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

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DECLARATION

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

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Abstract

Labrus bergylta (ballan wrasse) have recently emerged as a key resource to aquaculture through proven efficacy in controlling infestations of sea lice (Leclercq et al., 2014a). However, due to complex ecology, and a complete lack of sexual dimorphism gender identification endures as a key restriction to optimising broodstock management therefore male selection and establishing optimal sex ratios is difficult (Talbot et al., 2012). *L. bergylta*, are noted to demonstrate unusually coloured plasma ranging in hue from green to blue with the haem catabolite biliverdin established as the causal pigment in the majority of cases (Abolins, 1961). As most vertebrates excrete biliverdin, or rapidly metabolise it to prevent toxicity, accumulation to such excess is a phenomenon which merits attention. Notably, correlation between plasma biliverdin and gender has been reported in some Labridae. Although patterns vary between species, the abundance or characteristics were such that sexual identity could be established (Gagnon, 2006). Pigment analysis was therefore proposed as a potential sex-marker in *L. bergylta*. In the initial experimental phase (Chapter 3), the ultimate aim was to isolate and identify the blue pigment from *L. bergylta* plasma, and to develop a method of quantification. The initial phase confirmed the target pigment was biliverdin IXα by visible spectroscopy, TLC, HPLC, MSMS, and a series of reactions. Following this, a protocol was developed (Chapter 2) to quantify the pigment. This method was applied accross plasma sampled from four geographically distinct wild populations with established biometrics including age, mass, length, gender and external phenotype. Subsequent analysis revealed that although pigment abundance did not vary relative to ontogeny, and there was no difference in concentration between the binary genders, plasma biliverdin was depleted in individuals undergoing sex change. Although this conclusion was complicated by significant biliverdin variation relative to origin and phenotype, which were interrelated based on relative distributions across populations, further analysis of plasma pigment in related species identified that biliverdin

accumulation was associated with protogynous species. Considering the anti-oxidant capacity of biliverdin and other potentially relevant functions, this was indicative of association with the tissue remodelling processes which accompany inversion.

During Chapter 3 it was noted that the biliverdin appeared tightly bound to a protein moiety. Based on the hypothesis that the pigment was actively managed and accumulated in *L. bergylta* plasma by this association, the next phase of experiments (Chapter 4) was an exploration of biliverdin and its binding protein in *L. bergylta*. The experiments revealed plasma biliverdin comigrated with the protein such that it was depleted from solution at the same rate indicating that all of the pigment was associated. Subsequent electrophoretic experiments using the fractionation products supported this, and UV fluourescence identified fragments of interest in the 25-28 kDa region. To confirm observations from the previous cross species comparison, the study was similarly expanded to include other Labrini. This revealed that although the 25 kDa band was common to all species, and genders, the 28 kDa band was collocated with the protogynous, and as such hyperbiliverdinaemic species. The 28 kDa band was sequenced using MSMS, and was identified as similar to the lipocalin Apolipoprotein A1. In combination with the properties of biliverdin, and considering that ApoA1 is analogous to serum albumin in many telesots, this supported the chromoprotein association as the main mechanism of biliverdin accumulation in such species. Further to the proposed function of biliverdin with inversion processes, and considering relevant literature, the active properties of ApoA1 suggested additional associations with prolonged altered states of metabolism which considering the ecology of *L. bergylta* would include gender transition, overwintering torpor and prolonged micronutrient limitation, all of which occur simultaneously. Other potential roles include modulating inflammatory responses, inhibiting pathogenic incursions and acting as an external point of contact innate immune response. From this, it was concluded that the data fully supported the previous assertions of biliverdins relevance in protogynous species,

and identified a number of properties which could be of great interest to the industry in terms of welfare.

The final experimental phase (Chapter 5) had two main aims. The first was to establish whether protogynous inversion could be artificially induced in *L. bergylta* as a means of generating male fish, and whether size had any effect on the process. The second was then to utilise controlled induction for tracking biliverdin mobilisation across the process to test the previous hypothesis. The preliminary trial demonstrated that both androgen inhibition and non-aromatisable testosterone could stimulate inversion in female *L. bergylta*. From this, the second trial then determined that although there was a dose dependant effect in that high androgen dosages appeared to compress the inversion process, relative size was not a factor. Gonad histology was used to create a unified scale of protogynous transition which could be expressed as a gradient to structure the biliverdin analysis. Although the biliverdin data demonstrated cryptic trends at the higher resolution gender scales, when the endpoint was condensed back to the binary gender scale employed previously (Chapter 3), the prior assertion of depletion during transition, and therefore the association with sex change associated tissue remodelling was supported.

Ultimately this thesis revealed links between the biliverdin macromolecule and the highly unusual metabolic and physiological demands of gender transition in sequentially protogynous hermaphroditic temperate wrasse species.

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"Equipped with his five senses, man explores the universe around him and calls it science"

- Edward Powell Hubble

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List of Abbreviations

Abbreviation	Term
11-KT	11-ketotestosterone
17α -MT	17α -Methyltestosterone
ACTH	Adrenocorticotropin
AmSO4	Ammonium Sulphate
AP	Activator Protein
APO	Apomorphine
ApoA1	Apolipoprotein A1
BBP	Biliverdin Binding Proteins
BCs	Blue Chromoproteins
BSA	Bovine Serum Albumin
BV	Biliverdin
BV.HCl	Bilivedin Hydrochloride
BVR	Biliverdin Reductase
$C_2H_4O_2$	Acetic acid (Glacial)
$C_4H_4N_2O_3$	Barbituric acid
C_4H_9OH	Butanol
$C_6H_8O_6$	Ascorbic acid
CBR	Conjugated Bilirubin
$CHCl_3$	Chloroform

Abbreviation	Term
CNS	Central Nervous System
CPS	Cyanoproteins
CRP	C-Reactive Protein
DA	Dopamine
DAMP	Damage Associated Molecular Patterns
DTT	Dithiothreitol
E_2	17β-Estradiol
EFA	Exploratory Factor Analysis
ERK	Extracellular signal-Regulated Kinase
EtOH	Ethanol
FAD	Fadrazole
$FeCl_3$	Ferric chloride
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin Releasing Hormone
GST	Glutathione-S-Transferase
GH	Growth Hormone
GPx	Glutathione Peroxidase
GRB-2	Growth factor Receptor Bound protein 2
GSI	Gonado-somatic indices

Abbreviation	Term
GST	Glutathione-S-Transferase
GtH	Gonadotropins
HBV	Hyperbiliverdinaemic
HDL	High Density Lipoproteins
HO	Haem Oxygenase
HPA	Hypothalamic-Pituitary-Adrenal axis
HPG	Hypothalamic-Pituitary-Gonadal axis
HPLC	High Pressure Chromatography
HR	Hemoprotein Reductase
Hsp32	Heat Shock Protein 32
HPX	Haemopexin
IGF	Insulin like Growth Factor
IL-10	Interleukin 10
INS	Insectacyanin
IPM	Integrated Pest Management
IR	Ischemia-Reperfusion
IRec	Insulin Receptor
IRK	Insulin Receptor kinases
IRS	Insulin Receptor Substrates
LH	Leuteinising Hormone

Abbreviation	Term
L-PGDS	Lipo-Prostaglandin Synthase
MAPK	Mitogen-Activated Protein Kinase
MDHT	17α -Methyldihydrotestosterone
MeOH	Methanol
MeOH.HCl	Hydrochloric Methanol
MUP	Major Urinary Protein
MYA	Million Years Ago
NaOH	Sodium Hydroxide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NES	Nuclear Export Sequence
NE	Norepinephrine
NF-ƙB	Nuclear Factor-ƙB
$NH_4CH_3CO_2$	Ammonium acetate
NLS	Nuclear Localisation Sequence
OD	Over Dose
P13K	Phoshosphatidylinositol-3 kinase
PKC	Protein Kinase C
PIT	Passive Integrated Transponder
P-OA	Pre-Optic Area
PRL	Prolactin

Abbreviation	Term
PUFA	Poly-Unsaturated Fatty Acids
RES	Reticuloendothelial System
Rf	Retardation factor
ROS	Reactive Oxygen Species
RRf	Relative Retardation Factor
SA	Serum Albumin
SDS	Sodium Dodecyl Sulphate
SGR	Specific Growth Rate
SH2	Src homology binding sites
SOD	Superoxide Dismutase
T	Testosterone
TEMED	Tetramethylethylenediamine
TLC	Thin layer Chromatography
TLRs	Toll-Like Receptors
TSH	Thyroid Stimulating Hormone
UDPGT	Uridine-Diphosphate glucuronosyltransferase
UGT	Uridine 5'-diphospho-Glucoronide Transferase
UV	Ultra Violet

Species list

Common name	Binomial
African Lungfish	*Neoceratodus forsteri*
Ailanthus silk-moths	*Samia ricini*
Amazon mollies	*Poecilia formosa*
Artic sculpin	*Myoxocephalus scorpioides*
Axillary wrasse	*Crenilabrus mediterraneus*
Ballan wrasse	*Labrus bergylta*
Bambooleaf wrasse	*Pseudolabrus sieboldi*
Bean bugs	*Riptortus clavatus*
Bergall	*Tautoglabrus adspersus*
Black porgy	*Tautoga onitis*
Black-bream	*Spondyliosoma cantharus*
Blue throated wrasse	*Notolabrus tetricus*
Blue walleye	*Sander vitreus*
Blue-banded goby	*Lythrypnus dalli*
Blunt-head wrasse	*Thalassoma amblycephalum*
Blue-head wrasse	*Thalassoma bifasciatum*
Blue-side wrasse	*Cirrhilabrus cyanopleura*
Blue-spotted grouper	*Epinephalus fario*
Blue-streak wrasse	*Labroides dimidiatus*

Common name	Binomial
Broad barred goby	*Gobiodon histrio*
Brown crabs	*Cancer pagurus*
Brown wrasse	*Labrus merula*
Cabbage looper	*Trichoplusia ni*
Cabbage white butterflies	*Pieris xuthus and Pieris brassicae*
Channel catfish	*Ictalurus punctatus*
Cinnamon clownfish	*Amphiprion melanopus*
Cocktail wrasse	*Pteragogus aurigarius*
Common shore crabs	*Carcinus maenas*
Corkwing wrasse	*Symphodus melops*
Corn earworm	*Helicoverpa zea*
Cuckoo wrasse	*Labrus mixtus*
Doderleinis' wrasse	*Symphodus doderleini*
Dusky grouper	*Epinephalus marginatus*
Eelpout	*Zoarces viviparous*
Emerald cod	*Trematomus bernacchii*
Emerald wrasse	*Centrolabrus trutta*
European carp	*Cyprinus carpio*

Common name	Binomial
European eel	*Anguilla anguilla*
European perch	*Perca fluviatilis*
Five spotted wrasse	*Symphodus Roisalli*
Gar fish	*Belone belone*
Gilt-head bream	*Sparus aurata*
Goldsinny wrasse	*Ctenolabrus rupestris*
Greasy grouper	*Epinephalus tauvina*
Green wrasse	*Labrus viridis*
Green-eyed wrasse	*Symphodus ocellatus*
Guinea pig	*Cavia porcellus*
Guppy	*Poecilia reticulata*
Hawk moths	*Agrius convolvuli*
Heavybeak parrotfish	*Scarus gibbus*
Honeycomb grouper	*Epinephalus merra*
Hong Kong grouper	*Epinephalus akaara*
Humphead wrasse	*Cheilinus undulatus*
Japanese killifish	*Oryzias latipes*
Japanese puffer	*Takifugu rubripes*
Japanese rice eel	*Anguilla japonica*
Knob-snout parrotfish	*Callyodon ovifrons*

Common name	Binomial
Long-finned eel	*Anguilla dieffenbachii*
Lumpsuckers	*Cyclopterus lumpus*
Lyretail anthia	*Anthia squamipinnis*
Mackerel	*Scomber scrombrus*
Mediterranean black-tail wrasse	*Symphodus melanocercus*
Mediterranean rainbow wrasse	*Coris julis*
Migratory locust	*Locusta migratoria*
Mussel	*Mytilus edulis*
Orange spotted grouper	*Epinephalus coioides*
Oriental leafworm	*Spodoptera litura*
Ornate wrasse	*Thalassoma pavo*
Pacific saury	*Cololabis saira*
Peacock wrasse	*Crenilabrus tinca*
Pink-bream	*Pagellus centrodontus*
Plaice	*Pleuronectes platessa*
Potato grouper	*Epinephalus tukula*
Rainbow trout	*Oncorhynchus mykiss*
Rockcook wrasse	*Centrolabrus exoletus*
Sablefish	*Anaploma fimbria*

Common name	Binomial
Saddle wrasse	*Thalassoma duperrey*
Salmon	*Salmo salar*
Scale rayed wrasse	*Acantholabrus palloni*
Sea lice	*Lepeophtheirus salmonis*
Sea lice	*Caligus elongatus*
Sea scorpion	*Cottus scorpius*
Sea trout	*Salmo trutta*
Sevenband grouper	*Epinephalus septemfasciatus*
Short-finned eel	*Anguilla australis schmidtii*
Shovelnose sturgeon	*Scaphirhynchus platorynchus*
Small-mouth wrasse	*Centrolabrus caerelius*
Southern bluefin tuna	*Thunnus maccoyii*
Three-spotted wrasse	*Haliocheres trimaculatus*
Tobacco hornworm	*Manduca sexta*
Tricolour parrotfish	*Scarus cyanognathus*
Wild silkworm	*Antheraea yamamai*
Woolly sculpin	*Clinocottus analis*
Yellow croakers	*Larimichthys crocea*
Zebra fish	*Danio rerio*
Zebra mbuna	*Meylandia zebra*

Chapter 1:

General introduction

1.1 A context for cleanerfish

The copepodids *Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* (Nordmann) (collectively termed sea lice hereafter) represent the most damaging ectoparasites to salmonid aquaculture in terms of animal welfare, environmental impact and commercial significance (Boxaspen & Naess, 2000; Muncaster et al., 2010). In agreement with Soberon et al. (2014), "It is an unfortunate side effect of the high densities of organisms per unit area associated with intensive modern farming practices that the balance between pathogen and host can become severely uncoupled leading to the emergence of infections and parasites far beyond natural levels".

Sea lice use rasping mandibles to graze mucus, skin, underlying tissues and blood from hosts (Whelan, 2010). This results in increased mucus discharge, altered mucus biochemistry, decreased lymphocytes and proteins, ion imbalance, elevated cortisol and tissue necrosis (Bowers et al., 2000; Torrissen et al., 2013). Extreme infestations then cause mortality through epidermal erosion associated osmoregulatory dysfunction (Revie et al., 2002) and anaemia (Costello, 2006). As parasitized individuals are then physically compromised, chronically stressed and have impaired immuno-competence (Pickering & Pottinger, 1989), the risk of secondary pathogenesis is greatly increased as sea lice are also passive vectors of epizootics (Costello, 2006; Pike, 1989). Sub-lethal parasitism must also be considered (Grimnes & Jakobsen, 1996). Productivity is affected as host fish have reduced appetite and increased metabolic demand which impairs growth rate and food conversion efficiency through diversion of energy from somatic maintenance (Mustafa et al., 2001). Market value is also affected by lice attachment as external lesions reduce organoleptic quality (Bjordal, 1992; Costello, 2006). With estimated annual costs of £28 million in Scotland alone, there is significant interest in developing alternative strategies for preventing and managing parasite infestations over the last 40 years (Murray, 2014).

Another major concern is ecological fallout due to import and export of planktonic lice larvae by passive dispersal (Costello, 2006) as infection pressure is a function of the number of fish in a given system combined with the abundance of lice (Gjerde & Saltkjelvik, 2009). From this, as farmed fish exceed local counterparts by ~100:1 (Gjerde & Saltkjelvik, 2009), mariculture populations of salmonids can act as reservoirs for infection of wild populations due to proximity of out-migrating juveniles to cages during travel from freshwater to the open sea (Revie et al., 2009). The small and often scale-less juveniles which are already stressed by environmental transition, then become hosts to extensive populations of lice (Costello, 2009b) with the reduced survival probability having potentially far-reaching negative impacts on native stocks (Costello, 2009a; Whelan, 2010). Conversely, evidence of simultaneous occupation by multiple parasite generations (Boxaspen, 2006), observations of low level infestation in offshore environments, and year round residence of sea trout (*Salmo trutta*) (L. 1758) in sea lochs (Torrissen et al., 2013) support self-perpetuating cycles of parasitism (Costello, 2006). Parastic infestations are therefore initiated by remote import from wild populations, driven by cross-infection and amplification in farmed fish, then maintained through subsequent net export of lice from cages to wild populations (Costello, 2006).

Industry remains reliant on diagnostic protocols and chemotherapeutic interventions to control such infestations at present (Denholm et al., 2002). Monitoring systems are based upon regulated sampling regimes and specified protocols using threshold values calculated as average lice per fish (1 adult female louse per fish July-Jan or 0.5 Feb-June) (Denholm et al., 2002; Murray, 2014). Although this can be sensitive to artefacts including cage effects and local hydrology which make skewed estimations likely (Mustafa et al., 2001), it is still highly effective at determining treatment regimes (Cunningham, 2006). However; there is growing concern for the effective life span of such a limited range of topical agents, and such methods commonly receive negative public attention (Aaen et al., 2015; Torrissen et al., 2013).

Bath delousing using Deltamethrin (Alphamax™), Azamethos (Salmosan™), Cypermethrin (Excis™) and hydrogen peroxide (H_2O_2) with tarpaulin skirts to enclose net pens can be a major stressor for fish and presents numerous technical issues. These include material costs (Liu & Bjelland, 2014), crowding (Cunningham, 2006), oxygen saturation (anoxia) (Stien et al., 2012), labour costs (Roth, 2000), variable enclosed water volumes with unpredictable dispersal patterns (Denholm et al., 2002), postponed harvesting due to extended residence (half-life) (Raynard et al., 2002), and potential exposure to toxic chemicals and dangerous processes for workers (Bjordal, 1992). This is further compounded by low treatment sensitivity in juvenile lice stages so repeat applications are required to eliminate the residuals as they mature (Grant, 2002). This must also be considered in the context of legislative restrictions on discharge limits (set as quantity per unit time), which can prevent synchronous application in multi-pen farms hence promoting re-infection and perpetuating cyclical infection patterns (Grant, 2002). Moreover, as inadequate dosage or exposure results in incomplete treatment and subsequent re-infection (Jimenez et al., 2013), this incresaes selection for chemotherapeutically tolerant individuals and increases the risk of resistance development (Aaen et al., 2015). Finally, environmental impact must also be considered as the fate of spent topical agent discharged into the surrounding water body is unpredictable, and arthropod selective pesticides have high probabilities of affecting non-target crustaceans including crabs and lobsters (Grant, 2002).

At present, the most financially and ecologically sound method of application is the use of well boats as the efficiency of topical application increases drastically relative to reductions in the quantities of chemotherapeutant required (Raynard et al., 2002). Furthermore, mobility allows the contained water and associated agents to be applied across multi-cage systems to maximise cost-benefit and circumvent discharge regulations (Grant, 2002). Well boats also favour the use of H_2O_2 which offers an environmentally friendly alternative to the historical

compounds as the degradation products are largely harmless (Helgesen et al., 2015). There are however some limitations. There is still significant physical stress on the fish and gill damage can occur at therapeutic dosage levels (Grant, 2002). Similarly, this method is also less effective in early developmental stages and as lice recover and are able to re-attach, subsequent treatments are commonly required (Rae, 2002). Hence; in addition to fish being exposed to the associated stressors on subsequent occasions (Helgesen et al., 2015), when it is taken in the context of additional risk of louse populations becoming resistant as reported in some regions since 2000 (Grant, 2002), this method becomes limited in its scope of success (Grant, 2002; Helgesen et al., 2015).

As an alternative to bath treatment, medicated feeds such as Emamectin benzoate (Slice™) and Teflubenzuron (Ektobann™) have shown greater success and are safer to apply (Costello, 2009b). However, correspondingly with topical agents, prolonged applications of a limited range of compounds with variable inclusion rates (relative under/overdosing), and subsequent re-infection has driven reduced efficacy (sensitivity) over time (Horsberg, 2012). Further to this, withdrawal times are longer for subcutaneous medicines compared to epicutaneous treatments therefore harvest schedules can be significantly delayed (Liu & Bjelland, 2014). Hence, although it is true that long term residence and slow excretion of subcutaneous medication is advantageous, there is also potential for this to represent a gradient of treatment concentration where lice which attach later are exposed to sub-lethal levels which increases the risk of developed cross resistance greatly (Aaen et al., 2015). Half-life is also of environmental concern as avermectins have low water solubility and therefore tightly bind to any particulate matter so there is a strong potential for accumulation in sediments over repeated treatments with significant risk to benthic organisms (Haya et al., 2005). In short, these factors have led to development of Integrated Pest Management (IPM) strategies which use of short term applications of alternate compounds by rotation to reduce dependence on single chemical

classes (Haya et al., 2005), minimise repeat exposures to diminish the risk of resistant traits developing (Muncaster et al., 2010), and diminish any potential environmental impact (Haya et al., 2005). Furthermore, this is in conjunction with less intrusive measures which take into account site specific differences in currents and other hydrographic parameters including single year stocking on sites with synchronised fallowing periods to minimise peak infection, break the cycle of reinfection, and postpone resistance (Horsberg, 2012; Werkman et al., 2011).

In agreement with Grant (2002), "the climate in which farmers and pharmaceutical companies have to find solutions to these problems is determined by the commonly conflicting demands of producers, consumers, regulators and special interest groups which, while justifiable, can be difficult to reconcile". Consequently; the aquaculture industry is now focused on developing new technologies which would complement the existing IPM strategies whilst minimising the associated risks. There are a range of methods in development including, physical interventions like thermal baths, high pressure water jet systems or laser treatments, alterations in cage management e.g. surface skirts, submerged feeding systems or snorkel cages, as well as more traditional mitigation methods for disease management like selective breeding for lice resistance and vaccine development. However at present, the use of 'cleaner fish' as a pseudo-symbiotic biological control remains the solution closest to wide scale deployment across the industry. To quote Dr. Allison Alberts "nature has already solved many of the problems we're dealing with today".

1.2 Cleaner fish

With ~600 species and 82 genera (Sayer et al., 1993; Villegas-Ríos et al., 2013) the Labridae (including Scaridae and Ocacidae) are well known for high diversity, and represent the second largest family of reef associated marine perciforms (Cowman et al., 2009), which are abundant in shallow tropical and sub-tropical systems around the world (Wainwright et al.,

2004). In reflection of this, they are astonishing in their ecological diversity, which is underpinned by kaleidoscopic variations in morphology, colouration, pattern, social systems, reproductive biology and feeding strategies (Hanel et al., 2002).

Heterospecific-mutualistic behaviours have been reported in many vertebrate species, but are best documented in fish (Potts, 1973). In such cleaning symbioses, interactions involve small fish acting as 'cleaners' in the removal of ectoparasites, excess mucous, and other deleterious tissues from the body of larger (cooperative) repeat clients termed 'hosts' (Stummer et al., 2004). Cleaners benefit greatly through access to an abundant niche food source with greatly reduced competition and minimal foraging (Clague et al., 2011), while hosts demonstrate improved body condition and lower antibody response relative to conspecifics subjected to cleaner exclusion manipulations (Revie et al., 2009). The decrease in active immunological loading consequently liberates energy resources to enhance investment in somatic growth and reproduction (Clague et al., 2011). Such behaviours are best documented in the tropical reef dwelling blue-streak wrasse (*Labroides dimidiatus*) (Valenciennes 1839) (Hanel et al., 2002). The effects of cleaning are disproportionate to the size and relative abundance of *L. dimidiatus* hence they are considered a keystone species which influence local diversity, assemblage patterns, behaviours and habitat choices of a diverse range of species (Grutter et al., 2003). Thus; in consideration of the fore-mentioned parasitic infestation and treatment issues in salmonid aquaculture, such preventative and curative effects make the incorporation of cleaner-fish in IPMs a highly advantageous 'technology' (Leclercq et al., 2014).

The connection of temperate Labridae to salmonid aquaculture begins with field observations of the Mediterranean black-tail wrasse (*Symphodus melanocercus* (Risso 1810)) performing a similar service to *L. dimidiatus* with a correspondingly diverse range of species (Potts, 1968). Subsequently, similar observations were noted in corkwing wrasse (*Symphodus*

melops (L.)) at the Plymouth Public Aquarium which would commonly follow other species of fish including various wrasse, Plaice (*Pleuronectes platessa* (L.)), Pink-bream (*Pagellus centrodontus* (Delaroche 1809)), Mackerel (*Scomber scrombrus* (L.)) and Black-bream (*Spondyliosoma cantharus* (L.)), seemingly inspecting them and picking items from the body (Potts, 1973). Closer scrutiny revealed selective targeting of parasitic *Gnathia maxillaris* larvae and other 'ectophenomena' from host epidermis (Potts, 2009). Analogous behaviours were then described (Potts, 1968) in other resident labrids including ballan wrasse *(Labrus bergylta* (Ascanius 1767)) and goldsinny (*Ctenolabrus rupestris* (L.)). Captive cleaning behaviours were later confirmed in wild populations by stomach content analysis (Potts, 1973), and from this, the idea of employing such activity to delouse salmon in net pens was born.

Commercial trials began in Norway in 1987 with promising results which identified *C. rupestris* and *L. bergylta* were optimal in net pens with salmon of <2 kg and 2-4 kg respectively (Talbot et al., 2012; Skiftesvik, 2013). This success led to development of commercial wrasse fisheries in Scotland the following year and the rest of the UK in 1990 (Bjordal, 1992; Tully et al., 1996). As biological louse control (by wrasse) is a function of cleaning rate relative to parasite transmission, stocking density is therefore a key factor (Tully et al., 1996). Taking into account that ~40 million smolts are put to sea in the UK every year, even 1% cleaner fish stocking levels are equivalent to annual requirements for 400,000 individuals (Talbot et al., 2012), the recommended 4% stocking ratio would be 1.6 million, and must also take into account losses of approximately 7% at some sites (SARF, 2013). From this, estimates of wild capture harvests have grown from 100,000 in 1991 (Darwall et al., 1992), to exceed 10 million in Norway alone (D'Arcy et al., 2013). Hence, although wrasse are highly effective, there remain a number of questions regarding the sustainability of such practices due to removal of large numbers of individuals from wild populations, and the impact of this on population structures (D'Arcy et al., 2013). Although there is no information at the species specific level

as there are no conservation measures in place (D'Arcy, 2001), it is known that *L. bergylta* are particularly vulnerable to overfishing due to highly complex ecology and reproductive systems (Grant et al., 2015), and size selective harvest of mature individuals changing the demographic structure of communities (Talbot et al., 2012). From this, growing demand (D'Arcy, 2001), concerns regarding wild harvest sustainability (Darwall et al., 1992), and a requirement for a predictable supply of fish with a known health status (SARF, 2013), means that the future of wrasse utilisation in IPM systems is entirely dependent upon developing hatchery production (Talbot et al., 2012).

1.3 The Labrini

It has been said that with increasing latitude, "wrasse decline in both species richness, and relative abundance" (Hobson, 1969). The tribe Labrini which comprises 23 species from 8 genera including *Acantholabrus*, *Lapanella*, *Tautoga*, *Tautoglabrus*, *Centrolabrus*, *Symphodus*, *Ctenolabrus* and *Labrus* are an exception (Hanel et al., 2002). Excluding *Tautoga* and *Tautoglabrus* which are endemic to north-western Atlantic regions, and deep dwelling *Acantholabrus* and *Lapanella* species, they are predominantly in-shore species found associated with rocks and eel-grass beds of the north-eastern Atlantic seaboard (Hanel et al., 2002).

In terms of evolution and divergence from ancestral Labridae, the basal Labrini is thought to have entered the proto-Mediterranean basin in the early Miocene (20.5-19.5 MYA) from the Indo-pacific via the Red sea connection at the isthmus of Suez (Bianchi et al., 2012; Gómez, 2006; Hanel et al., 2002). The Mediterranean was part of the Tethys sea at this time, but the African, European and Adriatic plates were moving closer reducing the size, shape, and connectivity (Meynard et al., 2012). 12-18 Million Years Ago (MYA) tectonic movements completely closed the seaway between the two oceans (the Tethys closure) (Cowman &

Bellwood, 2013). This hard barrier to gene flow between the Mediterranean sea populations and those in the emergent Red Sea, then resulted in isolation of the basal labroid leading to emergence of the *Symphodus* lineage around 12 MYA with black porgy ((*Tautoga onitis*) (L.)) and scale rayed wrasse (*Acantholabrus palloni*) (Risso 1810) colonising Western Atlantic regions by 9 MYA via the Bering Strait (Domingues et al., 2005). Following this (7.5 MYA), the other Western Atlantic species (the) bergall (*Tautoglabrus adspersus*) began to diverge from the Eastern Atlantic sister taxa *C. rupestris*, with rockcook ((*Centrolabrus exoletus*) L.) and the remaining *Symphodus* species emerging soon after (Hanel et al., 2002).

Around this time, the Mediterranean basin closed at the Guadalhorce passage between the Iberian peninsula and the Rifian corridors of Northern Africa (Periáñez & Abril, 2015). This effectively isolated the sea from water exchange with the Atlantic causing desiccation and major decreases in sea level, which in combination with local geography limiting input from catchment areas, and altered hydrology cycles due to glaciation events, caused the Messanian Salinity Crisis (MSC) (Matschiner et al., 2010). This process eventually reduced the sea to a series of evaporitic lakes including anoxic, freshwater, brackish and hyper-saline lagoons (Hsü et al., 1977; Periáñez & Abril, 2015). For many years, it was thought that the hostile hydrological conditions had caused extinction of all marine species (Domingues et al., 2005). From this, it would follow that survival outside the Mediterranean basin and subsequent reintroduction would be the most likely scenario (Bianchi et al., 2012). However, recent fossil evidence has shown that numerous species including some Labridae had survived the crisis in-situ (Pawellek et al., 2012). Although it remains a contentious subject, it is thought that some fully marine refuges were maintained by persistent links with the Atlantic at the Betic portal, or in satellite basins, and that these isolated pockets containing the remnants of pre-MSC tethyan biota gave rise to the extant Mediterranean endemic biota (Hsü et al., 1977).

Around 5 MYA, the connection to the Atlantic Ocean was restored at the Strait of Gibraltar (Hanel et al., 2002). The basin was inundated in an event termed the Zanclean Flood, with the influx of new water from the Atlantic and reinstatement of circulation and productivity patterns allowing adaptive radiation of encapsulated species and/or reintroduction of excluded species with rapid colonisation of new habitats and ecological niches (Domingues et al., 2005; Matschiner et al., 2010). From this, it follows that combinations of sympatric and allopatric speciation driven by geographic, hydrographic and thermal isolation (Meynard et al., 2012), would then accelerate the emergence of diverse native and endemic fauna (Domingues et al., 2005).

Approximately 2.5 MYA (particularly last 700, 000 years), further isolation episodes linked to glaciation cycles caused very low water temperatures (Robalo et al., 2012). These events left isolated refugia in the Mediterranean, North Atlantic archipelagos, and Eastern Atlantic (Robalo et al., 2012). Such geographically isolated locations with thermally favourable conditions are thought to have favoured more recent speciation events for example the emergence of endemic species such as emerald wrasse (*Centrolabrus trutta* (Lowe 1834)) from five-spotted wrasse (*Symphodus roissali* (Risso 1810)), and similarly from small-mouth wrasse (*Centrolabrus caerelius* (Azevedo 1999)) which are endemic to the Canary Islands and the Azores respectively (Hanel et al., 2002). Correspondingly, species that were more thermally adaptive were able to inhabit far broader areas of the Mediterranean and Atlantic which is reflected by their thermal tolerance and cosmopolitan distribution ranges (Robalo et al., 2012). Supposition of recent speciation events in the Labrini is supported by minimal genetic distance between mitochondrial gene regions (both 12s and 16s) (Cowman et al., 2009), with single transversions in *C. exoletus* and *S. melanocercus,* or *S. melops* and S. *roissali*, or the identical 16s sequences of Brown wrasse (*Labrus merula (L.*)), Green wrasse (*Labrus viridis* (L.)) and

L. bergylta supporting that divergence occurred less than 1 MYA (Cowman et al., 2009; Matschiner et al., 2010).

In accordance with other Labridae the Labrini are also renowned for complex social, behavioural and reproductive patterns (Hanel et al., 2002). In contrast to gonochorists where pre-determined gender is fixed, many species are sequential hermaphrodites. Individuals therefore function as both male and female at different points during the life-cycle with variations in sexual pattern representing adaptations to a particular life history within a diverse and dynamic set of ecological habitats (Asoh, 2003; Robertson & Choat, 1974). This system can be described by a functional shift in gender so individuals will invert from male to female (protandry) or female to male (protogyny) (Asoh & Yoshikawa, 2003), where a particular direction is favoured as the optimal reproductive value occurs as one sex when young and small, and another when old or large (Allsop & West, 2003; Munday et al., 2006). Although bi-directional sex change and androdioecy (self-fertilisation) are reported in some lineages (Erisman & Allen, 2005), protogyny is the most common system in tropical and sub-tropical reef dwelling teleosts including Epinephelidae, Pomacentridae, Sparidae, Gobiidae, Serranidae and Labridae (Asoh, 2005; Erisman et al., 2013). Finally, some labrids and scarids (termed diandric) are able to produce males by both sex reversal and by initial maturation of juveniles as males (Shapiro, 1993). An example of this system would include the blue-head wrasse (*Thalassoma bifasciatum* (Bloch 1791)) where some individuals mature as male with full male tissues (termed Terminal Phase (TP)), while others follow the protogynous model by functioning as females and changing to male at a later stage (primarily termed Initial Phase (IP) then become TP in subsequent phase) (Munday et al., 2006). With the exception of gonochoristic species which are born as male or female and have fixed gender throughout their lives, the stimuli to undergo sex change is based on complex and highly variable cues integrated from external environments (Black et al., 2005).

Social control of gender expression in Labridae is one of the most complex examples of adaptive sex determination and reproductive plasticity in nature (Godwin, 1995; Lamm et al., 2015). Patterns of organisation are commonly based on the stability of relationships and hierarchies established over a period of time (Robertson & Choat, 1974). Baroiller et al. (1999) best described them as nested series of hierarchical subdivisions that are 'allocated' relative to social standing with access to area and resources a determinate of and status. At a practical level this means that the male (protogynous system) territory forms the boundary of the community and is superimposed over that of the lower social orders (Baroiller et al., 1999; Godwin et al., 1996). Regular visits and dominant interactions/behaviours then acts to reinforce the hierarchical structure and suppresses pro-inversion stimuli in subordinates (Black et al., 2005). Similarly, if the ultimate aim of the lower orders is considered as reaching the apex of the hierarchy (i.e. becoming male), then establishing a position and suppressing subordinates are key factors in success (Robertson & Choat, 1974). Finally, if another individual joining the group was considered as a risk to the existing status quo, then it would be in the whole communities interest to expel the interloper (Black et al., 2005). The basis of stability in haremic labrid communities is therefore found in their attachment to a particular site with strong territoriality towards conspecifics (Robertson & Choat, 1974).

These governing principles can be illustrated in a number of systems. Manipulation (removal) studies have directly stimulated transition in several protogynous species including *T. bifasciatum* (Warner, 1984), saddle wrasse (*Thalassoma duperrey* (Quoy and Gaimard 1824)) (Nakamura et al., 1998), and the blue-banded goby (*Lythrypnus dalli* (Gilbert 1890) (Black et al., 2005). In such systems, removal of the dominant male (and the presence of subordinate females) has been shown to initiate inversion in the dominant individual leading to major restructuring of tissues in the brain, genitalia and gonads accompanied by shifts in hormonal profiles with subsequent development of secondary sexual characteristics and

behaviours (Godwin, 2009; Black et al., 2005). Further to this, experimental mono-sex (female) groups of lyretail anthia (*Anthia squamipinnis*) (Peters 1885) are noted as taking longer to initiate sex change than natural haremic communities subjected to male removal (Shapiro, 1980). Similarly, experimentally introduced female pairs (large vs. small) of protandrous anemonefish (*Amphiprion* sp.) or protogynous *T. duperrey* results in aggressive interactions without inversion (Shapiro, 1993). This would then suggest that the threshold value is not only based on male removal but also takes into account relative proportions of the behaviours given and received within the hierarchy (Baroiller et al., 1999), where minimum numbers of the primary phase are required to induce inversion after male removal (Baroiller et al., 1999; Robertson, 1981; Shapiro, 1980). As the focal fish must therefore rapidly establish and maintain (male-type) dominance behaviours to establish dominance and suppress lower order females, this implies that social control of gender inversion represents a highly unusual system where the dominant female displays male behavioural phenotype prior to the involvement of testes or their hormonal products (Baroiller et al., 1999; Godwin et al., 1996).

Courtship, spawning and brood-care behaviours are also complex and highly variable. Seasonal and ephemeral colours and patterns are an important medium of communication in wrasse (Erisman & Allen, 2005). Gender phenotype can be permanently monochromatic, monochromatic with seasonal dichromatism or permanently dichromatic with shifts between states associated concurrent to specific gender identities, social and sexual signalling and physiological state (Erisman & Allen, 2005). Broadly, mating systems are known to include haremic communities with facultative pair spawning between the dominant male and a subordinate (but dominant within the nested hierarchy) female (or females) (Black et al., 2005), and lek-type behaviours with aggregations inter-individual competition and group spawning, but this can also be context dependent (Donaldson, 1995). For example, blue-head wrasse (*Thalassoma amblycephalum* (Bleeker 1856)) TP males (by protogynous inversion) show pair

spawning but IP males (born male but closely resemble females) participate in group spawning events (Donaldson, 1995). This means the mating system is most likely an adaptive determinate of species abundance where group spawning occurs in communities where male and female density is high, or paired spawning when density is low (Donaldson, 1995). Similarly, blue-side wrasse (*Cirrhilabrus cyanopleura* (Bleeker 1851)) are promiscuous at high density, but become haremic in low density communities (Donaldson, 1995). Thus; as sexual plasticity is reflected in the reproductive strategies it can be very difficult to define a unifying theory of sexuality and expression of sexuality in wrasse. With specific reference to temperate wrasse however, *L. bergylta, S. melops* and *L. mixtus* are protogynous hermaphrodites whereas *C. exoletus* and *C. rupestris* are gonochoristic with discrete sexual identities (Potts, 2009).

Although broadcast spawning is a general rule in tropical Labridae, and most species have planktonic eggs and larvae therefore parental investment is minimal, brood-care is well developed in the Labrini with a variety of strategies described (Hanel et al., 2002). Such behaviours range from formation of a crude spawning cavities by *L merula*, nesting with additional cleaning of the clutch surfaces in *L. bergylta*, and simple nest structures with ventilation of the eggs in *L. mixtus* (Hanel et al., 2002), to construction of complex nests with sequential spawning including a single male and a number of females with extensive parental care in *S. melops* (Potts, 2009), or extensive guarding and care from a primary territorial male supported by several 'helpers' in other taxa including *C. trutta* and *C. caerelius* (Hanel et al., 2002). It is thought that high levels of juvenile predation by planktivores and poor environmental conditions causing low settlement in the Mediterranean during the MSC, in conjunction with high mating success in haremic communities relative to other systems have led to complex behaviours described (Matschiner et al., 2010). Two exceptions to these nesting behaviours are *L. viridis* and Doderleinis' wrasse (*Symphodus doderleini* (Jordan 1890)). However, it appears that their close association with sea grass meadows allowed secondary

adaptations where nest building was not necessary as such an environment has comparatively low predator abundances and offers additional protection through provision of shelter (Hanel et al., 2002).

As mentioned previously, numerous species of wrasse are known to display symbiotic cleaning behaviours where ectoparasites, scales and mucous are removed from client fish (Arnal & Morand, 2001). Such behaviours can be highly specialised with ectoparasites forming the basis of the diet as seen in *L. dimidiatus*, non-specialised where benthic animals and alga are also consumed, or facultative in relation to life stage (Stummer et al., 2004). Although the most highly specialised cleaner fish such as *L. dimidiatus* are endemic to tropical distributions, *S. melanocercus* is the only obligate cleaner of the Labrini (Hobson, 1969). It is of interest however, that facultative cleaning activities are described within the Labrini including Mediterranean rainbow wrasse (*Coris julis* (L.)), ornate wrasse (*Thalassoma pavo* (L.)), oscillated wrasse (*Symphodus Ocellatus* (L.)), five spotted wrasse (*Symphodus Roisalli* (Risso 1810)), *C. tinca*, *C. rupestris*, *C. exoletus*, *L. mixtus*, *S. Melops* and *L. bergylta* (Bjordal, 1992; Hobson, 1969; Potts, 1968).

1.4 The ballan wrasse (*Labrus bergylta*)

As a predominantly in-shore species, adult *L. bergylta* are found closely associated with rock faces, boulder slopes, off-shore reefs and macro-alga beds in sublittoral zones to 30 meters (Treasurer, 1994). As with all Labridae, they are strongly diurnal and so are only active during the day, then hide at night in rock crevices or eel grass beds (Lipej et al., 2009). They are a slow growing (Artüz, 2005b), and long lived species which can achieve a total length of 65.9 cm and 4.35 kg over a 29 year lifespan (Ottesen et al., 2012). In terms of general morphology, they are a heavily built and deep set fish with a large head, gently fusiform face, and conspicuous protractible thick lips (Muncaster et al., 2010). Differentials in determinate features of jaw morphology between juveniles and adults promotes niche partitioning by

limiting the prey size/type hence minimising the potential for intraspecific resource competition (Asoh, 2005). The food spectrum then extends according to ontogenic stage as the pharyngeal apparatus is developed to allow harder prey items (Figueiredo et al., 2005). In adult *L. bergylta* extendable jaws, large conical teeth, two sets of pharyngeal teeth including two upper bilateral plates, and a lower tri-lobate plate provide excellent grinding surfaces to crush prey items (Dipper, 1977; Strohmeier et al., 2006). This includes brown crabs (*Cancer pagurus*), common shore crabs (*Carcinus maenas*), prawns, and bi-valve molluscs such as mussels (*Mytilus edulis*) and scallops (*Chlamys* spp.) (Deady & Fives, 2009). The carnivorous tendencies and preference for hard bodied prey are further supported by a short, straight and thick walled gut morphology reflecting preclusion of an acid medium by high levels of dietary calcium carbonate (Deady et al., 1995; Dipper, 1977).

Although there are some reports of *L. bergylta* in the Mediterranean, the established native range of *L. bergylta* (Fig. 1.1) extends from Norway, down the East Atlantic European coast to Portugal including the Western Baltic regions, Madeira, the Canaries and the Azores (D'Arcy et al., 2013).

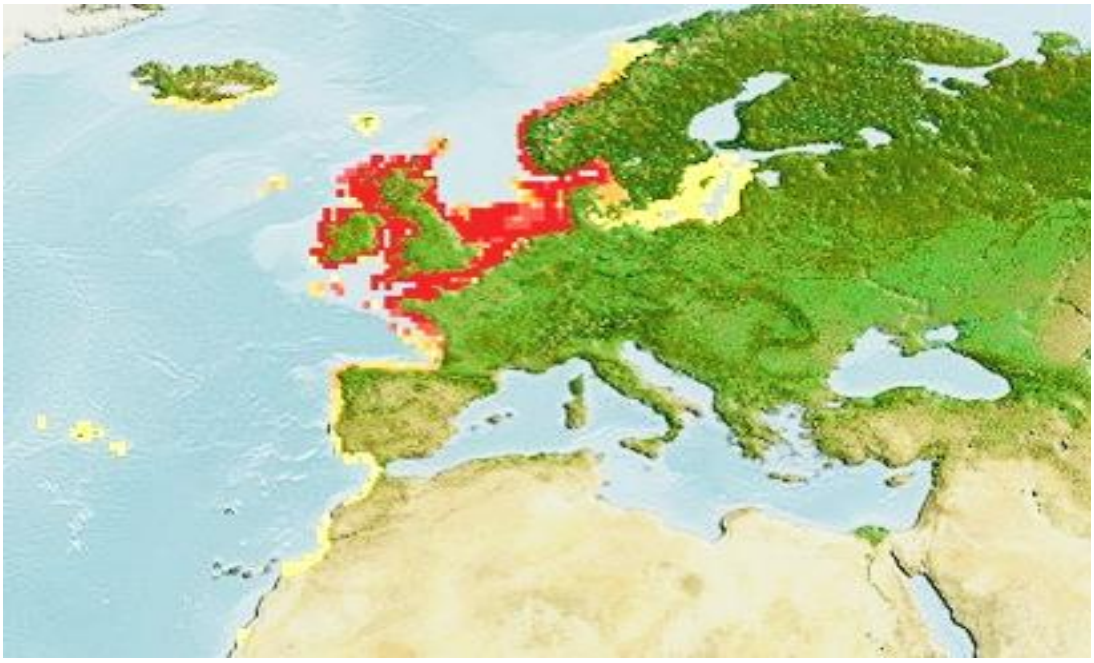
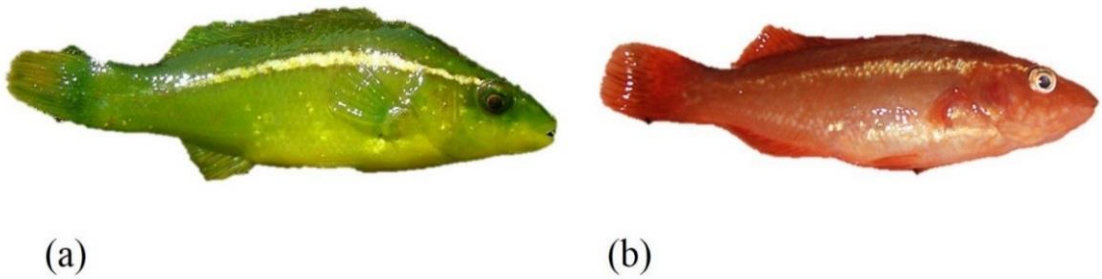


Figure 1.1: *Labrus bergylta* distribution (www.aquamaps.org).

Another notable *L. bergylta* characteristic is the highly variable colour and pattern morphisms (Dipper, 1981), which do not appear to relate to gender (Porteiro et al., 1996), origin (Villegas-Ríos et al., 2013) or seasonality (Porteiro et al., 1996; Villegas-Ríos et al., 2013), but does change as the fish become adults (Artüz, 2005a). To explain, wild juvenile fish are emerald green with few marking except a strong white lateral stripe (Fig. 1.2(a)) (Jeppesen & Cepeda, 2001; Quignard & Pras, 1986), whereas captive bred are predominantly a dusky pink/brown colour but retain the lateral white stripe (Fig. 1.2(b)).



(a) (b)

Figure 1.2: Juvenile *Labrus bergylta* showing (a) Wild caught specimen and (b) Captive bred specimen.

As the fish mature, three main colour types have been identified but it is of note that they can be sympatric in nature; namely red, intermediate brown which can have red/green tints, and green, with conspicuous pale centred scale morphologies with which confer a maculate appearance (Fig. 1.3) (termed spotty hereafter) (Villegas-Ríos et al., 2013). Basal colourations are then overlaid by striate accents, which can be highly variable or entirely absent in accordance with the communicative, physico-physiological and camouflage functions described by Ortolani (1999). The unpaired fins are commonly marked with grey or blue spots with a similarly coloured border (Jeppesen & Cepeda, 2001). Interestingly, the eyes also vary in colour and so may be red, yellow or blue depending on the individual (Porteiro et al., 1996). As a note, Fig. 1.3 is a good illustration of the lack of sexual differentiation..

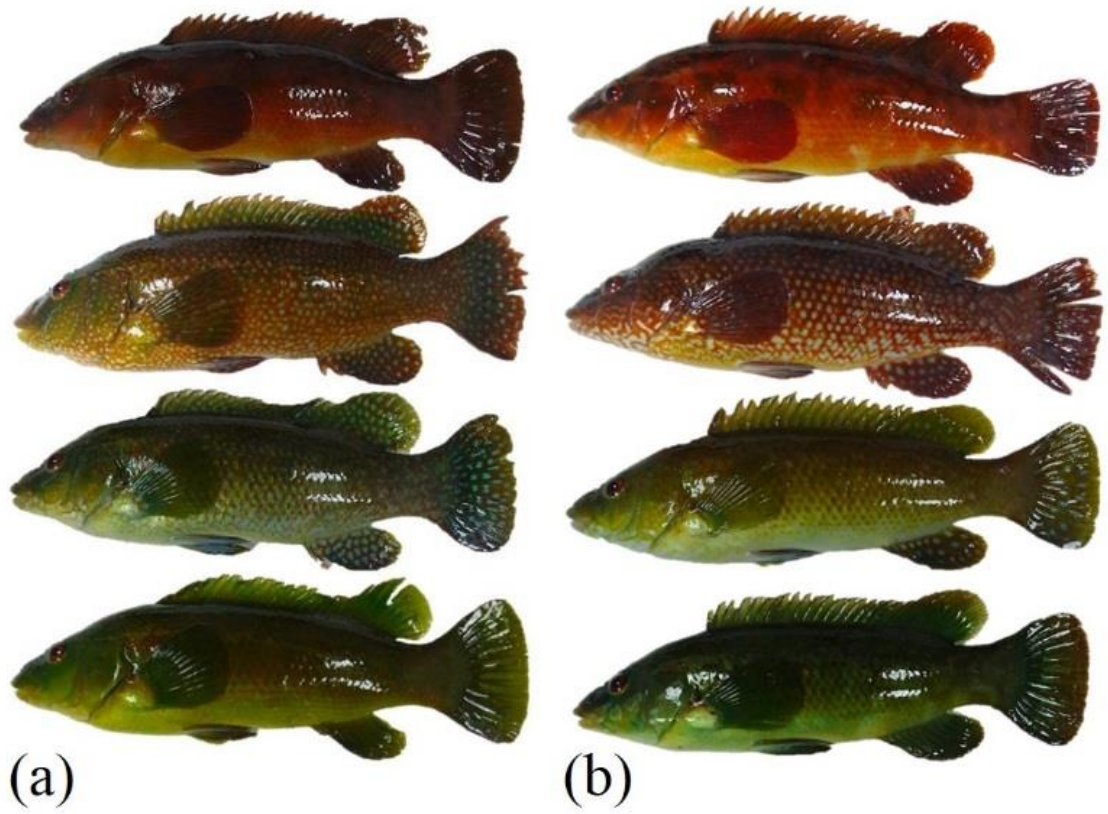


Figure 1.3: Phenotypic variation in mature *Labrus bergylta* showing (a) Males and (b) Females.

In accordance with other temperate, and high latitude teleosts, the reproductive cycle of *L. bergylta* in Northern Europe follows clear annual cycles with seasonal oscillations determining the optimal environmental conditions conducive to off-spring survival (Muncaster et al., 2010). The sex ratio is highly skewed (circa 10% male) in accordance with similar species (Leclercq et al., 2014b). As a serial spawning species (Grant et al., 2016), males build a nest then mate with with successive females a number of times over the season as demonstrated by the range of egg diameters observed (Artüz, 2005b; Muncaster et al., 2010). Gonad maturation and ovarian recrudescence begins in November with the appearance of oocytes including cortical alveoli and increasing sex steroid expression, followed by the oocytes progressing to vitellogenesis by late winter (Muncaster et al., 2010). Spawning occurs between April and June depending on local circumstances as demonstrated by peak Gonado-Somatic Indices (GSI) in both males and females, and maximal female gonadal steroid expression (T and 17-B

oestradiol) followed by a sharp decline (Deady & Fives, 2009; Talbot et al., 2012). Spawning is complete around the summer solstice with ovarian atresia observed by August (Muncaster et al., 2010). Eggs are demersal then post hatch juvenile fish become planktonic and subject to passive dispersal by the prevailing tide and dominant currents (Jeppesen & Cepeda, 2001). The pelagic stage lasts for up to 4 months depending on nutritional status and temperature until the fry reach approximately 10 mm and complete metamorphosis (Jeppesen & Cepeda, 2001). Young fish then migrate to inter-tidal regions and rock pools which are rich in green and fucoid algae (Wheeler, 1970), where they hide amongst vegetation and isopods and amphipods become the main food sources (Dipper, 1977). Very little is known about the subsequent recruitment process (pers.obs.)

During peak maturation the male *L. bergylta* become highly territorial and defend an approximate area of 300 m^2 (Deady & Fives, 2009) which forms the outer spatial boundary of a nested hierarchy where the males personal territory is situated centrally but is also superimposed over that of subordinates (Robertson & Choat, 1974). Regular patrols maintain territorial integrity with visits to females allowing interactions, and assertion of dominance (Baroiller et al., 1999). Amongst females, there is a simple dominance hierarchy based on relative size (Robertson & Choat, 1974). Continual testing and re-affirmation of relationships within the group then occurs through high levels of social interaction (Godwin et al., 2003). *L. bergylta* are monandric protogynous hermaphrodites and so are born female then become male at 5 years age and a length greater than 28 cm in response to the appropriate social and environmental cues including conspecific interactions and redress of the (10:1) gender ratio common to protogynists (Muncaster et al., 2013; Talbot et al., 2012). From this, and in agreement with other sequential hermaphrodites, it follows that the spatial distributions and dominance structures of older members in a community would be stable over time, and where death produces an in-stability, the group would then undergo re-organisation (Godwin et al.,

2003). Further to section 1.3, it is generally accepted that where the primary male is removed, the largest and most dominant female will undergo gender inversion to become the male and the lower social orders will reform a stable community based on relative size and dominance (Bass & Forlano, 2008). In accordance with similar species, the territoriality is relaxed after the breeding season so normal foraging activities are resumed (Thangstad, 1999).

Cleaning behaviours are well known and comprehensively described in tropical species such as *L. dimidiatus* but the origins of this symbiosis in temperate wrasse remain cryptic (Barber & Poulin, 1996). As cultured wrasse naïve of both salmon and lice have been demonstrated to show identical de-lousing efficacy to wild caught individuals in controlled experiments (Skiftesvik, 2013; Leclercq et al., 2014a), this suggests that such altruistic behaviours are innate and are most likely facultative in *L. bergylta* (Stummer et al., 2004). This is supported in observations of juvenile fish cleaning adults in the closely related Labrini *S. melanocercus* and *C. tinca* (Arnal & Morand, 2001). Further evidence is suggested by the well-defined white lateral stripe in juvenile *L. bergylta* (Fig. 1.2). To explain, heterospecific cleaning behaviours can be initiated by the cleaner approaching the host, or by the reverse (Potts, 1968). Until recently however, little was known about how protagonists recognised each other (Stummer et al., 2004). Although the basis of the idea has been around for a while (Potts, 1973), Stummer et al., (2004) theorise that convergent evolution has driven development of similar characters in such species which lead to a universal guild marker for such behaviours, where diminutive body size and a strong lateral stripe act as long distance signals of cleaning opportunities. It is therefore notable that juvenile *L. bergylta* conform to this hypothesis with the long white lateral stripe relative to body length serving as 'poster' signalling of cleaning as a foraging strategy. When taken in the context of *L. bergylta* showing a preference for more familiar food sources grazed from net fouling (Tully et al., 1997), this supports cleaning

behaviour in adult fish as an opportunistic adaptation of atavistic behavioural traits retained from the juvenile phase.

Although cleaner-fish have been proven more environmentally friendly alternatives to chemotherapeutant applications (Berg, 2012; Leclercq et al., 2014), the inshore fishery that has developed to meet demand in aquaculture has itself become of concern (Barber & Poulin, 1996; Muncaster et al., 2010; Skiftesvik, 2013). Initially, *L. bergylta* are the least abundant of the inshore wrasse species and are therefore most sensitive to increased pressure from fisheries (Leclercq et al., 2014a). They are effectively a sedentary species with predictable behaviours making them intrinsically vulnerable to target fisheries where aggregations are easy to find and repeat visits make it probable that larger proportions of the community will be removed (Sadovy & Domeier, 2005). Additional stress is found in minimum catch size management methods as females pass through an immature phase, and males are ultimately derived from the females, the removal of larger individuals then selects the larger and therefore most productive females and takes no account of male contribution (Villegas-Ríos, 2013). Such practices potentially disrupt a cascade of factors in wild communities including size at maturity in both genders, social interactions, sex change, operational sex ratios, population fecundity, egg quality and sperm production (Sadovy & Domeier, 2005). Further to this, net pens must be restocked with wrasse periodically due to predation, escape, disease and mortality to maintain optimal (~4%) stocking ratios, continued growth in the industry presents additional need for target fisheries and reiterates the requirement for optimising artificial production (Ottesen et al., 2012; Talbot et al., 2012).

It is of final note that the same intricacies and inter-related characters in the life history strategies of *L. bergylta* which amplify the effects of fishing practices, also represent major restrictions to the development and optimisation of broodstock management (Hamre et al., 2013). To explain, as there is no post-inversion differentiation it is difficult to accurately

identify male individuals which already form a minority in populations due to the skewed (1:10) sex ratios inherent to protogynous hermaphrodites. Similarly, as community structures and dominance interactions are major drivers of inversion processes, where males are difficult to select, and communities require long term interactions for stabilising hierarchies and generating dominant females (essentially primed as male) (Grober & Bass, 2002), it follows that hatchery personnel would have significant difficulties in establishing breeding groups and optimising production (Talbot et al., 2012). Improved understanding of all aspects of *L. bergylta* ecology, physiology and reproductive strategies is fundamental to advancing husbandry techniques and optimising hatchery production (Darwall et al., 1992; D'Arcy et al., 2013).

1.5 Other species of interest (The Labrini)

As mentioned previously, there are another 4 species from the Labrini tribe that are of interest to the salmon farming industry. *C. rupestris* (Fig. 1.4) is the smallest species of inshore wrasse at a (mature) average of 10-12 cm, and with a lifespan of 14 years in males and 18 years in females, it is a slower growing species than *S. melops*, *C exoletus* and *L. mixtus* (Matland, 2015). Adults are orange-red in colour with a distinctive black eye-spot on the base of the tail while juveniles are (dull/matt) green (Thangstad, 1999).

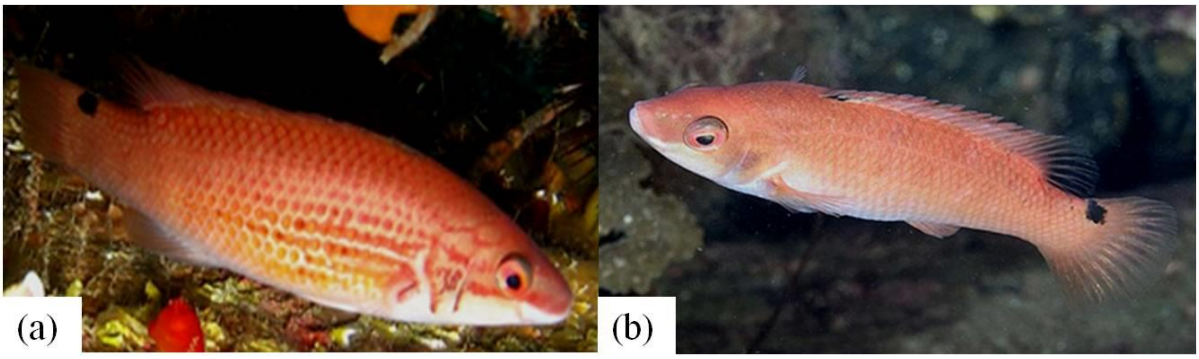


Figure 1.4: Goldsinny (*Crenilabrus rupestris*) **(a)** Male and (**b)** Female.

They are essentially gender monochromatic, but males (Fig. 1.4(a)) show reddish spots along the flanks in contrast to females (Fig. 1.4(b)) during the reproductive season (Thangstad, 1999). The limiting factor in distribution is the availability of suitable habitat as they have strong preference for substrates including brown alga, complex caves, crevices and holes (Matland, 2015; Sayer et al., 1993). High densities are also associated with turbulent water areas (Skiftesvik et al., 2013). In accordance with the other local labrids, they are highly territorial towards conspecifics during the reproductive season but otherwise aggregate in shoals above marginal substrata (Matland, 2015). During winter, *C. rupestris* migrate to deeper water and return to re-establish territories in May (Hilldén, 1981), with a reproductive season from April to September (Matland, 2015). Territories are consistant between years with the same males occupying the same spaces as previous, and new males settling in vacant plots where others were displaced or had disappeared (Hilldén, 1981; Matland, 2015). This is highly advantageous to the returning males as their diminutive size would potentially make extensive foraging a high risk strategy. Spawning events are polygynous with lek-like aggregations taking place midwater with pelagic eggs and no parental care (Matland, 2015). They are gonochoristic with discrete binary genders, but are also diandric with a secondary male subtype that closely resemble females (IP) to allow inclusion in a social group and participation in spawning events as 'satellites' (Matland, 2015). Depending on local hydrology, large proportions of eggs sink close to the original spawning site which is likely to be a good biotope for the species, while the remainder passively disperse (Hilldén, 1981). In considering the presumed lack of parental care in this species (Potts, 2009) in contrast to nest building Labrini, this may represent indirect protection to developing juveniles as territoriality continues after the reproductive season with assistance from tolerated females and other sub-adults (Hilldén, 1981). This would explain the advantage in expending energy on continued intraspsecific territoriality

as opposed to foraging in preparation for winter migration (Potts, 2009). Finally, *C. rupestris* is an opportunistic feeder with diets mainly consising of gastropods and amphipods, but also specialise in consuming bryozoans and hydrozoans from kelp leaves (Thangstad, 1999).

C. exoletus (Fig. 1.5) are the least well characterised species (Darwall et al., 1992), but are reported to have a maximal age of 8-9 years (Treasurer, 1994). Similarly to *C. exoletus*, female (Fig. 1.5(a)) age at maturity is 2 years, and the gender ratio is approximately equal (Skiftesvik et al., 2014; Thangstad, 1999). External colour morphisms are highly variable so can be green, brown or purple on the back, with lighter colouring on the belly which may be yellow, orange or blue with pronounced stripes on the head and caudal fin (Matland, 2015).

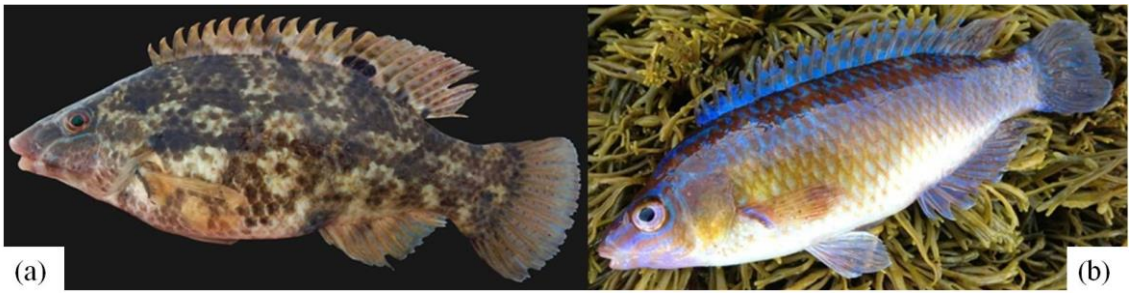


Figure 1.5: Rockcook (*Centrolabrus exoletus*) **(a)** Female and **(b)** Male.

This species is also gonochoristic with little sexual dimorphism beyond the reproductive season, but the scales of male fish (Fig. 1.5(b)) show blue iridescence at peak maturation (Thangstad, 1999). The reproductive season is later than the other species of interest occurring in May to August (Matland, 2015). This species is also highly territorial during the reproductive season and a simple nest is constructed, but after completion of spawning events, the fish then leave their territories and form aggregations (Thangstad, 1999). Like *C. rupestris*, *C. exoletus* is an opportunistic feeder but specialises on small polychaetes such as *Pomatocerus triqueter* (Thangstad, 1999).

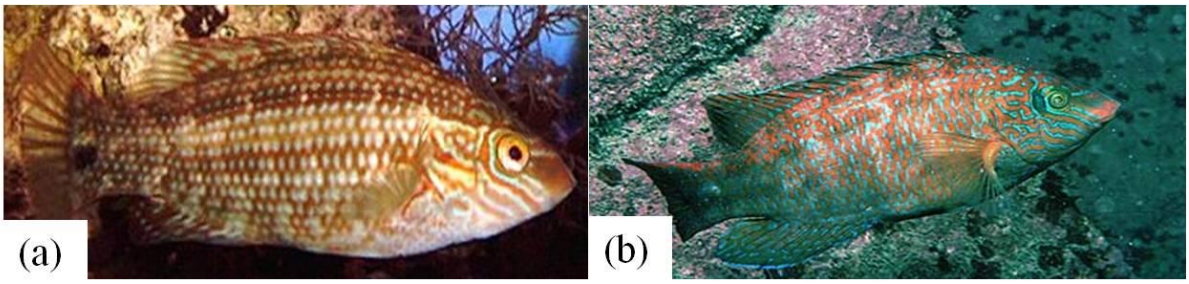


Figure 1.6: Corkwing (*Symphodus melops*) (a) Female and (b) Male

S. melops commonly reach lengths of 10 cm (potentially 28 cm), can live to the age of 9 years, and also mature at 2 years (Uglem, 2000). They prefer shallower water than the other species so are found at less than 5 m where they are associated with eel grass (*Posidonia oceanica*) beds and kelp forests (Matland, 2015; Matić-Skoko et al., 2013). They are protogynous (Skiftesvik et al., 2014), with batch spawning lasting from April to September (Matland, 2015; Uglem, 2001), but the sex ratio is lightly skewed in favour of females (60:40) (Skiftesvik et al., 2014). Genders are strongly dimorphic in the reproductive season with females showing brown or grey-green with conspicuous dark blue urogenital papilla (Fig 1.6(a)), and TP males with iridescent and brightly coloured green and red sinuous colourisms on the head (Fig. 1.6(b)) (Matland, 2015; Uglem, 2001). As with other diandric species, satellite (IP) males closely resemble the females in secondary sexual characteristics (Skiftesvik et al., 2014; Uglem, 2001). In this species, TP males more than 4 years old build elaborate nests (despite prior maturation the behaviour is only expressed at this age) in predominantly north facing crevices with the aspect and construction acting to increase reproductive success by helping to increase the probability of paternity by excluding interlopers, reducing predation and sheltering eggs against prevailing turbulence (Potts, 2009). Materials are site specific to a degree, but are carefully selected for textural properties and constructed such that the inherent stability and insulating properties of chosen constituents maintain the structural integrity and optimal thermal regime in the highly variable environment of littoral environments (Potts, 2009).

It is interesting that these same factors that act to protect the eggs, also increase the likelyhood of silting and the eggs becoming smothered therefore requiring extended fanning during the parental care phase as described for *C. melops* and similar nest building teleosts (Potts, 2009). Post spawning, the males engage in a further period of nest maintenance and cover the eggs from each mating event then become receptive to the next female (Matland, 2015; Uglem, 2001). This activity then enablies sequential spawning with numerous females in a single season, and nurture of several clutches of eggs simultaneously (Potts, 2009; Thangstad, 1999; Uglem, 2000).

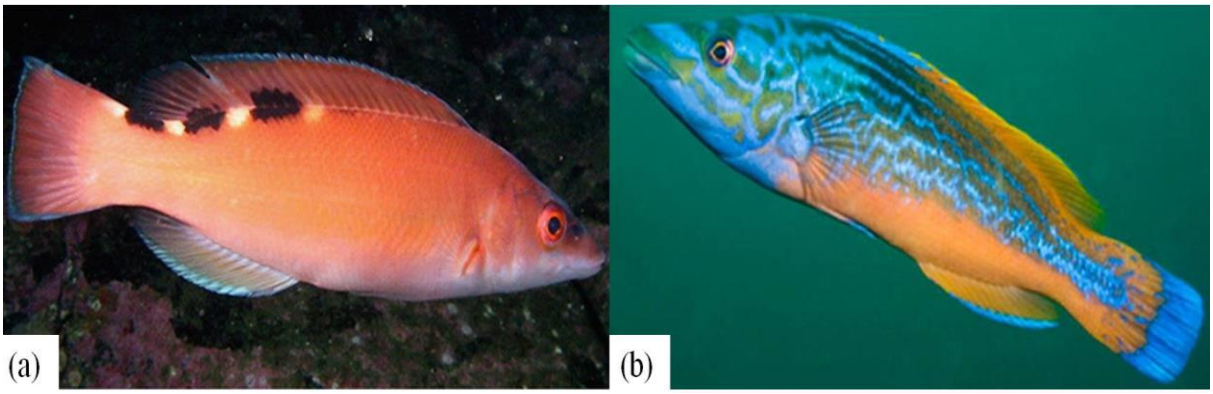


Figure 1.7: Cuckoo wrasse (*Labrus mixtus*) **(a)** Female and **(b)** Male.

L. mixtus (Fig. 1.7) have the distinction of being the most vibrantly coloured Labrini (in particular the males), but as yet, knowledge of their biology and ecology remains limited (Quigley, 2009). They prefer deeper distributions (40-80 m) (Quigley, 2009), that are rich in macroalga and seagrass beds (Matić-Skoko et al., 2013).

Again, this long lived (to 20 years) species, is sexually mature at 2 years, and are slow growing with maximum reported lengths of 30 and 40 cm for females and males respectively (Quigley, 2009). They are a protogynous species that change sex from female to male at older than 4 years old when there are no other males in a given community (Matić-Skoko et al., 2013). The reproductive season is from April to May with demersal eggs in a simple nest guarded by the primary male (Matić-Skoko et al., 2013). In this species, gender transition is accompanied by changes in external colouration so they appear highly sexually dichromatic

(Fig. 1.7(b)) (Matić-Skoko et al., 2013). Female preference in sexual selection favours males that are most brightly coloured (Dipper, 1981). In agreement with the other diandric species, IP *L. mixtus* males maintain an external phenotype that is identical to that of the females (Dipper, 1981). It is of note however, that the female livery changes prior to gonad re-structuring, and satellite males of this species can also change their external phenotype to that of a primary male (bright) therefore care must be taken when making generalisations regarding biophysiology, form and function relative to ontogenic stage (Dipper, 1981).

1.6 Gender identification

It is an obvious statement that gender identification is a fundamental pre-requisite to managing broodstock and optimising production of fin-fish in aquaculture (Chu-Koo et al., 2009). Numerous factors that are associated with the complexity of *L. bergylta* life history traits and reproductive patterns preclude use of many traditionally successful methods of gender identification. Although it is difficult to make generalisations about gender typical phenotype expression in teleosts, the key intrinsic roles are associated with, sexual selection including female choice, contest competition and maturation, but must also take into account the associations with other characteristics such as nutritional and immunological profiles (Ralls & Mesnick, 2008). Extrinsic functions would include intraspecific communication, photo-protection, and crypsis (Leclercq et al., 2009). Gender based dimorphism is a common feature of labrids with diverse patterns of colouration being correlated with reproductive success in many species (Fruciano et al., 2012). As many species are sequentially hermaphroditic the colour types of protogynous species are commonly described as “primary livery” (associated with IP phase), “secondary livery” (associated with TP phase) and “primary male livery” in diandric species which are born as male but retain the IP phenotype so they resemble females (Reinboth, 1988). This can be specific to a particular appendage as seen in cocktail wrasse

(*Pteragogus aurigarius* (Richardson 1845)) and/or include more general colouration changes such as that of *L. viridis* (Jeppesen & Cepeda, 2001), *C. julis* or *T. bifasciatum* (Magurran & Garcia, 2000). Returning to Fig. 1.3, as the colour patterns of *L. bergylta* are highly variable (Porteiro et al., 1996), and bear no relation to gender (Villegas-Ríos et al., 2013), alternative means of identifying gender became a priority (Talbot et al., 2012).

In contrast to the work of Higa et al. (2003) with three-spotted wrasse (*Haliocheres trimaculatus* (Quoy & Gaimard 1834)) which applied differentials in hormone and steroid profiles to accurately predict gender, although circulating E_2 and Testosterone T levels were higher in *L. bergylta* females and males respectively, there was no significant difference between genders (Godwin et al., 1996). This may have reflected demographic issues such as sample size and intermediate gender/male availability, or seasonal effects as *L. bergylta* reproduction and protogynous sex change are highly seasonal events which occur in a narrow timeframe (Muncaster et al., 2010). Hence, as background data was limited and repeated prospective samplings would be detrimental to assignation as broodstock, other non-invasive methods have been investigated. Ultrasound analysis has been proven successful for identifying gender in numerous species (Talbot et al., 2012). However, similarly to Shovelnose sturgeon (*Scaphirhynchus platorynchus* (Rafinesque 1820)), this appears to be stage dependent with sub-optimal survey timing, and retention of structural characteristics from primary (female) phases limiting this method to assessing maturation (Colombo et al., 2007). Vitellogenin is a precursor protein for the production of yolk in oviparous vertebrates (Pettersson et al., 2006). Non-invasive techniques of assaying vitellogenic processes can identify gender in many species of teleosts (Koo et al., 2009). In *L. bergylta* however, as relevant features from the female phase are retained for some time post-transition, ambiguous results suggest this method is also highly stage dependant (Talbot et al., 2012). Finally, morphometric analysis based on truss measurements and sexual size dimorphisms have proven

most successful for gender identification in *L. bergylta* (Leclercq et al., 2014b) but, correspondingly with the high degree of plasticity inherent in the stimuli for sex change protogynous systems, the overlap in differentiative profiles of midrange individuals can mask the specific identity.

Moving this forward, in contrast to typically pale yellow colouration of mammalian blood plasma (Gagnon, 2006), some teleosts including sculpins (Cottidae) and wrasse (Labridae), are observed to have coloured plasma ranging in hue from green through blue to maroon (Abolins, 1961; Low & Bada, 1974). Furthermore, marked gender dimorphism is reported in relation to pigment 'type' in the lumpsuckers (*Cyclopterus lumpus* (L.)) (Mudge & Davenport, 1986), and *C. tinca* (Abolins, 1961), or concentration in species including blue-throated wrasse (*Notolabrus tetricus* (Richardson, 1840)) (Gagnon, 2006), *C. mediterraneus* and *L. mixtus* (Abolins, 1961). In these cases, it was determined that the causative pigment of 'blue-green' sera was biliverdin (BV) (Fang, 1988). Although the mechanisms of plasma dimorphism remain to be fully characterised, relative differences between genders are thought to be a function of alternate hormonal profiles which cause disparity in expression levels of cyclic and open chain molecules (Abolins, 1961), and also through interactions with the binding regions of associated protein complexes (Fang & Bada, 1988). These differences were later established as an accurate methodology for differentiating gender (Abolins, 1961; Gagnon, 2006).

Figure 1.8: Plasma pigment variation in *Labrus bergylta*

Blue-green serum has been reported in *L. bergylta*, with strong variation in the degree of colouration (Fig. 1.4) in response to unknown factors (Abolins, 1961). This suggested that if the pigments and expression patterns were similar to those in the described plasma dimorphic species, then quantification may distinguish males from females to allow development of an in-situ applicable gender identification methodology.

1.7 The neuroendocrine basis of sex change

The neuroendocrine mechanisms by which social and environmental cues are integrated and translated to stimulus for sex change in sequentially hermaphroditic teleosts are of great interest in the context of broodstock management (Talbot et al., 2012), but remain poorly understood (Higa & Ogasawara, 2003). It is however safe to assume that the processes of sex reversal in teleosts requires a vast reorganisation of behavioural and biological systems (Lamm et al., 2015; Muncaster et al., 2013; Nakamura et al., 1989; Nozu et al., 2009), and that such changes are orchestrated and closely regulated by the activities of neurotransmitters, neuro-modulators and hormones (Larson et al., 2003). In mammals and birds, potentiality for expression of gender-typical behaviours is pre-determined by the organisational actions of

perinatally secreted steroids that induce sexual differentiation of the brain and tissues, and also by the activational effects of gonadal hormones in the adult phase (Munakata & Kobayashi, 2010). In this 'type', atypical sex differentiation of the brain is permanent so they exhibit a fixed gender specific hormone profile and the relevant behaviours (Munakata & Kobayashi, 2010). Contrastingly, it has been proposed that sequentially hermaphroditic teleosts have bi-potential brains which comprise both female and male persona with each expressed in response to a particular (male or female) hormonal template (Francis, 1992). Normal models of sexual differentiation which are based upon the brain and pituitary being influenced by patterns of differentiation in the gonads as communicated by androgens. The polarity would therefore be reversed in sequential hermaphrodites such that changes to the brains gender specific physiology, and hormonal expression patterns must therefore determine the fate of the gonads (Francis, 1992), which would allow the bipotential gender expressions as described, and facilitate epigenetically expressed gender as a function of social standing within the nested hierarchy (Baroiller et al., 1999).

The Hypothalamic-Pituitary-Gonadal (HPG) axis acts as an interface between social environments and stress and the resultant internal processes including reproduction (Lamm et al., 2015; Pankhurst, 2001). The anatomy, hormones and control mechanisms of the HPG axis are well conserved (Francis, 1992), but it is of note that precise mechanisms can vary depending on binary gender (Arcand-Hoy & Benson, 1998). Salient structures begin with the neuroendocrine regions of the Pre-Optic Area (P-OA) which form a direct interface between the Central Nervous System (CNS) and endocrine systems (Weltzien et al., 2004). The HPG is controlled via the Hypothalamic-Pituitary-Adrenal (HPA) axis in teleosts (Perry & Grober, 2003). Messenger molecules synthesised in the hypothalamus control the synthesis and release of pituitary hormones (Arcand-Hoy & Benson, 1998), including Adrenocorticotropin (ACTH), Prolactin (PRL), Growth Hormone (GH), Thyroid Stimulating Hormone (TSH), Gonadotropin

Releasing Hormone (GnRH) and two distinct but chemically related Gonadotropins (GtH I and GtH II) (Weltzien et al., 2004) which are analogous to mammalian Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH) respectively therefore GtH I mediates gametogenesis and steroidogenesis, whereas GtH II regulates the final maturation stages of gametogenesis (Arcand-Hoy & Benson, 1998).

GtH is subsequently responsible for modulating the synthesis of sex steroids including androgens, estrogens and progestins which in turn act upon systems that govern gametogenesis, reproduction, sexual phenotype and gender appropriate behavioural characteristics (Arcand-Hoy & Benson, 1998). Following this pathway, the monoamine neurotransmitters Dopamine (DA) and the antagonist Apomorphine (APO) regulate release of GnRH and GtH (Larson, 2003). This has been proven by exogenous DA intraperitoneal injections which reduced serum GtH levels and conversely, APO had the opposite effect (Larson, 2003). Further to this, Norepinephrine (NE) injections in the third cranial ventricle has a direct stimulatory action on GnRH and GtH release (Guiguen et al., 2010). Finally, serotonin has been shown to also increase GtH levels (Larson, 2003). In combination, as increasing NE or blocking GtH and DA lead to induction of sex reversal under non-permissive conditions, and as increases in serotonin blocked sex reversal under permissive conditions while inhibition of DA or NE retarded the process, this supports that the monoamines have a significant role in the induction process via interaction with the HPG (Larson, 2003). This is demonstrated by sex change in diandric *T. bifasciatum* which has been associated with increased levels of GnRH immuno-reactive neurons when compared to both females and intermediate males (Larson et al., 2003). It is also of interest that when this is considered in the context of the protandrous Cinnamon clownfish (*Amphiprion melanopus* (Bleaker 1852)), which shows identical GnRH patterns to *T. bifasciatum* in a species where females are the terminal phase, it would suggest that GnRH is most likely involved in maintaining male reproductive function as opposed to directly

controlling sex reversal (Larson et al., 2003). This is confirmed by the failure of GnRH to stimulate transition without the combinatory effect of a dopamine antagonist (Balment et al., 2006). GnRH are however important mediators of sex change associated neural signalling, gonadal remodelling, reproductive behaviours and sexual phenotype, as demonstrated by GtH binding to specific membrane receptors in the gonads regulating reproductive maturation, gamete release and maintenance of egg and sperm production (Elofsson et al., 1999). Hence, as it is known that chronic stress is accompanied by increased activity in the HPA axis, and decreases in reproductive functions as a function of preserving adrenal cortex function at the expense of reproductive investment this would potentially support suppression of reproduction and transition in lower order fish (Rivier & Rivest, 1991). This would at least in-part explain male typical behaviours in females immediately upon male removal (Lamm et al., 2015).

With specific consideration of the pro-transitional profile in protogynous sequential hermaphrodites, relative abundance and synthesis patterns of hormones including estrogens and androgens are known to regulate ovarian and testicular differentiation across vertebrates (Higa & Ogasawara, 2003; Lamm et al., 2015). In teleosts, 17β-Estradiol (E_2) and 11-ketotestosterone (11-KT) have emerged as a key mediators of transition at both the gonad and behavioural levels (Godwin, 1995). Although it has been shown that E_2 is present in both genders, E_2 is higher in females than males, whilst 11-KT demonstrates the opposite pattern (Lamm et al., 2015). From this; it follows that the activity of 11-KT and conversion of T to oestrogens by cytochrome P-450 aromatase is also important (Baroiller et al., 1999). In contrast to tetrapods, teleosts have two aromatase genes (cyp 19a1 and cyp 19a2) which are expressed in the gonads and the brain tissues respectively (Lamm et al., 2015). Studies have shown aromatase enzyme expression patterns are different between genders (Guiguen et al., 2010), with gonadal aromatase immuno-reactivity being higher in female tissues and declining rapidly after female to male transition (Godwin, 1995). This is in accord with the high E_2 levels in

ovarian tissue and supports it as the critical regulator of sex change in such species (Godwin, 1995). Similarly to the gonadal expression patterns of androgens, brain aromatase also shows far higher reactivity in the female phase then rapidly declines after transition, and expression of brain aromatase is negatively correlated with the emergence of male behavioural traits during this process (Godwin, 1995). Thus; through regulation of local E_2 levels, brain aromatase can actively participate in sexual differentiation of gonadotrophin secretion patterns and expression of sexual behaviours (Garcia-Segura, 2008). The targets of biosynthetic androgens are thought to be oestrogen response elements (EREs) in the brain which is consistent with stimulation by E_2 acting in maintenance of the female phenotype (Godwin, 1995). Hence, depletion of E_2 beyond a homeostatic set point is proposed as one of the major triggers of differentiation (Higa et al., 2003). With specific reference to *L. bergylta*, and similar observations in other protogynists including honeycomb groupers (*Epinephelus merra* (Bloch 1793)), and spinycheek groupers (*Epinephelus dicanthus* (Valenciennes 1828)) (Bhandari et al., 2003), this explains why there is greater potential for inversion at the end of the reproductive season as it coincides with naturally low levels of E_2 such that the threshold level is easier to attain (Muncaster et al., 2013). However, with the exception of Muncaster et al. (2013) there remains a lack of understanding of the sex change process in *L. bergylta* and by extension, the importance of social and/or environmental factors that drive endogenous change and ultimately the physiological demands that such a process entails.

1.8 Haem catabolism

As biliverdin is the intermediate product of haem catabolism (Takahashi et al., 2009), it is pertinent to understand the metabolic pathway as a whole and the key elements which regulate it.

Haem (protoporphyrin) is a biogenic pigment, which is fundamental to life through prosthetic incorporation in a suite of molecules which are themselves key to a wide array of biological processes including the transport and storage of oxygen, oxygen sensing, oxidative metabolism, controlling oxidative damage, and as a cofactor in the catalysis of redox reactions (Ajioka et al., 2006; Arruda et al., 2005; van Dooren et al., 2012). It is of note however that although it has many important regulatory roles (Khan & Quigley, 2011), free haem at local concentrations greater than 1 μM is shown to be physiologically deleterious due to intense pro-oxidant potential (Toh et al., 2010). Initially, it is present in large amounts in many cells and the highly hydrophobic nature allows transfer across cell membranes where it concentrates in the intercellular matrix (Stocker et al., 1987; Frei et al., 1988). Once within the cell, haem causes extensive cellular damage directly as the same properties used to catalyse redox reactions can also generate Reactive Oxygen Species (ROS) (Sawicki et al., 2015), or indirectly through the release of iron which has the potential to enter the hydrophobic lipid bilayer leading to uncontrolled Fenton reactions ($Fe^{2+} + H_2O_2 - Fe^{3+} + OH^- + OH°$), and Haber-Weiss reactions ($Fe^{3+} + O_2^{\circ-} - Fe^{2+} + O_2$) in the oxidation sensitive matrices of cell membranes (Balla et al., 2003; Valko et al., 2007). This generates hydroxide radicals which initiate reactions with Poly-Unsaturated Fatty Acids (PUFA) resulting in abstraction of further hydrogen atoms from other PUFA in the immediate vicinity and amplification of the oxidative stress (Mwebi, 2005). Negative effects then intensify through intercalation of the cellular phospholipid bilayer membrane with extensive lipid peroxidation and further propagation (Ryter & Tyrrell, 2000). This results in alterations to cell structure dynamics, and non-enzymatic generation of reactive species leading to extensive damage to a wide range of biological molecules and ultimately, cell death (Arruda et al., 2005; Valko et al., 2007). Hence, complex mechanisms have evolved to regulate the metabolism, catabolism and clearance of cellular haem through multistep enzymatic degradative processes

Haem Oxygenase (HO) is the rate limiting step in the biochemical pathway that governs haem catabolism and consequently, BV and bilirubin (BR) synthesis (Bucolo & Drago, 2009). The enzyme exists in two distinct isoforms. Under normal haemolytic (basal) turnover of scenescent erythrocytes and other haemoproteins, haem templates are degraded by constitutively expressed 36 kDa HO-2 which regulates normal physiological function and is predominantly found in the brain, gonads and vascular system (Arruda et al., 2005; Kirkby & Adin, 2006). Briefly, Haemopexin (HPX) (EC 3.2.1.35) binds free haem and directs it to the Reticuloendothelial Systems (RES) for degradation (Nowell et al., 1998). Within the RES which is predominantly localized to the spleen, liver and bone marrow, haem then undergoes phagocytosis by macrophages wherein enzymes act to degrade the haem (Lara et al., 2005). Contrastingly, 32 kDa HO-1 (also termed Heat Shock Protein 32 (Hsp32)) is induced in response to various forms of insult or injury including excess haemolysis (Poss & Tonegawa, 1997), UVA and visible light exposure (Poss & Tonegawa, 1997; Reeve & Tyrrell, 1999), oxidised lipoproteins (Poss & Tonegawa, 1997), hypoxia (Wang et al., 2008), hyperoxia (Tzaneva & Perry, 2014), thermal stress (Poss & Tonegawa, 1997), free haem and divalent metals (Poss & Tonegawa, 1997), sulfhydryl reagents (Panchenko et al., 2000) and inflammatory cytokines (Poss & Tonegawa, 1997).

In either case, HO activity generates equimolar amounts of CO, iron (Fe^{2+}) and the linear tetrapyrrole BV (Fig. 1.5) (Kirkby & Adin, 2006). In placental mammals, BV is then further processed by BV Reductase (BVR) to the terminal product BR (Cao, Inoue, Li, Drummond, & Abraham, 2009), but not in reptiles, fishes, birds and insects (Kikuchi et al., 2005). In such organisms, BV can be considered as the terminal product of this pathway and as such is excreted, or in some cases stored in tissues and plasma (Cornelius, 1981).

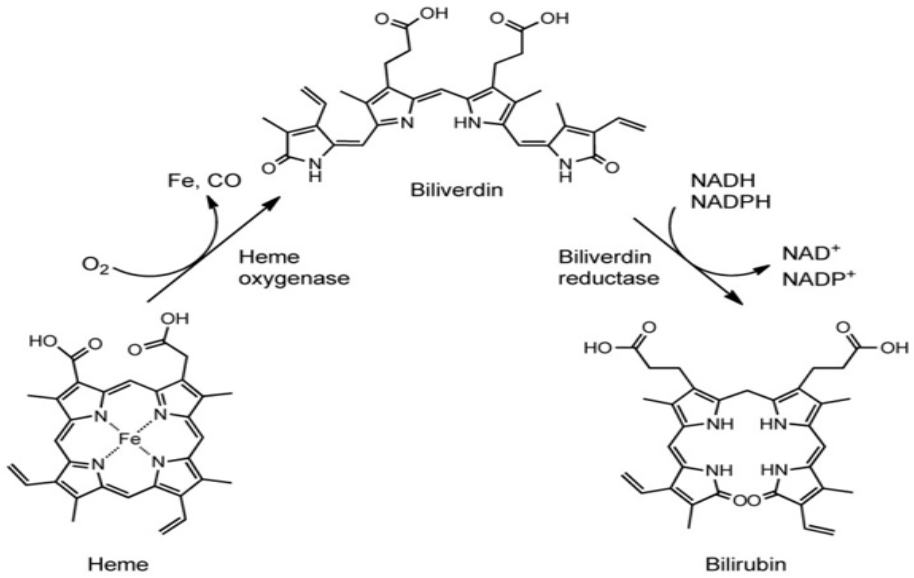


Figure 1.9: Catabolic pathway where HO catalyses conversion of haem, to BV releasing carbon monoxide (CO) and ferrous iron. Biliverdin is subsequently reduced to bilirubin by biliverdin reductase (Takahashi et al., 2009).

HO induction occurs by haem binding to the repressor protein Bach-1 which promotes upregulation of expression, but can also be regulated by redox sensitive pathways (Nath, 2006). The reaction begins with formation of a ferric haem-HO complex (1:1), where ferric-haem is reduced to a ferrous state with the transfer of an electron from Haemoprotein Reductase (HR) (Nicotinamide adenine dinucleotide phosphate (NADPH)-Cytochrome p450 Reductase) (EC 1.6.2.4) (Kikuchi et al., 2005; Toh et al., 2010). Subsequent O_2 binding hydroxylates the α–meso-carbon with oxygen dependent elimination of the hydroxylated meso-carbon as verdoheme (Paiva-Silva et al., 2006; Toh et al., 2010). The haem moiety is then enzymatically cleaved at the α-methenyl bridge between the pyrolic subunits by membrane bound microsomal HO (Lara et al., 2005; Nowell, 1998). Selective α-methene bridge cleavage by HO generates the most common physiological isoform (BV IXα) with the alternate (IXβ, IXγ and IXδ) isomers determined by the specifically eliminated carbonyl bridge in the haem precursor (Chen et al., 2012). It is of note that most species of birds, fish and reptiles excrete BV directly (Fang & Bada, 1988), whereas further reduction occurs in mammals where the terminal product can

be considered BR due to rapid BV reduction with no little or no accumulation (Ding & Xu, 2002; Nowell, 1998). In this reaction, water soluble BV ($C_{33}H_{34}N_4O_6$) (582.65 Da) is reduced to the terminal product BR ($C_{33}H_{36}N_4O_6$) (584.66 Da) by the activity of BV Reductase (BVR) (EC 1.3.1.24) which reduces the double bond between the pyrrole rings (Fig. 1.6) (Barañano & Rao, 2002).

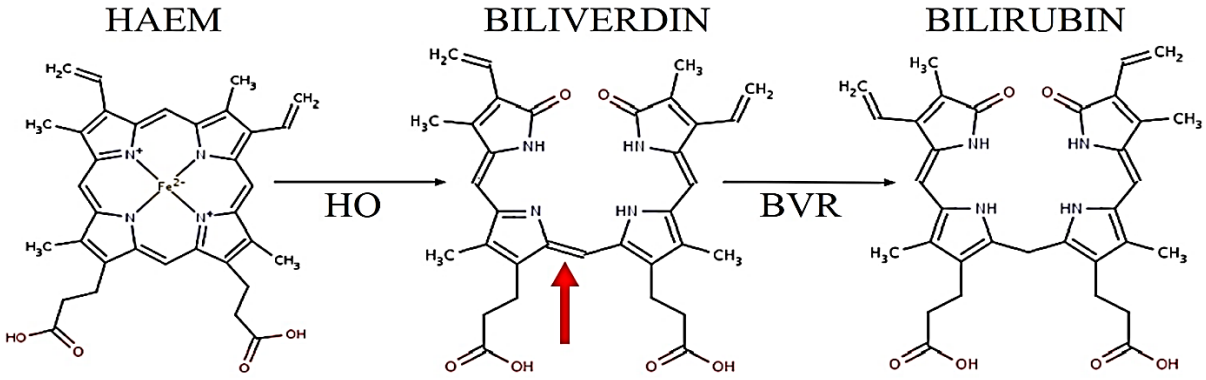


Figure 1.10: The Haem-bilirubin axis showing conversion of haem to biliverdin by Haem Oxygenase, and subsequent reduction of biliverdin to bilirubin by biliverdin reductase. Red arrow indicates the double bond biliverdin reductase acts upon. Haem Oxygenase (HO); Biliverdin Reductase (BVR).

Following the pathway to the terminal product, unconjugated (free) BR is insoluble in water due to polar propionic acid side-chains and amino acid residues with internalised hydrogen bonding (Wang et al., 2006). Further to this; as non-ionized BR can cross cell membranes and cause dysfunction in mitochondrial processes, it presents a wide range of cytotoxic effects at the cellular level (Kirkby & Adin, 2006; Wang et al., 2006). Deposition in brain tissues (kernicterus) has the most severe cytotoxic effects and is linked with reduced conjugative efficiency (Crigler-Najjar diseases/Gilberts syndrome) (Fevery, 2008), while deposition in skin and peripheral tissues (jaundice) is characteristic of liver disease (Weisiger et al., 2001). These effects are tightly regulated in mammals through tight binding of BR to Serum Albumin (SA), which also allows distribution around the circulatory system and extravascular spaces (Fang & Bada, 1988). The BR-SA complex is transported with the serum

fraction to the liver and dissociate at the plasma membrane of the hepatic sinusoids (Fang & Bada, 1988). BR then diffuses across the phospholipid bilayers into the hepatocyte (Kirkby & Adin, 2006), where it is bound by cystolic proteins including Glutathione-S-Transferase (GST) (ligandin) and fatty acid binding proteins within the hepatocyte to maintain homeostatic levels and abrogate cytotoxic effects (Fevery, 2008).

Within the hepatocyte cytosol, Uridine 5'-diphospho-Glucoronide Transferase (UGT) (EC 2.4.1.17) catalyses conjugation of glucoronic acid moieties to the propionate side chains (Kirkby & Adin, 2006) to form mono and di-glucuronides (Stillman, 1982). This disrupts internal hydrogen bonding resulting in structural alterations which forms the amphipathic form Conjugated BR (CBR) (Widmaier et al., 2008). This conjugation further reduces potential re-absorption due to the inherent inertia of the intestinal wall to the transit of hydrophilic molecules (Nowell, 1998). CBR is then excreted into the bile (Fevery, 2008), incorporated into mixed micelles including phospholipids, bile acids and cholesterol, and transported to the intestinal lumen (Kirkby & Adin, 2006). CBR is then de-conjugated by intestinal enzymes and anaerobic fauna which reduce the molecules to urobilinogens ($C_{33}H_{44}N_4O_6$) for reabsorption via enterohepatic recirculation for excretion in urine (Fevery, 2008). The remainder is converted to stercobilinogens ($C_{33}H_{48}N_4O_6$) (faecal urobilinogen) (Vítek, 2012; Wang et al., 2006).

1.9 Haem oxygenase

HO-1 activation is considered a protective pathway as upregulation is correlated with reduced free haem, decreased Reactive Oxygen Species (ROS) and marked cytoprotection (Cao et al., 2009). This can be illustrated in HO deficient experimental systems where pathological disease models of ischemia and reperfusion, pulmonary inflammation and hypertension, infectious microbial sepsis, cerebral malaria, xenograft rejection, auto-immune

inflammation, atherosclerosis and myocardial infarction were exacerbated by inactivated HO (Kikuchi et al., 2005; Otterbein et al., 2000; Soares & Bach, 2009). In consideration of this, and rapid upregulation of HO-1 in relevant stress conditions, activation of HO-1 can be considered as an adaptive response which enhances resistance to departure from cellular homeostasis and minimizes the risk of damage (Yao et al., 2009). It follows that although this generates free iron which is also strongly pro-oxidant, parallel ferritin activation serves to maintain homeostasis through sequestration (Yao et al., 2009), therefore HO activation is an instant method of protecting tissues from haem toxicity (Ferenbach et al., 2010; Yao et al., 2009). Under extreme haemolysis however, pro-oxidative molecules may exceed this capacity therefore additional systems become essential (Cao et al., 2009). HO activity is also linked to conserved patterns of retarded apoptosis and cellular survival in studies of upregulation, and conversely, increased levels of apoptosis and necrosis in inhibitory studies (Ferenbach et al., 2010). Although there is some debate as to the precise mechanisms of protection due to the highly pleiotropic nature of this pathway, and it is of note that HO expression and activation ultimately determines generation of any associated products and effects (Yao et al., 2009), there is strong evidence that it is in fact the downstream products BV and BR which mediate many of the beneficial properties.

1.10 The bile pigments: biliverdin and bilirubin

The bile pigments BV and BR display a diverse range of physiological functions. Evidence of this is found in exogenous administration experiments describing highly beneficial effects during Ischemia-Reperfusion (IR) insults (Tang et al., 2007), vascular injury (Wegiel & Otterbein, 2012), endotoxic shock (Soares & Bach, 2009; Wegiel & Otterbein, 2012), tissue graft rejection (Bilzer & Gerbes, 2000; Ferenbach et al., 2010; Wang et al., 2010), mutagenesis (Bulmer et al., 2008), HIV 1 (McPhee et al., 1996), HIV 2 (McPhee et al., 1996), cancer (Wang

et al., 2006), diabetes mellitus (Vítek, 2012), and hepatitis (Wegiel & Otterbein, 2012). It has been shown that although BV (Fig. 1.7(a)), lacks the reactive hydrogen atoms of BR (Fig. 1.7(b)), lability is similar due to the extended system of conjugated double bonds as it allows formation of a resonance stable carbon centred radical (Kaur et al., 2003).

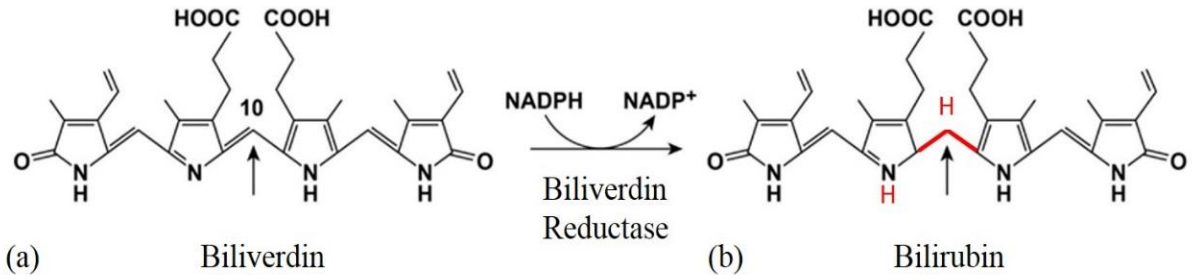


Figure 1.11: (a) Biliverdin, and **(b)** Bilirubin showing the C10 biliverdin double bond (arrow) being eliminated by biliverdin reductase activity, and reactive hydrogens of bilirubin (red).

The majority of these functions are directly based upon the anti-oxidant properties of the molecules. Although there are conflicting reports in literature with reference to the relative anti-oxidant activities of BV and BR (Asad et al., 2001), both have inherent properties that are similar to those of the carotenoid pigments (Morales et al., 2010). This is due to the ability to scavenge free radical species including superoxide anion and peroxide radicals, and the inhibition of free radical mediated cell lysis at concentrations much lower than more potent antioxidants such as trolox and ascorbate (Asad et al., 2001). Whilst BV is a water soluble and non-toxic compound (Kirkby & Adin, 2006), BR can be harmful in excess and is water insoluble and so requires conjugation for safe transport (Bulmer et al., 2008). At face value this presents the question of why an additional processing step is necessary when BV appears to represent an idealised format of the molecule in a biological context. In considering the fore-mentioned Hyperbiliverdinaemic (HBV) species which accumulate the pigment in their plasma and tissues, this would imply an endogenous store of a simple and safe anti-oxidant molecules, but as this represents a highly unusual trait amongst vertebrates, a likely explanation would

therefore be that not only bile pigments intrinsically valuable due to their anti-oxidant capacities, interconversion of BV to BR must also confer a potentially significant advantage.

It is of note however that the BV-BR oxidation reduction cycle makes separation of the bile pigments functions and properties more complex (Wang et al., 2010). Previous studies noted that nanomolar amounts of bilirubin could protect cells from intensely pro-oxidant concentrations of H_2O_2 (Doré & Takahashi, 1999). In this remarkable system, as BVR is present at functional excess in most tissues and has high affinity for its substrate (Doré & Takahashi, 1999), and the oxidation of bilirubin by reactive species generates a molecule of BV (Sedlak & Snyder, 2004), the reaction is coupled to the subsequent enzymatic reduction resulting in intrinsic amplification of the anti-oxidant effect. In agreement with Sedlak & Snyder (2004), the elegance of this system is found in the regenerative application of bilirubin's properties at augmented levels relative to the molecules present while maintaining tissue concentrations at sub-toxic levels.

1.11 Biliverdin binding proteins

Several previous studies have described forms of BV protein associations in animals (Jüttner et al., 2013). In contrast to algal biliproteins, which have well defined photosynthetic activities (Rudiger, 1970), experimental data has not yet fully determined the physiological significance. BV binding proteins have been described in the fins and scales of Labridae including *S. tinca* (Rudiger, 1970) and humphead wrasse (*Cheilinus undulatus*) (Rüppell 1835) (Yamaguchi & Matsuura, 1969); Scaridae including heavybeak (*Scarus gibbus*) (Rüppell 1829) (Yamaguchi, 1971), knob-snout (*Callyodon ovifrons*) (Temminck and Schlegel 1846) (Yamaguchi, 1971) and tricolour (*Scarus cyanognathus*) (Bleeker 1847) (Yamaguchi & Matsuura, 1969); Cottidae such as sea scorpions (*Cottus scorpius*) (L. 1758) (Yamaguchi, 1971), and Artic sculpin (*Myoxocephalus scorpioides*) (Fabricius 1780) (Yamaguchi &

Matsuura, 1969); and Scomberesocidae including Pacific saury (*Cololabis saira*) (Brevoort 1856) (Yamaguchi, 1971). These were for the most part observational papers based on the physical properties of macromolecules during solubility and precipitation experiments (Fang, 1986; Rudiger, 1970; Yamaguchi & Matsuura, 1969; Yamaguchi, 1971). Subsequent works (e.g. Abolins, 1961) were concentrated on elaboration of the chemical structure of the chromophore, but characterisation of the associated protein remained cryptic.

Further to this, BV is also found associated with proteins in the periosteum, spinal processes and scales of *B. belone* (L. 1761), and *Z. viviparus* (Jüttner et al., 2013). Similarly, blue forms of *S. vitreus* (Mitchill 1818) are observed express BV chromoproteins in mucous layers (Yu et al., 2007). Additionally; *C. analis* (Fang & Bada, 1988), *C. lumpus* (Mudge & Davenport, 1986), Japanese rice eel (*Anguilla japonica*) (Temminck & Schlegel, 1846) (Fang & Bada, 1988); and Labridae including *L. bergylta* (Abolins, 1961), *S. tinca* (Yamaguchi, 1971), *N. tetricus* (Gagnon, 2006), *S. melops* (Rudiger, 1970), *L. viridis* (Yamaguchi, 1971), *L. merula* (Rudiger, 1970), and green-eyed wrasse (*Symphodus ocellatus*) (L. 1758) (Yamaguchi, 1971) are known to accumulate BV in the plasma in a protein bound form.

1.12 Experimental aims

Chapters 2 and 3

- Isolate and identify the underlying pigment responsible for plasma colouration in *L. bergylta.*
- Develop an assay to quantify plasma biliverdin and to characterise it in relation to variation between individuals and establish if gender was the major driver of variation.

- Determine plasma biliverdin levels in other species of wrasse.

Chapter 4

- Isolate and identify the protein moiety associated with plasma biliverdin in *L. bergylta.*
- Explore biliverdin binding proteins in other species of wrasse.
- Advance our understanding of biliverdin's physiological relevance in *L. bergylta*.

Chapter 5

- Establish if hormonal induction can be used to generate male *L. bergylta.*
- Determine the effect of size relative to hormonally induced protogynous inversion.
- Use gonad histology from induced fish to develop a unified scale of transition.
- Explore biliverdin mobilisation across th inversion process.

Chapter 2:
General Materials and Methods and Method development for the qualitative and quantitative determination of Biliverdin

1.0 General materials sourcing statement

All chemicals and reagents used hereafter were obtained from Sigma-Aldrich, USA (www.sigmaaldrich.com) unless otherwise stated.

2.0 Experimental animals and facilities

The following work was based upon five main populations. All animal experimentation was performed in accordance with the Home Office Animals (Scientific Procedures) Act (1986), the sources and housing conditions where relevant can be summarised as follows:

2.1 Wild *Labrus bergylta* (Chapter 3)

Wild *L. bergylta* (n=397) were captured over a 6 week period corresponding to the end of the species spawning season (July - August 2012), from four distinct coastal locations: site A (Rong, Norway: 60∘ 33′N; 04∘ 53′ E; n=100) (Bergen hereafter), site B (Machrihanish, Scotland: 55∘ 25′ N; 05∘ 44′ W; n=100), site C (Ardtoe, Scotland: 56∘ 46′ N; 05∘ 53′ W; n=99), and site D (Scalloway, Shetland, Scotland: 60∘ 08′ N; 01∘ 17′ W; n=98) (Leclercq et al., 2014b). Stocks were maintained locally in flow-through tanks under ambient photo-thermal conditions until sampling within a few days.

2.2 Wild Labrini (Chapter 3)

Independently of the original fish collection, other native wrasse species including *C. exoletus* (n=10), *S. melops* (n=12), *C. rupestris* (n=12), *L. mixtus* (n=12) and *L. bergylta* (n=12) were collected by baited traps in the Lochaber region of Scotland (56°40’57”N, 5°18’2”W) on 20.08.15 with collection, demography, treatment and biometric data capture identical to the initial population (Section 2.1) as outlined below (Section 3.0).

2.3 Wild Labrini (Chapter 4)

C. rupestris (n=12), *C. Exoletus* (n=12), *S. melops* (n=12), *L. Mixtus* (n=12) and *L. bergylta* (n=12) were collected from Ardgour, Scotland (56°41'03.3"N 5°42'25.2"W) on 31/08/15 with collection, treatment and biometric data capture identical to the initial population (Section 2.1) as outlined below (Section 3.0). As no males were acquired on this occasion pooled and homogenised plasma from two confirmed males from the Machrihanish population (Section 2.1) were included to provide comparison.

2.4 *Labrus bergylta* sex reversal (Trial 1 (Chapter 5))

L. bergylta (n=18) were selected from a population of Passive Integrated Transponder (PIT) tagged broodstock at the Ardtoe Marine Laboratory. These animals had previously been wild caught in the Sound of Mull (56°37′N 6°04′W / 56.62°N 6.07°W) using prawn creels and lobster pots two years prior to their application in this study. In the interim period the fish had been held communally in a 14 m^3 tank with two confirmed males. Treatment and biometric data capture identical to the initial population (Section 2.1) as outlined below (Section 3.0).

2.5 *Labrus bergylta* Sex reversal (Trial 2 (Chapter 5))

L. bergylta (n=73) were wild caught in the mull of Kintyre region and held in captivity at the Machrihanish Marine Environmental Research Laboratory (55∘ 25′ N; 05∘ 44′ W) for one year prior to their application in this study. Fish were previously PIT tagged and based on body size and condition, only individuals which met the assumptions of the "female" morphometric definitions as described by (Leclercq et al., 2014a) were taken forward into the study. Treatment and biometric data capture identical to the initial population (Section 2.1) as outlined below (Section 3.0).

3.0 Sampling procedures

3.1 Anaesthesia and euthanasia

Experimental fish were starved for 24 hours and anaesthetised by immersion in tricane methanesulphonate (30 ppm; MS-222) prior to experimental procedures. In all cases, loss of equilibrium was induced within 1 minute with the anaesthetic bath refreshed regularly, and where fish were to be returned to stock they were transferred to clean well aerated water until recovered, then returned to original housing. When required, fish were euthanized in accordance with Home Office (ASPA; 1984) Schedule 1 guidelines (Section 1(4.3)) using lethal anaesthetic overdose (100ppm; MS-222), confirmed by destruction of the brain.

3.2 Blood sampling

Bloods were withdrawn from anaesthetised or euthanized fish by caudal puncture using 2 ml sterile syringes with 21G sterile hypodermic needles (Terumo N.V.; Leuven, Belgium) depending on size. Syringes were previously flushed with heparin (4mg.ml^{-1} in ddH_2O) to prevent coagulation. Blood was stored on ice in 1.5 ml tubes, centrifuged (1789 rcf, 8 mins), the plasma supernatant recovered, aliquots assigned to 0.5 ml tube, and stored at -70 °C.

3.3 External colour and pattern phenotype

Following euthanasia, specimens were placed in lateral recumbence for image acquisition using a high resolution digital camera (Panasonic Lumix DMC-FT20; 16 MP; www.panasonic.com) which was mounted to standardise the distance and light environment across individuals and between sites.

3.4 Biometric data capture

All specimens were measured for Body Mass ($M_B \pm 0.1$g) and Total Length ($L_T \pm$ 0.1cm) using electronic scales (Model BFS-242-020C, ± 0.1 g; Sartorius AG, UK) and an adapted measuring board respectively. Gonads were excised and weighed ($M_G \pm 0.1$g) prior to further processing. Left opercula were removed and frozen for later analysis with age determined by quantification of annuli based on successional hyaline rings, and the yearly age estimated from this by general consensus between operators (Leclercq et al., 2014b).

3.4.1 Equation 1: Specific Growth Rate

Specific Growth Rate (SGR) representing a point estimate of the percentage increase in size/weight per day (Lugert et al., 2014) was calculated as:

$SGR = ((Ln (M_B2)-(Ln (M_B1)*100)/Days$

Where M_B1 and M_B2 are mean-whole body wet weight (g) at times t1 and t2 respectively.

3.4.2 Equation 2: Gonado-Somatic-Index

Gonado-somatic indices (GSI) are a metric representing the proportionate weight of the gonads relative to the body mass of the organism (Rothschild, 2015) was calculated as:

$GSI = 100M_G (M_B-M_G)^{-1}$

Where Mg and M_B are gonad mass relative to the body mass (M_B).

3.5 Tissue preservation

After removal, the largest gonadal lobe from each individual was stored in 10% neutrally buffered formalin prior to histological analysis. The storage solution comprised deionised H_2O (900 ml), disodium hydrogen phosphate (6.5 g), sodium dihydrogen phosphate (3.5 g), and Formalin (40% formaldehyde) (100 ml).

3.6 Gonad histology

Tissue samples were excised from the anterior regions of the gonads for all individuals, and fixed in neutral phosphate buffered formalin solution (Section 3.5). The lobes were then wax embedded by conventional histological techniques then 5µm paraffin sections were cut. Slides were stained with haematoxylin and eosin, and then examined using a light microscope (Olympus BH- 2; Olympus Optical Co., London, UK). Exemplar images of gender phase dependant histology (Chapter 5; Section 3.1) were acquired using a Zeiss Axioscan model Z1 Bright-field digital slide reader (www.zeiss.com).

3.7 Gender determination

Gender was determined using gonadal histology with the ontogenic stage described as the degree of protogynous transition classified in accordance with Leclercq et al. (2014b), using a combination of methods developed from Muncaster et al. (2013) and Nozu et al. (2009) which established a scale of protogynous sex change based on leading differentiating stage (defined as most advanced transitional (towards male in protogynists). From this, and with consideration of the standardised terminology of (Brown-Peterson et al., 2011), individuals were generally classified as Female, Transitional (undergoing sex change), or Male (Chapters 3 and 5). It is notable that this scale of gender was further expanded to give greater resolution therefore additional subdivisions were developed but these are discussed in detail within the text (Chapter 5).

4.0 Plasma pigment preparation

All plasma samples were defrosted in darkness at 4 °C immediately prior to analysis. Exposure to any additional sources of oxidative processes was always kept minimal.

4.1 Chromophore extraction and analysis I

The first method of extracting the chromophore from *L. bergylta* plasma was based on the methods of Austin & Jessing (1994). 150 µl of MeOH.HCl was added to 30 µl of crude plasma and agitated for 1 hour at 4 °C in darkness to acidify the prosthetic group. 30 µl Chloroform ($CHCl_3$) was then added and the resultant solution stirred for 10 mins. A further 470 µl $CHCl_3$ was introduced and the serum proteins were removed by three 500 µl washes with ddH_2O. Following the method, the next step was to load the extracted pigment (20 µl) onto a silicic acid micro-column which was pre-equilibrated with $CHCl_3$, and then wash the column with additional $CHCl_3$ until a yellow band (presumed to be BR) eluted, then change the mobile phase to 2:1:3 volume to volume (v/v) Ethanol (EtOH): Methanol (MeOH): $CHCl_3$,

mixed with several drops of MeOH.HCl to elute the remaining BV fraction. The column was packed according to the methods of Barron & Hanahan (1957). Briefly, the silicic acid was dried for 12 hours at 110 °C prior to use. The column was pre-fitted with a fritted-glass disk with a thin layer of glass wool, and the silicic acid poured in as slurry in $CHCl_3$. Although this method made it difficult to maintain a constant packing volume, as a generalisation, the adsorption columns measured 6 mm in diameter and 100 mm long, into which 0.4 g of silicic acid would occupy 80 mm when fully packed. However, the silicic acid medium was noted to fracture under both positive and negative pressure on a number of occasions.

It was presumed that better control of the flow rate and as such, the localised pressure on the column surface would prevent this, so the system was redesigned to be pressurised, and a peristaltic pump (Watson Marlow 205U; www.watson-marlow.com) facilitated finite control of the mobile phase. The mobile phase flow rate was incrementally reduced from 1.0 to 0.2 $ml.min^{-1}$ to prevent fracturing the stationary phase. The column appeared stable at 0.2 ml/min^{-1} but the sample, which was visible in the dispersion volume, repeatedly failed to appear. Similarly, the loading volume of sera was varied to determine whether this effect was dose dependent. Initially, this was assumed to be a function of the solvent phase as the boiling point of $CHCl_3$ is similar to that of petroleum ether therefore it may cause similar problems to those described by Barron & Hanahan (1957), where the column is noted to bubble and become dry. The method was then modified to by increasing levels of Acetic acid: Chloroform ($C_2H_4O_2$:$CHCl_3$) (2%, 4%, 6%, 8% and 10%) to vary the polarity of the mobile phase during equilibration and elution but the problem persisted. After numerous reiterations, it was determined that regardless of the packing method, flow rate and loading volume the sample would fail to appear in the column.

There were a number of factors to consider. Initially, the original works had been in other species namely *C. analis* (Fang & Bada, 1988), and lizards of the Prasinohaema genus

(Austin & Jessing, 1994), therefore methods and results would potentially vary. Furthermore, as the pigment itself is highly labile (Mcdonagh, 2010) there was always potential to lose pigment to oxidation from light, oxygen exposure or excess temperature during transfer between processes. Finally, if the chromophore was indeed tightly bound to a protein moiety, the denatured proteins could form aggregates which then form a plaque at the stationary phase surface which would prevent the sample from loading properly and account for the structural stability issues in the stationary phase as described, and it must also be considered that the pigment may have still been bound to the protein, and as such, would also remain in the plaque. High Pressure Chromatography (HPLC) was determined as the best method to resolve this.

4.2 Chromophore extraction and analysis II

BV.HCl was dissolved in MeOH to 1 $mg.ml^{-1}$, then diluted in MeOH to 100 $\mu g.ml^{-1}$. Partially purified *L. bergylta* plasma from the previous method was then similarly diluted (1:10) in MeOH. In accordance with the standard method of BV analysis on the selected column, the apparatus used a membrane degasser with a Constametric 4100 solvent delivery system connected to a Thermo-Finnigan spectra-system UV3000 and FL3000 detector (www.thermo.com). The HPLC column (Supelco Discovery C-18 ((5 µm internal) 10 cm*2.1mm))) was at ambient temperature. The UV detector was set to 405nm with a runtime of 25 mins. Flow rate was 0.4 ml/min^{-1} and the system was equilibrated with the $NH_4CH_3CO_2$ mobile phase prior to use. 20 µl sample volumes were introduced via 20 µl injection loop. BV is known to elute just after 8min under these conditions (Chen et al., 2012).

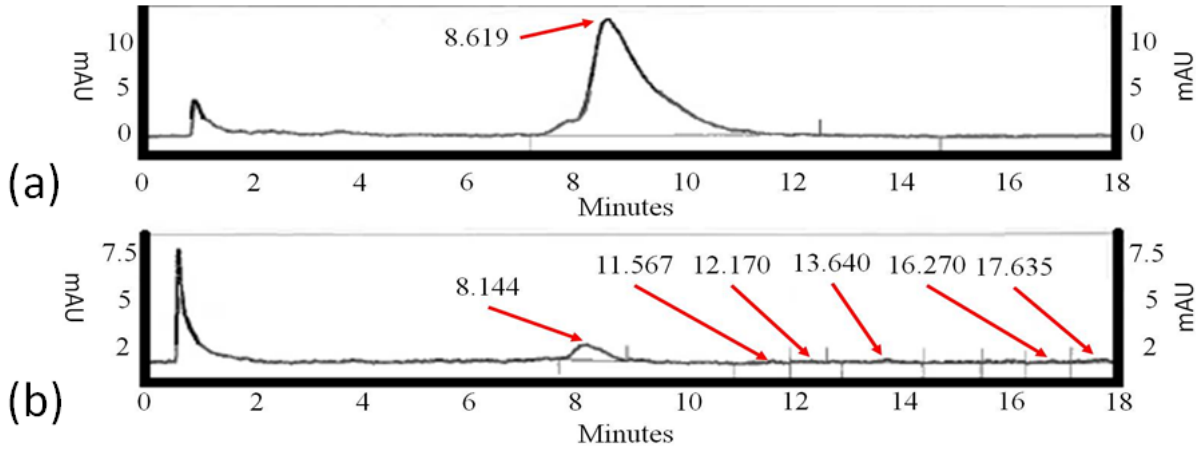


Figure 2.1: HPLC chromatograms of **(a)** 20 µl 100 µg.ml^{-1} biliverdin hydrochloride, and **(b)** 20 µl of *Labrus bergylta* plasma chromophore solution in MeOH.

The elution times of the 100 µg.ml^{-1} (8.53 ± 0.03 mins) (Fig 2.1(a)) BV.HCl, and the *L. bergylta* plasma chromophore solution (8.27 ± 0.09 mins) (Fig 2.1(b)) were all in good agreement with the expected elution time for BV as reported by Chen et al. (2012), and lends additional support to BV as the candidate pigment with the difference in the elution time potentially a reflection of the difference between the BV.HCl and the plasma BV. The area percent of the 100 µg.ml^{-1} (90.10 ± 2.71%) BV.HCl peaks was greater than that of the *L. bergylta* plasma chromophore (27.35 ± 1.45%). From this; and in considering the previous issues with the silicic acid chromatography, it was determined that the plasma purification method in the previous step was largely ineffective as demonstrated by the minimal peak of the plasma sample compared to the BV.HCl standards. This was indicative that the majority of BV was still bound to the protein after solvent purification by the described methods. Similarly, the additional fragments of molecules after 11 mins in the purified plasma suggested denaturation of the proteins during processing and ineffective purification. This once again suggested species specific differences in the mode of binding between the chromophore and the protein moiety, but as it could also symptomatic of denaturation and interactions between unfolded proteins in the solution (Bennion & Daggett, 2003) therefore Thin Layer

Chromatography (TLC) was identified as a relatively simple means of developing a more efficient and effective solvent phase for extracting BV from *L. bergylta* plasma.

4.3 Chromophore extraction and analysis III

From the work of Mudge & Davenport (1986), the assay used 250 µl 10% 3N MeOH.HCl added to each 1 ml of *L. bergylta* plasma to cleave the chromophore from the associated protein. This solution was agitated by vortex mixing and then centrifuged (1789 rcf, 2 mins). From this, the supernatant was decanted and 250 µl $CHCl_3$ added to extract the chromophore. The resultant mixture was again agitated, and centrifuged (1789 rcf, 2 mins) with the intention of retaining the pigment in the MeOH fraction in the bottom phase based on the relative molecular weights of the carrier solvent.

The original method resulted in a three phase separation (Fig. 2.2(a)) with the chromogen in the top layer, a thick light-blue layer of proteinaceous appearance at the interface, and a clear lower phase. The acidification phase was extended to 1 hour and an identical protocol followed which resulted in a 4 phase separation (Fig. 2.2(b) with the chromogen in the top layer, a biphasic protein layer between the solvent layers with a blue BV like top layer and a white bottom layer, the lower phase was clear.

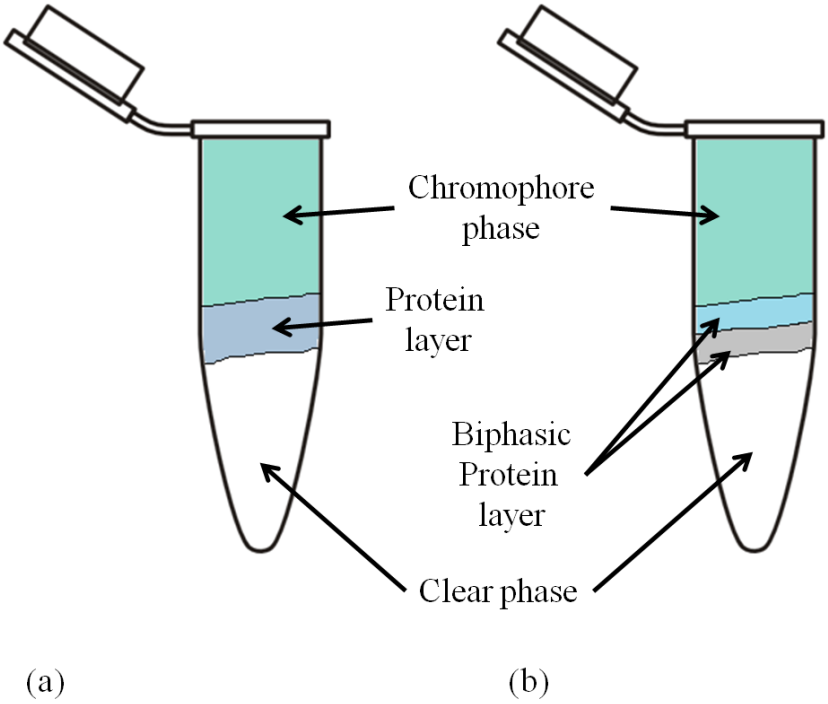


Figure 2.2: Illustration of solvent extraction methods showing **(a)** 3 phase separation using Mudge & Davenport (1986) method, and **(b)** 4 phase separation developed from Mudge & Davenport (1986).

Although this was somewhat puzzling given that the chromophore was expected to be associated with the bottom layer (Mudge & Davenport, 1986), but the rigidity of the denatured protein layer after centrifugation and the pigment leaching into the proteinaceous layer would suggest a physical barrier which prevented the pigment from crossing the central protein plug with the solvent. From this, notwithstanding the low recovery efficacy which would preclude using this extraction method for quantitative analysis, the pigment in the top layer was sufficiently purified to continue with much of the qualitative work hereafter.

4.4 Thin Layer Chromatography

All Thin layer Chromatography (TLC) used Merck (www.merck.com), Silica Gel-60 F_{254} plates with the origin line 1 cm from the bottom. *L. bergylta* plasma was derived from a

pooled source to minimise any intraspecific variation. Chambers were equilibrated for 10 mins with mobile phase at ambient temperature prior to exposure.

4.4.1 Retardation factor and Relative Retardation Factor

Retardation factor (R_f) as a measure of relative mobilisation (Bouwer, 1991) was calculated as: R_f = Migration distance of sample/ Migration distance of solvent front

Relative Retardation factor (RR_f) as a mobilisation metric (Blankinship & Hart, 2012) was then calculated as: RRf = (((Rf BV.HCl) (Rf_{BV})) / ((Rf (Plasma) (RfP))) * 100

4.5 TLC trial assay: biliverdin hydrochloride (standard)

The first experiment intended to establish loading volumes for the BV.HCl standard solution using a $CHCl_2$: MeOH (2:1 v/v) mobile phase with a 50 µl loading volume (Table 2.1).

Table 2.1: Trial TLC of biliverdin hydrochloride standard showing Retardation factor (R_f).

BV.HCl in MeOH (mg.ml)	Outcome	R_f
1	Streaked	0.93
0.1	Streaked	0.92
0.05	Streaked	0.97
0.025	Positive: Separated as single spot	0.96

From this, the BV.HCl standard showed good agreement across the loading volumes as described by the similarity of the R_f values, but only the 0.025 $mg.ml^{-1}$ sample demonstrated reasonable separation and a resolved product.

4.6 TLC trial assay: *Labrus bergylta* plasma loading medium

Following this, the next stage was to determine a loading medium and test the loading volume for the *L. bergylta* plasma under the same conditions.

Table 2.2: Trial TLC of *Labrus bergylta* plasma showing Retardation factor (R_f).

Loading medium (v/v)	Outcome	Rf
Crude plasma	Negative: no movement	0.0
1:10 v/v $CHCl_3$: MeOH (1:1)	Negative: denatured	N/A
1:10 v/v $CHCl_3$: MeOH (2:1)	Negative: no movement	0.0
1:20 v/v $CHCl_3$: MeOH (2:1)	Negative: no movement	0.0
1:10 v/v MeOH: H_2O	Negative: no movement	0.0

It was noted that the solvent phase had ran around the origin spots for the *L. bergylta* plasma solutions (Table 2.2), which suggested either polymerisation of the plasma as it dried on the plate or moisture being retained in the sample. The experiment was therefore repeated with the plates desiccated overnight under vacuum to minimize oxidation. The plate was developed the following day with identical results (data not shown), suggesting that the plasma would need a purification stage to minimise any potential cross linking in the denatured protein fraction, and the polarity of the mobile phase would need further development. The desiccation stage was included for all subsequent assays.

4.7 TLC Trial assay: alternative mobile phase systems

Table 2.3 summarises mobile phases derived from similar studies. Pooled *L. bergylta* plasma was purified using Extraction protocol III (Chapter 2; Section 4.3), and 75 µl of the

extract was spotted onto origins along with a BV.HCl standard and all plates dried overnight. In this case the solvent systems were applied dropwise.

Table 2.3: Trial mobile systems for TLC analysis of *Labrus bergylta* plasma.

Reference	Compound	Ratio (v/v)	Outcome
(Austin & Jessing, 1994)	EtOH: MeOH: H_2O	2:3:1	Negative
	EtOH: MeOH: H_2O + HCl	2:3:1	Negative
(Ding & Xu, 2002)	$CHCl_3$: MeOH: $C_2H_4O_2$	94:5:1	~Positive
(Fang, 1990)	C_4H_9OH: EtOH: H_2O	3:1:1.5	Negative
(Sakai, 1989)	$CHCl_3$: MeOH: H_2O	65: 35: 8	Negative

With the exception of the method applied by Ding & Xu (2002), none of the solvent systems had demonstrated any movement (Table 2.3). It was noted that this was the only system which contained CH_3COOH therefore similar systems became the focus of research. This identified the $C_4H_8O_2$: C_4H_9OH: CH_3COOH: H_2O (EBAW) mobile phase developed for use in the food industry (Zweig, 1978). The next series of experiments then tested this, and variants thereof to assess the efficacy of the solvent phase and optimise the polarity.

4.8 TLC trial assay: mobile phase development

L. bergylta plasma was purified as Method III (Chapter 2; Section 4.3) and 75 µl spotted along a 1 cm origin. $C_4H_8O_2$: C_4H_9OH: CH_3COOH: H_2O (EBAW) variants (Table 2.4) were developed by reducing the aqueous phase ($EBAW_1$ - $EBAW_3$), and by increasing the polarity ($EBAW_4$ – $EBAW_7$). Plates were desiccated under vacuum overnight and the chamber was equilibrated with the mobile phase at ambient temperature prior to exposing the plate. Upon completion, the plates were developed using a saturated environment of Iodine vapour.

Table 2.4: Trial assay of EBAW solvent system for application as mobile phase in *Labrus bergylta* plasma TLC showing Pigment Mobilisation (P_M), Solvent Front (S_F) and Retardation factor (Rf).

Variant	Ratio (v/v)	P_M (cm)	S_F (cm)	R_f	Notes
$EBAW_1$	75:10:5:10	N/A	18.9	N/A	Immiscible
$EBAW_2$	80: 10: 5: 5	N/A	18.9	N/A	No movement
$EBAW_3$	85: 5: 5: 5	N/A	18.9	N/A	No movement
$EBAW_4$	80: 10: 10: 0	17.2	18.9	0.92	Separated/Streaked
$EBAW_5$	80: 10: 10: 5	18.1	18.3	0.99	Separated/Streaked
$EBAW_6$	80: 10: 15: 0	18.2	18.3	0.99	Separated/Streaked
$EBAW_7$	80: 10: 20: 0	18.1	18.3	0.99	Separated/Streaked

From Table 2.4, $EBAW_1$ failed to form a solution. Reduced H_2O content relative to the other mobile phase components in $EBAW_2$ and $EBAW_3$ failed to move the sample from the origin suggesting that the partially purified BV product was water insoluble. There was reasonable movement in the $EBAW_4$ variant which increased CH_3COOH and omitted H_2O but the sample failed to separate (streaked). $EBAW_5$ used the same solvent levels as $EBAW_4$ but reinstated the H_2O with similar effect to $EBAW_4$ but the high R_f was indicative that the solution was too polar. Similarly, $EBAW_6$ and $EBAW_7$ demonstrated that increasing the CH_3COOH relative to the other solvents made the mobile phase too polar. From this, $EBAW_4$ was determined as most promising but as the chromatograph had failed to resolve this suggested that the purification method and loading medium could be modified further.

4.9 TLC assay: final method

As a final modification, the *L. bergylta* plasma protein content was simplified by Ammonium Sulphate ($AmSO_4$) fractionation (Scopes, 1994). For each 1 ml of pooled plasma,

0.056 g.ml^{-1} and 0.084 g.ml^{-1} was added with agitation over 20 mins and then mixed for another 10 mins to achieve 10% and 15% saturated solutions respectively. Samples were centrifuged (11180 rcf, 15 mins) to pelletise the precipitate. 800 µl and 600 µl supernatant was recovered at 10% and 15% respectively. The loading volumes were increased to 75 µl over the 1 cm origins and BV.HCl in MeOH included as a standard. Plates were desiccated under vacuum overnight to minimise oxidation. The chamber was equilibrated with the $EBAW_4$ mobile phase at ambient temperature for 10 mins before the plate was exposed.

Table 2.5: Thin layer chromatography of commercial biliverdin hydrochloride and plasma from *Labrus bergylta* using the $EBAW_4$ mobile phase variant.

Position	Sample	P_M (cm)	S_F (cm)	R_f
1	BV.HCl	18.8	19.4	0.97
2	10% saturated plasma	17.9	19.4	0.92
3	15% saturated plasma	18.1	19.4	0.93
4	BV.HCl	18.8	19.4	0.97

With reference to the data presented in Table 2.5; although the migration distance of the 10% saturated plasma was less than the 15% saturated sample, the Rf values were in good agreement with each other (RR_f = 98%). Furthermore, both the 10% (RR_f = 94%) and 15% (RR_f = 96%) were in agreement with the BV.HCl standard. From this, as the R_f of the plasma samples was comparable to those of the BV.HCl standard, and the results were similar to the findings of Fang & Bada (1988) regarding BV in *C. analis*, evidence from the TLC analysis under the conditions applied fully supported the identity of the plasma chromophore as biliverdin. The difference in R_f is presumed as a function of the relative change in polarity of the molecule due to the attached HCl moiety. Although this system works, and as R_f is

equivalent to Retention time, the next step would therefore ideally be HPLC with the $EBAW_4$ system but facilities were not available at the time.

5.0 Biliverdin quantification Method I

Initially, it was presumed that direct spectrophotometric analysis of the plasma pigment content based on a 650 nm diagnostic wavelength (Stocker et al., 1987) against a standard curve of BV would be an adequate means of quantification as demonstrated previously in *N. tetricus* (Gagnon, 2006).

Plasma from 8 individuals was pooled prior to analysis to provide a homogenous source, and centrifuged (11180 rcf, 2 mins) to eliminate residual cellular debris. The supernatant was recovered and aliquots stored at -20 °C when not in use. A 1 $mg.ml^{-1}$ reference solution was generated by dissolving 10 mg BV.HCl in 10 ml ddH_2O which was stored in darkness at 4 °C. The stock solution was then diluted to 0.02 $mg.ml^{-1}$ in 100mM potassium phosphate buffer which comprised 1 $mg.ml^{-1}$ Bovine Serum Albumin (BSA) and 1.8 mM nicotinamide adenine dinucleotide phosphate (NADPH) such that the final solution would approximate plasma. Pooled *L. bergylta* plasma was diluted (v/v) in ddH_2O prior to analysis. Samples were blanked against 100 mM potassium phosphate buffer which comprised 1 $mg.ml^{-1}$ BSA and 1.8 mM nicotinamide adenine dinucleotide phosphate (NADPH). Wavescans (350-750 nm) were recorded at 5 nm intervals.

Based upon preliminary analysis of the spectral signatures (Fig. 2.3(a)), as the 350 and 650 nm peaks of the standard solution and the pooled plasma were considered to be in good agreement, and with the exception of the 350 to 500 nm region, as the peaks were essentially superimposable at the diagnostic (650 nm) wavelength it was considered feasible to continue with this method by generating a standard curve of commercial BV.HCl to quantify the plasma fraction BV.

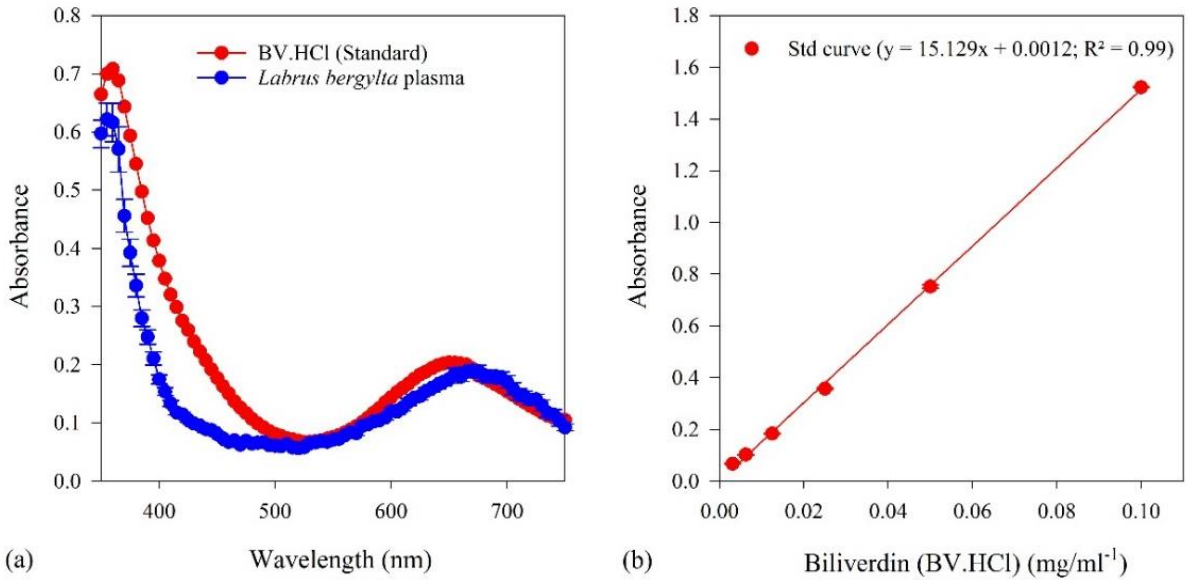


Figure 2.3: Initial biliverdin quantification method showing **(a)** Visible spectrum analysis (350-750 nm) of pooled *Labrus bergylta* plasma against a commercially obtained BV.HCl standard and **(b)** Standard curve of BV.HCl in physiological buffer.

In this case, the standard was based on 6 replicates, each of which was produced in triplicate daily. To generate the curve (Fig. 2.3(b)), 10 mg BV.HCL was dissolved in 10 ml ddH_2O to create a 1 mg.ml^{-1} pool which was then further diluted in 100mM potassium phosphate buffer which comprised 1 mg.ml^{-1} BSA and 1.8 mM NADPH to generate reference solutions (v/v 1:10, 1:20, 1:40, 1:80, 1:160 and 1: 320). As the curve was reproducible, and the coefficient of determination was strong (R^2 = 0.99), the equation (y = 15.129x + 0.0012) was considered adequate to interpolate the plasma BV content.

From this, the quantification protocol was developed such that plasma BV was measured by absorption using direct analysis of the 650 nm peak relative to a blank which comprised 100mM potassium phosphate buffer, 1 mg.ml^{-1} BSA, and 1.8 mM NADPH. As the intention was to develop and optimise the assay, readings were taken in triplicate, in tandem using a 1 cm path length cuvette and a 96 well plate (250 µl^{-1}) format for comparison. All values were interpolated from the standard curve (Fig. 2.3(b)) regression equation.

The Bergen samples were selected for preliminary analysis. However, when the Abs_{650} data was converted to absolute BV, discrepancies were noted between the two methods in that the 1 cm path-length assay consistently demonstrated higher estimations than the 96 well plate methods. Moreover, BV concentrations were considered low, and visible differences in the plasma hue between aliquots were not reflected in the magnitude or variation of the data. This suggested two factors of concern, initially the disparity between the methods suggested that there was an interaction between distance and transmission properties which would indicate there was interference from one or more of the plasma constituents, and that there is potentially large amounts of the pigment lost during transfer processes as the two methods used the same material for each individual therefore heat, light and O_2 exposure were unavoidable. Thus, it was considered prudent to test the behaviour of *L. bergylta* plasma under serial dilution, and to examine individual specific wavescans for any unaccounted variation.

To test the linearity by extended solution, *L. bergylta* plasma was serially diluted in potassium phosphate buffer (by factors of 1, 2, 4, 5, 10 and 20) and the Abs_{650} recorded in triplicate. Further to this, aliquots from five independent individuals were randomly selected from the population, centrifuged to remove any residual cellular debris then diluted (v/v 1:1) in potassium phosphate buffer. A 1 $mg.ml^{-1}$ reference solution was generated by dissolving 10 mg BV.HCl in 10 ml ddH_2O which was stored in darkness at 4 °C. The stock solution was then diluted to 0.02 $mg.ml^{-1}$ in 100mM potassium phosphate buffer which comprised 1 $mg.ml^{-1}$ BSA and 1.8 mM NADPH. Samples were blanked against 100mM potassium phosphate buffer which comprised 1 $mg.ml^{-1}$ BSA and 1.8 mM NADPH. Wavescans (350 -750 nm) were recorded at 5 nm intervals.

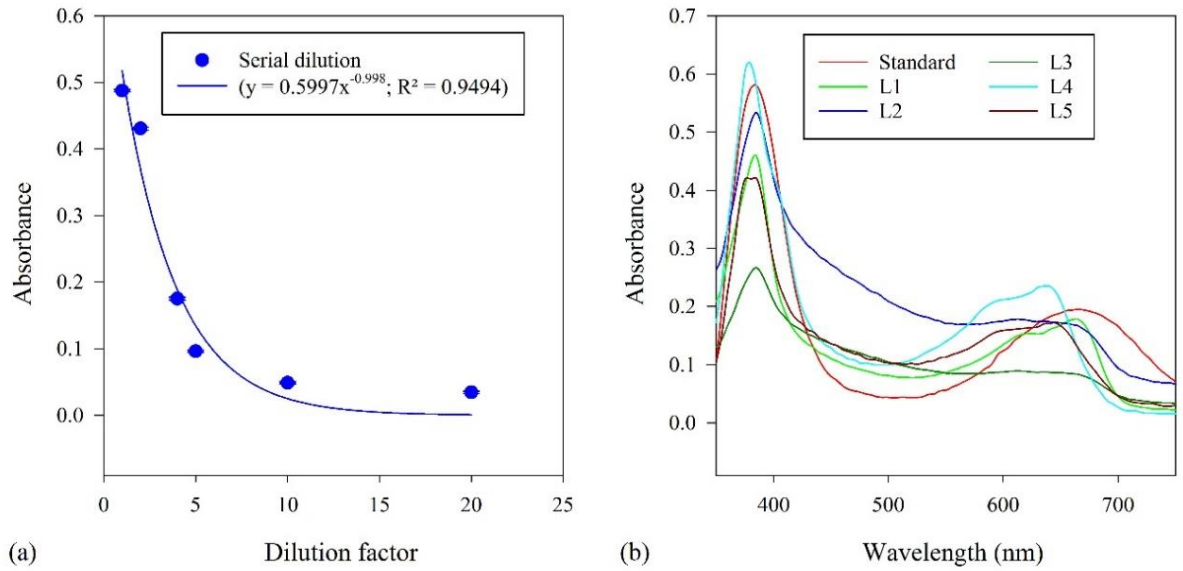


Figure 2.4: Exploration of the physical properties of *Labrus bergylta* plasma by **(a)** Serial dilution monitored at Abs_{650}, and **(b)** Comparative spectral analysis of commercially obtained BV.HCl with variation in *Labrus bergylta* plasma.

In contrast to the BV.HCl standard curve (Fig. 2.3(b)), serial dilution of *L. bergylta* plasma (Fig. 2.4(a)) demonstrated a curvilinear trend of exponential decay over the range of dilutions ($y = 0.5997x^{-0.998}$; $R^2 = 0.9494$). With reference to the absorbance spectra (Fig. 2.4(b)), the (650 nm region) peak wavelength of the plasma samples L1 (nm), L2 (nm), L3 (nm), L4 (nm), and L5 (nm) were noted to vary relative to each other, and the reference wavelength (nm). The 450 – 600 nm regions of L2 and L3 were also remarkable as there was prominent fronting on the expected 650 nm region peak. Similarly, L1, L4, and L5 show shoulders in the region of the diagnostic peak. Consequently, as there were issues with comparing the plasma signatures to the BV.HCl standard curve due to a range of variables which potentially included protein interactions, intra-individual bathochromic shifts, and oxidative degradation (Plikaytis et al., 1994) leading to formation of heterogenous reaction products, direct spectrophotometric quantification was abandoned in favour of alternate means.

5.1 Biliverdin quantification Method II

As direct measurement of plasma BV had been noted to introduce inaccuracies as a result of protein interactions, and separation of the chromophore from the protein made the molecule highly labile to oxidative processes, established methods of pigment analysis were therefore precluded. This lead to the work of Gutteridge & Tickner (1978), which applied the highly specific reaction of BV with Barbituric acid ($C_4H_4N_2O_3$) (Fig. 2.5) to form characteristic red 9-H dipyrrinones and complementary 9-CHO dipyrrinone adducts with an absorbance peak at 570 nm under alkaline conditions (Austin & Jessing, 1994).

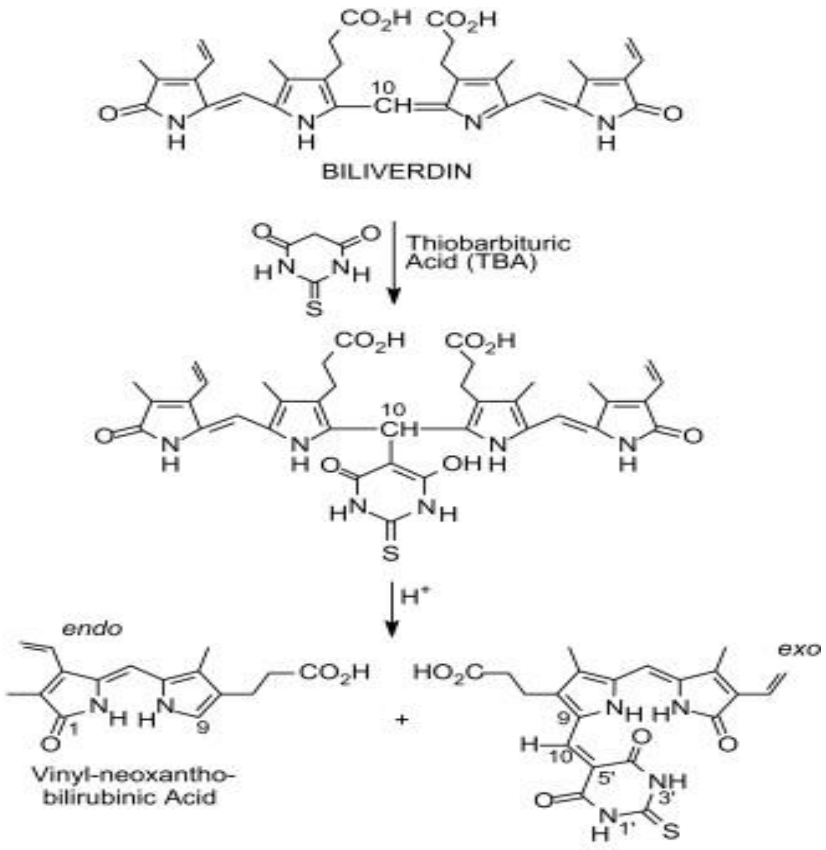


Figure 2.5: Barbituric acid cleavage of biliverdin into its component 9-H dipyrrinones and the complementary 9-CHO dipyrrinone thiobarbituric acid adducts.

5.2 Trial assay

The initial proof of concept assay was an adaptation from Austin & Jessing (1994). As a note, when the original paper was published BV was not commercially available therefore it was normal to generate biliverdin stock from BR. The initial step was to dissolve 0.6 mg

commercial BR in BSA base in 1780 µl 17.5 M glacial $C_2H_4O_2$, and 20 µl 4 mM Ferric Chloride ($FeCl_3$), with the resultant solution heated at 95 ºC for 2 hours to react, then cooled, and diluted to 20 ml with glacial $C_2H_4O_2$ to generate 50 µmol.l^{-1} stock. Moving on from this, as BR in BSA base was discontinued a number of years ago in favour of chemically pure BR due to advances in production methods, and BV.HCl was readily available, the protocol initially skipped the first stage such that BV.HCl was dissolved in 17.5 M glacial $C_2H_4O_2$ to generate 500 µmol.l^{-1} with serial dilution in 17.5 M glacial $C_2H_4O_2$ for standards ranging from 0-50 µmol.l^{-1}. Samples were heated at 95 ºC for 5 mins in the dark then cooled and 2.5ml n-Butanol (C_4H_9OH) with 1 ml 10 M NaOH added then agitated in the dark until the reaction was complete. A two phase solution formed after centrifugation (1789 rcf, 5mins) with the diagnostic red chromophore expected in the lower component.

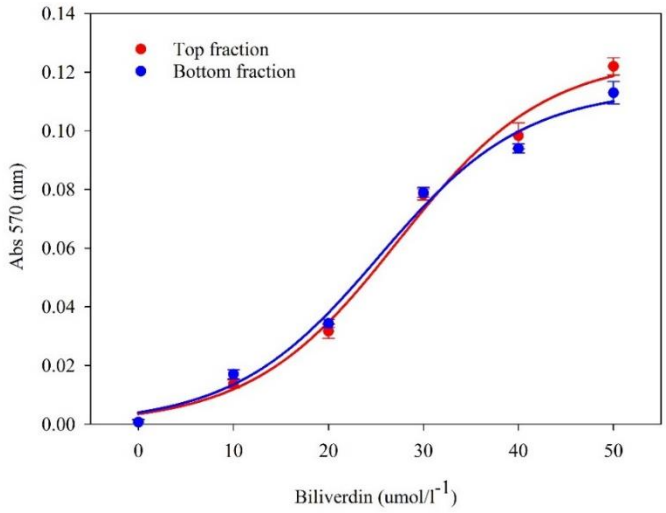


Figure 2.6: Standard curve generated by the barbituric acid method for quantification of *Labrus bergylta* plasma biliverdin.

With reference to the trial standard curve (Fig. 2.6), it was determined that as the red chromophore product and protein aggregates were present in both separation phases, the reaction products demonstrated second order polynomial curvilinear distributions, and that the two signatures were super-imposable suggested that the reaction was incomplete, or that some of the reagents were absent or imbalanced due to stokes shift altering sedimentation rate (i.e.

small concentrations/high concentrations behave similarly within integers but differently across the levels leading to the sigmoidal distribution).

5.3 Trial assay II

Based upon the previous experiment, all other aspects of the next assay were identical but the reaction time after the cooling stage was extended to test the specific issue.

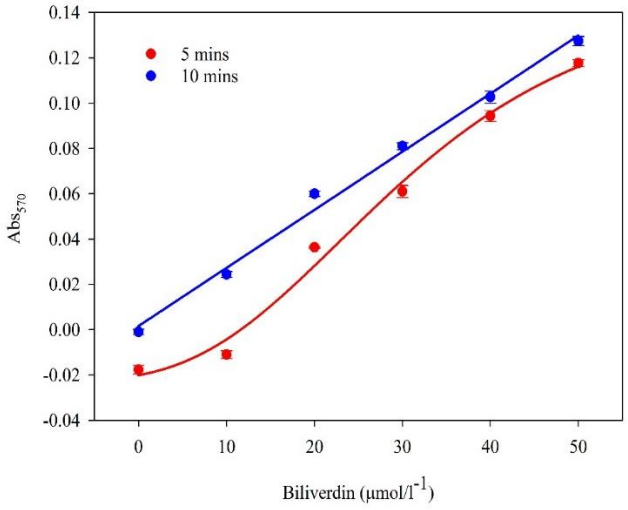


Figure 2.7: Biliverdin hydrochloride standard curve generated by the barbituric acid method for quantification of *Labrus bergylta* plasma biliverdin with 5 mins reaction time (red), and 10 mins reaction time (blue).

With reference to Fig. 2.7, the 5 mins assay was noted to have retained the curvilinear aspect observed in the initial experiment as demonstrated by the reduction of the Coefficient of determination score (R^2 = 0.9988 vs. 0.9838) when the data is described as a 4th order polynomial (y = -1E-09x4 - 8E-07x3 + 8E-05x2 + 0.0004x - 0.0002) compared to a straight line (y = 0.0025x - 0.0086). In addition, as processing determines centrifugal sedimentation rate via product solubility and density, this alters the ability of diagnostic chromogens to cross the interface causing pigment to occur in both phases and potentially contributing to observed trends (Manitto and Monti, 1980). Contrastingly, 10 mins reaction time produced a linear standard curve (y = 0.0026x + 0.0016) with a good Coefficient of determination score (R^2 =

0.9931). From this, and based on the results, there is a strong correlation between the biliverdin level, and Abs_{570} (Pearson's correlation coefficient $r = 0.9965$; $p < 0.05$). The time series was further extended to include 15 and 20 mins but the samples had denatured. From this, as the agitation stage was essentially a shift in pH to the desired alkaline conditions for generating the diagnostic chromogen, the optimal time was determined as 10 mins.

5.4 Assay to determine peak wavelength of reaction products

Following on from altering the reaction time, and based on previous experience, if the assay was indeed dependent on the pH shift, it was considered prudent to check if the peak (570 nm) wavelength had shifted due to the change in assay conditions (Helliwell & Helliwell, 2008). The 0 and 50 $\mu mol.l^{-1}$ samples were retained from the previous assay. Absorbance spectra from 350-750 nm were then recorded at 5 nm intervals in triplicate for the 50 $\mu mol.l^{-1}$ sample blanked against the 0 $\mu mol.l^{-1}$.

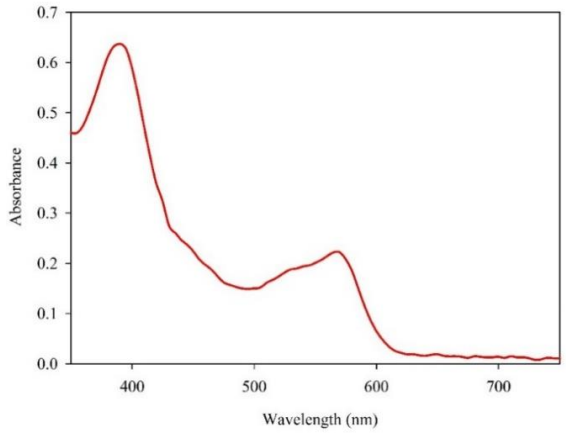


Figure 2.8: Confirmation of the cleavage of biliverdin to component 9-H dipyrrinones and the complementary 9-CHO dipyrrinone thiobarbituric acid adducts by wavescan (350-750 nm).

The wavescan (Fig. 2.8) had a major peak at 400 nm with a sharp secondary peak at 570 nm. This was in good agreement with Gutteridge & Tickner (1978), and confirmed that the experimental conditions were identical to those described by Austin & Jessing (1994).

5.5 Assay to determine the effect of BSA content in the assay

Further consideration of the original method also noted that the BR converted to BV was in a BSA base solution, and that it was carried through into the assay. As the precursor for the assay herein was BV.HCl there was no equivalent protein content in the assay. Research identified that no data was available on the BR in BSA solution, but based on a physiological level of 3.5-5.5% in humans (Busher, 1990) 0.1%, 0.25% and 0.5% were used as a starting point. A 10% stock solution of BSA in 100mM potassium phosphate buffer was generated by adding 10 g of crystalline BSA (Fraction 5) to the surface of 100 ml of buffer which had been pre-heated to 37 °C. The mixture was then gently stirred to prevent foaming by denaturation until the BSA was fully dissolved. Aliquots of 1 ml 10% BSA were then sealed and stored at -20 °C. The BSA was then added to the assay mixture as part of the aqueous addition such that the final levels in solution were 0.1, 0.2, and 0.5 %. The practicalities of the remaining protocol were then identical to the modified (Section 5.3) version of the original (Section 5.1) method.

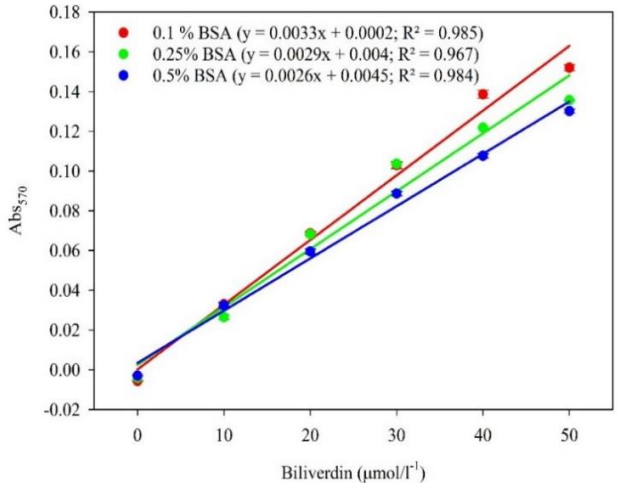


Figure 2.9: Analysis of variance in bovine serum albumin levels added to the aqueous phase.

With reference to the effect of BSA in the assay mix (Fig. 2.9), although the fitted regression lines are shown, the 0.1 and 0.25 % levels were noted to have a curvilinear aspect at the higher integers whereas the 0.5% level regression showed a superior regression fit and was therefore taken forward to be tested for reproducibility.

5.6 Reproducibility

BV.HCl was dissolved in 17.5 M glacial $C_2H_4O_2$ to generate 500 µmol.l^{-1} with serial dilution in 17.5 M glacial $C_2H_4O_2$ for standards ranging from 0-50 µmol.l^{-1} with 0.5% BSA. 500 µl distilled H_2O was added to 500 µl of each standard with 400 µl 40 mM Ascorbic acid ($C_6H_8O_6$) and 100 µl 200 mM $C_2H_4O_2$ in 1 M NaOH. Serum samples were prepared by addition of 450 µl glacial $C_2H_4O_2$ to 50 µl plasma, 500 µl distilled H_2O was added with 400 µl of 40 mM C6H8O6 and 100 µl 200 mM $C_4H_4N_2O_3$ in 1 M NaOH. Blanks were parallel samples with barbituric acid substituted with 1 M NaOH. Samples were heated at 95 ºC for 5 mins in darkness then cooled and 2.5ml C_4H_9OH with 1 ml 10 M NaOH added then agitated in the dark until the reaction was complete. A two phase solution formed after centrifugation (1789 rcf, 5mins) with the diagnostic red chromophore in the lower component. The top phase was discarded and A_{570} of the lower phase recorded in triplicate.

5.6.1: Validation

Tests of parallelism were based on the Bradley-Blackwood test (García-Pérez, 2013) which is derived from the observation that simultaneous testing for equality of the of the means and variances of X and Y if X and Y have a bivariate normal distribution is equivalent to simultaneous testing of:

Standard curve Residual SS = $\sum Y^2 - (\sum XY)^2 / \sum X^2$

and

Comparative curve Regression SS = $\sum Y^2 - (\sum XY)^2 / \sum X^2$

With the gradient of both calculated as $b = \sum XY / \sum X^2$ and Degrees of freedom as $D_f = n-1$

Covariance was then determined as:

($s^2_{y.x}$) p= (Residual SS + Regression SS) / (Residual D_f +Regression D_f)

And the pooled standard error of the slope calculated as:

$s_{b1-b2} = \sqrt{((s^2_{y.x})p / Res \sum X^2 + (s^2_{y.x})p / Reg \sum X^2)}$

Comparison of the slopes was calculated as:

$t = (b1 - b2) / s_{b1-b2}$

Then, parallelism or lack thereof was determined by comparison of the derived t value with Students t table using the null hypothesis of $b_1 = b_2$ (García-Pérez, 2013).

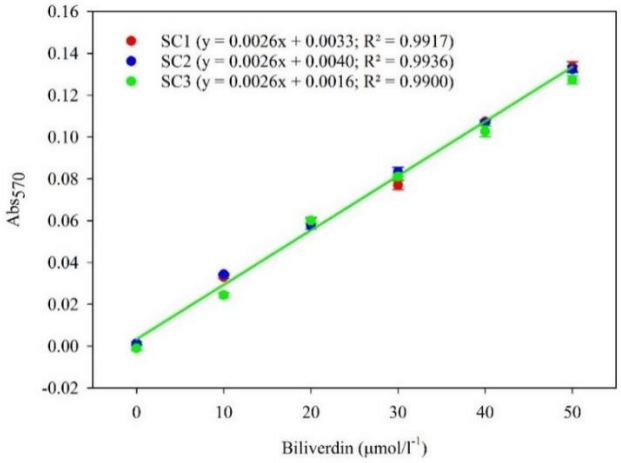


Figure 2.10: Initial test reproducibility of the barbituric acid method using commercial BV.HCl. SC1-SC3 represents replicates of the assay.

Although the standard curves (Fig. 2.10) were visibly superimposable, and demonstrated identical gradient coefficients (0.0026), they were tested for inter-assay repeatability (Lee et al., 2006). SC1 and SC2 were parallel ($t(34) = 0.083$, $p = 0.5342$). The two confirmed replicates were then pooled and used as a model to substantiate parallelism in SC3 ($t(34) = -0.1$, $p = 0.4605$).

5.7 Assay to test *Labrus bergylta* plasma behaviour under barbituric acid reaction

Plasma was pooled and homogenised prior to commencing the assay. Plasma samples were prepared by addition of 487.5 µl, 475 µl, 450 µl, 400 µl and 350 µl glacial $C_2H_4O_2$ to 12.5 µl, 25 µl, 50 µl, 100 µl and 150 µl plasma respectively. 500 µl distilled H_2O was added with 400 µl of 40 mM $C_6H_8O_6$and 100 µl 200 mM barbituric acid in 1 M NaOH. Blanks were

parallel samples with $C_4H_4N_2O_3$ substituted with 1 M NaOH. Samples were heated at 95 ºC for 5 mins in the dark then cooled and 2.5ml C_4H_9OH with 1 ml 10 M NaOH added then agitated in the dark until the reaction was complete. A two phase solution formed after centrifugation (1789 rcf, 5mins) with the diagnostic red chromophore in the lower component. The top phase was discarded and A_{570} of the lower phase recorded in triplicate, blanks were then subtracted from the samples.

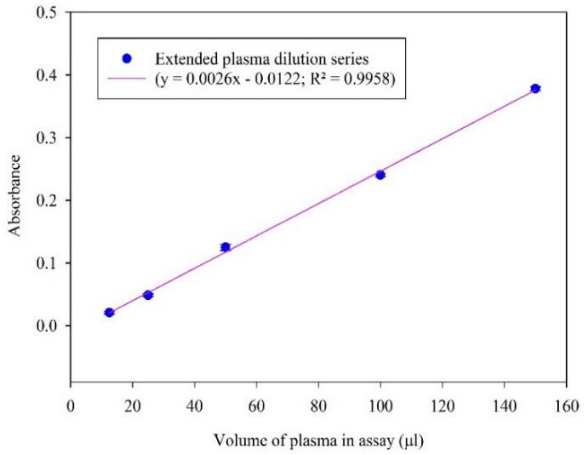


Figure 2.11: Extended plasma dilution series to assess linearity after processing with barbituric acid monitored at 570 nm.

The dilution series (Fig. 2.11) of *L. bergylta* plasma BV after barbituric fragmentation shows good linearity (R^2=0.9958). Further analysis of parallelism against the pooled standard curve revealed no significant difference in between the gradient coefficients (t (124) = -0.1, p = 0.5998), and confirmed that plasma BV can be extrapolated from the standard curve.

5.8: Spike and recovery

Plasma was obtained from Horse (*Equus ferus caballus*), Humans (*Homo sapiens*), Mice (*Mus musculus*), Cod (*Gadus morhua*), and Turbot (*Scophthalmus maximus*) for comparison. Similarly to other assays, *L. bergylta* plasma was selected randomly, pooled and homogenised prior to the assay. All variants were then treated identically. A standard curve

prepared by the normal protocol (Section 5.6) was included for each plate. Plasma samples were prepared by addition of 450 µl glacial $C_2H_4O_2$ comprising 0, 10, 20, 30, 40, and 50 µmol/l^{-1} to 50 µl plasma, 500 µl distilled H_2O was added with 400 µl of 40 mM ascorbic acid and 100 µl 200 mM barbituric acid in 1 M NaOH. Blanks were parallel samples with barbituric acid substituted with 1 M NaOH. Samples were heated at 95 ºC for 5 mins in the dark then cooled and 2.5ml C_4H_9OH with 1 ml 10 M NaOH added then agitated in the dark until the reaction was complete. A two phase solution formed after centrifugation (1789 rcf, 5mins) with the diagnostic red chromophore in the lower component. The top phase was discarded and A_{570} of the lower phase recorded in triplicate, blanks were then subtracted from the samples. As *L. bergylta* plasma was known to have BV present, and to ensure commonality of the 0 µmol/l^{-1} across the species for later comparison, the value obtained for non-spiked plasma was subtracted from the data across treatments within species.

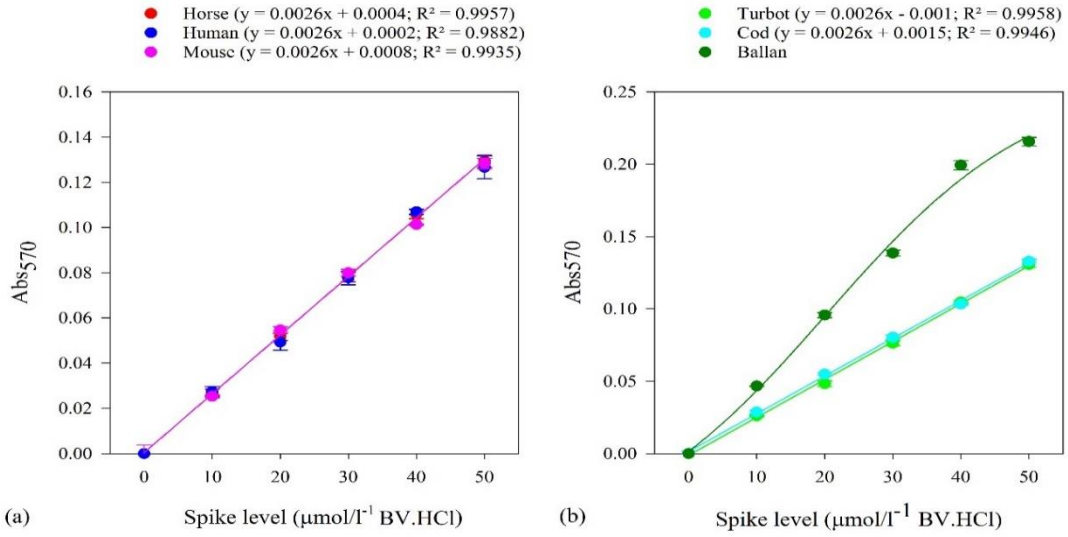


Figure 2.12: Spike and recovery of commercial BV.HCl from *Equus ferus caballus, Homo sapiens, Mus musculus, Gadus morhua, and Scophthalmus maximus.*

Based on visual comparison of the generated curves and the similarity of the gradient coefficients the spike and recovery were similar and the assumptions of normality were met. Further analysis revealed that the slopes of the mammals including *E. caballus* (t (70) = 0.9208, p = 0.8198), *H. sapiens* (t (70) = 1.454, p = 0.9248) and *M. musculus* (t (70) = 1.4797, p =

0.9283) (Fig 2.12(a)), and teleosts including *G. morhua* (t (88) = 0.013, p = 0.5052), and *S. maximus* (t (88) = 1.4277, p = 0.9211) (Fig. 2.12(b)) were not significantly different to the standard curve. Contrastingly, the *L. bergylta* plasma slope coefficient was noted as a significant departure (t (70) = 5.6793, p<0.05) which was best described as a polynomial curve (y = -2E-06x3 + 0.0001x2 + 0.0028x + 0.0063; R^2 = 0.9942). Further to this (Fig. 2.12), the data is also described in terms of absolute biliverdin recovery (Fig. 2.13).

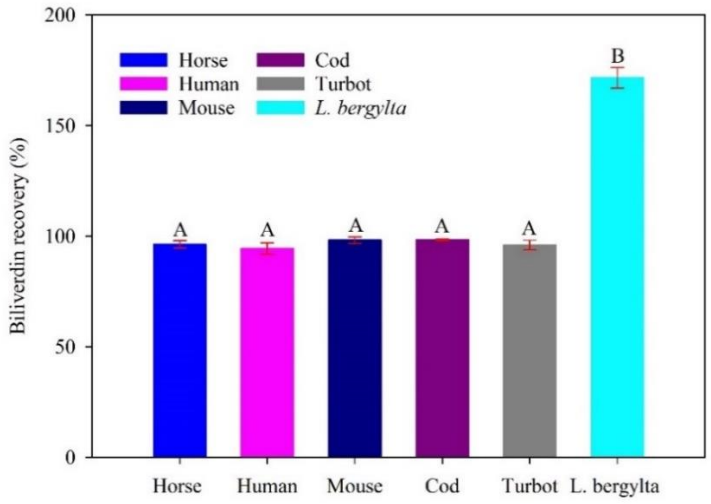


Figure 2.13: Mean biliverdin recovery from spiking assay including mammals (*Equus ferus caballus*, *Homo sapiens*, *Mus musculus*), teleosts (*Gadus morhua*, *Scophthalmus maximus*), and *Labrus bergylta*. Different superscript letters denote significant differences in mean levels.

The recovery results from the mammalian plasma including *E. caballus* (96.31 ± 1.68%), *H. sapiens* (94.46 ± 2.56%), *M. musculus* (98.24 ± 1.68%), and the teleosts *G. morhua* (98.39 ± 0.88%), and *S. maximus* (96.02 ± 2.20%) were all found comparable whilst the BV recovery of *L. bergylta* (171.57 ± 4.7%) was significantly greater (Kruskall-Wallace H (5) = 15.61, p<0.05). With reference to the consistency of the (non *L. bergylta*) spike and recovery assays, the comparability to the standard curve and linearity of the end-product demonstrated good precision and relative accuracy as defined by Lee et al. (2006), and indicated the method was robust, but the amplified recovery in *L. bergylta* was cryptic. As the trend was repeated

across assays (n=3), it suggested that there was potentially interference from an endogenous source but as the plasma is a relatively heterogeneous medium it was difficult to draw any conclusions with the exception that *L. bergylta* plasma behaves differently to the non-HBV species. This is discussed further in Chapter 6.

6.0 Final biliverdin quantification protocol

6.1 General statement

All chemicals were purchased in bulk such that the main reference materials and reagents were derived from a single source. All storage and subsequent application were conducted according to guidelines to prevent deterioration and minimise inter-assay variation. Similarly, collection, storage and processing was always consistent for the *L. bergylta* plasma analyte. Absorbance was recorded using a Biotek Synergy HT Multimode Microplate Reader (www.biotek.com) set at 20 °C.

6.2 Standardised plate design

Wells were allocated to samples and reference solutions using an identical layout at all times (Fig. 2.14), with Sarstedt 96 well tissue culture plates (Type F) (www.sarstedt.com) used throughout.

LANE	1	2	3	4	5	6	7	8	9	10	11	12
A	Line Std			Std 0	Std 10	Std 20	Std 30	Std 40	Std 50			Line Std
B				Std 0	Std 10	Std 20	Std 30	Std 40	Std 50			
C				Std 0	Std 10	Std 20	Std 30	Std 40	Std 50			
D												
E	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
F	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
G	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12
H	Line Std											Line Std

Figure 2.14: Standardised 96 well plate format showing BV.HCl standard levels (numbers

denote (µmol/l^{-1} starting material), L1-L12 were individual plasma samples, and Line standards.

6.3 Line standard

A line standard to monitor inter assay variation was established by mixing 10 mg crystalline BV.HCL with 10 ml of MeOH, then by diluting the resultant solution by a factor of 2 in MeOH to produce a 0.5 mg/ml solution with an Abs_{666} of 0.085. This reading was then used as a reference across all assays and between batches of standard. Line standards were recorded in quadruplicate. From this, analysis determined that the intra assay coefficient of variation was 2.1%, and the inter-assay coefficient of variation was 1.52% ± 0.002.

6.4 Precision and Limits of detection

Based on the variability of the CV data (Section 6.3), the precision and accuracy of the assay were acceptable (Tiwari & Tiwari, 2010). The Limit of Detection (LOD) was determined by serial dilution of the commercial BV.HCl standard solution in MeOH to produce a linear range of 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 5.0 µmol.l^{-1}. The assay was conducted as Section 5.6. The extended series and the normal calibration series were then repeated on three consecutive days in accordance with Berlec & Strukelj (2014), LOD was 0.2 µmol.l^{-1}.

6.5 BV.HCl standard curve preparation.

BV.HCl was dissolved in 17.5 M glacial $C_2H_4O_2$ to generate 500 µmol.l^{-1} with serial dilution in 17.5 M glacial $C_2H_4O_2$ for standards ranging from 0-50 µmol.l^{-1} with 0.5% BSA. 500 µl distilled H_2O was added to 500 µl of each standard with 400 µl 40 mM $C_6H_8O_6$ and 100 µl 200 mM $C_2H_4O_2$ in 1 M NaOH. This was carried out in triplicate on each plate (Section 6.2) across all assays (n=40) and the data pooled to produce the working standard curve (Fig. 2.15). From this, and as the pooled standard curve demonstrated good linearity (R^2=0.983) the

equation of the straight line (y = 0.0026x + 0.0014) was used to interpolate the *L. bergylta* plasma BV values.

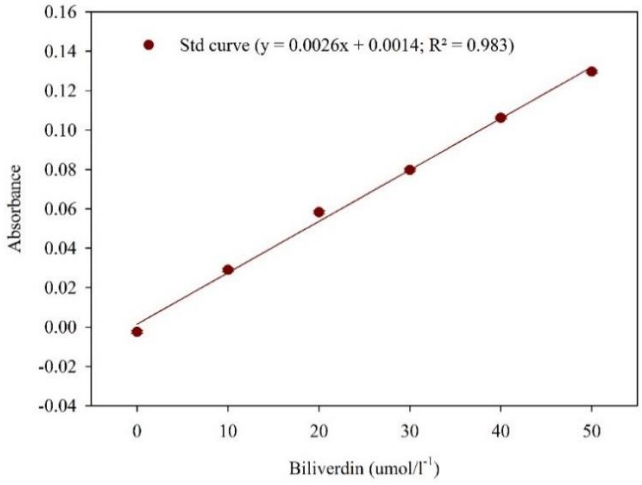


Figure 2.15: Representative standard curve of commercial BV.HCl processed by barbituric acid reaction and determined using Abs_{570} with the data presented derived as a cumulative mean calculated using the equation y = 0.0026x + 0.0014 (R^2 = 0.983) (n=40).

6.6 Plasma processing

Serum samples were prepared by addition of 450 µl glacial $C_2H_4O_2$ to 50 µl plasma, 500 µl distilled H_2O was added with 400 µl of 40 mM ascorbic acid and 100 µl 200 mM barbituric acid in 1 M NaOH. Blanks were parallel samples with barbituric acid substituted with 1 M NaOH. Samples were heated at 95 ºC for 5 mins in darkness then cooled and 2.5ml C_4H_9OH with 1 ml 10 M NaOH added then agitated in the dark until the reaction was complete. A two phase solution formed after centrifugation (1789 rcf, 5mins) with the diagnostic red chromophore in the lower component. The top phase was discarded and A_{570} of the lower phase recorded in triplicate, blanks were then subtracted from the samples. Variability in the reference solutions were accommodated by constructing a cumulative dataset each day then adjusting the data by use of a fully inclusive standard curve at completion.

7.0 Protein quantification

Bradford method (Bradford, 1976) was used to assess protein content. Briefly, the reagent was prepared by dilution of 0.06% w/v Coomassie G250 (Sigma-Aldrich, USA) in 1.9% perchloric acid and Abs_{465} adjusted to 1.3 - 1.5 with 1.9% perchloric acid. Protein (BSA) standards were prepared by dilution of a 10 $mg.ml^{-1}$ solution in PPB (pH 7.4) to generate 0, 5, 10, 15, 20 and 25 µg of BSA in 25µl of solution. Triplicates (25 µl) of each standard level were used to generate standard curves (Fig. 2.16).

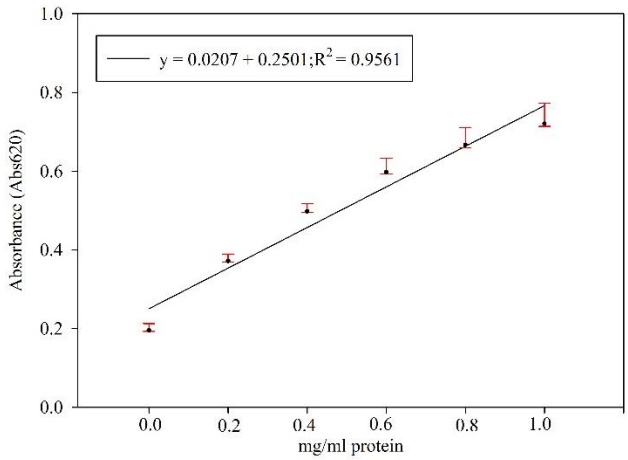


Figure 2.16: Bradford method standard curve (n=51) with standard protein content (mg/ml^{-1}) estimated from Abs_{620} and plasma protein content interpolated using the straight line equation $y = 0.0207x + 0.2501$ ($R^2 = 0.9561$).

For each saturation integer, and for both methods, 25 µl of plasma was transferred to a 96 well plate in triplicate. 225 µl Bradford reagent was introduced with agitation and allowed to react for 3 mins prior to measuring transmission properties.

8.0 Native biliverdin generation

The method for generating native BV (Chapter 3) was based on the methods of Austin & Jessing (1994). Briefly, 0.6 mg commercial crystalline BR was dissolved in 1780 µl 17.5 M glacial $C_2H_4O_2$, 200 µl 5% BSA solution and 20 µl 4 mM $FeCl_3$. The solution was heated at 95 ºC for 2 hours, cooled, and diluted to 20 ml with glacial $C_2H_4O_2$. The resultant native biliverdin

solution was centrifuged (7155 rcf, 2mins) and decanted to sealed flasks for storage (4 ºC). The end product (BRBV) was then examined by a comparative (350-750 nm) wavescan against commercial BV.HCl (Fig. 2.17)

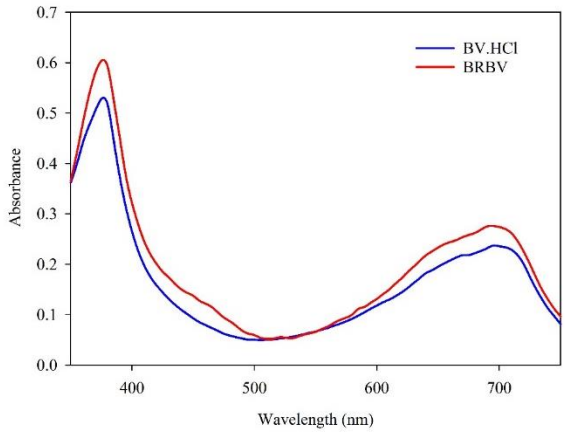


Figure 2.17: Comparative absorption spectra signatures of commercially obtained biliverdin hydrochloride (blue) with native biliverdin (red) generated from bilirubin oxidation.

9.0 Ionic fractionation

Ammonium sulphate ($AmSO_4$) ionic fractionation (Chapter 4) was determined the best method to test the relationship between BV and the proposed carrier protein (Chapter 4) as the non-chaotropic nature would minimise disruption. Plasma from the original survey (n=397) (Clark et al., 2016) of *L. bergylta* was randomly selected, then pooled to create a homogenous source of adequate volume. This pool was centrifuged to remove post freeze-thaw cellular debris then 5% integers of $AmSO_4$ were used to generate the precipitation gradient.

9.1 $AmSO_4$ calculations

$AmSO_4$ weights were determined for each increment using the following equation developed (Scopes, 1994):

Mass of $AmSO_4$ G = Sat(M2 - M1) / (SatM - (SpecV / 1000 * 132.14 * SatM * M2)).

Where G was the mass of $AmSO_4$ which would be added to 1 litre to reach a given increment, Sat was $AmSO_4$ mass in a saturated solution ($g.l^{-1}$), M_1 was starting molarity, M_2 was desired molarity, SatM was molarity of saturated $AmSO_4$ solution, SpecV was specific volume displaced by 1 g of $AmSO_4$ in an aqueous solution (0.54 ml), and 132.14 corresponds to $AmSO_4$ molecular weight. As Sat, SatM and SpecV all vary depending on temperature, the calculations and derived values were 20 ºC specific. Prior to the experiments, the laboratory, equipment, samples and reagents were acclimatised to 20 ºC. The salt was added gradually over 20 mins and agitated to minimise local solubility differentials. The resultant solutions were then further agitated for 1 hour to optimise precipitation. The Aliquots were then centrifuged (11180 rcf, 15 mins) to pelletise the precipitate, and the desired product recovered.

10.0 Assay buffers

Potassium phosphate buffer was premade in bulk and aliquots decanted to sterile flasks which were sealed and autoclaved prior to storage at 4°C.

10.1 1M Potassium Phosphate Buffer

Initially, 136.1 g of KH_2PO_4 was dissolved in 1 l^{-1} of ddH_2O to make a 1M solution of the monobasic precursor, and 174 g of K_2HPO_4 was dissolved in 1 l^{-1} of ddH_2O to make a 1M solution of the dibasic precursor. Following this, 200 ml of the dibasic precursor was mixed with 800 ml of the monobasic form to make a final 1M Potassium Phosphate Buffer solution of pH 7.4.

11.0 Biliverdin reductase sequence

The mRNA sequences for the two *D. rerio* biliverdin reductase genes, biliverdin reductase A (NM_001076601) and biliverdin reductase B (NM_001002686) were supplied to

O. Saele in the Norwegian National Institute of Nutrition and Seafood Research (NIFES). The sequence data was blast searched against a proprietary *L. Bergylta* mRNA sequence library not released in the public domain. The search returned three *L. Bergylta* specific mRNA sequences with high identity to the search sequences from zebrafish.

>Labrus_bergylta (BVRA1)

ATGTCAAAGGTCTGTTGTCAACAGTAAGAAGCGTAGCAGTCATTTTACTGTAGTTTCTATCAGAAT
GGTGGTCCATGGTGGCTGCGGTAACTGACAGCTCACCAAACCACTCAACCAAACAGGTGAGGCGA
GCAATGCCAGAGAATGATGGGAAACCAGCTCCAGGTTTCAGAGGTCCACCAGTAAAATGAAGGGT
TCCTTCTTGCAGGACTTTGCCCTCTACCTCTCTCTTAAGAGCTTTGTAGTCTTCTGATAGCAGCTCG
ATGTGTTCCTCATGGAGAATCACTCCTGATGTTCTCTTCATGAAACGCATTCTCAAGTGCAGATGA
AAGCCACATGAATGTCGTCTCTGCTCAAAGCCTCTTGAATTGGGATCTGACATATCCCCTGCTGAG
GGTCCAGGCTTCTCCTGGATATGAATCCTCTGA

> Labrus_bergylta (BVRA2)

ATGTCAAAGGTCTGTTGTCAACAGTAAGAAGCGTAGCAGTCATTTTACTGTAGTTTCTATCAGAAT
GGTGGTCCATGGTGGCTGCGGTAACTGACAGCTCACCAAACCACTCAACCAAACAGGTGAGGCGA
GCAATGCCAGAGAATGATGGGAAACCAGCTCCAGGTTTCAGAGGTCCACCAGTAAAATGAAGGGT
TCCTTCTTGCAGGACTTTGCCCTCTACCTCTCTCTTAAGAGCTTTGTAGTCTTCTGATAGCAGCTCG
ATGTGTTCCTCATGGAGAATCACTCCTGATGTTCTCTTCATGA

>Labrus_bergylta (BVRB)

ATGTGATTGTTATGTGACAGAAGAATCGTCTGAATCGCAACACCAATTTGACCACCAAGTTGTTCA
TGCACCGTGGATCCCGCTTCTTACAAATCATGTCGGACTCCATCAAAAACGTCGCGATATTTGGAG
CCACGGGAATGACCGGGCTGGCGACCCTGCCGCAAGCTGTGGCTGCAGGATACAATGTGACAGTG
CTGGTTCGGGACCCTGCCAGGCTGCCTGCAGACCACAAGGCATCCAGAGTGGTGGTGGGCGACGT
TGTTAATAAAGAGGATGTGAAGAAGGCCTTGGAGGGCCAGGACGCTGCAATCATCATCCTGGGCA
CCAGGAACGACCTGAGCCCGACCACCGTGATGTCTGAAGGCACCAAGAACATTATTGAAGTCATG
AAGGCTCGTGGGATCCGTAAAGTGATCGGCTGCATGTCAGTCTTTCTGCTCTGGGATCGTGCCAAA
GTCCCACCCCGAATGATTCCTGTGACAGAAGACCACGACAGGATGTACACAGCGCTGAAAACATC
TGGGCTGGACTATGTTGCTGTGATGCCGCCTCACATCGGTGGTGATCTTCCTCTGACGGGGAGTTA
CATGGCGTCAGAGAACATGCTGAAAGGAAGAGCCATCTCTGCACTCGACCTGGGACATTTCTTTGT
CCAGTGTCTGTCCAACACAGAGTGGGATGGCAAGACTGTGGGAGTCTGGGGAGAGTATAAATGAT
CTTAAATAA

The presumed derived amino acid sequences from *L. bergylta* BVR were then *in silico* compared to established BVR sequences using Clustal Ω software (www.ebi.ac.uk) and a neighbour joining tree (Fig. 2.18) generated using the maximum parsimony bootstrapping method provided by MEGA (www.megasoftware.net).

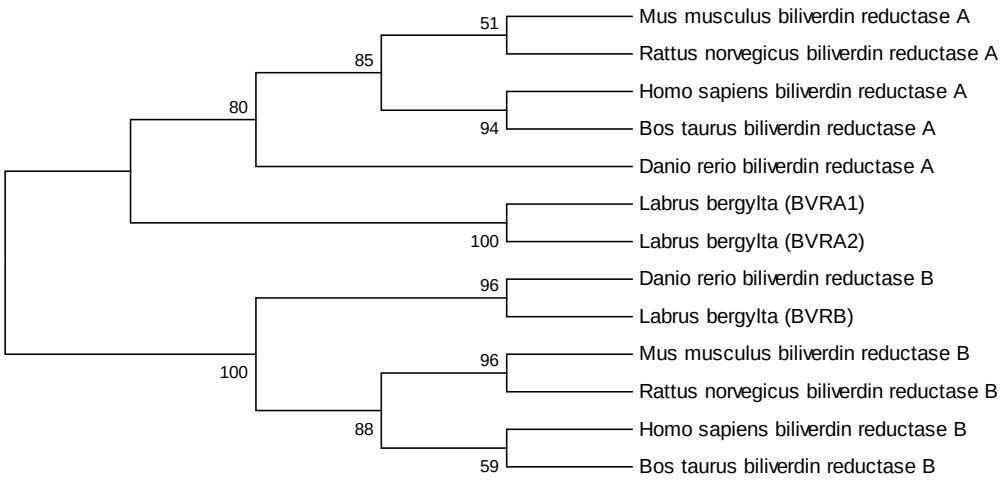


Figure 2.18 Neighbour joining tree of *Labrus bergylta* plasma compared to mammalian analogues.

Analysis revealed that the two partial sequences for *L. bergylta* BVRA were identical rather than separate isoforms, and that these were different from mammalian BVRA. Similarly, *L. bergy*lta BVRB was most closely related to *D. rerio* BVRB and grouped within the mammalian isoforms rather than native BVRA. Further research was not taken forward, however this is a clear demonstration that *L. bergylta* are capable of expression of BVRA and in so doing would be able to convert BV to the terminal product BR, but do not apparently do so in normal conditions. This further suggests the BV accumulation must represent an evolutionary and physiologically advantageous strategy relative to aspects of *L. bergylta* life history. The BVRB expression was somewhat surprising as BV-IXα is the physiologically relevant isomer in adult vertebrates (Smith et al., 2008) therefore it was expected that the corresponding enzyme (BVRA) would be present. From this, the IXα isomer remains the focus of this paper but as BVRB is known to reduce the alternate IXβ, IXδ and IXγ BV isoforms

(Franklin et al., 2009), the functions of which in higher vertebrates remains unknown (Smith et al., 2008), this observation may be of interest but remains to be explored in *L. bergylta*.

12.0 Protein gel electrophoresis

For analysing the plasma protein which was proposed as the BV carrier (Chapter 4), 10% Sodium Dodecyl Sulphate (SDS) running gel was combined with a 4.5% SDS stacking gel. 23 ml 30% acrylamide stock, 26 ml 1M Tris pH 8.8, 20 ml ddH_2O, 0.7 ml 10% w/v ammonium persulphate, 0.7 ml 10% SDS and 50ul Tetramethylethylenediamine (TEMED) formed the running gel. The stacking gel used 3.0 ml 30% acrylamide stock, 2.5 ml 1M Tris pH 6.8, 0.2 ml 10% w/v ammonium persulphate, 0.2 ml 10% SDS, 25ul TEMED. H_2O was added to make 20 ml. The loading buffer was made up using 125 mM Tris-Cl (pH 6.8), 5% SDS, 0.1 % bromophenol blue, 2.5 ml 25% glycerol, and H_2O. Loading buffer was combined (4:1 v/v) with Cleland's reagent (Dithiothreitol (DTT)). This solution was then combined (1:1 v/v) with the normalised plasma aliquots, and heated to 100 ºC for 3 mins prior to use, loading volume was 20 µl. For comparison, an unstained (Pageruler; Thermofisher scientific; (www.thermofisher.com) protein molecular weight marker was included as the first and last lanes in all gels. Apparatus was set at 20 mA-200 mV to stack, then run at 30 mA-200 mV. Staining colloid comprised 10% ammonium sulphate (w/v), ddH_2O, ortho-phosphoric acid, ethanol and Coomassie G250. Gels were stained for 8 hours then destained in ddH_2O for a further 8 hours. Visible and UV spectrum photographs were recorded for later analysis. Bands of interest were excised from the gel and preserved at -20 ºC for later analysis.

13.0 Mass spectrometry and protein identification

In an adaptation to the methods of Shevchenko et al. (1996), excised gel sections of interest (Chapter 4) were dissected into smaller fractions and de-stained by washing in

acetonitrile then subjected to reduction and alkylation prior to the experiment. The resultant suspension was then trypsin digested at 37 ºC and the peptides extracted with a 10% formalin solution. 0.5 ml of the digest solution was then applied to the MALDI target with an alpha-cyano-4-hydroxcinnamic matrix (0.5 μL, 10 mg/ml^{-1} in 50:50 acetonitrile: 0.1% TFA) and allowed to dry. The MALDI MS (4800 MALDI TOF/TOF Analyser (ABSciex)) was then acquired using a ND: YAG 355 nm laser which had been previously calibrated using a mixture of known peptides. The most intense parent ions were selected for MSMS analysis and the resultant MS spectra analysed using a GPS Explorer (ABS Sciex) with a direct interface to MASCOT (Matrix Science) 2.4 engine. Swiss-prot (www.uniprot.org) and NCBR (www.blast.ncbi.nlm.nih.gov) databases were interrogated with tolerances of 100ppm for the precursor ions and 0.5 Da for the fragment ions, trypsin as the cleavage enzyme assuming up to one missed cleavage, carbamidomethyl modification of cysteines as a fixed modification, and methionine oxidation selected as a variable modification. As a note, scores presented with this data are based on the MASCOT search engine probability based system which is derived as the chance that the observed data match is a random event. The data presented herein is therefore restricted to consensus comparisons which exceed the significance threshold of the datasets frequency distribution at the 5% significance.

Chapter 3:
Isolation, identification and characterisation of a blue-green plasma pigment from the ballan wrasse (*Labrus bergylta*, Ascanius 1767)

1.0 Introduction

L. bergylta are the largest and most robust of temperate Labrids, and have proven highly effective at delousing *S. salar* when deployed in sea cages as part of integrated pest management strategies (Leclercq et al., 2014a). Traditionally, wild captured fish have been used but increasing demand relative to limited wild stocks and sustainability concerns have driven an increase in hatchery production (Denholm et al., 2002). Current broodstock management practice is to establish harems of circa 20-30 individuals which spontaneously spawn over a natural two month window (Muncaster et al., 2010). The optimisation of

broodstock management practices are however limited through difficulties in confirming the broodstock gender. The colour and pattern phenotypes of *L. bergylta* are highly variable (Porteiro et al., 1996), but ultimately appears to have no relation to gender (Villegas-Ríos et al., 2013b). Furthermore, the restricted availability of males, as they represent only 10% of the population (Leclercq et al., 2014b), makes gender identification one of the key technical challenges limiting the expansion of production.

Research towards establishing reliable *in-vivo* identification methods has been difficult (Talbot et al., 2012). Direct ultrasound assessment of gonads has been effective for some species but is inconclusive in *L. bergylta* due to a lack of distinctive diagnostic features in gonadal tissue outside the spawning season (Talbot et al., 2012). Similarly, other analytical methods including Latex Bead Agglomeration assays to measure vitellogenin and sex steroid profiling showed limited success (Talbot et al., 2012). In these cases the ambiguous results most likely arose from the reproductive plasticity inherent in protogynous hermaphroditic species, and retention of features from the female phase (DeFalco & Capel, 2009). Thus, in agreement with Darwall et al. (1992), a better understanding of the species specific ecology, physiology and reproductive strategies is fundamental to advancing husbandry techniques and optimising hatchery production.

In contrast to typically pale yellow colouration of blood plasma in mammals, some teleosts including sculpins (Cottidae) and wrasse (Labridae) are observed to have coloured plasma ranging in hue from green through blue to maroon (Low & Bada, 1974). Marked gender dimorphism has been reported in relation to plasma pigment type in lumpsuckers *Cyclopterus lumpus* (L.) (Mudge & Davenport, 1986), and peacock wrasse *Crenilabrus tinca* (L.) (Abolins, 1961), and concentration in *L. mixtus* (L.), axillary wrasse *Crenilabrus mediterraneus* (L.) (Abolins, 1961), and *N. tetricus* (Gagnon, 2006). In most observations, blue-green sera was caused by the linear tetrapyrrole BV (Fang, 1988). Although the precise mechanisms of plasma

dimorphism remain cryptic, relative differences between genders are thought to be a function of alternate hormonal profiles which drive disparity in expression levels of cyclic and open chain molecules (Abolins, 1961), and through micro-environmental interactions with the binding regions of associated protein complexes (Fang & Bada, 1988). These differences have been established as an accurate methodology for differentiating gender in some wrasse species (Abolins, 1961, Gagnon, 2006).

Returning to Chapter 1, with particular reference to Figure 1.4, blue-green serum has been reported in *L. bergylta* with strong variation in the degree of colouration in response to unknown factors (Abolins, 1961). The initial aim of this study was therefore to isolate and identify the underlying pigment responsible for observed plasma colouration in *L. bergylta*, and to characterise it in relation to variation between individuals. Subsequently, with consideration of the described intra-specific differences in other species of wrasse, the secondary aim was to establish if gender was the major driver of variation, and to ascertain if plasma pigmentation could be used to determine sex.

2.0 Materials and methods

2.1 Experimental animals and facilities

For the main study population, *L bergylta* collection (n=397), demography, treatment and biometric data capture were as reported by Leclercq et al. (2014b). All sampling took place in a 6 week timeframe from July to August (2012). Gender was established by histological analysis of the gonads based on Muncaster et al. (2013) and Nozu et al. (2009) and delineated by consideration of the leading developmental edge such that the definition follows the most

advanced features of male gonad differentiation from the female phase. Further to this, and independently of the original fish collection, other native wrasse species including *C. exoletus* (n=10), *S. melops* (n=12), *C. rupestris* (n=12) and *L. mixtus* (n=12) were collected by baited traps in the Lochaber region of Scotland (56°40'57"N, 5°18'2"W) with collection, demography, treatment and biometric data capture identical to previous methods (Leclercq et al., 2014b). *L. bergylta* (n=12) were also sampled at this time to allow comparative analysis without additional complexity from seasonal variation.

2.2 Chromophore extraction for qualitative analysis

As there was significant intra-specific variation in the degree of plasma colouration aliquots were pooled and homogenised to create a large common source of plasma for the initial phase of study. Pooled plasma was then centrifuged (7155 rcf for 2 mins) to reduce post freeze-thaw cellular debris. To cleave the chromophore, 500 µl supernatant was decanted and 500 µl of Hydrochloric Methanol (MeOH.HCl) (3N) introduced to acidify prosthetic groups. After further centrifugation (7155 rcf, 2 mins), the supernatant was decanted and 500 µl $CHCl_3$ added with agitation. Following final centrifugation (7155 rcf, 2 mins), a two phase solution was formed with the chromophore bearing (top) layer extracted and retained for analyses.

2.3 Qualitative analysis

With biliverdin as the most likely pigment candidate based on characterisation in other wrasse species (Abolins, 1961), two biliverdin standards were prepared for comparative analysis against the extracted pigment. The first was commercially obtained BV.HCl (200 $\mu g.ml^{-1}$) in $C_2H_4O_2$, the second was laboratory-generated native biliverdin wherein 0.6 mg commercial BR was dissolved in 1780 µl 17.5 M glacial $C_2H_4O_2$, 200 µl 5% BSA solution and 20 µl 4 mM $FeCl_3$. The solution was heated at 95 ºC for 2 hours, cooled, and diluted to 20 ml

with glacial $C_2H_4O_2$. The resultant native biliverdin solution was centrifuged (7155 rcf, 2mins) and decanted to sealed flasks for storage (4 ºC) (Austin & Jessing, 1994). Absorbance spectra from 350-750 nm were recorded at 5 nm intervals (Ultro-spec 2100pro UV/Visible spectrophotometer, Beckman-coulter Inc., UK, www.beckman coulter.com) for the commercially obtained BV.HCl, the extracted pigment, and for the native biliverdin solution. Spectra were blanked against glacial $C_2H_4O_2$ and recorded at 20 ºC with means of three independent replicates superimposed. BV specific colorimetric assays based on adaptations to the bilatrene specific qualitative Gmelin reactions by Lemberg & Legge (1949), and the Austin & Jessing (1994) adaptation of the Gutteridge & Tickner (1978) were tested. In the first reaction (G_1), 30 % weight per volume (w/v) $(NH_4)2SO_4$ was added to 500 µl crude sera to compress the solvent layer and precipitate proteins. The solution was centrifuged (7155 rcf, 2 mins), supernatant recovered and equal volume fuming nitric acid HNO_3 introduced. In this reaction the blue-green BV is reduced in the presence of HNO_3 to yield a yellow product (BR) (Gray et al., 1961). In the second reaction (G_2), crude plasma (500 µl) was treated with suphuric acid (H_2SO_4) (500 µl) then heated (50 ºC) for 10 mins. In this case, the reaction is specific to BV (not meso-biliverdin) with a positive result observed by destruction (Lemberg & Legge, 1949). The final diagnostic assay which forms the basis of quantification methodologies used specificity of BV reactivity with barbituric acid ($C_2H_4O_2$) in the presence of ascorbic acid ($C_6H_8O_6$) in an alkaline solution to form a characteristic red di-pyrrole chromogen (Manitto & Monti, 1980).

2.4 ESI-TOF MS

The presence of BV in *L. bergylta* plasma was also determined using electro-spray time of flight mass spectrometry (ESI-TOF MS). Regions of interest were excised from an SDS-PAGE gel and the sample was subjected to digestion using a ProGest Investigator digestion

robot (Digilab) by standard protocol. The digest solution (20 µl) was desalted through a NOVAPAK MS C4 2.1x10 mm column (Waters), eluting with an increasing acetonitrile concentration (2% CH_3CN, 98% aqueous 1% CH_2O_2 to 98% CH_3CN, 2% aqueous 1% CH_2O_2) and delivered to a LCT electrospray ionisation mass spectrometer (Waters) which had previously been calibrated. An envelope of charged signals was obtained and de-convoluted using MaxEnt1 software to give the molecular mass of the molecule. Identical methodology was applied to commercial BV IXα (.HCl) to generate a known standard for comparative analysis. The most intense responses (up to 15) were selected for MSMS analysis and the MS data analysed, using GPS Explorer (ABSciex) to interface with the Mascot 2.4 search engine (Matrix Science) and the MSMS data using Mascot 2.4 directly. The data was searched with tolerances of 100 ppm for the precursor ions and 0.5 Da for the fragment ions, trypsin as the cleavage enzyme, assuming up to one missed cleavage, carbamidomethyl modification of cysteine as a fixed modification and methionine oxidation selected as a variable modification.

2.5 Thin layer chromatography

Finally, to determine that the chromatographic migration pattern of the plasma pigment was qualitatively similar to that of the predicted compound, equal volumes (75 µl) of extracted chromophore and BV.HCl in potassium phosphate buffer were spotted on a Thin Layer Chromatography (TLC) plate (Merck, Silica Gel-60 F_{254}). After 10 mins equilibration, plates were developed with $C_4H_8O_2$: C_4H_9OH: $C_2H_4O_2$ (80:10:10) then visualised with saturated iodine vapour. Relative Retardation factor (RRf) was calculated as RRf = ((Retardation factor (BV.HCl) (Rf_{BV}) = Migration distance of standard/ Migration distance of solvent front) / (Rf (Plasma) (RfP) = Migration distance of plasma/ Migration distance of solvent front))*100

2.6 Enzymatic reduction of chromophore

For enzymatic reduction of biliverdin to bilirubin by Biliverdin Reductase (BVR) (E.C.1.3.1.24), extracted pigment was dissolved in 1 ml potassium phosphate buffer and homogenised then 1.0 M NaOH added drop-wise to neutralise. Aliquots were then dried to a solid under vacuum and residuals re-suspended in 800 µl potassium phosphate buffer (pH 7.0) then agitated until full dissolution and re-combined. The assay mix comprised 100mM potassium phosphate buffer (pH 7.0), 10 µM BV.HCl, 1 $mg.ml^{-1}$ BSA, 1.8 mM nicotinamide adenine dinucleotide phosphate (NADPH) and 0.7 $U.ml^{-1}$ BVR. Absorption spectra ranging from 300-750 nm at 5 nm intervals were recorded at 0, 15, 30, 45, 60, 120 and 240 mins at 37 ºC. Activity was monitored as reduction in the NADPH specific peak at 340 nm, and an increase in the bilirubin product signal in the 460 nm region.

2.7 Chromophore quantification

Quantification protocols for plasma biliverdin were conducted as Austin & Jessing (1994) with the following adaptations. BV.HCl was dissolved in 17.5 M glacial $C_2H_4O_2$ to generate 500 $\mu mol.l^{-1}$ with serial dilution in 17.5 M glacial $C_2H_4O_2$ for standards ranging from 0-50 $\mu mol.l^{-1}$ with 0.5% BSA. 500 µl distilled H_2O was added to 500 µl of each standard with 400 µl 40 mM $C_6H_8O_6$ and 100 µl 200 mM $C_2H_4O_2$ in 1 M NaOH. Plasma samples were prepared by addition of 450 µl glacial $C_2H_4O_2$ to 50 µl plasma, 500 µl distilled H_2O was added with 400 µl of 40 mM ascorbic acid and 100 µl 200 mM barbituric acid in 1 M NaOH. Blanks were parallel samples with barbituric acid substituted with 1 M NaOH. Samples were heated at 95 ºC for 5 mins in the dark then cooled and 2.5ml C_4H_9OH with 1 ml 10 M NaOH added then agitated in the dark until the reaction was complete. A two phase solution formed after centrifugation (1789 rcf, 5mins) with the diagnostic red chromophore in the lower component. The top phase was discarded and A_{570} of the lower phase recorded in triplicate, blanks were

then subtracted from the samples. The standard solutions were used to construct calibration curves (Chapter 2, Section 6.5) and the BV quantifications extrapolated.

2.8 Statistical analyses

Concentration values calculated from the calibration curve were negative in some individuals. Although negative levels are not physiologically possible, this reflects difficulties in determining concentration by colorimetric methods and the oxidative lability of BV, hence non-detectible values were assigned an arbitrary value of 0 $\mu mol.l^{-1}$ for analyses. Absolute data was analysed using Minitab 17 Statistical Software (2010) (Minitab, Inc. Software, USA). Data was resistant to normalisation following transformation by any means therefore differences in parameters between treatments, variables or stages of maturity were analysed using Student's t-test where appropriate, non-parametric Kruskal-Wallis, and Tukey's HSD. Results are presented as median and range. As it was determined that the frequency and distribution of negative values resulted in strong kurtosis and discontinuity in the data, and as this was a function of conversion to absolute levels from colorimetric measurements, the Abs_{570} values (analogous to target molecule abundance) were used in further analyses described below.

2.9 Phenotypic analysis

External phenotypes were classified using digital photographs collected by Leclercq et al. (2014b). From this, fish were ascribed to colour-type groups with predominant red (Fig. 3.1(a)), and green phenotypes (Fig. 3.1(d)) forming extremities of the scale, with intermediate forms including red with some green inclusions (Fig. 3.1(b)) and green with some red inclusion (Fig. 3.1(c)). Designations were carried out by three independent operators with any classification discrepancies being resolved by assigning in favour of the majority.

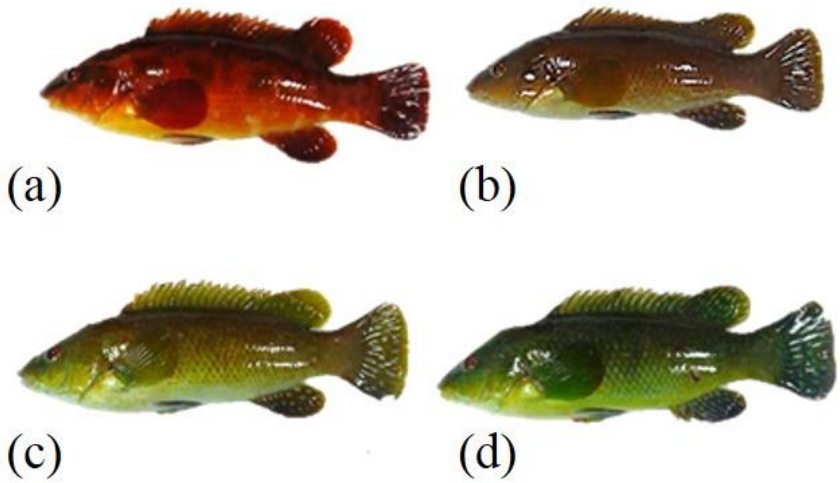


Figure 3.1: *Labrus bergylta* Colour phenotypes comprising: **(a)** Deep red/brown hue, **(b)** Red/brown hue with little green, **(c)** Less intense green with brown/red inclusions and **(d)** Deep green colouration.

The fish were also allocated to two pattern phenotypes which described individuals as Spotted (Fig 3.2(a)), or Plain (Fig. 3.2(b)) based on the overall appearance in accordance with (Villegas-Río et al., 2013b) by three independent operators. Any classification discrepancies were resolved by assigning in favour of the consensus majority.

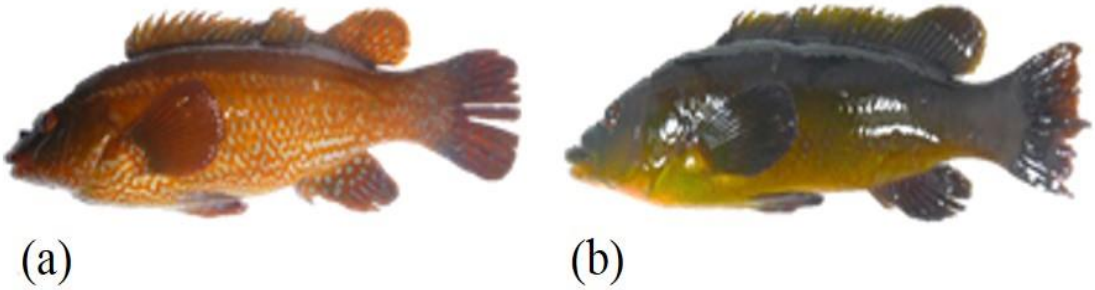


Figure 3.2: *Labrus bergylta* pattern phenotypes comprising **(a)** Spotted and **(b)** Plain

2.10 Factor analyses

As the initial statistical analysis of BV mobilisation was considered limited in the number of life history variables which could be included due to pooled populations (i.e.

captured across a number of sites then combined) and the inherent inter-individual variation between *L. bergylta* within populations, Exploratory Factor Analysis (EFA) (SPSS, Version 22.0, I.B.M., N.Y.) was applied to probe underlying relationships. The measured variables included Origin, Plasma BV (Abs570), Gender, Age, Body Mass (M_B) (g), Total Length (L_T) (mm), Colour, Pattern, and the latent constructs (Williams et al., 2012). Using a reductionist approach (Williams et al., 2012), Origin and Gender were determined as common factor internal attributes (Gorsuch, 1988) as a symptom of alternate sampling strategies across the sites. From this, the solution of best fit (Williams et al., 2012) determined development of the subsequent Origin Excluded (OE) and Origin and Gender excluded (OGE) models wherein component systems were developed through Kaiser conditioning in accordance with Kahn (2006), Cliff (1988) and Cattell (1983) then resolved using orthogonal rotation to simplify the structure of the analysis without affecting the communalities or the percent of variance explained (Williams et al., 2012).

3.0 Results

The identity of the blue-green chromophore in *L. bergylta* plasma was confirmed as BV IXα through comparison of extracted pigment to native biliverdin and commercial BV IXα (.HCl) by absorbance spectroscopy (Fig. 3.3(a)), and mass spectrometry (583.2 Da) (Fig. 3.3(b) and Fig. 3.3(c)).

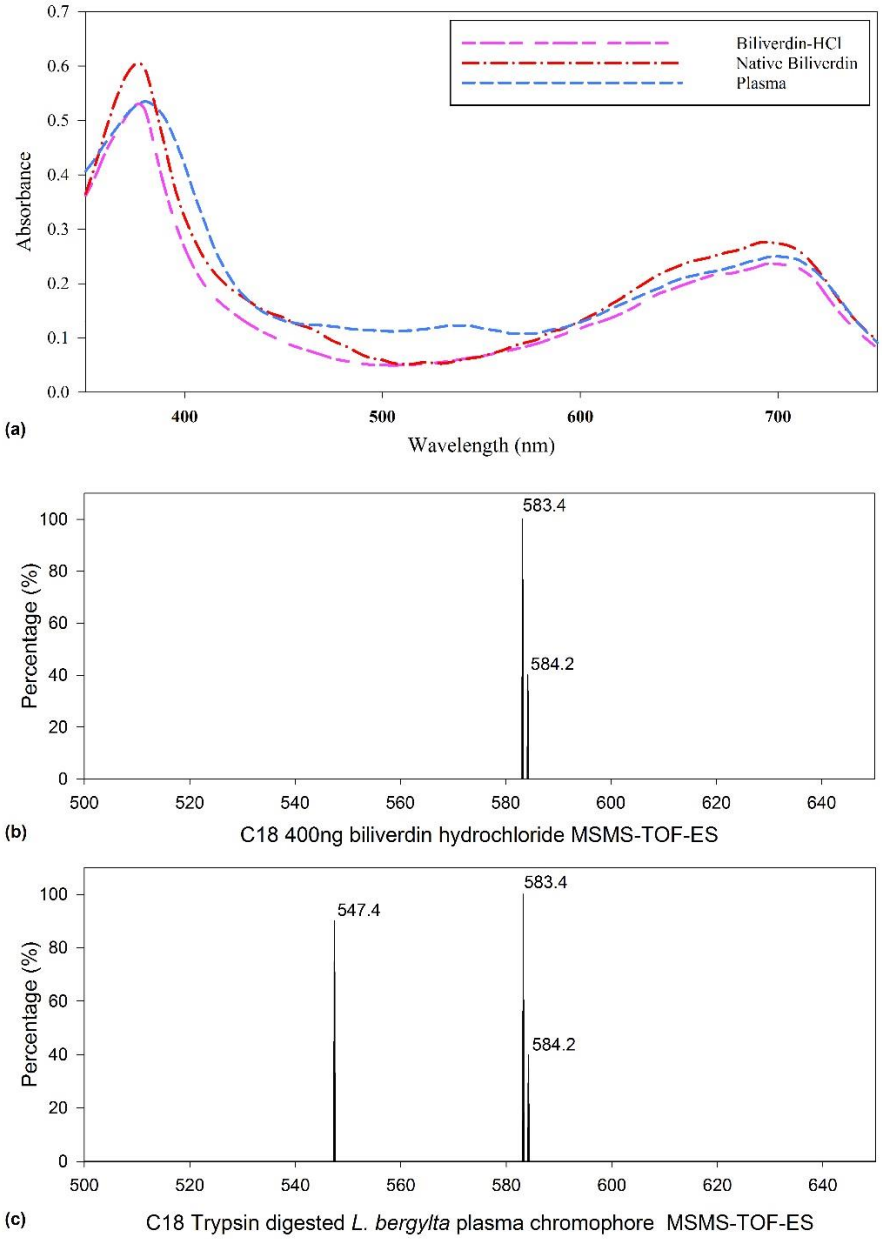


Figure 3.3: Confirmation of plasma chromophore by: **(a)** comparative absorption spectra of commercial biliverdin hydrochloride (pink) with native biliverdin (red) generated from bilirubin oxidation and *Labrus bergylta* plasma in $C_2H_4O_2$ (blue), and ion fragmentation spectra of **(b)** biliverdin hydrochloride and **(c)** trypsin digested *Labrus bergylta* plasma.

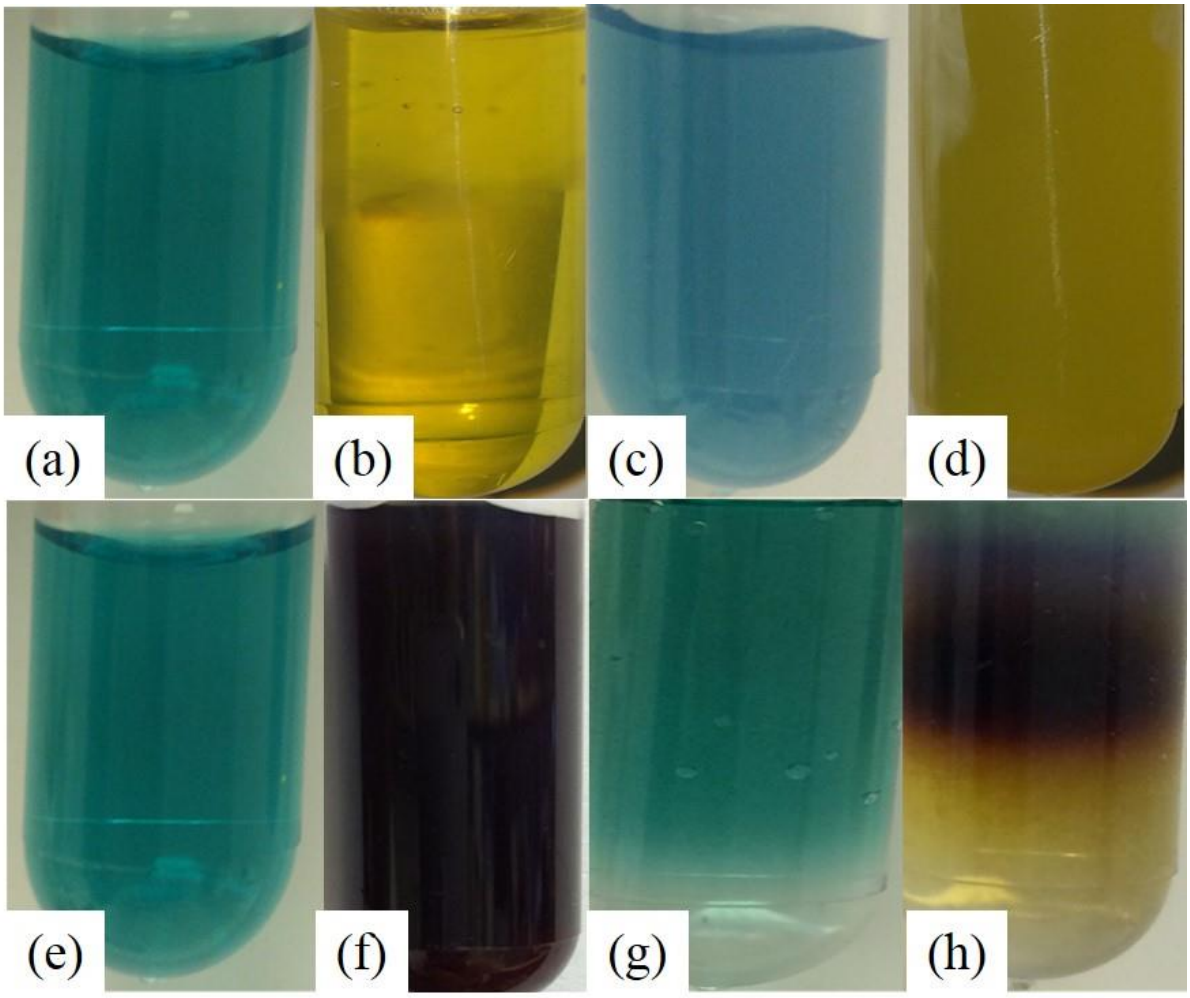


Figure 3.4: Paired Gmelin biliverdin IXα specific diagnostic tests for with HNO_3 treatments in *Labrus bergylta* plasma **(a-b)** and commercial biliverdin hydrochloride **(c-d)**; and H_2SO_4 treatments in *Labrus bergylta* plasma **(e-f)** and commercial biliverdin hydrochloride **(g-h)**.

The conclusion of the initial analysis was supported by the qualitative reactions which show commercial BV on the left (Fig. 3.4(a) and Fig. 3.4(e)) and *L. bergylta* plasma on the right (Fig. 3.4(c) and Fig. 3.4(g)), demonstrating identical reaction products in G_1 (Fig. 3.4(b) and Fig. 3.4(d)) and G_2 (Fig. 3.4(f) and Fig. 3.4(h)). Some variation was noted in the G_2 *L. bergylta* plasma products (Fig. 3.4(h)), which had a grey band, an identical band to the commercial pigment Fig. 3.4(f)), and a yellow band at the bottom. And similarly, the TLC Relative Retardation Factor (RR_f) returned as 90% consensus (data not presented).

Further confirmation was found in the enzymatic reduction data, which demonstrated conversion of NADPH to NADP by BVR activity as BV was converted to BR (Fig. 3.5(a), 3.5(b) and 3.5(c)), but the rate of reduction was lower than expected over the time period.

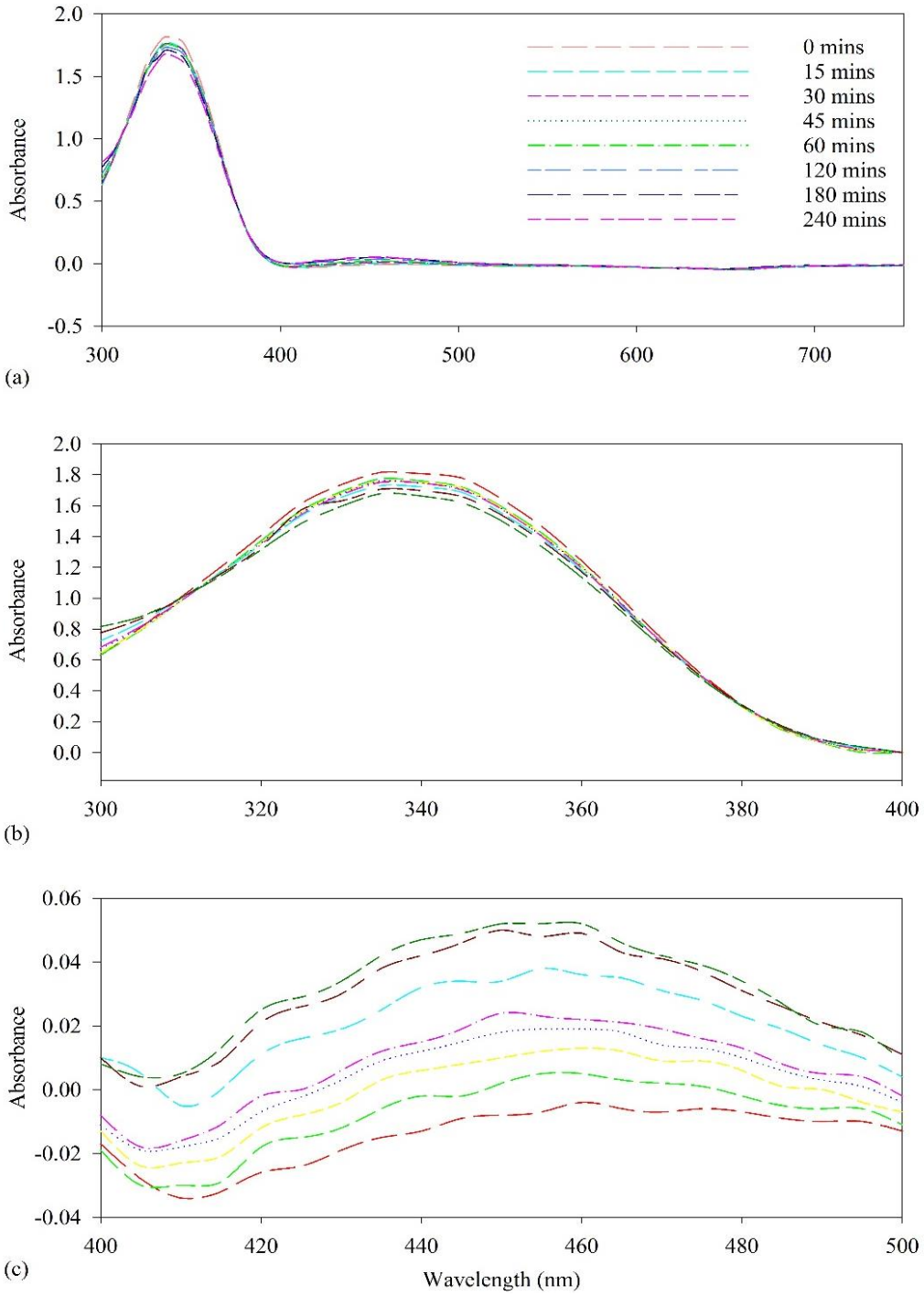


Figure 3.5: Enzymatic (biliverdin reductase) reduction at 0, 15, 30, 45, 60, 120, 180 and 240 minutes across 300-750nm **(a)** of *L. bergylta* plasma biliverdin to bilirubin monitored via conversion of NADPH (300-400nm) **(b)** to NADP (400-500) **(c)**.

The mean *L. bergylta* plasma BV concentration was 10.36 ± 0.4 µmol.l^{-1} (median = 10.96 µmol.l^{-1}; ranging from 0-32.05 µmol.l^{-1}). Plasma BV concentrations (Fig. 3.6) were lower in the Bergen population (median = 0.00 µmol.l^{-1}; ranging from 0–27.95 µmol.l^{-1}) than UK stock from Shetland (median = 12.18 µmol.l^{-1}; ranging from 0–33.01 µmol.l^{-1}), Ardtoe (median = 13.46 µmol.l^{-1}; ranging from 0–35.64 µmol.l^{-1}), and Machrihanish (median = 13.1 µmol.l^{-1}; ranging from 0–35.76 µmol.l^{-1}). Further comparative analysis of the BV levels revealed commonality between the UK groups and supported the assumption of significant difference between the Norwegian and the UK groups (Kruskall-Wallace H (3) = 91.25, $p<0.05$).

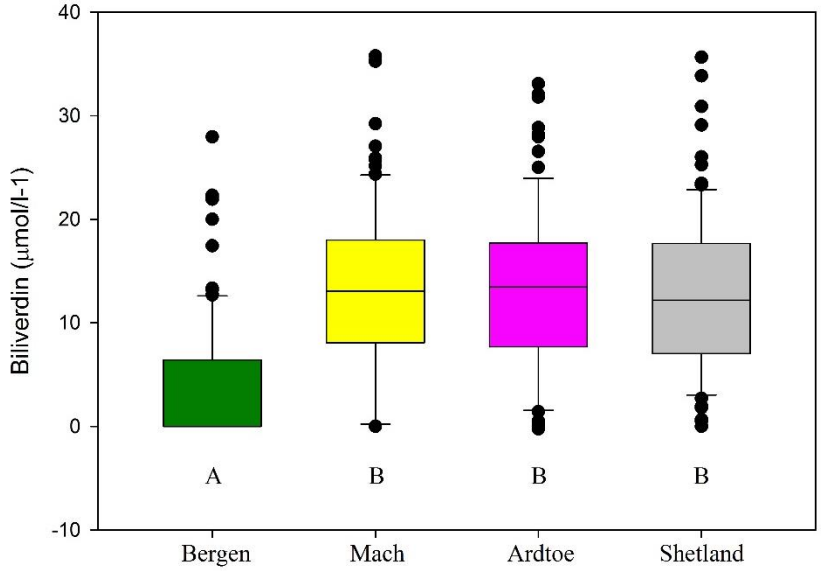


Figure 3.6: Variation in *Labrus bergylta* plasma biliverdin content as determined by absorbance spectrophotometry in relation to geographical origin including Bergen (n=100), Shetland (n=99), Ardtoe (n=98), and Machrihanish (n=100). Different subscript letters denote differences in mean levels.

It is notable that biliverdin did not vary relative to Age, M_B or L_T (data not shown). There was no significant difference in plasma BV between males (median = 10.38 µmol.l^{-1}; ranging from 0–35.77 µmol.l^{-1}) and females (median = 10.96 µmol.l^{-1}; ranging from 0–35.64 µmol.l^{-1}) (Kruskall-Wallace H (1) = 0.20, p=0.653), but transitional individuals (n=9) were

found to have significantly lower levels (median = 0.00 µmol.l^{-1}; ranging from 0–12.31 µmol.l^{-1}) than both genders repectively (Kruskall-Wallace H (1) = 5.95, $p<0.05$) (Kruskall-Wallace H (1) = 9.86, $p<0.05$) (Fig. 3.7).

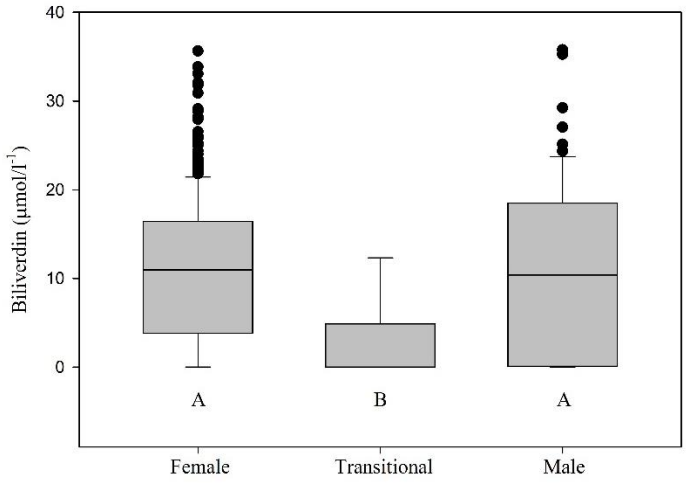


Figure 3.7: *Labrus bergylta* individual gender including Females (n=322), Transitional individuals (n=9), and Male (n=66). Different subscript letters denote significant differences in mean levels.

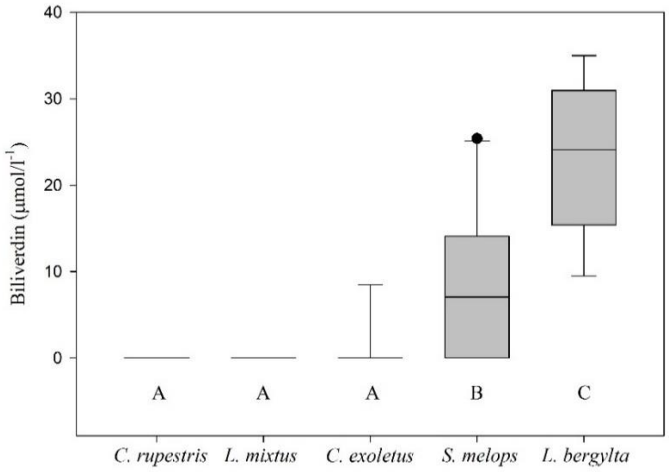


Figure 3.8: Plasma biliverdin concentration in Labridae including *Ctenolabrus rupestris*, *Centrolabrus exoletus*, *Labrus mixtus*, *Symphodus melops* and *Labrus bergylta*. Different subscript letters denote significant differences in mean levels.

Of the other UK native wrasse species tested (Fig. 3.8), plasma BV was at the detection limit of the assay in *C. exoletus* (median = 0.00 µmol.l^{-1}; ranging from 0–8.46 µmol.l^{-1}), and

was un-detectable in (female) *L. mixtus* or *C. rupestris*. Contrastingly, BV was present in *S. melops* (median = 7.05 µmol.l^{-1}; ranging from 0–25.38 µmol.l^{-1}) at lower magnitude than that of *L. bergylta* (median = 24.10 µmol.l^{-1}; ranging from 0–25.51 µmol.l^{-1}). Analysis revealed significant differences (Kruskall-Wallace H (4) = 36.40, $p<0.05$) between the species.

Table 3.1: Varimax rotated component matrix of Origin excluded dataset with Principle Component extraction and Kaiser-Normalisation to a 3 component model.

OE model	**Component 1**	**Component 2**	**Component 3**
Plasma biliverdin (Abs570)		0.701	0.419
Gender	-0.836		
Age	0.868		
M_B(g)	0.847	-0.441	
L_T (mm)	0.751	-0.515	
Colour			0.941
Pattern		0.735	

In the Exploratory Factor Analysis (EFA) of the manifest variables the OE model (Table 3.1) cumulatively described 79.14% of the total variance in plasma BV with the first component comprising 39.80%, the second component 23.38%, and the final component 15.95%. Component 1 showed the biometric variables of age, body weight and total length with strong positive loadings, and gender with strong negative loading. Component 2 had strong positive loading of plasma BV and pattern with negative loadings for M_B and L_T. The third component comprised a positive loading for plasma BV and strong positive loading for colour.

Biometric data is included (Table 3.2). In order of age, Ardtoe were youngest and smallest overall, Shetland were older and larger, followed by Bergen and Machrihanish which were relatively similar but well in excess of the other two groups. Later analysis determined differences in Age (Kruskall-Wallace H (3) = 281.80, $p<0.05$), Body Mass (M_B) (Kruskall-

Wallace H (3) = 295.38, $p<0.05$), and also Total Length (L_T) (Kruskall-Wallace H (3) = 293.61, $p<0.05$).

Table 3.2: Biometric data summary presented as median and range

Origin	Age (Y/O)	M_B (g)	L_T (mm)
Bergen	8 (5-18)	547.5 (317.0-1424.0)	324.5 (275.0-431.0)
Machrihanish	10 (4-20)	503.0 (221.9-1172.6)	319.0 (246.0-416.0)
Ardtoe	3 (1-6)	64.6 (20.5-253.8)	168.0 (117.0-259.0)
Shetland	4 (2-9)	165.0 (46.0-898.6)	220.5 (149.0-375.0)

Post-hoc Mood's median tests were then applied to the biometric data. This method identified that the Age of fish from Bergen was significantly different to Machrihanish (χ^2 (1, N = 200) = 13.8, $p<0.05$), Ardtoe (χ^2 (1, N = 198) = 187.20, $p<0.05$), and Shetland (χ^2 (1, N = 198) = 149.43, $p<0.05$). Similarly, the age of Machrihanish fish was significantly different to that of Ardtoe (χ^2 (1, N = 199) = 36.67, $p<0.05$) and Shetland (χ^2 (1, N = 198) = 112.37, $p<0.05$), and Ardtoe were different to Shetland (χ^2 (1, N = 197) = 15.87, $p<0.05$). The M_B of fish from Bergen was significantly different to Machrihanish (χ^2 (1, N = 200) = 3.92, $p<0.05$), Ardtoe (χ^2 (1, N = 198) = 195.04, $p<0.05$), and Shetland (χ^2 (1, N = 198) = 150.19, $p<0.05$). Similarly, the age of Machrihanish fish was significantly different to that of Ardtoe (χ^2 (1, N = 199) = 187.20, $p<0.05$) and Shetland (χ^2 (1, N = 198) = 104.70, $p<0.05$), and Ardtoe were different to Shetland (χ^2 (1, N = 197) = 36.67, $p<0.05$). The L_T of fish from Bergen was similar to Machrihanish (χ^2 (1, N = 200) = 2.43, $p=0.119$), but was different to Ardtoe (χ^2 (1, N = 198) = 195.04, $p<0.05$), and Shetland (χ^2 (1, N = 198) = 149.43, $p<0.05$). Similarly, the age of Machrihanish fish were significantly different to that of Ardtoe (χ^2 (1, N = 199) = 187.20, $p<0.05$) and Shetland (χ^2 (1, N = 198) = 104.70, $p<0.05$), and Ardtoe were different to Shetland (χ^2 (1, N = 197) = 30.09, $p<0.05$).

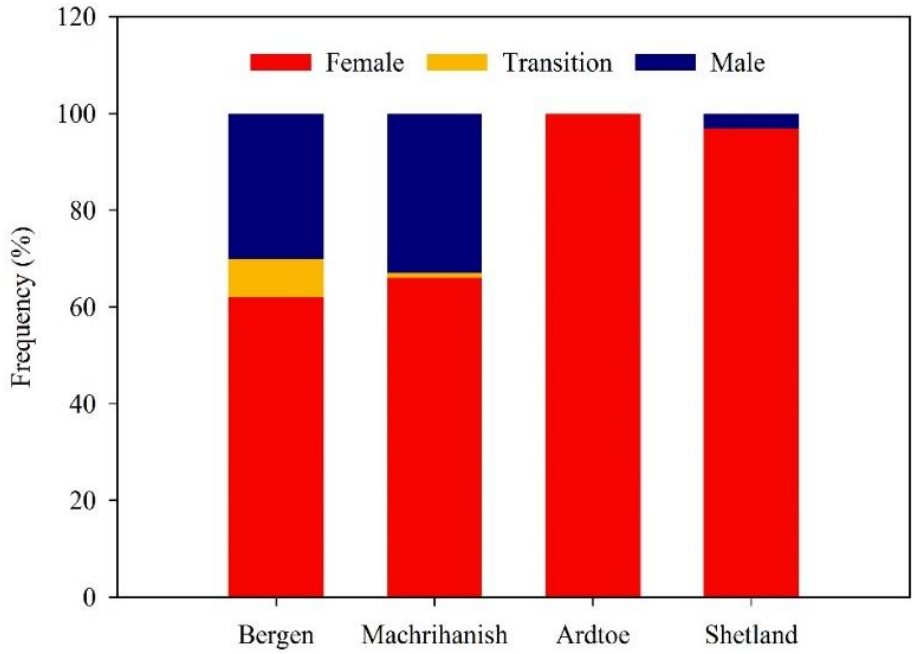


Figure 3.9: Gender phase frequency (delineated by histological analysis and leading edge of development) relative to origin including Bergen (Females (n=62), Transitional individuals (n=8) and Males (n=30)), Machrihanish (Females (n=66), Transitional individuals (n=1) and Males (n=33)), Ardtoe (Females (n=100)), and Shetland (Females (n=66), Males (n=3)).

Considering demographics (Fig. 3.9), 81% of the total population was female, 2% were undergoing transition and 17% were identified as male. As a reflection of the alternate size/age selective harvest methods between the study sites the majority of males and most of the transitional individuals were in the Bergen population. Machrihanish demonstrated a similar proportion of males but only two transitional individuals. The Ardtoe cohort were all female, and Shetland was predominantly female with a small male proportion. Further analysis revealed significant differences in gender phase across the origins (χ^2 (6, N = 397) = 89.783, $p<0.05$).

Table 3.3: Varimax rotated component matrix of Origin and Gender excluded (OGE) dataset with Principle Component extraction and Kaiser-Normalisation to a 3 component model.

OGE model	**Component 1**	**Component 2**	**Component 3**

Biliverdin (Abs_{570})		0.739	
Age	0.945		
Body weight (g)	0.932		
Total length (mm)	0.918		
Colour		0.836	
Pattern			0.958

The OGE model (Table 3.3) cumulatively described 85.01% of the variance with the first component comprising 46.04%, the second component 21.06%, and the final component 17.92%. Component 1 comprised very high loading magnitudes in the biometric parameters with Plasma biliverdin and external colour were strongly loaded in Component 2 and Pattern had resolved to a freestanding position in component 3 with high magnitude loading. In response to the OGE model, the plasma biliverdin and external coloration relationship was tested.

From this, it was determined that the plasma BV levels varied with external colour phenotype (Fig. 3.10) where Red individuals had the lowest concentration (median = 7.692 $\mu mol.l^{-1}$; ranging from 0–33.01 $\mu mol.l^{-1}$), the intermediate phenotypes classified as Red-Green (median = 11.15 $\mu mol.l^{-1}$; ranging from 0–35.64 $\mu mol.l^{-1}$) and Green-Red (median = 11.15 $\mu mol.l^{-1}$; ranging from 0–29.10 $\mu mol.l^{-1}$) were similar, and Green phenotype individuals had the most (median = 16.73 $\mu mol.l^{-1}$; ranging from 0–29.23 $\mu mol.l^{-1}$) . Further analysis identified significant differences in BV levels between the colour variants (Kruskall-Wallace H (3) = 21.52, $p<0.05$), but no difference in BV levels between pattern variants (Kruskall-Wallace H (1) = 27.67, $p=0.675$).

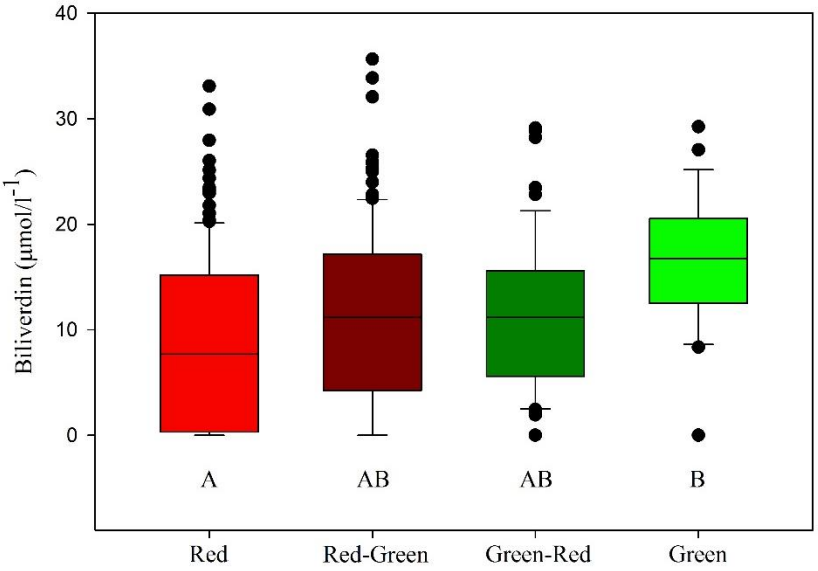


Figure 3.10: Variation in *L. bergylta* plasma biliverdin content determined by colorimetric spectrophotometry in relation to external colour phenotypes (Fig. 3.1) comprising: **(a)** Deep red/brown hue (n=169), **(b)** Red/brown hue with little green (n=136), **(c)** Less intense green with brown/red inclusions (n=55) and **(d)** Deep green colouration (n=28). Different subscript letters denote significant differences in mean levels.

In considering colour phenotype frequency (Fig. 3.11(a)), Bergen fish were predominantly Red with some Red-Green and few Green-Red, Machrihanish was noted to have approximately equal proportions of all variants, Ardtoe had similar proportions of Red and Red-Green individuals with Green-Red individuals and a few Green, and Shetland were similar to Bergen with predominantly Red individuals and a proportion of Red-Green with a slightly greater amount of Green-Red and a few Green. With reference to pattern (Fig. 3.11(b)), Bergen were mostly Spotty and UK locations were predominantly Plain, with Machrihanish and Shetland demonstrating approximately equal proportions of each, Ardtoe fish were essentially plain. Further analysis determined significant differences in colouration (χ^2 (6, N = 397) = 76.448, $p<0.05$), and pattern (χ^2 (3, N = 397) = 101.540, $p<0.05$) between locations.

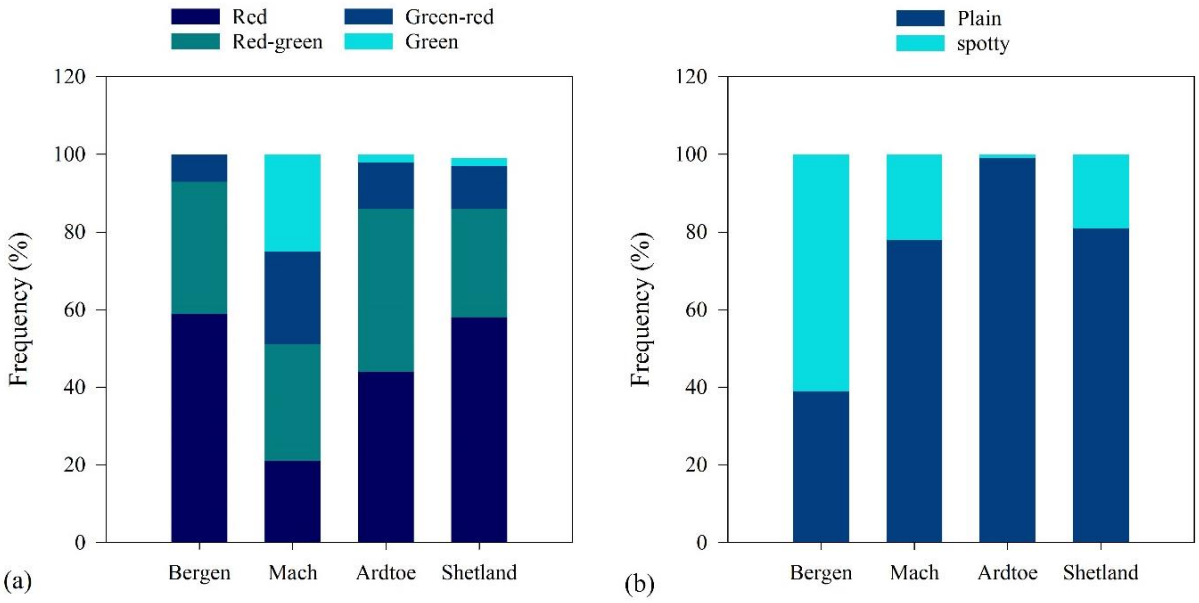


Figure 3.11: Geographic phenotype frequency describing **(a)** Colour including Bergen (Red (n=59); Red Green (n=34); and Green Red (n=7) (Green (n=0)), Machrihanish (Red (n=21); Red Green (n=30); Green Red (n=24); and Green (n=25)), Ardtoe (Red (n=44); Red Green (n=42); Green Red (n=12); and Green (n=2)),and Shetland (Red (n=57); Red Green (n=28); Green Red (n=11); and Green (n=2)), and **(b)** Pattern including Bergen (Plain (n=39); Spotty (n=61)), Machrihanish (Plain (n=78); Spotty (n=22)), Ardtoe (Plain (n=99); Spotty (n=1)), and Shetland (Plain (n=80); Spotty (n=18)).

When phenotype was considered with reference to gender it was noted that the Males and Females colour distributions were similar with some variation in relative proportions, and Transitional individuals were approximately equal amounts of Red and Red-Green (Fig. 3.12(a)). Females were noted as Plain with a small proportion of the Spotty type, Transitional individuals were Spotty with a smaller proportion of Plain, and Males demonstrated approximately equal amounts of both (Fig. 3.12(b)). Analysis revealed differences in Colour (χ^2 (4, N = 397) = 36.695, $p<0.05$), and Pattern (χ^2 (2, N = 397) = 77.943, $p<0.05$).

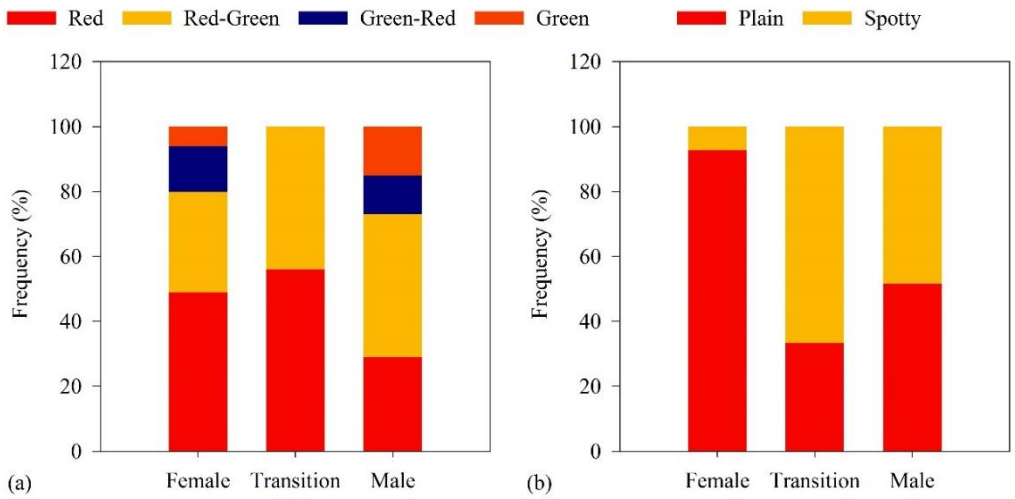


Figure 3.12: *Labrus bergylta* phenotypic frequency by gender with **(a)** Colour including Female (Red (n=144); Red-Green (n=91); Green-Red (n=41); Green (n=18)), Transitional individuals (Red (n=5); Red-Green (n=4); Green-Red (n=0); Green (n=0)), and Males (Red (n=19); Red-Green (n=29); Green-Red (n=8); Green (n=10)), and **(b)** Pattern including Female (Plain (n=273); Spotty (n=21)), Transitional individuals (Plain (n=3); Spotty (n=6)), and Males (Plain (n=34); Spotty (n=32)).

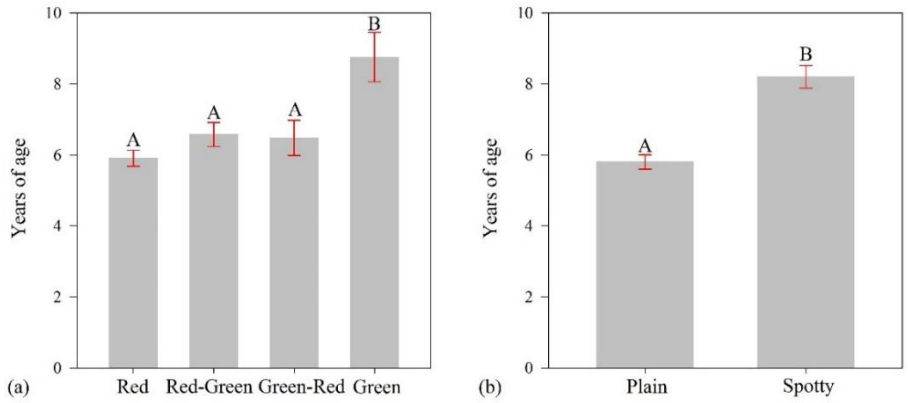


Figure 3.13: *Labrus bergylta* phenotype at age with **(a)** Colour including Red (n=181); Red-Green (n=133); Green-Red (n=54) and Green (n=29), and **(b)** Pattern including Plain (n=295) and Spotty (n=102). Different superscript letters denote significant differences in mean levels.

The mean age of Red individuals was lowest (5.91 ± 0.23 years), Red-Green (6.58 ± 0.34 years) and Green-Red (6.48 ± 0.49 years) fish were similar, and Green individuals (8.76 ± 0.23 years) were significantly older (Kruskall-Wallace H (3) = 13.62, $p<0.05$) (Fig. 3.13(a)). The pattern data (Fig. 3.13(b)) identified that Spotty individuals (8.21 ± 0.32 years) were

significantly older (Kruskall-Wallace H (1) = 41.11, $p<0.05$) than the Plain type (5.80 ± 0.20 years).

4.0 Discussion

It was determined that accumulation of biliverdin IXα in the plasma fractions of *L. bergylta* was the driver of reported blue-green colouration. This was consistent with findings in related species including *S. melops*, *L. mixtus* (Abolins, 1961) and *N. tetricus*, other teleosts such as woolly sculpin *Clinocottus analis* (Girard, 1858) (Fang, 1990), *C. lumpus* (Mudge & Davenport, 1986), gar fish *Belone belone* (L.), Eelpout *Zoarces viviparous* (L.) (Juettner, 2013), and lizards of the genus *Prasinohaema* (Austin & Jessing, 1994).

BV IXα biosynthesis is a ubiquitous process which can be constitutive during the catabolism of senescent erythrocytes and turnover of cytochrome p450 enzymes, or facultative in response to departure from haem homeostasis and when erythrocytes are damaged (McDonagh, 2006). The reaction is initiated is by NADPH dependent C-10 specific cleavage of the haem template with catalysis by HO1 (Morales et al., 2010). This generates equimolar quantities of CO, Fe^{2+} and BV (IXα) (Soares & Bach, 2009). In contrast to mammals where BV is an intermediate metabolite (Bulmer et al., 2008), and is rapidly further reduced to BR by region-specific quantitative BVR activity (McDonagh, 2006), it is the end product in birds, amphibians, reptiles and fish and is directly excreted in most species (Ding & Xu, 2002). HBV plasma is therefore a highly unusual observation in vertebrates and is most usually noted in pathological cases of biliary atresia, catarrhal jaundice or liver cirrhosis which act to increase circulating bilatrene levels by preventing further processing and elimination (Fang & Bada, 1990). From this, the evolutionary advantage and the mechanisms which allow accumulation are of great interest.

With reference to this, the inhibited reduction of *L. bergylta* plasma BV by BVR-A in the current study was of interest and reflected the work of Fang & Bada (1988). In Chapter 2 (Section 9.0), it was established that *L. bergylta* express BVR and therefore have the capability to reduce BV to BR. In contrast to mammals where BR (and endogenous BV (*in vitro*)) are found reversibly bound to albumin for transport in the blood prior to uptake at the hepatic sinusoids, *C. analis* BV was tightly bound to an unknown protein moiety (Fang, 1984). Closer analysis of the complex indicated BV associates with the binding pocket in a coiled helical formation with stabilisation via hydrogen bonding and hydrophobic interactions making the C-10 methene bridge inaccessible to BVR-A for reduction (Fang, 1984). This supports that excretion pathways are most likely modified in HBV species such as *L. bergylta* to prevent clearance by direct excretion or further catabolism to BR (Juettner, 2013). In theory therefore, as the association between BV and the protein would require dedicated protein metabolism, and long-term sequestration represents significant diversions of energy from the somatic budget, this is indicative of physiological functions beyond a chromogen.

The individuals undergoing inversion at the time of sampling were remarkable as plasma BV was notably lower than the binary gender counterparts suggesting some interaction with inversion associated processes such as tissue remodelling. Although the small (transitioning) subset makes it difficult to draw a definitive conclusion, and there is potential for a regional effect as most of the inverting fish originated from the Bergen subset which had comparatively lower BV, this appears corroborated by the additional Labridae in the expanded study with the observations of disparate BV expression between species supporting alternate metabolic strategies (Gagnon, 2006). To explain, the species in which BV was un-detectable (*C. rupestris* and *C. exoletus*) are both gonochoristic, whereas, *S. melops*, *L. mixtus* and *L. bergylta* are protogynous hermaphrodites. Although this is with reference to a limited number of species, and it is of note that all *L. mixtus* in the study were female therefore the assertion of

BV in the plasma is based upon earlier published works (Abolins, 1961), this implies that BV may occur in haemaphroditic species and further supports the hypothesis that BV accumulation is linked to remodelling processes during inversion (Yoshiga et al., 1997). Recent clinical research in humans would appear to support this (Barañano & Rao, 2002; Bisht, 2014; Jansen et al., 2010).

If Bergen and Machrihanish populations are considered as most representative of natural demography due to harvest methods being less selective, which is demonstrated by the increase in heterogeneity relative to the Ardtoe and Shetland groups, notwithstanding the departure from the expected 10% male to female ratio which was similar in both groups, that transitional individuals only represented 2% of the survey as a whole may be reasonably accurate. This illustrates the difficulty of studying sex changing species in a natural environment as the inherent plasticity and relative proportions of each gender subtype make it difficult to achieve truly representative samples. The ideal solution would be to induce sex change in a known population of female *L. bergylta* in a controlled environment, then track the physiological parameters across the differentiation process.

In direct contrast to the HBV species *L. mixtus* (Abolins, 1961) and *N. tetricus* (Gagnon, 2006) mentioned previously, which are proven to exhibit gender specific plasma dimorphism, the present study found no significant difference in the relative abundance of biliverdin relative to gender in *L. bergylta*. This most likely reflects inter-species differences in reproductive patterns arising from contrasting assemblage profiles. Similarly to *L. bergylta,* both *L. mixtus* and *N. tetricus* are both sequential hermaphrodites, but also exhibit strong external dimorphism in colouration with respect to gender (Abolins, 1961). In agreement with Mudge & Davenport (1986), sexual dimorphism in plasma pigmentation is closely linked with corresponding external colourations involved in sexual signalling. Thus, in considering that deposition of the chromophore in the skin (Abolins, 1961), or differentials in catabolic expression profiles

(Mudge & Davenport, 1986) act as the main drivers of reported gender dimorphism in plasma pigment, it would follow that strongly monomorphic species such as *L. bergylta* would show no discernible differential.

The ecological drivers of such a trait are of interest as the benefits of marked external dimorphisms are well known in lek type mating systems where inter-specific competition makes energy investment and the metabolic costs associated with advertising male status and courtship an advantageous strategy (Walker & McCormick, 2009). Contrastingly, it is thought that the long term stability of *L. bergylta* assemblages and high site fidelity of communities (Sayer et al., 1993; Villegas-Ríos et al., 2013a) provide a stable social context which means the male has no need to divert metabolism to invest in (reproductive) ornamentation as the group is essentially 'fixed' and the assertion of social hierarchies are constant (Black et al., 2005). Similarly, if we consider the ultimate aim of a wrasse is to become male, then a lack of secondary (sexual) colour differentiation is an advantage as it ameliorates increased predation risk through departure from primary (cryptic) colouration (Lailvaux & Irschick, 2006). These factors suggest that status dependent sexual selection and frequency dependent natural selection have driven external gender monomorphism and resulted in monandric reproductive strategies in *L. bergylta* (Uglem, 2000). This, the sympatric nature of phenotypes within sites, and the differential distributions across the sites would suggest that phenotypic variations in *L. bergylta* are regional adaptations of crypsis with local 'accents'.

As no obvious gender specific associations of *L. bergylta* plasma biliverdin variation was found in the manifest data, EFA was applied to ascertain any other latent relationships. This revealed clear inherent patterns to help guide future investigations. Prior to Kaiser conditioning, the model would not resolve as Origin and Gender were common factor internal attributes (Gorsuch, 1988), therefore the reductionist approach was applied to optimise factorial resolution (Williams et al., 2012). This was symptomatic of the differing harvest methods

between study sites as the Bergen and Machrihanish populations selected for larger individuals (Leclercq et al., 2014b), and further illustrates the interdependency of these factors as the largest individuals are most likely to be male. Similarly, in considering the mean ages of the populations, this would also explain the distribution of individuals undergoing inversion across sites and lack of males in the Ardtoe and Shetland cohorts. As a global view of the OE model it can be surmised that component 1 represents individual life history stage where age, body weight and total length are closely associated, and that gender is a function of these in accordance with the principles of protogyny and the Size Advantage Hypothesis (SAH) (Munday et al., 2006) as determined by Leclercq et al. (2014b). It is of note that the negative loading of gender in this system is explained by the allocation of numerical descriptors during statistical analyses where males and females were assigned the values of 1 and 2 respectively. The interpretation of this association lends additional support to the robustness of this component as high life history stage values (older and bigger) would therefore predict low gender value (male). Overall, the population size structure and gender analysis fully supports prior conclusions of functional protogyny in *L. bergylta* (Grant et al., 2015; Leclercq et al., 2014; Muncaster et al., 2013; Talbot et al., 2012; Villegas-Ríos et al., 2013).

The multi-factorial loading of life history traits and association of plasma biliverdin and pattern in the OE model was somewhat cryptic, but comparison with the resolution of variables in the OGE model with pattern as a free-standing variable would indicate this as an independent trait (Williams et al., 2012). Component 3 and component 2 of the OE and OGE models respectively were similar as they both comprised plasma BV and colour suggesting association between BV and external colour-type. Further statistical analyses supported this prediction of association between pigment and colour in green individuals but failed to differentiate the other ascribed phenotypes. Hence, although BV is interesting as a camouflage molecule as its conformational flexibility can vary colour from blue to green depending upon environment so

allowing fine tuning relative to a suitable cryptic hue (McDonagh, 2006), this may only be relevant in green *L. bergylta* colour-types. The lack of association in other phenotypic groupings would therefore illustrate the complexity of pigment interactions in the expression of phenotypic accents. This does however raise a rather interesting question of whether the BV interaction with colour is symptomatic of deposition in the skin as described in *N. tetricus* (Gagnon, 2006), or external expression as described in blue walleye (*Sander vitreus* (Hubbs 1926)) (Schaefer, Schmitz, Blazer, Ehlinger, & Berges, 2014) but this was beyond the scope of the current experiment. In addition, that the loading magnitudes increased in the reduced OGE model, and the resolution was improved reiterates the uncoupled association between gender and pigment in *L. bergylta*, and further supports that other drivers must be considered (Williams et al., 2012; Gorsuch, 1988).

Closer examination of the phenotype data revealed additional factors of interest. Returning to the capture demographics, the geographic phenotype frequency data identified significant differences between the locations in terms of both colour and pattern. An illustration of this would be a comparison of the Bergen group which was biased towards predominantly Red and Spotty, with the UK populations which demonstrated greater proportions of Green phenotypes which were mostly Plain. This is most likely a function of the high site fidelity and benthic spawning habits of *L. bergylta*, in conjunction with high juvenile retention and low adult dispersion would favour allopatric adaptive evolution towards an optimised phenotype for a given location (Villegas-Ríos et al., 2013a), but the sympatric distributions of alternate phenotypes within sites is indicative that additional drivers must also be considered. Initially, the demographics must be considered in the context of essentially mixed populations made up of individuals from numerous locations within the geographic locations therefore the broad trends are reliable but the responses may be diluted to a degree. Further to this, *L. bergylta* are a relatively young species therefore although a proportion of juvenile fish may settle in the

colony which they originated from as described in *C. rupestris* (Matschiner et al., 2010), passive dispersal of planktonic larval stages by the prevailing tides and dominant currents (Jeppesen & Cepeda, 2001), in conjunction with the pelagic stage lasting for up to 4 months (Hilldén, 1981), would naturally prevent the evolutionary bottlenecks which normally accelerate fixation of characters. Similarly, mixed phenotype communities and resultant interbreeding must also be considered as it would further retard this process by disrupting selection (Villegas-Ríos et al., 2013a). Moreover, diet may also influence the external phenotype as described in *G, morhua* which results in red and olive types through a benthic diet high in carotenoid content, and high amounts of forage fish respectively (Sherwood & Grabowski, 2010). There were also notable differences in the phenotype relative to gender which could suggest some shift post inversion but in considering the relationship with age then ontogeny would potentially confuse this observation. It is however notable that without exception all of the variants occur across all of the parameters therefore the identity and drivers of colour and pattern remain unresolved.

5.0 Conclusions

In closing, this is the first confirmation that the pigment driver of blue-green plasma in *L. bergylta* is Biliverdin IXα. Accumulation occurs through biliverdin associating with a protein moiety which prevents further processing or excretion to the extent that the pigment is visible in the plasma fraction. There was no association between BV abundance and gender but, that intersexual individuals demonstrated lower levels than male and female counterparts, and as BV was only found in hermaphroditic species the current study was strongly indicative that BV has biochemical functions connected with processes external to the associations of gender and colour phenotype. This was further supported by the suggested biological functions of linear tetrapyrroles play significant biological roles in animals (Cunningham et al., 2000).

HO-1 induction is thought to act as a rapid *in-vivo* anti-oxidant response which initially removes pro-oxidant haem from local tissues with the concurrent increases in the physiological reducing molecules BV and subsequent BR acting to confer longer term cellular defence mechanisms against oxidative damage (Abraham & Kappas, 2008). The future direction of this research is therefore to continue exploration of the physiological roles of biliverdin in *L. bergylta* and other hermaphrodite species, and to further investigate the mechanisms the species use to manage its availability and activity.

Chapter 4:
Investigating biliverdin and its binding protein in *Labrus bergylta* and other Labrini.

1.0 Introduction

In previous experiments (Chapter 3), it was determined that the occurrence of blue-green plasma in *L. bergylta* was caused by accumulation of chromogens proposed to be linked as a prosthetic group to an unknown protein moiety. In accordance with studies in related

species including *L. mixtus* (Abolins, 1961) and *N. tetricus* (Gagnon, 2006), other teleosts such as *C. analis* (Fang, 1987), Gar fish (*Belone belone*) (Jüttner et al., 2013), *C. lumpus* (Mudge & Davenport, 1986), and a range of additional organisms (Austin & Jessing, 1994; Chen et al., 2012; Cornelius, 1981; Marinetti & Bagnara, 1983; Morales et al., 2010), the prosthetic group was identified as IXα isomers of the linear tetrapyrrole BV ($C_{33}H_{34}N_4O_6$).

BV is the intermediate haem catabolite in most vertebrates (Gagnon, 2006). The pathway begins with stereospecific cleavage of haem units by HO in reactions requiring O_2 and NADPH (Abraham & Kappas, 2008). Reactions initiate by formation of ferric haem-HO complexes, which are reduced to the ferrous state by electrons derived from NADPH-Cytochrome P450 reductase (POR) (EC 1.6.2.4) (Kikuchi et al., 2005). Molecular oxygen binds the complex to create a metastable oxy-form, then converts to a hydroperoxide intermediate (Fe^{3+}-OOH) as it receives an electron from the POR, and a proton from distal pocket water (Kikuchi et al., 2005). The terminal oxygen of the hydroperoxide intermediate then alters the α-meso-carbon yielding ferric α-meso-hydroxyhaem (Yoshida & Kikuchi, 1978). Regioselective reaction with molecular O_2 at the C-10(α) mesocarbon of the porphyrin macrocycle forms a ferrous verdohaem (IXα)-HO complex, and releases Carbon monoxide (CO) (Emerit et al., 2004; Origassa & Câmara, 2013). Conversion to ferric BV (IXα)-iron chelate occurs when O_2 binds the ferrous verdohaem central iron (Kikuchi et al., 2005). Finally, the iron moiety is reduced by POR to promote release and liberate BV IXα (Yoshida & Kikuchi, 1978). In mammals, BV is reduced by quantitative regioselective modification by the pH dependant dual cofactor soluble enzyme BVR yielding BR ($C_{33}H_{36}N_4O_6$) for subsequent elimination and excretion (Bulmer et al., 2008). Contrastingly however, this pathway appears modified in some species such that BV is the terminal product (Fang & Bada, 1988).

Beginning at the apex, HO is traditionally designated as a protective protein due to poly-factorial stress induction pathways (Pachori et al., 2007), and selective over-induction (Yao et al., 2009), representing crucial cytoprotective mechanisms (Bucolo & Drago, 2009) through demonstrable in-vivo (Bulmer et al., 2011), and in-vitro (Sedlak & Snyder, 2004) anti-oxidant (Origassa & Câmara, 2013), anti-inflammatory (Bulmer et al., 2011), anti-apoptotic (Nath, 2006), anti-mutagenic (Valko et al., 2006) and anti-proliferative (Stocker & Perrella, 2006) capacities. Conversely, impairment of HO levels increases susceptibility to injury in a variety of stress models (Pachori et al., 2007; Siewert et al., 2013; Yao et al., 2009) due to cytotoxicity arising from haems intrinsic pro-oxidative reactivity with biological membranes (Tracz et al., 2007), and free Fe^{2+} which further accelerates and propagates oxidant injury (Otterbein et al., 2003; Tracz et al., 2007). However, although this is in part true as HO is the only means by which cells can catabolise haem (Otterbein et al., 2003), recent research suggests that it is in fact the downstream products which mediate beneficial effects (Wang et al., 2010). Hence, as the biochemistry and physiological significance of bile pigments is a relatively new field, much of this work has been BR focused due to the inherently transient nature of BV in humans (Cornelius, 1981), therefore little is known about the role of BV in other species (Fang et al., 1986).

In vitro experimental evidence using human erythrocytes (Frei et al., 1988; Stocker, 2005) and in-vivo work using rat hepatocytes has confirmed the protective capacity of physiological concentrations of both BV and BR surpasses α-tocopherol and ascorbic acid at protecting lipid membranes and proteins against oxidation. This anti-oxidant reactivity is due to both pigments containing extended systems of conjugated double bonds including reactive hydrogen atoms which make the REDOX potential of BV and BR equivalen to that of NADP/NADPH (Kaur et al., 2003). In fact, as a partial product of BR oxidation is BV (Liu et al., 2006), and any BV would be constitutively reduced to BR by BVR, this potentially suggests

a cyclical antioxidant network where the complementary molecules regenerate each other (Wei et al., 2006). Although this hypothesis remains controversial (McDonagh, 2010), it would reflect the ascorbate tocopherol antioxidant network (LePrell et al., 2007), and potentially account for reports of nanomolar volumes of BR protecting cultures from oxidative stress from 10000 times higher levels of H_2O_2 (Doré & Takahashi, 1999; Sedlak & Snyder, 2004). The presence of a peroxidase component to regenerate the BV element has been put forward (Liu et al., 2006), but seems unlikely in considering the literature.

Both BV and BR are conformationally flexible molecules which assume a range of forms in solution by slow rotation of atomic groups around interconnecting single bonds (McDonagh, 2010). In BR, stereoisomers form with the acidic carboxylate side chains internalised by hydrogen bonding to other groups in the molecule (McDonagh, 2002). This neutralises polar groups and accounts for BR as both water insoluble and very strongly lipophilic (McDonagh, 2010). Hence, BR solubility at physiological pH is improved by binding to proteins such as Glutathione-S-Transferase (GST) (EC 2.5.1.18) in the cytosol, and by association (98-99%) with Serum Albumin (SA) (Conjugated) in mammalian plasma (Kirkby & Adin, 2006; McDonagh et al., 1981). In addition to facilitating solubility to allow storage and transport, this also sequesters the potentially deleterious molecule which has the capacity to cross cell membranes and bind cell and mitochondrial membranes leading to disruption of mitochondrial processes and cell lysis (Kirkby & Adin, 2006; McDonagh, 2010). SA has one high affinity binding site and numerous low affinity sites for BR (Kirkby & Adin, 2006). Bound fractions are transported to the liver where BR dissociates and diffuses across the phospholipid bilayers into the hepatocytes, where it is bound by cystolic GST, glucoronic acid is then added to the propionic side chains by Uridine-Diphosphate glucuronosyltransferase (UDPGT) (EC 2.4.1.17) forming water soluble (conjugated) monoglucoronide and digluconoride species for excretion into the bile duct and final elimination (Kirkby & Adin, 2006).

Where BR exceeds the capacity of the SA system, such as during augmented haemolysis (Greenberg, 2002), or when excretory mechanisms are compromised (Dennery, McDonagh, Spitz, Rodgers, & Stevenson, 1995), or combinations of both, BR accumulates in the plasma and tissues and physiological jaundice is developed (Brito et al., 2006; Kremer, 1940). Toxicity develops when concentrations exceed of 200 μM (Kirkby & Adin, 2006; McDonagh, 2010) as accumulation in the central nervous system causes a wide range of neurotoxic effects leading to a fatal encephalopathy (Huang et al., 2012) termed kernicterus (Qaisiya et al., 2014). Although the unusual variant HBV (green jaundice) is rare in humans (Fang & Bada, 1990), it is associated with heterogeneous pathologies of poor prognosis including prolonged hepatic jaundice, catarrhal jaundice, biliary atresia and liver cirrhosis which act to increase circulating bilatrene levels by preventing processing and elimination (Huffman et al., 2009), resulting in green colouration of the plasma, soft tissues and urine (Gåfvels et al., 2009). Hence, as such an evolved trait is highly unusual, there is interest in the physiologies of HBV species, the mechanisms which manage accumulation and mobilisation of the pigment, and the underlying biochemical functions.

Several previous studies have described forms of BV protein associations in animals (Jüttner et al., 2013), but have not yet fully determined the physiological significance. BV binding proteins have been described in the fins and scales of Labridae including *S. tinca* (Rudiger, 1970) and humphead wrasse (*Cheilinus undulatus*) (Rüppell 1835) (Yamaguchi & Matsuura, 1969), Scaridae including heavybeak (*Scarus gibbus*) (Rüppell 1829) (Yamaguchi, 1971), knob-snout (*Callyodon ovifrons*) (Temminck and Schlegel 1846) (Yamaguchi, 1971) and tricolour (*Scarus cyanognathus*) (Bleeker 1847) (Yamaguchi & Matsuura, 1969), Cottidae such as sea scorpions (*Cottus scorpius*) (L. 1758) (Yamaguchi, 1971), and Artic sculpin (*Myoxocephalus scorpioides*) (Fabricius 1780) (Yamaguchi & Matsuura, 1969), and Scomberesocidae including Pacific saury (*Cololabis saira*) (Brevoort 1856) (Yamaguchi,

1971). These were for the most part observational papers based on the physical properties of macromolecules during solubility and precipitation experiments (Fang, 1986; Rudiger, 1970; Yamaguchi & Matsuura, 1969; Yamaguchi, 1971). Subsequent works (e.g. Abolins, 1961) were concentrated on elaboration of the chemical structure of the chromophore, but characterisation of the associated protein remained cryptic.

Further to this, BV is also found associated with proteins in the periosteum, spinal processes and scales of *B. belone* (L. 1761), and *Z. viviparus* (Jüttner et al., 2013). Similarly, blue forms of *S. vitreus* (Mitchill 1818) are observed express BV chromoproteins in mucous layers (Yu et al., 2007). Additionally, *C. analis* (Fang & Bada, 1988), *C. lumpus* (Mudge & Davenport, 1986), Japanese rice eel (*Anguilla japonica*) (Temminck & Schlegel, 1846) (Fang & Bada, 1988), and Labridae including *L. bergylta* (Abolins, 1961), *S. tinca* (Yamaguchi, 1971), *N. tetricus* (Gagnon, 2006), *S. melops* (Rudiger, 1970), *L. viridis* (Yamaguchi, 1971), *L. merula* (Rudiger, 1970), and green-eyed wrasse (*Symphodus ocellatus*) (L. 1758) (Yamaguchi, 1971) are known to accumulate BV in the plasma in a protein bound form.

It has been previously noted that although HBV species including *A. japonica* (Fang et al., 1986), *C. analis* (Fang & Bada, 1988), and *L. bergylta* (Chapter 2; Section 9.0) express BVR, and therefore have the capacity to reduce BV to BR, where it was tightly bound to a protein moiety in the serum it was not available for further metabolism, nor was it removed from circulation by the liver or kidney (Fang, 1988). Hence, it is retained in the blood and accumulated resulting in deeply blue-green coloured plasma (Fang & Bada, 1988). Traditionally, BV was thought to be associated with colouration (Cornelius, 1981). Paradis & Magnan (2005) noted enhanced mucal BV expression in blue forms of *S. vitreus*, when compared with the yellow forms and surmised that it was acting as an external component to enhance crypsis, but Yu et al. (2007) subsequently determined that although the distributions were separated there were no effective differences in prey type and abundance between the

phenotypes where a cryptic advantage would be expected between the blue and yellow morphisms. More recently, Jüttner et al. (2013) demonstrated that BV has high affinity for collagen in *B. belone* and *Z. viviparus*, and intimate some paralellisms with BV binding proteins in Lepidoptera, but did not go so far as postulate specific function. In agreement with Fang et al. (1986), very little is known about the nature of these protein complexes.

The previous chapter determined that *L. bergylta* plasma BV was tightly bound to an unidentified protein moiety, and proposed that this was the mechanism of accumulation similar to *A. japonica* and *C. analis* (Fang et al., 1986). Further to this, although male (10.71 ± 1.22 $\mu mol.l^{-1}$) and female (10.85 ± 0.45 $\mu mol.l^{-1}$) BV levels were not different as demonstrated in related species (Abolins, 1961; Gagnon, 2006), it was noted that the BV was signficantly reduced (2.58 ± 1.40 $\mu mol.l^{-1}$) in a small subset of individuals that were undergoing gender transition at the time of survey. Analysis was also expanded to include other local Labridae and determined that BV was absent from *C. rupestris* and female *L. mixtus*, was close to the detection limit in *C. exoletus*, was present in *S. melops* (8.30 ± 2.2 $\mu mol.l^{-1}$) and was highest in *L. bergylta* (22.82 ± 2.9 $\mu mol.l^{-1}$). Subsequent cross species comparison identified that although the species share much of their ecology, the reproductive strategies are highly variable (Dipper, 1979; Hilldén, 1981; Matland, 2015; Potts, 2009; Quigley, 2009; Skiftesvik et al., 2014; Thangstad, 1999; Uglem, 2001). To explain, *C. rupestris* are monochromatic (Thangstad, 1999) diandric gonochorists with discrete binary genders and satellite males (Matland, 2015), *C. exoletus* are essentially monochromatic gonochorists but males show some iridescence during the reproductive season (Thangstad, 1999), *S. melops* are monochromatic and protogynic (Potts, 1973), but become highly dimorphic in the reproductive season (Skiftesvik et al., 2014), and *L. mixtus* shares a protogynous strategy with *L. bergylta* (Matić-Skoko et al., 2013) but has the distinction of being both brilliantly coloured and highly dimorphic (Quigley, 2009). It is of note that the data regarding *L. mixtus* is somewhat

misleading as there were no males available at the time of study but they are known to express BV in the plasma while conspecific females do not (Abolins, 1961). Hence, this, and the observation that BV can be seen leaking from a cross sectioned *L. bergylta* gonad (Muncaster et al., 2013) lead to the theory that BV, and the associated protein moiety, were somehow linked to processes involved in the physiology of changing sex and associated tissue remodelling.

As this is such an unusual trait, teleost specific information are limited so the majority of work is with reference to the BV binding proteins of Lepidoptera. The Insectacyanin (INS) group comprise low molecular weight subunits (20-27 kDa) which form trimers or tetramers depending on species, and bind BV non-covalently (Saito & Shimoda, 1997). This series includes INS in Tobacco hornworm (*Manduca sexta*) (L. 1763) (Goodman et al., 1985) and Hawk moths (*Agrius convolvuli*) (L. 1758) (Saito & Shimoda, 1997), Biliverdin Binding Proteins (BBP) from the cabbage white butterflies (*Pieris xuthus* and *Pieris brassicae*) (L. 1758) (Yamanaka et al., 2000), and the blue biliproteins of wild silkworm (*Antheraea yamamai*) (Guérin-Méneville 1861) (Yamada & Kato, 2004) and Ailanthus silk-moths (*Samia ricini*) (Drury 1773) (Jüttner et al., 2013). The Cyanoproteins (CPS) group is found in hemimetabolous insects including bean bugs (*Riptortus clavatus*) (Thunberg 1783) and migratory locusts (*Locusta migratoria*) (L. 1758) (Saito & Shimoda, 1997). At 73 kDa and 83 kDa (Saito & Shimoda, 1997) respectively, the CPS subunits are far larger than the INS counterpart. The third group includes the Blue Chromoproteins (BCs) of three species of owlet moths (Noctuidae) namely corn earworm (*Helicoverpa zea*) (Boddie 1850), cabbage looper (*Trichoplusia ni*) (Hübner 1800), and oriental leafworm (*Spodoptera litura*) (Fabricius 1775) (Saito & Shimoda, 1997). With subunits of 150 kDa, BCs are the largest of the Lepidoptera bilin binding proteins (Saito & Shimoda, 1997). Although the Lepidopteran binding proteins are known to differ in various physico-chemical properties, the current consensus is that the INS group also complex with yellow carotenoids to modulate the externally expressed hue and

confer functionality in crypsis, whereas the CPS and BCS associations with lipids would allow them to function as storage proteins (Saito & Shimoda, 1997). Hence, although numerous studies have visited biliprotein associations, there are still significant gaps in understanding the specific identities and functionalities which evidently vary between species.

Plasma proteins have been extensively studied in vertebrates and are classified by their functions including transport proteins, immunoglobulins, coagulation related proteins and apolipoproteins (De Smet et al., 1998). In higher vertebrates, SA represents the most abundant protein in the plasma fraction, is the major soluble protein component of circulatory systems, and has numerous physiological functions (Wei et al., 2006). The most important of these are regulation of the colloidal osmotic pressure of the blood (Arroyo, 2009), and functioning as a transport mechanism for small molecules and water insoluble endogenous compounds (Wei et al., 2006). Heteroassociation complexes include Free Fatty Acids (FFA), hormones, various drugs, and both BV and BR (Stocker et al., 1987). In humans, the SA-BR complex is also well known as a key superoxide scavenger and peroxyl radical trapping antioxidant (Liu et al., 2006), but the presence of albumin in lower vertebrates is not ubiquitous (Ahmad et al., 2007). Some species express albumin in minute quantities, while it is entirely absent in others (Ahmad et al., 2007). Albumin 'like' proteins have been described in some species of amphibians, reptiles, birds and fish (De Smet et al., 1998). Although the specific drivers for the presence or absence, and also variation of serum albumin between species remains unclear (Ahmad et al., 2007), it has been suggested that albumin was evolved as an additional system to regulate osmotic homeostasis during the colonisation of land (De Smet et al., 1998). This is rather neatly illustrated by observations that albumin concentration in the plasma of reptiles and amphibians is negatively correlated with time spent in aquatic environments (De Smet et al., 1998), and also that African Lungfish (*Neoceratodus forsteri*) albumins are more similar to that of tetrapods than those of teleosts (Metcalf et al., 2007). With consideration of the evidence from

Chapter 3, whether such proteins are associated with *L. bergylta* plasma BV in a system which is analogous to the association of SA and BR in humans, or the BBP of Lepidopterans remains to be explored.

From this, the ultimate aim of this Chapter was therefore to provide a greater understanding of BV and BV binding proteins in *L. bergylta* through isolating the protein of interest from crude plasma, and then exploring the specific identity. Following this, the same methodologies would be applied across related temperate Labridae to ascertain abundance patterns for the protein of interest in the context of protogynous species relative to gonochoristic counterparts.

2.0 Methods and materials

2.1 Ionic fractionation

Ammonium sulphate ($AmSO_4$) ionic fractionation was determined the best method to test the relationship between BV and the proposed carrier protein as the non-chaotropic nature would minimise disruption. Plasma from the original survey (n=397) of *L. bergylta* was randomly selected, then pooled to create a homogenous source of adequate volume. This pool was centrifuged (1789 rcf, 5mins) to remove post freeze-thaw cellular debris then subdivided to allow sequential processing, and the parallel processes described hereafter. 5% integers of $AmSO_4$ were used to generate the precipitation gradient. $AmSO_4$ weights were determined for each increment using the following equation developed by (Scopes, 1994):

G = Sat(M2-M1)/(SatM-(SpecV/1000*132.14*SatM*M2)).

Where G was the mass of $AmSO_4$ which would be added to 1 litre to reach a given increment, Sat was $AmSO_4$ mass in a saturated solution ($g.l^{-1}$), M_1 was starting molarity, M_2 was desired molarity, SatM was molarity of saturated $AmSO_4$ solution, SpecV was specific volume displaced by 1 g of $AmSO_4$ in an aqueous solution (0.54 ml), and 132.14 corresponds to $AmSO_4$ molecular weight. As Sat, SatM and SpecV all vary depending on temperature, the calculations and derived values were 20 ºC specific. Prior to the experiments, the laboratory, equipment, samples and reagents were conditioned to 20 ºC.

As this method is normally used to purify proteins in solution by sequentially altering the ionic strength to precipitate proteins of a given solubility then recover them by centrifugal pelletisation (Nooralabettu, 2014), proteins of a given solubility are essentially 'cut' from the solution at each fraction (Fig. 4.1(a)), and prescribes that the supernatant is taken forward to the next step. Although this is an effective means of protein purification, as this study also intended to quantify BV in the supernatant the method was adapted to run as a parallel process (Fig. 4.1(b)).

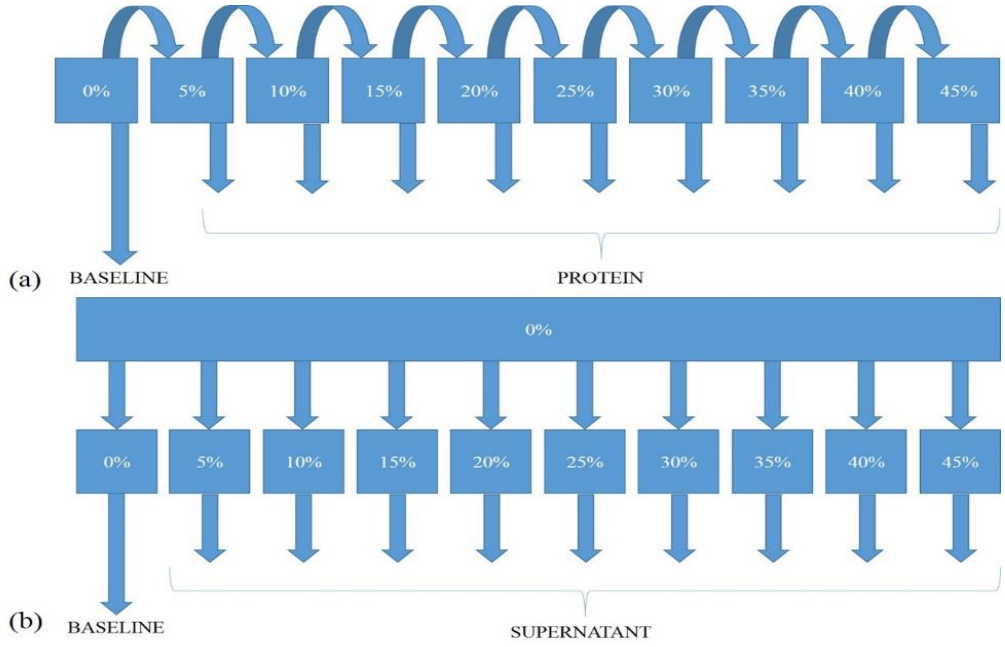


Figure 4.1: Workflow diagram of ammonium sulphate fractionation by **(a)** Standard sequential protocol and **(b)** Adapted parallel protocol.

From this, to examine the proteins present at each increment the sequential process (Fig. 4.1(a)) was applied therefore $AmSO_4$ was pre-measured to progress the terminal increment to the next (0-5% then 5-10% then 10-15% and so on.), with all parameters adjusted to allow for the change in volume. The salt was added gradually over 20 mins and agitated to minimise local solubility differentials. The resultant solutions were then further agitated for 1 hour to optimise precipitation. The aliquots were then centrifuged (11180 rcf, 15 mins) to pelletise the precipitate. Supernatant was recovered and transferred to a new tube for the next further processing, pellets were re-suspended in fixed volume of Potassium Phosphate Buffer (PPB) (pH 7.4). Samples were then generated for 0-5% (5%), 5-10% (10%), 10-15% (15%), 15-20% (20%), 20-25% (25%), 25-30% (30%), 30-35% (35%), 35-40% (40%) and 40-45% (45%). To explore the supernatant, the parallel process (Fig. 4.1(b)) was carried out to allow retention for each fraction. Supernatant samples were therefore generated by progressing aliquots for each integer from 0% to the saturation point so 0-5% (5%), 0-10% (10%), 0-15% (15%), 0-20% (20%), 0-25% (25%), 0-30% (30%), 0-35% (35%), 0-40% (40%) and 0-45% (45%).

2.2 Chromophore quantification

BV concentrations for the re-suspended pellet and supernatant series were measured by the modified method from Gutteridge & Tickner (1978). Commercially obtained BV.HCl was dissolved in 17.5 M glacial $C_2H_4O_2$ to 500 µmol l^{-1}, with serial dilutions in 17.5 M glacial $C_2H_4O_2$ for standards ranging from 0-50 µmol l^{-1} with 0.5 % BSA. 500 µl distilled H_2O was added to 500 µl of each standard with 400 µl 40 mM $C_6H_8O_6$ and 100 µl 200 mM $C_2H_4O_2$ in 1 M NaOH. Plasma samples were prepared by addition of 450 µl glacial $C_2H_4O_2$ to 50 µl plasma, 500 µl distilled H_2O was added with 400 µl 40 mM ascorbic acid and 100 µl 200 mM barbituric

acid in 1 M NaOH. Blanks were parallel samples with barbituric acid substituted for 1 M NaOH. Samples were heated at 95 ºC, 5 min in the dark then cooled to 20 ºC and 2.5 ml C_4H_9OH with 1 ml 10 M NaOH added, then samples were agitated in the dark until the reaction was complete. A two phase solution formed after centrifugation, with the diagnostic red chromophore in the lower component. The top phase was discarded and A_{570} of the lower phase recorded in triplicate, blanks were then subtracted from the crude readings and the working mean taken forward for analysis. Standard solutions were used to construct a calibration curve (Chapter 2; Section 6.5) and plasma BV extrapolated.

2.3 Protein quantification

Bradford method (Bradford, 1976) was used to estimate protein content according to the methods and resilts in Chapter 2 (section 7.0). Protein content was extrapolated for both the sequential (Fig. 4.2(a)), and parallel (Fig. 4.2(b)) series and the original samples were normalised by dilution in physiological buffer to 0.02 $mg.ml^{-1}$ for subsequent analysis.

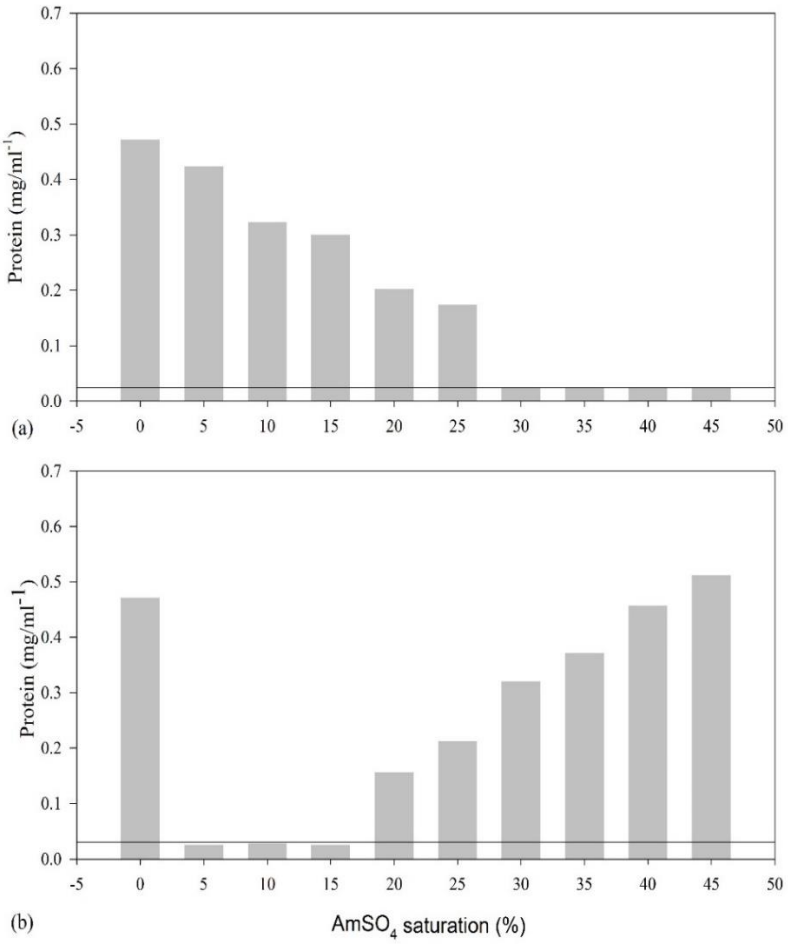


Figure 4.2: Plasma protein content (mg/ml^{-1}) in **(a)** Supernatant **(b)** Pellet ammonium sulphate fractionations with normalised levels taken forward for analysis superimposed. Black line shows normalisation level.

2.4 Gel electrophoresis

10% Sodium Dodecyl Sulphate (SDS) running gel was combined with a 4.5% SDS stacking gel. 23 ml 30% acrylamide stock, 26 ml 1M Tris pH 8.8, 20 ml ddH_2O, 0.7 ml 10% w/v ammonium persulphate, 0.7 ml 10% SDS and 50ul Tetramethylethylenediamine (TEMED) formed the running gel. The stacking gel used 3.0 ml 30% acrylamide stock, 2.5 ml 1M Tris pH 6.8, 0.2 ml 10% w/v ammonium persulphate, 0.2 ml 10% SDS, 25ul TEMED. H_2O was added to make 20 ml. The loading buffer was made up using 125 mM Tris-Cl (pH 6.8), 5% SDS, 0.1 % bromophenol blue, 2.5 ml 25% glycerol, and H_2O. Loading buffer was combined

(4:1 v/v) with Cleland's reagent (Dithiothreitol (DTT)). This solution was then combined (1:1 v/v) with the normalised plasma aliquots, and heated to 100 ºC for 3 mins prior to use, loading volume was 20 µl. For comparison, an unstained (Pageruler; Thermofisher scientific; www.thermofisher.com) protein molecular weight marker was included as the first and last lanes in all gels. Apparatus was set at 20 mA-200 mV to stack, then run at 30 mA-200 mV. Staining colloid comprised 10% ammonium sulphate (w/v), ddH_2O, ortho-phosphoric acid, ethanol and Coomassie G250. Gels were stained for 8 hours then destained in ddH_2O for a further 8 hours. Visible and UV spectrum photographs were recorded for later analysis. Bands of interest were excised from the gel and preserved at -20 ºC for later analysis.

2.5 Mass spectroscopy and protein identification

The mass spectroscopy and protein identification were carried out accordint to the methods stated in Chapter 2 (Section 13.0)

2.6 Cross species analysis

To determine the distribution of BV across temperate Labridae, *C. rupestris*, *C. Exoletus*, *S. melops*, *L. Mixtus*, and *L. bergylta* were collected from Ardgour, Scotland (56°41'03.3"N 5°42'25.2"W) on 20/08/15 and treated identically to those of the original study (Leclercq et al., 2014a). BV was qualitatively determined at the level of species and gender (excepting male *L. mixtus*) by spectroscopy using the BV specific peak at 650-670 nm (McDonagh & Palma, 1980), then quantified by colourimetric analysis of the barbituric reaction products (Chapter 2 section 6.0). Having noted that *L. bergylta* plasma appeared as two main colour species (blue and green), the electrophoretic method was initially applied to crude plasma from green 'type' and blue 'type' *L. bergylta*, and the other species available at

the time *C. rupestris*, *C. exoletus* and. *L. mixtus*. Protein concentrations were determined and normalised as per the previous methods (Fig. 4.3).

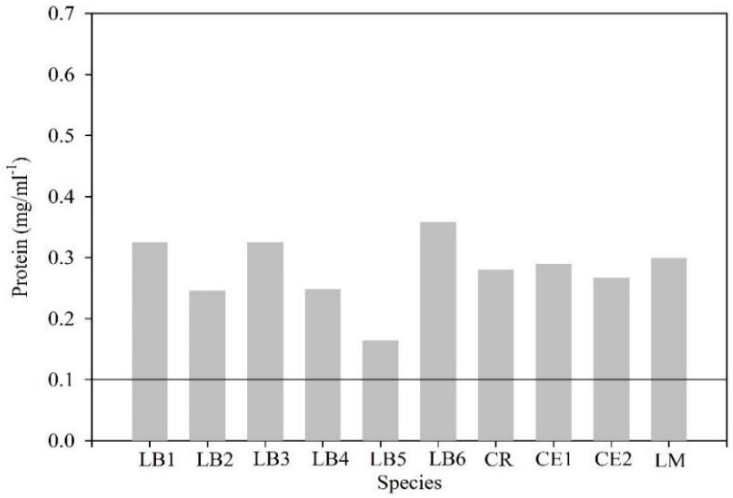


Figure 4.3: Cross species plasma protein concentration (mg/ml^{-1}) showing green 'type' *Labrus bergylta* (LB1-3), blue 'type' *Labrus bergylta* (LB4-6), *Ctenolabrus rupestris* (CR), *Centrolabrus exoletus* (CE1-2) and *Labrus mixtus* (LM). Black line shows normalisation level.

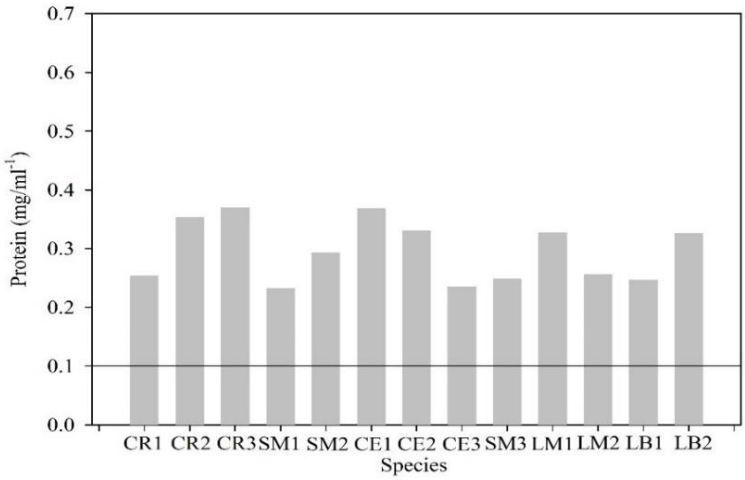


Figure 4.4: Cross species plasma protein concentration (mg/ml^{-1}) showing *Ctenolabrus rupestris* female (CR1-2), *Ctenolabrus rupestris* male (CR3), *Symphodus melops* female (SM1) *Symphodus melops* male (SM2-3), *Centrolabrus exoletus* female (CE1), *Centrolabrus exoletus* male (CE2-3), *Labrus mixtus* female (LM1-2), *Labrus bergylta* female (LB1) and *Labrus bergylta* male (LB2). Black line shows normalisation level.

A subsequent visit to the same survey site further expanded the library such that with the exception of (male) *L. mixtus and L. bergylta*, all genders and species could be represented (Fig. 4.4). All biometric data capture and subsequent treatment was identical to previous. In

accordance with the preparative methods in Section 2.3, the protein concentrations were calculated and diluted to approximately similar levels for subsequent analysis (Fig. 4.10 and Fig. 4.11).

3.0 Results

The $AmSO_4$ protein fractionation allowed reasonable separation of the protein fractions, supernatant fractions, and tracking of BV mobilisation (Fig. 4.5). The protein content of the supernatant fraction (described as % removed) reduced steadily from 0-15%, then rapidly from 15-30%, and was at the detection limit beyond 30% saturation. With the exception of a notable increase at 10% saturation, supernatant BV mobilisation (described as % recovered) was correlated with the protein with a sharp decrease between 15-30%, and levels at the detection limit thereafter. Finally, with reference to the pellet fractions, BV was at the detection limit from 0-15%, then showed a sharp increase with peak BV at 30% with a return to the detection limit beyond 35%.

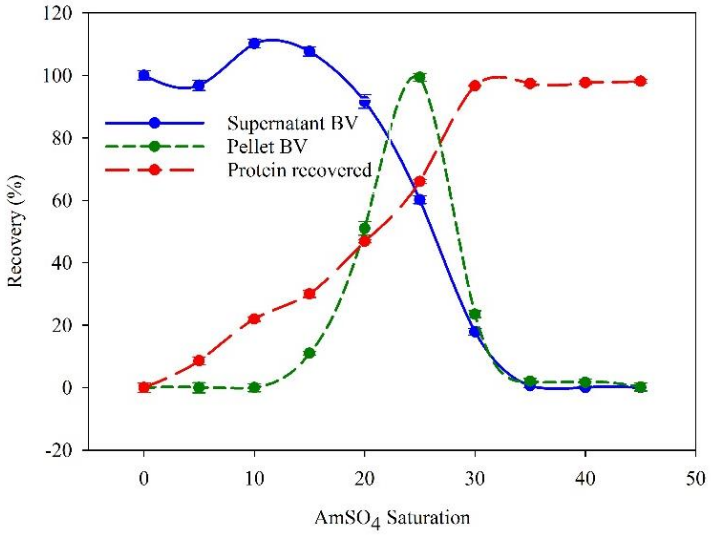


Figure 4.5: Mobilisation across a saturation gradient of ammonium sulphate describing: **(a)** the amount of biliverdin recovered from the supernatant (%), **(b)** Biliverdin in pellet fractions (%), and **(c)** the amount of protein removed from the supernatant (%).

Gel electrophoresis of the supernatant fraction (Fig. 4.6(a)) shows intense bands of 25 kDa and ~28 kDa are present in the 5%, then incrementally decrease in intensity, and are absent beyond the 30% integer. The protein fraction (Fig. 4.6(b)) demonstrated an opposite distribution with the same bands absent in the lower fractions (0-15%), with an increase in intensity peaking at 30%, and diminishing beyond this with none observed at 45%.

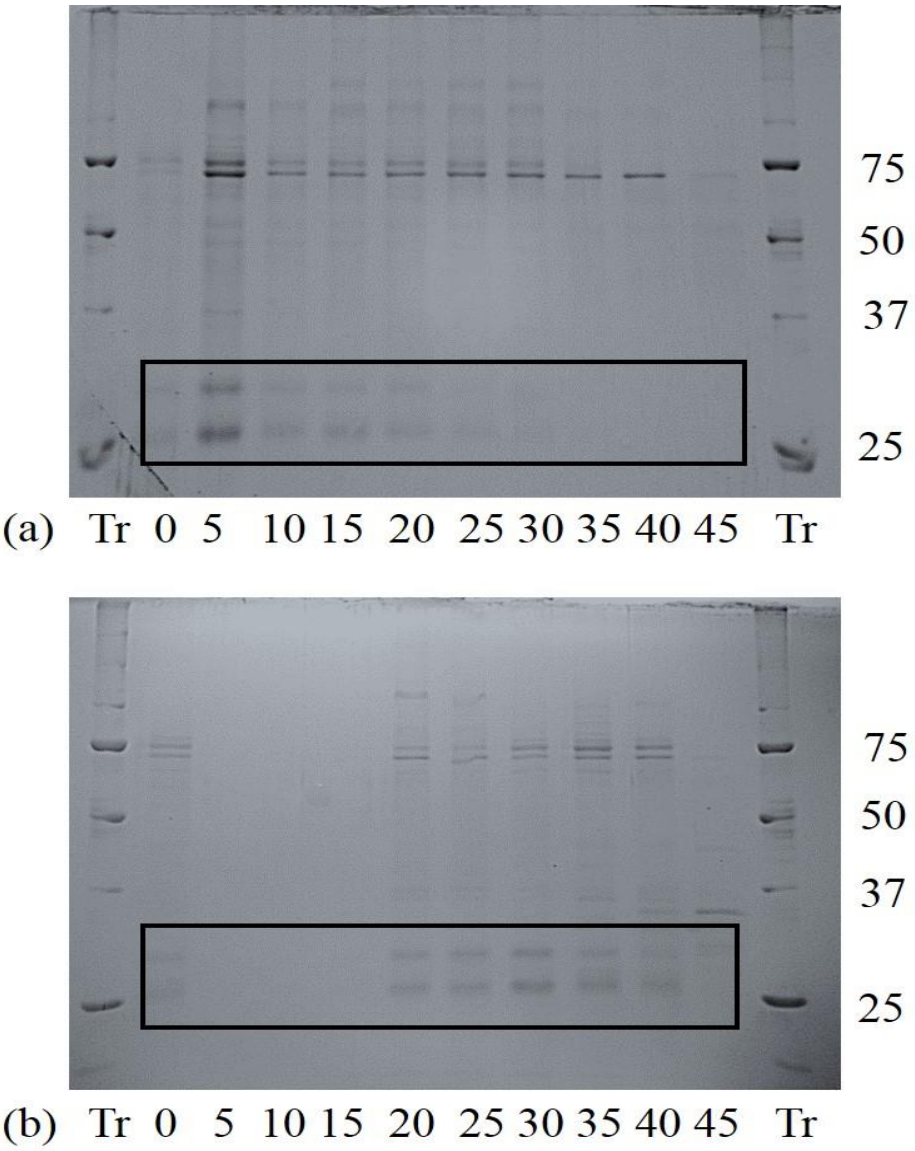


Figure 4.6: Denaturing gel electrophoresis of ammonium sulphate separations (Fig. 4.6) wherein; **(a)** shows the supernatant fraction, and **(b)** shows the pellet fraction. Lanes are marked with saturation increments. Molecular weights shown in the tracking lane (Tr) are kDa.

Although it is notable that the fluorescence was only observed on one occasion, the protein fractions at 25 and 28 kDa were visible under UV light in the second sample set of *L. bergylta* under $AmSO_4$ separation (Fig. 4.7). The fluorescence was not present in the 2-15% fractions, but increased in the 20-25% region, peaked in the 30-35% fractions, and decreased in the 40-45% fractions.

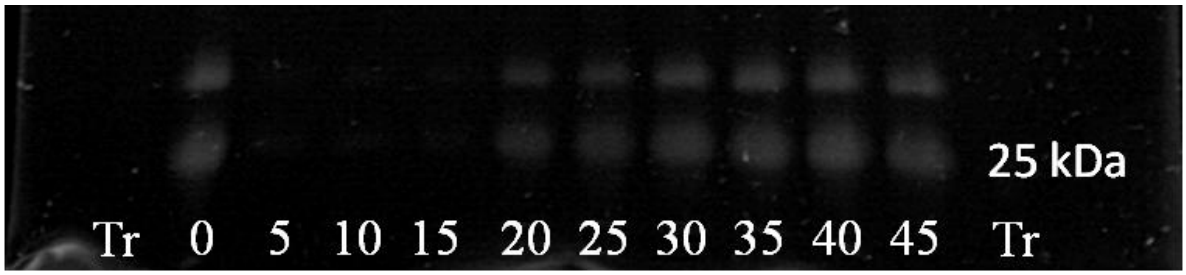


Figure 4.7: UV photograph showing denaturing electrophoresis of ammonium sulphate separations. Lanes are marked with saturation increments (%).Tracking lane (Tr) molecular weights are kDa.

Mass spectrometry analysis of excised 28 kDa band returned a diverse series of consensus sequences (Table 4.1). These included Immunoglobulin (IG) light chain from gilt-head bream (*Sparus aurata*) (L. 1758), yellow croaker (*Larimichthys crocea*) (Richardson 1846), sablefish (*Anaploma fimbria*) (Pallas 1814), and emerald cod (*Trematomus bernacchii*) (Boulenger 1902); Apolipoprotien A1 (ApoA1) from Hong Kong grouper (*Epinephalus akaara*) (Temminck & Schlegel 1842); complement component C3 from orange spotted grouper (*Epinephalus coioides*) (Hamilton 1822), and guppies (*Poecilia reticulata*) (Peters 1859); C3 like proteins from Japanese killifish (*Oryzias latipes*) (Temminck & Schlegel 1846), Amazon mollies (*Poecilia formosa*) (Girard 1859), zebra mbuna (*Meylandia zebra*) (Boulenger 1899), and the Japanese puffer (*Takifugu rubripes*) (Temminck & Schlegel 1850); and pro-C3 from *T. bernacchii*. The data is summarised in Table 4.1

Table 4.1: Analysis of 28 kDa bands at 30% saturation showing the MASCOT score and sequence coverage based on BLAST analysis.

Protein	Species	Accession	Score	Coverage (%)
Light chain immunoglobulin	*Sparus aurata*	gi|148806531	422 618	16
	Larimichthys crocea	gi|291165395	133 166	11
	Anaploma fimbria	gi|229366314	77	4
	Trematomus bernacchii	gi|111278861	124	22
Apolipoprotein A1	*Epinephalus akaara*	gi|529407265	100 90	4
Complement c3	*Epinephelus coioides*	gi|315570434	128	1
	Poecilia reticulata	gi|658830195	126	1
C3-like	*Oryzias latipes*	gi|765166967	129	1
	Poecilia formosa	gi|617491240	126 127	1
	Meylandia zebra	gi|498928208	64	N/A
	Takifugu rubripes	gi|410917321	50	1
Pro C3	*Trematomus bernacchii*	gi|573026040	117 127	2

Pigment quantification revealed BV was undetectable in either gender of *C. exoletus*, and female *L. mixtus* (no males were available) (Fig. 4.8). *C. rupestris* BV was at the detection limit in females and undetectable in males. Female *S. melops* plasma BV (median = 6.92 $\mu mol.l^{-1}$; ranging from 5.38–8.85 $\mu mol.l^{-1}$) was greater than that of the males (median = 3.84 $\mu mol.l^{-1}$; ranging from 3.46–5.00 $\mu mol.l^{-1}$). *L. bergylta* demonstrated higher levels where female plasma BV (median = 27.692 $\mu mol.l^{-1}$; ranging from 27.40–30.39 $\mu mol.l^{-1}$), and males plasma BV was similar (median = 27.692 $\mu mol.l^{-1}$; ranging from 26.83–28.85 $\mu mol.l^{-1}$). Further analysis reinforced the difference between the non-HBV and the HBV species (Kruskall-Wallace H (3) = 44.31, $p<0.05$), and supported significant difference between *S. melops* and *L. bergylta* plasma BV (Kruskall-Wallace H (3) = 44.40, $p<0.05$). There was no gender specific difference in *S. melops* (Kruskall-Wallace H (1) = 0.00, p=1). Similarly, although female *L. bergylta* plasma BV was greater than that of male there was no significant difference (Kruskall-Wallace H (1) = 1.13, p=0.288) in concentration.

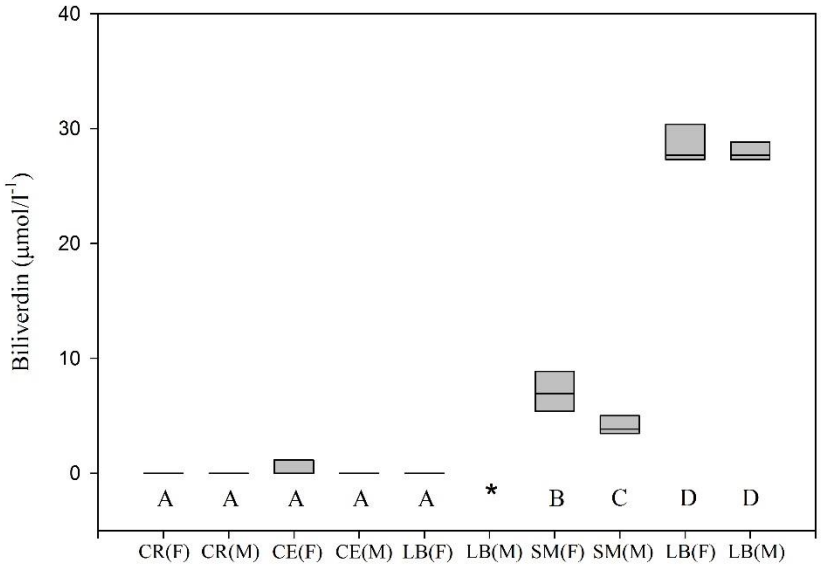


Figure 4.8: Plasma biliverdin quantification species comparison including *Ctenolabrus rupestris* (CR), *Symphodus melops* (SM), *Centrolabrus exoletus* (CE), *Labrus bergylta* (LB), and *Labrus mixtus* (LM). Gender is defined as Male (M) and Female (F). * denotes missing gender. Different subscript letters denote differences in mean levels.

Qualitative absorbance spectra demonstrated the BV distribution between species and genders using the diagnostic 650-670 nm peak. BV was clearly present in both *S. melops* (Fig. 4.9(b)) and *L. bergylta* (Fig. 4.9(e))) genders, and was absent in *C. rupestris* (Fig. 4.9(c)), and female *L. mixtus* (Fig. 4.9(d)). The *C. exoletus* (Fig. 4.9(a)) signatures were broad and of low magnitude. No *L. mixtus* males were available at the time of study.

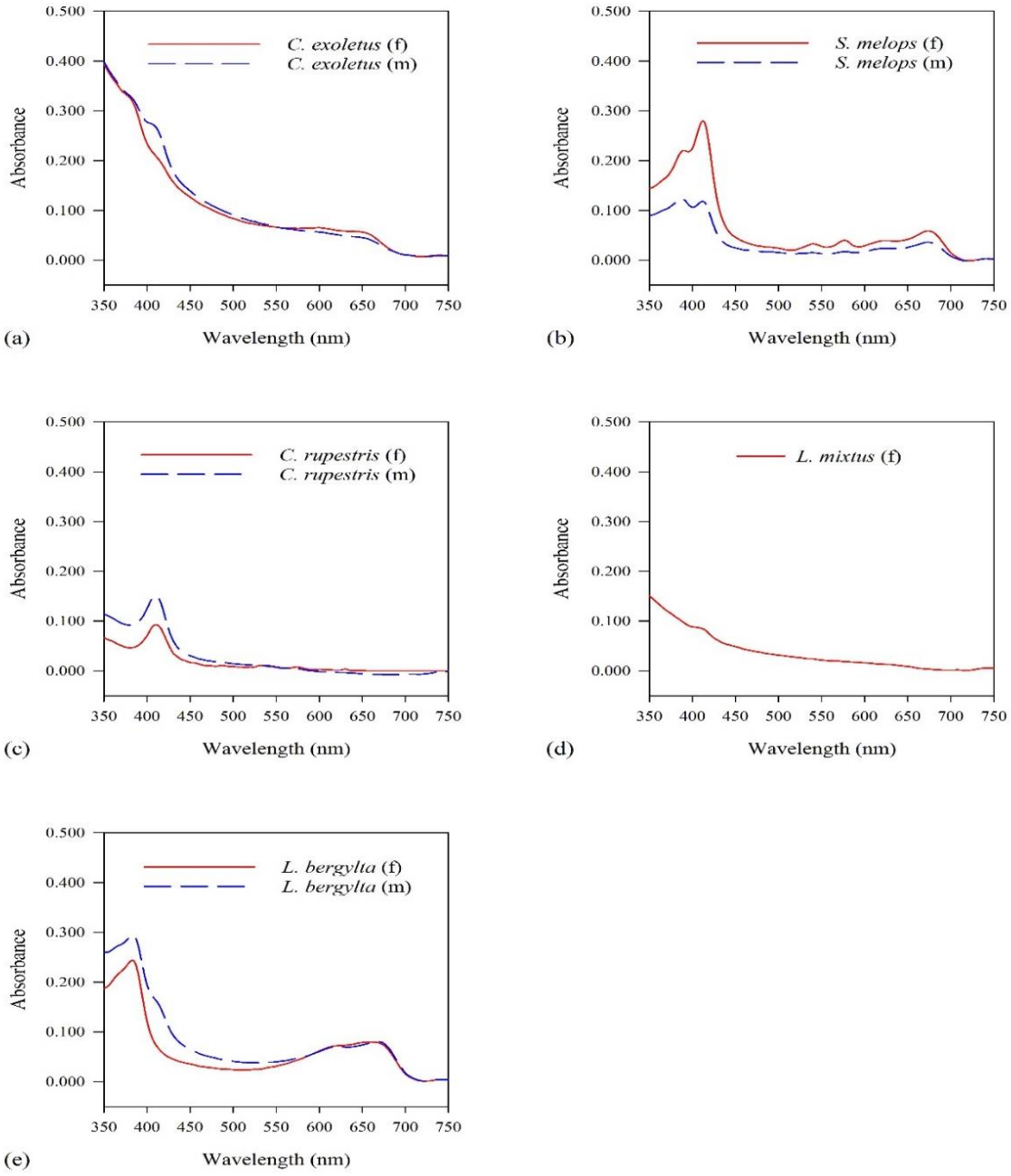


Figure 4.9: Gender specific absorption spectra (300-750 nm) in **(a)** *Ctenolabrus exoletus*, **(b)** *Symphodus melops*, **(c)** *Crenilabrus rupestris*, **(d)** *Labrus mixtus*, and **(e)** *Labrus bergylta*.

For the cross species comparison of plasma abundance (Fig. 4.10), crude plasma was pooled within species then homogenised, and the electrophoretic method was applied to *L. bergylta* (LB), *C. rupestris* (CR), *S. melops* (SM), *C. exoletus* (CE) and *L. mixtus* (LM).

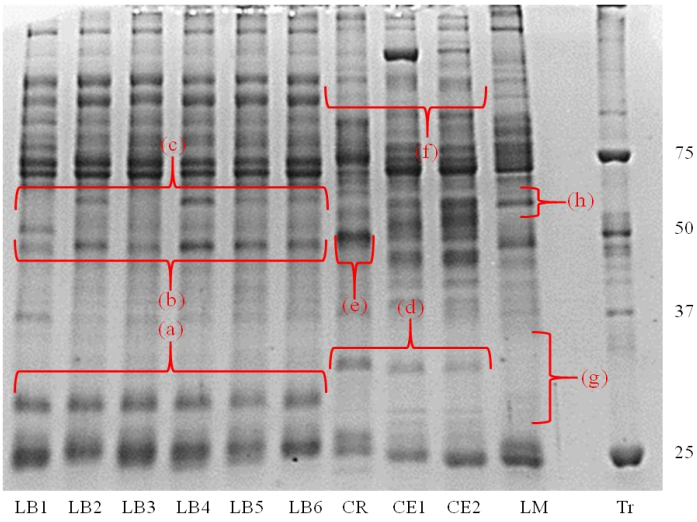


Figure 4.10: Denaturing gel electrophoresis in relation to species *Labrus bergylta* (LB), *Crenilabrus rupestris* (CR), *Ctenolabrus exoletus* (CE), and *Labrus mixtus* (LM). Individuals within species are denoted by an additional numerical descriptor. Letters and brackets in red are regions of interest discussed hereafter. Molecular weights shown in the tracking lane (Tr) are kDa.

The first 6 lanes (LB1-LB6) showed good agreement in the proteins present for the most part with some variation in relation to the 28 kDa (BV associated) band intensity (Fig. 4.10(a)). There were some differences in the 50kDa region within the LB group with a double band in LB1 and LB4 (Fig. 4.10(b)), and additional bands at approximately 60 kDa in LB2, LB4, LB5 and LB6 (Fig. 4.10(c)). CR and CE are similar in that the 28 kDa band is absent in both additional bands at around 45 kDa (Fig. 4.10(d)), with additional disparity at 50 kDa (Fig. 4.10(e)) and 80 kDa (Fig. 4.10(f)). CE1 had a strong additional band at 85 kDa. LM was noted to have no proteins present in the 30 kDa region (Fig. 4.10(g)), but shared the distinct band at 60 kDa (Fig. 4.10(h)) with LB 2, LB4, LB5 and LB6.

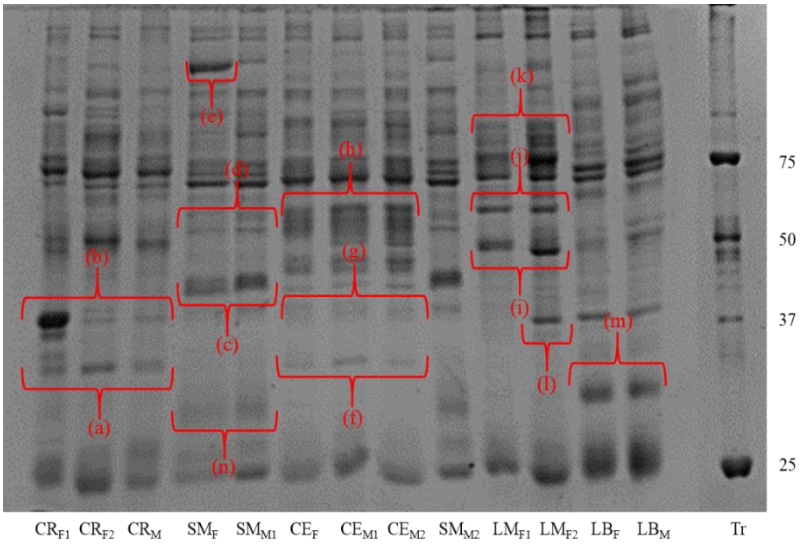


Figure 4.11: Denaturing gel electrophoresis in relation to species including *Crenilabrus rupestris* (CR), *Symphodus melops* (SM) *Ctenolabrus exoletus* (CE) *Labrus mixtus* (LM) and *Labrus bergylta* (LB). Subscript letters denote gender as Female (F) and Male (M), and numbers represent replicates of gender within species. Molecular weights in the tracking lane (Tr) are kDa.

The exploration of electrophoretic mobility was also extended to include the binary genders of each species (Fig. 4.11). In comparing *C. rupestris* genders, the male (CR_M) was similar to that of the females (CR_{F1} and CR_{F2}), but demonstrates a double band in the 30 kDa region (Fig. 4.11(a)) with a comparatively stronger intensity at the 37 kDa bands (Fig. 4.11(b)). *S. melops* plasma shows some differences relative to *C. rupestris* in that the 30-37 kDa and 50 kDa bands are absent, and there are additional signatures at approximately 40 kDa (Fig. 4.11(c)), and 60 kDa (Fig. 4.11(d)). In comparing the gender specific motility patterns of *S. melops*, the distributions of the bands are very similar but the female (SM_{F1}) demonstrates an additional band at the 85 kDa region (Fig. 4.11(e)), and males (SM_{M1} and SM_{M2}) show a more intense band at the 40 kDa signature (Fig. 4.11(c)). There were no species specific gender differences in the migration patterns of *C. exoletus*. The electrophoretic template does share some similarities with *C. rupestris* wherein there are single bands at 30-35 kDa region (Fig. 4.11(f)) and double bands at 37 kDa (Fig. 4.11(g)), but there is divergence around 50 kDa (Fig.

4.11(h)). *L. mixtus* share characters with *C. rupestris* at 50 kDa (Fig. 4.11(i)), and *S. melops* at 60 kDa (Fig. 4.11(j)) but the 75 kDa region (Fig. 4.11(k)) appears unique to the species. It is notable that although the *L. mixtus* individuals were both female there was an additional band in LM_{F2} (Fig. 4.11(k)) which is not present in LM_{F1}, but is common to the other species. The *L. bergylta* samples have many similarities to the migration patterns of the other species in that there were proteins present at 37 kDa and the 75kDa region as seen across all species, and the bands at 50 kDa as seen in *C. rupestris*, *C. exoletus* and *L. mixtus*. There were no gender specific differences in the electrophoretic templates of *L. bergylta* plasma. All of the species and genders show protein fragments in the 25 kDa region with some evidence of double bands but there are some distinctions of note with additional signatures appearing at 28 kDa in *L. bergylta* (Fig. 4.11(m)), and *S. melops* (Fig. 4.11(n)). This band was most intense in *L. bergylta*. In summary, the 28 kDa protein which is proposed to associate with BV was present in the species which the previous chapter established as hyperbiliverdinaemic which are known to undergo sex change, but were absent in the gonochoristic species.

4.0 Discussion

The association between BV and an endogenous protein moiety was supported by the mobilisation patterns in the $AmSO_4$ experiments. Plasma BV was noted to demonstrate co-mobilisation with the protein fraction of the supernatant under the parallel processing method. Additionally, almost 100% of the pigment was associated with the protein of interest in the coinciding sequential fraction. From this, it was concluded that there was a strong association between the pigment and the macromolecular component. The UV reactive gel sample was also of interest as similar observations (Bada, 1970; Mudge & Davenport, 1986) would further support the chromogen as BV, and strengthens the argument that the interaction serves to protect the complex structure as the sample had been processed through highly denaturing

condition but retained the pigment. However, as numerous gels were run with identical conditions, and only two showed this response (one of which was the second sampling) it suggests that there may be a seasonal effect, which is potentially masked by storage degradation. Further to this; in considering the cross-species analysis of BV distribution which is present in *S. melops, L. mixtus* and *L. bergylta,* and the proposed binding protein which was present in *S. melops* and *L. bergylta,* supports the proposed association with sex changing species. It is notable that the protein was absent from female *L. mixtus* in this study, but in considering only males express BV (Abolins, 1961), and none were available at the time of study, the contrasting absence of bands in the 27-50 kDa range makes interpretation difficult within that species, but does not affect the working hypothesis.

Comparison of BV mobilisation during ionic fractionation with electrophoretic mobilisation patterns, and consideration of similar studies (e.g Yu et al., (2007)) revealed that the 28 kDa band of the 30% $AmSO_4$ fraction was of interest. It is of note that the 25 kDa band also fluoresced (Fig. 4.7), which suggests that BV would also be present in that fraction, but as the 25 kDa band was present in all of the species and genders surveyed, whilst the 28 kDa band was noted as collocated with the protogynous HBV species (Fig. 4.10 and Fig. 4.11), the higher molecular weight protein was the focus of interest. However, with later consideration of Yamaguchi (1971) who demonstrated that similar proteins across a range of species vary in size from 20-100 kDa and can comprise multiple subunits therefore the 25 kDa fragment may represent a further component of the larger polypeptide carrier. The low consensus scores which were mainly single peptides per protein is an issue as the general consensus is that conservative recommendation is for ≥2 peptides for a positive identification (Carr et al., 2004). However, in agreement with Higdon & Kolker (2007), there are numerous factors including low concentration in the sample, few tryptic peptides an a small protein, or indeed masking by other more expressed proteins. There is always the possibility that the input sequence belongs

to a distantly related or *de novo* protein but the experimental design did not allow for a full exploration. The work of Higdon & Kolker (2007) is of great interest with reference to using predictive modelling to discern the probability of a single peptide sequence being a false positive (98% accuracy) but this would have to have been considered as part of the protocol from inception. Further work is necessary.

However, assuming that the peptide hits were accurate, subsequent comparison of the internal peptides with established protein sequences (NCBI database; www.ncbi.nlm.nih.gov) identified a range of candidates which included light chain immunoglobulins (IgL) from *S. aurata*, *L. crocea*, *T. bernacchii* and *A. fimbria*. Until recently, IgL was poorly understood in teleosts but the mass was in good agreement with southern bluefin tuna (*Thunnus maccoyii*) Castelnau) (L. 1758) (~28-29 kDa) (Watts et al., 2001), rainbow trout (*Oncorhynchus mykiss*) (Walbaum 1792) (~24, 26 kDa) (Salinas et al., 2011), *S. salar* (~25, 27 kDa) (Havarstein et al., 1988), European perch (*Perca fluviatilis*) (L. 1758) (~27-30 kDa) (Whittington, 1993), and channel catfish (*Ictalurus punctatus*) (Rafinesque 1818) (Salinas et al., 2011). From this, it was determined that IgL was most likely present by collocation and an extensive review of relevant literature revealed no evidence of direct association with BV. This left numerous matches with C3 and C3-like peptides from a similarly diverse range of species (Table 4.1), and Apolipoprotein A-1 (ApoA1) from *E. akaara.* In contrast to mammals, teleosts demonstrate multiple forms of C3 but the general structure is similar (Zarkadis et al., 2001). From this, and given the migration of the C3 fragments, these would be most similar to the α2 chain activation peptide (~25 kDa) (Bokisch et al., 1975), but similarly to IgL the molecular weight would result in electrophoretic co-location. Research around the complement system did reveal links between C3 and BV (Bisht, 2014), but in view of ApoA1 (together with ApoAII) constituting the most abundant circulating protein in teleosts (Braceland et al., 2013), and the 27 kDa bands comparative magnitude under electrophoresis, in conjunction with further investigation

identifying direct interactions between ApoA1 and BV as explained below, C3 was ruled out in favour of ApoA1 but will be revisited later in the discussion.

L. bergylta plasma BV was bound to a moiety of similar molecular weight to the 27 kDa BV binding protein named sandercyanin by Yu et al., (2007). It is notable however, that although both Yu et al., (2007) and the present study are in good agreement that the protein moiety was of the extracellular lipocalin protein superfamily, and comprised subunits of similar size, they differ regarding identity. Specifically, the internal peptides of the bound protein in *L. bergylta* were similar to those of *E. akaara* ApoA1, whereas Yu et al. (2007) identified the *S. vitreus* protein internal peptides as most related to Apolipoprotein D (ApoD) from zebra fish (*Danio rerio*) (Hamilton 1822), Guinea pig (*Cavia porcellus*) (L. 1758), *M. musculus* and *H. sapiens*. The shift in BV specific λmax from 660 nm in *L. bergylta* (Clark et al., 2016) to 630 nm in *S. vitreus* (Yu et al., 2007) is most likely a reflection of this structural difference in the carrier protein. Such disparity is however characteristic of the lipocalin superfamily (Wang et al., 2007), as they are generally defined on the basis of sequence and structural similarity and so have grown to encompass a large cohort (Flower, 1996). Hence, the group tends to have low pairwise conservation between species, and comparison identities are commonly below the 20% threshold for reliable alignments (Flower et al., 2000).

As a demonstration of the degree of heterogeneity, even in humans there are 28 recorded Apolipoprotein A size variants (Marcovina et al., 1999). Similarly, 11 ApoA1 genes and mature protein products have been identified in *A. japonica* which ranged in size from 23-29 kDa (Choudhury et al., 2011). Despite this, the predicted secondary structures of the group are surprisingly similar (Concha et al., 2004). To illustrate, although the *A. japonica* ApoA genes nucleotide sequences were divergent, the structural organisation is remarkably similar to the mammalian counterpart (Choudhury et al., 2011). From this, all lipocalins therefore retain characteristic motifs which form the basis of inclusion wherein kernel lipocalins share three

conserved sequence motifs, and outliers would typically have one or two in common (Flower et al., 1993; Flower et al., 2000). Thus, it is highly probable that the disparity is symptomatic of evolutionary divergence between species in conjunction with specific adaptations to alternate ecological drivers (Wang et al., 2007), but preservation of the overall structure conserves functions across evolutionary distance (Concha et al., 2004). In agreement with Choudhury et al. (2011), species specific whole genome duplications in teleosts has evolved a series of apolipoproteins with diverse functional domains which form a complex transport system for endogenous and exogenous lipids around the tissues.

There are numerous considerations supporting the *L. bergylta* chromoprotein as being a lipocalin, and similarly, some of the inherent structural properties and functionalities of a lipocalin moiety would confer the multi-functionality regarding both BV, and the BV-chromoprotein macromolecule as described. To explain, the lipocalin superfamily are a structurally and functionally diverse extracellular proteins which are involved in diverse physiological functions including binding small hydrophobic molecules, attaching to cell surface receptors, forming complexes with other macromolecules to promote physiological processes, transporting retinol proteins from the liver to target sites, olfaction, pheromone activity, immunomodulation, and the regulation of cellular homeostasis (Wang et al., 2007). Interestingly, Yamaguchi (1971) first noted that *A. Japonica* BV macromolecules shared characteristics of both BV binding proteins and lipocalins, but the specific identity and mode of association remained cryptic. Chapter 3 noted that difficulty separating BV from the protein moiety except under highly denaturing conditions was indicative of covalent bonding (Rudiger, 1970). This was demonstrated in the chromoproteins expressed in the muscle tissues of *C. undulatus* (Yamaguchi & Matsuura, 1969), the scales of some Scaridae (*C. ovifrons, S. cyanognathus and S. gibbus*) (Yamaguchi, 1971), and the skin and fins of *C. tinca* (Rudiger, 1970), but these appear to be the only such associations confirmed in teleosts to date. To

illustrate, the BV chromoprotein of *A. japonica* was found to be associated by hydrogen bonding and weak ester linkages (Fang, 1986). Similarly, the chromoprotein of *C. analis* is in free form, and the association is not covalent (Fang & Bada, 1988). With further consideration of Rudiger (1970), the described difficulty in isolating *L. bergylta* plasma BV (Chapter 2), and the inaccessibility of BVR to process the BV substrate (Chapter 3) the data could be indicative of covalent bonding but this remains to be proven. In agreement with Fang & Bada (1988), the most likely explanation is the protein complex envelops the BV in the hydrophobic carrier pocket of the protein, and that this association causes a conformational change which enhances bond strength greatly.

This is reflected in the common general structure of lipocalin folds highly symmetrically organised scaffolds comprising eight single stranded anti-parallel β-sheets which turn back on themselves to form a hydrogen bonded elliptical β-barrel with an internalised ligand binding site (Flower et al., 1993). Although there are no recent structure models available which show the mode of association between BV and ApoA1, the proposed association is very similar to the closely related Lipo-Prostaglandin synthase (L-PGDS) (Miyamoto et al., 2010). The long loop between the first two strands forms a cover that is conformationally held so that the internal ligand binding site is at the end of the barrel, while an N-terminal region α-helix covers the basal formation (Flower et al., 2000; Wang et al., 2007). Lipocalin binding sites are therefore highly variable to accommodate diverse ligands of varying shapes and size, as a function of alternate amino acid compositions in the binding pocket and scaffolding loops (Wang et al., 2007).

For example, when the binding modes of rodent Major Urinary Protein (MUP) and Lepidopteran BBPs are compared, the former binds its small hydrophobic ligands deep within the binding pocket where it is fully enclosed by the side chains, whereas the latter binds it's relatively large hydrophilic ligand at a solvent exposed region of the scaffold loop (Flower,

1996). From this, in considering that normal ligands for lipocalins are hydrophobic, and BV is inherently hydrophilic (Bulmer et al., 2008), the alternate affinities would require significant structural differences, which would also reflect in the relative amino acid compositions, and as such, influence the specific identity (Lakshmi et al., 2015). In agreement with Fang (1984), as the BV molecule would be effectively enclosed in the lipocalin fold, this could account for the inaccessibility of BV for reduction or further processing, while the structural integrity of the macromolecule would result in the strong binding characteristics as described. Further to this, returning to the comparison of MUP and BBP, as MUP is an excreted molecule which functions in external sexual signalling and mating synchronisation (Beynon & Hurst, 2003), and sequestered BBP remains systemic (Holden et al., 1987), the internalisation of the BV in MUP is such that the ligand is more stable as a reflection of intended exposure to external environments. Furthermore, the previous observation (Chapter 3) that BV may be excreted externally by *L. bergylta* may be analogous. Finally, analysis of epidermal histology would resolve the external excretion question given that *S. vitreus* have dedicated excretory cells for externalising sandercyanin, which were structurally similar to sacciform cells (Schaefer et al., 2014). Given the normal functions of sacciform cells (Salinas et al., 2011), this would support that the BV chromoprotein would have protective or regulatory functions, and explain some of the previously noted relationship with colour phenotype.

ApoA1 is best known as the major constituent of High Density Lipoproteins (HDL) and is active in the reverse transport (efflux) of cholesterol from the tissues to the liver for recycling or excretion (Delcuve et al., 1992), but has also recently been noted as an effector of innate immunity in teleosts (Concha et al., 2004). Several 25-29 kDa apolipoproteins have arisen in the fish evolutionary lineage with lipid binding properties and diverse functional domains associated with complex systems for transporting lipids around different tissues (Choudhury et al., 2011). The BV binding protein in *L. bergylta* may represent a further adaptation. Related

research in avian species established that enhanced ApoA1 levels were adaptations to the peculiarities of lipid metabolisms during uninterrupted migration when stored fatty acids are the primary source of fuel (Landys et al., 2005; Roman et al., 2009). Thus, there are two predominant, and potentially inter-related factors when such properties would be highly advantageous, the processes associated with tissue remodelling during gender transition which begins at the end of the reproductive season and is complete the following spring (Talbot et al., 2012; Villegas-Ríos, 2013), and temperature dependent obligate torpor in the course of overwintering.

It is established that during the initial phase of a pro-oxidative challenge, peroxidation of non-esterified fatty acids and other classes of lipids is effectively prevented by ascorbate until depletion, when damage is detected (Frei et al., 1988). Conversely, when the highly labile non-esterified fatty acids are bound to albumin in plasma, they are fully protected in a site specific manner by the associated BR (Frei et al., 1988; Stocker et al., 1987). Notwithstanding the toxic properties of free (unconjugated) BR, the association of BR and albumin not only sequesters the molecule in a non-toxic format, it allows distribution of the pigment around the entire circulatory system and throughout the extravascular spaces (Stocker et al., 1987). Furthermore, although the anti-oxidant capacity of SA bound BR is comparatively lower than that of ascorbate, it can be demonstrated that all radicals formed are scavenged by the bound BR, which allows complete protection of the albumin bound fatty acids and also the protein itself (Stocker et al., 1987). Interestingly, it is known that SA bound BV is easier to reduce than BR in a similar complex (Claret et al., 1995), therefore suggesting better protection in a BV system. From this, and considering the association between the pigment and the HDL transporter ApoA1, if BV is primarily acting as a protein bound antioxidant analogous to the BV binding proteins as described in Lepidoptera, it would allow *L. bergylta* to develop an

extensive reservoir of bioavailable amino acids with endogenous protection as a precursor to undergoing gender transition and facilitating the extensive tissue remodelling required.

Ellis & Poluhowich (1981) reported a similar system in the European eel (*Anguilla anguilla*) (L. 1758) and *A. japonica* which both have green plasma a result of a BV-glycoprotein-lipid complex. Observations that the pigment varied in relation to season, and difference in wild populations relative to captive groups then led to discovery that the concentrations correlated with ontogeny as diadromous life cycles means they cease feeding during the final (mature) stage with a concurrent reduction in the plasma content of BV (Ellis & Poluhowich, 1981). Hence, it is possible that the BV-lipocalin-lipid complex was acting as a storage molecule to facilitate the shift to the prolonged state of altered metabolism in adult *A. japonica*. Although the SAH (Erisman et al., 2013; Muñoz & Warner, 2003) does not consider this, the accrual of such resources to a level which would allow gender transition associated tissue remodelling may also be a factor in determining the minimum size at which induction can take place. This could potentially be resolved by using hormonal induction methods which are established in similar species (Bhandari et al., 2003), applied across a group of *L. bergylta* using a size gradient to test efficacy, but the specific effect would be difficult to measure as the hormonal induction methods could potentially bypass many of the internal regulatory systems. Further work is required.

Similarly, with reference to teleost ApoA1 reflecting the function of SA, if the BV is acting analogously to BR and albumin in humans, in considering that the liberation and deposition of lipids occurring during ovarian involution and testicular development during gender transition occur simultaneously (Muncaster et al., 2013), and in close proximity (Liarte et al., 2007), with the hyperlipidaemic state of the degenerating and remodelling tissues primed for oxidation and propagation of deleterious reactions (Garris, 2005), and as germ line cells are especially sensitive to oxidative stress (Bisht, 2014), having an extensive endogenous store of

an antioxidant molecule such as BV, which is readily available to scavenge any pro-oxidative molecules would also be highly advantageous. As a note, it may be of interest that HDL particles are known to demonstrate two main conformations, discoid HDL has the ApoA1 arranged in a double belt around the circumference, whilst spherical have a trefoil arrangement (Lund-Katz & Phillips, 2010). Structurally, in addition to stabilising the HDL particle, externalisation of the protective moieties would therefore shield the internalised lipid core (Babin & Vernier, 1989).

Hence, if BV is indeed acting as an endogenous antioxidant which buffers against oxidative processes incurred during transition, this would explain the mobilisation pattern in the transitional individuals relative to the binary genders as described in the previous chapter, and is supported by the presence of BV and the BV binding proteins in the protogynous species (i.e. *L. bergylta* and *S. melops*), and the absence of both in the gonochorists (i.e. *C. rupestris*, *C. exoletus*). Although it is always difficult to interpolate between experimental observations and physiological mechanisms, the spike and recovery data (Chapter 2) may be of interest with reference to this as the increase beyond 100% would suggest the presence of free BV in solution triggers mobilisation of the conjugated fractions into solution therefore increasing the local concentrations of BV independent of HO induction. As a note, in considering the depletion of BV must occur faster than HO produces the pigment during the inversion process (Chapter 3), this would confer a major advantage as the incurred oxidative stress of tissue remodelling during transition must therefore exceed the native anti-oxidant system. Hence, bearing in mind that fish are poikilothermic animals and as such have high degrees of unsaturated fatty acids (Babin & Vernier, 1989), which are inherently sensitive due to double bonds adjacent to methylene groups with a tendency to combine with peroxyl radicals and propagate a chain reaction by further abstraction of H^+ from neighbouring molecules (Repetto et al., 2012), the proposed enhancement of the anti-oxidant template with the BV-ApoA1 chromoprotein would

be highly advantageous. Interestingly, astaxanthine in Salmonids and α-tocopherol in trout are circulated by identical means and are thought to act in an analogous manner to that proposed for the BV complex in *L. bergylta* (Babin & Vernier, 1989).

In addition to the direct physiological roles for BV and ApoA1 as described, both have recently been linked to regulation of the teleost immune system (Choudhury et al., 2011). Although degeneration is for some part controlled by normal apoptotic signals and processes, there is also progressive necrosis which proliferates as inversion continues (Liarte et al., 2007). The cell structure disintegrates and the intracellular components are released along with Damage Associated molecular Patterns (DAMPs) (Cho & Seong, 2009) which then induce inflammation and lead to further localised cytotoxic damage (Medzhitov & Janeway, 2002). DAMPs are then recognised by innate immune system receptors including Toll-Like Receptors (TLRs) leading to transcription of Nuclear Factor-ƙB (NF-ƙB) and activation of the complement system (Cho & Seong, 2009). It is therefore of great interest that both ApoA1 (Zsila & Mády, 2008), and BV itself (Bisht et al., 2014) moderate this response at various levels. Initially ApoA1 is known to bind to the released hydrophobic portions and can therefore effectively 'quench' the pro-inflammatory DAMPs before TLR detection (Cho & Seong, 2009). Further to this, ApoA1 was found bound to the central C3 component in *G. morhua* (Magnadottir & Lange, 2004), and BV is known to modulate C5aR (Bisht et al., 2014). Thus, not only does ApoA1 inhibit activation of the inflammatory pathway, as ApoA1 and BV interact with the C3 and C5 anaphlotoxins, they effectively regulate the cascade (Magnadottir & Lange, 2004). Thus, in addition to potentially serving as a self-protecting reservoir of amino acids to facilitate tissue remodelling, and an endogenous source of antioxidant molecules, as the breakdown of initial phase gonad structures could be seen as tissue necrosis while expression of terminal phase cytological phenotype could also be considered non-self or

abnormal tissues (Medzhitov & Janeway, 2002), the proposed immunomodulatory properties of BV and the ApoA1 carrier would not only be advantageous, but most probably essential.

As mentioned previously, there are other hypotheses including torpor during overwintering which must be considered regarding the candidate protein and its proposed active properties. It is initially notable with reference to high latitude protogynous species that as they most commonly induce inversion at the end of the reproductive season and the process can take around 4 months (Muncaster et al., 2013), sex change and the associated processes normally take place overwinter. Returning to the inherent oxidative susceptibility of poikilotherms, as acclimatisation to colder temperatures is associated with cyclical patterns of elongation and de-saturation of phospholipids to maintain the plasma membrane fluidity, then overwintering fish are naturally primed for oxidative processes due to the increased sensitivity (Babin & Vernier, 1989). Although the phenotypic expression of hibernation varies greatly between species (Cotton & Harlow, 2010), it is universally recognised that dormancy (hypometabolism) is a physiological adaptation to maximise survival potential during unfavourable environmental episodes (Kelly et al., 2014). This behaviour is perhaps best known in several mammalian orders, but has also described in teleosts including *T. adspersus* and *C. rupestris* (Prendergast et al., 2002), and is suggested by anecdotal evidence in the *L. bergylta*, but remains to be confirmed experimentally. When respiration is minimal, the mitochondrial electron transport chain is disrupted (Storey & Storey, 2004). Leakage from mitochondrial matrices generates superoxide ions which react to form hydroxide radicals (Krivoruchko & Storey, 2010). As this mild oxidation of soluble proteins greatly enhances proteolytic susceptibility, and decreased metabolism causes aggregation of such proteins, further to the enhanced lability of the phospholipid fractions due to the preponderance of elongated and desaturated fatty acids (Babin & Vernier, 1989), the system is essentially primed to incur major damage upon reperfusion as the oxidative potential is increased (Almroth et al.,

2005). Further to this, prolonged periods of food abstinence (low insulin/high glucocorticoids), and inactivity (lack of neural input) cause an overall loss of muscle tissue due to decreases in protein synthesis combined with increased protein degradation (Cotton & Harlow, 2010). Thus, where the aforementioned processes are coupled to the pro-oxidative hyperlipidaemic transitional gonad in protogynous species such as *L. bergylta*, the oxidative potential is increased exponentially with the amount of substrate available (Page et al., 2009).

Torpor in teleosts is different to that of hibernation in that obligate metabolic reduction is correlated with declining water temperature (Campbell et al., 2008) in contrast to facultative downregulation in estivatory mammals (Ni & Storey, 2011). During this period, fish remain inactive, cease feeding, reduce protein synthesis, and stop growing (Campbell et al., 2008). Previous studies in hibernatory species have identified that transition from a hypo-metabolic state (torpor) to arousal is equivalent to a massive ishaemic reperfusion episode (Sayer & Davenport, 1996). Even relatively brief periods of torpor can be fatal to cells in highly oxidative tissues such as the heart and brain (Page et al., 2009). Thus, as such species remain resilient to skeletal muscle atrophy as predicted by traditional (non-hibernatory) disuse-atrophy and starvation models (Cotton & Harlow, 2010), they must have evolved alternate means of absorbing the oxidative damage accumulated as a by-product of altered metabolism (Cotton & Harlow, 2010). However, it has been shown that the intracellular anti-oxidant profiles of *C. rupestris* were not associated with upregulation of endogenous anti-oxidant enzymes in the major oxidative tissues (Page et al., 2009). This is somewhat intuitive as the transcriptional machinations required would necessitate significant departure from a supressed hypo-metabolic state as described (Campbell et al., 2008), but is of interest as it potentially explains some advantage of the additional energetic expense in protogynous species managing and accumulating an endogenous radical scavenging anti-oxidant such as BV as the pro-oxidative stimuli of transition combined with torpor could exceed the natural abrogative mechanisms

causing far reaching physiological damage. Thus, if we consider that the normative physiological response to such oxidative stress would be activation of the HO-BVR axis, that the enzymes are downregulated during torpor, and the protective effects are known to be mediated by the downstream products, then accumulation and management of BV by association with ApoA1 would confer the benefits of HO activation without disturbing iron homeostasis, and represents an endogenous antioxidant source which would be entirely functional during metabolic suppression. From this, and considering the association of HBV with sex change, it would appear that BV and BV binding proteins are an evolutionary adaptation to facilitate protogynous inversion concurrent to an altered state of metabolism during temperature dependent torpor in high latitude species. Similarly, the cessation of normative metabolism during winter could potentially explain the time difference of transition in temperate species such as *L. bergylta* relative to tropical counterparts, but this is considered further in the following chapter.

In closing, and to draw these thoughts together, fish are constantly subjected to contact with a range of microorganisms and pathogens where the aquatic media facilitates the transport and growth of opportunistic microflora (Tort et al., 2003). It is of note however, that their immune system is also conditioned by the poikilothermic state (Tort et al., 2003). Hence, in addition to cessation of physiological functions during torpor, cell mediated and humoral immune responses to pathogens are also compromised and the fish are subject to cumulative immunological insults upon arousal when the temperature exceeds the thermostatic immune response initialisation threshold (Prendergast et al., 2002). Thus, it is relevant that ApoA1 was demonstrated to inhibit growth of Gram positive and Gram negative bacteria at micromolar concentrations, suggesting they may be key effectors in teleost systemic innate responses (Concha et al., 2004). This putative association relates to previous observations regarding *L. bergylta* in a number of ways. In considering the phenotype observations (Chapter 3), although

there was no significant difference between the variants with the exception of those that were Green, as external secretion of the BV complex was observed in all populations of the original study (Chapter 3) as part of a response to wounding (pers. obs), this would support additional functionalities when the macromolecule is externalised. Further to this, and returning to structural considerations, although the primary form of ApoA1 is highly divergent between species, the secondary and tertiary structures are remarkably similar in that all share several amphipathic α-helices held in a horseshoe formation (McManus et al., 2000). As this includes a C-terminal cationic anti-microbial peptide in European carp (*Cyprinus carpio*) (L. 1758), then limited proteolysis would potentially contribute to enhancing innate immunity through bactericidal activity (Concha et al., 2004). Furthermore, as anti-inflammatory, anti-viral and anti-bacterial activities have been described in mammalian lipoproteins, the regulated proteolytic release of peptides from ApoA1 in the plasma and mucous of teleosts would potentially constitute an extrinsic 'point of contact' innate immune response allowing the fish to fight infection while remaining in torpor, and at least in part abrogate the physiological shock of restoring the immune system functions concurrent to the IR shock of arousal. Finally, as biliverdin is also photo-reactive (McDonagh, 2010), it may serve to protect the externalised protein from UV-B oxidation.

5.0 Conclusions

This chapter began with a realisation that BV has a fundamentally diverse range of functions which must be considered not only at the molecular level with associated properties, but must be expanded to include the massively complex and equally diverse array of interactions conferred by macromolecule formation. Based on the evidence and with

consideration of the relevant literature these experiments suggest that BV and ApoA1 are potentially key components of facilitating gender inversion and winter torpor by allowing *L. bergylta* to generate stores of bio-available HDL and amino acids with intrinsic anti-oxidant protection, and by absorbing and controlling the oxidative stress associated with tissue remodelling processes, environmentally driven states of altered metabolism, and systemic reperfusion episodes. However, in considering the additional anti-bacterial and anti-microbial properties as well as being important effectors of innate immunity and the primary defence of epithelial barriers in teleosts (Choudhury et al., 2011), it is likely that BV, and the binding protein may be fundamental to immunological modulation and anti-inflammatory functions associated with the processes of gender transition in a high latitude temperate protogynous Labrid such as *L. bergylta*. Looking to the future of this research, the protective capacity of the association could be further validated using methods such as electrochemical reduction of the complex monitored by absorbance and circular dichroism as a means of determining the protective capacity of BV (Claret et al., 1995). Similarly, the electrophoretic method could be expanded by isolation of Apo from different tissues using electrophoresis followed by Polymerase Chain Reaction (PCR) amplification and eventual sequencing with comparison to an established database to ascertain specific identities and divergent sequences (Choudhury et al., 2011). This would also allow modelling of the tertiary structures, allow greater comparison with other species, and potentially resolve the binding site. Additionally, it would also be of interest to pursue the fluorescent properties or absence thereof of the 25 kDa and 28 kDa bands (Fig. 4.8) as co-distribution suggested BV was present in both fractions, but difficulty in repeating this experiment limits the conclusions which can be drawn and the timeframe did not allow for repeated sampling and exploration of this anomalous character. As a final note, and considering the sentiment of Dr Albert's quote in the first chapter, it would be elegant indeed

if the protein carrier which allows BV accumulation, and controls mobilisation in *L. bergylta* is similarly protected by the molecule when externalised.

Chapter 5:
Exploring hormonal induction of gender reversal in female *Labrus bergylta* with biliverdin mobilisation tracking.

1.0 Introduction

Labrus bergylta have emerged as a key resource to salmon aquaculture through proven efficacy in controlling parasitic infestations of *L. salmonis* and *Caligus* spp. (Leclercq et al., 2014a). This is due to several factors including being the fastest growing, most robust (stress

tolerant), and most active (of available species) at low temperature (Berg, 2012). Additionally, their larger size means they are more efficient at cleaning 2-4 kg salmon (Talbot et al., 2012). From this, with ~40 million smolts put to sea in the UK every year, even 1% cleaner fish stocking levels are equivalent to a 'local' annual requirement for 400,000 individuals (Talbot et al., 2012). The recommended 4% wrasse stocking ratio would be 1.6 million, and must take into account losses of approximately 7% in some sites (SARF, 2013). Hence, wild capture 'wrasse' harvests have grown from 100,000 in 1991 (Darwall et al., 1992), to having exceeded 10 million by 2010 in Norway alone (D'Arcy et al., 2013). There is no information at the species level as there are no conservation measures (D'Arcy, 2001), but *L. bergylta* are particularly vulnerable to overfishing due to a long lifespan (>25 years) as females do not spawn until 6-9 years (Grant et al., 2015), and size selective harvest of mature individuals (Talbot et al., 2012). However, *L. bergylta* production is still very much in its infancy with only three hatcheries in the UK. In contrast to the harvest figures given previously, recent figures report deployment of 6000 *L. bergylta* and 3000 *C. rupestris* from farmed populations (Fishupdate, 2014). As a result, growing demand (D'Arcy, 2001), concerns regarding wild harvest sustainability (Darwall et al., 1992), and the need for predictable supply with a known health status (SARF, 2013), means that conservation of wild stocks, and the future of wrasse utilisation in Integrated Pest Management (IPM) systems is dependent upon developing hatchery production (Talbot et al., 2012).

Presently, aquaculture companies and marine farms continue to develop technologies to commercially cultivate *L. bergylta*. Typical management is based on haremic communities of 20-30 individuals with an ideal ratio of 1:10 males in reflection of wild communities (Muncaster et al., 2013), which are maintained in controlled photo-thermal conditions (Grant et al., 2015). Histological evidence supports that gonad maturation begins in November and the breeding season occurs from April to July (Muncaster et al., 2010). *L. bergylta* are

synchronous multiple batch spawning, so reproduction follows a rhythmic succession across the season with multiple females contributing on more than one occasion in some cases (Grant et al., 2015). Although hatchery productivity is restricted by the number of active spawning harems they can establish, it is male availability (~10% wild populations) which is the main limiting factor as the probability of capture is low (Darwall et al., 1992). Furthermore, as *L. bergylta* show no apparent sexual dimorphism (Clark et al., 2016), and many of the established methods of gender identification including ultrasound, vitellogenesis and hormone levels are ambiguous (Talbot et al., 2012), it can be very difficult to identify and select males to establish appropriate ratios of spawning capable individuals. Thus, a reliable method for generating male *L. bergylta* is currently a key priority within the industry.

As monandric protogynous hermaphrodites, *L. bergylta* develop initially as females then undergo transition to male (Francis, 1992). Based on observations in the related *T. bifasciatum* (Lamm et al., 2015), and similarly sequentially hermaphroditic species (Choat & Robertson, 1975), physical size and social dominance are most likely the main primers governing transition. This is based on a "fixed" context of temporally stable, haremic, hierarchical communities comprising approximate 1:10 male to female ratios (Muncaster et al., 2013), which is typical of monandric protogynous species (Robertson & Choat, 1974). In accordance with the Size Advantage Hypothesis (SAH) (Munday et al., 2006), where a hierarchy becomes unstable (male absence), the largest and most dominant female will undergo transition to redress balance (Semsar & Godwin, 2003), and thereby increase reproductive success relative to the primary gender (Erisman et al., 2013). This is also an advantage in the creation and maintenance of harems (Choat & Robertson, 1975). Thus, sex change normally occurs between 5-14 years old (Dipper, 1979), and before reaching 40cm (Muncaster et al., 2013), but as pro-inversion stimuli also considers other factors (Muñoz & Warner, 2003), greater size may not always be pre-requisite (Munday et al., 2006).

Work in similar species has identified other factors of interest. Patterns of social organisation are based on the stability of relationships established over a period of time (Robertson & Choat, 1974). Experimental mono-sex (female) groups of lyretail anthia (*Anthia squamipinnis*) (Peters, 1885) take longer to initiate sex change than natural haremic communities subjected to male removal (Shapiro, 1980), and experimental female pairs (large vs. small) of protandrous anemonefish (*Amphiprion* sp.) and protogynous *T. duperrey* (Quoy & Gaimard, 1824) results in aggressive interactions without inversion (Shapiro, 1993). This suggests that the threshold value is not only based on male removal but also takes into account relative proportions of the behaviours given and received within the hierarchy (Baroiller et al., 1999), where minimum numbers of the primary phase are required to induce inversion after male removal (Baroiller et al., 1999; Robertson, 1981; Shapiro, 1980). Hence, where artificial communities are abstracted from natural context, and the poly-factorial governing processes uncouple, natural inversion is highly capricious.

Whilst regulatory mechanisms are variable (Pandian & Sheela, 1995), the generalised patterns of sequential hermaphroditism and associated shifts in endocrinology show commonality across species (Munakata & Kobayashi, 2010). It is known that the social and environmental cues which initiate sex change in sequential hermaphrodites are transduced by the Hypothalamus-Pituitary-Gonad (HPG) axis (Nozu et al., 2009), which is in turn regulated by the Hypothalamus-Pituitary-Adrenal (HPA) axis (Perry & Grober, 2003). Perception of permissive stimuli acts as a trigger which initially causes a behavioural shift (Godwin, 2009), followed by radical restructuring of brain (Black & Grober, 2003), and reproductive tissues (Lamm et al., 2015). Steroid hormones are the proximal actuators of inversion (Nozu et al., 2009). E_2 is essential for maintenance of the female phase (Pandian & Sheela, 1995), oocyte development and vitellogenesis (Kroon et al., 2003). T and 11 11KT are the primary androgens in fish (Perry & Grober, 2003), where T is associated with gametogenesis, and 11-KT regulates

spermatogenesis and development of secondary sexual characteristics (Kroon et al., 2003). From this, it follows that the activity of 11-KT and conversion of T to oestrogens by cytochrome P-450 aromatase is also important (Baroiller et al., 1999), but protogynous initiation can ultimately be described by a rapid decrease of E_2 as cessation of the female phase hormonal template relative to 11KT expression causes a shift in gender specific endocrinology towards the male phase (Bhandari et al., 2003). Later stages can also be typified by further reductions in circulating E_2 with concurrent increases in 11KT until 11KT dominates in the terminal male phase (Muncaster et al., 2013). Consequently, as the combination of E_2 depletion relative to 11KT levels acts as the threshold for inversion, administration of synthetic androgens and aromatase inhibitors have been shown to induce inversion in numerous species (Higa & Ogasawara, 2003; Kroon et al., 2003; Nozu et al., 2009), where they are commonly used to generate monosex cultures (Pandian & Sheela, 1995). The relative ease and success of hormone induction has led to numerous publications and development of numerous methods (Table 5.1).

Table 5.1: Established hormone administration methods.

Method	Advantage	Limitations
Oral	Hormones are dissolved in alcohol and applied to food. Requires high dosage over long period. Remains most frequent method.	Hormones degrade over time and during intestinal transport, and vary in solubility relative to the carrier therefore feed/dosage uniformity may vary. Relative size and dominance can also cause differentials in dosage. Overdose (OD) can cause deformities, or skew sex ratios towards the non-target sex. Efficacy varies between species. Single dose can result in transient change. (Budd et al., 2015; Bhandari et al., 2006; Garcia et al., 2013; Pandian & Sheela, 1995)
Immersion	Frequently used in cold water species. Cheaper than dietary method. Uses less hormone than oral methods.	Mostly applied in embryo and post hatching stages. Not applicable in the field. Requires multiple treatments/long duration. Single dose can result in transient change with spontaneous reversion. High OD risk. Efficacy varies between species. (Garcia et al., 2013; Pandian & Sheela, 1995; Pandian & Kirankumar, 2003).
Injection	Requires less hormone. Drives inversion faster than previous methods	Most laborious, expensive and skilled method. Allows dosage to be adjusted relative to body weight so less OD risk but injury and infection possible. Applicable in species resistant to previous methods. (Pandian & Sheela, 1995; Pandian & Kirankumar, 2003).
Implantation	Single intervention with a uniform release of hormone over time.	Technique requires skill. Allows dosage to be adjusted relative to body weight so less OD risk but injury and infection possible. Initial high dosage followed by slow release over time. Only applicable in fish with late stage sexual differentiation. Applicable in species resistant to oral and immersion methods. (Pandian & Sheela, 1995; Pandian & Kirankumar, 2003).

Table 5.2: Established pharmaceutical androgens including Testosterone (T), 11-Ketotestosterone (11-KT), 17α-Methyltestosterone (17α-MT), 17-hydroxy-7,17-dimethylestr-4-en-3-one (Mibolerone), 4-(5,6,7,8-Tetrahydroimidazo[1,5-*a*]pyridin-5-yl)benzonitrile (Fadrazole), 4,4'-((1H-1,2,4-triazol-1-yl)methylene)dibenzonitrile (Letrazole), and 17 α - Methyldihydrotestosterone (MDHT) with the species of interest and publishing authors.

Mechanism	**Chemical**	**Species**
Natural steroids	**T**	Bambooleaf *wrasse (Pseudolabrus sieboldi)* (Ohta et al., 2012)
	11-KT	Three spot wrasse (*Halichoeres trimaculatus*) (Higa & Ogasawara, 2003) Honeycomb grouper (*Epinephalus merra*) (Bhandari et al., 2006b)
Aromatizable substrate analogue	**17α-MT**	Hong Kong grouper (*Epinephalus akaara*) (Li et al., 2006) Greasy grouper (*Epinephalus tauvina*) (Kailasam et al., 2007) Sevenband grouper (*Epinephalus septemfasciatus*) (Tsuchihashi et al., 2003)
Aromatase inhibitors	**Fadrazole**	Honeycomb grouper (*Epinephalus merra*) (Bhandari et al., 2005) Three spot wrasse (*Halichoeres trimaculatus*) (Higa & Ogasawara, 2003)
	Letrazole	Dusky grouper (*Epinephalus marginatus*) (Garcia et al., 2013)
Non-aromatizable substrate analogue	**(MDHT)**	Hong Kong grouper (*Epinephalus akaara*) (Li et al., 2006)

Because manipulating gender by hormonal induction to produce monosex cultures is such a valuable tool in aquaculture, there are multitudes of protocols for controlling gender which focus on food fish (Pandian & Sheela, 1995). For most of these, this involves treatment of post hatching or juvenile stages as a particular phenotype demonstrates commercially important traits such as optimised growth or desired ornamentation (Pandian & Kirankumar, 2003). In principle; as this means of controlling sexual differentiation is relevant to the species, stage of development, and the supplement type (Budd et al., 2015), Table 2 focuses on protogynous species as hormone interventions to actuate sex reversal are specific to mature fish (Pandian & Sheela, 1995; Pandian & Kirankumar, 2003). The methods of application and duration of treatments are highly variable between studies.

Ohta et al. (2012) implanted T and 11-KT applied over a month to stimulate inversion *P. sieboldi*. Contrastingly, Bhandari et al. (2006b) achieved similar results using 10 mg/kg 11-KT implants for 75 days in *E. merra*. Higa & Ogasawara (2003) caused complete sex reversal in *H. trimaculatus* by supplementing diets with 150 mg/kg feed 11-KT and 100 mg/kg feed Fadrazole over a six week period, while Kailasam et al. (2007) used a 17-MT supplement estimated at 24 mg/kg body mass monthly for 9 months in *E. tauvina*. Li et al. (2006) induced reversal in cohorts of 3 year old female *E. akaara* implanted with 10 mg/kg MT, 10 mg/kg MDHT or 10 mg/kg MT plus 1.0 mg/kg Fadrazole respectively for a 4 week period, which resulted in early transition gonads in the MT group, but more advanced development in the MDHT and MT-Fadrazole cohorts. Tsuchihashi et al. (2003) compared oral administration (10 mg/kg diet) to implantation (1 mg/kg and 4 mg/kg) of MT to drive inversion in *E. septemfasciatus,* and found that the dietary supplement group and the low dose implant cohorts reverted to the female phase when the treatments were withdrawn while the high dose group were fully functioning males. Quinitio et al. (2001) tested a range of treatment levels (0, 50, 100 and 200 µg/kg feed) of Mibolerone over an 18 week period in juvenile *E. coioides* with

initial success but fish began to revert to the primary gender phase upon withdrawal therefore the timing of treatment relative to development or the dosage may have required some adjustment. Further to Higa & Ogasawara (2003); Bhandari et al. (2005), and demonstrated Fadrazole was effective to cause sex reversal in *E. merra* at implantation doses of 10 mg/kg. And finally, Garcia et al. (2013) applied 100 mg/kg Letrazole by intraperitoneal injection with fish oil as a carrier every 4 weeks (over 9 weeks) to promote sex change in *E. marginatus,* but noted that the success of induction varied depending on the season.

In summary, although there is no question that exogenous applications of androgens can be used to control gender in protogynous species, there is a distinct lack of commonality across studies with regards to all of the parameters including treatment type, administration method, dosage and duration. Hence, the primary aim of this chapter was to investigate the validity of exogenous hormonal therapies that can reliably induce masculinisation, and to expand current knowledge of the remodelling processes associated with gender inversion. Further to this, in considering the available literature regarding protogynous species, and the lack of a universal histological scale of gender progression, the study is structured such that sex change in the gonads of *L. bergylta* was tracked with the intension of creating a detailed graduated series to resolve the sequential progression of female to male reproductive tissues.

In addition to this, previous studies (Clark et al., 2016) proposed protogynous tissue remodelling processes as the major driver of biliverdin depletion based on radical sequential restructuring throughout *L. bergylta* gonads during transition (Muncaster et al., 2013), as opposed to the partitioned delineated intersex gonads common to Sparidae including *S. aurata* (Liarte et al., 2007). Although many papers describe the shifting hormonal profiles and histology of the reproductive systems of sequential hermaphrodites (Miyake et al., 2012; Muncaster et al., 2010; Muncaster et al., 2013; Nozu et al., 2009; Nozu et al., 2013), and the cytological involution of testicular tissues in protandrous *S. Aurata* (Liarte et al., 2007; Liarte

et al., 2011), there are very few (Alonso-Fernández et al., 2011; Sadovy & Colin, 1995) descriptions of protogynous inversion at such high resolution. However, the changes that occur are analogous to the pathologies of persistent non-homeostatic interstitial hypercaloric metabolic syndrome (Garris, 2004; Garris, 2005).

During this inversion, the female reproductive system cytoarchitecture becomes severely compromised as alterations in follicular metabolism withdraw maintenance stimuli resulting in pronounced hypercytolipidemia and involution of the reproductive tract (Garris, 2004). The resulting reproductive incompetence is characterised by ovarian acyclicity (Freeman et al., 2009), compromised follicular development (Drummond, 2006), reduced sensitivity and responsivity to endocrine stimuli, depressed ovarian hormone synthesis, and accelerated follicular atresia (Garris, 2005). Simultaneously, changes to ovarian cyto-architecture are described by rapid increases in intra and inter cellular lipid depositions due to interstitial perivascular escape (Garris, 2005). Ultimately, this induces structural lipoatrophy including progressive organelle dissolution with subsequent cyto-transformation to adipocyte-like dysfunctional cells which are incapable of maintaining ovarian competency, and a suppressed oxidative metabolism (Garris, 2004) which is highly correspondent with the physiology of torpor as discussed previously (Chapter 4; Section 4.0). Thus, the working hypothesis based on previous experiments (Chapter 3) was that BV acts as an abundant endogenous molecular buffer to protect the naïve testis, associated developing male phase tissues, and the nascent spermatogonia from the oxidative processes, and through controlling the inflammation associated with gonadal transition and tissue involution from the transformative female phase ovaries. From this, the secondary aim of this study was to use the structured progression of individuals across the inversion process to track biliverdin mobilisation and further elucidate any correlation between the pigment and progression of sex change in *L. bergylta*.

2.0 Materials and Methods

All practicalities of sampling and methods applied are described in Chapter 2 Section 5.

2.1 Trial 1:

L. bergylta (n=18) were selected from a population of PIT-tagged broodstock held at the Ardtoe Marine Laboratory. These animals had previously been wild caught in the Sound of Mull using prawn creels and lobster pots two years prior to their use in this study. In the interim period the fish had been held communally in a 14 m^3 tank with two confirmed males (confirmed through observed release of milt in the previous spawning season). Total Length (L_T1) (cm) and Body mass (M_B1) (g) were recorded for the 18 individuals presumed as females at the start of the trial (based on the morphometric method developed by Leclercq et al. (2014b)) with individuals being assigned at random into three treatment groups. Blood plasma was collected at commencement by withdrawing a blood sample via caudal puncture using heparinised syringe, centrifuged (1789 rcf, 5mins) and the plasma frozen on dry ice for later storage at -70 °C. Androgenic hormones were infused in cocoa butter, then implanted into the peritoneal cavity as Poursaeid et al. (2012). The first group (n=6) received 5mg/Kg non-aromatizable 17α-methyl-dihydrotestosterone (MDHT), the second (n=6) received 5mg/Kg of the aromatase inhibitor Fadrazole (FAD), and the Control group (n=6) received the implant procedure without hormone/inhibitor infusion on the 21st February 2012 with fish being reared communally in a 14m^3 tank where Astroturf spawning substrates were provided along with plastic pipes and polyethylene sheeting hides. Fish were maintained at ambient temperature (6-15 ºC through the year) with a simulated natural photoperiod where the fish were fed daily to satiation with mussels and crabs. Fish were euthanized by anaesthetic overdose on the 23rd August 2012. Blood was gathered immediately by withdrawing a blood sample via caudal puncture and

plasma separated and preserved as previously. Total Length (L_T2) (cm) and Body mass (M_B2) (g) were recorded. Specific Growth Rate (SGR) was calculated as SGR = ((Ln (M_B2)-(Ln (M_B1)*100)/Days. The gonads were excised then weighed (M_G) to 0.001g for calculation of Gonado Somatic Indices (GSI) as GSI = $100M_G$ $(M_B2\text{-}M_G)^{-1}$ and preserved in 4% formalin solution and stored for later histological analysis.

2.2 Trial 2:

L. bergylta (n=73) were wild caught in the mull of Kintyre region and held in captivity at the Machrihanish Marine Environmental Research Laboratory for one year prior to their use in this study. Fish were previously PIT tagged and based on body size and condition, only individuals which met the assumptions of the "female" morphometric definitions as described by Leclercq *et al.* (2013) were taken forward into the study. L_T and M_B were recorded at the trial start, and the population divided evenly into three size matched cohorts. This experiment tested a dual androgen application of low (5mg/Kg FAD/5mg/Kg MDHT) (n=25), and high (5mg/Kg FAD/10mg/Kg MDHT) (n=25) levels with a Control group (n=23) receiving the implant procedure without androgen infusion. Treatment began 09th July 2013 with daily feeding to satiation and simulated natural photoperiod as well as ambient thermal conditions maintained for the trial duration. A randomised intermediate sample of 21 fish was taken 01st April 2014 with representative samples (Control (n=5); Low (n=8); High (n=7)). Fish were euthanized, with blood plasma samples taken as previously described. Biometric data capture and descriptors were recorded and calculated as before. At the end of the trial on 11th July 2014, the remaining fish (n=47) were euthanized. Blood sampled were withdrawn immediately and plasma extracted and stored treated as previously. Similarly, biometric data including L_T and M_B was recorded with SGR, and GSI calculated as before. The gonads were then stored in 10%

buffered formalin for histological analysis. Standardised photographs were taken for analysis of colour and pattern phenotype using the established scale (Chapter 3).

2.3 Histology

Tissue samples were excised from the anterior regions of the gonads for all individuals, and fixed in neutral phosphate buffered formalin solution. 5µm paraffin sections were cut and stained with haematoxylin, eosin and saffron by conventional methods and examined using a light microscope. Protogynous transition was then classified across both trials and using a combination of methods developed from consideration of Nozu et al. (2009) (Phase I gender), and Muncaster et al. (2013) (Phase II gender) which established gender based on leading differentiating stage.

2.4 Chromophore quantification

Quantification protocols for plasma biliverdin were adapted from Austin & Jessing (1994). BV.HCl was dissolved in 17.5 M glacial C2H4O2 to generate 500 µmol.l-1 with serial dilution in 17.5 M glacial C2H4O2 for standards from 0 - 50 µmol.l-1 with 0.5% BSA. 500 µl distilled H2O was added to 500 µl of each standard with 400 µl 40 mM C6H8O6 and 100 µl 200 mM C2H4O2 in 1 M NaOH. Plasma samples were prepared by addition of 450 µl glacial C2H4O2 to 50 µl plasma, 500 µl distilled H2O was added with 400 µl of 40 mM C6H8O6 and 100 µl 200 mM C4H4N2O3 in 1 M NaOH. Blanks were parallel samples with barbituric acid substituted with 1 M NaOH. Samples were heated at 95 ºC for 5 mins in the dark then cooled and 2.5ml C4H9OH with 1 ml 10 M NaOH added then agitated in the dark until the reaction was complete. A two phase solution formed after centrifugation (1789 rcf, 5mins) with the diagnostic red chromophore in the lower component. The top phase was discarded and A570 of the lower phase recorded in triplicate, blanks were then subtracted from the samples. The

standard solutions were used to construct a calibration curve (Chapter 2; Section 6.5) and the BV quantifications extrapolated.

2.5 Statistics

Data resisted normalisation therefore differences between treatments, variables or stages of maturity were analysed using Moods Median test, non-parametric Kruskal-Wallis, Pearsons Chi^2 and Tukey's HSD where appropriate.

3.0 Results

3.1 Standardised histological scale

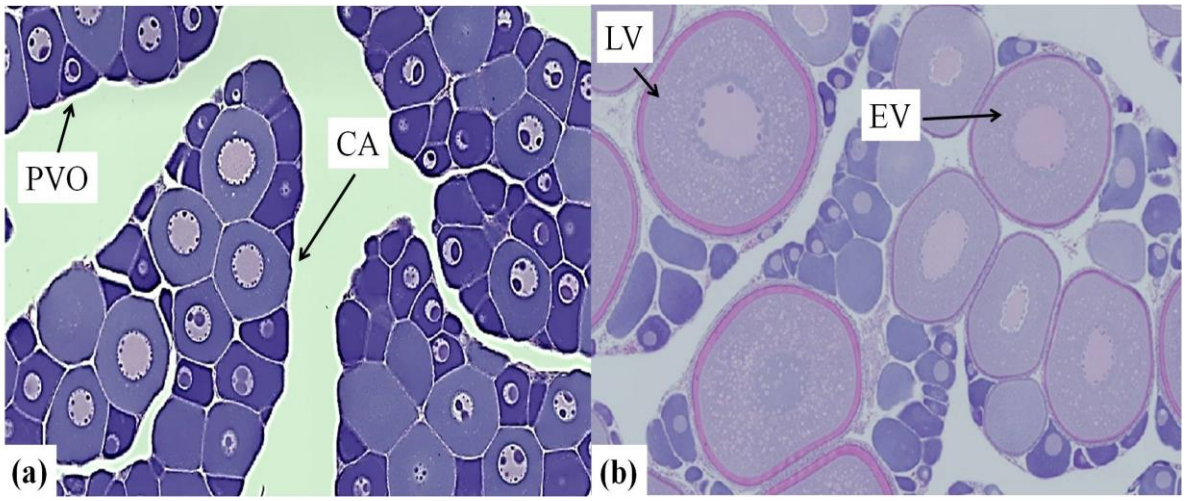


Figure 5.1: Female phase histology in *Labrus bergylta* showing: **(a)** Early maturing female phase, and **(b)** Late maturing stage. Pre-Vitellogenic Oocyte (PVO); Cortical Alveolus oocyte (CA); Early Vitellogenic oocyte (EV); Late Vitellogenic oocyte (LV).

Female gender was predominantly Pre-Vitellogenic Oocytes (PVO) with organised tissues and no evidence of male characters or spermatogenesis. Oocytes were present at stages of maturation including cortical alveolus oocytes (Fig. 5.1(a)), with some early vitellogenic and late vitellogenic oocytes showing visible yolk vesicle development (Fig. 5.1(b)). Some

oocytes were observed in the degenerative phase with evidence of atresia and post-ovulatory follicles in some (Fig. 5.2(a)), and apoptosis (Fig. 5.2(b))

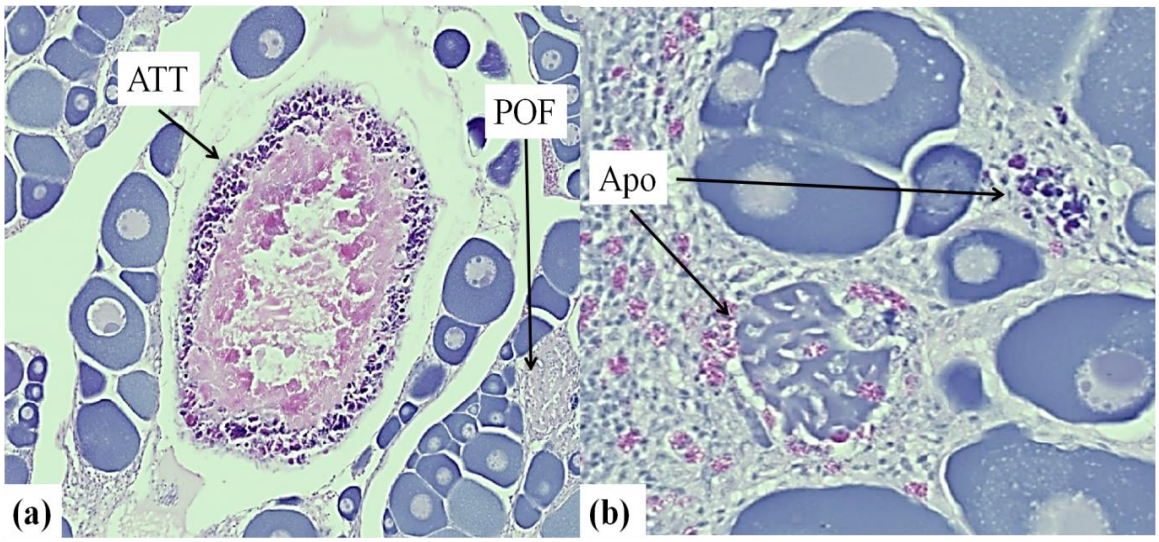


Figure 5.2: Ovarian development and Female phase histology in *Labrus bergylta* showing: **(a)** Resorbing stage and **(b)** Degenerating phase. Attretic oocyte (Att); Post-Ovulatory Follicle (POF); Apoptotic oocytes (Apo).

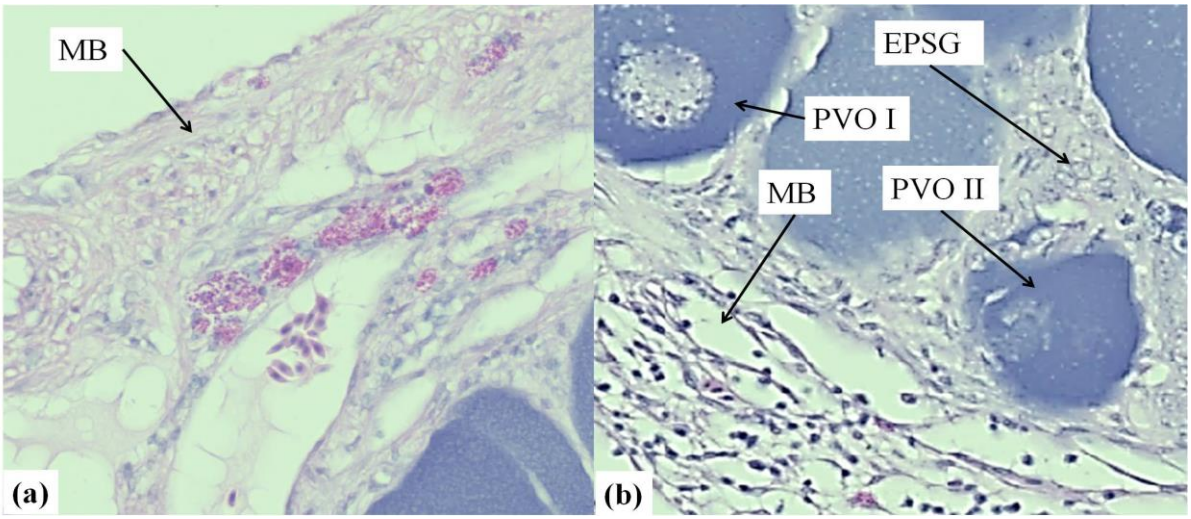


Figure 5.3: Initiation of inversion in *Labrus bergylta* showing: **(a)** Dissolution of muscle bundles and structural integrity, and **(b)** Further breakdown of the ovarian structures with developing pre-male phase characters. Muscle Bundles (MB); Pre-Vitellogenic Oocyte (PVO I); Degenerating Pre-Vitellogenic Oocyte (PVO II); Early presumed spermatogonia (EPSG).

Initiation of transition was typified by loss of structure in ovarian muscle bundles (Fig. 5.3(a)), with convolution of surfaces in PVO and the appearance of early presumed spermatogonia but no fully developed male phase characters (Fig. 5.3(b)).

Early Transition (ET) was described by proliferation of spermatogenic cells along the inner margins of transforming lamellae and degrading oocytes form yellow brown bodies (Fig. 5.4(a)), with appearance of early spermatogonia (Fig. 5.4(b)).

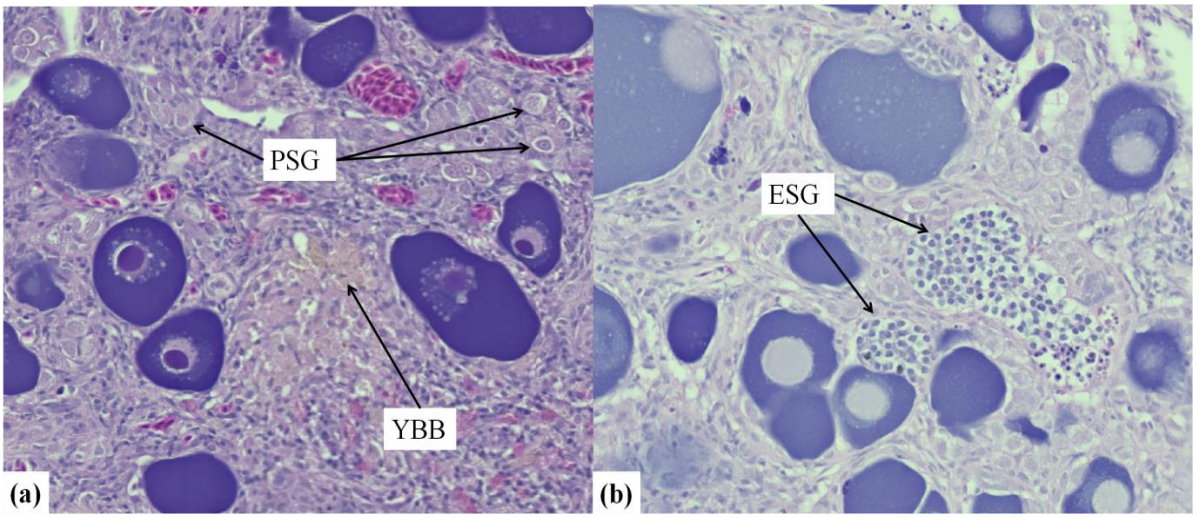


Figure 5.4: Early transition stage in *Labrus bergylta* showing: **(a)** Proliferation of primary spermatogonia and **(b)** Appearance of early spermatogonia. Primary spermatogonia (PSG); Yellow Brown Bodies (YBB); Early Spermatogonia (ESG).

Late Transition (LT) was described by further progression of oocyte degeneration and predominant spermatogenic tissues (Fig. 5.5(a)) as cysts become lobules containing advanced stages of sperm (Fig. 5.5(b)).

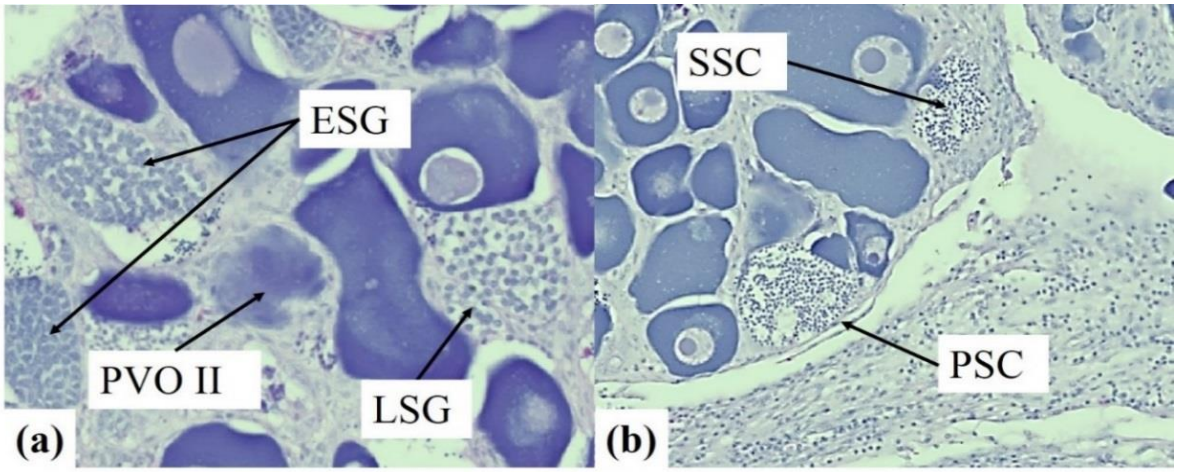


Figure 5.5: Late transition stage in *Labrus bergylta* showing: **(a)** Proliferation of primary spermatogenic tissues and **(b)** Appearance of later stages of spermatogonia. Degenerating Pre-Vitellogenic Oocyte (PVO II); Early Spermatogonia (ESG); Late Spermatogonia (LSG); Primary spermatocytes (PSC); Secondary spermatocytes (SSC).

Males were defined by fully differentiated spermatozoa (Fig. 5.6(a)) arranged in lobules along remnant ovarian lamellae with visible formation of efferent ducts (Fig. 5.6(b)).

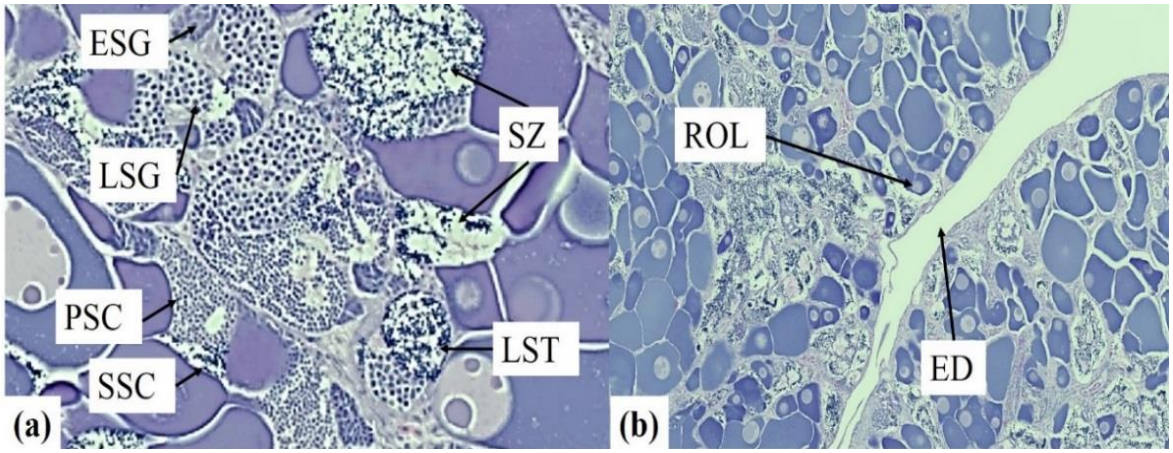


Figure 5.6: Male developmental stage in *Labrus bergylta* showing **(a)** Late spermatogenesis including fully differentiated spermatozoa, and **(b)** Progressive shift to male phase dominant structures. Early Spermatogonia (ESG); Late Spermatogonia (LSG); Primary spermatocytes (PSC); Secondary spermatocytes (SSC); Late spermatids (LST); Spermatozoa (SZ); Remnant Ovarian Lamellae (ROL); Efferent Ducts (ED).

There was a small subset of males within the study which had different gonad histology to the 'normal' intersex gonads which retain numerous PVO and reflect the female superstructure from the primary phase of development (Fig. 5.7(a)), as the testis demonstrated

complete male phase histology (Fig. 5.7(b)). Running males were defined by predominant spermatozoa which could be seen in the efferent ducts (Fig. 5.7(b)).

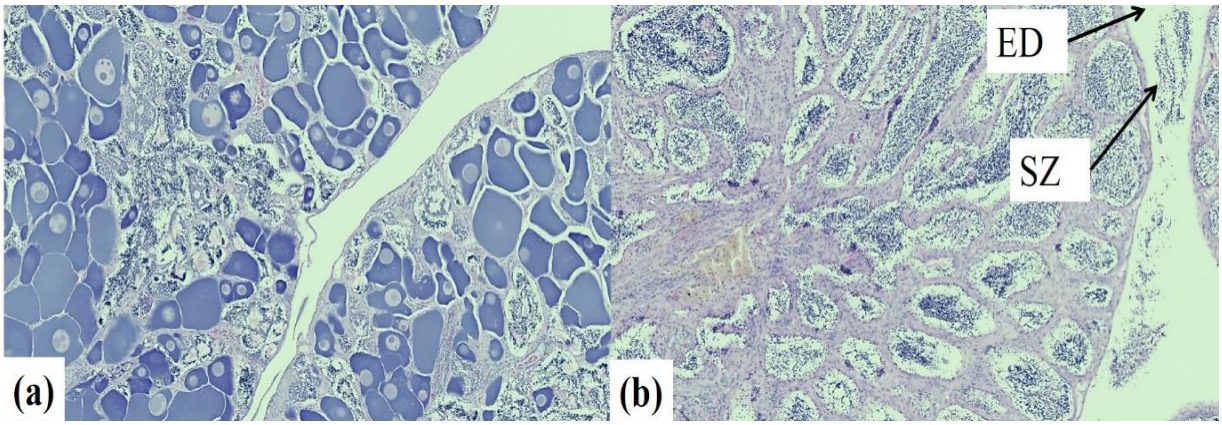


Figure 5.7: Male developmental stage in *Labrus bergylta* showing **(a)** Non-delineated intersex gonad and **(b)** Running male testis with no female characters. Spermatozoa (SZ); Efferent Ducts (ED).

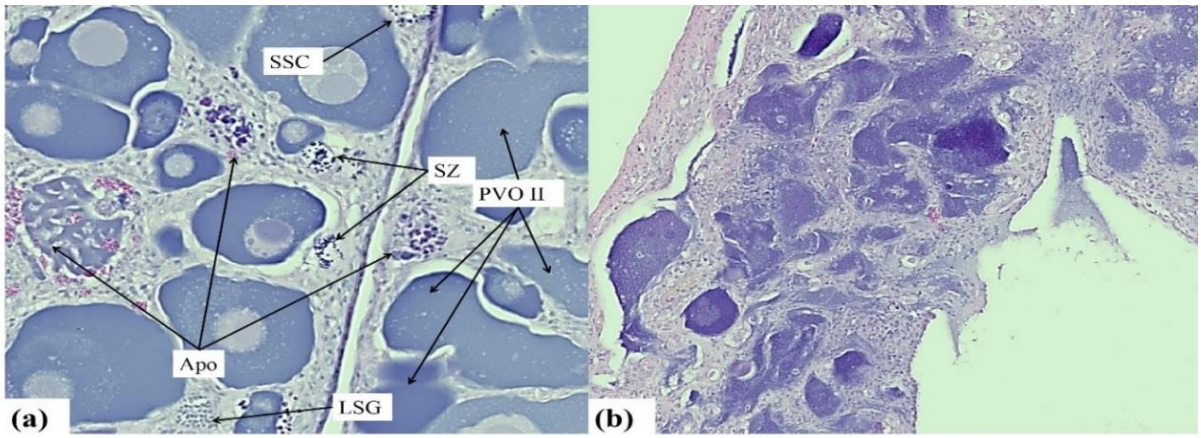


Figure 5.8: Non-delineated protogynous inversion in Labrus bergylta showing **(a)** Controlled processes and **(b)** Uncontrolled oocyte dissolution. Apoptotic oocytes (Apo); Late Spermatogonia (LSG), Secondary Spermatocytes (SSC), Degenerating PVO (PVO II).

Transition of female to male phase gonads occurred in a non-delineated manner with developing spermatogonia commonly adjacent to degenerating PVO and apoptotic oocytes (Fig. 5.8(a)), with extensive cyto-architectural breakdown in many cases (Fig. 5.8(b)). From this, it was evident from both trials that gonad differentiation followed similar progressions as described in Muncaster et al., (2013) however there were gaps in the descriptive scale. Following a review of the histological samples of both studies the following scale was used.

3.2 Standardised gender definitions

Table 5.3: Unified cross tabulated gender based on histological protogynous development in *Labrus bergylta*. Phase I describes the scale developed by Nozu et al. (2009), Phase II describes the scale developed by Muncaster et al., (2013), and Binary represents a simplified scale to unify the other parameters.

Phase I	Phase II	Binary	Description
Female	Female	Female	Immature ovarian histology demonstrated well organised tissues and PVO predominance with nests of oogonia. Mature hydrated oocytes were evident in the breeding season, with evidence of atretic unovulated oocytes. No evidence of spermatogenic cells.
Early	Initiating		Resembles female PVO dominant histology but was described by the appearance of loose connective tissues and somatic cells in central regions of the lamellae.
	Early	Transition	Appearance and proliferation of spermatogonia along the inner margins of lamellae. Many PVO remain and retain structure, but evidence of degradation can be seen from irregular perimeters with formation of Yellow Brown Bodies in some cases.
Late	Male		PVO still evident but show further degeneration. Apoptotic oocytes are observed and YBB are common. Spermatogonia become dominant relative to other germ cells with cysts progressing from the margins of the remnant lamellae towards the centre. More advanced spermatogenic cysts demonstrate later stages of germ cell development. Efferent ducts begin to develop at the outer regions of the gonad.
Male		Male	PVO of varying stages still evident but degeneration is advanced. The testis is well organised with defined lobules. Spermatogenic germ cells surrounded by sertoli cells predominate with differentiated spermatozoa present.

3.3 Results: Trial 1: Proof of concept

Trial 1 was carried out over 207 days. At the conclusion, one of the control fish had died during the trial and two of the fish treated with MDHT. Post-mortem investigation could not provide evidence of a disease condition, nor any other obvious cause of loss. Of the remaining fish, there was clear histological evidence of male induction in both the MDHT and FAD treatments, while no such evidence was apparent in the majority of control animals which received no hormone (Fig. 5.9).

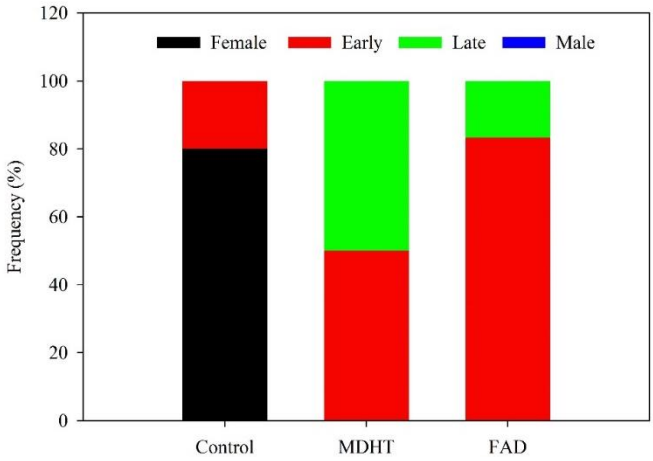


Figure 5.9: Trial 1 endpoint gender outcome frequency in Control group (n=5) showing Female (n=4) and Early transition (n=1), MDHT (n=4) with Early transition (n=2) and Late transition (n=2), and FAD (n=6) where Early transition (n=5) and Late transition (n=1). There were no individuals classified as Male in Trial 1 (n=0).

Broadly, one of the Control group was noted to have initiated sex change and had entered the early phase of transition, and both MDHT and FAD demonstrated full initiation of inversion, but MDHT had a larger proportion of individuals at the more advanced phase. Further analysis of the data (χ^2 (4, N = 15) = 288.515, $p<0.05$) revealed that there was a significant relationship between gender and hormone treatment.

With reference to growth, although the FAD group demonstrated increased growth relative to the controls and the MDHT group, there were no significant differences between the

treatments relative to M_B SGR (Fig. 5.10(a)) (Kruskall-Wallace H (2) = 1.40, p = 0.498), or L_T SGR (Fig. 5.10(b)) (Kruskall-Wallace H (2) = 0.75, p = 0.687).

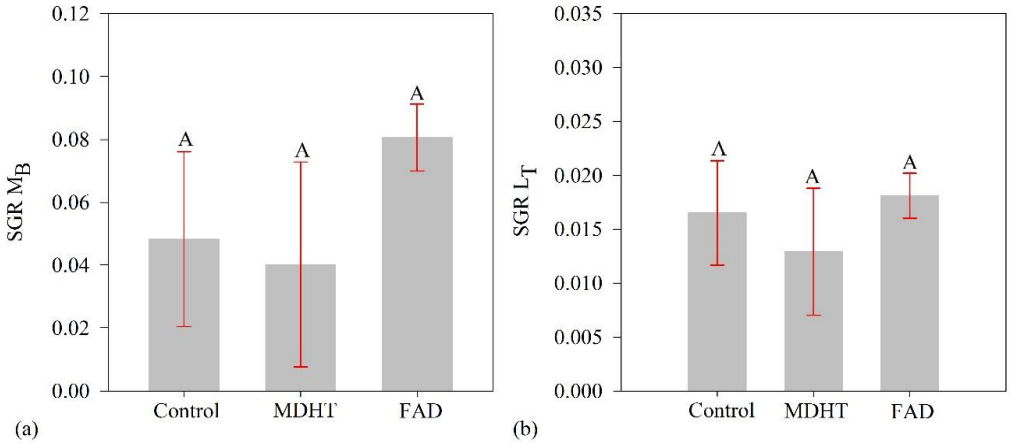


Figure 5.10: Trial 1 *Labrus bergylta* SGR of **(a)** Body Mass (M_B) and **(b)** Total Length (L_T) by treatment including Control (n=5), MDHT (n=4) and FAD (n=6). Different superscript letters denote differences in mean levels.

Similarly, there was no difference between treatments relative to M_B SGR (Fig. 12(a)) (Kruskall-Wallace H (2) = 1.19, p = 0.552), or L_T SGR (Fig. 5.11(b)) (Kruskall-Wallace H (2) = 0.88, p = 0.645).

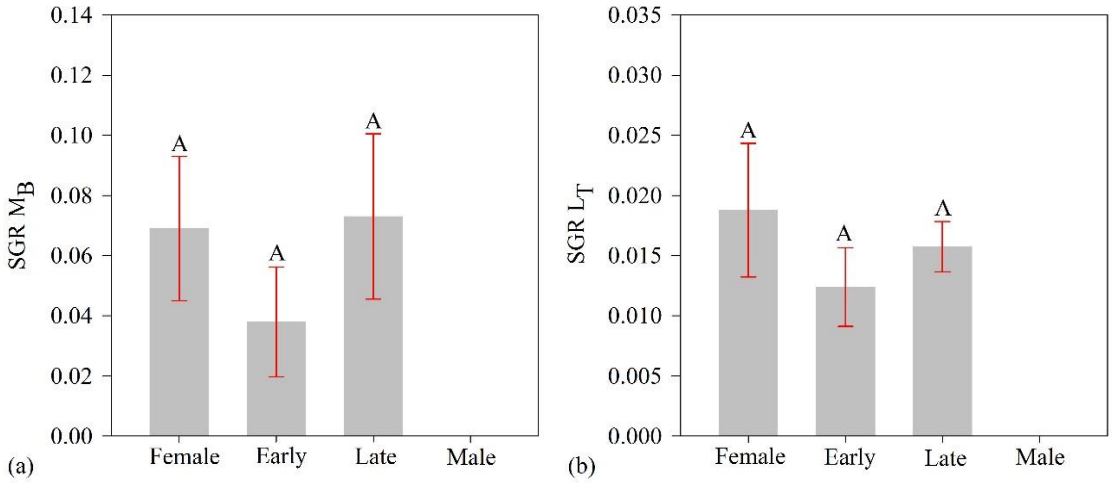


Figure 5.11: Trial 1 *Labrus bergylta* SGR of **(a)** Body Mass (M_B) and **(b)** Total Length (L_T) by gender phase including Female (n=4), Early transition (n=10) and Late transition (n=3). No individuals were classified as Male in Trial 1 (n=0). Different superscript letters denote differences in mean levels.

There were also no significant differences in the Trial 1 GSI in terms of treatment (Fig 5.12(a)) (Kruskall-Wallace H (2) = 2.46, p = 0.292), or gender (Fig 5.12(b)) (Kruskall-Wallace H (2) = 5.61, p = 0.061).

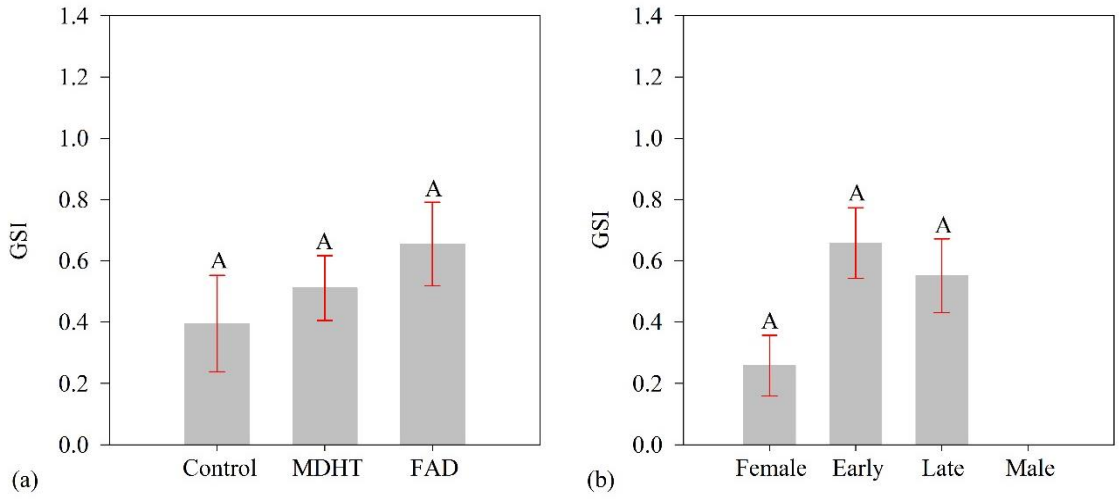


Figure 5.12: Trial 1 *Labrus bergylta* GSI of **(a)** Treatment including the Control group (n=5), the MDHT group (n=4) and the FAD group (n=6), and **(b)** Gender with Female (n=4), Early transition (n=8), and Late transition (n=3). No individuals were classified as Male in Trial 1 (n=0). Different superscript letters denote differences in mean levels.

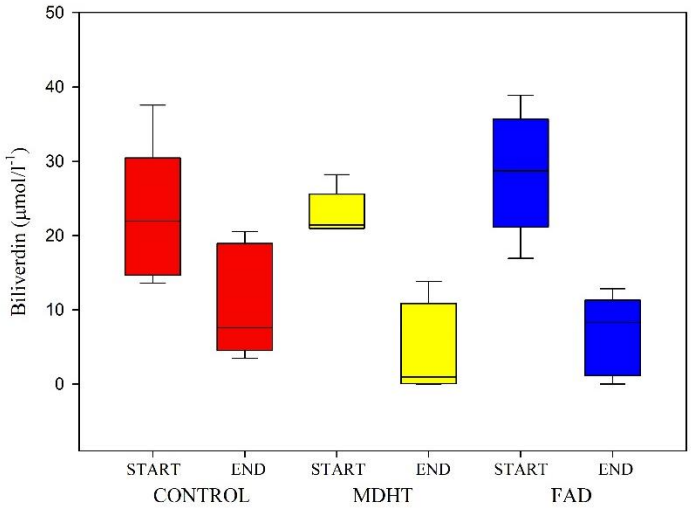


Figure 5.13: Comparison of *Labrus bergylta* plasma biliverdin concentration by treatment across the duration of Trial 1.

On commencement plasma BV concentration was 24.99 ± 2.28 µmol/l (median = 22.76 µmol.l^{-1}; ranging from 13.59–38.85 µmol.l^{-1}). Levels reduced over the time-course of the study

to 9.23 ± 1.97 µmol/l (median = 6.92 µmol.l^{-1}; ranging from 0–20.51 µmol.l^{-1}). There were no significant differences in BV levels between treatments at initiation of the trial (Kruskall-Wallace H (2) = 0.24, p = 0.889), or the endpoint (Kruskall-Wallace H (2) = 1.11, p=0.574), but there was a significant reduction across the duration (Kruskall-Wallace H (5) = 21.57, p<0.05) (Fig. 5.13).

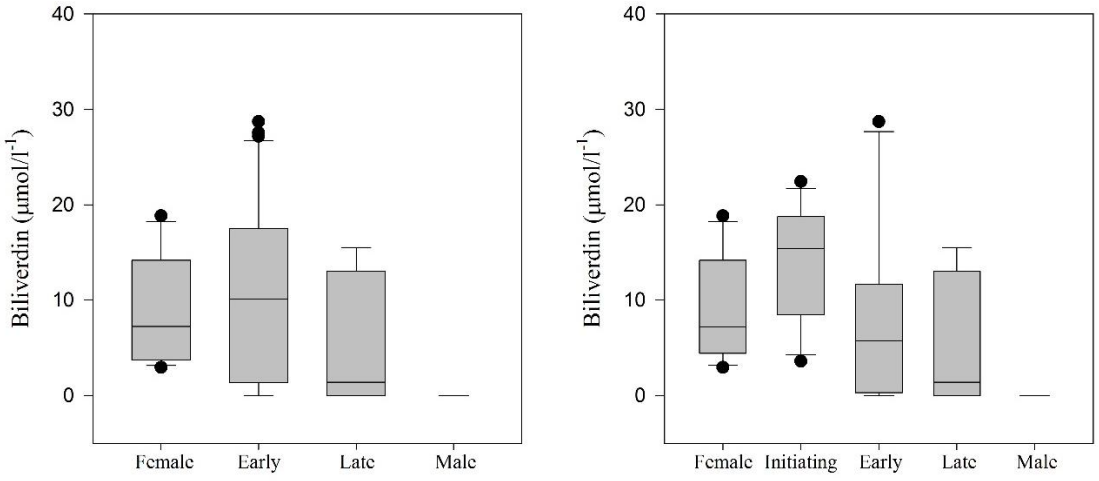


Figure 5.14: Trial 1 *Labrus bergylta* biliverdin concentrations as determined by **(a)** Low Phase I, and **(b)** Phase II histology (Table 5.3). Different superscript letters denote differences in mean levels.

Using Phase I gender (Fig. 5.14(a)), Female BV (median = 7.24 µmol.l^{-1}; ranging from 2.95–18.85 µmol.l^{-1}), ET (median = 10.13 µmol.l^{-1}; ranging from 0.00–28.72 µmol.l^{-1}), and LT (median = 1.41 µmol.l^{-1}; ranging from 0.00–15.51 µmol.l^{-1}) had similar distributions. With Phase II gender (Fig. 5.14(b)), Females (median = 7.18 µmol.l^{-1}; ranging from 2.95–18.85 µmol.l^{-1}), Initiating (median = 15.38 µmol.l^{-1}; ranging from 3.59–22.44 µmol.l^{-1}), ET (median = 5.71 µmol.l^{-1}; ranging from 0.00–28.72 µmol.l^{-1}), and LT (median = 1.41 µmol.l^{-1}; ranging from 0.00–15.51 µmol.l^{-1}) had similar distributions. There was no significant effect when the endpoint BV was determined by Phase I gender (Kruskall-Wallace H (2) = 3.49, p=0.175), or higher resolution Phase II gender (Kruskall-Wallace H (2) = 0.61, p=0.839).

3.4 Results: Trial 2: Induction level

With reference to the sampling timeframe of Trial 2, the Intermediate survey took place at 251 days and the Endpoint survey was at 353 days. At the end of Trial 2 two of the Control fish, one of the Low treatment fish, and three of the High treatment fish had died. Post-mortem investigation could not provide evidence of a disease condition, nor any other obvious cause of loss. Of the remaining fish, there was clear histological evidence of male induction at both time-points and in both treatments, while no such evidence was apparent in the Control animals (Fig. 5.15).

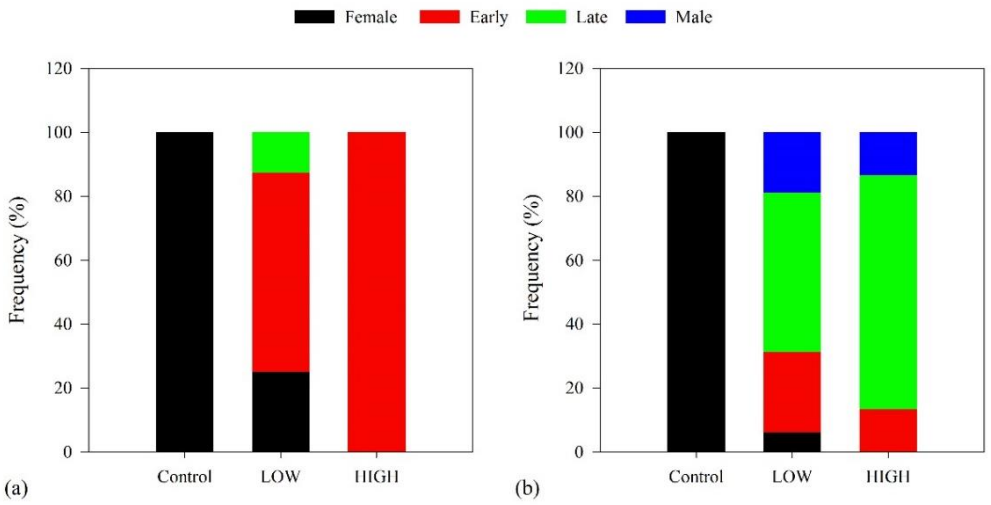


Figure 5.15: Trial 2 *Labrus bergylta* inversion outcome frequency in **(a)** Intermediate sample point Control group (n=6) showing Female (n=6); Low treatment (n=8) with Female (n=2), Early transition (n=5) and Late transition (n=1); and High treatment (n=6) where Early transition (n=6). There were no individuals classified as Male in Trial 1 (n=0), and **(b)** End sample point Control group (n=14) showing Female (n=14); Low treatment (n=16) with Female (n=1), Early transition (n=4), Late transition (n=8) and Male (n=3); and High treatment (n=15) where Early transition (n=2), Late transition (n=11) and Male (n=2).

Broadly; the Control groups were confirmed as Female at both timepoints. The intermediate sample demonstrated two individuals that remained female in the Low treatment cohort with a majority in ET and one in LT, whilst all of the High treatment level individuals were at LT (Fig. 15(a)). Contrastingly; the Endpoint cohort showed more mixed results with

one Low treatment individual which remained Female, four were in ET, eight were in LT and there were three complete Males. The High treatment cohort was mostly in the LT phase with a small proportion in ET, and two complete Males (Fig. 15(b)). Further analysis revealed that there was a significant effect of treatment relative to gender in the Intermediate group (χ^2 (4, N = 20) = 248.098, $p<0.05$), and the Endpoint group (χ^2 (4, N = 45) = 285.109, $p<0.05$). And similarly; there was a difference between the High and Low treatment groups at the Intermediate timepoint (χ^2 (3, N = 14) = 16.241, $p<0.05$), and the Endpoint (χ^2 (2, N = 31) = 45.399, $p<0.05$).

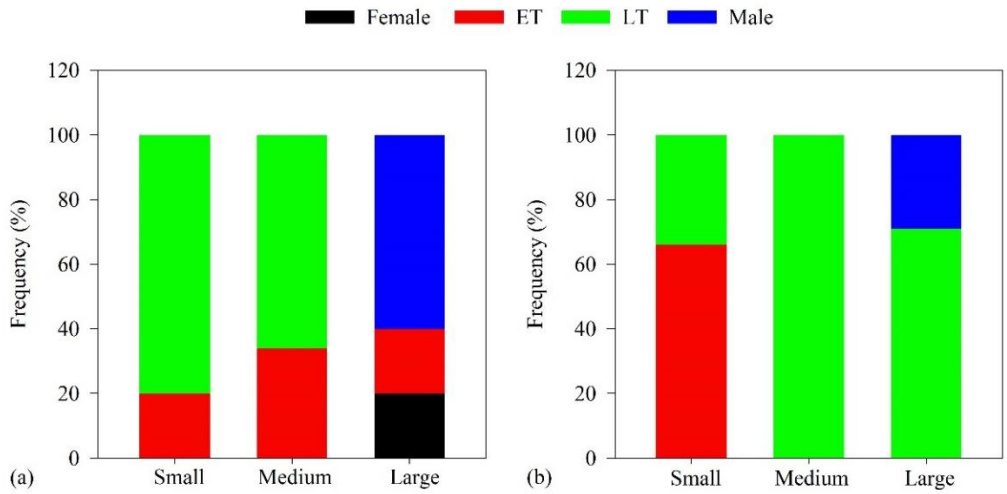


Figure 5.16: Trial 2 *Labrus bergylta* inversion outcome frequency relative to size sorted cohorts within the **(a)** Low treatment where Small (n=5) comprised Early transition (n=1) and Late transition (n=4), Medium (n=6) comprised Early transition (n=2) and Late transition (n=4), and Large (n=5) comprised Female (n=1), Early transition (n=1), and Male (n=3); and **(b)** High treatment where Small (n=5) comprised Early transition (n=2) and Late transition (n=1), Medium (n=5) comprised Early transition (n=5); and Large (n=7) comprised Early transition (n=5) and Male (n=2).

The Control group was excluded from size dependent analysis as all individuals remained female in both sampling subsets. Small, Medium and Large were defined by M_B as 300-450 g, 450-600g and 600-900 g. Both treatment levels demonstrated sex reverssal in the majority of individuals with some differences in the outcome when size at induction was

considered. With reference to the Low treatment (Fig. 5.16(a)), the Small and Medium groups were proportionally similar in terms of the phase distribution with some individuals in Early transition and a majority in Late phase. Contrastingly, one of the Large group remained Female, whilst there was a small proportion in the Early phase with a majority of Males. Further analysis identified there was a significant difference between the Small group and the larger groups (χ^2 (6, N = 16) = 240.311, p<0.05), and also between the Medium and Large levels (χ^2 (3, N = 11) = 149.630, p<0.05). The High treament cohort (Fig. 5.16(b)) were induced across all of the size parameters with the Small group showing mostly Early transition phase with some Late phase individuals, the Medium size group were all in the Late phase of transition, and the Larger individuals mostly in Late transition with some Male phase. Males were consistently in the largest size at induction cohort. There was a significant difference between the Small group and the larger groups (χ^2 (4, N = 17) = 222.029, p<0.05), and also between the Medium and Large levels (χ^2 (1, N = 13) = 33.918, p<0.05).

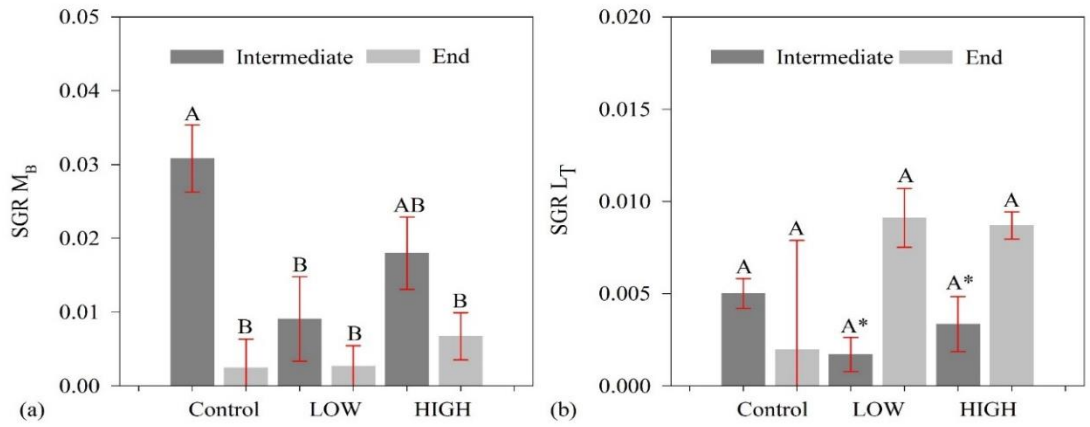


Figure 5.17: Trial 2 *Labrus bergylta* SGR of **(a)** Body Mass (M_B) and **(b)** Total Length (L_T) by sampling point and treatment level including Intermediate datasets with Control (n=6), Low (n=8) and High (n=7) and Endpoint datasets with Control (n=14), Low (n=16) and High (n=15). Different superscript letters denote differences in mean levels.

There was no significant difference across the study in M_B (Fig. 5.17(a)) (Kruskall-Wallace H (2) = 1.47, p=0.478) or L_T (Fig. 5.17(b)) (Kruskall-Wallace H (2) = 0.34, p=0.845).

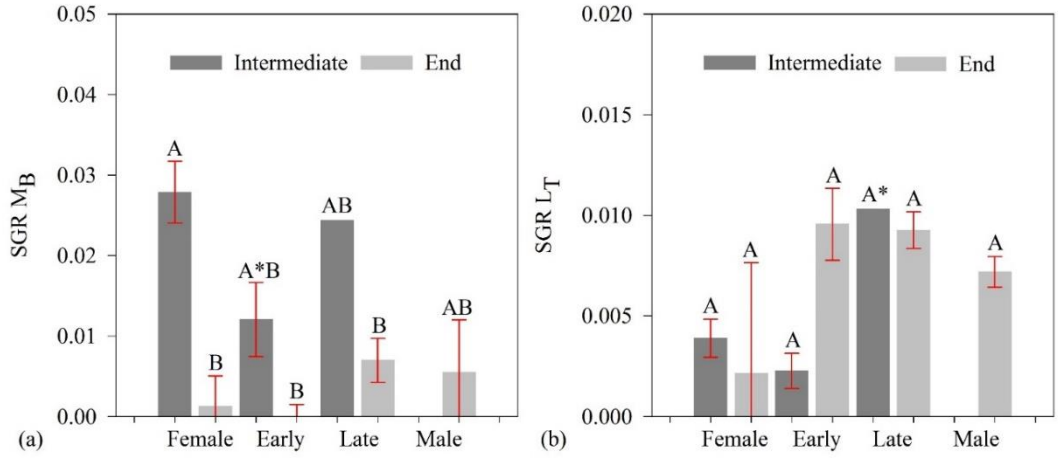


Figure 5.18: Trial 2 *Labrus bergylta* SGR of **(a)** Body Mass (M_B) and **(b)** Total Length (L_T) by sampling point and gender including Intermediate datasets with Female (n=8), Early transition (n=12), and Late Transition (n=1); and Endpoint datasets with Female (n=15), Early transition (n=6), Late Transition (n=19) and Male (n=5). No individuals were Male in the intermediate dataset (n=0). Different superscript letters denote differences in mean levels.

Similarly to the SGR data based on treatment levels, statistical analysis revealed no significant difference in M_B (Fig 5.18(a)) (Kruskall-Wallace H (3) = 3.11, p=0.376) or L_T (Fig 5.18(b)) (Kruskall-Wallace H (3) = 7.70, p=0.053) relative to gender at either of the sample points.

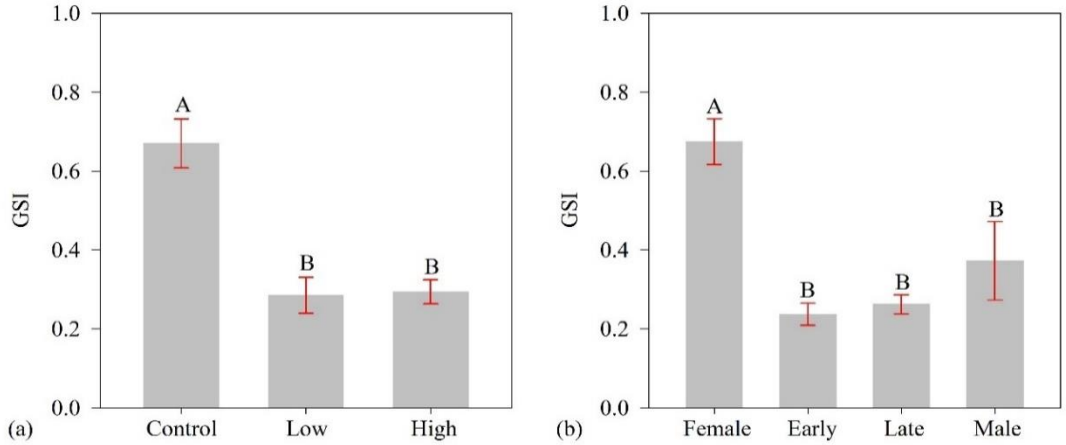


Figure 5.19: Trial 2 endpoint *Labrus bergylta* GSI of **(a)** Treatment including the Control group (n=14), Low treatment (n=16) and the High treatment (n=16); and **(b)** Gender with

Female (n=15), Early transition (n=6), Late transition (n=20) and Male (n=5). Different superscript letters denote differences in mean levels.

With reference to the GSI; the Control group was 0.67 ± 0.06, Low treatment was 0.26 ± 0.05 and High was 0.30 ± 0.03 (Fig. 5.19(a)). When this was considered by gender (Fig 5.19(b)), Female GSI was 0.68 ± 0.06, ET was 0.24 ± 0.03, LT was 0.26 ± 0.02 and Male was 0.37 ± 0.1. There were significant differences between the Control group and the Low and High treatment cohorts under comparison of the treatment levels (Kruskall-Wallace H (2) = 21.35, p<0.00), and also relative to the transition phase (Kruskall-Wallace H (2) = 26.07, p<0.00).

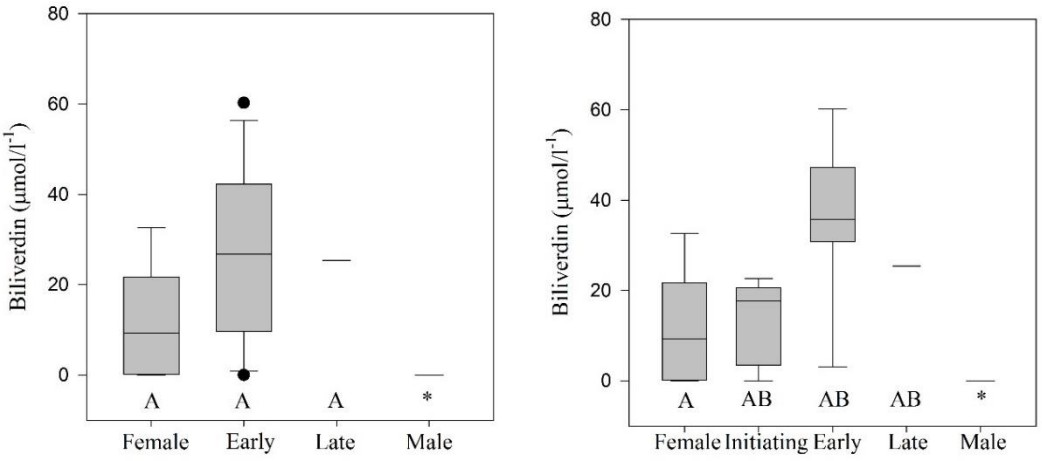


Figure 5.20: Trial 2 intermediate plasma biliverdin in *Labrus bergylta* by **(a)** Phase I gender and **(b)** Phase II gender. Different subscript letters denote differences in mean levels.

Mean plasma BV was 20.76 ± 3.77 µmol/l (median = 18.08 µmol.l^{-1}; ranging from 0.00–60.26 µmol.l^{-1}). When the data was explored by transition phase (Fig. 5.20(a)); Females BV concentration (median = 9.29 µmol.l^{-1}; ranging from 0.00–32.56 µmol.l^{-1}), ET (median = 17.69 µmol.l^{-1}; ranging from 0.00–22.69 µmol.l^{-1}) and LT (median = 25.39 µmol.l^{-1}; ranging from 25.39–25.39 µmol.l^{-1}) appeared broadly similar. There was a difference (Kruskall-Wallace H (3) = 8.75, p=0.032) in mean levels between phases wherein Females had less BV than those at ET (Kruskall-Wallace H (3) = 10.39, p>0.05), with no significant difference between ET and LT groups (Kruskall-Wallace H (3) = 0.33, p=0.578). When the Intermediate set was extrapolated to higher resolution (Fig. 5.20(b)), ET individuals had significantly more

BV (Kruskall-Wallace H (3) = 8.75, p<0.00) but there was no significant difference (Kruskall-Wallace H (2) = 3.71, p=0.156) between the Females and Initiating individuals (Kruskall-Wallace H (2) = 8.75, p=0.079). LT demonstrated commonalities with both.

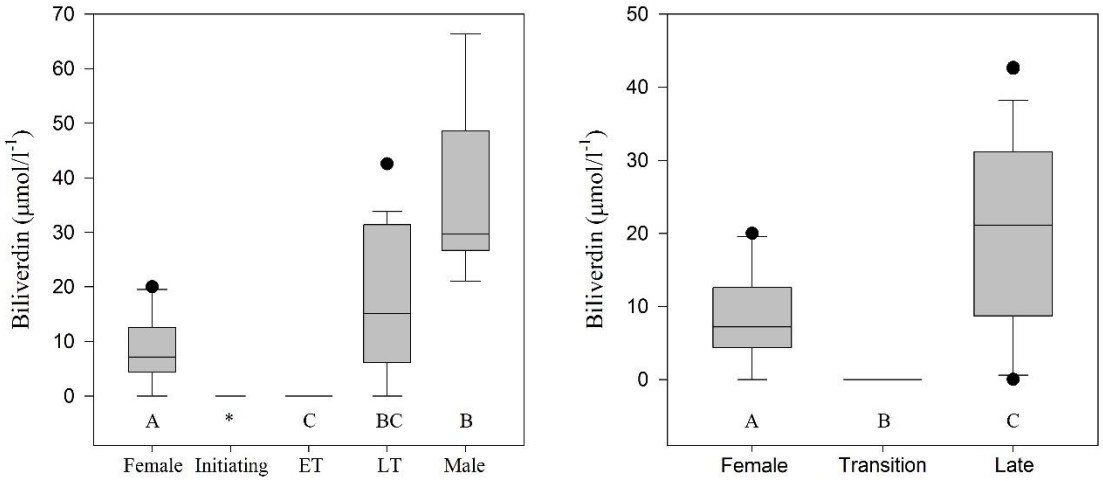


Figure 5.21: Trial 2 endpoint plasma biliverdin in *Labrus bergylta* as defined by **(a)** Phase II gender, and **(b)** Binary gender including transitional individuals. Different superscript letters denote differences in mean levels.

In the Trial 2 Endpoint dataset (Fig. 5.21(a)), Females demonstrated a mean BV concentration of 8.38 ± 1.52 µmol/l (median = 7.18 µmol.l^{-1}; ranging from 0.00–20.00 µmol.l^{-1}), ET were 0.00 ± 0.56 µmol/l (median = 0.00 µmol.l^{-1}; ranging from 0.00–0.00 µmol.l^{-1}), LT was 17.65 ± 3.01 µmol/l (median = 15.13 µmol.l^{-1}; ranging from 0.00–42.56 µmol.l^{-1}), and Males 37.79 ± 6.07 µmol/l (median = 26.68 µmol.l^{-1}; ranging from 0.00–66.41 µmol.l^{-1}). BV was significantly different (Kruskall-Wallace H (2) = 16.11, p<0.05) in the Trial 2 endpoint cohort where Post-hoc Mood's median tests revealed that Males had more than Females (χ^2 (1, N = 21) = 9.24, p<0.05), ET (χ^2 (1, N = 12) = 12.00, p<0.05) and LT (χ^2 (1, N = 25) = 3.95, p<0.05). Similarly, LT BV was greater than Females (χ^2 (1, N = 34) = 2.98, p<0.05) and ET (χ^2 (1, N = 25) = 7.29, p<0.05) . Where the scale was simplified as binary gender (Fig. 5.21(b)); the BV levels show a significant difference (Kruskall-Wallace H (3) = 20.11, p = 0.000) where

Females had more pigment than Transitional individuals (χ^2 (1, N = 20) = 6.67, $p<0.05$) , and Males more than Females (χ^2 (1, N = 39) = 8.05, $p<0.05$) .

4.0 Discussion

Successful induction of protogynous sex change by androgen treatment, and the long term effects have been noted to vary between species (Baroiller et al., 1999). Trial 1 demonstrated inversion could be induced in *L. bergylta* by both application of the aromatase inhibitor FAD to prevent E_2 expression and terminate the female endocrine template (Colburn et al., 2009), and by administration of the non-aromatizable androgen MDHT which presents as expression of a male specific endocrine template (Baroiller et al., 1999; Pandian & Sheela, 1995). Further to this, Trial 2 used a combination of the two methods to establish that silastic implantation of 5mg/Kg FAD and 5mg/Kg MDHT was an adequate dose of androgen to cause complete reversal, and that there was a potential size effect with regards the rate of reversal where larger individuals appeared to progress through the inversion process at a greater rate than smaller conspecifics. At the end of Trial 1, a greater proportion of the MDHT cohort was at the LT phase than the FAD group. This is indicative that MDHT intervention may be a more effective means of stimulating inversion than FAD as it bypasses the lag time resulting from physiological reduction of E_2 relative to T and 11-KT until the induction threshold is achieved. Contrastingly, Trial 2 used a highly successful combination of the two treatments such that the aromatase inhibitor FAD was constant, and the MDHT varied. From this, FAD acted to inhibit expression of the female endocrine template via down regulation of aromatase activity which increases endogenous T naturally, whilst MDHT supplementation drove forward the male phase endocrinology. It is however difficult to compare the induction efficacy between trials as the time limitation of Trial 1 prevented completion of the transition process. Logically therefore, in considering the outcome results of Trial 1 relative to Trial 2, although there may be an advantage in intervention via up-regulation of the male template as opposed to cessation

of the female phase, it is difficult to determine whether dual androgen supplements are more effective than MDHT alone. Following on from this, it could be argued that notwithstanding the individuals at advanced stages in the Low treatment cohort of the intermediate dataset in Trial 2, the phase distributions suggest a dose dependant increase in the rate of differentiation in the High level. This effect is more apparent in the endpoint dataset and as such, when it is considered over the alternate timeframes of study, this indicates that although increased hormone results in a stronger response and increased rate of transition, the process is to a degree limited in terms of timescale as the number of Male outcomes was similar. Thus, the High treatment level demonstrated a similar degree of success to that of the Low treatment, but compressed the inversion process suggesting that a second application of hormone could improve the outcome results as opposed to increasing the initial dosage further.

Although there was a relationship between size and the gender outcome, which can be illustrated by the relative proportions of each gender phase, there was no observed effect in terms of size being a limitation for artificial induction of sex change in *L. bergylta*. This is perhaps unsurprising given that exogenous androgenic supplementation by either means effectively bypasses the requirement for necessary priming (Baroiller et al., 1999), and perception of permissive stimuli (Lamm et al., 2015), therefore the natural ontogenic biometry and social contextual stimuli (Muncaster et al., 2013) become irrelevant. Two individuals were noted to have remained Female in the Low treatment group at both sample times, but this was most likely due to ineffective treatment as opposed to resistance. It is however an interesting question whether the presence of a dominant male would impede or retard the artificial transition process. Furthermore, there was one Trial 1 Control individual with ET histology, but in considering the degree of progression relative to the timeframe, induction must have occurred during the experiment. Thus, ‘natural’ inversion in an individual of diminutive size relative to the rest of the experimental group further illustrates the unpredictability of governing

processes in abstracted populations. It was also notable that there were two males in the Trial 2 endpoint cohort which demonstrated no evidence of female histology with well-formed lobular testes (Fig. 5.7 (b)) compared to the normal intersex gonad (Fig. 5.7 (a)) which would be expected considering the evidence in similar species (Bhandari et al., 2001; Bhandari et al., 2005; Garcia et al., 2013; Higa & Ogasawara, 2003; Kailasam et al., 2007; Li et al., 2006; Muncaster et al., 2013; Ohta et al., 2012; Quinitio et al., 2001; Tsuchihashi et al., 2003). This is somewhat controversial as it raises questions regarding the monandric status of *L. bergylta* (Leclercq et al., 2014a; Muncaster et al., 2013; Talbot et al., 2012). There are a few possible explanations. Initially, given the numbers of *L. bergylta* in this study and others which describe gonad histology during sex change it would be expected that more of this 'type' would have been noted considering 6% of the total population are satellite males in related diandric species (Munday et al., 2006; Trip et al., 2011). One theory of interest is based upon developmental plasticity where all individuals develop with a rudimentary bi-potential female gonad, then a small proportion differentiate as male prior to maturing in the female phase as a response to local environmental conditions, in conjunction with a particular individuals genetic sensitivity to those conditions (Warner, 1991). Thus, individuals that develop as males in this early phase of differentiation would have a testis which resembles that of a gonochoristic species and reflects the observations herein, while others would follow the prescribed route of protogynous development and as a result show the expected diagnostic mixed phase testis (Munday et al., 2006). Another possibility was described by Black & Grober (2003) where 'pirate' males that are larger and more dominant than most move between communities and temporarily displace the primary male such that they successfully breed with the female and leave parental care to the original male. This would be dependent on eventual disappearance of female characteristics in long living males, and would also present the unusual testis architecture as described. In

either case, further work including phenotype specific diagnostic hormone analysis (Perry & Grober, 2003) and expanded experimental populations would resolve the issue.

The SGR data showed no real patterns in terms of either treatments, or terminal gender phase in any of the studies. Although there were no significant findings, the data showed some suggestion that individuals remaining female across both studies had the highest SGR values, with lower values in ET and LT individuals, and the Male SGR demonstrating an increasing trend. This would be consistent with normative growth rates without inversion, followed by diversion of the somatic budget to processes such as tissue conversion at the expense of growth investment during inversion, and a reduced rate of diversion as the male end of the gender spectrum but the limited number of individuals for study, lack of reliable in-vivo methods and massive variability in the degree of histological progression between individuals causes a degree of interference.

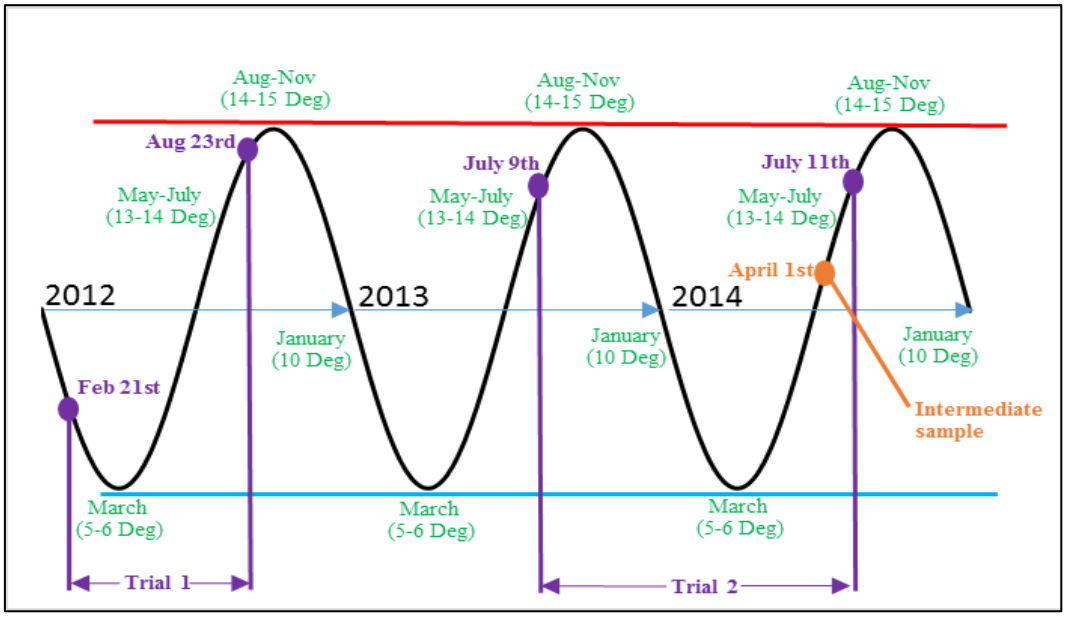


Figure 5.22: Visual summary of inversion trials showing induction timings and sampling relative to seasons.

The appearance of mature vitellogenic oocytes and attretic oocytes at the Trial 2 intermediate sample, and running males in the endpoint is indicative that the population's

reproductive dynamics were consistent with the normal seasonal cycles. Sequential sex change normally occurs at the end of the natural spawning window as it maximises the fecundity of the female phase (Lamm et al., 2015), and takes advantage of seasonal oscillations (Fig. 5.22) reducing E_2 at the end of the reproductive window (Muncaster et al., 2010). Thus, Trial 1 was induced to coincide with nominal E_2 during and continued until after breeding season the following summer, and Trial 2 ran for a year from the end of a reproductive season to allow a full seasonal cycle. Although it is true that individuals may gain full male functionality prior to complete inversion, in considering the degree of progression at the end of both trials relative to the time from induction, the data is in good agreement with a 5 months transition (Muncaster et al., 2013), with quiescence during winter as described in Chapter 4 (Section 4.0). However, bearing in mind that stochastic initiation by male removal may happen at any time, in view of 21 day inversion in *T. bifasciatum*) (Kramer & Imbriano, 1997), and 4-6 weeks in the broad barred goby (*Gobiodon histrio*) (Valenciennes 1837) (Kroon et al., 2003), more favourable thermal regimes may result in male functionality prior to complete sex change as reported by Muncaster et al. (2013).

Post-induction tissue remodelling followed a sequential pattern during transformation of the gonad from fully functional ovarian tissues with no evidence of the male phase, to fully functioning testes which retain some of the degenerating female phase (Nozu et al., 2013). The first histological evidence of inversion processes was degeneration of primary oocytes (Nozu et al., 2013). Degeneration appears to follow a structured process initially with the oocytes losing shape as the internal structures degenerate (Garris, 2004), followed by controlled apoptosis and eventual fragmentation as the ovarian tissues undergo involution (Liarte et al., 2007). As shown in Figure 5.8(b), this process can exceed regulation and presents the hyperlipidaemic oxidative potential described previously. Hence, the dissolution process is ongoing while isolated spermatogonia appear at the margins of the transforming lamellae of

the germinal epithelium, proliferate and undergo spermatogenesis (Miyake et al., 2012). Thus, further to the mechanism of depletion as described previously (Chapter 3), as BV is a water soluble scavenging anti-oxidant it would be expected that where a species such as *L. bergylta* is exposed to such a massive systemic oxidative stress, with inflammation due to released subcellular components, plasma BV would become depleted by direct reaction with aqueous pro-oxidative molecules (McDonagh, 2010), by reduction to the lipid soluble terminal anti-oxidant product bilirubin (Barañano & Rao, 2002), and utilisation to control inflammation (Bisht, 2014).

However, the BV data was of interest as it demonstrated some departure from the BV mobilisation patterns described in Chapter 3. In Trial 1, there were no significant differences between the gender transition phase BV concentrations but the data demonstrates a tentative trend where there is an initial increase from Female levels with a peak in the ET and relative reduction in LT. Trial 2 intermediate set demonstrates a similar distribution across the phases with an initial increase at the point of initiation with a peak in the ET and reduction in LT. In this case the magnitudes were greater than those of the 2012 group and ET BV peak was significant relative to other phases. It was unfortunate that there were no Males in either series for comparison. Contrastingly, although the Trial 2 endpoint Female BV concentration was comparable to that of the other experimental groups, and there were no individuals at the initiation stage, there was no BV in the ET phase with an increasing trend through LT to a peak in Males.

That the simplified binary gender scale appeared to give a clearer resolution is symptomatic of the complexity of histology in protogynous hermaphrodites. The BV distribution is reflective of the mobilisation patterns during sex change in wild *L. bergylta* wherein the pigment was depleted in individuals undergoing transition at the time of study (Chapter 3). Both inversion studies demonstrate similar starting levels to that of wild

counterparts, with reducing concentrations over the experimental timeframe. Similarly to studies in Anguilliforms, it is probable that data interpretation from *L. bergylta* becomes cryptic due to a hugely complex life cycle in conjunction with disturbed developmental programming as a result of acclimatisation to an ontogenically incompatible environment (Ellis & Poluhowich, 1981). Some dietary influence must be considered as biliproteins are a major constituent of many marine organisms (Fang & Bada, 1990), hence it is possible that a proportion of the pigment is derived from the prey/food types consumed (Yamaguchi, 1971). This may be of interest as dusky pink (pers. Obs.) cultured *L. bergylta* depart from the emerald green of wild fry counterparts, but further work is required and in agreement with Ellis & Poluhowich (1981), this does not preclude input of bile pigments as a derivative of haemoglobin turnover.

5.0 Conclusions

This study begins to characterise some of the parameters and limitations of artificially inducing protogynous transition in captive populations of female *L. bergylta* as a means of generating males for broodstock programmes, and explores BV mobilisation patterns across the associated processes. In conclusion, artificial induction of sex change is possible by artificially altering the endocrine template using both aromatase inhibitors and un-aromatizable testosterone regardless of biometric context and social primers. This has great potential in addressing the male deficit described previously but, it is of note that abstraction of environmentally and socially controlled ontogenically programmed organism such as the protogynous hermaphrodite *L. bergylta* may add further layers of complexity to the normal drivers of inversion. However, there is still a significant need to develop a gender specific diagnostic biomarker. It would have been interesting to extend the experiment to allow investigation of sperm quality considering reported reductions of fertilisation capacity in

artificially induced blue-spotted grouper (*Epinephalus fario*) (Kner 1864) (Kuo et al., 1988). And similarly, potato grouper (*Epinephalus tukula*) (Morgans 1959) have been shown to reverse the process and revert back to female the following year (Yeh et al., 2003), but such a longitudinal extension was beyond the capacity of the present study. Similarly, the study suggests that there is potential to improve the end outcome of masculinisation treatment by supplementary subsequent androgenic stimulatory intervention, but further study is required. Finally, in considering the proposed functions of BV in such species, the background reduction across both studies could potentially impact the welfare of induced fish as their anti-oxidant systems would seem impaired therefore some form of bilin precursor rich dietary supplement could be highly advantageous as it would allow priming of the system prior to, and during induction with improved biochemistry and anti-oxidant capacity, but this will require further elucidation.

Chapter 6:
General discussion

At conception, the general aim of this project was to develop a diagnostic test of specific gender in *L. bergylta* based on sexually dimorphic plasma BV levels in related species to

facilitate broodstock selection and optimise production for deployment as cleaner fish in the aquaculture industry. Although pigment analysis was ultimately disproven as a method due to a lack of dimorphism between the binary genders (Chapter 3), the successful hormonal induction of protogynous inversion in female *L. bergylta* (Chapter 5) affords the industry an artificial means of generating male fish, and therefore offers a finite control for developing broodstock populations and optimising production, but the requirement for a diagnostic marker for gender remains a priority for the industry. However, the BV story is far from over as this project revealed a surprising range of functionalities for a molecule which had previously been considered a waste product of haem catabolism in humans (Kirkby & Adin, 2006a), and a biogenic pigment in teleosts (Abolins, 1961; Gagnon, 2006; Schaefer et al., 2014; Yamaguchi & Matsuura, 1969; Yu et al., 2007).

This project is the first to isolate and identify the causative agent of blue-green pigmentation in the plasma and tissues of *L. bergylta* as the IXα isomer of the linear tetra-pyrrole BV. In contrast to placental mammals, where the conversion of BV to the terminal product BR is rapid, and HBV is symptomatic of disturbances in the enzymatic metabolism with a severely poor prognosis (Berlec & Strukelj, 2014), the catabolic axis is reduced in some vertebrates such that BV is the terminal (excretory) product (Cornelius, 1981). Within this, a fractional group of species which now includes *L. bergylta*, use tight binding of BV to a protein moiety to prevent further processing or elimination by BVR to facilitate accumulation of the pigment in the plasma fractions (Fang, 1987; Juettner, 2013). As noted previously, whilst research in related HBV species including other Labridae has identified that plasma dimorphism is commonly a function of sexual signalling with external deposition of the pigment (Gagnon, 2006) acting to advertise ‘maleness’ (Oliveira et al., 2001), this current work demonstrated that there was no consistently measurable difference in plasma BV between *L. bergylta* genders, but the pigment was depleted in the individuals which were changing sex at

the time of study (Chapter 3). From this; the theory that plasma BV, and the described mobilisation patterns were involved in the fundamental processes of inversion developed. Further analysis (Chapter 3) of the other 'local' Labrini lent further support to this, as BV accumulation (HBV) was co-distributed with protogynous reproductive systems, and absent in the gonochorists.

Hence, with consideration of the inherent cytoprotective properties of the HO1 axis which controls haem metabolism and therefore BV catabolism (Cao et al., 2009), in conjunction with the intrinsic anti-oxidant capacity of molecular BV (McDonagh, 2010), the most likely explanation for the described mobilisation patterns was that the tissue remodelling processes associated with gender inversion in protogynous hermaphrodites (Chapter 5) manifests as a massive pro-oxidative challenge, which is essentially balanced by endogenous stores of BV to protect the nascent testis and developing sperm from oxidative stress. With this in mind, BV depletion in transitional individuals (Chapter 3) is of some interest in the context of HO1 activation as it suggests that oxidative processes occur at a rate which exceeds the normal abrogative systems during transition in *L. bergylta*. Analogous events in mammalian systems would include hypoalbuminia (Rosenman et al., 1956) where SA becomes depleted and affected individuals have altered fatty acid and lipid metabolisms with severe hyperlipidaemia as management systems are impaired (Metcalf et al., 1999), leading to clinical manifestation as free cholesterol and lipids becoming trapped in the plasma fractions (Rosenman et al., 1956). Similarly, the catadromous, an-albuminic, and HBV New Zealand long-finned (*Anguilla dieffenbachii*) (Gray 1842), and short-finned *(Anguilla australis schmidtii*) (Phillipps 1925) eels demonstrate hypercholesterosis and hyperlipidaemia when they undergo transition from marine environments to freshwater or vice versa during spawning as the fatty acid and lipid transport systems become overloaded as a function of the shift in biochemistry which is accompanied by major changes in the cellular physiology (Metcalf et

al., 1999), and leads to major increases in free lipids (Rosenman et al., 1956). From this, and with specific reference to the work of Liarte et al., (2007) regarding testicular involution in *S. aurata*, descriptions of hypercytolipidemia during ovarian involution in humans (Garris, 2005), and the general histology of sex change in *L. bergylta* gonads (Chapter 5), it is highly likely that hyperlipidaemia and hypercholesterosis would accompany the process. Further to this, as fish are poikilothermic, and as such require a higher degree of unsaturated fatty acids in plasma membranes to maintain fluidity at lower temperatures (Babin & Vernier, 1989), teleost physiology presents greater oxidative potential than endothermic mammalian systems (Madamanchi et al., 2004), therefore enhanced anti-oxidant defences would be a great advantage. If this is indeed the case, then an endogenous store of BV would represent a fundamental component of switching gender as it would effectively prime the anti-oxidant profile to absorb excess pro-oxidative stress, and thus allow involution of the ovaries and non-delineated changes to gonad histology with development of full male reproduction prior to termination of the female phase characters (Muncaster et al., 2013). Notably, BV has also recently been linked to dorsal axis development by binding with a molecular partner as part of embryonic cytoplasmic determination during development in African clawed frogs (*Xenopus laevis*) (Daudin 1802) (Falchuk et al., 2002), which potentially suggests additional associations with gene regulation during protogynous gonadogenesis, but this remains to be explored in teleosts. From this, it may be of interest to study transition in BV deficient *L. bergylta*.

Looking to the future, there are a number of points to be revisted. Initially, the work of Berlec & Strukelj (2014) is of great interest as they report a high throughput BV assay which is resistant to interference from proteins and other biological contaminants. Briefly, infrared Fluorescent Protein (irfp) was inserted into *Escherichia coli* (DH5α) overexpression of irfp is driven by a constitutive promoter requiring no inducers, which were ultimately lysed and centrifuged to eliminate cellular debris such that the purified supernatant could be extracted.

The method then relies upon the highly specific reaction of the irfp with BV which is then monitored by measuring fluorescence in the infra-red region with the excitation wavelength of 690 nm and the emission wavelength at 713 nm. Absolute BV was interpolated from a standard curve of known concentration generated using commercially obtained BV. Although the method remains to be tested in *L. bergylta* and may well encounter the same substrate access issues as described for BVR in *C. analis* (Fang, 1987), and *L. bergylta* (Chapter 3), but the use of infra-red fluorescence is known to minimise interference by auto- fluorescence from endogenous biological molecules (Berlec & Strukelj, 2014). As a note, it is of great interest that Berlec & Strukelj (2014) report a 1.5 fold increase in expected BV under experimental conditions which reflects the results of the *L. bergylta* spike and recovery assay (Chapter 2) which they attributed to auto-oxidation of BR to BV in the samples. This was not considered at the time, but such factors may explain the observed disparity between HBV *L. bergylta*, the mammalian species, and the other non-HBV teleosts included for comparison. Interestingly, Berlec & Strukelj (2014) postulate that this may well complicate analysis in avian and reptilian species due to the preponderance of a BV centric system as opposed to the BR centric system of mammals, therefore *L. bergylta* would be expected to behave in a similar manner but this remains to be established.

Throughout the early experimental phase of this study (Chapter 3), the BV protein association was problematic as it precluded many of the purification techniques applied by previous studies. Similarly, when the molecule was isolated, the inherent reactivity of BV meant that many of the applied methods were restricted as the pigment was lost to light, oxygen and heat (Chapter 2). Whilst this was considered inconvenient at the time, it was later realised that this was in fact an illustration of the underlying mechanism of BV accumulation. In a system which resembles the BR-SA complex in humans, *L. bergylta* plasma BV was tightly bound to a 28 kDa protein which prevented further processing and protected the molecule from

reaction (Chapter 3). Similarly to the BV distribution across the related Labrini tribe included in the study, the protein was also noted as co-distributing with BV, and only occurring in protogynous species. At face value, and in considering the proposed mechanisms of plasma BV mobilisation and depletion in sex-changing individuals, this suggested a dedicated protein metabolism which facilitates BV accumulation as a pre-cursor to the pro-oxidative potentials associated with ovarian involution. However, when the protein was identified as potentially related to the lipocalin ApoA1, the range of possible functionalities expanded greatly.

Initially, ApoA1 is best known as a principal constituent (~70%) of High density Lipoprotein (HDL) and as such, has an important role in mediating lipid transport and metabolism (Lund-Katz & Phillips, 2010). This presented two main utilities. If the role of the association is primarily to sequester BV prior to transition, ApoA1 would be an ideal carrier as it is analogous to SA in mammals (Metcalf et al., 1999), and as such, is highly abundant in teleost plasma (Choudhury et al., 2011), therefore systemic distribution would maximise storage potential with the lipocalin fold internalising BV (Flower et al., 1993) such that it is protected from further processing and elimination to facilitate accumulation as described by in *C. analis* by Fang & Bada (1988), and supported by Chapter 3. Further to this, BV binding proteins which are similarly rich in HDL have been noted to function as amino acid storage in numerous Lepidoptera juveniles prior to ecdysis (Yoshiga et al., 1997). Although it is difficult to draw parallels between diverse species such as *L. bergylta* and Lepidoptera, it is notable that both undergo post differentiation tissue remodelling events which require fundamental organ dissolution (Sergio Liarte et al., 2007), and major lipid mobilisation (Chapter 5; Yoshiga et al., 1997). Thus, with consideration of this in the context of structural association of ApoA1in discoid and spherical HDL particles (Lund-Katz & Phillips, 2010), which acts to externalise the protein(s) and associated anti-oxidant BV component, there is potential for the *L. bergylta* BV-ApoA1 macromolecules to perform a similar role to the storage proteins of the

Lepidopterans (Yoshiga et al., 1997). Notwithstanding the difficulty in defining specific roles for such functionally diverse proteins (Choudhury et al., 2011), which commonly demonstrate interchangeable isomers and functionalities (Babin & Vernier, 1989), there is also potential for the BV-ApoA1 macromolecule to behave as a storage protein. Hence, the association could also represent a library of bioavailable amino acids developed as a pre-cursor to inversion, or indeed as a repository for lipids mobilised during transition. In either case, and returning to the functionalities of BR-SA complexes in humans (Neuzil & Stocker, 1993), the externalised BV-ApoA1 anti-oxidant molecule would act to protect the lipid rich centres of the HDL particles (McManus et al., 2000) which demonstrate enhanced pro-oxidative lability (Babin & Vernier, 1989), and would therefore act to mitigate the risk of uncontrolled Fenton and Haber-Weiss reactions (Balla et al., 2003; Valko et al., 2007) in a system which is essentially primed for massive oxidative stress (Mwebi, 2005) due to hyperlipidaemia as a result of lipid mobilisation during ovarian involution (Sergio Liarte et al., 2007).

Returning to the protein, its specific identity, and its functionalities, further work could use a more structured approach. Initially, in view of the work of Choudhury et al., (2011), it is likely that repeated centrifugation during initial processing and separation will have induced significant structural and compositional alterations in the ApoA1. Further to this, loss of ApoA1 from human serum has been recorded as a result of HDL centrifugation (Metcalf et al., 1999). From this, and bearing in mind that the primary function of ApoA1 is to bind the hydrophobic sequences of lipids (Lund-Katz & Phillips, 2010), this may explain the MSMS scores (Chapter 4) as the tertiary structure could have been altered of fragmented during processing and the proportion of ApoA1 would be reduced relative to other proteins which had co-migrated under electrophoresis including the IG light chains, and the C3 components may represent artefacts as there is potential for aggregate formation between the polar sequences. However, the methods described by Choudhury et al. (2011) would potentially allow better

resolution. To explain, ultra-centrifugal floatation methods based on the relative differences in molecular weights between the lipoprotein species was used to isolate the fractions then the proteins were further purified by electrophoretic separation. Following this, the N-terminal sequences were deduced, cDNA was extracted then Rapid Amplification of cDNA Ends (RACE) and PCR used to generate enough material for further analysis and sequencing with identification via the normal database channels (Choudhury et al., 2011). Application in both tissue specific and inversion tracking methods would advance knowledge of the species and this proteins functionality greatly.

In addition to the mechanistic processes of gonad remodelling during protogynous sex change, there are other factors associated with the life cycle of high latitude protogynous teleosts such as *L. bergylta* which must also be considered in the context of altered lipid metabolism and pro-oxidative potential including overwintering and torpor. Beginning with overwintering, although torpor remains as anecdotal evidence at present, restricted nutrition and potential starvation involves not only macronutrient limitation including fats, proteins and carbohydrates, but also results in sub-physiological levels of micronutrients (Okoro et al., 2011). From this, it follows that as many of the anti-oxidant defence mechanisms are derivatives of micronutrients or are themselves micronutrients, this affects some directly including the vitamins A and C, and indirectly including components of other abrogative systems including Superoxide Dismutase (SOD) (EC 1.15.1.1) through Mn, Cu and Zn limitation, or Glutathione Peroxidase (GPx) (EC 1.11.1.9) by Sn restriction (Evans & Halliwell, 2001). Thus, long term restriction of access to nutrition is well known to cause a gross derangement of the anti-oxidant profile which effectively translates as pro-oxidative state where production of reactive species exceeds the capacity of the natural abrogative systems (Okoro et al., 2011). Furthermore, complete fasting is accompanied by extensive lipolysis, increased fatty acid turnover, and a concurrent reduction in protein anabolism (Evans &

Halliwell, 2001). Hence, when this is considered in the context of concurrent gender inversion in *L. bergylta*, along with associated altered lipid metabolism, resultant enhanced pro-oxidative state, and energetic diversion from normative somatic budget including maintenance of the native anti-oxidant profile, the argument that an endogenous store of BV to buffer biological systems against the potentially synergetic and highly deleterious state is strengthened greatly. Similarly, returning to Chapter 4 and the hypothesis of torpor in high latitude *L. bergylta*, and bearing in mind that such obligate dormancy remains to be proven in the species of interest but has been confirmed in *C. rupestris* (M. Sayer & Davenport, 1996), the hypometabolic state would further exacerbate the pro-oxidative template and diminish the anti-oxidant capacity. Consequently, this reiterates the requirement for enhanced systems to mitigate the damage potential and further supports that HBV is a key facilitator of protogynous inversion in high latitude hermaphrodites. Accordingly, this would explain the co-distribution of HBV as a trait with the sequential hermaphrodites included in this study.

To draw these thoughts together, HBV appears to act as an intrinsic anti-oxidant network which is extrinsic to the normative HO axis and is therefore capable of operating independently during periods of altered metabolic state. An adaptation to the work of Sayer & Davenport (1996) would serve to resolve this. In brief, their method made use of temperature controlled flow through systems to induce obligate torpor in fish held in a customised restrictive chamber fitted with an oxygen probe, an ultrasound scanner, and a digital camera to measure the oxygen uptake, metabolic rate, opercula movement and heart rate. Subsequently, if this was applied to *L. bergylta*, it would initially resolve the question of obligate torpor in the study species, but were it extended to include BV quantification across the treatment, and combined with hormonal induction to control induction for a comparative analysis of 'fixed gender' against transitional individuals many of these issues could be clarified. This may be of relevance to the industry as it suggests overwintering wrasse in the net pens may not be an ideal

method, or that the wrasse could potentially be pre-conditioned to optimise their condition during and after winter but such work requires specialist equipment and facilities, and was therefore beyond the scope of the current work, but would be of great interest to develop further. It would also be useful to set physical limits for culture conditions. Time to deployment size in hatcheries is currently too long. A clearer understanding of thermal growth preference and by extension thermal minima to avoid torpor (if proven to exist) would be very valuable basic culture parameters.

Continuing with the association of ApoA1 and BV, both components have also been linked to anti-inflammatory, and anti-microbial functions (Choudhury et al., 2011). There are a number of pre-clinical trials which have demonstrated salutary effects in a range of animal models (Weigel and Otterbein, 2012). Initially, BV has been demonstrated to inhibit the C1 component of the classical complement cascade (Weigel and Otterbein, 2012), ApoA1 interacts with and modulates C3 (Magnadottir & Lange, 2004), and BV also regulates C5aR (Bisht et al., 2014) at low molecular concentrations suggesting BV-ApoA1 may represent a previously unknown anti-inflammatory mechanism (Bisht, 2014). Furthermore, REDOX proteomics have recently identified that ApoA1 regulates the pro-inflammatory cytokine TNF-α in the plasma by preventing monocyte T cell interactions and by interaction with the cholesterol transporter cellular plasma membrane sterol transporter ABCA1 (Butterfield, 2014). Conversely, oxidised APOA1 does not supress TNF-α (Butterfield, 2014) which leads to activation of (inducible) Nitric Oxide Synthase (iNOS) (Vakkala et al., 2000) with increases in NO generation and resultant nitrosative stress leading to mitochondrial dysfunction, Cytochrome -C release and eventual apoptosis (Butterfield, 2014). In short, this combination may be a key factor in managing the physiological shock of protogynous inversion. With reference to the work of Bisht et al., (2014), dose dependent activation/inhibition could be tested via quantification of IL-6 and TNF-α expression by LPS induction. In conjunction with qRT-PCR and Western

blotting, this has been demonstrated with commercial BV (Bisht et al., 2014) but remains to be tested in *L. bergylta.* Similarly, the histological analysis could include TUNEL staining to quantify the number of cells in apoptosis and further elucidate the specific activity. Returning to the anti-microbial properties of the complex, it is established that BR is able to uncouple the respiratory metabolism of bacteria resulting in significant membrane disruption (Nobles, 2013). Given the similarity of the molecules and notwithstanding the lipid soluble nature of BR which may have had some effect, there is potential for BV to have similar properties but this remains to be tested. This would be relatively simple as it could be determined by dose dependent growth inhibition on agar plates with quantification by densitometry (Nobles, 2013). Contrastingly however, this characteristic has been well studied with reference to ApoA1 (Concha et al., 2004). The experiment would therefore potentially benefit from comparative analysis under identical conditions between BV, ApoA1, and the BV-ApoA1 complex to elucidate the key functional component. Given that Chapter 3 demonstrated BV was indirectly associated with external colour phenotype, taking into account the theory that the association is due to the complex being externally secreted as previously demonstrated in *S. vitreus* (Schaefer et al., 2014), and the anti-microbial properties of externally secreted ApoA1 in (Concha et al., 2004), there is the potential for the complex to act as a extrinsic point of contact immune response as discussed previously (Chapter 4), but this remains to be confirmed. Establishing the presence, and nature of sacciform cells (Salinas et al., 2011; Schaefer et al., 2014) would therefore be of considerable interest. Finally, in considering that some labrids are able to detect gender by chemical means rather than visually (Warner, 1984), there may still be a non-invasive means of detecting gender based on the secreted complex but this remains a theory for now. With this in mind, another aspect of research that may be of interest would be to explore BV dynamics in response to bacterial infection using a standardised challenge model. If the levels fluctuate in response to a significant challenge then it would support that it

plays a meaningful role. If the BV doesn't clearly flux in response to a challenge then these possible bioactive properties are not necessarily significant. Further work is required.

In considering that this project was initially intended as a means of determining the specific gender of *L. bergylta* individuals, and that the BV concentrations lacked apparent dimorphisms as described in other species, the successful hormonal induction of female *L. bergylta* (Chapter 5) essentially supersedes the requirement for a diagnostic assay. It is thought provoking to note that there was some size advantage in the degree/rate of progression in the experimental groups as it may suggest that there are some characteristics which are attained across development and would therefore be expected to have some link to ontogenic advancement. This essentially suggests that an individual must accumulate "necessary priming" of particular characters prior to natural inversion. For example, enough BV to buffer the oxidative damage of transitional remodelling, and similarly, an energy budget which is adequate for overwintering/changing sex but this would be difficult to determine due to the highly plastic nature of socially controlled hermaphroditism, the pleiotropic nature of the governing stimulae, difficulty in establishing a colony which allows natural sex change, and the fact that artificial induction by hormone application bypasses the normative regulatory mechanisms. Moreover,, this must also be considered in the context of inter-individual variation therefore populations are always highly heterogeneous. From this, necessary priming may be a factor for consideration in determining the SAH (Chapter 1), but this remains to be verified. For the most part the experiments were fully successful as a proof of concept but there were some issues which would be interesting to revisit. Initially, temporal extension would have allowed analysis of sperm quality (Kuo et al., 1988) in such males, and similarly, some fish have been shown to reverse the process and revert back to female the following year (Yeh et al., 2003), but this was beyond the capacity of the present study.

Analogously, the inter-individual variation and polymorphisms may also have been a factor in the BV data when mobilisation was tracked across the inversion process (Chapter 5). This made the intermediate data somewhat difficult to interpret, however, it is noteworthy that where the binary gender scale was applied at the conclusion of Trial 2 (Chapter 5) was identical to the mobilisation patterns described previously (Chapter 3), and as such, supported the theory that BV is depleted during transition as part of the tissue remodelling process. In agreement with Robertson & Choat (1974), the requirement for application of differing gradiations of gender progression and the resultant effects on BV distribution was reflective of the considerable variability in the modes of reproduction, the mating systems, and the modes of differentiation which are inherent in such species. Thus, although the histological scales presented in Chapter 5 are robust, establishing a unified resolution is problematic as the patterns of sexual differentiation do not conform to any orthodox representation of discrete sexes. This could potentially be resolved by using non-invasive Magnetic Resonance Imaging (MRI) (Davenel et al., 2006; Davenel et al., 2010) which could be used to track the intra-specific remodelling processes at more regular intervals across the inversion process then individual data could be frameshifted relative to the rest of the cohort to normalise induction and differentiation. From this, as the method would remove artefacts resulting from the cross sectional histological analysis applied herein, it would allow a truly unified scale of gonad differentiation and potentially confer a cohesive scaffold to develop the BV analysis further. Admittedly, the welfare of the fish and effects of repetitive anaesthesia and blood abstraction would have to be considered and potentially mitigated but there is great potential for improvement.

In conclusion, this thesis is the first to isolate the blue pigment from *L. bergylta* plasma and confirm it as the scavenging antioxidant biliverdin. Furthermore, is is also the first to suggest the importance of BV with reference to sex change in protogynous hermaphrodites,

note the codistribution of HBV with protogynous species, and develop an assay for quantifying pigment variation in this species. The mechanism of accumulation in the plasma was by tight binding with a protein which appeared to be related to ApoA1 such that the molecule was protected from further processing or elimination. Moreover, it was shown that the plasma BV mobilisation is directly associated with the process of gender inversion. And finally, the research herein has suggested a number of physiological functions for the molecule and its associated protein which represent exiting and novel avenues of research which are rather neatly alighned with the current clinical application of BV to access the beneficial properties of the molecule while bypassing the HO system and avoiding any effect on homeostasis.

As mentioned previously, although this work also identified that androgenic supplementation can be used to drive inversion, and therefore affords the capacity to generate males for broodstock, the requirement for a reliable assay to determine gender must remain a priority. To close, in considering the recent realisation of the significance of BV and its metabolic pathway, the realisation of additional anti-inflammatory, anti-microbial and innate-immunological functions are perhaps obvious, but the field is as yet in its infancy and it is presumed that this will develop further with time.

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