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

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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.

Introduction

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), goldsinny *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 $CHCl_3$ 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.

***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 $C_2H_4O_2$; 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 $C_2H_4O_2$, 200 µl 5% bovine serum albumin (BSA) (Sigma-Aldrich, www.sigmaaldrich.com) solution and 20 µl 4 mM $FeCl_3$ (Sigma-Aldrich, www.sigmaaldrich.com). 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., www.beckmancoulter.com) for 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.

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) $(NH_4)2SO_4$ (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 HNO_3 introduced. In this reaction the blue-green biliverdin (de-hydrobilirubin) is reduced in the presence of HNO_3 to yield a yellow product (bilirubin) (Gray *et al.*, 1961). In the second Gmelin reaction, 500 µl crude plasma was treated with 500 µl H_2SO_4 (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 ($C_2H_4O_2$) in the presence of ascorbic acid ($C_6H_8O_6$) 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% 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, 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 F_{254}) (Merck, www.merckmillipore.com). After 10 mins equilibration, plates were developed using $C_4H_8O_2$: C_4H_9OH: $C_2H_4O_2$ (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

174 ml^{-1} BSA, 1.8 mM nicotinamide adenine dinucleotide phosphate (NADPH) (Sigma-Aldrich,
175 USA) and 0.7 U ml^{-1} BVR (Sigma-Aldrich, www.sigmaaldrich.com). Absorption spectra
176 ranging from 300-750 nm at 5 nm intervals were recorded at 0, 15, 30, 45, 60, 120 and 240
177 mins at 37 ºC. Activity was monitored as reduction in the NADPH specific peak at 340 nm,
178 and an increase in the bilirubin product signal in the 460 nm region.
179
180 **Chromophore Quantification**
181 Quantification protocols for plasma biliverdin were conducted as Austin & Jessing
182 (1994) with the following adaptations. BV.HCl was dissolved in 17.5 M glacial $C_2H_4O_2$ to
183 generate 500 µmol l^{-1} with serial dilution in 17.5 M glacial $C_2H_4O_2$ for standards ranging from
184 0-50 µmol l^{-1} with 0.5% BSA. 500 µl distilled H_2O was added to 500 µl of each standard with
185 400 µl 40 mM $C_6H_8O_6$ and 100 µl 200 mM $C_2H_4O_2$ in 1 M NaOH. Serum samples were
186 prepared by addition of 450 µl glacial $C_2H_4O_2$ to 50 µl plasma, 500 µl distilled H_2O was added
187 with 400 µl of 40 mM ascorbic acid and 100 µl 200 mM barbituric acid in 1 M NaOH. Blanks
188 were parallel samples with barbituric acid substituted with 1 M NaOH. Samples were heated
189 at 95 ºC for 5 mins in the dark then cooled and 2.5ml C_4H_9OH with 1 ml 10 M NaOH added
190 then agitated in the dark until the reaction was complete. A two phase solution formed after
191 centrifugation (1789 rcf, 5mins) with the diagnostic red chromophore in the lower component.
192 The top phase was discarded and A_{570} of the lower phase recorded in triplicate, blanks were
193 then subtracted from the samples. The standard solutions were used to construct a calibration
194 curve (r^2=0.94) and the BV quantifications extrapolated.
195 **Statistical analyses**
196 Concentration values calculated from the calibration curve were negative in some
197 individuals. Although negative levels are not physiologically possible, this reflects difficulties
198 in determining concentration by colorimetric methods and the oxidative lability of Biliverdin,

hence non-detectible values were assigned an arbitrary value of 0 µmol 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 ± 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

223 conditioning in accordance with the work of (Kahn, 2006), Cliff (1988) and Cattell (1983) then
224 resolved by orthogonal varimax rotation (Williams *et al.*, 2012).

225 **Results**

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

231 Mean *L. bergylta* plasma biliverdin concentration was 10.36 ± 0.4 μmol l^{-1} ranging
232 from 0 μmol l^{-1} to 32.05 μmol l^{-1}. Biliverdin concentrations were significantly lower (ANOVA:
233 $F(3,387) = 58.48$, $P = 0.000$) in the Bergen population (2.51 ± 0.4 μmol l^{-1}) compared to UK
234 stocks from Machrihanish (12.81 ± 0.74 μmol l^{-1}), Ardtoe (13.32 ± 0.81 μmol l^{-1}), and Shetland
235 (12.21 ± 0.74 μmol l^{-1}) (Fig. 5a). With reference to gender, there was no significant difference
236 in plasma biliverdin between males (n=66) (10.71 ± 1.22 μmol l^{-1}) and females (n=322) (10.85
237 ± 0.45 μmol l^{-1}) (ANOVA: $F(1,378) = 0.17$, $P = 0.683$), but transitional individuals (n=9) had
238 a significantly lower level (2.58 ± 1.40 μmol l^{-1}) (ANOVA: $F(1,386) = 7.56$, $P = 0.006$) (Fig.
239 5b). When presence of plasma biliverdin was tested in all UK native labrid species (Fig. 6),
240 biliverdin was observed in *S. melops* (n=12) (8.30 ± 2.2 μmol l^{-1}) at lower magnitude than *L.*
241 *bergylta* (n=12 samples independent of previous work) (22.82 ± 2.9 μmol l^{-1}), was at the
242 detection limit in *C. exoletus*, and was un-detectable in *L. mixtus* or *C. rupestris*.

243 In the Exploratory Factor Analysis (EFA) of the manifest variables the OE model
244 (Table I) cumulatively described 79.14% of the total variance in plasma biliverdin with the first
245 component comprising 39.80%, the second component 23.38%, and the final component
246 15.95%. Component 1 showed the biometric variables of age, body mass and total length with
247 strong positive loadings, and gender with strong negative loading. Component 2 had strong
248 positive loading of plasma biliverdin and pattern with negative loadings for BW and TL. The

249 third component comprised a positive loading for plasma biliverdin and strong positive loading
250 for colour.

251 The OGE model (Table I) cumulatively described 85.01% of the variance with the first
252 component comprising 46.04%, the second component 21.06%, and the final component
253 17.92%. Component 1 comprised very high loading magnitudes in the biometric parameters
254 with Plasma biliverdin and external colour were strongly loaded in Component 2 and Pattern
255 had resolved to a freestanding position in component 3 with high magnitude loading. In
256 response to the OGE model, the plasma biliverdin and external coloration relationship was
257 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 analis* (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, Fe^{2+} 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 un-detectable (*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 inter-specific 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.

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511 **Electronic resources**

512 Talbot, C., Medeiros, M. V., & Davie, A. (2012). In vivo gender determination in captive
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Table I : Varimax rotated component matrix of Origin excluded *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 *Labrus bergylta* dataset (OGE model) with principle component extraction and Kaiser-Normalisation to a 3 component model.

	Component 1	Component 2	Component 3
OE model			
Plasma Biliverdin (Abs570)		0.701	0.419
Gender	-0.836		
Age	0.868		
Body mass (g)	0.847	-0.441	
Total length (mm)	0.751	-0.515	
Colour			0.941
Pattern		0.735	
OGE model			
Plasma Biliverdin (Abs570)		0.739	
Age	0.945		
Body mass (g)	0.932		
Total length (mm)	0.918		
Colour		0.836	
Pattern			0.958

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 $C_2H_4O_2$; LC-MS spectra of (b) biliverdin hydrochloride and (c) trypsin digested *Labrus bergylta* plasma.

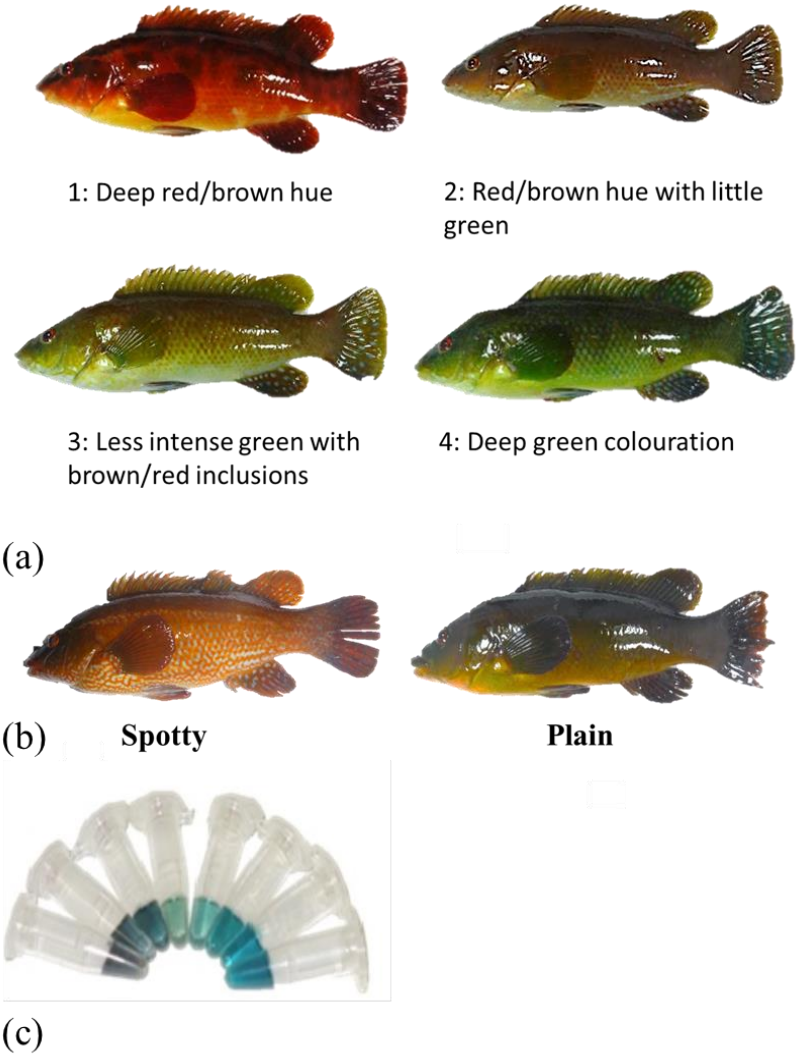
Figure 3: Paired Gmelin biliverdin IXα specific diagnostic tests for with HNO_3 treatments in *Labrus bergylta* plasma (a-b) and commercial biliverdin hydrochloride (c-d); and H_2SO_4 treatments in *Labrus bergylta* plasma (e-f) and commercial biliverdin hydrochloride (g-h).

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.



566

567 **Figure 1**

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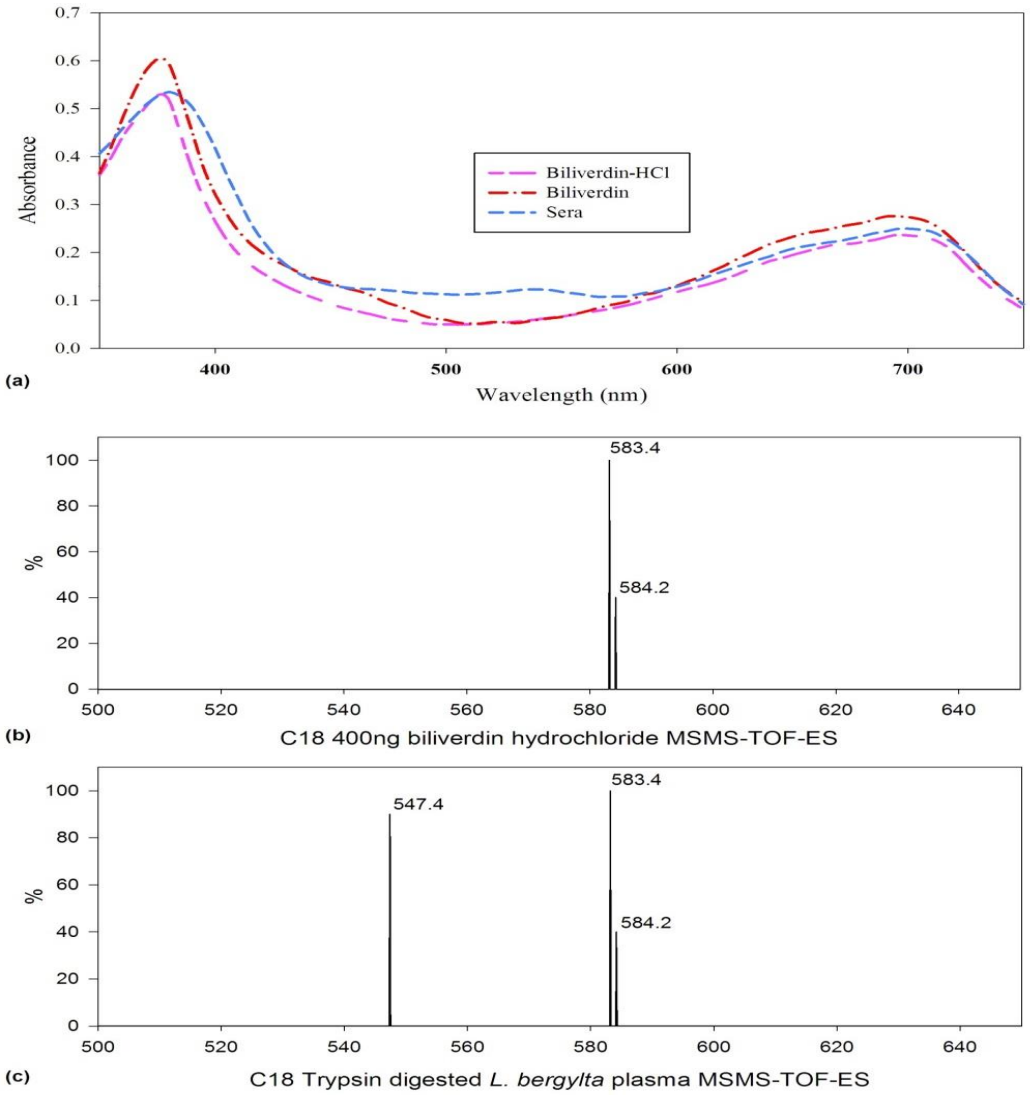

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570 **Figure 2**

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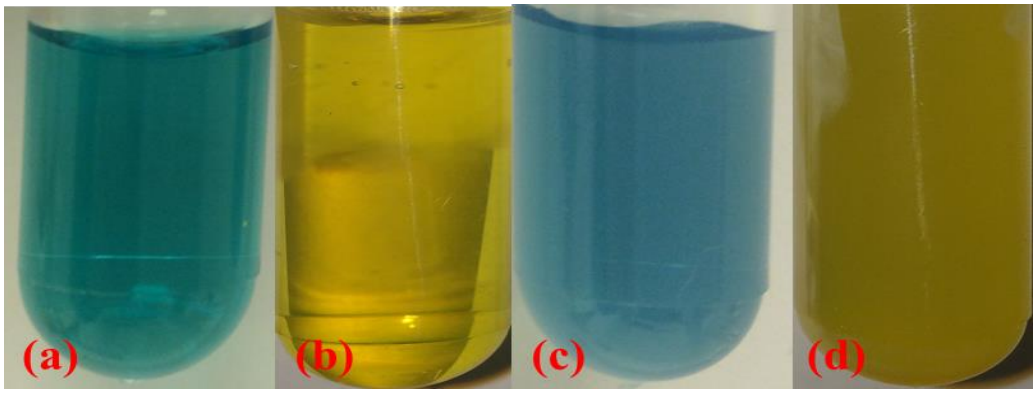
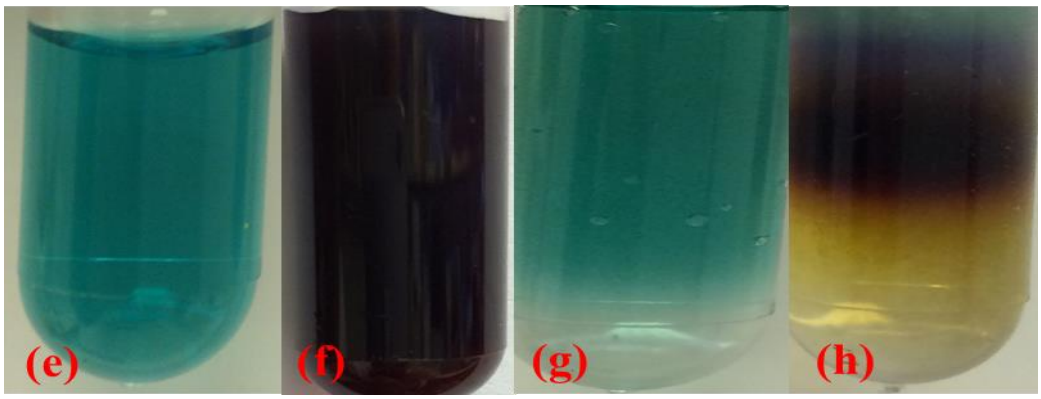
572 

573 

574

575 **Figure 3**

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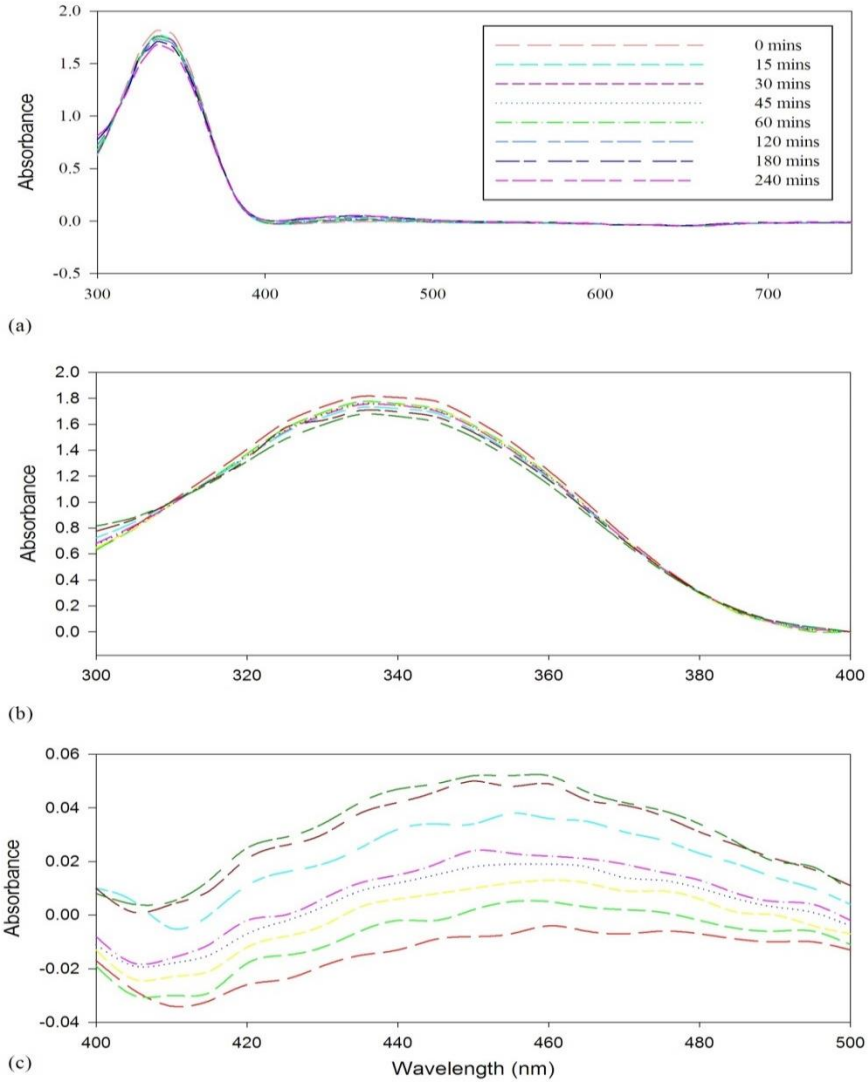

577

578 **Figure 4**

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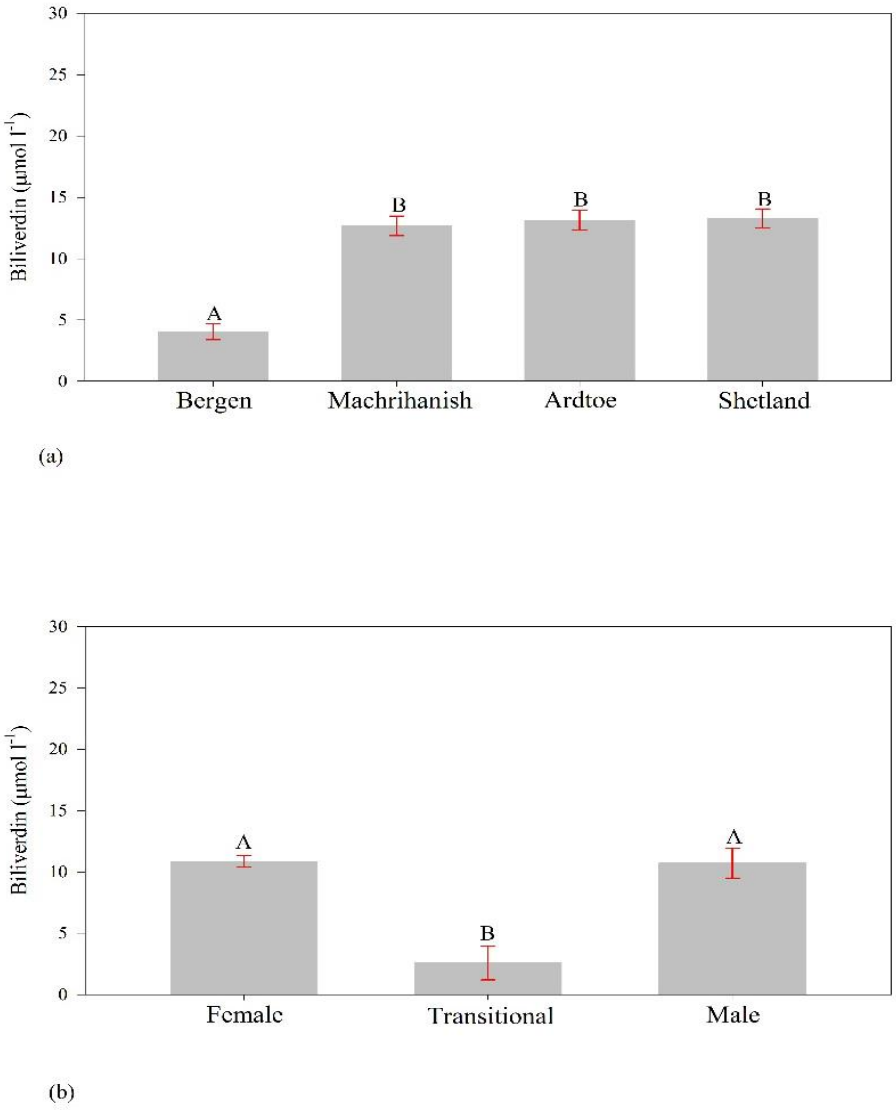

580

581 **Figure 5**

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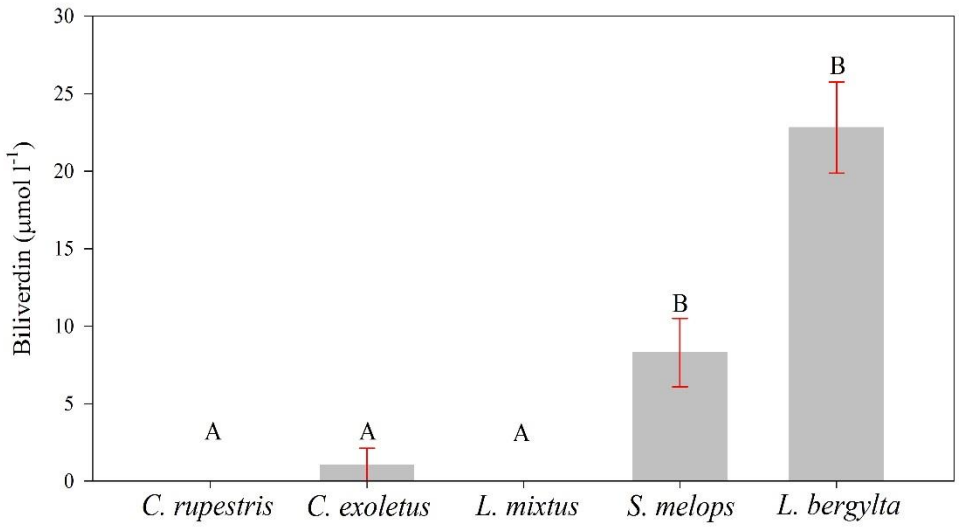

583

584 **Figure 6**

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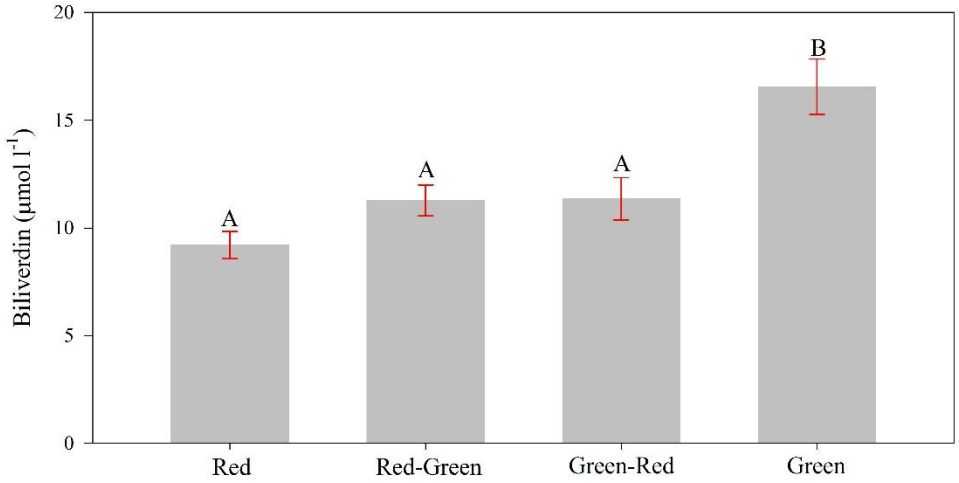

586

587 **Figure 7**

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