The bile pigments and genetics of ballan wrasse morphotypes

Thesis submitted to the National University of Ireland, Galway in a candidate for the degree of Doctor of Philosophy by

Sumeia Elgumati M.Sc. B.Sc.

Ryan Institute, National University of Ireland, Galway

December 2020

Supervisor: Dr Anne Marie Power
Martin Ryan Institute,
National University of Ireland, Galway

Table of Contents

List of Tables

Chapter 2

Chapter 3

List of Figures

Chapter 1

Chapter 3

Chapter 4

Declaration

I, Sumeia Elgumati, certify that this thesis is my own work and I have not obtained a degree in the National University of Ireland, Galway, or elsewhere, on the basis of the work described in this thesis.

Signed Sumeia Elgumati

Sumeia Elgumati

Date: 08/12/2020

Acknowledgements

First of all, I want to thank Almighty God, for making my dream a reality. I would like to thank all those who provided input, support, materials, and review for this thesis. I am eternally grateful that this work was supported by a PhD studentship which was funded by the Ministry of Higher Education Libya, with support from the National University of Ireland, Galway (NUIG) during my study journey.

First and foremost, many thanks and huge indebted to my supervisor, **Dr Anne Marie Power** whose give me an opportunity in realizing a PhD achievement in my life. Excellent credits to her for great supervising, constructive suggestions during this research work, constant support through many discussions, encouragement and informative feedback thought all years which we have had over the campus (NUIG).

In addition, I would like to express my appreciation to the members of my Graduate research committee, Professor Grace and Professor Louise for their encouragement, support and useful critiques of this research work. Huge thanks to the technicians of the Zoology Department, at Martin Ryan Institute Maeve Edwards and Eoin MacLoughlin who were amazingly kind with their time and a huge help thought all years of the project. I would also like to extend my thanks to Professor Mark Johnson for allowing me to use his Lab. I truly grateful also to Dr Alex Wan to the assistance and the support was given and the advice during my research. Huge thanks to Dr Robert Kennedy for with the assistance in analysed the data. I would like to thank you, Dr Maria Tuohy at the Chemistry Department to access her lab and to Dr Ryan Young for assistance and support.

Special thanks go to the staff for laboratory facilities at the Carna Research Station. I wish to thank various people for their contribution to this project, namely those who helped with sample collection, including Terry Callanan and Antaine Ó Conghaile as a fisherman who provided the live ballan wrasse, also for Leigh Barnwall, helping me in dissection the fish for taken the oranges. I'd like to thank which I couldn't have made it without whose help, travelling together by car to Carna named Kieran and Stephen.

Thank you to all the staff and students of the Ryan Institute who have helped and for encourage me, Nasa Aizen, Cesar Santana, Anne Bateman, Belinda Longakit, Jose Aguilar and Morag Taite. Although not directly linked to this thesis, I would like to thank Anne Cryne and Susan Gallagher for their administrative support to all postgraduate students. Special thanks should also be given to Dr Youssef Elgumati, for his professional guidance in cooperating with me during the statistical work.

Special thanks also to my Husband Ibrahim Elgahani and my sons which accompanied me along the study period, and for their love, patience and encouragement. Always deep in my heart to my mother which pray for me, my sisters and brothers, thank you for everything you have given me.

Finally, my wishes to dedicate this thesis to the memory of my father Salem Elgumati and Richard D. FitzGerald who helped and advice at the beginning of my journey to the PhD.

General abstract

All fish have the pigment bilirubin in their blood which results from the breakdown of haemoglobin, however, some Labridae and other species also have an alternative pigment known as biliverdin which results in blue-green coloured plasma. The functional role of alternative forms of blood pigment is still unclear. *Labrus bergylta* is a protogynous hermaphrodite, distinguished for its various morphotypes based on diverse colours and patterns. The current study has distinguished four different morphotypes sampled from two locations in Galway Ireland (n=57 from Carna 2015, n=21 from Carna 2016, n=37 from Spiddal 2015 and n=34 Spiddal 2016), which were classified as: 'plain', 'spotted', 'merle' and 'intermediate'. A series of hypotheses were tested, the first one being an association between the presence of biliverdin in blood plasma and the hermaphroditic reproductive mode. To determine presence of bili-pigments, blood plasma from several coastal fish species with either hermaphrodite or gonochoristic life histories were examined using colorimetry readings (*L* a* b** scores) and scanning UV spectrophotometry to examine absorbance spectra. *L* a* b** scores and peaks in absorbance spectra for gonochoristic fish species showed a range of plasma colourations: pinkish, blue-green and yellow/green, hence they did not support the hypothesis of a link between the hermaphroditic reproductive mode and plasma colouration. For *L. bergylta* only, the plasma pigments were extracted and confirmed to be biliverdin and bilirubin using Mass Spectrometry.

The next set of experiments examined links between biliverdin/bilirubin concentration in blood plasma (determined using a pigment extraction procedure known as the spectrometric method) and the four observed morphotypes in *L. bergylta*, along with possible connections to body size and a series of basic haematology variables (including packed cell volume (haematocrit), haemoglobin, blood cells count and blood indices). Overall, there were differences between the morphotypes in three variables: body size, biliverdin and total bilirubin concentrations (plasma), which when combined, were predictive of morphotype(s). Merle was the shortest and the lightest morph, while the spotted and intermediate morphotypes were the largest and the heaviest. In plain morphotypes, total bilirubin in the plasma was higher, while the other morphs overlapped in this variable. Plasma biliverdin concentration, on the other hand, was the highest for both plain and intermediate individuals and the lowest in merle. Despite these gross patterns, comparing pigment concentrations in the plasma between locations and years showed high variability: the bilirubin concentration in both locations and years was higher than biliverdin except for Carna 2015.

The next aim was to speculate as to the role of pigments in camouflage, to establish connections (or not) between morphotype and accumulation of biliverdin in external tissues (skin and scales) on the dorsal and ventral body surfaces. To do this, colorimetric readings (*L* a* b** scores) were taken, both from external tissue surfaces and in extracts made from these tissues. In addition, biliverdin was extracted from external tissues and its concentration measured using the spectrometric method. Correlations (if any) between biliverdin concentrations in external tissue extracts and concentrations in plasma for the same individuals were then examined. Overall (both locations combined), bilverdin concentrations in external tissues (skin and scales) and plasma of *L. bergylta* were positively correlated. In both locations, biliverdin concentrations extracted from skin was also positively correlated with biliverdin extracted from scales. Finally, comparison of biliverdin concentrations in external tissues between the morphotypes in Carna revealed variations in the skin and scales (in 2015 only), where the smaller morph (merle) had lowest concentrations, but larger morphs (spotted and intermediate) had highest biliverdin concentrations. The latter was a similar pattern to what was observed overall in plasma concentrations, however, this pattern did not apply to other years/locations.

In the final research chapter, the four different morphotypes were analysed using two genetic markers, i.e. the mitochondrial cytochrome oxidase subunit I (COI) gene and mitochondrial DNA control region (CR) sequences to investigate i) whether all morphotypes present can be identified genetically as *L. bergylta* species and also ii) to examine genetic variability of morphotypes. Genetic population structure was sampled at both Carna and Spiddal (2015 and 2016) and CR sequences were also compared with unknown morphs of *L. bergylta* from ten locations after accessing sequence data from Genbank. The latter were divided into 3 regions based on latitude ('Britain and Ireland', 'Azores' and 'Norway'). No genetic differences between the morphotypes was observed in haplotype networks (COI/CR genes) and AMOVA (CR). Ignoring morphotype and placing samples from this study within the broader geographic regional context (i.e. Ireland/UK, Norway and Azores) using the CR gene showed that Irish populations were separated from the Azores but were not completely separated from Norway populations.

Since biliverdin/bilirubin concentration in plasma is related to morphotypes and body size and since plasma biliverdin is somewhat linked to its concentration in external tissues, it is proposed that one of the roles of biliverdin may be in signalling of some sort to conspecifics. Alternatively, because concentrations were also quite variable across locations/years (varying from site to site), biliverdin may function in camouflage, or

because it is a strong antioxidant, may perform as a photoprotector or some other adaptation to life in shallow water. Further research is needed to understand the physiological role of biliverdin in certain morphs, years and locations. As the results do not indicate genetic isolation between the morphotypes, additional investigations are required to determine the reasons behind the morphotype variations in the life history of *L. bergylta*.

Keywords: Ballan wrasse, morphotypes, biliverdin, bilirubin, plasma, skin, scales, control region.

Chapter 1: General Introduction

1.1 Ballan wrasse (*Labrus bergylta*), a brief introduction

Wrasse are group of marine fish that belong to the family Labridae. This family contains over 600 species across 82 genera and can be found in both temperate and tropical waters (Sayer and Davenport 1996). Wrasse are found in many different habitats and exhibit a variety of body shape, size and colouration (Westneat, 2002). The majority of the species are small with a body length of less than 20 cm. Eastern central Atlantic wrasse vary from the small dwarf wrasse to the large ballan wrasse (Westneat, 2002). In general, the body is compressed, and the mouth is protrusible with prominent lips. The majority of species vary in colour, displaying bright and intricate colour morphotypes. Colours include various shades of brown, blue, green, red, yellow, and white, with a myriad of morphotype patterns including stripes, bars, blots, and ocelli (Westneat 2002). The morphotypes within this group often vary with age and sex inversion (Westneat 2002). However, in some protogynous wrasse such as ballan wrasse, the highly variable colour morphotypes are not related to sex (Costello 1991; Villegas-Ríos et al. 2013a).

Morphotypically, some ballan wrasse individuals have been described as exhibiting a greater degree of green, with white spots or a pattern of vertical dark bars, or a large lateral white stripe (Westneat 2002). In recent studies, two main colour morphotypes have been differentiated - plain and spotted (Villegas-Ríos et al. 2013b; Almada et al. 2016; Clark et al. 2016; Quintela et al. 2016; Seljestad et al. 2020). The plain morphotype is characterized by a uniform but variable body colour (predominately greenish, brownish or reddish), darker dorsally and whitish ventrally. The spotted morphotype has a dark orange or reddish body patterned with small white dots (Villegas-Ríos et al. 2013b). Several studies have examined demographic differences including growth rate (Villegas-Ríos et al. 2013b), genetic traits (Almada et al. 2016; Quintela et al. 2016; Seljestad et al. 2020), habitat preferences (Clark 2016), or other explanations for these morphotypes and their adaptive significance.

Meanwhile, Clark et al. (2016) focussed on the unusual blood plasma colouration in ballan wrasse. The colouration of blood plasma is typically pale yellow in the mammals (Gagnon 2006) but some teleost fish including sculpins (Cottidae) eels (Anguillidae) and wrasse (Labridae) have plasma colours that range from green through to blue and maroon (Abolins 1961; Low and Bada 1974). The cause of this 'blue-green' pigment in blood sera in anguillids was found to be biliverdin (Fang 1984), which is an antioxidant scavenging

molecule. Clark et al. (2016) isolated the blue pigment from ballan wrasse plasma and confirmed this to be biliverdin. They stated that accumulation in the plasma takes place via biliverdin's association with protein moiety that prevents its being further processed or excreted. No correlation was found between the concentration a of biliverdin and sex (male or female), although Clark et al (2016) suggested that biliverdin has biochemical functions associated with the hermaphroditic process. The various blood pigments and their biochemical pathways will be introduced in the next sections, before returning to ballan wrasse and its morphotypes in section 1.4 and 1.5.

1.2 Formation of bile pigments

Blood supplies the cells of the body with the necessary components including nutrients and oxygen and carries waste away from the cells (Cole and Kramer 2016). It consists mainly of erythrocytes (red blood cells), leukocytes (white blood cells) and platelets (Cole and Kramer 2016). Erythrocytes contain haemoglobin, an iron-binding protein, which functions in transportation of oxygen (Cole and Kramer 2016). Haemoglobin is a metalloprotein containing two α- and two β-chains which are connected to heme groups (Perutz 1983). When erythrocytes come to the end of their life or become damaged, they are removed by phagocytosis. The heme is then released into the circulatory system to be degraded by the liver (Kikuchi et al. 2005). During the course of this degradation in the liver the heme structure is split to produce the water-soluble tetrapyrrole bile pigment called biliverdin (Sedlak and Snyder 2004).

Bile pigments consist of a range of complex, highly-coloured biological compounds which are produced as a result of the breakdown the porphyrins, most notably heme. Heme is an iron-containing molecule which is notable due to its incorporation into significant biological compounds such as haemoglobin, myoglobin, cytochrome and catalase (Figure 1.1). These compounds are vital for a number of biological processes including oxygen transport and storage, in the case of haemoglobin and myoglobin, adenosine triphosphate (ATP) generation in the case of cytochrome, and reduction of oxidative stress to cells via the catalysis of various reactive oxygen species, such as hydrogen peroxide in the case of catalase.

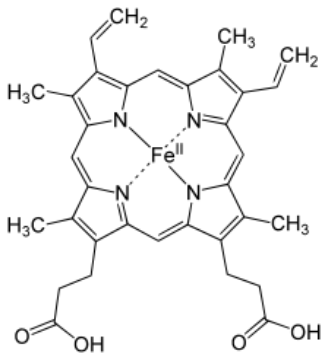


Figure 1. 1: Chemical structure of haemoglobin with the central ring structure consisting of an iron (Fe) ion bounded by a porphyrin ring (Anonymous 2018).

Haemoglobin consists of 4 pyrrolic rings that are bonded on a linear plane to form a larger ring (Figure 1.2). A pyrrole ring is a heterocyclic aromatic organic compound with the formula C_4H_4NH (Loudon 2002). The 4 pyrroles all contain a nitrogen atom which is positioned on the inside of the larger ring. It is to these 4 nitrogen atoms that the iron ($Fe2^+$) ion binds. Although it is a crucial part of many beneficial compounds, free heme is capable of causing both acute toxicity and excessive oxidative damage due to it possessing significant cytotoxic and pro-oxidant effects in high concentrations (Jeney 2002). Free heme, when oxidised is a powerful pro-oxidant which is capable of causing significant amounts of cellular damage through oxidative stress in high concentrations. Acute effects of excessive heme include hemolytic anaemia in mammals as well as heme-derived iron-forming arterial plaques (Jeney et al. 2014). Due to its toxic and pro-oxidative nature, excess heme must be degraded into a less toxic state. This process is typically controlled by the enzyme heme oxygenase that opens the porphyrin ring in heme, resulting in the formation of biliverdin.

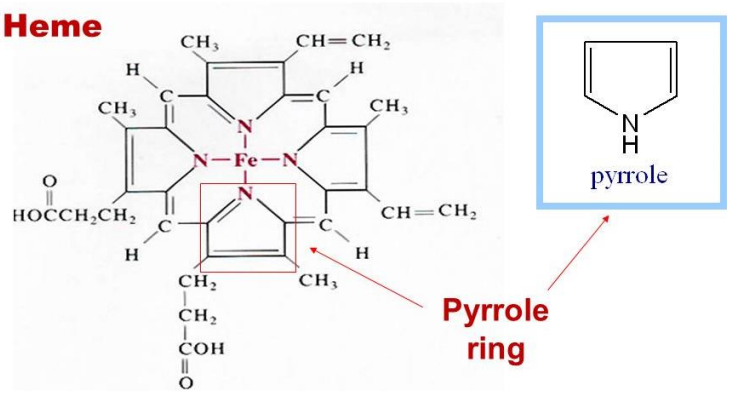


Figure 1. 2: Heme is a tetrapyrrole, a formation of 4 molecular structures known as porphyrins (Atkins, No date)

The first step of the heme degradation pathway occurs in the mononuclear phagocyte system within the spleen and liver and is an energy-dependent process with NADPH used as a reducing agent (Kikuchi et al. 2005). The terminal products of this first step of heme reduction are CO, ferrous iron and biliverdin. In some species including many fish and some reptiles (Figure 1.3), biliverdin is broken down no further and is incorporated into the blood serum and/or tissue instead. For the majority of vertebrates, biliverdin is converted to bilirubin by the enzyme, biliverdin reductase (Figure 1.4).

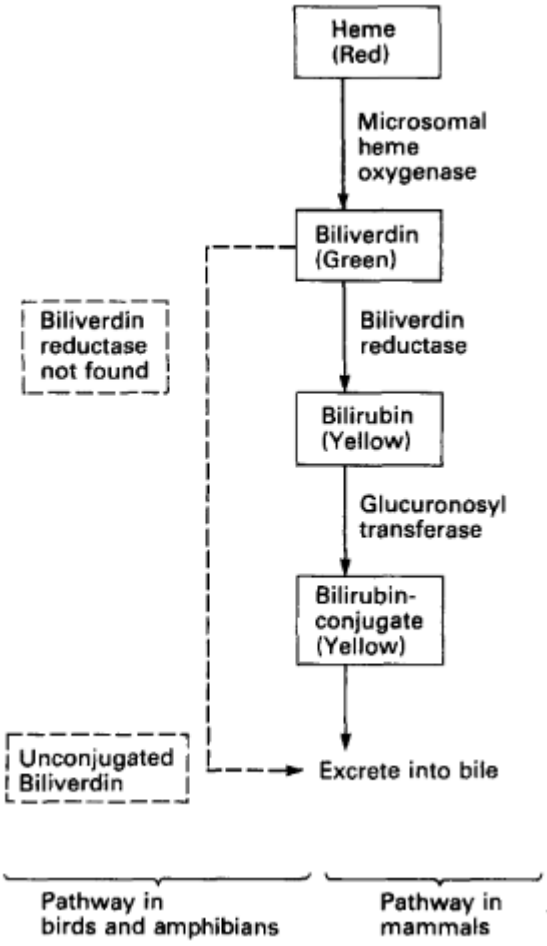


Figure 1. 3: The metabolic pathway of heme degradation in mammals and some other vertebrates i.e some spices of fish, birds and amphibians (Colleran and Heirwegh 1979; Fang 1982).

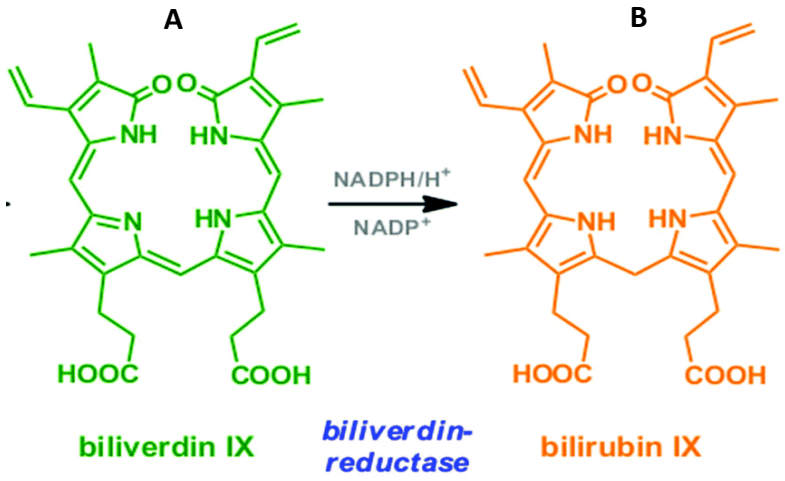


Figure 1. 4: Biliverdin (green colour) is converted to bilirubin (orange colour) by the biliverdin reductase enzyme: **A)** Chemical structure of biliverdin; **B)** Chemical structure of bilirubin (Yan et al. 2016).

Bilirubin is then transported to the liver where it is conjugated with glucuronic acid by the enzyme UDP-glucuronosyltransferase to become more water-soluble and is excreted. Whilst biliverdin is water soluble and not overly toxic (Kirkby and Adin 2006), bilirubin is capable of causing acute toxicity in excess and is water-insoluble, requiring conjugation for safe transport. As the reduction of biliverdin to bilirubin is an NADPH-dependant process, this is an energy-costly process, and the reason for this processing step to have evolved is not fully understood. Within placental mammals, the production of bilirubin may be explained by the fact biliverdin doesn't seem to be capable of crossing the placental barrier, leaving a developing embryo with no way of excreting the products of heme degradation. Bilirubin, on the other hand, is capable of crossing the placental barrier (McDonagh et al. 1981). But complete heme reduction to bilirubin occurs in most vertebrates, not just placental mammals, which indicates an alternative, cryptic reason for conversion of biliverdin to bilirubin. Bile pigments, or bilins, can be found in almost all forms of life, from cyanobacteria to higher vertebrates. However, of this group of compounds, biliverdin and bilirubin are the most widely studied and of most scientific interest. These pigments have been discovered in a wide range of organisms including plants and arthropods, but they are most commonly seen in vertebrates. Both biliverdin and bilirubin are linear in structure and occur in a number of different isomers, with the most common being biliverdin IXα and bilirubin IXα (Sedlak and Snyder, 2004).

Both bilirubin and biliverdin can have toxicological effects at high concentrations interfering with the normal physiological function in the animal, when they are not contained in the gallbladder. For example, jaundice is a human condition where significant amounts of bilirubin are deposited in skin tissue, eyes and mucous membranes (Dennery et al. 2001). These deposits can have an impact on the central nervous system, lead to deposits in the brain and lead to a form of kernicterus encephalopathy that creates severe issues, like hypertonia, seizures, brain paralysis, and postponed motor ability (Dennery et al. 2001).

Biliverdin and bilirubin play more significant roles in non-mammalian vertebrates which appear to be more tolerant to the presence of these compounds within their systems. In particular, fish species have been extensively documented incorporating bile pigments into their blood serum and tissue, using them for a number of novel purposes.

1.3 Biliverdin and bilirubin biological uses

In some species, the pathway of heme catabolism appears to be modified such that biliverdin is the terminal product which is then incorporated into the blood serum or tissue. The presence of biliverdin within serum or tissue is typically marked by a distinctive green/blue pigmentation. This has been documented extensively in fish and at least once in reptiles, with several lizard species of the genus *Prasinohaema* (skinks) discovered to accumulate significant amounts of biliverdin in their blood serum and tissue (Austin and Jessing 1994). A number of fish species such as the garfish (*Belone belone*) and the viviparous eelpout (*Zoarces viviparous*) have been observed to accumulate biliverdin in their connective tissue and bones, giving them a blue-green colour (Jüttner et al. 2013). The precise reason why these fish and lizards accumulate biliverdin in these areas and its adaptive significance is not fully understood. Biliverdin has been discovered in the blood serum of a number of hermaphroditic fish species including the ballan wrasse (*Labrus bergylta*) (Clark 2016) and blue-throated wrasse (*Notolabrus tetricus*) (Gagnon 2006), resulting in blue-green serum.

In ballan wrasse, biliverdin accumulation occurs through its association within the blood serum with a protein moiety of the lipocalin superfamily, which prevents further breakdown (Clark 2016). Ballan wrasse have also been shown to possess a biliverdin reductase enzyme and it has been further demonstrated that biliverdin reductase activity converted NADPH to NADP when biliverdin was converted to bilirubin (Clark et al. 2016). However, Clark et al. (2016) suggested that binding to lipocalin protein prevents the

action of this enzyme (Clark et al. 2016). Species such as *Anguilla japonica* (Fang et al. 1986), *Clinocottus analis* (Fang and Bada 1988), and *L. bergylta* all have biliverdin expressed in their blood, however, because *A. japonica* and *L. bergylta* have the biliverdin reductase enzyme, they are consequently capable of converting biliverdin to bilirubin (Fang and Lai 1987; Clark et al. 2016). Fang (1987) also suggested that, as the biliverdin is tightly connected to the protein moiety in the serum, the additional metabolic step is not possible, but neither was biliverdin eliminated from the circulatory system by the liver or kidney. Therefore, biliverdin was maintained in the blood, resulting in blue-green coloured plasma (Fang and Bada 1988). Some of this protein-bound biliverdin may also be stored in the fish's skin and tissue, resulting in strong pigmentation.

Like many wrasses, *L. bergylta* is a protogynous hermaphrodite meaning that all fish are born female with sex change to male occurring in certain individuals at no younger than 6 years of age (Muncaster et al. 2013). In fact, not all females will change sex, however a dominant female (or an individual that is larger than the other females) may convert to a male if a male is removed or if a harem recruits too many females for the male to dominate (Muncaster et al. 2013). During sex change, cytoarchitecture of the female reproduction system gets severely affected as changes in follicular metabolism remove maintenance stimuli leading to severe hypercytolipidemia and a reproductive tract involution (Garris 2004). In *L. bergylta*, the female's gonadal tissue is remodelled into its male form, and hyperlipidaemia and hypercholesterosis are extremely likely to occur during sex inversion, which results in the production of many free radicals such as reactive oxygen species leading to high amounts of oxidative stress (Clark 2016). An excess of free radicals within an organism can result in inflammation, lead to cell injury or death, and may contribute to diseases such as cancer (Schetter et al. 2009). Most species combat oxidative stress by maintaining a complex system of antioxidants which are either produced internally or are introduced via their diet. Biliverdin is known to possess powerful antioxidant properties (see above; Jansen and Daiber 2012). This antioxidant reactivity is due to the structure of biliverdin which contains extended systems of conjugated double bonds, resulting in a high redox potential, allowing it to effectively scavenge a range of free radicals including reactive oxygen species (Kaur et al. 2003; Stocker 2004). It has been suggested by Clark (2016) that ballan wrasse accumulate excess biliverdin in their blood serum, tissue and skin throughout their lives to be used as an antioxidant to combat free radicals and mitigate the damage caused during the sex change process. This is supported by the fact that biliverdin concentrations in the serum and skin of ballan wrasse significantly decreased during sex

change, suggesting that it was being actively used (Clark 2016). While this is an interesting hypothesis from Clark's (2016) research, it was based on a small number of transitioning individuals (transitioning n = 9, female n = 322 and male n = 66).

1.4 Ballan wrasse biology

Labrus bergylta is a temperate species common to rocky shore habitats throughout the North Atlantic, typically occurring at depths from 1 – 60 meters (Dipper et al. 1977). Its range extends from Morocco to Norway and includes the Mediterranean. *L. bergylta* is a robustly-built fish with a deep, powerful body, broad stocky fins and thick covering of robust scales, making it particularly well adapted to rocky reef environments and kelp beds (Figure 1.5). This is a slow growing and long-lived species, growing to a maximum length of 65 cm and weight of 4.4 kg and living upwards of 29 years (Dipper et al. 1977). This is also a sexually dimorphic species with larger fish being almost exclusively male (Villegas-Ríos 2013c). Within it's rocky habitat, *L. bergylta* are important predators of a wide range of benthic invertebrates. Bivalve molluscs such as the common mussel (*Mytilus edulis*) and decapods such as the common shore crab (*Carcinus maenas*) and brown crab (*Cancer pagurus*) are important prey items of ballan wrasse, with gastropods, echinoderms, bryozoans and amphipods also opportunistically taken (Deady and Fives, 1995). This diet of predominately hard-bodied organisms is facilitated by the specialised feeding biology of *L. bergylta*. Like most wrasses, *L. bergylta* possesses a projectile mouth with robust, outwards-pointing teeth allowing them to propel their jaw at rapid speeds and produce a powerful bite. This coupled with powerful pharyngeal jaws allows ballan wrasse to catch a myriad of hard bodied organisms and crush their armour with ease (Berkovitz and Shellis, 2016).

1.5 Morphotype variability

A notable characteristic seen in ballan wrasse is the highly variable colour patterns seen across individuals. Multiple colour morphs between individuals can be seen in other wrasse species but they are typically related to sex or origin (Villegas-Ríos 2013c). The varying morphotypes seen in *L. bergylta* have no correlation with sex or origin and have yet to be linked with any biological feature in this species (Villegas-Ríos 2013c).

Figure 1. 5: Ballan wrasse (*Labrus bergylta*) found in west coast of Ireland.

In the literature to date, *L. bergylta* have been divided into two distinctive morphotypes, plain and spotted (Almada et al. 2016; Quintela et al. 2016; Seljestad et al. 2020). Plain fish are characterised by a uniform, but variable, body colour while spotted fish are defined by a dark red to orange body with a covering of white spots. The precise disparity between morphotypes of this species is disputed in the literature. Differences in growth rate between morphotypes have been previously observed with spotted individuals displaying a more rapid growth rate as well as larger overall sizes, on average, compared to plain morphs (Villegas-Ríos 2013c). Recently the suggestion has been put forward that the two different morphotypes studied (i.e. plain and spotted) may represent distinctive taxonomic units, although the degree of genetic disparity between them is disputed. Quintela et al. (2016) used the mitochondrial cytochrome oxidase subunit I (COI) gene and microsatellite markers to determine gene flow and population differentiation and concluded that morphotypes represented distinctive taxonomic units. And also, Seljestad et al. (2020) who studied the genetic structure of *L. bergylta* using 82 single nucleotide polymorphisms (SNPs) markers from 18 locations in Norway, Sweden, and Galicia in Spain, found very distinguished genetic groups. Moreover, *L. bergylta* collected from Galicia was highly

differentiated from all Scandinavian locations. The authors found distinct genetic variations between plain and spotted morphs in Galicia, these were not apparent in Scandinavia. Villegas-Ríos (2013c) has also suggested the possibility of *L. bergylta* was in the process of splitting into two separate species, as defined by their slightly different life-history strategies. It was recognized that, while there were no geographical barriers (sympatric) or great gene flow, the combination of assortative mating and disruptive natural selection on a single trait (e.g. morphotypes) could lead to speciation in marine environments. These conclusions were somewhat contradicted by the findings of Almada et al. (2016) who used both mitochondrial and nuclear DNA markers but, ultimately, were unsuccessful in finding a genetic differentiation between morphotypes. Interestingly, both morphotypes appeared to be sympatric along their entire distribution, with proportions varying depending on local areas.

Spawning activity in this species is strictly seasonal, occurring in late spring to summer when water temperatures increase (Villegas-Ríos 2013c), with the precise timing of the single annual spawning event depending on the latitude (Muncaster et al. 2013; Mohd Yusof 2015). Although it takes place during a single annual event, batch spawning appears to be likely, with females releasing eggs in batches over the course of the spawning period (Clark 2016). As stated earlier, the main driver of the sex change process in *L. bergylta* seems to take place when a male is removed, or when a harem recruits too many females for the male to dominate, with the largest female undergoing sex change and becoming male (Muncaster et al. 2013). As is implied by the latter observation, not all individuals change sex, rather a small subset of individuals will have the opportunity to do this. During the spawning season, a male will claim a territory and aggressively defend it, mating with his harem of females who actively "accept" this male. There is strong site fidelity in both sexes, females use these areas as nesting sites where they deposit large clutches of sticky benthic eggs, which the male protects (Muncaster et al. 2010). Upon hatching, larval fish are passively dispersed by dominant tides and currents. After a short pelagic phase, young ballan wrasse migrate toward the coast to take refuge and mature in coastal microhabitats such as tide pools. Transition from female to male, and the associated re-organisation of tissues, seems to take place at the end of the spawning season as females either re-absorb unspawned ovary tissue or this tissue transforms to become testis (Muncaster et al. 2013; Mohd Yusof 2015).

1.6 Use as 'cleaner fish'

In Europe and North America, the Atlantic Salmon (*Salmo salar*) is rapidly becoming among the most important fish for human consumption, aided in part by its health benefits, including being a valuable source of omega-3 fatty acids (Jensen et al. 2012), which decreases the risk of behavioural disorders, cardiovascular and inflammatory diseases (Mozaffarian and Rimm 2006). Traditionally the demand for salmon flesh was met through the capture of wild fish, which in recent years has had negative effects on wild salmon populations (Inland Fisheries Ireland 2017). This, in turn, led to a dramatic market price increase for salmon products. Demand for salmon in world trade is increasing, especially for farmed Atlantic salmon and in recent years, overall prices have remained high (FAO 2016). In Ireland, salmon remained the most economically significant aquaculture sector in 2018 with production at 12,000 tonnes. The value of the Irish salmon sector was reported as €114.5 million with up to 464 persons directly employed (National Seafood Survey Aquaculture Report 2019). In Norway, the first-hand value of salmon aquaculture in 2019 was reported to be €6.24 billion (68.1 billion NOK) with production at 1,357,304 tonnes (Turi Baklien and Rundtom 2020). The value of farmed salmon in Scotland between 2014 and 2015 was reported to be £271 million GBP with production at approximately 171,722 tonnes. The gross added value to the Scottish economy was estimated to be £540 million (Marine Scotland 2017). It is important to note that many studies have revealed that high concentrations of sea lice associated with fish farms can have severe effects on wild salmon and sea trout populations, which can result in a reduction in the numbers of adult fish due to salmon lice induced mortality, resulting in reduced stocks and reduced opportunities for fisheries (Thorstad and Finstad 2018).

Like many other salmonid species, Atlantic salmon are anadromous: individuals are born in freshwater and migrate to seawater, where they grow into adults before returning to the freshwater environment to spawn. In farmed salmon operations, eggs are hatched in a freshwater hatchery and raised to the parr stage (~60g). These fish will undergo a physiological change (e.g. skin colour change, body shape and increase in Na+/K+-ATPase development in the gills) called smoltification that allows the fish to adapt and survive in the marine environment. These farmed salmon smolts will then be transferred to sea cages where they are grown at high density (e.g. EU legislation 710/2009; organic 15 kg m^3 and 25 kg m^3) until they reach market size (~2-3 kg).

A major issue faced when cultivating such dense populations of fish in such a restricted area is that parasites and disease can quickly become epidemics (Pike 1989). The

ectoparasites *Lepeophtheirus salmonis* and *Caligus elongatus* (Figure 1.6) represent a major problem to salmon aquaculture as they commonly proliferate in salmon cages. These parasitic copepods tightly cling to fish and use their rasping mandibles to feed on skin, tissue and blood, resulting in damaged fins and open sores on the skin which may create pathways for other viral, bacterial and fungal pathogens (Costello 2006). Even light infections can result in reduced fish health and impaired growth which also affect a fish's value at market. Heavier burdens of these parasites can lead to a myriad of complications including tissue necrosis, blindness, secondary infection, immune deficiency and in extreme cases, death (Mustafa et al. 2000; Costello 2006). Salmon cages act as hotspots of parasitic activities, which are also capable of effecting wild fish that venture too close to salmon cages at sea during their migratory activity into bays and estuaries prior to spawning (Torrissen et al. 2013). These sea lice may also introduce various bacterial, viral or fungal pathogens which have circulated in the crowded salmon farms to wild populations (Costello 2006).

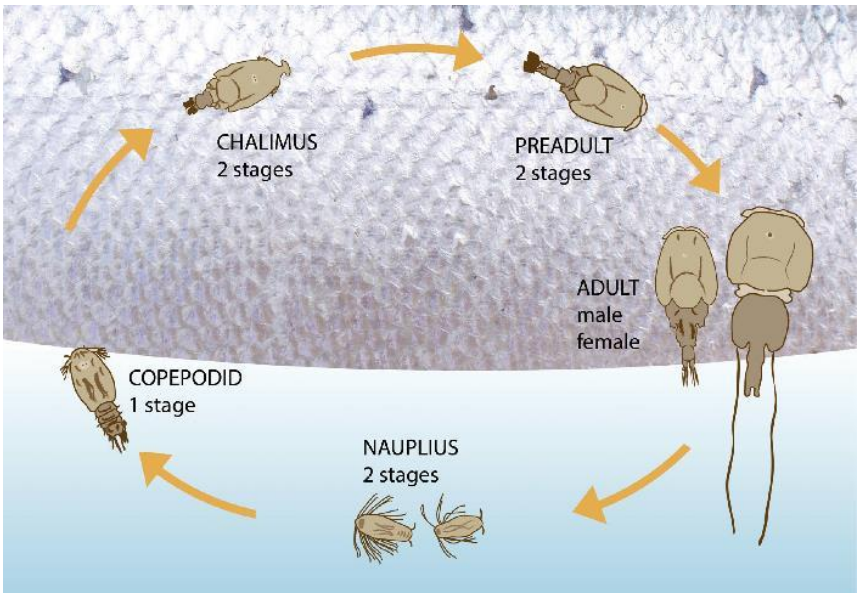


Figure 1. 6: The typical life cycle of sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus*) in relation to the parasitism to a fish host (Thorstad et al. 2015)

The aquaculture industry has traditionally relied heavily on chemical delousing agents and drugs to remove lice from fish with varying success (Denholm et al. 2002). These treatments are typically delivered orally via medicated feeds or as bath applications. Medicated feeds typically contain chemicals such as Emamectin or Teflubenzuron which

have both proven to be relatively safe and effective in the treatment of salmon lice (Costello 2009). Potential issues arise when fish are treated with incorrect doses which may lead to parasites gaining resistances to treatment, leading to the necessity for alternative methods to be developed (Aaen et al. 2015). Chemicals that are commonly used in bath treatments include insecticides such as cypermethrin, azamethiphos and deltamethrin as well as hydrogen peroxide (Torrissen et al. 2013). Issues arise when these chemicals start to affect non-target species such as copepods, bivalves and decapods which occur in close proximity to cages (Grant 2002). Problems with the use of delousing chemicals include increased labour costs (Roth et al. 1993), chemical runoff (Denholm et al. 2002), stressing fish and exposing workers to potentially toxic chemicals (e.g. hydrogen peroxide, azamethiphos). Consequently, the salmon industry is looking for alternatives which remove sealice, but which are environmentally friendly and safe for workers, thereby decreasing the number of fish lost while enhancing the public perception of salmon farming.

The use of cleaner fish has been extensively tested and has proven to be highly effective in selectively removing lice from salmon (Skiftesvik et al. 2013a). In particular, wrasses are a group with a number of species which display mutualistic cleaning behaviours that can be harnessed for potential cleaner-fish purposes (Skiftesvik et al. 2013b). As well as ectoparasites, cleaner fish routinely feed on excess cutaneous mucous and dead skin from larger ‘client’ fish to the benefit of both the cleaner and client. The cleaner fish is granted access to an abundant food source which requires very little foraging effort while the client fish enjoys improved overall health without the burden of ectoparasites. Perhaps the best known of cleaner fish is the tropical blue streaked cleaner wrasse (*Labroides dimidiatus*) (Freixial et al. 2018). Although lesser known, many temperate wrasse species have also proven to be highly proficient in the removal of ectoparasites including the ballan wrasse (*Labrus bergylta*) corkwing wrasse (*Symphodus melops*), goldshinny wrasse (*Ctenolabrus rupestris*) and rock cook (*Centrolabrus exoletus*) (Skiftesvik et al. 2013b; Deady et al. 1995). All of these species tolerate the same temperate water temperatures as *Salmo salar,* making them ideal candidates for cleaner fish in salmon farms. Both the ballan and corkwing wrasse are among the most popular wrasse species to be employed in salmon farms with the former being of particular value due to its larger size, resilient nature and ability to remove large volumes of ectoparasites from salmon (Imsland et al. 2014).

Fish to be deployed in salmon farms are regularly collected from wild stocks. The consequence of this method of controlling lice has led to a number of wrasse species becoming over-harvested in Ireland, Scotland and Norway (Skiftesvik et al. 2013b). For example, in 2010, it has been estimated that 10 million wrasse individuals were collected from the wild to be used as cleaner fish in Norway alone (Skiftesvik et al. 2013b), but this number may have increased more recently. This is of particular concern in longer-lived species such as *L. bergylta*, which may not become sexually active until 6-9 years of age (Muncaster et al. 2013; Grant et al. 2016). To relieve wild stocks from intensive fishing to meet the increasing demand from aquaculture, commercial cultivation of ballan wrasse in hatchery units is an expanding enterprise (Skiftesvik et al. 2013a).

In addition to the number of wrasse species used as cleaner fish, the lumpsucker (*Cyclopterus lumpus*) also displays mutualistic cleaning behaviours (Imsland et al. 2014). This species of temperate scorpaeniform has been recently trialled as a cleaner fish in salmon farms with promising results (Imsland et al. 2014). Lumpsucker displays a higher degree of resistance to colder waters when compared to many wrasse species, making the lumpsucker a favourable cleaner fish in Nordic countries where the climate is unsuitable for wrasse. However, there are also some disadvantages of to using lumpsuckers. For example, considerable mortality was observed in this species in Ireland following higher summer temperatures (Bolton-Warberg 2018). Observed diseases that have become a source of mortality include amoebic gill disease (AGD), piscirickettsia (Marcos-López et al. 2017) and microsporidian diseases (Scholz et al. 2016), which are also found in Irish hatchery conditions at ambient water temperatures (Bolton-Warberg 2018).

1.7 Study area

Galway Bay is a large inlet located on the west coast of Ireland bordering with Co. Clare to the south and Co. Galway to the north (Figure 1.7). The bay is 62 kilometres long and 33 kilometres wide but narrows to only 10 kilometres wide at Black Head (Anonymous, 2017). The dominant feature within the bay is the Aran Islands, Inishmore to the north, Inisheer to the south and Inishmaan in the middle. The river Corrib enters the bay through Galway City. Most of Galway Bay is relatively shallow with water depths rarely exceeding 20 meters. However, depths at the mouth of the Bay deepen towards the west, approaching the Aran Islands, with water > 60 meters depth in some areas (Anonymous, 2017). The seabed in Galway Bay is composed of two distinctive rock types: limestone in the Aran Islands and south in the Burren, Co. Clare, and granite in the region west and north of Galway and in Connemara. Much of the coastline in this area is dominated by rocky shores

and shallow reef systems, which are ideal habitats for a wide range of wrasse species, including *L. bergylta*.

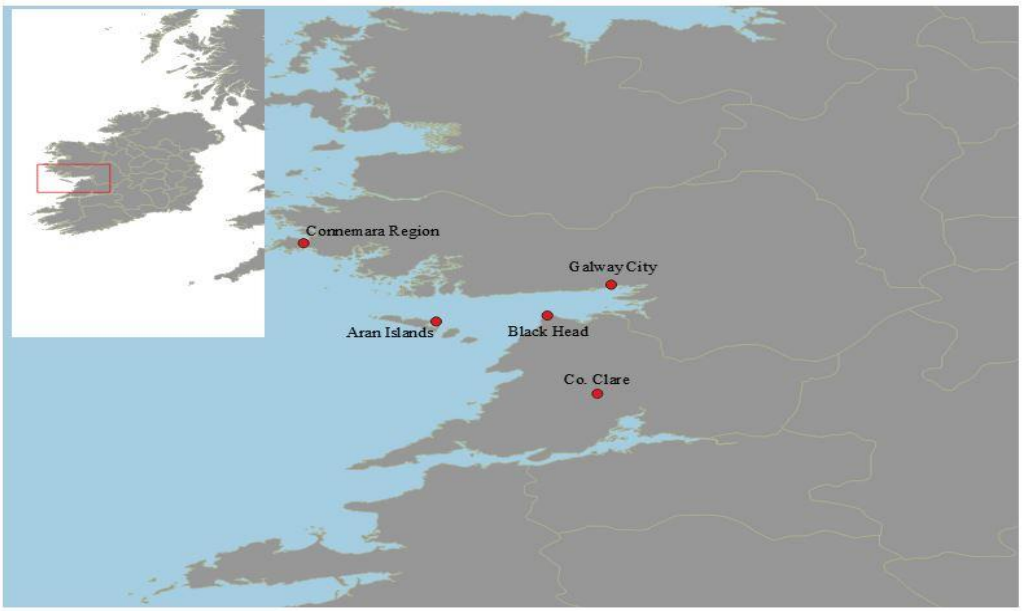


Figure 1. 7: Study area of Galway Bay where samples were collected.

1.8 Aims

As outlined above, the physiological role of biliverdin in fish such as ballan wrasse is still unknown. The general aims of the present study are to examine morphotypes of *L. bergylta* and determine their relationship, if any, to i) blood pigment concentration, ii) pigment in external tissues, or iii) genetic markers.

The thesis will be broken into three research chapters, as follows:

Chapter two investigates wrasse morphotypes, pigment (biliverdin and bilirubin) concentration in blood plasma in these morphotypes and a range of other blood parameters, to examine whether any correlation exists between these parameters and morphotype. Next, it examines pigment variation in relation to other variables such as sample locations and sampling year. This chapter also isolates and identifies the pigment responsible for observed plasma colouration in *L. bergylta*. A final hypothesis tested in this chapter is whether there is a relationship between the existence of biliverdin in blood plasma and the hermaphroditic reproductive mode (the latter examines a range of coastal fish species).

Chapter three investigates external tissues in wrasse, skin and scales, to determine biliverdin concentrations and compare with plasma biliverdin for the same individuals.

This was caried out to appreciate whether there is any relationship between circulatory pigments and those of external tissues. The general aim is to establish links (or not) between morphotype, accumulation of the pigment in external tissues, and to speculate as to the role of pigments in camouflage or conspecific signalling, etc.

Chapter four investigates whether all morphotypes present can be identified genetically as ballan wrasse species using the ‘barcoding gene’ i.e. mitochondrial cytochrome oxidase subunit I (COI) and also examine genetic variability of morphotypes using the mitochondrial DNA control region (CR) gene. The general aim is to test whether genetic variability (mtDNA CR gene) maps onto morphotypes (this aim is carried out in the context of contradictory claims about a possible link in the recent literature (Quintela et al. 2016; Almada et al. 2016; Seljestad et al. 2020).

References

Aaen, S. M., Helgesen, K. O., Bakke, M. J., Kaur, K., and Horsberg, T. E. (2015). Drug resistance in sea lice: a threat to salmonid aquaculture. *Trends in Parasitology*, **31**(2), 72-81.

Abolins, L. (1961). The existence of sexual dimorphism of blood plasma in fish of the family Labridae. *Arkiv Fur Zoology* **13**, 541–544.

Almada, F., Casas, L., Francisco, S. M., Villegas-Ríos, D., Saborido-Rey, F., Irigoien, X., and Robalo, J. I. (2016). On the absence of genetic differentiation between morphotypes of the ballan wrasse *Labrus bergylta* (Labridae). *Marine Biology*, **163**(4), 86.

Anonymous (2017). Infomar | Galway Bay. [online] Available at: http://www.infomar.ie/surveying/Bays/Galway.php [Accessed 7 Jun. 2017].

Anonymous (2018). Questions and answers in magnetic resonance imaging. [online] Available at: http://mriquestions.com/types-of-hemoglobin.html [Accessed 2 Jul. 2019].

Austin, C. C., and Jessing, K. W. (1994). Green-blood pigmentation in lizards. *Comparative Biochemistry and Physiology Part A: Physiology*, **109**(3), 619-626.

Atkins, J. (n.d.). Hemoglobin. [online] SlidePlayer. Available at: https://slideplayer.com/slide/11413924/ [Accessed 2 Jul. 2019].

Berkovitz, B. K., and Shellis, R. P. (2016). *The teeth of non-mammalian vertebrates*. 1st ed. London: Academic Press, 90-91.

Bolton-Warberg, M. (2018). An overview of cleaner fish use in Ireland. *Journal of Fish Diseases*, **41**(6), 935-939.

Clark, W. (2016). The isolation, identification and exploration of the biophysiological significance of plasma biliverdin in the ballan wrasse (*Labrus bergylta*). Ph.D. University of Stirling.

Clark, W., Leclercq, E., Migaud, H., Nairn, J., and Davie, A. (2016). Isolation, identification and characterisation of ballan wrasse *Labrus bergylta* plasma pigment. *Journal of Fish Biology*, **89**(4), 2070-2084.

Cole, L. A., and Kramer, P. R. (2015). *Human physiology, biochemistry and basic medicine*. Academic Press.

Colleran, E. and Heirwegh, K.P. (1979). Comparative aspects of bile pigment formation and excretion. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **64**(2), 133-139.

Costello, M. (1991). Review of the biology of wrasse (Labridae: Pisces) in Northern Europe. *Progress in Underwater Science*, **16**, 29-51.

Costello, M. J. (2006). Ecology of sea lice parasitic on farmed and wild fish. *Trends in Parasitology*, **22**(10), 475-483.

Costello, M. J. (2009). The global economic cost of sea lice to the salmonid farming industry. *Journal of Fish Diseases*, **32**(1), 115-118.

Deady, S., and Fives, J. M. (1995). Diet of ballan wrasse, *Labrus bergylta*, and some comparisons with the diet of corkwing wrasse, *Crenilabrus melops*. *Journal of the Marine Biological Association of the United Kingdom*, **75**(3), 651-665.

Deady, S., Varian, S. J., and Fives, J. M. (1995). The use of cleaner-fish to control sea lice on two Irish salmon (*Salmo salar*) farms with particular reference to wrasse behaviour in salmon cages. *Aquaculture*, **131**(1-2), 73-90.

Dennery, P. A., Seidman, D. S., and Stevenson, D. K. (2001). Neonatal hyperbilirubinemia. *New England Journal of Medicine*, **344**(8), 581-590.

Denholm, I., Devine, G. J., Horsberg, T. E., Sevatdal, S., Fallang, A., Nolan, D. V., and Powell, R. (2002). Analysis and management of resistance to chemotherapeutants in salmon lice, *Lepeophtheirus salmonis* (Copepoda: Caligidae). *Pest Management Science: Formerly Pesticide Science*, **58**(6), 528-536.

Dipper, F. A., Bridges, C. R., and Menz, A. (1977). Age, growth and feeding in the ballan wrasse *Labrus bergylta* Ascanius 1767. *Journal of Fish Biology*, **11**(2), 105-120.

Fang L. S. (1982) The blue-green pigment in the blood serum of a marine fish, *Clinocottus analis*: identification, metabolism and biological significance. Ph.D. Thesis, University of California, San Diego, CA. 176.

Fang, L. S. (1984). Identification and occurrence of the chromogen in the blue-green blood of Japanese eel, *Anguilla japonica. Bulletin of the Institute of Zoology, Academia Sinica*, **23**, 1-7.

Fang, L. S., Ong, C. W., and Hwang, W. S. (1986). A comparative study on the binding characteristics of the tight serum biliverdin-protein complexes in two fishes: *Anguilla japonica* and *Clinocottus analis*. *Comparative Biochemistry and Physiology. B, Comparative Biochemistry*, **84**(3), 393-396.

Fang, L. S. (1987). Study on the Heme Catabolism of Fish. *Comparative Biochemistry and Physiology B-Biochemistry and Molecular Biology*, **88**(2), 667–673.

Fang, L. S., and Bada, J. L. (1988). A special pattern of haem catabolism in a marine fish, *Clinocottus analis*, with green blood plasma. *Journal of Fish Biology*, **33**(5), 775-780.

Fang L. S. and Lai C. C. (1987) Characterization and purification of biliverdin reductase from the liver of eel, *Anguilla japonica*. *Comparative Biochemistry and Physiology. B, Comparative Biochemistry*, **88**(4), 1151-1155.

FAO (2016). The State of World Fisheries and Aquaculture 2016. Contributing to food security and nutrition for all. Rome. 200 pp. http://www.fao.org/3/a-i5555e.pdf

Freixial, C., Vasco-Rodrigues, N., Baylina, N. and Modesto, T. (2018). Cleaning behaviour of adult Bluestreak cleaner wrasse, *Labroides dimidiatus* (Perciformes: Labridae), in Oceanário de Lisboa. *Frontiers in Marine Science*, 5.

Gagnon, M. M. (2006). Serum biliverdin as source of colouration upon sexual maturation in male blue-throated wrasse *Notolabrus tetricus*. *Journal of Fsh Biology*, **68**(6), 1879-1882.

Garris, D. R. (2004). Ovarian hypercytolipidemia induced by obese (ob/ob) and diabetes (db/db) mutations: basis of female reproductive tract involution II. *Tissue and Cell*, **36**(3), 157-169.

Grant, A. N. (2002). Medicines for sea lice. *Pest Management Science: Formerly Pesticide Science*, **58**(6), 521-527.

Grant, B., Davie, A., Taggart, J. B., Selly, S. L., Picchi, N., Bradley, C., Prodohl, P., Leclercq, E.and Migaud, H. (2016). Seasonal changes in broodstock spawning performance and egg quality in ballan wrasse (*Labrus bergylta*). *Aquaculture*, **464**, 505-514.

Imsland, A., Reynolds, P., Eliassen, G., Hangstad, T., Foss, A., Vikingstad, E. and Elvegård, T. (2014). The use of lumpfish (*Cyclopterus lumpus* L.) to control sea lice (*Lepeophtheirus salmonis* Krøyer) infestations in intensively farmed Atlantic salmon (*Salmo salar* L.). *Aquaculture*, **424-425**, 18-23.

Inland Fisheries Ireland, (2017). The Status of Irish Salmon Stocks in 2016 and Precautionary Catch Advice for 2017. 96 pages. https://www.fisheriesireland.ie/documents/1412-the-status-of-irish-salmon-stocks-in-2016-with-precautionary-catch-advice-for-2017/file.html

Jansen, T. and Daiber, A. (2012). Direct Antioxidant Properties of Bilirubin and Biliverdin. Is there a Role for Biliverdin Reductase? *Frontiers in Pharmacology*, **3**, 30.

Jeney, V. (2002). Pro-oxidant and cytotoxic effects of circulating heme. *Blood*, **100**(3), 879-887.

Jeney, V., Balla, G. and Balla, J. (2014). Red blood cell, hemoglobin and heme in the progression of atherosclerosis. *Frontiers in Physiology*, **5**, 379.

Jensen, I. J., Mæhre, H. K., Tømmerås, S., Eilertsen, K. E., Olsen, R. L., and Elvevoll, E. O. (2012). Farmed Atlantic salmon (*Salmo salar* L.) is a good source of long chain omega-3 fatty acids. *Nutrition Bulletin*, **37**(1), 25-29.

Jüttner, F., Stiesch, M. and Ternes, W. (2013). Biliverdin: the blue-green pigment in the bones of the garfish *(Belone belone*) and eelpout (*Zoarces viviparus*). *European Food Research and Technology*, **236**(6), pp.943-953.

Kaur, H., Hughes, M., Green, C., Naughton, P., Foresti, R. and Motterlini, R. (2003). Interaction of bilirubin and biliverdin with reactive nitrogen species. *FEBS Letters*, **543**(1-3), 113-119.

Kikuchi, G., Yoshida, T. and Noguchi, M. (2005). Heme oxygenase and heme degradation. *Biochemical and Biophysical Research Communications*, **338**(1), 558-567.

Kirkby, K. A and Adin, C. A. (2006). Products of heme oxygenase and their potential therapeutic applications. *American Journal of Physiology-Renal Physiology*, **290**(3), F563-F571.

Loudon, M. (2002). *Organic Chemistry*. 4th ed. New York: Oxford University Press, 1135–1136.

Low, P., and Bada, J. L. (1974). Bile pigments in the blood serum of fish from the family Cottidae. *Comparative Biochemistry and Physiology-Part A: Physiology*, **47**(2), 411-418.

Marcos-López, M., Ruane, N. M., Scholz, F., Bolton-Warberg, M., Mitchell, S. O., Murphy O'Sullivan, S., Irwin Moore, A. and Rodger, H. D. (2017). Piscirickettsia salmonis infection in cultured lumpfish (*Cyclopterus lumpus* L.). *Journal of Fish Diseases*, **40**(11), 1625-1634.

Marine Scotland 2017. Scottish Government Topic Sheet No. 40.

McDonagh, A. F., Palma, L. A. and Schmid, R. (1981). Reduction of biliverdin and placental transfer of bilirubin and biliverdin in the pregnant guinea pig. *Biochemical Journal*, **194** (1), 273-282.

Mohd Yusof, M. F. (2015). Reproductive biology and growth of ballan wrasse *Labrus bergylta* in west of Ireland waters, (August). Available at: https://aran.library.nuigalway.ie/handle/10379/5350.

Mozaffarian, D., and Rimm, E. B. (2006). Fish intake, contaminants, and human health: evaluating the risks and the benefits. *Jama*, **296**(15), 1885-1899.

Muncaster, S., Andersson, E., Kjesbu, O., Taranger, G., Skiftesvik, A. and Norberg, B. (2010). The reproductive cycle of female ballan wrasse *Labrus bergylta* in high latitude, temperate waters. *Journal of Fish Biology*, **77**(3), 494-511.

Muncaster, S., Norberg, B. and Andersson, E. (2013). Natural sex change in the temperate protogynous Ballan wrasse *Labrus bergylta*. *Journal of Fish Biology*, **82**(6), 1858-1870.

Mustafa, A., MacWilliams, C., Fernandez, N., Matchett, K., Conboy, G. and Burka, J. (2000). Effects of sea lice (*Lepeophtheirus salmonis* Kröyer, 1837) infestation on macrophage functions in Atlantic salmon (*Salmo salar*). *Fish and Shellfish Immunology*, **10**(1), 47-59.

National Seafood Survey Aquaculture Report (2019). BIM Ireland's Seafood Development Agency, Dublin, Ireland, pp 48.

Perutz, M.F. (1983). Species adaptation in a protein molecule. *Molecular Biology and Evolution*. **1**, 1–28.

Pike, A. (1989). Sea lice — Major pathogens of farmed atlantic salmon. *Parasitology Today*, **5**(9), 291-297.

Quintela, M., Danielsen, E., Lopez, L., Barreiro, R., Svåsand, T., Knutsen, H., Skiftesvik, A. and Glover, K. (2016). Is the ballan wrasse (*Labrus bergylta*) two species? Genetic analysis reveals within-species divergence associated with plain and spotted morphotype frequencies. *Integrative Zoology*, **11**(2), 162-172.

Roth, M., Richards, R. and Sommerville, C. (1993). Current practices in the chemotherapeutic control of sea lice infestations in aquaculture: a review. *Journal of Fish Diseases*, **16**(1), 1-26.

Sayer, M. and Davenport, J. (1996). Hypometabolism in torpid goldsinny wrasse subjected to rapid reductions in seawater temperature. *Journal of Fish Biology*, **49**(1), 64-75.

Schetter, A., Heegaard, N. and Harris, C. (2009). Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis*, **31**(1), pp.37-49.

Scholz, F., Fringuelli, E., Bolton-Warberg, M., Marcos-López, M., Mitchell, S., Prodhol, P., Moffet, D., Savage, P., Murphy O'Sullivan, S., O'Connor, I., McCarthy, E and Rodger HD. (2016). First record of *Tetramicra brevifilum* in Lumpfish (*Cyclopterus lumpus*, L.). *Journal of Fish Diseases*, **40**(6), 757-771.

Sedlak, T. and Snyder, S. (2004). Bilirubin Benefits: Cellular Protection by a Biliverdin Reductase Antioxidant Cycle. *Pediatrics*, **113**(6), 1776-1782.

Seljestad, G. W., Quintela, M., Faust, E., Halvorsen, K. T., Besnier, F., Jansson, E., Dahle, G., Knutsen, H., André., Folkvord, A. and Glover, K. A. (2020) "A cleaner break": Genetic divergence between geographic groups and sympatric phenotypes revealed in ballan wrasse (*Labrus bergylta*). *Ecology and Evolution*, **10**, 6120-6135.

Skiftesvik, A., Blom, G., Agnalt, A., Durif, C., Browman, H., Bjelland, R., Harkestad, L., Farestveit, E., Paulsen, O., Fauske, M., Havelin, T., Johnsen, K. and Mortensen, S. (2013a). Wrasse (Labridae) as cleaner fish in salmonid aquaculture – The Hardangerfjord as a case study. *Marine Biology Research*, **10**(3), 289-300.

Skiftesvik, A., Bjelland, R., Durif, C., Johansen, I. and Browman, H. (2013b). Delousing of Atlantic salmon (*Salmo salar*) by cultured vs. wild ballan wrasse (*Labrus bergylta*). *Aquaculture*, **402-403**, 113-118.

Stocker, R. (2004). Antioxidant Activities of Bile Pigments. *Antioxidants and Redox Signaling*, **6**(5), 841-849.

Thorstad, E. B., Todd, C. D., Uglem, I., Bjørn, P. A., Gargan, P. G., Vollset, K. W., Halttunen, E., Kålås, S., Berg, M. and Finstad, B. (2015). Effects of salmon lice *Lepeophtheirus salmonis* on wild sea trout Salmo trutta a literature review. *Aquaculture Environment Interactions*, **7**(2), 91-113.

Thorstad, E.B. and Finstad, B. 2018. Impacts of salmon lice emanating from salmon farms on wild Atlantic salmon and sea trout. NINA Report 1449: 1-22.

Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O., Nilsen, F., Horsberg, T. and Jackson, D. (2013). Salmon lice - impact on wild salmonids and salmon aquaculture. *Journal of Fish Diseases*, **36**(3), pp.171-194.

Turi Baklien, A. and Rundtom, T., (2020). Aquaculture. [online] Statistics Norway. Available at: <https://www.ssb.no/en/fiskeoppdrett/> [Accessed 3 October 2020].

Villegas-Ríos, D. (2013c). Life-history and behaviour of "*Labrus bergylta*" in Galicia. Ph.D. University of Vigo, Galicia, Spain.

Villegas-Ríos, D., Alonso-Fernández, A., Domínguez-Petit, R., and Saborido-Rey, F. (2013a). Intraspecific variability in reproductive patterns in the temperate hermaphrodite fish, *Labrus bergylta*. *Marine and Freshwater Research*, **64**(12), 1156-1168.

Villegas-Ríos, D., Alonso-Fernández, A., Fabeiro, M., Bañón, R., and Saborido-Rey, F. (2013b). Demographic variation between colour patterns in a temperate protogynous hermaphrodite, the ballan wrasse *Labrus bergylta*. *PLoS One*, **8**(8), e71591.

Westneat, M.W. (2001). Labridae. Wrasses, hogfishes, razorfishes, corises, tuskfishes. In K.E.Carpenter and V.Niem (Eds.), *FAO species identification guide for fishery purposes. The living marine resources of the Western Central Pacific.*Vol. 6. Bony fishes part 4 (Labridae to Latimeriidae), estuarine crocodiles (pp.3381–3467). Rome: FAO.

Yan, D., Domes, C., Domes, R., Frosch, T., Popp, J., Pletz, M.W. and Frosch, T. (2016). Fibre enhanced Raman spectroscopic analysis as a novel method for diagnosis and monitoring of diseases related to hyperbilirubinemia and hyperbiliverdinemia. *Analyst*, **141**(21), 6104-6115.

Chapter 2: The relationship between morphotypes of ballan wrasse (*Labrus bergylta*) and their blood physiology

Chapter 2: The relationship between morphotypes of ballan wrasse (*Labrus bergylta*) and their blood physiology

2.1 Abstract

Biliverdin is a bile pigment that has been reported in a wide variety of species but whose adaptive significance is unknown. The hypothesis of an association between the existence of biliverdin in blood plasma and the hermaphroditic reproductive mode was investigated in several coastal fish species, i.e. in hermaphrodites such as ballan wrasse (*Labrus bergylta*) and gonochoristic (separate sexes) species (lumpfish, *Cyclopterus lumpus*; long-spined sea scorpion, *Taurulus bubalis*; short-spined sea scorpion, *Myoxocephalus Scorpius*; rockling *Gaidrops mediterraneus*; and Tompot blenny *Parablennius gattorugine*). To determine presence of bili-pigments, blood plasma from several coastal fish species with either hermaphrodite or gonochoristic were examined using colorimetry readings (*L * a * b ** scores) and scanning UV spectrophotometry to examine absorbance spectra. The significance of bili-pigments was investigated in more detail in ballan wrasse (*L. bergylta*), focussing on variation in different morphotypes collected from two locations in Co. Galway: Carna and Spiddal during 2015 and 2016. Particular attention was paid to comparisons of biliverdin and bilirubin concentrations between the morphotypes across years and locations. In addition, possible connections between pigment concentration and basic haematology of blood cells were investigated (packed cell volume (haematocrit), haemoglobin, blood cells count, blood indices, biliverdin and bilirubin).
Colorimetry readings (*L * a * b ** score) for several gonochoristic species showed a range of plasma colouration: where *G. mediterraneus, G. morhua* and *P. gattorugine* had pinkish plasma colour, *C. lumpus* and *T. bubalis* which are also gonochoristic species had blue-green plasma colour, and *M. scorpius* (also gonochoristic) had yellow / green plasma colour. Thus, the results didn't support the hypothesis of a link between the hermaphroditic reproductive mode and plasma colouration. Scanning UV spectrophotometry spectra for several coastal fish (e.g. *G. mediterraneus, G. morhua, P. gattorugine, C. lumpus, T. bubalis* and *M. scorpius*) shared a peak at 412 nm. However, *C. lumpus, T. bubalis* and *M. scorpius* had a second peak at 668 nm for *M. scorpius* and *C. lumpus,* and at 664 nm for *T. bubalis*. The plasma colouration of *L. bergylta* was observed by colorimeter (*L * a * b ** score), which varied between both locations and from 2015 to 2016. While the plasma colour of most Carna 2015 samples was blue with a few blue-green individuals, the Carna 2016 and Spiddal plasma (both years) was predominantly green. Spectrophotometric data showed that the blue plasma was linked with absorbance at 640-644 nm and the green plasma linked with absorbance at 668 nm.

Chapter 2: The relationship between morphotypes of ballan wrasse (*Labrus bergylta*) and their blood physiology

This is the first study which has reported additional morphotypes in *L. bergylta* to the two (plain and spotted) previously reported, these were: plain, spotted, merle and intermediate. There were no significant differences between morphotypes of *L. bergylta* across basic haematology parameters: haematocrit, haemoglobin, red blood cells, white blood cells and blood indices (Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC). However, there were significant differences between the morphotypes in total length, weight, conjugated bilirubin, unconjugated bilirubin, total bilirubin and biliverdin. The three variables: body size, biliverdin and total bilirubin concentrations, which when combined, were associated with morphotype (s). Merle was the shortest and the lightest morph, and the spotted and intermediate morphotypes were the largest and the heaviest. Total bilirubin concentration was higher in plain morphotypes while the other morphs overlapped. On the other hand, plain, intermediate and spotted individuals had the highest concentrations of biliverdin, and merle was the lowest. Analysis of locations and years demonstrated that bilirubin concentration was higher than biliverdin in both locations and years except in Carna 2015. The concentration of plasma biliverdin for samples from both locations combined in 2016 did not significantly differ between males and females. Mass spectrometry on the blood pigment in *L. bergylta* confirmed this colouration was due to the existence of biliverdin and bilirubin. Because the plasma concentration of biliverdin/bilirubin is linked to body size and morphotypes, it is suggested that one of the purposes of biliverdin may be in the signalling of some sort to conspecifics. However, However, for this idea to be developed, a link between plasma concentration and external tissues should first be investigated. Because pigment concentrations varied considerably across locations and years, biliverdin may have other functions, including functioning as camouflage, as an antioxidant, or as a type of sun screen.

Keywords: *Labrus bergylta*, morphotypes, biliverdin, bilirubin, plasma, hermaphroditic, gonochoristic, UV spectrophotometry, colorimetry, haematology.

2.2 Introduction

Respiratory pigments are critical features involved with red blood cells and the blood physiology of animals. Respiratory pigments are protein molecules which includes haemoglobin, hemocyanin, hemerythrin, and chlorocruorin. These metalloproteins possess metal ions (e.g. iron and copper) located at an "active site" that allows the molecule to bind onto oxygen from its environment (Manwell 1964). Of these, hemocyanin, hemerythrin, and chlorocruorin are found in the invertebrate respiratory system, hemocyanin contains a copper atom and is the major protein in Arthropoda and Mollusca (Burmester 2004). While hemerythrin contains two iron atoms and are found commonly in annelids (French et al. 2008; Stenkamp 1994). However, haemoglobin a red protein pigment is the only respiratory pigment present in all jawed vertebrates, which is found mainly in the erythrocytes (red blood cells) and gives rise to the cell's red colouration (Goodman et al. 1975). The haemoglobin contains a tetrapyrrole or 'heme' group that is broken down to tetrapyrrolic bile pigments bilirubin and biliverdin. The metabolic waste of heme degradation are bile pigments: biliverdin is a heme degradation intermediate metabolite. It is reduced to bilirubin in most mammals and excreted in conjugated form into the bile. In most jawed vertebrates including mammals, biliverdin is an intermediate breakdown product of heme that eventually degrades to bilirubin (Colleran and Heirwegh 1979). This degradation is carried out via the reducing action of nicotinamide adenine dinucleotide phosphate, and a two-stage enzyme-controlled pathway, i.e. heme oxygenase-1 and biliverdin reductase. However, in some species of vertebrate animals (e.g. fish, birds and reptiles), the heme catabolism pathway is modified such that biliverdin is the terminal product, which can then be incorporated into their blood serum or tissue.

For most fish species, the blood plasma is a light-straw colour due to the presence of bilirubin, which is comparable to those found in higher vertebrates. Although, there are a few exceptions where certain fish species including eels (Anguilleformes) and cottids (Scorpaeniformes) possess blood that is blue-green in colour due to the presence of biliverdin as the chromophore in the blood pigment protein (Low and Bada 1974; Cornelius 1991). Biliverdin has also been observed in tissues such as the yolk proteins in frogs (*Xenopus laevis*) (Redshaw et al. 1971) while several lizard species in the *Prasinohaema* genus have been discovered to accumulate significant amounts of biliverdin in their blood serum and tissue (Austin and Jessing 1994). A high level of biliverdin in a number of species of lizard from the island of New Guinea made the blood, muscle, bones,

and tongue a shiny green colour (Rodriguez et al. 2018). The responsible molecule for similar observations in Araucano fowl (*Gallus gallus*) shells were identified chemically as biliverdin-IX and protoporphyrin-IX (Kennedy and Vevers 1973). Biliverdin was also linked experimentally to eggshells of blue-shelled chickens when compared with brown-shelled morphs of the same species but was not found to differ in the blood, bile, excreta, and shell gland between these morphs (Zhao et al. 2006). The results indicated that biliverdin was most likely synthesized in the shell gland, and was then deposited on the shell (Zhao et al. 2006). The adaptive significance in eggshell colour variation could be linked to parasitism and crypsis. For example, brood-parasitic cuckoos (*Cuculus canorus*) lay their eggs in other species of bird's nest (Avilés 2008). The cuckoo must lay eggs that imitate the host's eggs colour, size and shape. In this case, the cuckoo performs crypsis to hide their eggs from competing cuckoos by producing biliverdin and protoporphyrin (Igic et al. 2012 (Gloag et al. 2014).

A number of fish species such as the garfish (*Belone belone*) and the viviparous eelpout (*Zoarces viviparous*) have been observed to accumulate biliverdin in their connective tissue and bones, giving them a blue-green colour (Jüttner et al. 2013). Other species of fish accumulated biliverdin in their plasma e.g. arctic sculpin (*Myoxocephalus scorpioides*) (Bada 1970). However, no links between pigment concentrations in different tissues have been examined in fish. Biliverdin, which was identified in various sculpin tissues (skin, muscle, digestive tract and eggs) was suggested to function variously as camouflage, in enabling colouration associated with sexual dimorphism, acting as a UV protector or a photoreceptor for hatching (Yamaguchi et al. 1976). In the latter, biliverdin might provide protection against isomerization and oxidation in the different parts of the body, having been stored temporarily and then transported to the skin and eggs by blood serum at the appropriate moment (Yamaguchi et al. 1976).

Biliverdin in the blood has also been identified as a common trait in a number of hermaphroditic fish species including *Labrus bergylta* (Clark et al. 2016) and blue-throated wrasse (*Notolabrus tetricus*) (Gagnon 2006). Within *L. bergylta*, biliverdin accumulation occurs through its association with a protein moiety of the lipocalin superfamily in the blood serum; this prevents further breakdown to bilirubin via oxidation (Clark et al. 2016). Despite biliverdin's association with this protein moiety, the pigment is visible in the plasma fraction, with the protein also preventing further processing or excretion (Clark et al. 2016). Some of this protein-bound biliverdin may then be stored in the fish's skin and

tissue, resulting in strong pigmentation. Like many wrasse species, *L. bergylta* is a protogynous hermaphrodite meaning that all fish are born female. However, later in their lives certain individuals undergo a change in sex, with this transition beginning at the end of the annual reproductive season (in spring) and ending by the following spring period (Talbot et al. 2012; Villegas-Ríos 2013a; Muncaster et al. 2013). The remodelling of the female gonad from egg-filled ovaries to a testis results in the production of many free radicals, such as reactive oxygen species, leading to oxidative stress (Clark et al. 2016). An excess of free radicals within an organism can lead to cell injury and death and may contribute to diseases such as cancer (Schetter et al. 2009). Most species combat oxidative stress by maintaining a complex system of antioxidants which they either produce internally (e.g., reduced glutathione, ubiquinol-10) or are introduced via their diet (e.g., vitamins C and E) (Stocker 2004).

Biliverdin is known to possess antioxidant properties (Jansen and Daiber 2012). This antioxidant activity is due to the structure of biliverdin, which contains extended systems of conjugated carbon double bonds, resulting in a high redox potential, allowing it to effectively scavenge a range of free radicals including reactive oxygen species (Kaur et al. 2003; Stocker 2004). It has been suggested that high levels of biliverdin in the blood, tissue and skin throughout their lifecycle may be used as an antioxidant to mitigate the damage caused during the sex change process. Clark et al.'s (2016) study suggests that biliverdin in *L. bergylta* is deposited in the serum when it is a female and stored in the skin until the spawning period. When the female undergoes transition to a male, the biliverdin is used as a free radical scavenger released during the reabsorption of eggs in the ovary. However, in that study (Clark et al. 2016), only nine individuals were observed to transition from female to male out of sample size of 397 individuals; as such, the theory is not conclusive. In addition, when biliverdin concentration was compared between protogynous hermaphrodites (*L. bergylta* and the cuckoo wrasse, *Labrus mixtus*) and gonochoristic (separate sexes) wrasse species, i.e. goldsinny (*Crenilabrus rupestris*), rockcook (*Centrolabrus exoletus*), and corkwing (*Symphodus melops*) (Dipper 1976; Clark et al. 2016), it was observed that *L.*

bergylta had higher concentrations of plasma biliverdin (22.82 ± 2.9 μmol.l^{-1}) than was found in *C. exoletus*, *C. rupestris* (did not meet the threshold of detection) and *S. melops* (8.30 ± 2.2 μmol.l^{-1}) females, but going against this hypothesis, it was also un-detectable in *L. mixtus* (Clark et al. 2016).

In addition to the role of biliverdin as an antioxidant, studies have shown there may also be a role for the molecule in mating. Where sexual dimorphism occurs, colour morphotype can also be related to the sex of an individual (male/female) due to some males being highly coloured when compared their female counterparts (Dipper 1981). This can complicate the understanding of morphotypes in species of fish which undergo sex-inversion, i.e. a single individual may become another morphotype when undergoing a sex change. Indeed, in cuckoo wrasse *L. mixtus*, gender transition was followed by changes in colour morphotype, therefore these species were described as being highly sexually dichromatic (Matić-Skoko et al. 2013). During sexual selection, female cuckoo wrasse will select males that have high skin colouration (Dipper 1981), therefore colouration, thanks to biliverdin, could function in sexual selection, as a way of attracting mates. If indeed this pigment can be linked to external colouration in male cuckoo wrasse, it would indicate a plausible function for biliverdin. In that case, it also ought to be established whether the biliverdin was associated more with the change of sex from female to male, or with sexual selection of males by females, or indeed, both roles.

In other protogynous wrasse species, for example, the male of *Notolabrus tetricus*, the bright blue colour only appears during transitional status in the reproduction period (Gagnon 2006). Clark, et al (2016) examined if there was any relationship between *L. bergylta* colour morphotypes and blood pigments. Morphotypes were classified based on colour, where red and green were the predominant colour morphs observed at the sampling locations in Scotland (Clark et al. 2016). The results showed that the red colour morph had the lowest biliverdin concentration and the green morph had the highest biliverdin concentration (Clark et al. 2016).

As there are several plausible hypotheses to explain the presence of alternative bili-pigments, the overall aims of this research chapter are quite broad: (**i**) examine a range of coastal fish species in the west coast of Ireland to determine which alternative blood pigment they possess (biliverdin or bilirubin) in relation to its reproductive strategy (hermaphrodite or gonochoristic); (**ii**) to examine blood pigment parameters and basic haematology of blood cells in *L. bergylta* and compare these across all identifiable morphs, in order to identify variations between *L. bergylta* morphotypes, and to determine the extent that morphotype could be predicted using blood pigments or haematological parameters. Finally, **(iii)** carry out a comparison of biliverdin and bilirubin concentrations between morphotypes sampled across years and locations.

To achieve these aims, I first examined a range of coastal fish that were either hermaphrodite: ballan wrasse (*L. bergylta*); or gonochoristic: (lumpfish (*Cyclopterus lumpus*), long-spined sea scorpion (*Taurulus bubalis*), short-spined sea scorpion (*Myoxocephalus scorpius*), shore rockling (*Gaidropsarus mediterraneus*), Atlantic cod (*Gadus morhua*) and Tompot blenny (*Parablennius gattorugine*), to examine the presence of blue/green plasma (assumed to be biliverdin) by reproductive mode (i.e. hermaphrodite or gonochoristic). The results were used to test the anti-oxidant theory of Clark et al. (2016).

Secondly, the measurement of haematological parameters can provide an insight into the physiological state of individual fish, which may vary according to age or sex of the fish, and the impact of different environmental factors (e.g. seasonal fluctuations, geographical distribution) (Jamalzadeh et al. 2009; Denton and Yousef 1975). As such, this study also examined a series of haematological parameters to give a deeper understanding of whether there are linkages between blood carrying capacity and the morphotype of *L. bergylta*. These included red blood cell count, haematocrit, haemoglobin levels and red blood cell indices. In addition, the pigment responsible for observed plasma colouration (determined with colorimeter) in *L. bergylta* was isolated and identified using mass spectrometry and wavelength characteristics (UV scanning spectrophotometry). Finally, quantitative measurements (using the spectrometric method) were made of biliverdin and bilirubin in four morphotypes of *L. bergylta* from two locations in Galway in the west of Ireland over two successive years (2015 and 2016). This was carried out to determine whether blood pigments map onto morphotypes, or put another way, whether these are associated with morphotype.

2.3 Materials and Methods

2.3.1 Locations and sampling

Seven species of coastal fish were sampled, including ballan wrasse, four other coastal species and the final two species were obtained from captive breeding / aquaculture programmes (see below for further details). All samples were collected in 2015 and 2016. All ballan wrasse individuals were captured from the wild. Due to the highly selective nature of *L. bergylta* regarding its habitat preferences, choosing an appropriate location to sample was very important. Rocky shore sites with an abundance of sub-surface cover from large seaweeds and moderate wave exposure were selected as possible sampling locations for *L. bergylta*. After an initial pilot trial, two locations were selected: A) Mweenish Island, Carna, Connemara, Co. Galway (53°18'01.9"N 9°51'09.6"W) B) Spiddal Pier, Co. Galway (53°14'24.8"N 9°18'39.9"W) (Figure 2.1). Specimens were collected during 2015 and 2016 over the summer period from June to October (see the appendix). The samples were collected by two techniques, by local fishermen using lobster pots (for the samples from Carna 2015) and using rod and line (for samples from Spiddal 2015, 2016 and Carna 2016). Initially, common shrimp, mussel flesh, shore crab and limpet flesh were used as bait, with mussel flesh being the preferred one on the basis of being the easiest to collect and the most practical bait to use. The samples from Carna 2015 (n=57) were held in a keep-box at sea, adjacent to the Carna Research Station Co. Galway, for a maximum period of 2 days prior to blood sampling being carried out. The remaining samples from Carna 2016 (n=21), Spiddal 2015 (n=37) and Spiddal 2016 (n=34) were not held in a keep-box at sea. In these cases, blood sampling was carried out immediately. The eventual total sample size was n=149 fish.

The four coastal fish species sampled from different sites included: long-spined sea scorpion (*Taurulus bubalis*) (n=11), short-spined sea scorpion (*Myoxocephalus scorpius*) (n=8), shore rockling (*Gaidropsarus mediterraneus*) (n=20), and tompot blenny (*Parablennius gattorugine*) (n=28). Some specimens were collected from National Aquarium of Ireland (Galway city, Ireland) and others were collected from Mweenish Bay (Carna, Connemara, Co. Galway) having been captured in lobster pots. The specimens were collected from mid - September through late November 2015, and these samples were also held in a keep-box at sea, adjacent to the Carna Research Station for a maximum period of 2 days prior to blood sampling being carried out. These species were captured from the wild, apart from the lumpfish (*Cyclopterus lumpus*) (n=10) and Atlantic cod

(*Gadus morhua*) (n=17) which were sourced from the lumpsucker breeding programme and EirCod breeding programme based at Carna Research Station. Atlantic cod samples were collected to provide a base line for a typical fish which does not possess pigmented blood.

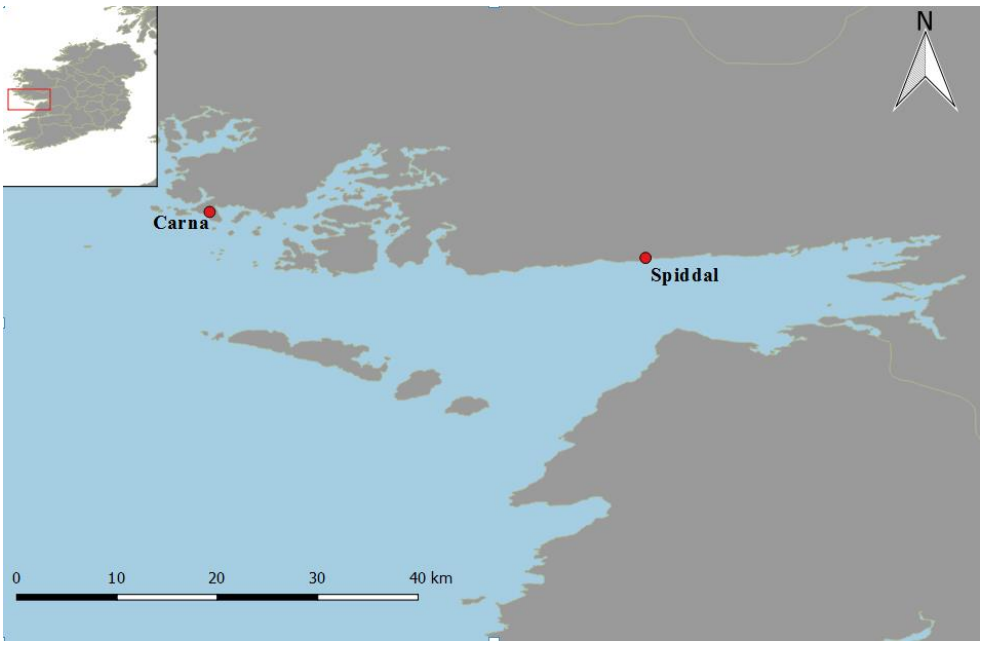


Figure 2. 1: Sample locations of the coastal fish: Mweenish Island, Carna, Connemara, Co. Galway Ireland; Spiddal, Co. Galway, Ireland.

Fish were euthanised immediately on capture using an overdose of MS222 (tricaine methanesulfonate, Pharmaq, Overhalla, Norway). Blood sampling was carried out immediately at the caudal vein by using heparinised needles and syringes and was subsequently decanted into storage vials. Once an individual fish was captured, morphometric measurements of total length (cm) and body weight (g) were taken and *L. bergylta* individuals were labelled and photographed (both in and out of water) in order to assign them to one of four morphotypes (plain, spotted, merle and intermediate). Fish were then stored on ice during transportation for further processing in the laboratory.

2.3.2 Morphotype characteristics of ballan wrasse (*Labrus bergylta*)

A total of 149 *L. bergylta* individuals were sampled during 2015 and 2016 at Carna and Spiddal and these presented some highly colourful patterns and variable morphotypes. In all, four different *L. bergylta* morphotypes could be distinguished, as follows: for Carna, ‘plain’ (n=6), ‘spotted’ (n=23), ‘merle’ (n=22) and ‘intermediate’ (n=27), and for Spiddal ‘plain’ (n=8), ‘spotted’ (n=7), ‘merle’ (n=51) and ‘intermediate’ (n=5) (Figure 2.2).

Spotted fish were identified as having a covering of white, blue or green spots, while plain fish featured a uniform but variable body colour. Merle fish were characterised as having a striped pattern, which occurred on their flanks (i.e. around the area of the lateral line). There was also an intermediate morph that was characterised as having both merle and spotted pattern features.

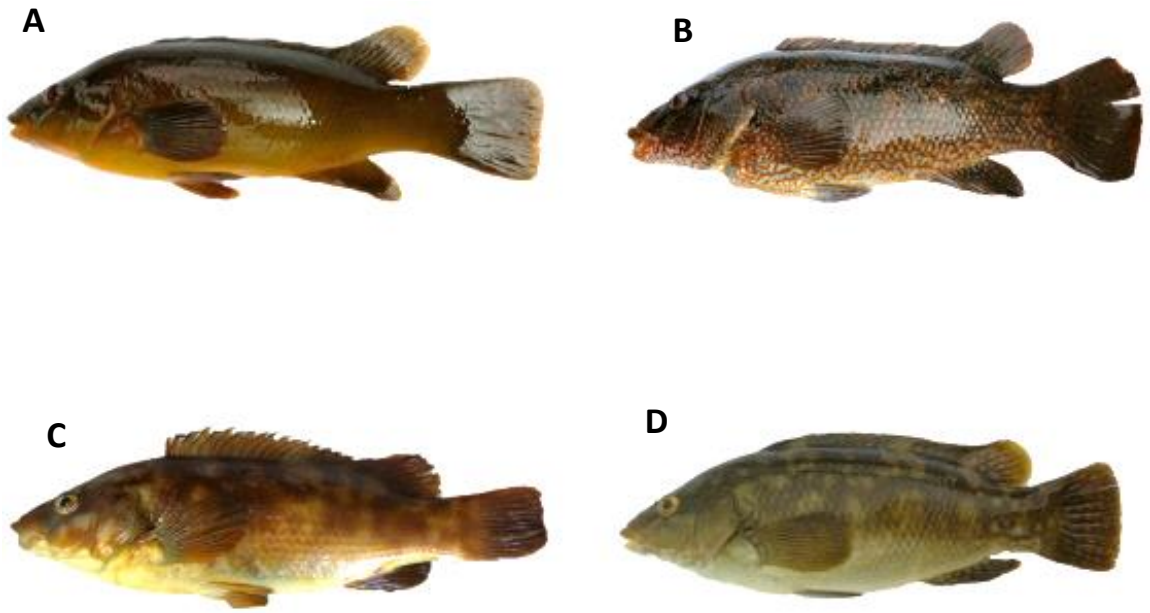


Figure 2. 2: Variation in the colour morphology of ballan wrasse (*Labrus bergylta*) morphotypes: **A**) Plain, **B**) Spotted, **C**) Merle, and **D**) Intermediate.

2.3.3 Blood and tissue sampling

Sampled whole blood was centrifuged at 13,000 *g* for 15 minutes. The plasma supernatant was then removed and further centrifuged for 2 minutes to remove any remaining blood cells. Plasma samples from individuals were then divided into aliquots and placed in individual microtubes and stored at -80°C for later analysis. Sex discrimination into males and females was carried out by dissection (in the breeding season) or using the equation of Leclercq et al. (2014) outside of the breeding season, based on the body mass (M_B in g), total body length (L_T in mm) and Fulton's condition factor (K) as discriminant variables to predict sex with 91% accuracy. The discriminant score (S_D) of a specimen was determined as $S_D = 0{\cdot}01M_B - 0{\cdot}016L_T - 3{\cdot}835K + 6{\cdot}252$; in females, S_D <1.459 and in males S_D is >1·504. Fulton's condition factor (K) was calculated using the equation given by Htun-Han (1978):

$$K=100\ (W/L^3)$$

Where *K* is the Condition Factor, *W* is body weight of the fish in grams (g), *L* is the total length of the fish in centimetres (cm), 100 is the factor which is used to bring K close to a value of one.

Female gonads were distinguished by being much bigger, puffy, with a creamy colour and at times were slightly translucent, whereas male gonads were much smaller and less puffy, and from brown to tan in colour as shown in Figure 2.3.

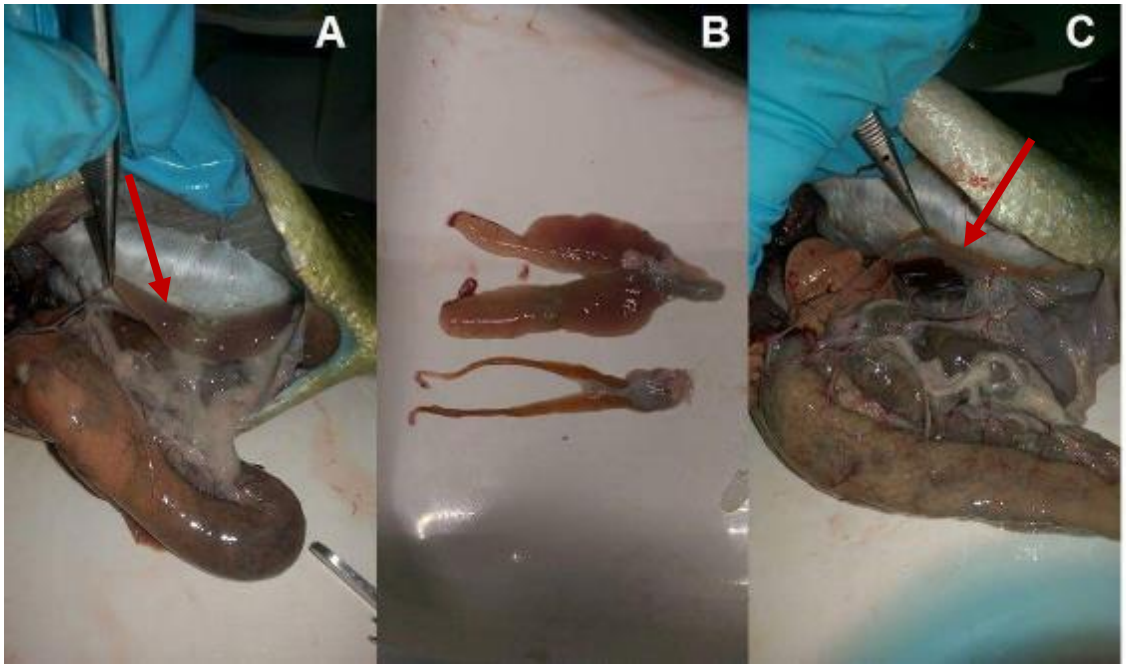


Figure 2. 3: Determination of sex in ballan wrasse (*Labrus bergylta*) through gonad differentiation. The gonads of a female **(A)** and male **(C)** in situ. Red arrows indicate where the gonads lie in-situ. **(B)** Both female (top) and male (bottom) gonads removed from *L. bergylta*.

2.3.4 Basic Haematology for ballan wrasse (*Labrus bergylta*)

2.3.4.1 Packed cell volume

For determining the percentage volume of erythrocytes to the rest of the blood components, Packed Cell Volume (PCV) was carried out (Bain et al. 2016). Pre-heparinised microhaematocrit tubes (~50 µl) were used to draw the blood sample through with capillary action until the tube was approximately three quarters full and sealed with putty sealant (Critoseal®, Wetzlar, Germany). Haematocrits were subsequently centrifuged for 12 minutes at 17,000 *g*. The volume percentage of the separated erythrocytes were measured using a haematocrit tube reader (Hawksley®, Lancing, United Kingdom).

2.3.4.2 Haemoglobin

Haemoglobin **(**Hb) concentration was determined through the cyanmethaemoglobin method (Bain et al. 2016). It is routinely used as an indicator of abnormal health e.g. anaemias. The method is carried out by reacting twenty microlitres of blood with 5 mL Drabkin's reagent (1 gram of sodium bicarbonate, 50 mg of potassium cyanide and 20 mg potassium ferricyanide in 1 L distilled water which is stored away from light) in a test tube for 5 minutes at ambient temperature. Hb in blood is oxidised to methaemoglobin via potassium ferricyanide. Absorbance of the samples were measured at 540 nm using a spectrophotometer (Genesys™ 10, Thermo Fisher Scientific, Waltham, Massachusetts, United States), which was blanked against unreacted Drabkin's reagent. To calibrate the Drabkin's reagent to the concentration of Hb in the blood of *L. berygylta*, a standard calibration curve was created using human haemoglobin as a standard (Table 2.1, Figure 2.4). The linear equation which was acquired from this process enabled the concentration of Hb samples from *L. bergylta* to be calculated.

Table 2. 1: The dilution series used for a haemoglobin standard calibration curve.

Haemoglobin concentration (mg ml^{-1})	**Tube No.**	**Distilled Water (µl)**	**Haemoglobin standard (µl)**
0.00	1	1000	0
39.50	2	750	250
79.00	3	500	500
118.50	4	250	750
158.00	5	0	1000

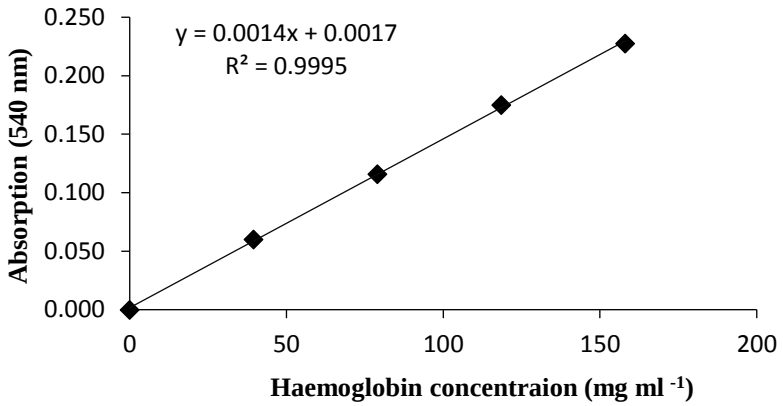


Figure 2. 4: A typical haemoglobin (human) standard calibration curve and the linear equation of the curve used to calculate the unknown haemoglobin content in *L. bergylta* samples.

2.3.4.3 Blood cell count

1980 µl of Dacie's fluid solution (Bain et al. 2016) (1 g brilliant cresyl blue, 31.3 g of trisodium citrate and 10 ml of formaldehyde in 1 L distilled water and filtered) was used to fix and stain 20 µl of fresh whole blood in microcentrifuge tubes, giving a 1:100 blood dilution. This was mixed by inverting the tubes several times. Approximately 10 µl of the mixed sample was introduced slowly into an improved Neubauer haemocytometer cell counting chamber with bright-line (Marienfeld Superior, Germany). Red blood cell (erythrocytes) counts were carried out in the central grid which is subdivided into 25 squares of 0.04 mm^2/ 4 nanolitres (nl) (one such small square shown in yellow in Figure 2.5 A) and five of these were counted in total (Figure 2.5 B). Meanwhile, white blood cells (leucocytes) were counted within the overall larger squares (in red, Figure 2.5 A) that make up the counting chamber giving a surface area of 1 mm^2/ 100 nanolitres (nl). Four of these squares were counted on each corner of the counting chamber (Figure 2.5 B). All cell counts were carried out in duplicate i.e. two counts from one sample on each of the upper chamber and lower chambers of the haemocytometer.

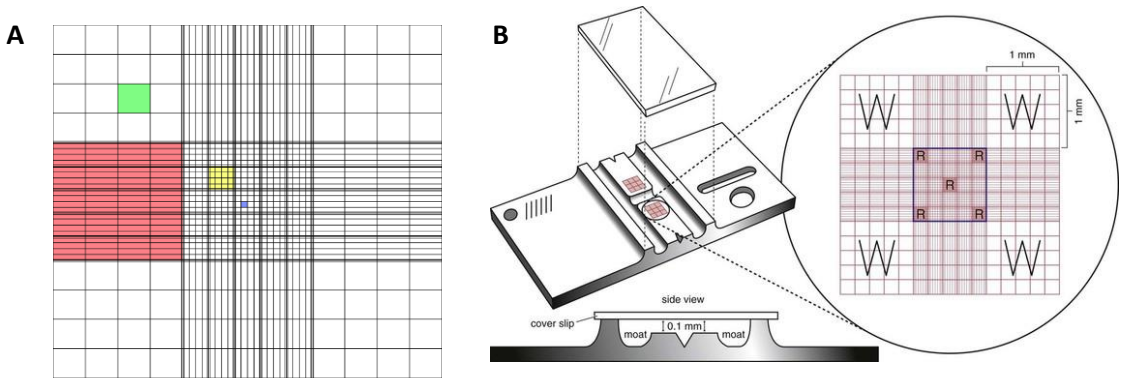


Figure 2. 5: A) Improved Neubauer haemocytometer grid surface area: [Red] 1 mm^2/ 100 nanolitres (nl) [Yellow] 0.04 mm^2/ 4 nanolitres (nl), height 0.1 mm (Holland, 2019); **B**) Areas where blood counts were carried out [W] white blood cells; [R] red blood cells (Anonymous, 2019).

The following formulae were used to calculate the blood cell concentration:

Red blood

$$\text{RBC per Litre} = \left(\frac{x \text{ cells per } 20 \text{ nL}}{20}\right)(\text{Dilution Factor})$$

White blood cell count

$$\text{WBC per Litre} = \frac{(x \text{ cells per } 400 \text{ nL})(\text{Dilution Factor})}{400}$$

2.3.4.4 Blood indices

Three common blood indices were examined (Bain et al. 2016): Mean red blood cell volume (Mean Corpuscular Volume), it can be calculated from packed cell volume (PCV) and the red blood cell count (RBC) as follows:

$$\text{MCV (fL)} = \frac{\text{PCV}}{\text{RBC}}$$

The concentration of Hb in red blood cells (Mean Corpuscular Haemoglobin)

$$\text{MCH (pg)} = \frac{(\text{Hb})}{\text{RBC}}$$

Mean Corpuscular Haemoglobin Concentration

$$\text{MCHC} \left(\frac{\text{g}}{\text{dl}}\right) = \frac{(\text{Hb})}{\text{PCV}}$$

2.3.5 Spectrophotometric measurement of the fish plasma

Crude plasma (50 µL, n=1 replicate readings for each fish individual) from individual *L. bergylta* (n=149), *C. lumpus* (n=10), *T. bubalis* (n=11), *M. scorpius* (n=8), *G. mediterraneus* (n=20), *G. morhua* (n=17) and *P. gattorugine* (n=28) were pipetted into a 96 well plate for preliminary analysis of the presence of biliverdin using a scanning spectrophotometer (Power Wave XS2, BioTek, Winooski, Vermont, United States). The samples were scanned at a wavelength of 300 nm to 900 nm with an interval of 4 nm.

2.3.6 Measurement of the plasma colour through colorimetry

Crude plasma (50 µL, n=1 replicate readings for each fish individual) was measured by a colorimeter (CR-400, Konica Minolta, Tokyo, Japan) using a 96 well plate. The colour of each sample was expressed using the industrial colour standard CIE 1976 *L**, *a**, *b**. The data were plotted with *a**, *b** as the XY axes respectively. *–a** denotes greenness *+a** denotes yellowness *–b** denotes blueness *+b** denotes redness. *L** refers to lighter or darker shades of the colour expressed in *a** and *b**. The plots enabled a graphical illustration comparing the trend in colours of plasma of each individual fish, depending on its i) sample location and ii) year. The colour values of the plasma were measured in triplicate for each sample.

2.3.7 Quantifying plasma biliverdin through the spectrometric method

The spectrometric method of Tickner and Gutteridge (1978) was used to quantify biliverdin concentrations in plasma samples. This method works by reacting the biliverdin with barbituric acid under alkaline conditions, which produces a specific chromogen with peak absorption at 570 nm (Tickner and Gutteridge, 1978). Biliverdin standards were prepared from biliverdin chloride. The concentration of the stock solution was 200 µmol/L, which was prepared by dissolving 117 mg biliverdin in 100 ml of 17.5 M glacial acetic acid. The solution was then stirred and heated at 45°C in a dark room for 1 hour to ensure complete dissolution and then the solution was left to cool down at room temperature. Concentrations of the standard ranging from 0-200 µmol/L were prepared by serial dilution with 17.5 M glacial acetic acid, with a final volume for each standard and blank of 1.5 ml. Approximately 500 µl from each concentration of standard and blank was added to 500 µl distilled water, 400 µl of 40 mM ascorbic acid, and 100 µl of 200 mM barbituric acid in 1 M NaOH. However, standard blanks included 1 M NaOH without the barbituric acid. Preparation of plasma samples and blank samples were exactly the same as for the standards except, this time, aliquots of 100 µl of plasma was added to 400 µl of distilled water, 500 µl glacial acetic acid, 400 µl of 40 mM ascorbic acid, and 100 µl of 200 mM

barbituric acid. All standards and samples with their corresponding blanks were covered with tin foil and incubated at 95 °C on a block heater in a dark room for five minutes. After allowing samples to cool, 2.5 ml n-butanol and 5 ml 2 M sodium hydroxide were added and vortexed followed by centrifugation for 10 minutes at 2500 *g*. After settling, two layers developed, the top layer was discarded, and the desired bottom pinkish-layer was extracted. Absorbance at 570 nm was measured on a spectrophotometer (Genesys™ 10, Thermo Fisher Scientific, Waltham, Massachusetts, United States) for two replicates of each standard and sample.

2.3.8 Quantification of Bilirubin (conjugated / unconjugated)

Bilirubin is an orange-yellow pigment existing in two forms: (i) conjugated bilirubin and (ii) unconjugated bilirubin. The concentration of bilirubin was estimated by the van den Bergh reaction, i.e., the estimation of conjugated bilirubin concentration is based on its reaction with diazotized sulphanilic acid, while for the unconjugated bilirubin, the concentration is estimated by the same reaction catalysed by alcohol. Both reactions produce a reddish-purple azo-derivative of dipyrrole at neutral pH and this colouration is measured photometrically at 540 nm. The reagents were prepared, reagent A called Diazo-reagent and it was prepared as follows: 1 g of sulphanilic acid was dissolved in 15 ml of concentrated HCl and volume was made up to 1L with distilled water. Reagent B, 0.5 % (w / v) sodium nitrite ($NaNO_2$) solution was prepared. Diazo- reagent was prepared fresh before use, by adding 0.8 ml of reagent B with 10 ml of reagent A. Also used for the experiment was 0.18 N HCl, 50 ml methanol (≥ 99.9%) and 100 ml chloroform (≥ 99.9%). The standard for bilirubin was prepared by 10 mg of pure bilirubin (commercial bilirubin, Sigma) transferred into a 100 ml volumetric flask. This was dissolved, and the volume made up with chloroform, then 1 ml of the stock was diluted with methanol to 50 ml in a volumetric flask to give a working standard of 2 μg/ml. The test procedure was as follows: 0.2 ml of the test plasma was dispensed into labelled test tubes (in duplicates). 1.8 ml distilled water and 0.5 ml of diazo-reagent were added, followed by addition of 2.5 ml methanol. The contents were mixed, and the colour allowed to develop for 30 minutes, at room temperature. The absorbance against blanks was measured at 540 nm. The absorbance of a set of standards was measured simultaneously, as shown in Table 2.2. Then a calibration curve was constructed by plotting the concentration of bilirubin (1.0 – 8.0 μg) on the x-axis and absorbance at 540 nm on the y-axis.

The concentration of bilirubin in the test sample was calculated from the calibration curve. In order to determine the concentration of serum conjugated bilirubin (bilirubin biglucuronide), 0.2 ml of the plasma was dispensed into test tubes (in duplicates). 4.3 ml of distilled water and 0.5 ml of diazo-reagent was added, the reaction was performed in the absence of methanol for 1 minute and the absorbance was immediately measured against the blank, then the concentration of bilirubin was calculated from the calibration plot. Then, total bilirubin was calculated by summing the unconjugated and conjugated bilirubin and values are expressed as μmol l^{-1}.

Table 2. 2: Bilirubin standard dilution protocol

Conc. bilirubin (μg ml^{-1})	**Standard bilirubin (ml)**	**Diazo-reagent (ml)**	**Methanol (ml)**	**Distilled water (ml)**	**HCL (0.18 N) (ml)**
-	Blank	-	2.5	2.0	0.5
1.0	0.5	0.5	4.0	-	-
2.0	1.0	0.5	3.5	-	-
4.0	2.0	0.5	2.5	-	-
6.0	3.0	0.5	1.5	-	-
8.0	4.0	0.5	0.5	-	-

- = Nothing added

2.3.9 Validating blood pigments using Tandem mass spectrometry

Plasma sample preparations: Proteins were removed from the plasma by adding a 50 μL aliquot of plasma to 200 μL of a saturated ammonium bicarbonate solution and allowing this to incubate at room temperature for 30 minutes. The precipitated proteins were pelleted using centrifugation and further diluted by adding 100 μL of supernatant to 100 μL of mobile phase B (see below). The resulting solution was filtered through 0.2 μm PTFE filters for UPLC-MSMS analysis.

Standard Plot: Biliverdin and Bilirubin standards (#30891 and #14370, respectively, Sigma Aldrich, St. Louis, Missouri, United States) were used to make aqueous stock solutions by dissolving 1 mg of the standard in 1 ml of distilled water. 100 μl of 2 M sodium hydroxide was also added to aid dissolution of the pigments. The dissolved product was brought to a final volume of 50 ml with 200 mM Tris-HCl buffer (pH 8.4). These stock solutions were serial diluted to create a standard calibration curve for each pigment. Plasma samples were analysed using an Agilent LC-QToF ESIMS 6540 (Santa Clara,

California, United States) coupled to an Agilent 1200 UPLC (Santa Clara, California, United States). Analytes (4 µL) were separated on a ZORBAX Eclipse Plus C18 column, 3.0 X 50 mm, 1.8 µm, 95 Å (Agilent, Santa Clara, California, United States) and eluted with a gradient of A (6.5 mM ammonium bicarbonate in water, pH 8.4) and B (6.5 mM ammonium bicarbonate, 95:5, methanol: water) at a flow rate of 200 µL minutes^{-1}. The initial mobile phase was 100 % A, which was changed to 100% B over 12 mins, and continued at this final gradient for an additional 3 minutes. Mass ions were created using a dual AJS ESI source with a sheath gas temperature of 300 ˚C and a flow rate of 11 L minutes^{-1}. The VCap and Nozzle voltages were set at 3500 and 1000 V, respectively. The bile pigments were qualitative using tandem MS monitoring for the molecular ions $[M+H]^+$ at a parent mass of m/z = 585.2699 and 583.2578 for bilirubin and biliverdin respectively. Bilirubin and biliverdin were fragmented at a collision energy of 25 and 40 eV, respectively.

2.3.10 Data and statistical analysis

2.3.10.1 Plasma colouration

Spectrophotometer and colorimetric (L^* a^* b^* values) readings were plotted in Excel for all coastal species in this study and for *L. bergylta* samples from Carna and Spiddal. A scatter plot was then made to illustrate "colour space" (i.e. a^* b^* values) of crude plasma in all species. To compare plasma colour readings, the normality and homogeneity of variance tests were performed then the T-test was carried out on the differences between Carna 2015 and Carna 2016 and Spiddal 2015 and Spiddal 2016, and also the differences between Carna and Spiddal in 2015 and Carna and Spiddal 2016, then finally the differences between Carna and Spiddal for 2015+2016 overall. A scatter plot was also performed for the scanning UV spectrophotometry in the coastal species and *L. bergylta* samples from Carna and Spiddal. Bar charts were used to compare the frequency of absorbance (wavelength as measured by spectrophotometer) in crude plasma between the morphotypes of *L. bergylta*.

2.3.10.2 Biological and haematology variables across morphotypes

Boxplots (box and whisker) were used to present variation within biological parameters (total length, weight and Fulton's condition factor) and all blood parameters (haemoglobin, haematocrit, red blood cells, white blood cell, MCV, MCH, MCHC, biliverdin, conjugated bilirubin, unconjugated bilirubin and total bilirubin) for each morphotype (plain, spotted, merle and intermediate). Boxplots (box and whisker) were used to show variation within a

particular variable for each morphotype (plain, spotted, merle and intermediate). The top edge (75 % quartile) and bottom edge (25 % quartile) of each box and the median position (also known as the 50 % percentile) is shown inside the box for each morphotype, so this plot is a very useful way of determining whether or not the distribution of the data is symmetrical. Moreover, the box itself represents interquartile range (middle half) of data values in each group, and the whiskers on the plot represent minimum and maximum values, while outliers may be shown below or above whiskers, in some cases. Boxes which do not overlap indicate that ~75 % of data are not shared and thus point towards possible statistical differences. As the variables were not normally distributed, the Kruskal-Wallis test was performed for 14 variables, followed by a Mann-Whitney test to see the differences between the morphotypes.

2.3.10.3 Multivariate analyses to determine which variables distinguish morphotype

Samples of n=110 *L. bergylta* from both locations and both sampling years which had been classified into four morphotype classes (plain, spotted, merle and intermediate) were included in an analysis to determine which explanatory variables could be associated with morphotype. To select the variables for inclusion, a correlation matrix was carried out to determine relationships amongst explanatory variables and to rule out simultaneous use of any highly correlated variables. This resulted in the inclusion of three explanatory variables: Total Length (TL), Biliverdin concentration (BV) and Total Bilirubin concentration (TBR). The subset of included variables were normalised and used to construct a similarity matrix with Euclidean Distance as the distance metric. A correlation-based principal component analysis (PCA) was also carried out. A three-way global analysis was carried out to assess the effects of Location, Year and Morphotype on interpoint distance in the distance matrix using Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson 2001, 2017). The fixed factor Morphotype (n=4) was included in the analysis to test the effect of Morphotype class on the blood chemistry (biliverdin and bilirubin) / size parameters measured. Locations (n=2) and Years (n=2) were also fixed factors in the analysis. P-values were determined by permutation. Type III sums of squares was used with permutation under a reduced model *sensu* Anderson (2017). A *post hoc* pairwise test of the different levels of the factor Morphotype was performed. . Analyses were carried out using PRIMER v6 (Clark and Gorley 2006) with the add-on package PERMANOVA+ (Anderson et al. 2008).

2.3.10.4 Comparison of biliverdin concentrations in plasma of *L. bergylta* between the sexes

The analysis to compare biliverdin concentrations in plasma between the sexes of *L. bergylta* was performed using T-test, with normality and homogeneity of variances testing being carried out prior to t-testing. The test was carried out with an alpha significance of 0.05 using SPSS (IBM SPSS v23) software package and the graphs were carried out in Excel and SPSS.

2.4 Results

2.4.1 Plasma colouration and UV- (scanning) spectrophotometer of coastal fish species

Plasma colouration for all species examined was first examined visually in Figure 2.6: (A) lumpfish (LF) (*Cyclopterus lumpus*), (B) long-spined sea scorpion (LS) (*Taurulus bubalis*), (C) short-spined sea scorpion (SS) (*Myoxocephalus scorpius*), (D) shore rockling (SR) (*Gaidropsarus mediterraneus*), (E) Atlantic cod (AC) (*Gadus morhua*) and (F) Tompot blenny (TB) (*Parablennius gattorugine*). Shore rockling, Atlantic cod and tompot blenny displayed a yellowish and pinkish coloured plasma, as shown in Figure 2.6 (D, E and F respectively). However, lumpfish, long-spined sea scorpion and short-spined sea scorpion presented a green, blue-green and purple plasma colour, with varing colouration between samples (Figure 2.6 A, B and C respectively). Interestingly, plasma colouration also varied between the colour morphotypes in lumpfish (LF) (Figure 2.6 A), with some variability also between individuals of long-spined sea scorpion (Figure 2.6 B) and possibly short-spined sea scorpion (Figure 2.6 C).

Colorimetry readings are plotted for all species of fish in Figure 2.7 and these confirmed the patterns observed by visual estimation of the haematocrit / Eppendorf tubes (in Figure 2.6). Atlantic cod, shore rockling and tompot blenny were found in the area between b^* and a^* which indicated yellow to red colour. Whereas long-spined sea scorpion and lumpfish (mostly) occupied the area between $-a^*$ and $-b^*$ which denotes blue and blue-green colour. Short-spined sea scorpion was found between $-a^*$ and b^* in the plot, which indicated green to yellow plasma colour.

Figure 2. 6: Plasma colouration of; **A**) lumpfish *Cyclopterus lumpus* (LF); **B**) long-spined sea scorpion *Taurulus bubalis* (LS); **C**) short-spined sea scorpion *Myoxocephalus scorpius* (SS); **D**) shore rockling *Gaidropsarus mediterraneus* (SR); **E**) Atlantic cod (AC) *Gadus morha* (image supplied by Anonymous, 2018) and; **F**) Tompot blenny *Parablennius gattorugine* (TB).

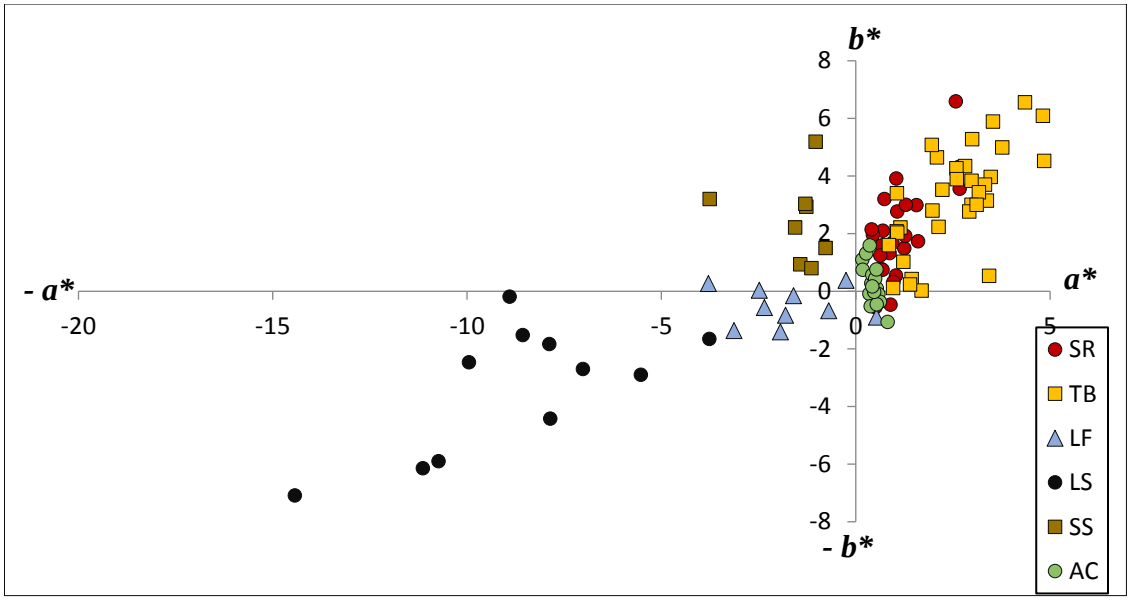


Figure 2. 7: *a*, b** colour space of crude plasma in: Shore rockling *Gaidropsarus mediterraneus* (SR) (n=20), tompot blenny *Parablennius gattorugine* (TB) (n= 28), Atlantic cod *Gadus morhua* (AC) (n=17), short-spined sea scorpion *Myoxocephalus Scorpius* (SS) (n= 8), long-spined sea scorpion *Taurulus bubalis* (LS) (n=11) and lumpfish *Cyclopterus lumpus* (LF) (n= 10), determined by a colorimeter. – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness.

The scanning UV spectrophotometry showed that all three species, long-spined sea scorpion, short-spined sea scorpion and lumpfish shared a peak at 412 nm. A second peak was located at 668 nm for short-spined sea scorpion and lumpfish, whereas this peak was found at 664 nm for long-spined sea scorpion as shown in Figure 2.8 A. The other species, Atlantic cod, shore rockling and tompot blenny also shared a peak at 412 nm, but no second peak was observed for those species as shown in Figure 2.8 B.

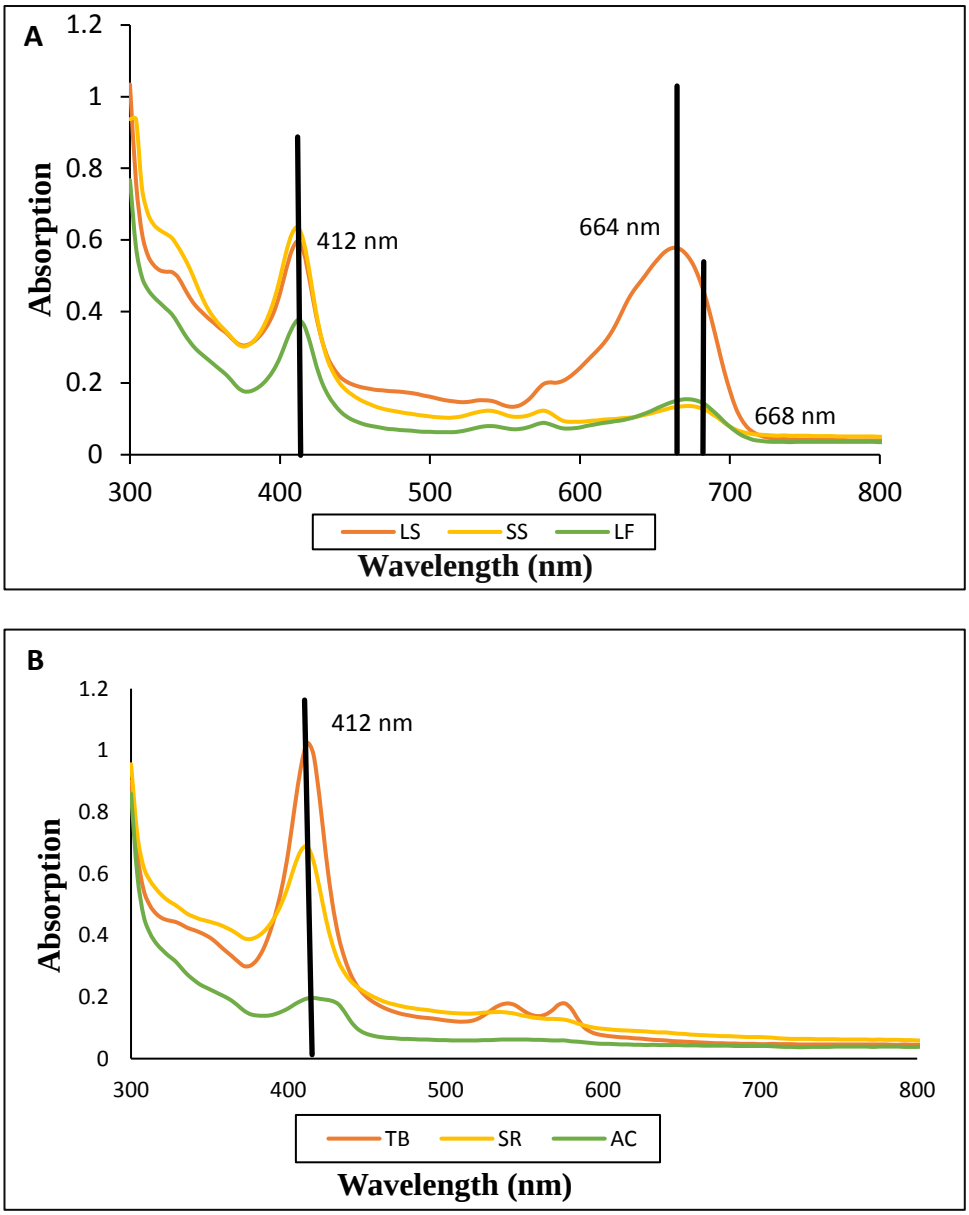


Figure 2. 8: A typical visible absorption spectrum of crude plasma of **A**) short-spined sea scorpion *Myoxocephalus scorpius* (SS) (n= 8), long-spined sea scorpion *Taurulus bubalis* (LS) (n=11) and lumpfish *Cyclopterus lumpus* (LF) (n= 10); **B**) shore rockling *Gaidropsarus mediterraneus* (SR) (n=20), tompot blenny *Parablennius gattorugine* (TB) (n= 28), Atlantic cod *Gadus morhua* (AC) (n=17).

2.4.2 Plasma colouration and UV – (scanning) spectrophotometer of *Labrus bergylta*

The colour of plasma in *L. bergylta* varied between two locations, Carna and Spiddal (Figure 2.9), and between the sampling years 2015 and 2016. Whereas the plasma colour of the majority of samples from Carna 2015 was blue (Figure 2.9 A) with a few plasma samples a more blue-green colour, the plasma colour was mostly green-ish from Carna 2016 and Spiddal for both years as shown in Figure 2.9 B-D. In the latter locations, only a

minority of blue or blue-green plasma samples were seen.

Table 2.3 presents a comparison of reproductive mode (i.e. hermaphrodite or gonochoristic) with existence of blue/green plasma (assumed to be biliverdin) across the sampled coastal fish species. The hypothesis of an association between plasma colouration and hermaphroditic reproductive mode was not upheld because of the presence of biliverdin in several gonochoristic species such as long-spined sea scorpion, short-spined sea scorpion and lumpfish (Table 2.3).

An important trend to note in this context were within-species differences in colour morphotype, which seemed to occur in all species possessing biliverdin. For example in lumpfish (*C. lumpus*), colour polymorphism appeared to be linked with the serum colour: the green body colour showed a green and blue-green serum and the yellowish body showed a purplish colour serum, although this difference was not apparent in UV scanning spectrophotometer readings. Similarly, long-spined sea scorpion (*T. bubalis*) morphs varied somewhat in external colour, as shown in Figure 2.6, which may be associated with sexual dimorphism. However, it was noted difficult to reliably determine sex of long-spined sea scorpion: males in breeding condition have redder colouration on their abdomen region (Anonymous, 2012).

Table 2. 3: A comparison of reproductive strategies and the presence of biliverdin in Irish coastal fish species.

Common name	Species name	Sample size	Hermaphrodite	Biliverdin	The references
Ballan wrasse	*Labrus bergylta*	n=149	Yes. Protogyny	Yes*	(Clark et al. 2016)
Long spined sea scorpion	*Taurulus bubalis*	n=11	No. Gonochoristic	Yes*	(King and Fives 1983)
Short spined sea scorpion	*Myoxocephalus scorpius*	n=8	No. Gonochoristic	Yes*	Froese and Pauly 2000)
Lumpfish	*Cyclopterus lumpus*	n=10	No. Gonochoristic	Yes*	Froese and Pauly 2000)
Shore rockling	*Gaidropsarus mediterraneus*	n=20	No. Gonochoristic	No	Froese and Pauly 2000)
Tompot blenny	*Parablennius gattorugine*	n=28	No. Gonochoristic	No	Froese and Pauly 2000)
Atlantic cod	*Gadus morhua*	n=17	No. Gonochoristic	No	Froese and Pauly 2000)

*Species with blue-green pigments (biliverdin) in their plasma.

Absorbance spectra in plasma samples, measured by UV scanning spectrophotometer are shown in Figure 2.10. At Carna, there was usually a peak at 384 nm in samples from both 2015 and 2016, with a second peak at 640-644 nm from Carna 2015, however in 2016 this peak shifted to 668 nm in some samples. Another peak observed in samples at Carna in 2015 and 2016 was around 412 nm. Figure 2.11 presents the frequency of *L. bergylta* morphotype presenting plasma with absorbance peaks at different wavelengths at Carna in 2015 and 2016. The highest frequency at Carna 2015 was of wavelengths 384 and 644 nm and these wavelengths applied to all morphotypes. The intermediate spotted merle and plain morphs were represented at 384 nm, 644 nm, and 668 nm peaks. The 412 nm peak was only seen in a negligible number of intermediate and spotted morphs (Figure 2.11 A). By contrast, most frequent at Carna in 2016 were wavelengths of 384 and 668 nm. These peaks applied to all morphotypes (however note that the plain morphotype wasn't available at Carna in 2016). Also, this time, the spotted morphotype didn't display any peak at 644 nm. Furthermore, the 412 nm peak applied to spotted, intermediate and merle morphotypes (Figure 2.11 B) and it was not restricted to a negligible number of individuals as seen in 2015.

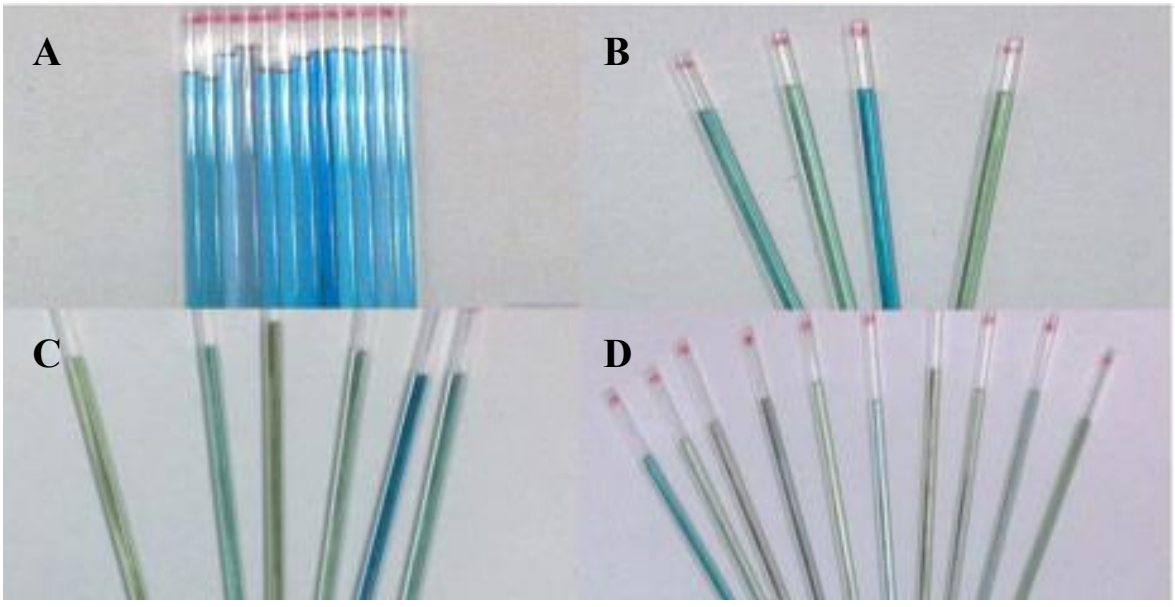


Figure 2. 9: Examples of the plasma colouration of ballan wrasse (*Labrus bergylta*) from two locations: (**A**) blue coloured plasma from Carna 2015 (n= 57); (**B**) green and blue-green coloured plasma from Carna 2016 (n=21); (**C**) green and blue-green coloured plasma from Spiddal 2015 (n=37) and (**D**) green and blue-green coloured plasma from Spiddal 2016 (n=34).

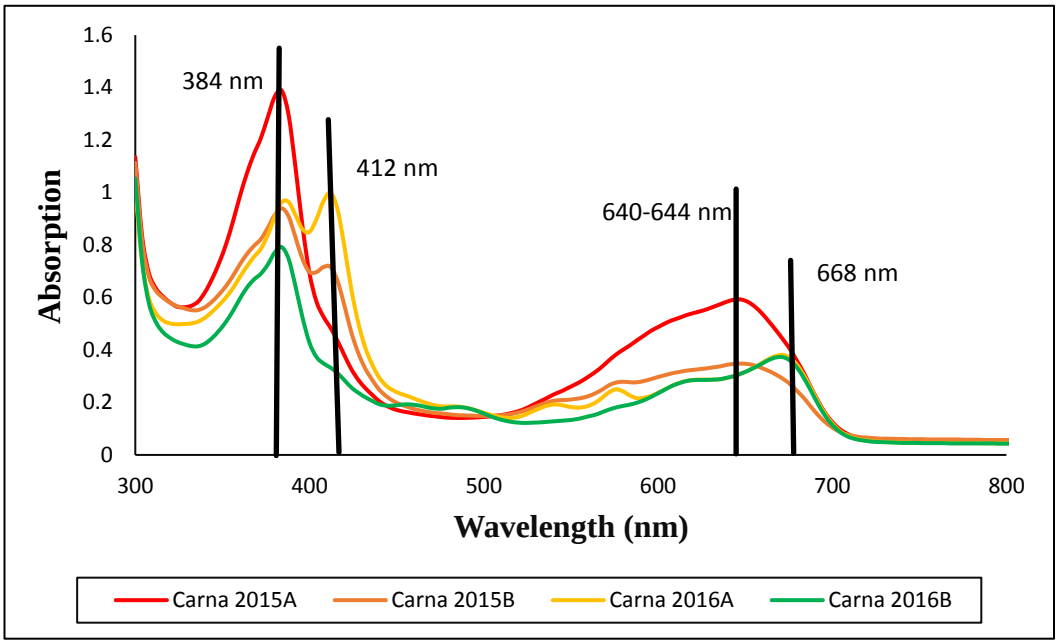


Figure 2. 10: A typical visible absorption spectrum of crude plasma of ballan wrasse (*Labrus bergylta*) for 2015 (n=57) and 2016 (n=21) at Carna as measured by scanning spectrophotometer, (|) peaks maximum Two patterns A and B were seen in that sample in both years.

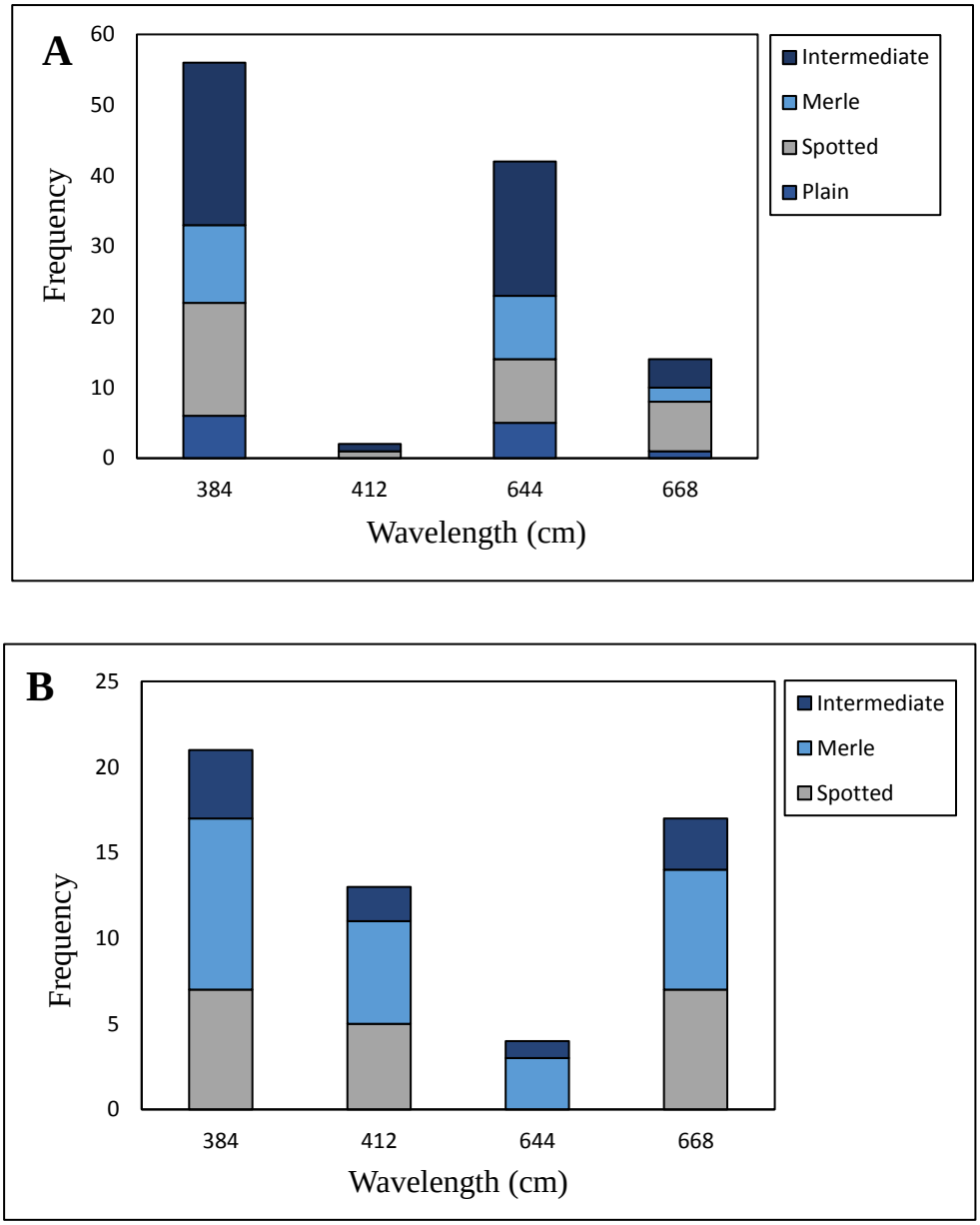


Figure 2. 11: The frequency of the absorbance (wavelength as measured by scanning spectrophotometer) between the morphotypes of ballan wrasse (*Labrus bergylta*) crude plasma at Carna for **A)** 2015 (n= 57) and **B)** 2016 (n= 21).

A typical scanning spectrograph in *L. bergylta* from Spiddal had a peak at 384 nm for 2015 and 2016, and a second peak at 668 nm for 2015 and 2016, but in two individuals' samples, the second peak shifted to 640-664 nm in 2015 and 2016 (Figure 2.12). Like Carna, another peak was observed at around 412 nm in samples from Spiddal in both 2015 and 2016. Figure 2.13 represents the frequency of *L. bergylta* individuals presenting plasma with absorbance peaks of different wavelengths according to morphotype at Spiddal 2015 (Figure 2.13 A) and at Spiddal 2016 (Figure 2.13 B). This time, highest frequencies were seen in both wavelength 384 nm and 668 nm and this applied to all

morphotypes. All of the morphotypes also possessed a peak at 412 nm, apart from intermediate in Spiddal 2015, and spotted in Spiddal 2016. All morphotypes apart from intermediate and plain in 2015 and intermediate and spotted in 2016 lacked a peak at 644 nm (both years). Overall, the results at Spiddal 2015 and 2016 were similar to Carna in 2016 but different from Carna in 2015 due to the high frequency of the 644 nm peak across different morphs at Carna in 2015. Also, the 412 nm peak was less frequent at Carna in 2015 than seen at Spiddal (both years) or Carna 2016.

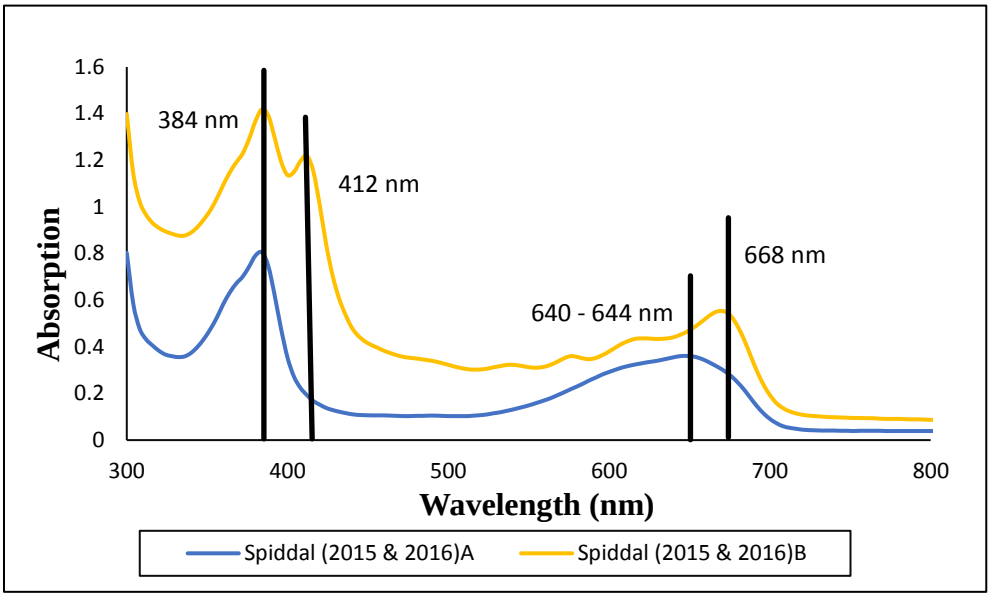


Figure 2. 12: A typical visible absorption spectrum of crude plasma of ballan wrasse (*Labrus bergylta*) for 2015 (n=37) and 2016 (n=34) at Spiddal as measured by scanning spectrophotometer**,** (|) peaks maximum Two patterns A and B were seen in that sample in both years together.

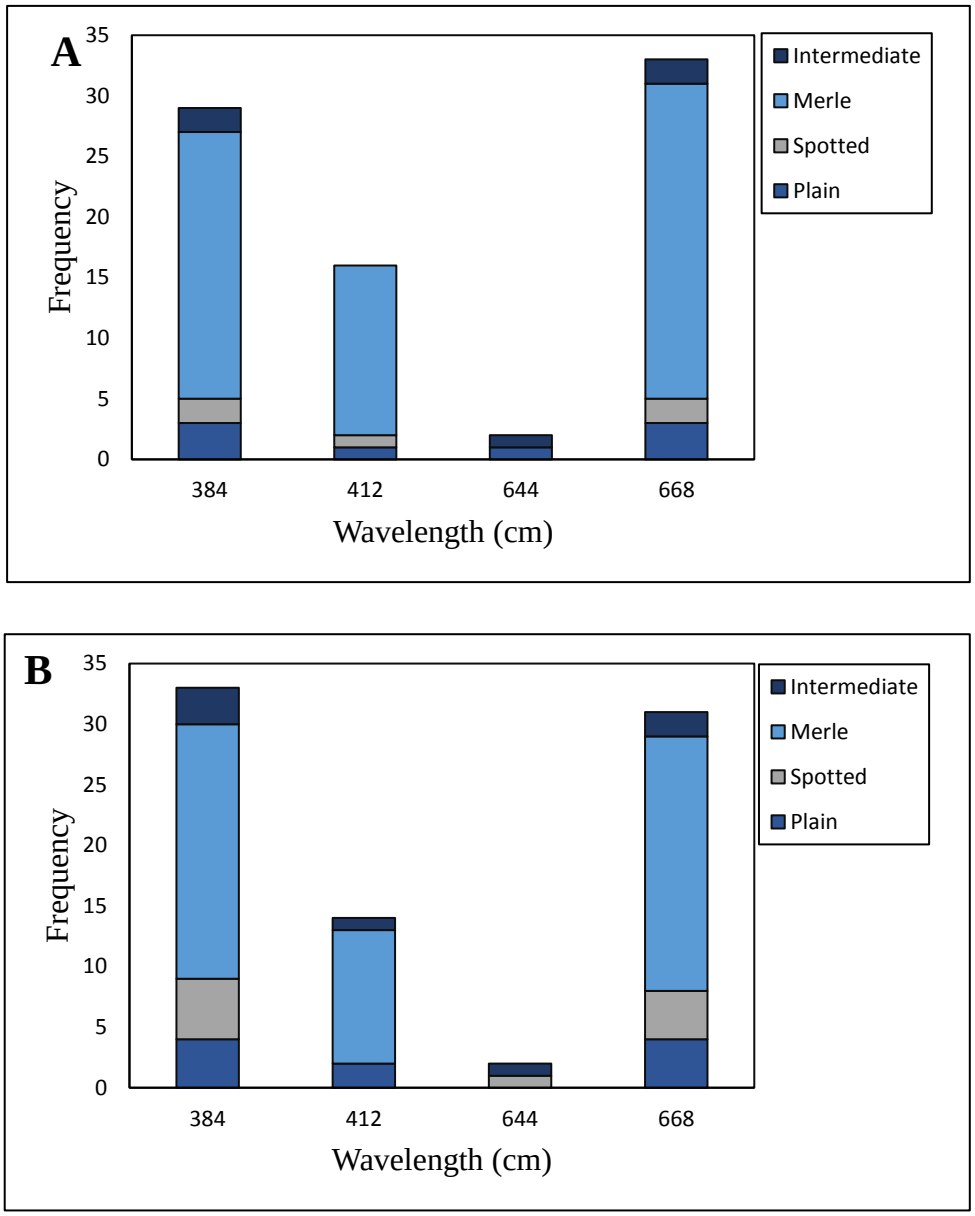


Figure 2. 13: The frequency of the absorbance (wavelength as measured by spectrophotometer) between the morphotypes of ballan wrasse (*Labrus bergylta*) crude plasma at Spiddal for **A)** 2015 (n=37) and **B)** 2016 (n=34).

Colorimeter readings of plasma are shown in Figure 2.14. Figure 2.14 A compares between years at Carna and shows that these were somewhat separate at this location. Mean values of plasma colour from Carna 2015 versus Carna 2016 were first compared using T-tests. Prior to this, normality and homogeneity of variance testing was performed, which showed that the L^* values were borderline/normally distributed with homogenous variances in Carna 2015 (Shapiro-Wilk = 0.983; $d.f$ = 56; P < 0.602) and Carna 2016 (Shapiro-Wilk =

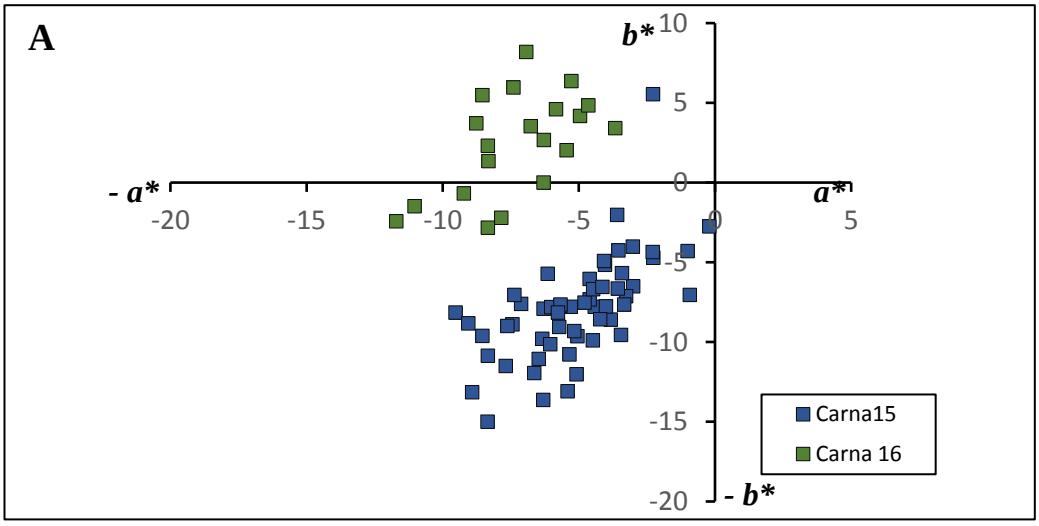
0.909; *d.f* = 19; *P* < 0.071). Similarly, tested was the *a** values in Carna 2015 (Shapiro-Wilk = 0.988; *d.f* = 56; *P* < 0.840) and Carna 2016 (Shapiro-Wilk = 0.973; *d.f* = 19; *P* < 0.831) and *b** values in Carna 2015 (Shapiro-Wilk = 0.980; *d.f* =56; *P* < 0.601) and Carna 2016 (Shapiro-Wilk = 0.947; *d.f* =19; *P* < 0.357). There were significant differences between both *a** values in plasma colour from Carna 2015 and Carna 2016 (*P* > 0.000, *df* = 74, -*a**= -5.09 ± 0.28 and -7.28 ± 0.46 respectively) and in *b** values in Carna 2015 and Carna 2016 (*P* > 0.000, *df* = 74, -*b**= -7.87 ± 0.43 and 2.57 ± 0.73) as shown in Table 2.4. The results showed the dominant plasma colour values existed between - *a** and - *b** on the plot in 2015, which denotes more blue, than in 2016. In 2016 the majority of values occupied - *a** and *b** on the plot, denoting green colours, with a few values falling into - *a** and - *b** colour space (Figure 2.14 A). There were differences too in the degree of colouration represented by *L**. There were significant differences in *L** values between Carna 2015 and 2016 (*P* > 0.015, *df* = 74, *L**= 42.32 ± 0.37, and 44.07 ± 0.51), this indicated that the plasma from Carna was darker in 2015 than in 2016.

Table 2. 4: Mean ± standard error *L**, *a**, *b** colorimeter readings of plasma colour of ballan wrasse (*Labrus bergylta*). Higher *L** value is lighter, – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness.

Lab colour space	Carna 2015	Carna 2016	Spiddal 2015	Spiddal 2016
*L**	42.32 ± 0.37	44.07 ± 0.51	43.81 ± 0.40	41.12 ± 0.56
*a**	-5.09 ± 0.28	-7.28 ± 0.46	-3.35 ± 0.40	-7.19 ± 0.49
*b**	-7.87 ± 0.43	2.57 ± 0.73	-0.01 ± 0.50	-0.89 ± 0.91

Colorimeter readings of plasma colour are also shown to compare between years at Spiddal (Table 2.4). Like Carna, mean values of plasma colour from Spiddal 2015 versus Spiddal 2016 were first compared using T-tests. Prior to this, normality and homogeneity of variance testing was performed, which showed that the *L** values were borderline/normally distributed with homogenous variances in Spiddal 2015 (Shapiro-Wilk = 0.957; *d.f* = 33; *P* < 0.214) and Spiddal 2016 (Shapiro-Wilk = 0.983; *d.f* = 33; *P* < 0.858). Similarly, tested was the *a** values in Spiddal 2015 (Shapiro-Wilk = 0.946; *d.f* = 33; *P* < 0.102) and Spiddal 2016 (Shapiro-Wilk = 0.978; *d.f* = 33; *P* < 0.730) and *b** values in Spiddal 2015 (Shapiro-Wilk = 0.966; *d.f* =33; *P* < 0.378) and Spiddal 2016 (Shapiro-Wilk = 0.954; *d.f* =33; *P* <

0.178). There were significant differences between *a** values in plasma colour from Spiddal 2015 and 2016 (*P* > 0.000, *df* = 64, -*a**= -3.35 ± 0.40 and -7.19 ± 0.49 respectively). However, there were not any significant differences between *b** values in plasma colour from Spiddal 2015 and 2016 (*P* < 0.339, *df* = 64, -*b**= -0.01 ± 0.50 and -0.89 ± 0.91) as shown in Table 2.4. There were differences too in the degree of colouration represented by *L**. There were significant differences in *L** values between Spiddal 2015 and 2016 (*P* > 0.000, *df* = 64, *L**= 43.81 ± 0.40 and 41.12 ± 0.56), this indicated that the plasma sampled at Spiddal was lighter in 2015 than in 2016. Overall, at Spiddal, a separation between years in plasma colour was not as clear as at Carna. Still, Spiddal 2015 was generally located to the right of Spiddal 2016 on the *a** axis i.e. further from the green end of this axis. Nevertheless, all values fell inside the green quadrants. Both years were extended across the area ranging from - *b** to *b** which indicates blue-green to yellow-green colours respectively, with more values of blue-green in 2016 but also a greater range of colours of plasma that year, and more consistency in plasma colours in 2015.



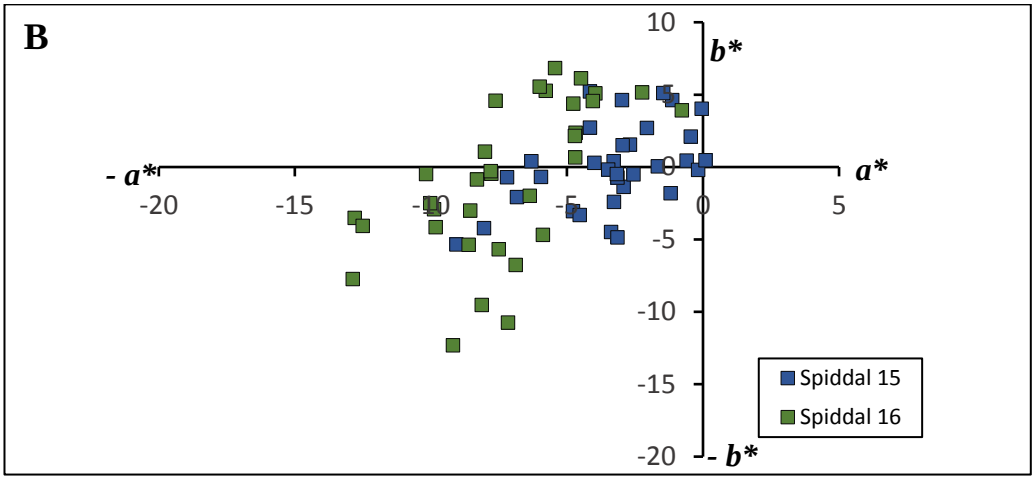


Figure 2. 14: *a**, *b** colour space of crude plasma in ballan wrasse (*Labrus bergylta*) determined by a colorimetry. Plots compare the sampling years at (**A**) Carna 2015 (n=57) and 2016 (n=21) (**B**) Spiddal 2015 (n=37) and 2016 (n=34). – *a**, greenness; *a**, redness; -*b**, blueness; *b**, yellowness.

For completeness, Figure 2.15 compares plasma colour between locations within sampling years. Colorimeter readings of plasma are shown in Figure 2.15. Figure 2.15 A compares between locations in 2015. The normality and homogeneity of variance testing was performed, which showed that the *L** values were normally distributed with homogenous variances in Carna 2015 (Shapiro-Wilk = 0.983; *d.f* = 56; *P* < 0.602) and Spiddal 2015 (Shapiro-Wilk = 0.957; *d.f* = 33; *P* < 0.214). Similarly, tested was the *a** values in Carna 2015 (Shapiro-Wilk = 0.988; *d.f* = 56; *P* < 0.840) and Spiddal 2015 (Shapiro-Wilk = 0.946; *d.f* = 33; *P* < 0.102) and *b** values in Carna 2015 (Shapiro-Wilk = 0.980; *d.f* =56; *P* < 0.601) and Spiddal 2015 (Shapiro-Wilk = 0.966; *d.f* =33; *P* < 0.378). T-tests showed that there were significant differences between *a** values in plasma colour from Carna 2015 and Spiddal 2015 (*P* > 0.001, *df* = 87, - *a**= -5.09 ± 0.28 and -3.35 ± 0.40 respectively) as well as in *b** values in Carna 2015 and Spiddal 2015 (*P* > 0.000, *df* = 87, -*b**= -7.87 ± 0.43 and -0.01 ± 0.50) as shown in Table (2.4). Very few values overlapped across locations as the majority of Carna samples were confined between – *a** and – *b** in the plots, whereas samples at Spiddal extended between both – *a** and – *b** and between – *a** and *b** on the plots. This indicated that plasma colour in 2015 at Carna was blue and blue-green, whereas at Spiddal it was blue-green and yellow-green (Figure 2.15 A). There were differences too in the degree of colouration represented by *L**. There were significant differences in *L**

values between Carna and Spiddal in 2015 ($P > 0.012$, $df = 87$, $L^* = 42.32 \pm 0.37$, and 43.81 ± 0.40), this indicated that the plasma was darker at Carna 2015 than Spiddal 2015. The same comparison of locations in 2016 showed superposition of values at Carna and Spiddal with many of the values tending to be around the $-a^*$ and b^* axis (yellow-green), along with quite a few blue-green values at Spiddal (Figure 2.15 B). Colorimeter readings of plasma are shown in Figure 2.15 B for the comparison between locations in 2016. The normality and homogeneity of variance testing was performed, which showed that the L^* values were normally distributed with homogenous variances in Carna 2016 (Shapiro-Wilk = 0.909; $d.f = 19$; $P < 0.071$) and Spiddal 2016 (Shapiro-Wilk = 0.983; $d.f = 33$; $P < 0.831$). Similarly, tested was the a^* values in Carna 2016 (Shapiro-Wilk = 0.973; $d.f = 19$; $P < 0.840$) and Spiddal 2016 (Shapiro-Wilk = 0.978; $d.f = 33$; $P < 0.730$) and b^* values in Carna 2016 (Shapiro-Wilk = 0.947; $d.f = 19$; $P < 0.357$) and Spiddal 2016 (Shapiro-Wilk = 0.954; $d.f = 33$; $P < 0.378$). T-tests showed that there were not any significant differences between a^* values in plasma colour from Carna 2016 and Spiddal 2016 ($P < 0.909$, $df = 51$, $-a^* = -7.28 \pm 0.46$ and -7.19 ± 0.49 respectively). However, there were significant differences between b^* values in Carna 2016 and Spiddal 2016 ($P > 0.005$, $df = 50$, $-b^* = 2.57 \pm 0.73$ and -0.89 ± 0.91) as shown in Table 2.4. This indicated that plasma colour in both Carna and Spiddal 2016 were green yellow with few blue-green values at Spiddal (Figure 2.15 B). There were also differences in the degree of colouration represented by L^*. There were significant differences in L^* values between Carna and Spiddal in 2016 ($P > 0.001$, $df = 51$, $L^* = 44.07 \pm 0.51$, and 41.12 ± 0.56), this indicated that the plasma from samples at Carna 2016 was lighter than Spiddal 2016.

Table 2. 5: Mean L^*, a^*, b^* colorimeter readings of plasma colour of ballan wrasse (*Labrus bergylta*) for comparing (Carna 2015+2016) and (Spiddal 2015+2016). Mean ± standard error is presented. Higher L^* value is lighter, $-a^*$, greenness; a^*, redness; $-b^*$, blueness; b^*, yellowness. Bold indicates a significant difference.

Lab colour space	**Carna 2015+2016**	**Spiddal 2015+2016**

*L**	42.78 ± 0.32	42.47 ± 0.38
*a**	-5.67 ± 0.26	-5.27 ± 0.39
*b**	**-5.22 ± 0.64**	**-0.45 ± 0.51**

Figure 2.15 C shows a comparison of all plasma colour data for both locations and years. Mean values of plasma colour from Carna 2015+2016 versus Spiddal 2015+2016 were first compared using T-tests. Prior to this, normality and homogeneity of variance testing was performed, which showed that the *L** values were normally distributed with homogenous variances in Carna 2015+2016 (Shapiro-Wilk = 0.993; *d.f* = 75; *P* < 0.945) and Spiddal 2015+2016 (Shapiro-Wilk = 0.984; *d.f* = 66; *P* < 0.553). Similarly, tested was the *a** values in Carna 2015+2016 (Shapiro-Wilk = 0.991; *d.f* = 75; *P* < 0.860) and Spiddal 2015+2016 (Shapiro-Wilk = 0.970; *d.f* = 66; *P* < 0.110) and *b** values in Carna 2015+2016 (Shapiro-Wilk = 0.981; *d.f* =75; *P* < 0.606) and Spiddal 2015+2016 (Shapiro-Wilk = 0.968; *d.f* =66; *P* < 0.084).

There were significant differences only between *b* * values in plasma colour from Carna 2015+2016 and Spiddal 2015+2016 (*P* > 0.000, *df* = 140, - *b* *= -5.22 ± 0.64 and -0.45 ± 0.51 respectively). However, there were not any significant differences between *a* * values in Carna 2015+2016 and Spiddal 2015+2016 (*P* < 0.408, *df* = 140, - *a* *= -5.67 ± 0.26 and -5.27 ± 0.39). There were also no differences in the degree of colouration represented by *L** values between Carna 2015+2016 and Spiddal 2015+2016 (*P* < 0.531, *df* = 140, *L**= 42.78 ± 0.32, and 42.47 ± 0.38) as shown in Table 2.5. These results indicated that Spiddal values tended to be towards the upper (yellow-green) area of the plot compared to the Carna values (bluer area), even though Carna values were also very variable, and included some of the most extreme cases at the blue-green and yellow-green extremes of the *a**, *b** colour space axes.

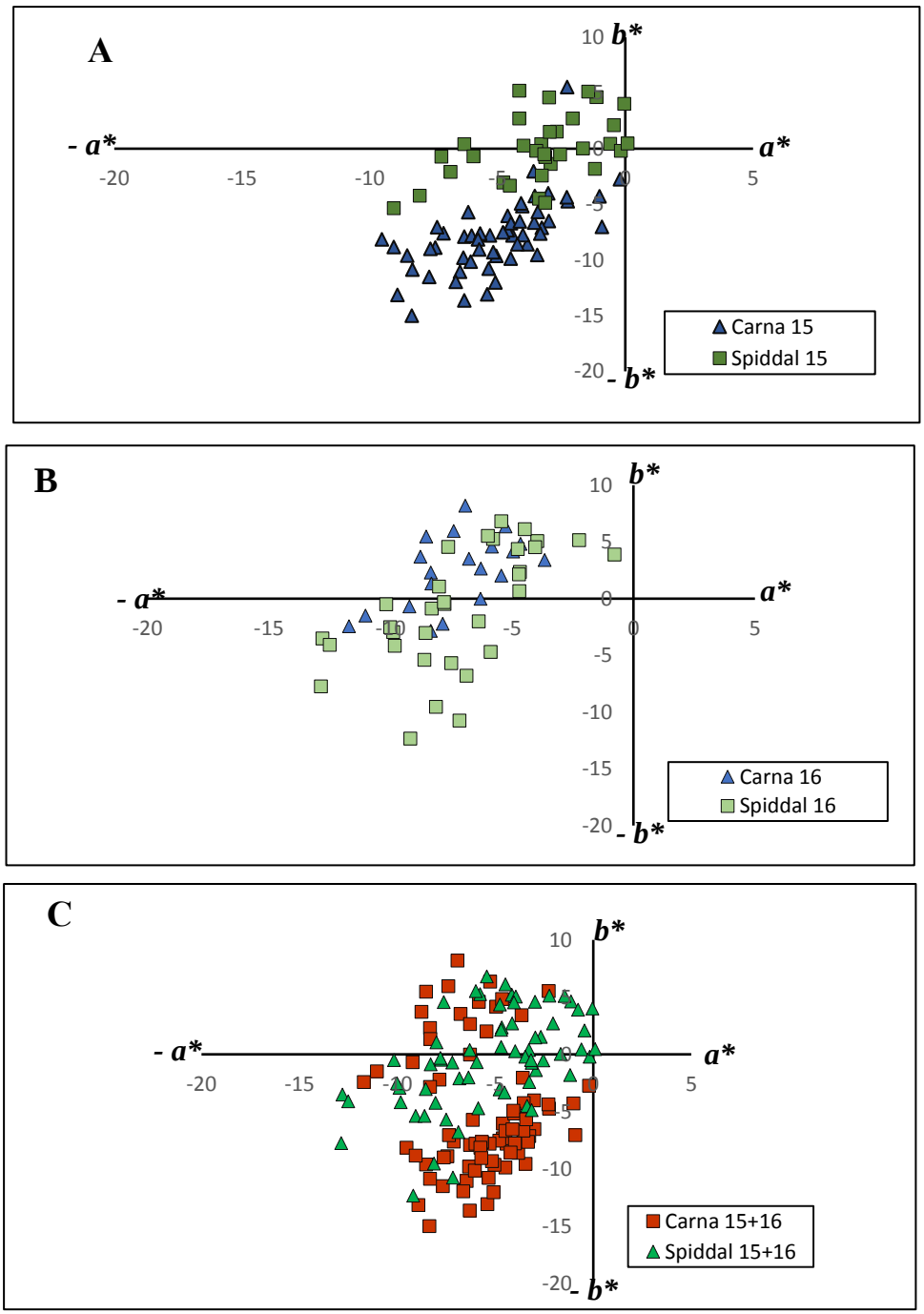


Figure 2. 15: *a**, *b** colour space of crude plasma of ballan wrasse (*Labrus bergylta*) determined by a colorimeter. Plots compare the locations in 2015 (**A**) Carna (n=57), Spiddal (n=37); in 2016 (**B**) Carna (n= 21), Spiddal (n=34); and in 2015+2016 (**C**) Carna (n= 78) Spiddal (n=71). – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness.

2.4.3 Correlation between the variables

A correlation matrix was performed for 14 variables: fish length, fish weight, Fulton's condition factor, haematocrit, haemoglobin, red blood cells, white blood cells, mean corpuscular volume, mean corpuscular haemoglobin concentration, mean corpuscular haemoglobin, conjugated bilirubin, unconjugated bilirubin, total bilirubin and biliverdin concentration. There were some correlated variables (significant at $P < 0.001$), as expected including: length and weight, haemoglobin and mean corpuscular haemoglobin concentration, conjugated bilirubin and total bilirubin, and unconjugated bilirubin versus total bilirubin. Other variables such as biliverdin versus conjugated bilirubin were moderately (0.292) correlated to each other, as was biliverdin to morphological variables length and weight (Table 2.6).

Table 2. 6: Pearson correlation matrix between morphological and haematological parameters in ballan wrasse (*Labrus bergylta*) (n=110). Total length, weight, Fulton's condition factor, haematocrit (Hct), haemoglobin (Hb), red blood cells (RBCs), white blood cells (WBCs), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular haemoglobin (MCH), conjugated bilirubin (CON BR), unconjugated bilirubin (UNCON BR), total bilirubin (TBR) and biliverdin concentration (BV). Any value > 0.25 is significant at 0.001 level. Bold indicates a significant difference

	Length	**Weight**	**K**	**Hct**	**Hb**	**RBCs**	**WBCs**	**MCV**	**MCH**	**MCHC**	**BV**	**CON BR**	**UNCON BR**	**TBR**
Length	1													
Weight	**0.960**	1												
K	**0.244**	**0.395**	1											
Hct	-0.018	-0.056	**0.319**	1										
Hb	-0.021	-0.008	-0.048	-0.078	1									
RBCs	0.103	0.128	0.190	**0.329**	0.159	1								
WBCs	**-0.235**	-0.195	-0.002	-0.014	-0.045	**0.374**	1							
MCV	-0.094	-0.114	-0.041	**0.307**	-0.137	**-0.735**	**-0.368**	1						
MCH	0.071	-0.076	-0.073	-0.160	**0.669**	**-0.476**	-0.195	**0.371**	1					
MCHC	-0.028	-0.038	-0.122	**-0.414**	**0.865**	-0.045	-0.033	**-0.247**	**0.707**	1				
BV	**0.370**	**0.398**	0.177	0.006	-0.053	-0.085	-0.107	-0.108	-0.025	-0.024	1			
CON BR	-0.153	-0.131	-0.015	-0.023	-0.027	-0.209	-0.107	0.197	0.107	-0.015	**0.291**	1		
UNCON BR	-0.190	-0.167	0.123	0.176	0.102	-0.186	-0.087	**0.289**	0.148	-0.024	-0.012	**0.453**	1	
TBR	-0.198	-0.172	-0.073	0.073	-0.034	-0.233	-0.115	**0.278**	0.147	-0.002	0.186	**0.891**	**0.808**	1

2.4.4 Biological and haematology variables across morphotypes

The Kruskal-Wallis test was performed for 14 variables: fish length, fish weight, Fulton's condition factor, haematocrit, haemoglobin, red blood cells, white blood cells, mean corpuscular volume, mean corpuscular haemoglobin concentration, mean corpuscular haemoglobin, conjugated bilirubin, unconjugated bilirubin, total bilirubin and biliverdin concentration. This test was to compare the variables across the morphotypes, which found significant differences in six variables: total length, weight, conjugated bilirubin, unconjugated bilirubin, total bilirubin and biliverdin concentration ($P > 0.000$, $P > 0.000$, $P > 0.001$, $P > 0.044$, $P > 0.011$ and $P > 0.011$ respectively).

Dealing first with size, a Mann-Whitney test was carried out to see differences between the morphotypes. It was shown that only merle was significantly shorter in length than all other morphs ($P < 0.05$). Figure 2.16 A shows that median total length of morphotypes were all similar apart from merle, whose median and even 75% quartile values were less than the 25% quartile for spotted and intermediate morphs. Plain and merle morphs overlapped slightly more, however median total length of merle was still much lower. Outliers of total length are shown below the whisker (25% quartile) of the intermediate morphotype. From this figure, the merle morphotypes had the lowest total length of all morphs and these were different from intermediate morphs (boxes did not overlap) and were also quite distinct from spotted morphs. All other morphs were not significantly different from each other.

Figure 2.16 B shows the boxplots of fish weight. A Mann-Whitney test was performed to see differences between the morphotypes. It was displayed that merle individuals was significantly the lowest of the morphs ($P < 0.05$), and again, this differed (was lower) compared to intermediate and was quite distinct from the spotted morph. Merle morphotypes were also more homogenous with lower variability than other morphotypes. The other morphotypes did not differ substantially in weight – spotted were only slightly heavier than plain morphs. Figure 2.16 C illustrates Fulton's condition factor across morphotypes. From this figure, it was clear that plain individuals had higher variability than the other morphotypes and also had the lowest median condition. However, the median condition factors were quite similar overall and there were likely no differences across morphotypes.

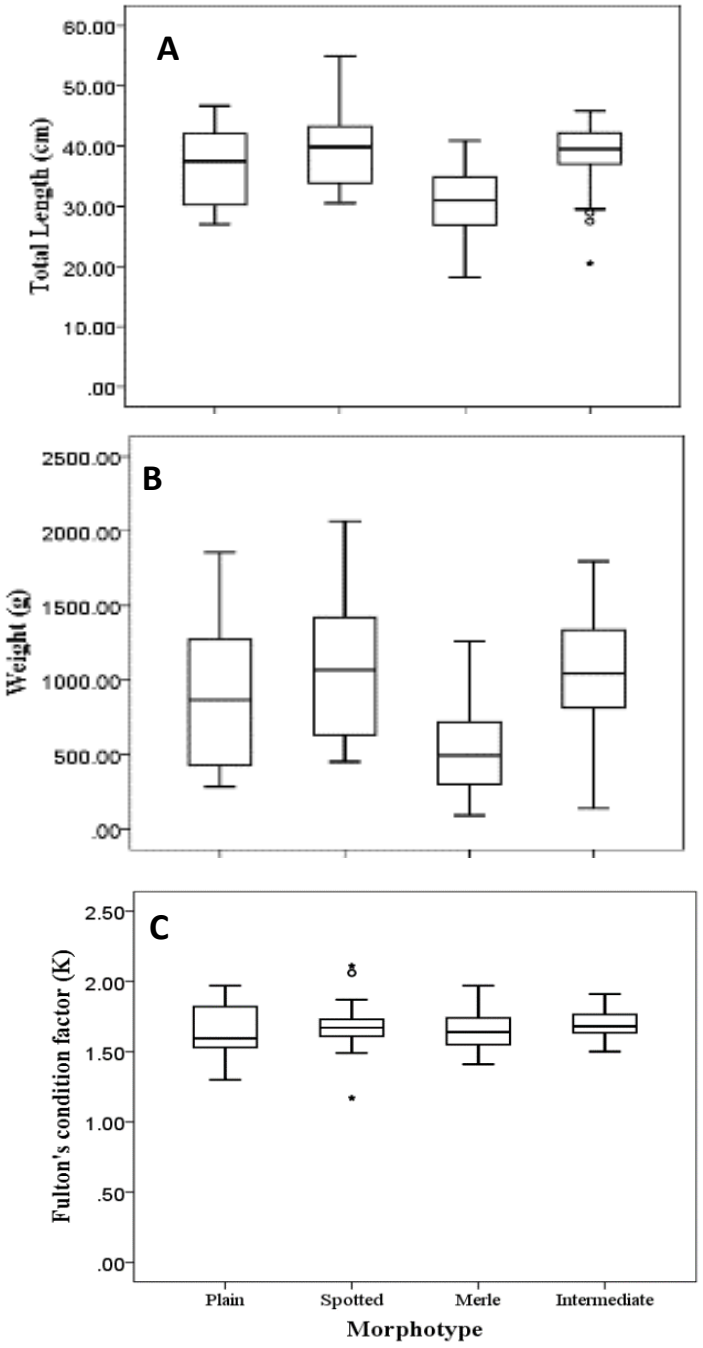


Figure 2. 16: Box and whisker plot for morphotypes of ballan wrasse (*Labrus bergylta*) (n= 110) (A) total length (cm), (B) weight (g) and (C) Fulton's condition factor (k).

Median haematocrit readings were lowest in the intermediate morph whereas spotted fishes had slightly elevated median haematocrit readings than other morphs (and a median above the 50 % percentile), but some outliers were evident (Figure 2.17 A). The merle fish also had a median which was relatively low for this variable. Plain individuals had much higher variability than the other morphotypes. The haemoglobin boxplot showed that morphotypes were nearly identical; boxes were highly overlapping with low variability

across groups, however there were slightly elevated values and elevated outliers in merle and intermediate morphotypes.

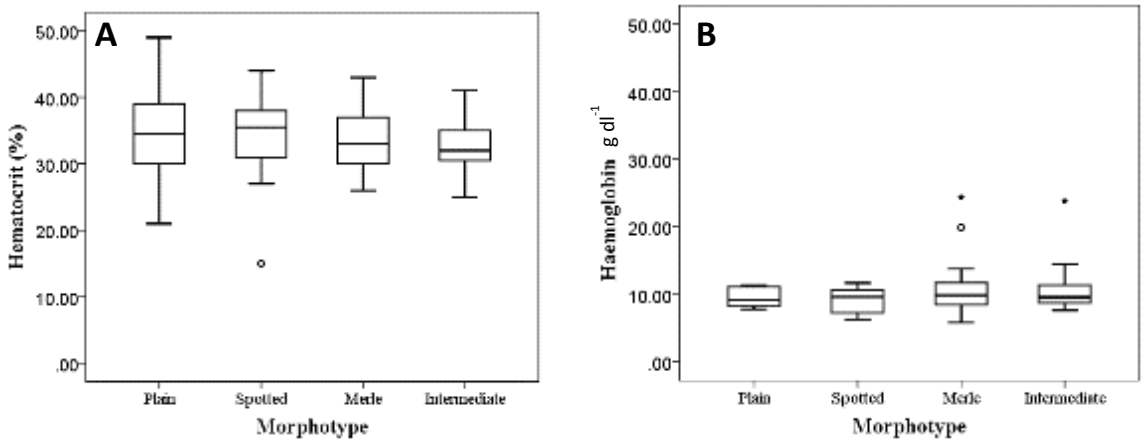


Figure 2. 17: Box and whisker plot for morphotypes of ballan wrasse (*Labrus bergylta*) (n= 110) (A) haematocrit (%) and (B) haemoglobin (g dl^{-1}).

Figure 2.18 illustrates the results of counts of RBCs and WBCs respectively, for different morphotypes. Intermediate morphs had slightly elevated median RBCs compared to the other groups (Figure 2.18 A). Intermediate morphs were also less variable, whereas merle was particularly variable in RBC. Some outliers were apparent in most morphs. Figure 2.18 B shows that WBC had a similar pattern in some respects, like RBCs, the median WBC in intermediate morphs was slightly elevated over other groups, and the medians otherwise followed a similar in trend to the RBCs. However, one difference was that there was a particularly high variability in intermediate morphs for the WBC variable.

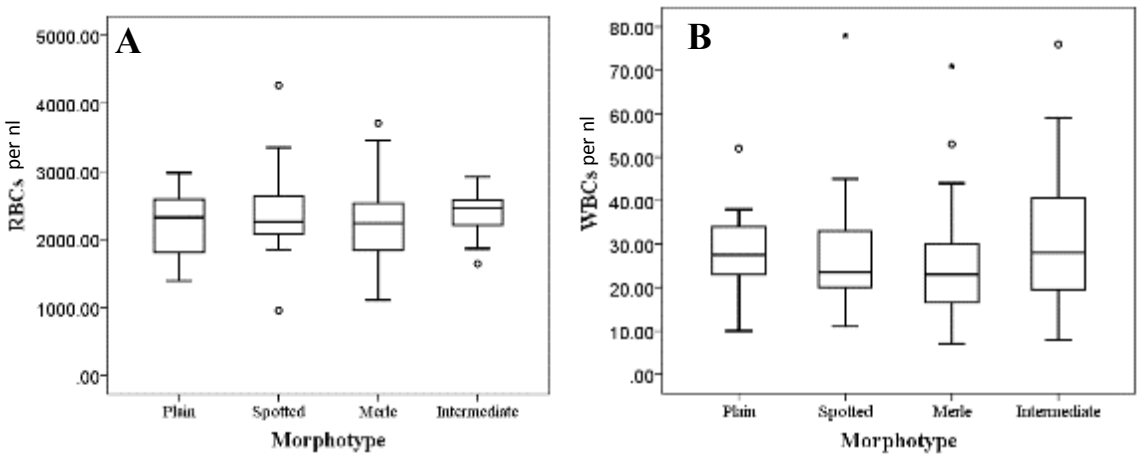


Figure 2. 18: Box and whisker plot for morphotypes of ballan wrasse (*Labrus bergylta*) (n= 110) (A) red blood cells (RBCs) and (B) white blood cells (WBCs).

Figure 2.19 A shows that median of the variable mean corpuscular volume (MCV) was lowest in the intermediate morph and highest in the plain morph, with intermediate levels of median MCV for spotted and merle morphotypes. However, the MCV data was quite variable and there were outliers in all morphs. Mean corpuscular haemoglobin concentration (MCHC) followed the opposite trend from MCV, the median was highest in the intermediate morph and lowest in the plain morph. MCHC was less variable than MCV (Figure 2.19 B). The final haematology index, mean corpuscular haemoglobin (MCH), was quite consistent across morphs and medians varied little across groups (Figure 2.19 C).

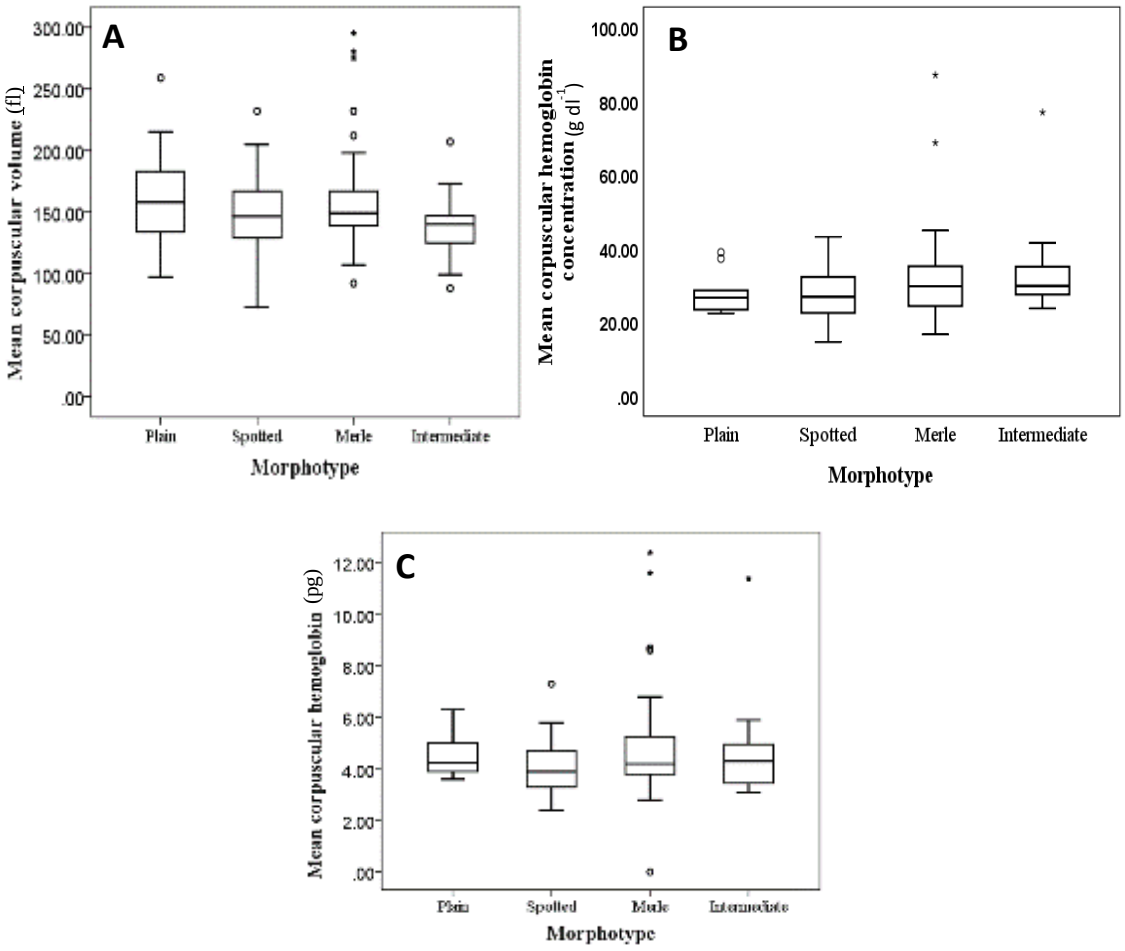


Figure 2. 19: Box and whisker plot for morphotypes of ballan wrasse (*Labrus bergylta*) (n= 110) **(A)** mean corpuscular volume (MCV), **(B)** mean corpuscular haemoglobin concentration (MCHC) and **(C)** mean corpuscular haemoglobin (MCH).

As previously mentioned, the Kruskal-Wallis test was performed these four variables (conjugated bilirubin, unconjugated bilirubin, total bilirubin and biliverdin concentration).

A Mann-Whitney test was carried out to see differences between the morphotypes. It was shown (Figure 2.20 A) that median conjugated bilirubin concentration in plain morphotypes was significantly highest of all groups ($P < 0.05$) and had low variability. In the same plot, the merle individuals had a bit more variability in conjugated bilirubin, including some outliers. However, merle did not show significant differences from spotted and intermediate morphs. Figure 2.20 B shows a similar trend for unconjugated bilirubin, again median values are highest in plain and merle morphotypes, with statistical differences from the spotted and intermediate morphs ($P < 0.05$) although this time the plain morph data is more variable than that of the other morphs. Total bilirubin in Figure 2.20 C is a function of the first two plots so this shows the same trend – the median is clearly significantly higher in plain morphs than the other morphs($P < 0.05$). Figure 2.20 D, on the other hand, illustrates biliverdin concentration across the morphotypes. Again, this time, it was clear that plain, spotted and intermediate individuals had significantly higher median concentrations of this pigment ($P < 0.05$), and merle was lowest. However, variability was quite high in plain and intermediate morphs, and there were outlier values in intermediate, spotted and merle morphs. So overall, the data for these pigments was quite variable over all morphotypes.

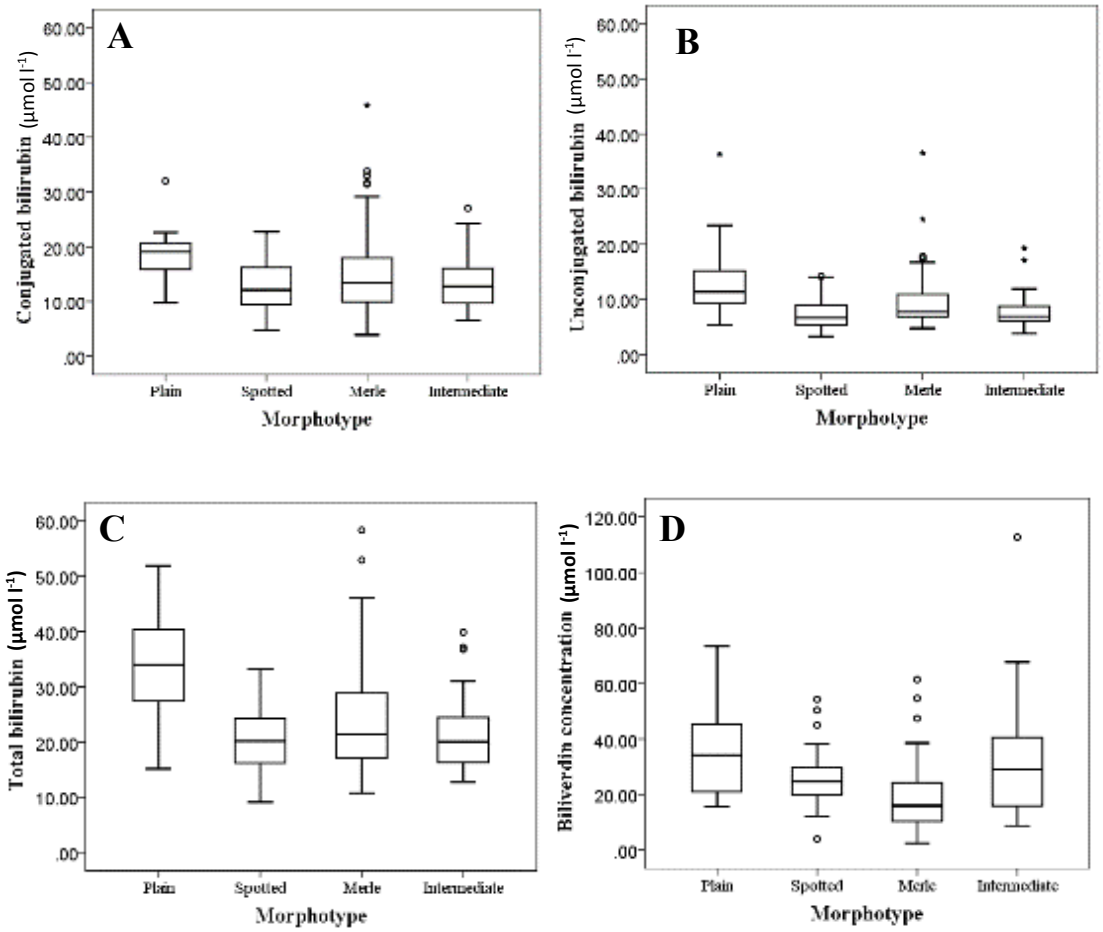


Figure 2. 20: Box and whisker plot for morphotypes of ballan wrasse (*Labrus bergylta*) (n= 110) (A) conjugated bilirubin, (B) unconjugated bilirubin, (C) total bilirubin and (D) biliverdin concentration in plasma (Measured using spectrometric method) of different morphotypes of *L. bergylta*.

2.4.5 Principal component analysis of morphotypes

To visualise the relationship between morphotypes and the measured variables, a principal component analysis (PCA) was carried out. The PCA analysis is not put forward as a predictive model of morphotype from the measured variables but rather it is a representation of how the four morphotypes (plain, spotted, merle and intermediate) are associated with the variables. Principal component analysis (PCA) was carried out using three explanatories variables: total length, biliverdin and total bilirubin concentration. Two dimensional PCA loading plots showed there were relationships among the four morphotypes (Figure 2.21). The three principal components (PCs): PC1, PC2 and PC3 accounted for 45.6 %, 38.4 % and 16.0 % of variance in the data, respectively (Table 2.7). From the plot in Figure 2.21 it is observed that there is some overlap between all the morphotypes with certain individuals of the merle morph separating out particularly in PC1

but also in PC2. The merle group were generally associated with shorter total lengths whereas some of the spotted morphs were associated with larger body lengths. The plain group was associated with higher biliverdin and total bilirubin concentrations. The spotted and intermediate groups showed less clear association in the two-dimensional plot but are generally associated with greater length than the merle group.

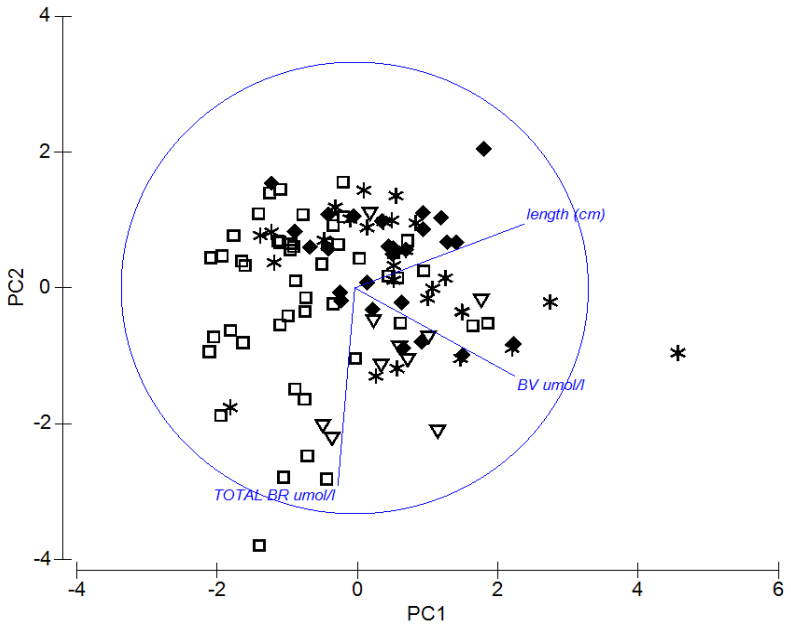


Figure 2. 21: Two-dimensional principal component analysis (PCA) plot of a normalised Euclidean distance matrix of ballan wrasse (*Labrus bergylta*) (n=110) traits symbolised by morphotype. The first two PCs account for 84% of variation in the distance matrix (Table 2.5). Spotted (♦), Plain (Δ), Merle (□) and (*) Intermediate.

Table 2. 7: Principal components analysis results for a normalised Euclidean distance matrix of *Labrus bergylta* traits.

	Eigenvectors	**Eigenvalues**	**% Variation**	**Cum. %Variation**
PC1	0.725 Length + 0.685 BV -0.072 BR	1.37	45.6	45.6
PC2	0.282 Length -.391 BV -.876 BR	1.15	38.4	84
PC3	0.629 Length -.614 BV + .477 BR	0.479	16	100

2.4.6 Three-way PERMANOVA to predict morphotypes

An analysis was performed using Permutational Multivariate Analysis of Variance (PERMANOVA) on Euclidean distances calculated as the distance metric. Three

explanatory variables were included in this analysis: biliverdin concentration, total bilirubin concentration and total length which were compared between four morphotypes (plain, spotted, merle and intermediate). These variables were chosen to show the relationship between the blood pigments and the morphotypes, but also because these showed some of the strongest differences in box and whisker plots, and they were not highly correlated with each other (Table 2.6). A three-way global analysis was carried out to assess the effects of morphotype, location and year on interpoint distance in the distance matrix. As mentioned above, the fixed factor morphotype (n=4) was included in the analysis to test the effect of morphotype on the blood chemistry/size parameters measured. Locations (n=2) and years (n=2) were also fixed factors in the analysis. For all tests, a subset of 999 permutations was used. The Location of *L. bergylta* sampling was not significant in the multivariate analysis, but the Year ($P > 0.007$, $F = 4.639$, $df = 1$) and Morphotype ($P > 0.002$, $F = 3.641$, $df = 3$) were significant, as shown in Table 2.8. The two interaction terms were also not significant (Location × Year, Location × Morphotype and Year × Morphotype) as well as the three-way interaction term (Location × Year × Morphotype) $P > 0.05$. Pairwise tests (Table 2.9) showed which groups of Morphotypes differed from each other in respect of the three explanatory variables. This displayed significant differences between pairwise comparisons of all morphotypes except plain and intermediate which were not significantly different (Table 2.9).

Table 2. 8: Main effects Permutational MANOVA (PERMANOVA) results for three-way analysis of the effects of Morphotype, Location and sampling Year on ballan wrasse (*Labrus bergylta*) traits for three explanatory variables TL, BV, TBR. Number of samples n=110. The three-way interaction matrix Location x Date x Morphotype had some empty cells.

Source of variation	*df*	Sum Squares	Mean Squares varies	Pseudo-F	P(perm)
Location	1	5.109	5.109	1.762	0.15
Year	**1**	**13.452**	**13.452**	**4.639**	**0.007**
Morphotype	**3**	**31.672**	**10.557**	**3.641**	**0.002**
Location * Year	1	4.916	4.916	1.695	0.148
Location * Morphotype	3	17.240	5.746	1.982	0.059
Year * Morphotype	3	14.928	4.976	1.716	0.098
Location * Year* Morphotype	2	7.937	3.968	1.368	0.207
Residual	95	275.430	2.899		
Total	109	436.000			

Pseudo-F F value by permutation, P-values are based on 999 permutations. Bold indicates a significant difference (P <0.05).

Table 2. 9: Pairwise posthoc comparisons for different levels of the factor in ballan wrasse (*Labrus bergylta*) morphotypes following permutational multivariate analysis of variance (PERMANOVA).

Groups	t	P(perm)	Unique perms
Spotted, Plain	**2.624**	**0.001**	**998**
Spotted, Intermediate	**1.935**	**0.021**	**999**
Spotted, Merle	**2.103**	**0.012**	**997**
Plain, Intermediate	1.133	0.281	998
Plain, Merle	**2.145**	**0.008**	**999**
Intermediate, Merle	**1.591**	**0.048**	**998**

Bold indicates a significant difference (P <0.05).

2.4.7 Comparison of morphotypes across years

Since years and morphotypes were both statistically significant in the PERMANOVA (Table 2.8), total lengths and blood pigments were plotted separately by year, to provide some extra information. The results in Table 2.10 showed normality and homogeneity of variance of total length (Shapiro-Wilk = 0.983; $d.f$ = 110; $P < 0.174$), biliverdin concentrations (Shapiro-Wilk = 0.991; $d.f$ = 110; $P < 0.715$) and total bilirubin concentrations (Shapiro-Wilk = 0.986; $d.f$ = 110; $P < 0.304$). However, the log10 transformation was performed prior to normality testing of biliverdin and total bilirubin concentration data. One-way ANOVA analysis was carried out for total length for each year separately which revealed significant differences in total length for 2015 ($P > 0.000$) and 2016 ($P > 0.009$). A Tukey test showed that the merle morphotype in 2015 had a significantly lower total length from all the morphs (mean = 32.44±1.05 cm, $P < 0.05$), while in 2016 the merle morphotype was significantly shorter than the spotted morphotype (mean = 27.93±1.18 cm, $P > 0.006$) (Table 2.11). The total length in Figure 2.22 showed that merle was the smallest morphotype in 2015 and 2016, but also that plain morphs were similar in length to merle in 2016. Spotted morphs were larger than the others morphotypes in 2016 but not in 2015. Overall, there was a consistent difference between merle (smaller) and spotted (larger) morphs in both years, while the relative size of plain and intermediate morphs was similar to each other in both years, but varied up or down in length relative to the other morphs.

Table 2. 10: Normality and homogeneity of variances test for total length, biliverdin concentrations and total bilirubin concentrations of ballan wrasse (*Labrus bergylta*) in both 2015 and 2016.

Variables	Shapiro-Wilk	*d.f*	*P* value	Levene's test	*d.f*	*P* value
Total length	0.983	110	0.174	1.301	7,102	0.257
Biliverdin	0.991	110	0.715	1.214	7,102	0.328
Total bilirubin	0.986	110	0.304	0.572	7,102	0.778

Table 2. 11: One-way ANOVA for comparison of the total length across the morphotypes of ballan wrasse (*Labrus bergylta*) in both years 2015 (n= 68), 2016 (n=42).

	Total length 2015					**Total length 2016**				
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	1284.233	3	428.078	19.449	**0.000**	381.731	3	127.244	4.394	**0.009**
Within Groups	1408.670	64	22.010			1100.394	38	28.958		
Total	2692.903	67				1482.125	41			

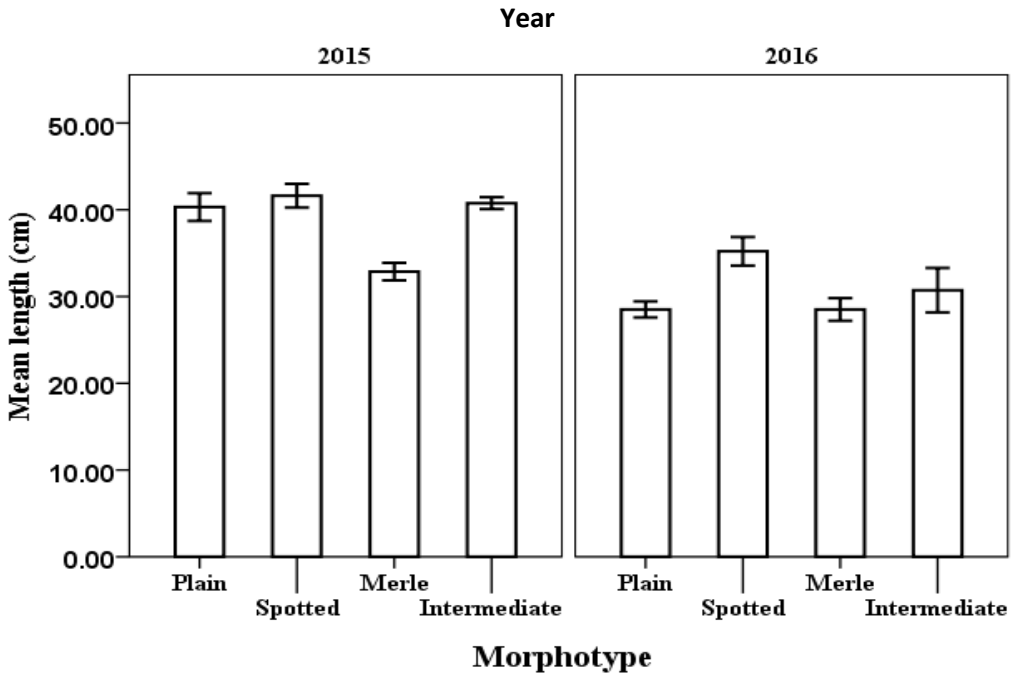


Figure 2. 22: Mean±SE total length across morphotypes of ballan wrasse (*Labrus bergylta*) in both years 2015 (n= 68), 2016 (n=42).

ANOVA showed significant differences in total bilirubin concentrations for 2015 ($P > 0.007$). The post-hoc comparisons showed that the plain morphotype in 2015 was significantly different from the intermediate morphotype in total bilirubin concentrations (mean = 1.50±0.06, $P > 0.037$). However, the same comparisons in 2016 did not show any significant differences ($P > 0.08$) (Table 2.12). Total bilirubin concentration varied somewhat between morphotypes as shown in Figure 2.23, this was highest of all in plain

morphs in both years. On the other hand, merle individuals were inconsistent in bilirubin concentrations between years, being higher in 2015 and lower in 2016. Spotted and intermediate morphs were very similar to each other in bilirubin concentration in both years. One-way ANOVA showed significant differences in biliverdin concentrations across morphotypes for 2015 ($P > 0.025$) and in 2016 ($P > 0.009$). A Tukey test showed that the merle morphotype in 2015 was significantly different from the intermediate morphotype (mean = 1.25±0.05, $P > 0.022$) and in 2016 also merle was significantly different from plain (mean = 1.12±0.05, $P > 0.006$) (Table 2.13). Biliverdin concentration varied a lot across morphs, this was exceptionally high in plain morphs in 2016 only. In 2015, intermediate morphs had the highest biliverdin concentration, followed by spotted and then plain morphs. Merle morphs were consistently low in biliverdin concentration (Figure 2.24).

Table 2. 12: One-way ANOVA for comparison of total bilirubin concentrations across the morphotypes of ballan wrasse (*Labrus bergylta*) in 2015 (n= 68) and 2016 (n=42).

	Total bilirubin 2015					**Total bilirubin 2016**				
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	0.318	3	0.106	4.395	**0.007**	0.182	3	0.061	2.428	0.080
Within Groups	1.546	64	0.024			0.948	38	0.025		
Total	1.864	67				1.130	41			

Table 2. 13: One-way ANOVA for comparison of biliverdin concentrations across the morphotypes of ballan wrasse (*Labrus bergylta*) in 2015 (n= 68) and 2016 (n=42).

	Biliverdin 2015					**Biliverdin 2016**				
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	0.639	3	0.213	3.316	**0.025**	1.019	3	0.340	4.483	**0.009**
Within Groups	4.111	64	0.064			2.878	38	0.076		
Total	4.749	67				3.897	41			

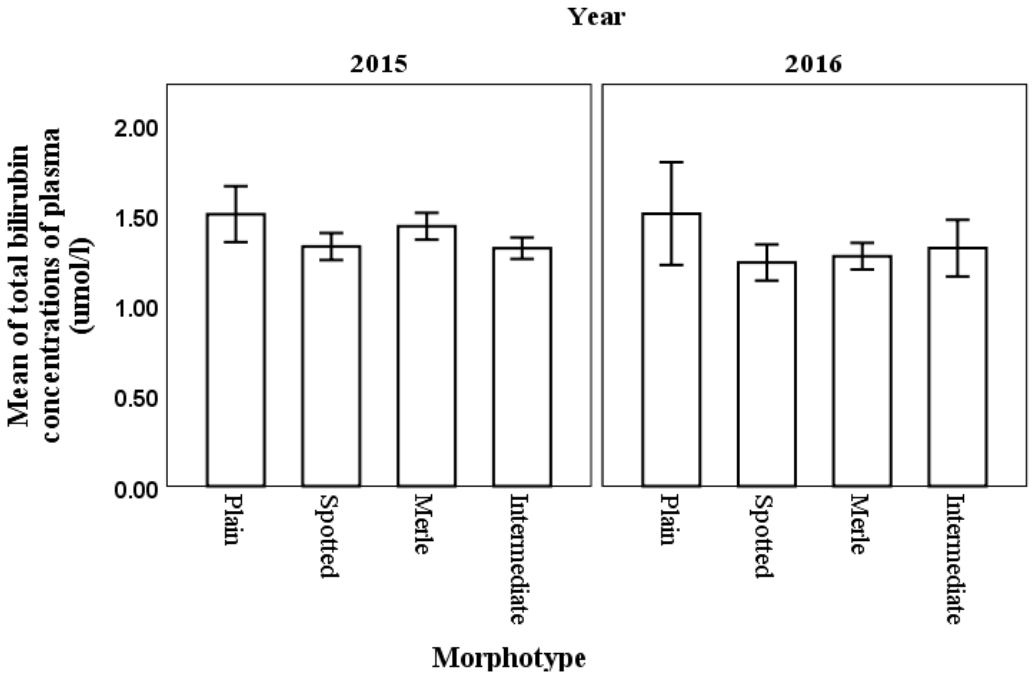


Figure 2. 23: Mean±SE total bilirubin concentrations across morphotypes of ballan wrasse (*Labrus bergylta*) in both years 2015 (n= 68), 2016 (n=42).

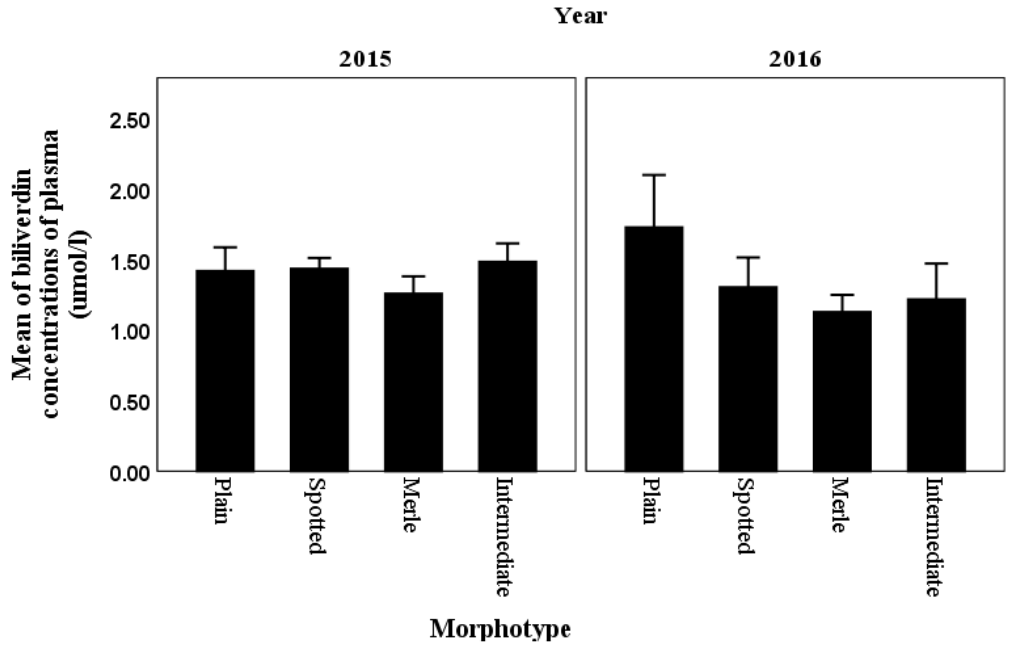


Figure 2. 24: Mean±SE biliverdin concentrations across morphotypes of ballan wrasse (*Labrus bergylta*) in both years 2015 (n= 68), 2016 (n=42).

Moreover, T-test was performed to compare the biliverdin and total bilirubin by locations and years. Prior to this, normality and homogeneity of variance testing was performed on biliverdin concentrations (Shapiro-Wilk = 0.991; *d.f* = 110; *P* < 0.715) and total bilirubin concentrations (Shapiro-Wilk = 0.986; *d.f* = 110; *P* < 0.304) (Note the log10 transformation was performed prior to normality testing on biliverdin and total bilirubin concentration data). There were significant differences in biliverdin concentrations in 2015 between Carna and Spiddal (*P* < 0.036, *df* =68, mean = 1.41± 0.03 and 1.26± 0.04 respectively) and the total bilirubin concentration also showed significant differences in 2015 between Carna and Spiddal (*P* > 0.000, *df* =68, mean = 1.33± 0.01 and 1.50± 0.04 respectively). There were also significant differences in biliverdin concentrations in 2016 between Carna and Spiddal (*P* > 0.041, *df* = 42, mean = 1.09± 0.06 and 1.29± 0.06 respectively) as well as in the total bilirubin concentration in 2016 between Carna and Spiddal (*P* > 0.021, *df* = 42, mean = 1.22± 0.02 and 1.32± 0.03 respectively). The results showed that the highest concentration of biliverdin was in Carna 2015 but in 2016 biliverdin was highest in Spiddal. However, bilirubin showed the highest concentration in Spiddal in both years. Comparisons of pigments in each location showed that bilirubin concentration was higher than biliverdin in both locations and years except Carna in 2015.

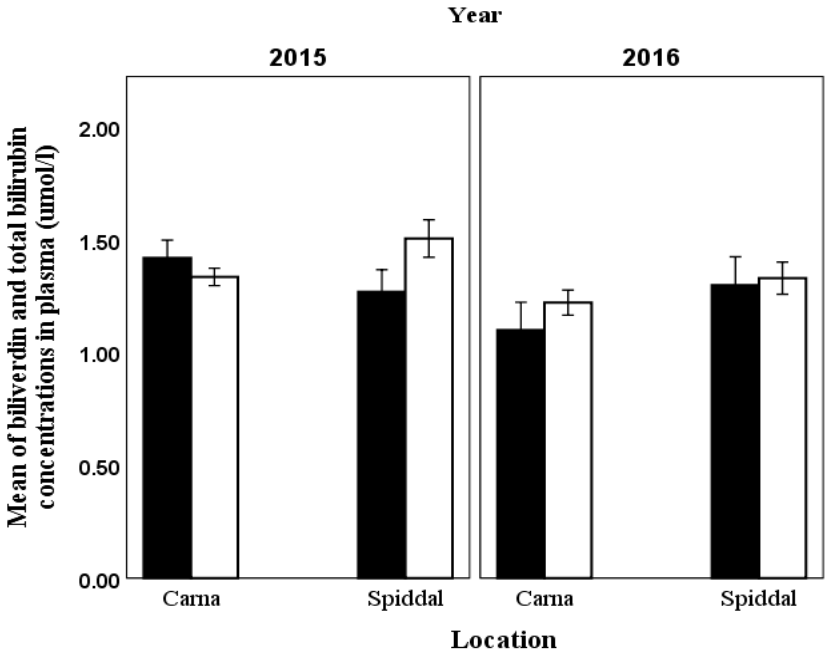


Figure 2. 25: Mean±SE biliverdin (■) and total bilirubin (□) concentrations of ballan wrasse (*Labrus bergylta*) across locations in 2015 (n= 68), 2016 (n=42).

2.4.8 Comparison of biliverdin concentrations in plasma of *L. bergylta* between the sexes

A discriminant score (S_D) was used to determine the sex, calculated as $S_D = 0{\cdot}01 M_B - 0{\cdot}016 L_T - 3{\cdot}835 K + 6{\cdot}252$. Sex is female if S_D is <1·459 or male if S_D is >1·504 (Leclercq et al. 2014). Sex determination of males and females (in the breeding season) was also carried out by dissection. A t-test was carried out to compare male (n=6) and female (n=49) *L. bergylta* with respect to biliverdin concentration in the plasma for both Carna 2016 and Spiddal 2016. Tests for data normality and homogeneity of variance testing were performed. The plasma biliverdin concentration was normally distributed with homogenous variances in both male and female (Shapiro-Wilk males = 0.986; *d.f.* = 5; $P <$ 0.965; Shapiro-Wilk females = 0.958; *d.f.* = 48; $P <$ 0.213). The t-test showed no significant difference ($P <$ 0.776) between males and females in the biliverdin concentration of the plasma for samples from both locations combined in 2016 (Figure 2.26).

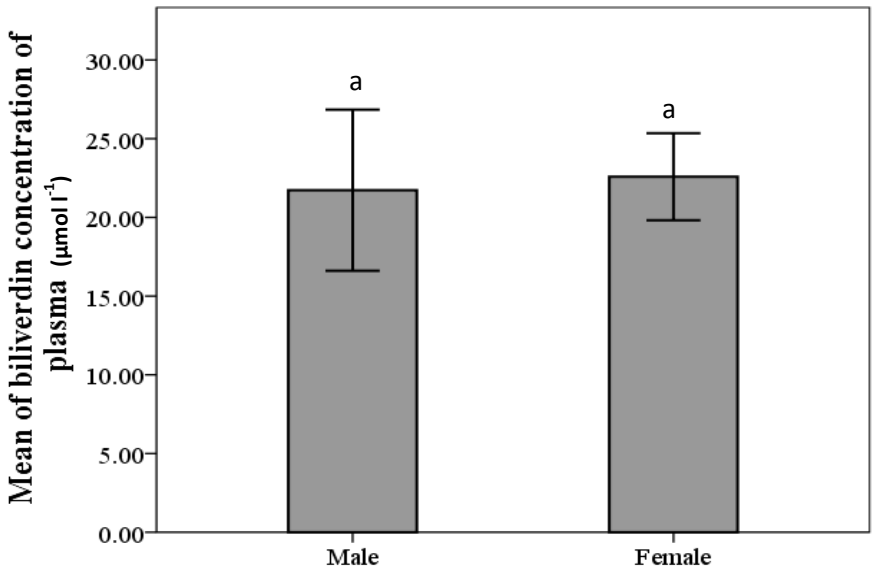


Figure 2. 26: Mean ± SE biliverdin concentration in plasma of ballan wrasse (*Labrus bergylta*) in males and females from both Carna and Spiddal combined in 2016, n = 55 (Male = 6, Female = 49). The letters above the bars show which bars are significantly different to the others (bars with the same letters are not significantly different to each other).

2.4.9 Biliverdin and bilirubin presence in plasma of *L. bergylta* by mass spectrometry (MS)

The spectrophotometer results indicate that pigment absorbance spectra (wavelengths) were consistent with biliverdin; however, many compounds may have a UV max at 640 nm. The use of tandem MS with chemical standards for each pigment allows us to state with 100% certainty that the plasma analysed indeed contains both biliverdin and bilirubin.

Figure 2.27 A shows total ion chromatogram (TIC) of *L. bergylta* plasma from Carna 2015, in ballan wrasse number 66 coded as 'BW66'. Specific retention times for biliverdin (8.583 minutes) and bilirubin (9.086 minutes) were evident in the chromatogram and they were virtually identical to standards for biliverdin (8.596 minutes) and bilirubin (9.091 minutes) (Figure 2.28 A and B). In the Carna sample BW66, the biliverdin peak was obvious, whereas the bilirubin peak was also present but much reduced, possibly because Carna 2015 had high biliverdin and low bilirubin as shown above (Figure 2.25). Figure 2.27 B also shows the tandem mass spectrum (MSMS) of fish from Carna 2015 ballan wrasse number 58 coded as 'BW58' using [M + H] = 583.2578 as the parent ion and collision energy set at 40 Ev, which also was identical to standards for biliverdin (Figure 2.28 C).

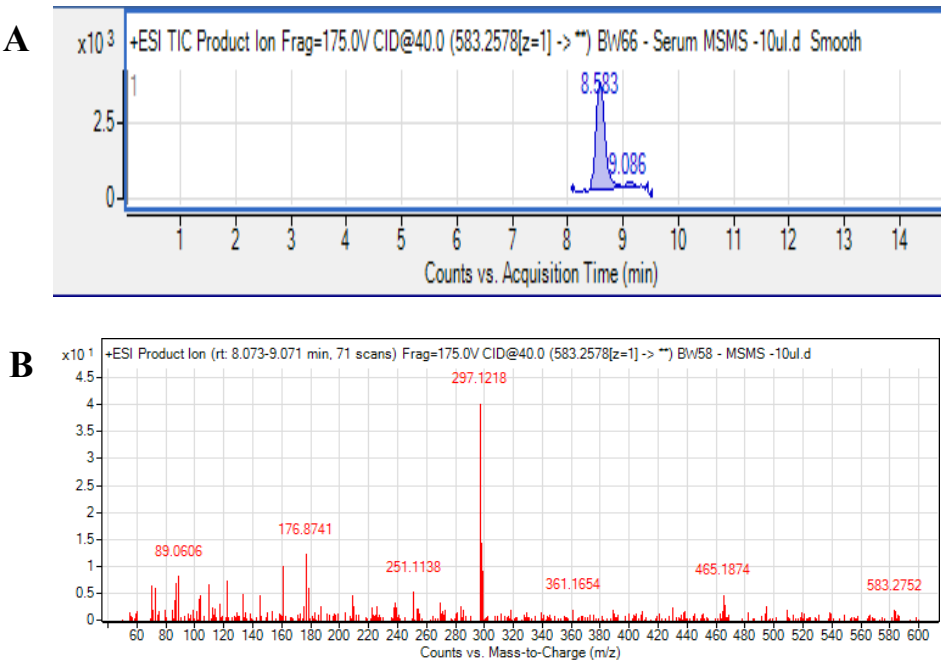


Figure 2. 27: (A) A typical mass spectrum of total ion chromatogram (TIC), counts vs. acquisition times of biliverdin (8.583 minutes) and bilirubin (9.086 minutes) in ballan wrasse (*Labrus bergylta*) plasma. **(B)** The targeted tandem mass spectrum (MSMS) for *L. bergylta* plasma from Carna 2015.

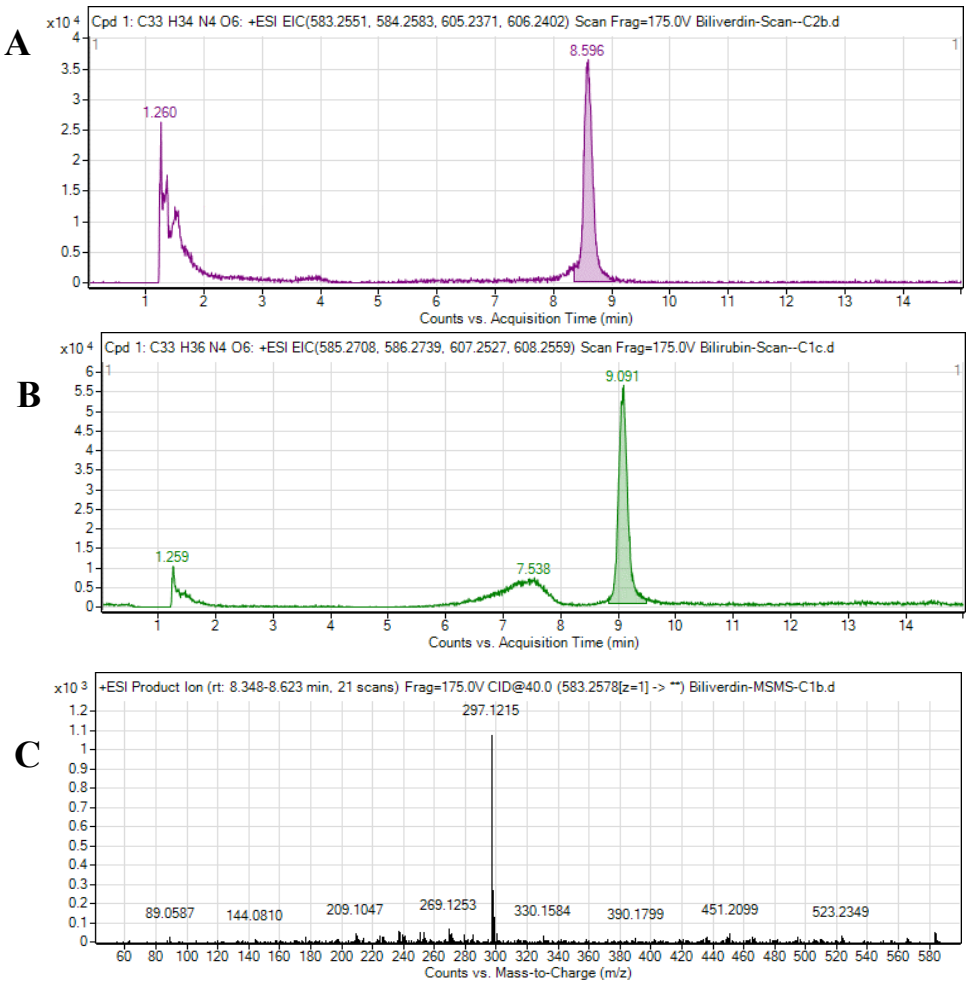


Figure 2. 28: A) Total ion chromatogram for biliverdin standard (TIC), **B)** Total ion chromatogram (TIC) for bilirubin standard, **(C)** The tandem mass spectrum (MSMS, the daughter ions using the parent ion as a precursor) for biliverdin standard.

2.5 Discussion

In the current study, the theme of hermaphroditism was explored by investigating blood pigments in a number of coastal fish, some of which were hermaphrodite and some of which were gonochoristic (=separate sexes). On the basis of Clark et al.'s (2016) hypothesis, it could be expected that fish species with a hermaphroditic reproductive strategy would possess high biliverdin concentrations in blood plasma. And logically, fish species that are gonochoristic would instead have bilirubin in their blood plasma. Instead, in the present study, gonochoristic fish species (lumpfish (*Cyclopterus lumpus*) and short-spined sea scorpion (*Myoxocephalus scorpius*)) possessed blue / green coloured plasma consistent with blood biliverdin, as measured by wavelength (scanning UV) and colorimeter. Furthermore, plasma from long-spined sea scorpion (*Taurulus bubalis*) was also found in the present study to display colouration consistent with biliverdin in the blood, which to the author's knowledge has not previously been reported.

A broad diversity of blood plasma colouration was observed in the coastal fish species surveyed in the present study. It was found that gonochoristic short-spined sea scorpion (*M. scorpius*), long-spined sea scorpion (*T. bubalis*) and lumpfish (*C. lumpus*) had a blue-green to green plasma, while the protogynous hermaphrodite ballan wrasse (*L. bergylta*) possessed similar colouration from blue to blue-green. Further investigation on the blood pigment composition in collected *L. bergylta* samples using scanning UV spectrophotometry and mass spectrometry, showed this colouration was due to the presence of biliverdin. Biliverdin has been reported in other protogynous hermaphrodite species (i.e. *Symphodus melops, Labrus mixtus*) and was hypothesised to be utilised as a free radical scavenger during the gonad remodelling process when the fish undergoes change in sex. Moreover, in some individuals of *L. bergylta* that were in the process of undergoing sexual conversion, it was noted that plasma biliverdin concentration was remarkably lower than that of gender specific equivalents (males and females), thus some interaction with conversion-linked processes such as tissue rearrangement was proposed (Clark et al. 2016). This finding is not supported by the results from three coastal fish species surveyed in the current study since these fish species possessed observable plasma biliverdin with sufficiently high concentration to deeply colour the plasma, even though they are gonochoristic. The latter was particularly true of lumpfish *C. lumpus* and long-spined sea scorpion *T. bubalis* (Figure 2. 6). It is possible, therefore, to suggest that

biliverdin has other advantages for the coastal fish besides acting as a free radical scavenger during sex change.

The present study showed two different colour morphs of lumpfish (green and yellow) which appeared to be linked with the serum colour; while the green-bodied morphs showed a green and blue-green plasma, the yellowish body morphs had a purplish blood plasma colouration. An earlier study has suggested this to be a developmental (i.e. ontogenetic) strategy: juvenile lumpfish use a cryptic default light green colouration when swimming as countershading, with the dorsal side being darker than the ventral side (Davenport and Bradshaw 1995). Elsewhere, Davenport and Thorsteinsson (1989) remarked that the body colours of wild-caught juvenile male and female lumpfish (*C. lumpus*) in Iceland had a 'quite bright' green or green-yellow body. However in adults, this might be more related to sexual dimorphism: the body colour of mature female lumpfish was blue-green and this was associated with blue-green serum (Mudge and Davenport 1986). Mature males, meanwhile, had a pink-red body colour especially during the breeding season and this was associated with red/magenta serum (Mudge and Davenport 1986). Males have elsewhere been reported to have violet serum colour from brown/red-bodied fish (Haugland et al. 2012). We can conclude, therefore, that lumpfish are sexually di-morphic and the blood plasma pigment profile appears to be related to the body colour. Moreover, the presence of biliverdin and colour polymorphism within the lumpfish species appears to be more relevant than the link between biliverdin and hermaphroditism.

In *L. bergylta*, it appeared that plasma colour as measured by colorimeter *L* a* b** score, differed between years, in particular at one of the sampling locations. At Carna, the 2015 sampling period was distinct from 2016, because the 2016 samples were greener and the 2015 were bluer (Figure 2.14 A). At Spiddal, plasma *L* a* b** scores were green in both years. As shall be discussed below, this was because bile pigment concentrations varied across the morphotypes, locations and years in the present study. Essentially, the blood plasma colouration patterns observed between locations and years was the result of a combination of the different proportions of bilirubin (yellow-orange) and biliverdin (blue-green) levels in plasma. Therefore, *L. bergylta* from Spiddal in 2015 and 2016 and Carna 2016 had high bilirubin (a yellow pigment) and low biliverdin levels (blue) resulting in many of the samples having green blood colouration. For Carna 2015, fish had high

biliverdin and low bilirubin concentrations which gave an overall blue colouration in the blood plasma in that case.

L a* b** score was also linked with wavelength data; the blue plasma was associated with absorbance at 640-644 nm and the green plasma linked with 668 nm. For example, the plasma samples taken from Spiddal in both 2015 and 2016 were greener and the plasma absorbance was at 668 nm. This result corresponded with Abolins' (1961) results on other species of Labridae: where *Crenilabrus melops* males showed green plasma and had three absorption spectra peaks at 383 nm, 614 nm and 668 nm, whereas the blue plasma of male *C. ocellatus* had peak absorbance at wavelengths of 381 nm, 582 nm and 640 nm. There can be slight variations in these wavelengths within and between species. For example, in the present study, long-spined sea scorpion (*T. bubalis*) had an absorbance peak at 664 nm, which was slightly different to that of short-spined sea scorpion (*M. scorpius*), lumpfish (*C. lumpus*) and *L. bergylta* (collected in Spiddal) all of whom had a peak at 668 nm. However, by contrast with all sampled *L. bergylta* in Carna and Spiddal, spectrophotometer spectra for long-spined sea scorpion (*T. bubalis*), short-spined sea scorpion (*M. scorpius*) and lumpfish (*C. lumpus*) did not have a second peak at 380 nm. Instead, another peak was found at 412 nm in all surveyed fish species, which was not generally found in *L. bergylta*, although a few individuals did possess it, however these exceptions were not associated with locations or sample years.

Spectra analysis of blood plasma in *L. bergylta*, *T. bubalis*, *M. scorpius*, *C. lumpus* in the present study, showed similar results with previous reports on other fish species. Pigment spectra results appear to be independent of how samples are processed (e.g. crude plasma versus extracted pigment) or sample source (e.g. blood versus bone). For example, extracted pigment from the bone of the garfish displayed two peaks at 376 and 666 nm (Jüttner et al. 2013), and blue-throated wrasse *Notolabrus tetricus* had peaks between 380-400 nm and 640–665 nm (Gagnon 2006). The absorbance spectrum in mucus from the blue-bodied forms of walleye, *Sander vitreus*, exhibited a peak at 380 nm, along with a wider peak at 640-680 nm, which was identical to commercial biliverdin IXa (Yu et al. 2007). Furthermore, lumpfish (*C. lumpus*) showed slightly different results from those in the present study with absorbance maxima at 406 and 678 nm for crude serum of males and females (Mudge and Davenport 1986). It is possible the slight variation in the spectral peaks between studies could be explained by differences in equipment type used and the methodology in spectra scanning. Previous studies have suggested the variation in the

spectral peaks (e.g from 660 nm in *L. bergylta* (Clark et al. 2016) to 630 nm in *S. vitreus* (Yu et al. 2007) reflects possible structural differences in the carrier protein. Therefore, it is essential that the precise identity of the pigment that is responsible for plasma colouration in *L. bergylta, T. bubalis, M. scorpius* and *C. lumpus* is isolated using appropriate techniques such as UV scanning spectrophotometry, confirming the presence of biliverdin. In addition, these methodologies have shown that pigment spectra observed in plasma can also be seen in other tissues of *L. bergylta,* which will be explored in further detail in Chapter 3.

Previous research classified the morphotypes of *L. bergylta* as plain and spotted and revealed variations between these morphs in growth, body mass at age, relationships between otolith length and body length, and mortality (Villegas-Ríos et al. 2013b), with sexual dimorphism being ruled-out as a link explaining these differences (Dipper and Pullin 1979; Villegas-Ríos et al. 2013c). Different life-history strategies were suggested, with the plain morphotype consuming more in reproduction at the expense of growth versus spotted morphs consuming less in reproduction but growing to a greater extent (Villegas-Ríos 2013a). The present study was the first to report further morphotypes, with four morphs of plain, spotted, merle and intermediate being distinguished. These findings were also mentioned by Hutchison (2014), who found that some *L. bergylta* are plain, while others are "mottled" or "spotted". Also, some *L. bergylta* have along the lateral line, a smaller row of scales in U.K. waters (Hutchison 2014). The results of blood physiology parameter investigations carried out in the present study showed that the four morphotypes didn't show any differences across the haematocrit, haemoglobin, red blood cell, white blood cell and blood indices (MCV, MCH and MCHC). However, they did show differences between the morphotypes in weight, conjugated bilirubin and unconjugated bilirubin. And also, across the three explanatories variables for morphotype, which were total length, biliverdin concentration and total bilirubin concentration. The merle morphotype was the shortest and the lightest, while the spotted and intermediate morphs had the largest body size in agreement with Villegas-Ríos et al. (2013b). The latter study found that spotted morphotypes had a larger mean size (based on fish which were also aged) compared with plain. Total bilirubin was higher in plain morphs compared with the other morphs, followed by merle, where levels were variable across individuals and overlapping with intermediate and spotted morphs. The latter two morphs had the lowest total bilirubin concentrations and were very similar to each other. Meanwhile, biliverdin

concentrations were lowest in merle morphs, were moderate in spotted and highest in plain and intermediate morphs, the latter two morphs were rather variable across years and very similar to each other for concentrations of this pigment.

This study was the first study to measure the bilirubin levels in *L. bergylta*. Clark et al. (2016) confirmed that *L. bergylta* possesses a biliverdin reductase enzyme, therefore is capable of converting biliverdin to bilirubin. This suggests that individual fish may adapt their blood pigment, hence plasma colour, on a continuous basis in response to stimuli which are, as yet, unknown. It has been suggested in the past that the differences in plasma colour from green to blue then red (Abolins 1961; Low and Bada 1974) is probably due to alternate hormonal profiles after the breeding season (Abolins 1961), or as a result of gender dimorphism (in *C. lumpus*) (Mudge and Davenport 1986). Moreover, it has been suggested that blood serum pigmentation from eels (*Anguilla japonica*) varies between individual fish, and according to both biological (e.g. age of the fish and degree of growth) and environmental (e.g. season) conditions (Yamaguchi et al. 1966).

L. bergylta from Carna 2015 had high biliverdin concentrations, which was distinct from Carna in 2016 or from Spiddal in both years. It was first suggested that variability in biliverdin concentrations in *L. bergylta* was related to the geographical origin of sampling by Clark et al. (2016). The authors found that the population sampled from Norway (Bergen) presented lower biliverdin concentrations than UK populations (Shetland, Ardtoe and Machrihanish). Results of the present study clearly show that variability between sampling locations also takes place at smaller spatial scales (i.e. Carna versus Spiddal). But geographic variability pattern does not explain the differences between fish sampled at Carna in 2015 and 2016. It is possible that this temporal difference could be the result of how the fish were collected i.e. sampled using lobster pots in 2015 whereas an angling method was used in 2016. It is possible, for example, that spending time in close proximity to conspecifics inside a lobster pot may have stressed the fish or altered hormonal or cortisol levels. Alternatively, these sampling methods may have sampled spatially slightly different populations with fish in lobster pots coming from slightly deeper waters. D'Arcy et al. (2013) found that the population of *L. bergylta* in the British Isles showed higher levels of genetic diversity than southern Norway, which appear to be the result of historical demographic events. Alternatively, Leclercq et al. (2010) reported that level of UV-light in the water column plays an essential role for the colour shift on the fish's skin and this

could also conceivably explain plasma colour differences from place to place and year to year.

The dietary history of the fish should also not be disregarded as an influencer of blood pigment composition, whether linked with the level of nutrients, or pigments in prey items, or some other factor. Crustaceans (Green 1959) and *Aplysia* sea hare molluscs (Chapman and Fox 1969) have all displayed the ability to store bile pigments from the algae in their food, and cottid fish do feed on crustaceans, therefore it is conceivable that accumulation of bile pigments may be obtained from the food chain. However, there has been no clear link found between diet and plasma colouration in the eight species from the family Cottidae (Cabezon (*Scorpaenichthys marmoratus*), lavender sculpin (*Leiocottus hirundo*), woolly sculpin (*Clinocottus analis*), sea scorpion (*Cottus Scorpius*), red Irish lord (*Hemilepidotus hemilepidotus*), grubby (*Myoxocephalus aenaeus*), short horn sculpin (*Myoxocephalus scorpius*) and Arctic sculpin (*Myoxocephalus scorpioides*) (Low and Bada 1974).

In the present study, we have ascertained that plasma colour variation in the sampled fish, including *L. bergylta*, is the result of the ratio of two pigments, bilirubin and biliverdin present in the blood. The present study also shows some association between morphotypes, mean size (merle were smallest) and blood biliverdin and bilirubin profile. It does not provide answers to why these pigments vary so much between sampling locations or sampling years, but this appears to be linked to morphotype and their associated size in some way (e.g. merle being a small morph and spotted being a rather large morph). However, the fact that there was a bias in sample size, both among morphs and among locations, must also be borne in mind. Further investigation will be needed to understand the physiological role of biliverdin in certain morphs, years and locations in *L. bergylta*, and the adaptive significance for this particular heme breakdown product in both hermaphrodite and gonochoristic coastal fish species.

References

Abolins, L. (1961). The existence of sexual dimorphism of blood plasma in fish of the family Labridae. *Arkiv Fur Zoology* **13**, 541–544.

Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. PRIMER-E, Plymouth, UK.

Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecology*, **26**, 32–46.

Anderson, M.J., 2017. Permutational Multivariate Analysis of Variance (PERMANOVA). Wiley Stats Ref: Statistics Reference Online. DOI: 10.1002/9781118445112.stat0784.

Anonymous, (2012). Fish, Tanks and Ponds A comprehensive guide to fish. *Taurulus bubalis* (Long Spined Sea Scorpion). [online] Available at: http://www.fishtanksandponds.co.uk/profiles/taurulus-bubalis.html> [Accessed 29 August 2018].

Anonymous, (2018). Atlantic cod (*Gadus morhua*). [online] Available at: http://www.mardon.com/atlantic-cod.php [Accessed 16 May 2019].

Anonymous, (n.d.). In manual white blood cell count using haemocytometer the dilution used is. [online] Available at: https://docs.google.com/document/d/17PcKCtHmEpThkUNcWs37HqvTALxFknIbbU59G5i_pnA/edit [Accessed 13 May 2019].

Avilés, A. (2008). Egg colour mimicry in the common cuckoo *Cuculus canorus* as revealed by modelling host retinal function. *Proceedings of the Royal Society B: Biological Sciences B* , **275,** 2345–2352. https://doi.org/10.1098/rspb.2008.0720

Austin, C. C., and Jessing, K. W. (1994). Green-blood pigmentation in lizards. *Comparative Biochemistry and Physiology*, **109A**(3), 619–626. https://doi.org/10.1016/0300-9629(94)90201-1

Bada, J. L. (1970). A blue-green pigment isolated from blood plasma of the Arctic Sculpin (*Myoxocephalus scorpioides*). *Specialia*, **15,** 251–252.

Bain, B. J., Bates, I., and Laffan, M. A. (2016). *Dacie and Lewis Practical Haematology* E-Book. Elsevier Health Sciences.

Burmester, T. (2004). Evolutionary history and diversity of arthropod hemocyanins. *Micron*, **35**(1–2), 121–122. https://doi.org/10.1016/j.micron.2003.10.034

Chapman, D. J., and Fox, D. L. (1969). Bile pigment metabolism in the sea-hare Aplysia. *Journal of Experimental Marine Biology and Ecology*, **4**(1), 71-78.

Clark, W. D. (2016). The isolation, identification and exploration of the biophysiological significance of plasma biliverdin in ballan wrasse (*Labrus bergylta*). PhD thesis. University of Stirling.

Clark, K. R., and Gorley, R. N. (2006). PRIMER v6: user manual. *Plymouth Marine Laboratory, Plymouth, United Kingdom.*

Colleran, E., and Heirwegh, K. P. (1979). Comparative aspects of bile pigment formation and excretion. *Comparative Biochemistry and Physiology Part B*:, **64**(2), 133-139.

Cornelius, C. E. (1991). Bile pigments in fishes: a review. *Veterinary Clinical Pathology*,

20, 106–115.

D'Arcy, J., Mirimin, L., and FitzGerald, R. (2013). Phylogeographic structure of a protogynous hermaphrodite species, the ballan wrasse *Labrus bergylta*, in Ireland, Scotland, and Norway, using mitochondrial DNA sequence data. *ICES Journal of Marine Science*, **70**(3), 685-693.

Davenport, J., and Bradshaw, C. (1995). Observations on skin colour changes in juvenile lumpsuckers. *Journal of Fish Biology*, *47*, 143–154. https://doi.org/10.1111/j.1095-8649.1995.tb01880.x

Davenport, J., and Thorsteinsson, V. (1989). Observations on the colours of lumpsuckers, *Cyclopterus lumpus* L. *Journal of Fish Biology*, **35**(6), 829-838.

Denton, J. E., and Yousef, M. K. (1975). Seasonal changes in hematology of rainbow trout, *Salmo gairdneri*. *Comparative Biochemistry and Physiology Part A*: *Physiology*, **51**(1), 151-153.

Dipper, F. A. (1976). *Reproductive biology of Manx Labridae (Pisces)*. Retrieved from https://ethos.bl.uk/OrderDetails.do?uin=uk.bl.ethos.536228%0A

Dipper, F. A. (1981). The strange sex lives of british wrasse. *New Scientist*, **90**(1253), 444–445.

Dipper, F. A., and Pullin, R. S. V. (1979). Gonochorism and sex-inversion in British Labridae (Pisces). *Journal of Zoology*, **187**(1), 97-112.

French, C. E., Bell, J. M. L., and Ward, F. B. (2008). Diversity and distribution of hemerythrin-like proteins in prokaryotes. *FEMS Microbiology Letters*, **279**(2), 131–145. https://doi.org/10.1111/j.1574-6968.2007.01011.x

Froese, R. and D. Pauly. Editors. 2019. FishBase. World Wide Web electronic publication. www.Fishbase.org, (08/2019)

Gagnon, M. M. (2006). Serum biliverdin as source of colouration upon sexual maturation in male blue-throated wrasse *Notolabrus tetricus*. *Journal of Fish Biology*, *68*, 1879–1882. https://doi.org/10.1111/j.1095-8649.2006.01033.x

Gloag, R., Keller, L., and Langmore, N. E. (2014). Cryptic cuckoo eggs hide from competing cuckoos. *Proceedings of the Royal Society B: Biological Sciences*, **281**, 1–7.

Goodman, M., Moore, G. W., and Matsuda, G. (1975). Darwinian evolution in the genealogy of haemoglobin. *Nature*, **253**, 603–608.

Green, J. (1959). Pigmentation of an ostracod, *Heterocypris incongruens*. *Journal of Experimental Biology*, **36**(3), 575-582.

Haugland, G. T., Jakobsen, R. A., Vestvik, N., Ulven, K., Stokka, L., and Wergeland, H. I. (2012). Phagocytosis and Respiratory Burst Activity in Lumpsucker (*Cyclopterus lumpus* L.) Leucocytes Analysed by Flow Cytometry. *PLoS ONE*, **7,** 1–11. https://doi.org/10.1371/journal.pone.0047909

Htun-Han, M. 1978. The reproductive biology of the dab *Limanda limanada* (L.) in the North Sea: gonadosomatic index, hepatosomatic index and condition factor. *Journal of Fish Biology* **13**(1): 351-377.

Hutchison, S., 2014. An Idiot's Guide To U.K. Wrasse Species. [online] World Sea Fishing. Available at: <https://www.worldseafishing.com/forums/threads/an-idiots-guide-to-u-k-wrasse-species.2357610/> [Accessed 15 June 2017].

Igic, B., Cassey, P., Grim, T., Greenwood, D. R., Moskat, C., Rutila, J., and Hauber, M. E. (2012). A shared chemical basis of avian host–parasite egg colour mimicry. *Proceedings of the Royal Society B: Biological Sciences*, **279**(1731), 1068-1076.

Jamalzadeh, H. R., Keyvan, A., Ghomi, M. R., and Gherardi, F. (2009). Comparison of blood indices in healthy and fungal infected Caspian salmon (*Salmo trutta caspius*). *African Journal of Biotechnology*, **8**(2).

Jansen, T., and Daiber, A. (2012). Direct antioxidant properties of bilirubin and biliverdin. Is there a role for biliverdin reductase?. *Frontiers in Pharmacology*, 3, 30.

Jüttner, F., Stiesch, M., and Ternes, W. (2013). Biliverdin: The blue-green pigment in the bones of the garfish (*Belone belone*) and eelpout (*Zoarces viviparus*). *European Food Research and Technology*, *236*, 943–953. https://doi.org/10.1007/s00217-013-1932-y

Kaur, H., Hughes, M. N., Green, C. J., Naughton, P., Foresti, R., and Motterlini, R. (2003). Interaction of bilirubin and biliverdin with reactive nitrogen species. *FEBS letters*, **543**(1-3), 113-119.

Kennedy, G. Y., and Vevers, H. G. (1973). Eggshell Pigments of the Araucano Fowl. *Comparative Biochemistry and Physiology*, **44,** 11–25.

King, P. A., and Fives, J. M. (1983). Littoral and Benthic Investigations on the West Coast of Ireland : XVI . The Biology of the Long-Spined Sea Scorpion *Taurulus bubalis* (Euphrasen , 1786) in the Galway Bay Area. *Royal Irish Academy*, **83B**, 215–239.

Leclercq, E., Grant, B., Davie, A., and Migaud, H. (2014). Gender distribution, sexual size dimorphism and morphometric sexing in ballan wrasse *Labrus bergylta*. *Journal of Fish Biology*, *84*, 1842–1862. https://doi.org/10.1111/jfb.12402

Leclercq, Eric, Taylor, J. F., and Migaud, H. (2010). Morphological skin colour changes in teleosts. *Fish and Fisheries*, **11,** 159–193. https://doi.org/10.1111/j.1467-2979.2009.00346.x

Low, S., and Bada, L. (1974). Bile pigments in the blood serum of fish from the family cottidae.*Comparative Biochemistry and Physiology* , **47A**, 411–418.

Manwell, C. (1964). *Chemistry, Genetics, and Function of Invertebrate Respiratory Pigments—Configurational Changes and Allosteric Effects*. *Oxygen in the Animal Organism*. PERGAMON PRESS LTD. https://doi.org/10.1016/B978-1-4831-9819-4.50010-8

Matić-Skoko, S., Bojanić Varezić, D., Šiljić, J., Tutman, P., and Pallaoro, A. (2013). The cuckoo wrasse, *Labrus mixtus* (Pisces: Labridae): biological indices for life history and conservation. *Scientia Marina*, **77,** 595–605. https://doi.org/10.3989/scimar.03884.07A

Mudge, M., and Davenport, J. (1986). Serum pigmentation in *Cyclopterus lumpus* L.*Journal of Fish Biology* , **29,** 737–745. https://doi.org/10.1111/j.1095-8649.1986.tb04989.x

Muncaster, S., Norberg, B., and Andersson, E. (2013). Natural sex change in the temperate protogynous Ballan wrasse *Labrus bergylta*. *Journal of Fish Biology*, **82**(6), 1858-1870.

Redshaw, M. R Follett, B. K Lawes, G. J. (1971). Biliverdin: a component of yolk proteins in *Xenopuslaevis*.*International Journal of Biochemistry* , **2,** 80–84.

Rodriguez, Z. B., Perkins, S. L., and Austin, C. C. (2018). Multiple origins of green blood in New Guinea lizards. *Science Advances*, **4**(5), 1–8. https://doi.org/10.1126/sciadv.aao5017

Schetter, A. J., Heegaard, N. H., and Harris, C. C. (2010). Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis*, **31**(1), 37-49.

Stenkamp, R. E. (1994). Dioxygen and Hemerythrin. *Chemical Reviews*, **94**(3), 715–726. https://doi.org/10.1021/cr00027a008

Stocker, R. (2004). Antioxidant activities of bile pigments. *Antioxidants and Redox Signaling*, **6**(5), 841-849.

Talbot, C., Medeiros, M. V., and Davie, A. (2012). In vivo gender determination in captive ballan wrasse (*Labrus bergylta* Ascanius 1767). *Final Report to the Scottish Salmon Producers Organisation*, 54. Retrieved from https://scholar.google.com/scholar?hl=en&as_sdt=0%2C5&q=In+vivo+gender+determination+in+captive+ballan+wrasse+%28Labrus+bergylta&btnG=

Tickner, T. R., and Gutteridge, J. M. C. (1978). A simple colorimetric method for the estimation of plasma biliverdin. *Clinica Chimica Acta*, *85*(2), 125–129. https://doi.org/10.1016/0009-8981(78)90231-0

Villegas-Ríos, D. (2013a). Life history and behaviour of *labrus bergylta* in Galacia. PhD thesis. University of Vago.

Villegas-Ríos, D., Alonso-Fernández, A., Fabeiro, M., Bañón, R., and Saborido-Rey, F. (2013b). Demographic variation between colour patterns in a temperate protogynous hermaphrodite, the ballan wrasse *Labrus bergylta*. *PloS One*, **8**, e71591. https://doi.org/10.1371/journal.pone.0071591

Villegas-Ríos, D., Alonso-Fernández, A., Domínguez-Petit, R., and Saborido-Rey, F. (2013c). Intraspecific variability in reproductive patterns in the temperate hermaphrodite fish, *Labrus bergylta*. *Marine and Freshwater Research*, **64**(12), 1156-1168.

Yamaguchi, K., Hashimoto, K., and Matsuura, F. (1976). Identity of blue pigments obtained from different tissues of the sculpin, *Pseudoblennius percoides* günther. *Comparative Biochemistry and Physiology*, **55B**, 85–87.

Yamaguchi, Katsumi, Hashimoto, K., and Matsuura, F. (1966). Studies on a Blue-Green Serum Pigment of Eel-VI. *Nippon Suisan Gakkaishi*. https://doi.org/10.2331/suisan.39.191

Yu, C.-L., Ferraro, D., Ramaswamy, S., Schmitz, M. H., Schaefer, W. F., and Gibson, D. T. (2007). Purification and properties of Sandercyanin, a blue protein secreted in the mucus of blue forms of walleye, *Sander vitreus*. *Environmental Biology of Fishes*, **82**(51–58). https://doi.org/10.1007/s10641-007-9252-3

Zhao, R., Xu, G., Liu, Z., Li, J., and Yang, N. (2006). A Study on Eggshell Pigmentation : Biliverdin in Blue-Shelled Chickens. *Poultry Science*, **85**(546–549).

Chapter 3: The relationship between ballan wrasse (*Labrus bergylta*) morphotypes and pigmentation in the tissues

Chapter 3: The relationship between ballan wrasse (*Labrus bergylta*) morphotypes and pigmentation in the tissues

3.1 Abstract

Ballan wrasse (*Labrus bergylta*) is well known for its many distinctive, colourful morphs. There is little knowledge about these morphs and no quantification has taken place of the colour of external tissues. This study focused on colouration and bile pigment content of external tissues on dorsal and ventral surfaces (both skin and scales) and also the muscle. This was carried out with the aim to determine these biliverdin concentrations and compare them with plasma biliverdin for the same individuals in order to appreciate whether there is any relationship between circulatory pigments and external tissues. Typically, *L. bergylta* has been divided in other studies into two separate morphotypes; plain and spotted. However, in this study, four different morphotypes were used to classify *L. bergylta*; plain, spotted, merle and intermediate. Another aim was to determine whether the concentration of pigments from external tissues mapped onto different *L. bergylta* morphotypes. *L. bergylta* were collected from two locations, Carna and Spiddal, during 2015 and 2016.

To determine presence of biliverdin, tissue extracts (skin and scales) and raw tissues from *L. bergylta* were examined using colorimetry readings (*L* a* b** scores) and scanning UV spectrophotometry to examine absorbance spectra. Based on colorimeter readings from both tissue extracts and raw tissues, there was an overall trend for the dorsal skin to be in the dark blue-green part of the colour spectrum. Ventral skin tended to be defined by a much lighter blue-green pigmentation, while the colour of raw muscle tissue varied from yellowish to a light greenish. The chemical identity of biliverdin in tissue extracts from skin and scales at Carna and Spiddal was confirmed by scanning spectrophotometer. Absorbance peaks of extracts from skin and scales in the sampled *L. bergylta* were similar to those from crude plasma, and peak wavelengths were consistent with biliverdin. Finally, there was a positive correlation between circulatory pigments (biliverdin) in external tissues and the plasma overall (both locations combined). There was also a moderately positive correlation between skin and scale biliverdin concentrations in both locations. Comparison of biliverdin concentrations between the morphotypes in Carna showed variations in the skin and scales (in 2015 only), where larger morphs (spotted and intermediate) had highest biliverdin concentrations, but the smaller morph (merle) had lowest concentrations. Since biliverdin in the plasma is associated with *L. bergylta* on external tissues and since the latter can in some instances be linked to morphotypes and their associated body sizes, it is suggested that one of the roles of biliverdin may be in

signalling of some sort to conspecifics. The fact that the effect is not consistent at all locations/years suggests biliverdin could alternatively function in providing photoprotection or in camouflage. As the results showed a correlation between biliverdin in the external tissues and the morphotypes, so further research is needed in certain morphs, years and locations, to understand the physiological role of biliverdin. For example, future studies might investigate if biliverdin is more abundant during the breeding season to allow morph recognition during courtship and reproduction.

Keywords: *Labrus bergylta*, morphotypes, biliverdin, bilirubin, skin, scales, spectrophotometer, colorimeter.

3.2 Introduction

The pigments associated with body fluids, exoskeleton and endoskeleton in marine animals can generally be grouped into three classes: (1) carotenoproteins, present in the external skeleton of some crustaceans and starfishes, (2) hemocyanin which was found in shrimp (*Litopenaeus vannamei*) (Zhang et al. 2009) and mud crab (*Scylla serrata*) (Yan et al. 2011), respiratory pigments found in shellfish and molluscs, and (3) other biological pigments (e.g. bilirubin and biliverdin) (Yamaguchi 1971). Biliverdin has been reported in blood serum and/or other tissues of many marine animals' bones, scales and also in the fin rays of garfish (*Belone belone*; Caglar 1945). Biliverdin is also reported from integument, bone, scales and fin rays of *B. belone* (Caglar 1945; Jüttner et al. 2013), in connective tissues and bone of garfish, in muscle of 'Hirosa' or humphead wrasse, *Cheilinus undulatus* (Yamaguchi and Matsuura 1969) and in plasma of *Labrus bergylta* (Abolins 1961; Clark et al. 2016). Another example can be seen in sculpin, *Myoxocephalus scorpiodes*, where the isoelectric point and absorption spectrum of the pigment was consistent with biliverdin, i.e. had a wavelength range between 658-660 nm (Bada 1970). Yamaguchi et al. (1976) described similar pigments that were named 'blue chromo-proteins' in the skin, muscles, digestive tract and eggs of sculpin, *Pseudoblennius percoides*. The presence of this chromophore in several tissues, including both the plasma and skin (Bada 1970) across a range of fish groups provides some clue to its presumed functional significance in fish biology.

Like many wrasse species, *Labrus bergylta* is a protogynous hermaphrodite meaning that all fish are born female and sex change occurs in a subset of individuals after ~ 6 years old. This occurs if a male is removed or when a harem recruits too many females for the male to dominate, then a dominant female (or largest female) may change sex to become a male (Muncaster et al. 2013). Sex change is suggested to take place after reproduction in Spring/early Summer in Norway, with early stages of gonad transition appearing between July and September and late stages of gonadal transition taking place in October and November (Muncaster et al. 2013). During sex change, the female gonad tissue for *L. bergylta* is rapidly changed into its male state, resulting in the creation of many free radicals such as reactive oxygen species that potentially lead to high amounts of oxidative stress (Clark 2016). It has been proposed by Clark (2016) that *L. bergylta* accumulate excess biliverdin in their blood serum, tissue and skin throughout their lives to be used as an antioxidant to combat free radicals and alleviate the damage they cause throughout the sex change process. This was supported by the fact that biliverdin concentrations in the

serum of *L. bergylta* significantly decreased throughout sex change, suggesting that it was being actively consumed in this period; however, there were no important differences in biliverdin concentrations between male and female *L. bergylta* (Clark 2016). Therefore, this hypothesis did not fully explain why concentrations should be similar in both sexes, chiefly why is an antioxidant required by males that have already transitioned? More support for the hypothesis of biliverdin acting as an antioxidant comes from a study on blue-shelled eggs in Korea by Sujiwo et al. (2017). The authors demonstrated that the capacity of blue-shelled eggs to scavenge free radicals (1,1-diphenyl-2-picryl- hydrazyl - DPPH) was significantly higher than that of conventional eggs (40.78 % vs. 35.35 %).

Animal colour morphotypes can be adapted for three purposes: camouflage, communication and physical-physiological functions (e.g. during sex change, as described above) (Ortolani 1999). Alternatively, it has been suggested that biliverdin is stored in the tissues to be used subsequently as a camouflage or in sexual dimorphism (Yamaguchi et al. 1976). Sexual dimorphism in plasma pigmentation is closely associated with corresponding external colourations involved in sexual signalling in Lumpfish *Cyclopterus lumpus*, where males develop a red pigmentation at time of sexual maturity prior to mating (Mudge and Davenport 1986; M. Bolton-Warberg, pers. comm.). Ortolani (1999) studied many morphological characteristics in animals such as dark spots, horizontal stripes and so on, and suggested that spotted, vertically striped and horizontally striped cover evolved for camouflage. Both background colour and animal behaviour are suggested to be important selective agents for the evolution of animal colour morphotypes (Ortolani 1999). For example, the stargazer fish *Genyagnus novaezelandiae* possesses a biliprotein complex in their plasma which may be connected to predator avoidance and acquisition of prey (Brittain and Wells 1989). These fish perform crypsis in the sand to avoid predators or ambush prey and their ability to adapt to the dotted green and brown background of the seafloor is suggested to be an adaptation helped by biliprotein complex (Brittain and Wells 1989). Similarly, when comparing the two forms (blue and yellow) of Walleye *Sander vitreus*, it was remarked that mucal biliverdin called sandercyanin in the blue form of *S. vitreus* (Yu et al. 2008) was produced as camouflage colouration (Paradis and Magnan 2005).

Clark (2016) found that plasma colour in *L. bergylta* varied amongst geographic locations (in another argument against a purely oxidative role in sex change). In that case, the colour morphotype showed a slight gradient effect, where green morphotypes had a higher biliverdin concentration compared with the same size of red morphotypes. For this reason,

besides its role as an antioxidant, the author suggests that pigment has a further role in camouflage (Clark 2016). It was suggested that because biliverdin can apparently be accumulated in the skin, its existence might have the further benefit of helping the fish to camouflage themselves to avoid predation and obtain prey. This author suggested that the sympatric nature of morphotypes found within sites and associated differential distributions across these sites would suggest that phenotypic variations in *L. bergylta* are regional adaptations of crypsis with local 'accents' (Clark 2016).

Diet may also explain geographic variations in fish morphotype so that correlations between bile pigments and colour morphotypes at a location may be due to locally available prey with particular associated pigments. For example, Sherwood and Grabowski (2010) mentioned that diet affects the external body colour as seen in Maine cod (*Gadus morhua*) which appears as red and olive patterns, where the red morphs fed preferentially on benthic invertebrates rich with carotenoid content and the olive morph fed more on shrimp. Prey species such as crustaceans (Green 1959) and *Aplysia californica* molluscs possess the ability to store pigments from algae (Chapman and Fox 1969). However, Low and Bada (1974) found no obvious link between the colour or intensity of pigment and food in family Cottidae. Although the food of cottid fish is not exclusively algae, they also feed on crustaceans, which possibly include pigments derived from algae. Consequently, feeding could not be ruled-out as a possible link to colour pigmentation (Low and Bada 1974). Recent research by Pita and Freire (2017) revealed that the majority of the diet in the stomach of *L. bergylta* from Galicia (NW Spain) was in the group Bivalvia *(*e.g. *Mytilus galloprovincialis),* followed by a broad range of prey such as crustaceans (Isopoda, Brachyura, Amphipoda and Balanidae). Although linked with diverse trophic sources, the plain and spotted morphotypes of *L. bergylta* display some dietary overlap. Both plain and spotted morphs eat the same food, with some differences only in the relative amount of food types consumed by each morphotype. Benthic prey was preferred by plain morphs over the spotted morphs (Pita and Freire 2017).

Finally, sexual selection depends on factors associated with female choice such as preference for very highly adorned males (e.g. the peacock's tail) or alternatively, females can choose stronger males that are able to show force by fighting with another male in a competition (e.g., large body size and antlers in deer) (Ralls and Mesnick 2008). As protogynous hermaphrodites, about one male in approximately ten *L. bergylta* females will undergo sex change. This male of *L. bergylta* will guard a harem of several females within

a patch at spawning, defending this territory until the eggs hatch (Dipper 1981). This indicates that males need to attract females away from competing males (Sjölander et al. 1972; Hillden 1981 a, b) as well as defend a temporary territory. In the breeding season, the male wrasse forms a nest of algae and carries out courtship of females with an excited courtship dance that is apparently stimulated by the colour of the female, the female deposits her eggs in the nest and the male defends them till they hatch (Dipper 1981).

The aim of this study is to investigate colouration of *L. bergylta* skin and scales to determine biliverdin concentrations and compare these with plasma biliverdin for the same individuals. This will be carried out to appreciate whether there is any relationship between circulatory pigments and external tissues. Further, to establish links (or not) between pigment concentration in various tissues and morphotype. Wrasse morphotypes/pigment variation was examined at two sample locations and sampling years to determine how 'stable' any trends may be in time and space. The ultimate aim is to establish whether biliverdin in external tissues is associated with divergence in morphotypes.

3.3 Materials and Methods

3.3.1 Locations and sampling

Due to the highly selective nature of *L. bergylta* regarding its habitat preferences, choosing an appropriate location to sample was important. Rocky shore sites with an abundance of sub-surface cover and moderate wave exposure were selected as possible sampling locations for *L. bergylta*. After an initial pilot fishing study, two locations were selected: A) Mweenish Island, Carna, Connemara, Co. Galway (53°18'01.9"N 9°51'09.6"W) and B) Spiddal Pier, Co. Galway (53°14'24.8"N 9°18'39.9"W) (Chapter 2, Figure 2.1). Specimens were collected during 2015 and 2016 in the summer period from June to October (see the appendix). The samples were collected by two techniques, by local fishermen using lobster pots for the samples from Carna 2015 and using rod and line for samples from Spiddal 2015, 2016 and Carna 2016. Initially, common shrimp, mussel flesh, shore crab and limpet flesh were used as bait, with mussel flesh being the preferred one as it was the easiest to collect and hence the most practical bait to use. The overall sample size was n=100 fish, i.e. n=46 from Carna 2015, n=19 from Carna 2016, n=15 from Spiddal 2015 and n=20 Spiddal 2016.

Fish were euthanised by overdosing in MS222 (tricaine methanesulfonate, Pharmaq, Overhalla, Norway). Blood sampling was carried out immediately after euthanasia at the caudal vein by using heparinised needles and syringes and was decanted into storage vials. Fish were then stored on ice during transportation for later processing in the laboratory. Morphometric measurements of total length (cm) and body weight (g) were taken and individual fish were labelled and photographed in order to assign them to one of four morphotypes (plain, spotted, merle and intermediate). The blood plasma was centrifuged at 13,000 *g* for 15 minutes. The plasma supernatant was then removed and further centrifuged for 2 minutes to remove any remaining blood cells. Plasma samples were then divided into several aliquots within microtubes and stored at -80 °C for later analysis. Sex discrimination into males and females was carried out by dissection (in the breeding season) as well as using the equation of LeClercq et al. (2014), as described in Chapter 2.

3.3.2 Sample preparation and colorimeter readings from raw tissues and tissue extracts

The skin was separated gently from the muscle using a scalpel. Immediately after all muscle tissue was cleaned off the skin, surgical scissors or a scalpel were used to cut and divide the skin from the ventral and dorsal sides of the individual. Thereafter, both skin sections and the muscle were placed on a white block away from direct sunlight. This

process guaranteed that the colorimeter readings would be stable no matter the time of the day. 'Colour' of each tissue was measured using a handheld colorimeter (CR-400, Konica Minolta, Tokyo, Japan). The colouration of each tissue was expressed by the industrial colour standard CIE 1976 *L**, *a**, *b**. The data were plotted with *a**, *b** as the XY axes respectively. –*a** denotes greenness +*a** denotes yellowness –*b** denotes blueness +*b** denotes redness. *L** refers to lighter or darker shades of the colour expressed in *a** and *b**. The plots enabled a graphical illustration comparing the trend in colours of each individual fish, depending on its i) sample location and ii) year. The colour value of each tissue was measured in triplicate for each sample.

3.3.3 Sample preparation (tissue extract and blending)

A small sample of muscle (about 1.5 g) was cut from each specimen and this was placed in a test tube containing 10 mL of distilled water. The two skin sections (i.e. dorsal/ventral, as introduced in Section 2.2 above) were de-scaled by moving a knife along the scales in a direction opposite to their growth. Again, a small piece (about 1.5g) of the de-scaled skin was cut and added to a test tube containing 10 mL of distilled water. The same process was applied to 1.5 g of the scales and all 3 tissues (i.e. muscle, skin (dorsal and ventral) and scales (dorsal and ventral)), were placed in the -80ºC freezer.

Five test tubes at a time were taken from the freezer, covered in tinfoil and kept in a dark cupboard or in the fridge until they reached a semi-thawed condition. Ice was used to fill a beaker with a hole in the centre to hold the test tube to keep the sample cooled and then the sample was positioned under a homogeniser-blender (T 25 Digital ULTRA, Fisher) that had been adjusted to beaker level on a retort stand. The sample was blended with half speed for no more than fifteen seconds. The blades were cleaned before repeating the procedure on the same sample to the point where most of the test tube's product had been liquidized. When adequately liquidized, balanced test tubes were placed in a centrifuge at 2,500 revolutions per minute (rpm) for 4 minutes to separate the solids and liquids within the sample. Liquid was pipetted off and 1.5 ml was added to each of 3 microtubes. All processed samples were then stored at -80 ºC prior to the analysis. 'Colour' of each tissue extract was measured using a handheld colorimeter (CR-400, Konica Minolta, Tokyo, Japan) and 200µl of tissue extracts were pipetted in 96 well plate to for colorimeter and also for further analysis (scanning spectrophotometer). The same protocol of the raw tissue was followed to measure the colour in extract tissue in section 2.3 above. The colour value of each tissue extracts was measured in triplicate for each sample.

3.3.4 Quantifying extracted pigment (skin and scales) biliverdin through the spectrometric method

The spectrometric method of Tickner and Gutteridge (1978) was used to quantify biliverdin concentrations on the extracted skin (dorsal and ventral side), scales (dorsal and ventral side) and muscle samples. This method works by reacting the biliverdin with barbituric acid under alkaline conditions, which produces the specific chromogen (i.e. biliverdin) with peak absorption at 570 nm (Tickner and Gutteridge 1978). Biliverdin standards were prepared from biliverdin chloride. The concentration of the stock solution was 200 μmol/L, which was prepared by dissolving 117 mg biliverdin in 100 ml of 17.5 M glacial acetic acid. The solution was then stirred and heated at 45°C in a dark room for 1 hour to ensure complete dissolution and then the solution was left to cool down at room temperature. Concentrations of the standard ranging from 0-200 μmol/L were prepared by serial dilution with 17.5 M glacial acetic acid, with a final volume for each standard and blank of 2 ml. Approximately 500 μl from each concentration of standard and blank was added to 1 ml distilled water, 400 μl of 40 mM ascorbic acid, and 100 μl of 200 mM barbituric acid in 1 M of NaOH. However, standard blanks included 1 M of NaOH without the barbituric acid. Preparation of skin / scales samples and blank samples were exactly the same as for the standards except, this time, aliquots of 1 ml of skin or scales extract were added to 500 μl glacial acetic acid, 400 μl of 40 mM ascorbic acid, and 100 μl of 200 mM barbituric acid in 1 M of NaOH. All standards and samples with their corresponding blanks were covered with tin foil and incubated at 95 °C on a block heater in a dark room for five minutes. After allowing samples to cool, 1.25 ml n-butanol and 5 ml 2 M sodium hydroxide were added and vortexed followed by centrifugation for 10 minutes at 2500 *g*. After settling, two layers developed, the top layer was discarded, and the desired bottom pinkish-layer was extracted. For two replicates of each standard and sample, absorbance at 570 nm was measured on a spectrophotometer (Genesys™ 10, Thermo Fisher Scientific, Waltham, Massachusetts, United States). Biliverdin concentration extracted for the tissue extracts of ventral skin, ventral scales and muscle were not analysed using this method due to the biliverdin in these tissues being un-detectable by spectrophotometer.

3.3.5 Scanning spectrophotometer of the extracted pigment from skin and scales

The extracted samples (200 μL) of dorsal skin and scales and ventral skin and scales were pipetted in 96 well plate and analysed for the presence biliverdin using a scanning spectrophotometer (BioTek, Power Wave XS2, Mason technology) at wavelengths from 300 nm-900 nm with an interval of 4 nm.

3.3.6 Statistical analyses

3.3.6.1 Tissue colouration

Colorimetric readings were plotted in Excel, for both the raw samples of skin (dorsal, ventral) and muscle, and for tissue extracts from skin (dorsal, ventral) and scales (dorsal ventral). A one-way ANOVA was performed to compare the difference between the means of colour values *L**, *a** and *b** for raw samples of dorsal skin, ventral skin and muscle of Carna and Spiddal samples after performing normality using a Shapiro-Wilk test and homogeneity of variance using a Levene's test. A Tukey test was used for post-hoc comparisons. To compare tissue extract readings, the normality and homogeneity of variance tests were performed then the T-test was carried out on the differences between the dorsal and ventral skin and dorsal and ventral scales extracts for Carna and a separate analysis was carried out on dorsal and ventral skin and dorsal and ventral scales extracts for Spiddal samples.

3.3.6.2 Morphotype comparisons

Labrus bergylta morphometric data, i.e. median weights and lengths were compared between four Morphotypes ('Plain', 'Spotted', 'Merle' and 'Intermediate') in both Carna and Spiddal. Because these data weren't normally distributed, a Kruskal-Wallis test was performed, and a Mann-Whitney test was used for comparisons of any significant results. Spectrophotometer readings for the concentration of biliverdin in skin and scales tissues between morphotypes were examined for normality using a Shapiro-Wilk test and homogeneity of variance using a Levene's test. To test the hypothesis that the concentration of the biliverdin in the dorsal skin and dorsal scales is different between morphotypes, biliverdin concentration was compared across morphotypes at Carna in 2015 using a one-way ANOVA. This was repeated for Carna 2016. A Tukey test was used for post-hoc comparisons of any significant ANOVA result. A T-test was performed to compare biliverdin concentration between years for each morphotype.

The spectrophotometer readings for the concentration of biliverdin in skin and scales tissues for the merle morphotype at Spiddal (the only morphotype with a sufficient sample size was merle) were examined for normality using a Shapiro-Wilk test (SW) and homogeneity of variance using a Levene's test. A T-test was then performed to compare biliverdin concentrations between years for the merle morphotype at Spiddal. Other morphs were too rare at Spiddal for this analysis.

3.3.6.3 Association between tissues in biliverdin concentrations

The hypothesis that tissues, e.g. dorsal skin and dorsal scales, express biliverdin in a similar way was tested by examining the relationship between the biliverdin concentration of skin and scales using Spearman's correlation. Similarly, biliverdin concentration of dorsal skin and dorsal scales were compared with plasma concentrations, all of which was carried out on both Carna and Spiddal populations.

3.3.6.4 Comparison biliverdin concentrations between males and females

A Mann-Whitney test was used to compare biliverdin concentration for males and females in dorsal skin, dorsal scales, and T-test for plasma for both locations in 2016 only, as the sex of fish was not recorded in 2015.

All statistical analyses were carried out with an alpha significance level of 0.05 using SPSS software (IBM SPSS 23) and the graphs were produced in Excel and SPSS.

3.4 Results

3.4.1 Tissue colouration in raw tissue at Carna

In *L. bergylta*, the colour of raw muscle tissue varied from yellowish to a light greenish colour (Figure 3.1). Dorsal skin was blue in colour and much darker than the ventral skin. Figure 1 displays both examples of raw tissues and extracts from those tissues illustrating that raw muscle tissues (Figure 3.1 A) were relatively pale, and more pigment was evident in raw dorsal and ventral skin tissues (Figure 3.1 B). The same pattern was evident for extracts of tissues; biliverdin extracted from dorsal skin (Figure 3.1 C) was more pigmented than in ventral skin (Figure 3.1 D).

Further data are not shown with regards to biliverdin concentration extracted for the ventral skin, ventral scales and muscle due to this being un-detectable by spectrophotometer.

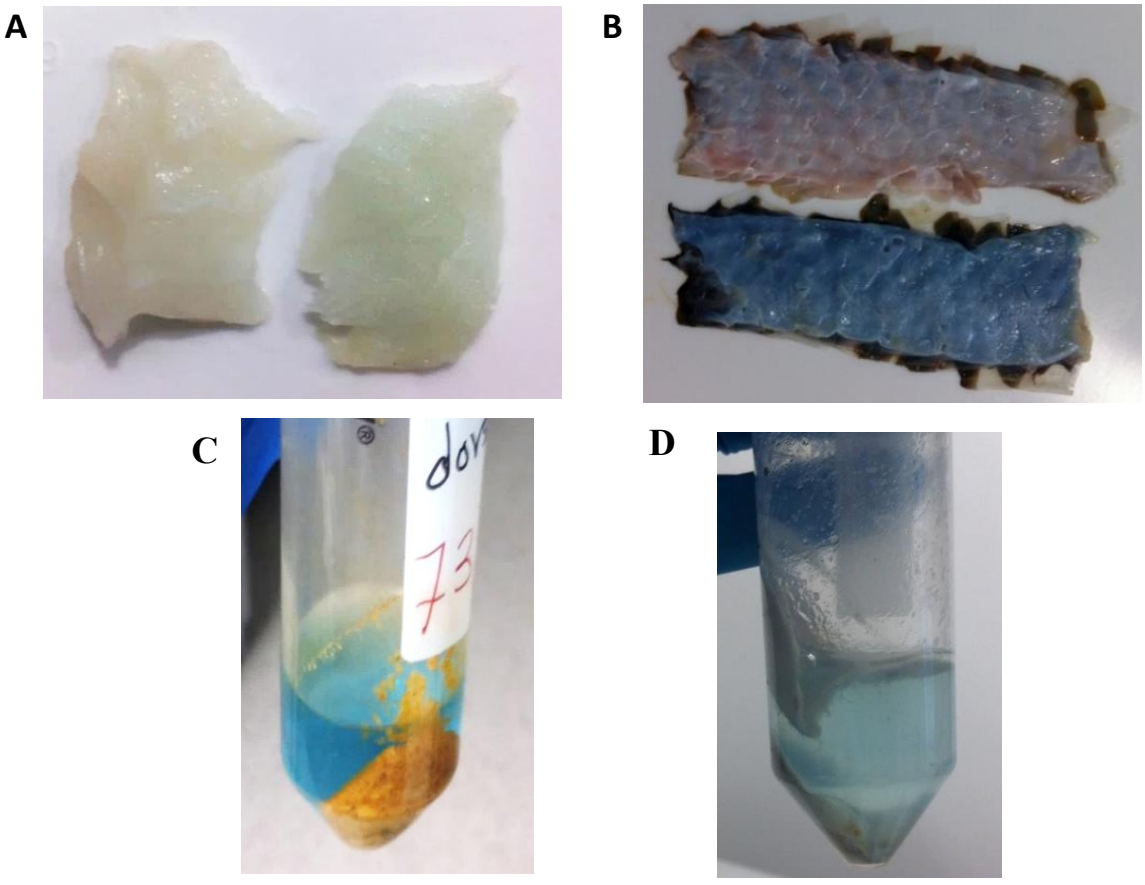


Figure 3. 1: Typical images showing the raw tissues of muscle, dorsal skin and ventral skin of ballan wrasse (*Labrus bergylta*) and tissue extracts of dorsal and ventral skin in individuals sampled at Carna. **A**) Unprocessed fillet muscle; **B**) raw ventral skin is shown on the top of this photo and dorsal skin is shown beneath; **C**) biliverdin extracted from dorsal skin; **D**) biliverdin extracted from ventral skin.

Figure 3.2 plots colorimeter readings for raw samples of muscle, ventral skin and dorsal skin in Carna 2015 and 2016. The majority of ventral and dorsal skin values occupied the quadrant between -*a** and -*b** on the plot, denoting a blue to green colour. The dorsal skin was consistently clustered at the bottom of this plot, however, which indicates bluer values and the ventral skin values were more blue-green. The colorimeter readings of raw muscle indicted greener to yellow colour as these were confined between -*a** and the *b**axis. These muscle values were highly consistent as can be seen from their being tightly grouped together. A few rare colorimeter readings of ventral skin were extreme, falling in the red to blue quadrant (*a** and -*b** axis). These readings were probably as a result of broken blood vessels in the ventral skin area which provided them with a reddish colour.

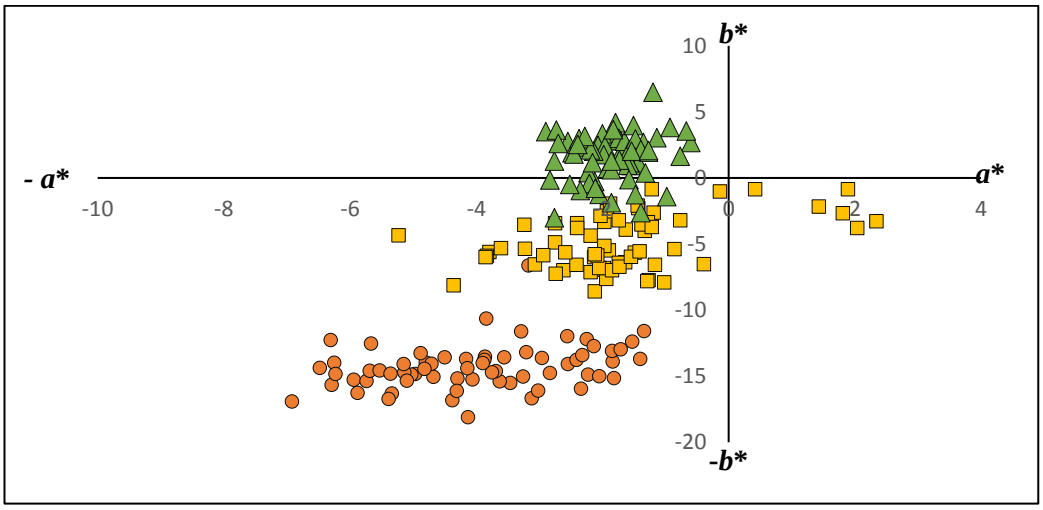


Figure 3. 2: *a**, *b** colour space of raw tissue of ballan wrasse (*Labrus bergylta*). Dorsal skin (●), ventral skin (□) and muscle (▲) from Carna population for 2015 (n=46) and 2016 (n=19), determined by a colorimeter. – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness.

The *L** values for raw tissue readings were normally distributed with homogenous variances in all tissues, including: dorsal skin (Shapiro-Wilk = 0.980; *d.f* = 64; *P* < 0.375), ventral skin (Shapiro-Wilk = 0.974; *d.f* = 64; *P* < 0.180) and muscle (Shapiro-Wilk = 0.970; *d.f* = 64; *P* < 0.119). Similarly, the *a** values in the dorsal skin (Shapiro-Wilk = 0.970; *d.f* = 64; *P* < 0.115), ventral skin (Shapiro-Wilk = 0.950; *d.f* = 64; *P* < 0.054), and muscle (Shapiro-Wilk = 0.987; *d.f* = 64; *P* < 0.717) were normal and homogenous. This also applied to the *b** measurements in the dorsal skin (Shapiro-Wilk = 0.977; *d.f* = 64; *P* < 0.350), ventral skin (Shapiro-Wilk = 0.961; *d.f* = 64; *P* < 0.056) and muscle (Shapiro-Wilk = 0.962; *d.f* = 64; *P* < 0.062).

Table 3.1 displays ANOVA results for the comparison of the *L**, *a* b** colour space scores in *L. bergylta* which was compared between the dorsal skin, ventral skin and muscle of raw tissues at Carna for both years overall. One-way ANOVA revealed significant differences in *L**, the degree of the lightness/darkness of the tissue colour ($F_{(2,192)} = 112.872$, $p = 0.001$). Similarly, *a** values also differed significantly between tissues $F_{(2,192)} = 69.607$, $P > 0.001$), as did the *b** values which were also significantly different between all tissues $F_{(2,192)} = 1228.532$, $P > 0.001$) (Table 3.1). A Tukey test was used for post-hoc comparisons of any significant ANOVA results (Table 3.2). Whereas, dorsal skin was significantly darker with $L^* = 61.25 \pm 0.56$ $P > 0.000$ than the ventral skin and muscle, ventral skin and muscle weren't significantly different from each other ($L^* = 71.37 \pm 0.54$ and 72.02 ± 0.59 respectively, $P < 0.699$). Similarly, *a** values of dorsal skin ($a^* = -3.99 \pm 0.18$, $P > 0.000$) were significantly different from ventral skin and muscle. But *a** values of ventral skin and muscle were not different from each other ($a^* = -1.73 \pm 0.17$ and -1.84 ± 0.06 respectively, $P < 0.870$). This indicates the degree of the colour on the 'green-yellow axis', meaning that dorsal skin was greener than the other tissues. Most interestingly, the *b** values were significantly different between all tissues ($P > 0.000$) indicating that this axis best separated the tissue types. The - *b** values which indicate 'blueness' of the samples were not as pronounced in the ventral skin and muscle, $b^* = -4.89 \pm 0.24$ and 1.65 ± 0.21 respectively, whereas the highest - *b** values were seen in dorsal skin ($b^* = -14.26 \pm 0.21$, Table 3.2). Overall the colorimeter results indicated the muscle tended towards yellowish, ventral skin towards greenish and dorsal skin towards a blueish hue, with differences in the degree of each colour represented by *L**.

Table 3. 1: *L*, a* b** colour space in ballan wrasse (*Labrus bergylta*): ANOVA for comparison of the dorsal skin, ventral skin and muscle of raw tissues obtained from individuals at Carna overall for both years (n=65).

		Sum of Squares	df	Mean Square	F-value	P-value
*L**	Between Groups	4734.426	2	2367.213	112.872	**0.001**
	Within Groups	4026.720	192	20.972		
	Total	8761.146	194			
*a**	Between Groups	210.835	2	105.417	69.607	**0.001**
	Within Groups	290.780	192	1.514		
	Total	501.614	194			
*b**	Between Groups	8312.283	2	4156.142	1228.532	**0.001**
	Within Groups	649.539	192	3.383		
	Total	8961.822	194			

Bold indicates a significant difference

Table 3. 2: Means *L*, a*, b** colorimeter readings of ballan wrasse (*Labrus bergylta*) from raw tissues of dorsal skin, ventral skin and muscle at Carna overall in 2015 and 2016. Mean ± standard error (n=65). Higher *L** value is lighter, *–a**, greenness; *a**, redness; *-b**, blueness; *b**, yellowness. Bold indicates a significant difference.

Lab colour space	Dorsal skin	Ventral skin	Muscle
*L**	**61.25±0.56**	71.37±0.54	72.02±0.59
*a**	**-3.99±0.18**	-1.73±0.17	-1.84±0.06
*b**	**-14.25±0.21**	**-4.89±0.24**	**1.65±0.21**

3.4.2 Tissue colouration: biliverdin extracts from tissues at Carna

Colorimeter results of extracted pigments for Carna overall (both 2015 and 2016) in dorsal and ventral skin, and dorsal and ventral scales are shown in Figure 3.3. Almost all samples occupied the quadrant between *-a** and *-b** on the plot, denoting blue to green colour. However, there were differences in hue between the dorsal and the ventral samples in both skin and scales tissues (Figure 3.3 A and B respectively), which indicated a more greenish colour in the ventral skin and scale tissue extracts and more blueish hue to dorsal skin and scales tissue extracts.

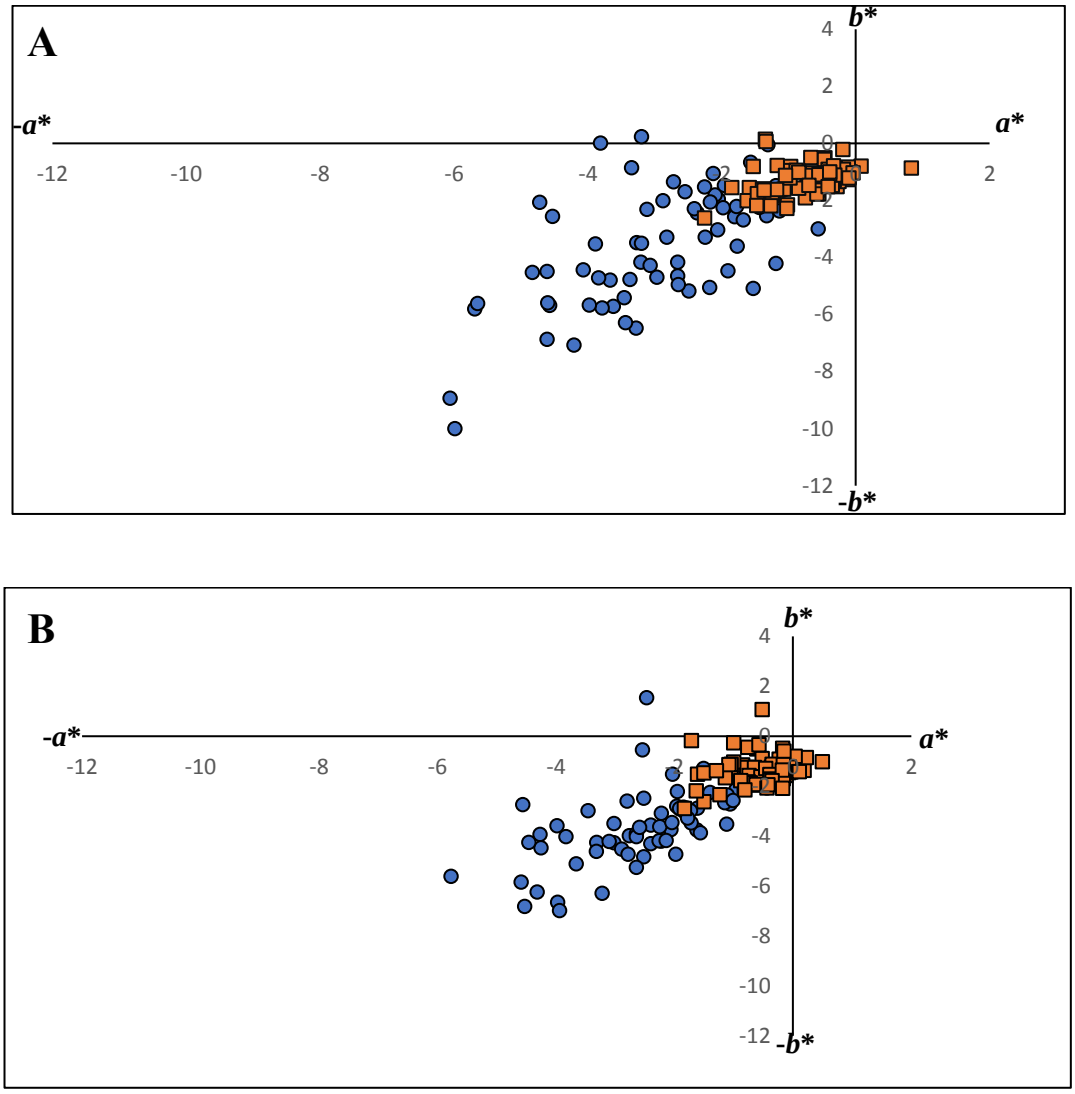


Figure 3. 3: *a**, *b** colour space of biliverdin tissue extracts of ballan wrasse (*Labrus bergylta*) at Carna in 2015 (n=46) and 2016 (n=19), determined by a colorimeter. (**A**) skin tissue extracts (**B**) scales tissue extracts. (●) dorsal skin and scales (□) ventral skin and scales. – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness.

Table 3.3 shows mean colorimeter values (*L**, *a**, and *b**) for biliverdin extracted from tissues at Carna (2015 and 2016). Mean values of biliverdin extracts of dorsal versus ventral surfaces in skin and dorsal versus ventral surfaces in scales were first compared using T-tests. Prior to this, normality and homogeneity of variance testing was performed, which showed that the *L** values were borderline/normally distributed with homogenous variances in both tissues, dorsal skin (Shapiro-Wilk = 0.980; *d.f* = 64; *P* < 0.407) and ventral skin (Shapiro-Wilk = 0.964; *d.f* = 64; *P* = 0.050). Similarly, tested was the *a** values in dorsal skin (Shapiro-Wilk = 0.967; *d.f* = 64; *P* < 0.085) and ventral skin (Shapiro-Wilk = 0.963; *d.f* = 64; *P* = 0.050) and *b** values in dorsal skin (Shapiro-Wilk = 0.963; *d.f*

= 64; $P < 0.053$) and ventral skin (Shapiro-Wilk = 0.988; *d.f* = 64; $P < 0.815$). Similarly, for scales, the *L** values were normally distributed with homogenous variances in both dorsal scales (Shapiro-Wilk = 0.984; *d.f* = 64; $P < 0.608$) and ventral scales (Shapiro-Wilk = 0.954; *d.f* = 64; $P < 0.176$). Similarly tested were the *a** values in dorsal scales (Shapiro-Wilk = 0.972; *d.f* = 64; $P < 0.169$) and ventral scales (Shapiro-Wilk = 0.972; *d.f* = 64; $P < 0.168$) and *b** values in dorsal scales (Shapiro-Wilk = 0.966; *d.f* = 64; $P < 0.124$) and ventral scales (Shapiro-Wilk = 0.963; *d.f* = 64; $P = 0.050$).

There were significant differences between *a** values in biliverdin extracts from dorsal and ventral skin ($P > 0.000$, *df* = 129, -*a**= -2.92 ± 0.16 and -0.72 ± 0.06 respectively) as well as in *b** values in dorsal and ventral skin ($P > 0.000$, *df* = 129, -*b**= -3.61 ± 0.26 and -1.29 ± 0.06). The results showed that extracts from dorsal skin tissues were more blue-green than ventral skin. There were similar significant differences between dorsal and ventral scales ($P > 0.000$, *df* = 129, -*a**= -2.54 ± 0.14 and -0.57 ± 0.06 respectively) as well as in *b** values in dorsal and ventral scales ($P > 0.000$, *df* = 129, -*b**= -3.58 ± 0.19 and -1.32 ± 0.07), as shown in Table 3.3. There were differences too in the degree of colouration represented by *L**. There were significant differences in *L** values between dorsal and ventral skin ($P > 0.000$, *df* = 129, *L**= 52.59 ± 0.29, and 55.58 ± 0.22), this indicated that the dorsal skin is darker than ventral skin. Also, in the scales extract, the *L** value was significantly darker on dorsal than ventral sides ($P > 0.000$, *df* = 129, *L**= 52.90 ± 0.17 and 56.36 ± 0.24 respectively –Table 3.3).

Table 3. 3: Mean *L**, *a**, *b** colorimeter readings of biliverdin tissue extracts of ballan wrasse (*Labrus bergylta*) for dorsal skin, dorsal scales and ventral skin and ventral scales in Carna (2015 and 2016 overall). Mean ± standard error is presented (n=65). Higher *L** value is lighter, – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness. Bold indicates a significant difference.

Lab colour space	Dorsal skin	Ventral skin	Dorsal scales	Ventral scales
*L**	**52.59±0.29**	55.58±0.22	**52.90±0.17**	56.36±0.24
*a**	**-2.92±0.16**	-0.72±0.06	**-2.54±0.14**	-0.57±0.06
*b**	**-3.61±0.26**	-1.29±0.06	**-3.58±0.19**	-1.32±0.07

3.4.3 The visible absorption spectrum of pigment extracted from tissues of Carna samples

A UV visible scanning spectrophotometer was used to examine the absorption spectra of pigment extracted from the dorsal skin and scales of *L. bergylta* in 2015 and 2016. The results in Figure 3.4 show samples for a representative selection of individuals at Carna. There were two peaks, one at 384 nm and the second peak was a wider one which varied between years i.e. this was between 640-644 nm for samples in 2015, and around 664-668 nm for samples in 2016.

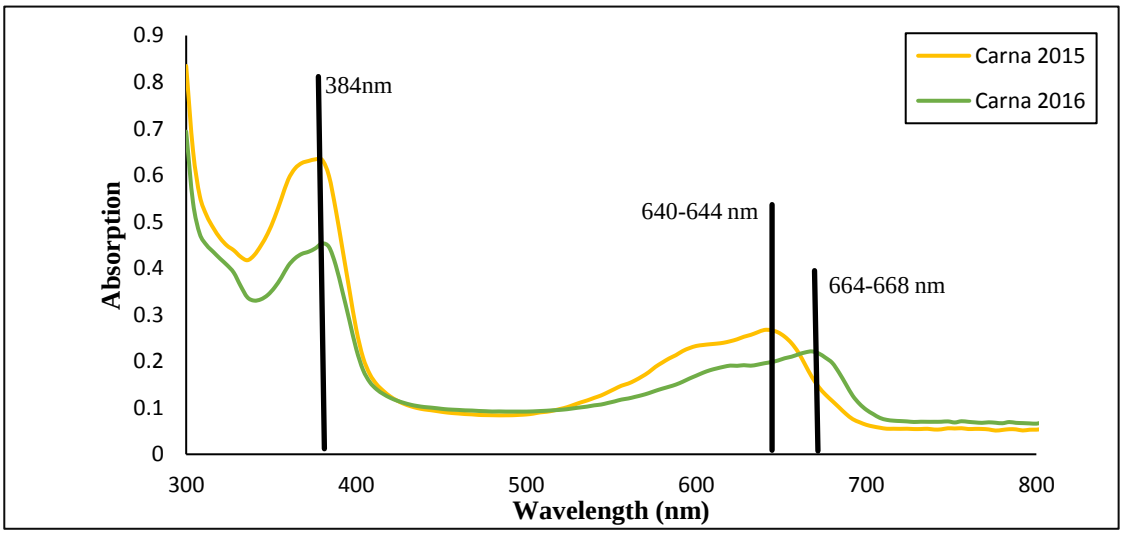
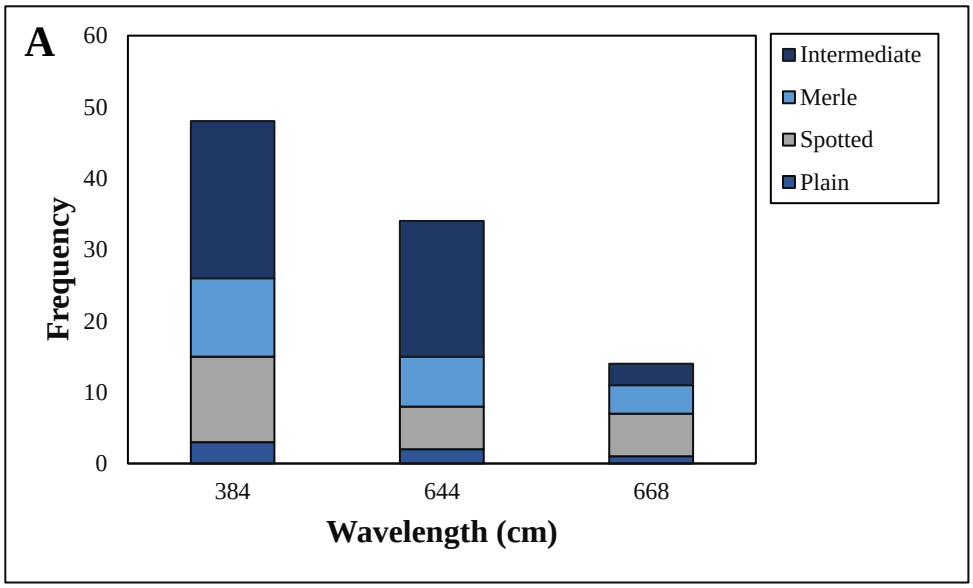


Figure 3. 4: A typical visible absorption spectrum of pigment extracted from skin and scales of ballan wrasse (*Labrus bergylta*) for 2015 (n=46) and 2016 (n=19), (|) peaks maximum.

Figure 3.5 presents the frequency of *L. bergylta* individuals presenting extracts (both dorsal skin and scales) with absorbance peaks at different wavelengths according to morphotype at Carna 2015 and 2016. The highest frequency at Carna 2015 was of wavelengths 384 and 644 nm and these applied to all morphotypes. All the morphotypes were represented by peaks at 384 nm, 644 nm, and 668 nm (Figure 3.5 A). Frequencies of the various absorbance peaks according to morphotype at Carna 2016 were different, with the highest frequency observed at wavelengths at 384 and 668 nm, which applied to all morphotypes (although the plain morphotype wasn't available in Carna 2016). Also, at Carna 2016, the 644 nm wavelength didn't apply to the spotted morphotype (Figure 3.5 B).



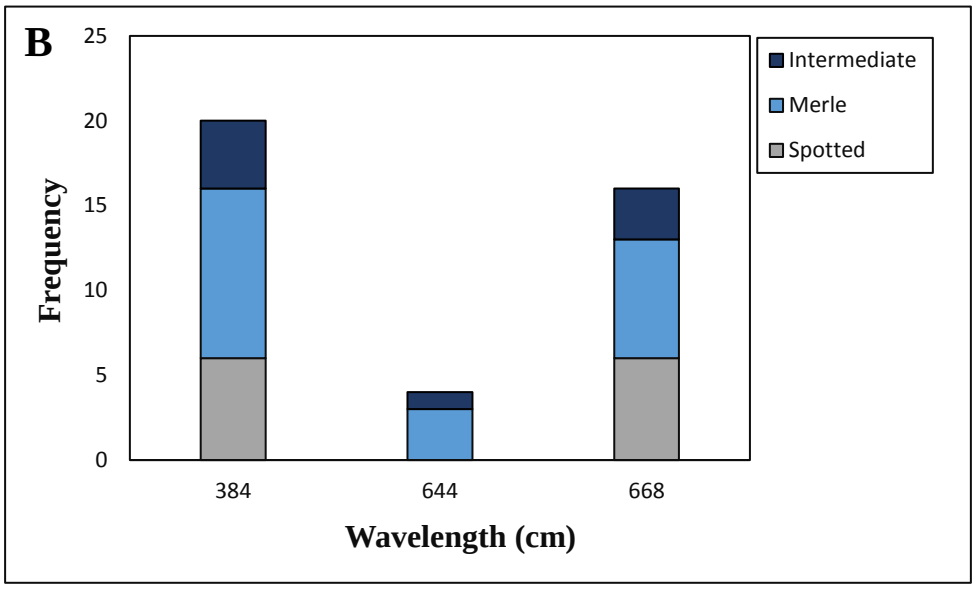


Figure 3. 5: The frequency of the absorbance wavelength, as measured by scanning spectrophotometer, from pigment extracted from both skin and scales for **A)** 2015 (n= 46) and **B)** 2016 (n=19) at Carna between morphotypes of ballan wrasse (*Labrus bergylta*).

3.4.4 Morphotype characteristics at Carna

A total of 65 fish from four different *L. bergylta* morphotypes, 'Plain', 'Spotted', 'Merle' and 'Intermediate' were captured at Carna during 2015 and 2016 as shown in Table 3.4. The captured *L. bergylta* presented some highly colourful patterns and variable

morphotypes. Spotted fish were identified as having a covering of white, blue or green spots, while plain fish featured a uniform but variable body colour. Merle fish were characterised as having a striped pattern, which occurs on their flanks (i.e. around the area of the lateral line). There was also an intermediate morph that is characterised as having both merle and spotted pattern features (Figure 2.2 in chapter 2).

Table 3.4 shows the number of samples of dorsal skin and scales samples for each morphotype in Carna with a total sample size of n = 46 in 2015, and n = 19 in 2016. The most common morphotype in Carna in 2015 was intermediate, followed by spotted and merle, then plain which was the least abundant morphotype. However, in 2016, the merle morphotype was the most common one, followed closely by spotted and then intermediate individuals. There were no plain morphs captured in Carna in 2016 as shown in Table 3.4.

Table 3. 4: Number of dorsal skin and scales samples for each morphotype of ballan wrasse (*Labrus bergylta*) at Carna from 2015 and 2016 (n = 65).

Morphotype	2015	2016
Plain	2	0
Spotted	12	6
Merle	11	9
Intermediate	21	4

Body size was compared across the morphotypes (Figure 3.6) by a Kruskal-Wallis test, which found significant differences in weight between morphotypes from combined samples from Carna 2015 and 2016 (Kruskal-Wallis = 10.234, $P > 0.017$, $df = 3$). Length of fish with different morphotypes from combined samples of Carna 2015 and 2016 were also significantly different from each other (Kruskal-Wallis = 10.948, $P > 0.012$, $df = 3$). A Mann-Whitney test was carried out to see which weights and lengths were different between the morphotypes. It was shown that merle was significantly lighter in weight than spotted and intermediate morphs ($P > 0.019$, $P > 0.003$ respectively), and also in length, merle was significant shorter than intermediate and spotted morphotypes ($P > 0.013$ and 0.004, respectively). Figure 3.6 showed median weight (726.50 g) and median total length (34.85 cm) of merle morphotypes was much smaller than the other morphs. Spotted and intermediate morphs were the heaviest fish with a median of 1148.50 and g respectively,

and the longest were spotted and intermediate (median=41.15 cm and 39.80 cm respectively).

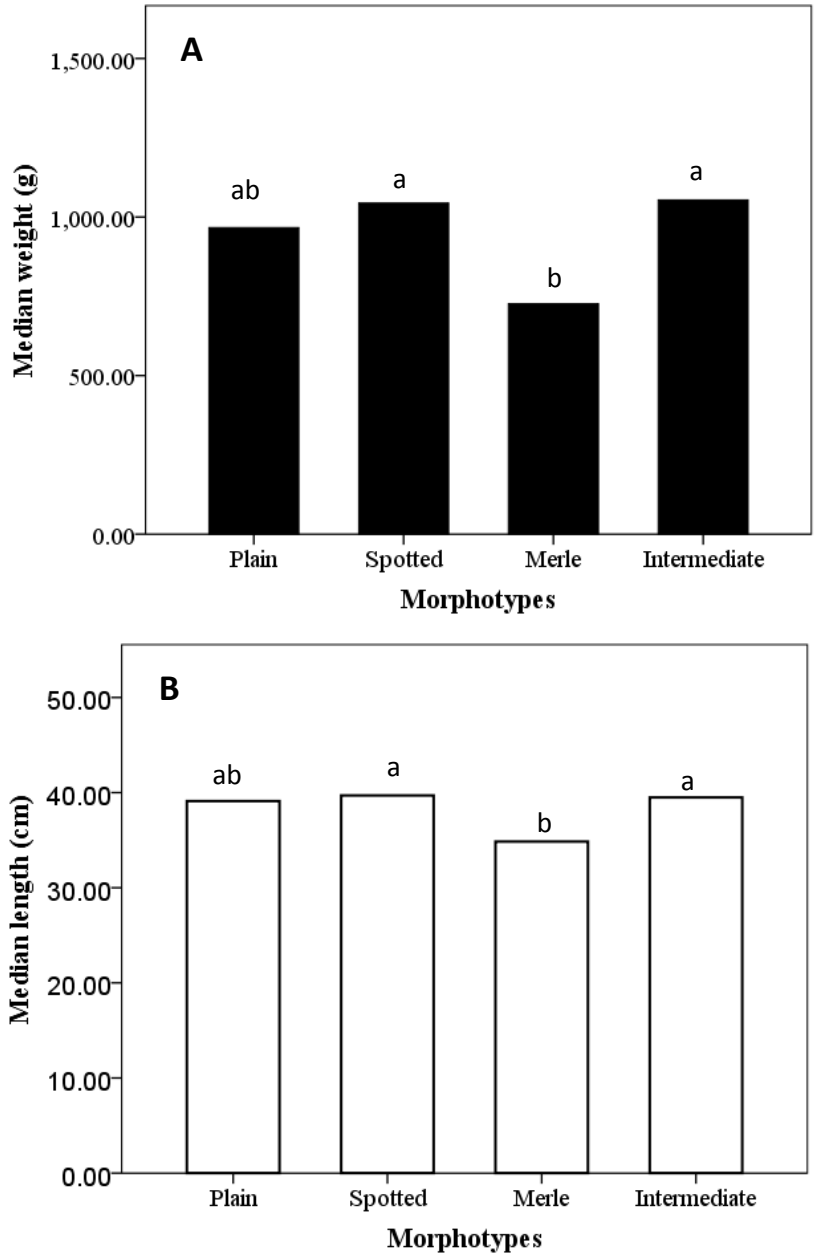


Figure 3. 6: Median weight and length in morphotypes of ballan wrasse (*Labrus bergylta*) at Carna overall in both years (n = 65). Median weight (g) between morphotypes in Carna (**A**); median total length (cm) between the morphotypes in Carna (**B**). The letters above the bars show which bars are significantly different to the others (bars with the same letters are not significantly different to each other).

3.4.5 Comparison of biliverdin concentration between morphotypes at Carna (2015 and 2016)

The results of normality and homogeneity of variance testing are shown in Table 3.5. The concentrations of biliverdin in dorsal skin for four morphotypes of *L. bergylta* in Carna 2015 (n = 46) and three morphotypes in 2016 (n = 19) were normally distributed with homogenous variances. Similarly, concentrations of biliverdin in dorsal scales for four morphotypes in Carna 2015 (n = 46) and three morphotypes in 2016 (n = 19) were normally distributed with homogenous variances (the log10 transformation was performed on dorsal scales in 2015 only).

Table 3. 5: Normality and homogeneity of variances test for biliverdin concentrations of ballan wrasse (*Labrus bergylta*) in dorsal skin and dorsal scales between the morphotypes in Carna 2015 and 2016.

Tissue/Year	Shapiro-Wilk	*d.f*	*P* value	Levene's test	*d.f*	*P* value
Skin 2015	0.958	46	0.099	1.041	3, 42	0.384
Skin 2016	0.968	19	0.741	2.878	2, 16	0.086
Scales 2015	0.974	46	0.402	0.092	3, 42	0.964
Scales 2016	0.950	19	0.392	0.235	2, 16	0.793

Biliverdin concentrations at Carna were compared between morphotypes by one-way ANOVA which found significant differences for dorsal skin samples between morphotypes in 2015 $F_{(42,3)} = 3.969$, $P > 0.014$), also in dorsal scales in 2015 $F_{(42,3)} = 7.488$, $P > 0.000$). However, the dorsal skin and dorsal scales were not significantly different between the morphotypes in 2016 ($F_{(16,2)} = 0.310$, $P < 0.738$; $F_{(16,2)} = 1.592$, $P < 0.234$ respectively), as shown in Table 3.6 and 3.7. A Tukey test was used for post-hoc comparisons of the significant ANOVA results in dorsal skin and dorsal scales for 2015. In dorsal skin 2015, merle was significantly lower in biliverdin concentration than spotted and intermediate morphotypes, the latter having the highest biliverdin concentrations with means of 0.88 ± 0.10, ($P > 0.022$) and 0.84 ± 0.07 ($P > 0.021$), respectively. There was no significant difference between the plain and all other morphs with mean concentrations of 0.61 ± 0.11 ($P > 0.05$). In the dorsal scales in 2015 at Carna, spotted and intermediate had significantly higher biliverdin concentration than merle morphotypes which had the lowest biliverdin

concentrations with means of 0.47 ± 0.07 ($P > 0.000$) and ($P > 0.025$) respectively,. However, biliverdin concentrations in dorsal skin and dorsal scales in 2016 didn't show any significant differences between the morphotypes as shown in Figure 3.7. (No plain morphs were available for testing in 2016).

Table 3. 6: ANOVA of biliverdin concentrations in ballan wrasse (*Labrus bergylta*) morphotypes in the dorsal skin and scales at Carna in 2015. Bold indicates a significant difference.

	Skin 2015					**Scales 2015**				
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	1.303	3	0.434	3.969	**0.014**	0.775	3	0.258	7.488	**0.000**
Within Groups	4.594	42	0.109			1.450	42	0.035		
Total	5.897	45				2.225	45			

Table 3. 7: ANOVA of biliverdin concentrations in ballan wrasse (*Labrus bergylta*) morphotypes in the dorsal skin and dorsal scales at Carna in 2016.

	Skin 2016					**Scales 2016**				
	Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**	**Sum of Squares**	**df**	**Mean Square**	**F**	**Sig.**
Between Groups	0.021	2	0.011	0.310	0.738	0.306	2	0.153	1.592	0.234
Within Groups	0.553	16	0.035			1.536	16	0.96		
Total	0.574	18				1.842	18			

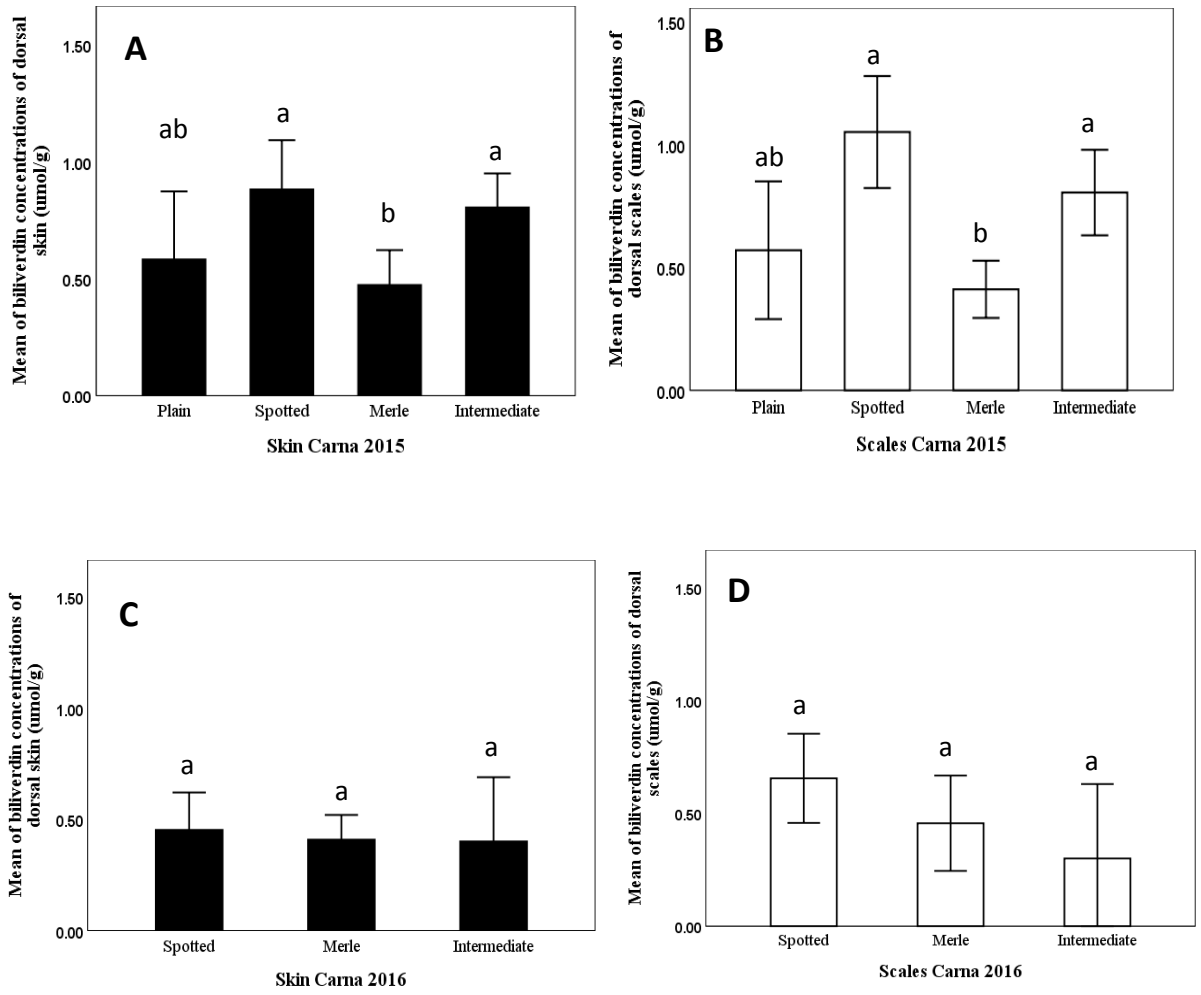


Figure 3. 7: Variation in biliverdin concentrations in dorsal skin and dorsal scales in ballan wrasse (*Labrus bergylta*) tested by ANOVA for four morphotypes in 2015 (n=46) and three morphotypes in 2016 (n=19) at Carna. Biliverdin concentrations were determined by absorbance spectrophotometry. **A)** skin Carna 2015, **B)** scales Carna 2015, **C)** skin Carna 2016 and **D)** scales Carna 2016. The error bars denote standard error (SE).

3.4.6 Comparison of biliverdin concentration between years for each morphotype at Carna

Table 3.8 shows mean biliverdin concentration between year for each morphotype at Carna using T-tests. Prior to this, normality and homogeneity of variance testing was performed, where required, data were log 10 transformed to satisfy homogeneity of variance requirements, especially merle in dorsal scales. The data showed that the spotted, merle and intermediate were normally distributed with homogenous variances in skin tissues (spotted: Shapiro-Wilk = 0.961; *d.f* = 18; *P* < 0.614, merle: Shapiro-Wilk = 0.963; *d.f* = 20;

$P < 0.610$, intermediate: Shapiro-Wilk = 0.937; $d.f = 25$; $P < 0.130$). Similarly, normality and homogeneity of variance testing was performed for scales tissues (spotted: Shapiro-Wilk = 0.945; $d.f = 18$; $P < 0.357$, merle: Shapiro-Wilk = 0.937; $d.f = 20$; $P < 0.212$, intermediate: Shapiro-Wilk = 0.980; $d.f = 25$; $P < 0.878$). The T-test showed significant differences in biliverdin concentration between years (Table 3.8). The spotted and intermediate morphs were significantly higher in biliverdin concentration in dorsal skin and dorsal scales in 2015. Merle morphs didn't present any significant differences between the years in dorsal skin or dorsal scales. The lack of data for plain morphotype in 2016 prevented comparison across years.

Table 3. 8: Mean biliverdin concentration comparisons of ballan wrasse (*Labrus bergylta*) between years in dorsal skin and dorsal scales for each morphotype at Carna analysed by T-test, Spotted (n = 18), Merle (n = 20) and Intermediate (n = 25) , n/a = no data. Bold indicates significant differences.

Morphotypes	**Year**	**Dorsal skin**		**Dorsal scales**	
		Mean±SE	***P*-Value**	**Mean±SE**	***P*-Value**
Plain	2015	0.61 ± 0.11	n/a	0.57 ± 0.14	n/a
	2016	n/a		n/a	
Spotted	2015	0.88 ± 0.10	**0.016**	1.05 ± 0.11	**0.040**
	2016	0.45 ± 0.08		0.65 ± 0.09	
Merle	2015	0.47 ± 0.07	0.810	-0.47 ± 0.06	0.729
	2016	0.49 ± 0.05		-0.48 ± 0.11	
Intermediate	2015	0.84 ± 0.07	**0.031**	0.73 ± 0.06	**0.013**
	2016	0.40 ± 0.14		0.30 ± 0.16	

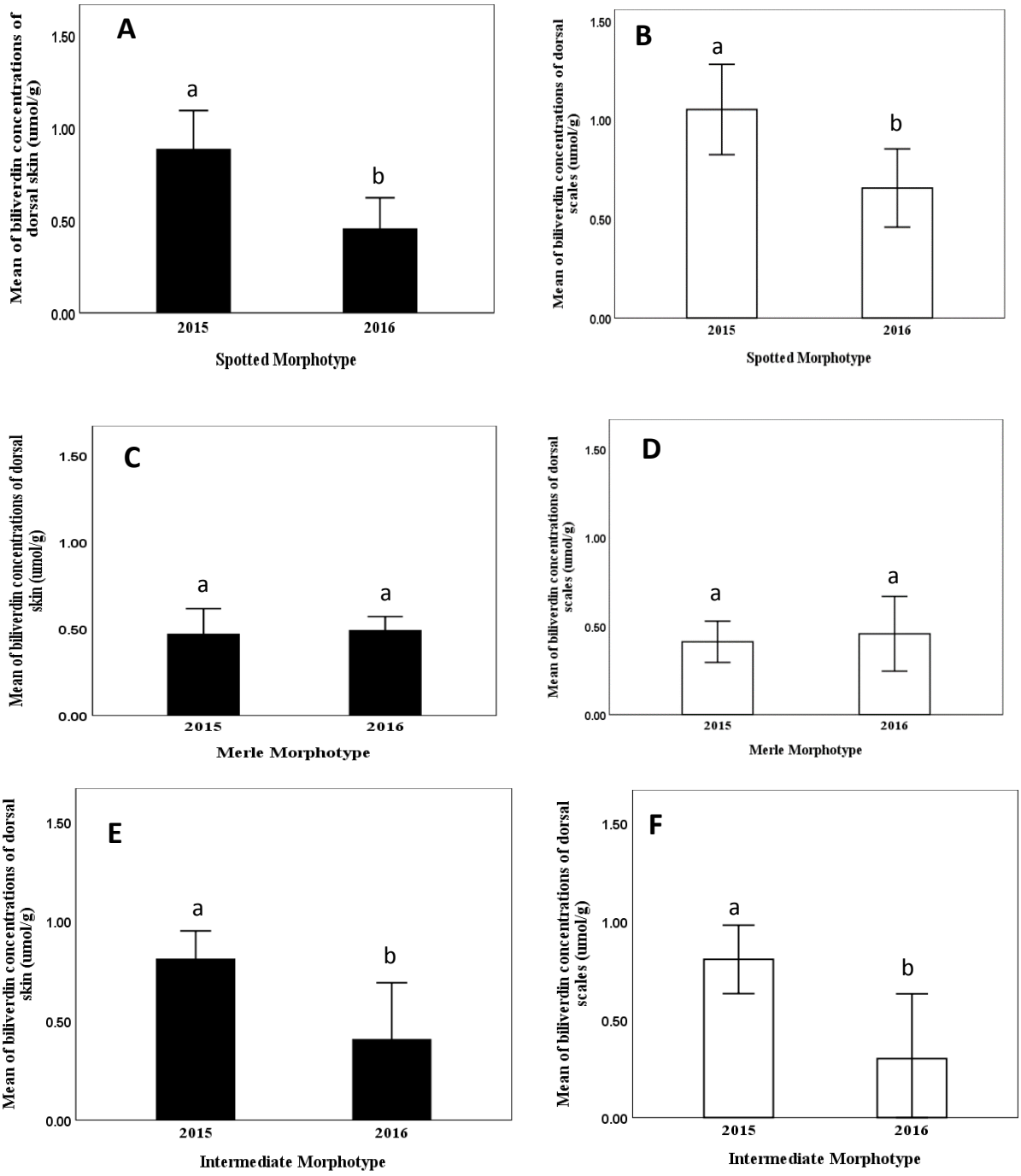


Figure 3. 8: Mean biliverdin concentration of ballan wrasse (*Labrus bergylta*) in the dorsal skin and scales between years at Carna. The error bars denote standard error (SE). **A)** dorsal skin of spotted morphs, **B)** dorsal scales of spotted morphs, **C)** dorsal skin of merle morphs, **D)** dorsal scales of merle morphs, **E)** dorsal skin of intermediate morphs and **F)** dorsal scales of intermediate morphs.

Graphic presentation of means ± SE biliverdin concentrations in Figure 3.8 demonstrate that 2015 had high biliverdin concentrations in dorsal skin and scales, particularly in spotted and intermediate morphs. However, in merle morphs, the concentrations in skin and scales were similarly low in both years.

3.4.7 The relationship between biliverdin concentrations in tissues at Carna

A scatter plot and Spearman's correlation was used to determine the relationship between biliverdin concentration across different tissues, i.e. in dorsal skin, scales and plasma at Carna (Figure 3.9). The total of 65 individual *L. bergylta* from Carna were analysed in this way (n = 65 for skin, n = 65 for scales and n = 65 for plasma overall in 2015 and 2016). There was a moderately significant positive correlation between biliverdin concentration in the dorsal skin and scales with a correlation coefficient of 0.632, and $P > 0.000$. Furthermore, the biliverdin concentration of the plasma and dorsal skin as well as the plasma and dorsal scales both demonstrated significant positive correlations, albeit with lower correlation coefficients.

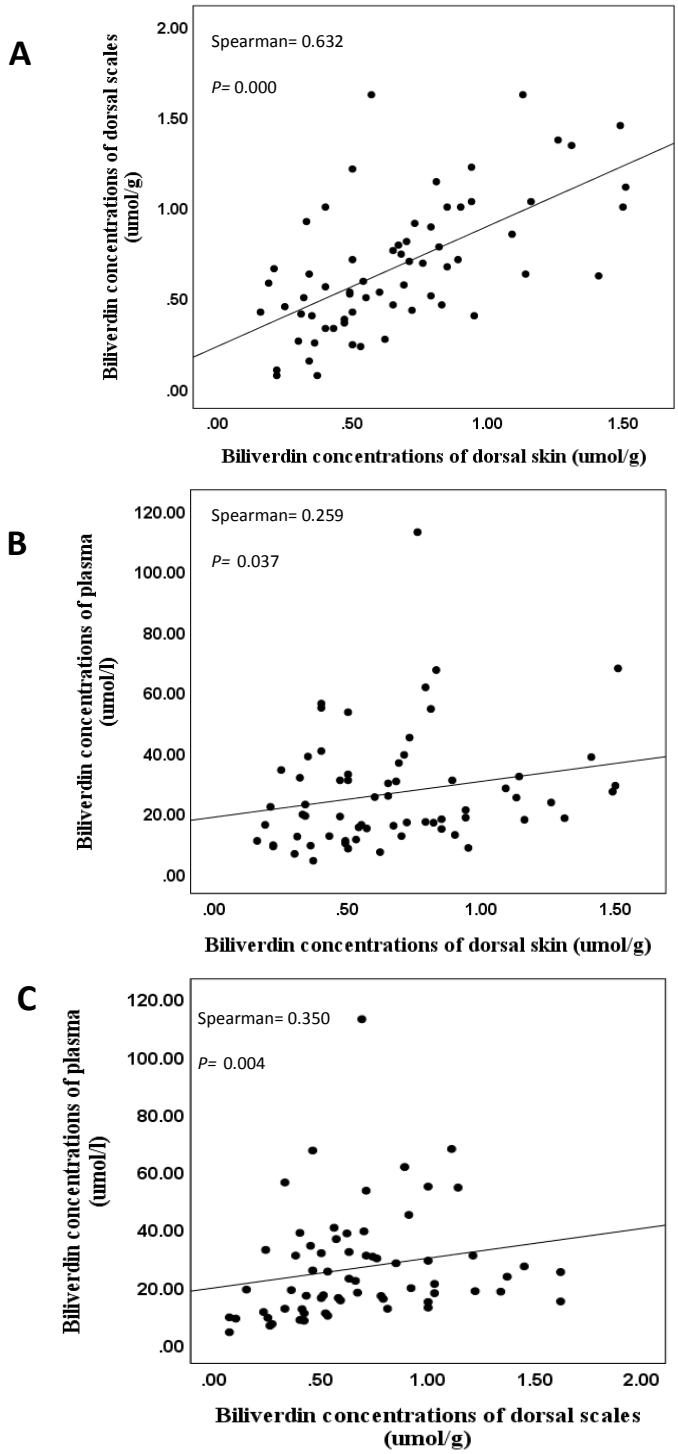


Figure 3. 9: Spearman's correlation between biliverdin concentrations in ballan wrasse (*Labrus bergylta*) tissues: dorsal skin, dorsal scales and plasma for 2015 and 2016 combined at Carna. (**A**) correlation between dorsal skin and dorsal scales. (**B**) correlation between dorsal skin and plasma. (**C**) correlation between dorsal scales and plasma. Sample size for all tissues n = 65.

3.4.8 Tissue colouration: raw tissue at Spiddal

At Spiddal, the colour of raw muscle tissue from *L. bergylta* varied from yellowish to a light greenish colour, while the dorsal skin was blue in colour and much darker than the ventral skin as was described previously in Figure 3.1. The colorimeter readings for samples of raw muscle are shown in Figure 3.10, as well as from unprocessed dorsal and ventral skin in Spiddal 2015 and 2016 (n= 15 individual fish for 2015 and n=20 for 2016). The colorimeter readings of muscle were mostly confined to the -*a** and *b** quadrant which denotes greenish to yellow, but there were some exceptional values occurring in the *b** and *a** quadrant (yellowish to red colour). The latter readings may have arisen as a result of broken blood vessels inside the muscle, providing it with a reddish colour. The ventral skin extended across three axes -*b**, -a* and *b** which indicated a wide variety of hues from blueish to green to yellowish. However, the dorsal skin was confined in the bottom area of -*a** and -*b** quadrant as a blue colour. The raw colorimeter measurements on the body of *L. bergylta* individuals' skin at Spiddal were taken slightly more laterally than fully ventral (as had been done for Carna), which may explain the more dispersed nature of the ventral skin readings in Figure 3.10 compared to Carna in Figure 3.2.

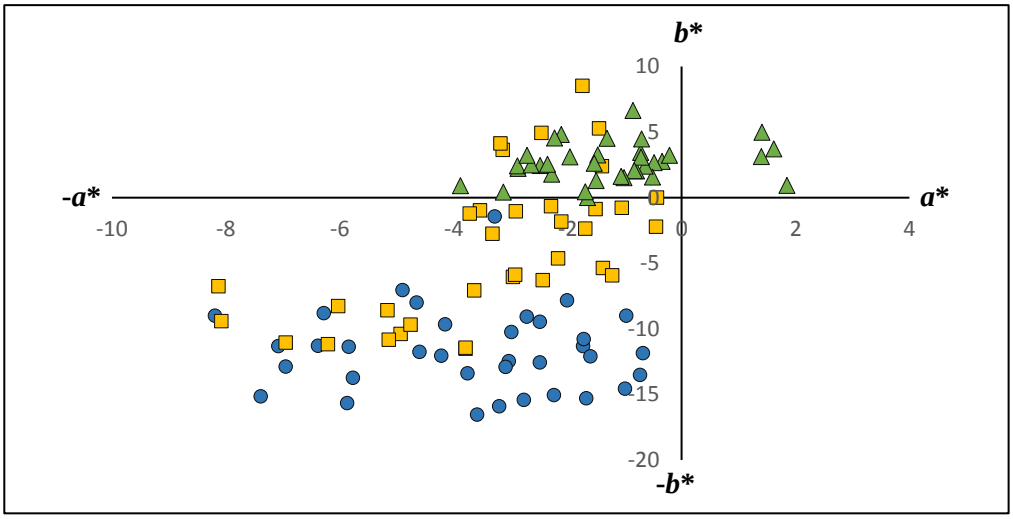


Figure 3. 10: *a**, *b** colour space in ballan wrasse (*Labrus bergylta*) raw dorsal skin tissue, ventral skin and muscle at Spiddal 2015 (n=15) and 2016 (n=20), determined by a colorimeter. Dorsal skin (●), ventral skin (□), muscle (▲). - *a**, greenness; *a**, redness; -*b**, blueness; *b**, yellowness.

The *L** values were normally distributed with homogenous variances in all raw tissues (dorsal skin: Shapiro-Wilk = 0.941; *d.f* = 34; *P* < 0.060; ventral skin: Shapiro-Wilk = 0.948; *d.f* = 34; *P* < 0.099, muscle: Shapiro-Wilk = 0.943; *d.f* = 34; *P* < 0.069). Similarly, the *a** values (dorsal skin: Shapiro-Wilk = 0.950; *d.f* = 34; *P* < 0.115, ventral skin: Shapiro-Wilk = 0.942; *d.f* = 34; *P* =0.050, muscle: Shapiro-Wilk = 0.948; *d.f* = 34; *P* < 0.102) and *b** values (dorsal skin: Shapiro-Wilk = 0.941; *d.f* = 34; *P* < 0.059, ventral skin: Shapiro-Wilk = 0.951; *d.f* = 34; *P* < 0.126, muscle: Shapiro-Wilk = 0.974; *d.f* = 34; *P* < 0.556).

Three one-way ANOVA analyses showed significant differences of all colour values (*L**, *a**, and *b**) in raw tissues colours in the three groups dorsal/ventral skin and muscle at Spiddal, as shown in Table 3.9. Tukey tests were used for post-hoc comparisons of any significant ANOVA results. When examining the lightness, or *L** values, dorsal skin was significantly darker than the ventral skin and muscle (*L** = 53.54 ± 1.32, *P* > 0.000; Table 3.10). However, the ventral skin and muscle weren't significantly different from each other (*L** = 64.69 ± 1.09 and 61.13 ± 0.57, *P* > 0.047 respectively) which indicated the ventral skin and muscle were much lighter in hue than the dorsal skin. Similarly, *a** value of muscle (-1.23 ± 0.23) was significantly different from dorsal skin and ventral skin (*a** = -3.71 ± 0.35, *P* > 0.000 and -3.32 ± 0.34, *P* > 0.000, respectively; Table 3.10) which indicated the colour of the skin was green-blueish and the colour of the muscle was yellowish. Like Carna, *b** values representing the blue-coloured hue were again significantly different between all tissues at Spiddal, the highest value was dorsal skin (-*b**= -11.66 ± 0.52, *P* > 0.000) (Table 3.10), which indicates the dorsal skin was blueish, the ventral skin was greenish (- *b**= -3.88 ± 0.92, *P* > 0.000), and muscle was yellowish (-*b**= 2.67 ± 0.24, *P* >0.000; Table 3.10).

Table 3. 9: One-way ANOVA results of *L**, *a** and *b** colour space between the dorsal skin, ventral skin and muscle of raw tissues in ballan wrasse (*Labrus bergylta*) at Spiddal in 2015 (n=15) and 2016 (n=20). Higher *L** value is lighter, – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness. Bold indicates a significant difference

		Sum of Squares	*df*	Mean Square	F	Sig.
*L**	Between Groups	2271.668	2	1135.834	29.693	**0.001**
	Within Groups	3901.729	102	38.252		
	Total	6173.398	104			
*a**	Between Groups	124.108	2	62.054	18.148	**0.001**
	Within Groups	348.770	102	3.419		
	Total	472.878	104			
*b**	Between Groups	3606.649	2	1803.325	130.577	**0.001**
	Within Groups	1408.659	102	13.810		
	Total	5015.308	104			

Table 3. 10: Means *L** *a** *b** scores of raw tissues on dorsal skin, ventral skin and muscle colour in ballan wrasse (*Labrus bergylta*) at Spiddal overall in 2015 and 2016 (n=35). Mean ± standard error. A higher *L** value denotes a lighter colour, – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness. Bold indicates mean(s) with a significant difference using the post-hoc Tukey test.

Lab colour space	Dorsal skin	Ventral skin	Muscle
*L**	**53.54 ± 1.32**	64.69 ± 1.09	61.13 ± 0.57
*a**	-3.71 ± 0.35	-3.32 ± 0.34	**-1.23 ± 0.23**
*b**	**-11.66 ± 0.52**	**-3.88 ± 0.92**	**2.67 ± 0.24**

3.4.9 Tissue colouration: biliverdin extracts from tissues at Spiddal

Colorimeter readings of biliverdin extracted from tissue of *L. bergylta* sampled at Spiddal (2015 and 2016) displayed unusually low organisation in terms of the colour space of individual tissue groups (Figure 3.11). However, almost all readings still fell into the blue-green colour space.

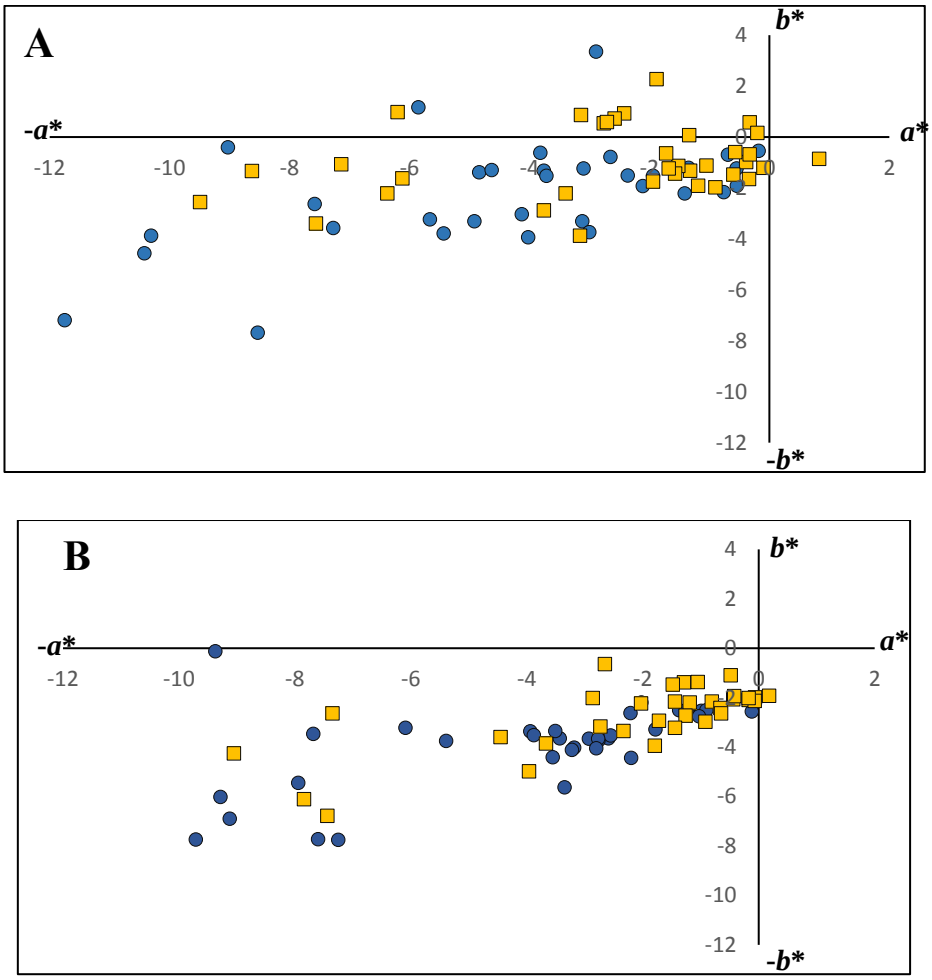


Figure 3. 11: *a**, *b** colour space of biliverdin tissue extracts of ballan wrasse (*Labrus bergylta*) at Spiddal overall in 2015 (n=15) and 2016 (n=20), determined by a colorimeter. Skin tissue extracts (**A**), scales tissue extracts (**B**), dorsal skin and scales (●) ventral skin and scales (□). – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness.

Biliverdin concentrations extracted from dorsal and ventral skin and dorsal and ventral scales tissues at Spiddal (2015 and 2016) were compared as follows: dorsal versus ventral in skin and dorsal versus ventral in scales using a T-test. Prior to testing, normality and homogeneity of variance testing was performed. The *L** values were normally distributed with homogenous variances in all tissues (dorsal skin: Shapiro-Wilk = 0.947; *d.f* = 34; *P* <

0.100, ventral skin: Shapiro-Wilk = 0.958; *d.f* = 34; *P* < 0.213). Similarly, the *a** values (dorsal skin: Shapiro-Wilk = 0.970; *d.f* = 34; *P* < 0.114, ventral skin: Shapiro-Wilk = 0.949; *d.f* = 34; *P* < 0.110) and *b** values (dorsal skin: Shapiro-Wilk = 0.946; *d.f* = 34; *P* < 0.094, ventral skin: Shapiro-Wilk = 0.975; *d.f* = 34; *P* < 0.604) were normal. Similarly, for scales, the *L** values were normally distributed with homogenous variances in all tissues (dorsal scales: Shapiro-Wilk = 0.953; *d.f* = 34; *P* < 0.158, ventral scales: Shapiro-Wilk = 0.954; *d.f* = 34; *P* < 0.176). Similarly, the *a** values (dorsal scales: Shapiro-Wilk = 0.980; *d.f* = 34; *P* < 0.116, ventral scales: Shapiro-Wilk = 0.950; *d.f* = 34; *P* < 0.115) and *b** values (dorsal scales: Shapiro-Wilk = 0.946; *d.f* = 34; *P* < 0.096, ventral scales: Shapiro-Wilk = 0.978; *d.f* = 34; *P* < 0.609) were normally distributed. The means of the colorimeter values (*L**, *a**, and *b**) for biliverdin extracts from dorsal and ventral skin and dorsal and ventral scales at Spiddal (2015 and 2016) are presented in Table 3.11. There were significant differences between *a** values in dorsal and ventral skin (*P* > 0.018, *df* = 69, -*a**= -4.54 ± 0.57 and -2.77 ± 0.44 respectively) as well as in *b** values in dorsal and ventral skin (*P* > 0.003, *df* = 69, -*b**= -2.31 ± 0.36 and -0.96 ± 0.23). This showed that biliverdin extracts from dorsal skin tissues were more greenish-blue than ventral skin extracts.

Similarly, there were significant differences between *a** in scores in dorsal and ventral scales (*P* > 0.017, *df* = 69, -*a**= -4.04 ± 0.27 and –2.73 ± 0.23 respectively) as well as in *b** values in dorsal and ventral scales (*P* > 0.001, *df* = 69, -*b**= -3.79 ± 0.47 and -2.23 ± 0.42), as shown in Table 3.11. This suggests that biliverdin extracts from dorsal scales were greener than ventral scales which tended to be more blue in colour, with differences too in the degree of colouration represented by *L**. There were significant differences in *L** values between dorsal and ventral skin (*P* > 0.048, *df* = 69, *L**= 55.13 ± 0.65, and 56.99 ± 0.47), that indicted the biliverdin extract from dorsal skin was darker than ventral skin. A similar significant difference between the *L** values of biliverdin extracts from dorsal and ventral scales (*P* > 0.046, *df* = 69, *L**= 57.64 ± 0.37 and 58.76 ± 0.32 respectively) is shown in Table 3.11.

Table 3. 11: Mean *L**, *a**, *b** colorimeter readings of biliverdin tissue extracts for dorsal and ventral skin and scales of ballan wrasse (*Labrus bergylta*) in Spiddal overall for 2015 and 2016 (n=35). Mean ± standard error A higher *L** value denotes lighter colour, – *a**, greenness; *a**, redness; - *b**, blueness; *b**, yellowness. Bold indicates a significant difference

Lab colour space	Dorsal skin	Ventral skin	Dorsal scales	Ventral scales
*L**	**55.13 ± 0.65**	56.99 ± 0.47	**57.64 ± 0.37**	58.76 ± 0.32
*a**	**-4.54 ± 0.57**	-2.77 ± 0.44	**-4.04 ± 0.27**	-2.73 ± 0.23
*b**	**-2.31 ± 0.36**	-0.96 ± 0.23	**-3.79 ± 0.47**	-2.23 ± 0.42

3.4.10 The visible absorption spectrum of pigment extracted from tissues in Spiddal

A UV visible scanning spectrophotometer was used to examine the absorption spectra of pigment extracted from the dorsal skin and scales of *L. bergylta* at Spiddal in 2015 and 2016. The results in Figure 3.12 shows a sample spectrum for a representative selection of individuals at Spiddal. This had two peaks, one at 384 nm which was identical to the same peak at Carna (also at 384 nm) and the second peak was a wider one between 664-668 nm (both 2015 and 2016), as also seen at Carna in 2016.

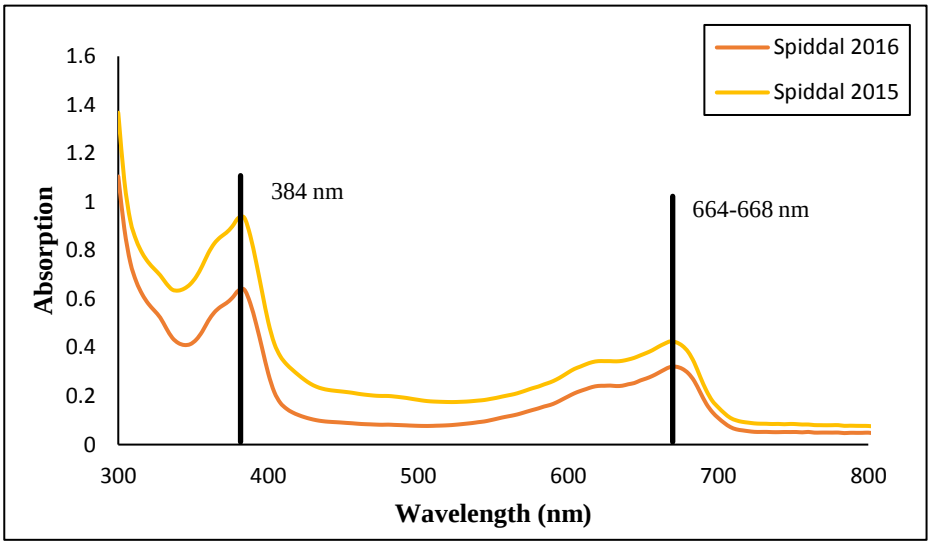


Figure 3. 12: A typical visible absorption spectrum of pigment extracted from skin and scales of ballan wrasse (*Labrus bergylta*) for 2015 (n=15) and 2016 (n=20) at Spiddal, (|) peaks maximum.

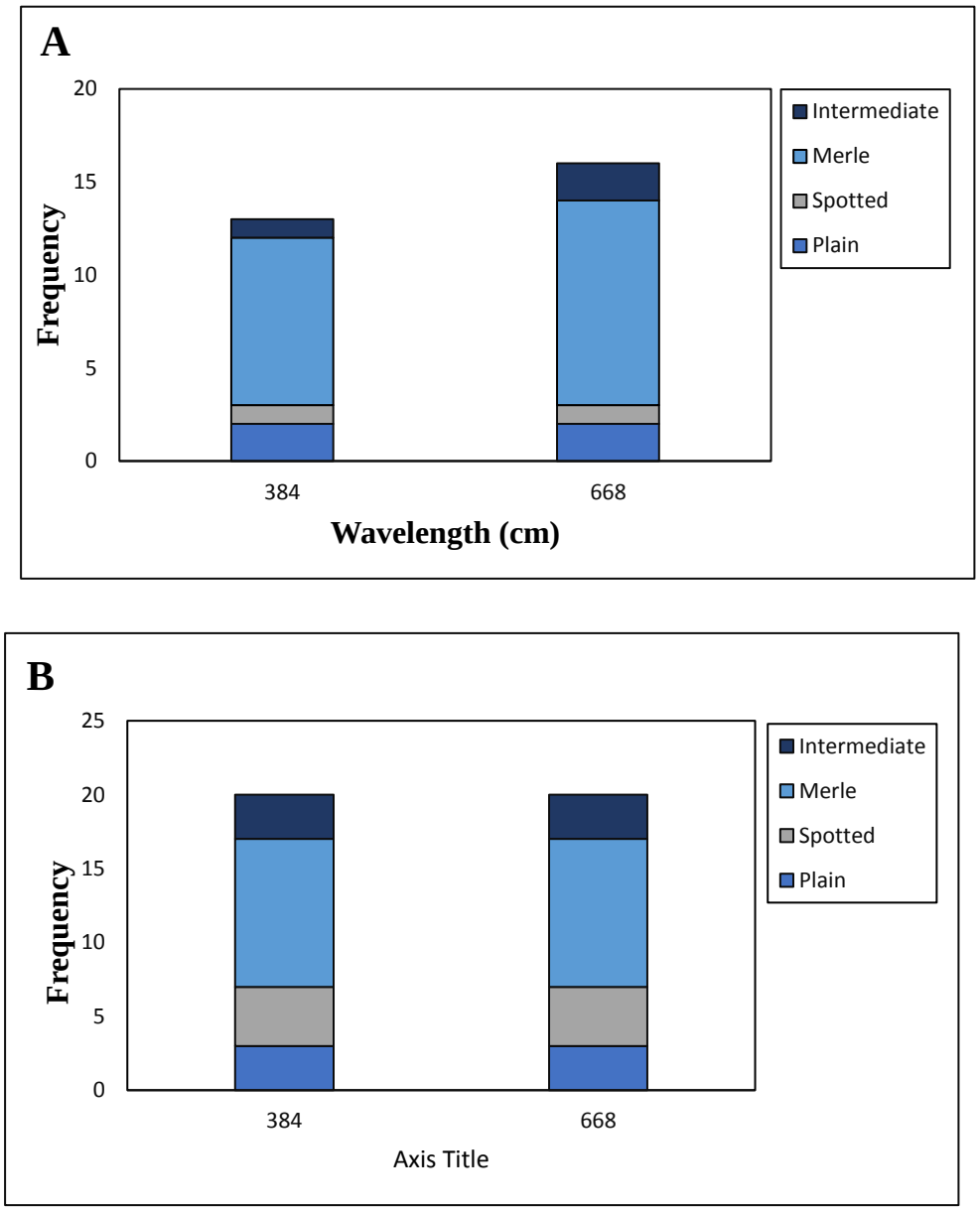


Figure 3. 13: The frequency of the absorbance wavelength, as measured by scanning spectrophotometer, from pigment extracted from both skin and scales for **A)** for 2015 (n= 15) and **B)** 2016 (n= 20) at Spiddal between morphotypes of ballan wrasse (*Labrus bergylta*).

Figure 3.13 represents the frequency of *L. bergylta* individuals presenting extracts (both dorsal skin and scales) with absorbance peaks at different wavelengths according to morphotype at Spiddal 2015 (Figure 3.13 A) and at Spiddal 2016 (Figure 3.13 B). The highest frequencies were seen in both wavelength 384 nm and 668 nm and this applied to all morphotypes. The merle morphotype was most common in both 2015 and 2016 and only wavelengths 384 nm and 668 nm applied to this morph (the 664 nm wavelength was

unrepresented in this morph). Morphotype associated with the rarer 664 nm wavelength varied between years at Spiddal; in 2015, the 664 nm wavelength applied to intermediate and plain morphs, whereas in 2016 this applied to intermediate and spotted morphs.

3.4.11 Morphotype characteristics at Spiddal

A total of 35 *L. bergylta* individuals from four different morphotypes were caught at Spiddal during 2015 and 2016 as shown in Table 3.12. By far the most abundant morphotype in Spiddal was merle in both 2015 and 2016, and all other morphotypes were more abundant in 2016 than 2015 (Table 3.12). Body size was compared across the four morphotypes ('Plain', 'Spotted', 'Merle' and 'Intermediate') (Figure 3.14) by Kruskal-Wallis Test which found significant differences in weight between morphotypes at Spiddal overall in 2015 and 2016 ($P > 0.016$, Kruskal-Wallis = 10.369, $df = 3$)), also in length at Spiddal in 2015 and 2016 ($P > 0.012$, Kruskal-Wallis = 11.006, $df = 3$). A Mann-Whitney test was carried out to see the differences the weight and length between the morphotypes. It was shown that merle fish were significantly lighter than spotted and intermediate fish ($P > 0.000$ for both), and also plain was significant lower in weight than spotted and intermediate ($P > 0.003$ in both cases). Furthermore, merle fish were significantly shorter than spotted and intermediate fish ($P > 0.006$ and 0.000, respectively). Plain fish were also significantly shorter in length than spotted and intermediate morphs ($P > 0.029$ and $P > 0.003$, respectively). As was the case at Carna, merle fish were the smallest morphotype at Spiddal with mean weight of 282 g, and total length 26.05 cm. As before, spotted and intermediate were the heaviest and the longest morphotypes, with weights of 635 g and 680.50 g respectively (Figure 3.14).

Table 3. 12: Number of samples of dorsal skin and dorsal scales for each morphotype of ballan wrasse (*Labrus bergylta*) at Spiddal for 2015 and 2016 (n =35)

Morphotype	**2015**	**2016**
Plain	1	3
Spotted	1	4
Merle	12	10
Intermediate	1	3

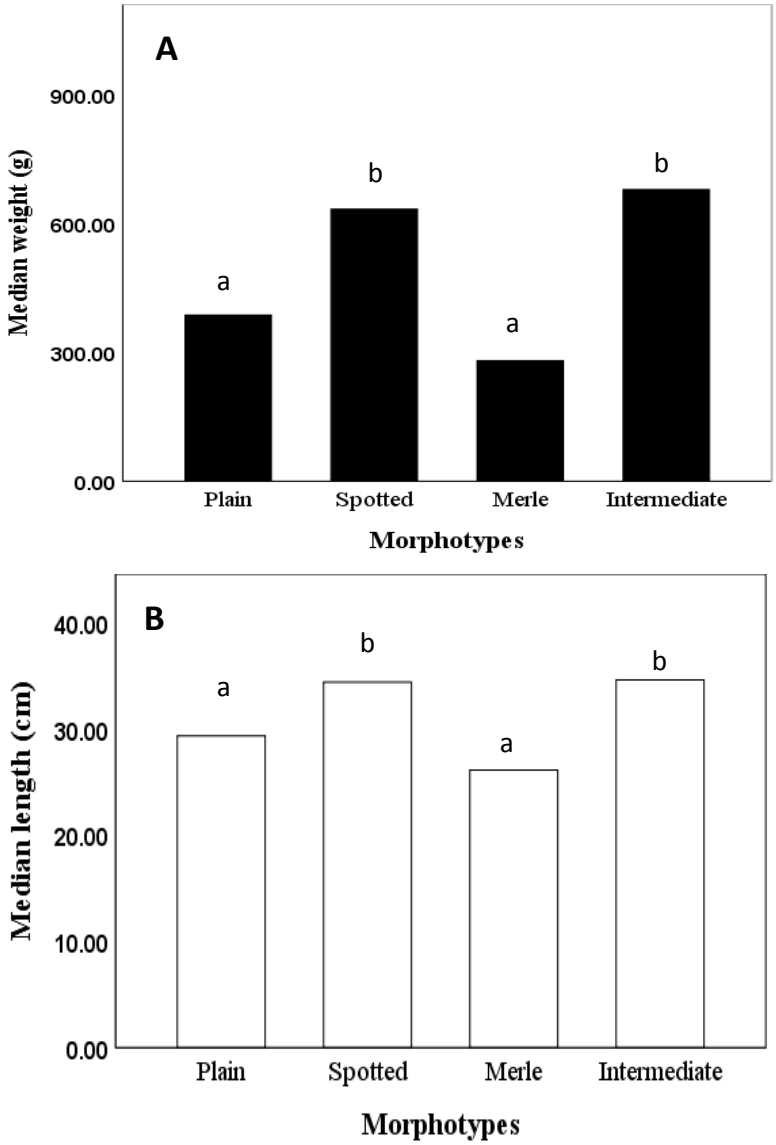


Figure 3. 14: Median weight (g) and length (cm) of four morphotypes of ballan wrasse (*Labrus bergylta*) at Spiddal overall in 2015 and 2016. Median weight at Spiddal (**A**) median total length at Spiddal (**B**), n = 35.

3.4.12 Comparison of biliverdin concentrations in the merle morphotype between 2015 and 2016 at Spiddal

Biliverdin concentrations measured by spectrophotometer in dorsal skin and scales between the years in the merle morphotype (i.e. the only morphotype with sufficient sample size, in 2015 n=12 and 2016 n=10) was analysed by T-test. Prior to this, normality testing was performed and both the skin and scales in 2015 and 2016 were normally distributed (skin 2015: Shapiro-Wilk = 0.898; $d.f$ = 11; P < 0.177, skin 2016: Shapiro-Wilk

= 0.848; *d.f* = 7; *P* < 0.118, scales 2015: Shapiro-Wilk = 0.909; *d.f* = 11; *P* < 0.236, scales 2016: Shapiro-Wilk = 0.875; *d.f* = 7; *P* < 0.204). The T-test showed a significant difference between the years in biliverdin concentration from dorsal skin and dorsal scales: both had highest biliverdin concentrations in 2016, as shown in Table 3.13 and Figure 3.15. As there were not enough samples for each morphotype as shown in Table 3.12, this test was performed only for merle morphs.

Table 3. 13: T-test of mean biliverdin concentration in dorsal skin and dorsal scales for the years 2015 and 2016 in the merle morphotype of ballan wrasse (*Labrus bergylta*) at Spiddal. Mean ± standard error,2015 (n=12) and 2016 (n=10) . Bold indicates a significant difference

	Dorsal skin		**Dorsal scales**	
	Mean±SE	***P*-Value**	**Mean±SE**	***P*-Value**
2015	0.44 ± 0.05		0.37 ± 0.05	
		0.016		**0.009**
2016	0.91 ± 0.15		0.93 ± 0.16	

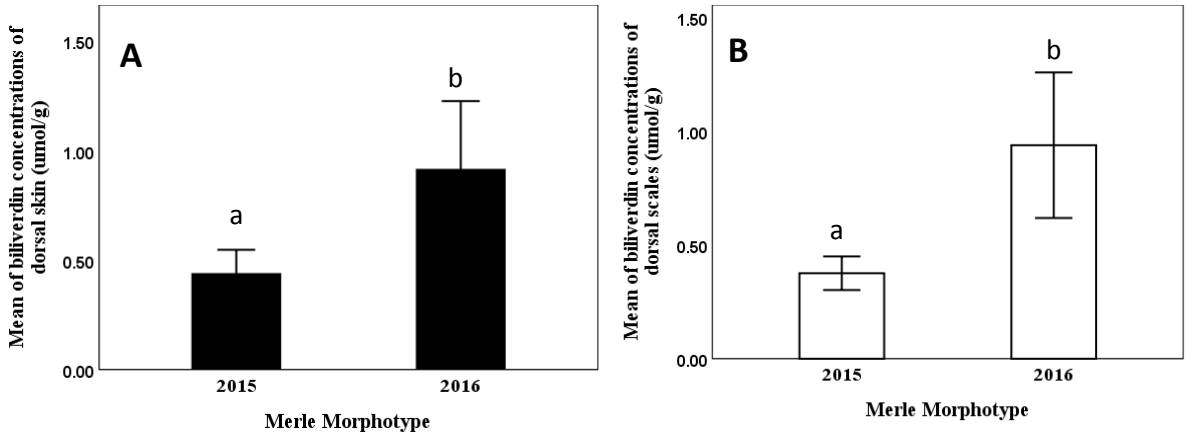


Figure 3. 15: Mean±SE biliverdin concentration of ballan wrasse (*Labrus bergylta*) in **(A)** dorsal skin and **(B)** dorsal scales between the years (2015 versus 2016) in the merle morphotype at Spiddal, n=22 for each year

3.4.13 The relationship between biliverdin concentrations in tissues at Spiddal

The relationship between biliverdin concentration in dorsal skin, dorsal scales and plasma was examined using Spearman's rho correlation analysis for a total of n = 35 *L. bergylta* from Spiddal combined for 2015 and 2016 (i.e. n = 35 skin, n = 35 scales and n = 35 plasma). The relationship between biliverdin concentration in dorsal skin versus dorsal scales was significant and moderately positive with correlation coefficient = 0.500 and $P > 0.009$. The plasma and dorsal scales also presented significant positive correlations, with slightly higher correlation coefficient and fit: Spearman rho = 0.555 and $P > 0.001$. However, the relationship between the plasma biliverdin and dorsal skin concentrations was not significant with correlation coefficient = 0.151 and $P < 0.442$, as shown in Figure 3.16.

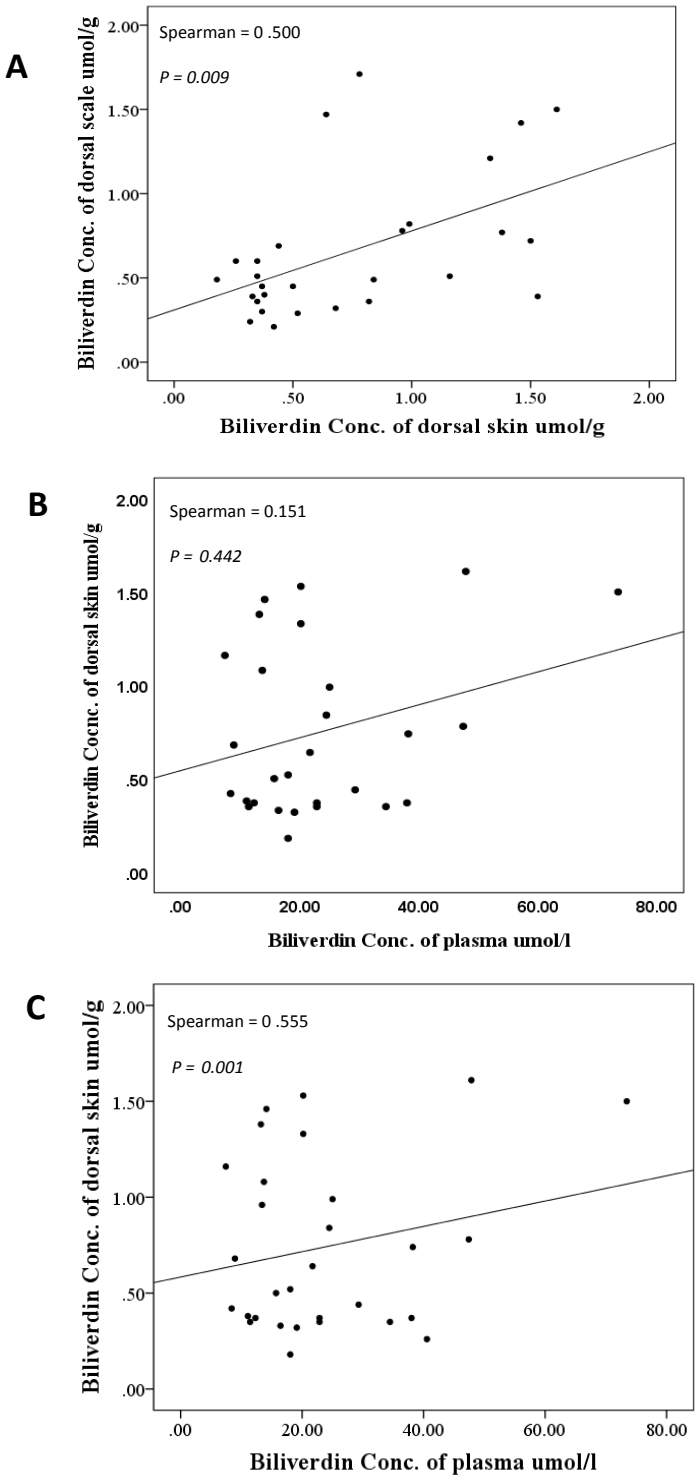


Figure 3. 16: Spearman correlation between biliverdin concentrations of dorsal skin, dorsal scales and plasma overall for 2015 and 2016 in ballan wrasse (*Labrus bergylta*) at Spiddal (n=35). (**A**) Dorsal skin versus dorsal scales. (**B**) Dorsal skin versus plasma. (**C**) Dorsal scales versus plasma.

A final Spearman correlation was performed on all samples at both Carna n = 65 and Spiddal n = 35 combined for both 2015 and 2016 to see whether this provided a better signal of relationships in biliverdin concentrations between different tissues than did separate plots within the locations. The relationship between dorsal skin and scales was again moderately positive and linear with correlation coefficient = 0.589 and $P > 0.000$, while plasma versus dorsal skin was now significant and also linear with correlation coefficient = 0.232 and $P > 0.025$, although with a lot of scatter and a less good fit than the other correlations (Figure 3.17). Plasma biliverdin versus dorsal scales again had a significant linear with correlation coefficient = 0.420 and $P > 0.000$.

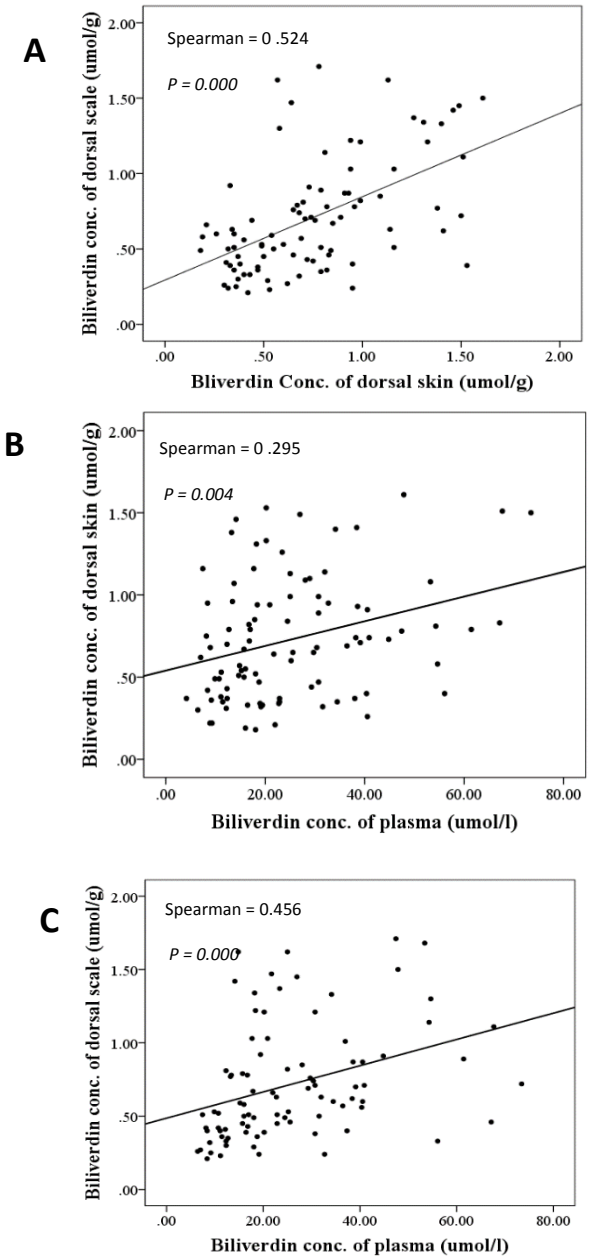


Figure 3. 17: Spearman's correlation between biliverdin concentrations of dorsal skin, dorsal scales and plasma in ballan wrasse (*Labrus bergylta*) overall for 2015 and 2016 at both Carna and Spiddal (n=100). (A) Dorsal skin versus dorsal scales (B) Dorsal skin versus plasma (C) Dorsal scales versus plasma.

3.4.14 Comparison of biliverdin concentrations in skin, scales and plasma between the sexes

A discriminant score (SD) was used to determine the sex, calculated according to the equation proposed by LeClercq et al. (2014) as in chapter 2. Sex determination of males and females was also carried out by dissection (in the breeding season) as described in chapter 2, with dissection/visual inspection confirming the SD when both methods were applied. A Mann-Whitney test was performed to determine the differences, if any, between males and females in biliverdin concentrations expressed in dorsal skin and scales at both locations combined (Carna and Spiddal) in 2016. A T-test was used to carry out the same comparison for biliverdin concentrations in plasma after first testing for normality and homogeneity of variance. The plasma biliverdin concentration was normally distributed with homogenous variances in both males and females (male: Shapiro-Wilk = 0.986; $d.f = 5$; $P < 0.965$, female: Shapiro-Wilk = 0.958; $d.f = 48$; $P < 0.213$). The T-test showed no significant difference ($P < 0.776$) between plasma biliverdin concentrations of male and female fish for samples from both locations combined in 2016, as shown in Figure 3.18. Moreover, Mann-Whitney tests showed no significant differences between males and females in biliverdin concentration in dorsal skin and scales for samples from both locations combined in 2016 as shown in Figure 3.18.

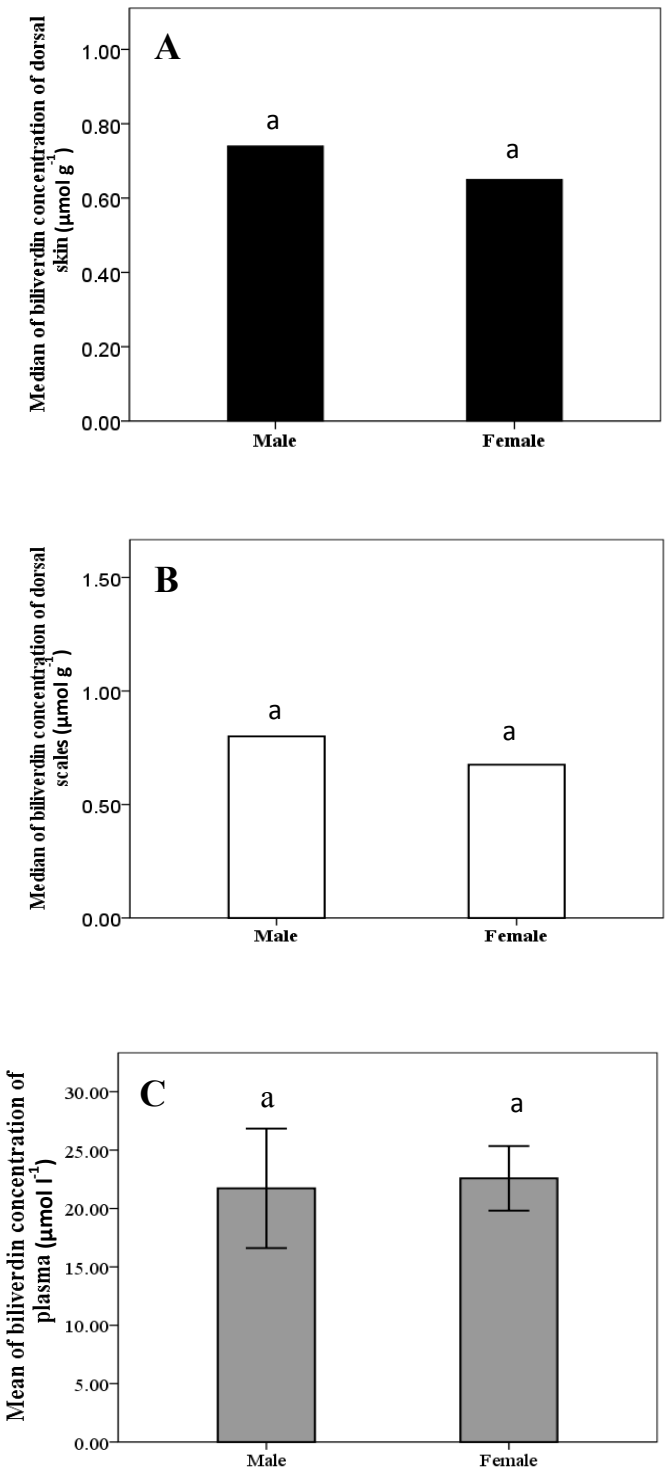


Figure 3. 18: Median biliverdin concentration in ballan wrasse (*Labrus bergylta*) between males and females combined from Carna and Spiddal populations in 2016. **A)** dorsal skin, **B)** dorsal scales **C)** Mean ± SE biliverdin concentration of plasma for the same comparison, n = 55 (Male = 6, Female = 49).

3.5 Discussion

The chemical identity of biliverdin in tissue extracts of *L. bergylta* populations at Carna and Spiddal was confirmed by scanning spectrophotometer with absorption maxima seen in two peaks: the first peak was seen at 384 nm for both Carna and Spiddal and the second at 640 - 644 nm for Carna and at 664 – 668 nm for Spiddal. These results agree with other studies on biliverdin in fish, such as Ellis and Poluhowich (1981) reporting on plasma of *Anguilla rostrata*, where the wavelengths were seen at 378 and 668 nm, and in Gagnon (2006) reporting on plasma of *Notolabrus tetricus,* with peaks in that case between 380–400 nm, and at 640–665 nm.

The absorption spectrum of pigment extracted from skin and scales of *Labrus bergylta* for Carna and Spiddal was related to the colour of the pigment since the wavelength at 640 nm indicated as a blue pigment and that at 668nm denoted a green or greenish (blue-green) pigment, as seen previously in plasma (chapter 2). These results were supported by previous studies by Yamaguchi et al. (1970) on the scales of *Scarus cyawgnathus* where the absorption maxima were at 383 nm and 666 nm, as well as in scales of *Cololabis saira* with absorption maxima at 390 nm and 670 nm (Tsuchiya and Nomura 1955). Both sets of readings were close to the observations in the present study. In contrast, Low and Bada (1974) provided the UV visible absorption spectra for Cabezon (*Scorpaenichthys marmoratus*) which have a blue chromoprotein that showed peaks at 370 and 667 nm and the green chromoprotein showed peaks at 357 and 635 nm. In both cases, the chromoprotein referred-to was biliverdin (Low and Bada 1974). Biliverdin was expressed as either a blue or green pigment, based on its state or environment on the protein (Low and Bada 1974). Its been suggested recently that the variation in biliverdin specific λmax is likely as a result of structural variation in the carrier protein (Clark et al. 2016).

The absorption spectrum of raw plasma of *L. bergylta* in chapter 2 was similar to that of skin and scale tissues in the present chapter. Abolins (1961) also remarked that the plasma spectrum of five species of Labridae demonstrated a similar spectrum to the skin of the East Atlantic peacock wrasse, *Crenilabrus pavo.* In a later study, Yamaguchi el al. (1976) demonstrated that a blue pigment, identified as biliverdin-protein, was the same in all tissues of sculpin. In that instance, Yamaguchi el al. (1976) suggested that the source of biliverdin came from haemoglobin and myoglobin and it combined with protein to safeguard against isomerization and oxidation. The pigment was understood to be temporarily stored in different parts of the body and then transported via the serum to the

skin and eggs at the appropriate time, perhaps for use in camouflage or sexual dimorphism (Yamaguchi el al. 1976). Since then, Clark et al. (2016) carried out some research on different geographic populations of *L. bergylta*, linking geography with morphotypes and colour morphotypes, as well as with plasma pigments. Traditionally *L. bergylta* have been classified into two distinct morphotypes: plain and spotted (Villegas-Ríos et al. 2013a; Almada et al. 2016; Quintela et al. 2016). However, it is clear that additional merle and intermediate morphotypes can be used to classify *L. bergylta*, as has been done in the present study.

The results showed that merle morphs were the smallest and spotted and intermediate morphs were the largest. It has been suggested in the literature that the morphotypes are more than superficial skin morph differences and that they may represent distinctive taxonomic units (Quintela et al. 2016). Although the latter studies provide conflicting evidence as to the validity of this claim, it is clear from these studies, as well as work by Villegas-Ríos (2013b), that major ecological differences exist between plain and spotted morphs, chiefly that growth (in body size) is not proportional between these two morphotypes. Villegas-Ríos (2013b) found that spotted individuals attained larger overall sizes and had an elevated growth rate when compared to their plain counterparts. Spotted morphs were also amongst the largest morph in the present study.

Comparison of biliverdin concentrations between the morphotypes in Carna shows variations in the skin and scales in 2015 only, spotted and intermediate morphs (the largest individuals) had highest biliverdin concentrations, but merle (the smallest individuals) and plain had lowest concentrations. It is likely that the high concentrations of biliverdin in plasma in these same morphs of Carna 2015 (chapter 2 figure 2.24) led to accumulation and expression of these pigments on skin and scales of spotted and intermediate morphotypes. Overall Spearman correlations between plasma and skin/scales (both years and both locations) showed that although both were positively correlated, they were only of moderate strength (plasma versus dorsal skin with Spearman correlation = 0.295, and plasma versus dorsal scales with Spearman correlation = 0.456,. The best evidence to support this finding comes from the studies of Clark (2016) who described colour morphotypes: red, green and ‘intermediate forms’: (a) deep red/brown hue, (b) red/brown hue with little green, (c) less intense green with brown/red inclusions and (d) dark green colouration. Like variation in colour morph, it was noted that there was a disparity in plasma biliverdin between geographic locations, and, like this study, the colour

morphotype analysis demonstrated a slight gradient effect, where green individuals had increased pigment (biliverdin) concentrations relative to the magnitude in red individuals. Colour variations may also be a feature of other fish species. There were slight variations in colour characteristics amongst populations of *Cyclopterus lumpus* (lumpsucker) collected from different areas (Mudge and Davenport, 1986), however, in this case a link with biliverdin was not investigated. It was noted that the roe and subcutaneous jelly were also very variable between populations, which suggested that differences probably come from the genotypic or phenotypic origin (Davenport and Thorsteinsson, 1989).

There were variations in frequency of *L. bergylta* morphotypes between the locations and the years, the most common morphotype in 2015 was the 'intermediate' morph in Carna, but in 2016 this was replaced by the merle morph, and plain morphs were absent or rare (depending on the sampling year) in Carna. In Spiddal, merle was by far the most common morphotype in both years. According to Clark (2016), the sympatric nature of morphotypes within sites and the differential distributions across the sites would suggest that morphotype variations in *L. bergylta* are regional adaptations of crypsis with local 'accents'; suggestive of a strong role in camouflage or a related function. Meanwhile, Gust (2004)showed that demographic parameters (e.g. growth) differed between populations of protogynous coral reef fishes (*Chlorurus sordidus* and *Scarus frenatus*) geographically separated by only tens to thousands of kilometres. The rationale suggested for this was that fish demography is influenced by a number of variables including temperature, the density of competitors, the presence of predators, resource availability, fishing pressure and habitat quality (Villegas-Ríos et al. 2013a). In the present study, there was variability in the body size of the morphotypes, the merle morph was the smallest fish whereas spotted and intermediate were the heaviest and the longest individuals overall for both years 2015 and 2016. This result was agreement with Villegas-Ríos et al. (2013a) that spotted and plain morphotypes displayed differences in the length-weight relationship.

The body colour morphotype of *L. bergylta* is highly diverse (Porteiro et al. 1996) and it has been suggested that the function of such colouration may be in sexual signalling (Mudge and Davenport 1986); however, no link has been made thus far to sex in this species (Villegas-Ríos et al. 2013c). *L. bergylta, Labrus mixtus* and *Notolabrus tetricus* are all hermaphrodites, but whereas these last two display strong external body colourations which are related to sex (Abolins 1961), *L. bergylta* display different colour morphotypes that are not related to sex (Clark et al. 2016). Recent studies by Clark et al. (2016) have noticed no notable difference in the relative concentration of plasma biliverdin related to

sex in *L. bergylta*. Nonetheless, biliverdin was suggested to have biochemical roles, which relate to processes linked to sex (notably hermaphroditism) and thus, were indirectly linked with colour morphotype (Clark et al. 2016). As noted before, this does not explain why males and females possessed similar biliverdin concentrations. The present study adds to this knowledge by showing that concentrations of biliverdin in external tissues (extracts of skin and scales), like plasma (i.e. Clark et al. 2016), also did not differ between males and females, although the method (formula) which was used in the current study to determine the sex of some individuals outside the breeding season was imperfect. It is notable that, in Clark et al. (2016), biliverdin concentration in plasma did not differ related to age, body weight or total length. This is unlike the present study where at Carna (2015), larger morphs (spotted and intermediate) had higher concentrations of biliverdin in external tissues/plasma (see also chapter 2 Figure 2.23, 2.24).

Although, the relationship between circulatory pigments and external tissues was not strong in the present study, there was a slight connection between the biliverdin concentrations in external tissues and the plasma, as well as between both external tissues, in both locations. Therefore, it is likely that increasing the biliverdin concentration in plasma encourages the pigment accumulation in these tissues (Figure 3.19), especially since absorbance spectra of extracts from all tissues were consistent with bilipigments. A high concentration of biliverdin pigments in the circulatory system can cause light green colour in muscles, bones and mucosal tissue in New Guinea lizards (Rodriguez et al. 2018). Also, Davenport and Thorsteinsson (1989) confirmed that skin and subcutaneous jelly colour in *Cyclopterus lumpus* were associated with plasma colour pigments.

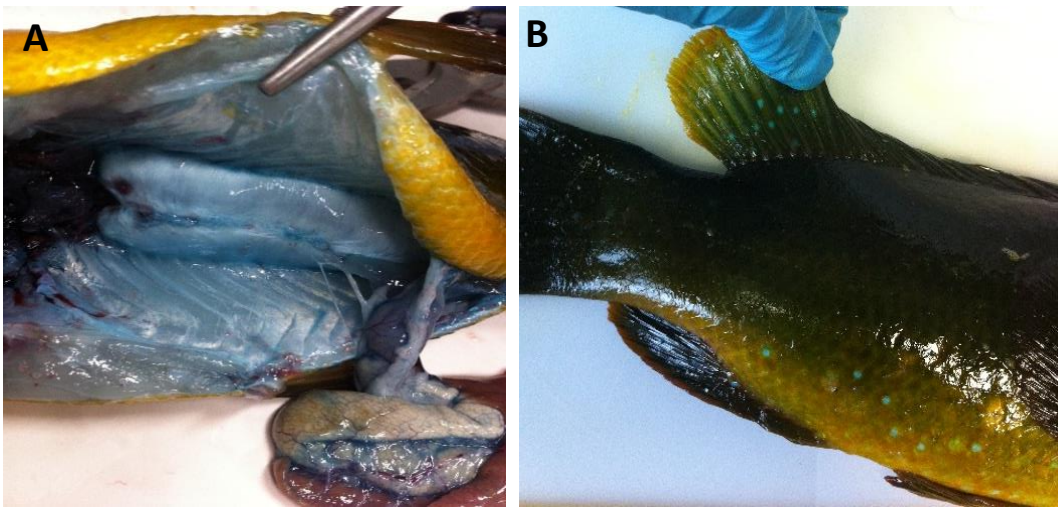


Figure 3. 19: Typical images showing the raw tissues of the stomach + intestine of *L. bergylta* (A) and some blue spots on body of *L. bergylta* (B).

Based on colorimeter readings of raw tissue samples from *L. bergylta* sampled at Carna, the highest degree of skin pigment disparity occurred between dorsal skin and muscle, followed by dorsal skin and ventral skin. Both ventral skin and muscle took up a similar colour space which corresponded to a blue-green to yellow colour, but the dorsal skin was measured with a more intense blue-green colour. By contrast, colorimeter readings of the raw tissue sampled from *L. bergylta* at Spiddal displayed far less organisation in terms of the colour space displayed by individual tissue groups. At Spiddal, the majority of colorimeter readings for muscle was in the green to yellow colour space. This differentiated muscle from skin tissue, due to the lack of blue-green pigment, likely as a result of minimal amounts of incorporated biliverdin in muscle at Spiddal.

The incorporation of biliverdin in muscle tissue as biliverdin-protein has been documented in another wrasse species, the Humphead wrasse (*Cheilinus undulatus*) (Yamaguchi and Matsuura, 1969). However, Yamaguchi and Matsuura (1969) reported there was little evidence of this occurring in *L. bergylta*. However, some muscle tissue of *L. bergylta* in the present study clearly showed blue colouration. There was a clearly observable blue-green colour in muscle and even in the stomach and intestinal region in three fish (Figure 3.19 A). Furthermore, blue spots appeared in some places on the external body surface of *L. bergylta* (Figure 3.19 B) and could be related to biliverdin. This suggests a possible role in crypsis or signalling of some sort (maybe sexual or dominance hierarchy between females who are pre-transition). However, it was only observed rarely and therefore does not explain all patterns between locations and morphs. This might indicate that there is occasionally a large amount of biliverdin mobilised in the body. It should be said that, although some colorimeter readings of the raw tissue samples from fish gathered at Spiddal (2015 and 2016) displayed far less organisation in terms of the colour space of individual tissue groups, it is thought that this was mainly due to operator error in the use of the colorimeter. An additional explanation is the merle morph dominated at Spiddal, and this was the smallest morph that tended to also have low biliverdin concentrations (in both years, but especially in 2015 at Spiddal).

Results from the tissue extracts and the raw tissues were in agreement with respect to *L* a* b** colour space results. The dorsal and ventral skin and dorsal and ventral scales tissue extracts in Carna and Spiddal displayed the same broad difference in the colour values of biliverdin as the raw values. Although there were some variations in colorimeter readings between areas, there was an overall trend for the dorsal skin to be definitively in the dark blue-green area of the colour spectrum. Ventral skin tended to be defined by much lighter

blue-green pigmentation, the origin of the blue-green pigments in the skin of these fish is likely due to the accumulation of the bile pigment biliverdin in the skin. *L. bergylta* have previously been suggested to accumulate biliverdin in their blood serum (Clark et al. 2016) and store it in their skin, resulting in a strong skin pigmentation (Abolins 1961), with the suggestion that body colour *via* concentrated skin biliverdin signals male sexual status (Mudge and Davenport 1986; Gagnon 2006). Although no sex-related patterns in biliverdin concentration of skin or scales was seen in the present study or that of Clark et al. 2016). The stronger blue-green pigmentation seen in the dorsal skin when compared to the ventral skin is an interesting observation for another reason. It is maybe this stark contrast of pigment intensity between dorsal and ventral skin contributes to cryptic countershading, which is a phenomenon that occurs in a huge range of fish in which there is a colour asymmetry between their dorsal and ventral pigmentation (with the dorsal side typically being darker) (Körner 1982). When viewed from above this darker pigmentation makes fish less conspicuous against the sea bed while viewed from below the lighter pigmentation gives the fish a less obtrusive silhouette against the surface (Körner 1982).

Alternatively, this pigment may act as a sort of sunscreen: a biliverdin-protein covering was found on only the skin and fins of *Crenilabrus pavo* through the Spring, and in *Crenilabrus mediterraneus* through the Autumn, has been suggested to have an important role in avoiding negative effects of invisible UV sunrays because the majority of these wrasse exist in tropical waters and are almost completely lacking melanin pigment in their tegument (Yamaguchi 1971). Although it was suggested that biliverdin in cyanobacteria (McDonagh 2001; Narikawa et al. 2015), plants and algae (McDonagh 2001), is used as a biosynthetic precursor for essential photoresponsive bilins, however, the biliverdin in animals and insects is mainly utilised as either camouflage or photoprotection (McDonagh 2001). This is because, as the biliverdin molecule has an ability to be conformationally flexible, their colours extend from blue to green according to the surrounding environment and can be 'tuned' to provide an appropriate cryptic colour (McDonagh 2001).

Sex determination was carried out during dissection, and although this isn't as precise as histological analysis for a sex-changing species, sex determination was approximated in each individual. There was no notable difference between the sexes in biliverdin concentration of any of the tissues examined; skin, scales or plasma. Clark (2016) also demonstrated no important differences in biliverdin concentrations between males and females of *L. bergylta*, but males had lower concentrations than female, transitional

individuals had the lowest concentration of all (i.e. between males, females, and transitional groups) and it was suggested that biliverdin in males was used during sex change for mopping-up harmful molecules released during remodelling of female gonad tissue. Regarding population demographics, Clark (2016) showed the majority of the total population was female with 81 %, 17 % were male and 2 % were identified as undergoing a transition. In the present study (2016 only), the number of females was 89 % and 11 % were male. This agrees with findings of Mohd Yusof, (2015) who found that the population sampled at Carna was dominated by females at every age group with a total of only 13 males plus 1 transitional individual seen overall in an overall sample containing 240 females.

To conclude, the pigment operator of blue-green scales and skin in *L. bergylta* is biliverdin, as shown by spectrum absorbance analysis. Although the main role of biliverdin is still unknown, a weak but significant relationship was identified between biliverdin concentration in circulatory fluids (plasma) and that in external tissues. Therefore, biliverdin was shown to be deposited in the skin and scales, with a possible role in camouflage, or in providing photoprotection, or in signalling of some sort between conspecifics. The latter could possibly include signalling male reproductive status, or dominance hierarchy between females who are pre-transition, or conspecific recognition which is a type of sexual selection. Sexual selection has been suggested as a major driving force beyond the speciation events that result in the spectacular diversity of cichlid fish found in large African lakes (Knight and Turner 2004) and the role of the colour in cichlid mate selection has been the subject of several studies (Seehausen and van Alphen 1998; Maan et al. 2004). It has further been demonstrated that under certain circumstances, sexual selection can lead to sympatric speciation (Arnegard and Kondrashov 2004). Biliverdin concentration in the present study was somewhat associated with the divergence in morphotypes (merle and plain had lowest concentrations in external tissues). Larger spotted and intermediate morphs had higher biliverdin concentrations, but only in some instances (e.g. at Carna 2015). To begin to answer questions about links between morphotypes and sexual selection or signalling in *L. bergylta*, the extent to which there is a link between morphotypes and genetic structure will be examined in the next chapter.

References

Abolins, L. (1961). The existence of sexual dimorphism of blood plasma in fishes of the family Labridae. *Arkiv Zool*, **13**, 541-544.

Almada, F., Casas, L., Francisco, S. M., Villegas-Ríos, D., Saborido-Rey, F., Irigoien, X., and Robalo, J. I. (2016). On the absence of genetic differentiation between morphotypes of the ballan wrasse *Labrus bergylta* (Labridae). *Marine Biology*, **163**(4), 86. doi.org/10.1007/s00227-016-2860-8

Arnegard, M.E. and Kondrashov, A.S. (2004). Sympatric speciation by sexual selection alone is unlikely. *Evolution*, **58**(2), 222-237.

Bada, J. L. (1970). A blue-green pigment isolated from blood plasma of the Arctic Sculpin (*Myoxocephalus scorpioides*). *Specialia*, **15**, 251–252. doi: 10.1007/BF01900075.

Brittain, T. and Wells, R. M. G. (1989). Characteristics of a bili-protein complex in the serum of the stargazer fish (Genyagnus novazelandiae: Uranoscopidae) and its possible role in camouflage. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **92B**(2), 303–306. doi.org/10.1016/0305-0491(89)90282-4

Caglar, M. (1945). Biliverdin as a pigment in a fish. *Nature*, **155**(3944), 670-670. doi.org/10.1038/155670a0

Chapman, D. J., and Fox, D. L. (1969). Bile pigment metabolism in the sea-hare *Aplysia*. *Journal of Experimental Marine Biology and Ecology* **4**(1), 71-78. doi.org/10.1016/0022-0981(69)90024-0

Clark, W., Leclercq, E., Migaud, H., Nairn, J., and Davie, A. (2016). Isolation, identification and characterisation of ballan wrasse *Labrus bergylta* plasma pigment. *Journal of Fish Biology*, **89**(4), 2070-2084.

Clark, W. D. (2016). The isolation, identification and exploration of the biophysiological significance of plasma biliverdin in ballan wrasse (*Labrus bergylta*).

Davenport, J. and Thorsteinsson, V. (1989). Observations on the colours of lumpsuckers, *Cyclopterus lumpus* L. Journal of Fish Biology, 35, 829–838. doi.org/10.1111/j.1095-8649.1989.tb03034.x

Dipper, F. A., Bridges, C. R. and Menz, A. (1977). Age, growth and feeding in the ballan wrasse *Labrus bergylta* Ascanius 1767. *Journal of Fish Biology*, **11**(2), 105–120. doi.org/10.1111/j.1095-8649.1977.tb04103.x

Ellis, M. J. and Poluhowich, J. J. (1981). Biliverdin concentrations of fresh and brackish in the plasmas water eels, *Anguilla Rostrata*. *Comparative Biochemistry and Physiology Part A: Physiology*, **70A**, 587–589. doi.org/10.1016/0300-9629(81)92580-9

Gagnon, M. M. (2006). Serum biliverdin as source of colouration upon sexual maturation

in male blue-throated wrasse *Notolabrus tetricus*. *Journal of Fish Biology*, **68**, 1879–1882. doi.org/10.1111/j.0022-1112.2006.01033.x

Green, J. (1959). Pigmentation of an ostracod, *Heterocypris incongruens*. *Journal of Experimental Biology*, **36**(3), 575-582.

Gust, N., 2004. Variation in the population biology of protogynous coral reef fishes over tens of kilometres. *Canadian Journal of Fisheries and Aquatic Sciences*, **61**(2), 205-218.

Hilldén, N.O. (1981a). Territoriality and reproductive behaviour in the goldsinny, *Ctenolabrus rupestris* L. *Behavioural Processes* **8**,87-90. doi.org/10.1016/0376-6357(81)90001-2

Hilldén, N.O. (1981b). Sociobiology of the labrids of the Swedish west-coast. *Memoranda Societatis pro fauna et flora Fennica*, **57**(7).

Jüttner, F., Stiesch, M. and Ternes, W. (2013). Biliverdin: The blue-green pigment in the bones of the garfish (*Belone belone*) and eelpout (*Zoarces viviparus*). *European Food Research and Technology*, **236**, 943–953. doi.org/10.1007/s00217-013-1932-y

Körner, H. K. (1982). Countershading by physiological colour change in the fish louse *Anilocra physodes* L. (Crustacea: Isopoda). *Oecologia*, **55**(2), 248-250. doi.org/10.1007/BF00384495

Knight, M. E. and Turner, G.F. (2004). Laboratory mating trials indicate incipient speciation by sexual selection among populations of the cichlid fish *Pseudotropheus zebra* from Lake Malawi. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **271**(1540), 675-680.

Leclercq, E., Grant, B., Davie, A., and Migaud, H. (2014). Gender distribution, sexual size dimorphism and morphometric sexing in ballan wrasse *Labrus bergylta*. *Journal of Fish Biology*, **84**(6), 1842-1862. doi: 10.1111/jfb.12402.

Low, S. and Bada, L. (1974). Bile pigments in the blood serum of fish from the family cottidae. *Comparative Biochemistry and Physiology Part A: Physiology*, **47A**, 411–418. doi.org/10.1016/0300-9629(74)90003-6

Maan, M.E., Seehausen, O., Söderberg, L., Johnson, L., Ripmeester, E.A., Mrosso, H.D., Taylor, M.I., Van Dooren, T.J. and Van Alphen, J.J. (2004). Intraspecific sexual selection on a speciation trait, male coloration, in the Lake Victoria cichlid *Pundamilia nyererei*. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, **271**(1556), 2445-2452.

McDonagh, A. F. (2001). Turning green to gold. *Nature Structural Biology*, **8**, 198–200. doi: 10.1038/84915.

Mohd Yusof, M. F. (2015). Reproductive biology and growth of ballan wrasse *Labrus bergylta* in west of Ireland waters, (August). Available at: https://aran.library.nuigalway.ie/handle/10379/5350.

Mudge, M. and Davenport, J. (1986). Serum pigmentation in *Cyclopterus lumpus* L. *Journal of Fish Biology*, **29**, 737–745. doi.org/10.1111/j.1095-8649.1986.tb04989.x

Muncaster, S., Norberg, B. and Andersson, E. (2013). Natural sex change in the temperate protogynous ballan wrasse *Labrus bergylta*. *Journal of Fish Biology*, **82**, 1858–1870. doi: 10.1111/jfb.12113.

Narikawa, R., Nakajima, T., Aono, Y., Fushimi, K., Enomoto, G., Itoh, S., Win, NI-NI., Sato, M. and Ikeuchi, M. (2015). A biliverdin-binding cyanobacteriochrome from the chlorophyll d–bearing cyanobacterium *Acaryochloris marina*. *Scientific reports*, **5**(1), 1-10. doi.org/10.1038/srep07950

Ortolani, A. (1999). Spots, stripes, tail tips and dark eyes: Predicting the function of carnivore colour patterns using the comparative method. *Biological Journal of the Linnean Society*, **67**(4), 433–476. doi: 10.1006/bijl.1998.0299.

Paradis, Y. and Magnan, P. (2005). Phenotypic variation of walleye, *Sander vitreus*, in Canadian Shield lakes: New insights on percid polymorphism. *Environmental Biology of Fishes*, **73**, 357–366. doi: 10.1007/s10641-005-2261-1.

Pita, P. and Freire, J. (2017). Trophic ecology of an Atlantic kelp forest fish assemblage (NW Spain) targeted by recreational fishers and implications for coastal management. *Journal of the Marine Biological Association of the United Kingdom*, 1–11. doi: 10.1017/S0025315417001862.

Porteiro, F. M., Barreiros, J. P. and Santos, R. S. (1996). Wrasses (Teleostei: Labridae) of the Azores wrasses. *ARQUIPÉLAGO. Ciências Biológicas e Marinhas= Life and Marine Sciences*, **14A**, 23–40.

Quintela, M., Danielsen, E. A., Lopez, L., Barreiro, R., Svåsand, T., Knutsen, H., Skiftesvik, A. B and Glover, K. A. (2016). Is the ballan wrasse (*Labrus bergylta*) two species? Genetic analysis reveals within-species divergence associated with plain and spotted morphotype frequencies. *Integrative zoology*, **11**(2), 162-172. doi.org/10.1111/1749-4877.12186

Ralls, K., and Mesnick, S. (2008). Sexual Dimorphism. *Encyklopedia of Marine Mammals*, 1005–1011.

Rodriguez, Z. B., Perkins, S. L., and Austin, C. C. (2018). Multiple origins of green blood in New Guinea lizards. *Science advances*, **4**(5), eaao5017. doi: 10.1126/sciadv.aao5017

Seehausen, O. and van Alphen, J.J. (1998). The effect of male coloration on female mate choice in closely related Lake Victoria cichlids (*Haplochromis nyererei* complex). *Behavioral Ecology and Sociobiology*, **42**(1), 1-8.

Sherwood, G. D. and Grabowski, J. H. (2010). Exploring the life-history implications of colour variation in offshore Gulf of Maine cod (*Gadus morhua*). *ICES Journal of Marine Science*, **67**(8), 1640–1649. doi: 10.1093/icesjms/fsq094.

Sjölander, S., Larson, H., and Engström, J. (1972). On the reproductive behaviour of two labrid fishes, the ballan wrasse (*Labrus bergylta*) and Jago's goldsinny (*Ctenolabrus rupestris*). *Revue du comportement* animal, **6**,43-51.

Sujiwo, J., Kim, D., Yoon, J. Y., Kim, H., Kim, J. S., Lee, S. K., and Jang, A. (2017). Physicochemical and functional characterization of blue-shelled eggs in Korea. *Korean Journal for Food Science of Animal Resources*, **37**(2), 181. doi: 10.5851/kosfa.2017.37.2.181

Tickner, T. R. and Gutteridge, J. M. C. (1978). A simple colorimetric method for the estimation of plasma biliverdin. *Clinica Chimica Acta*, **85**(2), 125–129. doi: 10.1016/0009-8981(78)90231-0.

Tsuchiya, Y., and Nomura, t. (1955). Studies on the blue green pigments of the integuments of fishes (1) the isolation of ichthyoverdin from the scales of saury, cololabis saira brevoort Studies on the blue green pigments of the integuments of fishes (1) the isolation of ichthyoverdin from the scales of sauries, *cololabis saira* brevoort. *Tohoku Journal of Agricultural Research*, **6**(1), 75-83.

Villegas-Ríos, D. (2013b). Life-history and behaviour of "*Labrus bergylta*" in Galicia. Ph.D. University of Vigo, Galicia, Spain.

Villegas-Ríos, D., Alonso-Fernández, A., Fabeiro, M., Bañón, R., and Saborido-Rey, F. (2013a). Demographic variation between colour patterns in a temperate protogynous hermaphrodite, the ballan wrasse *Labrus bergylta*. *PLoS One*, **8**(8), e71591. doi.org/10.1371/journal.pone.0071591

Villegas-Ríos, D., Alonso-Fernández, A., Domínguez-Petit, R., and Saborido-Rey, F. (2013c). Intraspecific variability in reproductive patterns in the temperate hermaphrodite fish, *Labrus bergylta*. *Marine and Freshwater Research*, **64**(12), 1156-1168. doi.org/10.1071/MF12362

Yamaguchi, K. (1971). Biliproteins of marine animals. *Bulletin of the Japanese Society of Scientific Fisheries*, **37**(4), 339–354. doi: 10.2331/suisan.37.339.

Yamaguchi, K., Hashimoto, K. and Matsuura, F. (1976). Identity of blue pigments obtained from different tissues of the sculpin, *Pseudoblennius percoides* Günther. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **55B**, 85–87. doi.org/10.1016/0305-0491(76)90177-2

Yamaguchi, K. and Matsuura, F. (1969). A blue pigment from the muscle of a marine teleost, "Hirosa", *Cheilinus undulatus*'. *Bulletin of the Japanese Society of Scientific Fisheries*, **35**(9), 920–927. doi: 10.2331/suisan.35.920.

Yan, F., Zhang, Y., Jiang, R., Zhong, M., Hu, Z., Du, H., Lun, J., Chen, J.and Li, Y. (2011). Identification and agglutination properties of hemocyanin from the mud crab (*Scylla serrata*). *Fish and Shellfish Immunology*, **30**(1), 354-360. doi.org/10.1016/j.fsi.2010.11.004

Yu, C. L., Ferraro, D., Ramaswamy, S., Schmitz, M. H., Schaefer, W. F., and Gibson, D.

T. (2008). Purification and properties of Sandercyanin, a blue protein secreted in the mucus of blue forms of walleye, *Sander vitreus*. *Environmental Biology of Fishes*, **82**(1), 51-58. doi.org/10.1007/s10641-007-9252-3

Zhang, Y., Yan, F., Hu, Z., Zhao, X., Min, S., Du, Z., Zhao, S., Ye, X., and Li, Y. (2009). Hemocyanin from shrimp *Litopenaeus vannamei* shows hemolytic activity. *Fish and Shellfish Immunology*, **27**(2), 330-335. doi.org/10.1016/j.fsi.2009.05.017

Chapter 4: Genetic discrimination between local populations and morphotypes of ballan wrasse (*Labrus bergylta*) in the West coast of Ireland

4.1 Abstract

Ballan wrasse, *Labrus bergylta* (Labridae), is a protogynous hermaphrodite that inhabits the eastern North Atlantic coastal waters, from Norway to Morocco. It is a commercially valuable species that is commonly used as a cleaner fish in salmon farms in northern Europe for the purpose of controlling parasites. Previous studies have shown differences in life history strategies between two morphotypes (plain and spotted), such as age and body size (growth). However, the genetic structure, particularly whether this varies between morphotypes, is currently unclear. In the present study, four different morphotypes; plain, spotted, merle and intermediate, were analysed using 2 genetic markers, i.e. the mitochondrial cytochrome oxidase subunit I (COI) gene and mitochondrial DNA control region (CR) sequences. Genetic population variation in *L. bergylta* morphotypes was examined at two locations in Co. Galway, Carna and Spiddal, sampled in 2015 and 2016 (n=119 individuals). CR sequences were also compared with unknown morphs of *L. bergylta* at ten locations that were grouped into 3 regions based on latitude ('Britain and Ireland', 'Azores' and 'Norway') after accessing sequence information from Genbank. Neither haplotype networks (COI/CR genes) nor AMOVA (CR) showed genetic differences between the morphotypes. Instead, pairwise comparisons showed significant F_{ST}s differences between particular morphs at Carna or Spiddal, or for a particular morph between Carna and Spiddal, but not in any consistent way that indicates differences by location or morphotype. Importantly, the COI gene grouped all morphs together relative to closely related *Labrus* species; suggesting good separation of species for this barcoding marker, despite the morphometric differences. From both Carna and Spiddal combined, spotted morphs showed the highest haplotype (h) and nucleotide (π) diversity values, whereas lower values were noted within the plain morphs (with the proviso that the latter could have been due to low number of samples). Ignoring morphotype and placing the samples from this study within the broader geographic regional context (i.e. Ireland/UK, Norway and Azores) using the CR gene showed that populations at the Azores and Norway regions were highly distinct and separated from other regions. However, Norway was not fully separated. Furthermore, the lowest value for haplotype diversity was within Azores (Santa Maria), although this was followed by one of the Irish shores (Carna). Similarly, nucleotide diversity (π) among locations was low in Azores (Corvo and Santa María), Carna, and Søgne (Norway); but all other shores including a Norwegian shore had higher

values (0.024 - 0.034). These values of genetic variability may be useful in future, given the extraction of a large biomass of wild *L. bergylta* by the salmon farming industry. Since the results from these genetic markers do not indicate genetic variation between the morphotypes, additional studies (including additional genetic markers) are necessary to examine the reasons behind the morphotype variations in ballan wrasse life history. Moreover, given high extraction of biomass by aquaculture industry and recreational fisheries, genetic variation in *L bergylta* from more geographic areas needs to be investigated in the future.

Keywords: *Labrus bergylta*, morphotypes, control region, cytochrome oxidase subunit I, regions.

4.2 Introduction

Taxonomic classification of the Labridae fish family has been revised considerably in recent years, with major lineage splits occurring in several genera including *Labrus* and *Symphodus* (Almada et al. 2002). Within these genera, a high degree of intraspecific polymorphism can be observed. This can typically be observed in the form of various skin colour and patterning differences that occur between individuals of the same species. Among the different species of coregonus, the occurrence of variable colour morphs is not uncommon, although many of these examples are typically related to sexual dimorphism or different ecotypes (Bernatchez et al 1996). Like many wrasse species, *L. bergylta* is known to have a number of distinctive colour morphs but, uniquely among the Labridae, these varying morphotypes have no correlation with sex, and have yet to be linked with any biological trait of this species (Villegas-Ríos 2013a).

Within the literature, *L. bergylta* is typically subdivided into two distinctive morphotypes: plain and spotted. Plain fish are defined by a uniform, but variable body colour, while spotted fish are characterised with a dark red or orange body covered with white spots (Villegas-Ríos et al. 2013b; Almada et al. 2016). The unique morphotypic variability within *L. bergylta* has recently attracted considerable attention, particularly with regards to the taxonomic significance of various colour and pattern morphs. Quintela et al. (2016) suggested that the variation in morphotypes may represent distinctive taxonomic units (i.e. incipient sub species), although some genetic overlap between morphotypes was not ruled out (Quintela et al. 2016). Seljestad et al. (2020) were also noted distinct genetic variations between plain and spotted morphs in Galicia, these were not apparent in Scandinavia. This was supported by findings of Villegas-Ríos (2013a) who showed that growth varied between plain and spotted morphotypes, with spotted individuals displaying a more rapid growth rate than plain individuals as well as larger overall sizes.

Quintela et al. (2016) found little evidence of separate breeding groups in *L. bergylta* based on the mitochondrial cytochrome oxidase subunit I (COI) gene, however a set of 20 microsatellite loci allowed further detailed analysis of gene flow and population differentiation (Quintela et al. 2016). Following statistical analysis, it was shown that there was a significant difference in the microsatellite markers between spotted and plain morphotypes. Quintela et al. (2016) concluded that, although the degree of genetic disparity was not sufficient to consider them separate breeding groups, nevertheless, the

observed generic structure was highly associated with the two morphotypes. These results agree with Seljestad et al. (2020) studies, who noted distinct genetic variations were between plain and spotted morphs in Galicia but were not apparent in Scandinavia. Again, this supports the findings of Villegas-Ríos et al. (2013b), that both morphotypes followed different life histories. However, the findings of Quintela et al. (2016) and Seljestad et al. (2020) are strongly contradicted by Almada et al. (2016), who found no genetic difference between the same two morphotypes. In the latter study, the mitochondrial control region (CR), COI, as well as the 18S ribosomal RNA gene and the nuclear S7 ribosomal protein gene were sequenced. Analysis of these yielded no significant differences between plain and spotted morphotypes. With such contradictory evidence of genetic disparity between morphotypes of *L. bergylta* in the literature, there is a clear need for additional studies to gain a comprehensive insight into the complex genetic structure of this highly polymorphic fish species.

Additional information was provided in an analysis of the phylogeographic structure of *L. bergylta,* in various regions in the North Atlantic conducted by D'Arcy et al. (2013). DNA samples from *L. bergylta* collected from three regions: Ireland, Scotland and Norway were analysed, also using the CR. Following this, the number and composition of polymorphic regions, haplotype diversity, and nucleotide diversity were calculated, as was the spatial genetic structure and date of the most recent common ancestor. The results of this study indicated that levels of haplotype diversity varied among regions, with Norwegian wrasse displaying lower levels when compared to their Irish and Scottish counterparts. D'Arcy et al. (2013) suggested that this low haplotype diversity indicated that wrasse, which come from a family of fish with origins in the tropics, were relatively recent colonisers of Scandinavian waters, and underwent a rapid recolonization by a low number of individuals following recent glacial retreats.

Almada et al. (2017) conducted a similar study where they analysed historical gene flow of *L. bergylta* across the north Atlantic. Samples were collected from regions as far south as Spain to a northern maximum of Scandinavia. From these samples, DNA was extracted and the CR and first intron of the nuclear S7 ribosomal protein gene, were amplified. Haplotype networks were built and analysis of haplotype diversity within each sample was estimated, to evaluate potential population differentiation. Following this, analysis of molecular variance (AMOVA) was used to compute pairwise FST estimates and corrected pairwise differences between populations. This study also found a significant decrease in

haplotype diversity as you move north, which agrees with the findings of D'Arcy et al. (2013). Additionally, the Azorean population of *L. bergylta* was found to be genetically distinct from all other regions. Almada et al. (2017) suggested that this distinct population could be explained by factoring-in discrepancies in oceanic circulation. These discrepancies can influence larval dispersal, resulting in pockets of genetically isolated subpopulations. This is especially true for the geographically distant Azorean population, with oceanic circulation preventing gene flow between them and the main European populations. The low degree of haplotype diversity seen in Scandinavia was explained by the recolonization of these waters following glacial retreat, just as D'Arcy et al. (2013) suggested.

All studies above recognised two morphotypes in *L. bergylta*, plain and spotted; however additional morphotypes exist in ballan wrasse, which are genetically unexamined to date. The primary aim of the present study was to investigate the genetic variation, including haplotype diversity and nucleotide diversity, in a range of *L. bergylta* morphotypes using the control region (CR) and the mitochondrial cytochrome oxidase subunit I (COI) gene. In addition, the study will examine how the genetic structure found in these morphotypes at two locations in the west of Ireland, Carna and Spiddal, compares to other parts of the geographic range.

4.3 Materials and Methods

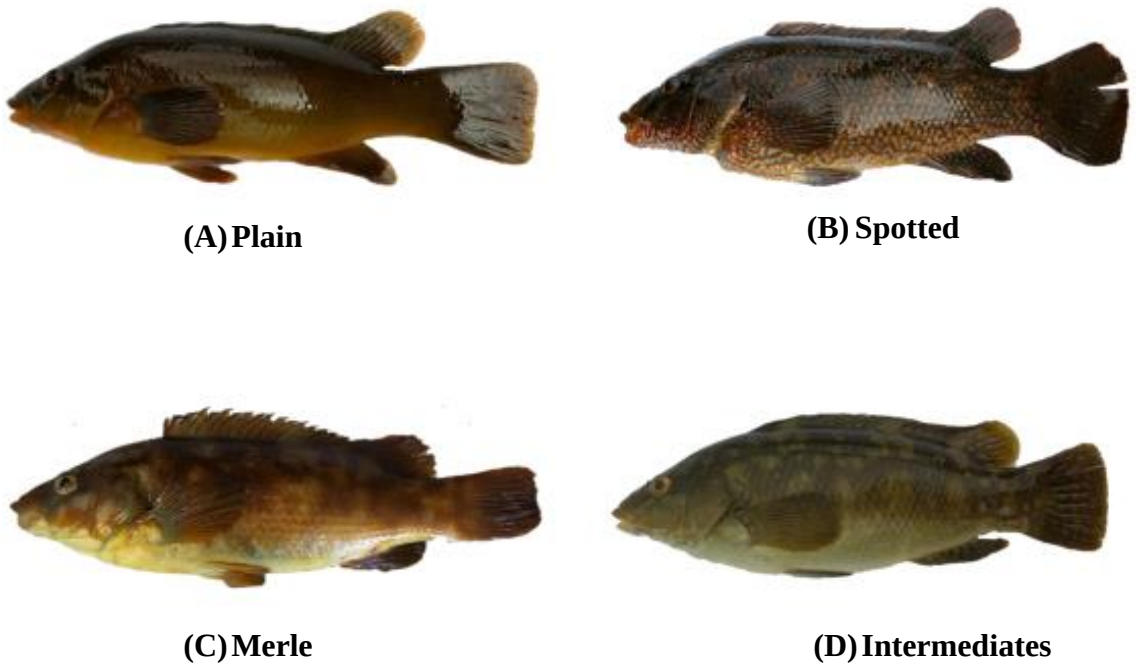
4.3.1 Locations and sampling

Two sampling locations were selected, Mweenish Island rocky shore near Carna (53°18'01.9"N 9°51'09.6"W) and Spiddal Pier (53°14'24.8"N 9°18'39.9"W) (-see Figure 2.1 in Chapter 2). Specimens were collected during 2015 and 2016 in the summer period from June to October. The samples were collected by two techniques, by local fishermen using lobster pots for the samples from Carna 2015 and using rod and line for samples from Spiddal 2015, 2016 and Carna 2016. Initially, common shrimp, mussel flesh, shore crab and limpet flesh were used as bait, with mussel flesh being the preferred one on the basis that this was the easiest to collect, or buy, and could be stored, making it the most practical bait to use. In total 119 fish were caught, with n=64 from Carna and n=55 from Spiddal. The sampled *L. bergylta* presented some highly colourful patterns and variable morphotypes. Spotted fish were identified as having a covering of white, blue or green spots, while plain fish featured a uniform but variable body colour. Merle fish were characterised as having a striped pattern, which occurs on their flanks (i.e. around the area of the lateral line). There is also an intermediate morph that is characterised as having features in common with both merle and spotted patterned fish (Figure 4.1).

Once an individual fish was captured, it was anaesthetised by transferring it to a tank of seawater containing MS-222 (Tricaine methanesulfonate, Pharmaq, Oranmore Business Park). Morphometric measurements of total length (cm) and body weight (g) were taken and individual fish were labelled and photographed in order to assign them to one of four morphotypes (plain, spotted, merle and intermediate). Morphotypes were recognised both in and out of the water as finer details of the fish colour morph were not always clear out of water. The fish were then immersed in an overdose of MS-222 until they were unresponsive. Selected individuals were stored in marked bags and placed on ice during transportation back to the laboratory for later processing. The fish were then dissected, removing the internal organs, along with a tissue section taken from the abdominal muscle which was preserved in 100 % ethanol at -20 °C for later use.

To broaden the molecular analysis for this chapter, 10-20 sequences of the CR from each of 8 locations from previous studies (D'Arcy et al. 2013; Almada et al. 2017) were included in the study as a comparison. This was carried out in order to place the genetic variation of the samples from Carna and Spiddal into geographic context. The sampled locations were Bertraghboy Bay and Mweenish Island Co. Galway (West coast of Ireland);

the islands of Santa María and Corvo in the Azores; Portaferry in Co. Down Northern Ireland and Loch Sunart in the West of Scotland (D'Arcy et al. 2013); Hidra and Søgne in the South of Norway (Almada et al. 2017). Ascension numbers of CR sequences of all the above in GenBank as shown in Table 4.1.



(A) Plain **(B) Spotted**

(C) Merle **(D) Intermediates**

Figure 4. 1: Ballan wrasse (*Labrus bergylta*) morphotypes: Photographs of (A) Plain (B) Spotted (C) Merle and (D) Intermediate.

Table 4. 1: GeneBank accession numbers of control region sequences of ballan wrasse (*Labrus bergylta*) used for broadening the molecular analysis.

Region	Accession number	Reference
Britain and Ireland	KC477846 –KC477936	(D'Arcy et al. 2013)
Norway	KC477846 –KC477936	(D'Arcy et al. 2013)
Azores	KU751889–KU752182	(Almada et al. 2017)

4.3.2 DNA extraction and PCR

DNA was extracted using a NucleoSpin® Tissue kit (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. Regions of the mitochondrial cytochrome oxidase subunit I (COI) gene and mtDNA control region (CR) were amplified and sequenced. For COI, the universal primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al. 1994) were used. For the control region, forward Primer A (5'-TTCCA CCTCT AACTC CCAAA GCTAG-3') (Lee et al. 1995) was used in conjunction with reverse primer TDKD of (5'- CCTGAAGTAGGAACCAGATG-3') (Kocher et al. 1993) (Eurofins Scientific, Brussels, Luxembourg). Each 25 µl PCR reaction mix comprised 2.5 µl of DNA, 12.5 µl Premix-Go Tag G2 Green Mix (Promega, Wisconsin, US), 9 µl sterile H_2O and 0.5 µl of each of the forward and reverse primers. PCR cycling conditions included a 5 minutes initial step at 94 °C followed by 35 cycles of 1 minute at 94 °C, 1 minute at 50 °C, 1 minute 30 second at 72 °C followed by a final extension step of 5 minutes at 72 °C. PCR yields were purified using a purification kit (Quick PCR purification kit, PureLink™, Thermo Scientific, Waltham, United States), and amplicons sequenced by GATC-Biotech (Konstanz, Germany) using the forward primers only.

4.3.3 Sequence analysis

The electropherograms were checked and any poor-quality sequences were discarded. Sequences were aligned using MEGA 6.0 software (Tamura et al. 2013). A separate alignment matrix was built for the COI and CR. All of the sequences were performed using DnaSP v6.12.01 (Rozas et al. 2017). The ARLEQUIN software v3.5.2.2 (Excoffier and Lischer 2010) was used to calculate the number of polymorphic sites, haplotype diversity, and nucleotide diversity. To explore the variation between the morphotypes at different locations, AMOVA was performed using ARLEQUIN, which calculated conventional *F* - statistics using haplotype frequency. In order to test the hypothesis of morphotype subdivision (i.e. all plain of Carna and Spiddal are in one group and spotted of Carna and Spiddal are in another group and so on), four morphs were specified at each of two locations (i.e. Carna plain, Carna spotted, Carna merle, Carna intermediate, Spiddal plain, Spiddal spotted, Spiddal merle and Spiddal intermediate) prior to AMOVA. *F* - statistics were calculated on the basis of haplotype frequency for CR between morphotypes, also within morphotypes across locations (Carna and Spiddal), as well as between individuals. Pairwise differences for any significant terms in the AMOVA were examined using

ARLEQUIN, where pairwise F-statistics (FST) were calculated using haplotype frequencies and significance tests were carried out after 10000 permutations.

AMOVA was also performed on CR sequences among the ten sampling 'Locations' organised into three 'Regions' including a total of n=226 sequences. These locations were organised into 'Regions' termed 'Britain and Ireland', 'Norway' and 'Azores' for the AMOVA. *F* - statistics were calculated on the basis of haplotype frequencies between sampling locations and regions. As before, pairwise differences for any significant terms in the AMOVA were examined using ARLEQUIN, where pairwise F-statistics (FST) were calculated using haplotype frequencies and significance tests were carried out after 10000 permutations.

In addition to analysing haplotype frequencies using AMOVA, haplotype networks were built from the sequences using TCS 2.1 (Clement et al. 2000) with default settings of a 95% connection limit and with gaps set as a 5th state. Networks were first built including morphotype information at Carna and Spiddal only (n=119 individuals), with separate networks being built for CR and COI. A further haplotype network was built to examine whether all morphs of *L. bergylta* were the same species. This haplotype network was based on COI and included all *L. bergylta* (n=119) from Carna and Spiddal as well as sequences of COI from Genbank for other *Labrus* species (green wrasse *Labrus viridis* n= 1 (Accession numbers: GQ341596) and cuckoo wrasse *Labrus mixtus* n=12 (Accession numbers: JN231248-JN231253, KJ768240- KJ768243 and KJ1285116- KJ1285117)) which are close relatives (Hanel et al. 2002), providing 'outgroup' sequence for this analysis. A fourth haplotype network was built without morphotype information and based on the CR at 10 locations, as described above (n=226 sequences). Results were visualised using PopART software version 1.7 (Leigh and Bryant 2015).

4.4 Results

The Control region (CR) alignment matrix was 347 bp long with 119 sequences in total (64 sequences from Carna and 55 sequences from Spiddal) for which morphotype information was available. CR sequences yielded 60 distinct haplotypes with 65 polymorphic sites and overall haplotype (gene) diversity was 0.961. Haplotype diversity within the morphotypes ranged from 0.985 - 0.888 (Table 4.2) and indicated only slight differences between morphotypes. The highest haplotype (*h*) and nucleotide (π) diversity values and mean number of pairwise differences were observed within the spotted morphotype, while the lowest of these were observed within the plain morphotype (Table 4.2). However, the merle morphotype had the highest number of haplotypes (*N*H = 36) and the highest number of polymorphic sites (PS) overall. Please note that fewer plain morphs were sampled compared to the other groups which possibly contributed to small sample artefacts.

Table 4. 2: Haplotype numbers and diversity indices for ballan wrasse (*Labrus bergylta*) of control region (CR) for morphotypes in Carna and Spiddal in 2015 and 2016 overall.

	Control region (CR)			
	Plain	Spotted	Merle	Intermediate
N	9	24	60	26
*N*H	6	21	36	16
PS	21	41	56	35
h	0.888	0.985	0.951	0.944
π	0.016	0.027	0.023	0.022
PD	5.833	9.590	8.006	7.633

N number of individuals, *N*H number of haplotypes, PS polymorphic sites, *h* Haplotype (gene) diversity, π Nucleotide diversity, PD mean number of pairwise differences.

The haplotype networks built using the CR revealed two distinct groups (clusters) within the haplotype network, separated by 12 mutational steps (Figure 4.2). Each cluster included representatives from both morphotypes and sampling locations, hence there was no structure associated with either of these factors. Cluster 1 was more branched and complicated than cluster 2.

Table 4.3 shows haplotype numbers for CR in morphotypes at Carna and Spiddal separately (2015+2016 overall), although please note that breaking this down meant that there were some very small sample sizes. The highest number of haplotypes was merle from Spiddal and represented 27 haplotypes. Haplotype diversity (*h*) within the

morphotypes at Carna ranged from 1.000 to 0.895, whereas at Spiddal this was lower, and ranged from 1.000 to 0.773 (Table 4.3). Low numbers of individuals for some of the morphotypes within locations may have introduced artefacts, e.g. only 3 – 6 individual wrasse were available for the plain morphs at Carna, or for plain, spotted and intermediate morphs at Spiddal. The nucleotide diversity (π) within morphotypes at Carna was higher in spotted and intermediate morphs (respectively 0.025, 0.024) than in the plain and merle (0.006, 0.017). Nucleotide diversity and mean number of pairwise differences within morphotypes at Spiddal was higher in spotted, merle and plain (respectively 0.038, 0.026, 0.022) than in the intermediate morphs (0.010), but again this could have arisen due to small sample sizes (Table 4.3).

Table 4. 3: Haplotype numbers and diversity indices for ballan wrasse (*Labrus bergylta*) of control region (CR) for morphotypes in Carna and Spiddal separately (2015+2016 overall).

	Control region							
	Carna				Spiddal			
	Plain	Spotted	Merle	Intermediate	Plain	Spotted	Merle	Intermediate
N	3	18	21	22	6	6	39	4
*N*H	3	17	14	15	3	6	27	3
PS	3	37	31	34	20	29	51	7
h	1.000	0.993	0.895	0.961	0.733	1.000	0.972	0.833
π	0.006	0.025	0.017	0.024	0.022	0.038	0.026	0.010
PD	2.000	8.555	5.881	8.277	7.600	13.400	9.056	3.500

N number of individuals, *N*H number of haplotypes, PS polymorphic sites, *h* Haplotype (gene) diversity, π Nucleotide diversity, PD mean number of pairwise differences.

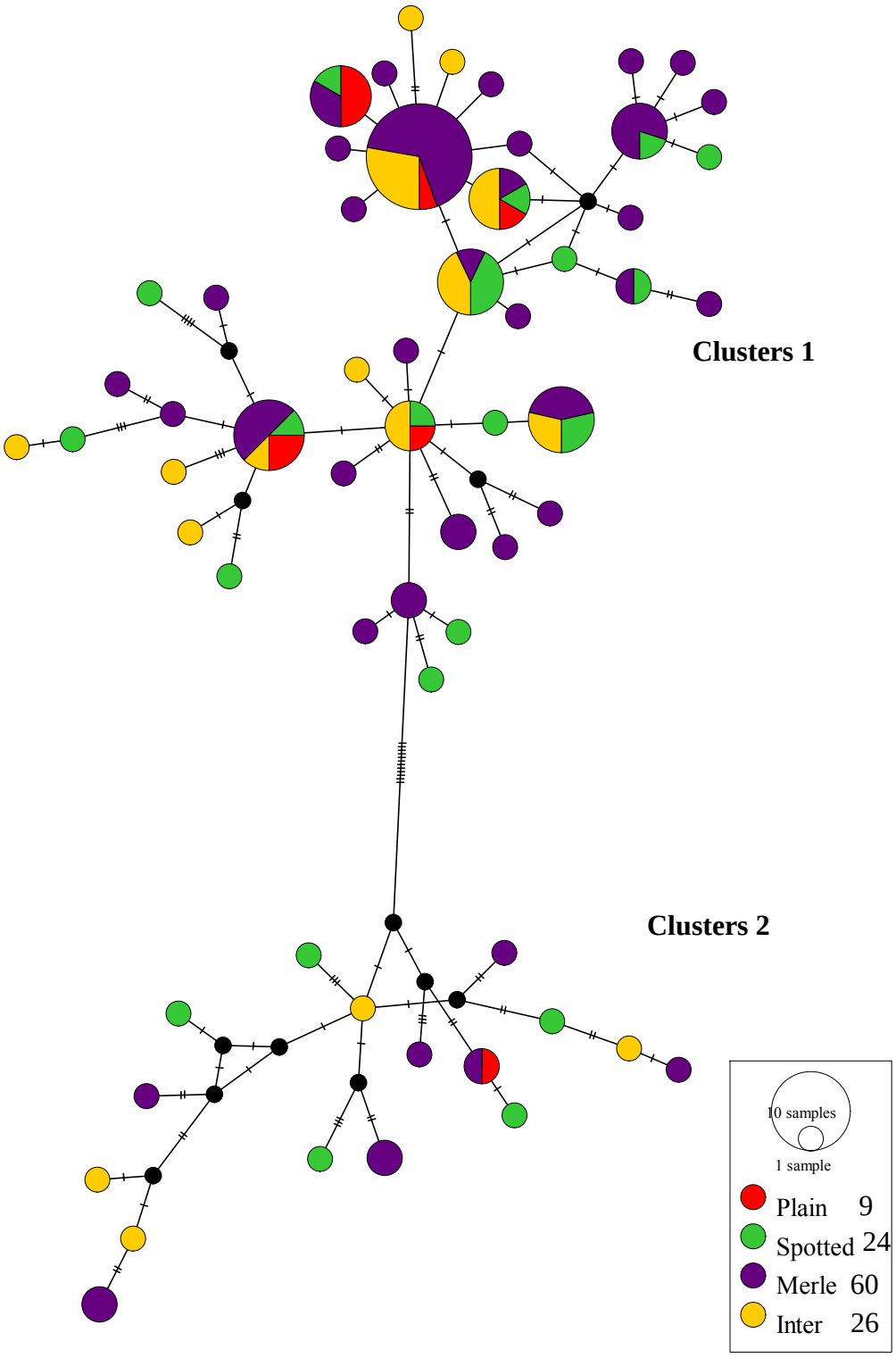


Figure 4. 2: Haplotype network for control region of ballan wrasse (*Labrus bergylta*) morphotypes. Colours denote the morphotypes sampled overall at Carna and Spiddal in 2015 and 2016. The area of the circle is proportional to each haplotype frequency and sample size of fish (n) is also indicated in the legend. A black circle is an inferred median. Dashes on the lines represent mutational steps between haplotypes.

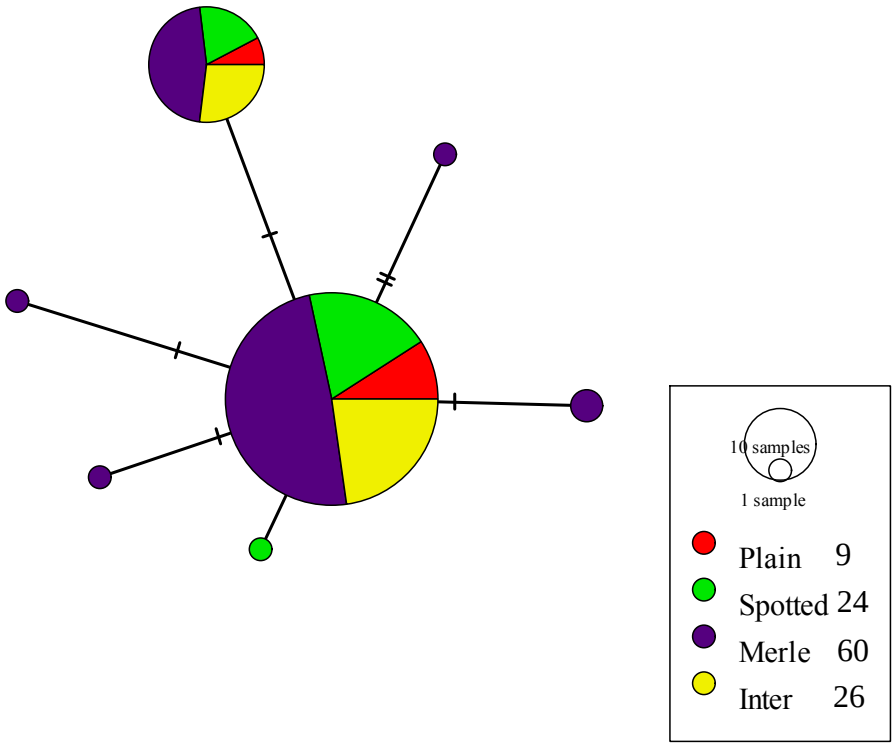


Figure 4. 3: Haplotype network for mitochondrial cytochrome oxidase subunit I (COI) gene of ballan wrasse (*Labrus bergylta*) morphotypes in both Carna and Spiddal during 2015 and 2016. Colours denote to the morphotypes and sample size of fish (n) is also indicated in the legend. The area of the circles is proportional to each haplotype frequency. Dashes on the lines represent mutational steps between haplotypes.

The COI alignment matrix was 654 base pairs (bp) long with 119 sequences in total for 4 morphotypes of *L. bergylta* (i.e. 64 sequences from Carna and 55 sequences from Spiddal). The haplotype network built using the COI showed no separation of the four morphotypes into distinct clusters (Figure 4.3). Instead, the COI haplotype network showed one dominant group (cluster) within the network, separated by only one mutational step from the other cluster or from individual haplotypes. Figure 4.4 shows that the haplotype network (also using COI gene) groups all morphs of *L. bergylta* together but separate from outgroup species *L. viridis* and *L. mixtus* in the network. This confirms that all morphs were more closely related to each other when compared with other closely related outgroup species.

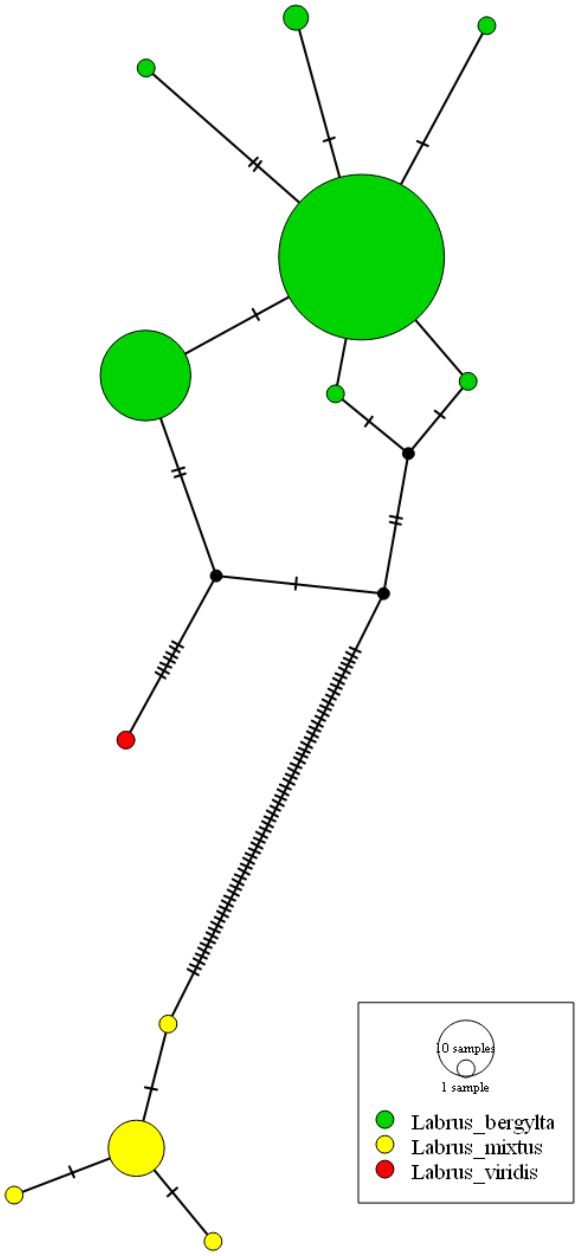


Figure 4. 4: Haplotype network for mitochondrial cytochrome oxidase subunit I (COI) gene of ballan wrasse (*Labrus bergylta*) in both Carna and Spiddal (all morphs, this study) compared with 'outgroup' Labrus species (green wrasse *Labrus viridis* and cuckoo wrasse *Labrus mixtus*) sequences from GenBank. Colours denote the species and sample size of fish (n) is also indicated in the legend. The area of the circles is proportional to each haplotype frequency. Dashes on the lines represent mutational steps between haplotypes.

Table 4. 4: AMOVA between the morphotypes of ballan wrasse (*Labrus bergylta*) for both Carna and Spiddal in 2015 and 2016 using the control region. Number of individuals sampled n=119

Source of variation	*d.f*	Sum of squares	Variance components	% variation	*F*-statistic	*P*- value
Between Morphs overall (i.e. Carna + Spiddal)	3	1.926	0.002	0.44	F_{CT} = 0.0043	0.2736
Within Morphs for each location	4	2.231	0.007	1.49	F_{SC} = 0.0149	0.1113
Between Individuals (irrespective of Morph or Location)	111	52.533	0.473	98.08	F_{ST} = 0.0192	0.0157
Total	118	56.689	0.482			

***F*ct** correlation of samples within a group of populations relative to samples from the whole dataset; F_{SC} correlation of samples within populations relative to samples from that subgroup of populations; F_{ST} correlation of samples within populations relative to samples from the whole data set (Lowe et al. 2004)

AMOVA was performed for the CR to investigate variation between and within the morphotypes of *L. bergylta*. There was no significant variation between the morphotypes (i.e. 'Between Morphs'; F_{CT} = 0.0043, P < 0.2736). There was also no significant genetic variation 'Within Morphs' including at different locations (F_{SC} = 0.0149, P < 0.1113) (Table 4.4). Instead, most of the variation (98%) resided 'Between individuals', irrespective of morphotype or location, with F_{ST} equal to 0.0192 and statistically significant P > 0.0157.

Pairwise comparisons for the significant F_{ST} term in the AMOVA revealed some differences when comparing individuals from certain location / morphotype combinations, however these did not vary consistently. So, for example, Spiddal plain differed from Carna plain and from Spiddal intermediate, which were strongly statistically different (Table 4.5). And also, some location / morphotype combinations were moderately different, e.g. (Spiddal plain differed from Spiddal spotted, Carna merle and Carna

intermediate). However, no consistent differences between morphs or locations were evident.

Table 4. 5: Pairwise comparisons for F_{ST} (the values calculated from a haplotype frequency) in Control Region gene between individuals (F_{ST} = 0.0192, $P < 0.0157$ see Table 4.4). The bold values are genetic difference based on Hartl and Clark (1997) classes for F_{ST} values between 0 and 1.0. A negative or zero value for F_{ST} means that there is no genetic diff. <0.05 = little genetic diff. 0.05-0.15 = moderate genetic diff. 0.15-0.25 = great genetic diff. >0.25 = very great genetic diff.

	CP	**SP**	**CS**	**SS**	**CM**	**SM**	**CI**	**SI**
CP	0							
SP	**0.163**	0						
CS	-0.034	0.074	0					
SS	0.000	**0.133**	-0.024	0				
CM	-0.050	**0.129**	0.051	0.052	0			
SM	-0.034	0.088	0.002	-0.014	0.013	0		
CI	-0.091	**0.118**	-0.010	-0.016	0.025	0.000	0	
SI	-0.213	**0.223**	0.054	0.074	-0.044	0.011	-0.002	0

CP = Carna Plain, CS = Carna Spotted, CM = Carna Merle, CI = Carna Intermediate, SP = Spiddal Plain, SS = Spiddal Spotted, SM = Spiddal Merle and SI Spiddal Intermediate.

In order to place genetic diversity of the samples from Carna and Spiddal within a broader context, a comparison was made with other locations using CR sequences from Genbank. The haplotype diversity (*h*) for the CR within locations at broader sampling regions was slightly lower in the Irish locations (Carna, Spiddal and Mweenish), as well as at both locations in the Azores, in comparison to the other locations/regions. For example, *h* was lowest at Santa Maria (Azores) (*h* = 0.933), followed by Carna (*h* = 0.942 Table 4.6) but was higher at Bertraghboy (IRE), UK and Norwegian locations. Nucleotide diversity (π) among locations was also low in Carna, Azores (Corvo and Santa María) as well as in Søgne (Norway), with respective values of π = 0.019, 0.017 and 0.013; but all other shores had higher values (0.024 - 0.034).

Table 4. 6: Diversity measures for the CR gene in ballan wrasse (*Labrus bergylta*) for locations in the present study (Carna, Spiddal) and locations/regions from GenBank.

			Control region (CR)					
Location/Region	**Label**	**Coordinates**	***N***	***NH***	**PS**	***h***	**π**	**PD**
Britain (UK) and Ireland (IRE)								
Carna IRE	CA	53°18' N 9°51' W	64	36	42	0.942	0.019	6.124
Spiddal IRE	SP	53°14' N 9°18' W	55	32	48	0.964	0.024	7.464
Mweenish IRE	MW	53°17′ N, 09°49′ W	20	19	40	0.994	0.034	10.568
Bertraghboy IRE	BB	53°19′ N, 09°51′ W	20	20	36	1.000	0.030	9.568
Portaferry UK	PF	54°23′ N, 05°33′ W	10	10	28	1.000	0.032	10.177
Loch Sunart UK	LS	56°40′ N, 05°56′ W	15	15	36	1.000	0.034	10.561
Norway								
Hidra	HI	58°13′ N, 06°31′ E	12	12	25	1.000	0.026	8.151
Søgne	SØ	58°04′ N, 07°48′ E	10	10	23	1.000	0.017	5.333
Azores								
Corvo	CO	39°41′ N, 31°05′ W	10	9	21	0.977	0.017	5.488
Santa María	SM	36°57′ N, 25°06′ W	10	8	14	0.933	0.013	4.088

N number of individuals, *N*H number of haplotypes, PS polymorphic sites, *h* Haplotype (gene) diversity, π Nucleotide diversity, PD mean number of pairwise differences.

AMOVA was performed to evaluate genetic structure along the distribution area of *L. bergylta* CR by specifying 'Locations' within three geographical 'Regions' (i.e. Britain and Ireland (four Irish locations, 2 UK locations) Norway (Hidra, Søgne) and Azores (Santa María, Corvo)). The highest variance was observed between the three geographic 'Regions' i.e. Britain and Ireland versus Norway versus Azores (61.55 %) with associated *F*-statistic (F_{CT} = 0.615, P > 0.001). Also, there was significant variation between 'Individuals' (F_{ST} = 0.618, P > 0.000) (Table 4.7). No significant variation was found between 'Locations' within regions (F_{SC} = 0.006, P < 0.225, Table 4.7). Pairwise comparisons for F_{ST} values calculated from a haplotype frequency in Table 4.8, which revealed significant differences between the locations. For example, Santa María and

Corvo (Azores) were differed from all the other locations, which were strongly statistically different with the highest Fst values range (0.801- 0.663) (Table 4.8). In addition, Søgne and Hidra were strongly statistically different from the other locations except Santa María and Corvo, but with lower Fst values range (0.571- 0.201).

Table 4. 7: AMOVA of CR in *Labrus bergylta* within and between 3 geographic 'Regions' (Britain and Ireland, Norway, Azores).

Source of variation	***d.f***	**Sum of squares**	**Variance components**	**% variation**	***F*-statistic**	***P*- value**
Between Regions	2	449.058	6.098	61.55	F_{CT} = 0.615	0.001
Within Regions (i.e. between 'Locations')	7	30.411	0.024	0.25	F_{SC} = 0.006	0.225
Between Individuals	216	817.434	3.784	38.20	F_{ST} = 0.618	0.000
Total	225	1296.903	9.90737			

***F*ct** correlation of samples within a group of populations relative to samples from the whole dataset; F_{SC} correlation of samples within populations relative to samples from that subgroup of populations; F_{ST} correlation of samples within populations relative to samples from the whole data set (Lowe et al. 2004)

Table 4. 8: Pairwise comparisons for F_{ST} (the values calculated from a haplotype frequency) in Control Region gene between individuals for all pairwise combinations of the ten locations (F_{ST} = 0.618, P > 0.000 see Table 4.7). The bold values are genetic difference based on Hartl and Clark (1997) classes for F_{ST} values between 0 and 1.0. A negative or zero value for F_{ST} means that there is no genetic diff. <0.05 = little genetic diff. 0.05-0.15 = moderate genetic diff. 0.15-0.25 = great genetic diff. >0.25 = very great genetic diff.

	CA	SP	MW	BB	PF	LS	Sø	HI	SM	CO
CA	-									
SP	-0.007	-								
MW	0.057	0.024	-							
BB	0.038	0.009	-0.031	-						
PF	0.054	0.019	-0.060	-0.051	-					
LS	0.047	0.011	-0.049	-0.028	-0.057	-				
Sø	**0.571**	**0.504**	**0.339**	**0.387**	**0.378**	**0.370**	-			
HI	**0.443**	**0.374**	**0.201**	**0.240**	**0.215**	**0.224**	-0.037	-		
SM	**0.775**	**0.738**	**0.681**	**0.702**	**0.715**	**0.694**	**0.801**	**0.738**	-	
CO	**0.764**	**0.727**	**0.663**	**0.682**	**0.688**	**0.672**	**0.772**	**0.712**	-0.008	-

CA= Carna, SP= Spiddal, MW= Mweenish, BB= Bertraghboy, PF= Portaferry, LS= Loch Sunart, Sø= Søgne, HI= Hidra, SM= Santa María, CO= Corvo.

The haplotype network in Figure 4.5 was designed to see where populations of *L. bergylta* at Carna and Spiddal are located in comparison to the populations from other locations and regions, as well as to identify phylogenetic relationships from CR sequence data and to deduce potential demographic events. The CR haplotype network shows a complex connecting pattern, within three highly divergent haplotype clusters. Representatives from all ten sampling locations were present in both clusters 2 and 3. However, cluster 1 exclusively contained Azores individuals (Figure 4.5). The clustering evident in this haplotype network clearly distinguishes Azores fish from the rest of Britain and Ireland and Norway. Furthermore, while most of the Norwegian samples are found in a sub cluster within cluster 3, some Norwegian haplotypes are also mixed together with samples from Ireland in cluster 2, more than 11 mutations away. Haplotypes associated with Carna and Spiddal were more commonly shown in cluster 2 than cluster 3.

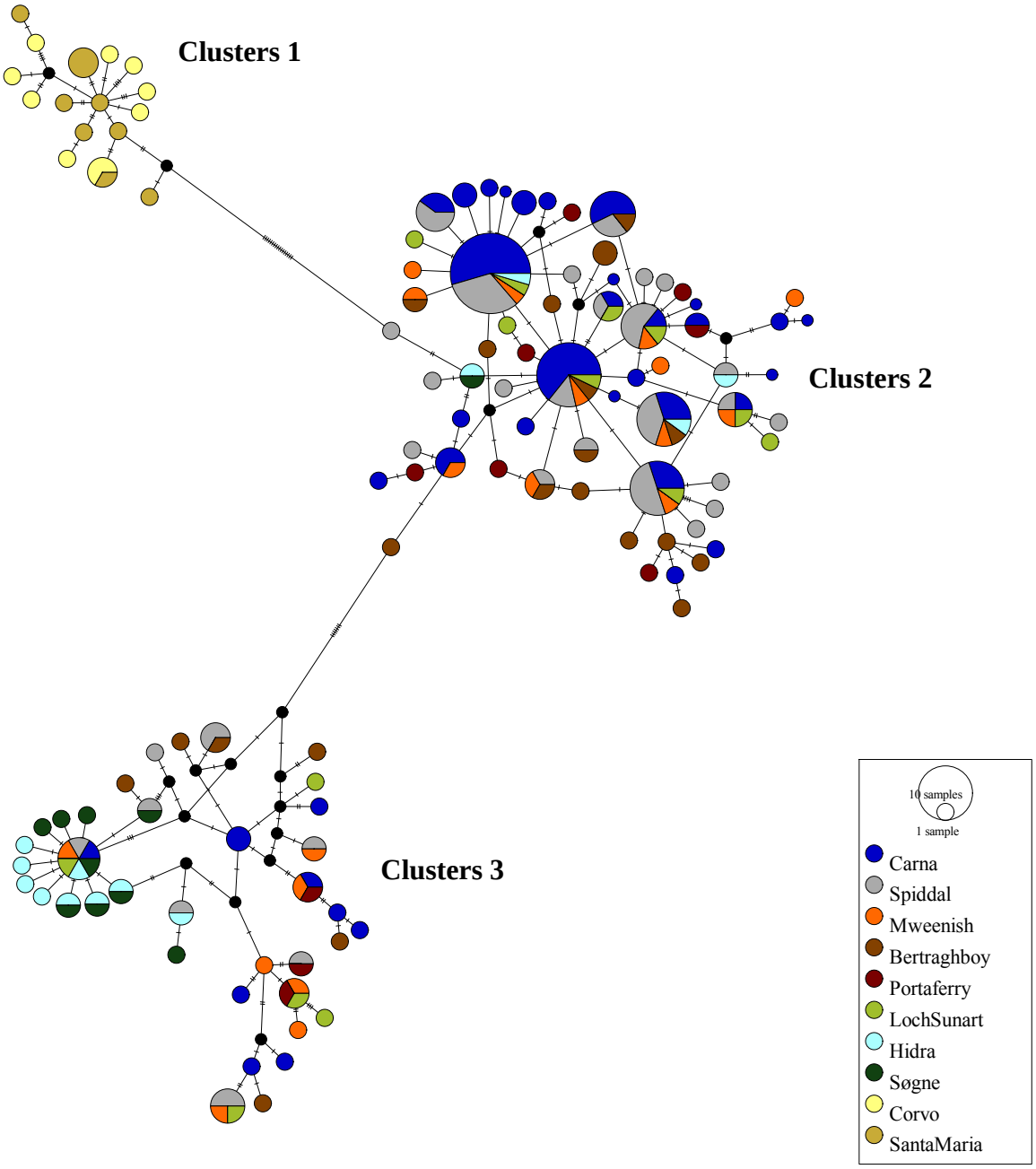


Figure 4. 5: Haplotype network for control region of ballan wrasse (*Labrus bergylta*) from different locations at 3 'Regions' ('Britain and Ireland', 'Azores' and 'Norway'). Colours denote to the 'Locations' within regions. The area of the circles is proportional to each haplotype frequency and sample size of fish (n) is also indicated in the legend. A black circle is an inferred median. Dashes on the lines represent mutational steps between haplotypes.

4.5 Discussion

No genetic variation was observed between the *L. bergylta* morphotypes (plain, spotted, merle and intermediate) using both the cytochrome oxidase subunit I (COI) gene and control region (CR) sequences. The CR haplotype network showed more divergence than the network built using COI, which is because the mutation rate is generally slower in the COI, meaning less variations arise over time. Interestingly, the COI confirmed that all the morphs were indeed *L. bergylta* (COI is often used to barcode species). This is important because, intermediate and merle morphs of *L. bergylta* that feature in the current study have not been previously described, genetically or otherwise, in the literature. The haplotype network results were confirmed by AMOVA, showing no genetic variation between the four morphotypes. Instead, most variation was observed between individual fish. Although strongly and moderately statistically significant pairwise differences were observed between some morphs within locations (Carna or Spiddal), or occasionally, morphs differed between Carna and Spiddal, neither morphs nor locations varied in a consistent manner using the CR marker.

In previous studies, genetic markers including the small-subunit (18S) ribosomal RNA gene, COI gene, CR and nuclear ribosomal protein (S7) gene were analysed for differences between plain and spotted morphotypes of *L. bergylta* (Almada et al. 2016). The study found that there were no genetic differences between these two morphotypes. However, in another study published that same year by Quintela et al. (2016), who used the mitochondrial COI gene and microsatellite markers, more contradictory evidence was presented. While that study showed no important significant genetic differences between plain and spotted using the COI marker, there were significant differences between morphs in microsatellite markers. And also, another study published recently by Seljestad et al. (2020), who used the single nucleotide polymorphisms (SNPs) markers found genetic differences between the two morphotypes (plain and spotted) which agreed with Quintela et al. (2016) findings.

Both the CR and nuclear DNA have been shown to be valuable in explaining the phylogeographic structure of corkwing wrasse (*Symphodus melops*) (Robalo et al. 2012). Moreover, the CR and nuclear ribosomal protein (S7) markers have been applied to phylogeographic investigations on various Atlantic fish (e.g. Blenniidae (*Coryphoblennius galerita*), Domingues et al. 2007a; (*Lipophrys pholis*) Francisco et al. 2011; Sparidae

(*Diplodus sargus*) Domingues et al. 2007b; Cottidae (*Taurulus bubalis*) Almada et al. 2012). D'Arcy et al.'s (2013) analysis of the CR region of *L. bergylta* suggested that low levels of genetic and nucleotide diversity indicated that populations in Scandinavian waters underwent a rapid recolonization by a low number of individuals following recent glacial retreat. Also, all Scandinavian samples presented remarkably less genetic diversity for microsatellites compared with a sample from Galicia (Seljestad et al. 2020). Furthermore, the intraspecific levels of haplotype diversity showed that populations of *L. bergylta* found in and around Scandinavian and British waters had been formed by distinct demographic histories (such as a population bottleneck accompanied by subsequent expansion) (D'Arcy et al. 2013). In the current study, highly complex clusters were observed in the haplotype network of CR at 10 locations in 3 geographic regions ('Britain and Ireland', 'Norway' and 'Azores'). D'Arcy et al (2013) noted that the cluster arrangements in *L. bergylta* suggested two extremely divergent clades. Both clusters showed evidence of expansion following the retention of major haplotypes in refugia (possibly more than one) during the last glacial maximum (D'Arcy et al. 2013).

The genetic structure shown in both the haplotype network and the AMOVA of the current study differentiated *L. bergylta* of the Azores first, followed by Norway, from those in other regions. This has also been shown in the past in the Azores region (Almada et al. 2016) and in Norway (D'Arcy et al. 2013). The Azores populations retain their uniqueness by being separated from the other European regions with a low average number of migrants (Nm) of 0.160 reported between Azorean and other regions (Almada et al. 2017). Several other species of fish are reported with similar findings regarding the peripheral position of Azorean haplotypes (Francisco et al. 2014; Stefanni et al. 2015). In the present study, nucleotide diversity was shown to be at low levels in Carna, Søgne and Corvo, which was comparable to the results reported by D'Arcy et al. (2013). However, high levels of haplotype diversity were present in all the regions, which did not agree with what D'Arcy et al. (2013) found, especially in Hidra and Søgne. However, the haplotype diversity of Hidra and Søgne in the present study was higher than previous studies because the twelve and ten sequences respectively selected from Genbank for Hidra and Søgne were all different, so this is a sampling artefact. More importantly, the highest haplotype (h) and nucleotide (π) diversity values at Carna and Spiddal was observed within the spotted morphotype, while the lowest of these were observed within the plain morphotype.

These results were similar to Almada et al. (2016), in which the nucleotide diversity was the highest in spotted but the haplotype diversity was the highest in plain morphotype.

Genetic differentiation is suggested to arise as a consequence of selective pressures and is often linked to the primary stage of sympatric speciation (Skúlason and Smith 1995). *L. bergylta* is the target of local fisheries over its entire distribution, particularly in recent years as it is used in aquaculture as a cleaner fish. It is also exploited by recreational fishers and sports fishers (Pawson et al. 2008: Pita and Freire 2017). Selection of the biggest specimens for this purpose involves a strong selective pressure on spotted individuals (Almada et al. 2016). Earlier research on two morphotypes of *L. bergylta* (plain and spotted) had reported variations in life-history such as mortality and growth rate, and otolith lengths (Villegas-Ríos et al. 2013b). Villegas-Ríos et al. (2013b) showed spotted morphs are larger, faster-growing, and are being marketed as a different species in regional fisheries. This could result in fishing selection for this particular morph. Since larger size-at-age is suggested to be achieved at the cost of lower breeding production (Villegas-Ríos et al. 2013c), this could also have consequences for genetic structuring. In addition, the fact that *L. bergylta* is a protogynous hermaphrodite and the largest and most dominant females can transition to become males, could give rise to male-biased over-exploitation since males are predicted to be larger than females, as well as resulting in unbalanced sex ratios (fewer males) (Almada et al. 2016). Similarly, because spotted individuals are of larger sizes, they might be expected to be at lower risk of predation, particularly during the male phase, throughout their lifetime (Villegas-Ríos et al. 2013b). Clearly, haplotype and nucleotide diversity was not low in spotted morphs in the present study, however, what is not known is whether recent selection of spotted morphs for use in aquaculture has had any impact on their population genetics over time, and this requires further research with larger sample sizes.

The diversity of *L. bergylta* morphotypes within populations throughout the range is suggested by Almada et al. (2016) to be as a result of a forked evolutionary process between sympatric populations that was created by assortative mating (prezygotic isolation). It has been noted that the combination of assortative mating and disruptive natural selection on a single characteristic (e.g. body colour morphotype) (Puebla et al. 2007; Elmer et al. 2009) can initiate speciation in marine environments, even without geographical boundaries (i.e. in sympatry) and in the presence of high gene flow (Puebla et

al. 2007; Barreto et al. 2008). *L. bergylta* males hold territories where they access 'harems' of females, hence there is some degree of sexual selection at breeding (Darwall et al. 1992). Sexual selection can lead to reproductive isolation, which, even in sympatry allows colour morphs to speciate (Elmer et al. 2009). Therefore, divergent selection can clearly be linked with gene flow for polymorphic colour keeping and speciation (Gray and Mckinnon 2006). It is still argued whether sexual selection can result in speciation in the absence of physical restrictions to gene flow (Elmer et al. 2009). However, it has been proposed that sexual selection through colour assortative mating may perform an essential role in the sympatric speciation of neotropical cichlid fish (Elmer et al. 2009). Moreover, in a study on parrotfishes (family Labridae), it was found that sympatric pairs displayed more difference in morphology and sexually dimorphic colour morphotype compared with allopatric pairs, and suggested that both genetic drift in allopatric species, and environmentally accommodative divergence between members of sympatric pairs, have performed a task in diversification of this group (Choat et al. 2012).

Overall, the present study shows a geographic trend in genetic structure rather than one based on morphotype forms. These results support the results of Almada et al. (2016), which does not indicate genetic isolation between the morphotypes. Hence there is no evidence of a role played by morphotype, and life history differences in morphotype (as demonstrated by Villegas-Rios et al. 2013a) in the genetic structuring of *L. bergylta*. Nevertheless, the results indicate some areas of potential concern with extraction of particular *L. bergylta* morphs for use in aquaculture and other activities (like sports fishing) due to a higher genetic variability in popular spotted morphs, which on the long term might have population genetic consequences.

References

Almada, V., Almada, F., Henriques, M., Santos, R. S., and Brito, A. (2002). On the phylogenetic affinities of *Centrolabrus trutta* and *Centrolabrus caeruleus* (Perciformes: Labridae) to the genus *Symphodus*: molecular, meristic and behavioural evidences. *Life and Marine Sciences*, **19A**, 85-92.

Almada, V. C., Almada, F., Francisco, S. M., Castilho, R., and Robalo, J. I. (2012). Unexpected high genetic diversity at the extreme Northern geographic limit of *Taurulus bubalis* (Euphrasen, 1786). *PLoS One*, **7**(8), 1-8. doi.org/10.1371/journal.pone.0044404

Almada, F., Casas, L., Francisco, S. M., Villegas-Ríos, D., Saborido-Rey, F., Irigoien, X., and Robalo, J. I. (2016). On the absence of genetic differentiation between morphotypes of the ballan wrasse *Labrus bergylta* (Labridae). *Marine Biology*, **163**, 1–6. doi.org/10.1007/s00227-016-2860-8

Almada, F., Francisco, S. M., Lima, C. S., FitzGerald, R., Mirimin, L., Villegas-Ríos, D., Saborido-Rey, F., Afonso, P., Morato, T., Bexiga, S., and Robalo, J. I. (2017). Historical gene flow constraints in a northeastern Atlantic fish: phylogeography of the ballan wrasse *Labrus bergylta* across its distribution range. *Royal Society Open Science*. **4**(2), 1-14. doi.org/10.1098/rsos.160773

Barreto, F. S., and McCartney, M. A. (2008). Extraordinary AFLP fingerprint similarity despite strong assortative mating between reef fish color morphospecies. *Evolution: International Journal of Organic Evolution*. **62**(1), 226-233. doi: 10.1111/j.1558-5646.2007.00285.x

Bernatchez, L., Vuorinen, J.A., Bodaly, R.A. and Dodson, J.J. (1996). Genetic evidence for reproductive isolation and multiple origins of sympatric trophic ecotypes of whitefish (Coregonus). *Evolution*, **50**(2), 624-635.

Choat, J. H., Klanten, O. S., Van Herwerden, L., Robertson, D. R., and Clements, K. D. (2012). Patterns and processes in the evolutionary history of parrotfishes (Family Labridae). *Biological Journal of the Linnean Society*. **107**(3), 529-557. doi.org/10.1111/j.1095-8312.2012.01959.x

Clement, M., Posada, D. C. K. A., and Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology*. **9**(10), 1657-1659. doi.org/10.1046/j.1365-294X.2000.01020.x

D'Arcy, J., Mirimin, L., and FitzGerald, R. (2013). Phylogeographic structure of a protogynous hermaphrodite species, the ballan wrasse *Labrus bergylta*, in Ireland, Scotland, and Norway, using mitochondrial DNA sequence data. *ICES Journal of Marine Science*. **70**(3), 685-693. doi.org/10.1093/icesjms/fst018

Darwall, W. R. T., Costello, M. J., Donnelly, R., and Lysaght, S. (1992). Implications of life-history strategies for a new wrasse fishery. *Journal of Fish Biology*. **41**, 111-123. doi.org/10.1111/j.1095-8649.1992.tb03873.x

Domingues, V. S., Faria, C., Stefanni, S., Santos, R. S., Brito, A., and Almada, V. C. (2007a). Genetic divergence in the Atlantic–Mediterranean Montagu's blenny, *Coryphoblennius galerita* (Linnaeus 1758) revealed by molecular and morphological characters. *Molecular Ecology*. **16**(17), 3592-3605.

Domingues, V. S., Santos, R. S., Brito, A., Alexandrou, M., and Almada, V. C. (2007b). Mitochondrial and nuclear markers reveal isolation by distance and effects of Pleistocene glaciations in the northeastern Atlantic and Mediterranean populations of the white seabream (*Diplodus sargus*, L.). *Journal of Experimental Marine Biology and Ecology*. **346**(1-2), 102-113. doi.org/10.1016/j.jembe.2007.03.002

Elmer, K. R., Lehtonen, T. K., and Meyer, A. (2009). Color assortative mating contributes to sympatric divergence of neotropical cichlid fish. Evolution: *International Journal of Organic Evolution*. **63**(10), 2750-2757. doi.org/10.1111/j.1558-5646.2009.00736.x

Excoffier, L., and Lischer, H. E. L. (2010). Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*. **10**(3), 564–567. doi.org/10.1111/j.1755-0998.2010.02847.x

Folmer, O., Black, M., HOEH, W., Lutz, R., and Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*. **3**(5), 294–299. doi.org/10.1371/journal.pone.0013102

Francisco, S. M., Almada, V. C., Faria, C., Velasco, E. M., and Robalo, J. I. (2014). Phylogeographic pattern and glacial refugia of a rocky shore species with limited dispersal capability: the case of Montagu's blenny (*Coryphoblennius galerita*, Blenniidae). *Marine Biology*. **161**, 2509–2520. doi.org/10.1007/s00227-014-2523-6

Francisco, S. M., Faria, C., Lengkeek, W., Vieira, M. N., Velasco, E. M., and Almada, V. C. (2011). Phylogeography of the shanny *Lipophrys pholis* (Pisces: Blenniidae) in the NE Atlantic records signs of major expansion event older than the last glaciation. *Journal of Experimental Marine Biology and Ecology*. **403**, 14–20. doi.org/10.1016/j.jembe.2011.03.020

Gray, S. M., and Mckinnon, J. S. (2006). Linking color polymorphism maintenance and speciation. *Trends in Ecology and Evolution*. **22**, 71–79. doi: 10.1016/j.tree.2006.10.005

Hanel, R., Westneat, M. W., and Sturmbauer, C. (2002). Phylogenetic relationships, evolution of broodcare behavior, and geographic speciation in the wrasse tribe Labrini. *Journal of Molecular Evolution*. **55**(6), 776-789. doi.org/10.1007/s00239-002-2373-6

Hartl, D. L., and Clark, A. G. (1997). Principles of population genetics (Vol. 116). Sunderland, MA: Sinauer associates.

Kocher, T. D., Conroy, J. A., McKaye, K. R., and Stauffer, J. R. (1993). Similar morphologies of cichlid fish in lakes Tanganyika and Malawi are due to convergence. *Molecular Phylogenetics and Evolution*. **2**(2), 158–165. doi.org/10.1006/mpev.1993.1016

Lee, W. J., Conroy, J., Howell, W. H., and Kocher, T. D. (1995). Structure and evolution of teleost mitochondrial control regions. *Journal of Molecular Evolution*. **41**(1), 54-66. doi.org/10.1007/BF00174041

Leigh, J.W. and Bryant, D. (2015). Popart: full-feature software for haplotype network construction. *Methods in Ecology and Evolution*, **6**(9), 1110-1116.

Lowe, A., Harris, S., and Ashton, P. (2004). Ecological genetics: design, analysis, and application. John Wiley and Sons.

Pawson, M. G., Glenn, H., and Padda, G. (2008). The definition of marine recreational fishing in Europe. *Marine Policy*. **32**(3), 339-350. doi.org/10.1016/j.marpol.2007.07.001

Pita, P., and Freire, J. (2017). Trophic ecology of an Atlantic kelp forest fish assemblage (NW Spain) targeted by recreational fishers and implications for coastal management. *Journal of the Marine Biological Association of the United Kingdom*. **99**(1), 19-29. doi.org/10.1017/S0025315417001862

Puebla, O., Bermingham, E., Guichard, F., and Whiteman, E. (2007). Colour pattern as a single trait driving speciation in Hypoplectrus coral reef fishes?. *Proceedings of the Royal Society B: Biological Sciences*. **274**(1615), 1265-1271. doi: 10.1098/rspb.2006.0435

Quintela, M., Danielsen, E., Lopez, L., Barreiro, R., Svåsand, T., Knutsen, H., Skiftesvik, A. and Glover, K. (2016). Is the ballan wrasse (*Labrus bergylta*) two species? Genetic analysis reveals within-species divergence associated with plain and spotted morphotype frequencies. *Integrative Zoology*, **11**(2), 162-172.

Robalo, J. I., Castilho, R., Francisco, S. M., Almada, F., Knutsen, H., Jorde, P. E., Pereira, A. M., and Almada, V. C. (2012). Northern refugia and recent expansion in the North Sea: the case of the wrasse *Symphodus melops* (Linnaeus, 1758). *Ecology and Evolution*. **2**(1), 153–164. doi.org/10.1002/ece3.77

Rozas, J., Ferrer-Mata, A., Sanchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., and Sanchez-Gracia, A. (2017). DnaSP 6: DNA sequence polymorphism analysis of large data sets. *Molecular Biology and Evolution*. **34**(12), 3299–3302.doi.org/10.1093/molbev/msx248

Seljestad, G. W., Quintela, M., Faust, E., Halvorsen, K. T., Besnier, F., Jansson, E., Dahle, G., Knutsen, H., André., Folkvord, A. and Glover, K. A. (2020) "A cleaner break": Genetic divergence between geographic groups and sympatric phenotypes revealed in ballan wrasse (*Labrus bergylta*). *Ecology and Evolution*, **10**, 6120-6135.

Skúlason, S. and Smith, T.B. (1995). Resource polymorphisms in vertebrates. *Trends in ecology & evolution*, **10**(9), 366-370.

Stefanni, S., Castilho, R., Sala-Bozano, M., Robalo, J. I., Francisco, S. M., Santos, R. S., Marques, N., Brito, A., Almada, V.C., and Mariani, S. (2015). Establishment of a coastal fish in the Azores: Recent colonisation or sudden expansion of an ancient relict population?. *Heredity*. **115**, 527–537. doi.org/10.1038/hdy.2015.55

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*. **30**, 2725–2729. doi.org/10.1093/molbev/mst197

Villegas-Ríos, D. (2013a). Life-history and behaviour of "*Labrus bergylta*" in Galicia. Ph.D. University of Vigo.

Villegas-Ríos, D., Alonso-Fernández, A., Fabeiro, M., Bañón, R., and Saborido-Rey, F. (2013b). Demographic variation between colour patterns in a temperate protogynous hermaphrodite, the ballan wrasse *Labrus bergylta*. *PLoS One*. **8**(8), 1-11. doi.org/10.1371/journal.pone.0071591

Villegas-Ríos, David, Alonso-Fernández, A., Domínguez-Petit, R., and Saborido-Rey, F. (2013c). Intraspecific variability in reproductive patterns in the temperate hermaphrodite fish, *Labrus bergylta*. *Marine and Freshwater Research*. **64**, 1156–1168. doi.org/10.1071/MF12362

Chapter 5: General discussion

A limited number of studies has been carried out on the relationship between the blue-green pigment, biliverdin, and the polymorphism exhibited in the protogynous species of *Labrus bergylta* (Abolins 1961; Clark et al. 2016). This study is the first to describe the four morphotypes; namely, plain, spotted, merle and intermediate in *L. bergylta* found on the west coast of Ireland. Previous research has shown distinctions in life history parameters, such as growth rate and reproduction, between plain and spotted morphotypes (Villegas-Ríos et al. 2013a).

5.1 Body size and biliverdin and bilirubin concentrations in *L. bergylta* plasma

This PhD thesis investigated possible connections between plasma colour pigments (biliverdin and bilirubin) and the morphotypes of *L. bergylta* including any links to blood physiology parameters (for example, haematocrit (%), haemoglobin concentration (g dl^{-1}) and red blood cell count (nanolitres (nl)) (Chapter 2). In fact, no difference between *L. bergylta* morphotypes was evident in blood physiology parameters, however three other parameters: body size (length, cm), biliverdin concentration (µmol l^{-1}) and total bilirubin concentration (µmol l^{-1}) in plasma, appeared to separate by morphotype and were associated with morphotype in a PERMANOVA and principal component analysis (PCA). PERMANOVA demonstrated differences in three explanatory variables between all morphotypes except plain and intermediate which were not different, while PCA results showed plain morphotypes, overall, had higher biliverdin and total bilirubin concentrations.

Comparing morphotypes across years (Figure 2.24, Chapter 2), not taking location into consideration, showed that in 2016, the biliverdin concentration of plasma was highest in plain morphs, while in 2015, intermediate and spotted and followed by plain morphs had the highest plasma biliverdin concentration. The merle morphs were consistently low in plasma biliverdin concentration in both years. Interestingly, the plasma total bilirubin concentration was also higher in plain morphotype in both years. Spotted and intermediate morphotypes exhibited similar bilirubin concentrations in both years. The total bilirubin concentrations in merle morphs were changeable between years, with 2015 being higher than 2016 (Figure 2.23, Chapter 2). Disregarding morphotype and comparing plasma biliverdin and total bilirubin by location across years (Figure 2.25, Chapter 2), the highest plasma biliverdin concentration was in Carna 2015, while the highest plasma biliverdin concentration was recorded in Spiddal 2016. Plasma bilirubin concentrations were highest

in Spiddal in both years. Comparison of pigments recorded from each location showed that bilirubin concentrations were higher than biliverdin in both locations and years with the exception of Carna in 2015 (Figure 2.25, Chapter 2).

Therefore, biliverdin and total bilirubin concentrations in plasma were variable across morphs. This is also apparent in the boxplots (Figure 2.20 C, Chapter 2), which showed that the plain morphs exhibited the highest total bilirubin. The plain and intermediate followed by spotted morphs displayed the highest biliverdin concentrations, while the merle morphs had the lowest plasma biliverdin concentrations (Figure 2.20 D, Chapter 2). Results relating to body size were somewhat inconsistent. Boxplot and principal component analyses showed that the merle morphs were smaller in body size, while the spotted morphs had the largest body size. A comparison of the total length across years showed merle was the smallest morph in 2015, but in 2016, both plain and merle morphs were the smallest. Spotted morphs were larger than the other morphotypes in 2016 but this was not the case in 2015. In summary, there was a consistent difference between merle (smaller) and spotted (larger) morphs in both years, while the relative size of plain and intermediate morphs were similar to each other in both years.

5.2 Biliverdin and bilirubin concentrations in *L. bergylta* external tissue (skin and scales)

In Chapter 3, the presence of biliverdin in external tissue (skin and scales) of morphotypes was examined, as was the potential relationship between pigment concentrations in circulatory (plasma) pigments (Chapter 2) and external tissues (Chapter 3). There was a positive relationship between the biliverdin concentrations in external tissues (skin and scales) and plasma in both locations (Carna and Spiddal). A comparison of biliverdin concentrations in both skin and scale tissues between the morphotypes at Carna revealed differences in 2015 only. The highest biliverdin concentrations were found in spotted and intermediate morphotypes (of both skin and scales). However, merle and plain morphs showed the lowest biliverdin concentrations of both skin and scales. The high plasma biliverdin concentrations in spotted and intermediate morphotypes of Carna 2015 are possibly responsible for build-up and expression of these pigments on the skin and scales. Further investigation will be required to understand the physiological role of biliverdin. One possible role is that of external signalling, although this does not appear to be sexual as there is no connection between the biliverdin concentrations and sex of *L. bergylta* (Clark et al. 2016).

5.3 The population genetic structure in *L. bergylta* including discussion of morphotypes

There have been some investigations in recent times regarding morphotypes and genotypes of *L. bergylta* and whether this species is splitting into separate species. Quintela et al. (2016) proposed that the variation in morphotypes may represent distinctive taxonomic units (i.e. incipient sub species), although some genetic overlap between morphotypes was not ruled out. Based on the mitochondrial cytochrome oxidase subunit I (COI) gene, and a set of 20 microsatellite loci, Quintela et al. (2016) found an important difference in the microsatellite markers between spotted and plain morphs. Moreover, Seljestad et al. (2020) studied the genetic structure of *L. bergylta* using 82 single nucleotide polymorphisms (SNPs) markers from 18 locations in Norway, Sweden, and Galicia in Spain. The authors found north western and south eastern Scandinavia were two very distinguished genetic groups. Moreover, *L. bergylta* which were collected from Galicia were highly differentiated from all Scandinavian locations but more alike to north western than south eastern locations. The separation in Scandinavia crossed an area that comprised sandy beaches, presenting a habitat discontinuity for *L. bergylta*. This 'distinct break' in population genetic structure and habitat were linked by the authors who suggested that sandy bottom substrates function as a habitat discontinuity with genetic consequences in *L. bergylta*. However, although distinct genetic variations were also noted between plain and spotted morphs in Galicia, these were not apparent in Scandinavia. Seljestad et al. (2020) suggested that extensive aquaculture-mediated translocation of *L. bergylta* from Sweden and southern Norway to western and middle Norway has the potential to merge genetically different populations. Regarding morph-specific variation in Galician samples, Seljestad et al. (2020) have suggested that it is possible, albeit subtle that the two morphotypes spawn at discrete times or areas. Alternatively, the two morphotypes may have different selective advantages along an environmental gradient. They also noted geographic differences in observed phenotypes with fewer spotted fish in northern latitudes, however a new 'intermediate' morph was seen in Scandinavia (Seljestad et al. 2020). In addition to the genetic differences shown using SNP markers in Galician spotted and plain *L. bergylta* (Seljestad et al. 2020). Villegas-Ríos, (2013b) found that these two morphotypes have different life history strategies such that spotted morphotypes showed a more rapid growth rate than plain morphotypes and larger overall sizes.

The results found by both Quintela et al. (2016) using mitochondrial cytochrome oxidase subunit I (COI) gene and 20 microsatellite loci, and Seljestad et al. (2020) using 82 SNP markers clearly dispute the findings of Almada et al. (2016), who used control region (CR), cytochrome oxidase subunit I (COI), the small-subunit (18S) ribosomal RNA gene and the first intron of the nuclear S7 ribosomal protein gene (S7). The latter study found that the plain and spotted morphotypes were not genetically different from each other. The present study examines additional *L. bergylta* morphotypes (i.e. plain, spotted, merle and intermediate) compared to previous work but also found no genetic differences between the morphotypes (COI and CR genes), as already detailed above (Chapter 4). Investigations of both markers (CR and COI) using AMOVA and haplotype networks showed no genetic variation among morphotypes. Disregarding morphotype and categorising the samples into a broader geographic and regional comparison (i.e. Ireland, UK, Norway and Azores) for the CR gene showed that populations in the Azores and Norway were highly distinct and separated from other regions. However, Norway was not fully separated from Ireland and UK. Early research on *L. bergylta* employed the CR to examine genetic diversity and population structuring of *L. bergylta* in waters around Ireland, the UK and southern Norway (D'Arcy et al. 2013). D'Arcy et al (2013) found that population differentiation between regions was strong, but within regions this was weak, important population genetic structuring between the two sites in Norway (Hidra and Søgne) was highlighted, which was attributed to historical demographic events (D'Arcy et al. 2013). Also, local environmental factors (e.g. water currents and deep water) and life-history can explain the difference in levels within-region (D'Arcy et al. 2013). Pita and Freire (2011) have found that *L. bergylta* is extremely sedentary and have very minimal movements which support the idea that 'local' populations may differ from others without much movement between. Nevertheless, our results do not indicate genetic isolation between the morphotypes, which seem not to represent separate breeding groups. Given disagreement in the literature, further knowledge on population genetic structure would be useful, particularly given the large biomass extraction of wild *L. bergylta* by the salmon farming industry. In Norway, it is permitted for a maximum of 18 million wrasse to be translocated within Norway and imported from Sweden for use in the aquaculture industry as cleaner fish in salmonid sea-cages (Seljestad et al. 2020). There is a lack of information on the fate of these wrasse, however, escapes, hybridization with wild populations and the deliberate release of wrasse from aquaculture facilities have been documented (Faust et al. 2018). These practices can result in gene flow between previously isolated and highly

distinct genetic populations (Seljestad et al. 2020). Clarifying the existence of disruptive selection and gene flow between morphotypes, would only be possible by means of detailed genetic analysis with various molecular markers (Planes and Doherty 1997; Puebla et al. 2007).

5.4 Variation of morphotypes in *L. bergylta* based on life history

Previous studies have reported variations in growth rate, body size, otolith size, age and mortality between plain and spotted morphotypes of *L. bergylta* (Villegas-Ríos et al. 2013a). The spotted morphotype was the least abundant and had a larger size than plain morphotypes (Villegas-Ríos et al. 2013a), and this trait was in agreement with the present study, the spotted (and intermediate) morphotypes were the largest morphs compared with other morphotypes (Chapter 3). Fewer resources were apparently invested in the reproduction process of the spotted morphotype, i.e. fewer eggs were produced when compared with plain morphotypes (Villegas-Ríos et al. 2013a). The spotted morphotype underwent changes in sex earlier than the plain morphotype as a result of a larger size-at-age (Villegas-Ríos et al. 2013b). Otolith sizes were also different for the same fish size in both morphotypes with otoliths from the plain morph being larger (Villegas-Ríos et al. 2013a). Based the size advantage hypothesis (Munday et al. 2006), and according to data in sequentially hermaphroditic species (Robertson and Choat 1974), as community structures and dominance hierarchies are major factors influencing sex change, communities require long term interactions to settle hierarchies and create dominant females (Grober and Bass 2002). Therefore, the morphs of *L. bergylta* could be some sort of signalling to do with dominance hierarchy (e.g. merle are smaller and spotted are larger). Possible explanations for all these differences between morphotypes were suggested to include evolutionary processes in populations of *L. bergylta*, perhaps as a result of an assortative mating (prezygotic isolation) in this species (Villegas-Ríos et al. 2013a). It was suggested that the combination of assortative mating and disruptive natural selection on a single trait, referred to as a 'magic trait' (Gavrilets 2004), can cause speciation in marine environments, even without geographical barriers (i.e. in sympatric populations) and in the presence of gene flow (Puebla et al. 2007; Barreto and McCartney 2008; Choat et al. 2012). While the role of colour variation in the speciation process has not yet been conclusively confirmed, the potential for this phenotypic feature has been demonstrated to promote diversification through assortative mating in marine and freshwater species (Puebla et al. 2007; Elmer et al. 2009; Puebla 2009). For example,

colour-based assortative mating has been shown to have an important role in the development of East African cichlids (Salzburger et al. 2006). Also, it was suggested that colour-based assortative mating combined with selection mating and colour disruptive patterns aids speciation in *Hypoplectrus* coral reef fishes (Puebla et al. 2007). Assuming genetic differences are discernible amongst morphotypes, all the above explain the possible underlying mechanisms. Genetic differences would, furthermore, be unsurprising, given the demographic differences outlined above.

5.5 Habitats of *L. bergylta* based on the morphotypes

All morphotypes of *L. bergylta*, plain, spotted, merle and intermediate, seem to overlap in their habitat in both locations studied, Carna and Spiddal (Chapter 3), although some morphotypes were more abundant than others at each site, depending on the year. In general, the most common morphotype was merle at Spiddal and intermediate at Carna. At Carna in 2015, the abundance of the morphotypes was: intermediate > spotted > merle > plain. But in Carna 2016, there were no plain morphotypes and the most common morphotypes were merle > spotted > intermediate. At Spiddal in 2015 and 2016, merle was also a highly abundant morphotype, and in 2016, spotted, plain and intermediate morphotypes were more abundant than in 2015. This result was in agreement with Villegas-Ríos (2013b) who found that the depth and habitat preferences of plain and spotted morphotypes appeared to be entirely overlapping, and any distinct use of space was also not observed (Villegas-Ríos 2013b).

5.6 The reasons potential behind morphotypes variations

Based on a limited number of observations, i.e. 14 reproductive groups, two spotted and twelve plain, preliminary observations during the spawning season indicated that some degree of assortative mating may occur between the morphotypes since mixed reproductive pairs (i.e. plain-spotted) have never been observed (Villegas-Ríos 2013b). Furthermore, benthic spawning and nesting behaviour in *L. bergylta* (Sjolander et al. 1972), in conjunction with larva retention and low adult dispersion (Villegas-Ríos 2013b), could intensify speciation (Palumbi 1992). These processes can contribute to genetic differentiation between neighbouring areas, and therefore, to the genetic structure of *L. bergylta* populations (D'Arcy et al. 2013).

A further plausible explanation is that colour morphotypes arise as a result of a difference in dietary composition or habitat preference without reproductive isolation, as was shown in other fish species (Webber et al. 1973). In a review of 97 teleost fish species, differences

in morphotype colouration and relationships between morphotypes and diet were frequently correlated to habitat separation between benthic and pelagic habits (Robinson and Wilson, 1994). An association between food and body colour morphotypes was also seen in Gulf of Maine cod (*Gadus morhua*) (Sherwood and Grabowski 2010). Some resident Gulf of Maine cod individuals had red colouration as a result of feeding mostly on benthic fauna (crabs, lobsters, and demersal fish) that were abundant in carotenoids. However, other Gulf of Maine cod individuals exhibited an olive colouration due to feeding on forage fish (including shrimp) and were transient in those habitats (Sherwood and Grabowski 2010). In this case, olive and red cod morphs were assumed to be two variants in life history since, as well as body form, they also showed diversity in growth and habitat preference (Sherwood and Grabowski 2010). No variations in migratory behaviour have ever been recorded in *L. bergylta* morphotypes and no differences in habitat preference have ever been observed between morphotypes (Villegas-Ríos 2013b). Some variation in diet may be apparent: the diet of *L.bergylta* has been analysed in relation to the different body colour morphotypes by Pita and Freire (2017) for *L. bergylta* in Galicia (NW Spain) who revealed that the majority of food in their stomach were Bivalvia (*Mytilus galloprovincialis*), followed by a range of crustacean prey (Isopoda, Amphipoda, Brachyura and Balanidae). Although they were weakly associated with various dietary sources, the plain and spotted morphs of *L. bergylta* demonstrated some overlap in diet. Nevertheless, benthic prey was favoured by plain morphotypes more than the spotted morphotypes (Pita and and Freire 2017). Despite these results, further research is clearly needed to determine the reasons behind the morphotype variations in *L. bergylta.*

The current study also focussed on links between the morphotypes of *L. bergylta* and blood pigments (Chapter 2). Total bilirubin concentration was higher in the plasma of plain morphotypes while the other morphotypes overlapped in this respect. Biliverdin concentrations in plasma were highest in both plain and intermediate morphs, with merle having the lowest concentrations. Comparisons of these pigments between locations and years (ignoring the morphotypes) showed that the bilirubin concentration in all locations and years was greater than biliverdin, except at Carna in 2015, where high biliverdin and low total bilirubin concentrations were generally noted (Chapter 2). Drilling down in a little more detail, comparison of pigments across morphotypes in Carna 2015 showed spotted and intermediate had high biliverdin concentrations in this particular year/location

while plain and merle showed contrary results with highest concentration of total bilirubin and the lowest biliverdin concentrations. It is important to note the small spatial scale involved, since Carna and Spiddal are within 40kms of each other. One of the possible reasons behind these differentiations at Carna 2015 is the different sampling methods (lobster pots were used rather than rod and line), plus these sampling methods may have sampled slightly different populations spatially, with fish in lobster pots coming from slightly deeper waters. Alternatively, sampling using lobster pots in Carna 2015 may have caused increased stress, as, following capture, these fish were held closely together in a storage tank before being brought to the University, which could conceivably have altered hormonal or cortisol levels. In previous studies, Clark et al. (2016) defined morphotypes using red, green and intermediate colour rather than in terms of pattern as were done in the present study. Clark et al. (2016) defined (a) deep red/brown hue, (b) red/brown hue with little green, (c) less intensively green with brown/red inclusions and (d) dark green colouration. These authors found that plasma colour in *L. bergylta* varied amongst geographic locations, and that colour morphotype showed a slight gradient effect, where green morphotypes had a higher biliverdin concentration compared with the same size of red morphotypes. Clark et al. (2016) suggested that variability in biliverdin concentrations was associated with the geographical origin of sampling. However, geographic variability does not resolve the differences between years at Carna. The level of UV light in the water column was found to play an essential role for colour change in the skin of teleost fish more generally (Leclercq et al. 2009), which could also explain plasma colour variations from place to place and year to year. The present study adds to this knowledge by demonstrating that biliverdin concentration in external tissues (extracts of skin and scales), like plasma (i.e. Clark et al. 2016), also did not vary between the sexes. Hence, a role in sexual selection signalling seems unlikely, but not impossible, given the existence of sex change in this species. It is noteworthy that, in Clark et al. (2016), the concentration of biliverdin in plasma did not differ depending on age or body size. This is unlike the current study, where at Carna (2015), smaller morphs (merle) had lower biliverdin concentration, and larger morphs (spotted and intermediate) had highest biliverdin concentrations in both plasma and external tissues (Chapters 2 and 3).

5.7 The correlation between pigments and morphotypes

Colour divergence is a trait of many fish species e.g. there was a slight divergence in colour characteristics between the populations of lumpsucker (*Cyclopterus lumpus*) sampled from various sites (Mudge and Davenport 1986). It was observed that the roe and subcutaneous jelly were also highly variable in colour amongst populations, possibly as a result of genotypic or phenotypic origin (Davenport and Thorsteinsson 1989). The present study (Chapter 2) presented results for two colour morphotypes of lumpfish (green and yellow) for which there appeared to be a link with plasma colour: the green body colour displayed green and blue-green plasma and the yellow body exhibited a purplish blood plasma colouration. Therefore, it appears that external colour morphotype may also be associated with serum colour in lumpfish, yet the evidence is incomplete as to whether this is in connection to sex (male or female) or development stage (juvenile or adult). Davenport and Bradshaw (1995) suggested that colour divergence was a developmental (i.e. ontogenic) strategy: juvenile lumpfish employ a cryptic default light green colouration while swimming, to provide countershading, with the dorsal side of the body being darker than the belly. Lumpfish are also sexually dimorphic, females are blue, whereas males have red-orange body colouration during the breeding season (Mudge and Davenport 1986) and our (semi-quantitative) colorimetry results suggest differences in the blood plasma pigment profile are present in lumpfish (Figure 2.6, Chapter 2). However, UV scanning spectrophotometer spectra did not show differences in the wavelengths associated with plasma pigments between the lumpfish morphs. Whether these are related to age or sex (lumpfish are gonochoristic) remains to be seen in future studies. Whether there are any parallels between lumpfish morphotype variation and that in *L. bergylta* is also unknown. However, what we do suggest is that biliverdin appears more linked with colour polymorphism than hermaphroditism.

5.8 The correlation between reproductive mode and biliverdin in blood plasma

The theme of hermaphroditism was examined by investigating blood pigments in a number of coastal fishes, some of which were hermaphrodite and some of which were gonochoristic (separate sexes) (Chapter 2). Clark et al. (2016) examined the 'local' Labrini species (Goldsinny (*Crenilabrus rupestris*), Rockcook (*Centrolabrus exoletus*), Corkwing (*Symphodus melops*) and Cuckoo wrasse (*Labrus mixtus*)) which were sampled from Ardgour, Scotland (56°41'03.3"N 5°42'25.2"W), as well as examining males, females and transitional individuals in *L. bergylta*. They found that mean biliverdin concentrations were

high in both males (n=66) (mean = 10.71 µmol.l^{-1}) and females (n=322) (mean = 10.85 µmol.l^{-1}), but transitional individuals (n=9) were found to have lower concentrations (mean = 2.58 µmol.l^{-1}) (Clark et al. 2016). The authors suggested that biliverdin has a possible role in mopping-up the free radicals arising from re-modelling of female gonads during sex-change (Clark et al. 2016). Thus these authors suggested that high concentrations of biliverdin in blood plasma are associated with a hermaphroditic reproductive strategy. Clark et al. (2016) proposed that the pigment was consumed during the sex inversion process while disposing of damaging molecules released through the reconstruction of female gonad tissue. Patterns of mobilisation of the plasma biliverdin molecule were also suggested and the interpretation was that the tissue remodelling operations connected with sex inversion in protogynous hermaphrodites presents such a huge pro-oxidative challenge, that this is balanced by endogenous stores of biliverdin to keep the nascent testis and developing sperm from oxidative stress (Clark et al. 2016). However, in the present and previous studies (Low and Bada 1974; Mudge and Davenport 1986), gonochoristic fish species such as the short-spined sea scorpion (*Myoxocephalus scorpius*) and lumpfish (*Cyclopterus lumpus*) possessed biliverdin in their blood plasma which was confirmed using UV scanning spectrophotometry (Figure 2.7 A, Chapter 2). Moreover, the present study found that the long-spined sea scorpion (*Taurulus bubalis*), which is also gonochoristic, had biliverdin in its blood plasma. These findings do not agree with the hypothesis that suggests a connection between the hermaphroditic reproductive mode and biliverdin in blood plasma (Clark 2016; Clark et al. 2016). Another idea examined previously in Labridae (Abolins 1961) and in *C. lumpus* (Mudge and Davenport 1986) was that serum colouration was linked with sexual dimorphism, including external pigment deposition, with the purpose of sexual signalling. In the present study, however, no variation was observed between the sexes (Male = 6, Female = 49) in the biliverdin concentration of any of the tissues studied: skin, scales or plasma (Chapter 2 and 3). Furthermore, there were no apparent variations in the concentrations of biliverdin between male and female *L. bergylta*, as reported in other wrasse species (Clark et al. 2016).

5.9 Interaction of biliverdin and bilirubin

High biliverdin concentrations have been described in several species including *A. japonica* (Fang et al. 1986), *Clinocottus analis* (Fang and Bada 1988), and *L. bergylta* (Clark et al. 2016), which all possess the enzyme biliverdin reductase and consequently have the ability to reduce biliverdin to bilirubin. The presence of this enzyme was motive

for further study to determine the bilirubin concentration of *L. bergylta* (Chapter 2). Furthermore, this suggests the rather interesting question of whether the bilirubin and biliverdin together interact to cause the variation across individuals in plasma colour. In the present study, these pigments differed across morphotypes, locations and years. Plasma colour varied between Carna and Spiddal and between 2015 and 2016 (Figure 2.9, Chapter 2). So, while the plasma colour for most of the 2015 Carna samples was blue with a few blue-green plasma samples, the plasma colour of Carna 2016 and Spiddal (both years) was mainly green-ish. Only a minority of blue or blue-green plasma samples were found in the latter groups. Comparisons of colorimeter readings and *L*, a*, b** scores between years at Carna shows that these were somewhat separate at this location (Figure 2.14, Chapter 2). While in 2015, the predominant plasma colour values denoted bluer colours, in 2016 the majority of values denoted green colours. A separation between years at Spiddal was not as clear. In both years at Spiddal, most of the values occupied the green quadrants but datapoints were extended across the area which indicated blue-green (2016) or yellow-green (2015) colours. The greatest total bilirubin concentrations across locations were in samples taken from Spiddal in 2015. We propose that this 'bilirubin spike' in the dataset was the major explanation for the different proportions of biliverdin (blue-green) and bilirubin (yellow-orange) concentrations across locations and years, and consequently, was responsible for the variation observed in plasma colouration.

5.10 The correlation between biliverdin/morphotypes and external tissues (skin and scales)

Biliverdin has been linked with proteins in scales, periosteum and spinal column of garfish *Belone belone* and eelpout *Zoarces viviparus* (Jüttner et al. 2013). Similarly, blue forms of walleye *Sander vitreus* express biliverdin chromoproteins in mucous tissues (Yu et al. 2007). In *L. bergylta*, it was previously established that biliverdin was indirectly linked with external colour morphotype (Clark et al. 2016), where green morphotypes had a higher biliverdin concentration when compared with the same size of red morphotypes. For this reason, in addition to its role as an antioxidant, the author proposes that pigment plays an extra role in camouflage (Clark et al. 2016). Similarly, in the present study, biliverdin in skin and scale samples taken at Carna in 2015 were linked to the morphotypes: spotted and intermediate, had highest biliverdin concentrations, whereas merle and plain had the lowest (Chapter 3). It is likely that the high concentrations of biliverdin in plasma, particularly in spotted and intermediate morphotypes (Chapter 2), led to the build-up of these pigments on

skin and scales in these morphs. Spearman's rank correlations in both years and at both sites showed a weak positive relationship between plasma biliverdin and dorsal skin biliverdin, and this was positively correlated but stronger in plasma versus dorsal scales (Chapter 3).

It would appear that biliverdin can be accumulated in the skin, therefore its existence might have a role in helping fish to camouflage themselves to avoid predation and capture prey (Clark et al. 2016). Biliverdin may be highly interesting as a camouflage molecule since it is conformationally flexible. Thus, depending on the environmental context, colour ranges from blue to green can enable organisms such as bird eggs, insects and lower organisms to adapt their camouflage to the local environment (McDonagh 2001). Changes in the skin colour of teleost fish are proposed by Leclercq et al. (2009) to be caused by two different phenomena: morphological and physiological, with physiological colour changes characterised as acute temporary operations occurring due to the motility of pigment vesicles or reflecting elements within their cells (Bowman 1942; Rodionov et al. 1998; Rodionov et al. 2003). The direct effect of environmental factors, such as light, on pigment migration are the main players in physiological changes in colour (Oshima 2001). Furthermore, physiological colour changes related to pigment translocation were under control of the nervous system and the endocrine system; this process is referred to as secondary physiological colour changes (Fujii 2000) with multiple factors involved including the hormones adrenocorticotropic (ACTH) and alpha-melanocyte-stimulating (α-MSH) hormone. Countershading is a type of camouflage colouration where the dorsal side of the animal is dark, and the ventral side is light. The adaptive feature of countershading has been defined by two mechanisms: 1) concealment of self-shadow; and 2) matching the background (Ruxton et al. 2004; Kiltie 1988). The dark dorsal side of the fish is obscured from above by matching the water darkness or a dark substrate underneath. The light ventral side matches the down-welling of light from above and further obscures the fish when seen from beneath (Cox et al. 2009). In the present study, dorsal and ventral skin of *L. bergylta* were notably different in biliverdin content which may hint at its usage as countershading camouflage (Chapter 3).

5.11 Future research

This research has been the first to define both biliverdin and bilirubin concentrations of *L. bergylta* plasma as a contribution to the understanding and classification of four morphotypes (plain, spotted, merle and intermediate). Dietary information would be a way to further understand this species and possibly provide a deeper understanding of the variations between morphotypes. For example, future studies could investigate the relationship between diet, blood pigment profile and morphotypes. Another interesting avenue for further study could be to investigate habitat preferences and fidelity of different morphotypes to different habitats (thereby using blood pigments as camouflage) or, indeed, as some sort of external signalling to maintain assortative mating in morphotypes (particularly in the breeding season, for example). Associated with this, further research into possible drivers of and mechanisms under-pinning morphotype-linked *L. bergylta* life history differences and demographic variations between the morphotypes – how are these maintained? To address this question, further research might include investigation of genetic differences including more subtle markers such as single nuleotide polymorphisms. Future research would also benefit from considering the diversity of morphotypes, including but not limited to plain, spotted, merle and intermediate varieties, across the entire geographic range: do certain morphs appear only in some locations and what may be the explanations for this? Given the variability observed in many of the parameters measured in the present study, not least between locations and years, sufficient numbers of individuals from each morphotype should be selected, along with equal sampling effort, where possible. In conclusion, this thesis provides a motive for additional study not only on *L. bergylta* but on different species which possess blood pigments and polymorphism, to continue the investigation of the physiological roles of biliverdin in fish physiology.

References

Abolins, L. (1961). The existence of sexual dimorphism of blood plasma in fish of the family Labridae. *Arkiv Fur Zoologi* **13**, 541–544.

Almada, F., Casas, L., Francisco, S. M., Villegas-Ríos, D., Saborido-Rey, F., Irigoien, X., and Robalo, J. I. (2016). On the absence of genetic differentiation between morphotypes of the ballan wrasse *Labrus bergylta* (Labridae). *Marine Biology*, **163**(4), 86.

Barreto, F. S., and McCartney, M. A. (2008). Extraordinary AFLP fingerprint similarity despite strong assortative mating between reef fish color morphospecies. *Evolution: International Journal of Organic Evolution*, **62**(1), 226-233.

Bowman, T. E. (1942). Morphological color change in the crayfish. *The American Naturalist*, **76**(764), 332-336.

Choat, J. H., Klanten, O. S., Van Herwerden, L., Robertson, D. R., and Clements, K. D. (2012). Patterns and processes in the evolutionary history of parrotfishes (Family Labridae). *Biological Journal of the Linnean Society*, **107**(3), 529-557.

Clark, W., Leclercq, E., Migaud, H., Nairn, J., and Davie, A. (2016). Isolation, identification and characterisation of ballan wrasse *Labrus bergylta* plasma pigment. *Journal of Fish Biology*, **89**(4), 2070-2084.

Cox, R. M., Stenquist, D. S. and Calsbeek, R. (2009). Testosterone, growth and the evolution of sexual size dimorphism. *Journal of Evolutionary Biology*, **22**, 1586–1598.

D'Arcy, J., Mirimin, L., and FitzGerald, R. (2013). Phylogeographic structure of a protogynous hermaphrodite species, the ballan wrasse *Labrus bergylta*, in Ireland, Scotland, and Norway, using mitochondrial DNA sequence data. *ICES Journal of Marine Science*, **70**(3), 685-693.

Davenport, J. and Bradshaw, C. (1995). Observations on skin colour changes in juvenile lumpsuckers, *Journal of Fish Biology*, **47**, 143–154.

Davenport, J. and Thorsteinsson, V. (1989). Observations on the colours of lumpsuckers, *Cyclopterus lumpus* L., *Journal of Fish Biology*, **35**, 829–838.

Elmer, K. R., Lehtonen, T. K., and Meyer, A. (2009). Color assortative mating contributes to sympatric divergence of neotropical cichlid fish. *Evolution: International Journal of Organic Evolution*, **63**(10), 2750-2757.

Fang, L. S., and Bada, J. L. (1988). A special pattern of haem catabolism in a marine fish, *Clinocottus analis*, with green blood plasma. *Journal of Fish Biology*, **33**(5), 775-780.

Fang, L. S., Ong, C. W., and Hwang, W. S. (1986). A comparative study on the binding characteristics of the tight serum biliverdin-protein complexes in two fishes:

Anguilla japonica and *Clinocottus analis*. *Comparative biochemistry and physiology. B, Comparative biochemistry*, **84**(3), 393.

Faust, E., Halvorsen, K. T., Andersen, P., Knutsen, H., and André, C. (2018). Cleaner fish escape salmon farms and hybridize with local wrasse populations. *Royal Society Open Science*, **5**(3), 171752.

Fujii, R. Y. O. Z. O. (2000). The regulation of motile activity in fish chromatophores. *Pigment Cell Research*, **13**(5), 300-319.

Gavrilets S (2004) Fitness landscapes and the origin of species (MPB- 41). Princeton University Press, Princeton

Grober, M.S., and Bass, A.H. (2002). Life history, neuroendocrinology, and behavior in fish. In: Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T. (Eds.), Hormones, Brain and Behavior. Academic Press, New York, **2**, 331–347.

Jüttner, F., Stiesch, M. and Ternes, W. (2013). Biliverdin: The blue-green pigment in the bones of the garfish (*Belone belone*) and eelpout (*Zoarces viviparus*), *European Food Research and Technology*, **236**, 943–953.

Kiltie, R. A. (1988). Countershading: universally deceptive or deceptively universal?. *Trends in Ecology and Evolution*, **3**(1), 21-23.

Leclercq, E., Taylor, J. F., and Migaud, H. (2009). Morphological skin colour changes in teleosts. *Fish and Fisheries*, **11**(2), 159-193.

Low, P., and Bada, J. L. (1974). Bile pigments in the blood serum of fish from the family Cottidae. *Comparative Biochemistry and Physiology, Part A: Physiology*, **47**(2), 411-418.

McDonagh, A. F. (2001). Turning green into gold. Nature Structural Biology, 8(3), 198–200.

Mudge, M. and Davenport, J. (1986). Serum pigmentation in *Cyclopterus lumpus* L, *Journal of Fish Biology*, **29**, 737–745.

Munday, P. L., Buston, P. M., and Warner, R. R. (2006). Diversity and flexibility of sex-change strategies in animals. *Trends in Ecology and Evolution*, **21**(2), 89-95.

Oshima, N. (2001). Direct reception of light by chromatophores of lower vertebrates. *Pigment Cell Research*, **14**(5), 312-319.

Palumbi, S. R. (1992). Marine speciation on a small planet. *Trends in Ecology and Evolution*, **7**(4), 114-118.

Pita, P., and Freire, J. (2011). Movements of three large coastal predatory fishes in the northeast Atlantic: a preliminary telemetry study. *Scientia Marina*, **75**(4), 759-770.

Pita, P. and Freire, J. (2017). Trophic ecology of an Atlantic kelp forest fish assemblage (NW Spain) targeted by recreational fishers and implications for coastal management, *Journal of the Marine Biological Association of the United Kingdom*, 1–11.

Planes, S., and Doherty, P. J. (1997). Genetic relationships of the colour morphs of *Acanthochromis polyacanthus* (Pomacentridae) on the northern Great Barrier Reef. *Marine Biology*, **130**(1), 109-117.

Puebla, O. (2009). Ecological speciation in marine v. freshwater fishes. *Journal of Fish Biology*, **75**(5), 960-996.

Puebla, O., Bermingham, E., Guichard, F., and Whiteman, E. (2007). Colour pattern as a single trait driving speciation in Hypoplectrus coral reef fishes?. *Proceedings of the Royal Society B: Biological Sciences*, **274**(1615), 1265-1271.

Quintela, M., Danielsen, E., Lopez, L., Barreiro, R., Svåsand, T., Knutsen, H., Skiftesvik, A. and Glover, K. (2016). Is the ballan wrasse (*Labrus bergylta*) two species? Genetic analysis reveals within-species divergence associated with plain and spotted morphotype frequencies. *Integrative Zoology*, **11**(2), 162-172.

Robertson, D. R., and Choat, J. H. (1974). Protogynous hermaphroditism and social systems in labrid fish. *In Proceeding of the Second International Coral Reef Symposium 1*. **1**, 217-225.

Robinson, B. W., and Wilson, D. S. (1994). Character release and displacement in fishes: a neglected literature. *The American Naturalist*, **144**(4), 596-627.

Rodionov, V. I., Hope, A. J., Svitkina, T. M., and Borisy, G. G. (1998). Functional coordination of microtubule-based and actin-based motility in melanophores. *Current Biology*, **8**(3), 165-169.

Rodionov, V., Yi, J., Kashina, A., Oladipo, A., and Gross, S. P. (2003). Switching between microtubule-and actin-based transport systems in melanophores is controlled by cAMP levels. *Current Biology*, **13**(21), 1837-1847.

Ruxton, G.D., Sherratt, T. N. and Speed,M. P. (2004) Avoiding Attack. Oxford, UK:Oxford University Press.

Salzburger, W., Niederstätter, H., Brandstätter, A., Berger, B., Parson, W., Snoeks, J., and Sturmbauer, C. (2006). Colour-assortative mating among populations of *Tropheus moorii*, a cichlid fish from Lake Tanganyika, East Africa. *Proceedings of the Royal Society B: Biological Sciences*, **273**(1584), 257-266.

Seljestad, G. W., Quintela, M., Faust, E., Halvorsen, K. T., Besnier, F., Jansson, E., Dahle, G., Knutsen, H., André., Folkvord, A. and Glover, K. A. (2020) "A cleaner break": Genetic divergence between geographic groups and sympatric phenotypes revealed in ballan wrasse (*Labrus bergylta*). *Ecology and Evolution*, **10**, 6120-6135.

Sherwood, G. D., and Grabowski, J. H. (2010). Exploring the life-history implications of colour variation in offshore Gulf of Maine cod (*Gadus morhua*). *ICES Journal of Marine Science*, **67**(8), 1640–1649.

Sjolander, S., Larson, H., and Engstrom, J. (1972). On the reproductive behaviour of two labrid fishes, the ballan wrasse (*Labrus bergylta*) and Jago's goldsinny (*Ctenolabrus rupestris*). *Revue du Comportement Animal*, **6**, 43-51.

Villegas-Ríos, D. (2013b). Life-history and behaviour of "*Labrus bergylta*" in Galicia. Ph.D. University of Vigo, Galicia, Spain.

Villegas-Ríos, D., Alonso-Fernández, A., Fabeiro, M., Bañón, R., and Saborido-Rey, F. (2013a). Demographic variation between colour patterns in a temperate protogynous hermaphrodite, the ballan wrasse *Labrus bergylta*. *PLoS One*, **8**(8), e71591.

Webber, R., Barlow, G. W., and Brush, Alan H. (1973). Pigments of a color polymorphism in a cichlid fish. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry.* **44B**,1 127-1135.

Yu, C.-L., Ferraro, D., Ramaswamy, S., Schmitz, M. H., Schaefer, W. F., and Gibson, D. T. (2007). Purification and properties of Sandercyanin, a blue protein secreted in the mucus of blue forms of walleye, *Sander vitreus*. *Environmental Biology of Fishes*, **82**(1), 51– 58.

Appendix

Sample No.	Location	Date	Fishing method	Morphs	length (cm)	Weight (g)
BW1	Carna	03/06/2015	Lobster pots	Spotted	40.7	1420
BW2	Carna	03/06/2015	Lobster pots	Spotted	32	612
BW3	Carna	03/06/2015	Lobster pots	Spotted	36.2	872
BW4	Carna	03/06/2015	Lobster pots	Intermediate	35.4	827
BW5	Carna	03/06/2015	Lobster pots	Plain	40.1	1271
BW6	Carna	03/06/2015	Lobster pots	Spotted	42.2	1186
BW7	Carna	03/06/2015	Lobster pots	Plain	43.5	1512
BW8	Carna	03/06/2015	Lobster pots	Plain	35.2	761
BW9	Spiddal	25/06/2015	Rod and line	Intermediate	37.5	921
BW10	Spiddal	25/06/2015	Rod and line	Merle	16.3	65
BW11	Spiddal	25/06/2015	Rod and line	Merle	32.6	542
BW12	Spiddal	25/06/2015	Rod and line	Merle	22.3	156
BW13	Spiddal	01/07/2015	Rod and line	Merle	25.2	197
BW14	Spiddal	01/07/2015	Rod and line	Merle	32	584
BW15	Spiddal	01/07/2015	Rod and line	Plain	36.8	611
BW16	Spiddal	28/07/2015	Rod and line	Merle	25	300
BW17	Spiddal	28/07/2015	Rod and line	Merle	30	500
BW18	Spiddal	28/07/2015	Rod and line	Merle	29.4	449
BW19	Spiddal	28/07/2015	Rod and line	Spotted	21.9	168
BW20	Spiddal	28/07/2015	Rod and line	Merle	31.5	590
BW21	Spiddal	28/07/2015	Rod and line	Merle	24	255
BW22	Spiddal	28/07/2015	Rod and line	Merle	25	278
BW23	Spiddal	28/07/2015	Rod and line	Merle	22.5	207
BW24	Spiddal	07/08/2015	Rod and line	Merle	27.3	336
BW25	Spiddal	07/08/2015	Rod and line	Merle	25.6	254
BW26	Spiddal	07/08/2015	Rod and line	Merle	31.3	551
BW27	Spiddal	07/08/2015	Rod and line	Merle	38	891
BW28	Spiddal	07/08/2015	Rod and line	Merle	30.6	454
BW29	Spiddal	07/08/2015	Rod and line	Merle	30.5	429
BW30	Spiddal	07/08/2015	Rod and line	Merle	30	445
BW31	Spiddal	07/08/2015	Rod and line	Merle	26.9	313
BW32	Spiddal	07/08/2015	Rod and line	Plain	42	1209
BW33	Spiddal	07/08/2015	Rod and line	Merle	30.8	473
BW34	Spiddal	07/08/2015	Rod and line	Merle	22	164
BW35	Spiddal	11/08/2015	Rod and line	Merle	28	342
BW36	Spiddal	11/08/2015	Rod and line	Merle	20.3	138
BW37	Spiddal	11/08/2015	Rod and line	Merle	18.9	105
BW38	Spiddal	11/08/2015	Rod and line	Plain	35	557
BW39	Carna	20/08/2015	Lobster pots	Spotted	39.7	1092
BW40	Carna	20/08/2015	Lobster pots	Spotted	44	1450
BW41	Carna	20/08/2015	Lobster pots	Spotted	40.5	1127

BW42	Carna	20/08/2015	Lobster pots	Plain	39.7	972
BW43	Carna	20/08/2015	Lobster pots	Intermediate	41	1300
BW44	Carna	20/08/2015	Lobster pots	Intermediate	38.8	1064
BW45	Carna	20/08/2015	Lobster pots	Merle	40.9	1121
BW46	Carna	20/08/2015	Lobster pots	Intermediate	40.7	1151
BW47	Carna	20/08/2015	Lobster pots	Intermediate	41.7	1197
BW48	Carna	20/08/2015	Lobster pots	Intermediate	39.5	1045
BW49	Carna	20/08/2015	Lobster pots	Plain	38.5	961
BW50	Carna	20/08/2015	Lobster pots	Intermediate	38.7	971
BW51	Spiddal	21/08/2015	Rod and line	Intermediate	44.6	1505
BW52	Spiddal	21/08/2015	Rod and line	Merle	20.6	126
BW53	Spiddal	21/08/2015	Rod and line	Spotted	33.9	635
BW54	Spiddal	21/08/2015	Rod and line	Merle	24	204
BW55	Spiddal	21/08/2015	Rod and line	Merle	25.6	247
BW56	Spiddal	21/08/2015	Rod and line	Merle	23.3	184
BW57	Carna	27/08/2015	Lobster pots	Spotted	46.5	1724
BW58	Carna	27/08/2015	Lobster pots	Spotted	41.8	1170
BW59	Carna	27/08/2015	Lobster pots	Intermediate	42	1347
BW60	Carna	02/09/2015	Lobster pots	Intermediate	45.8	1546
BW61	Carna	02/09/2015	Lobster pots	Spotted	46.5	2068
BW62	Carna	02/09/2015	Lobster pots	Intermediate	45.4	1753
BW63	Carna	02/09/2015	Lobster pots	Intermediate	45.5	1798
BW64	Carna	02/09/2015	Lobster pots	Intermediate	42.4	1430
BW65	Carna	02/09/2015	Lobster pots	Merle	36.3	832
BW66	Carna	02/09/2015	Lobster pots	Intermediate	42.8	1378
BW67	Carna	09/09/2015	Lobster pots	Spotted	55	1947
BW68	Carna	09/09/2015	Lobster pots	Intermediate	40.3	1096
BW69	Carna	09/09/2015	Lobster pots	Merle	40	1259
BW70	Carna	09/09/2015	Lobster pots	Spotted	40	1029
BW71	Carna	09/09/2015	Lobster pots	Intermediate	39.7	1054
BW72	Carna	09/09/2015	Lobster pots	Spotted	46	2000
BW73	Carna	09/09/2015	Lobster pots	Spotted	43.3	1454
BW74	Carna	09/09/2015	Lobster pots	Intermediate	45	1500
BW75	Carna	09/09/2015	Lobster pots	Spotted	41.9	1212
BW76	Carna	09/09/2015	Lobster pots	Intermediate	40.1	1200
BW77	Carna	09/09/2015	Lobster pots	Merle	40.9	1116
BW78	Carna	17/09/2015	Lobster pots	Intermediate	37.7	887
BW79	Carna	17/09/2015	Lobster pots	Merle	40	1165
BW80	Carna	17/09/2015	Lobster pots	Spotted	46.3	1623
BW81	Carna	17/09/2015	Lobster pots	Merle	38.3	866
BW82	Carna	17/09/2015	Lobster pots	Merle	34.7	711
BW83	Carna	17/09/2015	Lobster pots	Intermediate	36	768
BW84	Carna	17/09/2015	Lobster pots	Intermediate	37.6	870
BW85	Carna	17/09/2015	Lobster pots	Merle	39.3	1119

BW86	Carna	01/10/2015	Lobster pots	Intermediate	37.7	859
BW87	Carna	01/10/2015	Lobster pots	Intermediate	37.1	910
BW88	Carna	01/10/2015	Lobster pots	Merle	36.5	742
BW89	Carna	01/10/2015	Lobster pots	Merle	32.7	518
BW90	Carna	01/10/2015	Lobster pots	Merle	31	433
BW91	Carna	01/10/2015	Lobster pots	Merle	32.5	531
BW92	Carna	01/10/2015	Lobster pots	Plain	46.7	1857
BW93	Carna	01/10/2015	Lobster pots	Intermediate	42.7	1327
BW94	Spiddal	30/06/2016	Rod and line	Merle	23.4	198
BW95	Spiddal	30/06/2016	Rod and line	Intermediate	39.5	976
BW96	Spiddal	30/06/2016	Rod and line	Plain	25.7	246
BW97	Spiddal	30/06/2016	Rod and line	Plain	30.2	429
BW98	Spiddal	01/07/2016	Rod and line	Merle	22.7	185
BW99	Spiddal	01/07/2016	Rod and line	Merle	23.3	184
BW100	Spiddal	01/07/2016	Rod and line	Merle	21.3	139
BW101	Spiddal	01/07/2016	Rod and line	Merle	34.3	550
BW102	Spiddal	01/07/2016	Rod and line	Spotted	43.5	1420
BW103	Spiddal	01/07/2016	Rod and line	Merle	28.2	321
BW104	Spiddal	12/07/2016	Rod and line	Merle	29.0	419
BW105	Spiddal	12/07/2016	Rod and line	Merle	29.7	416
BW106	Spiddal	12/07/2016	Rod and line	Intermediate	29.5	385
BW107	Spiddal	12/07/2016	Rod and line	Merle	22.9	195
BW108	Spiddal	13/07/2016	Rod and line	Intermediate	25.0	300
BW109	Spiddal	13/07/2016	Rod and line	Spotted	33.1	602
BW110	Spiddal	13/07/2016	Rod and line	Merle	29.3	390
BW111	Spiddal	13/07/2016	Rod and line	Spotted	34.3	632
BW112	Spiddal	13/07/2016	Rod and line	Plain	27.0	283
BW113	Spiddal	24/08/2016	Rod and line	Merle	25.3	265
BW114	Spiddal	24/08/2016	Rod and line	Merle	24.3	224
BW115	Spiddal	24/08/2016	Rod and line	Merle	22.2	173
BW116	Spiddal	24/08/2016	Rod and line	Merle	18.2	93
BW117	Spiddal	24/08/2016	Rod and line	Spotted	30.5	457
BW118	Spiddal	24/08/2016	Rod and line	Merle	19.3	113
BW119	Spiddal	24/08/2016	Rod and line	Merle	22.0	171
BW120	Spiddal	25/07/2016	Rod and line	Spotted	45.3	1564
BW121	Spiddal	25/07/2016	Rod and line	Plain	21.2	150
BW122	Spiddal	25/07/2016	Rod and line	Merle	22.5	166
BW123	Spiddal	25/07/2016	Rod and line	Merle	33.6	584
BW124	Carna	31/08/2016	Rod and line	Spotted	31.6	514
BW125	Carna	31/08/2016	Rod and line	Merle	31.8	533
BW126	Carna	31/08/2016	Rod and line	Merle	28.0	405
BW127	Carna	31/08/2016	Rod and line	Merle	35.0	763
BW128	Carna	31/08/2016	Rod and line	Intermediate	27.5	368
BW129	Carna	31/08/2016	Rod and line	Merle	39.6	1086

BW130	Carna	31/08/2016	Rod and line	Spotted	28.5	411
BW131	Carna	31/08/2016	Rod and line	Merle	32.3	573
BW132	Carna	31/08/2016	Rod and line	Intermediate	29.7	475
BW133	Carna	31/08/2016	Rod and line	Merle	23.3	210
BW134	Carna	15/09/2016	Rod and line	Merle	26.6	337
BW135	Carna	15/09/2016	Rod and line	Spotted	31.8	552
BW136	Carna	15/09/2016	Rod and line	Merle	34.1	689
BW137	Carna	15/09/2016	Rod and line	Spotted	32.4	530
BW138	Carna	15/09/2016	Rod and line	Merle	26.4	289
BW139	Carna	15/09/2016	Rod and line	Spotted	36.3	713
BW140	Carna	15/09/2016	Rod and line	Intermediate	28.9	393
BW141	Carna	15/09/2016	Rod and line	Spotted	31.0	452
BW142	Carna	29/09/2016	Rod and line	Spotted	34.7	721
BW143	Carna	30/09/2016	Rod and line	Intermediate	39.5	1175
BW144	Carna	30/09/2016	Rod and line	Merle	34.0	700
BW145	Spiddal	11/10/2016	Rod and line	Merle	27.0	320
BW146	Spiddal	11/10/2016	Rod and line	Plain	28.3	347
BW147	Spiddal	11/10/2016	Rod and line	Merle	26.5	286
BW148	Spiddal	11/10/2016	Rod and line	Merle	21.2	153
BW149	Spiddal	11/10/2016	Rod and line	Merle	20.8	135