to what extent welfare criteria that work well for some species such as Atlantic salmon, *Salmo salar* L. (Pettersen et al., 2014, RSPCA, 2018) can also be applied to other farmed species, is not known (Treasurer and Feledi, 2014). Cleaner fish are novel species to aquaculture, used to control sea lice (*Lepeophtheirus salmonis*), one of the major threats to the salmon farming industry since 1970 (Torrissen et al., 2013). This ectoparasite not only results in significant economic losses, but has also an impact on fish welfare, the environment, and the public perception of aquaculture (Costello, 2009a, Aaen et al., 2015). Increasing resistance to chemotherapeutants traditionally used to combat sea lice has raised the interest in cleaner fish as environmentally friendly 'green' alternative to the use of medicines (Powell et al., 2018b). The public and retailers generally support the use of cleaner fish to control sea lice because of the environmental and efficacy benefits, but only if the welfare of cleaner fish is not compromised (Treasurer et al., 2018b).

Operational Welfare Indicators (OWIs) represent a practical approach to measure welfare in farmed species. These need to be tailored and be species-specific, although some OWIs can be adapted from other species. Many traits related to welfare, such as the presence of external injuries or deformities, have also potential to impact aquaculture production (Noble et al., 2012). Other criteria, such as a fin damage (or 'fin erosion') is a welfare issue common to many different species, particularly when they are reared at high densities (Hoyle et al., 2007, Noble et al., 2012). Fin damage can result from mechanical abrasion, nutritional deficiencies, systemic or external bacterial infections (Ellis et al., 2008), aggression (MacLean et al., 2000), but also from stress (Turnbull et al., 1996); and may have a detrimental effect upon production performance (growth and survival) and welfare, by increasing the susceptibility to infection and potentially impacting swimming ability (Noble et al., 2012). Eye darkening (ED), for instance, has been used as an indicator of social status in Nile tilapia, *Oreochromis niloticus* L. (Volpato et al., 2003, Cruz and Brown, 2007, Miyai et al., 2011, Vera Cruz and Tauli, 2015, Champneys et al., 2018) and Atlantic salmon (O'Connor et al., 1999, Suter and Huntingford, 2002). Eyedarkening is also a reliable,

easy, and inexpensive indicator of stress in fish as it can be induced by stressors like confinement and air exposure in Nile tilapia (Freitas et al.,2014). Darkening of the body in Atlantic salmon has also been associated with aggressive encounters with dominant fish, and both the eye sclera and the overall bodycoloration tended to darken in the fish that were losing territorial encounters, while victors retained their original coloration (O'Connor et al., 1999). Other traits are morespecific, for example, sucker deformity is unique to lumpfish *Cyclopterus lumpus* L. or fish that possess suctorial organs.

Commercial production of cleaner fish has grown exponentially over the past few years. Lumpfish are efficient for sea lice control in commercial salmon sea cages (Imsland et al., 2018a) due to their short production cycle (5-7 months to reach deployment size) compared to other cleaner fish species such as ballan wrasse *Labrus bergylta*, which needs around 18 months to reach a deployable size (Brooker et al., 2018). In addition, lumpfish continue foraging on sea lice under cold temperatures, unlike wrasse (Brooker et al., 2018, Imsland et al., 2014a, Morel et al., 2013). Only in the UK, lumpfish production reached 1.9 million in 2016 (Munro and Wallace, 2017) and it is estimated that 10M lumpfish will be required by 2020 (Powell et al., 2018a). However, the survival of cleaner fish in net pens cohabiting with Atlantic salmon is often poor, and there is increasing concern regarding their welfare (Treasurer and Feledi, 2014, Treasurer et al., 2018b). Recent surveys suggest that between 33% and 50% of lumpfish may die following deployment in salmon cages. Emaciation, poor welfare and lack of specific knowledge of their husbandry needs appear to be the principal challenges for lumpfish farming (Nilsen et al., 2014, Hjeltnes et al., 2019). Although several OWIs for lumpfish have been put together (Noble et al., 2019), there are no agreed lumpfish welfare standards, and it is important that these are developed for quality assurance (Brooker et al., 2018, Treasurer, 2018a). The aim of this study was to develop a scoring index for lumpfish by screening several individual-based OWIs to measure lumpfish welfare under farming conditions in a rapid but accurate manner.

## Material and Methods

### Development of the Lumpfish Operational Welfare Score Index (LOWSI)

Four steps were followed to develop this score index (Figure 2.1): (1) identification and selection of individual-based OWIs (including the exclusion of non-relevant ones); (2) validation of the selected OWIs against measures of plasma cortisol and body condition using relative weight (*Wr*); (3) refinement and simplification by assessing correlated traits, and (4) pilot trial to test the use of the index in the farm environment and its suitability through a questionnaire.

### Selection and screening of Operational Welfare Indicators (OWIs)

*Sample collection*

The welfare of 95 juvenile farmed lumpfish was examined at two different life stages: pre-deployment (n=60, ranging between 4.8 g and 152 g, from 2 different hatcheries in the UK) and post-deployment (n=35, between 22 g and 100 g, from a sea farm in Scotland). Measurements of body weight (g) and standard and total length (mm) were taken for each fish, as well as photographs of overall condition and detailed measurements of fins, eyes and ventral suction disc. Sampling to define the Welfare Score, detailed below, took less than 2 minutes per fish. Photographs were taken with a Canon EOS 800D (CANON EFS 18-55mm, TAMRON 90mm lens) mounted on a tripod against a black background, a ruler and a reference colour chart (Colour Checker Passport, X-rite) and stored in high resolution.

The results of a questionnaire given to 53 participants at the 1st Symposium on Welfare in Aquaculture (May 2019, Swansea) were used to select potential welfare indicators for testing. During the focus group, respondents were asked to rank the utility of 12 welfare indicators for lumpfish (Table 2.1). To select the OWIs for this study, all those indicators that were laboratory based or required specialized training (blood parameters, parasite/disease screening), or that had proved unreliable (body/eye darkening) or shown limited or no variation in pilot trials (operculum/gill damage, body deformities) were excluded. After the screening, six of the most representative parameters were selected to develop the Welfare Score, based on this previous

research: (1) external condition, (2) fin damage, (3) eye condition, (4) eye darkening, (5) suction disc deformity and (6) relative weight.

*Welfare Score description*

(1) External damage

External damage was assessed as the presence or absence of physical damage and/or skin lesions in a 2-point scale from 0 to 1 (Appendix, Figure S2.1). Lesions included any reddening, erosion, abrasion, inflammation, or ulcers.

(2) Fin damage

Fin damage of the 4-rayed fins: dorsal fin, caudal fin, anal fin and pectoral fins were recorded in a Likert scale (5-point) from 0 to 4 according to the extension of the tissue area affected (Appendix, Figure S2.2). The ventral section of the pectoral fin was also recorded and included in fin damage. Left and right pectoral fins were averaged and scored to the next highest score. All the fins combined resulted in a total fin damage score with values ranging from 0 to 20.

(3) Eye condition

Eye condition was scored from 0 to 6 and consisted in the measurement of three parameters: (a) eye damage, (b) exophthalmia or pop-eye and (c) eye cataracts (Appendix, Figure S2.3). Each of these parameters was measured in a 3-point scale from 0 to 2 depending on the extension of the condition (0: absence; 1: unilateral or one eye affected; 2: bilateral or both eyes affected).

(4) Eye darkening

For the assessment of eye darkening (ED), both eyes were drawn by hand in circular templates divided into 8 equal areas. The percentage of darkened area was estimated in each octant (with values between 0 and 1), then summed and multiplied by 12.5% (Volpato et al., 2003, Freitas et al., 2014, Champneys et al., 2018). Eye darkening was calculated as an average of left and right eyes and classified into a Likert-scale from 0 to 4 depending on the percentage of ED (Appendix, Figure S2.4).

(5) Suction disc deformity

Deformity of the ventral suction disc was assessed by measuring the extension of area affected based on five different parameters: (1) symmetry of the suction cup, (2) presence and severity of indentations, (3) depression of the centre of the suction cup, (4) papillae or muscular pad development and (5) deformity of the ventral section of the pectoral fin. Each of these parameters was assessed on a Likert scale (0-4). All the scores were combined into a total suction disc deformity score with values ranging from 0 to 20 (Appendix, Figure S2.5).

(6) Relative weight

Farmed lumpfish (n=2658) weights (g) and total lengths (mm) were collected from 2015 to 2019 from different hatcheries and sea farms and at different life stages. Length-weight relationships (LWRs) were examined to obtain relative weight (*Wr*) as a measure of body condition, due to the unusual shape lumpfish possess. Every individual was classified into the following life stages: (S1) Larvae (0-1g), (S2) Pre-deployment juveniles (1-10g), (S3) Pre-deployment juveniles (+10g) and (S4) Post-deployment. According to this, standard weight (*Ws*) was computed using the regression parameters from the respective fitted models following the formula: $Ws = 10^{a} \cdot TL^{b}$, where a is the intercept, b is the slope of the linear regression and TL is the total length (mm). Relative weight was then calculated as the ratio of the observed weight (*W*) and the length-specific standard weight value (*Ws*) (Quist et al., 1998, Blackwell et al., 2010) according to the following formula: $Wr = 100 \cdot (W/Ws)$ (Table 2.2).

**Validation of selected individual-based OWIs**

To validate the selected OWIs, both reliability and construct validity were used. Reliability measures the magnitude of the measurement error in relation to the inherent variability between subjects (Bartlett and Frost, 2008). Construct validity is the degree to which scores are consistent with a priori hypothesised differences between relevant groups, based on the assumption that the scale validly accurately captures the construct it purports to measure (Mokkink et al., 2010).

*Reliability*

Two trained raters (R1 and R2), working independently, blindly scored the images of the 95 lumpfish used for the OWI selection above. The images were allocated randomly. Rater 1 also scored the images twice (after 8 months) to provide a measure of intra-rater reliability. Reliability of the OWIs was measured as repeatability of the scores. The repeatability of each of the OWIs and the products of the PCA (PC1 and PC2) were calculated by the intraclass correlation coefficient (ICC) for both intra-rater (raters R1 $t_1$ vs R1 $t_2$) and inter-rater (raters R1 vs R2) reliability, using the *irr* R package (Gamer et al., 2019). ICC estimates were computed by a single- rating, absolute-agreement, 2-way random-effects model with 2 observers across 95 subjects. The ICC is a suitable tool to measure reliability as it considers both the degree of correlation and the agreement between measurements (Koo and Li, 2016).

*Construct validity*

The absence of an agreed gold standard test for assessing lumpfish welfare means that construct validity must be evaluated through measures of welfare that have been previously described in other farmed species (Noble et al., 2018). I used plasma cortisol, involved in the stress response, and relative weight, calculated as described above, as an indication of poor growth or emaciation. Individually, each of the OWI scores was summed in an aggregated welfare score (range:0-51), which was standardized and centred by subtracting the mean and dividing by the standard deviation before being analysed by Principal Component Analysis (PCA) using the *factoextra* R package (Kassambara and Mundt, 2019).

*Plasma cortisol analysis*

Lumpfish were humanely euthanised by an overdose of anaesthetic (tricaine methanesulfonate) according to Schedule 1 of the Animals (Scientific Procedures) Act 1986. Blood samples were obtained from a subsample of lumpfish (n=55, ranging between 22 and 152 g) by puncturing the caudal vein with a lithium-heparinized vacutainer blood collection system. To reduce sampling variation, all samples were collected within 30 seconds from cessation of opercular movement, by the same person, using the same equipment and mainly in the morning (9am-1pm). Samples were kept on ice until they were centrifuged at 1500rpm for 10 minutes at 15°C,

following the protocol from ThermoFisher Scientific (Thavasu et al., 1992). Plasma was separated and stored at -80°C until analysis. For cortisol analysis, a well-known multi-species competitive ELISA test (Arbor Assays DetectX® Cortisol Enzyme Immunoassay Kit, Michigan, USA) was employed. Each sample was treated with dissociation reagent to increase its yield and diluted with buffer before cortisol determination. Standards, blanks and test samples were loaded in duplicate and absorbance values (OD) were read with a SpectroStar Nano Plate Reader (BMG Labtech, Germany) at 450nm wavelength. The average of two duplicates was used to create a standard curve ($R^2$=0.994), and concentrations were multiplied by the dilution factor (1:100) to obtain total plasma cortisol values (in ng/ml). Intra and inter-assay precision (CV%) for duplicate samples, calculated following the protocol from Salimetrics (USA), was 15.4% and 6.8%, respectively. The total assay sensitivity was 24.7 pg/ml, and the lowest limit of detection was 18.01 pg/ml.

**Refinement and Simplification**

As the index had to be practical, all fins were assessed for correlation using the non-parametric pairwise matrix Spearman's rank correlation coefficients (ρ) in order to identify the most sensitive fins to score, as it was noted that lumpfish with damage in one fin, typically presented damage in others. The original welfare score (range:0-51), described above in step 1, was then simplified and refined to contain the most representative OWIs to include into the Lumpfish Operational Welfare Score Index (LOWSI, range:0-10), which included relative weight and the four most reliable OWIs (skin damage, caudal fin damage, eye condition and suction disc deformity), all scored on a 3-point scale to ensure equal weighting (Table 2.3).

**Pilot trial**

To test the application of the LOWSI, a pilot trial was performed at six different commercial sites: three hatcheries (H1-H3, n=120) and three sea farms (F1-F3,n=125), where 245 lumpfish were scored by one or two raters during 2018 and 2019.Lumpfish were classified into three different welfare classes, depending on theLOWSI points obtained (Table 2.3): (A) Good welfare (<3 points), (B) Moderately compromised welfare (3-5 points), and (C) Severely compromised welfare (>5 points). Both the reliability and the practicality of the LOWSI were tested by (1) using the scores of the author and eight fish farmers on a subsample (n=150) of lumpfish scoredtwice, and

(2) a Likert questionnaire (Table 2.4) given to the eight farmers that scored the lumpfish, respectively.

**Statistical analyses**

All data was analysed using R version 3.6.1 (R Core Team, 2019). To assess the results of the questionnaires (a - the utility of 12 welfare indicators for lumpfish, and b - the LOWSI performance), the Wilcoxon signed rank test with continuity correction was used to calculate (pseudo)medians, 95% confidence intervals and *p* values on the Likert-scale responses (Mangiafico, 2016). The *clmm2* function in the *ordinal* R package (Christensen, 2019) was computed into a cumulative link mixed model to evaluate the degree of agreement amongst participants. Model selection was performed based on the Akaike Information Criterion (AIC) values and the function *dredge* in the MuMIn R package (Barton, 2019). The variation of the two principal components (PC1 and PC2) was investigated against plasma cortisol and relative weight while statistically controlling for variation in size (total length). Model assumptions were tested by analysing the linearity, normality, homogeneity of variances and leverage of residuals. Overly influential observations were detected as outliers and excluded from the validation of relative weight (obs. #19) and plasma cortisol (obs. #17).

# Results

## Selection of Operational Welfare indicators (OWIs)

*Utility of welfare indicators for lumpfish*

The 12 welfare indicators tested by 53 participants on the questionnaire given during the 1st Symposium on Welfare in Aquaculture differed significantly in utility ($\chi^2$=47.11, df=11, p<0.001). Focusing only on individual based OWIs, fin and skin damage were the most useful, while body/eye darkening and blood parameters were the least useful (Appendix, Table S2.1). The consensus between participants was high, as 87% of them did not deviate from the response of the average rater (Appendix, Figure S2.7).

*Prevalence and variation in OWIs*

The prevalence of the different OWIs varied significantly depending on the life stage of the individuals (Figure 2.2). For instance, the prevalence of external body damage was higher in lumpfish in sea cages (45.7%) than in lumpfish in hatcheries (1.67%; z-test for equality of proportions with continuity correction, $\chi^2$=26.27, df=1, $p$<0.001), and the same occurred with eye damage, being more notorious in sea cages (22.9%) than in hatcheries (6.67%, $\chi^2$=3.89, df=1, $p$=0.048). In general, lumpfish in hatcheries were mostly affected by fin damage, in particular the ventral section of the pectoral fins (53.3%) and the caudal fin (51.7%), and by suction disc deformities (range: 36.7-58.3%). These differences between stages of development were confirmed by Principal Component Analysis. The two first Principal Components (PCs) of the PCA revealed eigenvalues higher than 1 (PC1=1.938 and PC2=1.037), being used as a cut-off point for which PCs were retained. PC1, which accounted for 38.8% of the variability in the data, was mainly associated with suction disc deformities (-0.78), eye darkening (0.67) and external body damage (0.64); while PC2 (20.7%) captured variation in eye condition (-0.81) and fin damage (-0.46). The relationship between all the OWIs and the contribution (contrib.) of the representation is showed in Figure 2.3 (A), along with the PCA biplot with variables and individuals, highlighting the different stages of development (B).

*Variation of body condition and incidence of underweight/emaciated fish*

The length-weight relationships of farmed lumpfish varied significantly between life stages (Table 2.1). Lumpfish growth was shown to be positively allometric ($b$>3) in hatcheries (at pre-deployment stage, S1-S3) and negatively allometric in sea cages (at post-deployment stage, S4), suggesting that lumpfish get fatter as they grow in the hatcheries, but they become progressively thinner over time once they have been deployed in sea cages. The length-weight relationship was assessed based on one (Ws1, all stages) and four equations (Ws4, four stages) and found that a single length-weight regression would overestimate relative weight in hatcheries and underestimate it in sea cages (mean absolute error of 5.2%).

The frequency of underweight (weights between 10-25% below their expected) and emaciated (weight below 25% expected) lumpfish differed significantly between life stages ($\chi^2$=73.86, df=1, p<0.001). In general, most of the lumpfish showed normal weights for each life stage (average 74%), but the proportion of lumpfish with poor body condition -both underweight and emaciated- was higher in life stage 1 (larvae 0-1g, 16 and 18.4%, respectively), which was the most variable stage (Figure 2.4). Overall, 28% of the 2658 sampled lumpfish had lower than normal weights for their respective length and 10% were emaciated.

*Variation in plasma cortisol*

Mean values of plasma cortisol varied significantly between life stages (Welch two sample t-test, t=6.56, df=35.98, $p$<0.001), being higher on lumpfish sampled in salmon sea cages (mean=84.70 ng/ml ± 10.99 SE) than juvenile lumpfish sampled in hatcheries (mean=11.61 ng/ml ± 1.88 SE, Figure 2.5). Cortisol values were also significantly more variable post-deployment (CV=76%) than pre-deployment (CV=72.6%; Fligner-Killeen test of homogeneity of variances, $\chi^2$=21.84, df=1, $p$<0.001). The highest cortisol value obtained was 261.71 ng/ml.

## Validation of OWIs

*Reliability*

Reliability of the OWIs was measured through repeatability of the scores. All of the selected individual-based OWIs showed good (ICC=0.75-0.90) or excellent (ICC>0.90) repeatability (Table 2.5), although eye condition was highly repeatable when measured by different raters (ICC=1.0, 95% CI=1.00-1.00, $p<0.001$), but not as significant as when assessed by the same rater at two different times (ICC=0.60, 95% CI=0.46-0.72, $p<0.001$). The OWI with the highest reliability (ICC=0.85 and ICC=0.94) for intra- and inter-rater repeatability values, respectively, was suction disc deformities.

*Construct validity*

Relative weight and plasma cortisol values differed significantly between pre- and post-deployment stages ($p<0.001$), so they were both assessed separately. PC1, PC2 and their interaction with total length, were significant predictors of relative weight during pre-deployment stages ($WR_{pre}$ ~ PC1 x PC2 x TL; $F_{5,54}=6.6$, $R^2_{adj}=0.32$, $p<0.001$), but relative weight was only dependent on total length during post-deployment ($WR_{post}$ ~ PC1; $F_{1,32}=6.9$, $R^2_{adj}=0.13$, $p=0.01$, Appendix, Table S2.3). In contrast, PC1 was a significant predictor of plasma cortisol during pre-deployment ($Cortisol_{pre}$ ~ PC1; $F_{1,17}=9.66$, $R^2_{adj}=0.31$, $p<0.01$), while plasma cortisol was dependent on PC2 and the interaction between PC2 and total length at post-deployment ($Cortisol_{post}$ ~ PC2 x TL; $F_{3,31}=4.58$, $R^2_{adj}=0.24$, $p<0.01$; Appendix, Table S2.4).

## Refinement and Simplification

The caudal was the most affected fin, with 47% of lumpfish showing mild to severe fin damage. It was also the easiest one to score when handling the fish and the one that showed the highest variation between individuals, with scores ranging from 0 to 4. The ventral section of the pectoral fins was also highly variable, but it was less affected (43%) than the caudal fin. Damage of the dorsal and anal fins was positively correlated (Spearman's $\rho=0.3017$, $p<0.01$), as well as damage on the anal fin and the ventral section of the pectoral fins (Spearman's $\rho=0.2222$, $p<0.05$), both positioned in the

ventral part of the longitudinal axis of the lumpfish. Scores of the ventral section of the pectoral fin and deformity of the suction disc were also positively correlated (Spearman's $\rho$=0.5309, $p$<0.01), suggesting that deformities or curling in the ventral section of the pectoral fin contributed significantly to the deformities in the suction cup. Most of the suction cup conditions were positively correlated, for instance, with papillae development (Spearman's $\rho$=0.786, p<0.001), highlighting redundancy and providing support to the use of a more simplified and practical Welfare Score Index (Table 2.3).

**Pilot trial: LOWSI testing and application**

The refined Lumpfish Operational Welfare Score Index, tested on 245 lumpfish across six commercial sites, showed a high repeatability (ICC=0.826, 0.767-0.871, $p$<0.001). Consensus amongst farmers who used the LOWSI to test its performance was high (Appendix, Figure S2.8) with 75% of them not deviating from the average response. The results of the questionnaire given to eight farmers supported that LOWSI is (1) practical to use, (2) quick, as each fish can be scored in less than 2 minutes, (3) easy to implement, and (4) simple enough that farm managers would be willing to train their staff to use it (Appendix, Table S2.2). Overall, 71% of the lumpfish were classified as class A (good welfare, LOWSI <3 points), 27% as class B (moderately compromised welfare, LOWSI=3-5 points) and 2% as class C (severely compromised welfare, LOWSI > 5 points), but the proportion of welfare classes varied significantly between the different sampled sites (6-sample for equality of proportions without continuity correction, $\chi^2$=44.69, df=5, $p$<0.001; Figure 2.6). The average value for LOWSI across all the sites was 1.9 and ranged from 0.82 (Farm 3) to 3.37 (Farm 1), indicating that the welfare of lumpfish at Farm 1 was significantly poorer than the rest of sites, with 57% of lumpfish classified as class B and 11% as class C. Poor welfare was mainly associated with fin damage and suction disc deformities in hatcheries and poor growth and eye damage in sea cages.

## Discussion

The salmon farming industry is nowadays under the spotlight for not maintaining an adequate lumpfish welfare in sea cages, and for allowing unacceptable high mortalities of these species at post-deployment (Imsland et al., 2016a, Compassion in World Farming, 2018, Stranden, 2020); which has instigated some pressure groups to discourage the use of cleaner fish until mortalities are addressed and their welfare is guaranteed (Marine Conservation Society, 2018, OneKind, 2018). Recently, the Norwegian Food Safety Authority has informed salmon farms to stop using cleaner fish until their welfare standards are met (Anon, 2020c), whose development has been flagged as a priority for lumpfish (FAWC, 2014, OneKind, 2018, Noble et al., 2019). With no agreed welfare standards for lumpfish, mortality might be the only proxy of poor welfare and by then is too late to take any corrective action (Stranden, 2020). Although several parameters have been proposed as welfare indicators for lumpfish (Noble et al., 2019, Imsland et al., 2020, Eliasen et al., 2020), some of them require sacrificial sampling or are just not easily applicable and can be subjective and time consuming. Therefore, a fast and simple scoring system would result more practical for the industry. A repeatable and practical Lumpfish Operational Welfare Score Index (LOWSI) has been developed, which is simple and practical to use, and suitable for routine assessment under farm conditions (Table 2.3). The application of this index at six commercial sites indicated that, although the welfare of lumpfish was generally good (71%), it was moderately compromised in 27% of the cases and undoubtedly poor in the remaining 2%. However, there was a significant variation in welfare scores between sites (with a difference of 4x), indicating that some farms are achieving high welfare standards, while others still not.

The high incidence of underweight lumpfish at all stages of development was a critical finding in this study. While 28% of the lumpfish had lower than expected weights for their length, 10% were emaciated, which indicates ethical concerns. Previous authors reported that up to 30% of lumpfish had empty stomachs in sea cages (Eliasen et al., 2018) and only 13 to 38% actually ate sea lice (Imsland et al., 2014a, Imsland et al., 2015a, Imsland et al., 2016a, Eliasen et al., 2018), indicating that lumpfish are susceptible species to suffer from malnutrition due to starvation. Also, sea lice are considered as ‘snacks’ for lumpfish and they are not highly nutritional to be their only

food source (Merakerås, 2020). The use of appropriate diets and feeding methods for supplementary feeding at sea cages (Imsland et al., 2019f) is key to reduce the risk of emaciation, but also to reduce stress and excessive energy expenditure. Thus, percentile length-weight charts (Appendix, Figure S2.6) were purposely created for fish farmers not only to monitor growth but also to control the incidence of underweight/emaciated lumpfish and take remedial actions before it becomes a real problem. Estimations of length-weight relationship in fish have been widely used by fisheries researchers and managers as an essential biological parameter, to predict biomass of a sample or estimate weight-at-age for fish distribution, but more importantly, as a practical index to assess fish condition (Le Cren, 1951, Mokhtar et al., 2015). Lumpfish possess an unusual body shape and their Fulton's condition index (K) is higher than in any other teleost (Imsland et al., 2014a, Brooker et al., 2018), which indicates that might not be representative enough. For this reason, the calculation of relative weight (Wr), which compensates for inherent changes in body form (Blackwell et al., 2010), was used as an estimation of lumpfish condition. Relative weight, which was selected as one of the indicators included in the LOWSI, is a useful index for monitoring health and welfare simultaneously (Blackwell et al., 2010, Kumolu-Johnson and Ndimele, 2010).

Four welfare conditions that affected lumpfish in a different manner depending on their stage of development were identified: suction disc deformities and fin damage were the most important determinants of lumpfish welfare in hatcheries, while body damage and eye damage were more significant in sea cages. The prevalence of suction disc deformities was relatively high (37-58%) and greater in hatcheries than in sea cages, probably due to the quality assessment carried out by lumpfish producers before sending lumpfish to salmon farms. Suction disc deformities have been reported in 65% of juvenile lumpfish in a rearing facility (Treasurer et al., 2018b), whose causes are not clear but could possibly be related to nutrition, environment and/or genetic factors, as it has been suggested in other type of deformities in different farmed species (Berillis, 2015). Hence, malformations of the suction disc in lumpfish might be exacerbated by high temperatures at incubation (Imsland et al., 2019b), poor nutritional status (Kousoulaki et al., 2018) and inbreeding depression of lumpfish populations (Whittaker et al., 2018). Independently of the causes, deformities are known to negatively impact the welfare and performance of farmed fish (Noble et al.,

2012) and they can represent a particular problem for lumpfish because they can affect their ability to swim or move (Davenport and Thorsteinsson, 1990), to rest (Imsland et al., 2015a, Imsland et al., 2018a, Johannesen et al., 2018, Leclercq et al., 2018) and possibly to cope with stress by clinging (Hvas et al., 2018) due to the lack of Mauthner cells, involved in the fast scape response (Hale, 2000).

Fin damage was also found as another common welfare problem in lumpfish in hatcheries, with 62% of the juveniles showing caudal fin damage across three different hatcheries (range: 50-93%), which is in accordance with the 69-87% prevalence that other authors (Johannesen et al., 2018) have reported. Most of the health challenges affecting lumpfish are related to stress (Brooker et al., 2018, Powell et al., 2018b), and secondary bacterial and fungal infections will be exacerbated by fin damage, so any actions that reduce the stress will also likely improve welfare and survival. In this sense, the combination of automatic and manual feeding can reduce the stress of competition for the feeding hotspot areas (Johannesen et al., 2018) and grading frequently in hatcheries may reduce fin nipping or cannibalism (European Union Reference Laboratory of Fish Diseases, 2016). Lumpfish are relatively sedentary beyond the spawning season (Powell et al., 2018b) but active swimming is required for delousing the salmon in sea cages (Imsland et al., 2015b, Imsland et al., 2016a, Leclercq et al., 2018) which will be compromised by damaged fins. This will also probably increase the energetic cost, promoting poor growth and expanding the risk of emaciation.

Eye damage and poor eye condition was observed in more than 15% of lumpfish in this study, and it was particularly significant in sea cages, with 26% of lumpfish affected. Lumpfish are visual feeders (Jonassen et al., 2017, Powell et al., 2018a) and any eye conditions such as eye damage, exophthalmia or cataracts will affect their ability to feed, which will increase the risk of emaciation. Eye cataracts, which are rare in wild lumpfish, are relatively common in lumpfish in captivity (20-100%) (Jonassen et al., 2017, Imsland et al., 2018d) and may be indicative of malnutrition (Jonassen et al., 2017) or nutritional deficiencies (Imsland et al., 2018d). However, overfeeding can also be a cause of cataracts (Imsland et al., 2019b). Cataracts were detected in 17% of lumpfish in sea cages (only 5% in lumpfish in hatcheries), which shows that eye

condition seems to deteriorate with time and highlights the need of appropriate diets also at post-deployment stage.

Plasma cortisol levels in fish increase by activation of the hypothalamic-pituitary-interrenal (HPI) axis after exposure to a stressor (Faught et al., 2016); however, different fish species have different cortisol tolerance. It seems that lumpfish are species with low cortisol response levels to acute stressors as authors reported cortisol basal levels in plasma of 10ng/ml before the stressor and 40-50 ng/ml after the stressor (Jørgensen et al., 2017). Other authors (Treasurer et al., 2018b) stated that, after 30 minutes crowding, lumpfish only showed a moderate increase in plasma cortisol (circa ~200nM) compared to Atlantic salmon *Salmo salar* L. and ballan wrasse *Labrus bergylta*, which had levels of 700nM and 600nM, respectively. Measurements of cortisol in plasma in this study provided some insights into the stress that lumpfish experience when cohabiting with salmon in sea cages. Cortisol levels in plasma were higher in lumpfish sampled from sea cages than in lumpfish sampled from hatcheries (means: 84.70 ng/ml ± 10.99 and 11.61 ng/ml ± 1.88, respectively), which were already higher than those reported for stressed lumpfish (36-63 ng/ml) in other studies (Iversen et al., 2015, Jørgensen et al., 2017, Hvas et al., 2018, Hvas and Oppedal, 2019, Staven et al., 2019). Though, the cortisol levels obtained in plasma for lumpfishin hatcheries were comparable to levels observed in non-stressed lumpfish (5.6-16 ng/ml) by other authors (Jørgensen et al., 2017).

A welfare index for farmed lumpfish, consisting in the visual assessment of five operational welfare indicators: skin damage, caudal fin damage, eye condition, suction disc deformities and body condition was developed, validated and tested at six commercial sites. The results show that one third of the lumpfish sampled had a compromised welfare and undoubtedly poor in 2% of the cases. A high prevalence of emaciated lumpfish, easy to monitor with the length-weight percentile charts, was also found at most of the stages of development. This practical index was aimed to be used by the aquaculture industry (mostly lumpfish producers and salmon farmers) but can also be helpful for health and quality personnel (biologists, veterinarians), policy developers, NGOs and welfare scheme assessors.

## Acknowledgements

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**Table 2.1**. Questionnaire given to 53 participants at the 1st Symposium on Welfare in Aquaculture, held on the 14th of May 2019 at Swansea.

| Question 1 - How useful are the following indicators for lumpfish welfare? | *Not useful* | *Rarely useful* | *Moderately useful* | *Useful* | *Very useful* |
|---|---|---|---|---|---|
| Fin damage | ○ | ○ | ○ | ○ | ○ |
| Skin damage | ○ | ○ | ○ | ○ | ○ |
| Mortality | ○ | ○ | ○ | ○ | ○ |
| Disease/Parasites | ○ | ○ | ○ | ○ | ○ |
| Eye damage | ○ | ○ | ○ | ○ | ○ |
| Suction disc deformities | ○ | ○ | ○ | ○ | ○ |
| Operculum/gill cover damage | ○ | ○ | ○ | ○ | ○ |
| Body deformities | ○ | ○ | ○ | ○ | ○ |
| Condition factor/Relative size | ○ | ○ | ○ | ○ | ○ |
| Poor growth | ○ | ○ | ○ | ○ | ○ |
| Body/eye darkening | ○ | ○ | ○ | ○ | ○ |
| Blood parameters | ○ | ○ | ○ | ○ | ○ |

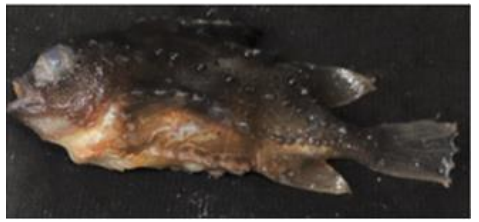
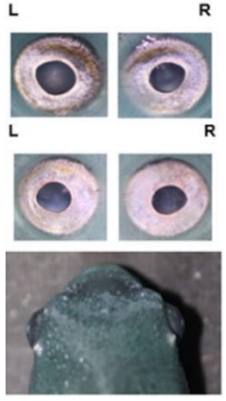
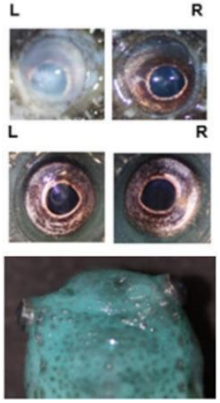
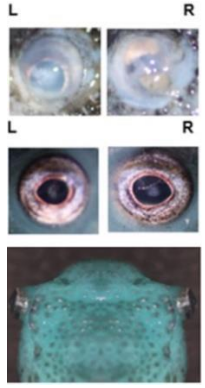
**Table 2.1**. (continuation)

| Question 2 – From the list below, tick the indicators that are used in your facility/business to assess the welfare of lumpfish. | |
|---|---|
| Fin damage | |
| Skin damage | |
| Mortality | |
| Disease/Parasites | |
| Eye damage | |
| Suction disc deformities | |
| Operculum/gill cover damage | |
| Body deformities | |
| Condition factor/Relative size | |
| Poor growth | |
| Body/eye darkening | |
| Blood parameters | |
| Others: | |

**Table 2.2**. Length-weight regression (LWR) coefficients for farmed lumpfish sampled at different life stages (S1-S4), where Ws = standard weight (g) and TL = total length (mm).

| Life stage | n | Intercept (a) | Slope (b) | Ws equation | Adjusted $R^2$ | *p* |
|---|---|---|---|---|---|---|
| S1. Larvae (0-1g) | 948 | -5.023 | 3.532 | $Ws = 10^{-5.02} \cdot TL^{3.53}$ | 0.903 | <0.001 |
| S2. Pre-deployment (1-10g) | 126 | -4.301 | 2.926 | $Ws=10^{-4.301} \cdot TL^{2.93}$ | 0.950 | <0.001 |
| S3. Pre-deployment (>10g) | 1229 | -4.737 | 3.181 | $Ws=10^{-4.74} \cdot TL^{3.18}$ | 0.970 | <0.001 |
| S4. Post-deployment | 355 | -3.516 | 2.559 | $Ws=10^{-3.52} \cdot TL^{2.56}$ | 0.847 | <0.001 |
| All stages | 2658 | -4.692 | 3.157 | $Ws=10^{-4.69} \cdot TL^{3.16}$ | 0.996 | <0.001 |

**Table 2.3**. Rapid visual scoring of the Lumpfish Operational Welfare Score Index (LOWSI).

| **OWI** | **Score 0** | **Score 1** | **Score 2** |
|---|---|---|---|
| *Skin damage* |  No skin damage present |  Moderate and localised skin damage |  Severe skin damage (abrasions/injuries) |
| *Caudal fin damage* |  No erosion, splitting or damage |  Moderate erosion, splitting or damage |  Severe erosion, splitting or damage |
| *Eye condition* |  |  |  |

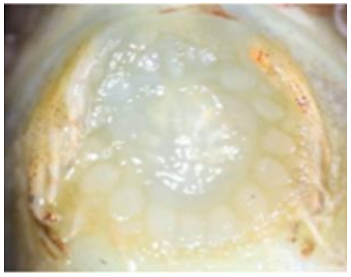
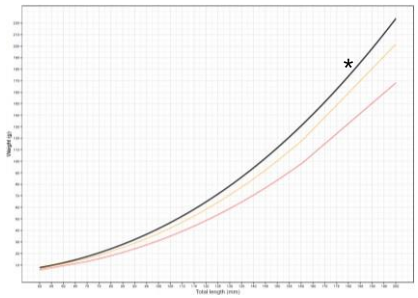
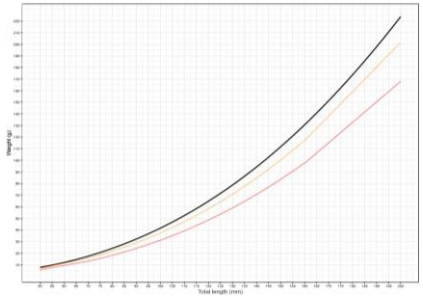
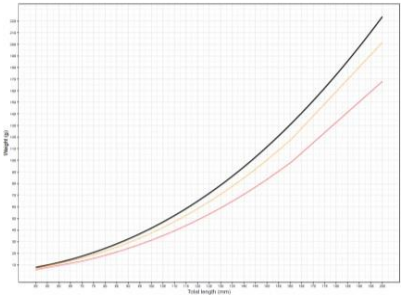
| | No damage, cataracts or exophthalmia | Unilateral eye damage, cataracts or exophthalmia (only one eye affected) | Bilateral eye damage, cataracts or exophthalmia (both eyes affected) |
|---|---|---|---|
| *Suction disc deformity* |  <br>Suction disc is fully functional, and no deformities are present |  <br>Some impairment is present in the suction disc |  <br>Suction disc is severely deformed and non-functional |
| *Relative weight* |  <br>Normal<br>Weight expected for its size<br>(Wr>90%) |  <br>Underweight<br>10-25% below expected weight<br>(Wr=75-90%) |  <br>Emaciated<br>25% or more below expected weight<br>(Wr<75%) |
| **LOWSI** | **<3 points**<br>Good welfare | **3-5 points**<br>Moderately compromised welfare | **>5 points**<br>Severely compromised welfare |
| ACTION | No action needed | Increase the frequency of welfare assessment and ensure the environment and food sources are adequate | Immediate corrective action (consultation with veterinary services) |

**Table 2.4**. Questionnaire given to fish farmers (n=8) to test the practicality of the LOWSI.

**Name:**_____ **Job position:** _____ **Site:** _____

| | | | | |
|---|---|---|---|---|
| Question 1 | I believe this Welfare Score Index is practical for my weekly/monthly farm routine | | | |
| *Strongly disagree* | *Disagree* | *Neutral* | *Agree* | *Strongly agree* |
| Question 2 | I believe this Welfare Score Index can be done in a significantly short period of time (less than 2 minutes/fish) | | | |
| *Strongly disagree* | *Disagree* | *Neutral* | *Agree* | *Strongly agree* |
| Question 3 | I believe this Welfare Score Index is easy to do and implement in the farm | | | |
| *Strongly disagree* | *Disagree* | *Neutral* | *Agree* | *Strongly agree* |
| Question 4 | I would train my staff to implement this Welfare Score Index in my farm | | | |
| *Strongly disagree* | *Disagree* | *Neutral* | *Agree* | *Strongly agree* |

**Table 2.5**. Repeatability of different operational welfare indicators (OWIs) and products from the PCA (PC1, PC2) based on the inter- and intra-class correlation coefficients (ICC) of two raters (R1 and R2) that randomly scored the lumpfish.

| OWIs | n | Inter-rater repeatability (R1 vs R2) | | | Intra-rater repeatability (R1, $t_1$ vs $t_2$) | | |
|---|---|---|---|---|---|---|---|
| | | ICC | 95 % CI | *p* | ICC | 95% CI | *p* |
| External damage | 95 | 0.83 | 0.76-0.88 | <0.001 | 0.82 | 0.74-0.88 | <0.001 |
| Fin damage | 95 | 0.93 | 0.90-0.96 | <0.001 | 0.79 | 0.68-0.86 | <0.001 |
| Eye darkening | 95 | 0.85 | 0.78-0.90 | <0.001 | 0.83 | 0.76-0.89 | <0.001 |
| Eye condition | 95 | 1.00 | 1.00-1.00 | <0.001 | 0.60 | 0.46-0.72 | <0.001 |
| Suction disc deformity | 95 | 0.94 | 0.91-0.96 | <0.001 | 0.85 | 0.78-0.91 | <0.001 |
| PC1 | 86 | 0.87 | 0.81-0.91 | <0.001 | 0.79 | 0.70-0.86 | <0.001 |
| PC2 | 86 | 0.86 | 0.80-0.91 | <0.001 | 0.44 | 0.26-0.60 | <0.001 |

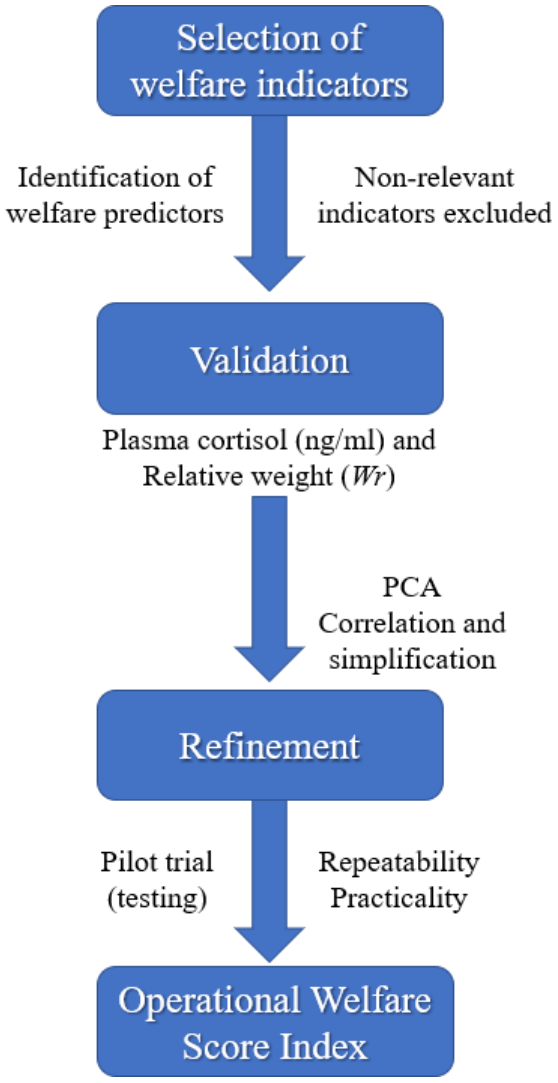



**Figure 2.1**. Flowchart used to develop an operational welfare score index for farmed lumpfish.

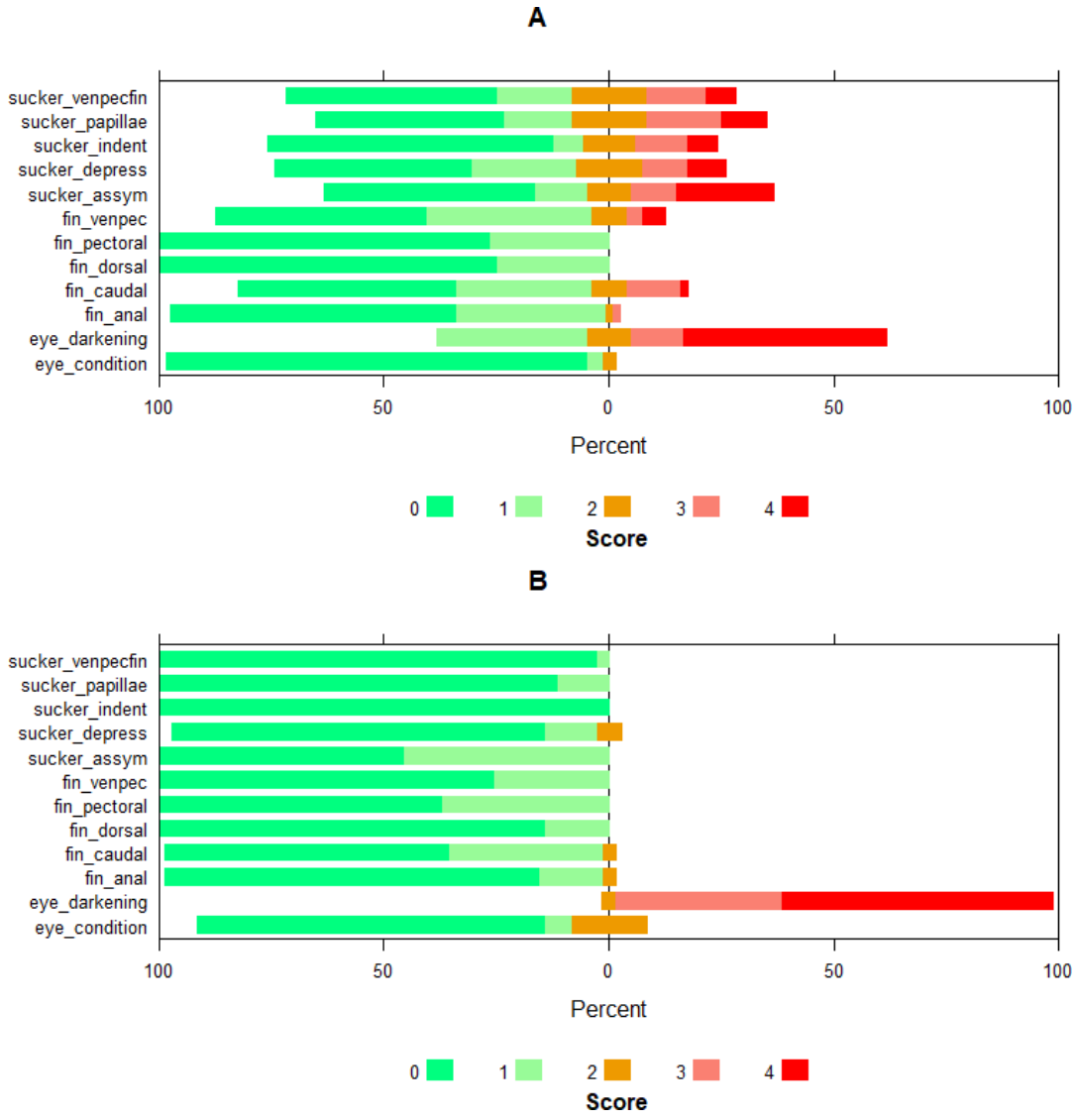



**Figure 2.2**. Diverging stacked bar plot showing the variation in operational welfare indicators (OWIs) in lumpfish sampled from hatcheries (A, n=60) and sea cages (B, n=35).

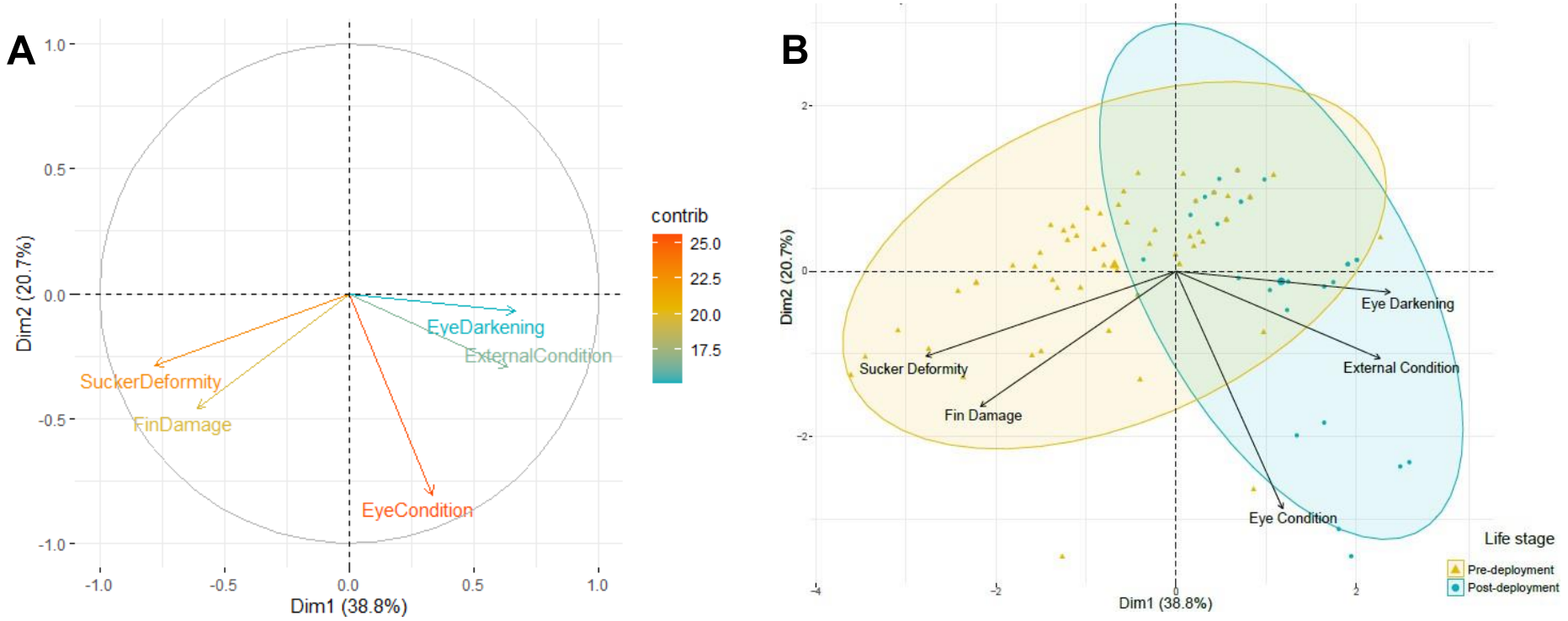



**Figure 2.3**. PCA plot with variables and their contribution (A) and PCA biplot showing variables and individuals (B), separated by stage of development: pre-deployment or hatchery stage (yellow triangles) and post-deployment or sea cage stage (blue circles).

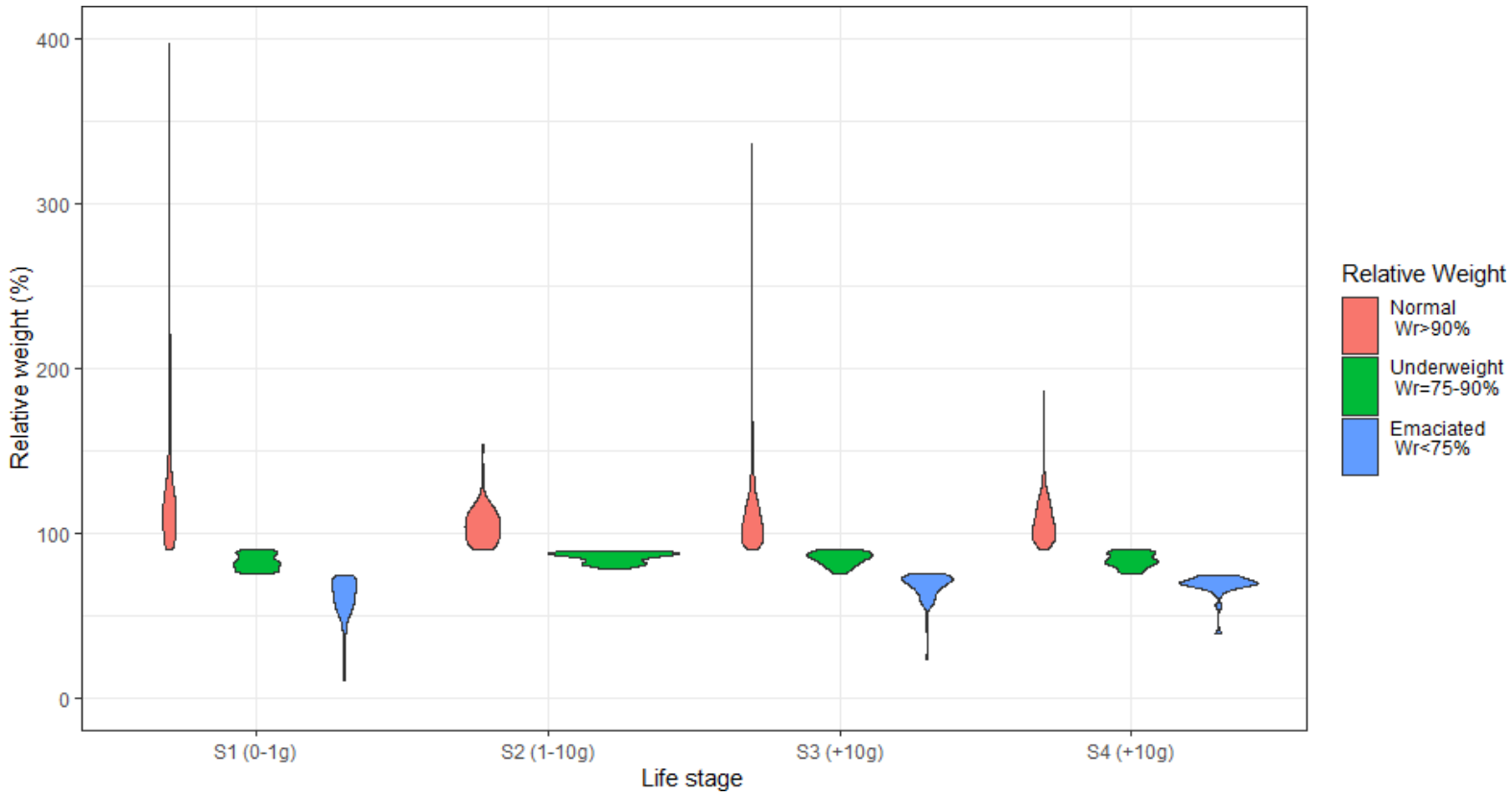



**Figure 2.4**. Variation of body condition (relative weight, %) by stage of development. Lumpfish with normal body condition (Wr>90%) are shown in pink, while underweight (Wr=75-90%) and emaciated (Wr<75%) lumpfish are shown in green and blue, respectively.

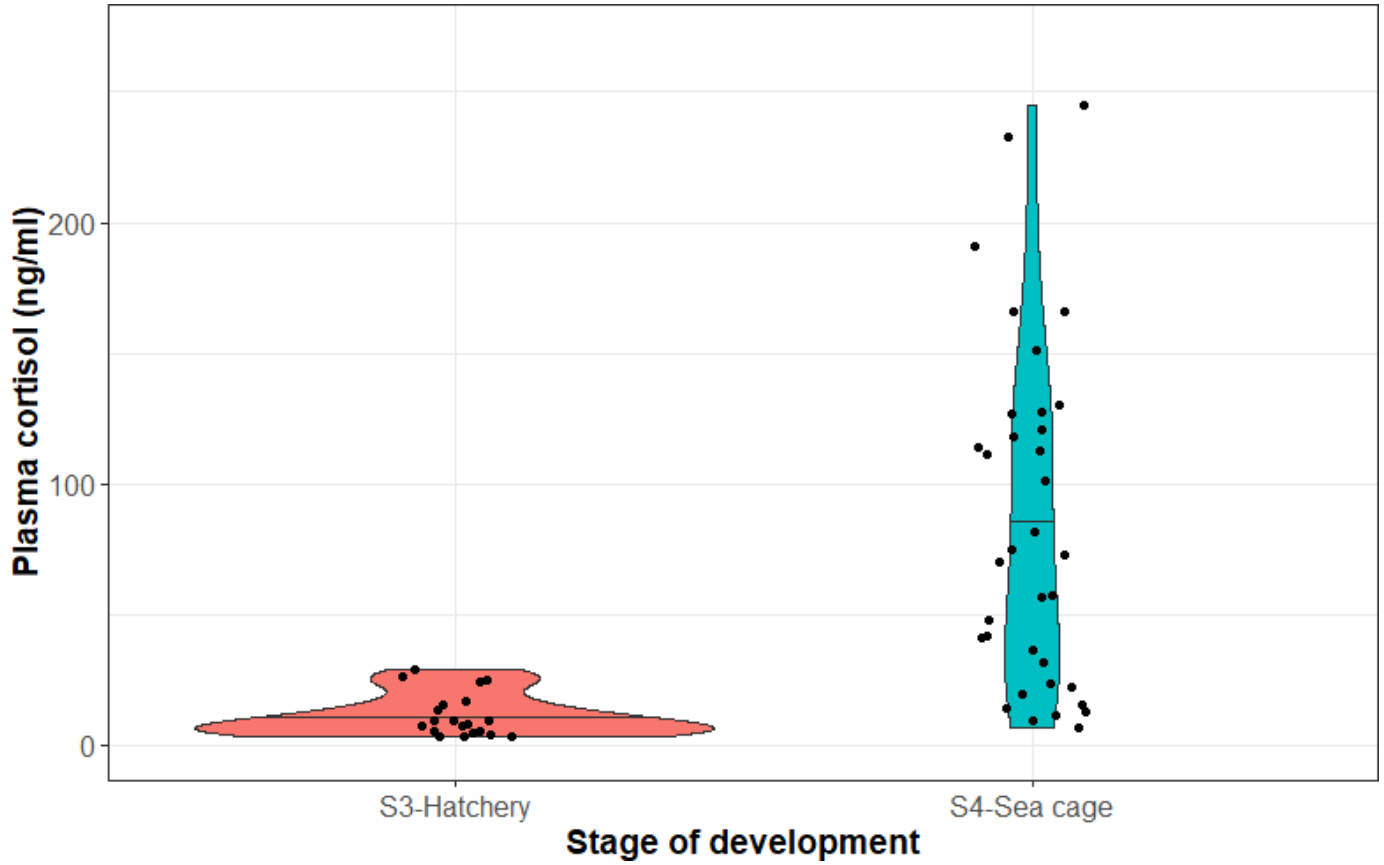



**Figure 2.5**. Variation in plasma cortisol (ng/ml) of lumpfish (n=55) sampled from two hatcheries (S3 – Pre-deployment) and one sea farm (S4 – Post-deployment).

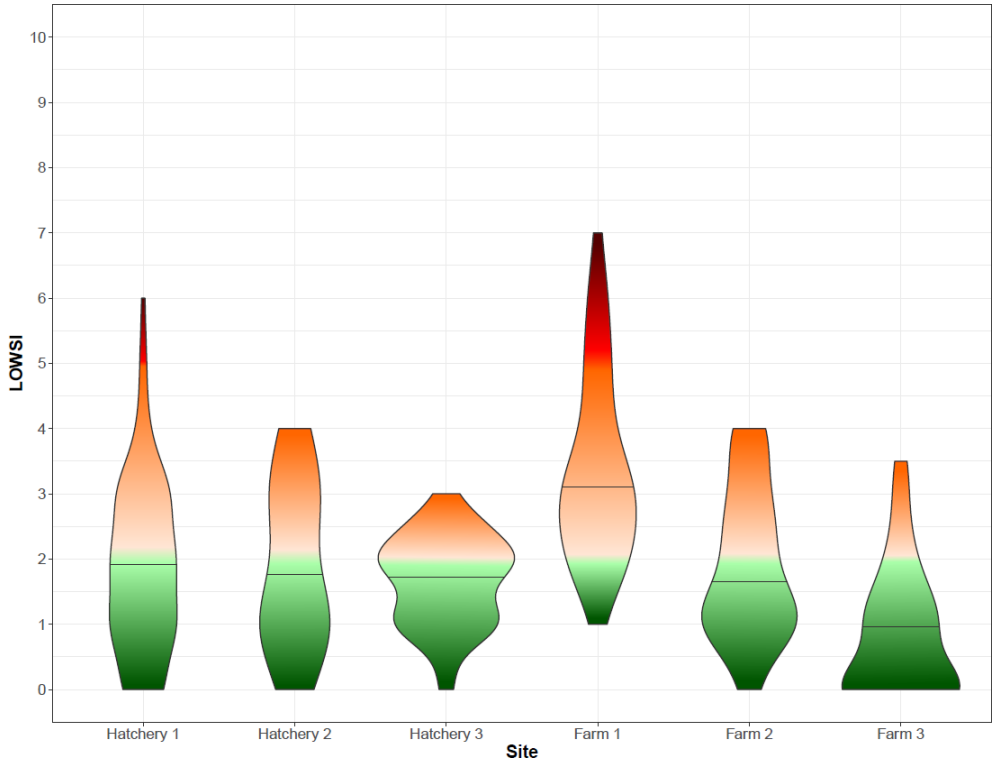



**Figure 2.6**. Variation in the Lumpfish Operational Welfare Score Index (LOWSI) pilot trial at six commercial sites (n=245 lumpfish): three hatcheries and three sea farms. The proportion of the lumpfish belonging to each of the welfare classes are represented by colour degradation in the violin plots: green for Class A (Good welfare), orange for Class B (Moderately compromised welfare) and red for Class C (Severely compromised welfare).

# Chapter 3 Changes in growth and welfare of lumpfish *Cyclopterus lumpus* L. over time in a longitudinal study

**Abstract**

The use of lumpfish (*Cyclopterus lumpus* L.) as cleaner fish is essential for the salmon industry, but lumpfish welfare can become compromised by cohabiting with salmon. A longitudinal study approach was used to investigate changes in size, welfare scores and feeding preferences of lumpfish over time. Mixed lumpfish populations from two different origins (Norwegian and English) and similar size were stocked at 10% of salmon density in quadruplicate (four cages), along with Atlantic salmon (salmon average weight ± SD: 165.13g ± 1.44). Lumpfish were randomly sampled twice from the surface of the pen, at three weeks and at three months post-deployment. English lumpfish had higher average weights and lengths three months after deployment, suggesting they grew faster than Norwegian lumpfish.Welfare scores did not differ between stocks but worsened with time spent at sea andwere worse for small fish. All measured Operational Welfare Indicators (OWIs) worsened except for caudal fin damage, which did not change significantly over time.The prevalence of ingested sea lice appeared to increase over time, but it was generallyvery low and also reflected the low infection levels of the farm. The prevalence of formulated pellets in stomachs was seen to increase over time, but it did not appear tobe related to welfare. Better understanding on how lumpfish welfare changes at sea over time can be valuable for the industry to identify critical periods and increase monitoring or take actions conveniently.

## Introduction

Lumpfish (*Cyclopterus lumpus* L.) is an economically important species used to control sea lice infestations in salmon aquaculture. Although lumpfish production may have reached a plateau in some areas in Norway (Berge, 2019), lumpfish worldwide production is currently over 39M annually (Norwegian Directorate of Fisheries,2021a, Anon, 2020b, Bolton-Warberg, 2018). This is not too far from the predicted 50M lumpfish needed to supply salmon farms in 2020 (Powell et al., 2018b), indicating the demand of lumpfish for salmon aquaculture will continue rising as partof an integrated cost-effective sea lice mitigation strategy. Salmon farmers stock and top-up their farms with lumpfish from different origins, including local and non-localstocks, based on season and availability. Logistically it is very challenging to rely on one single stock origin because lumpfish production still depends on wild broodstock.Also, lumpfish cannot be stocked at sea during the warmest months as it is detrimentalfor their health and welfare due to their low thermal range (Hvas et al., 2018).

The distribution of lumpfish covers a large area along both sides of the North Atlantic ocean (Davenport, 1985, Powell et al., 2018b). Mature adults show homing behaviour and return to the same areas to spawn (Kennedy et al., 2015), and larvae have limited dispersal as they attach to the substrate not long after hatching (Davenport, 1985); attributes that make stock differentiation strong. A recent study found five genetically different populations of lumpfish (West, Mid and East Atlantic, English Channel and Baltic Sea) with some substantial phenotypic variation in growth, condition and maturation between North Atlantic and Baltic regions (Whittaker et al., 2018). Also, differences have been reported in delousing ability, growth and disease resistance between lumpfish families, within the same stock origin, suggesting that variability could be explained genetically (Imsland et al., 2016a, Imsland et al., 2021), and possibly certain genotypes may be more suitable than others to be used as cleaner fish in salmon cages.

The commercial use of lumpfish in salmon farms is at present under scrutiny due to sea lice grazing variation, low survival rates and poor welfare, which appear to be important bottlenecks to achieve sustainability in the lumpfish industry. Reported lumpfish delousing efficacies are highly variable, ranging from 9 to 60%, although efficiency can be as high as 97% for adult female sea lice (Overton et al., 2020) in

land or small-scale trials. Only one study was performed at a large scale under real commercial conditions, showing lumpfish sea lice removal efficacies of 53-73% (Imsland et al., 2018a). About 13 to 38% of lumpfish are generally found with ingested sea lice in their stomachs (Imsland et al., 2015a, Imsland et al., 2018a, Eliasen et al., 2018). However, lumpfish are opportunistic feeders foraging on a wide variety of prey and seem to adapt to whatever food source is most readily accessible. They modify their diet according to prey availability, and do not rely on a single food source if others are present. Crustaceans have been found to make up to 91% of the diet of the juvenile lumpfish under natural conditions (Ingólfsson and Kristjánsson, 2002), but in sea cages lumpfish can consume crustaceans, hydrozoans, bivalves and fragments of macroalgae, among other sources (Imsland et al., 2015a). It is also very common to find a high proportion of lumpfish (61-96%) in salmon cages with formulated feed pellets in their stomachs (Imsland et al., 2014a, Imsland et al., 2015a, Imsland et al., 2016a).

Lumpfish mortalities in sea cages are an indicator of poor health and welfare (Brooker et al., 2018); hence maintaining good welfare in lumpfish is essential not only to promote cleaning behaviour, but also to reduce stress and improve survival. Although the welfare status of lumpfish appears to generally improve in small-scale trials after four months (Geitung et al., 2020), there are indications that lumpfish welfare deteriorates in commercial conditions and can be severely compromised in some commercial salmon farms (Gutierrez Rabadan et al., 2021), particularly not long after deployment at sea (Lopes, 2021). However, it has not been determined exactly at which point after sea deployment this happens or how other physiological changes over time affect lumpfish welfare. It is imperative that lumpfish health and welfare is continuously monitored to identify critical periods that can lead to detrimental welfare and take actions by correcting the husbandry at sea.

The aims of the present study were first to assess whether there were changes in size, welfare scores and feeding preferences over time by sampling lumpfish at two different time points under commercial conditions. Secondly, microsatellites markers and parental assignment were used to identify the origin where lumpfish originated from and differences between the different genetic stocks were investigated.

## Materials and methods

*Experimental fish*

The lumpfish used for this study originated from Norway and Dorset (South Coast of England) wild stocks. Norwegian lumpfish, belonging to a single family (same parentage), were shippedas eggs to hatchery 1 (Lat: 56° 1' 39.684" N, Long: 5° 18' 1.476" W) and reared in a flow-through system. English lumpfish juveniles, belonging to three different families(Eng-1, Eng-2 and Eng-4), were reared in a RAS hatchery 2 (Lat: 51° 36' 29.8" N, Long: 3° 58' 50.4" W), until all achieved deployment size. Three different males and three different females were used for each of the English family crosses, while Norwegian lumpfish originated from a cross between a single male and a single female.

*Experimental set up*

A similar proportion of mixed lumpfish from Norwegian and English origins (2754 and 2256,respectively) were stocked on the 19/10/2018 in quadruplicate in four 100m circular (10mnet depth) sea cages (n=20040; 11016 Norwegian and 9024 English) at a commercialsalmon farm in Scotland (Lat: 57° 32' 31.992" N, Long: 5° 42' 40.392" W). The studylasted 84 days, and sea water temperature decreased from 12.7°C in October to 9.6°Con 11/01/2019, where the study finished. Lumpfish were deployed at a stocking rate of 10% salmon density per cage and similar average weights (Norwegian: 30.2g, English1: 28.4g, English2: 29.3g, English4: 20.7g). The three different families withinthe English stock had the same representation on each cage (n=752/family). Other information is summarised in Table S3.2 (Appendix).

*Sample collection*

Lumpfish were randomly sampled with hand nets from the surface of the pen twice, at approximately three weeks (early November 2018, T1) and three months (mid-January 2019, T2) post-deployment, to assess changes over time. A total of 160 lumpfish (40 lumpfish per cage) were collected at each sampling point (total n=320). An overdose of tricaine methanesulfonate (MS-222) (Pharmaq, Hampshire, UK) and brain destruction were used to humanely euthanise the fish. For each individual sampled, body weight (g), total length (mm) and welfare scores for body damage, caudal fin damage, eye condition, suction disc deformity and relative weight (Gutierrez

Rabadan et al., 2021) were obtained. Individual stomachs were collected for content analysis and individualfin tissue was collected in 96% ethanol for genetic analysis. Both were stored at -20°C until analysis. Sea lice counts (average count per salmon; Appendix, Figure S3.1) wererecorded on a weekly basis for each of the sampled cages.

*Stomach content analysis*

Partially defrosted stomachs (n=320) were dissected and visually analysed for presence/absence (0: No, 1: Yes) and quantification of formulated pellets and sea lice. Fullness/distension ratio of the stomachs (0: empty, 0.25: partially full, 0.5: half-full, 0.75: moderately full, 1: completely full/bursting) was also individually recorded.

*DNA extraction and amplification*

Dorsal fin clip samples of approximately 5x5mm were obtained from lumpfish offspring of mixed origin (Norwegian/English, n=320). Additionally, fin clips from the wild English broodstock individuals (used as parents and caught with nets from the South Coast of England in March 2018) and 25 offspring (15 from England (English1 x5, English2 x5, English4 x5) and 10 from Norway) were also collected as controls for parents and offspring, respectively. Norwegian parents were not available for genotyping. DNA was extracted using Qiagen DNeasy 96 Tissue Kits (Manchester, UK) following the manufacturer's protocol. DNA concentration was calculated using a Nanodrop 2000 (Thermo Fisher Scientific Inc., USA) and DNA quality was assessed through the A260/A280 ratio. Samples were diluted when needed by adding DNA-free water to ensure all of them had similarly the same concentration.

*Microsatellite and genotype analysis*

Samples were sent to an external laboratory for analysis. In total, 30 genomic (g-STRs) and 30 expressed sequence tag (EST-STRs) candidate tetranucleotide microsatellites, evenly distributed between linkage groups, were identified from already published sequences (Maduna et al., 2020) and primers designed using Primer3 software (Untergasser et al., 2012). A subset of 25 yielding strongly amplifying clean amplicons were initially screened using M13 tailed primers (Blacket et al., 2012), resolved via an Applied Biosystems 3730XL DNA Analyser (Thermo Fisher Scientific, UK) and 10 polymorphic candidates suitable for multiplexing were identified. This new

multiplex *M1-10mix*, along with two existent multiplex panels, *M2-4mix* and *M3-6mix* (Skirnisdottir et al., 2013) were proposed for genotyping the lumpfish offspring. Loci names, sequences, length ranges and dyes are specified in Tables S3.2 and S3.3 (Appendix). After temperature titration and optimisation of primer concentrations, markers were amplified in 10 µl reactions containing 0.1U GeneOn DFS DNA polymerase (Ludwigshafen, Germany) and 1x proprietary multiplex PCR buffer (NoahGene Ltd., Alloa, UK) using a Biometra TRIO thermal cycler with the following profile: 1 min at 96°C followed by 32 cycles of 20s at 94°C, 20s at 58.4°C and 1min at 72°C. Diluted PCR products (1:10) were resolved using an Applied Biosystems 3730XL DNA Analyser including GeneScan 400HD ROX as size standard (Thermo Fisher Scientific, UK). Allele sizes were estimated by assigning the peaks on the electropherograms using both GeneMapper software 5 (Applied BioSystems™) and Genotyper (Applied Biosystems™) and binning allele peak sizes into genotypes following the Global Southern algorithm. Micro-checker v.2.2.3 (Van Oosterhout et al., 2004) was used to detect null allele frequencies, allelic dropouts and also stutter peaks for each microsatellite.

*Parental assignment*

All three genotype panel data were combined together and exported to the exclusion-based software VITASSIGN (Vandeputte et al., 2006), which allowed to identify mismatching loci in the genotypes (crucial for the dinucleotide panels). Only exclusions with not less than 3 loci were considered reliable. Due to the impossibility of fully reconciling di- and trinucleotide loci, trio assignments with one mismatch were allowed. All assignments were compared using CERVUS (Kalinowski et al., 2007) to assess the assignments to single parents and also to check for agreement with a trio delta>0.1. The total assignment achieved was 60%, considering that survival of both stocks would be similar and 50% of the samples would not be assigned as half of the parents (Norwegian origin) were unknown.

*Statistical analysis*

Statistical analysis was performed with R version 4.1.0 (R Core Team, 2021). A Chi-square goodness of fit test was performed to investigate whether the proportion of sampled fish belonging to each origin at time point 1 (T1) was different than what

would be expected from the proportion stocked at deployment, for each of the cages and all together. A two-proportion z-test was employed to assess if proportions of fish belonging to each stock (Eng1, Eng2, Eng4, Norwegian) differed over time between time points T1 and T2. As the sample size for some of the English families at recapture was relatively small (e.g., families 1 and 4), origin (English, Norwegian) instead of stock, was used for the rest of the statistical analyses. Variance in body weight, total length and body condition between time points was assessed using Levene's test. Origin and time (T1: three weeks, T2: three months) were used as predictors of changes in body weight using a linear model (LM) and changes in body size and condition using a linear mixed model (LMM), with cage (n=4) as a random factor. Total length and body condition (as relative weight, Wr%) were log transformed to meet model assumptions. A generalized linear model (GLM) was fitted with Poisson distribution using the lme4 package (Bates et al., 2007), for welfare scores (LOWSI counts) as the dependent variable and origin, time and size (total length) as predictors. The same approach was used for sea lice ingestion (counts) model, which was also tested against a null model with no predictors and also against a Zero-inflated Poisson (ZIP) regression model with the Vuong test (Desmarais and Harden, 2013) using the '*pscl*' package (Jackman et al., 2007). A GLM with quasi-Poisson family was used for pellet ingestion (counts) model due to data overdispersion. Model outputs are reported including estimates ± SE (standard error), statistic test and significance p-values. Models assumptions were assessed through linearity, homogeneity of variances and normal distribution of the residuals using the ggResidPanel package (Goode and Rey, 2019). Then, models were simplified using the *dredge* command in the MuMIn package (Barton, 2020) and selected by the lowest AIC.

## Results

*Relative abundance of lumpfish of different origins*

Norwegian (n=2754) and English (n=2256) lumpfish were deployed in each of the four cages of this study. The population sampled at the first time point (T1, three weeks post-deployment) was constituted by 73% of Norwegian lumpfish (117/160) and 27% of English lumpfish (43/160), which from those 22% (35/160) belonged to family 2, 4% (6/160) to family 1 and 1% (2/160) to family 4. At the second time point (T2, three months post-deployment), the proportion of sampled individuals was very similar (Norwegian: 74% and English: 26% - family 1: 5%, family 2: 19%, family 4: 2%), suggesting that the proportion of fish belonging to each stock did not differ over time (z-test; English1: $\chi^2$=0.08, df=1, $p$=0.8; English2: $\chi^2$=0.31, df=1, $p$=0.6; English4: $\chi^2$=1.00e-32, df=1, $p$=1; Norwegian, $\chi^2$=0.016, df=1, $p$=0.9), with similar representation at three weeks and at three months post-deployment (Table 3.1).

The relative abundance of Norwegian lumpfish (73%) was significantly higher than expected (2754/5010, 55%) while the relative abundance of English lumpfish (27%) was lower than expected (2256/5010, 45%; $\chi^2$=69.98, df=1, $p$<0.001; Appendix, Table S3.1).

*Changes in body size and condition between origins and over time*

Given that weights were all similar at deployment, lumpfish from different origins did not differ in weight at three weeks post-deployment (compared to deployment), but the average weight of English lumpfish (n=41, 84.2g ± 44.4) was higher than the average weight of Norwegian lumpfish (n=119, 53.5g ± 24.4; Table 3.1) at three months after deployment. There was also a significant interaction between time and stock origin, where both stocks increased their average weights between time points, although English lumpfish (t=10.25, df=316, $p$<0.001) gained more weight (increase in average body weight by 51.22g ± 4.99) in comparison to Norwegian lumpfish (-38.63g ± 5.82, t=-6.64, $p$<0.001) over time (Figure 3.1a). Similar changes occurred with the length of lumpfish, but in this case, origins differed in length at both time points (Table 3.2). English lumpfish average length increased over time by 34.81mm ± 5.76 (t=6.05, df=316, $p$<0.001) and were significantly longer (n=84, 125mm ± 25.1)

than Norwegian lumpfish (109.2mm ± 15.5) at three months post-deployment (t=11.55, $p$<0.001; Figure 3.1b). The effect of time spent at sea on total length was influenced by the stock origin, with faster growth in English compared to Norwegian lumpfish (-25.01 ± 3.6, t=-6.92, $p$<0.001). Negative estimate values for Norwegian lumpfish indicate that they grew slower in weight and length in comparison to English lumpfish. Levene's test results indicated unequal variances between time points for body weight ($F_{1,318}$=78.15, $p$<0.001) and total length ($F_{1,318}$=80.48, $p$<0.001), but homogeneity of variances for body condition ($F_{1,318}$=0.97, $p$=0.33).

Body condition differed between stocks (12.89 ± 6.27, t=2.06, $p$=0.04) and was influenced by the interaction between lumpfish origin and time spent at sea, with English lumpfish showing no differences over time and Norwegian lumpfish losing condition with time spent at sea (-9.59 ± 3.93, t=-2.44, $p$=0.02; Figure 3.2). The proportion of emaciated lumpfish (Wr <75%, between 10-25% below the expected weight for their length) increased over time ($\chi^2$=4.25, df=1, $p$=0.04) as none were observed at the first time point (three weeks) for any of the origins, while a small percentage (English: 7%, Norwegian: 3%) was found at three months post-deployment.

*Changes in welfare between origins and over time*

The full model included origin, time and size (total length) as predictors of welfare, but only time and total length, showing the lowest AIC, were retained (Appendix, Table S3.4). Welfare scores did not differ significantly between lumpfish origins. However, welfare was influenced by time spent in cages (z=11.07, df=316, $p$<0.001) and fish size (z=-5.14, df=316, $p$<0.001). LOWSI values increased (indicating worsened welfare) with increased time at sea, approximately by 1.22 points ± 0.10, being higher at three months (English: n=41, median ± interquartile (IQR): 2 ± 1; Norwegian: n=119, med ± IQR: 3 ± 1) than at three weeks post-deployment (English: n=43, median ± IQR: 1 ± 1; Norwegian: n=117, med ± IQR: 1 ± 1; Figure 3.3). All the scored welfare indicators (body damage: 3.06 ± 1.04, z=2.93, $p$=0.003; eye condition: 2.52 ± 0.23, z=11.02, $p$<0.001; suction disc deformity: 1.51 ± 0.61, z=2.46, $p$=0.01; relative weight: 1.46 ± 0.31, z=4.69, $p$<0.001) worsened over time, except for caudal fin damage that did not vary. Also, LOWSI values were higher in smaller than bigger lumpfish (-0.015 ± 0.003) whilst statistically controlling for stock origin and

time. These results suggest that welfare decreases with time spent at sea and is also worse for small fish, possibly reflecting poorer growth.

*Changes in the prevalence of sea lice and pellets in stomachs over time*

The number of sea lice found in stomachs was very low, accounting only for 1.5% (5/320) of the total population. English lumpfish belonging to family 1 (n=4, 1 sea lice each) and Norwegian lumpfish (n=1, 13 sea lice) were the two stocks that ingested sea lice. While controlling for stock origin and variation in size (Appendix, Table S3.5), counts of ingested sea lice varied with time spent at sea (3.11 ± 1.04, z=2.98, $p$=0.003) with slightly increased counts found at three months (mean ± SD: 0.1 ± 1.03) than at three weeks after deployment (mean ± SD: 0.006 ± 0.079). Sea lice infestation levels in salmon were slightly higher at three months (average 1.0·salmon$^{-1}$; Cage 3: 0.76, Cage 4: 0.9, Cage 7: 1.1, Cage 8: 1.25) than at three weeks post-deployment (0.6·salmon$^{-1}$; Cage 3: 0.9, Cage 4: 0.7, Cage 7: 0.45, Cage 8: 0.35); which could indicate that lumpfish ingested slightly more sea lice at three months than at three weeks because the infestation rates were slightly higher on the farm at that time.

Fish pellet counts in lumpfish stomachs differed between origins (z=-10.69, df=316, $p$<0.001), time spent at sea (z=-16.52, df=316, $p$<0.001) and size (z=5.08, df=316, $p$<0.001); with English lumpfish (44%, 37/84) eating more pellets than Norwegian lumpfish (12%, 27/236). More pellets were also eaten at three weeks (37%, 59/160) than at three months (3%, 5/160) and counts were also higher in larger size lumpfish (0.017 ± 0.003). Empty stomachs were found in almost 49% (156/320) of the total lumpfish in this study, 41% (66/160) at three weeks and 56% (90/160) at three months. The stomach fullness or distension rate was not significantly different over time (z=-1.38, df=316, $p$=0.17), but differed between stock origins (z=-4.38, $p$<0.001), with English lumpfish showing greater stomach fullness (mean ± SD: 0.51 ± .041) than Norwegian lumpfish (mean ± SD: 0.25 ± 0.36).

## Discussion

This study looked at how lumpfish changed in size, welfare and diet after deployment in sea cages. Body size, body condition, welfare scores and diet changed over time, between three weeks and three months post-deployment. Also, body size, body condition and the prevalence of pellets in stomachs varied also between lumpfish origins.

English lumpfish grew faster than Norwegian lumpfish, with higher average weights and lengths (Table 3.1), and an increase of mean weight of 2.5x and 1.3x in 66 days, respectively. There are no other studies comparing growth rates between English and Norwegian lumpfish in sea cages. However, average growth in Norwegian lumpfish here was similar to the growth recently reported by Imsland et al. (2021) in a Norwegian family in a 67-day trial in sea cages, with an order of 1.8x increase of mean weight. Differences in growth rates between genetic stocks have previously been reported in lumpfish; for instance, lumpfish from the East Atlantic (including Norway) were found to have a steeper length-weight relationship than lumpfish from the English channel, indicating that wild Norwegian lumpfish could grow faster than those caught from the English channel (Whittaker et al., 2018). However, here English lumpfish displayed faster growth rates than Norwegian lumpfish in cages. Lumpfish have a high growth potential as they are capable of increasing their mean body weight by ten times in only 76 days, when reared at optimum temperatures (Nytrø et al., 2014). In contrast to most other species, fast growth is not the focus for lumpfish usedas cleaner fish (Imsland et al., 2021). This is because, while lumpfish producers prefer stocks with faster growth rates in hatcheries, so lumpfish reach deployment size sooner (Powell et al., 2018b), salmon farmers prefer lumpfish with slower growth in cages since smaller lumpfish (20-30g) appear to be more effective at sea lice removal than larger ones (>75g) (Imsland et al., 2016a, Imsland et al., 2021). If there would be a trait for selection between growth rates and sea lice removal efficiency, the second should be prioritized over the first; but because mortality rates in lumpfish are sometimes close to 100% (Poppe, 2017), disease resistance and survival should be the selected trade-off to improve sustainability of the industry.

The proportion of stocks did not differ over time, with similar representation at three weeks than at three months. Sampling was performed randomly at the surface of the

cage and the number of individuals belonging to each stock was proportional when deployed; however, the observed proportion of Norwegian lumpfish was much greater than the expected, with the English proportion being much lower than expected. Due to their higher representation at both time points (T1 and T2), it would seem that Norwegian lumpfish had a better survival rate than English lumpfish. However, other factors such as the sampling technique or English lumpfish simply swimming deeper in the cage cannot be ruled out. Under natural conditions, adult lumpfish are usually found in deep waters (Kennedy et al., 2016), with daily vertical migrations greater than 100 metres, which is deeper than the maximum depth of most salmon cages.

Welfare scores for body damage, eye condition, suction disc deformities and relative weight did not differ between lumpfish origins but varied significantly with time spent at sea and fish size, with smaller lumpfish displaying worse welfare than larger lumpfish. This has also been found by other authors, where smaller lumpfish suffered also more damage than larger lumpfish in sea cages (Rey et al., 2021). Body damage occurrence here increased from 0.63% to 10% over time. This condition can be common in sea cages, especially when weather conditions are adverse in exposed sea sites (Hvas et al., 2021), and its prevalence needs to be closely monitored as external lesions make individuals more susceptible to secondary bacterial infections (Noble et al., 2012), one of the most common causes of mortality in lumpfish (Nilsen et al., 2014, Brooker et al., 2018, Erkinharju et al., 2021).

Poor eye condition (mostly cataracts) was also found to increase over time. Eye condition has been reported to deteriorate and worsen with time in sea cages (Geitung et al., 2020, Gutierrez Rabadan et al., 2021), especially with the increasing prevalence of cataracts as the time progresses (Imsland et al., 2021), which in-turn can increase the risk of emaciation as lumpfish depend on their vision to feed (Powell et al., 2018b). The risk of developing cataracts has been related to high growth (Jonassen et al., 2017, Imsland et al., 2018d), although in this case, cataracts affected similarly both lumpfish stocks. Although the prevalence of suction disc deformities was generally low, this condition also worsened over time (from 1.3% to 4.4%), increasing only its frequency but not its degree of affection. Farming conditions and routine procedures at sea often compromise fish health and welfare (Ashley, 2007), which can deteriorate quickly with time at sea. This can occur to both salmon and lumpfish, although lumpfish

welfare could worsen straight after transportation and deployment to sea cages, two conditions known to be very stressful for the species (Jonassen et al., 2018). Ideally, lumpfish should live in cages with salmon during their entire grow-out cycle, which can range from 12 up to 24 months (FAO, 2009). Unfortunately, this is not very common as lumpfish survival is significantly low in sea cages (Brooker et al., 2018, Klakegg et al., 2020, Erkinharju et al., 2021). Although the present study lasted for 84 days, which is not fully representative of the time expected for lumpfish to last at sea with salmon, it gives insights on how lumpfish welfare deteriorates at sea, suggesting that the more time they spend at sea, the more compromised welfare could get, especially if nothing is done to maintain good welfare.

Feeding preferences in lumpfish also changed over time, with the occurrence of sea lice in stomachs increasing from 0 to 17 specimens with time spent at sea, which also corresponded with a slight increase of the sea lice infection rate on the farm. This should be taken cautiously as the low prevalence of ingested sea lice is reflected by the low incidence of sea lice attached to salmon in this farm. However, several studies have reported that the efficiency of sea lice removal in lumpfish increases with time spent at sea (Imsland et al., 2014a, Imsland et al., 2015a, Imsland et al., 2018a), suggesting that lumpfish may need some time to adapt to cage conditions before they can show any cleaning behaviour. Previous habituation to salmon (Staven et al., 2019) and feeding live preys (Imsland et al., 2019e) before lumpfish are stocked in sea cages could potentially reduce this adaptation period, although the impact of the transfer and the deployment per se are not well documented yet. In contrast, the prevalence of formulated pellets in stomachs decreased over time, with more pellets eaten at three weeks than at three months post-deployment. English lumpfish (44%) ate considerably more pellets than Norwegian lumpfish (11%), and their stomachs were significantly more distended. This could be a potential reason why English lumpfish grew faster than Norwegian lumpfish in this study.

Overall, these results can be of interest for the industry. Since their growth rates are slower at sea, Norwegian lumpfish may be preferred by salmon farmers over English lumpfish to be used as cleaner fish, with the potential negative consequences of escapes and introgression. However, the low prevalence of sea lice found in stomachs (due to low infestation levels in salmon in the farm) could not make any of the stocks

better than the other at delousing. The higher prevalence of pellets in stomachs in the English stock during the first period could have contributed to its faster growth rate. Welfare worsened from three weeks to three months after deployment at sea, indicating that the first three months after the transfer are crucial for lumpfish. Welfare and health should be carefully monitored during this period, especially on the smaller size fish.

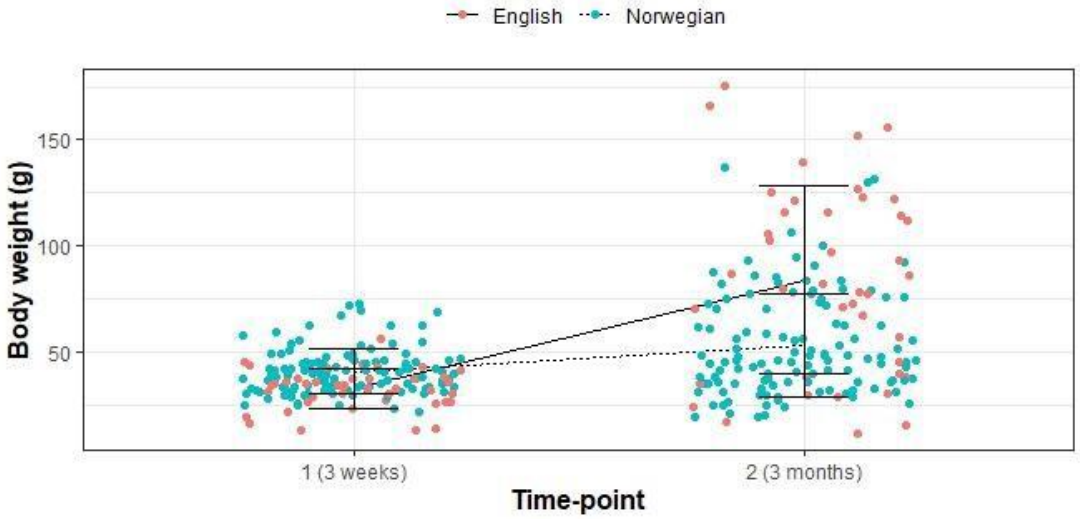
## Acknowledgements

I would like to thank Matthew Tomlinson†, Joel Ellis, Ali Duce and Iain Webster for organising the visit to the farm and providing essential information for this study. I thank the NoahGene team for analysing the samples. I also thank Ben Overland, Ben Jennings, Sergio Trevi and Ben Whittaker for their immense efficiency and support on collecting the samples in such a short time notice. I am also grateful to Paul Howesand Becky Stringwell at CSAR for being the contact point between Swansea University and the industry and for their help organising the design of the experiment.

**Table 3.1**. Summary of body size (BW and TL) and condition (Wr) data according to cage, origin, genetic stock and sampling time point. BW=body weight, TL=total length. Values are represented as mean ± SD=standard deviation. Variance between time-points was significantly different for body weight and total length, but not for body condition (Wr).

| | T1 (three weeks) | | | | T2 (three months) | | | |
|---|---|---|---|---|---|---|---|---|
| | Sample size (n) | BW (g) ± SD | TL (mm) ± SD | Wr (%) ± SD | Sample size (n) | BW (g) ± SD | TL (mm) ± SD | Wr (%) ± SD |
| *By cage* | | | | | | | | |
| Cage 3 | 40 | 37.8 ± 12.4 | 95.3 ± 10.0 | 103.3 ± 14.1 | 40 | 58.7 ± 28.2 | 111.6 ± 16.9 | 103.9 ± 17.2 |
| Cage 4 | 40 | 41.3 ± 9.3 | 97.1 ± 8.8 | 111.1 ± 18.1 | 40 | 53.2 ± 28.6 | 104.9 ± 13.7 | 109.5 ± 18.8 |
| Cage 7 | 40 | 38.1 ± 9.7 | 95.8 ± 7.3 | 105.3 ± 15.7 | 40 | 73.8 ± 37.9 | 122.9 ± 21.1 | 99.4 ± 12.3 |
| Cage 8 | 40 | 37.9 ± 11.5 | 95.5 ± 10.4 | 104.1 ± 11.7 | 40 | 59.8 ± 35.7 | 112.0 ± 21.8 | 98.5 ± 13.4 |
| *By origin* | | | | | | | | |
| English | 43 | 32.9 ± 9.2 | 90.9 ± 9.5 | 102.4 ± 12.7 | 41 | 84.2 ± 44.4 | 125.0 ± 25.1 | 106.0 ± 17.7 |
| Norwegian | 117 | 40.9 ± 10.5 | 97.7 ± 8.4 | 107.2 ± 15.9 | 119 | 53.5 ± 24.4 | 109.2 ± 15.5 | 101.4 ± 15.4 |
| *By stock* | | | | | | | | |
| English fam1 | 6 | 31.7 ± 9.3 | 85.8 ± 10.2 | 115.3 ± 12.8 | 8 | 24.3 ± 8.3 | 85.5 ± 6.5 | 87.7 ± 18.9 |
| English fam2 | 35 | 33.9 ± 8.8 | 92.7 ± 8.5 | 100.6 ± 11.3 | 30 | 99.4 ± 35.5 | 136.1 ± 16.9 | 109.1 ± 11.7 |
| English fam4 | 2 | 20 ± 9.9 | 76 ± 8.5 | 96.6 ± 22.1 | 3 | 92 ± 54.4 | 121.3 ± 19.0 | 129.1 ± 27.6 |
| Norwegian | 117 | 40.9 ± 10.5 | 97.7 ± 8.4 | 107.2 ± 15.9 | 119 | 53.5 ± 24.4 | 109.2 ± 15.5 | 101.4 ± 15.4 |

**a)**




**b)**

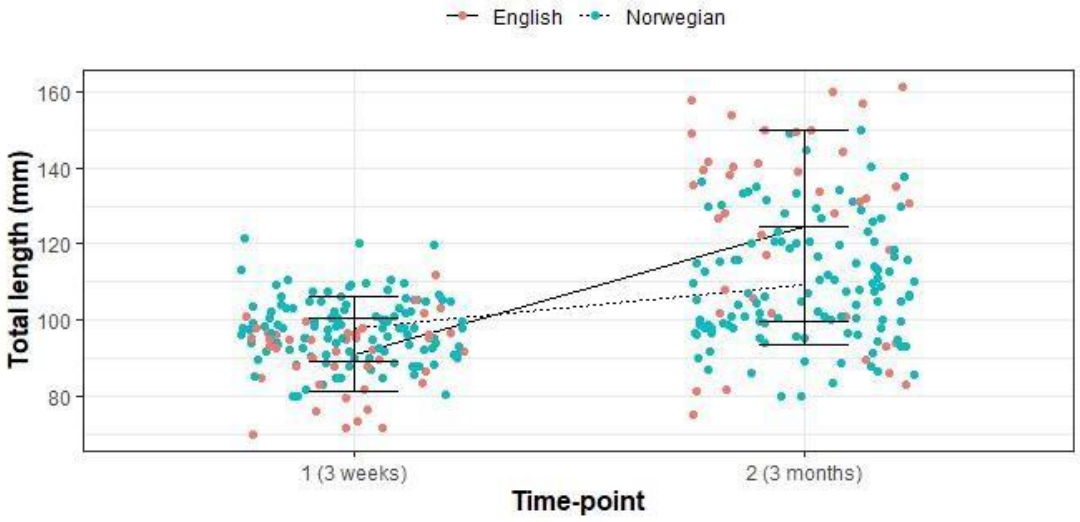
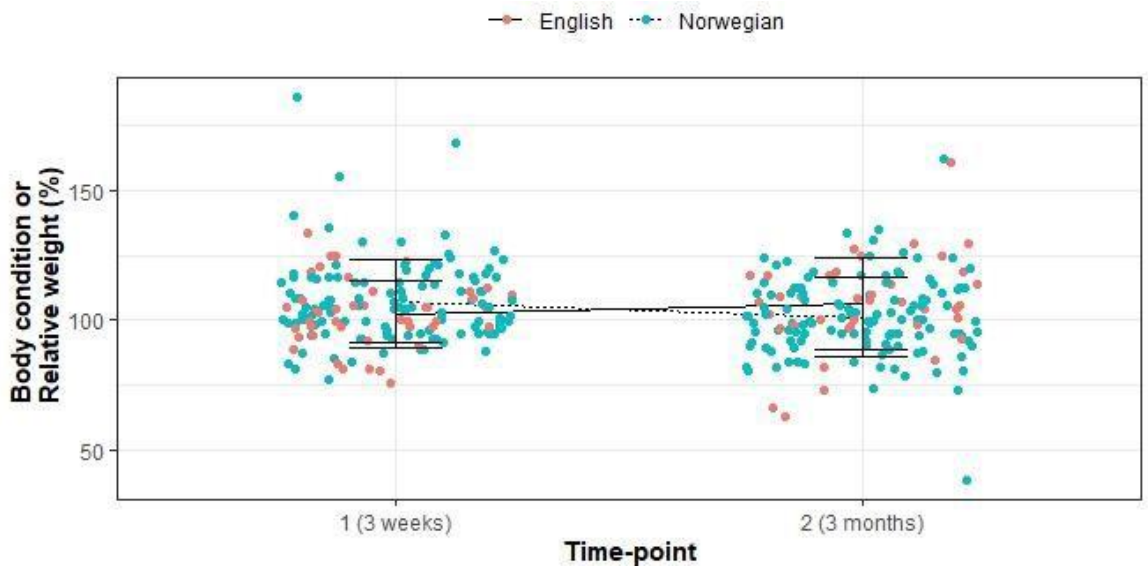



**Figure 3.1**. Lumpfish individual body weights **(a)** and total lengths **(b)** of each origin (English in pink, n=84; Norwegian in blue, n=236) at each of the time-point (1: Nov 2018, three weeks post-deployment; 2: Jan 2019, three months post-deployment). Error bars show the distribution of the data (-SD, +SD) with mean trajectories for each origin over time (continuous line for English and dotted line for Norwegian lumpfish). An interaction effect between the predictor variables (origin and time point) is shown.

**a)**




**b)**

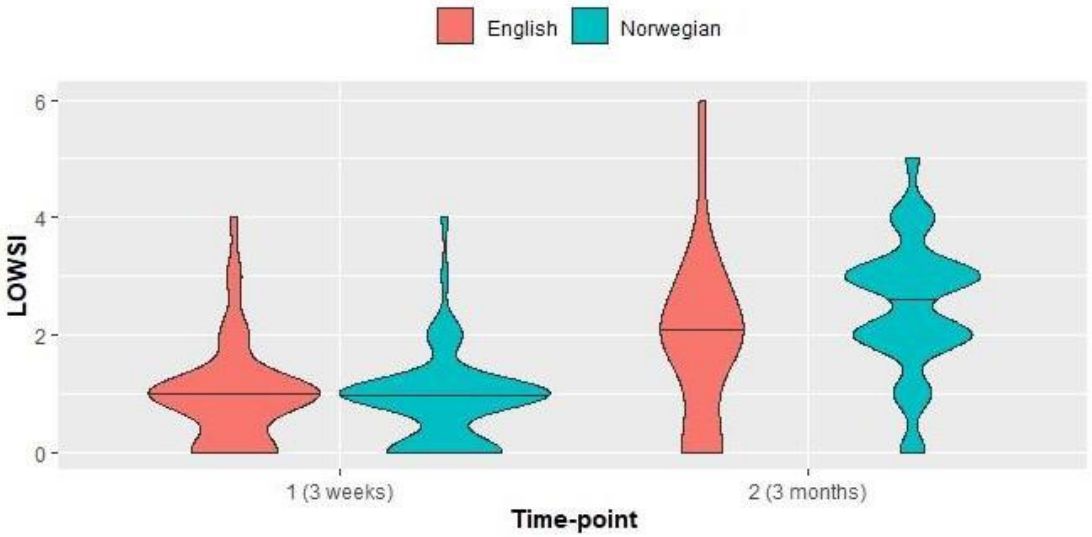



**Figure 3.2. a)** Body condition (or relative weight, % Wr) distribution in each of the genetic origins (English, n=84; Norwegian, n=236) at each time-point (1: three weeks, 2: three months). An interaction effect between the predictor variables of body condition is shown. **b)** Variation in welfare scores (LOWSI) between lumpfish origins and time-points (English: 1, n=43, 2, n=41; Norwegian: 1, n=117, 2, n=119).

# Chapter 4 Feeding preferences, growth and gut microbial communities of two stocks of lumpfish *Cyclopterus lumpus* L. deployed in a common sea cage

**Abstract**

Lumpfish are widely used in salmon farms as a biological control of sea lice. However, there are concerns about their suboptimal use and the large variation in performance between individuals when deployed in sea cages. It has been suggested that this variation could be genetically explained. To assess whether the genetic stock influences how lumpfish perform at sea, lumpfish from Icelandic and Scottish origins were stocked in a common sea cage of a commercial salmon farm. Body size and condition, welfare scores, cortisol level, diet and intestinal microbiome communities were compared between the two stocks. Icelandic lumpfish were significantly larger and grew faster than Scottish lumpfish and fed primarily on formulated feed while Scottish lumpfish fed mostly on crustaceans. Icelandic lumpfish also displayed better welfare scores than Scottish lumpfish. In terms of microbial communities, characterized by 16S rRNA gene sequencing, the Scottish lumpfish gut microbiota showed higher diversity and variability than the Icelandic lumpfish's, which was dominated (90%) by the genus *Mycoplasma*. A significant association was found between abundance of *Candidatus branchiomonas*, known to be involved in complex gill disease, and low lumpfish welfare, as well as between cortisol levels and high *Clostridium* abundance, suggesting that some bacteria may be used as welfare and stress biomarkers.

## Introduction

Lumpfish (*Cyclopterus lumpus* L.) are reared in hatcheries and deployed into commercial salmon farms for the control of the sea lice, *Lepeophtheirus salmonis*. Lumpfish have been classified as 'Near Threatened' by the International Union for Conservation of Nature (IUCN) Red List (Lorance et al., 2015). Although there have been some advances in captive breeding (Anon, 2020e), over 85% of all the lumpfish deployed in Scotland in 2017 were not locally sourced (Treasurer et al., 2018c) and importing non-native lumpfish eggs is still relatively common in the salmon industry. Baltic and North Atlantic lumpfish display phenotypic differences in skin lumps and tuberculation (Davenport, 1985), size (Lampart-Kaluzniacka and Heese, 2000) and growth (Pampoulie et al., 2014, Whittaker et al., 2018). There are also differences in body morphology, longevity, feeding preferences, growth rate and behaviour between Icelandic lumpfish and lumpfish from the English Channel (Whittaker, 2019). Thus, the movement of non-local stocks of lumpfish to supply salmon farms for sea lice control may have a negative impact for both translocated and native stocks, particularly if they interact as a consequence of farm escapes. Also, local stocks may be expected to perform and survive better than non-local ones, based on environmental conditions(ranges of optimal sea water temperatures, for example).

Once lumpfish reach a deployable size around 20 g (Brooker et al., 2018), they are transferred to commercial salmon cages by boats or lorries. Transport can sometimes take longer than 24 hours (Jonassen et al., 2018), depending on the distance between the rearing facility and the sea farm, and is considered one of the most stressful activities in lumpfish farming due to the difference in water quality parameters between environments, the variation in temperature, the accumulation of total ammonia in the transport tanks with no water exchange and the repeated handling, which affects both their health and welfare (Harmon, 2009, Sampaio and Freire, 2016, Jonassen et al., 2018, OIE, 2019b). Then, the first weeks at sea are also a critical period for lumpfish. Although pre-transfer health assessments are routinely carried out to ensure fish are healthy and fit to travel, acute mortalities after release into the sea are common, with some of them associated to transport (Bornø et al., 2016).

When lumpfish transfer to sea is smooth and straightforward and they are not

challenged by an outbreak of any infectious pathogen or husbandry issue, mortalities could then be explained by poor adaptation to the sea cage environment (personal observation). This may cause chronic stress that escalates to poor body condition and welfare, leading to emaciation, and posterior bacterial infections, usually caused by opportunistic *Tenacibaculum*-like bacteria (Småge et al., 2016). Stress, which is common under aquaculture settings (Conte, 2004, Davis, 2006, Mohapatra et al., 2013), is also a key driver of the diversity of fish gut microbiota (Legrand et al., 2020). The exposure to stressors in fish leads to an increase in the abundance of opportunistic pathogens and a reduction of beneficial microbiota (Legrand et al., 2020). Moreover, microbial communities have a dynamic nature in response to external conditions and any disruption is likely to impact fish health, welfare and performance (Uren Webster et al., 2020b). In addition, the characterization of microbiomes, to identify imbalances or dysbiosis, has been identified as a potential predictor of stress-related conditions and could be used as an important biomarker in aquaculture (Perry et al., 2020).

Microbiome species diversity and composition are largely influenced by environmental factors such as the diet, the habitat/geographic location (surrounding waters, salinity, temperature), the season or cohabiting hosts (Legrand et al., 2020). But host factors like the genetic background, sex, life stage, captive-state and phylogeny; and community factors, are also important in the microbial composition (Tarnecki et al., 2017, Talwar et al., 2018, Egerton et al., 2018, Huang et al., 2020). Lumpfish microbial studies are still scarce and have focused on bacteria present during the larval development (Dahle et al., 2017) or pathogens (Scholz et al., 2018), and more recently on bacterial communities of healthy lumpfish reared under different aquaculture systems (Roalkvam et al., 2019, Dahle et al., 2020), but there is no information about the influence of the geographical origin or genetic background on the lumpfish gut microbiome.

The present study had three aims. First, to compare two stocks of lumpfish toassess whether their performance in terms of growth, condition and welfare are different under the same environmental conditions. Secondly, to measure the impact of transport and deployment on welfare in tagged individuals. Lastly, to investigate lumpfish gutmicrobiota and assess the abundance of certain taxa as potential markers of welfare and stress.

## Material and Methods

*Sample collection and fish tagging*

Juvenile farmed lumpfish of similar age (11 months old) from two different genetic stocks (Icelandic, n=1154, reared at hatchery 1 (Lat: 57.840°N, Long: 5.586°W) and Scottish, n=923, reared at hatchery 2 (Lat: 51°36'29.8"N, Long: 3°58'50.4"W) were deployed into a commercial salmon net-pen in a farm in Scotland (Lat: 55°42'10.8"N, Long: 5°43'35.0"W), to compare and assess differences. Stocks differed in early-life rearing conditions with Icelandic lumpfish reared in a flow-through system (temp. range (°C): 9-11) while Scottish lumpfish were reared at a recirculation system (temp. range (°C: 8.5-9). Despite the two stocks hatching in the same week, the Icelandic lumpfish, stocked on 27/03/2019 (255 days post-hatch), achieved deployment size sooner than Scottish lumpfish, which were deployed on 26/04/2019 (291 dph). As Icelandic lumpfish grew faster and were more mature than Scottish lumpfish at the same age, probably due to a faster metabolism, they were deployed earlier and had spent 30 days more at sea than Scottish lumpfish before they were sampled. Lumpfish were stocked at a 4% salmon stocking density in a single 100m (10m deep) circular cage. Prior to deployment and in the hatchery stage, only the Scottish lumpfish were individually tagged in the musculature below the dorsal fin using ISO 11784 134.2 kHz passive integrated transponder (PIT) tags (Loligo Systems, Denmark). Lumpfish were examined with a portable tag reader (Agrident APR500, Germany) in situ to distinguish their genetic origin (Icelandic vs Scottish) and to estimate growth rate and changes in welfare scores before and 60 days after deployment. Lumpfish with no reads were considered Icelandic but the PIT tag injection location was carefully assessed to ensure there was no misclassification. Retention of PIT tags in lumpfish (D'Arcy et al., 2020) and in dorsal musculature has proven to be high (Mamer and Meyer, 2016). Wet weight (g), total and standard length(mm) and welfare scores (Gutierrez Rabadan et al., 2021), were recorded for all sampled individuals (n=142). A subsample of the total lumpfish sampled (n=60) was humanely euthanised (overdose of tricaine methanesulfonate (MS-222, Pharmaq, UK) followed by brain destruction under Schedule 1, according to UK Home Office regulations) for cortisol and stomach content analysis.

Fish in the sampled cage were fed a mix of salmon (Biomar® SSC LR 55 6.5mm and Biomar® SSC LR PE 9mm) and lumpfish (Biomar® Lumpfish Grower 2mm) formulated pellets at a 1.2% and 2% biomass rate daily, respectively. The biofouling on the cage net was last cleaned 10 days before the sampling, using a remote operated net cleaner (RONC), and the cleaning routine was every fortnight during the summer.

*Growth rate*

Individual growth rate (only for the individually tagged Scottish lumpfish) was calculated as Specific Growth Rate (Hopkins, 1992): SGR (%) = [(ln($BW_f$) – ln($BW_i$)/time interval], where $BW_f$ is final body weight, $BW_i$ is initial body weight (both in grams) and time interval is $\Delta t = t_2-t_1$, computed in days. This formula was also applied to length and relative weight data, the latter calculated following the same formula employed for Chapter 2: $Wr = 100 \cdot (W/Ws)$ (Blackwell et al., 2010) according to the life stage (Gutierrez Rabadan et al., 2021): lumpfish sampled at hatchery 1 or time 0 were treated as pre-deployment lumpfish (stage S3) while lumpfish sampled at the salmon farm or time 1 were treated as post-deployment lumpfish (stage S4). A schematic representation of the number of lumpfish used for each analysis is illustrated in Figure 4.1.

*Welfare scores*

Welfare status included scores for body damage (BD), caudal fin damage (CFD), eye condition (EC), suction disc deformities (SDD) and relative weight (RW), OWIs previously developed (chapter 2) and validated for lumpfish (Gutierrez Rabadan et al., 2021). The sum of the scores for all five indicators was computed to obtain LOWSI values for each individual and categorize the fish into three classes: *class A* (good welfare or LOWSI<2), *class B* (moderately compromised welfare or LOWSI=3-5) and *class C* (severely compromised welfare or LOWSI>5).

*Plasma cortisol determination*

Blood samples (n=60) were collected in lithium-heparinized tubes (BD Vacutainer® blood collection system, Becton, Dickinson and Company, USA) for plasma cortisol analysis within 30 seconds after fish death, and immediately placed on ice. They were then centrifuged for 10 minutes at 1500rpm (15°C), and plasma extracted with a

pipette for cortisol analysis using a multispecies DetectX Cortisol Enzyme Immunoassay kit (Arbor Assays, Michigan, USA). Plasma samples were treated with dissociation reagent and diluted 50% in buffer prior to cortisol determination. Cortisol concentration was calculated for each of the 60 plasma samples, run in duplicates across two plates, using the values obtained in a standard curve for each plate ($R^2$=0.99) and adjusted by the dilution factor (1:100) to obtain cortisol in ng/ml. The inter-assay precision, determined by 18 samples in duplicates across 2 plates, was 10.6% and the limit of detection was established at 59.9 pg/ml (0.06 ng/ml).

*Stomach content analysis*

Stomachs (n=60) were dissected and placed immediately on ice and stored at -20°C until analysis. Once partially defrosted, stomachs were dissected on enamel plates over ice to keep the content as preserved as possible during the analysis. All the stomachs were visually assigned to a fullness rating according to its distension level (0 = empty; 1 = partially full (<50%); 2 = half-full (50%); 3 = moderately full (>50% filled); 4 = completely full (100%) or bursting). After that, they were opened by an incision on the greater curvature to carefully remove all the food items, which were exposed in groups of the same food source along the plate for identification and quantification. All the content was weighted to the nearest 0.001g and inspected under a dissecting microscope (Leica Geosystems DFC290) outfitted with a Nikon SMZ800 camera and pictures taken for future reference. After identification, food items were pooled and categorized into nine food groups: unknown/unidentifiable, plastics, seaweed/algae, sea lice, bivalves, hydrozoans, crustaceans (gammarids, caprellids, copepods), fish (prey, scales, tissue) and formulated feed. The contribution of each food item to the diet composition was calculated as a combination of different quantitative metrics, calculated from the stomach content analysis: (1) the frequency of occurrence (%FO) with absence/presence data using the following formula: $\%FO_i = N_i/N$ (Baker et al., 2014) where $N_i$ is the number of stomachs containing item *i* and N is the total number of stomachs containing food, (2) the percentage by number (%N) with abundance data using $\%N_i = N_i/N_T$ (Hynes, 1950, Mahesh et al., 2019) where $N_i$ is the total number of item *i* and $N_T$ is the total number of food items, and (3) the percentage by weight (%W) using the gravimetric method and the formula $\%W_i = W_i/W_T$, where $W_i$ is the weight of food item *i* and $W_T$ is the total weight of food items (Hyslop, 1980, Mahesh et al., 2019). As none of these methods is accurate enough on its own, a combination of them

was used for standardised analysis as recommended by Amundsen and Sánchez-Hernández (2019). The prevalence of each food item was also calculated to visualize differences within the two stock populations. The Index of Relative Importance (IRI) was calculated to quantify the value of each food category in the diet (Mahesh et al., 2019) and was computed as $IRI = (\%N_i + \%W_i) \cdot \%FO_i$. Weekly sea lice counts, which are regularly monitored in all salmon farms following Scottish Government regulations (SRUC, 2018) were also obtained to quantify infestation levels, along sea temperatures for the whole production cycle. These included average counts of *Lepeophtheirus salmonis* of different stages: adult females (gravid and non-gravid), adult males, pre-adults and juveniles (*Chalimus*), and *Caligus elongatus* (Appendix, Figure S4.1).

*Microbiome analysis*

The distal section of the lumpfish intestine (n=60) was collected into sterile Eppendorfs, transported on ice and stored at -80°C until DNA extraction for microbiome analysis. DNA was extracted using DNeasy PowerLyzer PowerSoil kit (Qiagen, USA), with optimized bead-based tubes for the isolation of microbial DNA, according to the manufacturer's instructions. The library preparation for the 16S rRNA amplicon was carried out by the amplification of the V4 hypervariable region of the 16S rRNA gene using Earth Microbiome primers, previously selected as the best candidates for bacterial representation: F515 (5'-3':GTGCCAGCMGCCGCGGTAA) and R806 (5'-3': GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011), following the Illumina MiSeq 16S metagenomic sequencing library preparation protocol (Illumina Inc., 2013) with a few modifications. The first stage PCR, which had a total reaction volume of 25µl, contained 8µl of microbial DNA, 0.5µl of each of the amplicon PCR primers (10µM), 12.5 µl of 2x Platinum™ Hot Start PCR Master Mix (Invitrogen) and 3.5µl of molecular water. The thermal cycler program consisted in 3 minutes at 95°C to activate the polymerase enzyme, followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The elongation step involved 72°C for 5 minutes. The second stage PCR reaction was performed with 2.5µl of DNA product of the first PCR, 1.25µl of each of the Nextera XT Indexes (1: N7xx and 2: S5xx), 12.5µl of 2x Platinum™ Hot Start PCR Master Mix (Invitrogen) -as per the first PCR- and 10µl of molecular water, giving a total reaction volume of

27.5µl. The same thermal cycler program was used but, in this case, limited only to 7 cycles of amplification. Two microbial community standards (ZymoBIOMICS, USA) of well-defined composition (Table S4.1) were also used as controls, along with the samples and two extraction blanks. Products from the second PCR were then assessed in a 1D gel electrophoresis to validate a single band of expected amplicon size and posteriorly estimate concentration for the pooling step. Pools were purified with a ratio of 1:1 using Agencourt AMPure XP beads (Beckman Coulter, USA) and a magnetic rack. All pooled libraries were cleaned-up with 80% ethanol twice and eluted with elution buffer pH 8.5 (Qiagen, USA) according to the Illumina protocol. Pooled and cleaned libraries were then quantified using the NEBNExt Library Quant Kit for Illumina (New England Biolabs Inc., USA), normalized and diluted to 2nM before being sequenced with an Illumina MiSeq platform (250bp PE reads).

*Bioinformatic analysis*

Sequences were analysed using QIIME2 v.2019.4 (Bolyen et al., 2019). Demultiplexed sequence reads (n=96) were firstly assessed for quality scores using *fastqc* reports generated with fastx toolkit and then denoised using DADA2 (Callahan et al., 2016). Both forward and reverse reads were trimmed at 250bp length and at 19bp for primer and adapter removal to reduce spurious ASVs, correct sequencing errors, filter chimeras and reduce duplicates. Taxonomy assignment was performed using the SILVA database v.132 (Glöckner, 2019) as reference and sequence reads were filtered to exclude host DNA, chloroplasts and mitochondria. After rarefaction, 91 samples (94.79%) were retained at the specific sampling depth of 4008 reads. Alpha diversity was determined using two metrics: Chao's species richness (Chao and Chiu, 2006) and Shannon diversity (Shannon, 1948); while beta diversity was determined using the Bray-Curtis distance (Bray and Curtis, 1957) between samples. All metrics were used for further microbial diversity and composition analyses.

*Statistical analysis*

All statistical analyses were performed with R v.3.6.3 (R Core Team, 2019) and Bioconductor v.3.10 (BiocManager 1.30.10). Differences in body size (weight and length) and condition (relative weight) between stocks were tested with a Welch t-test; while a Kruskal-Wallis test was used for welfare scores (LOWSI), after assessing that size was not significant when used as covariate. Each of the individual OWIs (values:

0-2) included in the LOWSI were tested with a Mann-Whitney U test. Differences in plasma cortisol between stocks were analysed with a linear model (LM) while controlling for variation in size. Total length was used as a measurement of size as its coefficient of variation (CV=11.73%) was lower than that of body weight (CV=39.09%). Plasma cortisol levels were used as a continuous variable and also as a categorical variable called CortisolRange with two factors: Low (< 63 ng/ml) and High (>63 ng/ml), using the cut-off point described in Gutierrez Rabadan et al. (2021).

Changes in body size and condition in individually tagged lumpfish before and after deployment were tested with a linear mixed model (LMM) using time (before/after) as a fixed term and fish individual as a random term, with the lmerTest package (Kuznetsova et al., 2015). Changes in welfare were assessed with a generalized linear mixed model (GLMM), using the same fixed and random terms, but with Poisson as a family function and log as a link, under the lme4 package (Bates et al., 2011). Model assumptions for linearity, homogeneity of variance and normally distributed residuals, were checked with quantile-quantile (Q-Q) plots.

To assess dietary differences between genetic stocks, stomach content data was standardized and transformed using 'square root ($x$+1)' to obtain a Bray-Curtis similarity distance matrix, using the *vegan* package (Oksanen et al., 2013). Stomach content data consisted in three datasets containing absence/presence (FO), abundance (N) and gravimetric (W) data. Empty stomachs (4/60) were removed from the datasetbefore the analysis. The Bray-Curtis distance matrix of each dataset was visualized using non-metric multidimensional scaling (nMDS) and the one-way permutational multivariate analysis of variance (PERMANOVA) with 999 permutations was performed to statistically test the null hypothesis of no difference in diet compositionamong genetic stocks (Anderson, 2001), using size as covariate in the model. An additional permutation test of multivariate homogeneity of group dispersions (PERMDISP) was performed using the *betadisper* function to test the null hypothesisof no difference in dispersion between genetic stocks. This helped to identify if significant results from the PERMANOVA are driven by the data itself and not by thedispersion of the data. The similarity percentage analysis (SIMPER) was calculated with the *simper* function within the *vegan* package to identify the food categories thatcontributed most to the similarities within stocks and dissimilarities between stocks. The microbiome datasets extracted from QIIME2 (ASV and taxonomy tables) were compiled together with the sample metadata using the *phyloseq* package. All the analyses of

community data were performed at genus level. The microbial community diversity and structure analysis were also performed using the *vegan* package. Alpha diversity was treated as the dependent variable and genetic stock, welfare status and plasma cortisol levels were used as fixed predictors while statistically controlling for variation in fish size, which was used as a covariate. To identify differences in microbial community structure between genetic stocks, welfare status and cortisol levels, Bray-Curtis dissimilarity distances were computed in a non-metric multidimensional scaling (nMDS) ordination method using the '*metaMDS*' function. Mean distance to centroids was extracted with the '*meandist*' command within the *betadisper* function to obtain statistical differences in beta diversity between the groups. A permutational ANOVA (permutations=999) with the function '*adonis*' wasperformed to identify significant effects between stocks, welfare scores and cortisol levels, using size as a covariate. The multi-level pattern analysis through the '*multipatt*' function in the IndicSpecies package (De Caceres et al., 2020) wascomputed to assess the association between genera patterns and the different stocks, with the association function "r.g" that corrects for the unequal group sizes, significance level of $\alpha$=0.01 and False Discovery Rate (FDR) corrected p-values (Benjamini and Hochberg, 1995) with '*p.adjust*' function. *DESeq2* (Love et al., 2014)was used to assess differences in genera abundance (normalized counts) between groups of welfare status and cortisol levels, with the lowest adjusted p-value, exclusively in the Scottish stock (the only one with enough variability for the analysis).

## Results

*Comparison between stocks in body size, condition, welfare and cortisol levels*

Icelandic lumpfish were significantly heavier (153.1 g ± 62.1) and longer (158.8 mm ± 19.3) than Scottish lumpfish (129.5 g ± 42.4, 149.7 mm ± 15.0; weight: Welch t-test, t=2.69, df=139.7, $p$=0.008; length: Welch t-test, t=3.16, df=135.33, $p$=0.002), despite being almost a week younger (332 vs 338 dph, respectively). However, the stocks did not differ in body condition or relative weight (Welch t-test, t=0.17, df=101.6, $p$=0.87, Figure 4.2).

Stocks also differed in welfare scores (Kruskal-Wallis rank sum test, $\chi^2$=14.80, df=1, $p$<0.001), with the Scottish stock showing worse welfare (0.69 ± 0.19, t=3.48, $p$<0.001) than the Icelandic one (3.04 ± 0.86, t=3.53, $p$<0.001). However, size did not have an effect on welfare (-0.010 ± 0.005, t=-1.92, $p$=0.06). In the Icelandic stock, 91% of lumpfish were classified as class A (highest welfare), while only 61% of the Scottish lumpfish belonged to this class. Inspection of individual welfare metrics indicated that stocks differed in caudal fin damage (W=3076, $p$=0.002), eye condition (W=1702.5, $p$<0.001) and suction disc deformities (W=970, $p$<0.001). Size did not have an effect on any of the OWIs measured (BD: 0.001 ± 0.001, t=0.85, $p$=0.39; CFD: -0.006 ± 0.003, t=-1.78, $p$=0.07; EC: -0.0008 ± 0.003, t=-0.25, $p$=0.80; SDD: - 0.002 ± 0.002, t=-1.046, $p$=0.30 and RW: -0.003 ± 0.001, t=-1.74, $p$=0.08).

After adjusting for variation in size, no significant differences were found in mean plasma cortisol levels between Scottish (62.7 ng/ml ± 20.9) and Icelandic (72.5 ng/ml ± 34.2) lumpfish ($F_{1,58}$=1.85; $p$=0.18). Plasma cortisol average value within the entire population was 67.5 ng/ml ± 28.32.

*Growth and changes in welfare scores of indidually tagged lumpfish before and after deployment at sea*

Analysis of individually tagged lumpfish revealed significant changes in body weight (t=8.25, df=54, $p$<0.001), total length (t=17.51, df=54, $p$<0.001) and relative weight (t=4.89, df=54, $p$<0.001) before and after deployment. Although most of the population (93%) gained weight after deployment (36.15 g ± 4.38), 7% of lumpfish showed negative values for SGR (Figure 4.3), indicating post-deployment weight loss

of about 5.5 grams on average. However, all lumpfish grew in length (23.38 mm ± 1.34). Body condition or relative weight increased post-deployment (9.8 % ± 2.01) although 16% of lumpfish showed a reduction, and body condition declined on average by 8.4%. The overall growth of Scottish lumpfish during this period of 60 days (range: 55 – 64 days) was 0.56%·$d^{-1}$ at an average sea water temperature of 10.6 °C (range: 9.5 – 11.8 °C). The welfare scores of individual lumpfish, measured as LOWSI values, remained unaltered in 45.5% of lumpfish, while worsened in 32.7% and improved in 21.8% (Figure 4.4). Nevertheless, differences in LOWSI before and after deployment were not statistically significant (0.26 ± 0.14, z=1.91, $p$=0.06). However, the proportion of lumpfish with eye condition after deployment was significantly higher than the proportion before ($\chi^2$=26.61, df=1, $p$<0.001), increasing from 0/55 to 23/55 (42%).

*Stomach content analysis*

The stomachs of the lumpfish stocked in the salmon farm contained a wide range of food items and only 6% of the stomachs sampled (4/29 in Icelandic stock and 1/31 in Scottish stock) were completely empty (Figure 4.5). Considering the overall population (n=60) and the total number of items (%), crustaceans (mainly individuals from the Gammaridae family) accounted for the vast majority of prey items (73.4%), being the most frequent food item in lumpfish sampled at sea (Appendix, Table S4.2), while other items such as bivalves and fish prey accounted for less than 1% (0.09% and 0.04%, respectively). Sea lice infestation levels were relatively low in the sampled cage at the moment of the sampling, circa 0.2·$salmon^{-1}$ (0.1 average *L. salmonis* males and 0.1 average *L. salmonis* pre-adults, at 11.8°C), and only three sea lice, representing the 0.13% of the total food items, were found as two partially digested stages of *Lepeophtheirus salmonis* and a single *Caligus elongatus* (Appendix, Figure S4.2).

Based on the index of relative importance (IRI) of food categories (Table 4.1), Icelandic lumpfish primarily fed on formulated feed (%FO: 53.8%, %W: 73.3) while Scottish lumpfish mainly fed on crustaceans (%FO: 86.7%, %W: 51.1%). Results of the PERMANOVA test confirmed statistically significant differences in the composition of the diet between Icelandic and Scottish lumpfish at the presence/absence level (pseudo-$F_{1,53}$=3.54, $p$=0.008), while controlling for variation in size. Also, a significant size effect was found at gravimetric level only (stock:

pseudo-$F_{1,53}$=5.68, $p$=0.001; size: pseudo-$F_{1,53}$=2.79, $p$=0.03) suggesting that prey items weighted more in larger lumpfish. The PERMDISP test was not significant in any of the stomach content datasets (FO: $F_{1,54}$=1.87, $p$=0.21; N: $F_{1,54}$=1.13, $p$=0.28; W: $F_{1,54}$=0.91, $p$=0.35) indicating that dispersion was homogeneous within each group and differences in diet composition were solely attributed to the genetic stock. The SIMPER analysis revealed that the highest average dissimilarity in diet composition between Icelandic and Scottish lumpfish was with gravimetric data (W; 84.17%), followed by abundance (N; 80.21%) and absence/presence data (FO; 52.4%). The food categories that significantly contributed the most to the dissimilarity among genetic stocks were hydrozoans (FO, $p$=0.003; N, $p$=0.001; W, $p$=0.001), followed by copepods (W, $p$=0.01) and fish prey (W, $p$=0.04).

*Microbial analyses: diversity and structure*

After filtering and cleaning sequence reads, all retained samples were clustered into a total of 863 amplicon sequence variants (ASVs), of which there were 348 different at a genus level. From these, 307 (88.2%) were assigned to genus level, 29 (8.3%) to a family level, 7 (2%) up to an order level and 5 (1.4%) up to a class level. The most abundant bacterial genera were *Mycoplasma* (66.5%), *Vibrio* (12.5%), *Aliivibrio* (4.9%), *Photobacterium* (3%), *Clostridium sensu stricto 1* (2.1%), *Cetobacterium* (1.9%), *Aeromonas* (1.3%) and *Candidatus branchiomonas* (0.6%).

Microbiota alpha diversity differed between lumpfish stocks (both Chao richness and Shannon diversity, $p$<0.05). Icelandic lumpfish showed significantly lower Chao richness ($F_{2,54}$=5.1; $p$=0.009) and Shannon diversity ($F_{2,54}$=11; $p$<0.001) than Scottish lumpfish. Also, lumpfish with good welfare (*class* A, or LOWSI < 2 points) showed lower Shannon diversity (-1.1 ± 0.34; $F_{1,55}$=10.5; $p$=0.002) than lumpfish with compromised welfare (class B or C, LOWSI > 3 points). However, no significant differences in alpha diversity parameters were found for plasma cortisol groups (Low and High; Chao, $p$=0.38 and Shannon, $p$=0.56).

Microbial community structure for each genetic stock were plotted accordingly to their Bray Curtis dissimilarity distances (Figure 4.6) and statistically significant differences were confirmed between genetic stocks (PERMANOVA, pseudo-$F_{1,56}$=11.24, $p$=0.001). The Shepard plot showed increased dissimilarity when the ordination

distance increased, showing a good representation of the data in reduced dimensions (Stress=0.12; $R^2_{\text{non-metric fit}}$=0.985; $R2_{\text{linear fit}}$=0.966). The mean distance to centroids was 0.18 for the Icelandic stock and 0.73 for the Scottish stock, indicating that the gut microbiota of the Scottish lumpfish was much more diverse and variable in composition than the gut microbiota of Icelandic lumpfish (ANOVA, $F_{1,55}$=74.4; $p$<0.001; Figure 4.7). *Mycoplasma* spp. was the most abundant genera in both genetic stocks, although for Icelandic lumpfish represented 90% of the total microbiome and for Scottish only the 42%, which is the main reason why Icelandic lumpfish presented both lower alpha diversity and dispersion. *Vibrio* spp., *Photobacterium* spp. and *Aliivibrio* spp. followed *Mycoplasma* spp. counts in Scottish lumpfish with 23%, 6% and 6%, respectively; while Icelandic lumpfish had *Aliivibrio* spp. (4%), *Vibrio* spp. (2%) and *Francisella* spp. (0.5%). Welfare groups also differed significantly in community structure (PERMANOVA, $F_{1,56}$=3.22, $p$=0.019), with higher microbial diversity in lumpfish with compromised welfare (meandist=0.69) compared to lumpfish with good welfare (meandist=0.43; ANOVA, $F_{1,55}$=8.19, $p$=0.006).

*Microbial analyses: differential abundance of taxa*

The results of the multi-level pattern analysis revealed that *Mycoplasma* was significantly associated to the Icelandic lumpfish (0.60, $p_{adj}$<0.03), as this stock was dominated by this genus; while there were 15 different genera associated to the Scottish stock (Table 4.2), from which Gammaproteobacteria (0.544, $p_{adj}$=0.001), Clostridia (0.236, $p_{adj}$=0.005) and Bacteroidia (0.386, $p_{adj}$=0.005) were the most significant classes. Differential taxa abundance was computed within the Scottish stock of lumpfish (n=28) due to its greater diversity, using DESeq2 with normalized bacterial counts. One genus (0.51%) was underrepresented when comparing the two welfare status groups (Good -class A- and Compromised -class B-) and had a positive log2fold change indicating that bacterial counts for *Candidatus branchiomonas* were significantly higher (Wald test, $p_{adj}$<0.001) in lumpfish with compromised welfare compared to lumpfish with good welfare within the Scottish stock (Figure 4.8a). The same pattern was observed with *Tenacibaculum*, *Staphylococcus* and *Arenicella* counts at less but still significant levels (Wald test, $p_{adj}$<0.05). Additionally, lumpfish with high cortisol levels (>63 ng/ml) had significantly higher counts of *Clostridium*

*sensu stricto 1* (Wald test, $p_{adj}$<0.001) than lumpfish with low cortisol (< 63 ng/ml) values (Figure 4.8b).

## Discussion

Both stocks of lumpfish hatched at similardates (Scottish: 09/07/2018 and Icelandic: 15/07/2018) but, under a common garden design, Icelandic lumpfish were significantly heavier and longer than Scottish lumpfishwhen sampled at sea, suggesting a potential faster growth rate of the Icelandic stock. However, differences on the early-life rearing conditions (rearing systems and temperatures, stocking densities and diets) could have also intervened in these observed differences between genetic stocks in the sea cage stage. Welfare was significantly poorer (albeit only slightly) in the Scottish stock, particularly for caudal fin damage, suction disc deformities and eye condition. Both stocks had similar plasma cortisol levels (approximately 67.5 ng/ml), which is higher than expected basal levels (<20ng/ml) for unstressed lumpfish (Jørgensen et al., 2017). This could possibly highlight the stress that lumpfish experience in sea cages, even 60 days after deployment. Habituated lumpfish have shown lower cortisol levels than naïve lumpfish after an adaptation period (Staven et al., 2019). Although no intentional period of adaptation was followed in this case, cortisol levels in lumpfish would have been expected to be lowered after two months cohabiting with salmon. The sampling method may have also caused increases in plasma cortisol concentrations; however, all the fish were treated following the same protocol and variation in cortisol levels are not expected to result from handling.

Up to 13 different prey items were found in the stomachs of lumpfish at sea, which emphasises the opportunistic feeding behaviour of these species in salmon sea cages, where their choices are entirely based on what food source is available on their environment (Imsland et al., 2015a, Imsland et al., 2016a). Sea lice, however, accounted for less than 1% of the total food items as only 5% (3/60) of individuals ingested sea lice. This could be due to the low level of sea lice infestation rate in this cage at the time of sampling, although such a low incidence of sea lice in stomachs has been reported before in a study when comparing lumpfish families (Imsland et al.,

2016a). The Index of Relative Importance (IRI) indicated that Icelandic lumpfish mainly fed on formulated feed while Scottish lumpfish mostly fed on small crustaceans; which may suggest a relationship between the prey size and the size of the fish, where larger fish feed on larger prey. Significant differences were also found in feeding preferences for hydrozoans, being more representative within the Icelandic stock. The presence of biofouling growing from the cage nets (such as seaweed, crustaceans, hydrozoans/hydroids and bivalves) has been positively correlated with the presence of sea lice in stomachs, suggesting that lumpfish with foraging behaviour (biofoulers) are more predisposed to consume sea lice (Bannister et al., 2019, Eliasen et al., 2018). However, salmon farmers have been recommended to keep their nets as clean as possible to avoid 'distracting' the lumpfish from grazing on sea lice from the salmon (Powell et al., 2018b). In this study, although net-pens were cleaned 10 days before the sampling, the lumpfish with sea lice on their stomachs (5%) also presented crustaceans, adding evidence of the active foraging behaviour in lice-eating lumpfish.

Many biotic and abiotic factors can affect the fish gut microbiota (Butt and Volkoff, 2019). I assessed intrinsic factors such as the fish origin, the welfare status and the plasma cortisol level, as well as the diet composition (and sea lice consumption). The main finding was the difference in both microbial diversity and microbial composition between genetic stocks. At a similar age, the gut microbiota of Scottish lumpfish was significantly much more diverse and variable than the gut microbiota of the Icelandic lumpfish, which composition was considerably dominated by *Mycoplasma* (90%).

Low microbiome diversity has been associated with bad health and 'dysbiosis'(Talwar et al., 2018) as communities are less diverse in diseased than healthy fish (deBruijn et al., 2018), but this may not always be the case (Johnson and Burnet, 2016). Here, lumpfish with higher microbial diversity in the gut displayed worse welfare than lumpfish with a gut microbiota dominated by one specific genus (*Mycoplasma*). *Mycoplasma* was the most abundant genus in both genetic stocks, although forScottish lumpfish only accounted for 42%. This genus has been reported as the most predominant taxa (up to 96%) in the distal intestine of the Atlantic salmon (Holben et al., 2002) and several studies agree that its relative abundance becomes more prominent with time as fish mature and progress to the adult stage (Llewellyn et al., 2016, Heys et al., 2020, Wang et al., 2021). However, its role in the gastrointestinal tract of Atlantic salmon remains unclear, although a symbiotic relationship has been

recently suggested (Cheaib et al., 2020). Similarly, in comparison with smaller (1g) lumpfish, *Mycoplasma* dominated the gut microbiota of 25g lumpfish, where its abundance accounted for more than 50% of the microbiome in half of the sampled population (Christie et al., 2018). The difference in microbial communities and the dominance of *Mycoplasma* in Icelandic lumpfish could be the result of the different origin of the stocks including early rearing conditions, or longer time at sea cohabitating with salmon, or a combination of both. The preference of formulated feed by the Icelandic lumpfish is likely to have also influenced these microbial differences (Ringø et al., 2016), as well as the differences in size between the stocks (pelleted feed also promoting higher growth than crustaceans).

Within the differential taxa analysis, the genus *Candidatus branchiomonas* was significantly associated to lumpfish with compromised welfare. *Candidatus branchiomonas* sp. *cysticola* is an endosymbiont that has been associated to the presence of epitheliocysts in gills of Atlantic salmon with complex gill disease (Toenshoff et al., 2012, Mitchell et al., 2013, Gjessing et al., 2019). Gill health is a major indicator of fish health and welfare; however, the visual assessment of the gills in lumpfish can be anatomically very challenging and is not included in the practical welfare score system used in this study (Gutierrez Rabadan et al., 2021). Furthermore, lumpfish with high levels of cortisol presented the highest counts of *Clostridium*, suggesting a potential relationship between this genus and stress. Stress can shape the gut microbiome by promoting the abundance of specific operational taxonomic units (OTUs) within Gammaproteobacteria and Clostridia classes in Atlantic salmon (Uren Webster et al., 2020b). Although both Gammaproteobacteria and Clostridia classes were predominant in the Scottish stock compared to the Icelandic stock, they were not associated to welfare or cortisol levels in this case.

One of the most critical periods within the lumpfish production cycle is the one nearby the transfer, the deployment and the acclimatization to the new environment, including the introduction to Atlantic salmon. The impact of this transition in individually tagged Scottish lumpfish was mainly reflected in this study by loss of body condition, although it was not exclusively acquired after deployment as 15% of the lumpfish were already showing underweight before deployment. At sea, 2% of these lumpfishshowed emaciation, which has also been highlighted as a welfare problem in lumpfishin sea cages, affecting up to 10% of the population (Gutierrez Rabadan et al., 2021). In this

case, there were not significant changes in welfare before and 60 days after deployment as lumpfish welfare remained unaltered. However, the proportion of lumpfish with eye condition (including eye damage and cataracts) increased after deployment. Whilst no lesions were reported before deployment, 23 out of 55 lumpfish showed deterioration of eye condition after two months at sea. Eye condition has already been reported in lumpfish at cages in previous studies (Geitung et al., 2020, Eliasen et al., 2020), reaching up to 26% in some commercial sea sites (Gutierrez Rabadan et al., 2021). Hence, it is important that both the transfer and deployment of the lumpfish in sea cages is thoroughly planned, and the surrounding environment is conveniently prepared for their arrival, as conditions in sea cages are not as controlled as in hatcheries.

This study showed significant differences in body size between Icelandic and Scottish lumpfish, emphasizing different metabolism and growth rates. Welfare scores and feeding preferences, which are closely related to differences in intestinal microbial communities, were also different between lumpfish stocks. A relatively small loss of body condition (8% on average) was observed in this study over time, but welfare scores remained the same. Significant associations indicated that the abundance of specific taxa in the gut microbiome, such as *Candidatus branchiomonas* and *Clostridium* could be used as potential markers of welfare and stress, respectively.

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**Table 4.1**. Lumpfish diet composition indices by genetic stock (Icelandic, n=26; Scottish, n=30). %FO: frequency of occurrence (absence/presence method); %N: percentage by number (numerical abundance method); %W: percentage by weight (gravimetric method); IRI: Index of Relative Importance.

| | Icelandic lumpfish | | | | Scottish lumpfish | | | |
|---|---|---|---|---|---|---|---|---|
| Food categories | % FO | % N | % W | IRI | % FO | % N | % W | IRI |
| Unknown | 73.1 | 31.9 | 13.1 | 3284.6 | 73.3 | 5.1 | 21.3 | 1941.7 |
| Plastics | 0 | 0 | 0 | 0 | 3.3 | 0.3 | 0.1 | 1.3 |
| Seaweed | 3.8 | 0.5 | 0.03 | 2.2 | 26.7 | 2.0 | 2.6 | 121.6 |
| Sea lice | 3.8 | 0.2 | 0.006 | 0.7 | 6.7 | 0.1 | 0.04 | 1.0 |
| Bivalves | 0 | 0 | 0 | 0 | 6.7 | 0.1 | 0.04 | 1.0 |
| Hydrozoans | 65.4 | 3.1 | 11.2 | 934.2 | 30 | 0.5 | 4.1 | 139.8 |
| Crustaceans | 84.6 | 36.4 | 2.3 | 3276.9 | 86.7 | 86.2 | 51.1 | 11901.9 |
| Fish | 7.7 | 0.4 | 0.1 | 0.4 | 13.3 | 2.2 | 11.1 | 176.4 |
| Formulated feed | 53.8 | 27.5 | 73.3 | 5426.1 | 36.7 | 3.5 | 9.6 | 478.7 |

**Table 4.2**. Bacterial communities (n=15) associated to the Scottish stock with stats and *p* value, using multi-level pattern analysis within the IndicSpecies package (De Caceres et al., 2020) with association function='r.g' (Pearson's phi coefficient) and α=0.01. p-values were corrected using the False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995). ASV=Amplicon sequence variant. D5=Genus, D4=Family.

| ASV | Stats | *p* | *FDR-corrected p* |
|---|---|---|---|
| (D5) *Vibrio* | 0.425 | 0.0003 | **0.035** |
| (D5) *Aeromonas* | 0.370 | 0.0003 | **0.035** |
| (D5) *Acinetobacter* | 0.364 | 0.0006 | **0.045** |
| (D5) *Flectobacillus* | 0.332 | 0.0028 | 0.082 |
| (D5) *Cetobacterium* | 0.325 | 0.0040 | 0.098 |
| (D5) *Plesiomonas* | 0.319 | 0.0025 | 0.087 |
| (D5) *Dermacoccus* | 0.305 | 0.0083 | 0.124 |
| (D5) *Geobacillus* | 0.302 | 0.0052 | 0.098 |
| (D4) *Burkholderiaceae* | 0.290 | 0.0015 | 0.073 |
| (D5) *Escherichia/Shigella* | 0.266 | 0.0030 | 0.098 |
| (D4) *Peptostreptococcaceae* | 0.234 | 0.0078 | 0.124 |
| (D5) *Halomonas* | 0.231 | 0.0044 | 0.098 |
| (D5) *Clostridium sensu stricto 1* | 0.223 | 0.0031 | 0.098 |
| (D5) *Candidatus branchiomonas* | 0.188 | 0.0092 | 0.166 |
| (D4) *Rhodobacteraceae* | 0.155 | 0.0018 | 0.086 |

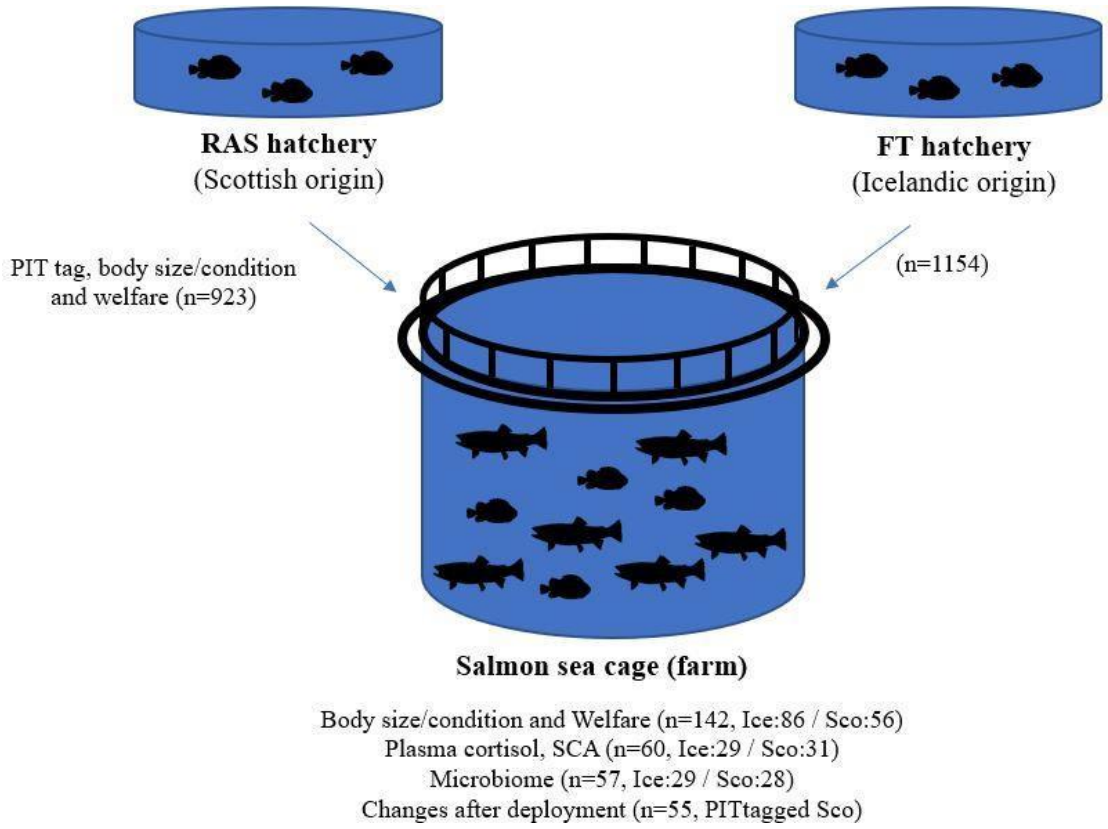



**Figure 4.1**. Schematic representation of the number of lumpfish used for each analysis within the common garden experiment. Credits: Salmon (clipart-library.com), lumpfish (Philopic).

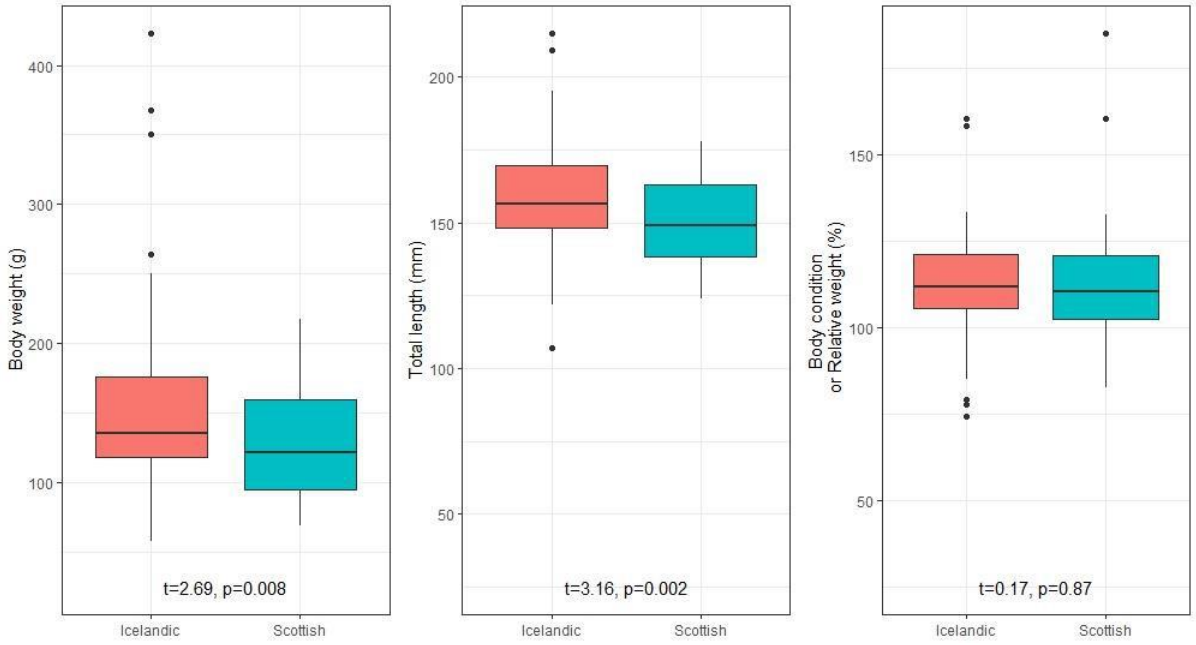



**Figure 4.2**. Distribution of body size (body weight and total length) and condition (Wr) in each stock of sampled lumpfish (n=142; Icelandic, n=86; Scottish, n=56).

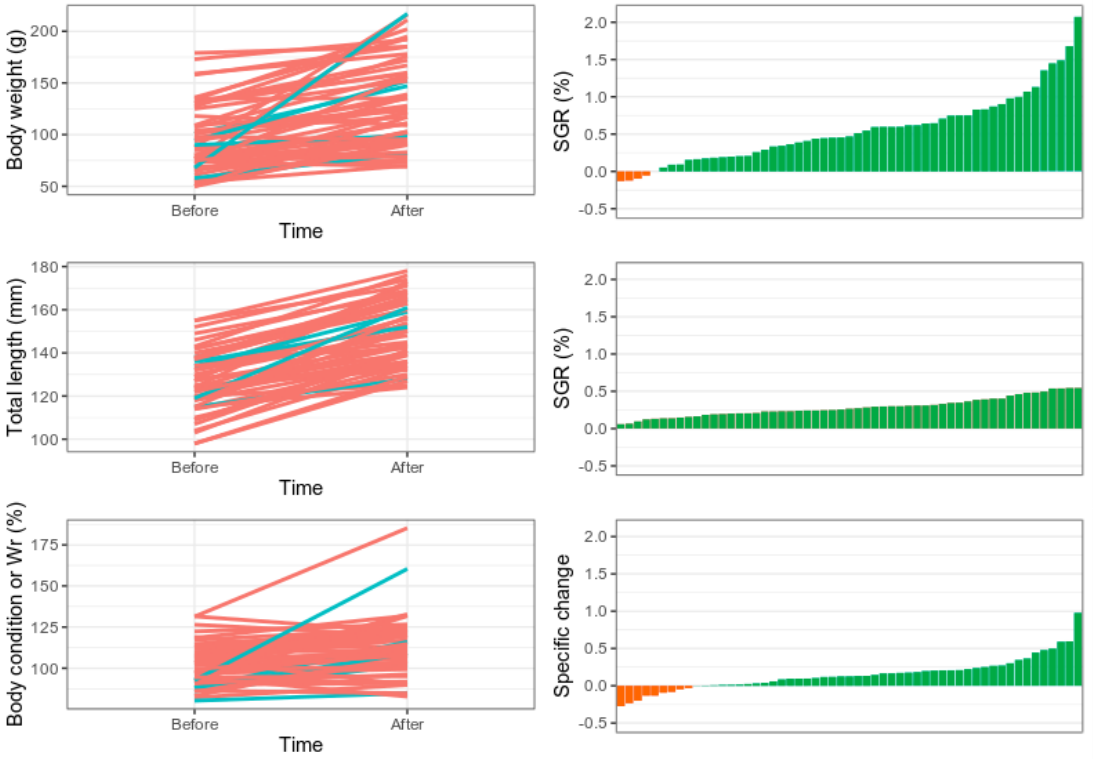



**Figure 4.3**. Spaghetti plots (left) showing individual trajectories in body weight (g), total length (mm) and body condition/relative weight (%) of individually tagged Scottish lumpfish (n=55) before and after deployment at sea. Pink lines show individuals belonging to Class A (n=28), while turquoise lines represent individuals from Class B (n=27). The bar plots (right) summarise the Specific Growth Rate (SGR,%) for body weight (g) and total length (mm) and the specific change of body condition for each individual (green bars for positive SGRs, orange bars for negative SGRs).

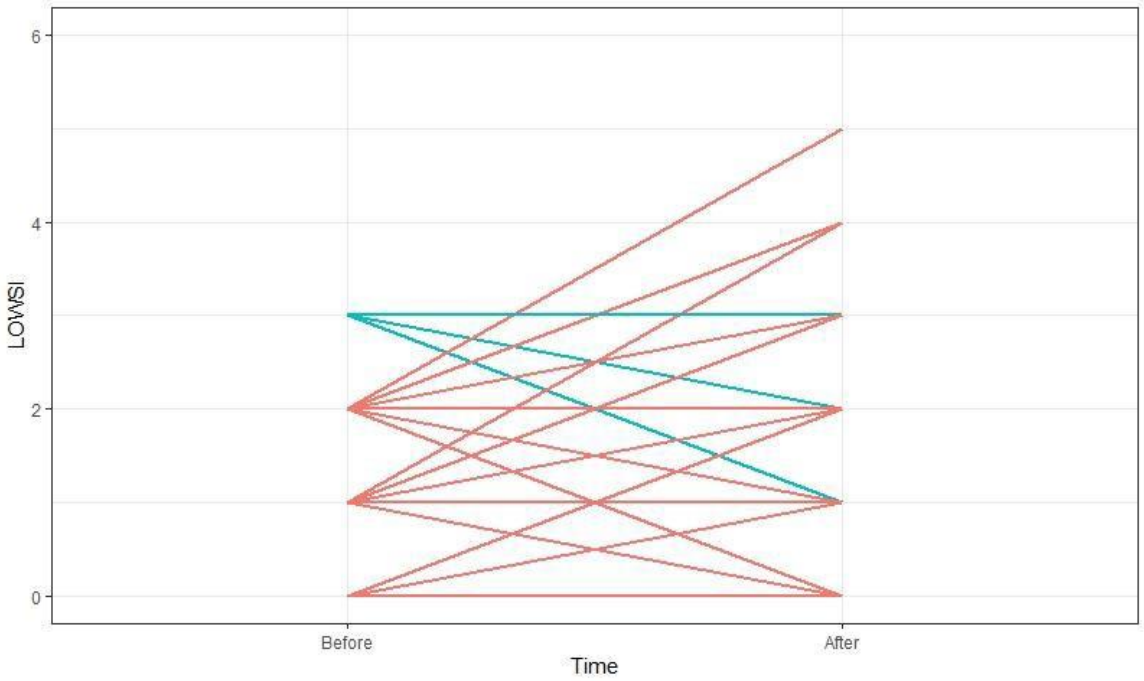



**Figure 4.4**. Spaghetti plot showing individual trajectories of welfare scores (LOWSI values) of tagged Scottish lumpfish (n=55) before and after deployment. Pink lines show individuals belonging to Class A (n=28), while turquoise lines represent individuals from Class B (n=27).

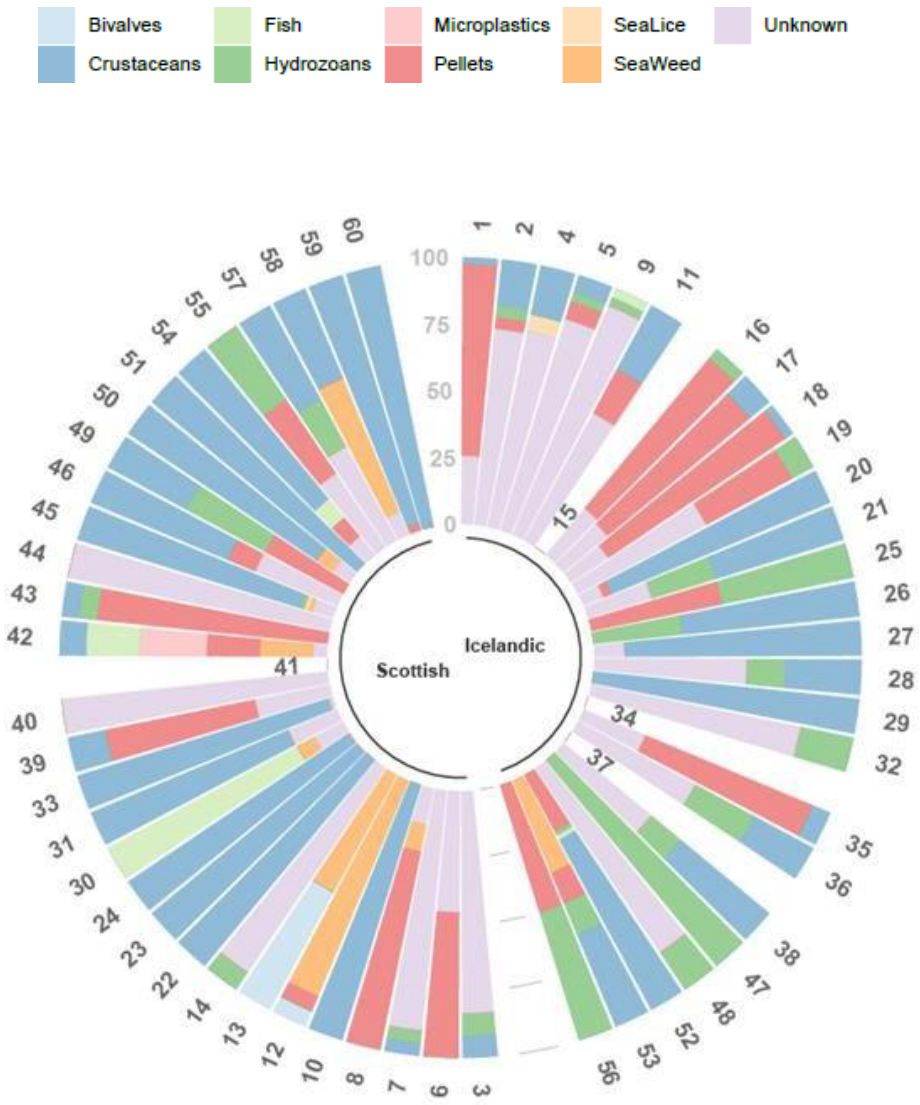



**Figure 4.5**. Feeding preferences of Icelandic (n=29) and Scottish (n=31) lumpfish sampled at sea cages, according to food category (expressed as a total number, %). Each number indicates an individual lumpfish. Note that four individuals -15, 34, 37 and 41- (representing 6% of the total population) had no content on their stomachs.

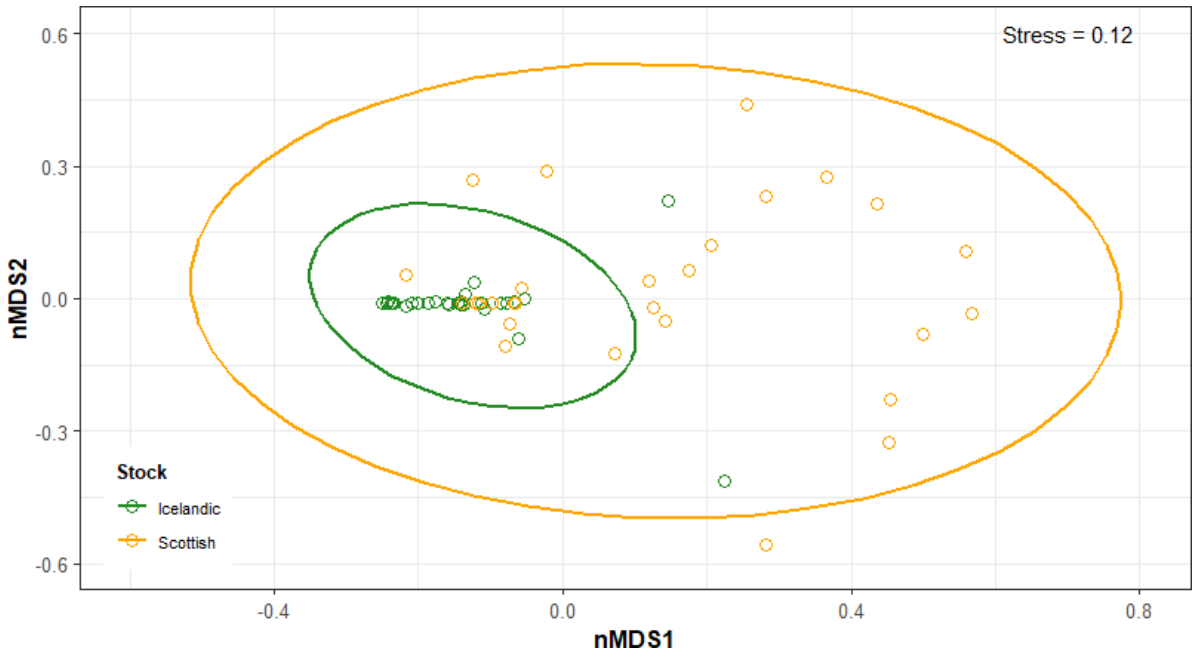



**Figure 4.6**. Non-metric multidimensional scaling (nMDS) ordination plot of gut microbial community structure from two different lumpfish genetic stocks: Icelandic (n=29, green) and Scottish (n=28, orange), based on Bray Curtis dissimilarity distances. Ellipses clustered similar amplicon sequence variants (ASVs) with 95% confidence estimates.

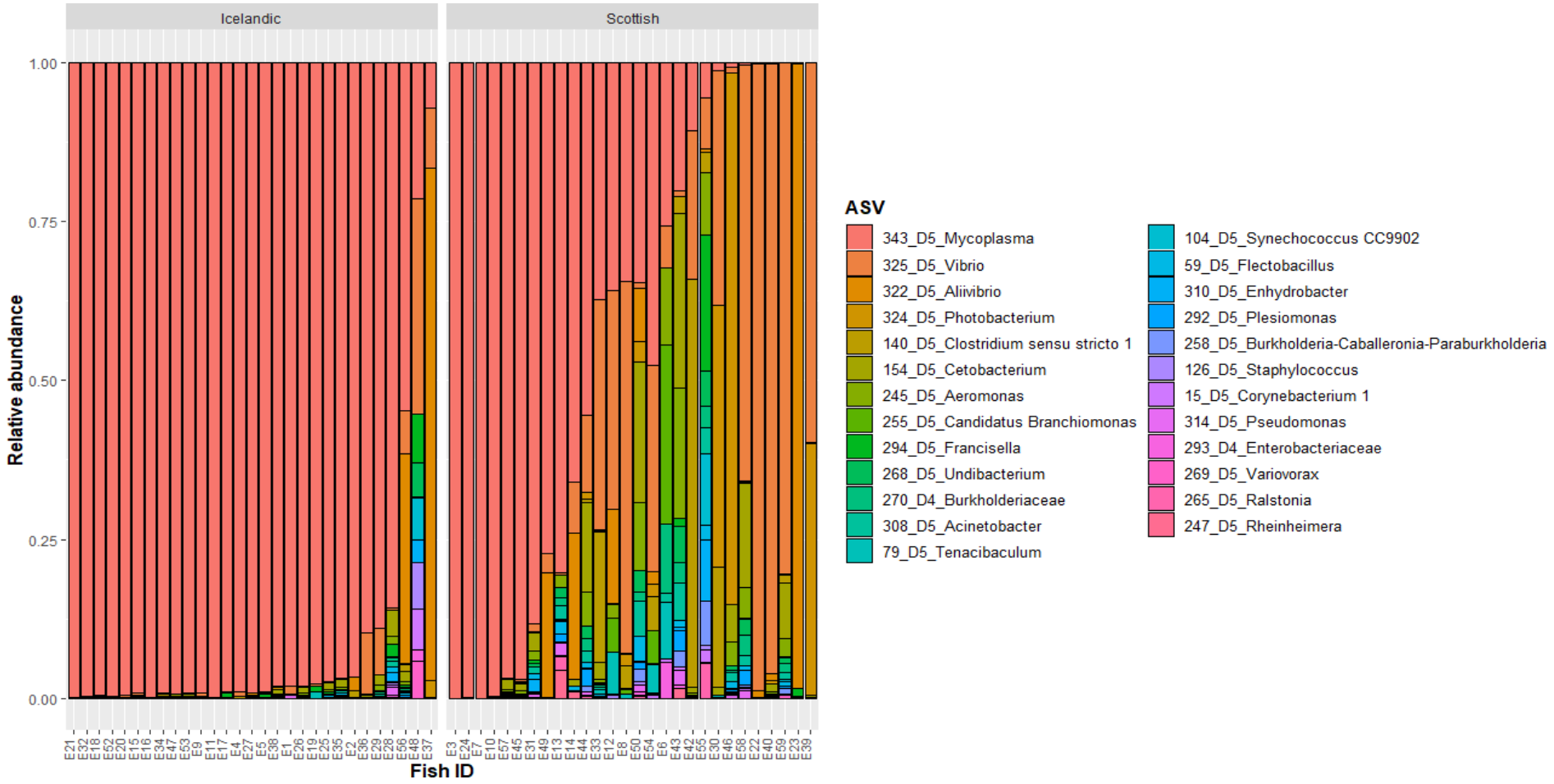
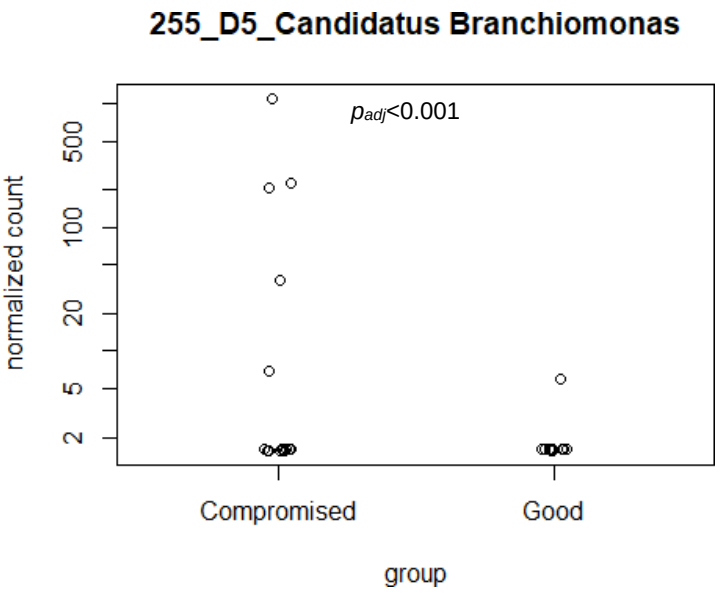



**Figure 4.7**. Relative abundance bar plot showing the 25 most common ASVs, at genus level, in each of the genetic stocks of lumpfish (Icelandic, n=29 and Scottish, n=28).

**a)**




**b)**

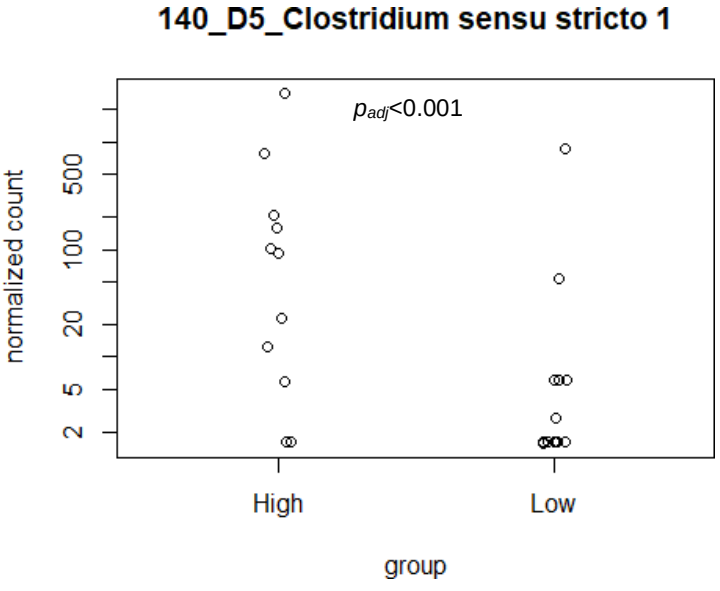



**Figure 4.8**. Normalized intestinal bacterial counts of **a)** *Candidatus branchiomonas* (Wald test, $p_{adj}$=0.0002) for welfare groups (Good and Compromised) and **b)** *Clostridium sensu stricto 1* (Wald test, $p_{adj}$<0.001) for cortisol range groups (Low or <63 ng/ml and High or >63 ng/ml) within the Scottish lumpfish stock (n=28) sampled at the salmon farm.

# Chapter 5 Effect of sea lice ingestion on lumpfish *Cyclopterus lumpus* L. welfare and gut health

**Abstract**

There has been a marked increase on lumpfish welfare research recently due to concerns on the ethical use of this species as cleaner fish in salmon cages. However, there is no information on how sea lice ingestion might affect lumpfish gut health and welfare. The aim of this study was to investigate whether delousing behaviour had any effect on lumpfish cortisol stress response, intestinal microbial communities and welfare. Lumpfish were sampled from a commercial farm in Scotland at the end of the summerwhen the production cycle of salmon was completed, and fish were harvested. Analysis of 35 stomach contents revealed that half of the fish (52%) had ingested sea lice, and 340 specimens (mixed *L. salmonis* and *C. elongatus*) were recovered. Sea lice eaters did not differ in size, body condition, plasma cortisol levels, welfare scores or gut microbial diversity or composition from lumpfish that did not eat sea lice. These results indicate that sea lice ingestion does not appear to compromise lumpfish welfare under commercial conditions.

# Introduction

Sea lice infestation by *Lepeophtheirus salmonis* is one of the major challenges the salmonid aquaculture has faced over the past 40 years. Countless control strategies have been implemented to confront these persistent copepod ectoparasites, whose annual economic cost is estimated around £460M only in Norway (Fletcher, 2019). Due to the development of sea lice resistance (Denholm et al., 2002), the use of biological control agents such as cleaner fish is seen as a valuable alternative to reduce the use and the environmental impact of chemotherapeutants at sea (Torrissen et al., 2013), as well as to maintain the welfare of the salmon (Overton et al., 2019).

Lumpfish (*Cyclopterus lumpus* L.) behave as suitable cleaner fish when stocked in salmon cages, efficiently reducing the counts of pre-adults and mature male and female lice stages (Imsland et al., 2014a). The feeding ecology of juvenile lumpfish has been studied (Ingólfsson and Kristjánsson, 2002), but lumpfish nutritional needs are still not well documented (Johannesen et al., 2018). The species have been considered as facultative cleaners (Vaughan et al., 2017, Treasurer et al., 2018c), although recently a study has found that certain personality profiles in lumpfish can lead to cooperative cleaning behaviour with salmon (Whittaker et al., 2021). Lumpish are generalist feeders and have shown to ingest a wide range of food items, if available, like crustaceans, hydrozoans, pellets, bivalves, etc., which indicates they have a strong opportunistic feeding behaviour in sea cages (Imsland et al., 2015a). Consequently, there is significant variation of the lumpfish lice removal ability, with lower efficacies at a small (9-60%) than at large (53-73%) scale trials (reviewed by Overton et al., 2020). Additionally, there is no information about the nutritional value of sea lice, although some studies have investigated the lipid and fatty acid composition of copepods from the *Lepeophtheirus* genus and have found that triacylglycerol (TAG) is the main energy store, and the quantities of polyunsaturated fatty acids (PUFAs) vary between life stages and sea lice recovered from wild or farmed salmon, due to the influence of feeds on louse composition (Tocher et al., 2010). The biochemical composition of generic copepods used in the production of marine fish at larval stages suggests that copepods contain macronutrients capable to satisfy the demands of fish larvae (van der Meeren et al., 2008) for a short period of time, for instance at weaning, when transitioning from live feed to formulated feed. However, sea lice are considered

"snack-like food" for lumpfish rather than a nutritional source (Merakerås, 2020, Johnsen, 2021), and it is thought that relying on sea lice as the unique source of food is not enough to support these fish. Hence, supplementary feeding is required to maintain lumpfish nutritional needs and welfare in sea cages (Imsland et al., 2018a, Imsland et al., 2018c). Diet (and feeding strategy) has a major effect on fish gut microbiota (Cahill, 1990), driving its composition and impacting fish metabolism. Many studies have explored the impact of dietary changes on the gastrointestinal (GI) microbiota of aquatic animals (Romero et al., 2014, Ringø et al., 2016). For example, certain microbes may contribute to the digestive process by delivering specific enzymes in coral reef fishes (Smriga et al., 2010), and members of the Fusobacteria phylum can synthetize vitamins that exert a positive effect on the health of zebra fish, *Danio rerio* (Roeselers et al., 2011).

Stressors can modulate and change the gut microbiota and, in turn, changes in the gut microbiota can regulate the stress response (reviewed by Butt and Volkoff, 2019). Probiotics are 'live microorganisms that confer a health benefit on the host by improving its intestinal microbial balance' (Fuller, 1989). These are being increasingly used to improve welfare, stress tolerance and boost the immune system, along other benefits such as the improvement of the aquaculture sustainability (Martínez Cruz et al., 2012, Carnevali et al., 2017). Farmed fish are often subjected to different stressors, which impact health and welfare (Conte, 2004) by promoting or disrupting specific microbial taxa in the GI tract. Atlantic salmon with elevated cortisol have shown specific gut microbiome signatures consisting of the dominance of genera within the Clostridia and Gammaproteobacteria classes while levels of beneficial bacteria, such as *Carnobacterium* sp., declined considerably (Uren Webster et al., 2020b). As it has been suggested in chapter 4, the potential relationship between Clostridia and stress also occurs in lumpfish deployed in sea cages (Gutierrez Rabadan, unpublished data).

Lumpfish sustainability is currently being threatened by ethical concerns of their use in the salmon industry (Anon, 2020c) and welfare awareness has significantly increased as a consequence. Microbiome research has also recently gained popularity within this species (Christie et al., 2018, Roalkvam et al., 2019, Dahle et al., 2020, Klakegg et al., 2020). However, and despite the widespread use of lumpfish in salmon cages (Overton et al., 2020), there are some fundamental unknowns that have not been

fulfilled. For instance, the sea lice ingestion effect on lumpfish gut health and welfare is unknown.

The present study aimed to characterise the gut microbiota of sea lice and non-sea lice-eating lumpfish sampled from a commercial salmon farm and assess the impact of sea lice consumption on the cortisol stress response and welfare, as well as on the diversity and composition of gut microbial communities.

## Material and Methods

*Fish sampling*

Lumpfish originating from Norwegian wild brood stock were reared in a recirculating (RAS) hatchery in North Wales and were deployed in a 100x10 m deep circular cage in a salmon farm in Scotland (Latitude: 55° 55.728 N, Longitude: 005° 04.594 W) at a stocking density of 4% (812 lumpfish transferred into a cage containing 20086 salmon) on 25/05/2018. Sampling took place at the end of the salmon's production cycle (11.5 months old, 352 days post-hatch) when lumpfish had spent four months (123 days) at sea. Both salmon and lumpfish were fasted for 48h prior to harvesting. A sample of lumpfish (n=35) were humanely euthanized with an overdose of tricaine methanesulfonate (MS-222, Pharmaq, UK) and fish size (wet body weight and total length) and condition and blood samples (to measure physiological stress by quantifying plasma cortisol) were collected individually as described in chapters 2 and 4. Stomachs were carefully dissected on site, kept on ice and stored at -20°C until analysis. The distal section of the intestine was taken through an incision of the vent, as aseptically as possible, for microbiome analysis.

*Welfare scores*

Welfare scores, recorded following the LOWSI methodology (Gutierrez Rabadan et al., 2021), were categorized into three classes (A, B, C) as described in Chapters 2 and 4. Lumpfish belonging to class A were categorized in the 'Good welfare' group while lumpfish from classes B and C were categorized as 'Compromised welfare'

*Sea lice ingestion analysis*

Once defrosted, lumpfish stomachs were analysed for content as detailed in chapter 4, but in this case the aim was to individually quantify the number of sea lice, both *Lepeophtheirus salmonis* and *Caligus elongatus*, rather than the identification of different items. Ingested sea lice were quantified, but sea lice binary data, as presence/absence, was used as a categorical predictor and lumpfish were split in two delousing groups (No, n=16; Yes, n=18) for statistical analysis. Salmon sea lice counts were recorded by farm staff during the complete production cycle and are shown in Figure S5.1 (Appendix).

*DNA extraction, 16S rRNA amplicon sequencing and bioinformatic analysis*

Intestinal tissue DNA from 35 lumpfish was extracted using DNeasy PowerLyzer PowerSoil kit (Qiagen, USA) following manufacturer's instructions. The amplicon library preparation with all the reagents and the volumes used for the two-step PCRs and the thermal cycler programs (summarised in Appendix, Table S5.1), as well as the downstream analysis, were performed following the same methodology employed in Chapter 4.

*Microbiome network construction and comparison*

Although relatively new, the network-based analytical approach used in other research disciplines, can be used as a tool to understand microbiomes as a system and to explore microbe-microbe and microbe-host interactions (reviewed by Layeghifard et al., 2017). To compare the microbial communities (Layeghifard et al., 2017) of the two delousing groups, microbiome networks were constructed and compared according to the welfare status (Good – lumpfish belonging to class A; Compromised – lumpfish belonging to classes B and C) and interactions at genus level were analysed using the NetCoMi package in R (Peschel et al., 2020). Pearson's correlation coefficient was used as an association measure (Weiss et al., 2016) with modified central log ratio (mclr) transformation to account for compositionality (Gloor et al., 2017), common in microbial data as counts represent proportions instead of absolute abundances; and a threshold or cut-off point of 0.3 to control for sparsity, which can lead to false associations (Matchado et al., 2021). Only the 50 taxa with the highest frequency were included in the microbiome network graphs to make visualization easier. Permutation tests (n=1000) were used for quantitative comparison between networks using the function '*createAssoPerm*', which allows block-wise execution (20 blocks, 5 repetitions) in parallel. Significance p-values were automatically adjusted within the function for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg, 2000). The ARI or Adjusted Rand Index (Hubert and Arabie, 1985) is an evaluation metric used to determine whether two dimension-reduced cluster results or partitions are similar to each other. ARI values can range from -1 to 1, with ARI=1 when there is a perfect agreement between clusterings, ARI=0 when the clusterings are completely random and negative values of ARI if the agreement is less than what is expected for a random result (complementary).

*Statistical analysis*

Statistical analyses were computed using R version 4.1.0 (R Core Team, 2021) and BiocManager version 3.13. A Welch t-test was used to identify differences between delousing groups while using body weight, total length and body condition as dependent variables. To investigate the effect of sea lice ingestion on welfare scores (LOWSI, count data), a generalized linear model (GLM) with Poisson distribution and size (total length) as covariate was used. A general linear model (LM) was used for stress response (plasma cortisol) and microbiome (alpha and beta diversity), with size (total length) as a covariate, to determine differences between delousing groups. One influential outlier (obs. #5) was detected and removed from the analysis of the cortisol dataset. Model residuals were plotted using the function 'plot' to check whether model assumptions of normality, linearity, heteroscedasticity and presence of influential points were met. A PERMANOVA was employed to explore differences in microbiome composition (Bray Curtis distance matrix) between delousing groups. To explore differentially abundant (DA) taxa, normalized bacterial counts were compared between delousing groups using DESeq2 (Love et al., 2014).

## Results

*Lumpfish welfare*

Lumpfish ranged between 22 and 100 g in weight and between 91 and 150 mm in total length. More than half of the population (54%) were emaciated (Wr<75%, 68.8% ± 3.9) and 40% of lumpfish were underweight (Wr=75-90%, 80.8% ± 5.3); only 6% of the lumpfish were considered to have a 'normal' body condition (Wr>90%, 91.7% ± 1.8). LOWSI scores for welfare (Gutierrez Rabadan et al., 2021) ranged from 1 to 7, where 31% of lumpfish were categorized as class A (best welfare) and 46% and 23% belonged to classes B (moderately compromised welfare) and C (poor welfare), respectively. The mean LOWSI value was 3.37 (median=3, IQR=2), indicating that the general welfare of these fish was moderately compromised. Low body condition was the most frequent welfare problem (94%), followed by suction disc deformity (60%) and body damage (46%).

*Variation in delousing efficiency*

Lumpfish were sampled when the sea lice levels in the farm were approximately 1.8 females/salmon (Appendix, Figure S5.1). Sea lice was found in 52% (18/35) of lumpfish stomachs and 340 individual sea lice in total (mixed *L. salmonis* and *C. elongatus*; Appendix, Figure S5.2a) were recovered. Species identification was not possible due to sea lice being partially digested, but based on the sea cage prevalence, expected proportions would approximately be 255 *L. salmonis* (75%) and 85 *C. elongatus* (25%) recovered. The number of sea lice ingested per lumpfish varied between 1 and 118 specimens. The incidence of empty stomachs was 11% (4/35), and other food sources found were mainly crustacean gammarids (65%), hydroid fouling (52%) and crustacean caprellids (42%; Appendix, Figure S5.2b), based on the total number of full stomachs.

*Relationship between sea lice ingestion, body size and condition, welfare scores and cortisol stress response*

Lumpfish size (weight: t=0.59, df=32.74, $p$=0.56; length: t=0.21, df=31.14, $p$=0.83) and body condition (t=1.44, df=27.94, $p$=0.16) did not differ between delousing

groups. Whilst controlling for variation in body size, sea lice eaters and non-sea lice eaters did not differ either on plasma cortisol levels (t=1.5, *p*=0.14), although no control group or stress test was available in this case.

Sea lice ingestion did not have any significant effect on lumpfish welfare scores (0.17 ± 0.19, z=0.95, *p*=0.34), adjusting for size variation; however, there was a significant effect of size on lumpfish welfare, indicating that smaller size lumpfish had worse welfare than larger lumpfish (-0.017 ± 0.007, z=-2.6, *p*=0.009; Figure 5.1). Lumpfish classified as having good welfare (class A) were significantly larger (132 mm ± 14.1) than lumpfish showing compromised welfare (classes B and C; 120 mm ± 12.8; t=2.3, df=17.96, *p*=0.03).

*Relationship between sea lice ingestion and diversity of microbiome communities*

No differences were found between delousing and non-delousing lumpfish in alpha microbial diversity (Chao richness: $F_{2,31}$=1.29, *p*=0.29; Shannon diversity: $F_{2,31}$=0.45, *p*=0.64; Figure 5.2a). Microbial composition (or beta diversity) was also similar between delousers and non-delousers, while adjusting for body size (PERMANOVA, Sea lice ingestion: pseudo-F=0.76, *p*=0.6; Size: pseudo-F=1.57, *p*=0.13; Figure 5.2b). No differences were observed on mean distances to centroids between delousing groups neither (ANOVA, nperm=999, *p*=0.99).

*Characterization of lumpfish gut microbial communities and DA analysis*

A total of 389 ASVs and 191 different microbial species at a genus level were identified, classified as Bacteria (98.9%) and Archaea (1.1%). The most representative genera were *Vibrio* (27.8%), *Photobacterium* (26.6%), *Clostridium* (12.4%), *Piscirickettsia* (9.1%), *Mycoplasma* (6.3%), *Tenacibaculum* (3.8%) and *Aliivibrio* (3.6%; Figure 5.3). In terms of differential abundance (DA), no significant taxa were associated with the variables of interest sea lice ingestion and welfare (stat=2.53, $p_{adj}$=0.99).

Microbiome networks for sea lice ingestion and welfare were constructed and analysed separately, and finally combined for comparison. Microbiome communities in non-delousing lumpfish were selected for welfare comparison, where 33 taxa remained for each of the groups (Compromised welfare, n=10; Good welfare, n=6;

Figure 5.4). While the strongest positive associations were found between 7 and 11 taxa (corr=1.000) for compromised and good welfare, respectively (Table 5.1), the strongest negative associations were found between *Staphylococcus* and *Mycoplasma* (corr=-0.598) in lumpfish with compromised welfare and between *Piscirickettsia* and *Photobacterium* (corr=-0.945) in lumpfish with good welfare. Delousing lumpfish microbial communities consisted of 34 taxa in each welfare group (Compromised welfare, n=13; Good welfare, n=5; Figure 5.2). The strongest positive association in lumpfish with compromised welfare was found between *Burkholderia-Caballeronia-Paraburkholderia* and *Pseudoxanthomonas* (corr=1.000), while lumpfish with good welfare had positive associations between 16 taxa (corr=1.000; Table 5.6). Competition was observed between *Aliivibrio* and *Tenacibaculum* (corr=-0.698) and *Vibrio* and *Photobacterium* (corr=-0.998) for lumpfish with compromised and good welfare, respectively. The phylum Gammaproteobacteria was the most representative between cooperative (18/35, 51%) and competitive (5/8, 63%) taxa, with *Photobacterium* as the most competitive bacteria.

*Network quantitative comparison*

Within the non-delouser lumpfish group, vertex ($p$=0.029) and edge connectivity ($p$=0.039) were significantly higher in lumpfish with good welfare (14.000) than in lumpfish with compromised welfare (3.000), possibly indicating that the good welfare associated taxa have more resilience as a network than the one for compromised welfare, as connectivity measures the minimum elements to be removed to isolate nodes of taxa. Permutation test results, however, did not show significant quantitative differences when comparing the microbial community networks. The adjusted rand index (ARI), considered one of the best performing methods for cluster agreement or similarity between two partitions (Hoffman et al., 2016), was 0.055 ($p$=0.15) when analysing welfare groups within non-delousing lumpfish, and -0.022 ($p$=0.63) when comparing welfare groups within lumpfish that ingested sea lice. The quantitative comparison properties for each of the networks are summarised in detail in Tables 5.3 (non-delousing lumpfish) and 5.4 (delousing lumpfish).

## Discussion

Lumpfish are widely used as biological agents to control sea lice infestations on salmon farms. However, lumpfish are not obligate cleaner fish and do not naturally eat sea lice in the wild, hence it is unknown if ingesting sea lice has any impact on their welfare or gut health. This is the first study performed at a commercial scale that investigates the effect of sea lice ingestion on lumpfish gut microbiota and welfare. The results indicate that sea lice ingestion did not have an effect on body size, condition, physiological stress, welfare scores or gut microbiota.

Most of the lumpfish of this study were classified as class B and C, showing moderately to severely compromised welfare (Gutierrez Rabadan et al., 2021). There was a positive association between welfare and size of the lumpfish, where larger lumpfish showed better welfare scores than smaller size lumpfish (Figure 5.1). Lumpfish with good welfare were 12 mm larger on average than those with compromised welfare. This could be explained by the most frequent conditions observed in smaller lumpfish: suction disc deformities and body damage, two conditions known to result in poor growth (Gutierrez Rabadan et al., 2021).

Despite lumpfish were fasted (of supplementary feed pellets) prior to sampling for 48h following best practice harvest guidelines (Waagbø et al., 2017), most of them (89%) continued foraging and ingested different preys and food items available in their environment, while only a small proportion (11%) had their stomachs empty. Lumpfish are not obligate cleaners and are known to be opportunistic feeders that will not rely only on one food source if others are readily available around. In this study, half of the population was emaciated (54%) and also half of the population ingested sea lice (52%). However, there was no evidence of a relationship between ingesting sea lice and emaciation, although there are no indications that sea lice play an important role in terms of nutrition for lumpfish (Johannesen et al., 2018), as they are mostly eaten as ‘snacks’ (Merakerås, 2020, Johnsen, 2021). It is likely that the only way to maintain nutritional condition, health and welfare of lumpfish during the entire salmon grow-out stage at sea, is to provide lumpfish with supplementary feeding with extruded pellets or feed blocks (Imsland et al., 2020). The use of cleaner fish and their implications (supplementary feeding, additional shelter/enrichment, additional staff, vet visits and husbandry) are still considered a very low-cost effective sea lice control

measure, as long as health and welfare are well managed (Overton et al., 2019, Toma et al., 2020). Some studies suggest that body condition in lumpfish can be rapidly lost if husbandry conditions are not optimal and lumpfish cannot be expected to feed solely on sea lice in sea cages (Johannesen et al.,2018, Garcia de Leaniz et al., 2021). The previous chapter showed decrement in body condition after two months of being transferred into sea cages (Gutierrez Rabadan, unpublished data), which highlights the need of an adequate feed management plan, especially at sea, to cover lumpfish nutritional needs.

Variation in sea lice grazing can be high among individuals of the same family (Imsland et al., 2016a). In the present study half of the population (52%) had sea lice in their stomachs, a value similar to that found by Imsland et al. (2018a). Although dissection is one of the easiest and most practical methods to analyse sea lice ingestion in the farm environment, there are concerns about its lethality and also its reliability as an indicator of how many lumpfish are delousers, due to variation in sea lice digestion times (Eysturskarð et al., 2017). In this sense, the use of non-lethal alternative methods, such as the use of stomach fluid real-time PCR (Eysturskarð et al., 2017, Imsland et al., 2019c) or gastric lavage/flushing (Hartleb and Moring, 1995), currently in violation of the Norwegian Animal Welfare Act (Mattilsynet, 2016), may have provided different results. The fact of not finding sea lice in some of the lumpfish stomachs in this study does not necessarily mean that these lumpfish are not consuming them, especially considering that sea lice can be digested and transited to the intestine as shortly as 6 hours post-ingestion in some individuals (Eysturskarð et al., 2017, Imsland et al., 2019c). Thus, even if no differences in microbiome networks were observed between non-delousing and delousing lumpfish, it cannot be ruled out that some lumpfish may have been feeding on sea lice, without these being found in their stomachs at the time of sampling.

The presence of specific gut microbial communities can also give insights into how diet can impact gut health. In this study, 6 out of 7 most common genera (*Tenacibaculum*, *Piscirickettsia*, *Vibrio*, *Aliivibrio*, *Photobacterium* and *Clostridium*) are considered pathogenic or opportunistic in lumpfish (Småge et al., 2016, Marcos-López et al., 2017, Tolås, 2020). Hence, it is not unexpected that these microorganisms dominated the gut microbiota of lumpfish with moderately compromised welfare, although they were also present in lumpfish with good welfare. Microbial

communities were represented using graphical networks, which allowed to explore positive and negative associations between taxa. Proteobacteria clearly dominated the gut microbiota of lumpfish in this study, which appears to be in accordance with other recent fish gut microbiome studies (Roeselers et al., 2011, Kim et al., 2021). Both cooperation and competition were dominated by taxa from the Gammaproteobacteria (Proteobacteria) phylum, although cooperation occurred between 9 different phyla and competition only between 4 (Tables 5.3 and 5.4), suggesting that diverse bacteria can co-exist for the indirect benefit of all the microbial taxa in the community. It is important to note that except *Mycoplasma,* possibly considered to be a commensal (Rasmussen et al., 2021), all the genera found to compete in the microbial networks (*Photobacterium, Piscirickettsia, Vibrio, Aliivibrio* and *Staphylococcus*) are infectious pathogens of fish (Sudheesh et al., 2012, Småge et al., 2016, Marcos-López et al., 2017, Tolås, 2020). Also, a higher number of cooperative taxa were involved in lumpfish showing good welfare than in lumpfish with compromised welfare, although further study on functional analysis of bacterial communities will be required to better understand these interactions. When comparing microbial networks, ARI values may have suggested that networks between delousing groups were clustering differently based on the welfare status of the fish, but they could be slightly more similar (0.055 vs -0.022) when measured between lumpfish with compromised welfare. However, p-values for the two-tailed test were non-significant which would indicate no significant differences between the compared networks. The results of the analysis of microbiome networks did not differentiate between delousers and non-delousers, perhaps due to the limitations of stomach content analysis or because the microbiome associated with feeding on sea lice is not very different from the microbiome signature associated with feeding on other crustaceans (found to be ingested by 82% of the sampled lumpfish). In any case, the results suggest that feeding on sea lice does not appear detrimental for lumpfish welfare, as no differences in welfare were found between the delousing groups neither.

This study investigated the effect of sea lice ingestion on lumpfish gut microbiota and welfare through the analysis of stomach content in a commercial salmon farm. The results indicate that sea lice ingestion did not have any impact on lumpfish body size and condition, physiological stress levels, welfare scores or intestinal microbial communities, suggesting that sea lice ingestion is not harmful for lumpfish welfare.

The lumpfish studied were sampled close to the end of the salmon grow-out cycle and presented moderately compromised welfare, but this does not appear to be associated with sea lice consumption. Future research should, in general terms, target a thorough evaluation of gut microbial communities depending on different specific diets in a more controlled environment; for instance, investigating communities when using formulated pellets or live feeding in larval stages. The study of microbiome markers could identify beneficial practices for the industry. In more specific terms, the industry could also benefit from the use of probiotics in hatcheries several weeks before sea transfer to ensure lumpfish have the best chance at survival in sea cages.

## Acknowledgements

I thank Matthew Tomlinson[†] and Iain Webster for their support organising the visit to the salmon farm and Ben Jennings for helping with the sampling and data collection.

**Table 5.1.** Most representative (strongest) microbial associations within non-delouser lumpfish. Positive (+) associations indicate cooperation while negative (-) associations indicate competition.

| Taxa | Phylum | Association | Correlation |
|---|---|---|---|
| Compromised welfare | | | |
| *Enhydrobacter* | Gammaproteobacteria | (+) | 1 |
| *Pseudoxanthomonas* | Gammaproteobacteria | (+) | |
| *Flavobacteraceae* | Bacteroidetes | (+) | |
| *Tepidimonas* | Gammaproteobacteria | (+) | |
| *Arcobacter* | Epsilonbacteraeota | (+) | |
| *Cloacibacterium* | Bacteroidetes | (+) | |
| *Synechococcus* | Cyanobacteria | (+) | |
| *Staphylococcus* | Firmicutes | (-) | -0.598 |
| *Mycoplasma* | Tenericutes | (-) | |
| Good welfare | | | |
| *Cetobacterium* | Fusobacteria | (+) | 1 |
| *Shewanella* | Gammaproteobacteria | (+) | |
| *Staphylococcus* | Firmicutes | (+) | |
| *Pseudoxanthomonas* | Gammaproteobacteria | (+) | |
| *Francisella* | Gammaproteobacteria | (+) | |
| *Plesiomonas* | Gammaproteobacteria | (+) | |
| *Cloacibacterium* | Bacteroidetes | (+) | |
| *Synechococcus* | Cyanobacteria | (+) | |
| *Tepidimonas* | Gammaproteobacteria | (+) | |
| *Aliivibrio* | Gammaproteobacteria | (+) | |
| *Piscirickettsia* | Gammaproteobacteria | (-) | -0.945 |
| *Photobacterium* | Gammaproteobacteria | (-) | |

**Table 5.2.** Most representative (strongest) microbial associations within delouser lumpfish. Positive (+) associations indicate cooperation while negative (-) associations indicate competition.

| Taxa | Phylum | Association | Correlation |
|---|---|---|---|
| Compromised welfare | | | |
| *Burkholderia-Caballeronia-Paraburkholderia* | Gammaproteobacteria | (+) | 1 |
| *Pseudoxanthomonas* | Gammaproteobacteria | (+) | |
| *Aliivibrio* | Gammaproteobacteria | (-) | -0.696 |
| *Tenacibaculum* | Bacteroidetes | (-) | |
| Good welfare | | | |
| *Arcobacter* | Epsilonbacteraeota | (+) | 1 |
| *Enterobacteriaceae* | Gammaproteobacteria | (+) | |
| *Uncultured* | Alphaproteobacteria | (+) | |
| *Glutamicibacter* | Actinobacteria | (+) | |
| *Thermus* | Deinococcus-Thermus | (+) | |
| *Pseudoalteromonas* | Gammaproteobacteria | (+) | |
| *Acinetobacter* | Gammaproteobacteria | (+) | |
| *Burkholderia-Caballeronia-Paraburkholderia* | Gammaproteobacteria | (+) | |
| *Brevundimonas* | Alphaproteobacteria | (+) | |
| *Cloacibacterium* | Bacteroidetes | (+) | |
| *Pseudoxanthomonas* | Bacteroidetes | (+) | |
| *Francisella* | Gammaproteobacteria | (+) | |
| *Tepidimonas* | Gammaproteobacteria | (+) | |
| *Paracoccus* | Alphaproteobacteria | (+) | |
| *Sphingobium* | Alphaproteobacteria | (+) | |
| *Vibrio* | Gammaproteobacteria | (-) | -0.998 |
| *Photobacterium* | Gammaproteobacteria | (-) | |

**Table 5.3**. Results for quantitative network comparison in non-delouser lumpfish (Compromised welfare, n=10; Good welfare, n=6) on genus-level via permutation tests (n=1000).

| | Compromised welfare | Good welfare | Absolute difference | $p_{adj}$ |
|---|---|---|---|---|
| Global network properties | | | | |
| Number of components | 1.000 | 1.000 | 0.000 | 1.000 |
| Clustering coefficient | 0.742 | 0.766 | 0.025 | 0.881 |
| Moduarity | 0.147 | 0.061 | 0.086 | 0.267 |
| Positive edge percentage | 89.020 | 72.527 | 16.492 | 0.366 |
| Edge density | 0.483 | 0.689 | 0.206 | 0.297 |
| Natural connectivity | 0.272 | 0.337 | 0.064 | 0.564 |
| Vertex connectivity | 3.000 | 14.000 | 11.000 | **0.029** |
| Edge connectivity | 3.000 | 14.000 | 11.000 | **0.039** |
| Average dissimilarity | 0.740 | 0.667 | 0.072 | 0.426 |
| Average path length | 1.021 | 0.950 | 0.071 | 0.178 |
| Degree (normalized) | | | | |
| *Photobacterium* | 0.094 | 0.906 | 0.812 | 0.323 |
| *Francisella* | 0.125 | 0.688 | 0.562 | 0.989 |
| *Uncultured* | 0.312 | 0.812 | 0.500 | 0.989 |
| *Clostridium sensu stricto* | 0.156 | 0.594 | 0.438 | 0.989 |
| *Clostridiaceae* | 0.219 | 0.656 | 0.438 | 0.969 |
| Betweenness centrality (normalized) | | | | |
| *Psychrobacter* | 0.107 | 0.006 | 0.101 | 0.312 |
| *Burkholderiaceae* | 0.091 | 0.006 | 0.085 | 0.468 |
| *Shewanella* | 0.087 | 0.002 | 0.085 | 0.312 |
| *Staphylococcus* | 0.058 | 0.001 | 0.057 | 0.468 |
| *Enterobacteriaceae* | 0.014 | 0.054 | 0.040 | 0.779 |
| Eigenvector centrality (normalized) | | | | |
| *Francisella* | 0.035 | 1.000 | 0.965 | 0.793 |
| *Enhydrobacter* | 1.000 | 0.191 | 0.809 | 0.963 |
| *Aeromonas* | 0.967 | 0.218 | 0.749 | 0.793 |
| *Plesiomonas* | 0.376 | 1.000 | 0.624 | 0.963 |
| *Arcobacter* | 1.000 | 0.388 | 0.612 | 0.793 |

**Table 5.4**. Results for quantitative network comparison in delouser lumpfish (Compromised welfare, n=13; Good welfare, n=5) on genus-level via permutation tests (n=1000).

| | Compromised welfare | Good welfare | Absolute difference | $p_{adj}$ |
|---|---|---|---|---|
| Global network properties | | | | |
| Number of components | 1.000 | 1.000 | 0.000 | 1.000 |
| Clustering coefficient | 0.568 | 0.825 | 0.258 | 0.327 |
| Moduarity | 0.183 | 0.138 | 0.045 | 0.941 |
| Positive edge percentage | 72.093 | 79.339 | 7.246 | 0.881 |
| Edge density | 0.383 | 0.647 | 0.264 | 0.634 |
| Natural connectivity | 0.147 | 0.359 | 0.211 | 0.257 |
| Vertex connectivity | 2.000 | 11.000 | 9.000 | 0.198 |
| Edge connectivity | 2.000 | 13.000 | 11.000 | 0.089 |
| Average dissimilarity | 0.834 | 0.643 | 0.191 | 0.198 |
| Average path length | 1.063 | 0.964 | 0.099 | 0.198 |
| Degree (normalized) | | | | |
| *Burkholderiaceae* | 0.212 | 0.970 | 0.758 | 0.073 |
| *Undibacterium* | 0.182 | 0.909 | 0.727 | 0.073 |
| *Clostridiaceae* | 0.242 | 0.848 | 0.606 | 0.073 |
| *Arenicella* | 0.303 | 0.909 | 0.606 | 0.117 |
| *Aliivibrio* | 0.364 | 0.909 | 0.545 | 0.073 |
| Betweenness centrality (normalized) | | | | |
| *Sphingobium* | 0.108 | 0.000 | 0.108 | 0.311 |
| *Undibacterium* | 0.000 | 0.085 | 0.085 | 0.518 |
| *Piscirickettsia* | 0.019 | 0.095 | 0.076 | 0.518 |
| *Pseudomonas* | 0.055 | 0.000 | 0.055 | 0.840 |
| *Psychrobacter* | 0.057 | 0.004 | 0.053 | 0.678 |
| Eigenvector centrality (normalized) | | | | |
| *Glutamicibacter* | 0.109 | 1.000 | 0.891 | 0.376 |
| *Undibacterium* | 0.108 | 0.989 | 0.882 | 0.376 |
| *Arenicella* | 0.172 | 0.962 | 0.790 | 0.376 |
| *Aeromonas* | 0.172 | 0.951 | 0.780 | 0.376 |
| *Thermus* | 0.261 | 1.000 | 0.739 | 0.376 |

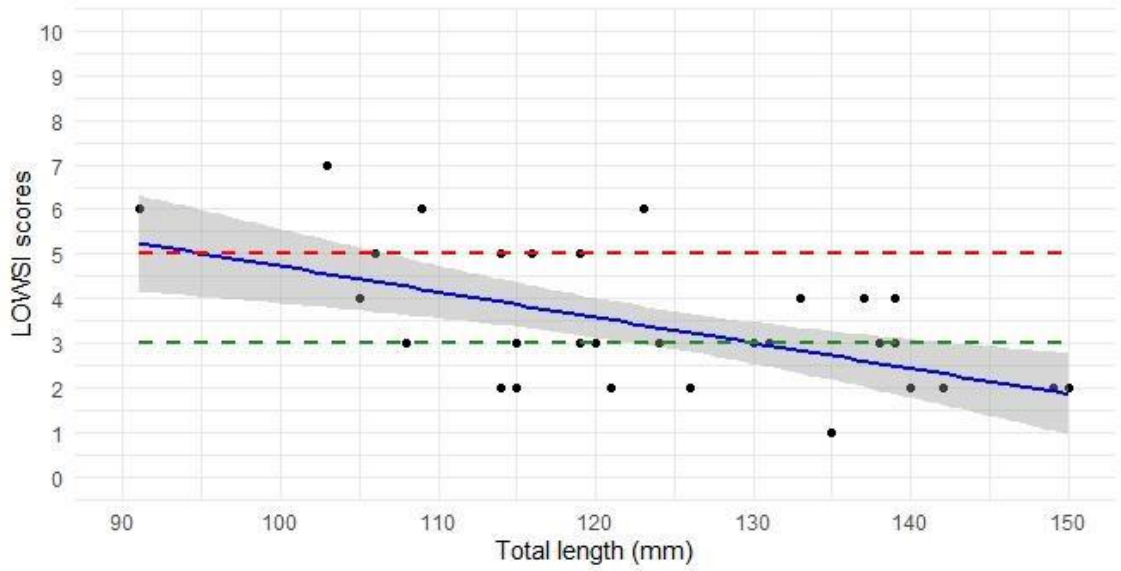



**Figure 5.1**. Effect of size (total length, mm) on lumpfish welfare . The grey shadow represents confidence intervals (CI=95%). The green dashed line divides lumpfish from classes A and B, while the red dashed line separates lumpfish from classes B and C.

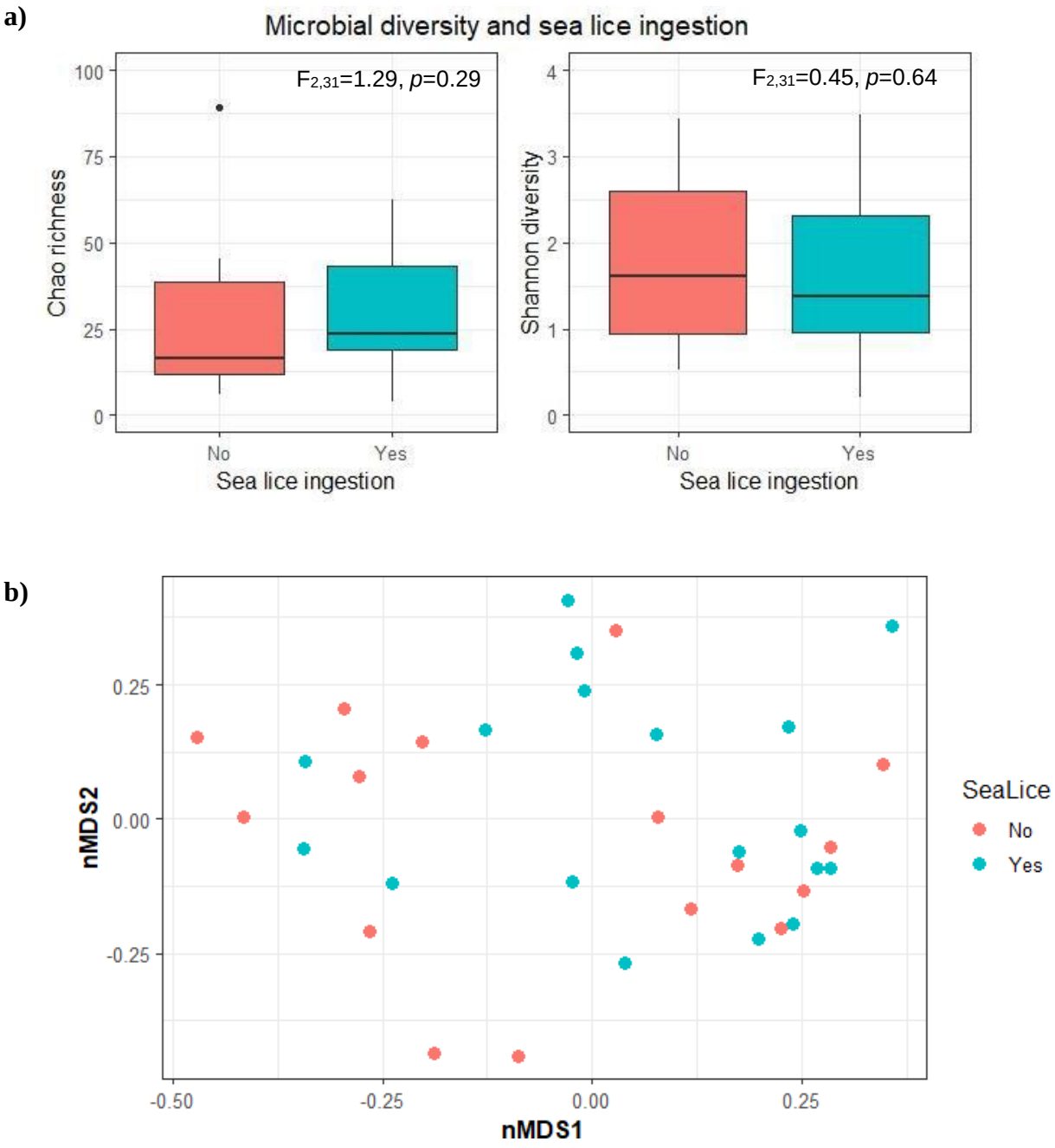



**Figure 5.2**. Microbial diversity and composition analysis, adjusted for body size. **a)** Boxplots of alpha diversity values (Chao's species richness and Shannon diversity), according to non-sea lice eating-lumpfish (No, n=16) and sea lice eating-lumpfish (Yes, n=18) groups. **b)** Non-metric dimensional scale (nMDS) of beta diversity (Bray-Curtis distances) for the same delousing groups.

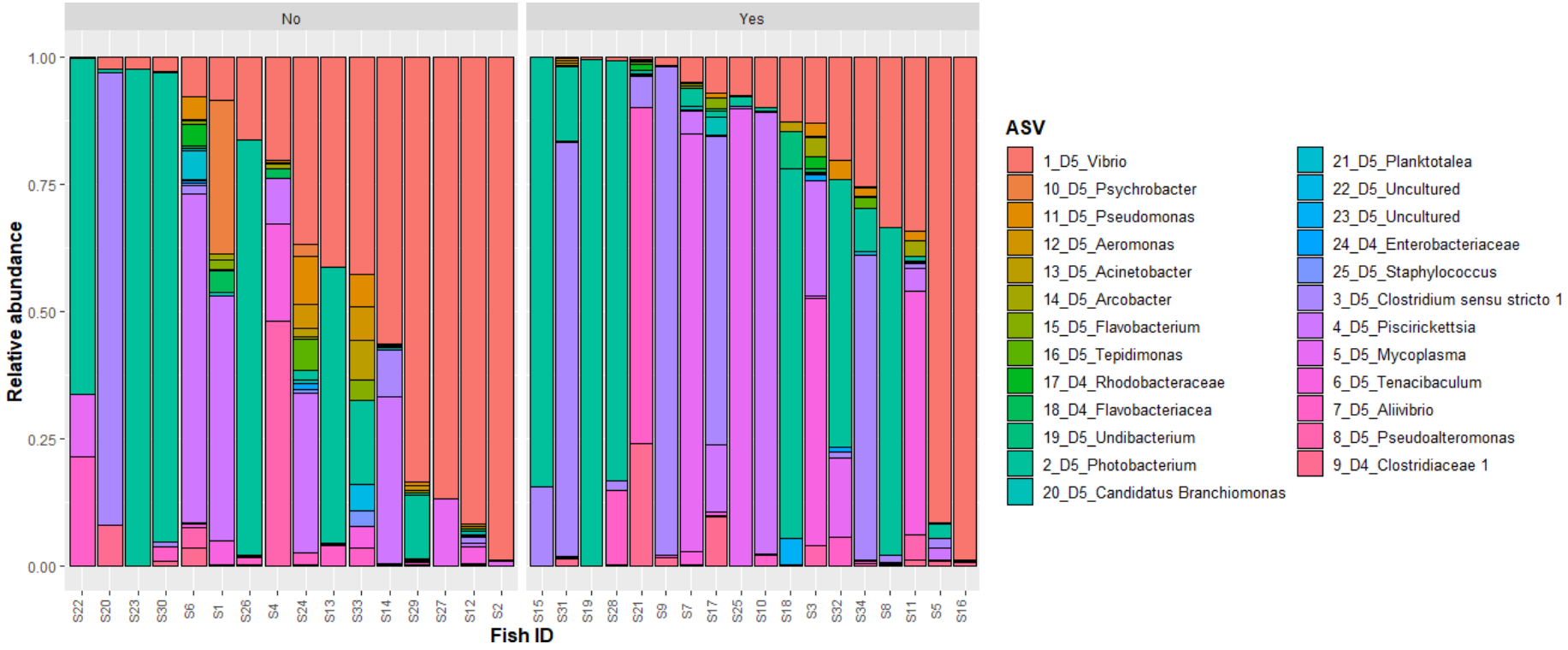



**Figure 5.3**. The 25 most representative ASVs (at genus level) according to each delousing group (Sea lice ingestion: No, n=16 and Yes, n=18).

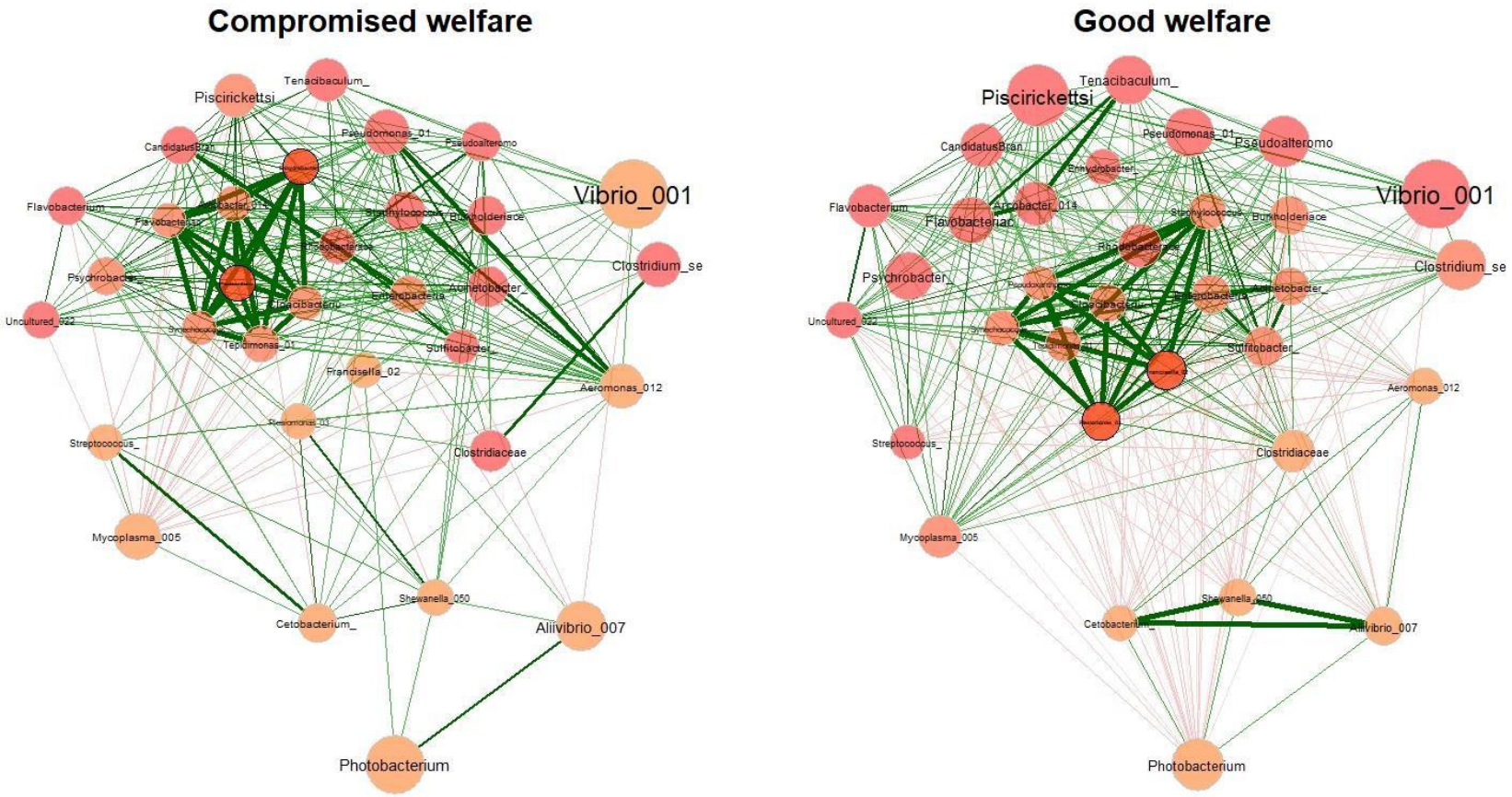



**Figure 5.4**. Microbiome network comparison for non-delouser lumpfish (Compromised welfare, n=10; Good welfare, n=6) on genus-level with 33 taxa per group, using Pearson's correlation coefficient as association measure and Fruchterman & Reingold layout. Positive associations are shown by green lines while negative interactions are shown by red lines. The thickness of the connection lines represents the correlation strength between taxa.

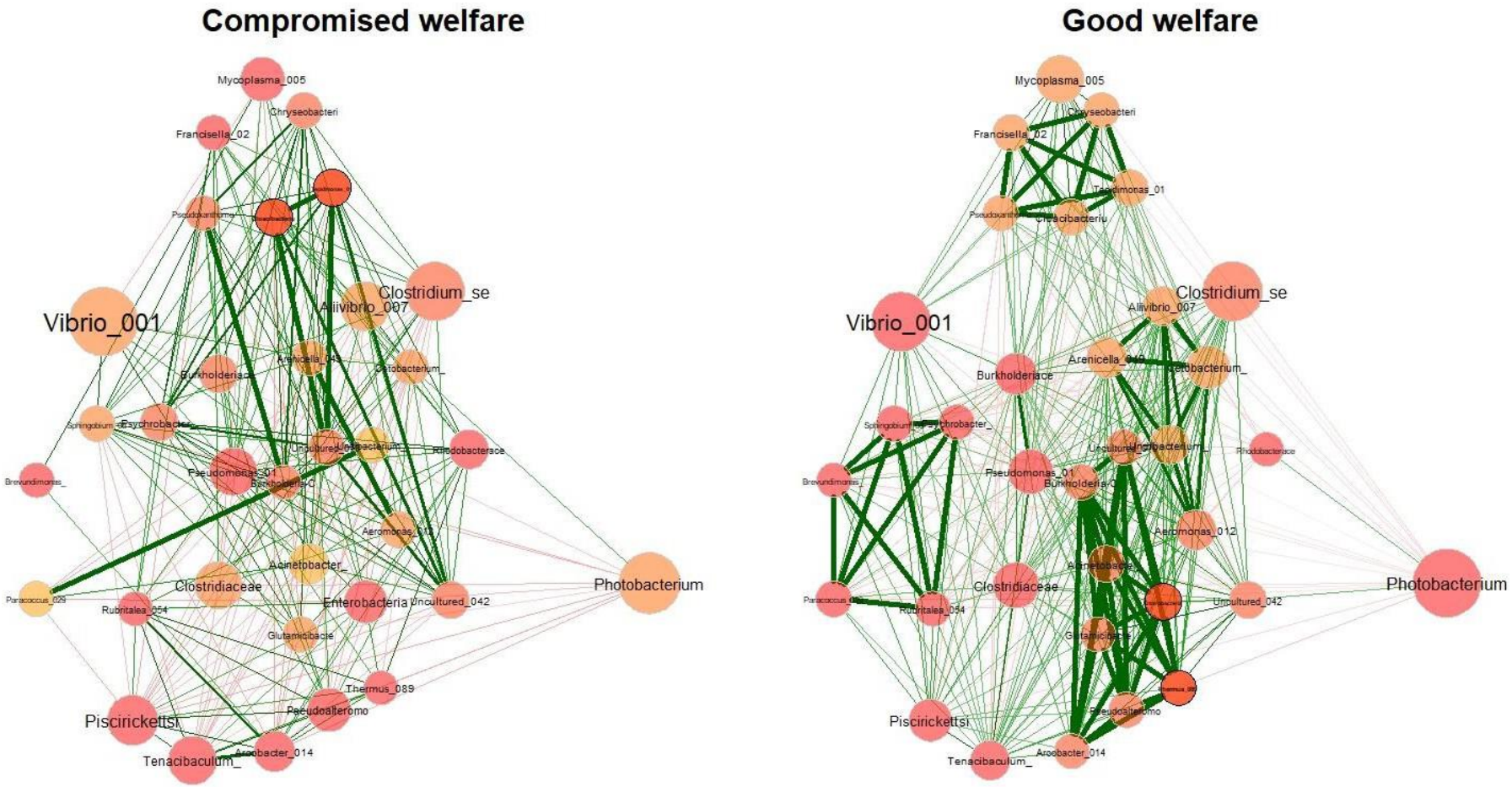



**Figure 5.5**. Microbiome network comparison for delousing lumpfish (Compromised welfare, n=13; Good welfare, n=5) on genus-level with 34 taxa per group, using Pearson's correlation coefficient as association measure and Fruchterman & Reingold layout. Positive associations are shown by green lines while negative interactions are shown by red lines. The thickness of the connection lines represents the correlation strength between taxa.

# Chapter 6 General discussion and conclusions

Lumpfish (*Cyclopterus lumpus* L.) have been farmed for the past 10 years and are now being widely used as cleaner fish in commercial salmon farms in many countries like Norway (Imsland et al., 2018a), Iceland (Steinarson and Árnason, 2018), Ireland (Bolton-Warberg, 2018), Scotland (Treasurer, 2018a), Faroe Islands (Eliasen et al., 2018) and Canada (Haugland et al., 2020). *Lepeophtheirus salmonis* continues being a major threat for the salmonid aquaculture (Igboeli et al., 2014), compromising fish welfare and the environment, and becoming resistant to almost every possible treatment (Denholm et al., 2002, Aaen et al., 2015). The stress associated to medicinal baths and the high mortalities due to mechanical and thermal treatments (Overton et al., 2019), make the use of cleaner fish a green and cost-effective alternative. However, the use of lumpfish comes with welfare implications.

Fish farmers have been reproved by the NFSA (Stranden, 2020), who claims their job may be not good enough as many lumpfish still disappear or die in cages. Lumpfish mortalities in cages are undoubtedly high and can range from 27% to 100% (Nilsen et al., 2014, EURLFD, 2016, Poppe, 2017, OneKind, 2018, Geitung et al., 2020, Erkinharju et al., 2021), indicating that lumpfish welfare is undoubtedly deteriorated. Hence, there are ethical concerns of whether the use of lumpfish continue to be acceptable and appropriate, due to the difficulties this species have at surviving in cages (Merakerås, 2020). Another issue is the fact that, despite salmon and lumpfish sharing the same environment at sea, salmon farming has developed in a way that covers well the needs of salmon, but not those of lumpfish (Garcia de Leaniz et al., 2021), and lumpfish are seen less valuable than salmon as they are not destined for human consumption. Some changes have been introduced in the salmon cages to accommodate the lumpfish needs for shelter (Imsland et al., 2015b, Imsland et al., 2018b, Imsland and Conlon, 2019a), but satisfying the needs of two different species under farming conditions has proven to be challenging.

***Lumpfish welfare and stress***

Lumpfish welfare is now a priority and the focus of some of the farmed fish welfare schemes (RSPCA, 2018). A practical way to measure, assess and lately improve welfare under farm conditions, is the use of Operational Welfare indicators (OWIs). Several OWIs have been developed for lumpfish during the past three years but many are unpractical, need training and instruments (laboratory-based OWIs) or require lethal sampling, which is not sustainable. Chapter 2 screened and selected different OWIs, already used in lumpfish or adapted from other farmed fish species (Freitas et al., 2014), with the aim to find the most practical and repeatable ones to combine them into an easy-to-use scoring index (LOWSI). The application of LOWSI (Table 2.3) allows assessing welfare in lumpfish populations under farming conditions in a rapid but still accurate manner and is very valuable for the aquaculture industry (mainly lumpfish producers and salmon farmers that use lumpfish as cleaner fish). Moreover, this index could benefit policy makers, NGOs and animal welfare associations that need scientific references to develop welfare standards and accreditations. The use of LOWSI in six commercial sites, including hatcheries and sea farms, revealed that lumpfish welfare can easily deteriorate, with 27% of lumpfish suffering from compromised welfare and 2% from severely poor, meaning that although some farms achieve welfare standards there are still some others that do not (Gutierrez Rabadan et al., 2021), and there is scope for improvement. Welfare can be monitored in regular basis to obtain a significant representation of the welfare status on lumpfish stocks and act as an early warning of health and welfare issues; but also, to assess specific farming operations such as grading, vaccination, disease treatments, transport, deployment at sea, etc. where is likely that welfare is compromised due to stress.

To better understand when welfare could begin to deteriorate in sea cages, lumpfish were monitored at three weeks and three months after deployment. Chapter 3 found that welfare significantly deteriorates with time spent at sea, which translated into worse LOWSI scores over time. It was also suggested that lumpfish size was a predictor of welfare, and this was also demonstrated in Chapter 5 where lumpfish with good welfare were nearly as 10% larger in average than lumpfish with compromised welfare. These results may suggest that poor welfare in lumpfish may have a negative impact on growth, as reduced growth in fish is often seen as indicative of poor welfare (Compassion In World Farming, 2009).

Lumpfish have shown modest cortisol stress responses in comparison to Ballan wrasse and Atlantic salmon (Treasurer et al., 2018b), which are more susceptible to stress and present higher levels of plasma cortisol (around 218 and 254 ng·ml-1, respectively) than those observed in lumpfish (circa 72.5 ng·ml-1) one hour after crowding stress (Iversen et al., 2015). Unlike other species, lumpfish apparently lack acute cortisol stress response, and seem not to reflect their stress levels externally. Chapter 2 used plasma cortisol levels to validate the scoring index and was found to be lower in hatcheries than in sea cages, which also gave insights into the stress experienced by lumpfish when cohabiting with salmon in sea farms. An adaptation period may be highly recommendable as lumpfish that have previously exposed to salmon have less plasma cortisol levels and calmer behaviour than naïve lumpfish (Staven et al., 2019), suggesting lumpfish may need some time to acclimate to cage conditions before they start showing any cleaning behaviour. Some acclimatization cages, previously trialled for farmed ballan wrasse (Brooker et al., 2020), have been successful and could be likely applied to lumpfish as well.

Ideally, lumpfish should live long enough at sea to match the salmon's grow out stage in cages until harvest to ensure sea lice levels are under control, unless their health or welfare is compromised. Salmon's production cycle at sea can last for 12-14 months, depending on the input, which indicates that lumpfish will be around 500g-1kg if they survive. However, it is thought that lumpfish may decrease their delousing efficacy as they become older and may prefer to eat salmon pellets to have a better satiation than feeding on sea lice. In that case, lumpfish could be removed from sea cages and kept in captivity (and quarantine) to be used as a brood stock in the future, which will improve the sustainability of the industry.

At the moment this has not been possible in significant numbers due to low survival at the end of the salmon's production life. If survival was significant, there would be a need to deal with lumpfish at the slaughterhouse, and proper methods for humanely culling this species, along a market for the use of their flesh should be in place.

### *Lumpfish transfer and deployment at sea*

The transition of lumpfish from hatchery to sea cages involves a series of stressors: from the handling to be loaded into lorries or wellboats and the transfer *per se* (Sampaio and Freire, 2016); to deployment into sea cages, a new unknown environment with new fast-

swimming species (salmon). It is then not surprising that some of the acute mortalities, occurring immediately or few weeks after release in sea cages, are associated to transport (Bornø et al., 2016), although the exact numbers are unknown. Chapter 4 investigated the effect of the transfer and the first two months at sea in lumpfish welfare. Although mean LOWSI did not vary in this period, eye condition deteriorated in 41% of lumpfish, and body condition decreased in 16% after deployment. These results may be related as visual impairment could increase the risk of emaciation, being lumpfish visual feeders (Jonassen et al., 2017, Powell et al., 2018b). Future research on lumpfish feeds at the sea cage stage is also crucial to avoid nutritional deficiencies that can lead to the presence of cataracts.

One of the best strategies to safeguard the welfare of lumpfish during transfer and deployment at sea is to ensure all staff are competent and well trained on Specific Operational Procedures (SOPs) at each of the stages (hatchery pre-conditioning, loading, transport and delivery). Understanding all the steps, good communication between the different parties involved and commitment is critical for a good deployment and a high chance of survival. For complying with these, best practice guidelines are available (Sigstadstø, 2017).

***Lumpfish microbial communities***

Microbiome applications are promising as they have the potential for monitoring health and welfare in farmed animals and can be a predictor of stress-related conditions and biomarkers in aquaculture (Perry et al., 2020). Studies of lumpfish gut microbiota are still rare. Lumpfish gut microbiota was characterized in Chapter 4 and compared between stocks showing main differences in diversity. Low microbial diversity in fish gut has popularly been associated with poor health (Talwar et al., 2018), although this is not necessarily always the case (Johnson and Burnet, 2016). *Mycoplasma* dominated the gut microbiota of Icelandic lumpfish, which were larger and showed better welfare than Scottish lumpfish. The role of *Mycoplasma* in the gut microbiota of salmon has recently been described as symbiotic or commensal (Cheaib et al., 2020) and has characterized the gut of healthy salmon as well as its relative abundance has been positive correlated with the fish weight (Bozzi et al., 2021).

This thesis found that some specific taxa could be used as potential biomarkers such as *Candidatus branchiomonas* and *Clostridium*, found to be associated to compromised welfare and high plasma cortisol levels, respectively. *Candidatus*

*branchiomonas* has been associated with complex gill disease in salmon (Gjessing et al., 2019), while genera from the Clostridia class were promoted in stressed salmon (Uren Webster et al., 2020b). In this case, microbial communities were sequenced using intestine tissue, but non-lethal approaches (using faeces or even skin mucus) could be used instead to reduce the number of sampling casualties and increase sustainability. The use of accurate microbial biomarkers (to measure stress, welfare or even health status) can be a valuable diagnostic tool for the industry, not only to understand the biology of fish but also to see how they perform under different conditions and environments. Currently it may not be economical, but next generation sequencing seems to be expanding in a way that microbiome sampling will be possible in the farm environment on regular basis fairly soon.

***Sea lice ingestion***

Chapter 5 assessed the effect of sea lice ingestion in welfare and microbial communities and found that the fact of eating or not eating sea did not have any influence on welfare and gut microbiome.

Cleaning behaviour in lumpfish has proven to have a genetical component (Imsland et al., 2016a), which means that some genetic stocks may be more suitable to be used as cleaner fish than others. It could seem that selecting lumpfish families or stocks for slow growth rate in sea cages, as suggested in Chapter 3, could be a potential strategy to maximise lumpfish delousing efficiency, described to be 40% better in smaller size than larger size lumpfish (Imsland et al., 2016b, Imsland et al., 2021).

High variation in sea lice grazing is extremely common, which poses doubts about the efficiency of cleaner fish (Anon, 2020d). In this thesis, the prevalence of ingested sea lice found in lumpfish stomachs was very variable and ranged from 1.5 to 52%. Other authors, however, have found this prevalence to range from 13 to 38% (Eliasen et al., 2018, Imsland et al., 2018a). Sea lice ingestion in lumpish can be affected by factors like the infection levels on the farm or the sampling method, among others. Chapters 3 and 4 showed relatively low infection levels at the sea farms (1 and 0.2 average sea lice/salmon) and sea lice ingestion occurred in 1.5% and 5% of the population; whereas Chapter 5 showed higher infection pressure (5.5 sea lice/salmon) and 52% of lumpfish ingested sea lice. Sea lice ingestion was quantified by assessing lumpfish stomach

contents, although the fact of not finding sea lice in the stomachs does not necessarily mean that lumpfish did not ingest them, as digestion times can vary between individuals (Eysturskarð et al., 2017, Imsland et al., 2019c). It appears that there is a widespread suboptimal use of lumpfish (Overton et al., 2020), reflecting that many producers may excel at rearing lumpfish in hatcheries while husbandry is inadequate for lumpfish to thrive at sea. Robust evidence of their efficacy is needed to justify their use, constrained by the limited number of studies performed under commercial conditions (Overton et al., 2020). It is important that research is applicable into the industry, mimicking the conditions of a real farm.

***Stock differentiation***

Phenotypic differences have been reported between lumpfish from different genetical backgrounds, where Northern lumpfish grew faster than Southern lumpfish(Whittaker, 2019). Chapter 3 compared English and Norwegian lumpfish whileChapter 4 assessed the performance of Icelandic and Scottish lumpfish. Growth rateswere reported to be faster in Icelandic than Scottish lumpfish in Chapter 3, which arein accordance with results found by Whittaker et al. (2018). In contrast, English lumpfishgrew faster than Norwegian lumpfish in Chapter 4, oppositely than expected. This thesis results suggest that faster growth rates were observed on those lumpfish that predominantly fed formulated pellets, e.g., Icelandic and English stocks, indicating that formulated feed has a high nutritional value and promotes growth, although fast growth is not an aim for lumpfish in sea cages. Further research in the genetic structure of lumpfish as well as in feed management at sea cages is needed.

***Lumpfish underweight and emaciation***

The length-weight percentile charts (Appendix, Figure S2.6), developed in Chapter 2, are a useful tool to monitor body condition and identify the proportion of underweight and emaciated individuals in lumpfish populations. Chapter 2 found 28% of underweight lumpfish and 10% emaciated when assessing six commercial sites, indicating that there is a need for a suitable feed management plan, especially after deployment in sea cages (Gutierrez Rabadan et al., 2021), as it cannot be expected that lumpfish rely only on feeding sea lice. Lumpfish can easily lose body condition in four weeks if not optimally reared (Johannesen et al., 2018). Chapter 3 showed that the

proportion of emaciated lumpfish increased with time at sea, highlighting the importance of providing supplementary feeding (for instance, the use of feed blocks) in sea cages to maintain lumpfish nutritional needs, condition and welfare. Further research into lumpfish nutritional requirements, specific needs during the deployment stage (including the use of probiotics to boost the immune system) as well as an agreed criteria for supplemental feeding in cages is needed.

## Conclusions

- A reliable scoring index for lumpfish was developed, validated and tested under commercial conditions and proved to be a practical tool to monitor welfare in lumpfish. This index consisted in the visual assessment of body damage, caudal fin damage, eye condition, suction disc deformities and body condition and its usage revealed that welfare can deteriorate at any stage of thelumpfish life cycle.

- Lumpfish welfare worsened with time spent at sea, with increasing prevalenceof most of the OWIs and with fish size, with smaller fish showing the worst welfare scores. These results showed that the three first months at sea can be acritical period for lumpfish and welfare monitoring should be particularly regular.

- Under a common commercial environment, Icelandic and Scottish lumpfish differed in growth rates, diet and welfare scores with Icelandic lumpfish growing faster, ingesting more formulated pellets and showing better welfare scores than Scottish lumpfish. These differences were also reflected in their gut microbial communities, where Scottish lumpfish gut microbiota was muchmore diverse. Significant associations found indicated that the abundance of specific taxa such as *Candidatus branchiomonas* and *Clostridium* and welfarestatus and cortisol levels, suggesting these could be used as potential markers of welfare and stress, respectively.

- Sea lice ingestion did not influence lumpfish body size or condition, plasma cortisol levels, welfare scores or intestinal microbial communities, suggesting that sea lice ingestion is not harmful for lumpfish welfare. The lumpfish studied were sampled close to the end of the salmon grow-out cycle and presented moderately compromised welfare, but this does not appear to be associated with sea lice consumption.

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# Supplementary tables

**Table S2.1**. Reported use and utility of lumpfish welfare indicators based on the results of a questionnaire given to 53 participants attending the 1st Symposium on Welfare in Aquaculture (Swansea, 2019). Pseudo-medians and 95% confidence intervals are shown for the Likert-scale responses (1: Not useful, 2: Rarely useful, 3: Moderately useful, 4: Useful, 5: Very useful).

| **Welfare indicator** | **Reported use** | **n** | **(Pseudo) Median** | **95% CI** | ***p*** |
|---|---|---|---|---|---|
| Fin damage | 96.8 % | 53 | 5.0 | 4.5-5.0 | <0.001 |
| Mortality | 80.6 % | 52 | 4.5 | 4.5-5.0 | <0.001 |
| Suction disc deformities | 80.6 % | 50 | 4.5 | 4.5-5.0 | <0.001 |
| Skin damage | 77.4 % | 53 | 4.5 | 4.5-5.0 | <0.001 |
| Eye damage | 77.4 % | 51 | 4.5 | 4.5-5.0 | <0.001 |
| Condition factor | 61.3 % | 53 | 4.0 | 4.0-4.5 | <0.001 |
| Poor growth | 61.3 % | 53 | 4.0 | 4.0-4.0 | <0.001 |
| Disease/Parasites | 61.2 % | 51 | 4.5 | 4.5-5.0 | <0.001 |
| Body deformities | 58.1 % | 52 | 4.0 | 4.0-4.5 | <0.001 |
| Operculum/gill damage | 51.6 % | 50 | 4.5 | 4.0-4.5 | <0.001 |
| Body/eye darkening | 32.2 % | 43 | 4.0 | 4.0-4.5 | <0.001 |
| Blood parameters | 29.0 % | 48 | 4.0 | 3.5-4.0 | <0.001 |

**Table S2.2**. Lumpfish Operational Welfare Score Index (LOWSI) performance based on the results of a questionnaire given to 8 farmers that scored 150 lumpfish at 2 commercial sites. Pseudo-medians and 95% confidence intervals are shown for the Likert-scale responses (1: Strongly disagree, 2: Disagree, 3: Neutral, 4: Agree, 5: Strongly agree).

| **Performance trait** | **n** | **(Pseudo) Median** | **95% CI** | ***p*** |
|---|---|---|---|---|
| Q1. Practicality | 8 | 4.0 | 4.0-4.0 | 0.008 |
| Q2. Efficiency | 8 | 5.0 | 5.0-5.0 | 0.008 |
| Q3. Ease of implementation | 8 | 4.0 | 4.0-4.0 | 0.008 |
| Q4. Willingness to use | 8 | 4.0 | 3.5-4.5 | 0.012 |

**Table S2.3.** Model selection for the validation of the individual-based OWIs, using principal components (PC1 and PC2) and size (TL) as predictors of relative weight at pre- ($WR_{pre}$) and post- ($WR_{post}$) deployment. Only models with delta < 2 are represented. Included are degrees of freedom (df), log likelihood (logLik), corrected Akaike information criterion (AICc), delta and model weight.

| Model | PC1 | PC2 | TL | PC1:PC2 | PC1:TL | PC2:TL | PC1:PC2:TL | df | logLik | AICc | Delta | Weight |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Relative weight at pre-deployment ($WR_{pre}$) | | | | | | | | | | | | |
| $WR_{pre}56$ | x | x | x | | x | x | | 7 | -265.69 | 547.5 | 0.00 | 0.579 |
| $WR_{pre}64$ | x | x | x | x | x | x | | 8 | -265.20 | 549.2 | 1.71 | 0.246 |
| | | | | | | | | | | | | |
| Relative weight at post-deployment ($WR_{post}$) | | | | | | | | | | | | |
| $WR_{post}5$ | | | x | | | | | 3 | -120.98 | 248.7 | 0.00 | 0.407 |

**Table S2.4**. Model selection for the validation of the individual-based OWIs, using principal components (PC1 and PC2) and size (TL) as predictors of plasma cortisol levels at pre- ($Cort_{pre}$) and post- ($Cort_{post}$) deployment. Only models with delta < 2 are represented. Included are degrees of freedom (df), log likelihood (logLik), corrected Akaike information criterion (AICc), delta and model weight.

| Model | PC1 | PC2 | TL | PC1:PC2 | PC1:TL | PC2:TL | PC1:PC2:TL | df | logLik | AICc | Delta | Weight |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cortisol at pre-deployment ($Cort_{pre}$) | | | | | | | | | | | | |
| $Cort_{pre}1$ | x | | | | | | | 2 | -71.66 | 148.03 | 0.00 | 0.385 |
| $Cort_{pre}2$ | | | | | | | | 3 | -70.77 | 149.04 | 1.01 | 0.232 |
| Cortisol at post-deployment ($Cort_{post}$) | | | | | | | | | | | | |
| $Cort_{post}39$ | | x | x | | | x | | 5 | -190.89 | 393.8 | 0.00 | 0.376 |
| $Cort_{post}3$ | | x | | | | | | 3 | -194.39 | 395.6 | 1.73 | 0.158 |
| $Cort_{post}40$ | x | x | x | | | x | | 6 | -190.34 | 395.7 | 1.84 | 0.150 |

**Table S3.1.** Number of lumpfish stocked at deployment/cage (n=4) and sampled at each time-point (T1: three weeks, n=160; T2: three months, n=160) for each of the genetic stocks and families.

| Stock/family | Deployment | T1 (three weeks) | T2 (three months) |
|---|---|---|---|
| Norwegian | 2754 | 117 | 119 |
| English | 2256 | 43 | 41 |
| English family 1 | 752 | 6 | 8 |
| English family 2 | 752 | 35 | 30 |
| English family 4 | 752 | 2 | 3 |

**Table S3.2.** Summary information about the cohabiting salmon and lumpfish stocking densities in each cage at deployment and each of the time-points (T1,T2). pd = post-deployment.

| Date | Sea temperature (°C) | Cage | No. of salmon | Avg. weight (g) | No. of lumpfish | Stocking density (%) |
|---|---|---|---|---|---|---|
| 19/10/2018 (Lumpfish deployment) | 12.70 | 3 | 48,227 | 165.9 | 5,010 | 10.4 |
| | | 4 | 49,661 | 163.9 | 5,010 | 10.1 |
| | | 7 | 51,143 | 166.8 | 5,010 | 9.8 |
| | | 8 | 51,237 | 163.9 | 5,010 | 9.8 |
| 06/11/2018 (T1: three weeks pd) | 11.69 | 3 | 48,052 | 225.6 | 4,962 | 10.3 |
| | | 4 | 49,312 | 221.3 | 4,909 | 9.9 |
| | | 7 | 50,711 | 229.2 | 4,976 | 9.8 |
| | | 8 | 50,876 | 225.6 | 4,986 | 9.8 |
| 11/01/2019 (T2: three months pd) | 9.46 | 3 | 47,393 | 692.1 | 4,723 | 9.9 |
| | | 4 | 48,532 | 655.5 | 4,625 | 9.7 |
| | | 7 | 50,032 | 638.4 | 4,767 | 9.5 |
| | | 8 | 50,165 | 633.1 | 4,785 | 9.5 |

**Table S3.3**. Lumpfish tetranucleotide sequences used to generate the microsatellite panel M1-10 mix. $N_A$: Number of alleles per locus.

| Locus ID | Primer sequence (5' 3') | Repeated motifs | Allele size range (bp) | Origin | Dye | $N_A$ |
|---|---|---|---|---|---|---|
| NG_Clu121 | F: GGTGCACATCACCTCACATC<br>R: TGCATCTCCTCACCATGAAA | 4 | 250-286 | EST | NED | 11 |
| NG_Clu133 | F: GCAGAGGTGAGCCTTAGGAC<br>R: CACAAGTCTCTGCGCCATAA | 4 | 198-314 | Genomic | 6-FAM | 22 |
| NG_Clu202 | F: AATGAAAGAGGGAGCCACAG<br>R: CATCACAGTTGGCGAGAGTG | 4 | 308-360 | Genomic | 6-FAM | 8 |
| NG_Clu226 | F: GTTCGATTTCCAGGAACGAC<br>R: ATATCCAACACCCGGATGAA | 4 | 140-200 | EST | 6-FAM | 14 |
| NG_Clu277 | F: CCAAAGCAGCATGGGATATT<br>R: ATGGGCGTGTTATCCTGAAG | 4 | 290-362 | EST | VIC | 7 |
| NG_Clu330 | F: TCCTCCTCTTCCTCCCTTTC<br>R: CGGCGGAGCATAAAGATAAA | 4 | 100-128 | EST | VIC | 6 |
| NG_Clu344 | F: GATGACTGAGGAAGAAGCGG<br>R: AACTGGACCTCCTTGTGTCG | 4 | 185-241 | EST | VIC | 8 |
| NG_Clu355 | F: TCCATCCTCAACCCACTTTC<br>R: AACAAATTAGCAACCCACGC | 4 | 280-380 | EST | NED | 17 |
| NG_Clu410 | F: AGATCAGCGTCCTTAAGCCA<br>R: CGCGACCCTAATGAGGATAA | 4 | 120-172 | EST | NED | 6 |
| NG_Clu800 | F: AGCCATCACTCCCTCTTCCT<br>R: AAGACCGGATGTTTCCCATT | 4 | 240-292 | Genomic | VIC | 8 |

**Table S3.4**. Lumpfish microsatellite sequences used to generate the microsatellite panels M2-4 mix and M3-6 mix .

| Multiplex | Locus ID | Primer sequence (5' 3') | Repeated motifs | Allele size range (bp) | Dye |
|---|---|---|---|---|---|
| M2-4mix | Clu29 | F: CGCGCGGTCAGCTCATCCTTAG<br>R: TCGCGTGACGGACAGGTTTCG | 2 | 136-150 | PET |
| | Clu34 | F: TCTGCGATAGTAGCGTCAGGGTTC<br>R: AGGCCGGCTGATCAAGAGCAC | 2 | 191-225 | NED |
| | Clu36 | F: CACGGCGAGTCAGACGAGGC<br>R: GCTGCCGCTACTCCGCACAG | 3 | 191-211 | 6-FAM |
| | Clu45 | F: GCGCAGGAATGCGCCTGAAG<br>R: ACCGCAGCTTGTTGGGCAGG | 2 | 274-298 | PET |
| M3-6mix | Clu12 | F: CCACAACCGGTGGGTCCCG<br>R: ACGCTCCTTCTGATCTTCGCCC | 2 | 200-208 | 6-FAM |
| | Clu26 | F: CGAGAGAGGAGAACGCACGGC<br>R: GGCACAAGTGCATGGGCACG | 2 | 103-125 | 6-FAM |
| | Clu33 | F: TCATGCAAGCATTTGAGCGCCG<br>R: TGTTGCCTTGTAACTGCGCTTGAG | 2 | 179-197 | VIC |
| | Clu37 | F: CTTCACAGGTCGGGCGACGG<br>R: GCACAGCGATGACGCTTGCAG | 2 | 218-234 | PET |
| | Clu40 | F: TGGGCATACAGGTCTGAACACGC<br>R: GCCACCTGCTGCAGCCTCTC | 2 | 254-276 | NED |
| | Clu44 | F: CCGGCCCAGCCTGCCTTATG<br>R: TGCCTGGAAACAGTGTATGGCAC | 2 | 279-295 | 6-FAM |

**Table S3.5**. Model selection table for welfare model (Welf, LOWSI scores) with predictors of lumpfish origin (Or), sampling time point (SP) and size as total length (TL). Included are degrees of freedom (df), log likelihood (logLik), corrected Akaike information criterion (AICc), delta and model weight. Retained model is shown in bold.

| Model | Or | SP | TL | Or:SP | Or:TL | SP:TL | Or:SP:TL | df | logLik | AICc | Delta | Weight |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Welf.7** | | **x** | **x** | | | | | **3** | **-439.79** | **885.7** | **0.00** | **0.282** |
| Welf.39 | | x | x | | | x | | 4 | -439.19 | 886.5 | 0.83 | 0.186 |
| Welf.8 | x | x | x | | | | | 4 | -439.69 | 887.5 | 1.83 | 0.113 |
| Welf.40 | x | x | x | | | x | | 5 | -442.58 | 895.3 | 2.51 | 0.09 |

**Table S3.6**. Model selection table for sea lice ingestion model (SLI, counts) with predictors of lumpfish origin (Or), sampling time point (SP) and size as total length (TL). Included are degrees of freedom (df), log likelihood (logLik), corrected Akaike information criterion (AICc), delta and model weight. Retained model is shown in bold.

| Model | Or | SP | TL | df | logLik | AICc | Delta | Weight |
|---|---|---|---|---|---|---|---|---|
| **SLI.7** | | **x** | **x** | **3** | **-79.91** | **165.9** | **0.00** | **0.462** |
| SLI.3 | | x | | 2 | -81.47 | 167.0 | 1.08 | 0.270 |
| SLI.8 | x | x | x | 4 | -79.89 | 167.9 | 2.01 | 0.169 |

**Table S3.7**. Model selection table for pellet ingestion model (PI, counts) with predictors of lumpfish origin (Or), sampling time point (SP) and size as total length (TL). Included are degrees of freedom (df), log likelihood (logLik), corrected Akaike information criterion (AICc), delta and model weight. Retained model is shown in bold.

| Model | Or | SP | TL | df | logLik | AICc | Delta | Weight |
|---|---|---|---|---|---|---|---|---|
| **PI.8** | **x** | **x** | **x** | **4** | **-1190.27** | **2388.7** | **0.00** | **1** |
| PI.4 | x | x | | 3 | -1203.20 | 2412.5 | 23.80 | 0 |

**Table S4.1**. Defined microbial composition (theoretical %) in the ZymoBIOMICS® Microbial Community Standards (I and II), with species and concentrations contained, used as a control for the preparation of 16S metagenomic libraries.

| | *Microbial Community DNA Standard I* | | *Microbial Community DNA Standard II* | |
|---|---|---|---|---|
| Species | gDNA | 16S only | gDNA | 16S only |
| *Listeria monocytogenes* | 89.1 | 95.9 | 12 | 14.1 |
| *Pseudomonas aeruginosa* | 8.9 | 2.8 | 12 | 4.2 |
| *Bacillus subtilis* | 0.89 | 1.2 | 12 | 17.4 |
| *Saccharomyces cerevisiae* | 0.89 | NA | 2 | NA |
| *Escherichia coli* | 0.089 | 0.069 | 12 | 10.1 |
| *Salmonella enterica* | 0.089 | 0.07 | 12 | 10.4 |
| *Lactobacillus fermentum* | 0.0089 | 0.012 | 12 | 18.4 |
| *Enterococcus faecalis* | 0.00089 | 0.000067 | 12 | 9.9 |
| *Cryptococcus neoformans* | 0.00089 | NA | 2 | NA |
| *Staphylococcus aureus* | 0.000089 | 0.0001 | 12 | 15.5 |

**Table S4.2**. Percentage of each food item (ordered by abundance) contributing to the diet composition of lumpfish (n=60) sampled at the sea cage. The 'Crustacean' category included gammarids, caprellids and copepods while the 'Fish' category was constituted by tissue, scales and prey.

| Food item | Percentage |
|---|---|
| Crustaceans – Gammarids | 73.4 % |
| Unknown/unidentifiable | 11.5 % |
| Formulated feed (pellets) | 9.21 % |
| Seaweed | 1.65 % |
| Fish – Tissue | 1.43 % |
| Hydrozoans | 1.13 % |
| Crustaceans – Copepods | 0.87 % |
| Fish – Scales | 0.26 % |
| Plastics | 0.20 % |
| Sea lice | 0.13 % |
| Crustaceans – Caprellids | 0.09 % |
| Bivalves | 0.09 % |
| Fish – Prey | 0.04% |

**Table S5.1**. Detailed reagents and volumes for the 16S rRNA metagenomic library preparation with a few adjustments , employed for both chapters 2 and 3. The number of cycles varied between PCR 1 (x30) and PCR2 (x7).

| **1st stage PCR (25µl)*** | **2nd stage PCR (27.5µl)**** | **Thermal Cycler program** |
|---|---|---|
| 12.5µl 2x InvitrogenPlatinum™ | 12.5µl 2x Invitrogen Platinum™ | Activation: 95°C (3 min) |
| 8µl microbial DNA | 2.5µl DNA (product PCR 1) | 95°C (30 sec) ⎫ ⎧ |
| 0.5µl F515 primer (10µM) | 1.25µl Nextera N7xx Index | 55°C (30 sec) ⎬ x30* ⎨ x7** |
| 0.5µl R806 primer (10µM) | 1.25µl Nextera S5xx Index | 72°C (30 sec) ⎭ ⎩ |
| 3.5µl molecular water | 10µl molecular water | Elongation: 72°C (5 min) |

## Supplementary figures

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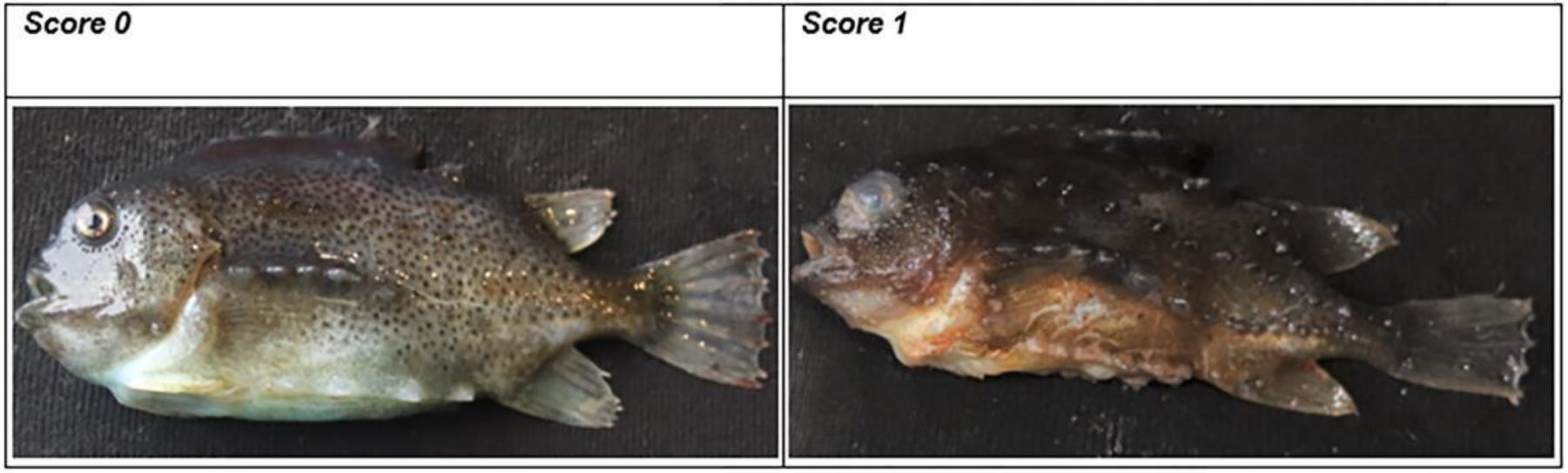



**Figure S2.1.** Scoring (0–1) of external body damage (0: no external lesions, 1: presence of skin lesions such as reddening, abrasion, wounds and ulcers).

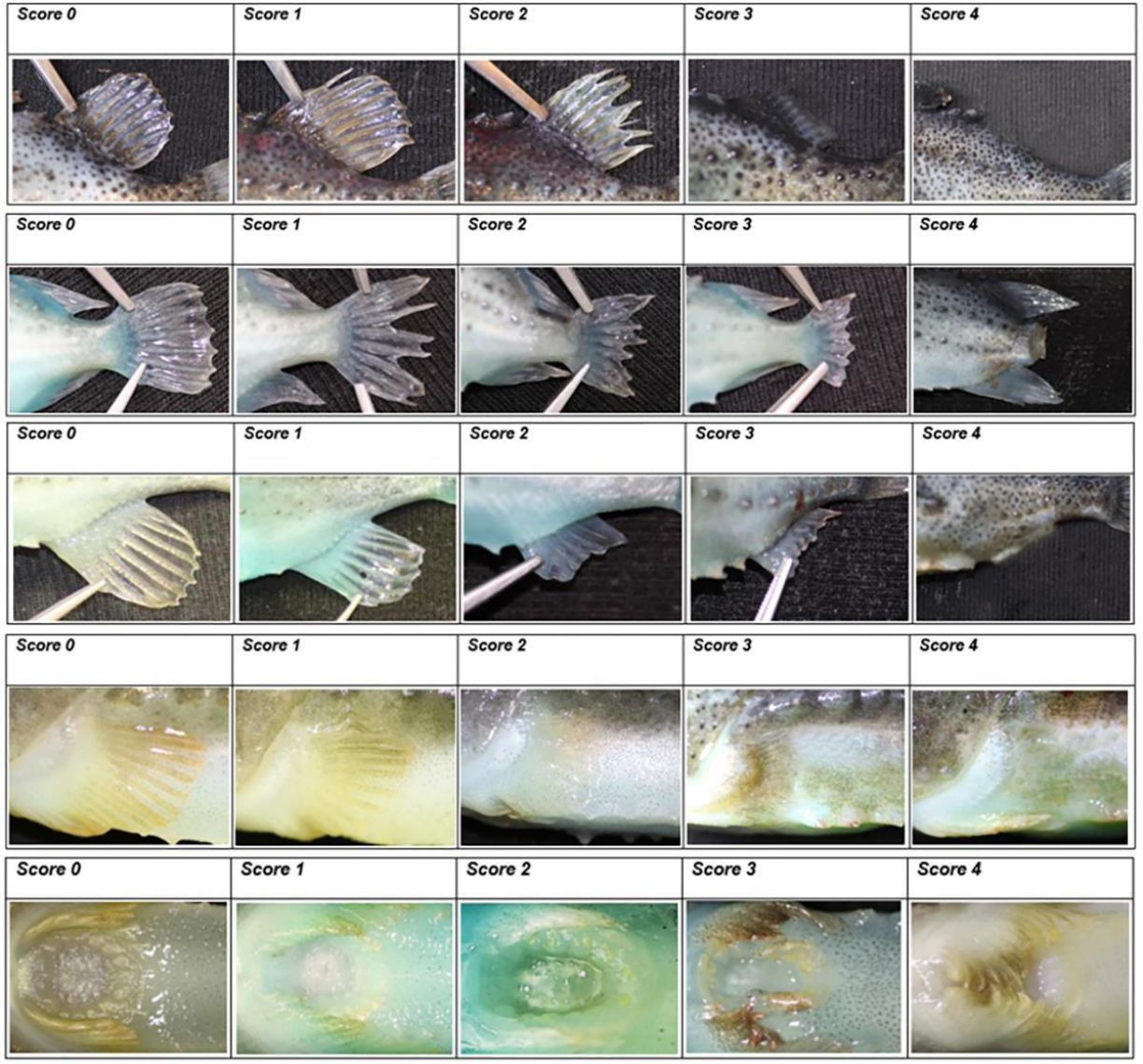



**Figure S2.2.** Scoring (0–4) of fin damage (0: fin in good condition, with no damage, splitting, thickening or deformities present; 1: minor damage affecting up to 25% of the fin area; 2: moderate damage affecting 25–50% of the fin; 3: substantial damage affecting 50–75% of the fin; 4: severe damage, muscle tissue frequently exposed, affecting more than 75% of the fin). Image forscores 3–4 for the dorsal, anal, and pectoral fins were reconstructed with Photoshop, as these scoreswere not found in the test.

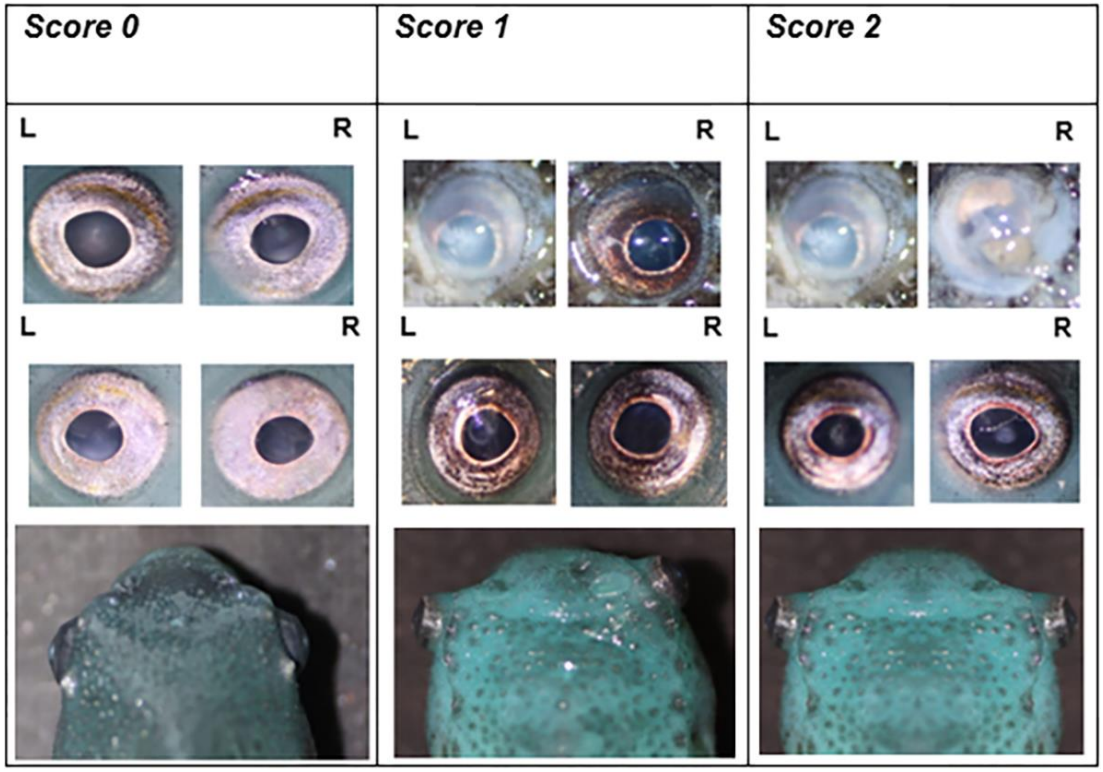



**Figure S2.3.** Scoring (0–2) of three eye conditions (0: eyes in good condition with no damage, exophthalmia or cataracts; 1: unilateral damage, exophthalmia or cataract; 2: bilateral damage, exophthalmia or cataract). Image for score 2 was reconstructed in Photoshop as this score was not found in the test sample.

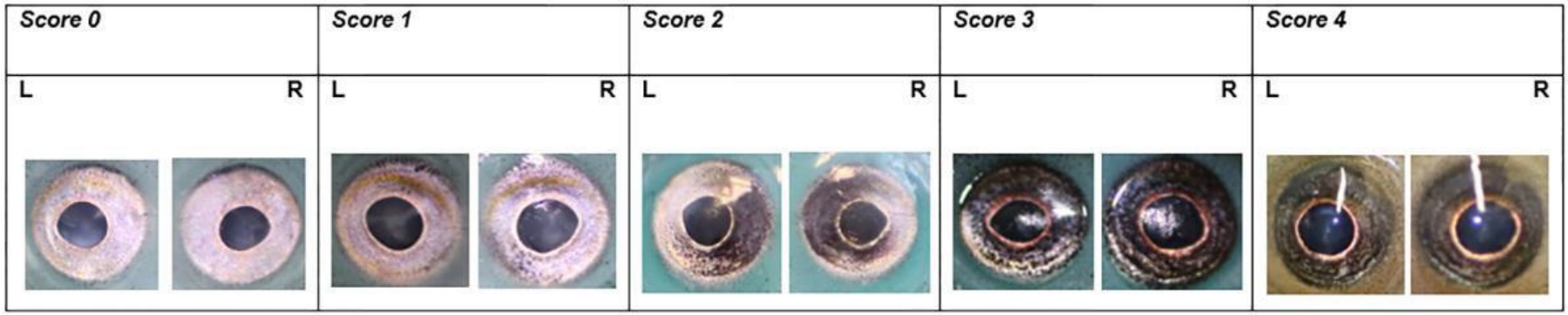



**Figure S2.4.** Scoring (0–4) of eye darkening (0: absence of darkening in the eye sclera; 1: average darkening affects up to 25% of the eye sclera; 2: average darkening affects 25–50% of the eye sclera; 3: average darkening affects 50–75% of the eye sclera; 4: average darkening affects 75–100% of the eye sclera). Images for score 0 was reconstructed in Photoshop as this score was not found in the test sample.

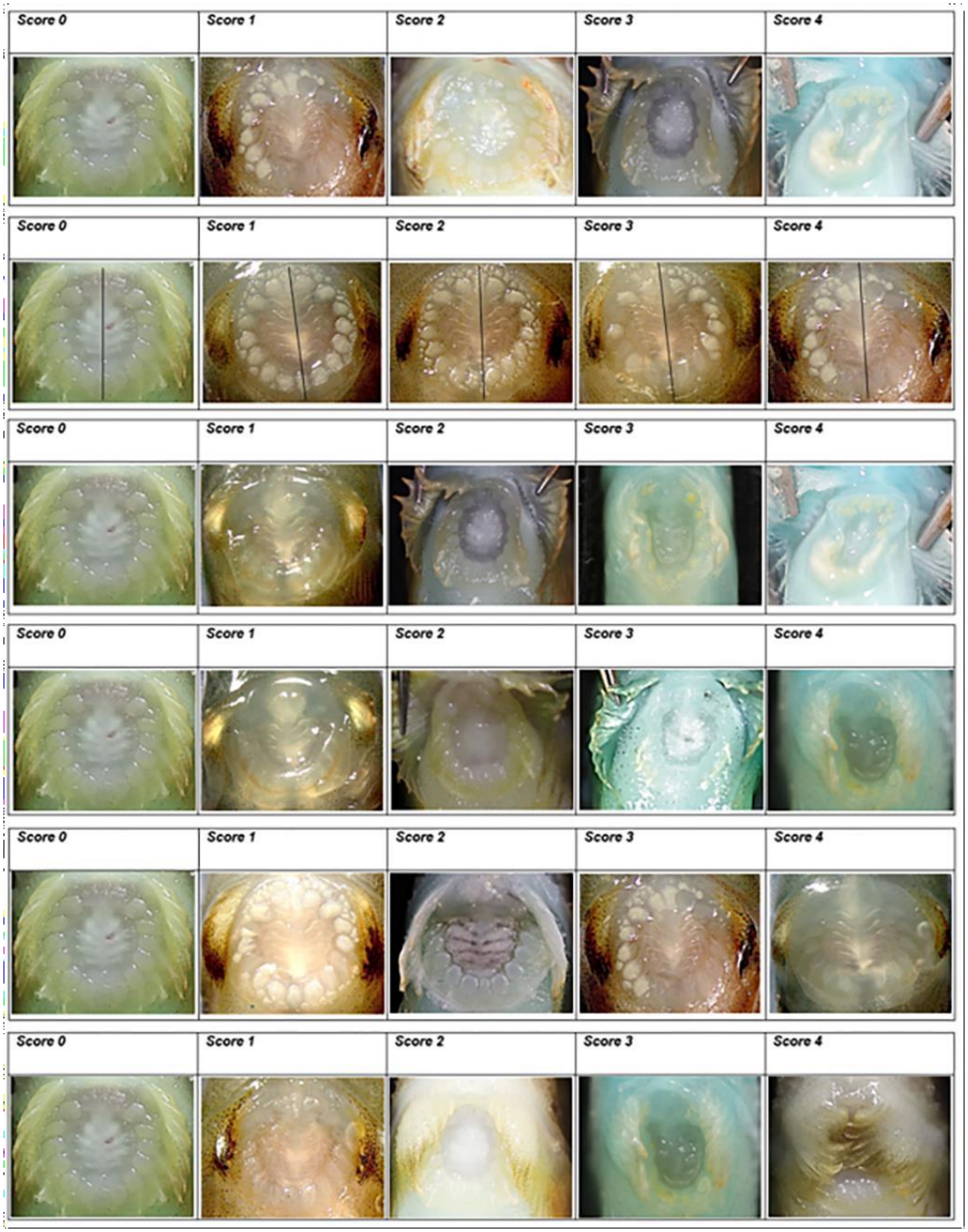



**Figure S2.5.** Scoring (0–4) of five suction disc conditions (symmetry, indentations, depressions, papillae development and curling of pectoral fins). *a. Symmetry*. 0 - right and left sides of the suction disc are symmetrical; 1 - minor asymmetry affecting up to 25% of the suction disc; 2 - moderate asymmetry affecting 25–50% of the suction disc; 3 - substantial asymmetry affecting 50–75% of the suction disc; 4 - severe asymmetry affecting more than 75% of the suction disc area. *b. Indentation* (i.e. inward bending or folding of the suction cup, usually towards the centre). 0 – no indentations present; 1 - minor indentation affecting up to 25% of the suction disc; 2 -moderate

indentation affecting 25–50% of the suction disc; 3 - substantial indentationaffecting 50–75% of the suction disc; 4 - severe indentation affecting more than 75%of the suction disc area. *c. Depression*. 0 - suction disc is flat, with no depression); 1 -minor depression affecting up to 25% of the suction disc; 2 - moderate depression affecting 25–50% of the suction disc; 3 - substantial depression affecting 50–75% of the suction disc; 4 - severe depression affecting more than 75% of the suction disc area. *d. Papillae development.* 0 – all muscular papillae (pads) are present and well developed; 1 – minor underdevelopment, up to 25% of the papillae are under-developed or absent; 2 – moderate under-development, 25–50% of the papillae are under-developed or absent; 3 – substantial under-development, 50–75% of the papillae are under-developed or absent; 4 – severe under-development, more than 75% of the papillae are under-developed or absent. *e. Curling/deformity of pectoral fins.* 0 – the ventral side of pectoral fins is well developed, not curled, and expose the entire suctioncup; 1 – minor deformity, up to 25% of the pectoral fin area is deformed or curled; 2 – moderate deformity, 25–50% of the pectoral fin area is deformed or curled; 3 – substantial deformity, 50–75% of the pectoral fin area is deformed or curled; 4 – severedeformity, more than 75% of the pectoral fin area is deformed or curled and cover theentire suction disc. Aggregated suction disc scores (0−20): Class A – Perfect suction disc (total score = 0). Symmetrical, without indentations, flat, with well-developed papillae and with pectoral fins that do not obliterate the suction cup. Class B – Mild deformity (total score 1–5). Slight asymmetry, with some depression and/or indentations and minor under-development of the papillae or slight curling of the pectoral fins. Class C – Moderate deformity (total score 6–10). Moderate asymmetry, depressions and indentations, and moderate under-development of the papillae and curling of the pectoral fins that hide parts of the suction cup. Class D – Substantial deformity (total score 11–15). Substantial asymmetry, with deep depressions and indentations, substantial under-development of the papillae, and marked curling of thepectoral fins that hide most of the suction cup. Class E – Severe deformity (total score > 15), non-functional suction disc. Severe asymmetry, with severe depressions,indentations and under-development of the papillae and totally deformed or curled pectoral fins that cover all the suction cup.

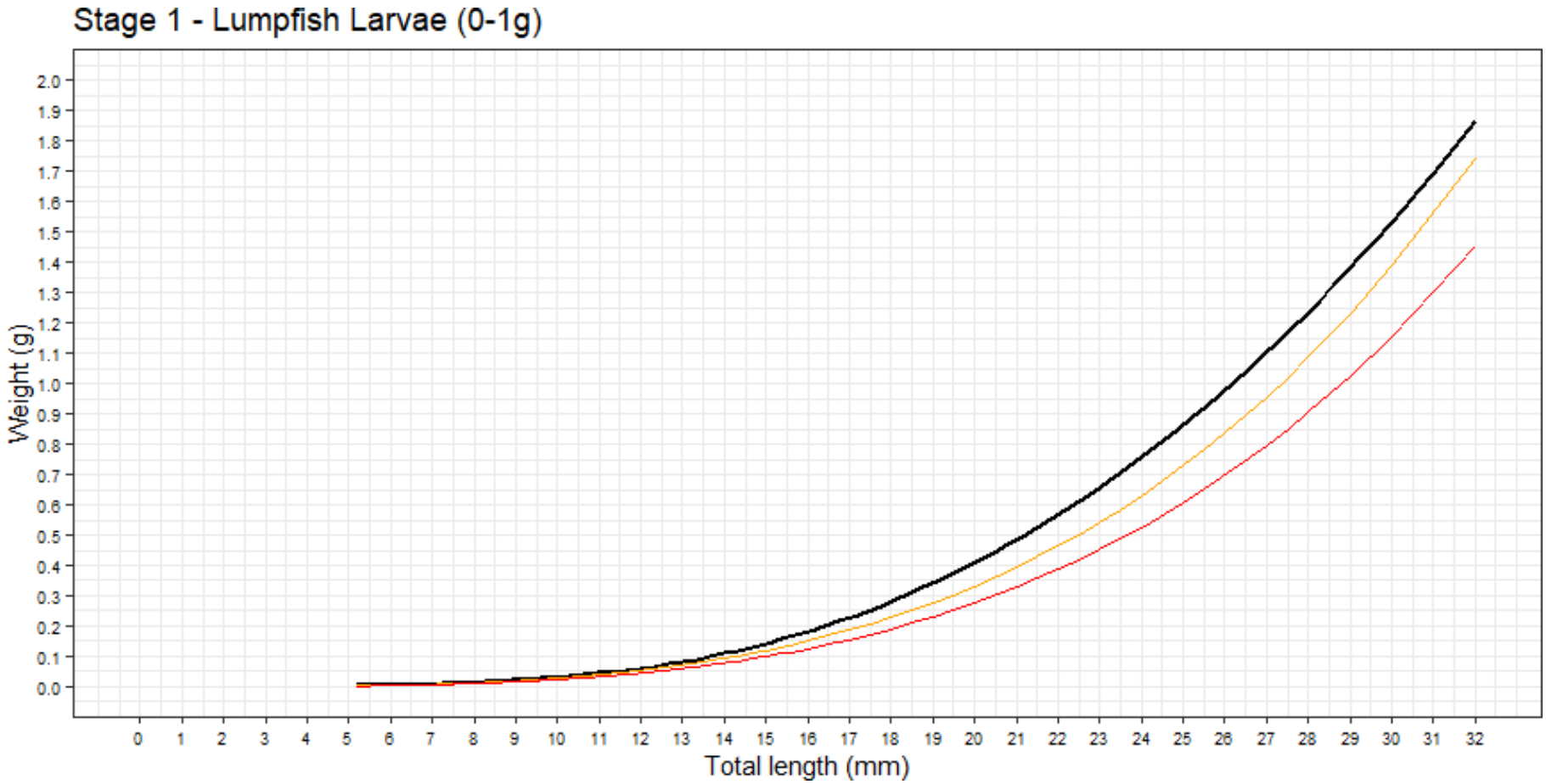



**Figure S2.6. a.** Percentile length-weight charts for farmed lumpfish at larval stage (S1: 0-1g) to allow the quick identification of normal (blackline, $W_R > 90\%$), underweight (orange line, $W_R = 90–75\%$) and emaciated (red line, $W_R < 75\%$) fish.

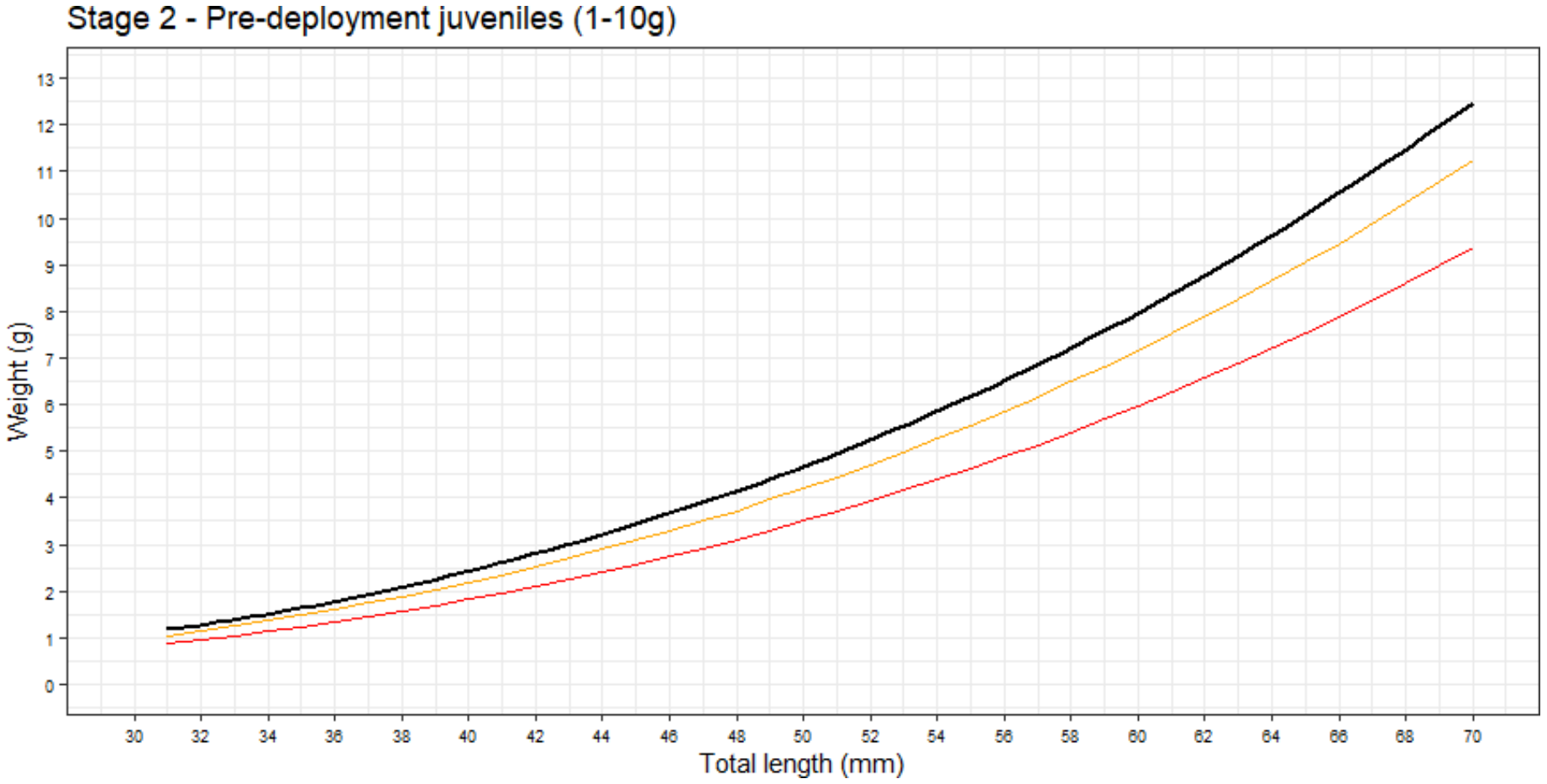



**Figure S2.6. b.** Percentile length-weight charts for farmed lumpfish at pre-deployment stage (S2: 1-10g) to allow the quick identification of normal (black line, $W_R > 90\%$), underweight (orange line, $W_R = 90–75\%$) and emaciated (red line, $W_R < 75\%$) fish.

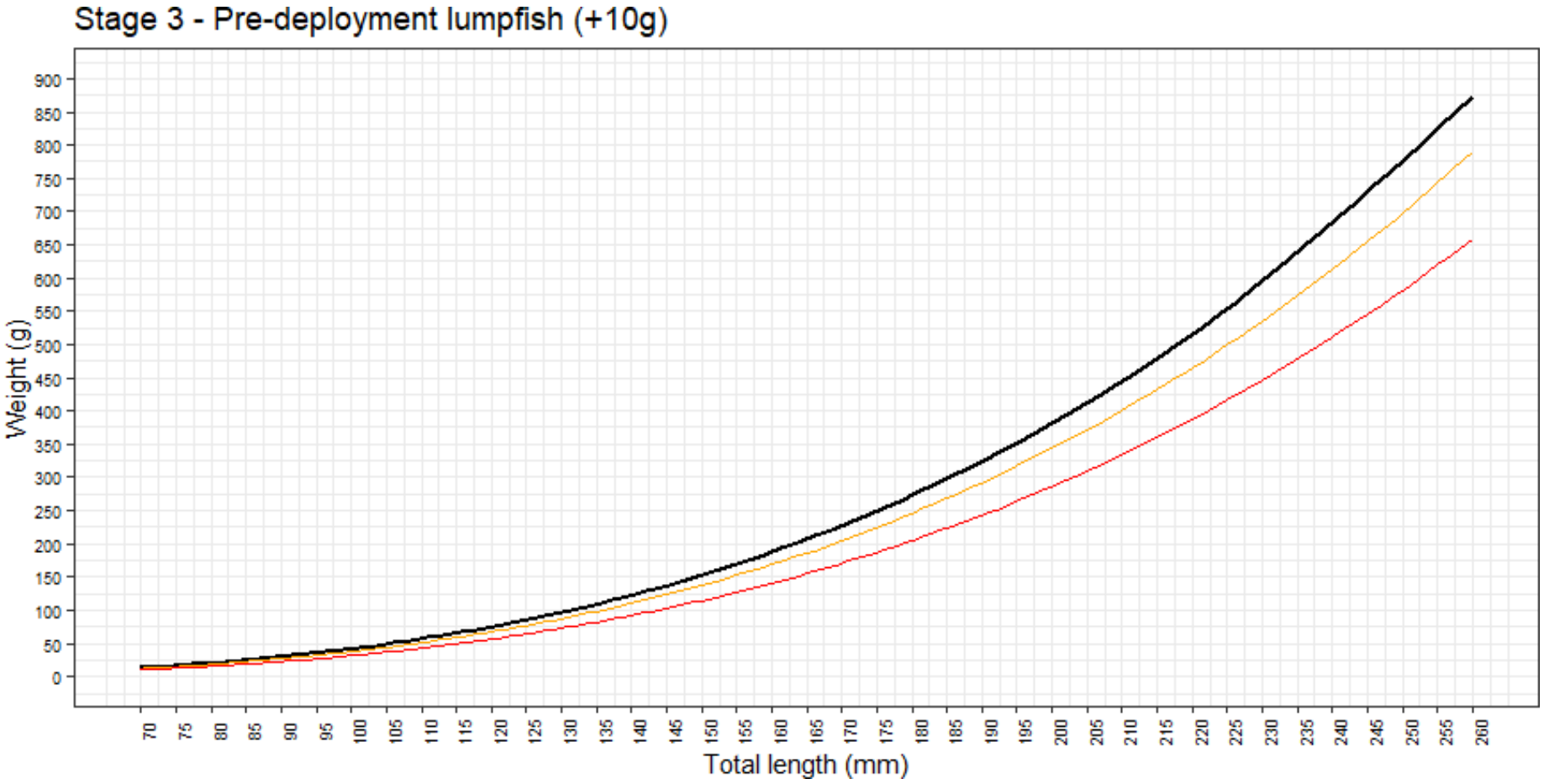



**Figure S2.6. c.** Percentile length-weight charts for farmed lumpfish at pre-deployment stage (S3: >10g) to allow the quick identification of normal (black line, $W_R > 90\%$), underweight (orange line, $W_R = 90–75\%$) and emaciated (red line, $W_R < 75\%$) fish.

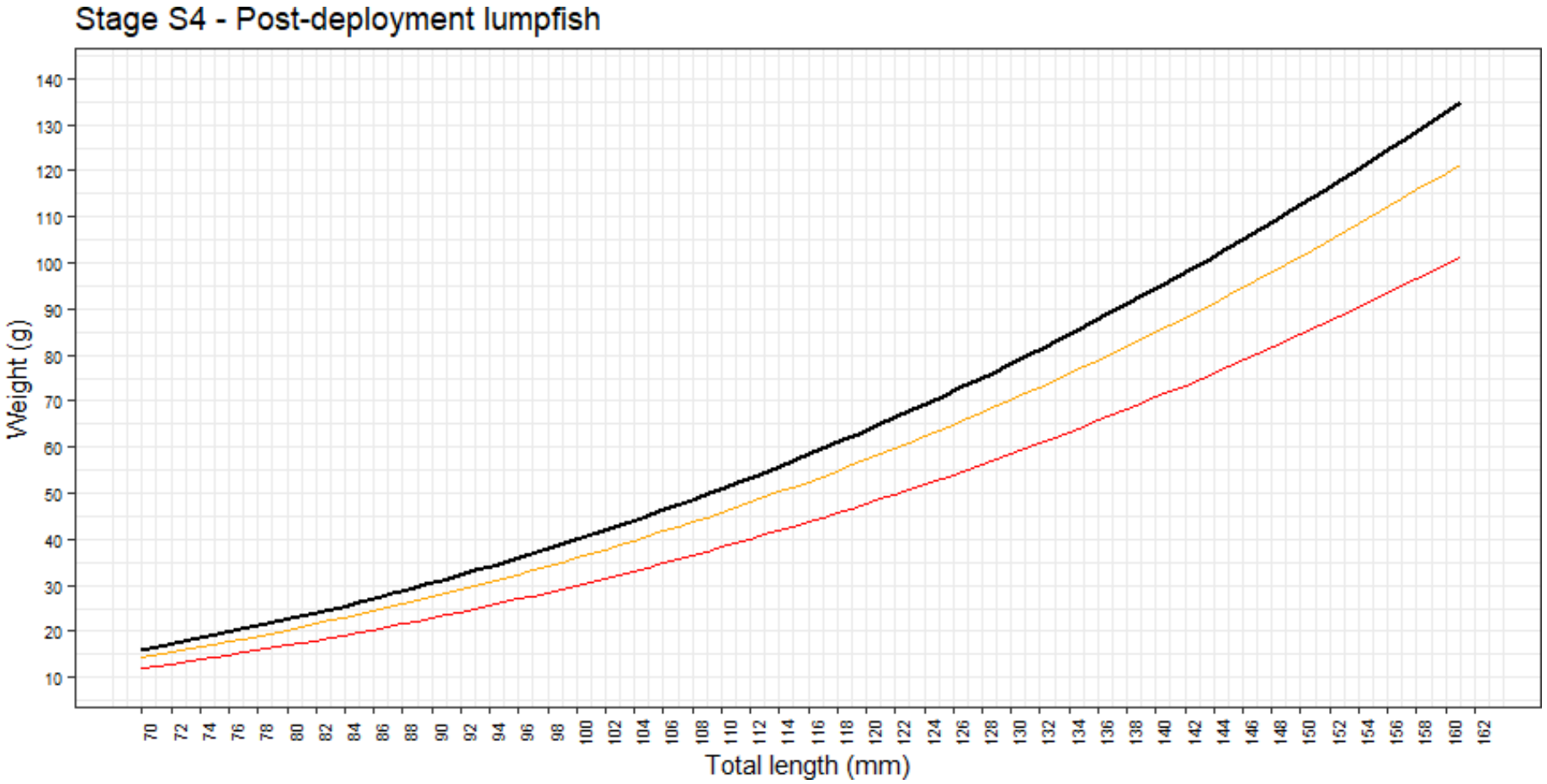



**Figure S2.6. c.** Percentile length-weight charts for farmed lumpfish at post-deployment stage (S4: sea cages) to allow the quick identification of normal (black line, $W_R > 90\%$), underweight (orange line, $W_R = 90–75\%$) and emaciated (red line, $W_R < 75\%$) fish.

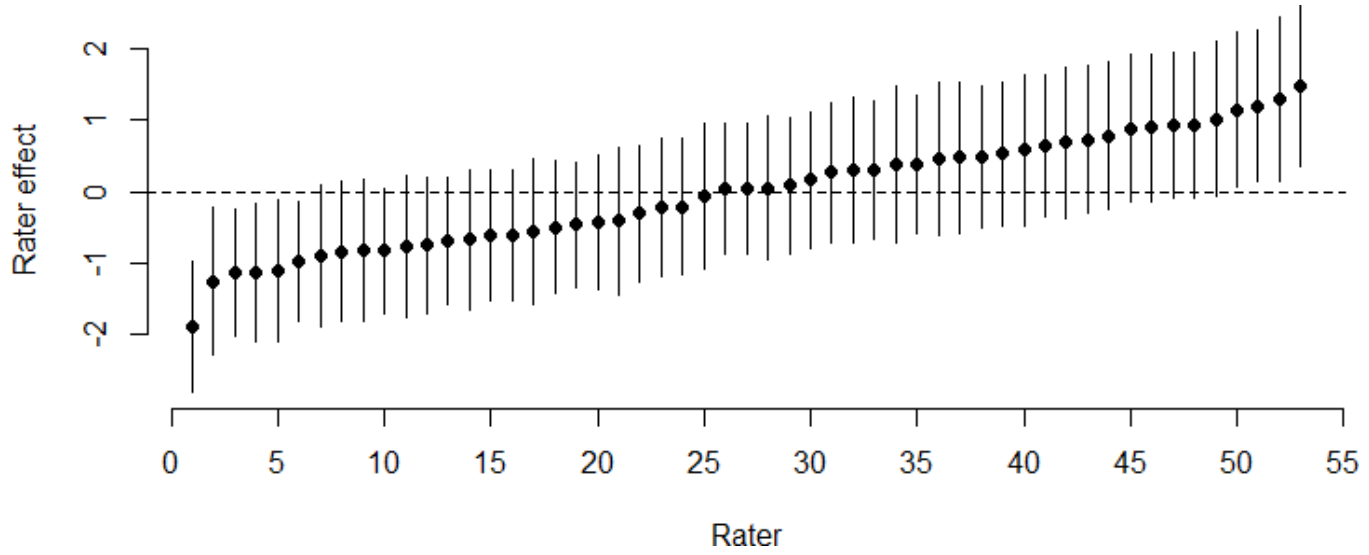



**Figure S2.7**. Rater effect obtained from the questionnaire given at the 1st Symposium on Welfare in Aquaculture to 53 participants to assess the utility of 12 welfare indicators for lumpfish.

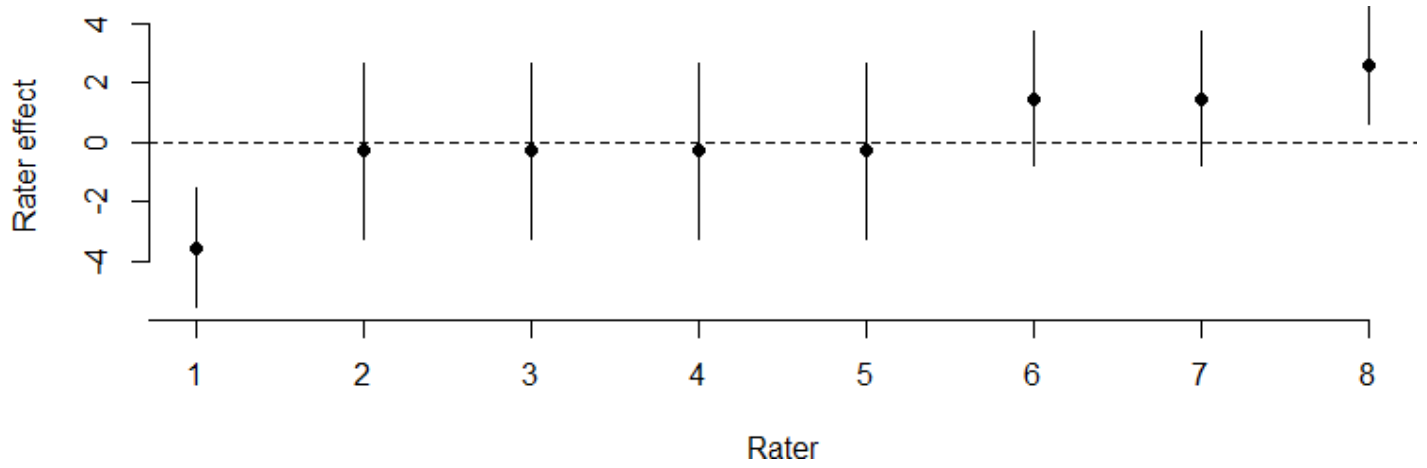



**Figure S2.8**. Rater effect obtained from the questionnaire given to 8 farmers assessing the performance of the LOWSI through four traits: practicality, efficiency, implementation and willingness to use.

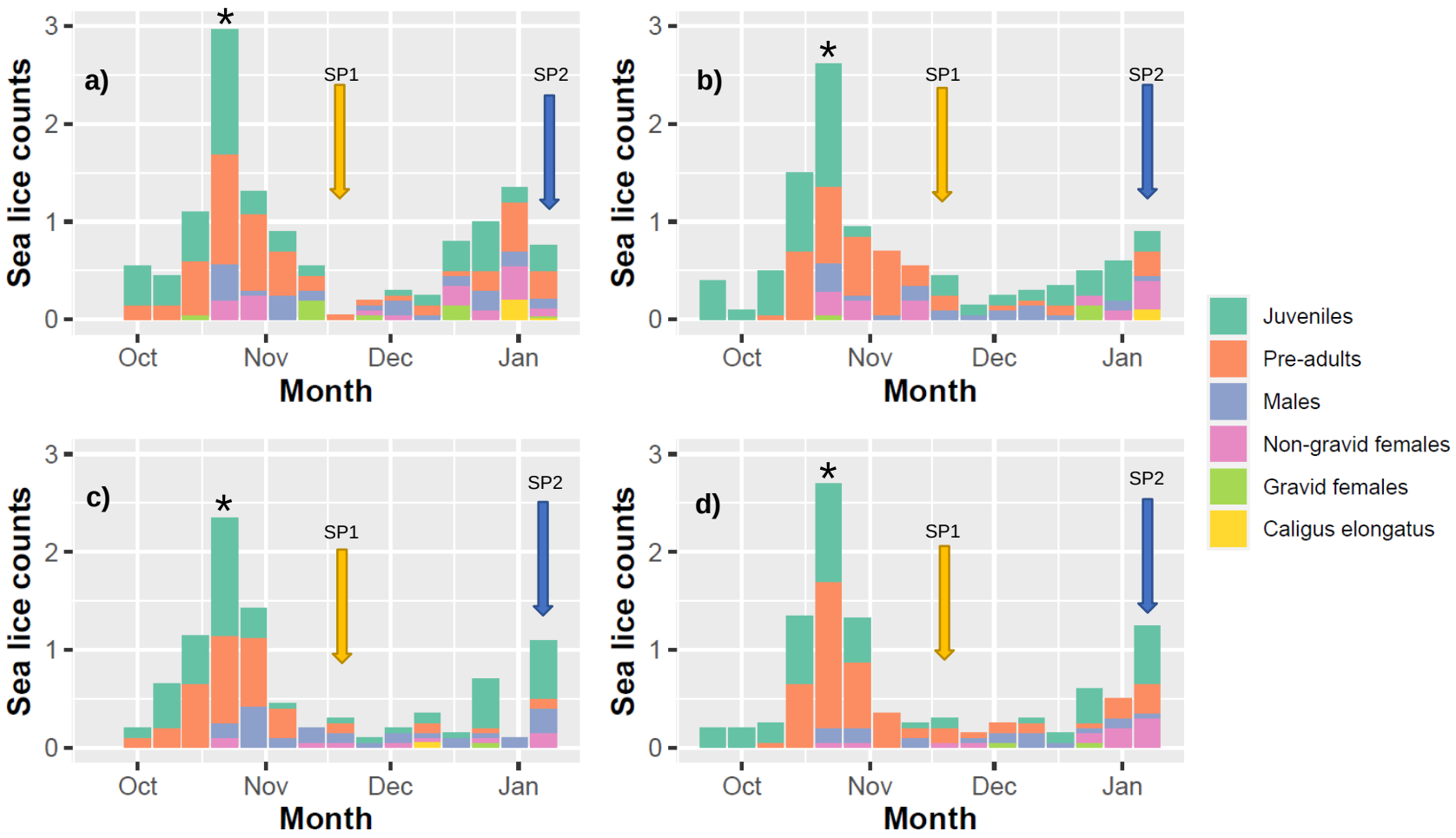



**Figure S3.1**. Average sea lice count (by life stages) in salmon: (a) Cage 3, (b) Cage 4, (c) Cage 7 and (d) Cage 8. Sampling time points 1 (three weeks) and 2 (three months) are shown by the yellow and blue arrows, respectively. Lumpfish were stocked on 19/10/2018 (*).

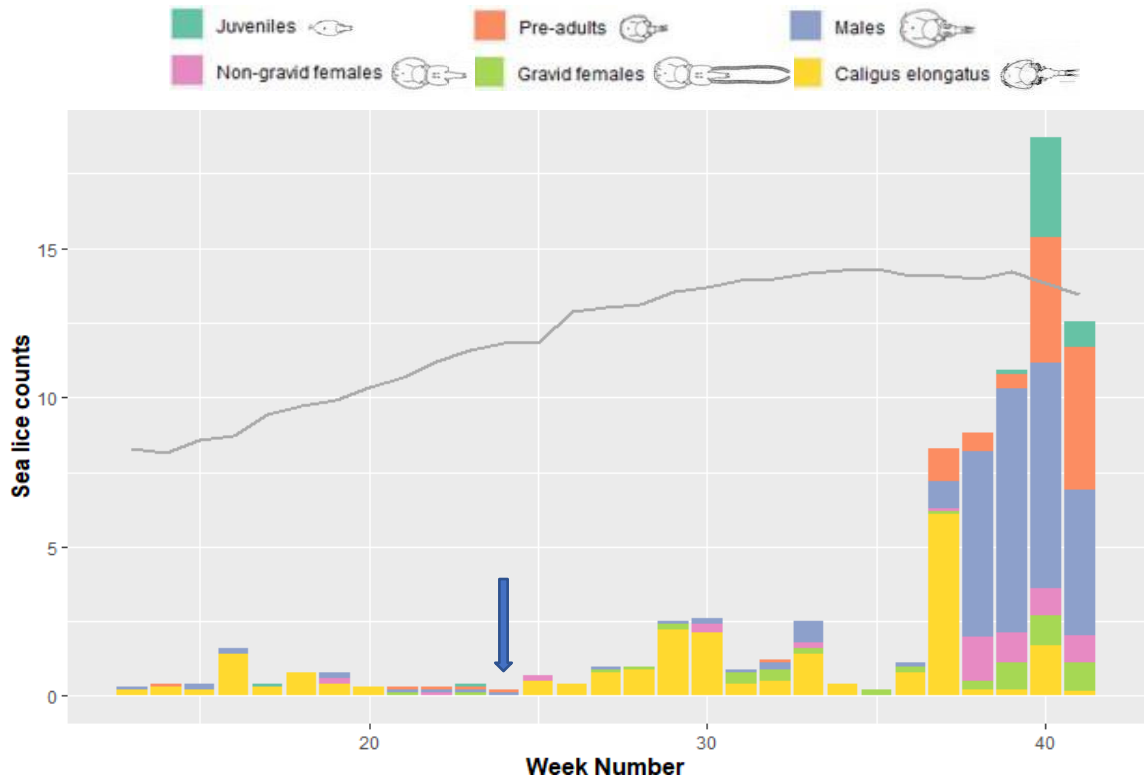



**Figure S4.1**. Weekly average counts (sea lice counts/total fish counted) of *Lepeophtheirus salmonis* (juveniles or *Chalimus*, pre-adults, males and females -gravid and non-gravid-) and *Caligus elongatus* of the commercial Scottish cage used in the study, from the start (25/03/2019) to the end (13/10/2019) of the production cycle. Sea water temperatures are represented by the grey line. Lumpfish were sampled on week 24 (blue arrow).

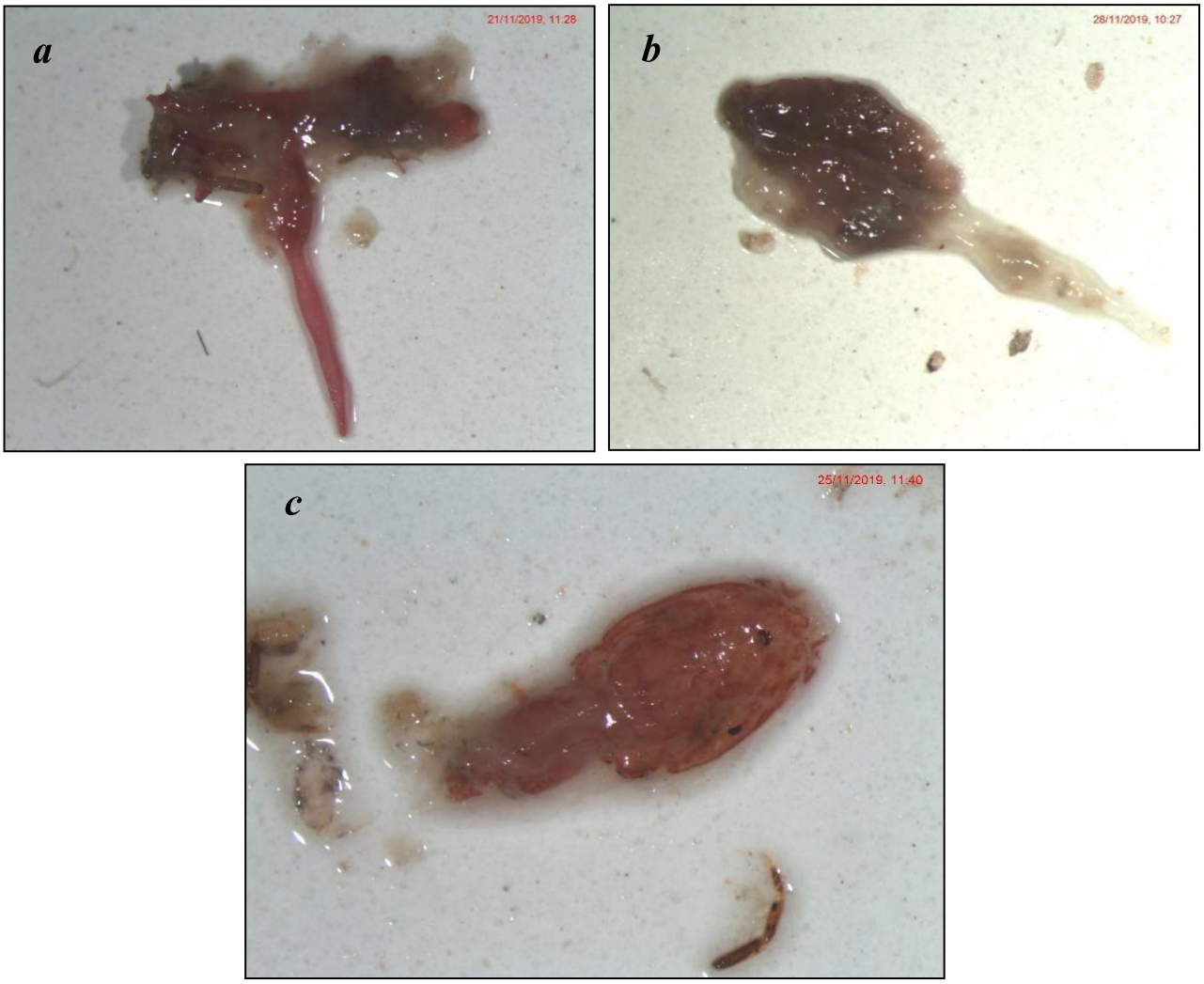



**Figure S4.2**. Sea lice found in lumpfish stomach contents. Partially digested *Lepeophtheirus salmonis* (a and b). Intact *Caligus elongatus* (c).

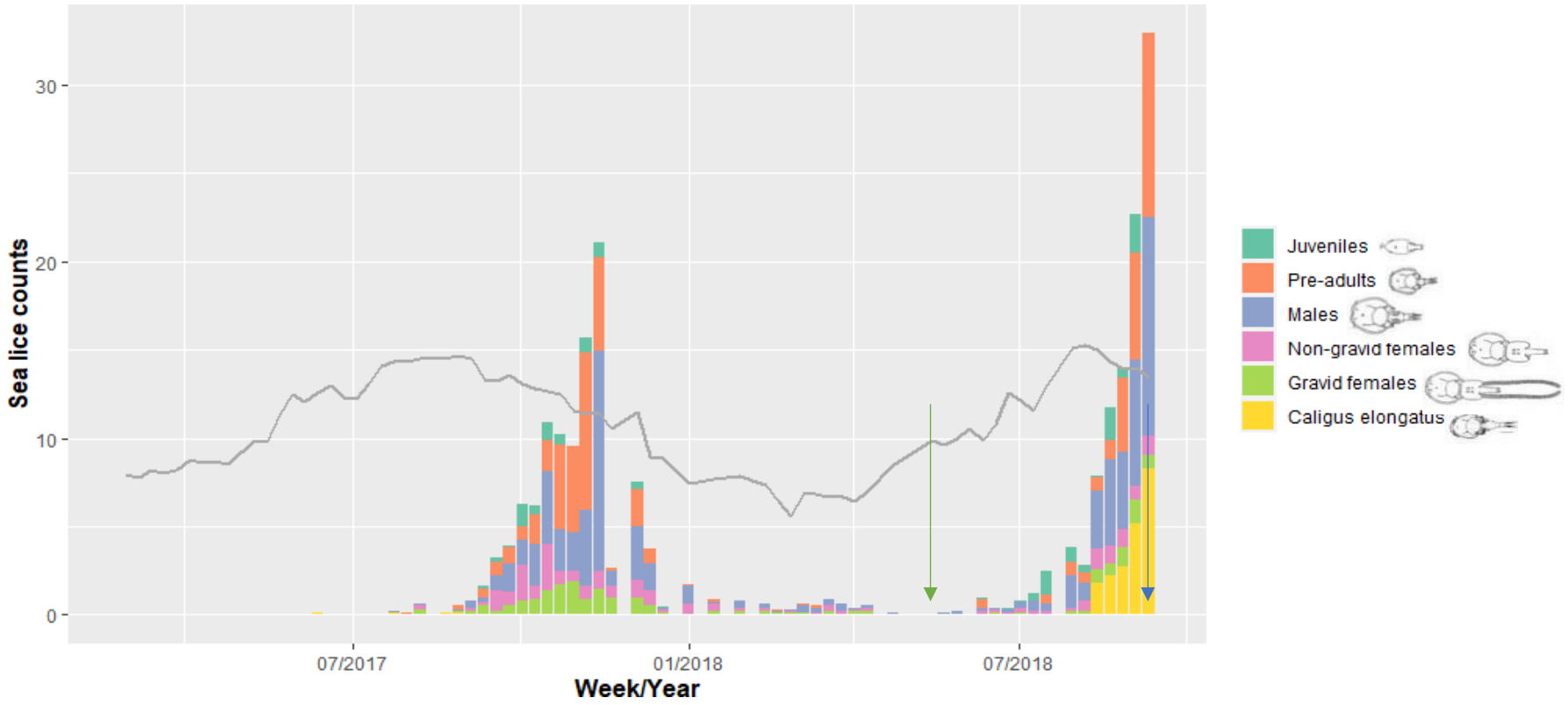



**Figure S5.1**. Weekly sea lice count (average/total salmon counted) at the studied salmon cage, from the start (27/02/2017) to the end (25/09/2018) of the production cycle. Lumpfish were stocked at 4% on 25/05/2018 (green, first arrow) and sampled at the end of the salmon cycle (blue, second arrow). All stages of *Lepeophtheirus salmonis* (juveniles, pre-adults, adult males and adult females) as well as *Caligus elongatus*, with a strong seasonal pattern, were recorded. The grey line shows sea water temperatures.

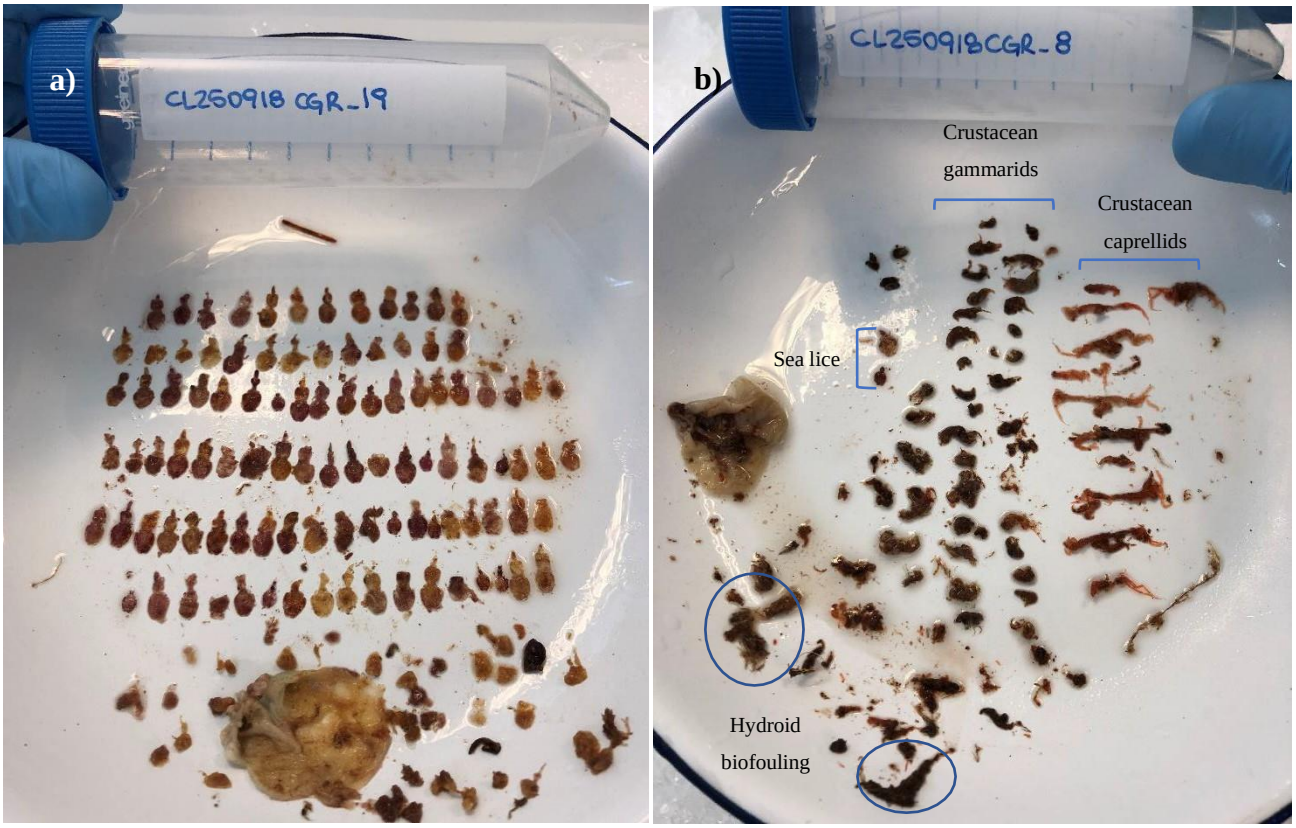
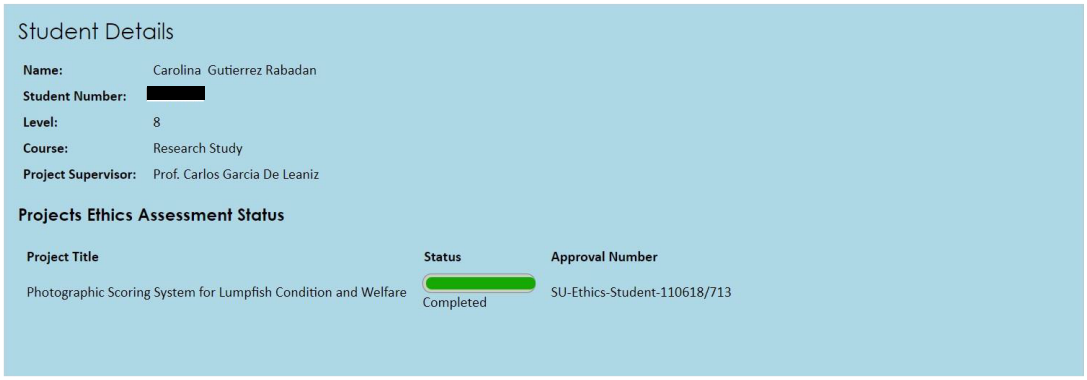



**Figure S5.2**. a. Mixed *Lepeophtheirus salmonis* and *Caligus elongatus* partially digested specimens recovered from lumpfish stomachs. b. Crustacean gammarids/caprellids, hydroid biofouling and sea lice found in lumpfish stomachs.

# Ethics Approval

Ethics Approval Number: SU-Ethics-Student-110618/713

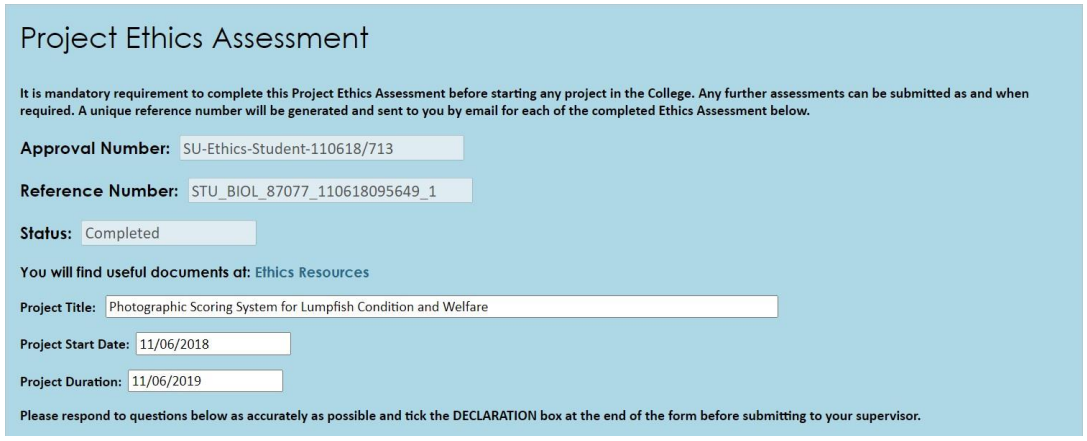


Student Details

**Name:** Carolina Gutierrez Rabadan
**Student Number:** [redacted]
**Level:** 8
**Course:** Research Study
**Project Supervisor:** Prof. Carlos Garcia De Leaniz

**Projects Ethics Assessment Status**

| Project Title | Status | Approval Number |
|---|---|---|
| Photographic Scoring System for Lumpfish Condition and Welfare | Completed | SU-Ethics-Student-110618/713 |



Project Ethics Assessment

**It is mandatory requirement to complete this Project Ethics Assessment before starting any project in the College. Any further assessments can be submitted as and when required. A unique reference number will be generated and sent to you by email for each of the completed Ethics Assessment below.**

**Approval Number:** SU-Ethics-Student-110618/713

**Reference Number:** STU_BIOL_87077_110618095649_1

**Status:** Completed

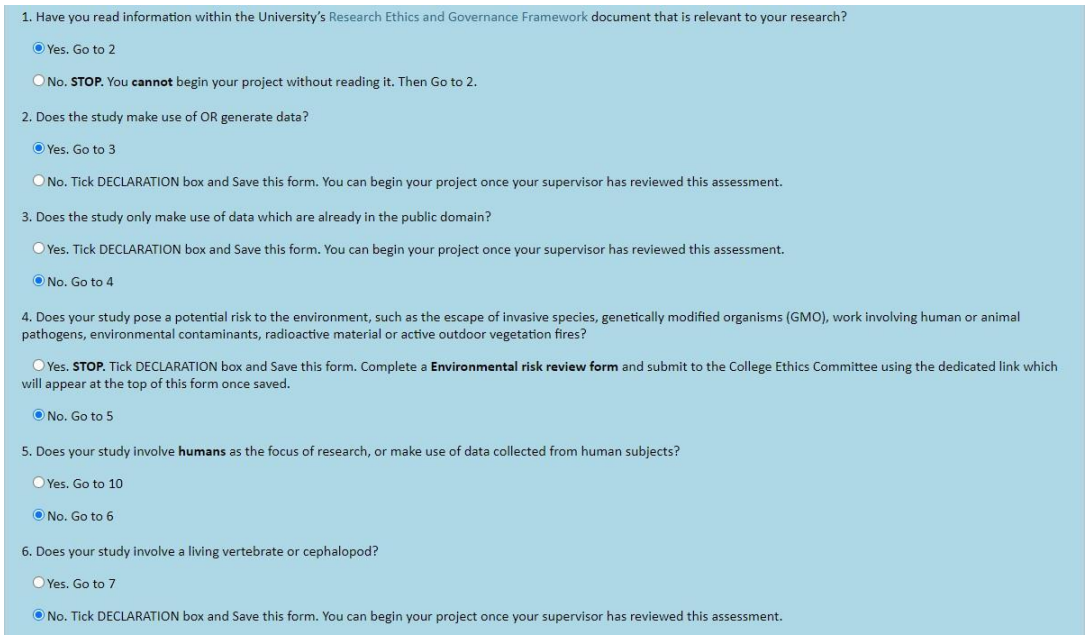
**You will find useful documents at:** Ethics Resources

**Project Title:** Photographic Scoring System for Lumpfish Condition and Welfare

**Project Start Date:** 11/06/2018

**Project Duration:** 11/06/2019

**Please respond to questions below as accurately as possible and tick the DECLARATION box at the end of the form before submitting to your supervisor.**



1. Have you read information within the University's Research Ethics and Governance Framework document that is relevant to your research?

- (●) Yes. Go to 2
- ( ) No. **STOP.** You **cannot** begin your project without reading it. Then Go to 2.

2. Does the study make use of OR generate data?

- (●) Yes. Go to 3
- ( ) No. Tick DECLARATION box and Save this form. You can begin your project once your supervisor has reviewed this assessment.

3. Does the study only make use of data which are already in the public domain?

- ( ) Yes. Tick DECLARATION box and Save this form. You can begin your project once your supervisor has reviewed this assessment.
- (●) No. Go to 4

4. Does your study pose a potential risk to the environment, such as the escape of invasive species, genetically modified organisms (GMO), work involving human or animal pathogens, environmental contaminants, radioactive material or active outdoor vegetation fires?

- ( ) Yes. **STOP.** Tick DECLARATION box and Save this form. Complete a **Environmental risk review form** and submit to the College Ethics Committee using the dedicated link which will appear at the top of this form once saved.
- (●) No. Go to 5

5. Does your study involve **humans** as the focus of research, or make use of data collected from human subjects?

- ( ) Yes. Go to 10
- (●) No. Go to 6

6. Does your study involve a living vertebrate or cephalopod?

- ( ) Yes. Go to 7
- (●) No. Tick DECLARATION box and Save this form. You can begin your project once your supervisor has reviewed this assessment.

# R Scripts

```
#### Chapter 2 ####

### Perceived utility of WI for lumpfish ############################
setwd("~/PhD/Chapter 2. LOWSI/Datasets")
Quest <- read.csv("QuestSWELA_53.csv")
library(psych)

# Check the data frame
headTail(Quest)
str(Quest)
summary(Quest)

# Remove rows containing 'Don't know' or Score 0 as an answer
Quest <- Quest[!grepl("0", Quest$Score),]
summary(Quest)

# Utility variation by OWI
mod1 <- glm (Score ~ WI, data=Quest)
summary(mod1)
anova(mod1, test="Chisq", type=3)

mod2 <- lm (Score ~ WI, data=Quest)
summary(mod2)
anova(mod2, test="Chisq")

# Summarise data
xtabs(~ Participant + LikertScore, data=Quest)
XT = xtabs( ~ Participant + LikertScore, data =Quest)
prop.table(XT, margin = 1)

# Welfare Indicator frequency barplot
XT = xtabs(~ LikertScore + WI, data=Quest)
barplot(XT, col="dark gray", xlab="WI Likert", ylab="Frequency")

# Utility variation by OWI
mod1 <- glm (Score ~ WI, data=Quest)
summary(mod1)

mod2 <- glm (Score ~ WI + Participant, data=Quest)
summary(mod2)

anova(mod1,mod2)
anova(mod1, test="Chisq", type=3)
anova(mod2, test="Chisq")

# Subset dataset by each welfare indicator
Findam <- subset(Quest, WI=="Fin damage")
Skidam <- subset(Quest, WI=="Skin damage")
Mort <- subset(Quest, WI=="Mortality")
Dispar <- subset(Quest, WI=="Disease/Parasites")
Eyedam <- subset(Quest, WI=="Eye damage")
Sucdef <- subset(Quest, WI=="Suction cup deformities")
Opdef <- subset(Quest, WI=="Operculum/Gill damage")
Bodef <- subset(Quest, WI=="Body deformities")
```

```
Condfac <- subset(Quest, WI=="Condition factor/Relative size")
Growth <- subset(Quest, WI=="Poor growth")
Dark <- subset(Quest, WI=="Body/eye darkening")
Blood <- subset(Quest, WI=="Blood parameters")

# Apply Wilcoxon signed-rank test to obtain pseudo-medians with 95%
CI (Table S2.1)
wilcox.test(Findam$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Skidam$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Mort$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Dispar$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Eyedam$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Sucdef$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Opdef$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Bodef$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Condfac$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Growth$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Dark$Score, mu=1, conf.int=TRUE, conf.level=0.95)
wilcox.test(Blood$Score, mu=1, conf.int=TRUE, conf.level=0.95)

### Consensus between participants and rater effect on WI ##########
Quest_SWELA <- read.csv("QuestSWELA_53.csv")
library(ordinal)

# Remove rows containing 'Don't know' or Score 0 as an answer
Quest_SWELA <- Quest_SWELA[!grepl("0", Quest_SWELA$Score),]

# Create a new variable for ordered Likert scores (factor)
Quest_SWELA$Score <- factor(Quest_SWELA$Score, ordered=TRUE)
Quest_SWELA$ordered.Score <- factor(Quest_SWELA$Score,
                                    levels=c("1","2","3","4","5"),
                                    ordered=TRUE)
# Rater effect SWELA

# Model
m1 <- clm(ordered.Score ~ WI, data=Quest_SWELA)
summary(m1)

# Mixed model
m1_mixed <- clmm2(ordered.Score ~ WI, random=factor(Participant),
Hess=TRUE,
                  data=Quest_SWELA)
summary(m1_mixed) # AIC=1238.3

m2_mixed <- clmm2(ordered.Score ~ WI + (1|Participant),
data=Quest_SWELA)
summary(m2_mixed) # AIC=1287.8

ci <- m1_mixed$ranef + qnorm(0.975) * sqrt(m1_mixed$condVar) %o% c(-
1,1)
ord.re <- order(m1_mixed$ranef)

ci <- ci[order(m1_mixed$ranef),]

# Plot rater effect (Fig.S2.7)
par(mfrow=c(1,1))
plot(1:53, m1_mixed$ranef[ord.re], axes=FALSE, ylim=range(ci),
     xlab="Rater", ylab="Rater effect", pch=19)
axis(1, at=seq(0,55,by=5), labels=seq(0,55,by=5))
```

```
axis(2, at=seq(-2,2,by=1), labels=seq(-2,2,by=1))
for(i in 1:53) segments(i, ci[i,1], i, ci[i,2])
abline(h=0, lty=2)

### Prevalence and variation in OWIs ################################
Welfare <- read.csv("Welfare.csv")

# Subset by stage of development
Welfare_pre <- subset(Welfare, stage=="pre-dep") # n=60
Welfare_post <- subset(Welfare, stage=="post-dep") # n=35

# Prevalence of Body damage
BodyDam_pre <- table(Welfare_pre$BodyDam!=0)/60*100
BodyDam_post <- table(Welfare_post$BodyDam!=0)/35*100
filter(Welfare_pre, BodyDam!=0) #n=1
filter(Welfare_post, BodyDam!=0) #n=16
prop.test(x=c(1,16), n=c(60,35))

# Prevalence of Fin damage
FinDam_pre <- table(Welfare_pre$FinDamage!=0)/60*100
FinDam_post <- table(Welfare_post$FinDamage!=0)/35*100

Dorsal_pre <- table(Welfare_pre$dorsalfin_sco!=0)/60*100
Dorsal_post <- table(Welfare_post$dorsalfin_sco!=0)/35*100

Caudal_pre <- table(Welfare_pre$caudalfin_sco!=0)/60*100
Caudal_post <- table(Welfare_post$caudalfin_sco!=0)/35*100

Anal_pre <- table(Welfare_pre$analfin_sco!=0)/60*100
Anal_post <- table(Welfare_post$analfin_sco!=0)/35*100

Pect_pre <- table(Welfare_pre$pecfin_sco!=0)/60*100
Pect_post <- table(Welfare_post$pecfin_sco!=0)/35*100

Venpec_pre <- table(Welfare_pre$pelfin_sco!=0)/60*100
Venpec_post <- table(Welfare_post$pelfin_sco!=0)/35*100

# Prevalence of Eye darkening
ED_pre <- table(Welfare_pre$EyeDarkening!=0)/60*100
ED_post <- table(Welfare_post$EyeDarkening!=0)/35*100

# Prevalence of Eye condition
Eyecond_pre <- table(Welfare_pre$EyeCondition!=0)/60*100
Eyecond_post <- table(Welfare_post$EyeCondition!=0)/35*100
filter(Welfare_pre, EyeCondition!=0) n=4
filter(Welfare_post, EyeCondition!=0) n=8
prop.test(x=c(4,8),n=c(60,35))

# Prevalence of Suction disc deformities
SuckDef_pre <- table(Welfare_pre$SuckerDef!=0)/60*100
SuckerDef_post <- table(Welfare_post$SuckerDef!=0)/35*100

Sym_pre <- table(Welfare_pre$sym_sco!=0)/60*100
Sym_post <- table(Welfare_post$sym_sco!=0)/35*100

Ind_pre <- table(Welfare_pre$ind_sco!=0)/60*100
Ind_post <- table(Welfare_post$ind_sco!=0)/35*100

Dep_pre <- table(Welfare_pre$dep_sco!=0)/60*100
```

```
Dep_post <- table(Welfare_post$dep_sco!=0)/35*100

Pap_pre <- table(Welfare_pre$pap_sco!=0)/60*100
Pap_post <- table(Welfare_post$pap_sco!=0)/35*100

Pel_pre <- table(Welfare_pre$pel_sco!=0)/60*100
Pel_post <- table(Welfare_post$pel_sco!=0)/35*100

### Principal Component Analysis ###################################
PCA <- read.csv("PCA.csv")
library(factoextra)

# Data standardization (normalisation of variables)
PCA$ExternalCondition <- scale(PCA$ExternalCondition)
PCA$FinDamage <- scale(PCA$FinDamage)
PCA$EyeDarkening <- scale(PCA$EyeDarkening)
PCA$EyeCondition <- scale(PCA$EyeCondition)
PCA$SuckerDeformity <- scale(PCA$SuckerDeformity)
# Principal Component Analysis
PRCOMP1 <- prcomp(~ ExternalCondition + FinDamage + EyeDarkening +
                    EyeCondition + SuckerDeformity,
                  data=PCA, na.action=na.omit, scale=TRUE)
summary(PRCOMP1)

#Scree plot
fviz_eig(PRCOMP1)

# Get coordinates of variables
var <- get_pca_var(PRCOMP1)
var
var$coord
var$cos2

# PCA - Contribution of variables
fviz_pca_var(PRCOMP1, col.var="contrib",
             gradient.cols=c("#00AFBB", "#E7B800", "#FC4E07"),
             repel=TRUE) #Fig.2.3A

# PCA biplot - Variables and individuals by life stage
PCA$stage <- factor(PCA$stage, levels=c("pre-dep","post-dep"))
fviz_pca_biplot(PRCOMP1, geom.ind="point", col.ind=PCA$stage,
                         palette=c("#00AFBB", "#E7B800", "#FC4E07"),
                         addEllipses=TRUE, label="var",
col.var="black",
                         repel=TRUE, legend.title="Life stage") #
Fig.2.3B
```

```
# Extraction of eigenvalues and eigenvectors
PRCOMP1$sdev^2 # to extract eigenvalues
PRCOMP1$rotation # to extract the eigenvectors
eig.val <- get_eigenvalue(PRCOMP1)
eig.val

# Contribution of each variable to the PC
res.var <- get_pca_var(PRCOMP1)
res.var$contrib

# Obtain coordinates and add PC1 and PC2 values to the dataset
PC1 <- predict(PRCOMP1)[,1]
PCA$PC1 <- predict(PRCOMP1)[,1]
PC1
PC2 <- predict(PRCOMP1)[,2]
PCA$PC2 <- predict(PRCOMP1)[,2]
PC2

### Variation in LWR and body condition ############################
LWR_DE <- read.csv("LWR_differentEquations.csv")

### Using 1 equation for all stages (WS1, n=2658) ###
library(ggplot2)
ggplot(LWR_DE, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

WS1 <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=LWR_DE)
summary(WS1)

### Using 2 equations for pre- and post- stages (WS2) ###
LWR_pre <- subset(LWR_DE, time=="pre-dep") # n=2303
LWR_post <- subset(LWR_DE, time=="post-dep") # n=355

ggplot(LWR_pre, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

ggplot(LWR_post, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

WS2_pre <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=LWR_pre)
summary(WS2_pre)

WS2_post <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=LWR_post)
summary(WS2_post)

### Using 4 equations for each of the stages S1-S4 (WS4) ###
LWR_S1 <- subset(LWR_DE, life_stage=="S1") # n=948
LWR_S2 <- subset(LWR_DE, life_stage=="S2") # n=126
LWR_S3 <- subset(LWR_DE, life_stage=="S3") # n=1229
LWR_S4 <- subset(LWR_DE, life_stage=="S4") # n=355

ggplot(LWR_S1, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

ggplot(LWR_S2, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

ggplot(LWR_S3, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)
```

```
ggplot(LWR_S4, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

WS4_S1 <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=LWR_S1)
summary(WS4_S1)

WS4_S2 <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=LWR_S2)
summary(WS4_S2)

WS4_S3 <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=LWR_S3)
summary(WS4_S3)

WS4_S4 <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=LWR_S4)
summary(WS4_S4)

# Table 2.2. Length weight regression coefficients #
LWR <- read.csv("Lumpfish_Length_weight.csv")

WS <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=LWR)
summary(WS)

### Using 4 equations for each life stage ###
# S1. Larvae (0-1g)
StageS1 <- subset(LWR, life_stage=="S1")
ggplot(StageS1, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

WS_S1 <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=StageS1)
summary(WS_S1)

# S2. Pre-deployment (0-10g)
StageS2 <- subset(LWR, life_stage=="S2")
ggplot(StageS2, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

WS_S2 <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=StageS2)
summary(WS_S2)

# S3. Pre-deployment (+10g)
StageS3 <- subset(LWR, life_stage=="S3")
ggplot(StageS3, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

WS_S3 <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=StageS3)
summary(WS_S3)
# S4. Post-deployment
StageS4 <- subset(LWR, life_stage=="S4")
ggplot(StageS4, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

WS_S4 <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=StageS4)
summary(WS_S4)

# All stages
ggplot(LWR, aes(x=log(tl_mm, 10), y=log(bw_g, 10))) +
  geom_point() + geom_smooth(method=lm, se=FALSE)

WS_All <- lm(log(bw_g, 10) ~ log(tl_mm, 10), data=LWR)
summary(WS_All)
```

```
### Incidence of underweight/emaciated fish ########################
Emaciation <- read.csv("Emaciation.csv")
library(Hmisc)

p <- cbind(Emaciation$Positive, Emaciation$Negative)
binomprops <- binconf(Emaciation$Positive, Emaciation$Total)

ggplot(Emaciation, aes(x=Stage, y=binomprops[,1], fill=Measure)) +
  geom_bar(position=position_dodge(), stat="identity",
  colour="black") + geom_errorbar(aes(ymin=binomprops[,1],
ymax=binomprops[,3]),width=0.3,
                position=position_dodge(0.9)) +
  ylab("Frequency") + xlab("Stage of development") +
  scale_fill_discrete(name="Relative Weight",
                      labels=c("Emaciated \n Wr<75% \n",
                               "Underweight \n Wr=75-90% \n")) +
ylim(0,0.4) +
  scale_x_discrete(labels=c("S1" = "S1 (0-1g)",
                                          "S2" = "S2 (1-10g)",
                                          "S3" = "S3 (+10g)",
                                          "S4" = "S4 (+10g)")) +
theme_bw()
# Frequency/proportion test for U/E fish
prop.test(x=c(484,265),n=c(2658,2658))

### Variation in plasma cortisol ###################################
Cortisol <- read.csv("Lumpfish_Blood_plasma_Cortisol.csv")
hist(Cortisol$Cortisol)

# Subset for stages
Cortisol_S3 <- subset(Cortisol, stage=="S3")
Cortisol_S4 <- subset(Cortisol, stage=="S4")

# Mean cortisol +/- SE for pre-deployment stage (S3)
mean(Cortisol_S3$Cortisol)
sd(Cortisol_S3$Cortisol)
sqrt(20)
se_S3 = sd(Cortisol_S3$Cortisol)/sqrt(20)

## Mean cortisol +/- SE for post-deployment stage (S4)
mean(Cortisol_S4$Cortisol)
sd(Cortisol_S4$Cortisol)
sqrt(35)
se_S4 = sd(Cortisol_S4$Cortisol)/sqrt(35)

# Plasma cortisol between life stages
t.test(Cortisol_S4$Cortisol, Cortisol_S3$Cortisol)

# Cortisol variability
library(raster)
cv(Cortisol_S3$Cortisol)
cv(Cortisol_S4$Cortisol)

# Cortisol distribution: not normally distributed
hist(Cortisol$Cortisol)

# Plasma cortisol variability (Fligner-Killeen test)
fligner.test(Cortisol ~ stage, Cortisol)
```

```
### Validation OWIs against relative weight and plasma cortisol ####
PC1PC2_C_pre <- read.csv("PC1PC2_C_pre.csv")

### Model for cortisol at pre-deployment ###
Cort_pre<-lm(cortisol_ng.ml_new~PC1*PC2*tl_mm,data=PC1PC2_C_pre)
step(Cort_pre)
library(MuMIn)
options(na.action = "na.fail")
dredge(Cort_pre)

Cort_pre_clean <- lm(cortisol_ng.ml_new ~ PC1 + PC2 + tl_mm +
PC1:PC2 + PC2:tl_mm, data=PC1PC2_C_pre)
dredge(Cort_pre_clean)

# Model averaging from MuMIN documentation
msA_pre <- dredge(Cort_pre)
msA_pre

# Models with delta.aicc < 2
summary(model.avg(msA_pre, subset = delta < 2))

# Final model
Cort_final_pre<-lm(cortisol_ng.ml_new~PC1, data=PC1PC2_C_pre)
summary(Cort_final_pre)
plot(Cort_final_pre) # Observation #17 is very influent

# Using robust regression to correct for increasing variance
library(estimatr)
Cort_final_rob_pre<-lm_robust(cortisol_ng.ml_new~PC1,
data=PC1PC2_C_pre)
summary(Cort_final_rob_pre)

# Try withouth obs #17
Cort_final_rob_17<-lm_robust(cortisol_ng.ml_new~PC1,
data=PC1PC2_C_pre[-17, ])
summary(Cort_final_rob_17)

PC1PC2_C_post <- read.csv("PC1PC2_C_post.csv")

### Model for cortisol at post-deployment ###
Cort_post<-lm(cortisol_ng.ml_new~PC1*PC2*tl_mm,data=PC1PC2_C_post)
step(Cort_post)
dredge(Cort_post)

# Model averaging from MuMIN documentation
msA_post <- dredge(Cort_post)
msA_post

# Models with delta.aicc < 2
summary(model.avg(msA_post, subset = delta < 2))

# Final model
Cort_final_post<-
lm(cortisol_ng.ml_new~PC2+tl_mm+PC2:tl_mm,data=PC1PC2_C_post)
summary(Cort_final_post)
plot(Cort_final_post)

PC1PC2_WR_pre <- read.csv("PC1PC2_WR_pre.csv")
```

```
### Model for relative weight at pre-deployment ###
WR4_pre<-lm(WR4~PC1*PC2*tl_mm,data=PC1PC2_WR_pre)
step(WR4_pre)
dredge(WR4_pre)

# Model averaging from MuMIN documentation
ms4_pre <- dredge(WR4_pre)
# Final model
WR4_final<-lm(WR4~PC1*tl_mm+PC2*tl_mm,data=PC1PC2_WR_pre)
summary(WR4_final)
plot(WR4_final)

PC1PC2_WR_post <- read.csv("PC1PC2_WR_post.csv")

### Model for relative weight at post-deployment
WR4_post<-lm(WR4~PC1*PC2*tl_mm,data=PC1PC2_WR_post)
step(WR4_post)
dredge(WR4_post)

# Model averaging from MuMIN documentation
ms1_post <- dredge(WR4_post)

# Final model
WR4_final<-lm(WR4~tl_mm,data=PC1PC2_WR_post)
summary(WR4_final)
plot(WR4_final)

# Remove observation #19 and use robust regression to correct for
variance
WR4_final_rob<-lm_robust(WR4~tl_mm,data=PC1PC2_WR_post[-19, ])
summary(WR4_final_rob)

### Simplification - Correlation between measured OWIs #############
# Correlation between fins
Correlation <- read.csv("FinCorrelation.csv")
library(irr)

# Dorsal and caudal fins
ggplot(Correlation, aes(x=dorsalfin_sco, y=caudalfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')

dorVScau <- cor.test(x=Correlation$dorsalfin_sco,
y=Correlation$caudalfin_sco, method='spearman')

# Dorsal and anal fins
ggplot(data=Correlation, aes(x=dorsalfin_sco, y=analfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')

dorVSana <- cor.test(x=Correlation$dorsalfin_sco,
y=Correlation$analfin_sco, method='spearman')

# Dorsal and pectoral fins
ggplot(data=Correlation, aes(x=dorsalfin_sco, y=pecfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')

dorVSpec <- cor.test(x=Correlation$dorsalfin_sco,
```

```
y=Correlation$pecfin_sco,
                     method='spearman')

# Dorsal and ventral section of pectoral fins
ggplot(data=Correlation, aes(x=dorsalfin_sco, y=venpecfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')
dorVSven <- cor.test(x=Correlation$dorsalfin_sco,
y=Correlation$venpecfin_sco, method='spearman')

# Caudal and anal fins
ggplot(data=Correlation, aes(x=caudalfin_sco, y=analfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')

cauVSana <- cor.test(x=Correlation$caudalfin_sco,
y=Correlation$analfin_sco, method='spearman')

# Caudal and pectoral fins
ggplot(data=Correlation, aes(x=caudalfin_sco, y=pecfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')

cauVSpec <- cor.test(x=Correlation$caudalfin_sco,
y=Correlation$pecfin_sco,
                     method='spearman')

# Caudal and ventral section of pectoral fins
ggplot(data=Correlation, aes(x=caudalfin_sco, y=venpecfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')

cauVSven <- cor.test(x=Correlation$caudalfin_sco,
y=Correlation$venpecfin_sco, method='spearman')

# Anal and pectoral fins
ggplot(data=Correlation, aes(x=analfin_sco, y=pecfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')

anaVSpec <- cor.test(x=Correlation$analfin_sco,
y=Correlation$pecfin_sco,
                     method='spearman')

# Anal and ventral section of pectoral fins
ggplot(data=Correlation, aes(x=analfin_sco, y=venpecfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')

anaVSven <- cor.test(x=Correlation$analfin_sco,
y=Correlation$venpecfin_sco, method='spearman')

# Pectoral and ventral section of pectoral fins
ggplot(data=Correlation, aes(x=pecfin_sco, y=venpecfin_sco)) +
  geom_point(color='#2980B9', size=4) +
  geom_smooth(method=lm, se=FALSE, fullrange=TRUE, color='#2C3E50')

pecVSven <- cor.test(x=Correlation$pecfin_sco,
y=Correlation$venpecfin_sco,
                     method='spearman')
```

```
# Correlation between sucker deformity conditions
p <- ggplot(data=Correlation, aes(x=venpecfin_sco, y=SuckDef.PF)) +
  geom_point(color="grey", size=4) +
  geom_smooth(method="loess", se=FALSE, fullrange=TRUE,
color="blue") +
  xlab("Ventral section pectoral fins Score") + ylab("Sucker
deformity
  Score")

p + theme(axis.title.x = element_text(color="black",size=14,
face="bold"),
          axis.title.y = element_text(color="black",size=14,
face="bold"))

venpecVSsucker <- cor.test(x=Correlation$venpecfin_sco,
                           y=Correlation$SuckDef.PF,
method='spearman')

### Repeatability of LOWSI ########################################
# Skin damage
SD <- read.csv("Skindamage_ICC.csv")
icc(SD, model=c("twoway"), type=c("agreement"), unit=c("single"),
    r0=0, conf.level=0.95)

# Caudal fin damage
CFD <- read.csv("Caudalfindamage_ICC.csv")
icc(CFD, model=c("twoway"), type=c("agreement"), unit=c("single"),
    r0=0, conf.level=0.95)

# Eye condition
EC <- read.csv("Eyecondition_ICC.csv")
icc(EC, model=c("twoway"), type=c("agreement"), unit=c("single"),
    r0=0, conf.level=0.95)

# Suction disc deformity
SDD <- read.csv("Suctiondiscdeformity_ICC.csv")
icc(SDD, model=c("twoway"), type=c("agreement"), unit=c("single"),
    r0=0, conf.level=0.95)

# Relative weight
RW <- read.csv("Relativeweight_ICC.csv")
icc(RW, model=c("twoway"), type=c("agreement"), unit=c("single"),
    r0=0, conf.level=0.95)

# LOWSI
LOWSI <- read.csv("LOWSI_ICC.csv")
icc(LOWSI, model=c("twoway"), type=c("agreement"), unit=c("single"),
    r0=0, conf.level=0.95)

### LOWSI proportions by site #####################################
LOWSI <- read.csv("Lumpfish_Welfare_Score_Index_LOWSI.csv")

# Subset by site to extract proportions of welfare classes
LOWSI_H1 <- subset(LOWSI, site=="H1")
summary(LOWSI_H1)

LOWSI_H2 <- subset(LOWSI, site=="H2")
summary(LOWSI_H2)
```

```
LOWSI_H3 <- subset(LOWSI, site=="H3")
summary(LOWSI_H3)

LOWSI_F1 <- subset(LOWSI, site=="F1")
summary(LOWSI_F1)

LOWSI_F2 <- subset(LOWSI, site=="F2")
summary(LOWSI_F2)

LOWSI_F3 <- subset(LOWSI, site=="F3")
summary(LOWSI_F3)

LOWSI_props <- read.csv("LOWSI_props.csv")

# Proportion between sites
prop.test(x=c(51,10,29,11,45,28),n=c(74,16,30,35,60,30)) # Class A
prop.test(x=c(20,6,1,16,15,2),n=c(74,16,30,35,60,30)) # Class B
prop.test(x=c(3,0,0,8,0,0),n=c(74,16,30,35,60,30)) # Class C


#### Chapter 3 ####

### Stock proportion of sampled individuals ########################
Lumpfish_STR <- read.csv("Lumpfish_STR.csv")

# Explore data
head(Lumpfish_STR)
str(Lumpfish_STR)
Lumpfish_STR$SP <- factor(Lumpfish_STR$SP, levels=c("1","2"))

# Get summary statistics
Library(dplyr)
Lumpfish_STR %>%
  group_by(Cage, SP) %>%
  summarise(meanBW=mean(BW_g),
            meanTL=mean(TL_mm),
            meanWr=mean(Wr),
            sdBW=sd(BW_g),
            sdTL=sd(TL_mm),
            sdWr=sd(Wr))

Lumpfish_STR %>%
  group_by(Origin, SP) %>%
  summarise(meanBW=mean(BW_g),
            meanTL=mean(TL_mm),
            meanWr=mean(Wr),
            sdBW=sd(BW_g),
            sdTL=sd(TL_mm),
            sdWr=sd(Wr))

Lumpfish_STR %>%
  group_by(Stock, SP) %>%
  summarise(meanBW=mean(BW_g),
            meanTL=mean(TL_mm),
            meanWr=mean(Wr),
            sdBW=sd(BW_g),
            sdTL=sd(TL_mm),
            sdWr=sd(Wr))
```

```
# By each stock
zEng1 <- prop.test(x=c(14,306), n=c(320,320)) #Sign
zEng2 <- prop.test(x=c(65,255), n=c(320,320)) #Sign
zEng4 <- prop.test(x=c(5,315), n=c(320,320)) #Sign
zNorw <- prop.test(x=c(236,84), n=c(320,320)) #Sign
# Over time
zEng1SP <- prop.test(x=c(6,8), n=c(160,160)) #No sign
zEng2SP <- prop.test(x=c(35,30), n=c(160,160)) #No sign
zEng4SP <- prop.test(x=c(2,3), n=c(160,160)) #No sign
zNorwSP <- prop.test(x=c(117,119), n=c(160,160)) #No sign

### Changes in body size and condition between origins and over time
######

### Body weight (BW) ###
library(lmerTest)
# Full model
BW.m1 <- lmer (BW_g ~ Origin * SP + (1|Cage),
REML=FALSE,data=Lumpfish_STR)
BW.m2 <- lm (BW_g ~ Origin * SP, data=Lumpfish_STR)
anova(BW.m1, BW.m2, test="Chisq") # No differences, use LM

# Model assumptions
plot(BW.m2)

# Model reduction
dredge(BW.m2, options(na.action="na.fail"))

# Final model
BW.m3 <- lm (BW_g ~ Origin * SP, data=Lumpfish_STR)
summary(BW.m3)
plot(BW.m3)

Lumpfish_STR$SP <- factor(Lumpfish_STR$SP, levels=c("1","2"))
Lumpfish_STR$Origin <- factor(Lumpfish_STR$Origin,
levels=c("English","Norwegian"))

# Plot (Fig.3.1a)
Lumpfish_STRsum <- Lumpfish_STR %>%
  group_by(SP, Origin) %>%
  summarise(sd = sd(BW_g, na.rm=TRUE),
            BW_g = mean(BW_g))

ggplot(Lumpfish_STRsum, aes(SP, BW_g)) +
  geom_line(aes(linetype = Origin, group = Origin)) +
  geom_jitter(aes(x=SP, y=BW_g, color=Origin), data=Lumpfish_STR,
              position = position_jitter(0.25)) +
  geom_errorbar(aes(ymin = BW_g-sd, ymax = BW_g+sd, group =
Origin),width = 0.2) +
  xlab("Time-point") + ylab("Body weight (g)") + theme_bw() +
  scale_x_discrete(breaks=c("1","2"), labels=c("1 (3 weeks)","2 (3
months)")) +
  theme(axis.title.x=element_text(size=12, face="bold"),
        axis.title.y=element_text(size=12, face="bold"),
        legend.position="top", legend.title=element_blank())

### Total length (TL) ###
# Full model
TL.m1 <- lmer (TL_mm ~ Origin * SP + (1|Cage),
```

```
REML=FALSE,data=Lumpfish_STR)
TL.m2 <- lm (TL_mm ~ Origin * SP, data=Lumpfish_STR)
anova(TL.m1, TL.m2, test="Chisq") # LMM (TL.m1) is better than LM
(TL.m2)
AIC(TL.m1, TL.m2)
# Model reduction
dredge(TL.m1, options(na.action="na.fail"))
# Model refit
TL.m3 <- lmer (TL_mm ~ Origin * SP + (1|Cage), REML=TRUE,
data=Lumpfish_STR)
summary(TL.m3)
plot(TL.m3)
ranef(TL.m3)
drop1(TL.m3, test="Chisq")

# Model assumptions #
# Linearity #
Linearity <- plot(resid(TL.m3), Lumpfish_STR$TL_mm) # Non-random
pattern

# Homogenetiy of variance
Lumpfish_STR$TL.m3_res <- residuals(TL.m3)
Lumpfish_STR$Abs.TL.m3_res <-abs(Lumpfish_STR$TL.m3_res)
Lumpfish_STR$TL.m3_res2 <- Lumpfish_STR$Abs.TL.m3_res^2
Levene.TL.m3 <- lm (TL.m3_res2 ~ Cage, data=Lumpfish_STR)
anova(Levene.TL.m3) # Equal variance of residuals (homoscedasticity
ok)

# Residuals normally distributed
library(lattice)
qqmath(TL.m3, id=0.05) # looks ok

# Transform TL to meet model assumptions
Lumpfish_STR$lgTL <- log10(Lumpfish_STR$TL_mm)
lgTL.m4 <- lmer (lgTL ~ Origin * SP + (1|Cage), REML=TRUE,
data=Lumpfish_STR)
summary(lgTL.m4)

# Assumptions
library(ggResidpanel)
resid_panel(lgTL.m4) #looks OK
plot(cooks.distance(lgTL.m4))

# Plot (Fig.3.1b)
Lumpfish_STRsum <- Lumpfish_STR %>%
  group_by(SP, Origin) %>%
  summarise(sd = sd(TL_mm, na.rm=TRUE),
            TL_mm = mean(TL_mm))

ggplot(Lumpfish_STRsum, aes(SP, TL_mm)) +
  geom_line(aes(linetype = Origin, group = Origin)) +
  geom_jitter(aes(x=SP, y=TL_mm, color=Origin), data=Lumpfish_STR,
              position = position_jitter(0.25)) +
  geom_errorbar(aes(ymin = TL_mm-sd, ymax = TL_mm+sd, group =
Origin),width = 0.2) +
  xlab("Time-point") + ylab("Total length (mm)") + theme_bw() +
  scale_x_discrete(breaks=c("1","2"), labels=c("1 (3 weeks)","2 (3
months)")) +
  theme(axis.title.x=element_text(size=12, face="bold"),
        axis.title.y=element_text(size=12, face="bold"),
```

```
        legend.position="top", legend.title=element_blank())

### Body condition (Wr) ###
# Full model
Wr.m1 <- lmer (Wr ~ Origin * SP + (1|Cage),
REML=FALSE,data=Lumpfish_STR)
Wr.m2 <- lm (Wr ~ Origin * SP, data=Lumpfish_STR)
anova(Wr.m1, Wr.m2, test="Chisq") # LMM (Wr.m1) is better than LM
(Wr.m2)
AIC(TL.m1, TL.m2)

# Model reduction
dredge(Wr.m1, options(na.action="na.fail"))

# Model refit
Wr.m3 <- lmer (Wr ~ Origin * SP + (1|Cage), REML=TRUE,
data=Lumpfish_STR)
summary(Wr.m3)
drop1(Wr.m3, test="Chisq")

# Model assumptions #
# Linearity
Linearity <- plot(resid(Wr.m3), Lumpfish_STR$Wr) # Not-random
pattern

# Homogeneity of variance
Lumpfish_STR$Wr.m3_res <- residuals(Wr.m3)
Lumpfish_STR$Abs.Wr.m3_res <-abs(Lumpfish_STR$Wr.m3_res)
Lumpfish_STR$Wr.m3_res2 <- Lumpfish_STR$Abs.Wr.m3_res^2
Levene.Wr.m3 <- lm (Wr.m3_res2 ~ Cage, data=Lumpfish_STR)
anova(Levene.Wr.m3) # Homoscedasticity NOT met
# Residuals normally distributed
qqmath(Wr.m3, id=0.05) # looks more or less ok

# Transform Wr to meet model assumptions
Lumpfish_STR$lgWr <- log10(Lumpfish_STR$Wr)
lgWr.m4 <- lmer (lgWr ~ Origin * SP + (1|Cage), REML=TRUE,
data=Lumpfish_STR)
summary(lgWr.m4)
ranef(lgWr.m4)

# Assumptions
resid_panel(lgWr.m4) #looks better
plot(cooks.distance(lgWr.m4))

# Plot (Fig.3.2)
Lumpfish_STRsum <- Lumpfish_STR %>%
  group_by(Origin) %>%
  summarise(sd = sd(Wr, na.rm=TRUE),
            Wr = mean(Wr))

ggplot(Lumpfish_STRsum, aes(SP, Wr)) +
  geom_line(aes(linetype = Origin, group = Origin)) +
  geom_jitter(aes(x=SP, y=Wr, color=Origin), data=Lumpfish_STR,
              position = position_jitter(0.25)) +
  geom_errorbar(aes(ymin = Wr-sd, ymax = Wr+sd, group =
Origin),width = 0.2) +
  xlab("Time-point") + ylab("Body condition or \n Relative weight
(%)") + theme_bw() +
```

```
  scale_x_discrete(breaks=c("1","2"), labels=c("1 (3 weeks)","2 (3
months)")) +
  theme(axis.title.x=element_text(size=11, face="bold"),
        axis.title.y=element_text(size=11, face="bold"),
        legend.position="top", legend.title=element_blank())

# Frequency/proportion test for underweight+emaciated lumpfish over
time
prop.test(x=c(16,28),n=c(160,160))

# Frequency/ proportion test for emaciated lumpfish over time
prop.test(x=c(0,6),n=c(160,160))

# Test for variance between sampling time points
shapiro.test(Lumpfish_STR$BW_g) # not normally distributed
shapiro.test(Lumpfish_STR$TL_mm) # not normally distributed
shapiro.test(Lumpfish_STR$Wr) # not normally distributed

# Use Levene's test to compare variances
Lumpfish_STR$SP <- factor(Lumpfish_STR$SP, levels=c("1","2"))

library(car)
leveneTest (BW_g ~ SP, data=Lumpfish_STR)
leveneTest (TL_mm ~ SP, data=Lumpfish_STR)
leveneTest (Wr ~ SP, data=Lumpfish_STR)

### Changes in welfare (LOWSI) between origins and over time #######

# Full model
library(lme4)
welf.m1 <- glmer (LOWSI ~ Origin * SP * TL_mm + (1|Cage),
                  family="poisson", data=Lumpfish_STR)

welf.m2 <- glm (LOWSI ~ Origin * SP * TL_mm,
                family="poisson", data=Lumpfish_STR)

lrtest(welf.m1, welf.m2) # Adding a random factor does not fit the
model better
dredge(welf.m2)
summary(welf.m2)

# Reduced model
welf.m3 <- glm (LOWSI ~ Origin + SP + TL_mm,
                family="poisson",data=Lumpfish_STR)
summary(welf.m3)

# Model Assumptions #
plot(welf.m3)
resid_panel(welf.m3)
plot(cooks.distance(welf.m3))

ggplot(data=Lumpfish_STR, aes(x=SP, y=LOWSI, fill=Origin)) +
  geom_violin(draw_quantiles=0.5) +
  ylab ("LOWSI") + xlab("Time-point") +
  scale_x_discrete(breaks=c("1","2"), labels=c("1 (3 weeks)","2 (3
months)")) +
  theme(axis.title.x=element_text(size=11, face="bold"),
        axis.title.y=element_text(size=11, face="bold"),
        legend.position="top", legend.title=element_blank())
```

```
# Summary statistics for welfare
Lumpfish_STR %>%
  group_by(SP, Origin) %>%
  get_summary_stats(LOWSI, type="median_iqr")

ggplot(data=Lumpfish_STR, aes (x=TL_mm, y=LOWSI)) +
  geom_point() +
  geom_smooth(method='lm', formula=y~x) +
  geom_line(aes(y=3), size=1, colour="pale green",
linetype="dashed") +
  geom_line(aes(y=5), size=1, colour="orange", linetype="dashed") +
  xlab("Total length (mm)") + ylab("Welfare (LOWSI points)") +
  scale_y_continuous(breaks=seq(0,10,by=1), limits=c(0,10)) +
  scale_x_continuous(breaks=seq(70,170,by=10), limits=c(70,170)) +
  theme_bw()

### Body damage ###
BD.m1 <- glm (BD ~ SP + TL_mm,
              family="poisson",data=Lumpfish_STR)
dredge(BD.m1)
summary(BD.m1)
resid_panel(BD.m1)

### Caudal fin damage ###
CFD.m1 <- glm (CFD ~ SP + TL_mm,
               family="poisson", data=Lumpfish_STR)
dredge(CFD.m1)
summary(CFD.m1)
resid_panel(CFD.m1)

### Eye Condition ###
EC.m1 <- glm (EC ~ SP + TL_mm,
              family="poisson", data=Lumpfish_STR)
dredge(EC.m1)
summary(EC.m1)
resid_panel(EC.m1)

### Suction Disc Deformity ###
SDD.m1 <- glm (SDD ~ SP + TL_mm,
               family="poisson", data=Lumpfish_STR)
dredge(SDD.m1)
summary(SDD.m1)
resid_panel(SDD.m1)

### Relative weight ###
RW.m1 <- glm (RW ~ SP + TL_mm,
              family="poisson", data=Lumpfish_STR)
dredge(RW.m1)
summary(RW.m1)
resid_panel(RW.m1)

### Changes in ingested sea lice between origins and over time #####
SCA <- read.csv("StomachContent_STR.csv")
hist(SCA$SeaLiceC)

# Sea lice ingestion (counts) full model
SLI.m1 <- glm (SeaLiceC ~ Origin * SP * TL_mm,
               family=poisson (link=log),
               data=SCA)
```

```
SLI.m2 <- glm (SeaLiceC ~ Origin + SP + TL_mm,
               family=poisson (link=log),
               data=SCA)

anova(SLI.m1, SLI.m2) # no diffs
dredge(SLI.m2, options(na.action="na.fail"))

# Reduced model
SLI.m3 <- glm (SeaLiceC ~ SP + TL_mm,
               family=poisson (link=log),
               data=SCA)

resid_panel(SLI.m3)
summary(SLI.m3)
resid_panel(SLI.m3) # assumptions not met

# Assess over/under dispersion
Ov.SLIm3 <- SLI.m3$deviance/SLI.m3$df.residual
Ov.SLIm3 # 0.465 (under-dispersion)

# Alternative model: Zero-Inflated Poisson regression (ZIP)
library(pscl)
SLI.m4 <- zeroinfl(SeaLiceC ~ TL_mm + SP,
                   data=SCA)
summary(SLI.m4)

# Null model with no predictors
mnull <- update(SLI.m4, . ~ 1)
pchisq(2 * (logLik(SLI.m4) - logLik(mnull)), df = 2, lower.tail =
FALSE)

# Test if ZIP model is better than GLM
SLI.m3 <- glm (SeaLiceC ~ TL_mm + SP,
               family=poisson (link=log),
               data=SCA)
summary(SLI.m3)

vuong(SLI.m3, SLI.m4) #ZIP model is no better than GLM

# Summary statistics
SCA %>%
  group_by(SP) %>%
  get_summary_stats(SeaLiceC, type="mean_sd")

### Changes in ingested pellets between origins and over time ######
SCA <- read.csv("StomachContent_STR.csv")
# Pellet ingestion (counts) full model #
PI.m1 <- glm (PelletsC ~ Origin * SP * TL_mm,
              family=poisson (link=log),
              data=SCA)

PI.m2 <- glm (PelletsC ~ Origin + SP + TL_mm,
              family=poisson (link=log),
              data=SCA)

anova(PI.m1, PI.m2) # no diffs
dredge(PI.m2, options(na.action="na.fail"))
resid_panel(PI.m2)
```

```
# Assess overdisperison/underdispersion
Ov.PIm2 <- PI.m2$deviance/PI.m2$df.residual
Ov.PIm2 # 6.74 (overdispersion)

# Try with family=quasipoisson
PI.m3 <- glm (PelletsC ~ Origin + SP + TL_mm,
              family=quasipoisson (link=log),
              data=SCA)

resid_panel(PI.m3)
dredge(PI.m3)
summary(PI.m3)

# Summary statistics #
SCA %>%
  group_by(Origin) %>%
  get_summary_stats(PelletsC, type="mean_sd")

SCA %>%
  group_by(SP) %>%
  get_summary_stats(PelletsC, type="mean_sd")

### Fullness/distension ratio of stomachs ##########################
FR.m1 <- glm(StomachContent ~ SP + Origin + TL_mm,
             family=binomial(link="logit"),
             data=Lumpfish_STR)

FR.m2 <- glm(StomachContent ~ SP * Origin * TL_mm,
             family=binomial(link="logit"),
             data=Lumpfish_STR)

anova(FR.m1,FR.m2)
summary(FR.m1)
summary(FR.m2)
plot(FR.m1)
resid_panel(FR.m1)

summary(Lumpfish_STR$StomachContent)

Lumpfish_STR %>%
  group_by(Origin) %>%
  summarise(SC=mean(StomachContent),
            sd=sd(StomachContent))


#### Chapter 4 ####

### Comparison between Icelandic and Scottish stocks ###############
Lumpfish_ETB <- read.csv("Lumpfish_ETB.csv")
library(gvlma)
library(qqplotr)
library(performance)
library(olsrr)
options(ggrepel.max.overlaps = Inf)

Lumpfish_ETB %>%
  group_by(Stock) %>%
  summarise(meanBW=mean(BW_g),
```

```
            sdBW=sd(BW_g))

Lumpfish_ETB %>%
  group_by(Stock) %>%
  summarise(meanTL=mean(TL_mm),
            sdTL=sd(TL_mm))

Lumpfish_ETB %>%
  group_by(Stock) %>%
  summarise(meanWr=mean(Wr),
            sdWr=sd(Wr))

### Body size (BW,TL) and condition (Wr) ############################
t.test(BW_g ~ Stock, data=Lumpfish_ETB)
t.test(TL_mm ~ Stock, data=Lumpfish_ETB)
t.test(Wr ~ Stock, data=Lumpfish_ETB)

### Welfare (LOWSI) #################################################
ggplot(data=Lumpfish_ETB, aes(LOWSI,BW_g)) +
  geom_point() +
  facet_grid(~Stock)

ggplot(data=Lumpfish_ETB, aes(TL_mm, LOWSI)) +
  geom_point() +
  facet_grid(~Stock)

# Coefficient of variation for BW and TL
cv(Lumpfish_ETB$BW_g) # more variable
cv(Lumpfish_ETB$TL_mm) # less variable

# Model with LOWSI #
welf_1 <- glm(LOWSI ~ Stock + TL_mm, data=Lumpfish_ETB)
summary(welf_1) # LOWSI differed significantly between stocks, but
TL not significant
plot(welf_1)
check_outliers(welf_1)

# Non-parametric Kruskal-Wallis test
kruskal.test(LOWSI ~ Stock, data=Lumpfish_ETB)

# Assessing distribution of LOWSI scores
hist(Lumpfish_ETB$LOWSI)

# Welfare problems by stock #
Lumpfish_ETB$CountBD <- ave(Lumpfish_ETB$BD, Lumpfish_ETB$Stock,
                            FUN = function(x) sum(x!=0))

Lumpfish_ETB$CountCFD <- ave(Lumpfish_ETB$CFD, Lumpfish_ETB$Stock,
                             FUN = function(x) sum(x!=0))

Lumpfish_ETB$CountEC <- ave(Lumpfish_ETB$EC, Lumpfish_ETB$Stock,
                            FUN = function(x) sum(x!=0))

Lumpfish_ETB$CountSDD <- ave(Lumpfish_ETB$SDD, Lumpfish_ETB$Stock,
                             FUN = function(x) sum(x!=0))

Lumpfish_ETB$CountRW <- ave(Lumpfish_ETB$RW, Lumpfish_ETB$Stock,
                            FUN = function(x) sum(x!=0))
```

```
### Percentage of welfare problems by stock ########################
# Subset Icelandic (n=86) and Scottish (n=xx) lumpfish
Icelandic <- subset (Lumpfish_ETB, Stock=="Icelandic")
Scottish <- subset (Lumpfish_ETB, Stock=="Scottish")

# Welfare problems Icelandic stock
BD_Ice <- (10/86)*100
CFD_Ice <- (61/86)*100
EC_Ice <- (13/86)*100
SDD_Ice <- (8/86)*100
RW_Ice <- (6/86)*100

# Welfare problems Scottish stock
BD_Sco <- (3/56)*100
CFD_Sco <- (25/56)*100
EC_Sco <- (24/56)*100
SDD_Sco <- (39/56)*100
RW_Sco <- (6/56)*100

# 2-proportion z-test #
zBD <- prop.test(x=c(10,3), n=c(86,56))
zCFD <- prop.test(x=c(61,25), n=c(86,56))
zEC <- prop.test(x=c(13,24), n=c(86,56))
zSDD <- prop.test(x=c(8,39), n=c(86,56))
zRW <- prop.test(x=c(6,6), n=c(86,56))

#### Welfare (by OWI) ##############################################

### Body damage (BD) ###
BDmod <- lm(BD ~ Stock + TL_mm, data=Lumpfish_ETB)
summary(BDmod) # Stock and size not significant
par(mfrow=c(2,2))
plot(BDmod)
par(mfrow=c(1,1))

# Non-parametric Mann-Whitney U test
wilcox.test(BD ~ Stock, conf.int=TRUE, data=Lumpfish_ETB) #No sign
diffs

### Caudal fin damage (CFD) ###
CFDmod <- lm(CFD ~ Stock + TL_mm, data=Lumpfish_ETB)
summary(CFDmod)
par(mfrow=c(2,2))
plot(CFDmod)
par(mfrow=c(1,1))

# Non-parametric Mann-Whitney U test
wilcox.test(CFD ~ Stock, conf.int=TRUE, data=Lumpfish_ETB) #Sign
diffs

### Eye condition (EC) ###
ECmod <- lm(EC ~ Stock + TL_mm, data=Lumpfish_ETB)
summary(ECmod)
par(mfrow=c(2,2))
plot(ECmod)
par(mfrow=c(1,1))

# Non-parametric Mann-Whitney U test
```

```
wilcox.test(EC ~ Stock, conf.int=TRUE, data=Lumpfish_ETB) #Sign
diffs

### Sucker deformity (SDD) ###
SDDmod <- lm(SDD ~ Stock + TL_mm, data=Lumpfish_ETB)
summary(SDDmod)
par(mfrow=c(2,2))
plot(SDDmod)
par(mfrow=c(1,1))

# Non-parametric Mann-Whitney U test
wilcox.test(SDD ~ Stock, conf.int=TRUE, data=Lumpfish_ETB) #Sign
diffs

### Relative weight (RW) ###
RWmod <- lm(RW ~ Stock + TL_mm, data=Lumpfish_ETB)
summary(RWmod)
par(mfrow=c(2,2))
plot(RWmod)
par(mfrow=c(1,1))

# Non-parametric Mann-Whitney U test
wilcox.test(RW ~ Stock, conf.int=TRUE, data=Lumpfish_ETB) #No sign
diffs

#### Plasma Cortisol ################################################

# Subset data (only first n=60)
Cortisol <- Lumpfish_ETB[1:60,]

#Range of cortisol values
min(Cortisol$Cortisol)
max(Cortisol$Cortisol)

# Cortisol differences between stocks
mcort <- lm(Cortisol ~ Stock + TL_mm, data=Cortisol)
summary(mcort) # No differences between stocks, with size adjustment
plot(mcort) #looks ok

# Distribution of cortisol
hist(Cortisol$Cortisol)

LumpfishETB <- na.omit(Lumpfish_ETB)
hist(LumpfishETB$Cortisol)

# Plasma cortisol summary statitics
Cortisol %>%
  group_by(Stock) %>%
  summarise(meanCort=mean(Cortisol), sdCort=sd(Cortisol))
mean(Cortisol$Cortisol)
sd(Cortisol$Cortisol)

### Growth and changes before and after deployment (tagged, n=55) ##
TAGtime <- read.csv("TAG_time.csv")
TAGtime$Time <- factor(TAGtime$Time, levels=c("Before","After"))

#### Body size and condition ####
TAGtime %>%
  group_by(Time) %>%
  get_summary_stats(BW_g, TL_mm, Wr, type="mean_sd")
```

```
library(ggpubr)
ggboxplot(TAGtime, x="Time", y=c("BW_g", "TL_mm", "Wr"), merge=TRUE,
          ylab="", xlab="Time")

# Linear Mixed Models (LMMs) for repeated measures data
library(lme4)
library(lmerTest)

### LMM for BW ###
modBW <- lmer (BW_g ~ Time + (1|FishID),
               REML=TRUE, data=TAGtime)
summary(modBW)
drop1(modBW, test="Chi")
anova(modBW) #Sign diffs

# Model Assumptions #
# Linearity
modBW.Linearity <- plot(resid(modBW),TAGtime$BW_g) #ok

# Variance homogeneity
TAGtime$modBW.res <- residuals(modBW)
TAGtime$modBW.absres <- abs(TAGtime$modBW.res)
TAGtime$modBW.res2 <- TAGtime$modBW.absres^2
Levene.modBW <- lm(modBW.res2 ~ FishID, data=TAGtime)
anova(Levene.modBW) #p=0.4 (equal variance residuals,
homoscedasticity ok)
plot(modBW)

# Normality
qqmath(modBW, id=0.05) #ok

### LMM for TL ###
modTL <- lmer (TL_mm ~ Time + (1|FishID),
                REML=TRUE, data=TAGtime)
summary(modTL)
drop1(modTL, test="Chi")
anova(modTL) #Sign diffs

# Model Assumptions #
# Linearity
modTL.Linearity <- plot(resid(modTL),TAGtime$TL_mm) #ok

# Variance homogeneity
TAGtime$modTL.res <- residuals(modTL)
TAGtime$modTL.absres <- abs(TAGtime$modTL.res)
TAGtime$modTL.res2 <- TAGtime$modTL.absres^2
Levene.modTL <- lm(modTL.res2 ~ FishID, data=TAGtime)
anova(Levene.modTL) #p=0.6 (equal variance residuals,
homoscedasticity ok)
plot(modTL)

# Normality
qqmath(modTL, id=0.05) #ok

### LMM for WR ###
modWR <- lmer (Wr ~ Time + (1|FishID), REML=TRUE, data=TAGtime)
summary(modWR)
drop1(modWR, test="Chi") #Sign diffs

# Model Assumptions #
# Linearity
```

```
modWR.Linearity <- plot(resid(modWR),TAGtime$Wr) #ok

# Variance homogeneity
TAGtime$modWR.res <- residuals(modWR)
TAGtime$modWR.absres <- abs(TAGtime$modWR.res)
TAGtime$modWR.res2 <- TAGtime$modWR.absres^2
Levene.modWR <- lm(modWR.res2 ~ FishID, data=TAGtime)
anova(Levene.modWR) #p=0.3 (equal variance residuals,
homoscedasticity ok)
plot(modWR)

# Normality
qqmath(modWR, id=0.05) #ok

### Welfare (LOWSI) ###
TAGtime %>%
  group_by(Time) %>%
  get_summary_stats(LOWSI, type="median_iqr")

ggboxplot(TAGtime, x="Time", y=c("LOWSI"), merge=TRUE,
          ylab="", xlab="Time")

# Generalised Linear Mixed Models (GLMMs) for repeated measures data
### GLMM for LOWSI ###
modLOWSI <- glmer (LOWSI ~ Time + (1|FishID),
                   family=poisson (link="log"),
                   data=TAGtime)
summary(modLOWSI)

isSingular(modLOWSI, tol=1e-4)
drop1(modLOWSI, test="Chi")

### Welfare (by OWI) ###

# Summary statistics for welfare (LOWSI)
TAGtime %>%
  group_by(Time) %>%
  get_summary_stats(BD, CFD, EC, SDD, RW, type="median_iqr")

ggboxplot(TAGtime, x="Time", y=c("BD","CFD","EC","SDD","RW"),
merge=TRUE,
          ylab="", xlab="Time")

### GLMM for Body Damage (BD)  ###
modBD <- glmer (BD ~ Time + (1|FishID),
                   family=poisson (link="log"),
                   data=TAGtime)
summary(modBD)
isSingular(modBD, tol=1e-4)
drop1(modBD, test="Chi")

### GLMM for Caudal Fin Damage (CFD) ###
modCFD <- glmer (CFD ~ Time + (1|FishID),
                  family=poisson (link="log"),
                  data=TAGtime)
summary(modCFD)
### GLMM for Eye Condition (EC) ###
modEC <- glmer (EC ~ Time + (1|FishID),
                  family=poisson (link="log"),
```

```
                 data=TAGtime)
summary(modEC)

### GLMM for Suction Disc Deformity (SDD) ###
modSDD <- glmer (SDD ~ Time + (1|FishID),
                family=poisson (link="log"),
                data=TAGtime)
summary(modSDD)

### GLMM for Relative Weight (RW) ###
modRW <- glmer (RW ~ Time + (1|FishID),
                 family=poisson (link="log"),
                 data=TAGtime)
summary(modRW)

### Lumpfish growth rate during 60 days ############################
TAGdata <- read.csv("TAGdata.csv")

meanSGR.BW <- mean(TAGdata$SGR_BW)
meanSGR.BW # Overall growth of lumpfish for 60-day trial: 0.56%/d

meanSGR.TL <- mean(TAGdata$SGR_TL)
meanSGR.TL # Overall growth of lumpfish for 60-day trial: 0.29%/d

meanSGR.WR <- mean(TAGdata$SGR_Wr)
meanSGR.WR # Overall growth of lumpfish for 60-day trial: 0.15%/d

### Stomach Content Analysis #######################################
SCA_N <- read.csv("SCA_N.csv", header=TRUE, row.names=1)

# Calculate total of each food item
Pellets_T <- sum(SCA_N$Pellets)
Bivalves_T <- sum(SCA_N$Bivalves)
Hydrozoans_T <- sum(SCA_N$Hydrozoans)
Gammarids_T <- sum(SCA_N$Gammarids)
Caprellids_T <- sum(SCA_N$Caprellids)
Copepods_T <- sum(SCA_N$Copepods)
SeaLice_T <- sum(SCA_N$SeaLice)
FishPrey_T <- sum(SCA_N$FishPrey)
FishScales_T <- sum(SCA_N$FishScales)
FishTissue_T <- sum(SCA_N$FishTissue)
SeaWeed_T <- sum(SCA_N$SeaWeed)
Microplastics_T <- sum(SCA_N$Microplastics)
Unknown_T <- sum(SCA_N$Unknown)

# Total food items
Food_T=sum(Pellets_T,Bivalves_T,Hydrozoans_T,Gammarids_T,Caprellids_
T,Copepods_T,SeaLice_T,FishPrey_T,FishScales_T,FishTissue_T,SeaWeed_
T,Microplastics_T,Unknown_T)

# Calculate each food item % (ordered by abundance)
Gammarids <- (Gammarids_T/Food_T)*100
Unknown <- (Unknown_T/Food_T)*100
Pellets <- (Pellets_T/Food_T)*100
Seaweed <- (SeaWeed_T/Food_T)*100
FishTissue <- (FishTissue_T/Food_T)*100
Hydrozoans <- (Hydrozoans_T/Food_T)*100
Copepods <- (Copepods_T/Food_T)*100
FishScales <- (FishScales_T/Food_T)*100
```

```
Microplastics <- (Microplastics_T/Food_T)*100
Sealice <- (SeaLice_T/Food_T)*100
Caprellids <- (Caprellids_T/Food_T)*100
Bivalves <- (Bivalves_T/Food_T)*100
FishPrey <- (FishPrey_T/Food_T)*100

### Lumpfish diet composition (absence/presence) ###################
SCA_FO <- read.csv("SCA_FO.csv", header=TRUE, row.names=1)


# Subset by genetic stock
SCA_Ice <- subset(SCA_FO, Stock=="Icelandic")
SCA_Sco <- subset(SCA_FO, Stock=="Scottish")

# Remove individuals with empty stomachs
SCA_Ice.SC <- SCA_Ice[-c(7,20,23),]
SCA_Sco.SC <- SCA_Sco[-17,]

# %FO for each food item/group for Icelandic lumpfish
FO.Pellets_Ice <- (sum(SCA_Ice.SC$Pellets)/nrow(SCA_Ice.SC))*100
FO.Bivalves_Ice <- (sum(SCA_Ice.SC$Bivalves)/nrow(SCA_Ice.SC))*100
FO.Hydrozoans_Ice <-
(sum(SCA_Ice.SC$Hydrozoans)/nrow(SCA_Ice.SC))*100
FO.Gammarids_Ice <- (sum(SCA_Ice.SC$Gammarids)/nrow(SCA_Ice.SC))*100
FO.Caprellids_Ice <-
(sum(SCA_Ice.SC$Caprellids)/nrow(SCA_Ice.SC))*100
FO.Copepods_Ice <- (sum(SCA_Ice.SC$Copepods)/nrow(SCA_Ice.SC))*100
FO.SeaLice_Ice <- (sum(SCA_Ice.SC$SeaLice)/nrow(SCA_Ice.SC))*100
FO.FishPrey_Ice <- (sum(SCA_Ice.SC$FishPrey)/nrow(SCA_Ice.SC))*100
FO.FishScales_Ice <-
(sum(SCA_Ice.SC$FishScales)/nrow(SCA_Ice.SC))*100
FO.FishTissue_Ice <-
(sum(SCA_Ice.SC$FishTissue)/nrow(SCA_Ice.SC))*100
FO.Seaweed_Ice <- (sum(SCA_Ice.SC$SeaWeed)/nrow(SCA_Ice.SC))*100
FO.Microplastics_Ice <-
(sum(SCA_Ice.SC$Microplastics)/nrow(SCA_Ice.SC))*100
FO.Unknown_Ice <- (sum(SCA_Ice.SC$Unknown)/nrow(SCA_Ice.SC))*100
FO.Crustaceans_Ice <-
(sum(FO.Gammarids_Ice,FO.Caprellids_Ice,FO.Copepods_Ice))
FO.Fish_Ice <-
(sum(FO.FishPrey_Ice,FO.FishScales_Ice,FO.FishTissue_Ice))

# %FO for each food item/group for Scottish lumpfish
FO.Pellets_Sco <- (sum(SCA_Sco.SC$Pellets)/nrow(SCA_Sco.SC))*100
FO.Bivalves_Sco <- (sum(SCA_Sco.SC$Bivalves)/nrow(SCA_Sco.SC))*100
FO.Hydrozoans_Sco <-
(sum(SCA_Sco.SC$Hydrozoans)/nrow(SCA_Sco.SC))*100
FO.Gammarids_Sco <- (sum(SCA_Sco.SC$Gammarids)/nrow(SCA_Sco.SC))*100
FO.Caprellids_Sco <-
(sum(SCA_Sco.SC$Caprellids)/nrow(SCA_Sco.SC))*100
FO.Copepods_Sco <- (sum(SCA_Sco.SC$Copepods)/nrow(SCA_Sco.SC))*100
FO.SeaLice_Sco <- (sum(SCA_Sco.SC$SeaLice)/nrow(SCA_Sco.SC))*100
FO.FishPrey_Sco <- (sum(SCA_Sco.SC$FishPrey)/nrow(SCA_Sco.SC))*100
FO.FishScales_Sco <-
(sum(SCA_Sco.SC$FishScales)/nrow(SCA_Sco.SC))*100

FO.FishTissue_Sco <-
(sum(SCA_Sco.SC$FishTissue)/nrow(SCA_Sco.SC))*100
FO.Seaweed_Sco <- (sum(SCA_Sco.SC$SeaWeed)/nrow(SCA_Sco.SC))*100
FO.Microplastics_Sco <-
```

```
(sum(SCA_Sco.SC$Microplastics)/nrow(SCA_Sco.SC))*100
FO.Unknown_Sco <- (sum(SCA_Sco.SC$Unknown)/nrow(SCA_Sco.SC))*100
FO.Crustaceans_Sco <-
(sum(FO.Gammarids_Sco,FO.Caprellids_Sco,FO.Copepods_Sco))
FO.Fish_Sco <-
(sum(FO.FishPrey_Sco,FO.FishScales_Sco,FO.FishTissue_Sco))

### Lumpfish diet composition (abundance) ##########################
SCA_N <- read.csv("SCA_N.csv", header=TRUE, row.names=1)

# Subset by genetic stock
SCA_Ice <- subset(SCA_N, Stock=="Icelandic")
SCA_Sco <- subset(SCA_N, Stock=="Scottish")

# %N for each food item/group for Icelandic lumpfish (Nt=549)
N.Pellets_Ice <- (sum(SCA_Ice$Pellets)/549)*100
N.Bivalves_Ice <- (sum(SCA_Ice$Bivalves)/549)*100
N.Hydrozoans_Ice <- (sum(SCA_Ice$Hydrozoans)/549)*100
N.Gammarids_Ice <- (sum(SCA_Ice$Gammarids))
N.Caprellids_Ice <- (sum(SCA_Ice$Caprellids))
N.Copepods_Ice <- (sum(SCA_Ice$Copepods))
N.SeaLice_Ice <- (sum(SCA_Ice$SeaLice)/549)*100
N.FishPrey_Ice <- (sum(SCA_Ice$FishPrey))
N.FishScales_Ice <- (sum(SCA_Ice$FishScales))
N.FishTissue_Ice <- (sum(SCA_Ice$FishTissue))
N.Seaweed_Ice <- (sum(SCA_Ice$SeaWeed)/549)*100
N.Microplastics_Ice <- (sum(SCA_Ice$Microplastics)/549)*100
N.Unknown_Ice <- (sum(SCA_Ice$Unknown)/549)*100
N.Crustaceans_Ice <-
(sum(N.Gammarids_Ice,N.Caprellids_Ice,N.Copepods_Ice)/549)*100
N.Fish_Ice <-
(sum(N.FishPrey_Ice,N.FishScales_Ice,N.FishTissue_Ice)/549)*100

# %N for each food item/group for Scottish lumpfish (Nt=1752)
N.Pellets_Sco <- (sum(SCA_Sco$Pellets)/1752)*100
N.Bivalves_Sco <- (sum(SCA_Sco$Bivalves)/1752)*100
N.Hydrozoans_Sco <- (sum(SCA_Sco$Hydrozoans)/1752)*100
N.Gammarids_Sco <- (sum(SCA_Sco$Gammarids))
N.Caprellids_Sco <- (sum(SCA_Sco$Caprellids))
N.Copepods_Sco <- (sum(SCA_Sco$Copepods))
N.SeaLice_Sco <- (sum(SCA_Sco$SeaLice)/1752)*100
N.FishPrey_Sco <- (sum(SCA_Sco$FishPrey))
N.FishScales_Sco <- (sum(SCA_Sco$FishScales))
N.FishTissue_Sco <- (sum(SCA_Sco$FishTissue))
N.Seaweed_Sco <- (sum(SCA_Sco$SeaWeed)/1752)*100
N.Microplastics_Sco <- (sum(SCA_Sco$Microplastics)/1752)*100
N.Unknown_Sco <- (sum(SCA_Sco$Unknown)/1752)*100
N.Crustaceans_Sco <-
(sum(N.Gammarids_Sco,N.Caprellids_Sco,N.Copepods_Sco)/1752)*100
N.Fish_Sco <-
(sum(N.FishPrey_Sco,N.FishScales_Sco,N.FishTissue_Sco)/1752)*100


### Lumpfish diet composition (volume) ############################
SCA_W <- read.csv("SCA_W.csv", header=TRUE, row.names=1)

# Subset by genetic stock
SCA_Ice <- subset(SCA_W, Stock=="Icelandic")
SCA_Sco <- subset(SCA_W, Stock=="Scottish")
```

```
# %W for each food item/group for Icelandic lumpfish (Wt=43.73)
W.Pellets_Ice <- (sum(SCA_Ice$Pellets)/43.73)*100
W.Bivalves_Ice <- (sum(SCA_Ice$Bivalves)/43.73)*100
W.Hydrozoans_Ice <- (sum(SCA_Ice$Hydrozoans)/43.73)*100
W.Gammarids_Ice <- (sum(SCA_Ice$Gammarids))
W.Caprellids_Ice <- (sum(SCA_Ice$Caprellids))
W.Copepods_Ice <- (sum(SCA_Ice$Copepods))
W.SeaLice_Ice <- (sum(SCA_Ice$SeaLice)/43.73)*100
W.FishPrey_Ice <- (sum(SCA_Ice$FishPrey))
W.FishScales_Ice <- (sum(SCA_Ice$FishScales))
W.FishTissue_Ice <- (sum(SCA_Ice$FishTissue))
W.Seaweed_Ice <- (sum(SCA_Ice$SeaWeed)/43.73)*100
W.Microplastics_Ice <- (sum(SCA_Ice$Microplastics)/43.73)*100
W.Unknown_Ice <- (sum(SCA_Ice$Unknown)/43.73)*100
W.Crustaceans_Ice <-
(sum(W.Gammarids_Ice,W.Caprellids_Ice,W.Copepods_Ice)/43.73)*100
W.Fish_Ice <-
(sum(W.FishPrey_Ice,W.FishScales_Ice,W.FishTissue_Ice)/43.73)*100

# %W for each food item/group for Scottish lumpfish (Wt=16.42)
W.Pellets_Sco <- (sum(SCA_Sco$Pellets)/16.42)*100
W.Bivalves_Sco <- (sum(SCA_Sco$Bivalves)/16.42)*100
W.Hydrozoans_Sco <- (sum(SCA_Sco$Hydrozoans)/16.42)*100
W.Gammarids_Sco <- (sum(SCA_Sco$Gammarids))
W.Caprellids_Sco <- (sum(SCA_Sco$Caprellids))
W.Copepods_Sco <- (sum(SCA_Sco$Copepods))
W.SeaLice_Sco <- (sum(SCA_Sco$SeaLice)/16.42)*100
W.FishPrey_Sco <- (sum(SCA_Sco$FishPrey))
W.FishScales_Sco <- (sum(SCA_Sco$FishScales))
W.FishTissue_Sco <- (sum(SCA_Sco$FishTissue))
W.Seaweed_Sco <- (sum(SCA_Sco$SeaWeed)/16.42)*100
W.Microplastics_Sco <- (sum(SCA_Sco$Microplastics)/16.42)*100
W.Unknown_Sco <- (sum(SCA_Sco$Unknown)/16.42)*100
W.Crustaceans_Sco <-
(sum(W.Gammarids_Sco,W.Caprellids_Sco,W.Copepods_Sco)/16.42)*100
W.Fish_Sco <-
(sum(W.FishPrey_Sco,W.FishScales_Sco,W.FishTissue_Sco)/16.42)*100

# Index of Relative Importance (IRI) for Icelandic lumpfish
IRI.Pellets_Ice <- (sum(N.Pellets_Ice,W.Pellets_Ice))*FO.Pellets_Ice
IRI.Bivalves_Ice <-
(sum(N.Bivalves_Ice,W.Bivalves_Ice))*FO.Bivalves_Ice
IRI.Hydrozoans_Ice <-
(sum(N.Hydrozoans_Ice,W.Hydrozoans_Ice))*FO.Hydrozoans_Ice
IRI.Crustaceans_Ice <-
(sum(N.Crustaceans_Ice,W.Crustaceans_Ice))*FO.Crustaceans_Ice
IRI.SeaLice_Ice <- (sum(N.SeaLice_Ice,W.SeaLice_Ice))*FO.SeaLice_Ice
IRI.Fish_Ice <- (sum(N.Fish_Ice,W.Fish_Ice))*FO.Fish_Ice
IRI.Seaweed_Ice <- (sum(N.Seaweed_Ice,W.Seaweed_Ice))*FO.Seaweed_Ice
IRI.Microplastics_Ice <-
(sum(N.Microplastics_Ice,W.Microplastics_Ice))*FO.Microplastics_Ice
IRI.Unknown_Ice <- (sum(N.Unknown_Ice,W.Unknown_Ice))*FO.Unknown_Ice

IRI_Ice = sum(IRI.Pellets_Ice, IRI.Bivalves_Ice, IRI.Hydrozoans_Ice,
IRI.Crustaceans_Ice,
              IRI.SeaLice_Ice, IRI.Fish_Ice, IRI.Seaweed_Ice,
IRI.Microplastics_Ice,
              IRI.Unknown_Ice)

# Index of Relative Importance (IRI) for Scottish lumpfish
```

```
IRI.Pellets_Sco <- (sum(N.Pellets_Sco,W.Pellets_Sco))*FO.Pellets_Sco
IRI.Bivalves_Sco <-
(sum(N.Bivalves_Sco,W.Bivalves_Sco))*FO.Bivalves_Sco
IRI.Hydrozoans_Sco <-
(sum(N.Hydrozoans_Sco,W.Hydrozoans_Sco))*FO.Hydrozoans_Sco
IRI.Crustaceans_Sco <-
(sum(N.Crustaceans_Sco,W.Crustaceans_Sco))*FO.Crustaceans_Sco
IRI.SeaLice_Sco <- (sum(N.SeaLice_Sco,W.SeaLice_Sco))*FO.SeaLice_Sco
IRI.Fish_Sco <- (sum(N.Fish_Sco,W.Fish_Sco))*FO.Fish_Sco
IRI.Seaweed_Sco <- (sum(N.Seaweed_Sco,W.Seaweed_Sco))*FO.Seaweed_Sco
IRI.Microplastics_Sco <-
(sum(N.Microplastics_Sco,W.Microplastics_Sco))*FO.Microplastics_Sco
IRI.Unknown_Sco <- (sum(N.Unknown_Sco,W.Unknown_Sco))*FO.Unknown_Sco

IRI_Sco = sum(IRI.Pellets_Sco, IRI.Bivalves_Sco, IRI.Hydrozoans_Sco,
IRI.Crustaceans_Sco,
              IRI.SeaLice_Sco, IRI.Fish_Sco, IRI.Seaweed_Sco,
IRI.Microplastics_Sco,
              IRI.Unknown_Sco)

### Differences in diet composition between stocks (PERMANOVA) #####
adonis2 (SCA_FO.bc ~ SCA_FO.$Stock + SCA_FO.$TL_mm) #Sign
adonis2 (SCA_N.bc ~ SCA_N.$Stock + SCA_N.$TL_mm) #No sign
adonis2 (SCA_W.bc ~ SCA_W.$Stock + SCA_W.$TL_mm) # TL and stock
significant

### Test variance within groups (PERMDISP) ##########################

### SC by absence/presence (FO) ###
SCA_FO.$group <- paste(SCA_FO.$Stock)
FO.bc <- as.dist(SCA_FO.bc)
permdisp_FO <- betadisper(FO.bc, SCA_FO.$group)
plot(permdisp_FO)
anova(permdisp_FO)
permutest(permdisp_FO)

### SC by abundance (N) ###
SCA_N.$group <- paste(SCA_N.$Stock)
N.bc <- as.dist(SCA_N.bc)
permdisp_N <- betadisper(N.bc, SCA_N.$group)
plot(permdisp_N)
anova(permdisp_N)
permutest(permdisp_N)

### SC by weight (W) ###
SCA_W.$group <- paste(SCA_W.$Stock)
W.bc <- as.dist(SCA_W.bc)
permdisp_W <- betadisper(W.bc, SCA_W.$group)
plot(permdisp_W)
anova(permdisp_W)
permutest(permdisp_W)
### Average dissimilarity in diet composition (SIMPER) #############
```

```
# SIMPER function to be applied in the Bray-Curtis distance matrix
library(vegan)
SC.env <- SCA_N.[,1:7]

# SC by FO
simper.FO <- simper(SCA_FOdata, SC.env$Stock, permutations=999)
simper.FO
summary(simper.FO, ordered=TRUE, digits=3)

# SC by N
simper.N <- simper(SCA_Ndata, SC.env$Stock, permutations=999)
simper.N
summary(simper.N, ordered=TRUE, digits=3)

# SC by W
simper.W <- simper(SCA_Wdata, SC.env$Stock, permutations=999)
simper.W
summary(simper.W, ordered=TRUE, digits=3)

# Calculate the overall dissimilarity between the two genetic stocks
lapply(simper.FO, FUN=function(x)[x$overall]) # 52.4% different
lapply(simper.N, FUN=function(x)[x$overall]) # 80.21% different
lapply(simper.W, FUN=function(x)[x$overall]) # 84.17% different

### Microbiome analysis: diversity and structure ###################

# Install core packages of Bioconductor
if (!requireNamespace("BiocManager", quietly = TRUE))
  install.packages("BiocManager")
BiocManager::install()

#Install specific packages
BiocManager::install(c("GenomicFeatures", "AnnotationDbi"))
BiocManager::install("DESeq2")

# Load packages
library(grid)
library(gridExtra)
library(lme4)
library(nlme)
library(permute)
library(lattice)
library(vegan)
library(pheatmap)
library(patchwork)
library(reshape2)
library(stringr)
library(DESeq2)

metadata <- read.csv("metadata.csv")
alpha <- read.table("alpha_results.text", header=T)



### Alpha Diversity ################################################
# Subset datasets for Scottish and Icelandic lumpfish (n=57)
metadata <- metadata[-c(58:91),]
alpha <- alpha[-c(58:91),]
# Double check samples are in the same order
```

```
all.equal(metadata$SampleID, alpha$sample)

# Add information from metadata to alpha
alpha$Fish <- metadata$Fish
alpha$Site <- metadata$Site
alpha$Stock <- metadata$Stock
alpha$Cage <- metadata$Cage
alpha$BW_g <- metadata$BW_g
alpha$TL_mm <- metadata$TL_mm
alpha$SL_mm <- metadata$SL_mm
alpha$Size <- metadata$Size
alpha$Cortisol <- metadata$Cortisol
alpha$CortisolRange <- metadata$CortisolRange
alpha$SD <- metadata$SD
alpha$CFD <- metadata$CFD
alpha$EC <- metadata$EC
alpha$SuD <- metadata$SuD
alpha$WR <- metadata$WR
alpha$LOWSI <- metadata$LOWSI
alpha$Class <- metadata$Class
alpha$Welfare <- metadata$Welfare
alpha$SeaLice <- metadata$SeaLice
alpha$Pellets <- metadata$Pellets
alpha$Content <- metadata$Content
alpha$Ws <- metadata$Ws
alpha$Wr <- metadata$Wr

# Establish rank levels in some of the variables:
alpha$Stock <- factor(alpha$Stock, levels=c("Scottish","Icelandic"))
alpha$Size <- factor(alpha$Size, levels=c("Smaller","Bigger"))
alpha$SD <- factor(alpha$SD, levels=c("0","0.5","1"))
alpha$CFD <- factor(alpha$CFD, levels=c("0","0.5","1","2"))
alpha$EC <- factor(alpha$EC, levels=c("0","0.5","1","1.5","2"))
alpha$SuD <- factor(alpha$SuD, levels=c("0","0.5","1","1.5","2"))
alpha$WR <- factor(alpha$WR, levels=c("0","1","2"))
alpha$LOWSI <- factor(alpha$LOWSI,
levels=c("0","0.5","1","1.5","2","2.5",

"3","3.5","4","5","6","7"))
alpha$Class <- factor(alpha$Class, levels=c("A","B","C"))
alpha$Welfare <- factor(alpha$Welfare,
levels=c("Compromised","Good"))
alpha$CortisolRange <- factor(alpha$CortisolRange,
levels=c("Low","High"))
alpha$SeaLice <- factor(alpha$SeaLice, levels=c("No","Yes"))
alpha$Pellets <- factor(alpha$Pellets, levels=c("No","Yes"))
alpha$Content <- factor(alpha$Content, levels=c("Empty","Full"))

### Alpha Diversity Linear Models ##################################
# Chao Richness
chao_m1 <- lm(chao1 ~ Stock + TL_mm, data=alpha)

chao_m2 <- lm(chao1 ~ Stock, data=alpha)
summary(chao_m1)
shapiro.test(residuals(chao_m1)) # not normally distributed
anova(chao_m2)

chao_m3 <- lm(chao1 ~ Welfare, data=alpha)
summary(shan_m3)
```

```
chao_m4 <- lm(chao1 ~ TL_mm, data=alpha)
summary(chao_m4)

chao_m5 <- lm(chao1 ~ Cortisol, data=alpha)
summary(chao_m5)

# Shannon Diversity
shan_m1 <- lm(shannon ~ Stock + TL_mm, data=alpha)

shan_m2 <- lm(shannon ~ Stock, data=alpha)
summary(shan_m1)
shapiro.test(residuals(shan_m2)) # not normally distributed
anova(shan_m2)

shan_m3 <- lm(shannon ~ Welfare, data=alpha)
summary(shan_m3)

shan_m4 <- lm(shannon ~ TL_mm, data=alpha)
summary(shan_m4)

shan_m5 <- lm(shannon ~ Cortisol, data=alpha)
summary(shan_m5)

### Beta Diversity Models ##########################################
# PERMANOVA
adonis2(bc_dist ~ metadata$Stock * metadata$Welfare *
metadata$TL_mm)
adonis2(bc_dist ~ metadata$Stock + metadata$Welfare +
metadata$TL_mm)
adonis2(bc_dist ~ metadata$Stock + metadata$TL_mm)

adonis2(bc_dist ~ metadata$CortisolRange) # no differences
adonis2(bc_dist ~ metadata$Welfare) # significant differences

# Extract mean distance to centroids
bc_dist.S <- as.dist(bc_dist)
betadisper_stock <- betadisper(bc_dist.S, metadata$Stock,
type="centroid", bias.adjust=TRUE)
summary(betadisper_stock)
plot(betadisper_stock)
anova(betadisper_stock)
permutest(betadisper_stock)

MDTC.stock <- meandist(bc_dist.S, metadata$Stock)
MDTC.stock

### ASV Analysis - Relative abundance between stocks ###############
ASV <- read.csv("asv259_table.csv", check.names = F, row.names = 1)
meta <- read.csv("metadata.csv", row.names=1)

# Sum rows and sort by most abundant ASVs
ASV <- cbind(ASV, rowSums(ASV))
ASV <- ASV[order(rowSums(-ASV)),]
ASV[,c("rowSums(ASV)")] <- list(NULL)

# Subset metadata for Icelandic/Scottish stock
meta <- meta[1:57,]

# Transpose matrix so samples are in rows and species in columns
```

```
(requirement)
tASV <- t(ASV)

# Create relative abundance matrix from a total abundance matrix
library(funrar)
abs_abund = as.matrix(tASV)
head(abs_abund) #absolute abundances
rel_abund = make_relative(abs_abund)
head(rel_abund) #relative abundances
ASVra <- rel_abund

# Select the 25 most abundant ASVs
tASVra <- t(ASVra)
Bar25ra <- head(tASVra, 25)

# Transpose data back so species are in columns and samples in rows
tBar25ra <- t(Bar25ra)

# Create SampleID name so they can be split later:
meta$name2 <- paste(meta$Fish, meta$Stock, meta$Welfare,
meta$CortisolRange)
all.equal(row.names(tBar25ra), row.names(meta)) #check same order
row.names(tBar25ra) <- meta$name2 #change header name in rows

# Convert data frame from a "wide" format to a "long" format where
# Var1=SampleID, Var2=ASV, Var3=Value(count):
mtop25ra = melt(tBar25ra, id=c("ASV"))
mtop25ra$Var1 #57 levels=samples arranged in the new format
# Split names into extra columns for annotations:
fish_annot <- str_split_fixed(mtop25ra$Var1," ", 4)
fish_annot <- fish_annot[,1]
stock_annot <- str_split_fixed(mtop25ra$Var1," ", 4)
stock_annot <- stock_annot[,2]
welfare_annot <- str_split_fixed(mtop25ra$Var1," ", 4)
welfare_annot <- welfare_annot[,3]
cortisol_annot <- str_split_fixed(mtop25ra$Var1," ", 4)
cortisol_annot <- cortisol_annot[,4]

# Add separate colums to the mtop25 dataset:
mtop25ra$fish <- fish_annot
mtop25ra$stock <- stock_annot
mtop25ra$welfare <- welfare_annot
mtop25ra$cortisol <- cortisol_annot

mtop25ra$stock <- factor(mtop25ra$stock,
levels=c("Icelandic","Scottish"))
mtop25ra$welfare <- factor(mtop25ra$welfare,
levels=c("Compromised","Good"))
mtop25ra$cortisol <- factor(mtop25ra$cortisol,
levels=c("Low","High"))

ggplot(mtop25ra, aes(x=fish, y=value, fill=Var2)) +
  geom_bar(stat="identity", position="fill", colour="black") +
  facet_grid(rows=NULL, cols=vars(stock), scales="free",
space="fixed") +
  theme(axis.text.x=element_text(angle=90, size=8, vjust=0),
        axis.title.x=element_text(size=12, face="bold"),
        axis.title.y=element_text(size=12, face="bold"),
        legend.title=element_text(size=12, face="bold"),
        legend.text=element_text(size=10)) +
```

```
  labs(x="Fish ID", y="Relative abundance", fill="ASV")

### Differential Abundance Analysis with IndicSpec #################
library(indicspecies)
ASVab = read.csv("ASV.csv", header=TRUE, row.names=1)

# Split ASV abundance in a dataframe and the vectors for the factors
to group
abund = ASVab[,16:ncol(ASVab)]
stock = ASVab$Stock
welfare = ASVab$Welfare
cortisol = ASVab$CortisolRange

# Run indicator species command multipatt
IS_stock = multipatt(abund, stock, func="r.g",
control=how(nperm=9999))
summary(IS_stock, alpha=0.01)

# Re-analyse with Scottish classes (Clostridia related to stress)
ASVclass = read.csv("ASV_Class.csv", header=TRUE, row.names=1)

# Split ASV abundance in a dataframe and the vectors for the factors
to group
abund = ASVclass[4:ncol(ASVclass)]
stock = ASVclass$Stock
welfare = ASVclass$Welfare
cortisol = ASVclass$CortisolRange

# Run indicator species command multipatt
IS1 = multipatt(abund, stock, func="r.g", control=how(nperm=9999))
summary(IS1, alpha=0.01)

# Extract p-values
IS1.all <- IS1$sign
IS1.all.pvals <- IS1.all[,"p.value"]

## Correct p-values (FDR correction) and bind the results to
original output
fdr.p.value.IS1 <- p.adjust(IS1.all.pvals, method="fdr")
IS1.all.fdr <- cbind(IS1.all, fdr.p.value.IS1)

## Omit NA values and print only those with p <= 0.05
attach(IS1.all.fdr)
IS1.all.fdr.nona.sort <- IS1.all.fdr[order(fdr.p.value.IS1, p.value,
na.last=NA),]
IS1.all.fdr.nona.sort
detach(phi.all.fdr)

# Re-analyse by subsetting only the Scottish stock (greater
diversity)
ASVclass_Sco = read.csv("ASV_Class_Sco.csv", header=TRUE,
row.names=1)

# Split ASV abundance in a dataframe and the vectors for the factors
to group
abund_Sco = ASVclass_Sco[5:ncol(ASVclass_Sco)]
welfare_Sco = ASVclass_Sco$Welfare
cortisol_Sco = ASVclass_Sco$CortisolRange
```

```
m1 <- lm (Gammaproteobacteria ~ Cortisol, data=ASVclass_Sco)
summary(m1)

m1 <- lm (Clostridia ~ Cortisol, data=ASVclass_Sco)
summary(m1)

m1 <- lm (Bacteroidia ~ Cortisol, data=ASVclass_Sco)
summary(m1)

### Differential Abundance Analysis with DESeq2 ####################
countData <- as.matrix(read.csv("asv259_table.csv",
                          row.names=1, check.names=F))
countData <- countData+1 #sums 1 to each cell
colData <- read.csv("metadata.csv", row.names=1)
colData <- colData[-c(58:91),]

all(rownames(colData) %in% colnames(countData))
countData <- countData[,rownames(colData)]
all(rownames(colData) == colnames(countData))

### Deseq2 by Stock ###
Sco_stock_dseq <- DESeqDataSetFromMatrix(countData=countData,
                                         colData=colData,
                                         design=~Stock)

Sco_stock_dseq <- Sco_stock_dseq[rowSums(counts(Sco_stock_dseq)) >
58,]

design(Sco_stock_dseq) <- formula(~Stock)
Sco_stock_dseq <- DESeq(Sco_stock_dseq)

# Apply pairwise comparison and get output results
Sco_stock_res <- summary(results(Sco_stock_dseq, alpha=0.01,

contrast=c("Stock","Icelandic","Scottish")),
                           alpha=0.01)

setwd("~/PhD/Chapter 3. Common Garden Experiment/Datasets")
write.csv(results(Sco_stock_dseq, alpha=0.01,
                  contrast=c("Stock","Icelandic","Scottish")),
          "Deseq_out/Results_Sco_Stock.csv")

# Load result (output) file to analyse
setwd("~/PhD/Chapter 3. Common Garden
Experiment/Datasets/Deseq_out")
results <- read.csv("Results_Sco_Stock.csv", header=T, row.names=1)
summary(results)

res <- results(Sco_stock_dseq, alpha=0.01)
head(results(Sco_stock_dseq, tidy=TRUE))
summary(res)

# Summary list by adjusted p-val (in order):
res <- res[order(res$padj),]
head(res)
# Subsetting by Scottish Stock
Scotland_colData <- subset(colData, colData$Stock =="Scottish")
#n=28
Scottish_countData <-
countData[,c(1,3,7,8,9,10,11,12,13,14,18,19,20,21,22,26,
```

```
  28,29,30,31,32,34,41,42,44,46,47,50,57)]

### Deseq2 by Welfare ###
Sco_welfare_dseq <-
DESeqDataSetFromMatrix(countData=Scottish_countData,
                                            colData=Scotland_colData,
                                            design=~Welfare)

Sco_welfare_dseq <-
Sco_welfare_dseq[rowSums(counts(Sco_welfare_dseq)) > 29,]

design(Sco_welfare_dseq) <- formula(~Welfare)
Sco_welfare_dseq <- DESeq(Sco_welfare_dseq)

# Apply pairwise comparison and get output results
Sco_welfare_res <- summary(results(Sco_welfare_dseq, alpha=0.01,

contrast=c("Welfare","Compromised","Good")),
                           alpha=0.01)

setwd("~/PhD/Chapter 3. Common Garden Experiment/Datasets")
write.csv(results(Sco_welfare_dseq, alpha=0.01,
                  contrast=c("Welfare","Compromised","Good")),
          "Deseq_out/Results_Sco_Welfare.csv")

# Load result (output) file to analyse
setwd("~/PhD/Chapter 3. Common Garden
Experiment/Datasets/Deseq_out")
results <- read.csv("Results_Sco_Welfare.csv", header=T,
row.names=1)
summary(results)

res <- results(Sco_welfare_dseq, alpha=0.01)
head(results(Sco_welfare_dseq, tidy=TRUE))
summary(res)

# Summary list by adjusted p-val (in order):
res <- res[order(res$padj),]
head(res)

# Plotcounts to compare the normalized counts:
par(mfrow=c(1,1))
plotCounts(Sco_welfare_dseq, gene="ASV008_Candidatus Branchiomonas",
           intgroup="Welfare")
### Deseq2 by CortisolRange ###
Sco_cortisol_dseq <-
DESeqDataSetFromMatrix(countData=Scottish_countData,
                                            colData=Scotland_colData,
                                            design=~CortisolRange)

Sco_cortisol_dseq <-
Sco_cortisol_dseq[rowSums(counts(Sco_cortisol_dseq)) > 29,]

design(Sco_cortisol_dseq) <- formula(~CortisolRange)
Sco_cortisol_dseq <- DESeq(Sco_cortisol_dseq)

# Apply pairwise comparison and get output results
Sco_cortisol_res <- summary(results(Sco_cortisol_dseq,

contrast=c("CortisolRange","Low","High")),
```

```
                                    alpha=0.01)

setwd("~/PhD/Chapter 3. Common Garden Experiment/Datasets")
write.csv(results(Sco_cortisol_dseq, alpha=0.01,
                  contrast=c("CortisolRange","Low","High")),
          "Deseq_out/Results_Sco_Cortisol.csv")

# Load result (output) file to analyse
setwd("~/PhD/Chapter 3. Common Garden
Experiment/Datasets/Deseq_out")
results <- read.csv("Results_Sco_Cortisol.csv", header=T,
row.names=1)
summary(results)

res <- results(Sco_cortisol_dseq, alpha=0.01)
head(results(Sco_cortisol_dseq, tidy=TRUE))
summary(res)

# Summary list by adjusted p-val (in order):
res <- res[order(res$padj),]
head(res)

# Plotcounts to compare the normalized counts:
par(mfrow=c(1,1))
plotCounts(Sco_cortisol_dseq, gene="ASV005_Clostridium sensu stricto
1",
           intgroup="CortisolRange")


#### Chapter 5 ####

Lumpfish_SD <- read.csv("Lumpfish_SD.csv")
str(Lumpfish_SD)
summary(Lumpfish_SD)

# Sea lice variability
library(raster)
cv(Lumpfish_SD$SeaLiceC)

# Summary statistics
library(dplyr)
Lumpfish_SD %>%
  group_by(SeaLicePA) %>%
  summarise(meanBW=mean(BW_g),
            sdBW=sd(BW_g))

Lumpfish_SD %>%
  group_by(SeaLicePA) %>%
  summarise(meanTL=mean(TL_mm),
            sdTL=sd(TL_mm))

Lumpfish_SD %>%
  group_by(SeaLicePA) %>%
  summarise(meanWr=mean(Wr),
            sdWr=sd(Wr))

Lumpfish_SD %>%
  group_by(SeaLicePA) %>%
  summarise(meanCortisol=mean(Cortisol),
            sdCortisol=sd(Cortisol))
```

```
Lumpfish_SD %>%
  group_by(SeaLicePA) %>%
  summarise(meanLOWSI=mean(LOWSI),
            sdLOWSI=sd(LOWSI))

# Body condition: Underweight and emaciated lumpfish #
Lumpfish_SD %>%
  group_by(NutritionStatus) %>%
  summarise(meanWr=mean(Wr),
            sdWr=sd(Wr))

### Relationship between SLI and body size and condition ###########
t.test(BW_g ~ SeaLicePA, data=Lumpfish_SD)
t.test(TL_mm ~ SeaLicePA, data=Lumpfish_SD)
t.test(Wr ~ SeaLicePA, data=Lumpfish_SD)

# Subset dataset according to sea lice ingestion (SeaLicePA =
factor)
Lumpfish_SD$SeaLicePA <- factor(Lumpfish_SD$SeaLicePA,
levels=c("0","1"))

SLC_No <- subset(Lumpfish_SD, SeaLicePA=="0")
SLC_Yes <- subset(Lumpfish_SD, SeaLicePA=="1")

# Morphometric range and Mean&SE for non sea lice eaters
summary(SLC_No)

median(SLC_No$LOWSI)
range(SLC_No$LOWSI)

# Morphometric range and Mean&SE for sea lice eaters
summary(SLC_Yes)

median(SLC_Yes$LOWSI)
range(SLC_Yes$LOWSI)

### Relationship between SLI and welfare ############################
welf.m1 <- glm(LOWSI ~ SeaLicePA + TL_mm,
               family=poisson(link="log"),
               data=Lumpfish_SD)
summary(welf.m1) #TL significant
plot(welf.m1)
drop1(welf.m1) #better AIC if dropping SeaLicePA

welf.m2 <- glm(LOWSI ~ TL_mm,
               family=poisson(link="log"),
               data=Lumpfish_SD)
summary(welf.m2) #LOWSI is predicted by size of the fish
plot(welf.m2)

# Mean/median LOWSI value
library(rstatix)
Lumpfish_SD %>%
  get_summary_stats(LOWSI, type="median_iqr")

mean(Lumpfish_SD$LOWSI) # 3.37 (already classified as compromised
welfare)
sd(Lumpfish_SD$LOWSI)/sqrt(length(Lumpfish_SD$LOWSI))
```

```
median(Lumpfish_SD$LOWSI) #3
iqr(Lumpfish_SD$LOWSI)

### Lumpfish welfare ################################################
# Welfare problems (%)
ave(Lumpfish_SD$BD, FUN = function(x) sum(x!=0))
BodyDamage=(16/35)*100
ave(Lumpfish_SD$CFD, FUN = function(x) sum(x!=0))
CaudalFinDamage=(13/35)*100
ave(Lumpfish_SD$EC, FUN = function(x) sum(x!=0))
EyeCondition=(8/35)*100
ave(Lumpfish_SD$SDD, FUN = function(x) sum(x!=0))
SuctionDiscDeformity=(21/35)*100
ave(Lumpfish_SD$RW, FUN = function(x) sum(x!=0))
RelativeWeight=(33/35)*100

# Welfare problems by delousing groups (0=No, 1=Yes)
ave(SLC_No$BD, FUN = function(x) sum(x!=0))
BodyDamage_0=(5/17)*100
ave(SLC_No$CFD, FUN = function(x) sum(x!=0))
CaudalFinDamage_0=(6/17)*100
ave(SLC_No$EC, FUN = function(x) sum(x!=0))
EyeCondition_0=(3/17)*100
ave(SLC_No$SDD, FUN = function(x) sum(x!=0))
SuctionDiscDeformity_0=(9/17)*100
ave(SLC_No$RW, FUN = function(x) sum(x!=0))
RelativeWeight_0=(17/17)*100

ave(SLC_Yes$BD, FUN = function(x) sum(x!=0))
BodyDamage_1=(11/18)*100
ave(SLC_Yes$CFD, FUN = function(x) sum(x!=0))
CaudalFinDamage_1=(7/18)*100
ave(SLC_Yes$EC, FUN = function(x) sum(x!=0))
EyeCondition_1=(5/18)*100
ave(SLC_Yes$SDD, FUN = function(x) sum(x!=0))
SuctionDiscDeformity_1=(12/18)*100
ave(SLC_Yes$RW, FUN = function(x) sum(x!=0))
RelativeWeight_1=(16/18)*100

# Tests for welfare differences in delousing groups
wilcox.test(BD ~ SeaLicePA, data=Lumpfish_SD, paired=FALSE)
wilcox.test(CFD ~ SeaLicePA, data=Lumpfish_SD, paired=FALSE)
wilcox.test(EC ~ SeaLicePA, data=Lumpfish_SD, paired=FALSE)
wilcox.test(SDD ~ SeaLicePA, data=Lumpfish_SD, paired=FALSE)
wilcox.test(RW ~ SeaLicePA, data=Lumpfish_SD, paired=FALSE)

# Size effect on lumpfish welfare (Fig.5.1)
library(ggplot2)
library(tidyverse)
ggplot(data=Lumpfish_SD, aes (x=TL_mm, y=LOWSI)) +
  geom_point() +
  geom_smooth(method='lm', formula=y~x) +
  geom_line(aes(y=3), size=1, colour="pale green",
linetype="dashed") +
  geom_line(aes(y=5), size=1, colour="orange", linetype="dashed") +
  xlab("Total length (mm)") + ylab("Welfare (LOWSI points)") +
  scale_y_continuous(breaks=seq(0,10,by=1), limits=c(0,10)) +
  scale_x_continuous(breaks=seq(90,150,by=10), limits=c(90,150)) +
  theme_bw()
```

```
t.test(TL_mm ~ Welfare, data=Lumpfish_SD)

Lumpfish_SD %>%
  group_by(Welfare) %>%
  summarise(TL=mean(TL_mm),
            sd=sd(TL_mm))

### Relationship between SLI and cortisol response #################
library(performance)
cort_1 <- lm (Cortisol ~ SeaLicePA + TL_mm, data=Lumpfish_SD[-5,])
summary(cort_1) #p=0.3
plot(cort_1)
check_outliers(cort_1)

cort_2 <- lm (Cortisol ~ SeaLiceC + TL_mm, data=Lumpfish_SD[-5,])
summary(cort_2) #p=0.4
plot(cort_2)

AIC(cort_1, cort_2)

Lumpfish_SD$SeaLicePA <- factor (Lumpfish_SD$SeaLicePA,
                                 levels=c("0","1"),
                                 labels=c("No","Yes"))

ggplot(Lumpfish_SD, aes(x=SeaLicePA, y=Cortisol, fill=SeaLicePA)) +
  geom_boxplot() +
  xlab("Sea lice ingestion") +
  ylab("Plasma cortisol (ng/ml)") +
  ylim(0,260) + theme_bw()

# Relationship cortisol and LOWSI
ggplot(Lumpfish_SD, aes(x=LOWSI, y=Cortisol)) +
  geom_point() +
  geom_smooth(method='lm', formula=y~x)

### Relationship between SLI and diversity of microbiome ###########
alpha <- read.table("alpha_results.text", header=TRUE)
metadata <- read.csv("metadata.csv")
library(ggplot2)
library(grid)
library(gridExtra)
library(lme4)
library(nlme)
library(permute)
library(lattice)
library(vegan)
library(pheatmap)
library(patchwork)
library(reshape2)
library(stringr)
library(DESeq2)

# Double check samples are in the same order
all.equal(alpha$sample, metadata$SampleID)

# Add variables to alpha dataset
alpha$SeaLicePA <- metadata$SeaLicePA
alpha$SeaLicePA <- factor(alpha$SeaLicePA, levels=c("0","1"),
```

```
labels=c("No","Yes"))
alpha$TL_mm <- metadata$TL_mm
alpha$Welfare <- metadata$Welfare
alpha$Welfare<- factor(alpha$Welfare,
levels=c("Compromised","Good"))
alpha$LOWSI <- metadata$LOWSI
alpha$LOWSI <- factor(alpha$LOWSI,
levels=c("1","2","3","4","5","6","7"))

### Alpha Diversity linear models ###################################
alpha1 <- lm(chao1 ~ SeaLicePA + TL_mm, data=alpha)
summary(alpha1)
plot(alpha1)

alpha2 <- lm(shannon ~ SeaLicePA + TL_mm, data=alpha)
summary(alpha2)
plot(alpha2)

### Beta Diversity linear models ####################################
beta <- read.table("braycurtis_distance.tsv", header=TRUE)
beta <- beta[-c(1:57),-c(1:57)]
metadata <- read.csv("metadata.csv")

# Double check samples are in the same order:
all.equal(rownames(metadata), rownames(beta))

# Subset metadata by sea lice ingestion
Sealice_No <- subset (metadata, SeaLicePA=="0")
Sealice_Yes <- subset (metadata, SeaLicePA=="1")

# Betadisper
bcMDS <- metaMDS(beta) #beta is BrayCurtis distance matrix
bcMDS
stressplot(bcMDS) # Stress=0.21; Non-metric fit (R2)=0.956; Linear
fit (R2)=0.76

# Extract points (NMDS1 and NMDS2) from MDS
NMDS1 <- bcMDS$points[,1]
NMDS2 <- bcMDS$points[,2]

# Add them to the metadata dataset
nMDS.plot <- cbind(NMDS1,NMDS2,metadata)

# Plot NMDS ordination with sea lice consumption groups
nMDS.plot$SeaLicePA <- factor(nMDS.plot$SeaLicePA,
levels=c("0","1"),
                              labels=c("No","Yes"))
ggplot(nMDS.plot, aes(NMDS1,NMDS2,colour=SeaLicePA)) +
  geom_point(position=position_jitter(0.1), size=3) +
  theme_bw(base_size=12) +
  labs(x="nMDS1",y="nMDS2") +
  theme(axis.title.x=element_text(face="bold"),
        axis.title.y=element_text(face="bold"))

# PERMANOVA
adonis2(beta ~ metadata$SeaLicePA * metadata$TL_mm)
adonis2(beta ~ metadata$SeaLicePA + metadata$TL_mm)

# Extract p-values from adonis model
```

```
adonis.coeffs <- adonis$aov.tab
adonis.pvals <- adonis.coeffs[,"Pr(>F)"]

# Correct p-values (FDR correction) and bind results to original
output
fdr.pvals <- p.adjust(adonis.pvals, method="fdr")
adonis.all.fdr <- cbind(adonis.coeffs, fdr.pvals)

# Stats for beta diversity differences #
# Sea lice ingestion
# Extract mean distance to centroids
beta.SL <- as.dist(beta)
betadisper_SL <- betadisper(beta.SL, metadata$SeaLicePA,
                            type="centroid", bias.adjust=TRUE)
summary(betadisper_SL)
plot(betadisper_SL)
anova(betadisper_SL)
permutest(betadisper_SL)

MDTC.SL <- meandist(beta.SL, metadata$SeaLicePA)
MDTC.SL

### Characterization and relative abundance of ASVs ################
ASV_gen <- read.csv("asv191_table.csv", row.names=1)
metadata <- read.csv("metadata.csv", row.names=1)

# Transpose matrix so samples are in rows and species in columns
tASV_gen <- t(ASV_gen)

# Create relative abundance matrix from a total abundance matrix
library(funrar)
abs_abund = as.matrix(tASV_gen)
head(abs_abund) #absolute abundances
rel_abund = make_relative(abs_abund)
head(rel_abund) #relative abundances

ASVra <- rel_abund

# Select the 25 most abundant ASVs
tASVra <- t(ASVra)
Bar25ra <- head(tASVra, 25)

# Transpose data back so species are in columns and samples in rows
tBar25ra <- t(Bar25ra)

# Create SampleID name so they can be split later:
metadata$name2 <- paste(metadata$Fish, metadata$SeaLicePA,
metadata$Welfare,
                        metadata$NutritionStatus,
metadata$CortisolRange)
all.equal(row.names(tBar25ra), row.names(metadata)) #check same
order
row.names(tBar25ra) <- metadata$name2 #change header name in rows

# Convert data frame from a "wide" format to a "long" format where
library(reshape)
mtop25ra = melt(tBar25ra, id=c("ASV"))
mtop25ra$X1 #34 levels=samples arranged in the new format

# Split names into extra columns for annotations
```

```
library(stringr)
fish_annot <- str_split_fixed(mtop25ra$X1," ", 5)
fish_annot <- fish_annot[,1]
sealice_annot <- str_split_fixed(mtop25ra$X1, " ", 5)
sealice_annot <- sealice_annot[,2]
welfare_annot <- str_split_fixed(mtop25ra$X1, " ", 5)
welfare_annot <- welfare_annot[,3]
wr_annot <- str_split_fixed(mtop25ra$X1, " ", 5)
wr_annot <- wr_annot[,4]
cort_annot <- str_split_fixed(mtop25ra$X1," ", 5)
cort_annot <- cort_annot[,5]

# Add separate columns to the mtop25 dataset
mtop25ra$fish <- fish_annot
mtop25ra$sealice <- sealice_annot
mtop25ra$welfare <- welfare_annot
mtop25ra$wr <- wr_annot
mtop25ra$cort <- cort_annot

mtop25ra$sealice <- factor(mtop25ra$sealice, levels=c("0","1"),
labels=c("No","Yes"))
mtop25ra$welfare <- factor(mtop25ra$welfare, levels=c("Compromised",
"Good"))
mtop25ra$wr <- factor(mtop25ra$wr,
levels=c("Normal","Underweight","Emaciated"))
mtop25ra$cort <- factor(mtop25ra$cort, levels=c("Low","High"))

# Relative abundance according to the ingestion of sea lice
(Fig.5.3)
ggplot(mtop25ra, aes(x=fish, y=value, fill=X2)) +
  geom_bar(stat="identity", position="fill", colour="black") +
  facet_grid(rows=NULL, cols=vars(sealice), scales="free",
space="fixed") +
  theme(axis.text.x=element_text(angle=90, size=8, vjust=0),
        axis.title.x=element_text(size=12, face="bold"),
        axis.title.y=element_text(size=12, face="bold"),
        legend.title=element_text(size=12, face="bold"),
        legend.text=element_text(size=10)) +
  labs(x="Fish ID", y="Relative abundance", fill="ASV")

### Differential Abundance Analysis (IndicSpec) ####################
ASVab = read.csv("asv191_metatable.csv", header=TRUE, row.names=1)
library(indicspecies)

# Split ASV abundance in a dataframe and the vectors for the factors
to group
abund = ASVab[,8:ncol(ASVab)]
sealice = ASVab$SeaLicePA
welfare = ASVab$Welfare
cortisol = ASVab$CortisolRange
condition = ASVab$NutritionStatus

# Run indicator species command multipatt
IS1 = multipatt(abund, sealice, func="r.g", control=how(nperm=9999))
summary(IS1, alpha=0.05)

# Extract p-values for each analysis
IS1.all <- IS1$sign
IS1.all.pvals <- IS1.all[,"p.value"]
```

```
    # Correct p-values (FDR correction) and bind the results to original
    output
    fdr.p.value.IS1 <- p.adjust(IS1.all.pvals, method="fdr")
    IS1.all.fdr <- cbind(IS1.all, fdr.p.value.IS1)

    # Omit NA values and print only taxa with p < 0.05
    attach(IS1.all.fdr)
    IS1.all.fdr.nona.sort <- IS1.all.fdr[order(fdr.p.value.IS1, p.value,
    na.last=NA),]
    IS1.all.fdr.nona.sort
    detach(IS1.all.fdr) # No DA taxa after FDR correction

    ### DA with DESeq2 ################################################
    if (!requireNamespace("BiocManager", quietly = TRUE))
      install.packages("BiocManager")
    BiocManager::install("DESeq2")
    library(DESeq2)

    # Convert phyloseq object to DeSeq format
    library(DESeq2)
    DES <- phyloseq_to_deseq2(LumpMic1, ~ SeaLicePA * Welfare)

    # Run DESeq2 analysis (negative binomial GLM framework)
    dds <- DESeq(DES)

    # Investigate results
    res <- results(dds)
    deseq.results <- as.data.frame(res)
    df <- deseq.results
    df$taxon <- rownames(df)
    df <- df %>% arrange(log2FoldChange, padj)

    # Print the results; flitered and sorted by pvalue and effectsize
    library(knitr)
    df <- df %>% filter(pvalue < 0.05 & log2FoldChange > 1.5) %>%
      arrange(pvalue, log2FoldChange)
    kable(df, digits = 5)

    ### Microbiome Networks ###########################################
    devtools::install_github("stefpeschel/NetCoMi", dependencies = TRUE,
    force=TRUE,repos = c("https://cloud.r-project.org/",
BiocManager::repositories()))
    # Load packages
    library(NetCoMi)
    library(readxl)
    library(phyloseq)
    library(dplyr)

    # Load datasets for non-delousing lumpfish
    asv_mat1 <- read_excel("phyloseq1_SLINo.xlsx", sheet="ASVtable")
    tax_mat1 <- read_excel("phyloseq1_SLINo.xlsx", sheet="ASVtaxonomy")
    samples_df1 <- read_excel("phyloseq1_SLINo.xlsx", sheet="Samples")
    samples_df1$Welfare <- factor(samples_df1$Welfare,
    levels=c("Compromised","Good"))

    # Define row names for each dataset
    asv_mat1 <- asv_mat1 %>% tibble::column_to_rownames("ASV")
    tax_mat1 <- tax_mat1 %>% tibble::column_to_rownames("ASV")
    samples_df1 <- samples_df1 %>% tibble::column_to_rownames("Samples")
```

```
# Transform asv_mat and tax_mat into matrices
asv_mat1 <- as.matrix(asv_mat1)
tax_mat1 <- as.matrix(tax_mat1)

# Transform to phyloseq object
OTU = otu_table(asv_mat1, taxa_are_rows=TRUE)
TAX = tax_table(tax_mat1)
samples = sample_data(samples_df1)

LumpMic_NoSLI <- phyloseq(OTU, TAX, samples)
LumpMic_NoSLI

# Load datasets for delousing lumpfish
asv_mat2 <- read_excel("phyloseq1_SLIYes.xlsx", sheet="ASVtable")
tax_mat2 <- read_excel("phyloseq1_SLIYes.xlsx", sheet="ASVtaxonomy")
samples_df2 <- read_excel("phyloseq1_SLIYes.xlsx", sheet="Samples")
samples_df2$Welfare <- factor(samples_df2$Welfare,
levels=c("Compromised","Good"))

# Define row names for each dataset
asv_mat2 <- asv_mat2 %>% tibble::column_to_rownames("ASV")
tax_mat2 <- tax_mat2 %>% tibble::column_to_rownames("ASV")
samples_df2 <- samples_df2 %>% tibble::column_to_rownames("Samples")

# Transform asv_mat and tax_mat into matrices
asv_mat2 <- as.matrix(asv_mat2)
tax_mat2 <- as.matrix(tax_mat2)

# Transform to phyloseq object
OTU = otu_table(asv_mat2, taxa_are_rows=TRUE)
TAX = tax_table(tax_mat2)
samples = sample_data(samples_df2)

LumpMic_SLI <- phyloseq(OTU, TAX, samples)
LumpMic_SLI

### Non-delousing lumpfish Network Comparison ######################
# Split phyloseq object according to welfare
NoSLI <- metagMisc::phyloseq_sep_variable(LumpMic_NoSLI, "Welfare")

# Network construction
net_NoSLI <- netConstruct(data=NoSLI$Compromised,
                          data2=NoSLI$Good,
                          filtTax="highestFreq",
                          filtTaxPar=list(highestFreq=50),
                          measure="pearson",
                          normMethod="mclr",
                          zeroMethod="none",
                          sparsMethod="threshold",
                          thresh=0.3)

# Network analysis
props_NoSLI <- netAnalyze(net_NoSLI)
summary(props_NoSLI)

# Identify the strongest positive and negative associations of each
network
# Group 1 - Compromised welfare
```

```
assomat1 <- net_NoSLI$assoMat1
diag(assomat1) <- 0
max(assomat1)
which(assomat1 == max(assomat1), arr.ind = TRUE)
min(assomat1)
which(assomat1 == min(assomat1), arr.ind = TRUE) # no negative
associations

# Group 2 - Good welfare
assomat2 <- net_NoSLI$assoMat2
diag(assomat2) <- 0
max(assomat2)
which(assomat2 == max(assomat2), arr.ind = TRUE)
min(assomat2)
which(assomat2 == min(assomat2), arr.ind = TRUE)

# Network plot (Fig.5.4)
plot(props_NoSLI, sameLayout=TRUE, layoutGroup="union",
     rmSingles="inboth", nodeSize="mclr", nodeColor="cluster",
     labelScale=TRUE, shortenLabels="intelligent", labelLength=14,
     labelPattern=c(10,"'",3), cexNodes = 4, cexLabels = 5.5,
     cexHubLabels = 3, cexTitle = 1.8,
     groupNames = c("Compromised welfare", "Good welfare"),
     showTitle=TRUE, colorVec=rainbow(30), nodeTransp=50,
repulsion=1.05,
     hubBorderCol  = "black")

# Network quantitative comparison (permutation tests) with block-
wise execution (in parallel)

# Create the matrix with permuted group labels
permGroupMat <- createAssoPerm(props_NoSLI,
                               nPerm = 100,
                               computeAsso = FALSE,
                               seed = 123456)

nPerm_all <- 100
blocksize <- 20
repetitions <- nPerm_all / blocksize  # 5 repetitions

# Execute in parallel
library("foreach")

cores <- 2 # depending on PC power
cl <- snow::makeCluster(cores)
doSNOW::registerDoSNOW(cl)

# Create progress bar:
pb <- utils::txtProgressBar(0, repetitions, style=3)
progress <- function(n){
  utils::setTxtProgressBar(pb, n)
}

opts <- list(progress = progress)
tmp <- foreach(i = 1:repetitions,
               .packages = c("NetCoMi"),
               .options.snow = opts) %dopar% {

                 blocksize,
```

```
p                ress(i) NetCoMi::createAssoPerm(props_NoSLI,
r                nPerm =
o
g                                                permGroupMat =
permGroupMat[(i-1) * blocksize

+ 1:blocksize, ],
                                                 computeAsso = TRUE,
                                                 fileStoreAssoPerm =
paste0("assoPerm", i),
                                                 storeCountsPerm = FALSE,
append = FALSE)
               }

# Close progress bar
close(pb)

# Stop cluster
snow::stopCluster(cl)
```

```
# Combine the matrices and store them into a new file (netCompare
needs ext file)
library(filematrix)

assoPerm_all <- NULL
for(i in 1:repetitions){

  assoPerm_tmp <- fm.open(filenamebase = paste0("assoPerm", i) ,
readonly = TRUE)

  assoPerm_all <- rbind(assoPerm_all, as.matrix(assoPerm_tmp))

  close(assoPerm_tmp)
}

dim(assoPerm_all)

# Combine the permutation association matrices
fm.create.from.matrix(filenamebase = "assoPerm", mat = assoPerm_all)

# Pass the combined matrix to netCompare function
comp_noSLI <- netCompare(props_NoSLI, permTest = TRUE, nPerm = 1000,
                         fileLoadAssoPerm = "assoPerm",
                         storeCountsPerm = FALSE, seed = 123456)
summary(comp_noSLI)

### Delousing lumpfish Network Comparison ##########################
# Split phyloseq object according to sea lice ingestion
YesSLI <- metagMisc::phyloseq_sep_variable(LumpMic_SLI, "Welfare")
# Network construction
net_YesSLI <- netConstruct(data=YesSLI$Compromised,
                           data2=YesSLI$Good,
                           filtTax="highestFreq",
                           filtTaxPar=list(highestFreq=50),
                           measure="pearson",
                           normMethod="mclr",
                           zeroMethod="none",
                           sparsMethod="threshold",
                           thresh=0.3,
                           seed=123456)

# Network analysis
props_YesSLI <- netAnalyze(net_YesSLI)
summary(props_YesSLI)

# Identify the strongest positive and negative associations of each
network

# Group 1 - Compromised welfare
assomat1 <- net_YesSLI$assoMat1
diag(assomat1) <- 0
max(assomat1)
which(assomat1 == max(assomat1), arr.ind = TRUE)
min(assomat1)
which(assomat1 == min(assomat1), arr.ind = TRUE) # no negative
associations

# Group 2 - Good welfare
assomat2 <- net_YesSLI$assoMat2
```

```
diag(assomat2) <- 0
max(assomat2)
which(assomat2 == max(assomat2), arr.ind = TRUE)
min(assomat2)
which(assomat2 == min(assomat2), arr.ind = TRUE)

# Network plot
plot(props_YesSLI, sameLayout=TRUE, layoutGroup="union",
     rmSingles="inboth", nodeSize="mclr", nodeColor="cluster",
     labelScale=TRUE, shortenLabels="intelligent", labelLength=14,
     labelPattern=c(10,"'",3), cexNodes = 4, cexLabels = 5.5,
     cexHubLabels = 3, cexTitle = 1.8,
     groupNames = c("Compromised welfare", "Good welfare"),
     showTitle=TRUE, colorVec=rainbow(30), nodeTransp=50,
repulsion=1.05,
     hubBorderCol  = "black")

# Network quantitative comparison (permutation tests) with block-
wise execution (in parallel)

# Create the matrix with permuted group labels
permGroupMat <- createAssoPerm(props_YesSLI,
                               nPerm = 100,
                               computeAsso = FALSE,
                               seed = 123456)

nPerm_all <- 100
blocksize <- 20
repetitions <- nPerm_all / blocksize  # 5 repetitions

# Execute in parallel
library("foreach")

cores <- 2 # depending on PC power
cl <- snow::makeCluster(cores)
doSNOW::registerDoSNOW(cl)

# Create progress bar:
pb <- utils::txtProgressBar(0, repetitions, style=3)
progress <- function(n){
  utils::setTxtProgressBar(pb, n)
}

opts <- list(progress = progress)
tmp <- foreach(i = 1:repetitions,
               .packages = c("NetCoMi"),
               .options.snow = opts) %dopar% {

                 progress(i)
                 NetCoMi::createAssoPerm(props_YesSLI, nPerm =
blocksize,
                                         permGroupMat =
```

```
permGroupMat[(i-1) * blocksize
+ 1:blocksize, ],

pasteO("assoPerm", i),append = FALSE)
                                        }
# Close progress barclose(pb)
```

```
computeAsso = TRUE,fileStoreAssoPerm =

storeCountsPerm = FALSE,

# Stop cluster
snow::stopCluster(cl)
# Combine the matrices and store them into a new file (netCompare
needs ext file)
library(filematrix)

assoPerm_all <- NULL
for(i in 1:repetitions){

  assoPerm_tmp <- fm.open(filenamebase = paste0("assoPerm", i) ,
readonly = TRUE)

  assoPerm_all <- rbind(assoPerm_all, as.matrix(assoPerm_tmp))

  close(assoPerm_tmp)
}

dim(assoPerm_all)

# Combine the permutation association matrices
fm.create.from.matrix(filenamebase = "assoPerm", mat = assoPerm_all)
# Pass the combined matrix to netCompare function
comp_YesSLI <- netCompare(props_YesSLI, permTest = TRUE, nPerm =
1000,
                          fileLoadAssoPerm = "assoPerm",
                          storeCountsPerm = FALSE, seed = 123456)

summary(comp_YesSLI)
###
```

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## Development, validation and testing of an Operational Welfare Score Index for farmed lumpfish *Cyclopterus lumpus* L

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ABSTRACT

Lumpfish (*Cyclopterus lumpus* L.) are widely used for controlling sea lice in salmon farming, but their welfare is often challenged by poor husbandry, stress, and disease outbreaks, which compromise their ability to delouse salmon and cause public concern. It is hence important to identify when the welfare of lumpfish is being compromised in a simple and effective manner so that remedial actions can be taken. We developed, validated and tested a Lumpfish Operational Welfare Score Index (LOWSI) based on a visual assessment of skin and fin damage, eye condition, sucker deformities and relative weight, operational welfare indicators that fish farmers considered to be the most informative and were validated against cortisol measurements. We also present percentile length-weight charts to enable fish farmers to detect underweight and emaciated lumpfish at different stages of development. The lumpfish welfare score index was quick and easy to score and was highly repeatable (intra class correlation coefficient = 0.83 ± 0.05). Most lumpfish (71%) displayed good welfare, but significant differences were found between six commercial sites and 28% of lumpfish had lower than normal weights for their length, and 10% were emaciated. The most common welfare problems were sucker deformities and fin damage in hatcheries, and poor eye condition and body damage in sea cages, conditions that may increase the risk of emaciation. Being able to score the welfare of lumpfish quickly and accurately will help improve their welfare, reduce stress-related mortalities, and improve the sustainability of the salmon farming industry.

### 1. Introduction

Growing consumer demand for ethically-produced food has led to the development of specific welfare standards for a few farmed fish such as Atlantic salmon (Pettersen et al., 2014; RSPCA, 2018), but only generic guidelines exist for most farmed species (Cooke, 2016). Given the large diversity of fish, and their very different habitat and social requirements, welfare criteria that may work well for some species may not be applicable to others (Toni et al., 2019; Treasurer and Feledi, 2014).

The lumpfish (*Cyclopterus lumpus* L.) is a novel species to aquaculture that is increasingly being used to control sea lice (*Lepeophtheirus salmonis*), an ectoparasite that represents one of the major threats to salmon farming (Torrissen et al., 2013). Sea lice cause substantial economic losses to industry (Costello, 2009b), impact the survival and welfare of wild and farmed salmon alike (Costello, 2006; Costello, 2009a), and tarnish public's perception of salmon farming (Hersoug, 2015; Jackson et al., 2018). Increasing resistance to chemotherapeutants traditionally used to combat sea lice (Aaen et al., 2015) has prompted an interest on the use of cleaner fish as an environmentally-friendly 'green' alternative to medicines (Powell et al., 2018b). Commercial production of lumpfish has grown exponentially over the past few years, and reached 4.8 million in 2017 in the UK alone (Treasurer et al., 2018b). However, their survival is often poor, and there is increasing concern regarding their welfare (Brooker et al., 2018; Treasurer and Feledi, 2014). Studies suggest that between 33% and 50% of lumpfish may die following deployment in salmon cages (Imsland et al., 2016; Noble et al., 2019; Stien et al., 2020), reaching 100% in some cases (European Union Reference Laboratory for Fish Diseases, 2016; OneKind, 2018). Infectious diseases are a common cause of mortality in lumpfish (Brooker et al., 2018; Powell et al., 2018b), but they are not the only ones. Starvation, poor husbandry, high water temperatures, strong currents, low oxygen, and traumatic injuries caused by rough handling have also been cited as sources of mortality (Anon, 2020; Grefsrud et al., 2019; Stien et al., 2020) and compromise the welfare of lumpfish (Hjeltnes et al., 2018; Treasurer et al., 2018a). The public and retailers generally support the use of lumpfish for controlling sea lice because of the environmental and efficacy benefits that they provide (Anon, 2013), but only as long as the welfare of cleaner fish is not compromised (Treasurer et al., 2018a).

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Thus, the development of suitable metrics of lumpfish welfare is important, not only for identifying those activities that compromise welfare, but also for quality assurance (Brooker et al., 2018; Treasurer et al., 2018a) and for restoring public confidence on the salmon farming industry and its ability to tackle the threat posed by sea lice (Hersoug, 2015; Jackson et al., 2018).

To be effective, operational welfare indicators need to be practical and easy to use, or they will not be used by fish farmers (North et al., 2008; van de Vis et al., 2012). The use of Operational Welfare Indicators (OWIs) represents a practical approach to achieve this, as these indicators are designed to be easily scored at the farm (Folkedal et al., 2016; Noble et al., 2018). Welfare indicators need to be fit for purpose and be tailored to particular species and uses (Gismervik et al., 2018; Kolarevic et al., 2018), although some OWIs are more generic than others and may be used across species and contexts. For example, a high prevalence of deformities, external injuries, and fin damage may signal low welfare in many species, particularly those reared at high densities (Hoyle et al., 2007; Noble et al., 2012). Although genetic factors should not be excluded, fin damage can result from aggression, but also from stress (Turnbull et al., 1996) and may cause detrimental effects on growth and survival by increasing the susceptibility to infection, potentially impacting swimming ability (Noble et al., 2012). In contrast, some welfare indicators, like eye and body darkening, can be indicative of social stress in territorial or aggressive species like Nile tilapia (Champneys et al., 2018; Freitas et al., 2014) or Atlantic salmon (O'Connor et al., 1999; Suter and Huntingford, 2002), but may be totally unsuitable for shoaling fish. Other individual based indicators, such as plasma cortisol (Pavlidis et al., 2013), expression of stress-related genes (Rodriguez-Barreto et al., 2019; Uren Webster et al., 2018), or the presence of bacterial biomarkers (Uren Webster et al., 2020), require analytical equipment and training that are not typically available within an aquaculture setting; they are laboratory-based welfare indicators, not operational ones (Noble et al., 2018).

Assessing the welfare of lumpfish under farm conditions poses particular challenges. Lumpfish are weak swimmers (Hvas et al., 2018) and lack a swim bladder, which makes them particularly vulnerable to exhaustion and barotrauma if they cannot attach to a suitable substrate (Powell et al., 2018a). Juveniles tend to aggregate in clumps, display a low cortisol response (Treasurer et al., 2018a) and lack Mauthner neurons (Hale, 2000), which makes it difficult to assess their stress response and determine optimal rearing densities (Powell et al., 2018b). In addition, lumpfish can easily suffer from malnutrition, as they cannot survive grazing on sea lice alone and need supplemental feeding (Imsland et al., 2018b; Treasurer et al., 2018a). Thus, some measure of body condition should be included in an operational welfare indicator for this species (Johannesen et al., 2018). A recent report has reviewed some potential welfare indicators for lumpfish (Noble et al., 2019), but there is no validated index that can be used under farm conditions. Here we used an aggregated welfare indicator approach (Rousing et al., 2001) to develop, validate, refine and test a Lumpfish Operational Welfare Score Index (LOWSI) based on the visual assessment of several operational individual-based welfare indicators that can be easily scored in hatcheries and sea cages.

## 2. Materials and methods

To develop a practical index of welfare for lumpfish (LOWSI) we adopted a workflow that consisted of four steps (Fig. 1): (1) selection and screening of individual-based OWIs in collaboration with lumpfish farmers; (2) validation of OWIs against measures of cortisol and body condition; (3) refinement and simplification of OWIs, and (4) testing of an aggregated operational welfare score index at six commercial sites.

### 2.1. Selection and screening of individual-based OWIs

To select potential welfare indicators for testing, a questionnaire

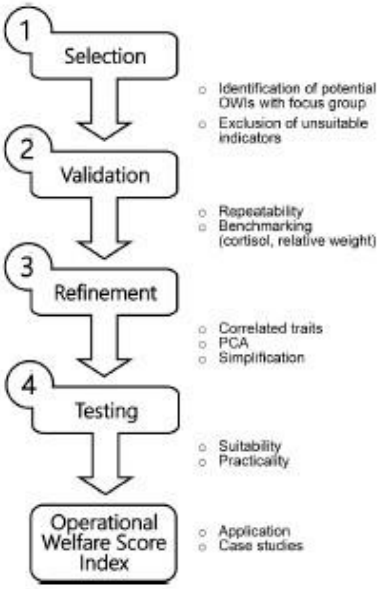



**Fig. 1.** Work-flow used to develop an operational welfare score index for farmed lumpfish.

was given to 53 lumpfish farmers and other participants in the Lumpfish Welfare Workshop at the First Welfare in Aquaculture Symposium (Swansea, 14th May 2019). During the focus group, respondents were asked to assess the potential utility of 12 welfare indicators for lumpfish (Table S1). We excluded from further testing those indicators that were group based (mortality, growth), laboratory based (blood analysis), or required specialized training (parasite/disease screening), or had proved unreliable (body darkening) or shown limited or no variation in pilot trials (operculum erosion, body deformities). We focussed on 6 potential OWIs (external body damage, fin damage, eye condition, eye darkening, suction cup deformities, and relative weight) as described below. For these, we scored high resolution photographs (Canon EOS 800D; EFS 18-55 mm, TAMRON 90 mm lens) of the body, eyes, fins and suction cup of 95 freshly euthanized lumpfish (overdose of anaesthesia, UK HO schedule 1) sampled at two hatcheries ($n = 60$, 5-152 g) and one salmon cage ($n = 35$, 22-100 g) in the UK. A tripod and a standard black background fitted with a scale and a reference colour chart (Colour Checker Passport, X-rite) were used to ensure consistency between photographs.

### 2.2. External body damage

External body damage was assessed on a 2-point scale (absence: 0, presence: 1) depending on the presence of skin lesions, including erosion, reddening, abrasion and body ulcers (Fig. S1).

### 2.3. Fin damage

We scored damage of the 4-rayed fins (dorsal, caudal, anal and pectoral) on a 5-point Likert scale according to the extension of the tissue area affected (Fig. S2). Left and right pectoral fins were averaged, and an aggregated total fin damage score ranging between 0 and 20 was obtained by summing the erosion of the four fins.

### 2.4. Eye condition

Three eye conditions (eye damage, exophthalmia and cataracts) were scored on a 3-point scale, depending on the extension of the condition (0: absence; 1: one eye affected; 2: both eyes affected; Fig. S3). An aggregated eye condition score was obtained by adding the three scores, with values ranging from 0 to 6.

### 2.5. Eye darkening

To quantify eye darkening, we divided photographs of the eye sclera into eight equal sections and assessed the percentage of darkening in each octant (Champneys et al., 2018). The average of the left and right eyes was converted to a 5-point Likert scale depending on the extent of the darkened area (Fig. S4).

### 2.6. Suction cup deformities

Deformity of the ventral suction cup was assessed according to five parameters that were found to vary on a pilot screening: (1) symmetry of the suction cup, (2) indentations, (3) depressions, (4) papillae development, and (5) deformity or curling of the ventral section of the pectoral fins. Each condition was assessed on a 5-point Likert scale depending on severity and extent (Fig. S5), and all scores were summed to provide a suction cup deformity score, with values ranging from 0 to 20, that classified the fish into five suction cup deformity classes: Class A – Perfect suction cup (total score = 0). Symmetrical, without indentations, flat, with well-developed papillae and with pectoral fins that do not obliterate the suction cup. Class B – Mild deformity (total score 1–5). Slight asymmetry, with some depression and/or indentations and minor under-development of the papillae or slight curling of the pectoral fins. Class C – Moderate deformity (total score 6–10). Moderate asymmetry, depressions and indentations, and clear under-development of the papillae or curling of the pectoral fins that hide parts of the suction cup. Class D – Substantial deformity (total score 11–15). Substantial asymmetry, with deep depressions and indentations, substantial under-development of the papillae, and significant curling of the pectoral fins that hide most of the suction cup. Class E – Severe deformity (total score > 15), non-functional suction cup. Severe asymmetry, with severe depressions, indentations and under-development of the papillae and totally deformed or curled pectoral fins that cover all the suction cup.

### 2.7. Relative weight

Relative weight ($W_r$) was used as an index of body condition, rather than Fulton's condition factor (Blackwell et al., 2000), because it is more appropriate for fish like lumpfish that have an unusual body shape (Al Nahdi et al., 2016). We collected data on total length (TL, mm) and body mass (W, wet weight, g) of 2658 farmed lumpfish sampled during 2015–2019 at four stages of development: (S1) Larvae (0-1 g), (S2) Pre-deployment juveniles (1–10 g), (S3) Pre-deployment juveniles (+10 g) and (S4) Post-deployment. From these, expected standard weights ($W_s$) were computed for each stage of development using the parameters of the fitted regressions $\log10\ W_s = a + (b * \log10\ TL)$, where $a$ is the intercept, $b$ is the slope and TL is the total length. Relative weight was then calculated as $W_r = 100*(W/W_s)$, where $W$ is the observed weight and $W_s$ is the standard (i.e. expected) weight for fish of that length and that stage of development (Blackwell et al., 2000). We considered that fish were underweight if they were 10–25% below their expected weight (i.e. Wr = 90–75%) and severely underweight or emaciated if they were 25% or more below their expected weight (i.e. $Wr < 75\%$), when their head typically becomes the widest part of the body (Noble et al., 2019). To aid farmers to quickly identify underweight fish we constructed percentile length-weight charts for each stage of development (Fig. S6).

### 2.8. Validation of individual-based OWIs

We used two criteria for OWI validation: (1) reliability, and (2) construct validity. Reliability measures the magnitude of the measurement error in relation to the inherent variability between subjects (Bartlett and Frost, 2008) and was calculated by scoring the same fish twice by the same and different raters. Construct validity is the degree to which scores are consistent with a priori hypothesised differences between relevant groups, based on the assumption that the scale validly accurately captures the construct it purports to measure (Mokkink et al., 2010).

### 2.9. Reliability

Two raters (A and B) working independently scored the images of the 95 lumpfish used for the OWI screening above. Images were allocated at random. Observer A also scored the same images after 8 months, to provide a measure of intra-rater reliability. For each OWI, we calculated the intraclass correlation coefficient (ICC) using the *irr* R package (Gamer et al., 2019) and the single-rating, absolute-agreement, 2-way random-effects model. The ICC is a suitable tool to measure reliability, as it considers both the strength of the correlation and the agreement between measurements (Koo and Li, 2016).

### 2.10. Construct validity

In the absence of an agreed standard for measuring lumpfish welfare, construct validity was evaluated via two surrogate welfare measures previously tested on other species (Noble et al., 2018): relative weight (as an indication of poor growth) as described above, and plasma cortisol (involved in the stress response). An aggregated welfare score was calculated for each fish by adding the scores of each OWI (range: 0 to 51), these were standardized and centred by subtracting the mean and dividing by the standard deviation before being analysed by principal component analysis (PCA) using the *factoextra* R package (Kassambara and Mundt, 2019).

To measure plasma cortisol, we collected blood through a puncture of the caudal vein (lithium-heparinised Vacutainer Blood Collection System) in a sample of recently euthanized lumpfish used in the OWI scoring above ($n = 55$, range = 22–152 g). Blood was collected mainly in the morning (0900–1300 h), by the same person, using the same equipment, and within 30 s from cessation of opercular movement to reduce unwanted variation. We employed the plasma preparation protocol from ThermoFisher Scientific to separate plasma from whole blood (Thavasu et al., 1992) consisting of centrifugation at 1500 rpm for 10 min at 15 °C and storage at −80 °C until analysis. For cortisol quantification we used a competitive ELISA test (DetectX Cortisol Enzyme Immunoassay Kit, Arbor Assays, Michigan, USA), that has been widely used before to measure plasma cortisol in fish (Huyben et al., 2019; Uren Webster et al., 2020).

Each plasma sample was treated with a dissociation reagent to increase its yield (mean recovery rate 96.7%) and diluted with buffer (1:50) before cortisol determination. Standards, blanks and test samples were loaded in duplicate and absorbance values (OD) were read with a SpectroStar Nano Plate Reader at 450 nm wave-length. The average of two duplicates was used to create a standard curve ($R^2 = 0.994$), and concentrations were multiplied by the dilution factor (1:100) to obtain plasma cortisol values (ng/ml). Assay sensitivity was 24.7 pg/ml, while detection limit was 18.01 pg/ml. Intra-assay and inter-assay precision (CV%) for duplicate samples was 15.4% and 6.8%, respectively.

### 2.11. Refinement & simplification

As fish that had one eroded fin typically showed erosion in others, we examined the pairwise matrix of Spearman's rank correlation coefficients between fin erosion scores to identify the most sensitive fins for

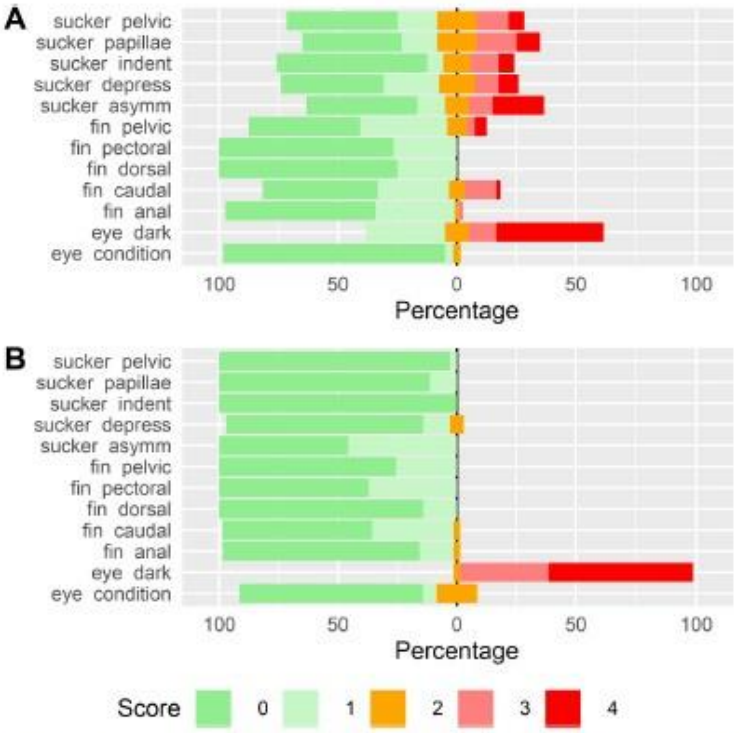



**Fig. 2.** Variation in operational welfare indicators (OWIs) in lumpfish sampled from (A) hatcheries (pre-deployment, $n = 60$) and (B) in sea cages (post-deployment, $n = 35$). Shown are the percentage of fish scored on a 5-point Likert scale (score 0–4) for each indicator, depending on the extent and severity of each condition.

damage scoring. We then simplified the original OWI scores into a 10-point Lumpfish Operational Welfare Score Index (LOWSI) that included relative weight and the four most reliable OWIs, all scored on a 3-point scale to ensure equal weighting.

### 2.12. Testing

To test the application of the LOWSI, 245 lumpfish from three hatcheries (H1-H3, $n = 120$) and three sea farms (F1-F3, $n = 125$) were scored by one or two raters during 2018–2019. Rearing temperature ranged between 8 and 12 °C, density between 4.5 and 24 kg/m$^3$ in hatcheries and between 4 and 15% of salmon numbers in sea cages, and photoperiod between 12 and 24 h light at hatcheries, depending on stage of development. Lumpfish were classified into three welfare classes depending on the values of the LOWSI: (A) Good welfare (< 3 points), (B) Moderately compromised welfare (3–5 points), and (C) Severely compromised welfare (> 5 points). ICC estimates of reliability were computed as above, using the scores of the lead author and 8 fish farmers on a subsample of 150 fish that were scored twice. To assess the practical implementation of the LOWSI, a questionnaire (Table S2) consisting of four questions and five possible responses (1-Strongly disagree, 2-Disagree, 3-Neutral, 4-Agree, 5-Strongly agree) was given to 8 fish farmers who had previously scored the welfare of lumpfish.

### 2.13. Statistical analysis

All data were analysed using R version 3.6.1 (R Core Team, 2019). We used the Wilcoxon test to compute pseudo-medians and 95% CI on the Likert scale to estimate the perceived utility of each welfare indicator (Mangiafico, 2016), and a cumulative link mixed model with the *clmm2* function in the R package *ordinal* to assess the degree of consensus among participants, having tested the proportional odds assumption via the *nominal_test* and the *scale_test* (Christensen, 2019). Consensus among lumpfish farmers on the practical application of the aggregated welfare score index was analysed with a cumulative link mixed model, as above.

To asses construct validity, we used changes in the Akaike Information Criterion (AIC) and the *MuMIn* package (Barton, 2019) to investigate how the first two principal components of the aggregated welfare score (PC1, PC2) varied in relation to plasma cortisol and relative weight while statistically controlling for variation in body size. We tested model assumptions by examining the distribution of residuals with respect to linearity, normality, homogeneity of variances, and leverage using the *plot* command and the *gvlma* package in R (Pena and Slate, 2019). Two observations were identified as overly influential outliers by the *olsrr* R package (Hebbali, 2020) and were excluded from the validation of relative weight (obs. #19, Cook's distance = 0.142, Studentized residual = 2.77) or cortisol (obs #17, Cook's distance = 2.31, Studentized residual = 2.84).

### 2.14. Ethical statement

The study was conducted following approval by Swansea University Ethics Committee (Permits SU-Ethics-Student-130718/692, SU-Ethics-Student-110618/713).

## 3. Results

### 3.1. Selection of operational welfare indicator (OWIs)

#### 3.1.1. Perceived utility

The 12 OWIs considered by participants at the Lumpfish Welfare Workshop group differed significantly in perceived utility (type III analysis of deviance, $\chi2 = 68.041$, df = 11, $P < .001$); model assumptions were met (tests of nominal effects, background LRT = 10.2, $P = .12$; tests of scale effects, trait LRT = 17.03, $P = .11$; background LRT = 1.13, $P = .57$). Fin erosion and body damage were considered to be the most useful, while body/eye darkening and blood parameters were considered to be the least useful (Table S1). Although participants differed in opinion about the utility of different OWIs for lumpfish, depending on their background ($\chi2 = 11.504$, df = 2, $P = .003$), consensus was high, and 87% of them did not deviate significantly from the responses of the average rater.

#### 3.1.2. Variation in OWIs

The prevalence of different welfare conditions varied significantly between stages of development (Fig. 2). For example, the prevalence of fish with external body damage was rare in hatcheries (2%), but common in sea cages (46%; z-test with continuity correction, $\chi2 = 26.27$, df = 1, $P < .001$). In general, lumpfish in hatcheries were mostly affected by fin erosion, particularly of the caudal (52%) and pelvic fins (53%), and by suction cup deformities (37–58%), whereas lumpfish in the sea cages were more affected by eye damage (23%), which was significantly less common in hatcheries (7%, $\chi2 = 3.89$, df = 1, $P = .048$). Such separation was confirmed by Principal Component Analysis (Fig. 3). PC1 accounted for 39% of variation and was mainly associated with sucker deformity (−0.78), eye darkening (0.67) and external body damage (0.64), while PC2 (21%) mostly captured variation in eye condition (−0.81) and fin damage (−0.46).

#### 3.1.3. Incidence of underweight and emaciated fish

The length-weight relationships of farmed lumpfish differed significantly between life stages (Table 1; life stage x total length interaction, $F_{3,2650} = 61.346$, $P < .001$). Growth was positively allometric (i.e. b > 3.0) in hatcheries, i.e. fish became progressively fatter as they grew, and negatively allometric (i.e. b < 3.0) in the sea, i.e. fish became progressively thinner over time. Using a common length-weight regression for all stages of development (instead or four) would introduce a mean absolute error of 5.2% in the estimation of relative weight, but this varied between 1.1% for stage S3 (just prior to deployment) to 9.5% for stage 1 (larvae). In general, a single length-weight regression would overestimate relative weight in hatcheries (i.e. lumpfish would appear to be fatter than they really are) and underestimate it in sea cages (i.e. lumpfish would appear to be thinner).

The frequency of underweight fish (i.e. those with weights between 10% and 25% below their expected value) varied significantly between life stages ($\chi2 = 8.235$, df = 3, $P = .041$), being highest prior to deployment (stage S3, 20.3%). In contrast, the incidence of emaciated fish (i.e. fish weighing 25% or less below the expected value) was significantly higher during the larval S1 stage (18.4%) than at any other stage ($\chi2 = 121.51$, df = 3, $P < .001$; Fig. 4). Overall, 28% of the 2658 farmed lumpfish we sampled had lower than normal weights for their length, and 10% were emaciated.

#### 3.1.4. Variation in plasma cortisol

Mean plasma cortisol differed significantly between life stages (Welch two sample t-test for unequal variances, $t = 6.56$, df = 35.975, $P < .001$), being approximately 7 times higher among post-deployment lumpfish sampled in salmon sea cages (mean = 84.70 ng/ml ± 10.99 SE) than among pre-deployment juveniles sampled in hatcheries (mean = 11.61 ng/ml ± 1.88 SE; Fig. 5). Cortisol values were also significantly more variable post-deployment (CV = 76.7%) than pre-deployment (CV = 72.6%; Fligner-Killeen test, $\chi2 = 21.84$, df = 1, $P < .001$).

### 3.2. Validation of OWIs

#### 3.2.1. Reliability

All welfare indicators showed good (ICC = 0.75–0.90) or excellent reliability (ICC > 0.9; Table 2) except for eye condition, which was

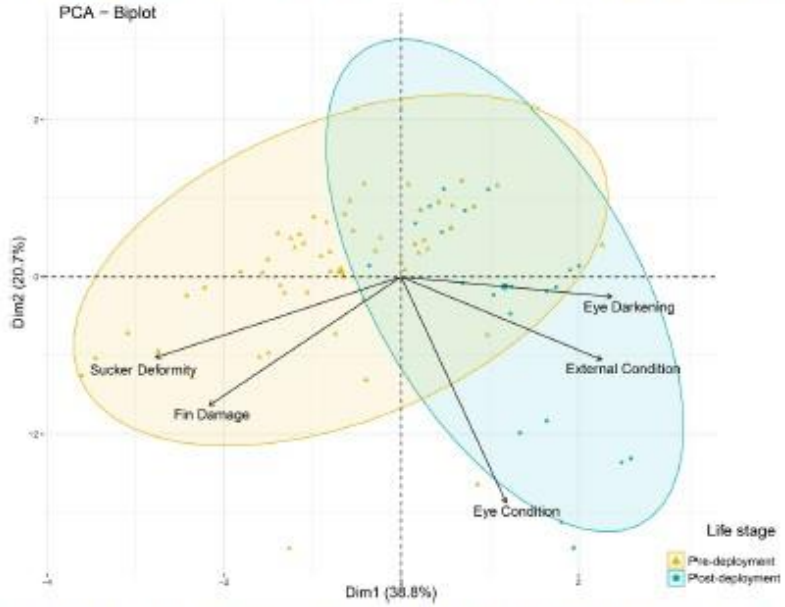



Fig. 3. Principal Components Analysis biplot of lumpfish welfare showing separation of individuals depending on their stage of development (pre-deployment vs. post-deployment) and relative influence of different welfare indicators.

**Table 1**
Length-weight regression coefficients ( ± SE) for farmed lumpfish at different stages of development (log10 Ws = a + b·(log10 TL), where Ws = standard weight (g) and TL = total length (mm).

| Stage | Weight range (g) | *N* | *a* | *b* | $R^2$ | *P* |
|---|---|---|---|---|---|---|
| S1. Larvae | 0–1 | 948 | −5.023 ± 0.035 | 3.532 ± 0.038 | 0.903 | < 0.001 |
| S2. Pre-deployment | 1–10 | 126 | −4.301 ± 0.101 | 2.926 ± 0.060 | 0.950 | < 0.001 |
| S3. Pre-deployment | +10 | 1229 | −4.737 ± 0.034 | 3.181 ± 0.016 | 0.970 | < 0.001 |
| S4. Post-deployment | +10 | 355 | −3.516 ± 0.117 | 2.559 ± 0.058 | 0.847 | < 0.001 |
| All Stages | 0–1380 | 2658 | −4.692 ± 0.007 | 3.157 ± 0.004 | 0.996 | < 0.001 |

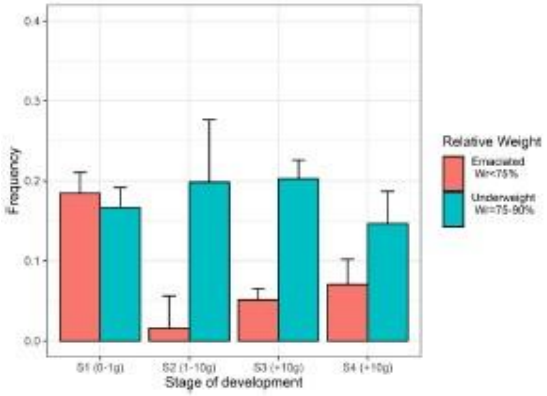



**Fig. 4.** Variation in the proportion ( ± binomial 95% CI) of underweight (Wr = 90–75%) and emaciated (Wr < 75%) lumpfish (n = 2658) at different stages of development sampled from three hatcheries (stages S1-S3) and three sea farms (stage S4).

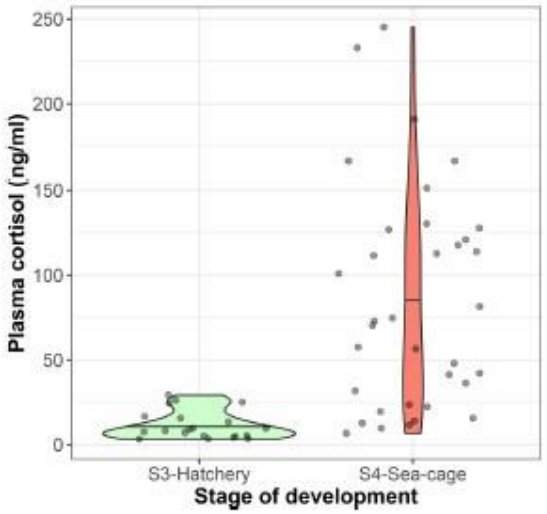



**Fig. 5.** Variation in blood plasma cortisol (ng/ml) of lumpfish (n = 55) sampled in two hatcheries (stage S3 - pre-deployment) and one sea farm (stage S4 - post-deployment).

highly repeatable when measured by different raters (ICC = 1.0), but had a modest repeatability when it was assessed at two different times by the same rater (ICC = 0.60, 95% CI = 0.46–0.72).

#### 3.2.2. Construct validity against relative weight and plasma cortisol

As both relative weight and cortisol differed significantly between stages of development ($P < .001$), construct validity was assessed separately for the pre- and post-deployment stages. Relative weight was found to be dependent on PC1 and PC2 and their interactions with total length during the pre-deployment stages ($F_{5,54} = 6.6$, $R^2_{adj} = 0.32$, $P < .001$), but was only dependent on total length during post-deployment ($F_{1,32} = 6.19$, $R^2_{adj} = 0.14$, $P = .02$). PC1 was a significant predictor of plasma cortisol during pre-deployment ($F_{1,17} = 8.98$, $R^2_{adj} = 0.31$, $P < .01$), while PC2 and the interaction between PC2 and total length were significant cortisol predictors at post-deployment ($F_{3,31} = 4.58$, $R^2_{adj} = 0.24$, $P < .01$).

### 3.3. Refinement and simplification

The caudal was the fin most commonly damaged (47%), the easiest to score, and also the one that showed the highest variation among individuals, with scores ranging from 0 to 4. The ventral section of the pectoral fins was also highly variable, but it was less affected by erosion (43%) than the caudal fin. Damage of the dorsal and anal fins was positively correlated (Spearman's $\rho = 0.302$, $P < .01$), as was damage on the anal fin and the ventral section of the pectoral fins (Spearman's $\rho = 0.222$, $P < .05$), both positioned in the ventral part of the longitudinal axis of the lumpfish. Scores for the ventral section of the pectoral fin and deformities of the suction disc were also positively correlated ($\rho = 0.531$, $P < .001$). Inspection of the data indicated that scoring of the caudal fin would identify 74% of individuals that had also damage in other fins, and that scoring of the suction papillae was positively correlated with other suction cup conditions ($\rho = 0.786$, $P < .001$), indicating redundancy and lending support to the use of a simplified Welfare Score Index (Table 3).

### 3.4. Testing and application

The simplified Lumpfish Operational Welfare Score Index was tested on 245 farmed lumpfish from six commercial sites (three hatcheries and three sea farms), showing high repeatability (ICC = 0.826, 0.767–0.871, $P < .001$). Consensus among farmers on the performance of the index was high, as 75% of them did not deviate significantly from the average response; model assumptions were met (tests of nominal effects, trait LRT = 3.12, $P = .37$). Farmers agreed that it was easy to score, lasting less than 2 min per fish, and that it was practical and easy to implement at the farm. Moreover, they were also willing to train their staff to implement it (Table S2).

Overall, 71% of lumpfish were classified as class "A" (good welfare, unlikely to be compromised), 27% as class "B" (moderately compromised welfare) and 2% as class "C" (severely compromised welfare) but this differed significantly among farms (analysis of deviance on binomial proportions, $\chi2 = 47.397$, df = 5, $P < .001$; Fig. 6). Mean LOWSI values ranged from 0.82 to 3.37 among the six sites, suggesting there was a 4× difference in welfare conditions. Post-hoc Tukey contrasts indicated that the welfare at one of the sea farms (Farm 1) was significantly poorer than the rest with 57% of fish classified as class "B" and 11% classified as class "C"; poor welfare was typically associated with fin damage and suction cup deformities on hatcheries, and poor

**Table 2**
Repeatability of different operational welfare indicators (OWI) based on the inter- and intra-class correlation coefficients (ICC) of two raters (R1 and R2) independently scoring 95 farmed lumpfish.

| OWI | Intra-rater repeatability (R1 at time 1 & time 2) | | | Inter-rater repeatability (R1 vs R2) | | |
|---|---|---|---|---|---|---|
| | ICC | 95 CI | P | ICC | 95 CI | P |
| Body damage | 0.82 | 0.74–0.87 | < 0.001 | 0.83 | 0.76–0.88 | < 0.001 |
| Fin damage | 0.79 | 0.68–0.86 | < 0.001 | 0.93 | 0.90–0.96 | < 0.001 |
| Eye darkening | 0.83 | 0.76–0.89 | < 0.001 | 0.85 | 0.78–0.90 | < 0.001 |
| Eye condition | 0.60 | 0.46–0.72 | < 0.001 | 1.00 | 1.00–1.00 | < 0.001 |
| Sucker deformity | 0.85 | 0.78–0.90 | < 0.001 | 0.94 | 0.91–0.96 | < 0.001 |

growth and eye damage in sea cages (Fig. 6).

## 4. Discussion

The salmon farming industry has been criticized for not doing enough to maintain the welfare of lumpfish (Compassion in World Farming, 2018; 2019; Stranden, 2020) and for causing unacceptably high mortalities in some cases (European Union Reference Laboratory for Fish Diseases, 2016; Imsland et al., 2016; OneKind, 2018). This has caused concern among consumers and prompted some pressure groups to discourage the use of cleaner fish until high mortalities are addressed and welfare standards can be guaranteed (Marine Conservation Society, 2018; OneKind, 2018), Compassion in World Farming, pers. comm. 27/02/2020). The Norwegian Food Safety Authority has recently warned salmon farms that they may have to stop using cleaner fish if welfare standards are not met (Anon, 2020). However, it is difficult to maintain good welfare if farmers do not know what to measure. The development of welfare standards has been flagged as an urgent priority for lumpfish (FAWC, 2014; Noble et al., 2019; OneKind, 2018), because without standards, mortality is often the only indicator of compromised welfare, which is of course too late to take remedial action (Stranden, 2020). Although several welfare indicators have recently been proposed for lumpfish (Imsland et al., 2020; Noble et al., 2019), these have not been validated and there is a need for a simple index that fish farmers can use under working conditions.

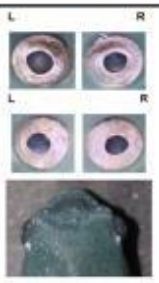
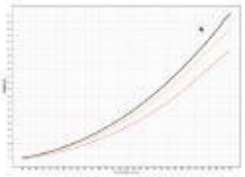
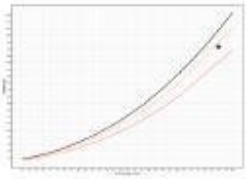
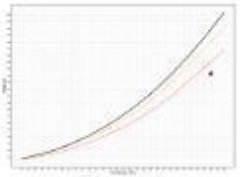
We developed and validated a repeatable lumpfish operational welfare score index (LOWSI) that is easy to score and can be used for routine welfare assessment under commercial conditions with minimal training (Table 3). Our welfare index is based on the same OWIs recently employed to assess variation in growth and mortality of lumpfish in sea cages in Norway (Imsland et al., 2020), indicating that the welfare metrics we used are meaningful across contexts. Our screening at six commercial sites indicates that although the welfare of most lumpfish (71%) was not compromised (class "A"), it was likely compromised in 27% of cases (class "B"), and was clearly poor in the remaining 2% (class "C"). However, welfare scores varied by a factor of 4× among sites, and the prevalence of fish with good welfare varied three-fold (from 31% to 97% class A). This indicates that while some farms are already achieving high welfare standards, others are not.

One key finding from our study was the relatively high incidence of under-weight lumpfish at all stages of development, which raises ethical concerns. Our results indicate that 28% of lumpfish had lower than normal weights for their length, and 10% were clearly emaciated. In previous studies, between 10% and 30% of lumpfish were found to have empty stomachs in sea cages (Eliasen et al., 2018), and only 13–38% were found to eat sea lice (Eliasen et al., 2018; Imsland et al., 2014; Imsland et al., 2015b; Imsland et al., 2016), although this can be as low as 0% during the summer (Eliasen et al., 2018). Clearly, reducing the risk of emaciation is a major challenge for the ethical use of lumpfish. This could be achieved by supplementary feeding, better diets, and novel feeding methods (Imsland et al., 2019a; Imsland et al., 2019d), but also by reducing stress and excessive energy expenditure. In this sense, our length-weight charts could be used by fish farmers to regularly monitor growth, and to take remedial actions before emaciation becomes a problem. They could also be used to select elite lines that are efficient sea lice eaters (Imsland et al., 2016; Imsland et al., 2018d; Powell et al., 2018b), as they provide a benchmark against which growth can be easily compared.

Four welfare conditions were identified in our study that affected lumpfish differently depending on the stage of development, and which may have also increased the risk of emaciation: suction cup deformities and fin erosion in hatcheries, and eye damage and external body injuries in sea cages. The prevalence of fish with suction disk deformities was relatively high (mean 37%, range 3–69%), and was higher in hatcheries than in sea cages, probably because juveniles are typically screened for deformities before they are deployed. Treasurer et al. (2018a) reported that 65% of juveniles had deformed suction disks at one rearing facility, but as deformed fish are more likely to die (Hustad, 2008), the true frequency of deformities at birth is probably higher. The causes of suction cup deformities are unclear but nutritional, environmental, and genetic factors have been implicated in deformities in other species (reviewed in Bertilis (2015). Over one third of lumpfish larvae may show different types of malformations upon hatching (Hustad, 2008), which may be exacerbated by high temperatures during development (Imsland et al., 2019b). It has also been suggested that suction cup deformities may be associated with poor nutritional status (Kousoulaki et al., 2018), although it is more likely that deformities result in poor growth, rather than the other way around, as the suction cup is completely formed at hatching (Hanssen, 2018). Deformities may also result from inbreeding depression, as some lumpfish populations are very small and have gone through genetic bottlenecks (Whittaker et al., 2018). There is some evidence that deformities may vary among families (Danielsen, 2016), which might make it easier to select for deformity-free lines. Whatever the reasons, deformities are known to compromise the welfare of many farmed fish (Noble et al., 2012), and represent a particularly acute problem for lumpfish because they can affect the ability to rest (European Union Reference Laboratory for Fish Diseases, 2016; Imsland et al., 2018a; Imsland et al., 2015a; Johannesen et al., 2018; Leclercq et al., 2018), move (Davenport and Thorsteinsson, 1990), and perhaps also to cope with stress (Hvas et al., 2018). Unlike most other fish, lumpfish lack Mauthner neurons involved in the fast startle response, so their primary response to threat is to cling and hide, rather than to escape (Hale, 2000). A deformed, non-functional suction disc, therefore, will likely increase stress and energy expenditure.

Fin damage was another common welfare problem observed in our study. We found that 62% of juveniles had caudal fin damage in three hatcheries (range 50–93%), which is similar to the 69–87% prevalence reported by Johannesen et al. (2018). Many of the health conditions that affect lumpfish are stress related (Brooker et al., 2018; Powell et al., 2018b), and secondary bacterial and fungal infections will be exacerbated by fin and body damage, so any actions that reduce stress will likely improve welfare and survival. For example, manual feeding combined with automated pulse feeding may be used to reduce stress caused by competition (Johannesen et al., 2018), while regular grading may also reduce fin nipping and fin erosion (European Union Reference

**Table 3**
Rapid visual scoring of the Lumpfish Operational Welfare Score Index (LOWSI).

| OWI | 0 points | 1 point | 2 points |
|---|---|---|---|
| *Skin damage*<br>• reddening<br>• abrasion<br>• wounds<br>• ulcers |  <br>No damage |  <br>Moderate damage |  <br>Severe damage |
| *Caudal fin damage*<br>• ray splitting<br>• fin erosion |  <br>No damage |  <br>Moderate damage |  <br>Severe damage |
| *Eye condition*<br>• cataracts<br>• exophthalmia<br>• injuries |  <br>No damage |  <br>One eye damaged |  <br>Both eyes damaged |
| **OWI** | **0 points** | **1 point** | **2 points** |
| *Suction disc*<br>• asymmetry<br>• indentation<br>• depression<br>• papillae<br>• curling |  <br>No deformities, fully functional |  <br>Moderate deformity, some impairment |  <br>Severe deformity, non-functional |
| *Relative weight* |  <br>Normal<br>Normal weight for its size<br>(Wr>90%) |  <br>Underweight<br>10-25% below expected weight<br>(Wr = 75-90%) |  <br>Emaciated<br>25% or more below expected weight<br>(Wr = <75%) |
| **Average LOWSI** | **Class A: <3 points**<br>**Good welfare** | **Class B: 3-5 points**<br>**Moderately compromised welfare** | **Class C: >5 points**<br>**Severely compromised welfare** |
| **ACTION PLAN** | • No action needed<br>• Continue monitoring | • Increase frequency of monitoring<br>• Check mortality<br>• Check diet and food delivery<br>• Check use of shelters<br>• Check diseases & parasites<br>• Check sources of stress<br>• Check environmental parameters | • Consider immediate corrective actions<br>• Consult with Veterinary Services<br>• Consider culling (under veterinary advice)<br>• Continue monitoring & reassess |

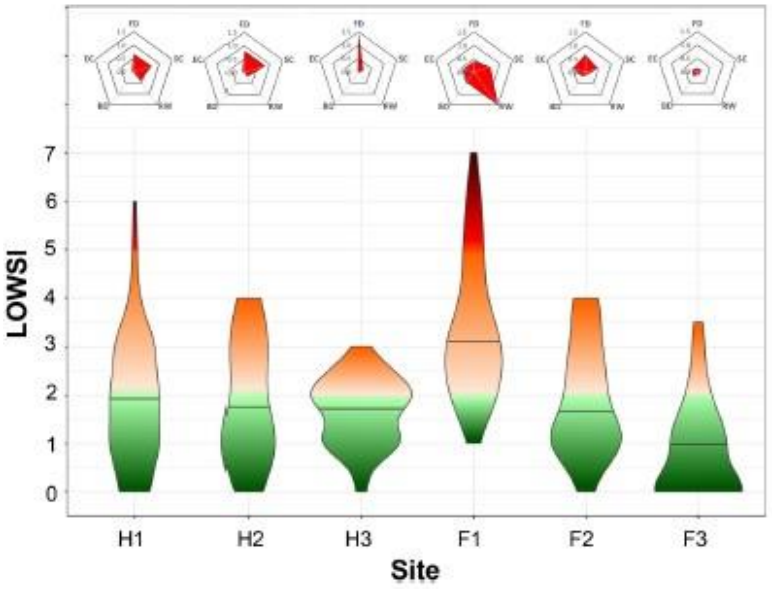



**Fig. 6.** Variation in the Lumpfish Operational Welfare Score Index (LOWSI) at six commercial sites (three hatcheries – H1-H3, and three sea farms, F1-F3; n = 245). The proportion of fish falling into each of the three welfare classes (A-C) is shown by the violin plots in different colours (class A, green: welfare unlikely to be compromised; class B, orange: moderately compromised welfare; class C, red: poor welfare). The radar plots at the top indicate the mean site scores for each of the five OWIs (scored from 0 to 2) that make up the LOWSI (FD – caudal fin damage; SC = suction cup deformity; RW – relative weight; BD – body damage; EC – eye condition).

Laboratory for Fish Diseases, 2016). Although lumpfish are relatively sedentary outside the spawning migrations (Powell et al., 2018a), delousing requires active swimming (Imsland et al., 2015b; Imsland et al., 2016; Leclercq et al., 2018), which may be compromised by damaged or eroded caudal fins. Salmon net pens are often thermally stratified which forces salmon to undertake vertical migrations (Oppedal et al., 2011), which cleaner fish must also follow to graze on sea lice. Some salmon pens may also be exposed to high current velocities (Johansson et al., 2014), which may exceed the 70–110 cm/s maximum current velocity lumpfish can withstand (Hvas et al., 2018). Damaged fins will likely make swimming less efficient and more energetically costly, leading to poor growth and increasing the risk of emaciation.

More than 15% of lumpfish displayed eye damage and poor eye condition in our study, particularly in sea cages, where this figure reached 26%. Maintaining healthy eyes is essential for sit-and-wait, visual feeders like the lumpfish (Powell et al., 2018a), which depend on having unimpaired vision to feed (Jonassen et al., 2017). Eye damage, cataracts and exophthalmia will likely affect feeding and may therefore also increase the risk of emaciation. While exophthalmia may be symptomatic of several underlying diseases (Austin et al., 2012), other conditions like cataracts may be improved by changes in diet (Imsland et al., 2018c) and feeding regimes (Imsland et al., 2019c). Eye cataracts are rare among wild lumpfish, but can affect 20–100% of lumpfish in captivity (Imsland et al., 2018c; Jonassen et al., 2017). Cataracts may be indicative of malnutrition (Jonassen et al., 2017), but also of overfeeding (Imsland et al., 2019c) and nutritional deficiencies (Imsland et al., 2018c). In our study, cataracts were detected in 17% of fish in sea cages, but only in 5% of juveniles in hatcheries (where nutrition is probably better controlled), which serves to highlight the importance of ensuring that lumpfish have access to suitable diets at all stages of development, and not just in hatcheries.

Seasonal changes in the welfare of lumpfish need to be monitored, and critical periods identified. For example, heavy mortalities have been reported during the summer (MOWI, 2019), when lumpfish tend to be more active (Leclercq et al., 2018), food is less abundant (Eliasen et al., 2018), and temperatures may exceed the species' optimum (Mortensen et al., 2020), making conditions more stressful. In this sense, our measurements of blood plasma cortisol provide some insights into the stress experienced by lumpfish in salmon net-pens. We found a mean cortisol value of 85 ng/ml ± 11 in sea cages, which is higher than that found for unstressed (5.6–16 ng/ml, or even stressed (36–63 ng/ml) lumpfish in other studies (Hvas and Oppedal, 2019; Hvas et al., 2018; Iversen et al., 2015; Jørgensen et al., 2017; Staven et al., 2019). Using a plasma cortisol cut-off of 63 ng/ml for stressed fish, our results suggest that 54% of the lumpfish we sampled in salmon net-pens might have been chronically stressed.

## 5. Conclusions

We developed and validated an operational welfare index for farmed lumpfish and tested its application across six commercial sites. The results indicate that the welfare of one third of the lumpfish we sampled was probably compromised, and in 2% of cases was undoubtedly poor.

## 6. Recommendations to improve the welfare of farmed lumpfish

Approximately one in four lumpfish was underweight, and one in ten was severely undernourished or emaciated. These figures appear unacceptably high, and highlight the need for a suitable feed management plan (lumpfish cannot be expected to rely on sea lice alone), as well as for the provision of suitable shelters where lumpfish can rest and be sheltered from strong currents. They also highlight the need for the artificial selection of elite lines that adapt well to captivity and are efficient at eating sea lice. Lumpfish are farmed to feed on sea lice, so underweight fish represent a system failure in every way, not just from a welfare and ethical angle, but also from an economic perspective.

Three of the four welfare conditions that affected lumpfish in our study may be expected to impact growth. Thus, loss of weight is a useful welfare metric for lumpfish because it results from multiple welfare insults. The percentile length-weight charts we developed should enable farmers to identify underweight and emaciated fish rapidly and easily at different stages of development.

A large proportion of lumpfish (37%) displayed suction cup deformities, both in hatcheries and in sea pens, and it is likely that this results in excessive energy expenditure, poor growth and compromised survival. A better understanding of the environmental and genetic basis of sucker deformities may help alleviate this problem, but rapid

screening methods are also needed to identify larvae with deformed suckers and exclude them from commercial production.

Almost half of the lumpfish in sea pens were affected by eye or skin damage, which represent potential routes of infection and may be indicative of underlying pathologies, but also of physical injury. While the incidence of eye cataracts can be reduced by changes in diet, improvements are also needed in the way lumpfish are handled during farm operations in order to reduce the risk of physical injury.

Fin damage appears widespread in lumpfish hatcheries, in common with many other intensively farmed fish. Frequent grading, provision of shelters, improvements in diet, use of on-demand feeders, and in general husbandry practices that reduce stress and aggression, have proved beneficial in other species and may also reduce fin damage in lumpfish.

A 4× fold difference in welfare scores was found between the best and worst farms, indicating considerable scope for improvement. There are now more than 530 salmon farms using lumpfish in Europe, each facing slightly different welfare challenges, but most of which source their lumpfish from a small number of hatcheries. This provides unique opportunities for ensuring that juveniles sent for deployment are free of suction cup deformities and other conditions that compromise welfare. In this sense, it is recommended that a Code of Best Welfare Practices is drawn with farmers, regulators and NGOs to ensure that the welfare of lumpfish is properly monitored, that farmers are trained in the use of operational welfare indicators, and that best practices are agreed and shared. Improving the welfare standards of lumpfish will result in better survival, better delousing efficacy, and ultimately, in fewer cleaner fish and a more sustainable and ethically sound industry.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2020.735777.

## Statement of relevance

The operational welfare index presented here will enable fish farmers to monitor and improve the welfare of farmed lumpfish, making the industry more sustainable.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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