

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

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

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14 **Abstract**

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

25 **Keywords:** Ballan wrasse, Blue serum, Biliverdin, Biliverdin IX α , Biliverdin reductase,
26 Bilirubin.

27 **Introduction**

28 Ballan wrasse *Labrus bergylta* (Ascanius 1767) are the largest and most robust of temperate
29 Labridae, and have proven highly effective at delousing Atlantic salmon *Salmo salar* (L. 1758)
30 when deployed in sea cages as part of integrated pest management strategies (Leclercq *et al.*,
31 2014a). Traditionally, wild captured fish have been used but increasing demand relative to
32 limited wild stocks and sustainability concerns have driven an increase in hatchery production
33 (Denholm *et al.*, 2002). Current broodstock management practice is to establish harems of circa
34 20-30 individuals which spontaneously spawn over a natural two month window (Muncaster
35 *et al.*, 2010). The optimisation of broodstock management practices are however limited
36 through difficulties in confirming the broodstock gender. The colour and pattern phenotypes
37 of *L. bergylta* are highly variable (Porteiro *et al.*, 1996), but ultimately appears to have no
38 relation to gender (Villegas-Ríos *et al.*, 2013b). Furthermore, the restricted availability of
39 males, as they represent only 10% of the population (Leclercq *et al.*, 2014b), makes gender
40 identification one of the key technical challenges limiting the expansion of production.

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

52 In contrast to typically pale yellow colouration of blood plasma in mammals, some
53 teleosts including Cottidae and Labridae are observed to have coloured plasma ranging in hue
54 from green through blue to maroon (Low & Bada, 1974). Marked gender dimorphism has been
55 reported in relation to plasma pigment type in lumpsuckers *Cyclopterus lumpus* (L. 1758)
56 (Mudge & Davenport, 1986), and peacock wrasse *Symphodus tinca* (L. 1758) (Abolins, 1961);
57 and concentration in cuckoo wrasse *Labrus mixtus* (L. 1758), axillary wrasse *Symphodus*
58 *mediterraneus* (L. 1758) (Abolins, 1961), and the blue-throated wrasse *Notolabrus tetricus*
59 (Richardson 1840) (Gagnon, 2006). In most observations, blue-green sera was caused by the
60 linear tetrapyrrole biliverdin (Fan88). Although the precise mechanisms of plasma dimorphism
61 remain cryptic, relative differences between genders are thought to be a function of alternate
62 hormonal profiles which drive disparity in expression levels of cyclic and open chain molecules
63 (Abolins, 1961), and through micro-environmental interactions with the binding regions of
64 associated protein complexes (Fang & Bada, 1988). These differences have been established
65 as an accurate methodology for differentiating gender in some Labrids (Abolins, 1961;
66 Gagnon, 2006).

67 Blue-green serum has been reported in *L. bergylta* with strong variation in the degree
68 of colouration in response to unknown factors (Abolins, 1961). The initial aim of this study
69 was therefore to isolate and identify the underlying pigment responsible for observed plasma
70 colouration in *L. Bergylta*, and to characterise it in relation to variation among individuals.
71 Subsequently, with consideration of the described intra-specific differences in other Labridae,
72 the secondary aim was to establish if gender was the major driver of variation, and to ascertain
73 if plasma pigmentation could be used to determine sex.

74 **Materials and Methods**

75 For the main study population ($n=397$), wild *L. bergylta* collection, demography,
76 treatment and biometric data capture were as reported by Leclercq *et al.* (2014b) with all
77 sampling being completed within a 6 week timeframe. Gender was established by histological
78 analysis of the gonads based on Muncaster *et al.* (2013) and Nozu *et al.* (2009) and delineated
79 by consideration of the leading developmental edge. External colour and pattern phenotype
80 were classified using digital photographs collected by Leclercq *et al.* (2014b) wherein fish were
81 ascribed to colour-type groups (Fig. 1a), and the pattern-type groups (Fig. 1b). Classification
82 was carried out by three independent operators with any classification discrepancies being
83 resolved by assigning in favour of the majority.

84 Further to this, and independently of the original fish collection, other native Labrid
85 species including rockcook *Centrolabrus exoletus* (L., 1758) ($n=10$), corkwing *Symphodus*
86 *melops* (L. 1758) ($n=12$), goldsinny *Ctenolabrus rupestris* (L. 1758) ($n=12$) and cuckoo wrasse
87 *L. mixtus* (L. 1758) ($n=12$) were collected by baited traps in the Lochaber region of Scotland
88 ($56^{\circ}40'57''N$, $5^{\circ}18'2''W$) with collection, treatment and biometric data capture identical to
89 previous methods (Leclercq *et al.*, 2014b). *L. bergylta* ($n=12$) were sampled at this time to
90 allow comparative analysis without additional complexity from seasonal variation.

91

92 ***L. bergylta* chromophore extraction**

93 As there is significant intra-specific variation in the degree of plasma colouration (Fig.
94 1c), to create a large common source of plasma for the initial phase of study, aliquots were
95 pooled and homogenised (JKMS2 mini-shaker; Gemini Systems, www.geminibv.nl). Pooled
96 plasma was then centrifuged (Microcentaur MkII; MSE, www.mseuk.co.uk at 7155 *rcf* for 2
97 minutes to reduce post freeze-thaw cellular debris. To cleave the chromophore, 500 μ l
98 supernatant was decanted and 500 μ l of MeOH.HCl (3N) (Sigma-Aldrich,

99 www.sigmaaldrich.com) introduced to acidify prosthetic groups. After further centrifugation
100 (7155 rcf, 2 mins), the supernatant was decanted and 500 μl CHCl_3 added with agitation.
101 Following final centrifugation (7155 rcf, 2 mins), a two phase solution was formed with the
102 chromophore bearing top layer extracted and retained for analyses.

103

104 ***L. bergylta* chromophore characterisation**

105 With biliverdin being the most likely pigment candidate, based on previous
106 characterisation in other Labrids (Abolins, 1961), two biliverdin standards were prepared for
107 comparative analysis against the extracted pigment. The first was commercially obtained
108 BV.HCl (Sigma-Aldrich, www.sigmaaldrich.com) ($200 \mu\text{g ml}^{-1}$) in $\text{C}_2\text{H}_4\text{O}_2$; the second was
109 laboratory-generated native biliverdin wherein 0.6 mg commercial bilirubin (BR) (Sigma-
110 Aldrich, www.sigmaaldrich.com) was dissolved in 1780 μl 17.5 M glacial $\text{C}_2\text{H}_4\text{O}_2$, 200 μl 5%
111 bovine serum albumin (BSA) (Sigma-Aldrich, www.sigmaaldrich.com) solution and 20 μl 4
112 mM FeCl_3 (Sigma-Aldrich, www.sigmaaldrich.com). The solution was heated at 95 $^\circ\text{C}$ for 2
113 hours, cooled, and diluted to 20 ml with glacial $\text{C}_2\text{H}_4\text{O}_2$. The resultant native biliverdin solution
114 was centrifuged (7155 rcf, 2mins) and decanted to sealed flasks for storage (4 $^\circ\text{C}$) (Austin &
115 Jessing, 1994).

116 Absorbance spectra from 350-750 nm were recorded at 5 nm intervals (Ultra-spec
117 2100pro UV/Visible spectrophotometer, Beckman-coulter Inc., www.beckmancoulter.com)
118 for commercially obtained BV.HCl, the extracted pigment, and for the native biliverdin
119 solution. Spectra were blanked against glacial $\text{C}_2\text{H}_4\text{O}_2$ and recorded at 20 $^\circ\text{C}$ with means of
120 three independent replicates superimposed.

121 Biliverdin specific colorimetric assays based on adaptations to the bilatrene specific
122 qualitative Gmelin reactions by Lemberg & Legge (1949), and Austin & Jessing's (1994)
123 adaptation of the Gutteridge & Tickner (1978) biliverdin specific assays were tested. In the

124 first Gmelin reaction, 30 % weight per volume (w/v) $(\text{NH}_4)_2\text{SO}_4$ (Sigma-Aldrich,
125 www.sigmaaldrich.com) was added to 500 μl crude sera to compress the solvent layer and
126 precipitate proteins. The solution was centrifuged (7155 rcf, 2 mins) with supernatant
127 recovered and equal volume HNO_3 introduced. In this reaction the blue-green biliverdin (de-
128 hydrobilirubin) is reduced in the presence of HNO_3 to yield a yellow product (bilirubin) (Gray
129 *et al.*, 1961). In the second Gmelin reaction, 500 μl crude plasma was treated with 500 μl H_2SO_4
130 (Sigma-Aldrich, www.sigmaaldrich.com) then heated in a water-bath (50 $^\circ\text{C}$) for 10 mins. In
131 this case, the reaction is specific to biliverdin (not meso-biliverdin) with a positive result
132 observed by destruction of the pigment (Lemberg & Legge, 1949). The final diagnostic assay
133 which forms the basis of quantification methodologies developed from Gutteridge & Tickner
134 (1978) and Austin & Jessing (1994), used specificity of biliverdin reactivity with barbituric
135 acid ($\text{C}_2\text{H}_4\text{O}_2$) in the presence of ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$) in an alkaline solution to form a
136 characteristic red chromogen (Manitto & Monti, 1980).

137 The presence of biliverdin in crude sera was also determined using electro-spray time
138 of flight mass spectroscopy (ESI-MALDI-TOF). Crude plasma was subjected to digestion
139 using a ProGest Investigator digestion robot (Digilab, www.digilabglobal.com) by standard
140 protocol (Shevchenko *et al.*, 1996). The digest solution (0.5 μl) was applied to the MALDI
141 target along with alpha-cyano-4-hydroxycinnamic acid matrix (0.5 μl , 10 mg ml^{-1} in 50:50
142 acetonitrile: 0.1% TFA) and allowed to dry. MALDI MS was acquired using a 4800 MALDI
143 TOF/TOF Analyser (ABSciex, www.sciex.com) equipped with an Nd: YAG 355 nm laser and
144 calibrated using a mixture of peptides. The most intense responses (up to 15) were selected for
145 MSMS analysis and the MS data analysed, using GPS Explorer (ABSciex, www.sciex.com) to
146 interface with the Mascot 2.4 search engine (Matrix Science, www.matrixscience.com) and the
147 MSMS data using Mascot 2.4 directly. The data was searched with tolerances of 100 ppm for
148 the precursor ions and 0.5 Da for the fragment ions, trypsin as the cleavage enzyme, assuming

149 up to one missed cleavage, carbamidomethyl modification of cysteine as a fixed modification
150 and methionine oxidation selected as a variable modification. The protein sample (20 μl , 10
151 μM μl^{-1}) was desalted on-line through a NOVAPAK MS C4 2.1x10 mm column (Waters,
152 www.waters.com), eluting with an increasing acetonitrile concentration (2% CH_3CN , 98%
153 aqueous 1% CH_2O_2 to 98% CH_3CN , 2% aqueous 1% CH_2O_2) and delivered to a LCT
154 electrospray ionisation mass spectrometer (Waters, www.waters.com) which had previously
155 been calibrated. An envelope of multiply charged signals was obtained and de-convoluted
156 using MaxEnt1 software to give the molecular mass of the molecule. Identical methodology
157 was applied to commercial Biliverdin IX α (.HCl) to generate a known standard for comparative
158 analysis.

159 Finally, to determine that the chromatographic migration pattern of the pigment was
160 similar to that of the predicted compound, equal volumes (75 μl) of extracted chromophore and
161 BV.HCl in potassium phosphate buffer were spotted on a Thin Layer Chromatography (TLC)
162 plate (Silica Gel-60 F₂₅₄) (Merck, www.merckmillipore.com). After 10 mins equilibration,
163 plates were developed using $\text{C}_4\text{H}_8\text{O}_2$: $\text{C}_4\text{H}_9\text{OH}$: $\text{C}_2\text{H}_4\text{O}_2$ (80:10:10) (Sigma-Aldrich,
164 www.sigmaaldrich.com) then visualised with saturated iodine vapour.

165

166 **Enzymatic reduction of chromophore**

167 For enzymatic reduction of biliverdin to bilirubin by biliverdin reductase (BVR)
168 (E.C.1.3.1.24) (Sigma-Aldrich, www.sigmaaldrich.com), extracted pigment was dissolved in
169 1 ml potassium phosphate buffer and homogenised then 1.0 M NaOH added drop-wise to
170 neutralise. Aliquots were then dried to a solid under vacuum (miVac Quattro Concentrator,
171 Genevac Ltd., www.genevac.com) and residuals re-suspended in 800 μl potassium phosphate
172 buffer (pH 7.0) then agitated until full dissolution and re-combined. The assay mix comprised
173 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 °C. 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 C₂H₄O₂ to
183 generate 500 μmol l⁻¹ with serial dilution in 17.5 M glacial C₂H₄O₂ for standards ranging from
184 0-50 μmol l⁻¹ with 0.5% BSA. 500 μl distilled H₂O was added to 500 μl of each standard with
185 400 μl 40 mM C₆H₈O₆ and 100 μl 200 mM C₂H₄O₂ in 1 M NaOH. Serum samples were
186 prepared by addition of 450 μl glacial C₂H₄O₂ to 50 μl plasma, 500 μl distilled H₂O 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 °C for 5 mins in the dark then cooled and 2.5ml C₄H₉OH 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 ref, 5mins) with the diagnostic red chromophore in the lower component.
192 The top phase was discarded and A₅₇₀ 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 (r²=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,

199 hence non-detectable values were assigned an arbitrary value of $0 \mu\text{mol l}^{-1}$ for analyses.
200 Absolute data was analysed using Minitab 17 Statistical Software (2010) (Minitab, Inc.
201 Software, www.minitab.com). Data was resistant to normalisation following transformation by
202 any means therefore differences in parameters between treatments, variables or stages of
203 maturity were analysed using Student's t-test where appropriate, non-parametric Kruskal-
204 Wallis one-way ANOVA and Tukey's HSD. If individual sample values were more than 1.5
205 interquartile ranges below or above the "treatment" first or third quartile respectively they were
206 considered outliers and removed from the analysis. Outliers ($n=10$) were identified during the
207 "geographic origin" and the "colour phenotype" analysis of plasma biliverdin which reduced
208 the total sample pool to $n=387$ in these analyses. All results are presented as mean \pm SD. As it
209 was determined that the frequency and distribution of negative values resulted in strong
210 kurtosis and discontinuity in the data, and as this was a function of conversion to absolute levels
211 from colorimetric measurements, the Abs₅₇₀ values (analogous to target molecule abundance)
212 were used in further analyses described below.

213

214 **Factor analyses**

215 Exploratory Factor Analysis (EFA) (SPSS, Version 22.0, I.B.M. www.ibm.com) was
216 applied to probe underlying relationships between the measured variables including Origin,
217 Plasma Biliverdin (Abs₅₇₀), Gender, Age, Body mass (g), Total length (mm), Colour, Pattern,
218 and the latent constructs (Williams *et al.*, 2012). Origin and Gender were determined as
219 common factor internal attributes (Gorsuch, 1988) therefore a reductionist approach was
220 adopted to find the solution of best fit and optimise factorial resolution (Williams *et al.*, 2012).
221 This determined development of the subsequent Origin excluded (OE) and Origin and Gender
222 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 IX α through comparison of extracted pigment to native biliverdin and commercial
228 biliverdin IX α (.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 R f = 90% similarity).

231 Mean *L. bergylta* plasma biliverdin concentration was $10.36 \pm 0.4 \mu\text{mol l}^{-1}$ ranging
232 from $0 \mu\text{mol l}^{-1}$ to $32.05 \mu\text{mol l}^{-1}$. Biliverdin concentrations were significantly lower (ANOVA:
233 $F(3,387) = 58.48, P = 0.000$) in the Bergen population ($2.51 \pm 0.4 \mu\text{mol l}^{-1}$) compared to UK
234 stocks from Machrihanish ($12.81 \pm 0.74 \mu\text{mol l}^{-1}$), Ardtoe ($13.32 \pm 0.81 \mu\text{mol l}^{-1}$), and Shetland
235 ($12.21 \pm 0.74 \mu\text{mol l}^{-1}$) (Fig. 5a). With reference to gender, there was no significant difference
236 in plasma biliverdin between males ($n=66$) ($10.71 \pm 1.22 \mu\text{mol l}^{-1}$) and females ($n=322$) (10.85
237 $\pm 0.45 \mu\text{mol l}^{-1}$) (ANOVA: $F(1,378) = 0.17, P = 0.683$), but transitional individuals ($n=9$) had
238 a significantly lower level ($2.58 \pm 1.40 \mu\text{mol l}^{-1}$) (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* ($n=12$) ($8.30 \pm 2.2 \mu\text{mol l}^{-1}$) at lower magnitude than *L.*
241 *bergylta* ($n=12$ samples independent of previous work) ($22.82 \pm 2.9 \mu\text{mol l}^{-1}$), 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).

258 **Discussion**

259 It was determined that accumulation of biliverdin IX α in the plasma fraction of *L.*
260 *bergyta* was the driver of reported blue-green colouration. This was consistent with findings
261 in related species including *S. melops*, *L. mixtus* (Abolins, 1961) and *N. tetricus* (Gagnon,
262 2006); other teleosts such as woolly sculpin *Clinocottus analis* (Girard 1858) (Fang, 1990), *C.*
263 *lumpus* (Mudge & Davenport, 1986), Gar-fish *Belone belone* (L. 1758), Eelpout *Zoarces*
264 *viviparous* (L. 1758) (Juettner, 2013), and lizards of the genus *Prasinohaema* (Austin & Jessing,
265 1994).

266 Biliverdin IX α biosynthesis is a ubiquitous process which can be constitutive during
267 the catabolism of senescent erythrocytes and turnover of cytochrome p450 enzymes, or
268 facultative in response to departure from haem homeostasis and when erythrocytes are
269 damaged (McDonagh, 2006). The reaction is initiated is by NADPH dependent C-10 specific
270 cleavage of the haem template with catalysis by Heme-oxygenase 1 (HO-1; EC 1.14.99.3)
271 (Morales *et al.*, 2010). This generates equimolar quantities of CO, Fe²⁺ and biliverdin (IX α)
272 (Soares & Bach, 2009). In contrast to mammals where biliverdin is an intermediate metabolite
273 (Bulmer *et al.*, 2008), and is rapidly further reduced to bilirubin by region-specific quantitative
274 biliverdin reductase (BVR-A; EC 1.3.1.24) activity (McDonagh, 2006), it is the end product in
275 birds, amphibians, reptiles and fish and is directly excreted in most species (Ding & Xu, 2002).
276 Hyperbiliverdinaemic plasma is therefore a highly unusual observation in vertebrates and is
277 most usually noted in pathological cases of biliary atresia, catarrhal jaundice or liver cirrhosis
278 which act to increase circulating bilatrene levels by preventing further processing and
279 elimination (Fang & Bada, 1990).

280 With reference to this, the inhibited reduction of *L. bergylta* plasma biliverdin by BVR-
281 A in the current study was of interest and reflected the work of Fang & Bada (1988). In contrast
282 to mammals where bilirubin (and endogenous biliverdin (*in vitro*)) are found reversibly bound

283 to albumin for transport in the blood prior to uptake at the hepatic sinusoids, in *C. analis* the
284 biliverdin was tightly bound to a protein moiety (Fang 1984). Closer analysis of the complex
285 indicated biliverdin associates with the binding pocket in a coiled helical formation with
286 stabilisation via hydrogen bonding and hydrophobic interactions making the C-10 methene
287 bridge inaccessible to BVR-A for reduction (Fang, 1984). This supports that excretion
288 pathways are modified in hyperbiliverdinaemic species such as *L. bergylta* to prevent clearance
289 by direct excretion or further catabolism to bilirubin (Juettner, 2013). In theory therefore, as
290 the association between biliverdin and the protein would require a dedicated protein
291 metabolism and long-term sequestration represents significant diversions of energy from the
292 somatic budget, and there is evidence of active management, this would suggest physiological
293 functions well beyond that of a simple chromogen.

294 The small subset of individuals undergoing sexual inversion at the time of sampling
295 were remarkable as plasma biliverdin was significantly lower than that of gender specific
296 counterparts, suggesting some interaction with inversion associated processes such as tissue
297 remodelling. This appears corroborated by the additional labrid species in the expanded study
298 with the observations of disparate biliverdin expression between species supporting alternate
299 metabolic strategies (Gagnon, 2006). To explain, the species in which biliverdin was un-
300 detectable (*C. rupestris* and *C. exoletus*) are both gonochoristic, whereas, *S. melops*, *L. mixtus*
301 and *L. bergylta* are all protogynous hermaphrodites. Although this is with reference to a limited
302 number of species, and it is of note that all *L. mixtus* in the study were female therefore the
303 assertion of biliverdin in the plasma is based upon earlier published works (Abolins, 1961),
304 this would imply that biliverdin may occur in species that undergo sex change and supports the
305 hypothesis that biliverdin accumulation is linked to remodelling processes during inversion
306 (Yoshiga *et al.*, 1997).

307 In direct contrast to the hyperbiliverdinaemic species *L. mixtus* (Abolins, 1961) and *N.*
308 *tetricus* (Gagnon, 2006) mentioned previously, which are proven to exhibit gender specific
309 plasma dimorphism; the present study found no significant difference in the relative abundance
310 of biliverdin relative to reproductive status in *L. bergylta*. This most likely reflects inter-
311 specific differences in reproductive patterns arising from contrasting assemblage profiles.
312 Similarly to *L. Bergylta*, both *L. mixtus* and *N. tetricus* are both sequential hermaphrodites, but
313 also exhibit strong external dimorphism in colouration with respect to gender (Abolins, 1961).
314 In agreement with Mudge & Davenport (1986), sexual dimorphism in plasma pigmentation is
315 closely linked with corresponding external colourations involved in sexual signalling. Thus; in
316 considering that deposition of the chromophore in the skin (Abolins, 1961), or differentials in
317 catabolic expression profiles (Mudge & Davenport, 1986) act as the main drivers of reported
318 gender dimorphism in plasma pigment, it follows that a strongly monomorphic species such as
319 *L. bergylta* would show no discernible differential.

320 The ecological drivers of such a trait are of interest as the benefits of marked external
321 dimorphisms are well known in lek type mating systems where inter-specific competition
322 makes energy investment and the metabolic costs associated with advertising male status and
323 courtship an advantageous strategy (Walker and McCormic, 2009). In contrast to this, it is
324 thought that the long term stability of *L. bergylta* assemblages and high site fidelity of
325 communities (Sayer *et al.*, 1993; Villegas-Ríos *et al.*, 2013a) provide a stable social context
326 which means the male has no need to divert metabolism to invest in reproductive ornamentation
327 as the group is essentially ‘fixed’ and the assertion of social hierarchies are constant (Black *et*
328 *al.*, 2005). In considering that the ultimate aim of a protogynous species is to become male,
329 then a lack of secondary (sexual) colour differentiation ameliorates increased predation risk
330 through departure from primary (cryptic) colouration (Lailvaux & Irschick, 2006). These
331 factors suggest that status dependent sexual selection and frequency dependent natural

332 selection have driven external gender monomorphism and resulted in monandric reproductive
333 strategies in *L. bergylta* (Uglem, 2000).

334 As no obvious gender specific associations of *L. bergylta* plasma biliverdin variation
335 was found in the manifest data, EFA was applied to ascertain any other latent relationships.
336 This revealed clear inherent patterns to help guide future investigations. As a global view of
337 the OE model it can be surmised that component 1 represents individual life history stage where
338 age, body mass and total length are closely associated, and that gender is a function of these in
339 accordance with the principles of protogyny and the size advantage hypothesis (Munday *et al.*,
340 2006) as determined by (Leclercq *et al.*, 2014b). It is of note that the negative loading of gender
341 in this system is explained by the allocation of numerical descriptors during statistical analyses
342 where males and females were assigned the values of 1 and 2 respectively. The interpretation
343 of this association lends further support to the robustness of this component as high life history
344 stage values (older and bigger) would therefore predict low gender value (male).

345 The multi-factorial loading of life history traits and association of plasma biliverdin and
346 pattern in the OE model was somewhat cryptic, but comparison with the resolution of variables
347 in the OGE model with pattern as a free-standing variable would indicate this as an independent
348 trait (Williams *et al.*, 2012). Component 3 and component 2 of the OE and OGE models
349 respectively were similar as they both comprised plasma biliverdin and colour suggesting
350 association between biliverdin and external colour-type. Further statistical analyses supported
351 this prediction of association between pigment and colour in green individuals but failed to
352 differentiate the other ascribed phenotypes. Hence, although biliverdin is of great interest as a
353 camouflage molecule as it is conformationally flexible and can therefore vary in colour from
354 blue to green depending upon environmental influence and allowing fine tuning by organisms
355 relative to a suitable cryptic hue (McDonagh, 2006). This may only be relevant in green colour-
356 types with the lack of association in other phenotypic groupings illustrating the complexity of

357 pigment interactions in the expression of phenotypic accents in *L. Bergylta*. This is perhaps
358 best illustrated by comparison of the Bergen subset with the Scottish cohort as the
359 predominantly red phenotype in Norway (Data not shown) most likely drives the location
360 difference in biliverdin levels between origins. Furthermore; improved resolution under
361 application of the optimised OGE model as demonstrated by the relative increase in loading
362 magnitudes, reiterates the uncoupled association between binary gender and pigment in *L.*
363 *bergylta*, and further supports that other drivers must be considered (Williams *et al.*, 2012;
364 Gorsuch, 1988).

365 In closing, this is the first confirmation that the pigment driver of blue-green plasma in
366 *L. bergylta* is biliverdin IX α . Accumulation occurs through biliverdin associating with a protein
367 moiety which prevents further processing or excretion to the extent that the pigment is visible
368 in the plasma fraction. There was no association between biliverdin abundance and gender but;
369 that intersexual individuals demonstrated lower levels than male and female counterparts, and
370 as biliverdin was only found in hermaphroditic species, the current study was strongly
371 indicative that biliverdin has biochemical functions connected with processes out-with the
372 associations of gender and phenotype. This was further supported by the biological functions
373 that linear tetrapyrroles play in animals (Cunningham *et al.*, 2000). HO-1 induction is thought
374 to act as a rapid *in-vivo* anti-oxidant response which initially removes pro-oxidant haem from
375 local tissues with the concurrent increases in the physiological reducing molecules biliverdin
376 and subsequent bilirubin acting to confer longer term cellular defence mechanisms against
377 oxidative damage (Abraham & Kappas, 2008). The future direction of this research is therefore
378 to continue exploration of the physiological roles of biliverdin in *L. bergylta* and other
379 hermaphrodite species, and to further investigate the mechanisms the species use to manage its
380 availability and activity.

381

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387

388

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516 **Table I** : Varimax rotated component matrix of Origin excluded *Labrus bergylta* dataset (OE
517 model) with principle component extraction and Kaiser-Normalisation to a 3 component
518 model as well as the varimax rotated component matrix of Origin and Gender excluded
519 *Labrus bergylta* dataset (OGE model) with principle component extraction and Kaiser-
520 Normalisation to a 3 component model.

521

	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

522

523

524

525 **Figure legends:**

526

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

532

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

537

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

541

542 **Figure 4:** Enzymatic (biliverdin reductase) reduction over time 0 (---- ---- ----), 15 (--- ---
543 ---), 30 (--- --- ---), 45 (.....), 60 (--- . ---), 120 (- ---- ----), 180 (--- --- -) and 240 (- --- -)
544 minutes across 300-750nm (a) of *Labrus bergylta* plasma biliverdin to bilirubin monitored via
545 conversion of NADPH (300-400 nm) (b) to NADP (400-500 nm) (c).

546

547 **Figure 5:** Variation in *Labrus bergylta* plasma biliverdin content ($\mu\text{mol l}^{-1}$) as determined by
548 absorbance spectrophotometry in relation to (a) geographical origin ($n=94$ (Bergen); 98
549 (Machrihanish); 99 (Ardtoe) 96 (Shetland)) of and (b) individual gender ($n=322$ (female); 9
550 (transitional); 66 (male)). Different superscript letters denote significant differences in mean
551 levels.

552

553 **Figure 6:** Variation in plasma biliverdin content ($\mu\text{mol l}^{-1}$) in species of Labridae native to the
554 UK including *Ctenolabrus rupestris*, *Centrolabrus exoletus*, *Labrus mixtus*, *Symphodus melops*
555 and *Labrus bergylta* as determined by absorbance spectrophotometry. Different superscript
556 letters denote significant differences in mean levels.

557

558 **Figure 7:** Variation in plasma biliverdin content ($\mu\text{mol l}^{-1}$) as determined by colorimetric
559 spectrophotometry in relation to external colour phenotypes comprising: (1) Deep red/brown
560 hue ($n=168$), (2) Red/brown hue with little green ($n=136$), (3) Less intense green with
561 brown/red inclusions ($n=55$) and (4) Deep green colouration ($n=28$). Different superscript
562 letters denote significant differences in mean levels.

563

564

565



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

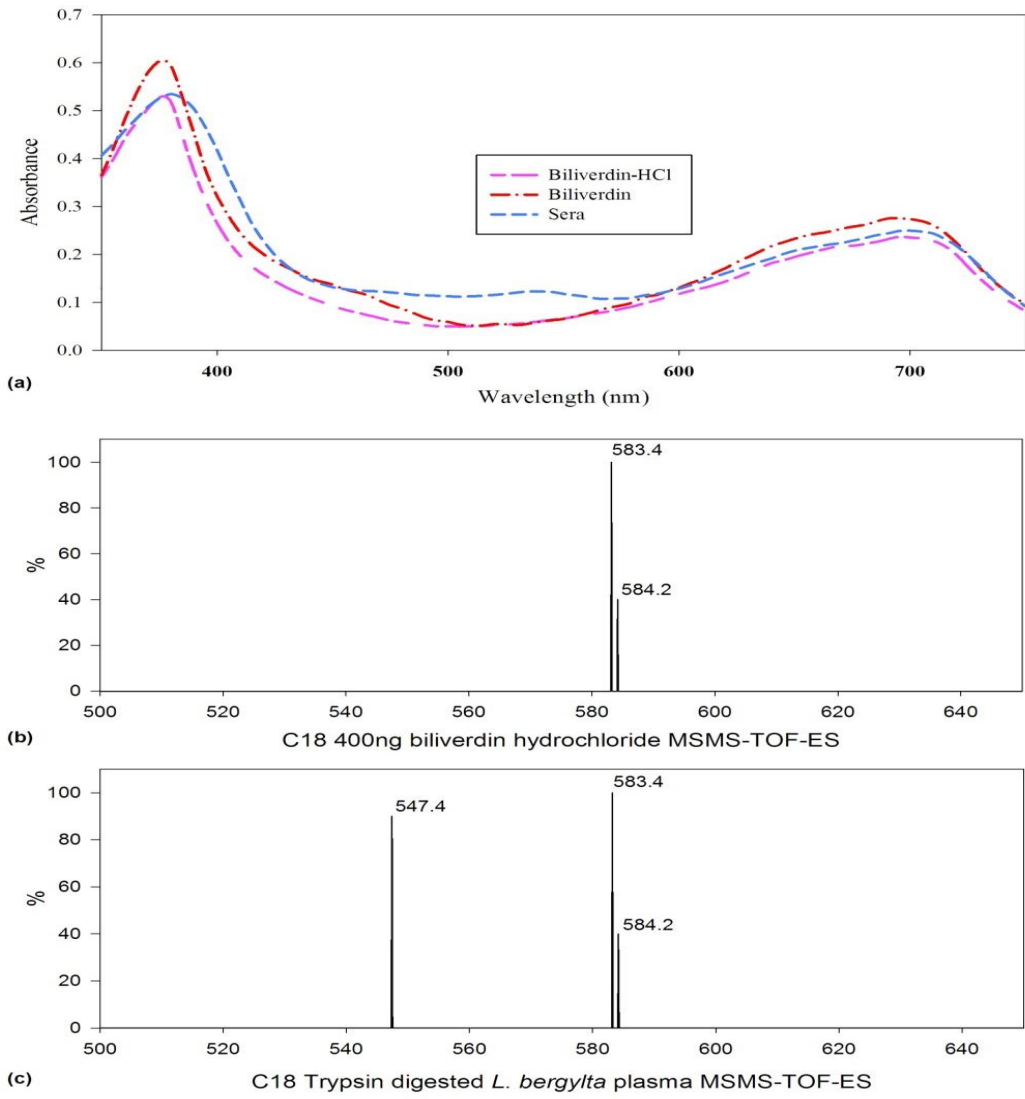


(c)

566

567 **Figure 1**

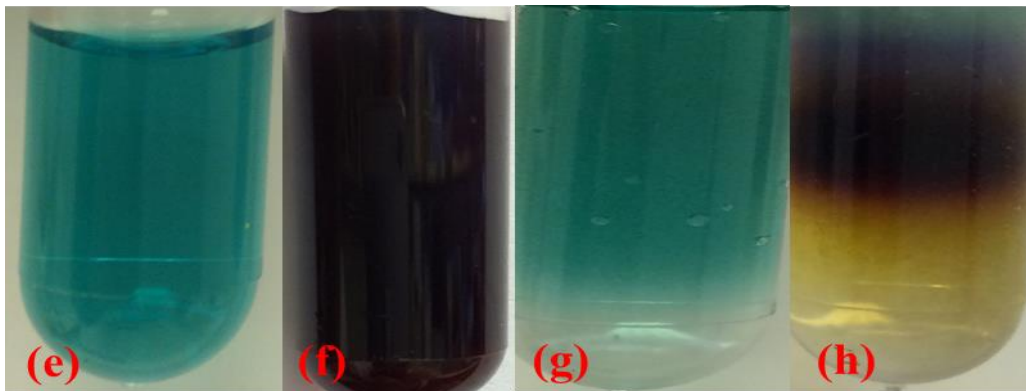
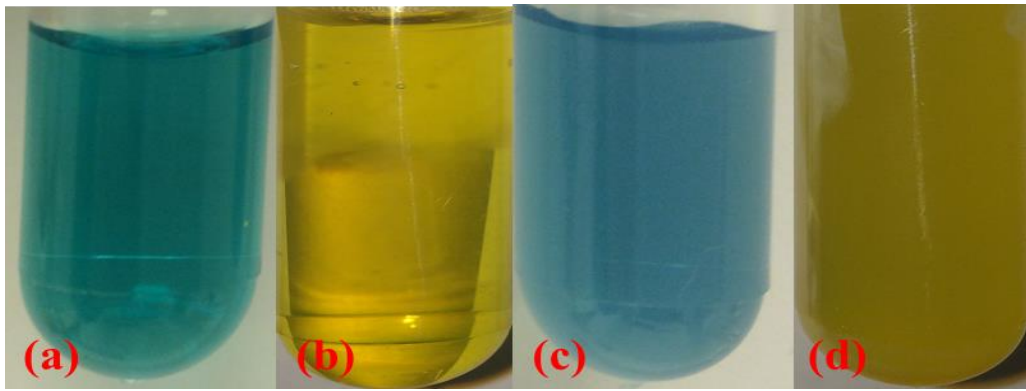
568



569

570 **Figure 2**

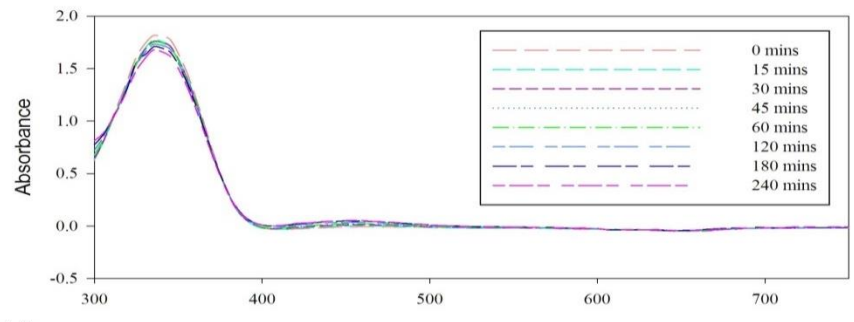
571



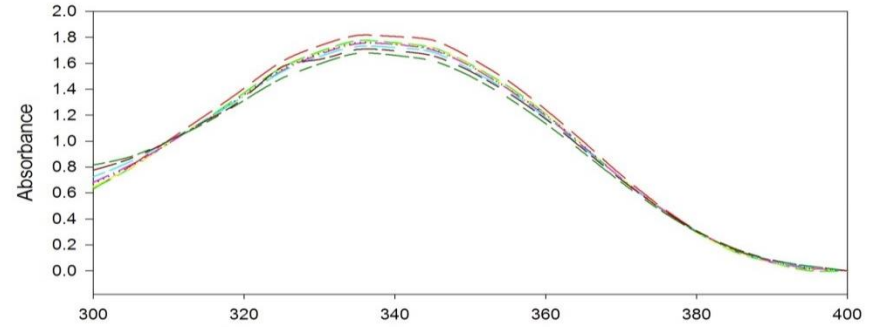
574

575 **Figure 3**

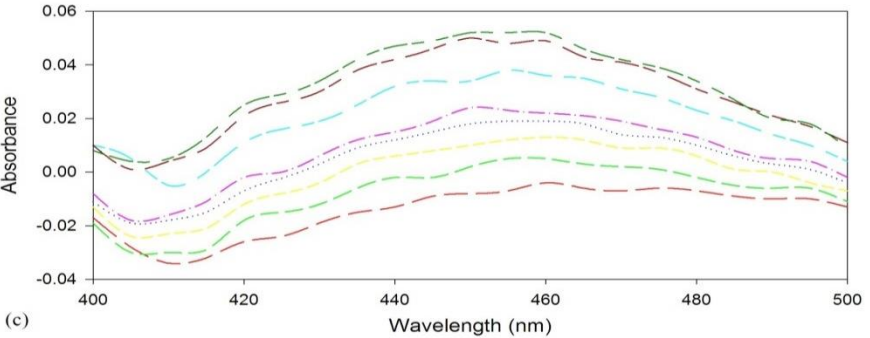
576



(a)



(b)

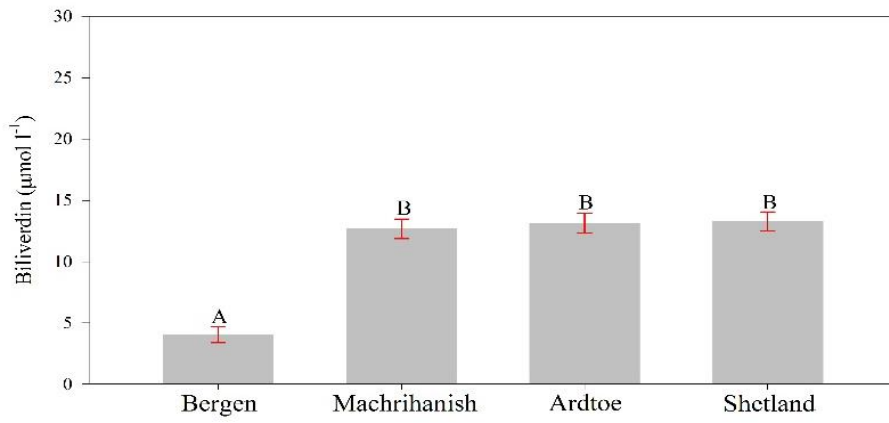


(c)

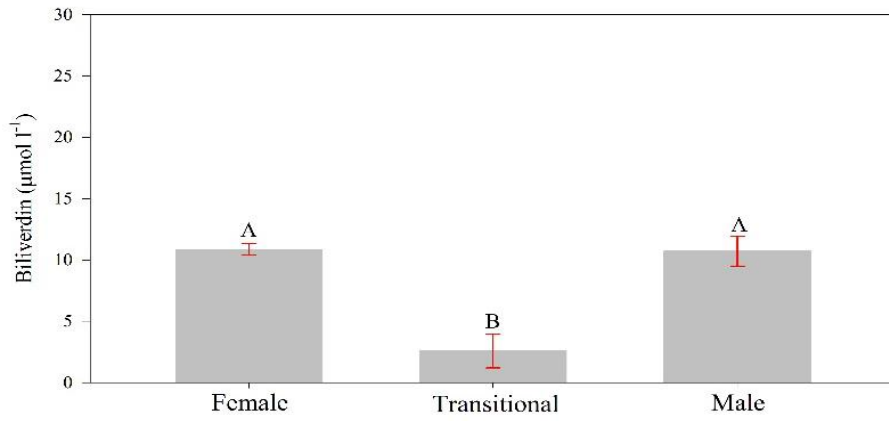
577

578 **Figure 4**

579



(a)

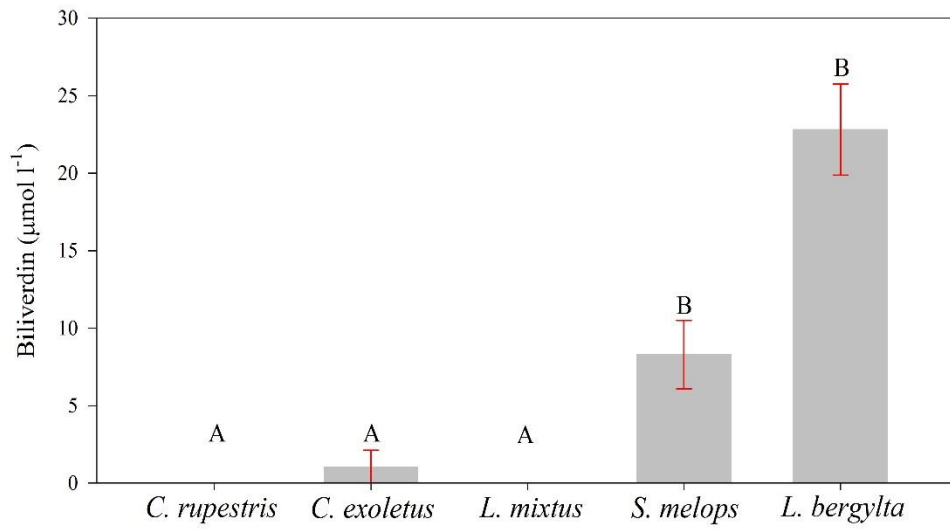


(b)

580

581 **Figure 5**

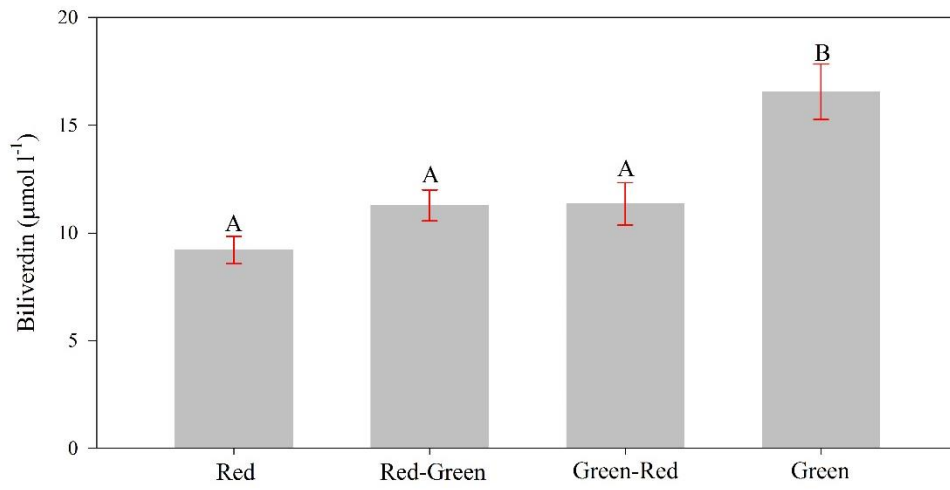
582



583

584 **Figure 6**

585



586

587 **Figure 7**

588