A quantitative analysis of objective feather colour assessment:

measurements in the lab are more reliable than in the field

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1 **Abstract**

2   The evolution of animal colouration is importantly driven by sexual selection operating on traits used to

3 transmit information to rivals and potential mates, which therefore, have major impacts on fitness.

4 Reflectance spectrometry has become a standard colour-measuring tool, especially after the discovery of

5 tetrachromacy in birds and their ability to detect UV. Birds' plumage patterns may be invisible to humans,

6 necessitating a reliable and objective way of assessing colouration not dependent on human vision.

7 Plumage colouration measurements can be taken directly on live birds in the field or in the lab (e.g. on

8 collected feathers). Therefore, it is essential to determine which sampling method yields more repeatable

9 and reliable measures, and which of the available quantitative approaches best assess the repeatability of

10 these measures. Using a spectrophotometer, we measured melanin-based colouration in barn swallows'

11 (*Hirundo rustica*) plumage. We assessed the repeatability of measures obtained with both traditional

12 sampling methods separately to quantitatively determine their reliability. We used the ANOVA-based

13 method for calculating the repeatability of measurements from two years separately, and the GLMM-

14 based method to calculate overall adjusted repeatabilities for both years. We repeated the assessment for

15 the whole reflectance spectrum range and only the human-visible part, to assess the influence of the UV

16 component on the reliabilities of sampling methodologies. Our results reveal very high repeatability for lab

17 measurements and a lower, still moderate to high repeatability, for field measurements. Both increased

18 when limited to only the human-visible part, for all plumage patches except the throat, where we observed

19 the opposite trend. Repeatability between sampling methods was quite low including the whole spectrum,

20 but moderate including only the human-visible part. Our results suggest higher reliability for

21 measurements in the lab and higher power and accuracy of the GLMM-based method. They also suggest

22 UV reflectance differences amongst different plumage patches.

23   **Key words:** Adjusted repeatability, bird plumage, colourful displays, sexual selection,

24 spectrophotometry, tetrachromacy, ultraviolet.

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Introduction

Colour vision involves the capacity to discriminate amongst different wavelengths of light, independent of their intensity [1,2]. Although colouration traits expressed in animals have proven essential components to understand the nature of selection, sexual selection in particular, only relatively recently have scientists appreciated the importance of a systematic understanding of both function and evolution of colouration, as well as the mechanisms that underpin it [3]. Birds in particular, due to their colourful displays and the role of their colour signals in fitness differentials, have traditionally been employed as prime model systems to understand the causes and implications of colour evolution. However, the mechanisms of colour vision and spectral information processing needed to understand how birds perceive colours remain areas with more questions than answers.

Mate choice theory predicts that elaborately ornamented males can provide female birds with direct (if ornamental traits reflect individual condition, useful individual attributes or somatic quality independent of condition) and/or indirect fitness benefits ('good genes' or attractiveness for offspring – as conspicuous and costly male traits indicate highly heritable viability) [4-6]. Therefore, birds with more elaborate colourful displays are expected to enjoy a selective advantage given their higher mating chances [4,7].

Given the paramount significance of studies of bird plumage colouration in behavioural and evolutionary ecology, methods for reliably and objectively quantifying such colouration are critical. Methods traditionally used for colour assessment include colour ranks on an arbitrary scale [8], tristimulus colour models based on human vision, such as the Munsell system [9,10], reference colour swatches [11], or digital photography [12-14]. However, although all these methods offer simple and affordable ways of colour measurements in different analytical settings, they lack reliability and objectivity [15], as they are tuned to the human visual system instead of the bird visual system.

Birds do not perceive colours in the same way as humans [16]. Birds have evolved a fourth cone type in their eyes, with a pigment that is sensitive to ultraviolet light. And although we are still far from understanding exactly how colours are perceived by birds [3], progress is being made towards understanding how colour vision works in general and how spectral information is processed by birds and other non-human animals [2,17,18]. Methods have been developed for comparing colour patterns as birds see them, using known properties of bird eyes and generating detailed formal descriptions of colour spaces and the equations used to plot them [19].

Since the 1990s, a wide range of further methods for analyzing spectrophotometry data have emerged [3]. This development stems largely from the revival of interest in UV vision and tetrachromacy in birds and the fact that birds can see colours that humans cannot experience [20,21]. This raises the possibility

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65   of the existence of plumage patterns invisible to the human eye, and mate choice behaviours based on

66   the ultraviolet part of the bird reflectance spectrum have been discovered [22,23]. Therefore, reliable and

67   objective ways of quantifying bird coloration, not dependent on human vision, are at a premium. Miniature

68   diode-array spectroradiometer systems, lighter, more portable and affordable than previous spectrometry

69   systems but as precise and objective in colour quantification, have provided popular tools for colour

70   communication studies [3].

71   Two traditional ways of assessing bird plumage colouration with spectrophotometers have been

72   reported in the literature. Measurements may be taken directly on the bird, applying the pointer of the

73   spectrophotometer to plumage patches as they occur *in situ* [24-29]. Alternatively, measurements may be

74   taken in the lab, with feather samples collected from the field, applying the pointer (the cone-shaped piece

75   of black plastic on top of the probe used to direct the light from the source in a given angle) to "plumage

76   patches" created by mounting these feathers on a flat surface in a way that mimics the original plumage

77   structure [30-38]. Despite the popularity of the use of spectrophotometers for colour assessment and the

78   growing number of studies on bird colouration, few studies have rigorously assessed the consistency of

79   both methods for measuring the colouration of plumage patches, and the repeatability of results obtained

80   when using either one or the other (see [39] for a comparison of carotenoid-based plumage coloration in

81   great tits).

82   Additionally, there is little consensus on how to best quantify the reliability, or repeatability, of spectral

83   measurements. The most common measure of repeatability, or more precisely, the coefficient of intraclass

84   correlation (r_i), can be formally defined as the proportion of the total variance explained by differences

85   among groups [40,41]:

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87    $r_i = \sigma_\alpha^2 / (\sigma_\alpha^2 + \sigma_\varepsilon^2)$ (eqn 1)

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89   where σ_α^2 is the between-group variance and σ_ε^2 the within-group variance, whereas the sum of both

90   comprises the total phenotypic variance [41]. Until recently, the most common ways to estimate

91   repeatabilities from data with Gaussian errors have employed the correlation-based method [40] or the

92   ANOVA-based method, commonly used by behavioural and evolutionary ecologists [42,43]. However,

93   Nakagawa and Schielzeth [41] developed an innovative R-based function for calculating GLMM-based

94   repeatability estimates, which allows for confounding variables to be factored out and calculates the

95   confidence intervals for each repeatability calculation, inferred from distributions of repeatabilities obtained

96   by parametric bootstrapping.

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97   Our aim is twofold. We first compare two different methods for measuring melanin-based plumage

98   ornamentation to determine which one allows to obtain more repeatable and reliable measures. The

99   methods consist in measuring the feather colouration either directly on the bird in the field or on feather

100   samples in the laboratory. We then compare two statistical methods to assess repeatability, one ANOVA-

101   based and another one GLMM-based, and determine the pros and cons of each of them. We hypothesize

102   that measuring feather colouration in the lab will yield more repeatable and reliable measures, as it avoids

103   the logistic, technical and animal welfare limitations imposed by the field method and provides more

104   controlled conditions during the measurements. Also, we predict more realistic and accurate repeatability

105   estimates with the GLMM-based statistical method, as it allows us to introduce more sources of variation

106   in each analysis. We use the European barn swallow *Hirundo rustica* as a model species. To the best of

107   our knowledge, this is the first time GLMM-based repeatability estimates have been used to assess the

108   reliability of melanin-based plumage colouration measurements.

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110   **Methods**

111   **Field work and data collection**

112   Field work was carried out during May-August 2009 and March-August 2010 in multiple sites, mostly

113   farmlands, around the Falmouth area in Cornwall, UK. A total of 59 adult European barn swallows (21 in

114   2009 and 38 in 2010)*,* were caught using mist nets, ringed, morphometric measurements taken, plumage

115   reflectance spectra quantified in the field and feather samples collected for subsequent assessment in the

116   lab.

117   Colour was quantified based on Endler and Mielke's [19] approach, using a USB2000

118   spectrophotometer (Ocean Optics, Dunedin, Florida), and a xenon flash lamp (Ocean Optics). Before

119   using the spectrophotometer, we calibrated it by setting the white and black references, i.e., we "told" the

120   machine which colour we want to be considered as the 100% reflectance (white) standard, and the 0%

121   reflectance (dark) standard, so that the rest of the colour measurements are determined in relation to

122   those maximum and minimum possible reflectance values. We used a WS-1 SS Diffuse Reflectance

123   Standard, a diffuse white plastic >98% reflective from 250-1500 nm, as the white reference (100%

124   reflectance), and a piece of black velvet as the dark standard (0% reflectance) to correct for the noise

125   when no light is reaching the sensor. At the far end of the reflection probe/light source, we put a non-

126   reflective black pointer cut in a 45 degree angle, to avoid mismeasurement derived from the white light

127   directly reflected by the plumage reaching the sensor [15]. Using the spectra acquisition software package

128   OOIBase (Ocean Optics), we measured the reflectance of four body regions, namely the throat, breast,

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129   belly and vent of each bird. For the measurements of feather samples in the lab, we collected enough

130   feathers from live birds as to being able to mount them one on top of the other and simulate the original

131   pattern found on live birds. We mounted the feathers on a piece of black velvet to avoid background noise.

132   Once we had tested for the reliability of the measures obtained with both sampling methods separately,

133   we averaged the three measurements for each method and used these average values to test the

134   comparability between field and lab measurements.

135   We also used Endler and Mielke's [19] method to calculate brightness, chroma and hue, parameters

136   generally used to specify a colour. Using their equations and the mathematical software Matlab (The

137   MathWorks Inc., Natick, MA), we got the spectral sensitivity functions of the cones corrected for the cut

138   points of oil droplets, calculated the quantal catch for each photoreceptor and converted those quantal

139   catches into dimensional colour space coordinates in a tetrahedral colour space (Fig. 1). Chroma is

140   defined as the strength of the colour signal or the degree of difference in stimulation among the cones,

141   and it is proportional to the Euclidean distance from the origin, that is, the distance from the bird grey

142   (achromatic) point to each point, specified by three space coordinates. Perception of hue depends of

143   which cones are stimulated, and in tetrahedral colour space, it is defined by the angle that a point makes

144   with the origin. As bird colour space is 3D, hue is defined by two angles, analogous to latitude and

145   longitude in geography [19].

146   Brightness is defined as the summed mean reflectance across the entire spectral range ($R_{300-700}$;

147   [44,45]). As well as these parameters, we included UV chroma, a measure of spectral purity, into our

148   analysis, which was calculated as the proportion of reflectance in the UV part of the spectrum ($R_{300-400}$) in

149   relation to the total reflectance spectrum ($R_{300-700}$; [46]). Cone sensitivities and oil droplet cut points were

150   taken from Bowmaker et al. [16], Hart [47], Vorobyev et al. [48], Govardovskii et al. [49], and Hart and

151   Vorobyev [50].

152   Although all the avian families investigated show plumages reflecting significant amounts of UV light

153   (see [51] for a review), in the particular case of barn swallows, ventral plumage shows a noisy reflectance

154   pattern in the UV part of the spectrum and does not exhibit a clear ultraviolet reflectance peak (Fig. 2;

155   [34]). For this reason, we calculated the same colour variables both including and not including the UV

156   part of the reflectance spectrum, and carried out a repeatability assessment using either the whole

157   reflectance spectrum (300-700 nm) or only the human-visible part (400-700 nm). When using only the

158   visible part, we did not include UV chroma, for obvious reasons, nor hue, as values are identical in both

159   cases.

160   Due to the way data were collected, the three plumage colouration measurements taken in the field in

161   2009 covered a wider area of each plumage patch than the measurements made on feather samples,

162   which were restricted to the area covered by the bunch of feathers plucked from each patch on each

163   individual. In 2010, however, the three field measurements were taken approximately in the same

164   plumage area for each patch.

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166   **ANOVA-based method**

167   In order to test for the reliability of both sampling procedures (described above) separately, we

168   calculated the repeatability for colour variables in the four patches for the different procedures according

169   to Lessells and Boag [43], Senar [52] and Quesada and Senar [39]. Repeatabilities were computed from

170   the mean squares of ANOVA on three repeated measures per individual. Both in field and lab procedures,

171   the second and third measurements were made after removing the reflection probe/light source and the

172   black pointer on top of it and placing it again on the colouration patch. IV took all the measurements.

173   Once we calculated the "within-method" repeatabilities, i.e., the repeatabilities for each sampling

174   method, we averaged the three measurements per patch per individual and assessed the "between-

175   method" repeatability, i.e. the repeatability of measurements across procedures, but this time the ANOVA

176   was carried out on two repeated measures per individual, one from the field and another one from the lab.

177   We repeated this process for both 2009 and 2010 data separately.

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179   **GLMM-based method**

180   We used a modified version of the R function *R.Anson*, which is itself a modification of rpt.remlLMM

181   function [41]. We fitted two random-effect terms (individual identity and year) in our linear mixed-effects

182   models, and calculated the adjusted repeatability estimate as:

183

184    $r_i = \sigma_\alpha^2 / (\sigma_\alpha^2 + \sigma_\varepsilon^2 + \sigma_r^2)$ (eqn 2)

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186   where σ_r^2 is the year variance.

187   In order to have a general idea of repeatability for each patch, we included all the colour variables in a

188   principal component analysis (PCA) and calculated the repeatability (and confidence intervals) for the first

189   component (PC1) within each plumage patch.

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191   As we conducted multiple statistical tests on data subsets that are not likely to be biologically

192   independent of each other (i.e. different components of the spectra, same metrics on different years, or

193   same metrics in the lab and in the field), there was an increased probability of type I error rates. To control

194   for this increased probability, we corrected our p-values for multiple testing based on the sequentially

195   rejective Bonferroni procedure of Holm [53], using the *p.adjust* function from the *stats* package in R [54].

196   All the statistical analyses were carried out using R [54,55].

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198   **Results**

199   **ANOVA analyses**

200   In 2009, when including the whole spectrum in the analyses, measuring plumage colouration in the lab

201   proved to be a reliable method. Brightness, UV chroma, chroma and hue latitude and longitude being

202   highly repeatable for all the patches, and hue latitude in the throat being less repeatable (r_i=0.418,

203      $F_{21,44}$=3.157, P=0.01; Table 1). The method of measuring the plumage colouration in the field (in three

204   different points, covering a wider area of each patch) was also quite consistent but with overall lower

205   values of repeatability, although still reasonably high, for all the variables and patches, being especially

206   low for hue latitude in breast (r_i=0.382, $F_{20,42}$=2.856, P=0.025) and vent (r_i=0.394, $F_{21,44}$=2.955, P=0.017;

207   Table 1).

208   When including only the visible part of the spectrum in the analysis, overall repeatability declined. The

209   lab method again proved to be the most reliable, with high values of repeatability for brightness and

210   chroma in all the patches. The field procedure was moderately repeatable for belly and throat, but showed

211   low repeatability for brightness in the breast (r_i=0.36, $F_{20,42}$=2.6885, P=0.038) and for chroma in the vent

212   (r_i=0.428, $F_{21,44}$=3.241, P=0.007; Table 2).

213   The values of repeatability across the field and laboratory procedures were very low for all the patches

214   measured, both considering the whole reflectance spectrum or only the visible part (r_i<0.35 and P>0.05 in

215   all cases), suggesting a lack of consistency across the two assessment methods for melanin-based

216   plumage colouration. Repeatabilities of brightness measurements were slightly higher for the whole

217   spectrum than for only the visible part, in all the patches except from the vent. However, in this case,

218   including only the visible part of the spectrum yielded much more repeatable chroma values than including

219   the whole range, sometimes even turning negative repeatability values into positive, e.g. for the belly

220   (whole range: r_i=-0.507, $F_{20,21}$=0.327, P=1; only visible range: r_i=0.346, $F_{20,21}$=2.056, P=0.599), or the vent

221   (whole range: r_i=-0.758, $F_{21,22}$=0.138, P=1; only visible range: r_i=0.296, $F_{21,22}$=1.842, P=0.657; Table 1 and

222   Table 2).

223

224   In 2010, when including the whole spectrum in the analyses, repeatability measurements in the field

225   (taken approximately in the same point within each patch) yielded considerably higher results than in

226   2009, with all the r_i values above 0.60, except from hue latitude in the throat (r_i=0.515, $F_{37,75}$=4.186,

227   P<0.0001), and with most of the values ranging from 0.74 to 0.91, except for brightness in the breast

228   (r_i=0.611, $F_{37,76}$=5.710, P<0.0001), hue latitude in the belly (r_i=0.63, $F_{37,76}$=6.1, P<0.0001), hue latitude in

229   the vent (r_i=0.629, $F_{37,76}$=6.088, P<0.0001) and hue longitude in the vent (r_i=0.679, $F_{37,76}$=7.356,

230   P<0.0001). In the lab, repeatability values ranged from 0.71 to 0.95 in most of the patches, except from

231   hue latitude in the throat (r_i=0.65, $F_{37,76}$=6.569, P<0.0001), and repeatability was overall higher than when

232   measuring it on live birds, except from UV chroma in the belly (r_i=0.857, $F_{37,76}$=18.986, P<0.0001), breast

233   (r_i=0.788, $F_{37,76}$=12.117, P<0.0001) and vent (r_i=0.819, $F_{37,76}$=14.571, P<0.0001) and hue latitude in the

234   breast (r_i=0.722, $F_{37,76}$=8.808, P<0.0001), where it was slightly lower. Repeatability values were similar to

235   the ones obtained in 2009 (Table 3).

236   When doing the analysis including only the visible part of the spectrum, measuring colouration in the lab

237   was again the most reliable method of both, with all the r_i values above 0.91, except for chroma in the

238   throat (r_i=0.881, $F_{37,76}$=22.964, P<0.0001). Field procedure still yielded high repeatability values, with

239   brightness in the breast (r_i=0.569, $F_{37,76}$=4.959, P<0.0001) and chroma in the vent (r_i=0.696, $F_{37,76}$=7.839,

240   P<0.0001) being the only measurements with values below 0.81. For both methods, repeatability values

241   were higher than in 2009 for all the variables within all the plumage patches (Table 4).

242   Repeatabilities across field and lab methods in 2010 were quite heterogeneous including the whole

243   spectrum in the analyses: moderately high for hue longitude in the belly (r_i=0.794, $F_{37,38}$=8.732, P<0.0001)

244   and breast (r_i=0.657, $F_{37,38}$=4.818, P<0.0001), moderate for vent hue latitude (r_i=0.463, $F_{37,38}$=2.723,

245   P=0.018) and longitude (r_i=0.561, $F_{37,38}$=3.553, P=0.001), belly hue latitude (r_i=0.431, $F_{37,38}$=2.515,

246   P=0.034) and breast brightness (r_i=0.482, $F_{37,38}$=2.861, P=0.012), and low for breast chroma (r_i=0.326,

247    $F_{37,38}$=1.966, P=0.21), throat UV chroma (r_i=0.321, $F_{37,38}$=1.944, P=0.312) and chroma (r_i=0.274,

248    $F_{37,38}$=1.755, P=0.576) and vent brightness (r_i=0.349, $F_{37,38}$=2.070, P=0.141). For the rest of the cases,

249   repeatabilities were very low (r_i<0.23 and P>0.05 for all the cases). When including only the visible part of

250   the spectrum, repeatability was moderate to high and had a significant effect for both brightness and

251   chroma in the breast, and for chroma in the vent and in the belly, whereas it was quite low and non-

252   significant for brightness in the belly (r_i=0.217, $F_{37,38}$=1.556, P=0.899), and very low for both variables in

253   the throat. Repeatability values for chroma in all the patches except for the throat were much higher than

254   when we included the whole spectrum range, e.g. in the belly (whole range: r_i=-0.049., $F_{37,38}$=0.906, P=1;

255 only visible range: r_i=0.75, $F_{37,38}$=7.005, P<0.0001) and in the vent (whole range: r_i=0.224, $F_{37,38}$=1.579,

256 P=0.657; only visible range: r_i=0.416, $F_{37,38}$=2.428, P=0.047; Table 3 and Table 4).

257

258 **Generalized Linear Mixed Model analyses**

259 When we included the whole spectrum in the analyses, for all the principal component analyses carried

260 out within each patch for field and lab measurements, PC1 accounted for more than a 53% of the total

261 variance, except for vent measurements in the field (where it explained a 49% of the total variance) and

262 for measurements in the throat (where it explained between 44% and 47%). When including only the

263 visible part of the spectrum, PC1 explained a 67% of the total variance for vent measurements in the field,

264 and between 75% and 93% in the rest of the cases.

265 Repeatability of feather colour measurements was much higher and confidence intervals smaller when

266 quantifying colouration from measurements taken in the lab than when doing it on measurements taken on

267 live birds in the field, both including the whole spectrum in the analyses or only the visible part, being

268 particularly high in the breast (whole range: r_i=0.916, 95%CI=0.855, 0.943, P<0.0001; visible range:

269 r_i=0.927, 95%CI=0.872, 0.95, P<0.0001). All the repeatability values from lab measurements ranged

270 between 0.71 and 0.93 and were highly significant (P<0.0001).

271 When using field measurements, repeatabilities were still moderately high (all r_i values above 0.50) and

272 higher when including only the visible spectrum range in the analyses than including the whole range,

273 except in the throat (whole range: r_i=0.629, 95%CI=0.303, 0.874, P<0.0001; visible range: r_i=0.564,

274 95%CI=0.266, 0.816, P<0.0001; Fig. 3).

275 Repeatabilities across both field and lab methods yielded higher results when we included only the

276 visible spectrum in the analyses than when we included the whole spectrum. Leaving the values for throat

277 apart, as here repeatability was not significantly different from zero no matter the spectrum range we

278 included in the analyses, r_i ranged between 0.19 and 0.41 when including the whole spectrum, and

279 between 0.44 and 0.62 when including only the visible part (Fig. 3). All the repeatabilities in belly, breast

280 and vent were significant except for the belly when including the whole spectrum (r_i=0.189, 95%CI=0,

281 0.415, P=0.069), but it became significant and higher when only the visible spectrum was included in the

282 analyses (r_i=0.503, 95%CI=0.281, 0.667, P<0.0001, Fig. 3).

283

284 **Discussion**

285 **Measuring plumage ornamentation to gain repeatable and reliable measures**

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286   Measuring plumage colouration from feather samples in the lab proved to be a highly reliable method,

287   with high values of repeatability in general for all the variables and patches in 2009, 2010 and when

288   applying the GLMM-based method for both years. Measurements taken directly on specimens in the field

289   showed a reasonable extent of reliability, but with overall lower values of repeatability compared to the lab

290   for most of the variables measured on different patches. As an exception, some variables in the throat in

291   2009 and UV chroma measurements in the belly, breast and vent in 2010 when considering the whole

292   spectrum, yielded higher values of repeatability when measured in the field, only when applying the

293   ANOVA-based method.

294   A potential explanation for our findings is that the throat patch is smaller and much darker than the rest

295   of the patches. The feathers of the throat patch are also considerably smaller. Therefore, it is often quite

296   difficult to obtain a reliable reflectance measurement with such a limited amount of photons reaching the

297   spectrophotometer probe. Also, it is more difficult to create a "plumage patch" in the lab with a feather

298   arrangement similar to the bird's original one and big enough to be able to apply the spectrophotometer

299   pointer to it. An alternative explanation is that the UV part of the spectrum shows a highly noisy pattern,

300   thus highly consistent UV chroma repeatabilities across field or lab measurements may not necessarily be

301   expected. This latter alternative may explain why repeatability values in the lab for UV chroma

302   measurements were higher in the field in 2010, whereas the rest of the repeatability values consistently

303   tended to be higher in the lab.

304   When applying the GLMM-based method, repeatability was moderate to high within all the patches for

305   field measurements, whereas it was considerably higher, and the confidence intervals considerably

306   narrower, for lab measurements. This finding suggests that lab-based measures are more reliable ways of

307   assessing melanin-based colouration.

308   When comparing both methods, the values obtained in 2009 for different variables measured in different

309   plumage patches directly on field birds were poorly repeatable compared to the values obtained for the

310   same variables measured from feather samples in the lab, and non-significant in all cases. In 2010, in

311   contrast, repeatabilities were higher and significant for certain metrics in certain patches only. These

312   results stand in marked contrast to the positive results of another study, which compared the

313   repeatabilities between both colouration assessment procedures for carotenoid-based plumage [39].

314   There can be several reasons for this difference: for example, due to the different characteristics of the

315   two types of pigments, carotenoid-derived colouration is more variable among individuals than melanin-

316   based colouration [56], and repeatability of a character increases with variability [52]. In order to increase

317   the repeatability of some measurements, a possible solution could be to increase the number of

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318 measurements, for example from three to five, as it has already been done by several authors [22,28,32].

319 Unfortunately, this is not an option when working with live birds in the field, as we would be increasing the

320 manipulation times and, consequently, the stress levels to an unacceptable degree, although it can be

321 applied when assessing colouration in the lab on feather samples [39].

322 As mentioned above, the three plumage colouration measurements taken in the field in 2009 covered a

323 wider area of each plumage patch than in 2010, when the three field measurements were taken

324 approximately in the same plumage area for each patch, being this area also the one from which the

325 majority of feathers for lab measurements were taken. Due to the different ways in which data were

326 collected in the field each year, repeatability of 2009 field measurements can be taken as an estimate of

327 colouration consistency within the plumage patches. Our results suggest a moderate to high within-patch

328 consistency for melanin-based ventral colouration in our model species. The comparability of both

329 procedures in 2009 may have been compromised, although the repeatability of the 2010 samples was

330 higher even for lab measurements, especially when considering only the visible part of the spectrum. This

331 may be indicative of higher patch colouration homogeneity in 2010. Also, the higher lab repeatabilities in

332 2009 than the field ones could be argued to be a function of field measures being taken over bigger

333 plumage areas. But the fact that we still get higher lab than field measurements in 2010 suggests practical

334 limitations to repeatability measurements in the field.

335 Consequently, collecting feathers from birds and assessing their colouration in the lab, as well as being

336 more convenient, minimising risk to a sensitive device like a spectrophotometer and reducing handling

337 times of the animals [39], is a more reliable method for assessing melanin plumage colouration than

338 doing so directly on live birds, according to our results.

339

340 **Assessing repeatability**

341 The GLMM-based method [41], applied to data from both years, allowed us to control for year effects by

342 adding the year variance into the total variance calculation, so that we could obtain the adjusted

343 repeatability for data from both years. The PCA allowed to create composite variables accounting for

344 almost 50% of the total variance in the metrics taken from each patch, which therefore, made it possible to

345 estimate the overall repeatability within each patch for both methods separately and across methods.

346 The possibility of calculating adjusted repeatabilities by including year as a random factor, together with

347 the reduction in the number of variables accounting for a great proportion of the total variance achieved by

348 the PCA, considerably reduced the amount of multiple tests necessary for repeatability calculation. Thus,

349 the p-values obtained with this method were less affected by Bonferroni corrections than those obtained

12

350   with the ANOVA-based method, reducing the probability of type II errors and increasing the power of this

351   repeatability-calculation method.

352   Additionally, this reduction in the number of variables offered us the possibility of getting a general view

353   of the colour measurement repeatability within each patch. As our variables are all derived from the

354   reflectance spectra obtained on each colouration patch through the spectrophotometer, comparing the

355   repeatability of all the different variables within the patches, as well as increasing the probability of type II

356   errors, can be redundant. The PCA-GLMM combination, in contrast, made it possible to compare the first

357   principal components within each patch, which can be regarded as whole spectrum estimates, as they

358   gather a great proportion of the total variance contained in all the variables originally extracted from the

359   reflectance spectra measurements. And it also allowed us to do so with data from both years.

360   Finally, thanks to the use of the GLMM-based method, we could calculate confidence intervals, useful

361   indicators of the reliability of our repeatability estimates, additionally to just p-values. That way, and

362   together with the advantages mentioned above, it was possible to get a more complete and reliable overall

363   perspective of the question being studied.

364   The fact that almost all the repeatability measurements, and especially the repeatabilities across field

365   and lab methods (in patches other than the throat), were higher when including only the human-visible

366   spectrum in the analyses , suggests that the noisy reflectance pattern in the UV part of the spectrum may

367   be decreasing the repeatability and underestimating the comparability of the two methods. For throat

368   plumage, however, we observed the opposite trend, with higher repeatability values when including the

369   whole spectrum, which could be indicative that the UV part of the spectrum is more important in the throat

370   than in the rest of the patches. We find support for this idea when looking at reflectance spectra plots for

371   different patches (Fig. 2): throat reflectance spectra, although showing also quite a noisy pattern for the

372   UV part, and unlike the rest of the patches' spectra, tends to show UV reflectance peaks in both sexes.

373   Further work is needed to find out whether there are UV reflectance differences amongst different

374   plumage patches.

375

376   **Conclusions**

377   Our results suggest that collecting feathers from live animals and assessing colouration in the lab is a

378   better approach for measuring plumage ornamentation in order to gain repeatable and reliable results

379   compared to direct measures on live birds in the field. In addition, since it is easier on equipment and

380   minimises the length of time birds need to be handled (minimising the stress levels inflicted on them),

381   feather sampling would appear to be the best method available.

13

382   In addition, from a statistical point of view, our results support the superiority of the GLMM-based method

383   [41] for repeatability calculation, as it enables random factors to be accounted for and can calculate

384   adjusted repeatability values, which are more accurate than those calculated using other (e.g., ANOVA)

385   methods and increase the power of the tests. The reduction in the number of variables gives us a general,

386   patch by patch overview of the problem being studied, and the confidence intervals allow us to test the

387   reliability of our own repeatability estimates.

388   Finally, we have also shown that it is important to check for the effect that the UV part of the spectrum

389   could be exerting on repeatability calculations, as the capability of the plumage to reflect the UV light could

390   have different biological implications in different plumage patches.

391

14

References

1. Cuthill IC (2006) Color perception. In: Hill GE, McGraw KJ, editors. Bird coloration Mechanisms and measurements Volume 1. Cambridge: Harvard University Press. pp. 3-40.

2. Kelber A, Vorobyev M, Osorio D (2003) Animal colour vision: behavioural tests and physiological concepts. Biological Reviews 78: 81-118.

3. Hill GE, McGraw KJ (2006) Bird coloration. Mechanisms and measurements. Volume 1. Cambridge: Harvard University Press.

4. Andersson M (1994) Sexual selection. Princeton: Princeton University Press.

5. Garamszegi LZ, Hegyi G, Heylen D, Ninni P, de Lope F, et al. (2006) The design of complex sexual traits in male barn swallows: associations between signal attributes. Journal of Evolutionary Biology 19: 2052-2066.

6. Pomiankowski A (1987) Sexual selection: the handicap principle does work sometimes. Proceedings of the Royal Society of London B, Biological Sciences 231: 123-145.

7. Hill GE (2006) Female mate choice for ornamental coloration. In: Hill GE, McGraw KJ, editors. Bird coloration Function and evolution Volume 2. Cambridge: Harvard University Press. pp. 137-200.

8. Promislow DEL, Montgomerie R, Martin TE (1992) Mortality costs of sexual dimorphism in birds. Proceedings of the Royal Society of London B, Biological Sciences 250: 143-150.

9. Peiponen VA (1992) Colour discrimination of two passerine bird species in the Munsell system. Ornis Scandinavica 23: 143-151.

10. Zuk M, Decruyenaere JG (1994) Measuring individual variation in colour: a comparison of two techniques. Biological Journal of the Linnean Society 53: 165-173.

11. Hill GE (1990) Female house finches prefer colourful males: sexual selection for a condition-dependent trait. Animal Behaviour 40: 563-572.

12. Loyau A, Saint-Jalme M, Sorci G (2005) Intra- and intersexual selection for multiple traits in the peacock (*Pavo cristatus*). Ethology 111: 810-820.

13. Bortolotti GR, Blas J, Negro JJ, Tella JL (2006) A complex plumage pattern as an honest social signal. Animal Behaviour 72: 423-430.

14. Stevens M, C.A. P, Cuthill IC, Partridge JC, Troscianko TS (2007) Using digital photography to study animal coloration. Biological Journal of the Linnean Society 90: 211-237.

15. Andersson S, Prager M (2006) Quantifying colors. In: Hill GE, McGraw KJ, editors. Bird coloration Mechanisms and measurements Volume 1. Cambridge: Harvard University Press. pp. 41-89.

16. Bowmaker JK, Heath LA, Wilkie SE, Hunt DM (1997) Visual pigments and oil droplets from six classes of photoreceptor in the retinas of birds. Vision Research 37: 2183-2194.

17. Smith EL, Greenwood VJ, Bennett ATD (2002) Ultraviolet colour perception in European starlings and Japanese quail. Journal of Experimental Biology 205: 3299-3306.

18. Kelber A, Osorio D (2010) From spectral information to animal colour vision: experiments and concepts. Proceedings of the Royal Society of London B, Biological Sciences 277: 1617-1625.

19. Endler JA, Mielke PWJ (2005) Comparing entire colour patterns as birds see them. Biological Journal of the Linnean Society 86: 405-431.

20. Bennett ATD, Cuthill IC, Norris KJ (1994) Sexual selection and the mismeasure of color. American Naturalist 144: 848-860.

21. Cuthill IC, Partridge JC, Bennett ATD, Church SC, Hart NS, et al. (2000) Ultraviolet vision in birds. In: Slater PJB, Rosenblatt JS, Snowdon CT, Roper TJ, editors. Advances in the study of behavior Volume 29. London: Academic Press. pp. 159-214.

22. Bennett ATD, Cuthill IC, Partridge JC, Lunau K (1997) Ultraviolet plumage colors predict mate preferences in starlings. Proceedings of the National Academy of Sciences USA 94: 8618-8621.

23. Maier EJ (1994) Ultraviolet vision in a passeriform bird: from receptor spectral sensitivity to overall spectral sensitivity in *Leiothrix lutea*. Vision Research 34: 1415-1418.

24. Senar JC, Figuerola J, Pascual J (2002) Brighter yellow blue tits make better parents. Proceedings of the Royal Society of London B, Biological Sciences 269: 257-261.

25. Bize P, Piault R, Moureau B, Heeb P (2006) A UV signal of offspring condition mediates context-dependent parental favouritism. Proceedings of the Royal Society of London B, Biological Sciences 273: 2063-2068.

26. Herrera G, Zagal JC, Diaz M, Fernández MJ, Vielma A, et al. (2008) Spectral sensitivities of photoreceptors and their role in colour discrimination in the green-backed firecrown hummingbird (*Sephanoides sephaniodes*). Journal of Comparative Physiology A 194: 785-794.

27. Catoni C, Peters A, Schaefer HM (2009) Dietary flavonoids enhance conspicuousness of a melanin-based trait in male blackcaps but not of the female homologous trait or of sexually monochromatic traits. Journal of Evolutionary Biology 22: 1649-1657.

28. Doucet SM, Hill GE (2009) Do museum specimens accurately represent wild birds? A case study of carotenoid, melanin, and structural colours in long-tailed manakins *Chiroxiphia linearis*. Journal of Avian Biology 40: 146-156.

29. Del Cerro S, Merino S, Martínez-de la Puente J, Lobato E, Ruiz-de-Castañeda R, et al. (2010) Carotenoid-based plumage colouration is associated with blood parasite richness and stress protein levels in blue tits (*Cyanistes caeruleus*). Oecologia 162: 825-835.

30. Cuthill IC, Bennett ATD, Partridge JC, Maier EJ (1999) Plumage reflectance and the objective assessment of avian sexual dichromatism. American Naturalist 153: 183-200.

31. Keyser AJ, Hill GE (2000) Structurally based plumage coloration is an honest signal of quality in male blue grosbeaks. Behavioral Ecology 11: 202-209.

32. Perrier C, de Lope F, Møller AP, Ninni P (2002) Structural coloration and sexual selection in the barn swallow *Hirundo rustica*. Behavioral Ecology 13: 728-736.

33. McGraw KJ, Safran RJ, Evans MR, Wakamatsu K (2004) European barn swallows use melanin pigments to color their feathers brown. Behavioral Ecology 15: 889-891.

34. Safran RJ, McGraw KJ (2004) Plumage coloration, not length or symmetry of tail-streamers, is a sexually selected trait in North American barn swallows. Behavioral Ecology 15: 455-461.

35. Komdeur J, Oorebeek M, van Overveld T, Cuthill IC (2005) Mutual ornamentation, age, and reproductive performance in the European starling. Behavioral Ecology 16: 805-817.

36. McGraw KJ, Safran RJ, Wakamatsu K (2005) How feather colour reflects its melanin content. Functional Ecology 19: 816-821.

37. Safran RJ (2007) Settlement patterns of female barn swallows *Hirundo rustica* across different group sizes: access to colorful males or favored nests? Behavioral Ecology and Sociobiology 61: 1359-1368.

38. Vaquero-Alba I (2011) Animal sexual signals: do they maximise or optimise information content? Exeter: University of Exeter.

39. Quesada J, Senar JC (2006) Comparing plumage colour measurements obtained directly from live birds and from collected feathers: the case of the great tit *Parus major*. Journal of Avian Biology 37: 609-616.

40. Sokal RR, Rohlf FJ (1995) Biometry: the principles and practice of statistics in biological research. California: W. H. Freeman.

41. Nakagawa S, Schielzeth H (2010) Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. Biological Reviews 85: 935-956.

42. Donner A (1986) A review of inference procedures for the intraclass correlation coefficient in the one-way random effects model. International Statistical Review 54: 67-82.

43. Lessells CM, Boag PT (1987) Unrepeatable repeatabilities: a common mistake. Auk 104: 116-121.

44. Montgomerie R (2006) Analyzing colors. In: Hill GE, McGraw KJ, editors. Bird coloration Mechanisms and measurements Volume 1. Cambridge: Harvard University Press. pp. 90-147.

45. Galván I, Møller AP (2009) Different roles of natural and sexual selection on senescence of plumage colour in the barn swallow. Functional Ecology 23: 302-309.

46. Siefferman L, Hill GE (2005) Evidence for sexual selection on structural plumage coloration in female eastern bluebirds (*Sialia sialis*). Evolution 59: 1819-1828.

47. Hart NS (2001) The visual ecology of avian photoreceptors. Progress in Retinal and Eye Research 20: 675-703.

48. Vorobyev M, Osorio D, Bennett ATD, Marshall NJ, Cuthill IC (1998) Tetrachromacy, oil droplets and bird plumage colours. Journal of Comparative Physiology A 183: 621-633.

49. Govardovskii VI, Fyhrquist N, Reuter T, Kuzmin DG, Donner K (2000) In search of the visual pigment template. Visual Neuroscience 17: 509-528.

50. Hart NS, Vorobyev M (2005) Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. Journal of Comparative Physiology A 191: 381-392.

51. Eaton MD, Lanyon SM (2003) The ubiquity of avian ultraviolet plumage reflectance. Proceedings of the Royal Society of London B, Biological Sciences 270: 1721-1726.

52. Senar JC (1999) La medición de la repetibilidad y el error de medida. Etologuía 17: 53-64.

53. Holm S (1979) A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6: 65-70.

54. R Development Core Team (2014) R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.

55. Crawley MJ (2007) The R book. Sussex: John Wiley & Sons.

56. Badyaev AV, Hill GE (2000) Evolution of sexual dichromatism: contribution of carotenoid- versus melanin-based coloration. Biological Journal of the Linnean Society 69: 153-172.

Table 1: ANOVA-derived Repeatabilities in 2009 plumage colouration measurements taken from live birds in the field, feather samples in the lab, and across both procedures (UV+Visible spectrum).

| | UV+Visible range | | | | | | | |
| | Belly | | Breast | | Throat | | Vent | |
	F	r_i	F	r_i	F	r_i	F	r_i
Repeatability field								
Brightness	6.0446	**0.627***	3.1183	**0.414***	12.273	**0.79***	6.1868	**0.634***
UV Chroma	10.203	**0.754***	14.171	**0.814***	13.861	**0.811***	5.3196	**0.59***
Chroma	9.0161	**0.728***	11.238	**0.773***	10.714	**0.764***	4.5611	**0.543***
Hue latitude	4.0921	**0.508***	2.8564	**0.382***	4.4146	**0.532***	2.9545	**0.394***
Hue longitude	13.025	**0.8***	9.1422	**0.731***	24.348	**0.886***	4.6771	**0.55***
Repeatability lab								
Brightness	13.188	**0.802***	14.777	**0.821***	8.2092	**0.706***	20.212	**0.865***
UV Chroma	46.493	**0.938***	23.015	**0.88***	34.895	**0.919***	25.561	**0.892***
Chroma	42.489	**0.932***	26.975	**0.896***	62.481	**0.954***	29.052	**0.903***
Hue latitude	11.986	**0.785***	6.5101	**0.647***	3.1573	**0.418****	23.024	**0.88***
Hue longitude	25.986	**0.893***	9.2833	**0.734***	6.0119	**0.625***	27.291	**0.898***
Comparison field-lab								
Brightness	1.4636	**0.188**	2.0702	**0.349**	0.865	**-0.072**	1.8043	**0.287**
UV Chroma	1.3153	**0.136**	1.3591	**0.152**	0.7499	**-0.143**	0.9006	**-0.052**
Chroma	0.327	**-0.507**	1.3715	**0.157**	1.0143	**0.007**	0.1376	**-0.758**
Hue latitude	0.9084	**-0.048**	0.8635	**-0.073**	1.2523	**0.112**	0.706	**-0.172**
Hue longitude	1.7364	**0.269**	1.7513	**0.273**	0.6709	**-0.197**	1.1055	**0.05**

'***' $P<0.001$; '**' $P<0.01$; '*' $P<0.05$ '.' $P<0.1$

20

Table 2: ANOVA-derived Repeatabilities in 2009 plumage colouration measurements taken from live birds in the field, feather samples in the lab, and across both procedures (Only Visible part of the spectrum).

	Visible range							
	Belly		Breast		Throat		Vent	
	F	r_i	F	r_i	F	r_i	F	r_i
Repeatability field								
Brightness	4.8958	**0.565*****	2.6885	**0.36***	11.221	**0.773*****	5.6072	**0.606*****
Chroma	6.7724	**0.658*****	6.4868	**0.646*****	6.6207	**0.652*****	3.2416	**0.428****
Repeatability lab								
Brightness	11.818	**0.783*****	15.892	**0.832*****	6.9936	**0.666*****	19.326	**0.859*****
Chroma	28.865	**0.903*****	21.117	**0.821*****	29.402	**0.873*****	23.838	**0.901*****
Comparison field-lab								
Brightness	1.461	**0.187**	2.0097	**0.335**	0.6804	**-0.19**	1.8902	**0.308**
Chroma	2.0563	**0.346**	1.6683	**0.25**	1.1396	**0.065**	1.8415	**0.296**

'***' $P<0.001$; '**' $P<0.01$; '*' $P<0.05$ '.' $P<0.1$.

21

Table 3: ANOVA-derived repeatabilities in 2010 plumage colouration measurements taken from live birds in the field, feather samples in the lab, and across both procedures (UV+Visible spectrum).

	UV+Visible range							
	Belly		Breast		Throat		Vent	
	F	r_i	F	r_i	F	r_i	F	r_i
Repeatability field								
Brightness	16.422	**0.837*****	5.7104	**0.611*****	17.398	**0.846*****	14.534	**0.819*****
UV Chroma	25.333	**0.89*****	15.853	**0.832*****	12.357	**0.791*****	16.829	**0.841*****
Chroma	24.037	**0.885*****	14.749	**0.821*****	22.212	**0.876*****	12.377	**0.792*****
Hue latitude	6.0997	**0.630*****	9.7411	**0.744*****	4.1857	**0.515*****	6.0878	**0.629*****
Hue longitude	23.669	**0.883*****	31.363	**0.910*****	9.8914	**0.748*****	7.3556	**0.679*****
Repeatability lab								
Brightness	55.197	**0.947*****	31.387	**0.910*****	30.9	**0.909*****	44.036	**0.934*****
UV Chroma	18.986	**0.857*****	12.117	**0.788*****	17.544	**0.847*****	14.571	**0.819*****
Chroma	25.375	**0.890*****	25.936	**0.893*****	27.679	**0.899*****	39.854	**0.928*****
Hue latitude	8.3908	**0.711*****	8.8076	**0.722*****	6.5692	**0.650*****	9.1422	**0.731*****
Hue longitude	37.357	**0.924*****	31.683	**0.911*****	11.664	**0.781*****	37.517	**0.924*****
Comparison field-lab								
Brightness	1.3041	**0.131948**	2.8612	**0.482***	0.7642	**-0.134**	2.0703	**0.349**
UV Chroma	0.5572	**-0.284**	1.059	**0.029**	1.9441	**0.321**	1.5873	**0.227**
Chroma	0.9059	**-0.049**	1.9662	**0.326**	1.755	**0.274**	1.5788	**0.224**
Hue latitude	2.5152	**0.431***	1.2479	**0.110243**	0.7839	**-0.121**	2.7232	**0.463***
Hue longitude	8.7322	**0.794*****	4.8175	**0.657*****	**0.9731**	**-0.014**	3.5525	**0.561****

'***' $P<0.001$; '**' $P<0.01$; '*' $P<0.05$ '.' $P<0.1$

Table 4: ANOVA-derived Repeatabilities in 2010 plumage colouration measurements taken from live birds in the field, feather samples in the lab, and across both procedures (Only Visible part of the spectrum).

	Visible range							
	Belly		Breast		Throat		Vent	
	F	r_i	F	r_i	F	r_i	F	r_i
Repeatability field								
Brightness	14.408	0.817***	4.959	0.569***	16.59	0.839***	13.527	0.806***
Chroma	22.566	0.878***	22.163	0.876***	20.007	0.864***	7.8386	0.696***
Repeatability lab								
Brightness	59.713	0.951***	34.778	0.918***	34.133	0.917***	49.55	0.942***
Chroma	51.455	0.944***	57.897	0.950***	22.964	0.881***	72.314	0.959***
Comparison field-lab								
Brightness	1.5559	0.217	3.1819	0.522**	0.679	-0.191	2.3696	0.406.
Chroma	7.0047	0.750***	7.0261	0.751***	1.0876	0.042	2.4275	0.416*

'***' $P<0.001$; '**' $P<0.01$; '*' $P<0.05$ '.' $P<0.1$

23
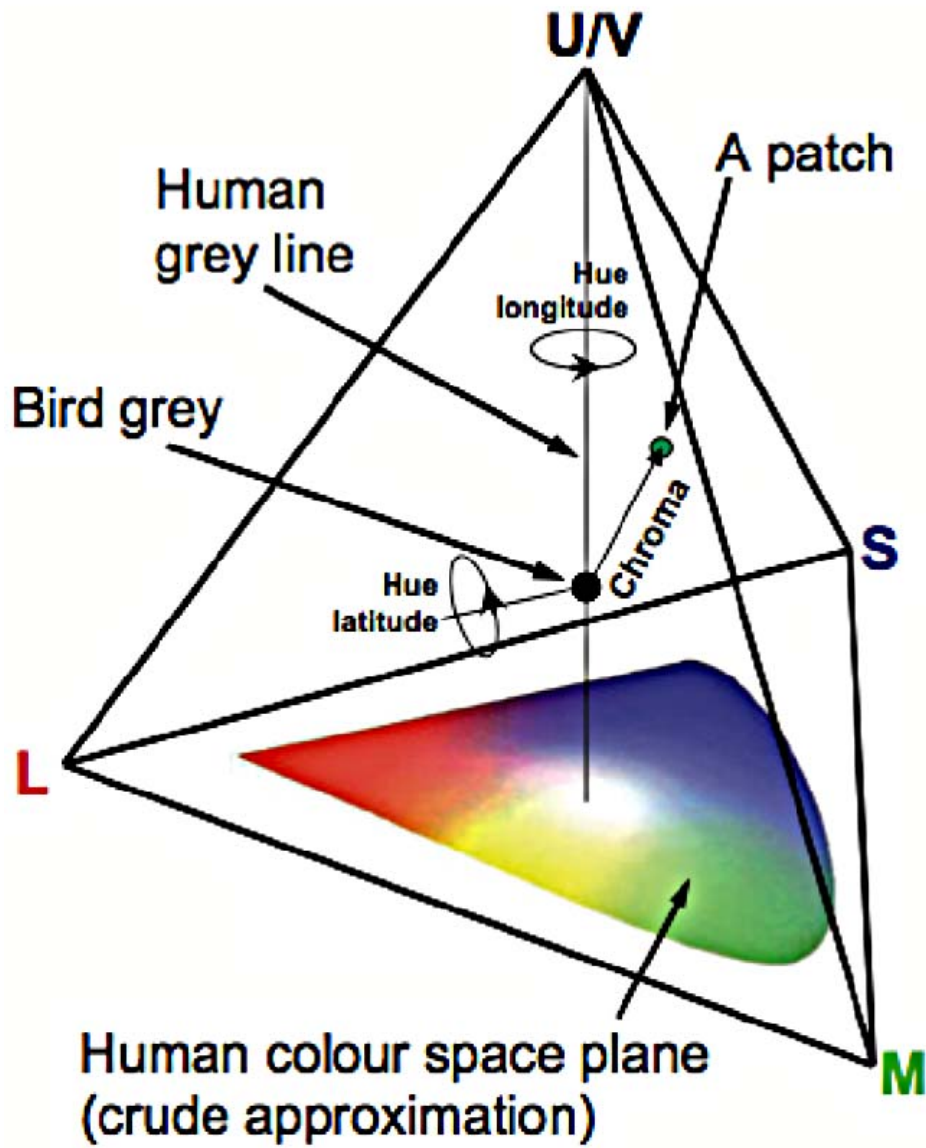
Figure 1: The avian tetrahedral colour space (from Endler and Mielke, 2005).
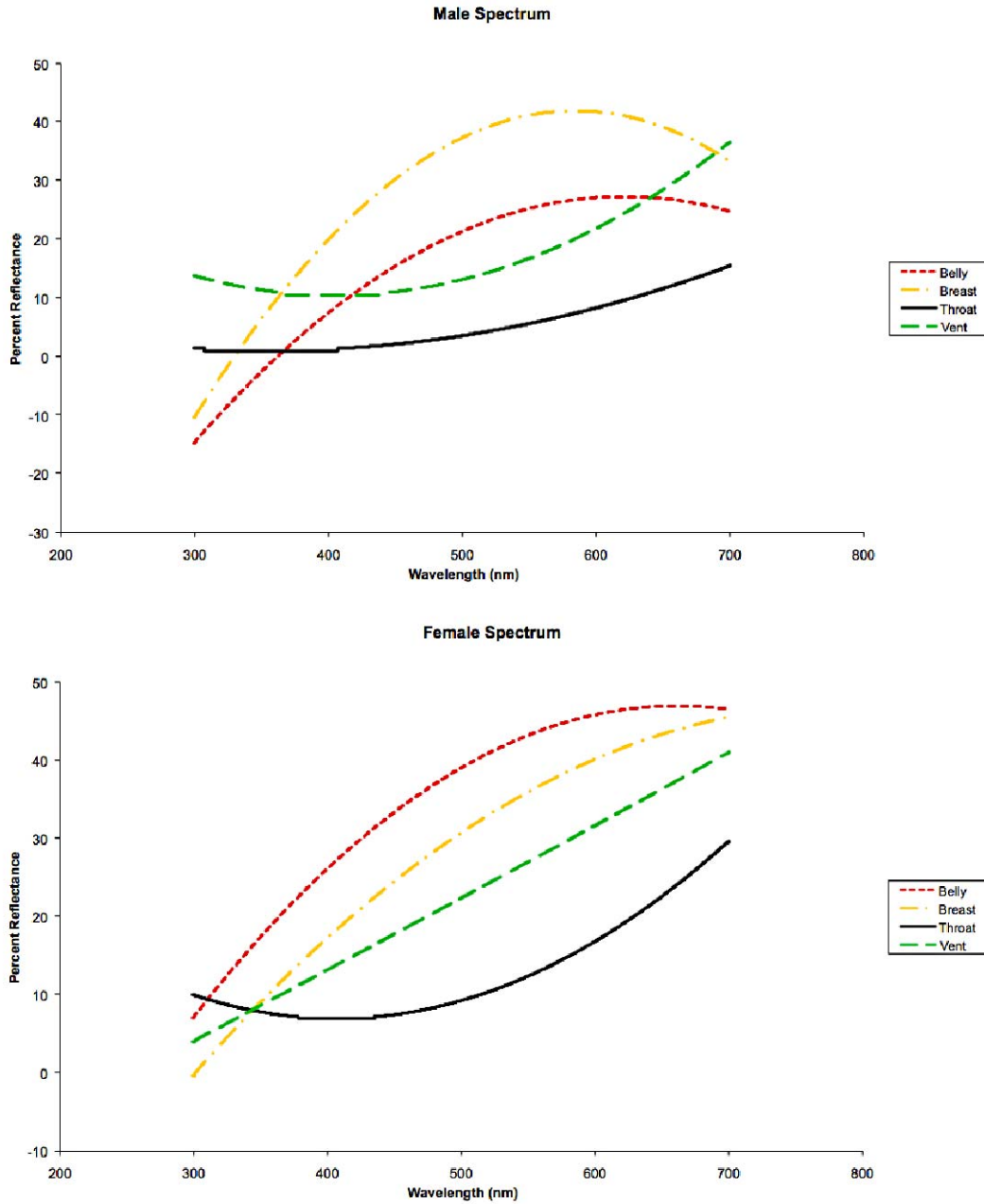
Figure 2: Reflectance spectra for belly, breast, throat and vent patches of male and female barn
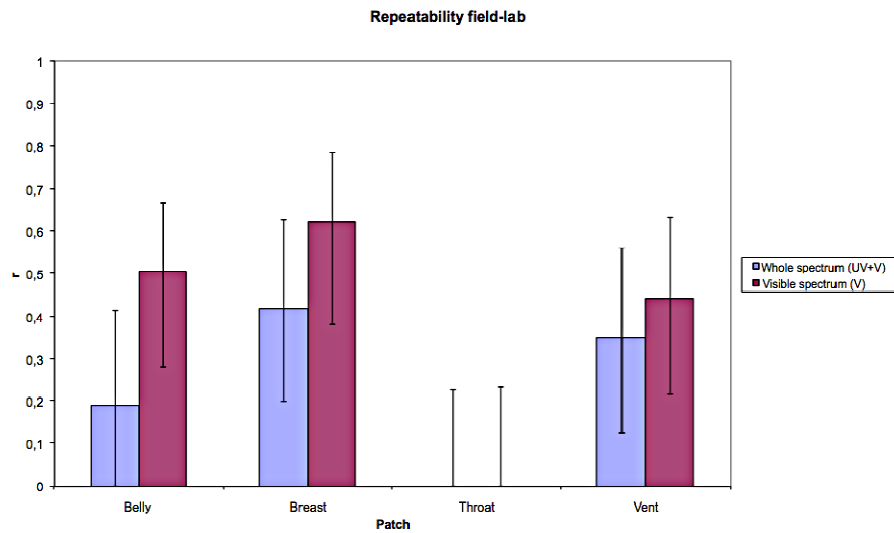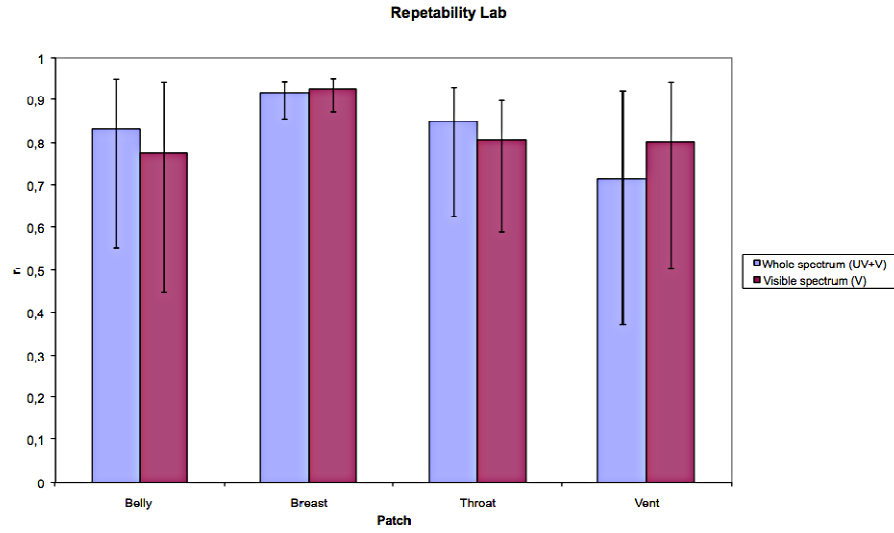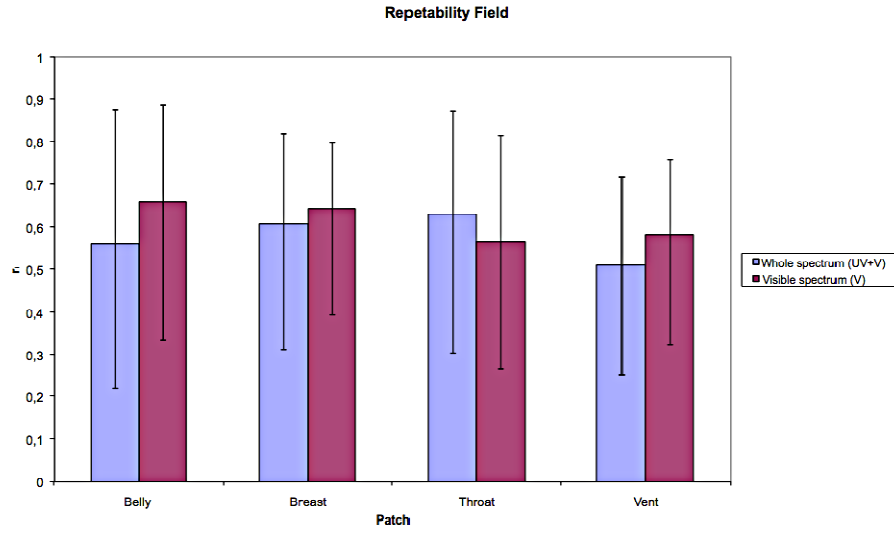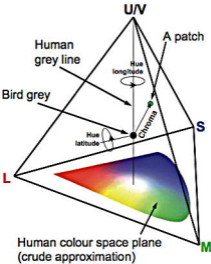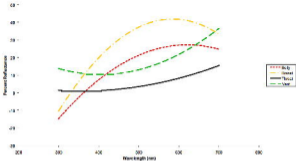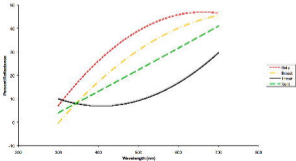
swallow (regression lines).

Figure 3: GLMM-derived repeatabilities (± 95% CI) in 2009-2010 plumage colouration measurements taken a) from live birds in the field, b) from feather samples in the lab, and c) across both procedures, both when including the whole light spectrum or only the human-visible spectrum in the analyses.
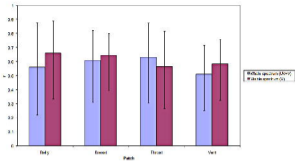
U/V

A patch
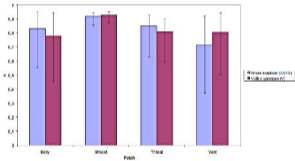
Human grey line

Hue longitude

Bird grey

Chroma

Hue latitude

S

L

M

Human colour space plane (crude approximation)

Male Spectrum

Female Spectrum

Repeatability Field

Repeatability Lab

Repeatability field/lab