Isolation, identification and characterisation of ballan wrasse *Labrus bergylta* plasma pigment.

W. Clark*, E. Leclercq*, H. Migaud*, J. Nairn‡ and A. Davie*†

* Institute of Aquaculture, University of Stirling, Stirling, Scotland. FK9 4LA. Scotland, U.K. ‡ School of Biology, University of St Andrews, St Andrews, Fife KY16 9TS, Scotland, UK

**Running title: L. BERGYLTA PLASMA PIGMENT.**

†Author to whom correspondence should be addressed. Tel.: +44 1786 467988; email: andrew.davie@stir.ac.uk
Abstract

This study confirmed that observations of blue-green colouration in plasma fractions of the ballan wrasse *Labrus bergylta* were caused by the linear tetra-pyrrole biliverdin, and that the molecule was of the physiologically relevant IXα isomer. Accumulation appears driven by chromogenic association with an unknown protein moiety which precludes enzymatic reduction and would suggest active management. It was demonstrated that the pigment did not fluctuate relative to ontogeny, or indeed binary gender in the species of interest, but mobilisation and depletion in the subset of individuals undergoing sex change at the time of study supports a potential association with gender inversion processes. It is of note that although biliverdin does have some effect on external colouration, the evidence is indicative that crypsis is a supplementary function thus other factors must be considered.

Keywords: Ballan wrasse, Blue serum, Biliverdin, Biliverdin IXα, Biliverdin reductase, Bilirubin.
Ballan wrasse *Labrus bergylta* (Ascanius 1767) are the largest and most robust of temperate Labridae, and have proven highly effective at delousing Atlantic salmon *Salmo salar* (L. 1758) 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 out-with spawning (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 Cottidae and 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. 1758) (Mudge & Davenport, 1986), and peacock wrasse Symphodus tinca (L. 1758) (Abolins, 1961); and concentration in cuckoo wrasse Labrus mixtus (L. 1758), axillary wrasse Symphodus mediterraneus (L. 1758) (Abolins, 1961), and the blue-throated wrasse Notolabrus tetricus (Richardson 1840) (Gagnon, 2006). In most observations, blue-green sera was caused by the linear tetrapyrrole biliverdin (Fan88). 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 Labrids (Abolins, 1961; Gagnon, 2006).

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 among individuals. Subsequently, with consideration of the described intra-specific differences in other Labridae, 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.
**Materials and Methods**

For the main study population (n=397), wild *L. bergylta* collection, demography, treatment and biometric data capture were as reported by Leclercq et al. (2014b) with all sampling being completed within a 6 week timeframe. 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. External colour and pattern phenotype were classified using digital photographs collected by Leclercq et al. (2014b) wherein fish were ascribed to colour-type groups (Fig. 1a), and the pattern-type groups (Fig. 1b). Classification was carried out by three independent operators with any classification discrepancies being resolved by assigning in favour of the majority.

Further to this, and independently of the original fish collection, other native Labrid species including rockcook *Centrolabrus exoletus* (L., 1758) (n=10), corkwing *Symphodus melops* (L. 1758) (n=12), goldsinnny *Ctenolabrus rupestris* (L. 1758) (n=12) and cuckoo wrasse *L. mixtus* (L. 1758) (n=12) were collected by baited traps in the Lochaber region of Scotland (56°40’57”N, 5°18’2”W) with collection, treatment and biometric data capture identical to previous methods (Leclercq et al., 2014b). *L. bergylta* (n=12) were sampled at this time to allow comparative analysis without additional complexity from seasonal variation.

**L. bergylta chromophore extraction**

As there is significant intra-specific variation in the degree of plasma colouration (Fig. 1c), to create a large common source of plasma for the initial phase of study, aliquots were pooled and homogenised (JKMS2 mini-shaker; Gemini Systems, www.geminibv.nl). Pooled plasma was then centrifuged (Microcentaur MkII; MSE, www.mseuk.co.uk at 7155 rcf for 2 minutes to reduce post freeze-thaw cellular debris. To cleave the chromophore, 500 µl supernatant was decanted and 500 µl of MeOH.HCl (3N) (Sigma-Aldrich,
www.sigmaaldrich.com) introduced to acidify prosthetic groups. After further centrifugation (7155 rcf, 2 mins), the supernatant was decanted and 500 µl CHCl₃ 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.

**L. bergylta chromophore characterisation**

With biliverdin being the most likely pigment candidate, based on previous characterisation in other Labrids (Abolins, 1961), two biliverdin standards were prepared for comparative analysis against the extracted pigment. The first was commercially obtained BV.HCl (Sigma-Aldrich, www.sigmaaldrich.com) (200 µg ml⁻¹) in C₂H₄O₂; the second was laboratory-generated native biliverdin wherein 0.6 mg commercial bilirubin (BR) (Sigma-Aldrich, www.sigmaaldrich.com) was dissolved in 1780 µl 17.5 M glacial C₂H₄O₂, 200 µl 5% bovine serum albumin (BSA) (Sigma-Aldrich, www.sigmaaldrich.com) solution and 20 µl 4 mM FeCl₃ (Sigma-Aldrich, www.sigmaaldrich.com). The solution was heated at 95 ºC for 2 hours, cooled, and diluted to 20 ml with glacial C₂H₄O₂. 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., www.beckmancoulter.com) for commercially obtained BV.HCl, the extracted pigment, and for the native biliverdin solution. Spectra were blanked against glacial C₂H₄O₂ and recorded at 20 ºC with means of three independent replicates superimposed.

Biliverdin specific colorimetric assays based on adaptations to the bilatrene specific qualitative Gmelin reactions by Lemberg & Legge (1949), and Austin & Jessing's (1994) adaptation of the Gutteridge & Tickner (1978) biliverdin specific assays were tested. In the
first Gmelin reaction, 30 % weight per volume (w/v) (NH₄)₂SO₄ (Sigma-Aldrich, www.sigmaaldrich.com) was added to 500 µl crude sera to compress the solvent layer and precipitate proteins. The solution was centrifuged (7155 rcf, 2 mins) with supernatant recovered and equal volume HNO₃ introduced. In this reaction the blue-green biliverdin (de-hydrobilirubin) is reduced in the presence of HNO₃ to yield a yellow product (bilirubin) (Gray et al., 1961). In the second Gmelin reaction, 500 µl crude plasma was treated with 500 µl H₂SO₄ (Sigma-Aldrich, www.sigmaaldrich.com) then heated in a water-bath (50 ºC) for 10 mins. In this case, the reaction is specific to biliverdin (not meso-biliverdin) with a positive result observed by destruction of the pigment (Lemberg & Legge, 1949). The final diagnostic assay which forms the basis of quantification methodologies developed from Gutteridge & Tickner (1978) and Austin & Jessing (1994), used specificity of biliverdin reactivity with barbituric acid (C₂H₄O₂) in the presence of ascorbic acid (C₆H₈O₆) in an alkaline solution to form a characteristic red chromogen (Manitto & Monti, 1980).

The presence of biliverdin in crude sera was also determined using electro-spray time of flight mass spectroscopy (ESI-MALDI-TOF). Crude plasma was subjected to digestion using a ProGest Investigator digestion robot (Digilab, www.digilabglobal.com) by standard protocol (Shevchenko et al., 1996). The digest solution (0.5 µl) was applied to the MALDI target along with alpha-cyano-4-hydroxycinnamic acid matrix (0.5 µl, 10 mg ml⁻¹ in 50:50 acetonitrile: 0.1% TFA) and allowed to dry. MALDI MS was acquired using a 4800 MALDI TOF/TOF Analyser (ABSciex, www.sciex.com) equipped with an Nd: YAG 355 nm laser and calibrated using a mixture of peptides. The most intense responses (up to 15) were selected for MSMS analysis and the MS data analysed, using GPS Explorer (ABSciex, www.sciex.com) to interface with the Mascot 2.4 search engine (Matrix Science, www.matrixscience.com) 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. The protein sample (20 μl, 10 pM μl⁻¹) was desalted on-line through a NOVAPAK MS C4 2.1x10 mm column (Waters, www.waters.com), eluting with an increasing acetonitrile concentration (2% CH₃CN, 98% aqueous 1% CH₂O₂ to 98% CH₃CN, 2% aqueous 1% CH₂O₂) and delivered to a LCT electrospray ionisation mass spectrometer (Waters, www.waters.com) which had previously been calibrated. An envelope of multiply charged signals was obtained and de-convoluted using MaxEnt1 software to give the molecular mass of the molecule. Identical methodology was applied to commercial Biliverdin IXα (HCl) to generate a known standard for comparative analysis.

Finally, to determine that the chromatographic migration pattern of the pigment was 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 (Silica Gel-60 F₂₅₄) (Merck, www.merckmillipore.com). After 10 mins equilibration, plates were developed using C₄H₈O₂: C₄H₉OH: C₂H₄O₂ (80:10:10) (Sigma-Aldrich, www.sigmaaldrich.com) then visualised with saturated iodine vapour.

**Enzymatic reduction of chromophore**

For enzymatic reduction of biliverdin to bilirubin by biliverdin reductase (BVR) (E.C.1.3.1.24) (Sigma-Aldrich, www.sigmaaldrich.com), 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 (miVac Quattro Concentrator, Genevac Ltd., www.genevac.com) 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 (Sigma-Aldrich, USA), 1 mg
ml⁻¹ BSA, 1.8 mM nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma-Aldrich, USA) and 0.7 U ml⁻¹ BVR (Sigma-Aldrich, www.sigmaaldrich.com). 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 °C. 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.

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 C₂H₄O₂ to generate 500 µmol l⁻¹ with serial dilution in 17.5 M glacial C₂H₄O₂ for standards ranging 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. Serum 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 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 °C for 5 mins in the dark then cooled and 2.5ml C₆H₉OH 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 (r²=0.94) and the BV quantifications extrapolated.

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 Biliverdin,
hence non-detectible values were assigned an arbitrary value of 0 \( \mu \text{mol l}^{-1} \) for analyses. Absolute data was analysed using Minitab 17 Statistical Software (2010) (Minitab, Inc. Software, www.minitab.com). 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 one-way ANOVA and Tukey’s HSD. If individual sample values were more than 1.5 interquartile ranges below or above the “treatment” first or third quartile respectively they were considered outliers and removed from the analysis. Outliers \((n=10)\) were identified during the “geographic origin” and the “colour phenotype” analysis of plasma biliverdin which reduced the total sample pool to \(n=387\) in these analyses. All results are presented as mean \(\pm\) SD. 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.

**Factor analyses**

Exploratory Factor Analysis (EFA) (SPSS, Version 22.0, I.B.M. www.ibm.com) was applied to probe underlying relationships between the measured variables including Origin, Plasma Biliverdin (Abs\(_{570}\)), Gender, Age, Body mass (g), Total length (mm), Colour, Pattern, and the latent constructs (Williams *et al.* 2012). Origin and Gender were determined as common factor internal attributes (Gorsuch, 1988) therefore a reductionist approach was adopted to find the solution of best fit and optimise factorial resolution (Williams *et al.* 2012). This 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 the work of (Kahn, 2006), Cliff (1988) and Cattell (1983) then resolved by orthogonal varimax rotation (Williams et al., 2012).
**Results**

The identity of the blue-green chromophore in *L. Bergylta* plasma was confirmed as biliverdin IXα through comparison of extracted pigment to native biliverdin and commercial biliverdin IXα (.HCl) by absorbance spectroscopy (Fig. 2a), mass spectrometry (583.2 Da) (Fig. 2b and Fig. 2c)), qualitative chemical reactions (Fig. 3), enzymatic reduction (Fig. 4a, 4b and 4c), and by TLC (Relative Rf = 90% similarity).

Mean *L. bergylta* plasma biliverdin concentration was 10.36 ± 0.4 µmol l⁻¹ ranging from 0 µmol l⁻¹ to 32.05 µmol l⁻¹. Biliverdin concentrations were significantly lower (ANOVA: F(3,387) = 58.48, P = 0.000) in the Bergen population (2.51 ± 0.4 µmol l⁻¹) compared to UK stocks from Machrihanish (12.81 ± 0.74 µmol l⁻¹), Ardtoe (13.32 ± 0.81 µmol l⁻¹), and Shetland (12.21 ± 0.74 µmol l⁻¹) (Fig. 5a). With reference to gender, there was no significant difference in plasma biliverdin between males (n=66) (10.71 ± 1.22 µmol l⁻¹) and females (n=322) (10.85 ± 0.45 µmol l⁻¹) (ANOVA: F(1,378) = 0.17, P = 0.683), but transitional individuals (n=9) had a significantly lower level (2.58 ± 1.40 µmol l⁻¹) (ANOVA: F(1,386) = 7.56, P = 0.006) (Fig. 5b). When presence of plasma biliverdin was tested in all UK native labrid species (Fig. 6), biliverdin was observed in *S. melops* (n=12) (8.30 ± 2.2 µmol l⁻¹) at lower magnitude than *L. bergylta* (n=12 samples independent of previous work) (22.82 ± 2.9 µmol l⁻¹), was at the detection limit in *C. exoletus*, and was un-detectable in *L. mixtus* or *C. rupestris*.

In the Exploratory Factor Analysis (EFA) of the manifest variables the OE model (Table I) cumulatively described 79.14% of the total variance in plasma biliverdin 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 mass and total length with strong positive loadings, and gender with strong negative loading. Component 2 had strong positive loading of plasma biliverdin and pattern with negative loadings for BW and TL. The
third component comprised a positive loading for plasma biliverdin and strong positive loading for colour.

The OGE model (Table I) 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 and shown to be significant with particular reference to the green phenotype (Fig. 7).
Discussion

It was determined that accumulation of biliverdin IXα in the plasma fraction 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* (Gagnon, 2006); other teleosts such as woolly sculpin *Clinocottus analyis* (Girard 1858) (Fang, 1990), *C. lumpus* (Mudge & Davenport, 1986), Gar-fish *Belone belone* (L. 1758), Eelpout *Zoarces viviparous* (L. 1758) (Juettner, 2013), and lizards of the genus *Prasinohaema* (Austin & Jessing, 1994).

Biliverdin 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 Heme-oxygenase 1 (HO-1; EC 1.14.99.3) (Morales *et al.*, 2010). This generates equimolar quantities of CO, Fe²⁺ and biliverdin (IXα) (Soares & Bach, 2009). In contrast to mammals where biliverdin is an intermediate metabolite (Bulmer *et al.*, 2008), and is rapidly further reduced to bilirubin by region-specific quantitative biliverdin reductase (BVR-A; EC 1.3.1.24) activity (McDonagh, 2006), it is the end product in birds, amphibians, reptiles and fish and is directly excreted in most species (Ding & Xu, 2002). Hyperbiliverdinaemic 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).

With reference to this, the inhibited reduction of *L. bergylta* plasma biliverdin by BVR-A in the current study was of interest and reflected the work of Fang & Bada (1988). In contrast to mammals where bilirubin (and endogenous biliverdin *(in vitro)*) are found reversibly bound
to albumin for transport in the blood prior to uptake at the hepatic sinusoids, in *C. analis* the biliverdin was tightly bound to a protein moiety (Fang 1984). Closer analysis of the complex indicated biliverdin 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 modified in hyperbiliverdinaemic species such as *L. bergylta* to prevent clearance by direct excretion or further catabolism to bilirubin (Juettner, 2013). In theory therefore, as the association between biliverdin and the protein would require a dedicated protein metabolism and long-term sequestration represents significant diversions of energy from the somatic budget, and there is evidence of active management, this would suggest physiological functions well beyond that of a simple chromogen.

The small subset of individuals undergoing sexual inversion at the time of sampling were remarkable as plasma biliverdin was significantly lower than that of gender specific counterparts, suggesting some interaction with inversion associated processes such as tissue remodelling. This appears corroborated by the additional labrid species in the expanded study with the observations of disparate biliverdin expression between species supporting alternate metabolic strategies (Gagnon, 2006). To explain, the species in which biliverdin was undetectable (*C. rupestris* and *C. exoletus*) are both gonochoristic, whereas, *S. melops*, *L. mixtus* and *L. bergylta* are all 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 biliverdin in the plasma is based upon earlier published works (Abolins, 1961), this would imply that biliverdin may occur in species that undergo sex change and supports the hypothesis that biliverdin accumulation is linked to remodelling processes during inversion (Yoshiga *et al.*, 1997).
In direct contrast to the hyperbiliverdinaemic 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 reproductive status in *L. bergylta*. This most likely reflects interspecific 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 follows that a 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 and McCormic, 2009). In contrast to this, 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). In considering that the ultimate aim of a protogynous species is to become male, then a lack of secondary (sexual) colour differentiation 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).

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. As a global view of the OE model it can be surmised that component 1 represents individual life history stage where age, body mass 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 (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 further support to the robustness of this component as high life history stage values (older and bigger) would therefore predict low gender value (male).

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 biliverdin and colour suggesting association between biliverdin 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 biliverdin is of great interest as a camouflage molecule as it is conformationally flexible and can therefore vary in colour from blue to green depending upon environmental influence and allowing fine tuning by organisms relative to a suitable cryptic hue (McDonagh, 2006). This may only be relevant in green colour-types with the lack of association in other phenotypic groupings illustrating the complexity of
pigment interactions in the expression of phenotypic accents in *L. Bergylta*. This is perhaps best illustrated by comparison of the Bergen subset with the Scottish cohort as the predominantly red phenotype in Norway (Data not shown) most likely drives the location difference in biliverdin levels between origins. Furthermore; improved resolution under application of the optimised OGE model as demonstrated by the relative increase in loading magnitudes, reiterates the uncoupled association between binary gender and pigment in *L. bergylta*, and further supports that other drivers must be considered (Williams *et al.*, 2012; Gorsuch, 1988).

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 biliverdin abundance and gender but; that intersexual individuals demonstrated lower levels than male and female counterparts, and as biliverdin was only found in hermaphroditic species, the current study was strongly indicative that biliverdin has biochemical functions connected with processes out-with the associations of gender and phenotype. This was further supported by the biological functions that linear tetrapyrroles play 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 biliverdin and subsequent bilirubin 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.
Acknowledgements

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References


**Electronic resources**

Table I: Varimax rotated component matrix of Origin excluded \textit{Labrus bergylta} dataset (OE model) with principle component extraction and Kaiser-Normalisation to a 3 component model as well as the varimax rotated component matrix of Origin and Gender excluded \textit{Labrus bergylta} dataset (OGE model) with principle component extraction and Kaiser-Normalisation to a 3 component model.

<table>
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<tr>
<th></th>
<th>Component 1</th>
<th>Component 2</th>
<th>Component 3</th>
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<tr>
<td><strong>OE model</strong></td>
<td></td>
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<tr>
<td>Plasma Biliverdin (Abs570)</td>
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<td>Gender</td>
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<td>Age</td>
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<td>Total length (mm)</td>
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Figure legends:

Figure 1: Typical images showing the classification of *Labrus bergylta* external (a) colour phenotypes comprising: (1) Deep red/brown hue, (2) Red/brown hue with little green, (3) Less intense green with brown/red inclusions and (4) Deep green colouration; (b) pattern phenotypes comprising (1) Spotted and (2) Plain; as well as (c) showing a demonstration of the variation observed in blue-green colour intensity of plasma from *Labrus bergylta*.

Figure 2: Confirmation of plasma chromophore by: (a) comparative absorption spectra of commercially obtained biliverdin hydrochloride (--- - ---) with native biliverdin generated from bilirubin oxidation (--- * ---) and *Labrus bergylta* plasma (--- --- ---) in glacial C₂H₄O₂; LC-MS spectra of (b) biliverdin hydrochloride and (c) trypsin digested *Labrus bergylta* plasma.

Figure 3: Paired Gmelin biliverdin IXα specific diagnostic tests with HNO₃ treatments in *Labrus bergylta* plasma (a-b) and commercial biliverdin hydrochloride (c-d); and H₂SO₄ treatments in *Labrus bergylta* plasma (e-f) and commercial biliverdin hydrochloride (g-h).

Figure 4: Enzymatic (biliverdin reductase) reduction over time 0 (---- ---- ----), 15 (--- --- ---), 30 (--- --- ---), 45 (-----), 60 (--- - --), 120 (----- ----), 180 (--- --- -) and 240 (--- - -) minutes across 300-750nm (a) of *Labrus bergylta* plasma biliverdin to bilirubin monitored via conversion of NADPH (300-400 nm) (b) to NADP (400-500 nm) (c).
Figure 5: Variation in *Labrus bergylta* plasma biliverdin content (µmol l⁻¹) as determined by absorbance spectrophotometry in relation to (a) geographical origin (n=94 (Bergen); 98 (Machrihanish); 99 (Ardtoe) 96 (Shetland)) of and (b) individual gender (n=322 (female); 9 (transitional); 66 (male)). Different superscript letters denote significant differences in mean levels.

Figure 6: Variation in plasma biliverdin content (µmol l⁻¹) in species of Labridae native to the UK including *Ctenolabrus rupestris, Centrolabrus exoletus, Labrus mixtus, Symphodus melops* and *Labrus bergylta* as determined by absorbance spectrophotometry. Different superscript letters denote significant differences in mean levels.

Figure 7: Variation in plasma biliverdin content (µmol l⁻¹) as determined by colorimetric spectrophotometry in relation to external colour phenotypes comprising: (1) Deep red/brown hue (n=168), (2) Red/brown hue with little green (n=136), (3) Less intense green with brown/red inclusions (n=55) and (4) Deep green colouration (n=28). Different superscript letters denote significant differences in mean levels.
Figure 1

1: Deep red/brown hue
2: Red/brown hue with little green
3: Less intense green with brown/red inclusions
4: Deep green colouration

(a)

(b) Spotty  Plain

(c)
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7