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The chemical basis of a signal of individual identity:

Shell pigment concentrations track the unique appearance of Common Murre eggs

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20 **Abstract:**

21 In group-living species with parental care, the accurate recognition of one's own young is critical to
22 fitness. Because discriminating offspring within a large colonial group may be challenging, progeny of
23 colonial breeders often display familial or individual identity signals to elicit and receive costly parental
24 provisions from their own parents. For instance, the Common Murre (or Common Guillemot: *Uria aalge*)
25 is a colonially breeding seabird that does not build a nest and lays and incubates an egg with an
26 individually unique appearance. How the shell's physical and chemical properties generate this
27 individual variability in coloration and maculation has not been studied in detail. Here, we quantified
28 two characteristics of the avian-visible appearance of murre eggshells collected from the wild:
29 background coloration spectra and maculation density. As predicted by the individual identity
30 hypothesis, there was no statistical relationship between avian-perceivable shell background coloration
31 and maculation density within the same eggs. In turn, variation in both sets of traits was statistically
32 related to some of their physico-chemical properties, including shell thickness and concentrations of the
33 eggshell pigments biliverdin and protoporphyrin IX. These results illustrate how individually unique
34 eggshell appearances, suitable for identity signaling, can be generated by a small number of structural
35 mechanisms.

36

37 **Key words:** coloration, coloniality, discrimination, egg recognition, individual recognition, maculation

38 **Introduction:**

39

40 Caring for dependent young often incurs energetic and temporal costs for the parents (1). Recognition
41 mechanisms that enable the delivery of parental care to genetically related young, therefore, are
42 favored by natural selection (2). In many parental species, identifying one's own progeny reliably can be
43 achieved indirectly through the recognition of the site at which young are housed and developing (e.g.,
44 hive, nest, or den) (3). However, in colonially breeding species, especially those without fixed dens or
45 nests, or with mobile offspring, the direct recognition of own offspring is necessary for accurate parental
46 investment (4). In birds, individually unique coloration and maculation of eggs provides a potential
47 mechanism for direct offspring recognition (5).

48 Avian eggs are often highly variable in appearance, both among and within species (6,7). This
49 variation includes diversity in size, shape, background coloration and, when present, maculation (8,9).
50 Several adaptive functions may explain intraspecific variability of avian eggshell appearance (10,11),
51 including direct individual egg recognition by parents in species without nests (12). A notable textbook
52 (e.g.: 13) exemplar of this is the highly variable egg of the Common Murre (or Common Guillemot: *Uria*
53 *aalge*, hereafter: murre) (Fig. 1 inset). Murres are seabirds that breed in dense cliff colonies, and do not
54 build a nest but instead individually recognise and retrieve their own egg when faced with a choice
55 between it and the unattended egg of a neighbor or other conspecific (14) (Fig. 1).

56 Traits that subserve individual recognition have a characteristic set of qualities that discriminate
57 them from display trait sets that function in different recognition context, such as kinship or Fisherian
58 attractiveness (5). Specifically, individual recognition traits would show a high level of variability,
59 multimodal distribution, lack of correlation between component traits, and strong degree of genetic
60 determination (15). Murre eggs appear to conform to these trait qualities, in that they show

61 multimodality and lack of correlation between their shell background colours' hue and saturation (16),
62 and high variability between individuals with strong high putative genetic control within individuals
63 (*sensu* 17) for egg shape (18), coloration and maculation: (19, 20), and size (20).

64 Here, we address the structural basis of individual recognition signaling in egg coloration and
65 maculation in murre. Chemically, avian eggshell colour variation is under the influence of just two
66 known pigments: the blue-green biliverdin and the rusty-black protoporphyrin IX (21), and their
67 interaction with the white calcite of the eggshell matrix (8). These two pigments are ubiquitous across
68 the avian radiation (22), and had originated once in non-avian dinosaurs (23), and so our study is
69 relevant to understanding the structural bases in variation of avian and reptilian eggshell coloration and
70 maculation more generally. Additional structural and environmental constituents of eggshells can
71 contribute to variation in avian egg appearance, including a vaterite layer covering the shell background
72 (24), or dirt accumulating during incubation (25). However, these mechanisms do not explain the
73 structural origin of individually consistent but interindividually variable murre egg phenotypes (20).

74 Here we quantified the relationship between avian-perceivable visual components of murre
75 eggshells (background coloration and maculation density) with two of the shells' structural elements
76 known to be related to coloration and maculation (pigment concentrations: 8, and thickness: 26). These
77 analyses tested two predictions related to the *Eggshell Identity Signal Hypothesis*:

78 **Prediction A: shell background colour and maculation density contributing to avian-perceivable egg**
79 **appearance are statistically uncorrelated within the same eggs, and**

80 **Prediction B: variation in the physico-chemical structure of the shell generates avian-visible diversity**
81 **across murre eggs.**

82 This work, thus, complements our recent report of intraindividual repeatability of eggshell phenotypes
83 laid by known females across different years (20). Specifically, this new study helps to uncover the
84 structural bases underlying uniquely high phenotypic variability to yield a signal that facilitates individual
85 recognition (5).

86

87 **Methods:**

88

89 *Murre Eggshell Collection:*

90 We collected murre eggs (n=50) from Gull Island, Witless Bay, Newfoundland & Labrador, Canada.
91 Sympatric gulls, notably the American Herring Gull *Larus (argentatus) smithsonianus* and the Great
92 Black-backed Gull *L. marinus*, depredate murre eggs regularly at this site (27). We collected only recently
93 depredated eggs (within the last week) from gull territories because depredated eggs show human-
94 visible signs of weathering/fading after a few weeks of exposure (GJR pers. obs.). The depredated murre
95 eggs might not represent a random subset of the population of breeding birds at the site because
96 disproportionately more eggs may be from younger/inexperienced murre, relegated to nesting at the
97 margins of the colony (28). However, our sample of collected eggshells did display the full range of
98 human-visible egg coloration present in the colony as a whole (GJR pers. obs.), and, thus, were deemed
99 suitable for representative pigment analyses. After collecting the eggs, we washed off any surface debris
100 and fouling from each egg with seawater and then packaged them in a box for shipment to New York
101 City, NY, USA for laboratory analysis at Hunter College. Upon delivery, the eggshells were stored in a
102 dark container at 4°C until processing.

103

104 *Eggshell Measurements:*

105 For each egg, we broke the shell into two $\sim 1 \text{ cm}^2$ fragments/egg from different parts of the egg. We
106 washed the fragments with double distilled water, measured each fragment's thickness to an accuracy
107 of 0.01 mm with a point micrometer (Series 112 Mitutoyo), and then weighed each fragment to an
108 accuracy of 1 mg (XS403S Mettler Toledo). We then took digital photos of the fragments that included a
109 size scale.

110 We collected avian-visible spectral (300-700 nm) reflectance data using an Ocean Optics
111 USB2000 spectrometer, illuminated by a DT mini-lamp (Ocean Optics, Inc., Dunedin, FL, USA). All
112 measurements were taken at a 90° angle (following 29). Spectral reflectance measurements were taken
113 in triplicate from an average of 2 fragments from their shell background (N=50 eggs), excluding areas
114 with maculation. We did not analyze spot coloration as this trait is not statistically repeatable across
115 eggs laid by the same female in different years (20).

116 Averaged reflectance spectra for the separate fragments of each egg (Fig. 2) were transformed
117 into avian tetrahedral colourspace coordinates (30) using the *tcspace* function from the *pavo* package
118 v.1.3.1 in R 3.5.0 (31). We then calculated the repeatability (R) of *r.achieved* (a measure of avian-
119 perceived "colourfulness"), *hue.theta* and *hue.phi* (colour hue angles), using the *rpt* function from the
120 *rptR* v.0.9.21 package in R. For all repeatabilities, we performed significance tests against a null
121 distribution of N=2000 permuted samples and estimated the 95% confidence interval of R with N=2000
122 bootstrap samples. Because repeatabilities of modelled avian-perceived within-egg fragment colours
123 were all significant (all $P < 0.001$) and highly repeatable (all $R > 0.83$; data not shown), we took the mean
124 of the two egg fragments' background reflectance spectra for each egg to obtain a "whole egg"
125 background reflectance spectrum for each egg.

126 We used a receptor-noise limited visual model to model avian perceived colour discriminability
127 between all collected murre eggs' background spectral reflectance (32). Visual models were run using
128 the *coldist* function from the *pavo* package in R. Because no published data of murre visual physiology
129 exist, we constructed a general avian visual model using available knowledge of predicted species-
130 specific vision. Murres likely possess a violet sensitive (*VS* vision) rather than ultra-violet sensitive (*UVS*
131 vision) visual system, based on predicted amino acid spectral tuning sites using *SWS1* opsin gene
132 fragments for closest relatives in the order Charadriiformes (33). Therefore, we used an average avian
133 *VS* visual system for subsequent perceptual modelling (*avg.v*, peak cone sensitivities= 416nm, 478nm,
134 542nm, 607nm), with photoreceptor densities of the Red-billed Leiothrix (*Leiothrix lutea*) (34; *VS*-
135 cone=1, *SWS*-cone=2, *MWS*-cone=2, *LWS*-cone=4), a receptor noise Weber fraction of 0.1 with the long-
136 wave sensitive cone (*LWS*) as the reference cone, and excluded any effect of light transmission through
137 ocular media (*ideal* transmission). The visual model also assumed that murre eggs were illuminated by
138 full sunlight (*D65*) and perceived colour differences were independent of the viewing background
139 appearance (*ideal* background). Quantum cone catch values for all murre egg colours were transformed
140 into chromatic perceivable differences from one another (chromatic just noticeable differences,
141 hereafter: JNDs). These chromatic JND values were then mapped into perceptually-corrected Cartesian
142 coordinates using the *jndxyz* function in *pavo*, with the mean murre egg colour in our sample set as the
143 central origin point in 3D-space and Euclidean distances from the origin equal to JNDs from the mean
144 egg colour for each egg. For each egg background colour, we obtained X, Y, and Z coordinates in 3D JND
145 space.

146 We quantified eggshell maculation density by visualizing the shell fragments in Image J (v. 1.51;
147 35) and dividing the total number of unconnected human-visible marks (i.e. spots and/or lines) by the
148 surface area of each shell fragment. Repeatability was high between fragments sourced from the same
149 shell ($R = 0.87$), and so we used the mean value per egg in the statistical analyses.

150

151 *Eggshell Pigment Concentrations:*

152 We washed the fragments with 70 % ethanol and then, after drying, manually pulverised them. We used
153 an ethylenediaminetetraacetic acid (EDTA) protocol (21) to extract pigments from the eggshell
154 fragments, resulting in 1 ml of dissolved sample in acetonitrile-acetic acid (4:1 v/v). Samples were run in
155 a Cary 300 UV-Vis spectrophotometer for UV absorbance, with biliverdin and protoporphyrin
156 absorbance observed at 377 nm and 405 nm, respectively (22).

157 We performed ultra-high performance liquid chromatography (UHPLC) using the same method
158 as described in similar avian eggshell extraction studies from our laboratory (e.g., 24, 36). Briefly,
159 samples were run at a flow rate of 0.4 mL/min using as solvents A and B water with 0.01 M formic acid
160 or acetonitrile with 0.1 M formic acid, respectively. The linear gradient was set to 2% A and 98% B at
161 6.5min. Biliverdin eluted at ~ 3.5 min and protoporphyrin IX at ~ 5.6 min. We calculated relative
162 proportions of the two pigments using Beer Lambert's law ($A=\epsilon lc$) (following 24). Additionally, pigment
163 presence or absence was independently confirmed through mass spectrometry (following 36). Samples
164 were standardised by dividing the amount of pigment (in moles) with the mass of the initial eggshell
165 sample (M/g) (following 24). Repeatability of pigment concentrations was moderately high between
166 fragments sourced from the same shell ($R_{biliverdin} = 0.65$ and $R_{protoporphyrin} = 0.61$), and we used the mwan
167 value per egg in our analyses.

168

169 *Statistical Tests:*

170 Murre eggshell thickness, as well as patterning, varies among different shell regions (20, 28). We did not
171 keep track of which eggshell fragment yielded which spectral and maculation vs. thickness and pigment

172 concentration data point, and therefore we averaged each set of data types per egg in the subsequent
173 statistical analyses.

174 A statistical inspection of the correlations between the X, Y, and Z coordinates revealed strong
175 covariations (all pairwise $|R| \geq 0.48$, all $P \leq 0.0004$) and visual inspection of the eggshell JND space
176 coverage indicated a planar distribution (*sensu* [37]). Nevertheless, we opted to analyse the data using
177 each of these coordinates separately as their dimensions can be directly interpreted as perceptual JND
178 distances within the avian visual system.

179 To test the prediction of the individual recognition hypothesis, namely that different
180 components of multiple visible cues are independent of each other within eggs, we used Standard Least
181 Squares Models in JMP 12.0 (SAS Institute, Cary, NC).

182 To analyze the putative relationship between physico-chemical properties of each eggshell and
183 its background coloration JND scores or maculation density, we used a second set of Standard Least
184 Squares Models. Predictors in these models were the ln-transformed average concentrations of each of
185 the two pigments and the untransformed value of the average shell thickness per egg because raw
186 pigment concentration data did (Shapiro-Wilk tests, both $W > 0.58$, $P < 0.0001$) but thickness did not
187 differ ($W = 0.99$, $P = 0.93$) from normal distributions. The response variables were the three JND
188 coordinate scores from averaged background coloration analysis and the average maculation density.
189 We omitted non-significant terms and interactions from the final model statistics reported for the
190 significant terms. For illustrative purpose we plotted the relationships of eggshell properties using
191 colored data points that reflect the human-visible appearance of the eggs as calculated from each shell's
192 average reflectance spectrum (Figs 3-6).

193

194 **Results:**

195

196 As predicted by (A), the individual recognition signal hypothesis, there was no statistical relationship
197 within the same egg between any one of its X, Y, and Z JND coordinates and its maculation density
198 metric (all $|R| < 0.05$, $P > 0.60$).

199 For prediction (B) regarding the shell's physico-chemical properties, there was no statistically
200 significant relationship between JND coordinate X and shell thickness or pigment concentrations (all $F <$
201 1.7 , all $P > 0.20$) (Fig. 3). In contrast, coordinate Y was negatively correlated with thickness ($F = 3.8$, $P =$
202 0.05), not correlated with biliverdin ($F = 0.1$, $P = 0.76$), and negatively correlated with protoporphyrin IX
203 ($F = 4.6$, $P = 0.048$) concentrations (Fig. 4). The JND coordinate Z was also not statistically related to
204 thickness, biliverdin or protoporphyrin IX (all $F < 1.5$, $P > 0.23$) (Fig. 5). Finally, maculation density was
205 negatively correlated with thickness ($F = 7.5$, $P = 0.0086$), negatively correlated with biliverdin ($F = 4.4$, P
206 $= 0.042$), and positively correlated with protoporphyrin IX ($F = 4.2$, $P = 0.047$) (Fig. 6).

207

208 **Discussion:**

209

210 *Identity Signalling Hypothesis:*

211 Contrary to American Coots *Fulica americana* (38) and Common Moorhens *Gallinula chloropus* (39), the
212 context of individual egg recognition, and of the resulting conspecific egg discrimination, is unlikely to be
213 conspecific brood parasitism, as murrelets lay only a single egg and do not build nests. Nonetheless,
214 behavioural experimentation shows that murrelets can reliably recognise and retrieve their own eggs when

215 facing a choice of unattended conspecific eggs in their dense breeding colonies (4, 14) (Fig. 1). They do
216 so likely through the unique combination of their own egg's visible traits (though critical experiments
217 with manipulated real or model murre eggs remain to be conducted; *sensu* 40).

218 As assumed by the individual identity signaling hypothesis (5), the appearance of eggs of
219 common murres is repeatable both within the same shell (this study) and across eggs laid by the same
220 female in different years (20). However, as predicted by (A) of the same hypothesis, the multiple
221 components of each murre egg's visible appearance, including its avian-perceivable background shell
222 coloration and maculation density, are not statistically related to each other even within the same eggs
223 (16, this study).

224

225 *Pigmentary Basis of Egg Morphology:*

226 Here, we also assessed the structural basis underlying the immense variation in visible traits of murre
227 eggs. As predicted by (B), even when analyzing just two physico-chemical properties of each shell,
228 namely its thickness and pigment concentrations, these covaried statistically with some of our metrics of
229 avian perceivable background colour spectra and maculation density taken from the same egg.
230 Specifically, using the avian perceivable color space coordinates (X, Y, and Z) in units of JND (Fig. 3-5)
231 allowed us to measure the pigment-based co-variation of colour appearance of the shells within our
232 data set. This variation reached, on average, an extent of up to ~ 2.0 JNDs, which is predicted to be
233 discriminable by the visual system of the murres. The results, therefore, illustrate how individually
234 unique eggshell appearances, suitable for individual identity signaling, can be generated by a small
235 number of structural and physiological mechanisms involved in the formation of avian eggshells and
236 their pigmentation during oviposition (9).

237 Regarding the statistical relationships we found between eggshell thickness with shell
238 background coloration and maculation density, earlier work on murre eggs, also collected at Gull Island,
239 showed no significant covariation between these traits (28). However, such differences may have arisen
240 due to divergent methodologies used between the two studies, i.e. using human-assessed (background
241 colour, spot pattern) vs. avian perceivable metrics (JND coordinates based on background reflectance
242 spectra, count-based maculation density), respectively. In turn, prior work on Great Tit *Parus major*
243 eggs from England also showed an ecological and structural linkage between thinner shells and
244 pigmentation leading to denser spotting patterns (26). Here, we found a statistically negative
245 relationship of biliverdin and a positive relationship of protoporphyrin IX with maculation density (Fig.
246 6A,B), which is in support of the latter known as the pigmentary basis of avian eggshell maculation
247 patterns in other taxa (41).

248 Specifically, as seen in the Great Tit, we also found a negative relationship between eggshell
249 thickness and maculation density (Fig. 6C). However, given that the thinnest murre eggshells in our
250 sample were already 30% thicker than size-matched average chicken *Gallus domesticus* shell's thickness
251 (42), it therefore remains a relevant subject for future research whether the co-variation in murre
252 eggshell thickness with spotting patterns translates into biologically relevant variation in structural
253 strength. For example, the known overall thicker-than-predicted-by-size shells of murre, and the
254 thicker equatorial than blunt end shell regions, may be an adaptation to incubation and rolling on a
255 rough cliff-edge breeding surface (14) and/or to withstand microbial contamination through contact
256 with the feces-soiled breeding substrate in the colony (43).

257

258 *Conclusions:*

259 Previous work suggested that the pigmentary composition of avian eggshells might be poor predictors of
260 the visible appearance of shells across and within species (36, 44, 45, but see 29). In contrast, here we
261 imply the opposite: the extraordinary amount of interindividual variation in murre egg appearance
262 seems to be generated by consistent differences in some aspects of the pigment compositions and
263 concentrations among eggs. However, whether and how a putatively tighter physico-chemical control of
264 eggshell appearance in murre is under more direct genetic control so as to generate adaptively
265 variable, but individually consistent, shell colors and spotting patterns remain to be investigated in the
266 future. In general, across different bird species and lineages, the two eggshell pigments may interact
267 with diverse physical properties of the eggshell differently to produce avian perceivable variation in
268 eggshell colours and maculation patterns (8). Further micro- and nanostructural analyses of the shell
269 matrix structure in murre and other avian taxa (following 46, 47) should also be informative to evaluate
270 these alternatives.

271

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380

381 **Figures**

382 **Fig. 1.** Common Murres at their breeding colony off Gotland, Sweden. Inset: Representative human-
383 visible shape, colour, and pattern variation between eggs, collected from different individual common
384 murrelets in Iceland. Photo credits: M. Hauber and B. Stauffer.

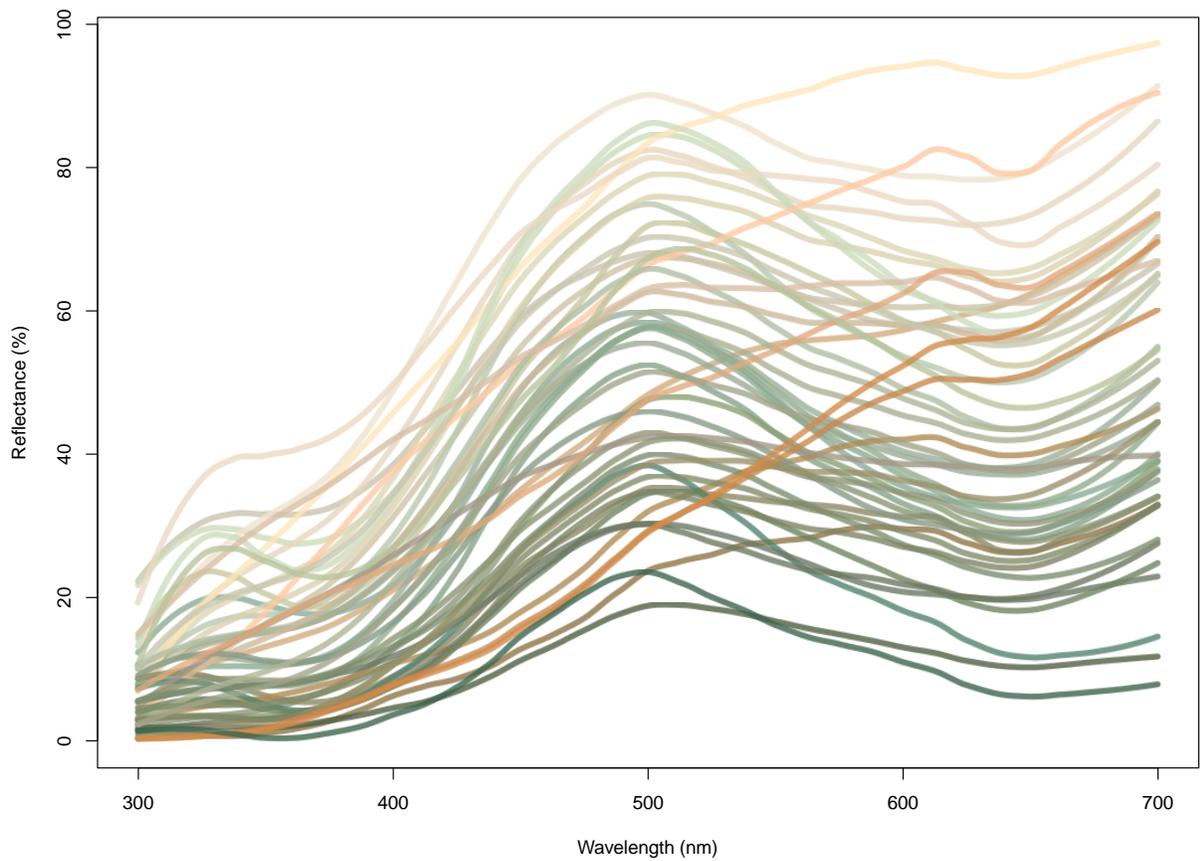
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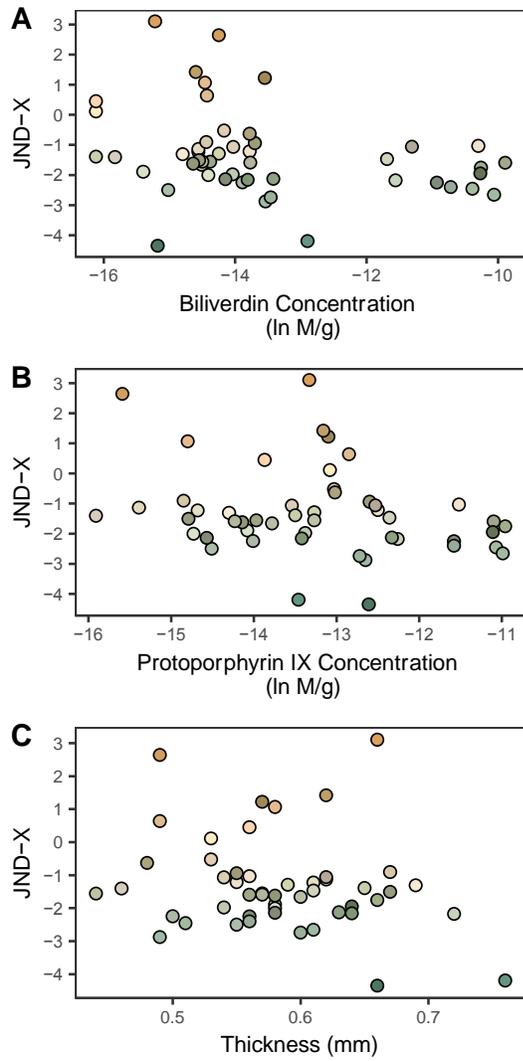
388 **Fig. 2.** Avian-visible reflectance spectra of eggshell fragments utilized for pigment extractions in this
389 study. For illustrative purpose we plotted spectra to reflect the human-visible appearance of the eggs as
390 calculated from each shell's average reflectance spectrum.



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392 **Fig. 3.** Relationship between murre eggshells' avian perceivable colour space coordinate X (in units of
393 JND) with their physico-chemical traits (A-B: pigment concentrations, C: thickness).

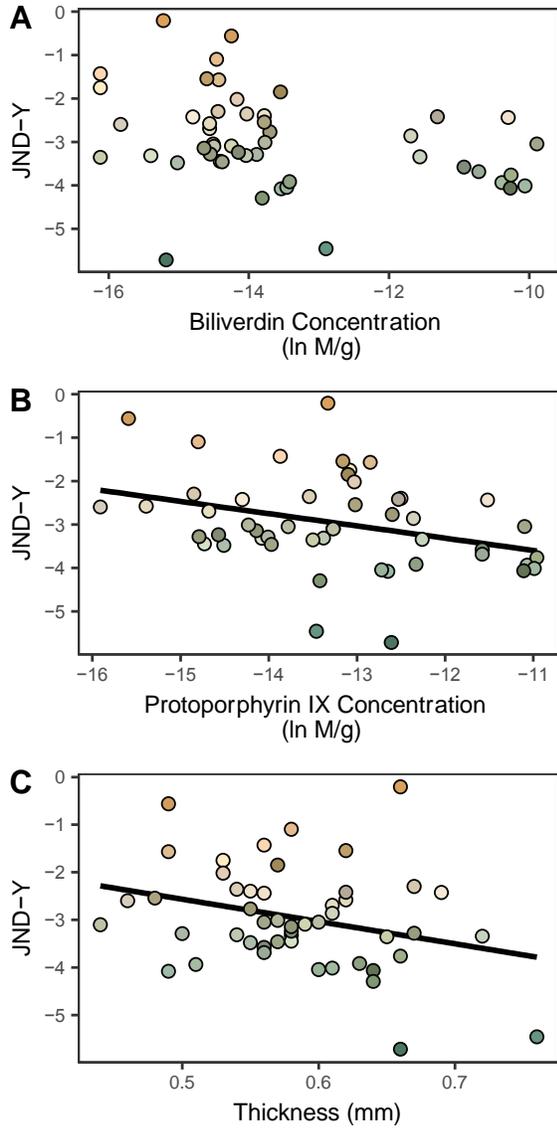
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396 **Fig. 4.** Relationship between murre eggshells' avian perceivable colour space coordinate Y (in units of
397 JND) with their physico-chemical traits (A-B: pigment concentrations, C: thickness). Significant
398 relationships from the Results are shown with linear regression lines.

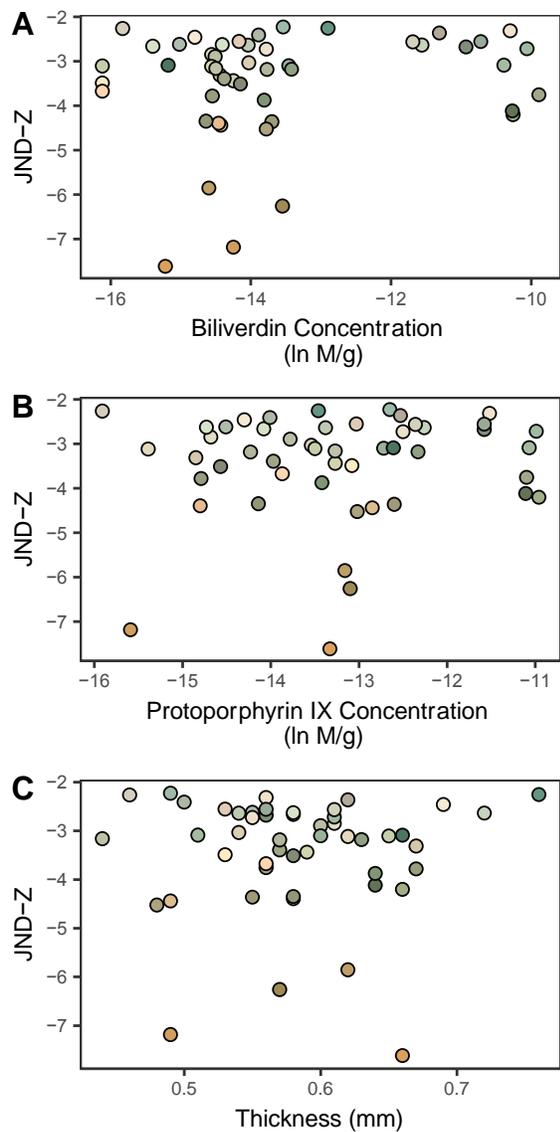
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401 **Fig. 5.** Relationship between murre eggshells' avian perceivable colour space coordinate Z (in units of
402 JND) with their physico-chemical traits (A-B: pigment concentrations, C: thickness).

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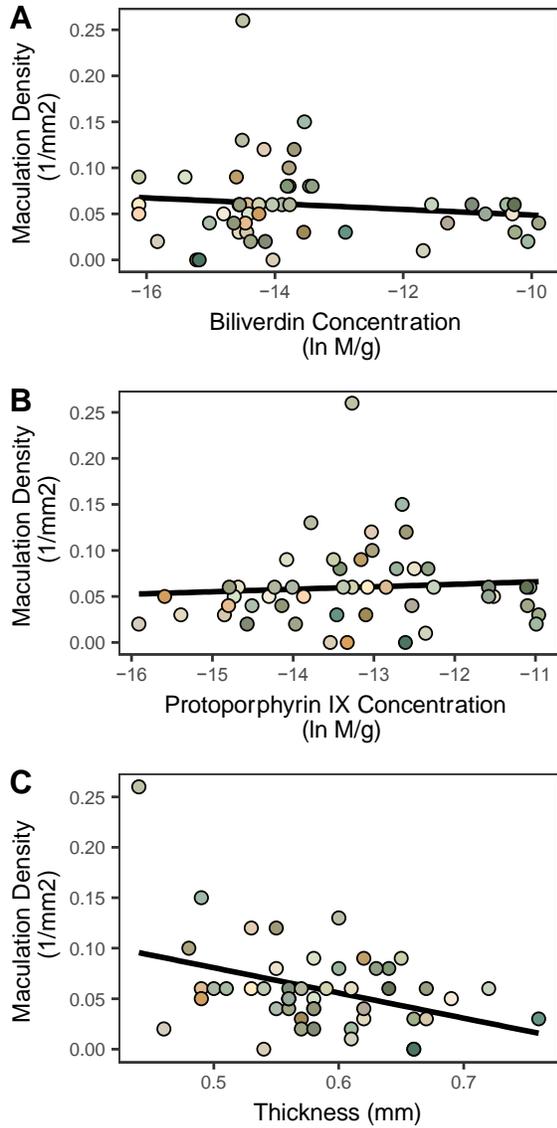


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405

406 **Fig. 6.** Relationship between murre eggshells' maculation density with their physico-chemical traits (A-B:
407 pigment concentrations, C: thickness). Significant relationships from the Results are shown with linear
408 regression lines.

409



410